Selection and Structure of Ion-selective Ligands for Platelet Integrin $\alpha_{IIb}\beta_3$* **

Integrins contain a number of divalent cation binding sites that control ligand binding affinity. Ions such as Ca$^{2+}$ and Mg$^{2+}$ bind to distinct sites on integrin and can have opposing effects on ligand binding. These effects are presumably brought about by alterations of the shape of the ligand binding pocket. To gain insight into the nature of these structural differences, we probed the integrin ligand binding site with an RGD-based library of unparalleled complexity. A cysteine-constrained phage library containing six random amino acids and the RGD motif present in seven different registers was used to select for ligands that exhibit ion-selective binding to integrin $\alpha_{IIb}\beta_3$. The library was used to select for peptides that bind to the integrin $\alpha_{IIb}\beta_3$ preferentially in Ca$^{2+}$ versus Mg$^{2+}$. Peptides were identified which bound selectively in each ion. The Ca$^{2+}$-selective peptides had a range of sequences, with the only obvious consensus involving a motif that had four cysteine residues bonded in a 1,4:2,3 arrangement. Interestingly though, the Mg$^{2+}$-selective peptides exhibited a well defined consensus motif containing Cys-X-aromatic-L/G-R-G-D-hydrophobic-R/K-Cys. As a first step toward understanding the structural basis for this selectivity, solution NMR structures were obtained for representatives of both sets of peptides. All peptides formed turns, with the RGD motif at the apex. The Mg$^{2+}$-selected peptides contained a unique basic patch that protrudes from the base of the turn.

Integrin-mediated cell adhesion is regulated tightly by extracellular divalent cations such as Ca$^{2+}$ and Mg$^{2+}$ (for review, see Ref. 1). Interestingly though, divalent ions can have opposing effects on ligand binding, promoting both cell adhesion and cell detachment (2–6). This can be explained by the fact that integrins contain two classes of divalent ion binding sites. One class of sites, called ligand-competent (LC)* sites, must be filled for ligand to bind (7). The LC site(s) can bind to a number of different ions including Ca$^{2+}$, Mg$^{2+}$, and Mn$^{2+}$. A number of studies (8), including recent co-crystals of integrin I domains with bound ligands (9), suggest that the LC sites may actually sit within the ligand binding pocket and make direct contact with ligand.

A separate class of ion binding sites is allosteric to the ligand binding site and inhibits ligand binding by increasing the rate of ligand dissociation. Because of their effects on ligand dissociation, we called these allosteric sites inhibitory, or “I” sites (10). These I sites appear to bind selectively to Ca$^{2+}$. The effects of the I site can be pronounced, as an integrin with Ca$^{2+}$ bound at the I site can have a dissociation rate that is 20-fold higher than when the I site is unoccupied. Because the affinities of the LC and I sites for divalent ions are within the physiologic ranges, these sites act cooperatively, and in an opposing manner, to regulate the function of the integrin ligand binding pocket.

There are several physiologic and pathophysiologic circumstances in which divalent ions play an important role in regulating cell adhesion. For example, osteoclasts, the primary bone-resorbing cell, adhere to the bone surface through the $\alpha_{IIb}\beta_3$ integrin. The adherent osteoclast resorbs mineralized bone, liberating free Ca$^{2+}$, which can rise above 10 mM (11). This elevation in [Ca$^{2+}$] is then sensed by the osteoclast via a receptor (12, 13), and this ultimately causes the osteoclast to detach from the bone surface. The identity of this osteoclast Ca$^{2+}$ receptor remains unclear, but it appears to have many of the properties of the allosteric I site on the $\alpha_{IIb}\beta_3$ integrin (4, 10). Consequently, we proposed that the liberated Ca$^{2+}$ binds to this site on integrin, accounting for the effects of this ion on osteoclast detachment. A similar mechanism can be proposed in wound healing, where keratinocyte integrins play a major role. In wound fluids the ratio of Mg$^{2+}$ to Ca$^{2+}$ is substantially higher than under normal circumstances (14). This scenario is expected to favor the action of the integrin LC ion binding sites over the I site and favor adhesion over detachment. Indeed, the elevated levels of Mg$^{2+}$ appear to promote integrin-mediated migration of keratinocytes and healing of the wounds (14).

Divalent ions also influence the ligand binding properties of the integrin that is the subject of this study, platelet integrin $\alpha_{IIb}\beta_3$ (15). The $\alpha_{IIb}\beta_3$ integrin is somewhat unique in that it contains two physically distinct but interacting ligand binding pockets (16). One ligand binding pocket binds to the carboxy-terminal region of fibrinogen (Fg), called the $\gamma$-chain site. The binding of $\alpha_{IIb}\beta_3$ to the $\gamma$-chain is necessary for the aggregation of
of platelets in blood (17–19). Importantly, the γ-chain of Fg lacks the RGD motif. Interestingly, the effects of divalent ions on the binding of the γ-chain to αιββ is different from the effects on RGD binding. Binding between the Fg γ-chain and αιββ proceed faster in Ca2+ than in any other ion, indicating that the 1 site is not involved in this interaction.

