Structure-function analysis of RGD-helix motifs in αvβ6 integrin ligands

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Summary

Data relating to the structural basis of ligand recognition by integrins are limited. Here we describe the physical requirements for high-affinity binding of ligands to αvβ6. By combining a series of structural analyses with functional testing we show that 20mer peptide ligands, derived from high affinity ligands of αvβ6 (foot-and-mouth-disease virus, latency associated peptide), have a common structure comprising an Arg-Gly-Asp motif at the tip of a hairpin turn followed immediately by a C-terminal helix. This arrangement allows two conserved Leu/Ile residues at Asp+1 and Asp+4 to be presented on the outside face of the helix enabling a potential hydrophobic interaction with the αvβ6 integrin, in addition to the RGD interaction. The extent of the helix determines peptide affinity for αvβ6 and potency as an αvβ6 antagonist. A major role of this C-terminal helix is likely to be the correct positioning of the Asp+1 and Asp+4 residues. These data suggest an explanation for several biological functions of αvβ6 and provide a structural platform for design of αvβ6 antagonists.

Keywords: RGD, Helical Peptides, Structure-function, alpha-v-beta-6, integrin
Introduction

Understanding of the molecular basis of the binding interface of integrins with their ligands still is relatively poor. The most detailed information available comes from X-ray crystallography of RGD peptide binding to αvβ3, where the RGD motif bridged the α and β subunits, the arginine associating with the αv subunit and the aspartate co-ordinating with the bivalent metal ion on the β3 subunit (1). However, the RGD motif occurs in many ECM ligands so specificity is modified by other residues, often flanking the RGD site (2), though distant residues also can affect ligand-binding (3). Detailed comparison of the different ligands of one integrin could illuminate the essential elements that determine specificity and affinity, improve biological understanding of integrin-ligand interactions and allow rational design of targeting peptides and peptidomimetics (4, 5, 6); we have made just such a comparison for alpha v beta 6 (αvβ6).

The integrin αvβ6 is an epithelial-specific integrin that is expressed at low or undetectable levels in adult tissues but which can be upregulated during tissue remodelling. Thus increased αvβ6 expression occurs during wound healing, development and inflammation (7) and in more severe pathologies, including chronic skin wounds (8) and cancer (9). The ability of αvβ6 to promote migration and invasion, in part through protease upregulation (10,11,12), may explain why αvβ6 expression is an independent indicator of colon cancer aggressiveness (13). These data suggest that reagents designed to specifically antagonize αvβ6 could have clinical utility in colon cancer and possibly other diseases.

αvβ6 binds to the Arginine-Glycine-Aspartate (RGD) motif in its ligands which include fibronectin, tenascin, the latency associated peptides (LAP) of TGFβ1 (14) and TGFβ3 (15), and the VP1 coat protein of Foot-and-Mouth-Disease Virus (FMDV) (16). In addition, the motif DLXXL was identified by phage display of 7- and 12- residue peptides (17) as an αvβ6-specific motif, and shown to be crucial to the ability of a 15mer peptide to inhibit infection of BHK cells by FMDV (18).

We have characterized specific peptide antagonists to αvβ6 and have discovered the basis of high affinity binding of natural ligands for this integrin. Such ligands (including the GH-loop of the VP1 protein of foot-and-mouth-disease virus and latency associated peptide of TGFβ1) possess a C-terminal post-RGD helix which positions leucine or isoleucine residues in the Asp +1 and +4 positions as adjacent amino-acids on the outer face of the helix, allowing for a potential hydrophobic interaction with αvβ6. Our data present a structural explanation for certain biological functions of αvβ6 and provide a structural platform for design of αvβ6 antagonists.
Experimental Procedures

Cell lines and antibodies
Retroviral transduction generated αvβ6-positive, from αvβ6-negative, mouse NIH 3T3 fibroblasts (3T3β6.19) as described previously (10). Recombinant soluble αvβ6 (rsαvβ6) was purified from CHOβ6 cells, a kind gift from Dr. D. Sheppard (University of California SF, USA), as described previously (19). VB6 is an αvβ6-expressing oral squamous carcinoma cell line (10). Antibody 10D5 to αvβ6 was from Chemicon International, (Harrow, UK). 6.3G9, (anti-αvβ6) (20) was supplied by BiogenIdec. All other reagents were from Sigma-Aldrich unless stated otherwise.

A375Ppuro and A375Pβ6puro cell lines were generated from the human melanoma cell line A375P which was infected with pBabe retroviruses encoding puromycin resistance or, in addition, cDNA for human β6, as described previously (10). Cells were selected in puromycin (1.25ug/ml) and then αvβ6 expressing cells were selected by magnetic bead sorting, using 10D5 (anti-αvβ6; Chemicon International), according to the manufacturer’s instructions (Dynal, Invitrogen; Paisley, UK).

Cell Adhesion Assays
Adhesion of [51Cr]-labeled cells to 96-well flexible plates coated with ECM ligands has been described previously (10).

