

# Structural elements of the osteopontin SVVYGLR motif important for the interaction with $\alpha_4$ integrins

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**Abstract** The osteopontin SVVYGLR motif binds the integrins  $\alpha_4\beta_1$  and  $\alpha_9\beta_1$ . We show that  $\alpha_4\beta_7$  also interacts with this motif and that an SVVYGLR-OH peptide antagonises the  $\alpha_4\beta_7$  MAdCAM interaction. The important elements of this motif required to bind  $\alpha_4\beta_1$  and  $\alpha_4\beta_7$  were probed using a series of mutated peptides based around SVVYGLR. Leu167 is important for the interaction with  $\alpha_4$  integrins, as is the C-terminal carboxylic acid of Arg168 exposed by thrombin cleavage. The importance of the acidic group means that SVVYGLR has structural elements in common with other  $\alpha_4$  integrin-binding motifs and suggests why thrombin cleavage activates this motif. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

**Key words:** Osteopontin; Integrin;  $\alpha_4\beta_1$ ;  $\alpha_4\beta_7$ ;  $\alpha_9\beta_1$

## 1. Introduction

The integrins comprise a large family of heterodimeric transmembrane receptors that mediate both cell–cell and cell–matrix interactions [1]. They engage numerous ligands and regulate a variety of cellular and physiological processes such as cell proliferation, apoptosis, migration, differentiation, inflammation and tissue remodelling [2]. The integrins  $\alpha_4\beta_1$ ,  $\alpha_4\beta_7$  and  $\alpha_9\beta_1$  form a subfamily of receptors based on sequence homology and ligand-binding repertoire [3]. They mediate the migration of leukocytes into, and maintain their activation at sites of inflammation [4–8]. Therefore antagonists of these integrins have potential as anti-inflammatory agents. Indeed blocking  $\alpha_4$  integrins reduces the inflammatory infiltrate in a number of models of inflammation, while blockade of  $\alpha_9\beta_1$  reduces neutrophil chemotaxis [8–14].

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**Abbreviations:** TPA, phorbol 12-myristate 13-acetate; MAdCAM, mucosal addressin cell adhesion molecule; VCAM, vascular cell adhesion molecule; CS-1, connecting segment-1; CS-5, connecting segment 5; FN-III5, fibronectin type three repeat 5; FN-III14, fibronectin type three repeat 14; GST, glutathione *S*-transferase; Opn17–168, GST–osteopontin amino acid residues 17–168; Opn153–168, GST–osteopontin amino acid residues 153–168; BCECF-AM, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester

Osteopontin binds a number of integrins via two contiguous but distinct motifs within the sequence GRGD-SVVYGLR (amino acid residues 158–168). The integrins  $\alpha_v\beta_3$ ,  $\alpha_v\beta_1$ ,  $\alpha_v\beta_5$ ,  $\alpha_8\beta_1$  and  $\alpha_5\beta_1$  bind via a classical RGD motif [15–20], while  $\alpha_4\beta_1$  and  $\alpha_9\beta_1$  bind via the cryptic SVVYGLR motif generated by thrombin cleavage at Arg168 [21,22]. The SVVYGLR motif is unusual as it lacks a critical acidic residue present in other binding motifs for  $\alpha_4\beta_1$  and  $\alpha_9\beta_1$ . For example  $\alpha_4\beta_1$  binds various Asp-containing motifs in fibronectin, namely connecting segment-1 (CS-1), CS-5, fibronectin type three repeat (FN-III)5 and FN-III14, as well as vascular cell adhesion molecule (VCAM) and the disintegrin EC3 [23–28]. Similarly  $\alpha_9\beta_1$  binds VCAM and tenascin via Asp-containing motifs [8,29,30].

The third member of this integrin subfamily,  $\alpha_4\beta_7$ , binds VCAM, CS-1 and FN-III5, as well as the  $\alpha_4\beta_7$ -specific ligand mucosal addressin cell adhesion molecule (MAdCAM). Again these interactions are all dependent on a critical Asp residue [24,28,31,32]. Given that  $\alpha_4\beta_1$  and  $\alpha_9\beta_1$  bind a number of common ligands, including osteopontin, it was of interest to determine whether  $\alpha_4\beta_7$  also bound osteopontin, and to determine the residues in the SVVYGLR motif required to interact with  $\alpha_4$  integrins.