Integrin αιββ also contains a separate RGD binding pocket that binds to ligands such as von Willebrand factor, fibronectin, as well as RGD peptides and their mimetics. This RGD binding site is governed cooperatively by the LC and I ion binding sites as discussed above. Another important feature of the RGD binding site is that it is allosterically connected to the Fg γ-chain binding site. When αιββ is occupied with RGD ligands, the rate of dissociation for Fg is increased dramatically. Altogether then, the ligand binding properties of αιββ are regulated in a complex manner, with both divalent ions and the presence of additional ligands making key contributions. In general though, the current understanding of these interactions suggests that platelet aggregation to soluble Fg will be enhanced by Ca2+ and that platelet adhesion to immobilized RGD ligands such as von Willebrand factor will be enhanced by Mg2+.

Given the clinical importance of dissecting these two functions of αιββ and the lack of a structural basis for ion-selective binding to integrins, we sought to engineer RGD ligands for αιββ which exhibit Mg2+- and Ca2+-selective binding. To address these issues we constructed a phage library of cyclic RGD peptides of unparalleled complexity. The library is comprised of ninemers that are conformationally constrained by two terminal cysteine residues. The RGD motif and six randomized residues lie between the two cysteines. The library takes full access of all possible conformations of RGD by displaying the motif in every register. In solution, all of the peptides that bind to αιββ adopt the conformation of a general β-turn, with the RGD sequence near the apex of the turn. Yet, the study reveals key differences in the primary structure of ion-selective RGD peptides. A distinguishing feature of the Mg2+-selective peptides is the presence of a protruding basic patch adjacent to the turn.

MATERIALS AND METHODS

Proteins and Peptides—Platelet integrin was purified from outdated human platelets using RGD affinity columns as described previously (20, 21). It is important to emphasize that the form of αιββ used in these studies is active in binding RGD ligands or is present in Activation State-2, as we have reported previously (20). Peptides based on the sequences of phage A1, A22, D16, and D18 were synthesized by Anaspec. Peptides were cyclized via disulfide bonds through terminal cysteine residues. The amino terminus of each peptide was acetylated, and the carboxyl terminus was amidated. Before analysis all peptides were purified by reverse phase HPLC and judged to be greater than 90% pure based on elution profiles of HPLC. Peptide mass was confirmed by mass spectrometry. Peptides based on the sequence of phage A22 were synthesized in both the 1,3,3,4 and 1,2,3,4 disulfide bond configuration, where the numerals indicate the order of appearance of the cysteine residues in the peptide sequence.

Construction of RGD Superlibrary—The phagemid pDV-FL was constructed from the phagemid pBlueScript SK. The phagemid encodes the pEb leader sequence, the FLAG octapeptide epitope, the cloning sites for random peptides, and amino acids 198–406 of the β3 chain protein from M13 bacteriophage. Amino acids 198–406 of pIII of M13 bacteriophage were PCR amplified using the oligonucleotides 5’-GAATTCACGGCCTCTG-3’ and 5’-GGAGGAGGATCATGT(NGG)-3’ which exhibit Mg2+-turn, with the C terminal cysteine residues when expressed on phage. The template DNA was digested with Restriction enzymes BamHI and XhoI and ligated into EcoR HindIII-digested pBlueScript to give pDV. The FLAG epitope was generated by annealing oligonucleotides 5’-CA-TGGCCCCTGATCCATGACACAGCGACGCAGACAAACG-3’ and 5’-GATCCGTGGTTGTCGCTGGTTTGTGATGGCGATGGC-3’ and ligating them into NeoI/BamHI-cleaved pET20b. The pEb leader sequence and FLAG epitope were subcloned from pET20b into pDV at the NeoI/ EcoRI sites to yield pDV-FL.

The seven RGD-containing random peptide libraries were constructed by flanking cysteine residues when expressed on phage. The DNA encoding random hexamers and RGD in seven registers were generated by PCR amplification of template oligonucleotides. An example of one of the seven oligonucleotides is 5’-ATATAGCGGGTACCGAGATATACGGCATGATVYNRGGTGGCCGGACTCCT-AGACCG-3’, where N is a random nucleotide and Y is the RGD sequence (CGTGGCGAC) is expressed in the fourth register. The template oligonucleotides were amplified by PCR using the two flanking oligonucleotides 5’-ATATAGCGGGATTGC-3’ and 5’-CGGTCTAGGAATTTCC-3’. The PCR-amplified DNA encoding the random RGD peptides was digested with EcoRI and BamHI and ligated into BamHI/EcoRI-digested pDV.