ELISA
80 ng/well rsαvβ6, in 100µl, was immobilized on 96-well plates (18h at 4°C). Plates were washed with Wash Buffer (20mM Tris, 150mM NaCl, 0.5 mM MgCl2, 1 mM CaCl2) before blocking for approximately 2 hours with Conjugate Buffer (1% casein (w/v) in Wash Buffer). All subsequent washes were with Wash Buffer. After washing, 100µl biotinylated peptide was added to triplicate wells in Conjugate Buffer, bound for 1 hour before washing and the addition of 50ml ExtrAvidin HRP, diluted 1:500 in Conjugate Buffer. After 1 hour plates were washed and developed with TMB+ (DAKO; according to manufacturer’s instructions). IC50s were generated using GraphPad Prism software; data shown represent mean ± standard deviation of three independent experiments.

Peptide synthesis
20mer peptides derived from the latency associated peptide of TGFβ1 (A20LAP: GFTTGRRGLATIHGMNRPF), and the GH-loop of VP1 protein of FMDV serotypes C-S8c1 (A20FMDV1:YTASARGDLAHLTTHARHL; ref. 18) and 01 BFS (A20FMDV2: NAVPNRLGDLQVLQKVART; ref. 21) were generated on an automatic synthesizer (Model 431A updated and 433A Applied Biosystems Solid Phase Synthesizer) by the Cancer Research UK Peptide Synthesis Laboratory using standard procedures. Where stated peptides were biotinylated in situ on resin support using standard procedures.

Circular Dichroism
CD spectra were recorded (Jasco J-600 spectropolarimeter) at room temperature using 0.4 mM peptide in NMR buffers containing TFE between 0-50% (v/v). Each solution was loaded onto 5 mm path length quartz cuvettes and spectra obtained from an average of 4 scans (range between 190 and 260 nm), recorded 20 nm/min, 1 nm bandwidth a 2 s response and a resolution of 0.2 nm. Spectra shown have no baseline correction. Spectropolarimeter OD values were converted into ellipticity and adjusted to the relative peptide concentrations by J-700 windows standard analysis (v.1.50.01) software. The maximal helicity ($\theta_{\text{max}}$) was calculated by methods as described (22).

NMR Sample Preparation
All NMR samples were prepared to a final volume of 300 µL for use in a Shigemi BMS005V NMR tube by dissolving purified, freeze-dried peptide in 2 mM phosphate buffered saline (PBS); phosphate concentration of 25 mM and saline concentration of 100 mM. For structural studies, trifluoroethanol-d$_3$ (TFE) was added to a final concentration of 30% (v/v). Saturation Transfer Difference NMR (STDNMR) samples were prepared with additional components: 28 µM integrin αvβ6, 0.5 mM Mg$^{2+}$ (MgCl$_2$) and 1.0mM Ca$^{2+}$ (CaCl$_2$) but without TFE.
Structural and Saturation Transfer Difference NMR Spectroscopy

Experiments were recorded (Varian Unity INOVA 600 MHz NMR spectrometer) with a z-shielded gradient triple resonance probe. Structural experiments, at 10°C, included two-dimensional (2D) nuclear Overhauser effect spectroscopy (NOESY), total correlation spectroscopy (TOCSY), rotating frame Overhauser effect spectroscopy (ROESY) and double-quantum-filtered correlated spectroscopy (DQFCOSY). Mixing times of 250, 70.0 and 100 ms for NOESY, TOCSY and ROESY respectively. Experiments were collected with 512/1024 and 2048 complex points with acquisition times of 64/128 and 256 ms in the indirectly and directly acquired $^1$H dimensions. Slow exchanging amide protons were detected from the fingerprint region of a 50 ms mixing time 2D NOESY experiment. Data processing analysis used Sun Silicon Graphics and Linux workstations with NMRPipe (23) to process and NMRView (24) to analyze spectra. Spin systems were identified using standard sequential assignment methods (25).

Saturation Transfer Difference NMR (STDNMR) experiments were run as described (26,27) but incorporating a 30 ms Hahn-echo filter (28). STD control and difference data were obtained at 25°C with 600 µl sample volumes of: (a): A20-FMDV-2 (2 mM) + αvβ6 (28 µM), (b): A20-FMDV-2 (2 mM) + carbonic anhydrase (30 µM) and (c): peptide GYQQGDYQQYNPD + αvβ6 (28 µM). All STD difference spectra were with a spectral width of 6000 Hz, data points and transients of 8192 and 16384 respectively. On resonance irradiation was set to −2.5 ppm and off resonance irradiation was set to −70.0 ppm with irradiation applied using a train of 9.4 ms Gaussian pulses, applied for 2.0 s, with each pulse having a 100 Hz bandwidth and separated by a 1.7 ms delay. STDNMR transfer data was assigned using peptide NMR assignments obtained in no TFE at 25°C. Resonance volume integrals were obtained using VNMR software (Varian Inc. Palo Alto, CA USA) Data were analyzed as outlined (27) to obtain the STD amplification factor using a ligand excess of 71.4. Individual amplification factors for each amino acid residue, from a sum of amplification factors from each $^1$H resonance for each residue, were converted to percentage STD amplification factors to compare with the highest residue factor (assigned 100%).