## 2. Materials and methods

### 2.1. Cell culture

RPMI8866 cells and Jurkat cells were maintained in 1:1 RPMI1640 HEPES modification (Gibco):DMEM HEPES modification (Sigma) supplemented with Glutamax (Gibco) and 10% FCS.

### 2.2. Antibodies and other reagents

Antibody clones were as follows; anti- $\beta_1$  integrin antibody (4B4) (Coulter). Anti- $\alpha_4$  integrin (HP2/1), anti- $\alpha_5$  integrin (SAM-1), anti- $\alpha_v$  integrin (69-6-5) (Immunotech). The anti- $\beta_7$  integrin antibody (Fib504) was purified in house. The synthetic peptides Ac-SVVYGLR-OH (SVVYGLR-OH, parent), Ac-GRVLYSV-OH (GRVLYSV-OH, scrambled control) and Ac-SVVYGLR-NH<sub>2</sub> (SVVYGLR-NH<sub>2</sub>, capped) were synthesised at GlaxoSmithKline. The series of peptides in which each residue of the SVVYGLR motif was individually mutated to Ala were purchased from Cambridge Research Biochemicals Ltd. (Stockton-on-Tees, UK).

### 2.3. Production of recombinant protein fragments

All glutathione *S*-transferase (GST) bacterial expression constructs were as previously described [26]. The recombinant zz-VCAM and zz-MAdCAM, comprising the extracellular domain of each molecule coupled to the immunoglobulin-binding domain of protein-A, were expressed in baculovirus and purified using an IgG-coupled affinity column.

#### 2.4. Adhesion assays

All bacterial proteins were coated in PBS onto Maxisorp plates (Nunc) overnight at 4°C, then blocked with 3% BSA/PBS for 1 h at 37°C. The GST–osteopontin fusion proteins GST–osteopontin amino acid residues 17–168 (Opn17–168) and Opn153–168 were coated as indicated, GST-CS-1 was coated at 5 µg/ml, 100 µl per well. The zz-VCAM and zz-MAdCAM were adsorbed on to Maxisorp plate pre-coated with 100 µl of human IgG at 50 µg/ml. For capture zz-VCAM and zz-MAdCAM were diluted to 1 µg/ml and 0.2 µg/ml respectively in 3% BSA/PBS, and incubated overnight at 4°C. No adhesion occurred to GST, BSA or IgG alone under any assay condition (data not shown).

For the  $\alpha_4\beta_7$  adhesion assay RPMI8866 cells were washed in cation-free HBSS with 1 mM EDTA, pelleted and labeled with 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxyethyl ester (BCECF-AM) (Molecular Probes) in HBSS, 37°C for 10 min. Fc receptors were blocked by co-incubation with 1 µg/ml human IgG. After washing in cation-free HBSS, cells were used in the adhesion assay at a final concentration of  $3 \times 10^6$  cells ml<sup>-1</sup> in the presence of the appropriate treatment as indicated (100 µl per well). Assays were performed in 50 mM HEPES pH 7.5 buffered HBSS supplemented with 2 mM MgCl<sub>2</sub> and 50 ng/ml phorbol 12-myristate 13-acetate (TPA) unless indicated. To prevent aggregation, plates were centrifuged at 400 rpm for 3 min without braking. For antibody inhibition cells were pre-incubated with the appropriate antibody at 20 µg/ml for 5 min at room temperature. Cells were adhered for 20 min at 37°C, washed twice in PBS and adhesion quantitated by BCECF fluorescence measurement. For the  $\alpha_4\beta_1$  assay Jurkat cells were pelleted, washed once in HBSS (Sigma) then spread in 50 mM HEPES pH 7.5 HBSS with 0.2 mM MnCl<sub>2</sub> (unless otherwise indicated) at a final concentration of  $2 \times 10^6$  ml<sup>-1</sup> (100 µl per well). Cells were adhered for 35 min at 37°C, washed twice in PBS, once in ethanol, and fixed in ethanol for 20 min at room temperature. For quantitation cells were stained with 0.1% crystal violet (Sigma) for 10 min then lysed in 0.5% Triton X-100 (Sigma) and read at 570 nm.

