Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

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1.1 Experimental Procedures

1.1 Reagents

Recombinant human αvβ6 was from R&D Systems (Minneapolis, MN); wild-type human integrins αvβ3, αvβ5, and α5β1 (octyl β-D-glucopyranoside preparation) were obtained from Immunological Sciences (Rome, Italy); anti-αvβ6 monoclonal antibody (clone 10D5, IgG2a) was from Millipore (Billerica, MA); anti-αvβ8 polyclonal antibody (EM13309, IgGs) was form Absolute Antibodies (Oxford, UK); normal rabbit immunoglobulins (IgGs, purchased from Primm, Italy) were purified by affinity chromatography on protein A-sepharose; mouse IgG1, (clone MOPC 31C) was from Sigma (Missouri, USA); goat anti-mouse and goat anti-rabbit Alexa Fluor 488-labeled secondary antibodies were purchased from Invitrogen. Synthetic peptides **1**, **2** and **6** were purchased from Biomatik (Delaware, USA).

1.2 Expression and purification of recombinant $^{13}C/^{15}N$ peptide 1

*Preparation of the expression vector coding for recombinant peptide **1***

Recombinant peptide **1** was produced by recombinant DNA technology as a fusion product with ketosteroid isomerase (KSI), by cloning the peptide **1** sequence downstream the KSI gene and upstream of a His(6x)-tag sequence. 5'-phosphorylated forward and reverse complementary DNA oligonucleotides coding for peptide **1** were synthesized by PRIMM (Italy).

Forward:

5'-

TTTGAGACACTCCGAGGAGATGAACGGATCCTTTCCATTCTGAGACATCAGAATT
TACTGAAGGAGCTCCAAGACATG-3';

Reverse:

5'-

GTCTTGGAGCTCCTTCAGTAAATTCTGATGTCTCAGAATGGAAAGGATCCGTTCA
TCTCCTCGGAGTGTCTCAAACAT-3'.

The oligonucleotides produced had a three-base 3' overhangs (underlined) coding for a methionine residue, necessary for cloning strategy and for CNBr cleavage. The oligonucleotides (10 μM each) in 40 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$ were annealed as follows: 10 min at 99 °C, 15 min at 30 °C and 20 min at 4 °C. The annealed

product (0.26 pmol) was then ligated with 0.026 pmol of a pET31b(+) plasmid (Novagen), previously digested with *AlwNI* enzyme. *E. coli* cells (DH5α) were then transformed with the ligation product and ampicillin-resistant colonies were selected and screened for the correct incorporation of the insert by restriction digestion using *XhoI* and *Xbal* enzymes. The identity of the selected clone (called KSI-P1 plasmid) was confirmed by DNA sequencing (Eurofins Genomics, Germany). The KSI-P1 plasmid was then used to transform BL21 DE3 *E. Coli* cells for protein expression.

*Expression of the KSI-peptide **1** fusion protein*

BL21 DE3 cells containing KSI-P1 plasmid were grown in 50 mL LB medium containing ampicillin (100 μg/ml) overnight at 37 °C under shaking. Five mL of overnight culture were then inoculated in 0.5 L of M9 medium supplemented with ampicillin, ^{13}C-D-glucose (2 g) and $^{15}NH_4Cl$ (1.5 g), as unique sources of carbon and nitrogen, and left to grow at 37 °C under shaking. When the culture reached an optical density at 600 nm of 0.8 Units, 1 mM isopropyl β-D-1-thiogalactopyranoside was added to induce protein expression. The cells were then incubated for additional 16 h at 28 °C under shaking.

*Purification of KSI-peptide **1** from inclusion bodies*

The cells were pelleted, resuspended in 10 mL of lysis buffer (50 mM Tris-HCl pH 8, containing 10 mM EDTA, 0.1% Triton X-100, 20 μg/mL DNAse, 20 μg/mL RNAse and 50 μg/ml lysozyme) and broken by sonication using an Ultrasonic Processer (Sonopulse, Bandelin) (3 cycles of 1.5 minute each, alternating 30 s of pulses and wait periods). The cell lysate was centrifuged (14000 x g, 15 min 4 °C), and the resulting pellet was washed twice with washing buffer (50 mM Tris HCl pH 8, containing 10 mM EDTA and 0.5% Triton X-100) followed by two additional washes with water. The pellet was then resuspended with 20 ml of refolding buffer (20 mM Tris-HCl pH 8, containing 150 mM NaCl, 10 mM imidazole pH 8, 1 mM 2-mercaptoethan-1-ol, 6 M guanidinium chloride) and loaded onto a chromatography column filled with 10 ml of Ni^{2+}-NTA resin (Qiagen) (flow rate 0.5 ml/min at 4 °C), previously equilibrated with refolding buffer. The column was washed with 50 ml of refolding buffer followed by 50 ml of refolding buffer-1 (20 mM Tris-HCl pH 8, containing 150 mM NaCl, 20 mM imidazole pH 8, 1 mM 2-mercaptoethan-1-ol, 6 M guanidinium chloride); the protein was then eluted from the resin after incubation with 5 mL of elution

buffer (20 mM Tris-HCl pH 8, containing 150 mM NaCl, 300 mM imidazole pH 8, 1 mM 2-mercaptoethanol, 6 M guanidinium chloride) at 4 °C for 15 minutes; small volumes of elution buffer were then gradually added until the protein was completely eluted (checked on small aliquots using Bradford reagent). The product (30 ml) was then dialyzed using a 3.5 kDa membrane (CelluSep) against 2 L of water at 4 °C for 16 h. The dialysis product, consisting of insoluble protein, was then centrifuged (500 x g, 30 min, at 4 °C), washed twice with water and stored at -80 °C.

*CNBr cleavage of KSI-peptide **1** fusion protein and purification of recombinant peptide **1***

The pellet containing recombinant KSI-peptide **1** was dissolved with 5 ml of TFA (80%, v/v) containing 0.2 g of CNBr, and stirred for 18-24 h at 25 °C in the dark. CNBr cleavage led to the formation of a peptide with the expected N-terminal residue and with a homoserine lactone residue at the C-terminus (**Figure S12a**). The solution was partially evaporated by bubbling N_2 gas, diluted with 5 volumes of water, and freeze-dried. The product was resuspended in 20 mM phosphate buffer. pH 7.5-8, 100 mM NaCl and left to stir at 25 °C for 16 h in the dark. The suspension was then centrifuged at 14000 x g for 15 min. The resulting supernatant was loaded onto a preparative Shimadzu Shim-pack G15 column and purified as described below. Fractions with a purity >95% were pooled and lyophilized. Product identity were assessed by mass spectrometry (**Figure S12b,c**).

1.3 Synthetic Peptides

Peptides Synthesis

Peptides **3**, **4** and the linear peptide precursor of **5** (with propargylglycine and azidolysine in positions 54 and 58, respectively, **Figure S6a**) were assembled by stepwise microwave-assisted Fmoc-SPPS on a Biotage ALSTRA Initiator+ peptide synthesizer, operating in a 0.12 mmol scale on a Rink-amide resin (0.5 mmol/g). Resin was swelled prior to use with an NMP/DCM mixture. Activation and coupling of Fmoc-protected amino acids were performed using Oxyma 0.5 M / DIC 0.5 M (1:1:1), with a 5 equivalent excess over the initial resin loading. Coupling steps were performed for 7 min at 75 °C. Deprotection steps were performed by treatment with a 20% piperidine solution in DMF at room temperature (1 x 10 min). Following each coupling or deprotection step, peptidyl-resin was washed with DMF (4 x 4 ml). Upon complete chain assembly, peptides were cleaved from the resin using a 90%

TFA, 5% water, 2.5% thioanisole, 2.5% TIS (triisopropyl silan) mixture (2 hours, room temperature). Following precipitation in cold diethyl ether, crude peptide was collected by centrifugation and washed with additional cold diethyl ether to remove scavengers. Peptides were then dissolved in 50% acetonitrile containing 0.07% TFA and purified by preparative RP-HPLC.

*Intramolecular Cu^{I} -catalyzed azido-alkyne 1,3- cycloaddition to obtain **5***

To the linear peptide precursor of **5** (with propargylglycine and azidolysine in positions 54 and 58, respectively) (0.5 mg/ml in degassed water) were added $CuSO_4 \cdot 5\ H_2O$ (10 eq) and ascorbic acid (10 eq) in order to originate *in situ* Cu^{I} catalyst (**Figure S6a,b**). The reaction was left to stir at room temperature until the complete conversion of linear precursor into the desired heterodetic 1,2,3-triazolyl-containing peptide occurred (monitoring by RP-HPLC). The resulting stapled peptide was RP-HPLC purified as described below.

Peptides purification and characterization

Peptides were purified by reversed phase high performance liquid chromatography (RP-HPLC) using a Shimadzu Prominence HPLC system, equipped with a Shimadzu Prominence preparative UV detector, connected to Shimadzu Shim-pack G15 10µ C18 90Å (250 x 20 mm). The column was eluted with mobile phase A (3% acetonitrile, 0.07% trifluoroacetic acid in water) and mobile phase B (70% acetonitrile, 0.07% trifluoroacetic acid in water) using the following chromatographic method: 0% B (7 min), linear gradient (0−30% B), 40 min; flow rate, 14 ml/min. Peptides purity was ≥ 95% as determined by analytical RP-HPLC using a Shimadzu Shim-pack GWS 5µ C18 90Å column (150 x 4.6 mm) connected to diode array detector (**Figure S12b**, **S13**). Peptides identity was confirmed by mass spectrometry analysis (**Table S3**).

1.4 NMR experiments

NMR spectra were recorded on a Bruker Avance-600 spectrometer (Bruker BioSpin) equipped with a triple-resonance TCI cryo-probe with an x, y, z shielded pulsed-field gradient coil. All the spectra were acquired at 280 K. Peptides were dissolved in NMR buffer (20 mM phosphate buffer *pH* 6.5, 100 mM NaCl, 20 mM $MgCl_2$, 0.5 mM $CaCl_2$, 90% H_2O, 10% D_2O or 100% D_2O) to a concentration of 0.5-1 mM. Each sample was transferred in a 3 mm NMR

tube for NMR analysis. Resonance assignments were obtained from the analysis of twodimensional homonouclear (2D ^{1}H-^{1}H TOCSY, TOtal Correlation SpectroscopY, t_{mix}=60 ms; 2D ^{1}H-^{1}H NOESY, Nuclear Overhauser Effect SpectroscopY, t_{mix}=100-600 ms) and heteronuclear (2D-^{1}H-^{13}C-HSQC, Heteronuclear Single Quantum Coherence, 2D ^{1}H-^{13}C HMBC, Heteronuclear Multiple Bond Correlation) experiments (**Table S1**). Complete ^{1}H, ^{13}C and ^{15}N resonance assignment of recombinant peptide **1** was obtained from the following 2D and three-dimensional (3D) experiments acquired for ^{13}C/^{15}N recombinant peptide **1**: 2D-^{1}H-^{15}N-HSQC, 3D-HNCA, 3D-HNCO. 3D-^{1}H-^{13}C-HSQC-NOESY (t_{mix}=180 ms) and 3D-^{1}H-^{15}N-HSQC-NOESY (t_{mix}=120 ms) (**Table S1**). All spectra were processed using Topspin 3.2 NMR software from Bruker. Spectral analysis was performed using CCPNmr Analysis2.4 software.[1] Chemical shifts of recombinant peptide **1** have been deposited in BioMagResBank (accession code 34381). Chemical shift assignment of **3**, **4** and **5** are reported in **Supplementary Table S4-S6**.