Phage particles expressing the RGD-containing peptides were generated by transforming ligated DNA into XLI-blue bacteria (Stratagene, La Jolla, CA). The transformation efficiency was assessed by plating some of the material onto LB agar plates containing 100 µg/ml ampicillin. The rest of the bacteria were grown further in SB supplemented with 50 µg/ml carbenicillin and then infected with 1 × 10^8 VCSM13 helper phage (Stratagene). Bacteria expressing recombinant RGD peptides on phage were selected by the addition of kanamycin to a final concentration of 70 µg/ml. The bacteria culture-expressing phage were grown overnight with shaking at 37 °C. The overnight bacterial cultures were centrifuged to remove bacteria and phage particles were precipitated by the addition of polyethylene glycol 8000 and NaCl to final concentrations of 4 and 3%, respectively. The 3% precipitated phage were resuspended 50 mM Tris, pH 7.4, 150 mM NaCl, 1% bovine serum albumin (BSA), and 20% glycerol and stored at –80 °C.

Phage Selections—RGD-selective RGD motifs were isolated from the RGD superlibrary using the following procedure. Microtiter wells of a Linbro/Titertek 96-well ELISA plate (ICN, Aurora, OH) were coated with 10 µg/ml purified αιββ at 4 °C in coating buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 1 mM MnCl2) (7). Additional wells of the plate were coated with 3 µg of BSA so that specific phage enrichment could be assessed. After coating, the bound integrin was depleted of endogenously bound divalent cation by six washes with ice-cold TBS buffer (50 mM Tris, pH 7.4, 100 mM NaCl). Then, the wells were blocked with 30 mg/ml BSA for 1 h at 37 °C. An aliquot of each sublibrary containing 1 × 10^11 transforming units was pooled together to generate the superlibrary. 60 µl of this library containing a total of 7 × 10^11 transforming units was incubated with immobilized integrin in TBS containing either 1 mM CaCl2 or 1 mM MgCl2 for 3 h at 37 °C. The unbound phage were removed by washing 10 times with TBS buffer containing 0.5% Tween 20. Bound phage were eluted with 0.2 M glycine buffer, pH 2.2, containing 1 mg/ml BSA. Eluted phage were amplified in XLI-blue Escherichia coli using VCSM13 helper phage (Stratagene). Phage were partially purified by precipitation with 4% polyethylene glycol 8000 and 3% NaCl and resuspended in TBS buffer with 10 mg/ml BSA and 20% glycerol. Three subsequent pannings were performed to enrich for ion-selective binders by incubating 7 × 10^11 transducing units of phage with immobilized integrin αιββ. After the fourth panning, colonies from the output phage titration were grown into large cultures and the phage DNA was purified and sequenced. Sequences were determined using the ABI 377 DNA sequence in combination with the Prism Big Dye sequencing kit (ABI, Foster City, CA) and the primer 5’-CGCAAACGCCCTCTCCCC-3’.

Phage Binding Assays—The binding of individual phage to purified αιββ was measured using a modification of the ligand binding assay that we have described previously (7). Wells of a Linbro/Titertek 96-well ELISA plate were coated with 100 ng of purified αιββ (RGD-binding form) as described above. In preparation for binding studies, the plate was incubated for 5 min in cation-free TBS to allow dissociation of bound ion and then washed six consecutive times with ice-cold TBS. We have shown that under these conditions all ligand binding to αιββ is eliminated and can then be reconstituted by readdition of divalent ions (7). The plate containing αιββ was blocked with 30 mg/ml BSA in TBS for 1 h at 37 °C. Then, individual phage were added to the wells in binding buffer containing either 1 mM CaCl2 or 1 mM MgCl2. Phage (1.2 × 10^12 colony-forming units/ml), or ~2 nm, were allowed to bind to integrin as a function of time. Nonspecific binding was measured by adding 2.0 mM K4[ 35]GDP as a competing ligand. Unbound phage was removed by washing with TBS containing 0.5% Tween 20 and phage were detected by incubating the wells with an anti-M13 monoclonal antibody (Amersham Biosciences, Inc.) and then horseradish peroxidase-conjugated anti-mouse IgG antibody (Bio-Rad).

Competition Binding Assays—Competition binding experiments were performed in an ELISA-type format. Integrin αιββ was immobilized in microtiter wells, and the plates were subsequently blocked with 1% BSA to limit nonspecific binding. Binding was measured at con-
trations of Fg and Fab-9 which just saturate the binding sites on immobilized integrin. Binding was measured across a range of competing cyclic peptide. Bound Fg or Fab-9 was detected with a horseradish peroxidase- or alkaline phosphatase-conjugated secondary antibody.