Structural Calculations and Analysis

All structural calculations were obtained using Crystallography and NMR System (CNS) version 1.1 running on Silicon Graphics Octane2 and Transtec X2100 SuSE 8.1 Linux workstations (29). NOE and ROE contacts were classified between 2.5-5.0Å with final structures calculated from extended coordinates using the standard CNS NMR anneal protocol with sum averaging for dynamic annealing with restraints from both extended and folded precursors. A final structural ensemble of 40 structures per peptide was produced with all structures used to produce statistical energy and root mean square (r.m.s.) deviation structural information. Backbone and heavy atom r.m.s. deviation values were obtained using MOLMOL version 2k.2 (30) on a PC running Microsoft Windows 2000. The structural integrity of each ensemble was evaluated using PROCHECK-NMR (31) run on a Transtec X2100 Linux workstation. Energy comparisons between structure ensembles created in CNS were made using GROMOS96 43Bl parameter set (32) within DEEPVIEW version 3.7 (33).
Results

**RGDLXXL/I 20mer peptides can have significantly different potencies**

In initial studies we tested a panel of 7-12mer RGD peptides, derived from high affinity natural ligands of αvβ6 (foot-and-mouth-disease virus and latency associated peptide), for their ability to inhibit αvβ6-dependent cell adhesion. Results (not shown) revealed that the most potent peptides tended to be longer and all had an RGDLXXL or RGDLXXI motif. We therefore analyzed further three 20mer peptides: A20FMDV1 (YTASARGDLAHLTTHARHL), A20FMDV2 (NAVPLNRGDQLVLAQKVART) and A20LAP (GFTTGRGDLATIHGMNRPF). Each of these peptides exhibited a dose-dependent inhibition of αvβ6-dependent cell adhesion using two different cell models. Thus, the IC50 for inhibiting αvβ6-dependent adhesion of 3T3β6.19 for A20FMDV2 was 1.2±0.2μM, for A20LAP was 13.8±3.3μM and for A20FMDV1 86.5±49.9μM (Figure 1a). We obtained a similar response using VB6 cells (Supplementary Fig 1). We also observed that there was a dose-dependent adhesion of biotinylated-peptides to immobilized recombinant αvβ6 that followed the same order of activity; thus 50% maximal binding to rscαvβ6 occurred at 0.5±0.15 nM for A20FMDV2, 1.4±0.93 nM for A20LAP and 6.8±3.49 nM for A20FMDV1. These data indicate that A20FMDV2 has the highest affinity of binding to αvβ6 followed by A20LAP and then A20FMDV1 (Figure 1b); the same order of activity as shown for their behaviour as antagonists.

**RGDLXXL/I Peptide potency correlates with helical propensity**

Although all three peptides possessed the previously identified αvβ6-specific RGDLXXL/I motif (17) it was not apparent why there should be such large differences in potency. Logan et al (21) had noted that if a di-sulfide bond was reduced in the GH-loop of the VP1 protein of FMDV O serotype, from which A20FMDV2 was derived, it was then able to form a helix in the post-RGD region. Therefore we analyzed the sequences of our peptides in the helix-predictive software Agadir (http://www.embl-heidelberg.de/Services/serrano/agadir/agadir-start.html) and found that A20FMDV2 and A20LAP were predicted to form helices and that the probability of this was greatest for A20FMDV2; A20FMDV1 was not predicted to form a helix (Figure 2a). To confirm the ability of these peptides to form helices we performed Far-ultraviolet Circular Dichroism (CD) analysis in increasing concentrations of the helix-promoting solvent TFE (trifluoroethanol-d3) (34). Figure 2b confirms, by the presence of ellipticity at 222nm, that there is a strong propensity for A20FMDV2 to form helices followed by A20LAP; A20FMDV1 formed a helix but only weakly. Thus experimental results confirmed the *in silico* predictions by Agadir software. In order to determine the extent, and position, of the helix in each of the peptides we conducted structural analysis of these peptides using NMR spectroscopy.