### 3. Results

#### 3.1. The osteopontin SVVYGLR motif supports adhesion via $\alpha_4\beta_7$

Both  $\alpha_4\beta_1$  and  $\alpha_9\beta_1$  bind to the SVVYGLR motif between amino acid residues 162–168, terminating at the thrombin cleavage site, Arg168 [21,22]. As  $\alpha_4\beta_1$  and  $\alpha_4\beta_7$  are related integrins which recognise common sites in certain ligands [31] we used RPMI8866 cells to assess whether  $\alpha_4\beta_7$  also binds osteopontin. These cells express primarily  $\alpha_4\beta_7$ , a small amount of  $\alpha_4\beta_1$  and do not express any detectable  $\alpha_v$  integrins or  $\alpha_9\beta_1$  (data not shown).

GST–osteopontin fusion proteins support adhesion as efficiently as purified human protein [24]. To determine the binding conditions required to support maximal binding, RPMI8866 cells were adhered to bacterially expressed protein mimicking thrombin cleaved osteopontin (Opn17–168) under a range of conditions. RPMI8866 cells bound Opn17–168 maximally in Mn<sup>2+</sup> and Mg<sup>2+</sup>/TPA, but only showed weak adhesion in the presence of Mg<sup>2+</sup> alone (Fig. 1A), a pattern that was similar to MAdCAM adhesion (Fig. 1B). The difference in coating concentration of ligands required to support maximal binding is due to the different methods employed to immobilise the ligands. Similar binding profiles were obtained in the presence of function blocking anti- $\beta_1$  integrin antibodies indicating that the differences in binding were not due to differential binding via  $\alpha_4\beta_1$  integrin (data not shown).

The SVVYGLR motif is the primary  $\alpha_4\beta_1$ -binding motif in osteopontin [21] and is proximal to the osteopontin RGD motif, which binds  $\alpha_v$  integrins and  $\alpha_5\beta_1$ . To investigate  $\alpha_4\beta_7$  binding to the SVVYGLR motif, RPMI8866 cells were adhered to Opn153–168 which also contained a RGD to