*Relaxation experiments on recombinant peptide **1***

NMR experiments for the determination of longitudinal and transverse ^{15}N relaxation rates (R_1=1/T_1 and R_2=1/T_2) and the ^{1}H-^{15}N heteronuclear NOE (hetNOE)[2] were recorded on ^{13}C/^{15}N recombinant peptide **1**. Solvent suppression was achieved using pulsed field gradients with a flip-back pulse to avoid saturation of water magnetization which could affect signal intensity of exchangeable amide protons. A series of ^{1}H-^{15}N-HSQC experiments using different time intervals were recorded for the determination of ^{15}N relaxation rates. T_1 measurement, based on inversion-recovery type experiments, were recorded using variable delays 50, 100, 150, 250 (repeated twice for error analysis), 350, 500 (repeated twice for error analysis), 700, 900, 1100, 1400, 2000 ms. T_2 measurement, based on a Carr–Purcell–Meiboom–Gill (CPMG) spin-echo pulse sequence, were acquired using variable delays (8.5 (repeated twice for error analysis), 17, 34, 68 (repeated twice for error analysis), 85, 136, 170, 212.5, 238 ms). T_1 and T_2 values were obtained using Dynamics Center Bruker software, by fitting the peak intensity to a 2- parameter exponential decay (**Figure S14**). For ^{1}H-^{15}N heteronuclear NOE measurements, two HSQC spectra were acquired in interleaved fashion with and without 4 s of proton saturation during the relaxation delay. The heteronuclear NOE values were obtained from the ratio between the saturated and unsaturated peak intensities. The uncertainty was calculated as the standard deviation of the noise in the spectrum divided

by the intensity of the reference peak. Acquisition parameters of multidimensional experiments are summarized in **Table S1**.

1.5 Structure calculation

Recombinant peptide **1** structures were calculated with ARIA 2.3.2[3] in combination with CNS using experimentally derived restraints. All NOEs were assigned manually and calibrated by ARIA, the automated assignment was not used. A total of eight iterations was performed, computing 20 structures in the first seven iterations and 300 in the last iteration. The 15 best structures from the last iteration were used for the final default ARIA water refinement step. The quality of the structures was assessed using PROCHECK-NMR software.[4] Statistics of the 15 lowest energy structures are reported in **Table S2**. The family of the 15 lowest energy structures (no distance or torsional angle restraints violations >0.5 Å or >5°, respectively) has been deposited in the PDB (PDB accession code 6R2X). Chemical shift and restraints lists used for structure calculations have been deposited in BioMagResBank (accession code: 34381).

1.6 NMR binding experiments

1D ^{1}H STD and WaterLOGSY experiments

1D ^{1}H STD measurements (pulse sequence: stddiffesgp.3) were acquired in NMR buffer on peptide **1** (0.5 mM) in the presence of 4 μM recombinant human αvβ6 extracellular domain (R&D Systems) using a pulse scheme with excitation sculpting with gradients for water suppression and spin-lock field to suppress protein signals.[5] The spectra were acquired using 800-4000 scans. For protein saturation, a train of 60 Gaussian shaped pulses of 50 ms was applied, for a total saturation time of 3 s. Relaxation delay was set to 3 s. On-resonance irradiation was set at 12 ppm; off-resonance irradiation was applied at 107 ppm. STD spectra were obtained by internal subtraction of the on-resonance spectrum from the off-resonance spectrum. WaterLOGSY[6] experiments were acquired on the same samples using 256 scan with 20 ppm spectral width, using a t_{mix} of 1 s and a relaxation delay of 2 s.

2D-STD-HSQC experiments

2D-STD-^{1}H-^{13}C-HSQC experiments (stdhsqcetgpsp) were recorded on ^{13}C/^{15}N recombinant peptide *1* (0. 5mM) in NMR buffer (100% D_2O) the presence of 4 μM recombinant human

αvβ6 extracellular domain (R&D Systems), by applying on ^{1}H on-resonance and off-resonance irradiation at 10.5 ppm and 107 ppm, respectively. Protein saturation was obtained using a train of Gaussian shaped pulses of 50 ms each. A total saturation time of 2.5 s and a relaxation delay of 2.5 s were used, with a time domain of 2048 points in the direct dimension and 160 complex points in the indirect dimension with a total 128 scans. The spectral width was set to 12 ppm for the direct dimension and 80 ppm for carbon dimension. The STD difference was obtained internally by phase cycling. 2D-STD-^{1}H-^{15}N-HSQC experiments [7] were acquired on $^{13}C/^{15}N$ recombinant peptide **1** (0. 5mM) in NMR buffer containing 10% D_2O in the presence of 4 μM recombinant human αvβ6 extracellular domain (R&D Systems). The experiment consists of a ^{15}N HSQC with echo/antiecho coherence selection and water flip back pulses in both inept steps to which a saturation transfer element[5] is prepended. The latter consists of a train of Gaussian shaped pulses of 50 ms, executed multiple times to achieve 3 s of saturation period. The relaxation delay was set to 3 s. 2048 points were acquired for the direct dimension and 40 complex points on the indirect dimension. In total 224-312 scans were used with a spectral width of 12 and 21 ppm for proton and nitrogen dimensions respectively. The saturation element is applied on- and off-resonance (-3 ppm and 107 ppm, respectively) in alternating scans which are kept in separate blocks of the memory until the chosen number of scans is reached. The data are then stored on the disk and the t1 delay is incremented to obtain the final interleaved 2D. The data are preprocessed using the c-program split (Bruker TopSpin 3.2 software) with the argument 2 to get the off-resonance reference spectrum and with the argument *ipap 2* to obtain the difference spectrum in which only signals that did experience saturation transfer are visible. For negative control experiments similar spectra were acquired on labelled recombinant peptide **1** in the absence of αvβ6, in the presence of αvβ6 which was previously incubated with 20 mM EDTA-d16 (Cambridge Isotope Laboratories, Inc), or in the presence of 4 μM Bovine Serum Albumin (Merck) (**Figure S3**). For each non overlapping resonance the relative STD% was evaluated as follows:

$$STDfactor = \frac{I_{STD}}{I_{ref}} \qquad \textit{(eq.1)}$$

$$relative\ STD\% = \frac{STDfactor}{STDfactor_{max}} 100 \qquad (eq.2)$$

where I_{STD} is the peak intensity in the 2D-STD-^{1}H-^{15}N-HSQC spectrum, I_{ref} is the peak intensity of the reference (off-resonance) 2D-STD-^{1}H-^{15}N-HSQC spectrum, and $STDfactor_{max}$ is the maximum value of the STDfactor. [7]

1.7 Docking Calculations

HADDOCK2.2 [8,9] was used for the docking calculation of peptides **1**, **4** and **5** into αvβ6. As input structures for peptide **1**, an ensemble of 10 out of the 30 best NMR structures in terms of energy were acetylated at the N-terminus and amidated at the C-terminus using Maestro (Schrödinger, LLC, New York, NY, 2019). Input structures for **4** and **5** were first generated modifying the ensemble of structures of peptide **1** and then minimized using Maestro. Missing topologies and parameters were determined with PRODRG2 web server.[10] The structure of the extracellular head of αvβ6 (β-propeller of the αv subunit and βI domain of the β6 subunit) when bound to proTGF-β1 (PDB: 5FFO) [11] was prepared using the Protein Preparation Wizard tool of Maestro.[12] All the crystallographic water molecules were removed. Missing side-chains, hydrogen atoms and loops were added; the orientation of the hydroxyl groups of Serine, Threonine and Tyrosine, the side chains of Asparagine and Glutamine residues, and the protonation state of Histidine residues were optimized. A restrained minimization was run using the OPLS-AA force field [13] with a root mean square deviation (RMSD) tolerance on heavy atoms of 0.3 Å.

In order to maintain a stable coordination of the ions in the metal ion-dependent adhesion site (MIDAS), adjacent to MIDAS (ADMIDAS) and ligand-associated metal binding site (LIMBS), unambiguous restraints were applied throughout the whole docking protocol between Mg^{2+} ions and the coordinating residues in the MIDAS ($Asp123_{\beta6}$, $Ser125_{\beta6}$, $Ser127_{\beta6}$, $Thr221_{\beta6}$, $Glu223_{\beta6}$, $Asp254_{\beta6}$), ADMIDAS ($Ser127_{\beta6}$, $Asp130_{\beta6}$, $Asp131_{\beta6}$, $Asp254_{\beta6}$) and LIMBS ($Glu162_{\beta6}$, $Asn218_{\beta6}$, $Asp220_{\beta6}$, $Pro222_{\beta6}$, $Glu223_{\beta6}$). Furthermore, as the binding of CgA to αvβ6 is RGD dependent additional unambiguous restraints were applied during it0 and it1 between: R43 of the input peptides and αv residue $Asp150_{\alpha v}$ and $Asp218_{\alpha v}$; D45 of the input peptides and the Mg^{2+} ion in the MIDAS. For αvβ6, active and passive residues were selected from the 5FFO PDB structure as follows: residues involved in the RGD electrostatic clamp and residues within a radius of 5 Å from the interacting fragment

of TGF-β1 (F210 – P227), with a water accessibility of the main chain and side chain higher than 10%, as determined by Naccess 2.1.1.[14] For peptide **1**, RGD and the residues which gave a relative STD% > 75% in the 2D-STD-^{1}H-^{15}N-HSQC experiment, were chosen as active; the remaining residues of the peptide were used as passive. For **4** and **5**, G44, D45, L46 residues were chosen as active; the remaining residues of the peptide were used as passive. The list of active and passive residues for the definition of the AIRs is summarized in **Table S7**. The HADDOCK protocol involves three main steps. After the rigid body docking the best 1000 structures in terms of HADDOCK score were then subjected to the semi-flexible refinement step. In this stage, for all peptides, the backbone of residues from F39 to E46 was maintained fully flexible. In this case the best 500 structures, according to the HADDOCK score, were selected for the water refinement stage. Also for the last water refinement stage, residues F39 to E46 were maintained fully flexible. OPLS force field [13] and TIP3P water model[15] were applied. The best 500 decoy poses in terms of HADDOCK score were then clusterized based on geometrical criteria. Poses were aligned on the αvβ6 backbone, and the RMSD was calculated on the backbone of the ligand from R43 to L52 and side chains of the R43 and D45. RMSD cutoff was set to 3.5 Å, and only clusters containing more than five structures were considered. To remove any bias of the cluster size on the cluster statistics, the final overall score of each cluster was calculated on the four lowest HADDOCK scores models in each cluster (**Figure S15)**.

1.8 Circular Dichroism (CD) spectroscopy

CD spectra were recorded on a Jasco J-815 spectropolarimeter equipped with a Peltier temperature control system. Typical peptides concentration was 30-40 μM, in phosphate buffer 20 mM, NaF 100 mM, pH 6.5. Spectra were acquired in a 1 mm quartz cuvette, at 280 K using an average of four scans between 190 and 260 nm, with a scanning speed of 20 nm/min, 0.5 s of data integration time and a resolution of 0.1 nm.

1.9 Competitive Integrin binding assays

Peptide binding was measured by a competitive binding assay using as integrin probe a complex made by a N-terminal acetylated isoDGR peptide biotinylated at the ε-amino group of the lysine, acetyl-CisoDGRCGVRSSSRTPSDKY-bio, and a streptavidin-peroxidase conjugate (called isoDGR/STV-HRP)[16]. The equilibrium dissociation constants (K_d) of the

isoDGR/STV-HRP was determined by direct binding assay to 96-well microtiterplates coated with $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, and $\alpha_v\beta_8$ (1 μg/ml) or with $\alpha_5\beta_1$ (4 μg/ml) and were calculated by non-linear regression analysis using "*One site -Specific binding*" equation of the GraphPad Prism Software. The following K_d values were obtained: αvβ3, 1.3 nM; αvβ5, 1.7 nM; αvβ6, 1.4 nM; αvβ8, 1.6 nM and α5β1, 1.3 nM.