**NMR Analysis of αvβ6-binding 20mer peptides**

Using the lowest concentration of TFE required to support helix formation (30% TFE) we performed structural NMR on all three peptides. NMR solution structures were determined using 1H chemical shift assignments for each peptide (Supplementary Table 1). Through-space assignment data were from two-dimensional NOESY and ROESY spectra in 30% TFE (v/v). Specific NMR experiments highlight 1H nuclei proximal in space (typically separated by less than 6 Å) by showing an NOE or ROE contact between these nuclei. These contacts provide the major restraints used to calculate particular structural models. Additional restraint data from the assignment of hydrogen bond donors and dihedral angles were obtained using NMR NOESY data and high-resolution DQF-COSY spectra respectively (Supplementary Table 2). Distributions of NOE and ROE contacts were more numerous between nuclei in residues on the C-terminal side of the RGD for each peptide. Figure 3 summarizes contact types and additional restraints that indicate standard helix formation directly C-terminal to the RGD motif. Figure 4 highlights the main helical contact regions of the NOESY spectra for all three peptides (the number of contacts and resonance dispersion is greatest with A20FMDV2, least with A20FMDV1).
Structure Calculations and Analysis

All structures were determined using CNS. None gave violations greater than 0.2Å or bond angle violations greater than 5°. Structural restraint data were incorporated to generate the structural ensembles (Figures 5a, 5d ad 5g) and average structures (Figures 5b, 5e and 5h) and defined the structural limits of the α-helices. NMR and structural calculations confirmed the observations from the CD data. Residue i-j contacts, as shown for NH-NH in Figure 3, identify restraints that make all three peptides adopt a turn conformation that enables the RGD motif to be presented at the turn of a hairpin structure. NOE contacts used to confirm the turn-helix arrangement were completely unambiguous. We thus excluded many potentially ambiguous NH-αH turn contacts from i+i+2 residues which could artificially influence calculated structures. However, the turn was still capable of being defined from a combination of short (i+i+2 and i+i+3) and long-range NOE side chain contacts as can be seen from NOE contact maps (Supplementary Data Figure 7). This turn is well-defined and can be simulated from short range NOE’s (i+i+3 or less) alone, excluding the long range NOE’s from the calculation. These data provide assurance that this structural characteristic is not forced by the analysis. Helix associated residues for each peptide were Leu10-Val17 for A20FMDV2; Leu10-Gly15 for A20LAP and Ala10-Thr14 for A20FMDV1. ^3_1H values less than 5 Hz were used to restrain ϕ for that residue to −60° ± 30° because ^3_1H values obtained by DQF-COSY always are larger than those obtained by more accurate heteronuclear NMR methods (25). Structural energy statistics and backbone r.m.s. deviations are shown in Supplementary Table 3. Backbone r.m.s. deviations are quoted over residues DLXX[L/I]XX (Supplementary Table 3). PROCHECK-NMR analysis of the 40-structure ensembles identified 94.3, 94.8 and 93.6% of residues fell in the allowed regions of the Ramachandaran plot for A20FMDV1, A20FMDV2 and A20LAP respectively. Residues outside the allowed regions were from the first four amino acids in each ensemble. Helix limits and the turn definitions for each peptide structure were identified from a combination of dihedral angles, hydrogen bond geometries and NOE contacts. This approach proved to be invaluable for cases where overlap from chemical shift degeneracy prevented complete unambiguous NOE assignment which was particularly apparent in the NOE data from the A20FMDV peptide. Thus both the length and definition of the helix increases in the order A20FMDV1 > A20LAP > A20FMDV2, which correlates with peptide potency and further supports the CD and Agadir data. The data also predict that the residues at Asp+1 and Asp+4 are exposed almost in apposition on one face of the helix (represented in Figures 5c, 5f, 5i), particularly in A20FMDV2 and A20LAP; these residues are implicated strongly in the efficacy of peptide inhibitors of αβ6-dependent functions (17, 18).

A post-RGD helix increases affinity and potency of RGDLXXL/I peptides

To confirm the role that a post-RGD helix had for binding to αβ6 we generated A20DV1217, a derivative of A20FMDV2 that replaced the two L-valines at positions 12 and 17 with D-valines. This substitution was designed to maintain the biochemical nature and charge distribution over the length of the peptide while completely disrupting the helix. Analysis by CD (Supplementary Figure 2) and solution NMR (Supplementary Figure 3a and 3b) confirmed that A20DV1217 was unable to form a stable helix. In flow cytometry assays with biotinylated peptides, more than 1000-fold concentrations of A20DV1217 were required to obtain similar fluorescence signals on A375Pβ6puro cells compared with A20FMDV2 (Figure 6a). Thus the affinity of binding of the A20FMDV2 to cellular αβ6 is significantly higher than the helix-deficient A20DV1217. Inhibition of cell adhesion assays the concentration of A20DV1217 required to inhibit 50% αβ6-dependent adhesion by 3T3β6.19 cells, compared with A20FMDV2, was 48.5 ± 37.0 µM versus 1.2 ± 0.2 µM, respectively, a 40-fold reduction in activity (Figure 6b). A similar result was seen with αβ6-dependent adhesion of VB6 cells (Supplementary Figure 4). These data confirm that a post-RGD helix promotes the affinity of peptides for αβ6 and their potency as αβ6 antagonists.