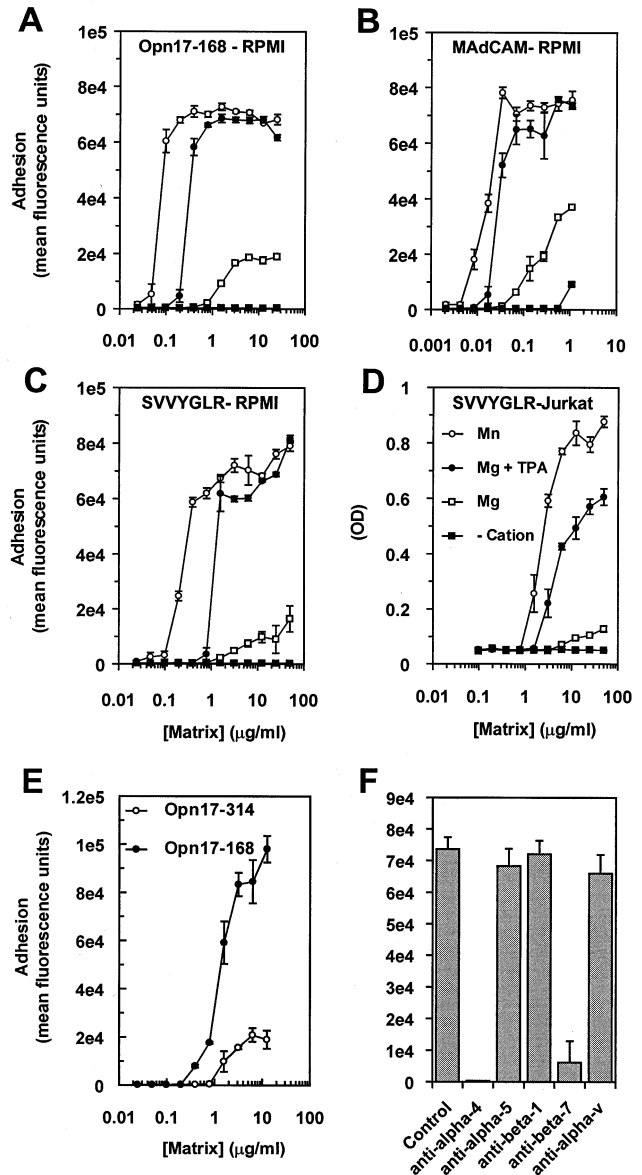


Fig. 1. Interaction of  $\alpha_4\beta_7$  with the SVVYGLR motif of osteopontin. RPMI8866 were adhered to (A) Opn17–168, (B) zz-MAdCAM and (C) Opn153–168 (SVVYGLR) in the presence of 2 mM MgCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>+50 ng/ml TPA or 0.2 mM MnCl<sub>2</sub> as indicated. Cells were incubated at 37°C for 20 min. D: Jurkat cells were adhered to coated Opn153–168 (SVVYGLR), in the presence of 2 mM MgCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>+50 ng/ml TPA or 0.2 mM MnCl<sub>2</sub> as indicated at 37°C for 30 min. E: RPMI8866 cells were adhered to Opn17–314 or Opn17–168 in the presence of 2 mM MgCl<sub>2</sub> and 50 ng/ml TPA at 37°C for 20 min. F: RPMI8866 cells were adhered to Opn153–168 in the presence or absence (control, no antibody) of anti-functional integrin antibodies (20 µg/ml), as indicated at 37°C for 20 min in the presence of 2 mM MgCl<sub>2</sub>+50 ng/ml TPA. All the data are representative of at least four experiments, each point represents the mean  $\pm$  S.D. of duplicate points.

RAD mutation which abrogated any interaction via RGD dependent integrins (data not shown). The profile of RPMI8866 adhesion to Opn153–168 was similar to Opn17–168, maximal binding occurring in Mn<sup>2+</sup> and Mg<sup>2+</sup>/TPA (Fig. 1C). Jurkat cells bind the SVVYGLR motif solely through  $\alpha_4\beta_1$  [21]. The profile of  $\alpha_4\beta_1$  mediated Jurkat adhesion was slightly different to that of  $\alpha_4\beta_7$ , with maximal adhesion only