NMR Spectroscopy—Peptides A1, A22, D16, and D18 were studied by solution NMR methods. Peptides representing the sequences were synthesized and cyclized via disulfide bonds formed by oxidation of the cysteine residues that flank each motif. NMR spectra were acquired on a Varian UNITY-plus 500-MHz spectrometer equipped with 5- and 3-mm triple resonance probes with a single axis pulse field gradient accessory along the z axis. For peptides A1, D16, and D18, data were acquired at 4 °C in phosphate buffer, pH 6. Because of its limited solubility, the data for peptide A22 were acquired in MeSO at 25 °C. The amount of peptide used was 1.5 mg for A22 in 220 μl of buffer, 2.5 mg for D16, 3.9 for A1, and 4.2 mg for D18 in 600 μl. For the peptides in aqueous solutions, homonuclear two-dimensional NMR experiments including jump return NOESY (τmax = 75, 150, and 300 ms), TOCSY (τmax = 60 ms) and DQF-COSY (acquired at 5, 10, 15, and 25 °C to determine the temperature coefficients of amide protons) were acquired. For peptide A22, which was examined in Me2SO, the amide chemical shifts are reported with respect to external TSP-d4 and trace Me2SO-d5. NMR data were processed and analyzed using Felix2000 software (MSI, San Diego) on an SGI Indigo work station. For each of the peptides studied, only one set of NMR resonances was observed.

Experimental Constraints—Distance constraints were extracted from two-dimensional NOESY spectra with mixing times of 75 and 150 ms. NOE cross-peaks with strong intensities were classified to correspond with interproton distances of 1.8–2.7 Å, where the lower bound, 1.8 Å, is the closest possible interproton distance consistent with van der Waals repulsion. NOE cross-peaks with medium, weak, and very weak intensities in 150-ms mixing time spectra were classified into interproton distances of 1.8–3.3 Å, 1.8–5.0 Å, and 3.0–6.0 Å, respectively. Pseudoatom corrections were used to modify the upper bound distance constraints for NOEs involving aromatic, guanidinyl, methyl, and methylene groups (22). JHN, coupling constants were obtained from DQF-COSY spectra and used to calculate the backbone dihedral angle ϕ based on Equation 1,

\[ J_{\text{HN}} = 6.68 \cos^2 \theta - 1.38 \cos \theta + 1.72 \]  

where \( \theta = 60^\circ \) (23). The \( \chi_1 \) dihedral angles were defined based on the E-COSY spectra and NOE intensities between H2–H3 and H2–H3 compared with the NOE intensities between H2–H3 and H2–H3 (Keating et al. 1990a). For the cysteine residue, if NOE intensities of H3–H4 > H2–H3 and H3–H4 < H2–H3, the values for \( \chi_1 \) can thus be defined as \( \chi_1 = +60 \pm 30^\circ \). For NOE intensities of H2–H3 > H3–H4 and H2–H3 < H3–H4, then the values for \( \chi_1 \) were defined as \( \chi_1 = -60 \pm 30^\circ \).

Structure Calculations—A full conformational space search was undertaken by generation of 200–400 starting structures for each peptide using distance geometry methods based on NMR-derived constraints. Each of the subembdeded coordinates was regularized with template fitting, correct enantiomer testing, high temperature dynamics (2000 K), and simulated annealing (24, 25). Structures with relatively low empirical energies and low distance and dihedral angle constraint violations were selected for further refinement with additional constraint data from the chemical shifts of Cα, Cα, and Hα as well as the coupling constants. Hydrogen bond constraints were also enforced in the calculations based on the amide proton temperature coefficients less than 2.0 ppb/K. All distance geometry and restrained molecular dynamics calculations were performed on SGI work stations using the program X-PLOR-3.851 (24).

RESULTS

Construction of Directed RGD Phage Libraries—Several groups, including ours, have applied phage display to select integrin-binding peptides and antibodies (26–32). Here, we significantly extend the approach to incorporate far more diversity into the library. Seven sublibraries were constructed to display the RGD loops as fusion proteins with the gene 3 protein and were displayed as monovalent ligands on phage (33). Each sublibrary contains the RGD motif and six randomized residues that are flanked by amino- and carboxyl-terminal cysteines. Importantly though, each sublibrary displays the RGD in a different register. The terminal cysteines are referred to as positions 0 (P0) and 5 (P5). Residues between these cysteines, which comprise the RGD loop, are referred to as position 1 (P1) through position 9 (P9). A high representation of all possible sequences was ensured by achieving high ligation and transformation efficiencies. The seven sublibraries were combined prior to selection on \( \alpha_1 \beta_3 \), creating a working superlibrary. To our knowledge, this is the most diverse library of RGD compounds reported to date.

Identification of RGD Peptides That Bind Integrin \( \alpha_1 \beta_3 \) in Ca2+- and Mg2+-selective Fashion—A total of \( 2 \times 10^8 \) phage from the superlibrary were incubated with \( \alpha_1 \beta_3 \) in either 1 mM Ca2+ or 1 mM Mg2+. Bound phage were eluted with acid, amplified, and subjected to three additional rounds of selection. 40 phage from each panning were randomly selected, and the amino acid sequence of the RGD motifs was derived from the phage DNA sequence (Tables II and III). Most of the selected phage contained the RGD motif in P4–6 or P5–7 within the cysteine constrained loop. In addition, almost all of the phage contained at least one aromatic residue within the RGD loop, and many of the phage have aromatic residues that flank the RGD.