Next, to determine the K_i values for each peptide we performed competitive binding assays using a fixed concentration of the isoDGR/STV-HRP probe (1.68 nM, for $\alpha_v\beta_3$ and $\alpha_v\beta_5$; 1.00 nM for αvβ6; 2.00 nM for αvβ8 and 7.12 nM for α5β1) mixed in binding buffer (25 mM Tris-HCl, pH 7.4, containing 150 mM sodium chloride, 1 mM magnesium chloride, 1 mM manganese chloride and 1% BSA) with each competitor at various concentrations (6 dilution in duplicate or triplicates).

Each mixture was then added to integrin-coated wells and left to incubate for 2 h at room temperature. After washing with 25 mM Tris-HCl, pH 7.4, containing 150 mM sodium chloride, 1 mM magnesium chloride, 1 mM manganese chloride, each well was filled with a chromogenic solution (o-phenylenediamine dihydrochloride) and left to incubate for 30 min at room temperature. The chromogenic reaction was stopped by adding 1 N sulfuric acid. The absorbance at 490 nm was then measured using a microtiterplate reader. K_i values were calculated by non-linear regression analysis of competitive binding data using the "*One site - Fit K_i*" equation of the GraphPad Prism Software using the K_d values of the probe indicated above.

1.10 Cell culture

Human bladder 5637 cancer cells (ATCC HTB-9, grade II carcinoma) were cultured in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Human skin keratinocytes cells (HaCaT) were kindly provided by Dr. Alessandra Boletta (San Raffaele Scientific Institute, Italy). HaCaT were cultured in DMEM containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

1.11 Flow Cytometry Analysis

Flow cytometry analysis of αvβ6 integrin was carried out as follows: 5637 or HaCaT cells were detached with Dulbecco's Phosphate Buffered Saline (DPBS, without $CaCl_2$ and $MgCl_2$)

containing 5 mM EDTA pH 8.0 solution (DPBS-E), washed twice with DPBS and resuspended with 25 mM Hepes buffer, pH 7.4, containing 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1% bovine serum albumin (binding buffer) in presence of various amount of peptides 1, 2, 4, 5 or 6 and mAb 10D5 (5 µg/ml, 33 nM), for 1 h on ice ($5x10^5$ cells/100 µl). After washing with 25 mM Hepes buffer, pH 7.4, 150 NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, the cells were incubated with a goat anti-mouse Alexa Fluor 488 conjugated secondary antibody (5 µg/ml in binding buffer containing 1% normal goat serum) for 1 h on ice. After washing, the cells were fixed with 4% formaldehyde in DPBS and analyzed by flow cytometry. Flow cytometry analysis of αvβ8 was performed essentially as described above using a rabbit anti-αvβ8 monoclonal antibody (clone EM13309, 1 µg/ml) and a goat anti-rabbit Alexa Flour 488 conjugated secondary antibody (5 µg/ml).

1.12 Preparation of Quantum dots (Qdot) labelled with 5.

Amine-modified Qdot nanoparticles (2 nmol of Qdot605 ITK Amino (PEG), Invitrogen, Carlsbad, CA) were buffer-exchanged in PBS (10 mM sodium phosphate buffer, pH 7.4, 138 mM NaCl, 2.7 mM KCl, Sigma, P-3813) containing 5 mM EDTA (PBS-E) by ultrafiltration using Ultra-4 Ultracel-100K (Amicon) according to the manufacturer's instructions. Qdot were then activated with 200 µg of sulfo-SMCC sulfosuccinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (Sulfo-SMCC; Pierce, Rock- ford, IL), a heterobifunctional cross-linking reagent, 1 h at room temperature. The maleimide-tagged nanoparticles were purified from the unreacted cross-linking reagent by gel-filtration chromatography on NAP-5 column (GE Healthcare) using PBS-E as eluent buffer. The product was then divided into 2 aliquots (~300 µl each) and mixed with **5** or a control peptide (cyclic head-to-tail c(CGARAG)) (480 µg in 96 µl of water) and incubated for 2 h at room temperature. 2-mercaptoethanol was then added (0.1 mM final concentration) and left to incubate for 0.5 h at room temperature. Conjugates (called **5**-Qdot and ***Qdot**) were separated from free peptide by ultrafiltration using Ultra-4 Ultracel-100 K, resuspended in 100 mM Tris–HCl, pH 7.4 (300 µl).

The concentrations of **5**-Qdot and ***Qdot** used in the binding assay were determined spectrofluorimetrically using unconjugated Qdot in 25 mM Tris-HCl, 150 mM NaCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$ supplemented with 1% BSA as reference standard (1:5 dilution, in triplicate, 200 µl/well). The fluorescence of samples and standards were then measured using a Victor Wallac3 instrument (PerkinElmer, excitation filter F355 nm; emission filter 590 nm,

bandwidth ±10 nm).

1.13 Binding assay of 5-Qdot to human 5637 cells

Binding assays of **5**-Qdot and ***Qdot to 5637 cells were carried out as follows: 5637 cells were grown in chamber slides ($6x10^4$ cells/ well). The cells were washed with 25 mM HEPES buffer, pH 7.4, containing 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and incubated with **5**-Qdot or ***Qdot solution (3.3 nM in binding buffer) for 2 h at 37 °C, 5 % CO_2. The cells were washed again with binding buffer, fixed with paraformaldehyde for 20 min, counterstained with DAPI (0.05 µg/ml, Invitrogen), and analyzed using a fluorescence microscopy (Carl Zeiss, Axioscop 40FL; excitation, filter, BP 560/40 nm; beam splitter filter, FT 585 nm; emission filter, 630/75 nm). FACS analysis was carried out as follows: the cells were detached with DPBS-E solution, washed with DPBS, resuspended in binding buffer containing **5**-Qdot or ***Qdot (11-3.7 nM, $5x10^5$ cells/100 µl tube), and left to incubate 2 h at 37 °C. After washing with 25 mM Hepes buffer, pH 7.4, containing 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $MgCl_2$, the cells were fixed with formaldehyde and analyzed using the CytoFLEX S (Beckman Coulter).

1.14 Peptide stability assay

Peptide stability in human serum

The stability of **4** and **5** in serum was assessed by ELISA using mAb 5A8, an anti-CgA_{54-57} antibody (against the sequence HQNL)[17] that can cross-react with **5** (See **Figure S10 for** experimental set up). To this aim, both peptides were synthetized with an additional N-terminal cysteine residue (**4a** and **5a**) (**Table S3**) to allow coupling to maleimide-activated HRP (Expedeon). **4**- and **5**-HRP conjugate (called **4**-HRP and **5**-HRP) were prepared by mixing 24 µg of peptide (5 µl) with 528 µg (108 µl) of maleimide-activated HRP (1:1 ratio) in PBS containing 5 mM EDTA (150 µl final volume) followed by incubation for 3 h at room temperature. To test its stability in serum, 20 µl aliquots of 100 nM of **peptide**-HRP were added to 200 µl aliquots of human serum (Sigma, precleared by centrifugation at 15000 g, 10 min, 4 °C) and incubated at 37 °C. Aliquots were collected at different times (0, 1, 2, 4, 8 and 24 h), blocked by adding a solution consisting of Inhibitor Protease Cocktail III (1:100, final

dilution, Calbiochem) and 10 mM EDTA, pH 8.0 (final concentration). The products were then diluted (1-0.25 nM final concentration) with 50 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and 1% w/v heat denatured BSA, and added to microtiterplates pre-coated with mAb 5A8 (5 µg/ml in 50 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl, 50 µl/well, overnight at 4°C). After washing, the peptide-peroxidase conjugate bound to the plate was determined using the o-phenylendiamine chromogenic substrate of HRP. In parallel, the effect of serum on the peroxidase activity of the conjugate was also checked by measuring the enzyme activity in all samples using the same chromogenic substrate.

Peptide stability in mouse liver microsomal preparations

The stability of compound **4** and **5** in mouse liver microsomal preparations was assessed by HPLC analysis. To this aim microsomes were prepared as follows: 11 g of mouse liver tissue (C57BL/6 mice) was homogenized in cold PBS supplemented with 0.25 M sucrose (3 ml/g of tissue) using a Potter-Elvehjem homogenizer (10 strokes), followed by additional homogenization using a rotor-stator homogenizer (40 sec). The homogenate was filtered through 70 µm cell-strainers, centrifuged three times to remove insoluble materials (500 x g, 5 min; 3000 x g, 30 min; 110000 x g, 90 min, 4°C). The clear part of the final pellet (i.e. the microsome fraction) was gently resuspended with cold PBS (14 ml) and centrifuged again. The final pellet was resuspended with 8.25 ml of cold PBS (0.75 ml/g of original tissue), aliquoted and stored at -80°C. Protein concentration was measured by measuring the absorbance at 280 nm with a NanoDrop spectrophotometer (Thermo Scientific).

Peptide stability studies were performed as follows: each peptide (100 µg in 20 µl of water) was added to 200 µl aliquots of the microsomal preparation (2.5 mg/ml protein concentration) and incubated at 37 °C. Aliquots were collected at different times (0, 1, 2, 4, 8, 24, 48 and 120 h), diluted with 200 µl of 90% acetonitrile containing 0.1% TFA and stored at -80°C for subsequent analyses. After thawing, samples were centrifuged (14000 x g, 10 min, 4°C) and analyzed by HPLC using a C18 LiChrospher column (100 RP-18, 125 mm × 4 mm, 5 µm; Merck), as follows: *buffer A*, 0.1% TFA in water; *buffer B,* 90% acetonitrile, 0.1% TFA , 0% B (5 min), linear gradient (0−100% B) in 20 min; 100 % B (4 min); 0 % B, 8 min; flow rate, 0.5 ml/min.

1.15 Images

All 2D structure images reported were prepared using BIOVIA draw (Dassault Systèmes BIOVIA, BIOVIA draw, Release 2017, San Diego: Dassault Systèmes, 2018).

All 3D structure images were prepared using pymol-1.8.4.2 (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

Graphs were prepared using Matplotlib,[18] XMGRACE (Turner PJ. XMGRACE, Version 5.1.19. Center for Coastal and Land-Margin Research, Oregon Graduate Institute of Science and Technology, Beaverton, OR; 2005), or Adobe Illustrator 2017.

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3. Supplementary Figures

Figure S1. **Multiple Sequence alignment of human CgA with αvβ6 interacting proteins.** Alignment of residues 42-53 of human CgA (Uniprot: P10645) and E46L mutant with TGF-β1 (Uniprot: P01137, residues 243-254), TGF-β3 (Uniprot: P10600, residues 260-271), VP1 coat protein of FMDV (Uniprot: B2MZQ8, residues 144-155), tenascin C (Uniprot: P24821, 876-887), vitronectin (Uniprot: P04004, residues 63-74). The alignment was performed with ClustalX[19] on residue 42-53 of CgA and plotted with ESPript3.0.[20] Completely conserved, highly conserved and highly homologous residues are highlighted with a red background, colored in red or boxed, respectively.