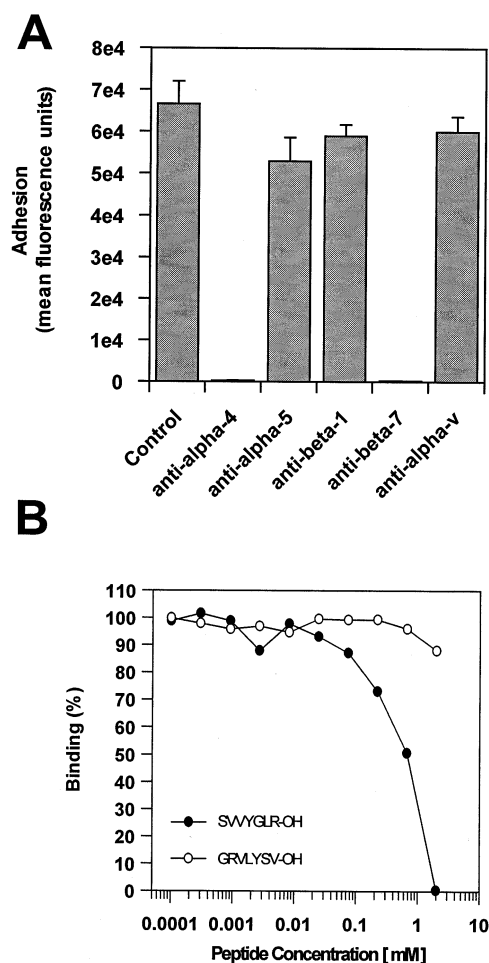


Fig. 2. A peptide SVVYGLR inhibits  $\alpha_4\beta_7$  binding to MAdCAM. A: RPMI8866 cells were adhered to zz-MAdCAM in the presence or absence of anti-functional integrin antibodies (20  $\mu\text{g/ml}$ ), as indicated, at 37°C for 20 min in the presence of 2 mM  $\text{MgCl}_2$ +50 ng/ml TPA. The data are representative of four experiments and each data point is the mean  $\pm$  S.D. of duplicate points. B: RPMI8866 cells were adhered to zz-MAdCAM in the presence of varying concentrations of parent SVVYGLR-OH or scrambled GRVLYSV-OH peptides. These data are representative of at least three experiments, and each point is the mean  $\pm$  S.D. of duplicate points.

occurring the presence of  $\text{Mn}^{2+}$ , suggesting that  $\alpha_4\beta_7$  binds at a lower activation state than  $\alpha_4\beta_1$  (Fig. 1D). Truncation of osteopontin to Arg168 modulates the binding of RPMI8866 cells as in the presence of  $\text{Mg}^{2+}$ /TPA the full length protein Opn17–314 bound cells poorly compared to the thrombin cleaved fusion protein Opn17–168 (Fig. 1E). We previously found that two sites within osteopontin could support  $\alpha_4\beta_1$  binding [21]. This second site, within amino acid residues 132–146 will also support weak binding of  $\alpha_4\beta_7$ , but only under assay conditions that generate a maximal integrin-activation status (data not shown).  $\alpha_4\beta_1$  and  $\alpha_4\beta_7$  integrins can therefore access this site in the intact protein, but upon thrombin cleavage bind SVVYGLR motif, which is the stronger binding site for  $\alpha_4$  integrins.

To conclusively demonstrate that RPMI8866 bind the SVVYGLR motif through  $\alpha_4\beta_7$ , cells were adhered to Opn153–168 in  $\text{Mg}^{2+}$ /TPA and with function blocking antibodies (Fig. 1F). Adhesion of RPMI8866 cells to Opn153–168 was blocked by  $\alpha_4$  and  $\beta_7$  integrin function blocking antibodies,

but not by antibodies against  $\beta_1$ ,  $\alpha_v$  or  $\alpha_5$  integrin, demonstrating that RPMI8866 cells bind the SVVYGLR motif exclusively via  $\alpha_4\beta_7$ . Collectively these data establish  $\alpha_4\beta_7$  as a receptor for the osteopontin SVVYGLR motif.

### 3.2. An SVVYGLR peptide inhibits $\alpha_4\beta_7$ binding to MAdCAM

An SVVYGLR-OH peptide inhibits  $\alpha_4\beta_1$  binding to a GST fusion protein corresponding to the CS-1 alternatively spliced fragment of fibronectin [21]. The ability of SVVYGLR-OH to antagonise RPMI8866 cell adhesion to MAdCAM was assessed. RPMI8866 cells bind MAdCAM exclusively through  $\alpha_4\beta_7$ , as blocking antibodies against the  $\alpha_4$  integrin subunit and the  $\beta_7$  integrin subunit but not the  $\beta_1$  integrin subunit blocked adhesion to zz-MAdCAM (Fig. 2A). The SVVYGLR-OH peptide inhibited adhesion of the RPMI8866 cells to MAdCAM, while the scrambled control peptide had no effect (Fig. 2B). That the SVVYGLR-OH peptide antagonises the  $\alpha_4\beta_7$ /MAdCAM interaction suggest it is a bona fide ligand for  $\alpha_4\beta_7$ . Moreover it also suggests either that MAdCAM and osteopontin bind within the same region of  $\alpha_4\beta_7$ , or that binding of each ligand is mutually exclusive.