There are notable distinctions between the phage selected in Ca2+ versus Mg2+. For example, 25 of the 39 sequenced phage selected in Ca2+ displayed the RGD at P5–7. In contrast, only 11 of 39 phage selected in Mg2+ contained the RGD in positions P5–7. Rather, 28 of 39 of the Mg2+-selective phage displayed the RGD at P4–6. Two other distinctions among the two groups of phage are evident. First, a few of the phage selected in Ca2+ contained additional cysteine residues. These cysteines are likely to form a second disulfide bridge within the peptide. No additional cysteines were observed in the phage selected in Mg2+. Second, many of the phage selected in Mg2+ contain basic residues (Lys or Arg) at P7 and P8, which were absent in the phage selected in Ca2+.

Among the Binding Properties of Selected Phage—Initial binding assays were performed with whole phage to determine the apparent affinity of selected RGD loops. For these measurements individual phage were amplified and purified and then used as ligand in ELISA-type binding assays with immobilized \( \alpha_1 \beta_3 \). Because the display system was designed to express one copy of the peptide/phage, the concentration of peptide ligand was derived from the phage titer. This may be

| RGD sublibrary | Position of the RGD motif | Complexity | Representation |
|----------------|--------------------------|------------|---------------|
| 1              | CRGDXXXXXX       | 1.4 x 10³ | 75            |
| 2              | CXXXRGDXXXXX     | 2.5 x 10³ | 73            |
| 3              | CXXRGDXXXXC     | 2.6 x 10³ | 75            |
| 4              | CXXRXRGDXXC     | 2.0 x 10³ | 69            |
| 5              | CXXXRXRGDXXC     | 2.0 x 10³ | 99            |
| 6              | CXXXRXRGDXXC     | 2.1 x 10³ | 71            |

There is notable distinction between the phage selected in Ca2+ versus Mg2+. The data from the chemical shifts of Cα, Cα, and Hα as well as the coupling constants were used to calculate the backbone dihedral angles. The values for \( \chi_1 \) can thus be defined as \( \chi_1 = +60 \pm 30^\circ \). For NOE intensities of H2–H3 > H3–H4 and H2–H3 < H3–H4, then the values for \( \chi_1 \) were defined as \( \chi_1 = -60 \pm 30^\circ \). This is the most diverse library of RGD compounds reported to date.
Thirteen phage exhibited Mg$^{2+}$ in Ca$^{2+}$. These phage there was not a well defined consensus motif. The amino acid sequence of the random insert was derived from cDNA sequence analysis of each phage.

| Structural category and phage no. | Amino acid sequence |
|----------------------------------|---------------------|
| RGD in P5–7; aromatic in P3 and P8 | 31 H1FVPRGDYF |
|                                   | 19 RVPMPRGDWF |
|                                   | 33 KPWPRGDWF |
|                                   | 26 KGMPFRGDWF |
|                                   | 24 N1W5SRGDWF |
|                                   | 27 L1W5SRGDWF |
|                                   | 20 VR5MPRGDWF |
|                                   | 37 PAWHRGDNS |
|                                   | 3 LRVVRGDWDA |
|                                   | 34 IGWRVRGDWA |
|                                   | 17 STWL5RGDFL |
|                                   | 16 S2FVRGDWF |
| RGD in P5–7; aromatic in P8       | 2 TRLIRGDFF |
|                                   | 14 ERLHRGDVFV |
|                                   | 30 WRA5MRGDWFV |
|                                   | 7 R1HR5RGDFWF |
|                                   | 15 LRP5SRGDWF |
|                                   | 21 PVIPRGDWA |
|                                   | 39 TPVPRGDWA |
|                                   | 40 TSSFRGDWF |
| RGD in P5–7                       | 11 NHVVRGDLP |
|                                   | 36 YRLIRGDGA |
|                                   | 38 HRRLLRGDQV |
| RGD in P4–6, Pro at P8            | 35 YIRBGMPW |
|                                   | 32 RTRGDMPR |
|                                   | 28 YERKGMPF |
|                                   | 18 H1PRGDRPS |
|                                   | 10 FGVRGDSPR |
| RGD in P4–6; aromatic in P7       | 1 RVRVRGDVL |
|                                   | 9 RMVRGDPYT |
|                                   | 4 RMVRGDPFP |
|                                   | 29 SLRVRGDPFR |
|                                   | 16 KVRGDPWPT |
|                                   | 23 KVRGDRPST |
|                                   | 5 VVR5MGDPFG |
|                                   | 25 PHEPGDRSR |
| Inclusion of additional cysteines   | 8 A1ARGDCPT |
|                                   | 13 KCAIRGDCY |
|                                   | 11 YC5LRGDCY |

An overestimate of the concentration of peptide because it is possible that not all infective phage display the fusion protein. Virtually all of the phage we tested showed apparent affinities that range from 1 to 10 nM. The notable exceptions were phage A22 and A13, which had apparent affinities of ~0.1 nM (data not shown). These phage contain four cysteines that are likely to constrain the RGD motif more tightly through additional disulfides (see below).