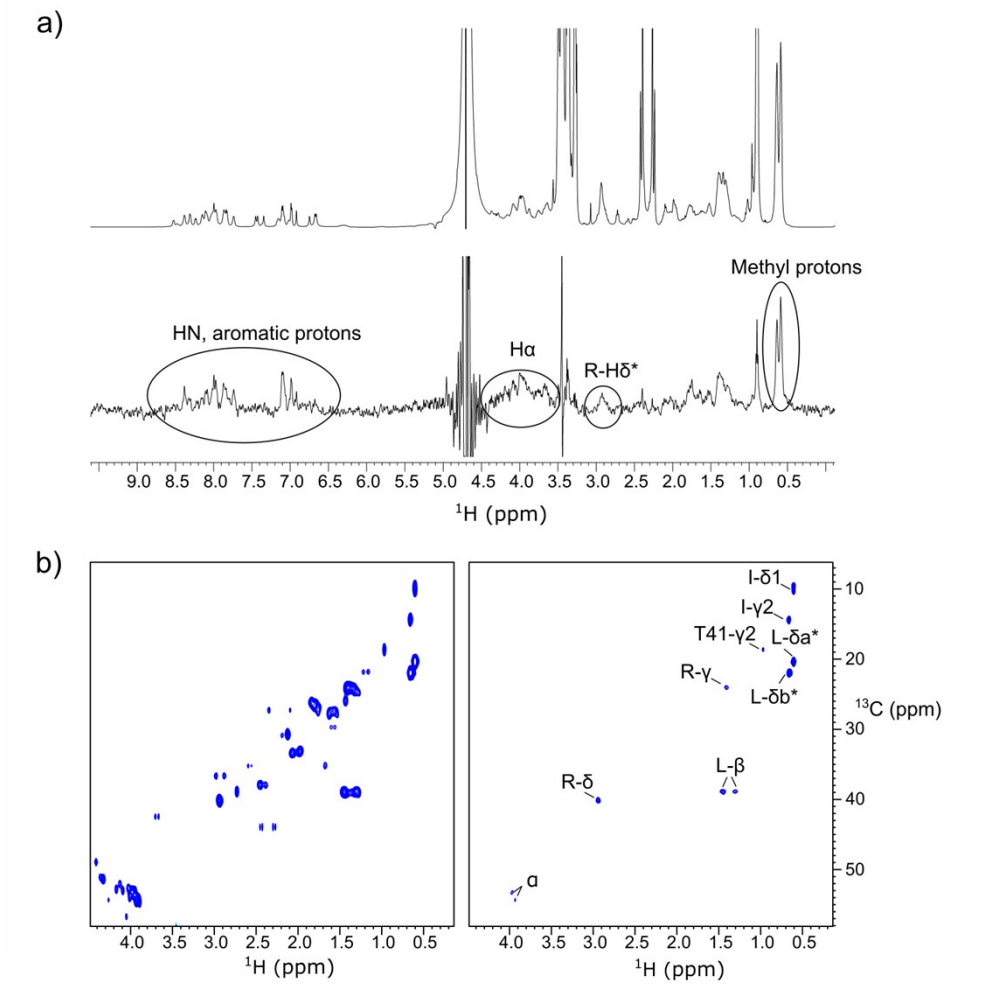

Figure S2. **STD experiments of peptide 1 in the presence of recombinant human αvβ6**. a) ^{1}H 1D-STD experiment (lower panel) and corresponding off-resonance spectrum (upper panel) performed on peptide **1** (0.3 mM) in the presence of recombinant extracellular αvβ6 (1.3 μM). b) ^{1}H-^{13}C-HSQC reference spectrum (left) and 2D-STD-^{1}H-^{13}C-HSQC spectrum (right) performed on ^{13}C/^{15}N recombinant peptide **1** (0.5 mM) and recombinant extracellular αvβ6 (4 μM). Detectable groups of signals are labelled.

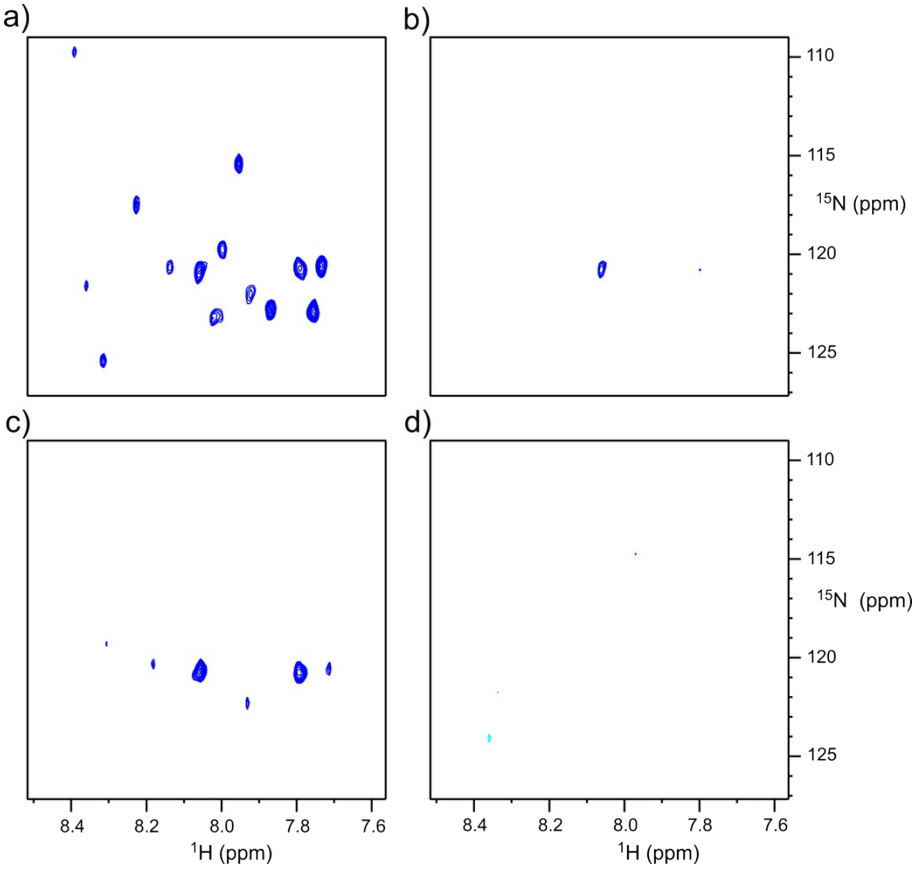

Figure S3. **2D-STD-^{1}H-^{15}N-HSQC spectra of peptide 1.** Experiments on ^{15}N labelled peptide **1** (0.5 mM) a) in the presence of recombinant extracellular αvβ6 (4 μM); b) in the presence of bovine serum albumin (4 μM), c) **1** in the presence of recombinant extracellular αvβ6 (4 μM previously treated with 20 mM of EDTA d16, and d) **1** alone.

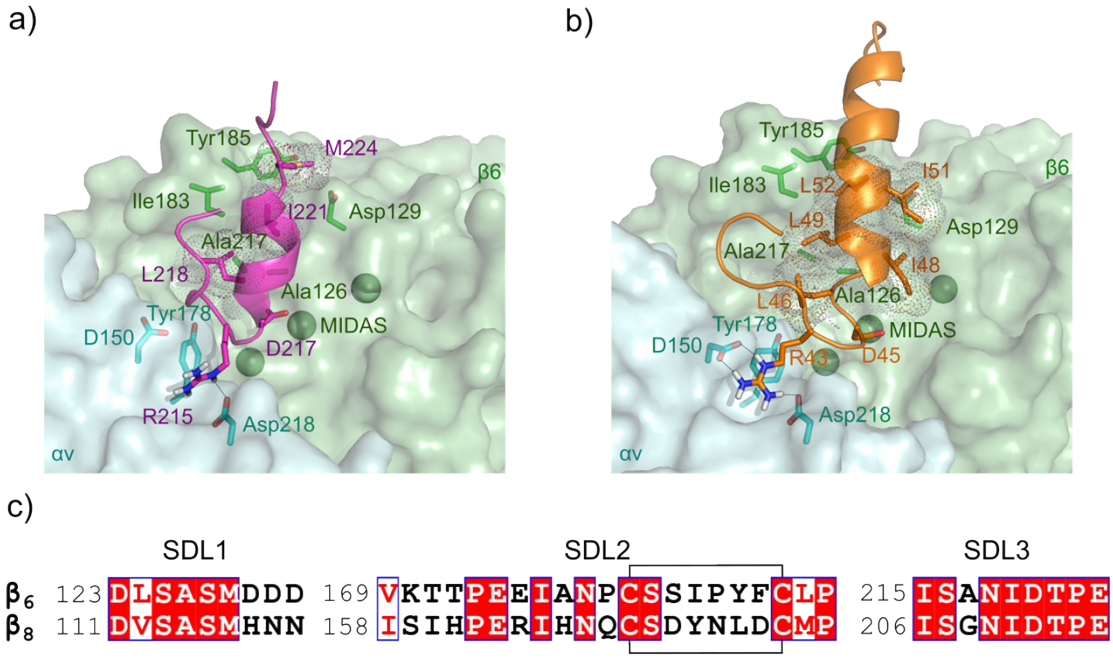

***Figure S4*. Structural comparison between TGF-β1 and 4/αvβ6 binding mode and alignement of SDL sequences of β6 and β8.** a) Crystal structure of αvβ6 together with TGF-β1 (PDB:5FFO)[11]. b) HADDOCK model of peptide **4**/αvβ6 interaction; TGF-β1 (magenta) from residue F210 to P227 and peptide **4** (orange) are shown in cartoon representation. αv and β6 subunits are represented as pale cyan and green surfaces, respectively, with metal ions shown as spheres. Ligand residues side chains involved in the interaction are shown in sticks and labeled with one-letter code, with side chains of hydrophobic residues highlighted with dots; receptor interacting residues are shown in sticks and labeled with three-letter code; electrostatic interactions are represented with dashed lines. c) Sequence alignment of SDL1, 2, and 3 of β6 and β8 was performed with ClustalX [19] and plotted with ESPript3.0.[20] Completely conserved, highly conserved and highly homologous residues are highlighted with a red background, colored in red or boxed, respectively.

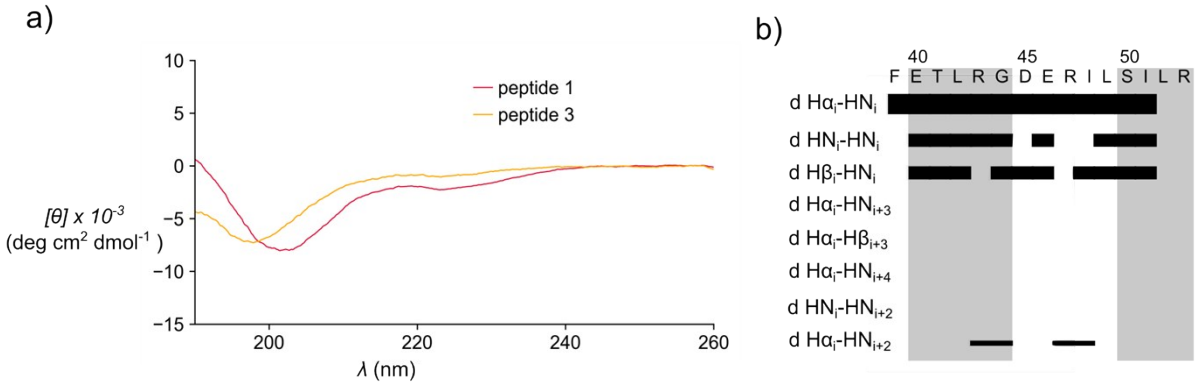

***Figure S5*. Circular Dichroism and NMR analysis of 3.** a) Overlay of CD spectra of peptide **1** (red) and **3** (orange) (30 µM), in phosphate buffer 20 mM, NaF 100 mM, *pH* 6.5, *T*=280K. b) Schematic representation of medium and short NOE contacts identified in **3**. The height of the box is proportional to the NOEs intensities.