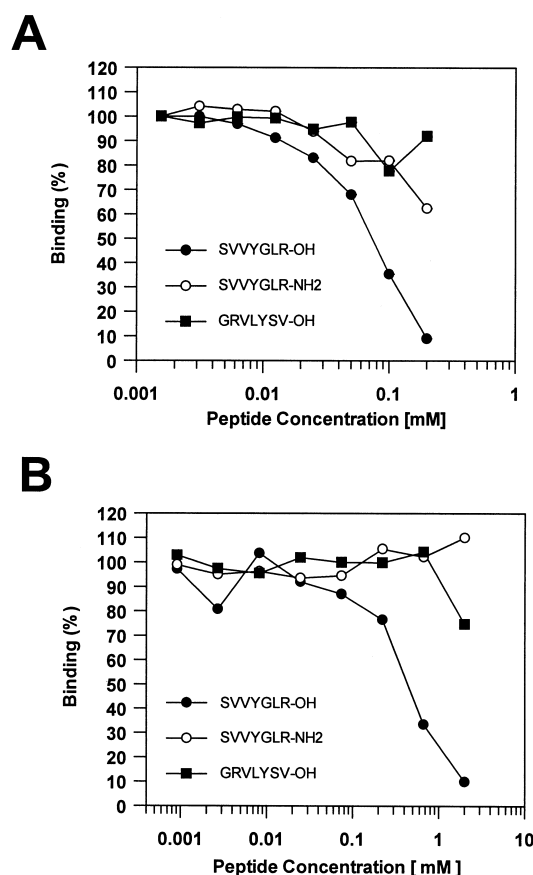


Fig. 3. The C-terminal COOH group is required for inhibition of  $\alpha_4\beta_1$  and  $\alpha_4\beta_7$ . A: Jurkat cells were adhered to GST-CS-1 in the presence of 0.2 mM  $\text{MnCl}_2$  and (B) RPMI8866 cells were adhered to zz-MAdCAM in the presence of 2 mM  $\text{MgCl}_2$ +50 ng/ml TPA with varying concentrations of parent SVVYGLR-OH, capped SVVYGLR-NH2 or scrambled GRVLYSV-OH peptides. These data are representative of at least three experiments, each point is the mean  $\pm$  S.D. of duplicate points.

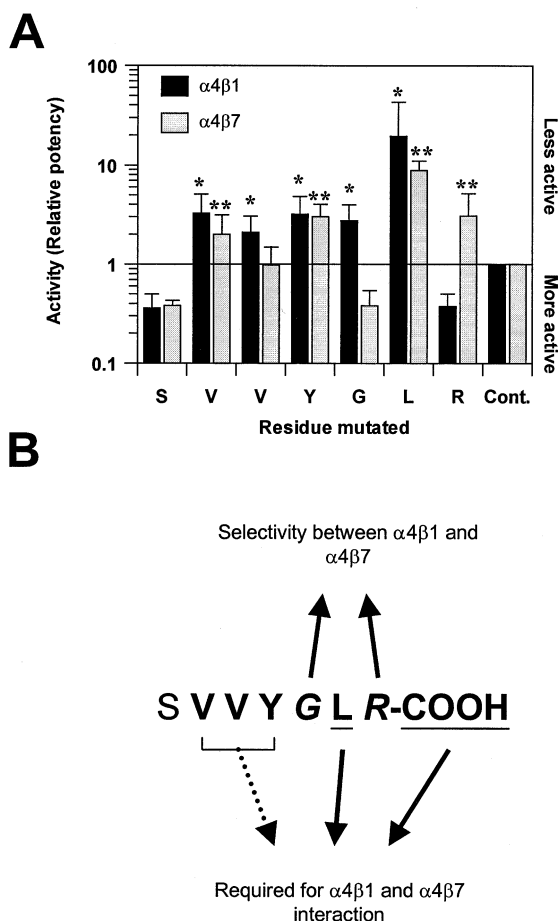


Fig. 4. Identification of key residues within SVVYGLR required for inhibition of  $\alpha_4\beta_1$  and  $\alpha_4\beta_7$ . A: Jurkat (black bars) and RPMI8866 cells (grey bars) were adhered to CS-1 and zz-MAdCAM in 0.2 mM  $\text{MnCl}_2$  and 2 mM  $\text{MgCl}_2$ +50 ng/ml TPA respectively and a series of individual alanine-substituted SVVYGLR-OH based peptides. The data are expressed as a RP compared to the  $\text{IC}_{50}$  of parent peptide SVVYGLR-OH in each individual experiment ( $\text{RP} = (\text{IC}_{50} \text{ of mutant peptide})/(\text{IC}_{50} \text{ of SVVYGLR-OH})$ )  $>1$  = less active and  $<1$  = more active than the parent peptide), and represents the mean of four experiments  $\pm$  S.E.M. (\* $P \leq 0.05$ ; \*\* $P \leq 0.001$ ). B: Summary of the key elements of the SVVYGLR motif.