Because it is conceivable that phage without any ion preference could be selected, further binding experiments were performed to identify those phage that exhibit ion-selective binding. As an initial screen, phage binding was measured in either 1 mM Ca$^{2+}$ or 1 mM Mg$^{2+}$ at phage concentrations that approximated the apparent $k_d$ measured above (data not shown). Phage that exhibited a 2-fold higher binding in one ion versus that other were considered to be selective binders. Based on these initial binding studies seven phage were found to have Ca$^{2+}$-selective binding (underlined in Table II). Yet, among these phage there was a not a well defined consensus motif. Thirteen phage exhibited Mg$^{2+}$-selective binding (underlined in Table III), and these generally fell within two closely related structural families. Within these families the RGD sequence occupies P4–6, a hydrophobic residue occupies P7, and two polar (often basic) residues occupy P8 and P9. Phage D16 and D18 are representative of this family.

To illustrate the ion-selective nature of the binding of these RGD motifs, the binding of two phage from each of these functional groups was measured as a function of time. These binding studies were performed in either 1 mM Ca$^{2+}$ or 1 mM Mg$^{2+}$ (Fig. 1). Phage A1 and A22 represent the Ca$^{2+}$-selective phage, and D16 and D18 represent the Mg$^{2+}$-selective motifs.

**Characterizing the Behavior of Synthetic Peptides Containing Ion-Selective Binding Motifs—Peptides based on the sequences of phage A1, A22 and D16 and D18 were synthesized and purified by reverse phase HPLC. The peptide derived from phage A22 contains four cysteine residues. Consequently, two peptides were synthesized, one with a 1,4:2,3 disulfide bond pattern and another with a 1,3:2,4 disulfide bond pattern. The**
peptide in the 1,3,2,4 conformation lacked biological activity and was not analyzed further.

To determine whether the synthetic peptides recapitulated the ion-selective binding observed for the corresponding phage RGD loop, each peptide was tested for the ability to block the binding of macromolecular ligands for α\text{III}β\text{3} in an ion-dependent manner. Fab-9, an antibody engineered to contain an RGD in the antigen binding site (28), was used as a model RGD interaction. Fab-9, an antibody engineered to contain an RGD loop, each peptide was tested for the ability to block the binding of Fab-9 binding. We suspect that the aberrant behavior of peptide A1 arises because it mimics the structure of the γ-chain of Fg (as opposed to RGD) and thereby binds to the Fab binding site on integrin (see "Discussion").

**Solution Structures of the Cyclic RGD Peptides**—To probe the structural basis of ion-selective binding, solution structures of the four ion-selective RGD peptides were solved by NMR. Representative ensembles of these structures are shown in Fig. 3. The structure of peptide A1 was based on 45 intraresidue, 39 sequential, and 33 longer range restraints with an imposed hydrogen bond between the carbonyl of Val at P3 and the amide of Asp at P6. Within this peptide, the VRGD sequence was found in a turn that could generally be ascribed to a type II β-turn based on Lewis criteria (34) (i.e. with one expected ± 30 Phi/Psi for Gly and Asp with an extra 15 degrees of freedom for one of the four angles). Peptide A22, which contains four cysteines linked in a 1,4,2,3 arrangement, was freely soluble only in Me\text{SO}. Although one might anticipate solvent effects to alter molecular dynamic trajectories, prior work with cyclic RGD pharmacophores shows that a subset of the conformations observed in Me\text{SO} is similar to those observed in water for the same peptide (35). The structure of A22 was based on 55 intraresidue, 45 sequential, and 23 longer range restraints. Although this peptide clearly forms a tight turn, with the RGD at the apex, it does not fall into any of the classic definitions of β-turns by strict criteria.