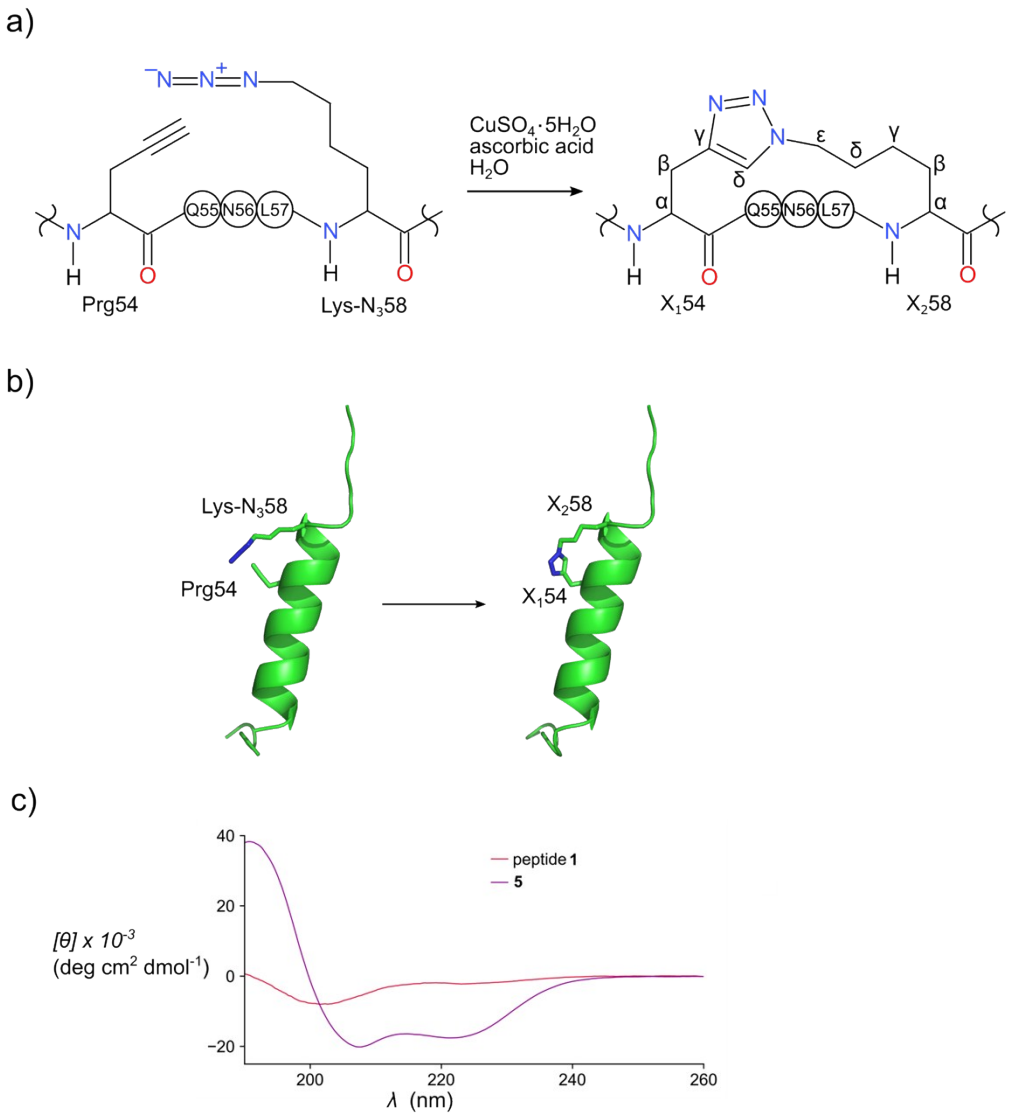

Figure S6. **Effect of stapling on the conformation of 1.** a) Schematic representation of the "click" reaction used to obtain **5**; b) cartoon representation of the precursor and product, with propargylglycine and azidolysine in position 54 and 58 on the left and triazole bridge on the right shown as sticks. c) CD spectra of peptides **1** (red) and **5** (purple) (30 μM), in phosphate buffer 20 mM, NaF 100 mM, *pH* 6.5, *T*=280K.

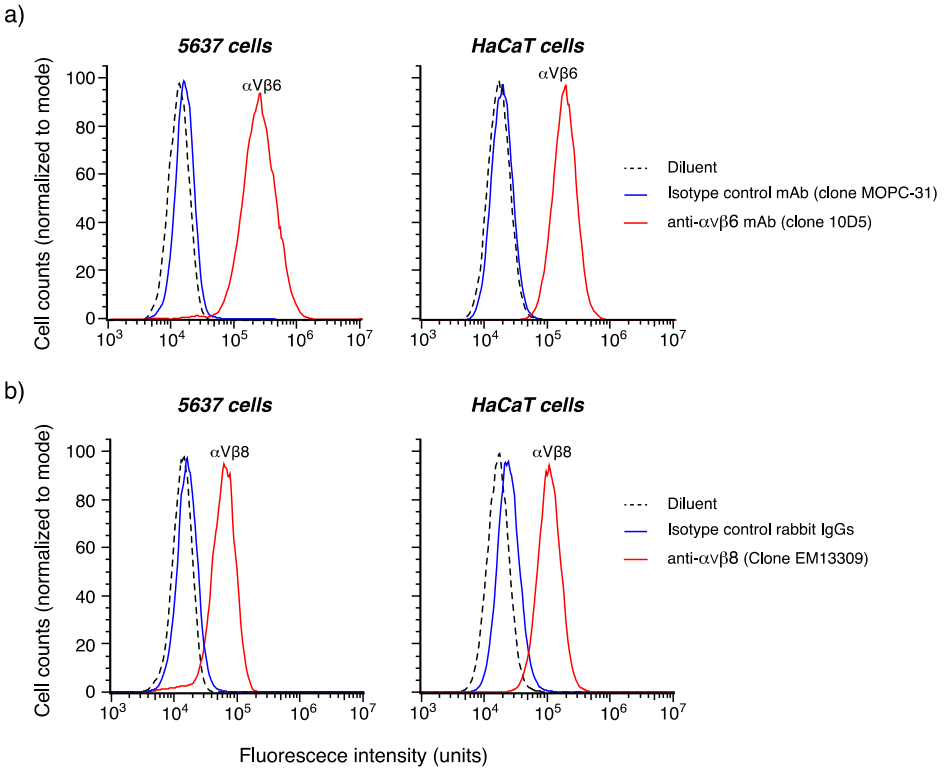

***Figure S7*. αvβ6 and αvβ8 integrin expression on human bladder carcinoma 5637 cells and human skin keratinocytes (HaCaT).** Representative flow cytometry analysis of the expression of αvβ6 (a) and αvβ8 integrin (b) as detected by FACS analysis using an anti-αvβ6 mAb (clone 10D5, 5 µg/ml) and an anti-αvβ8 antibody (clone EM13309, 1 µg/ml), followed by a goat anti-mouse or an anti-rabbit Alexa Fluor 488-labeled secondary antibodies (5 µg/ml), respectively. Binding of isotype control antibodies is also shown.

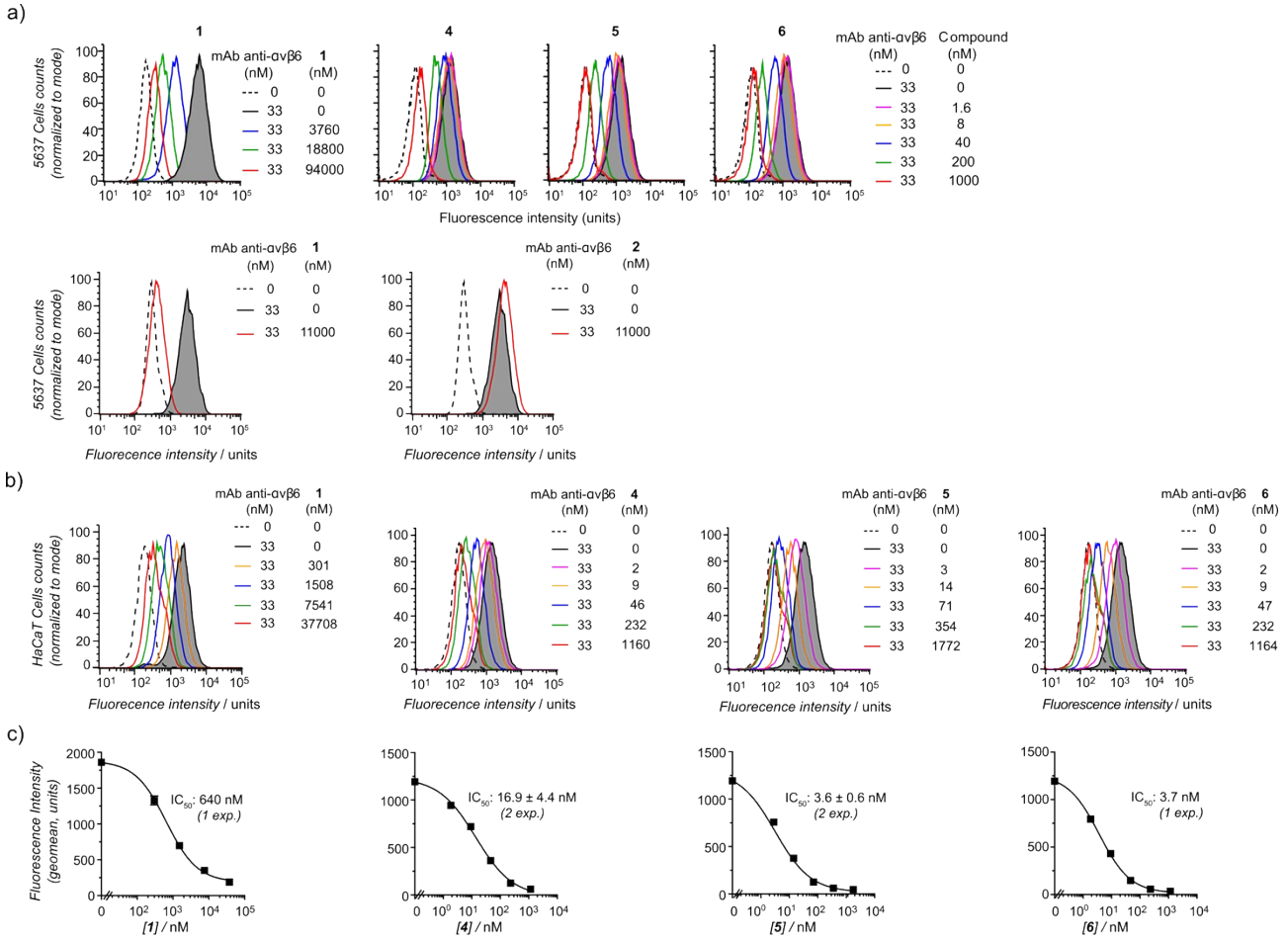

Figure S8*. Effect of 1, 2, 4, 5 and 6 on the binding of anti-αvβ6 mAb 10D5 to human bladder carcinoma 5637 cells and human skin keratinocytes (HaCaT)**. a) Representative flow cytometry analysis showing the effect of peptide **1**, **2**, **4**, **5** and **6** on the binding of mAb 10D5 to αvβ6 positive 5637 cells. Compounds were mixed with mAb 10D5 and added to cells; mAb binding was detected by FACS. Quantification of mAb binding is reported in ***Figure 3A. b) Representative flow cytometry analysis and c) quantification of antibody binding showing the effect of **1, 4, 5** and **6** on the binding of mAb 10D5 to αvβ6 positive HaCaT cells. Squares show mean ± SD of duplicates. Inhibitory concentration 50 (IC_{50}, mean ± SD) of the indicated number of independent experiments is shown.