### 3.3. Structural elements of the SVVYGLR motif important for the interaction with $\alpha_4$ integrins

As SVVYGLR-OH effectively blocks adhesion of  $\alpha_4\beta_1$  to CS-1 and  $\alpha_4\beta_7$  to MAdCAM, this approach was utilised to determine the structural elements of the SVVYGLR motif that interact with  $\alpha_4\beta_1$  and  $\alpha_4\beta_7$ . A number of the  $\alpha_4$  integrin motifs contain a critical acidic residue, normally an Asp [24–29,31,32]. One significant feature of the SVVYGLR motif is that it lacks an obvious acidic group, therefore understanding how this motif binds  $\alpha_4$  integrins could have important implications for the design of small molecule antagonists. The SVVYGLR site is located immediately N-terminal to the thrombin cleavage site, Arg168, with truncation of osteopontin at Arg168 modulating the interaction with both  $\alpha_4\beta_1$  and  $\alpha_9\beta_1$  [21,22]. It is possible that cleavage at Arg168 reveals a carboxylic acidic group, enabling the SVVYGLR motif to engage  $\alpha_4$  integrins. To test this hypothesis a capped carboxamide peptide SVVYGLR-NH<sub>2</sub> was synthesised and assayed

for the ability to inhibit Jurkat and RPMI8866 cell adhesion to GST-CS-1 and zz-MAdCAM respectively (Fig. 3A,B). Removing the carboxylic acid group at the free C-terminus abrogated the ability of this peptide to inhibit adhesion via both  $\alpha_4\beta_1$  and  $\alpha_4\beta_7$ . Therefore the free C-terminus of SVVYGLR provides an acidic group essential for its interaction with  $\alpha_4$  integrins. Moreover this data suggest thrombin cleavage of osteopontin at Arg168 is required for  $\alpha_4\beta_1$  and  $\alpha_4\beta_7$ -dependent ligation of the SVVYGLR motif.

To further determine which other elements of the SVVYGLR motif are required for the interaction with  $\alpha_4$  integrins, a series of synthetic peptides were generated in which each amino acid was individually mutated to alanine. The ability of each peptide to block adhesion of Jurkat and RPMI8866 cells to GST-CS-1 and zz-MAdCAM respectively was then assayed. The activity of each peptide is shown in Fig. 4 expressed as a relative potency (RP) compared to the activity of the parent peptide SVVYGLR-OH. This analysis clearly shows that Leu167 is also important. Mutating this residue results in a RP similar to that observed with the capped peptide SVVYGLR-NH<sub>2</sub> or the scrambled control. A number of other residues also make a minor contribution to binding in particular Val163 and Tyr165. Mutation of these residues reduced the potency against both integrins. The observation that Tyr165 makes only a minor contribution to  $\alpha_4$  binding is interesting as this residue appears to be critical for the interaction with  $\alpha_9\beta_1$  [22]. Surprisingly mutation of Arg168 has opposite effects on the potency of the peptide against  $\alpha_4\beta_1$  and  $\alpha_4\beta_7$ . Mutating this residue to Ala results in a peptide that is more potent against  $\alpha_4\beta_1$  but less potent against  $\alpha_4\beta_7$ . Therefore the residue at this position may confer some selectivity between  $\alpha_4\beta_1$  and  $\alpha_4\beta_7$ . Collectively these data show that Leu167 and the C-terminal carboxylic acid of Arg168 are the most critical elements within the SVVYGLR motif for binding to  $\alpha_4$  integrins. These data are summarised in Fig. 4B.

## 4. Discussion

Here we have shown that in addition to  $\alpha_4\beta_1$  and  $\alpha_9\beta_1$  the related integrin  $\alpha_4\beta_7$  also binds osteopontin through the SVVYGLR motif. The SVVYGLR-OH peptide acts as an  $\alpha_4\beta_7$  antagonist capable of inhibiting  $\alpha_4\beta_7$  adhesion to MAdCAM, demonstrating that this motif binds with sufficient affinity to compete with known  $\alpha_4\beta_7$  ligands.