STD NMR suggests peptide ligands for αβ6 bind in helical form.
Our data indicate that the presence of an RGDLXXL/I motif is not sufficient for high affinity binding to αvβ6 (compare A20FMDV1 with A20FMDV2- Fig1a and Fig1b). The studies of Mateu et al (18) and Kraft et al (17) identified, in addition to the RGD motif, the Asp+1 and Asp+4 residues as critical in peptide efficacy as αvβ6 antagonists. In contrast single-residue substitution studies did not identify any essential residues C-terminal to the LXXL motif (18). Thus we speculated that the role of the extended helix in A20FMDV2 and A20LAP is to stabilize the RGD-proximal helix region, ensuring the appropriate presentation of the leucines/isoleucines in the Asp+1 and Asp+4 positions. Structural NMR had confirmed that these residues are appropriately located on one external face of the post-RGD helix for such an interaction to occur. Thus if this hypothesis is correct residues at Asp+1 and Asp+4 would necessarily be physically close to the integrin to allow for direct interaction with αvβ6. To investigate this question we performed Saturation Transfer Determination NMR (26,27). This technique combines receptor and peptide ligand and analyzes the NMR signal transfer from the receptor to residues in the ligand. Transfer signals generally imply a separation distance from the receptor of ≤6 Å and the higher the transfer signal, the smaller the separation distance compared with other residues in the ligand. In addition, STD NMR has the significant advantage of being conducted in physiological buffer unlike solution NMR, described above, which required non-physiological TFE. In the histograms shown in Fig 7 (derived from spectral data shown in Supplementary Figures 5 and 6) the RGD residues have been highlighted in yellow and the Asp+1 and Asp+4 residues have been highlighted in red. It can be seen that the Asp+1 and Asp+4 residues usually gave the highest signal of any of the residues in the RGDLXXL motif indicating very close proximity to the surface of αvβ6. These data suggest that the Asp+1 and Asp+4 residues actually bind to αvβ6, presumably through a hydrophobic interaction. Since the Asp+1 and Asp+4 residues are not adjacent these data are also consistent with the likelihood that the peptide is in an helical form when bound to αvβ6 in a physiological setting. Moreover, as shown in the space-fill models (5d-f) that highlight the RGD (yellow) and Asp+1 and Asp+4 (red) residues, there is a common binding interface for all three peptides; the A20FMDV2 and A20LAP peptides form a similar stereo-chemical arrangement of the key residues whereas A20FMDV1 is less well structured; again, correlating with affinity and potency.

The histograms in Figure 7 show also that there are additional residues, outside of the RGDLXXL region, exhibiting significant transfer signals. Most of these additional signals are C-terminal for A20FMDV2 and A20LAP. These data suggest that additional interactions are occurring along the length of the post-RGD helix. Leu6 is highlighted in the STD NMR results for A20FMDV2 in Figure 7a. This is due to ¹H chemical shift overlap making data from this residue merge with that from Leu10 and Leu13. Since previous analyses has not revealed that a leucine preceding the RGD motif is critical either in αvβ6-specificity (17) or FMDV infection (18) it is unlikely that Leu6 is significantly involved in the interaction with αvβ6 as compared with Leu10 and Leu13. The STD NMR data for A20FMDV1 (Figure 7c) show a relatively even dispersion of transfer signals across the whole of the molecule. These results are consistent with functional and binding data showing that this peptide binds relatively weakly and thus is interacting with αvβ6 in multiple conformations.

Note that the RGD motif in all contact maps (Figure 5) appears in different orientations as these residues occur at the turn in the peptides with few NOE's defining their position. The position of the aspartate residue is well-defined as it occurs at the N-terminus of the helix, the most structured part of the peptides. However, the structures calculated show that arginine and glycine have a high degree of variation. Additionally, as there are few structural restraints observed for the N-termini of the peptides, a similar degree of structural variation also applies to these regions and such variability must be considered when viewing the structural models. Within the RGD motif, the arginine gave a higher transfer signal than the aspartate residue, presumably because the
aspartate is acting to co-ordinate a cation (1) and thus, compared with arginine, proton contacts will be more distant from the integrin. In fact the RGD transfer signal is lower than that compared with transfer signals determined for many other peptide residues in accordance with that observed from the STD NMR of cyclic peptides associating with αIIbβ3 on platelets (35).