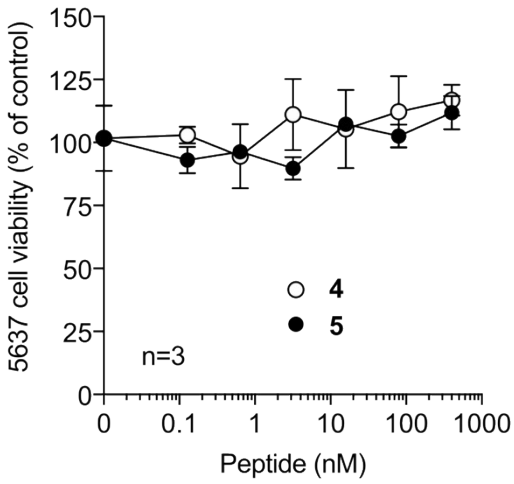

***Figure S9*. Effect of 4 and 5 on human bladder 5637 carcinoma cell viability.** 5637 cells were seeded in a 96-well microtiterplate (20,000 cell/well) and cultured for 16 h at 37°C, 5% CO_2. The day after the indicated doses of peptide **4** and **5** were added to the cells and left to incubate for additionally 48 h at 37°C, 5% CO_2. Cell viability was assessed using the *PrestoBlue® cell viability reagent* (ThermoFisher) according to the manufacturer's instructions. Viability of the treated cells was normalized to that of untreated cells and is reported as a percentage (mean±SE of triplicate wells).

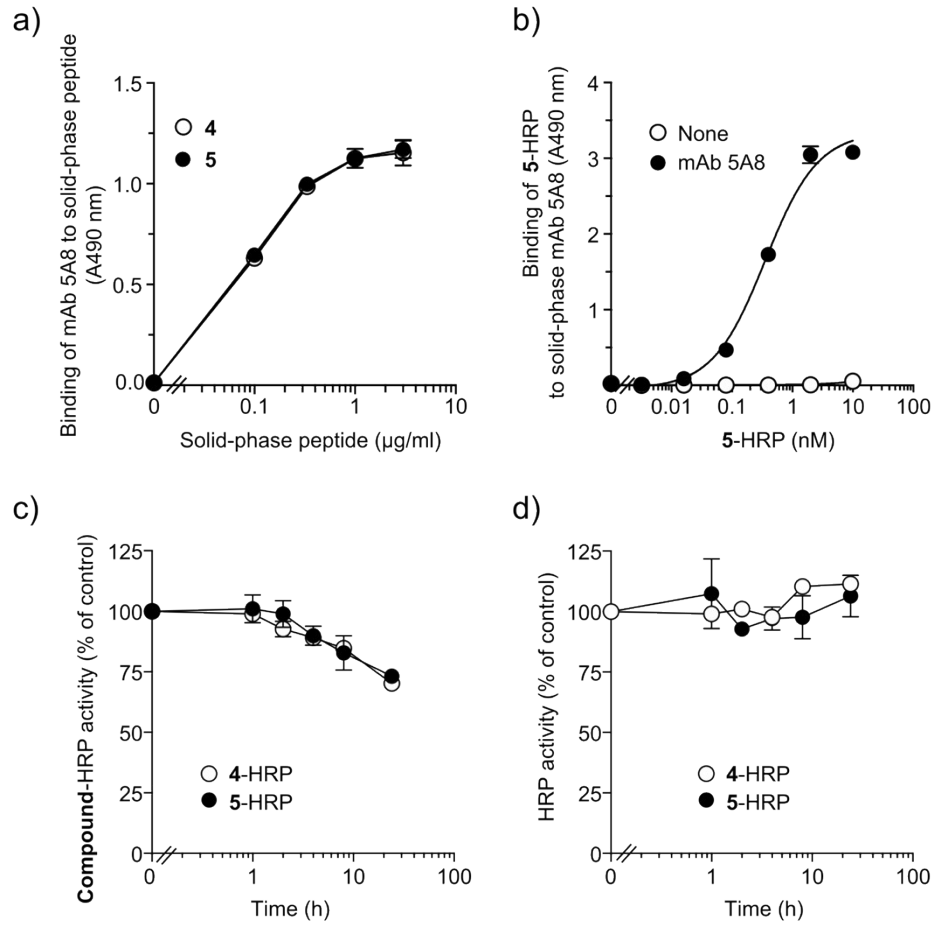

Figure S10. **Stability of 4-HRP and 5-HRP in human serum as determined by ELISA**.
a) Experimental set up of ELISA assays to monitor the stability of **4** and **5** in human serum. Binding of mAb 5A8 (anti-CgA_{54-57}) to microtiterplates coated with peptide **4** or **5**. The binding of mAb 5A8 was detected using a peroxidase-labeled goat-anti-mouse antibody and o-phenylendiamine as a chromogenic substrate. The results show that peptide stapling does not impair mAb 5A8 binding. b) Peptide **5**-horseradish peroxidase conjugate (**5**-HRP) assay dose-response curve. Binding of **5**-HRP at various concentrations to a microtiterplate coated with or without mAb 5A8 (5 µg/ml) is shown. Each point represents mean ± SEM of quadruplicates. c) **4**- or **5**-horseradish peroxidase conjugates (**4**-HRP and **5**-HRP, respectively) were incubated in human serum at 37 °C, collected at different times (0, 1, 2, 4, 8 and 24 h) and added to microtiterplates pre-coated with mAb 5A8 (5 µg/ml). Compound-peroxidase conjugate bound to the plate was determined using the o-phenylendiamine chromogenic substrate of HRP (left panel). d) In parallel, the effect of serum on the peroxidase activity (HRP) of the conjugate was also checked by measuring the enzyme activity using the same chromogenic substrate. Each point represents mean ± SEM of quadruplicates.

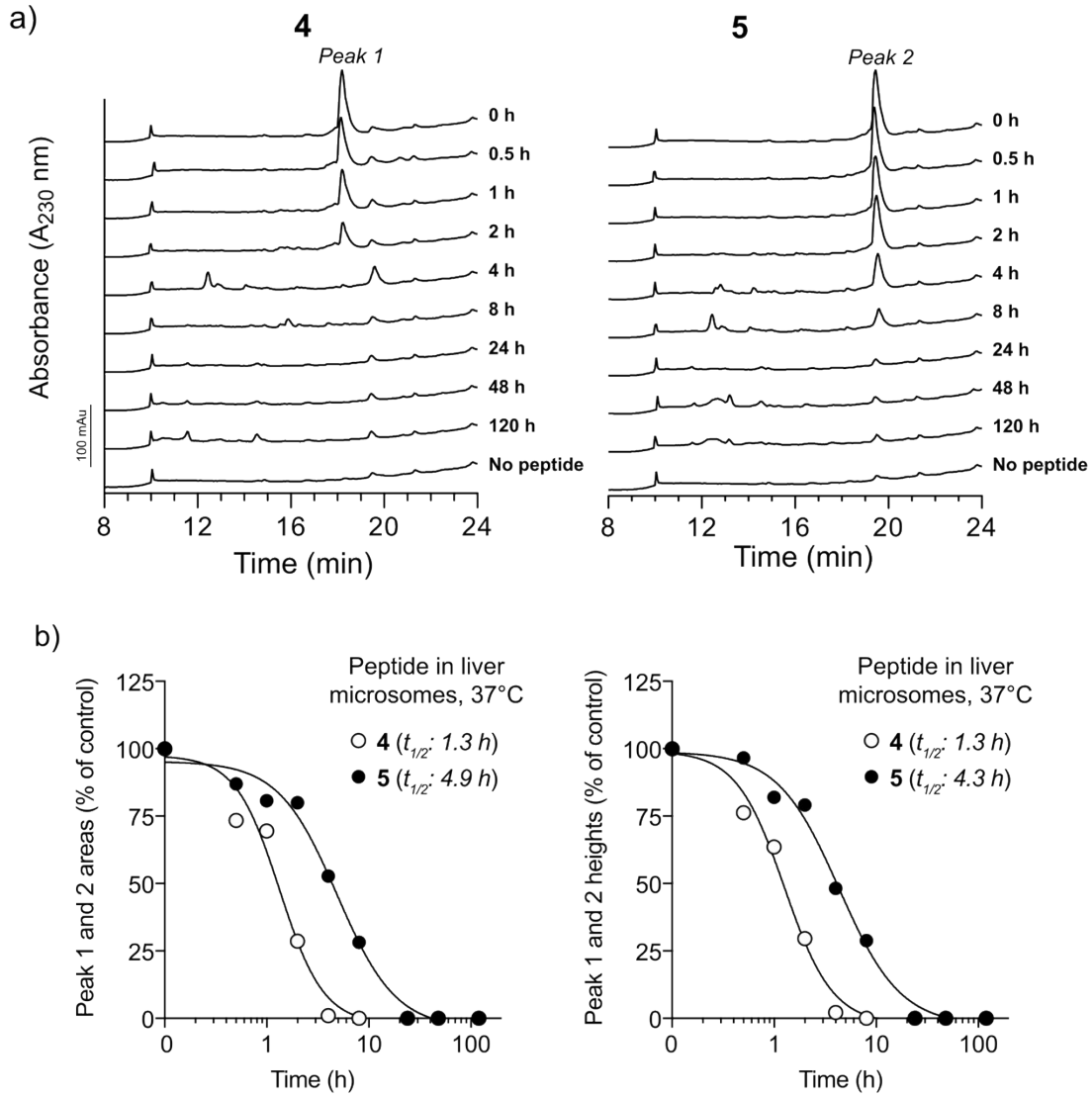

Figure S11. **Stability of 4 and 5 in murine liver microsomes as determined by RP-HPLC.**
a) RP-HPLC of 4 and 5 after incubation at 37 °C in murine liver microsomes. *Peak 1* corresponds to 4 and 5. The peptides were added to murine liver microsomes (454 µg/ml, final concentration) and incubated for the indicated time, diluted with an equal volume of 90% acetonitrile containing 0.1% TFA and analyzed onto a LiChrospher C18 column (16 µg). *No peptide* indicates liver microsome aliquot without the peptide. b) Quantification of Peak 1 area (*left panel*) and height (*right panel*) of the indicated peptides. The corresponding half-life is also shown.