Importantly the SVVYGLR peptide requires the free C-terminal carboxylic acid of Arg168 to interact with  $\alpha_4$  integrins, as a peptide SVVYGLR-NH<sub>2</sub> in which the carboxylic acid was replaced with a carboxamide failed to inhibit cell adhesion mediated by either  $\alpha_4\beta_1$  or  $\alpha_4\beta_7$ . This suggests that in vivo osteopontin must be cleaved by thrombin at Arg168 to enable  $\alpha_4$  integrins to engage this motif, this is supported by the observation that truncation of osteopontin to Arg168 modulates the interaction with  $\alpha_4\beta_7$  and also  $\alpha_4\beta_1$  [21]. Moreover, like the classical  $\alpha_4$  integrin-binding sites, SVVYGLR requires a critical acidic moiety. This is provided by Asp in motifs such as LDVP, I/LDAP, or IDSP found in [24–27,31,32] as well as the disintegrin EC3 [28,29]. The data presented here raise the novel suggestion that the critical acidic group can also be provided by the free C-terminus of a protein fragment generated by proteolytic cleavage.

The inhibition studies using a series of Ala-substituted pep-

tides revealed that Leu167 is also important for the interaction with  $\alpha_4$  integrins. A number of other residues make minor contributions, in particular mutating Val163 and Tyr165 reduces the ability of the peptide to block both  $\alpha_4\beta_1$  and  $\alpha_4\beta_7$ . Yokosaki et al. [22] showed that Tyr165 was critical for osteopontin to bind  $\alpha_9\beta_1$ , raising the possibility that although  $\alpha_4$  integrins and  $\alpha_9\beta_1$  bind similar motifs in common ligands, the importance of individual residues may differ between the integrins. Mutating Arg168 to Ala increased the activity against  $\alpha_4\beta_1$  but reduced the activity against  $\alpha_4\beta_7$ , suggesting that peptides with selectivity between receptors could be generated.

It has been suggested that ligands occupy different regions of  $\alpha_9\beta_1$  as SVVYGLR only inhibits  $\alpha_9\beta_1$ -mediated adhesion to osteopontin, but not VCAM or tenascin. Our data suggest that for  $\alpha_4\beta_7$  SVVYGLR can occupy a binding site similar to that for MAdCAM and for  $\alpha_4\beta_1$  similar to CS-1. However SVVYGLR will only partially inhibit  $\alpha_4\beta_1$  binding to VCAM, and does not block  $\alpha_4\beta_7$  binding (data not shown), whether this reflects occupancy of different binding sites or the affinity of the peptide is unclear.

The interaction between  $\alpha_4$  integrins and osteopontin is highly regulated. Not only does the SVVYGLR motif require activation by thrombin, but neither  $\alpha_4\beta_1$  or  $\alpha_4\beta_7$  bind osteopontin unless the integrin is 'activation'.  $\alpha_4\beta_7$  exhibits maximal binding in the presence of  $Mn^{2+}$  or  $Mg^{2+}$ /TPA, while  $\alpha_4\beta_1$  only binds maximally in  $Mn^{2+}$ , and binds sub-maximally in  $Mg^{2+}$ /TPA. These conditions are thought to stimulate the activation state of the integrin. This would suggest that osteopontin is only engaged by  $\alpha_4$  integrins when cells are stimulated in a proteolytic environment, for example during an inflammatory response. In vivo the interactions of  $\alpha_4\beta_1$  are highly regulated, as  $\alpha_4\beta_1$  can occupy a variety of activation states [33,34]. This basal activation state differs between cell types and is rapidly altered by various cytokines. This suggests that the interaction of  $\alpha_4\beta_1$  and  $\alpha_4\beta_7$  with osteopontin is not one primarily involved in mediating cell transmigration from the vasculature but may fulfil some other function such as eliciting  $\alpha_4$ -dependent responses in a target tissue or mediating cell migration.

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