To confirm that the STDNMR signals were derived from a real integrin:ligand interaction we performed several controls. Firstly we examined STD NMR signals from various control combinations (A20FMDV2 (2mM) + IgG (Supplementary Figure 6d), A20FMDV2 (2 mM) + carbonic anhydrase (30 µM; data not shown) and an unrelated peptide GYQQGDYQQYNPD + αvβ6 (28 µM; Supplementary Figure 6e). In no case were significant transfer signals detected. Secondly, we repeated the STD NMR signals with A20FMDV2 (Supplementary Fig 6a-c), but suspended the αvβ6 in 20mM EDTA in order to chelate the cations that are essential for integrin:ligand binding. Supplementary Figure 6 shows that EDTA essentially abrogates saturation transfer signals. Because cations are essential for RGD binding, the ability of EDTA to inhibit peptide binding completely indicates that the RGD region of the peptide must bind first. Thus the proposed hydrophobic binding of the post-RGD helix, which would be cation-independent, acts to stabilize this interaction since functional experiments above show that the helix increases affinity and potency of the peptides.
Discussion
The expression of the integrin \( \alpha v \beta 6 \) is significantly upregulated on many carcinomas compared with the corresponding non-transformed tissues where expression is usually undetectable (reviewed in ref. 9). This differential expression, together with accumulating experimental (10,11,12) and clinical (13) data, suggests that \( \alpha v \beta 6 \) is promoting cancer progression and identifies this integrin as a promising candidate for imaging and therapy of carcinoma. However, there are no clinically useful reagents available for investigating these avenues. We therefore began by developing \( \alpha v \beta 6 \)-specific peptides designed from high affinity ligands of \( \alpha v \beta 6 \), namely latency associated peptide of TGF\( \beta 1 \) (14) and the GH-loop of the VP1 protein of foot-and-mouth-disease virus (16). Our lead peptides, which were all 20mers (as shorter peptides were much less effective -data not shown) each include the motif RGD…XXL/I. The sequence DLXXL had previously been identified in peptide phage-display studies as an \( \alpha v \beta 6 \)-specific motif (17) and in earlier studies, this same motif was required for the ability of peptides to inhibit FMDV infection (18). However, our data showed that there were large differences in potency of the lead 20mers indicating that the presence of the residues RGDLXXL/I was insufficient to predict the behavior of an antagonist. As the peptides were linear, initially we did not consider secondary structure as a possible explanation. In fact, in the context of native FMDV, the RGD-containing GH-loop is unstructured in solution (21,36). However, Logan et al (21) had reported that if a disulfide bond was reduced in the GH-loop of the VP1 protein of FMDV O\(_3\) serotype, from which our most potent peptide (A20FMDV2) was derived, it was then able to form a helix in the post-RGD region.

We therefore investigated whether our linear peptides possessed secondary structure. Far-UV circular dichroism analysis confirmed that all of the lead peptides had varying degrees of helical propensity and that this propensity correlated with their potential as antagonists of \( \alpha v \beta 6 \). Structural NMR revealed the location of the helix within the peptide and showed that all the peptides possessed a common structure: a hairpin with RGD at the tip followed immediately on the C-terminal side by the helix. NMR also showed that the extent of the helix correlated with peptide potency, the longest helix being in the most potent peptide, A20FMDV2. The structural data generated here for A20FMDV2 peptide are supported strongly by earlier X-ray crystallography studies of the same sequence in the reduced GH-loop of FMDV O\(_3\) that identified a post-RGD helix (21). Thus the potential of three linear peptides to form secondary structures that included a post-RGD helix seemed to be an important component of their efficacy. This was confirmed by analysis of A20DV1217, a peptide conservatively designed to be an almost identical biochemical match of A20FMDV2 but which lacked the propensity to form a helix. In flow cytometry 1000-fold higher concentrations of biotinylated A20DV1217 were required to give signals of the same levels as biotinylated A20FMDV2. These data suggest that the affinity of binding of A20FMDV2 to \( \alpha v \beta 6 \) is significantly increased by the presence of the post-RGD helix and this was reflected also in a dramatic drop in efficacy as an antagonist.

Earlier studies (18) identified the leucines at positions Asp+1 and Asp+4 as critical for peptide inhibitors of FMDV infection. We examined the DLXXL/DLXXI region of the NMR structures and noted that presence of a post-RGD helix caused the normally non-adjacent pair of leucines in A20FMDV2 (leucine/isoleucine in A20LAP) to come into juxtaposition on the outer-face of the helix (Figure 5c and 5f). The residues at these positions are highly conserved in FMDV (18,37) and it is likely that they contribute to the binding. As A20FMDV1 is less well structured (Fig 5h) the position of the Asp+1 and Asp+4 leucines are not likely to be as close to each other as the corresponding residues in A20FMDV2 and A20LAP, as represented in Fig 3i. The likelihood that the Asp+1 and Asp+4 residues are involved in binding to \( \alpha v \beta 6 \) is supported by our STD NMR analyses. By extrapolation from previous X-ray crystallography studies examining the binding of RGD peptides to \( \alpha v \beta 3 \), we can assume that both the arginine and aspartate residues of the RGD motif in our peptides are participating in binding to the integrin. The high energy transfer to Asp+1

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and Asp+4 residues indicates a very close proximity >6Å to the \(\alpha v\beta 6\) surface, closer in fact than either the arginine or aspartate residues in A20FMDV2 and A20LAP. The STD NMR data therefore also support the likelihood that the peptides bind as RGD-helix structures to \(\alpha v\beta 6\). This conclusion is supported also by previous reports that, when bound to infection-blocking antibodies, fragments of the GH-loop formed a post-RGD helix (38, 39, 40).