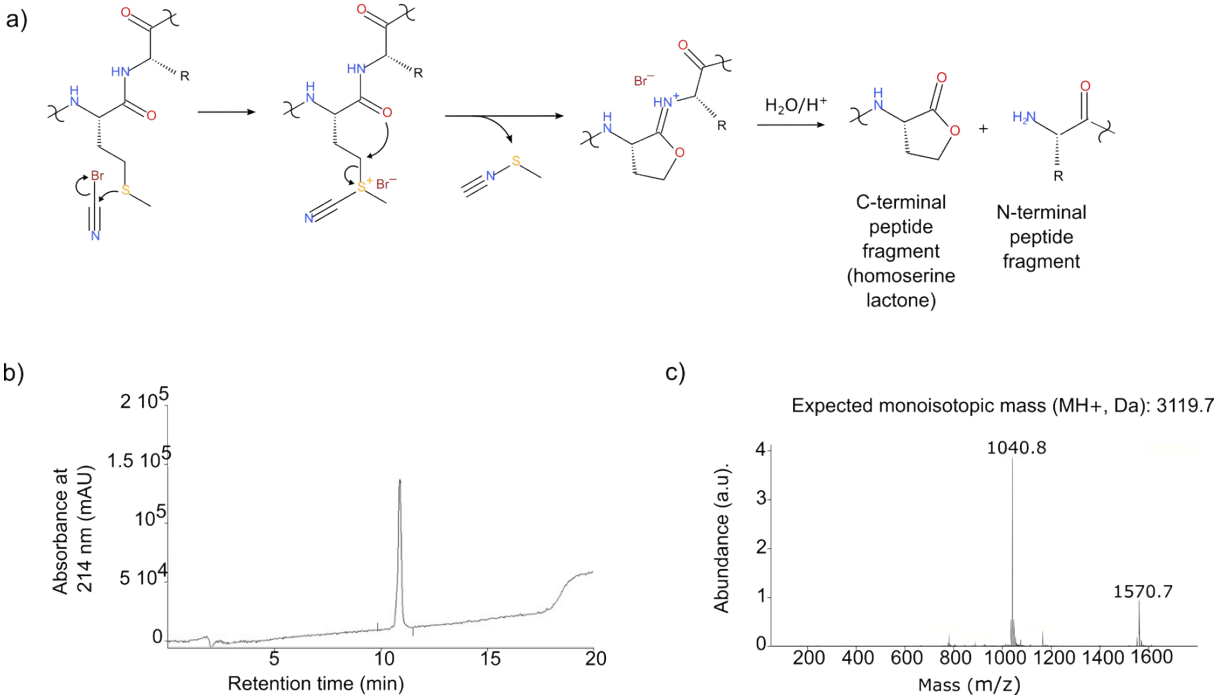

Figure S12. **Reaction mechanism and purification of recombinant peptide 1.** a) Reaction mechanism of the cleavage of methionine-containing peptide with cyanogen bromide. The product of the reaction is a homoserine lactone C-terminal residue. b) Analytical RP-HPLC and c) electro-spray mass spectrometry (ESI-MS) analysis of recombinant peptide **1**. ESI-MS was performed using a Bruker Esquire 3000+ instrument equipped with an electro-spray ionization source and quadrupole ion trap detector. The mass of the peptide including the lactone $[M+H]^+$ is 3119.7 Da and the peaks at 1040.8 Da and 1570.7 Da correspond to $[M+3H]^{3+}$ and to $[M+H+Na]^{2+}$, respectively.

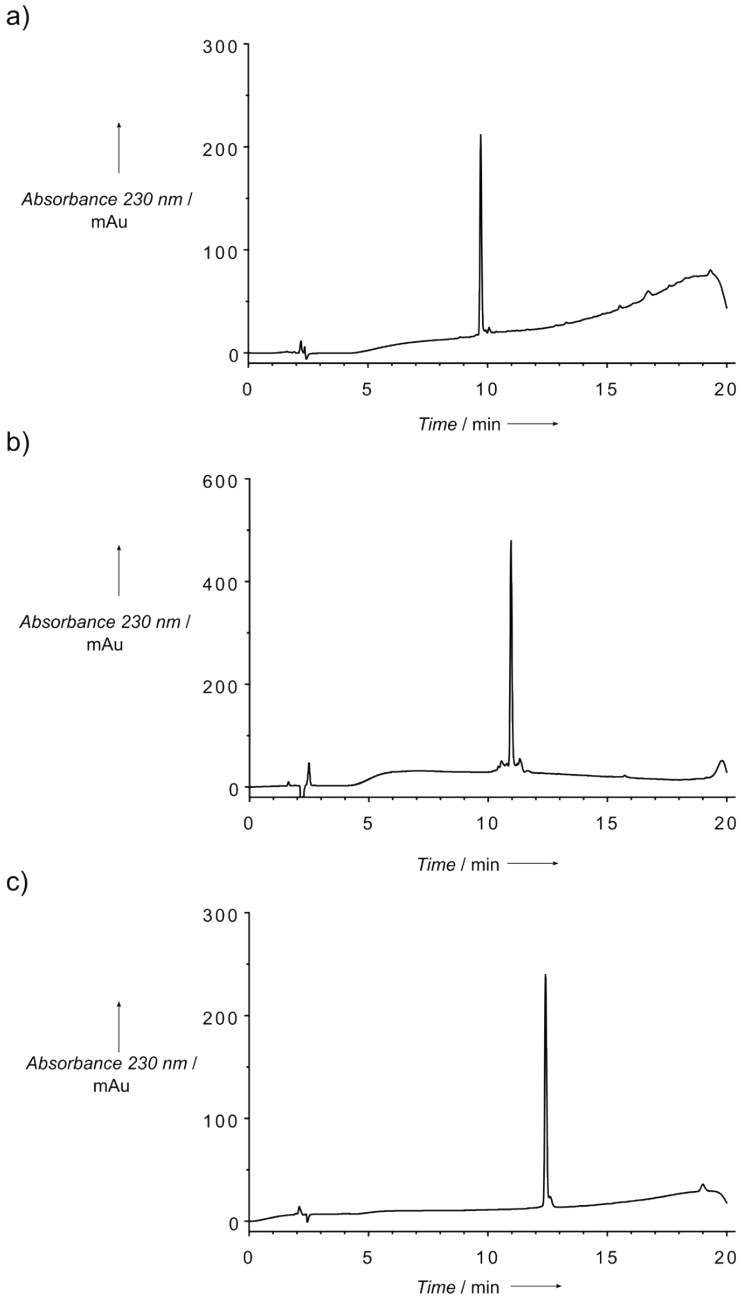

Figure S13. **Analytical RP-HPLC.** RP-HPLC of a) **3**, b) **4**, and c) **5** was carried out on a Shim-pack GWS C18 (5 µm, 4.6 x 150 mm) using a Shimadzu Prominence HPLC.

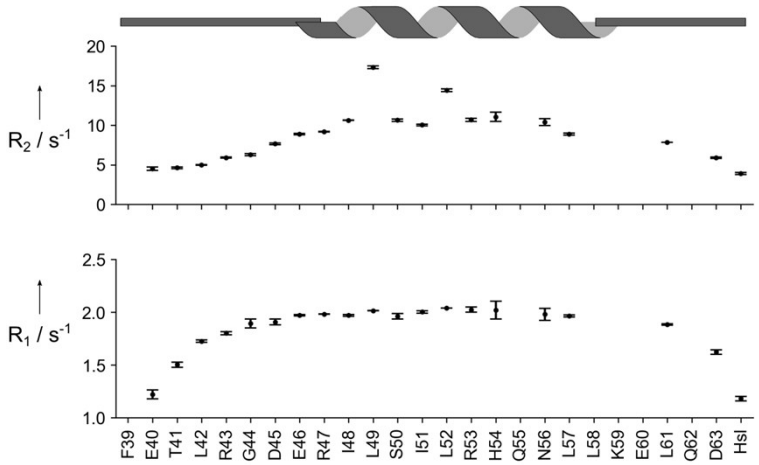

Figure S14. **^{15}N Relaxation analysis.** ^{15}N R_1 (bottom) and R_2 (top) relaxation rates measured for recombinant peptide **1**; elements of secondary structure are indicated on the top of the figure.

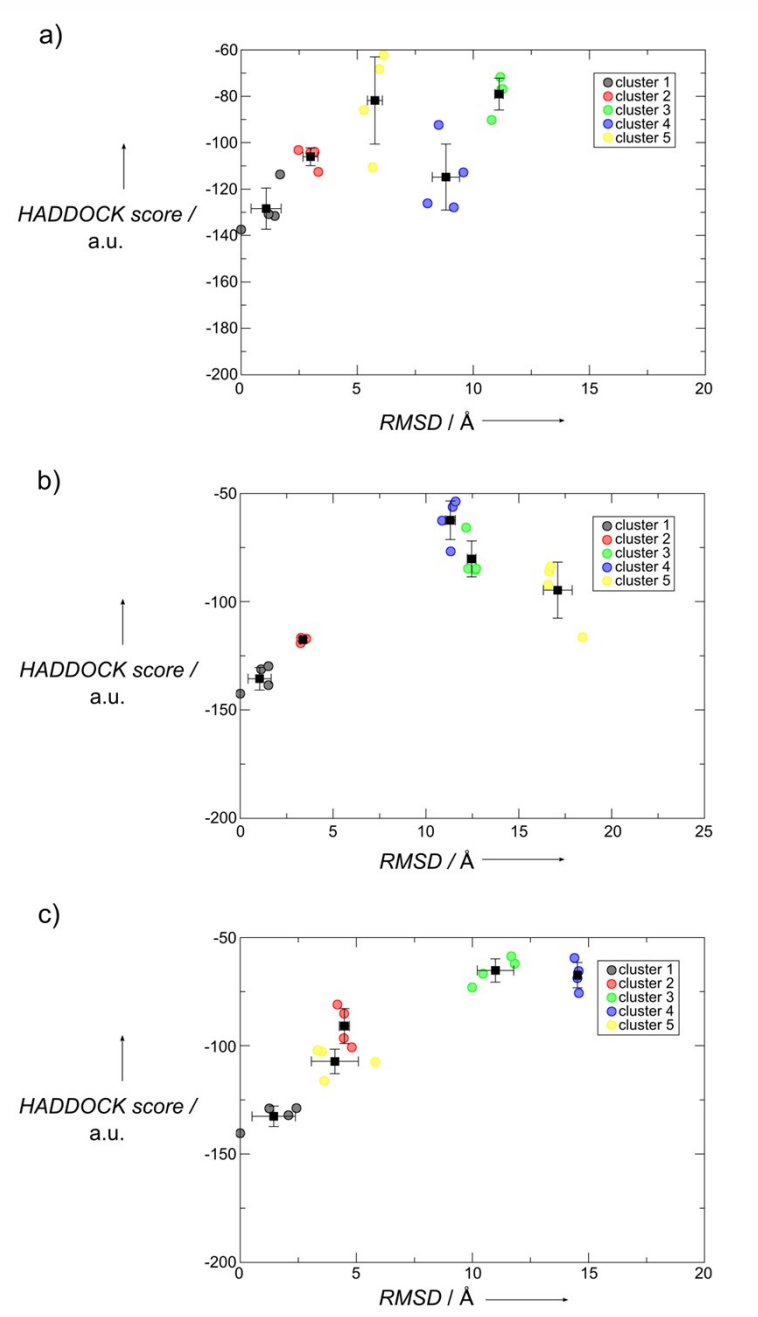

Figure S15. **HADDOCK score of the clusters as a function of their RMSD from the lowest energy structure.** Graphs represent HADDOCK score vs RMSD from the lowest energy complex structures in terms of HADDOCK score (a.u.) for the clustered decoy poses of: a) αvβ6/**1**, b) αvβ6/**4**, and c) αvβ6/**5**. Circles correspond to the best four structures of each cluster; black squares correspond to the cluster averages with the standard deviation indicated by bars. The first best 5 clusters in terms of HADDOCK score are represented.

4. Supplementary Tables

Table S1. **NMR experiments**. List of 2D and 3D NMR experiments performed for the characterization of the peptides, where TD is the total number of points acquired, SI the total number of points used for processing after zero filling, SW the spectral width, and t_{mix} the mixing time.