The structures of the two Mg\text{2+}-selective peptides, D16 and D18, appeared to be very similar from P2-P6. Their ensembles...
FIG. 2. Competition binding studies with ion-selective cyclic peptides. Peptides based on the sequences of phage A1 (panel A), A22 (panel B), D16 (panel C), and D18 (panel D) were synthesized and tested for the ability to block the binding of Fab-9 to purified αbβ3. The binding of Fab-9 was measured using an ELISA-type binding assay described under “Materials and Methods.” The binding of Fab-9 was measured across a range of competing peptide in buffer containing either 1 mM Ca2+ (■) or 1 mM Mg2+ (○). Each point is the average of triplicate measurements in which the S.E. was less than 12%. Results are representative of at least four similar experiments, each yielding nearly identical observations.

| Peptide | Ion   | Competing ligand IC50 (µM) |
|---------|-------|---------------------------|
| A1      | Ca2+  | 65 ± 10                   |
| A22     | Ca2+  | 140 ± 20                  |
| D16     | Mg2+  | 2 ± 0.5                   |
| D18     | Mg2+  | 1 ± 0.1                   |

were derived from fewer long range constraints. The scarcity of NOEs from these peptides may reflect a greater inherent flexibility. The structure of D16 was based on 40 intraregion, 27 sequential, and 4 long range restraints. The structure of D18 was based on 48 intraregion, 18 sequential, and 2 long range constraints. In both cases a hydrogen bond between the carboxyl at P3 and the amide of Asp at P6 was deduced from the protection of the amide proton of Asp to exchange with solvent. Both D16 and D18 display a basic patch that protrudes from the core of the peptide. This protrusion is comprised of two polar/basic residues that are found in virtually all of the motifs that bind selectively in Mg2+. This cationic surface is a necessary structural feature in achieving selective binding in this ion and may make contact with integrin that are absent when Ca2+ is present (see “Discussion”).

**DISCUSSION**

The RGD motif is the key recognition element for a large segment of the integrin protein family. However, there is an underlying binding specificity between integrins and RGD for which we still have no structural basis. In most cases, integrin binding RGD motifs are presented at the apex of β-turns (36–41). Yet, this body of information falls short of providing a systematic data set of key structure-activity relationships because the RGD loops are embedded within different protein backbones. We reasoned that such data could be obtained by using biochemical selection to obtain RGD loops with a given biochemical property and then probing the solution structures of these loops with NMR. As a test of the strategy, we created the most diverse library of RGD loops assembled to date and used this library to identify RGD loops that distinguish subtle conformational differences in the ligand binding pocket of a single integrin. Our findings indicate that even subtle differences in the shape of a single RGD binding pocket can be discerned using a library of sufficient diversity.

We screened for RGD motifs that can distinguish the αbβ3 integrin when its cation binding sites are occupied with either Ca2+ or Mg2+. RGD loops were identified with both biochemical properties. Loops from both selections could be grouped into well defined subfamilies with rather obvious consensus motifs. There are several common features among the peptides selected in the two ions. First, the RGD motif is observed most frequently at positions 4–6 and 5–7, slots that put the RGD at the apex of a loop or β-turn. Second, most of the RGD motifs contain at least one and often two, aromatic residues. In the vast majority of cases an aromatic residue follows directly after RGD. Tryptophan, phenylalanine, and tyrosine residues were all observed at this position. This observation is consistent with prior reports showing that the affinity of linear RGD peptides for αbβ3 is enhanced greatly by inclusion of a hydrophobic residue just after RGD (42, 43). Aromatic residues were often found at other positions, both preceding and following the RGD motif.

Despite the similarity in the positioning of RGD within the loops, there are other features that distinguish the two functional families. The primary distinguishing feature of the Mg2+-selected loops is the presence of two basic, or polar, residues that fall on the carboxyl-terminal side of the RGD. Two subfamilies of phage from the Mg2+ selection display this feature (Table III). Both subfamilies contain the RGD at P4–6. The small distinc-
tions arise at P7, which is occupied by a hydrophobic residue in one family and an aromatic residue in the other. There is also a distinction at P8, which is populated exclusively by Arg or Lys in the first subfamily but can be occupied by noncharged, but usually polar, residues in the second family. The NMR structures of peptides that represent both subfamilies, D16 and D18, show the basic patch to protrude from the base of the RGD loop. The findings presented here suggest that this patch is necessary for Mg$^{2+}$-selective binding.

The inherent flexibility of the loops is a second feature that appears to distinguish the Ca$^{2+}$- and Mg$^{2+}$-selective peptides. The two phage loops with the highest binding affinity and the highest degree of selectivity for binding in Ca$^{2+}$ are A13 and A22. Both of these loops contain two additional cysteine residues that presumably constrain the peptide further by forming an additional disulfide bond. This extra disulfide is expected to impose more rigidity onto the RGD loop, and the NMR data for A22 support this concept.

The NMR spectra for the peptides containing a single disulfide bridge are also consistent with enhanced flexibility for the Mg$^{2+}$-selected loops. Both D16 and D18 display fewer NOEs than their Ca$^{2+}$-selective counterpart, A1. The dearth of NOEs in D16 and D18 may be interpreted as increased conformational flexibility as has been observed for highly flexible or unfolded regions (44). It is also conceivable that NOEs for D16 and D18 are undetectable under the experimental conditions chosen. We view this latter possibility as unlikely because D16 and D18 are the same size as A1, where NOEs are clearly evident, and all three populate the same NOE regime. Furthermore, studies on D16 and D18 were performed under solvent and temperature conditions identical to those for A1. The strong sequential $\alpha$N NOEs and large spin-spin coupling constants ($^2J_{HN}$, above 9 Hz) of the A1 peptide (A1 spectrum and calculations in the supplementary material) may also be interpreted to indicate that this peptide has a more extended conformation (22). Spin-spin coupling constants were significantly lower for D16 and D18, suggesting enhanced flexibility compared with the Ca$^{2+}$-selective A1. Although the measurement of reduced heteronuclear NOEs is the method of choice for probing flexibility (45, 46) it requires $^{15}$N-labeled peptides, which are not available in our case.