Although the STD NMR data indicate significant amounts of energy transfer to residues in the C-terminus it is not possible to conclude from our data whether the peptides form additional contacts with \(\alpha v\beta 6\) along the length of the helix or whether the role of a more stable C-terminal helix is required only for optimal presentation of the Asp+1 and Asp+4 residues in the RGD-proximal helix. Certainly the observation that EDTA inhibits both peptide binding and STD NMR signals indicate that the primary binding event is the binding of the RGD-motif to the integrin. As the helical propensity enhances affinity and potency of peptides, together these data show that one important function of the helix is to stabilize this RGD interaction, probably through a hydrophobic interaction of the Asp+1 and Asp+4 residues with the surface of the \(\beta 6\) subunit.

Our data, together with the work of others (38, 39, 40), suggest that when the GH-loop binds to its natural receptor, \(\alpha v\beta 6\) (16,41), a helix forms in this loop stabilizing this interaction. We can predict therefore that a post-RGD helix will also form when LAP binds to \(\alpha v\beta 6\), thereby contributing to an important biological function of \(\alpha v\beta 6\). Integrin \(\alpha v\beta 6\) can activate latent TGF\(\beta\) by binding to LAP and, through actin-dependent traction forces, expose the TGF\(\beta\) molecule to its receptors (14). Our data suggest that a post-RGD helix in LAP would serve to enhance binding affinity and provide the high stability of interaction with \(\alpha v\beta 6\) required for this physical activation of TGF\(\beta\).

In summary, we have defined the structural basis of high affinity binding of physiological ligands to \(\alpha v\beta 6\): an RGD motif at the tip of a hairpin loop followed immediately by a C-terminal helix. Structural NMR analyses showed also that the highly conserved Leucine or Isoleucine residues at Asp+1 and Asp+4 positions in the binding site are presented as adjacent residues on the exterior face of the loop and STD NMR showed that these residues bind very closely to the \(\alpha v\beta 6\) surface, possibly through a hydrophobic interaction with the integrin. These structurally determined conclusions are supported by the recent findings of Burman et al (37), who predicted a similar conclusion based on the use of peptides to block virus infection. Furthermore, a combination of STD NMR and the use of a helix-deficient mutant peptide confirmed the strong likelihood that, when bound, \(\alpha v\beta 6\) interacts with an RGD-helix motif in its high affinity ligands. These data provide a structural explanation for the necessity of the DLXXL motif (17) for binding to \(\alpha v\beta 6\) and establish a structural template upon which \(\alpha v\beta 6\)-targeting antagonists can be designed. In addition, these data raise the possibility that structural motifs flanking integrin-binding sites in other ligands may also participate in determining ligand affinity and potency.
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Figure 1: αvβ6-dependent adhesion is inhibited with 20mer peptides derived from αvβ6 ligands. Inset shows that binding of 3T3β6.19 cell to LAP is abrogated entirely by six 2G9 (αvβ6-blocking antibody) versus control antibody W6/32 (anti-MHC class 1). Radiolabeled [51Cr] 3T3β6.19 in various concentrations of peptides A20LAP, A20FMDV1 or A20FMDV2 were added to 96-well plates coated with 50µl (0.25 µg/ml) LAP.

b) 20mer peptides bind with varying affinities to rsαvβ6. Biotinylated peptides were added to plates coated with rsαvβ6. After 1h unbound peptide was removed by washing and bound peptide detected with ExtrAvidin-HRP. Peptides did not bind significantly to BSA-coated wells (data not shown). Data show the mean and standard deviation of triplicate wells, and are representative of three separate assays with similar results.

Figure 2: a) Helical propensity of A20FMDV1, A20LAP and A20FMDV2 predicted by Agadir software (http://www.embl-heidelberg.de/Services/serrano/agadir/agadir-start.html). Note that the prediction is that A20FMDV2 has a high probability of helix formation followed by A20LAP. A20FMDV1 has little helical propensity. (b-d) Far-UV circular dichroism spectra of (b) A20FMDV1, (c) A20LAP and (d) A20FMDV2 in increasing proportions of trifluoroethanol-d3 (TFE). All spectra are the average of 4 scans. Wavelength at 222 nm is indicated by the vertical bar. Proportions of TFE (v/v) are indicated for each spectrum trace with the line pattern shown as 0% (-----), 10% (- - - - -), 20% (--- ---), 25% (--------) and 30% (· · · · · · · · · · · ·). Percentage helicity in 30% (v/v) TFE calculated from these data were 20%, 14% and 10% for A20FMDV2, A20LAP and A20FMDV1 respectively.

Figure 3: Schematic of main NOE and ROE contact types, hydrogen bond acceptors and residues giving rise to φ restraints for (a) A20FMDV1, (b) A20FMDV2 and (c) A20LAP. Helical regions are indicated as a black box. NH-NH i-j contacts in the N-terminal region of peptide support the turn observed in these peptides that creates the structural arrangement.