Experiment	Pulse sequence	TD	SI	SW(ppm)	t_{mix} (ms)	solvent suppression
^{1}H-^{1}H-TOCSY[a]	mlevesgpph	2048x400	2048x1024	11x11	60	excitation sculpting
^{1}H-^{1}H-NOESY	noesyesgpph	2048x512	2048x1024	11x11	200-400	excitation sculpting
^{1}H-^{1}H-NOESY (100% D_2O)	noesyesgpph	2048x512	2048x1024	11x11	200	excitation sculpting
^{1}H-^{13}C-HSQC	hsqcetgpsisp2	2048x256	2048x512	11x80	-	gradient selection
^{1}H-^{15}N-HSQC	hsqcfpf3gpphwg	2048x256	2048x512	11x21	-	water gate
3D-HNCA	hncagp3d	2048x60x110	2048x256x256	11x21x32	-	gradient selection
3D-HNCO	hncogp3d	2048x60x72	2048x128x128	11x21x22	-	gradient selection
3D-HNHA	hnhagp3d	2048x180x46	2048x256x128	12x12x21	-	gradient selection
^{1}H-^{13}C-HSQC-NOESY (100% D_2O)	noesyhsqcetgp3d	2048x46x176	2048x128x512	11x27x11	180	gradient selection
^{1}H-^{15}N-HSQC-NOESY[b]	noesyhsqcetf3gp3d	2048x44x200	2048x256x512	12x21x12	120	gradient selection
^{1}H-^{15}N-HSQC (T_1)	hsqct1etf3gpsi3d	2048x110	2048x256	11x21	-	gradient selection
^{1}H-^{15}N-HSQC (T_2)	hsqct2etf3gpsi3d	2048x110	2048x256	11x21	-	gradient selection
^{1}H-^{15}N-HSQC (hetNOE)	hsqcnoef3gpsi	2048x200	2048x512	11x21	-	gradient selection

***Table S2*. Statistics of the 30 lowest energy structures of 1**. Ramachandran quality parameters were assessed using the PROCHECK-NMR software.[4]

Restraints information	
Total number of experimental distance restraints	360
NOEs (intraresidual/sequential/short/medium)	112/121/115/12
Dihedral angle restraints (phi/psi)	7/7
Deviation from idealized covalent geometry	
Bonds (Å)	0.0026 ± 0.0001
Angles (°)	0.434 ± 0.016
Coordinate r.m.s.d. (Å)[a]	
Ordered backbone atoms (N, Cα, CO)	0.208
Ordered heavy atoms	0.715
Ramachandran quality parameters	
Residues in most favored regions (%)	79.2 (100) [b]
Residues in additional allowed regions (%)	19.1
Residues in generously allowed regions (%)	0.9
Residues in generously allowed regions (%)	0.9

[a] Root mean square deviation between the ensemble of structures and the lowest energy structure calculated on residues E46 to N56.
[b] Values obtained for residues E46–N56.

***Table S3*. Molecular mass of synthetic peptides as determined by mass spectrometry analysis (ESI-MS).**

Peptides[a]	Code	Monoisotopic mass (Da)	
		Expected	Found
CgA derived peptides			
FETLRGDERILSILRHQNLLKELQD	1	3035.7	3035.5
FETLRG***E***ERILSILRHQNLLKELQD	2	3049.7	3051.0
FETLRGDERILSILR	3	1817.0	1817.4
FETLRGD***L***RILSILRHQNLLKELQD	4	3022.5	3022.5
FETLRGD***L***RILSILRX_1QNLX_2KELQD[b]	5	3059.7	3059.7
CFETLRGD***L***RILSILRX_1QNLX_2KELQD	5a	3121.7	3121.7
Foot and mouth disease virus-derived peptide			
NAVPNLRGDLQVLAQKVART[c]	6	2163.2	2162.4

[a] Single letter code; mutated residues (italics, bold); triazole-stapled residues (bold X1 and X2).
[b] N-terminal acetylated and C-terminal amidated.
[c] Also known as A20FMDV2 peptide.

***Table S4*. Chemical shift assignment of 3.** Assignment was determined in 20 mM phosphate buffer, 100 NaCl mM, pH 6.5 (10% D_2O) at 280 K.

Residue	HN	Hα	Hβ	Hγ	Hδ	Hε/HNε
F39	-	4.28	3.25 3.15	-	7.26	-
E40	8.76	4.45	2.03 1.89	2.24 2.24	-	-
T41	8.51	4.29	4.15	1.23	-	-
L42	8.59	4.38	1.66 1.57	1.66	0.93 0.87	-
R43	8.82	4.34	1.79 1.88	1.63 1.63	3.21 3.21	7.42
G44	8.68	3.95 3.95	-	-	-	-
D45	8.36	4.60	2.70 2.68	-	-	-
E46	8.60	4.21	2.06 1.98	2.29 2.31	-	-
R47	8.41	4.27	1.80 1.80	1.62 1.62	3.19 3.19	7.40
I48	8.20	4.12	1.87	0.90 (1a) 1.48 (1b) 1.18 (2)	0.85	-
L49	8.41	4.37	1.64 1.58	1.64	0.87 0.93	-
S50	8.33	4.44	3.84 3.84	-	-	-
I51	8.16	4.19	1.88	1.18 (1a) 1.43 (1b) 0.90 (2)	0.86	-
L52	8.36	4.39	1.62 1.62	1.62	0.93 0.87	-
R53	8.00	4.17	1.84 1.71	1.58 1.58	3.17 3.18	7.24

Table S5. **Chemical shift assignment of 4.** Assignment was determined in 20 mM phosphate buffer, 100 NaCl mM, pH 6.5 (10% D_2O) at 280 K.

Residue	HN	Hα	Hβ	Hγ	Hδ	Hε/HNε
F39	-	4.29	3.15 3.25	-	7.26	7.38
E40	8.76	4.45	1.90 2.02	2.25 2.25	-	-
T41	8.52	4.28	4.15	1.24	-	-
L42	8.61	4.38	1.57 1.67	1.66	n.a.[a]	-
R43	8.55	4.33	1.79 1.89	1.64 1.67	3.21 3.21	7.45
G44	8.55	3.92 3.92	-	-	-	-
D45	8.38	4.56	2.65 2.75	-	-	-
L46	8.29	4.27	1.61 1.61	1.67	n.a.	-
R47	8.31	4.21	1.83 1.83	1.60 1.66	3.21 3.21	7.48
I48	8.03	4.04	1.91	1.22 (1a) 1.50 (1b) 0.92 (2)	0.87	-
L49	8.26	4.27	1.57 1.68	1.68	n.a.	-
S50	8.21	4.36	3.91 3.91	-	-	-
I51	8.06	4.06	1.92	1.17 (1a) 1.52 (1b) 0.91 (2)	0.86	-
L52	8.20	4.26	1.55 1.68	1.68	n.a.	-
R53	8.28	4.24	1.81 1.81	1.57 1.66	3.18 3.18	7.29
H54	8.42	4.60	3.23 3.30	-	7.28	8.51
Q55	8.48	4.25	2.02 2.06	2.37 2.37	-	6.94 7.70

N56	8.61	4.67	2.78 2.86	-	7.03 7.74	-
L57	8.29	4.30	1.62 1.62	1.67	n.a.	-
L58	8.15	4.29	1.58 1.68	1.68	n.a.	-
K59	8.16	4.25	1.79 1.84	1.43 1.43	1.69 1.69	3.00 3.00
E60	8.45	4.24	1.96 2.06	2.26 2.31	-	-
L61	8.29	4.35	1.61 1.61	1.67	n.a.	-
Q62	8.38	4.36	1.98 2.15	2.37 2.37	-	6.94 7.70
D63	8.11	4.38	2.58 2.67	-	-	-

[a] n.a.: not assigned

***Table S6*. Chemical shift assignment of 5.** Assignment was determined in H_2O (10% D_2O) at 280 K.

Residue	HN	Hα	Hβ	Hγ	Hδ	Hε/HNε	Hζ
F39	8.39	4.53	3.01 3.12	-	7.25	-	-
E40	8.66	4.26	2.03 1.93	2.26 2.26	-	-	-
T41	8.20	4.26	4.21	1.22	-	-	-
L42	8.24	4.36	1.55 1.68	1.68	n.a.[a]	-	-
R43	8.39	4.32	1.79 1.89	1.64 1.64	3.21 3.21	7.51	-
G44	8.53	3.96 3.89	-	-	-	-	-
D45	8.45	4.54	2.70 2.79	-	-	-	-
L46	8.33	4.20	1.63 1.81	1.81	n.a.	-	-
R47	8.21	4.13	1.95 1.84	1.59 1.68	3.22 3.22	7.48	-
I48	7.87	3.90	1.98	1.30 (1a) 1.58 (1b) 0.96 (2)	0.88	-	-
L49	8.11	4.18	1.74 1.60	1.81	n.a.	-	-
S50	8.11	4.29	4.00 4.05	-	-	-	-
I51	8.03	3.92	2.01	1.15 (1a) 1.72 (1b) 0.96 (2)	0.87	-	-
L52	8.15	4.15	n.a.	n.a.	n.a.	-	-
R53	8.32	4.10	1.82 1.98	1.66 1.66	3.25 3.25	7.36	-
$X_1$54[b]	8.26	4.54	2.76 2.76	-	7.32	-	-
Q55	7.98	4.18	2.14 2.14	2.44 2.44	-	6.91 7.63	-
N56	8.14	4.46	2.87 2.84	-	6.96 7.71	-	-

L57	7.81	4.11	1.68 1.62	1.68	n.a.	-	-
$X_2$58[b]	8.15	3.86	n.a.	n.a.	n.a.	n.a.	n.a.
K59	7.70	4.10	1.93 1.96	1.53 1.45	1.70 1.70	2.99 2.99	7.65
E60	7.89	4.14	2.16 2.16	2.53 2.39	-	-	-
L61	7.98	4.19	1.59 1.77	1.77	n.a.	-	-
Q62	8.67	3.78	2.13 2.31	2.47 2.63	-	6.94 7.63	-
D63	8.26	4.19	3.57 3.39	-	-	-	-

[a] n.a.: not assigned
[b] See Supplementary Figure S6A for the chemical structure of $X_1$54and $X_2$58.

Table S7. **List of active and passive residues.** Residues selected for the generation of the Ambiguous Interaction Restraints (AIRs)s for HADDOCK calculations.

		Active residues	Passive residues
αvβ6	αv subunit	Asp150, Asp218	Asp148, Phe177, Tyr178, Gln180, Ala215
	β6 subunit	MIDAS (Mg^{2+}), Ala126, Pro179, Ile183, Ala217, Asn218	Ser127, Asp129, Asp130, Glu175, Cys180, Ser181, Ser182, Pro184, Tyr185, Cys187, Ile215, Thr221
Peptide 1		R43, G44, D45, I48, L49, I51, L52	F39, E40, T41, L42, E46, R47, S50, R53, H54, Q55, N56, L57, L58, K59, E60, L61, Q62, D63
Peptide 4		R43, G44, D45, L46	F39, E40, T41, L42, E46, R47, I48, L49, S50, I51, L52, R53, H54, Q55, N56, L57, L58, K59, E60, L61, Q62, D63
Peptide 5		R43, G44, D45, L46	F39, E40, T41, L42, E46, R47, I48, L49, S50, I51, L52, R53, $X_1$54[a], Q55, N56, L57, $X_2$58[a], K59, E60, L61, Q62, D63

[a] Stapling residue