The presumed flexibility of D16 and D18, superimposed to a local signature provided by their additional cationic patch, may well be an important regulator of the binding of physiologic ligands. Such inherent flexibility would allow for protein-protein interactions subsequent to initial contact with the RGD sequence. The precedent for differential rigidity in RGD-containing loops from homologous cell adhesion domains has been demonstrated for homologous fibronectin type III domains from tenascin and fibronectin (47). In the context of small peptidic inhibitors, other examples have been reported in which increased flexibility is needed for binding, e.g. for factor Xa inhibitors (48).

Our study also raises another important issue concerning recognition of ligands by the two distinct ligand binding pockets on IIb$3$. We showed previously that IIb$3$ contains two distinct ligand binding pockets that are linked allosterically.

InsightII modeling programs (Molecular Simulations, Inc.). Ensembles of peptides A1, A22, D16, and D18 are superimposed about C$\alpha$, N, O, and C' (positions P2–P8) along with their averaged backbone trajectories. Side chains of the RGD motif are annotated. Other side chains are absent for clarity. Panel C, the most representative conformers in the ensemble of each peptide is used to illustrate aspects of the electropositive surfaces, which were generated with the OLDERADO program (http://neon.chem.le.ac.uk/olderado/) and the GRASP program (http://trantor.bioc.columbia.edu/grasp/). The arginine of the RGD sequence is noted in each peptide. The protruding basic patch present in D16 and D18 is noted with ++.
(16). One binding pocket binds to the physiologic ligand Fg, and the other binding pocket binds to RGD ligands. An important and unappreciated aspect of the allosteric connection between these two binding sites is the fact that RGD ligands can bind to α1β3 even when Fg occupies its binding site. In doing so RGD ligands induce the dissociation of bound Fg. One observation from the present study may relate to this mechanism. Most of the RGD peptides from the current study inhibit Fg with far lower IC50 values than they exhibit for Fab-9. This is expected given that the RGD peptides and Fab-9 are anticipated to bind integrin. Interestingly however, peptide A1 behaved differently. It inhibited Fab-9 binding at far lower concentrations than required for inhibition of Fg binding. The reason for this difference in behavior is not entirely clear, but one is tempted to speculate that this peptide could actually be a mimic of the Fg γ-chain motif rather than a true RGD mimic. It would then compete directly with Fg for association with α1β3 but not Fab-9. Given the molecular diversity within the RGD superlibrury, nearly 500 million different motifs, it is not unreasonable to expect that some of these motifs could approximate the shape of the fibrinogen γ-chain, a motif of between 11 and 15 residues (18, 49). This idea is also consistent with the fact that both peptide A1 and the γ-chain peptide adopt type II β-turns in solution, with an aspartic acid near the apex of the turn.

Because α1β3 is an excellent paradigm for most other members of the integrin family, and because many other integrins also exhibit Ca2+- and Mg2+-regulated binding of physiologic ligands, the results presented here could potentially be extrapolated to other integrins. The availability of peptides that bind to the two conformations of α1β3 is likely to make it possible to determine the structures of ion-selective ligands while bound to integrin. A comparison of the peptide solution structures with the integrin-bound structures should provide a structural basis for the way that ions influence ligand binding specificity. Such information will provide additional framework for understanding ion-regulated binding of all integrins.

This study also lays the groundwork for two future lines of investigation. First, the ion-selective ligands identified here can be applied to test hypotheses regarding the role of ions in regulating platelet adhesion functions. It is well known that certain RGD-based antagonists have different effects on platelet aggregation versus bleeding times. These differences have often been attributed to a distinction in binding kinetics which is still not understood. We have speculated that thrombosis, which is driven by aggregation, is a Ca2+-controlled event, and that hemostasis, which is driven by adhesion to RGD-containing proteins, is a Mg2+-controlled event. The availability of peptides with well defined selectivity for binding in the two ions should make it possible to test this hypothesis.

Finally, the study validates the use of the RGD superlibrury for the selection of high affinity binders with exquisite specificity. We have shown previously that RGD-containing loops can be grafted into proteins, endowing them with integrin binding function. The availability of the superlibrary for identifying highly selective RGD loops should enhance our ability to design proteins that are targeted to specific integrin targets. This type of highly specific target could potentially enhance the therapeutic efficacy of protein-based drugs.

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