Figure 4: Sections of 2D NOESY NMR Spectra for A20FMDV1: (a), (d) and (g); A20FMDV2: (b), (e) and (h) and A20LAP: (c), (f) and (i). Regions of NOESY spectra are shown where helical contacts are observed: (a-c) showing the Hα-Hβ region, (d-f) the NH-NH region and (g-i) the NH-εH region. All chemical shifts are referenced externally to a 100µM solution of dimethylsilapetane sulfonic acid (DSS) in PBS/30% (v/v) TFE.

Figure 5: Analysis of peptide structure using Nuclear Magnetic Resonance (NMR) Spectroscopy. Calculated structures for A20FMDV2 (a, b, c); A20LAP (d, e, f) and A20FMDV1 (g, h, i) in 30% TFE. Ensembles of 40 structures (a, d, g) show all backbone bonds (residues 1-20). The bonds colored red identify the DLXX[L/T]XX region used to fit the ensembles. Ribbon diagrams (b, e, h and c, f, i) are of a single structure from the ensemble that is closest to the mean structure calculated for each peptide from the ensemble data. b, e and h show a side-view with the RGD residues in ball-and-stick mode. c, f and i show the end-on view and the Asp+1 and Asp+4 residues are in ball-and-stick mode. All figures were created in MOLMOL 2k.2 (30).

Figure 6: The post-RGD helix is required to improve peptide affinity and potency. Flow cytometry was used to measure binding of biotinylated peptides to A375Pβ6puro (a,c,e) or A375Ppuro (b,d,f). a.b: White-fill: IgG control, black solid: 10D5 (mouse anti-αvβ6). c,d: A20FMDV2 at 1nM (white-fill), 10nM (grey-fill) and 100nM (black-fill). e,f: A20DV1217 at 100nM (white-fill), 1µM (grey-fill) and 10µM (black-fill). Note that A20DV1217 requires more than 1000-fold more peptide than A20FMDV2 to achieve the same level of binding to A375Pβ6puro.
Radio-labeled [$^{51}$Cr] VB6 cells were added to 96-well plates coated with 50µl (0.25 µg/ml) LAP in various concentrations of peptides A20FMDV2 or A20DV1217. Binding to BSA-coated wells and binding in presence of 10µg/ml αvβ6-blocking antibody 63G9 was not significant (data not shown). Data shown are from one experiment using triplicate samples, and are representative of three separate experiments with similar results. Data show a significant reduction in potency as an antagonist to αvβ6-specific cell adhesion compared with A20FMDV2. Since the only significant difference between these two peptides is the absence of a helix in A20DV1217, these data highlight the importance of the helix.

Figure 7: Analysis of peptide binding to rsαvβ6 using $^1$H Saturation Transfer Difference Nuclear Magnetic Resonance (STD NMR) Spectroscopy

STD NMR was performed using 70-molar excess of A20FMDV2 (a, d), A20LAP (b, e) or A20FMDV1(c, f) mixed with 1mg of HPLC-purified (>95% purity) rsαvβ6 in phosphate buffered saline supplemented with 1mM Ca$^{2+}$ and 0.5mM Mg$^{2+}$. Histograms (a-c) show absolute energy transfers, represented as a percentage for each residue, normalized to the residue with the highest transfer energy. The RGD motif is highlighted in yellow and the Asp+1 and Asp+4 residues are highlighted in red. d-f show the corresponding CYK space-fill diagrams of the peptides predicted from solution NMR with the RGD and Asp+1 and Asp+4 residues highlighted. Note that A20FMDV2 and A20LAP have a similar binding face.
Figure 1
Figure 2
Figure 3

(a)

\[
\begin{align*}
\alpha H-NH & \quad i+1 \\
NH-NH & \quad i+1 \\
\alpha H-NH & \quad i+3 \\
\alpha H-\beta H & \quad i+3 \\
NH-NH & \quad i-j \\
& \quad H-Bond Acceptors \\
& \quad \phi \ restraint
\end{align*}
\]

(b)

\[
\begin{align*}
\alpha H-NH & \quad i+1 \\
NH-NH & \quad i+1 \\
\alpha H-NH & \quad i+3 \\
\alpha H-\beta H & \quad i+3 \\
NH-NH & \quad i-j \\
& \quad H-Bond Acceptors \\
& \quad \phi \ restraint
\end{align*}
\]

(c)

\[
\begin{align*}
\alpha H-NH & \quad i+1 \\
NH-NH & \quad i+1 \\
\alpha H-NH & \quad i+3 \\
\alpha H-\beta H & \quad i+3 \\
NH-NH & \quad i-j \\
& \quad H-Bond Acceptors \\
& \quad \phi \ restraint
\end{align*}
\]
Figure 4
Figure 5
Figure 6
Figure 7
Structure-function analysis of RGD-helix motifs in αvβ6 integrin ligands
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