Directed protein evolution, which employs a combination of random mutagenesis, phage display, and in vitro selection, was used to identify second-site suppressors of the fibronectin (Fn) cell binding domain mutation Asp1585Ala (RGD). The mutations in the Fn 9th (3fn9) and 10th (3fn10) type III repeats obtained after selection on purified integrins zIIb/z3(D1585Y) and z5/z1 are reported. The 3fn9–10(D1585A) phage with substitution mutations at Asp1585, which is located within the linker region between 3fn9 and 3fn10, enhanced binding to the integrins zIIb/z3 and z5/z1, but not zx/z3. The substitution mutations identified at residue Asp1585 were introduced into the native recombinant 3fn9–10 sequence and found to augment binding to zIIb/z3, demonstrating that the observed gain-of-function phenotype was independent of the multivalent character of the phage. These results support the following conclusions. First, regions of Fn in addition to the RGD loop are in close proximity to zIIb/z3 and z5/z1 and are capable of participating in the binding to these integrins. Secondly, the conformational relationship between the 3fn9 and 3fn10 modules may be an important factor in the binding of Fn to these two integrins. Thirdly, other altered properties of Fn-integrin interactions, such as integrin specificity, may also be selected. This is the first description of Fn mutations that augment binding to integrins. The ability to select for particular phenotypes in vitro and the subsequent characterization of these mutations should further our understanding of the molecular details involved in the association of integrins and their ligands. Additionally, these higher-affinity 3fn9–10 ligands provide a starting point for further in vitro evolution and engineering of integrin-specific modules.

Key words: cell adhesion, integrin, platelet, phage display, type III repeat.

INTRODUCTION

The extracellular matrix molecule fibronectin (Fn) is a dimeric protein that functions in a variety of important physiological processes involving cell adhesion and migration [1]. Individual subunits of Fn are mosaic polypeptides that consist of three types of repeating structural elements designated as type I, type II, and type III repeats or modules (3fn) [2]. These structural modules combine to make up functional domains in Fn that interact with cell adhesion receptors, glycosaminoglycans, fibrin, and gelatin. The central cell-binding domain (CCBD) which is recognized by the integrin family of adhesion receptors is composed of multiple 3fn modules. Similar to the Ig module, each individual 3fn module forms a /-sandwich that possesses two /-sheets composed of three and four anti-parallel /-strands [3]. The folded module results in the amino and carboxyl termini on opposite ends allowing for end-on-end linkage [4–6]. In addition to Fn, the 3fn module is found in many functionally diverse proteins [7]. It appears that these structural building blocks can tolerate substantial sequence variation while maintaining the /-sandwich structure.

Integrins are heterodimeric glycoprotein receptors that play critical roles in cell adhesion and signal transduction [8,9]. The CCBD of Fn is recognized by zIIb/z3, z/3, and z5/z1, as well as five other members of the integrin family. These integrins utilize the sequence Arg1493-Gly1494-Asp1495 (RGD) located in the 10th type III (3fn10) module of Fn for binding. However, optimal binding of Fn by zIIb/z3 and z5/z1, but not zx/z3, also requires the presence of the 9th type III repeat (3fn9) [10–13]. In addition to the RGD site in Fn, zIIb/z3 and z5/z1 share a requirement for Fn residues Arg1569, Arg1571, and Arg1579 in 3fn9 and Arg1445 in the 3fn10 module [14,15]. Thus, the binding site on Fn for the integrins zIIb/z3 and z5/z1 involves multiple contacts on two adjacent 3fn modules.

Integrin-dependent binding of Fn to cells in solution can be relatively poor, yet when Fn is coated onto a solid surface its avidity for the same cells is significantly enhanced [16,17]. This suggests that there are conformational aspects to integrin-Fn binding in addition to the involvement of specific residues. The conformation or module–module solution dynamics of the 3fn9–10 module pair may be an important factor in Fn-zIIb/z3 or Fn-z5/z1 binding. Indeed, increasing the linker region between 3fn9 and 3fn10 by insertion of glycine residues reduced the avidity of the 3fn9–10 modules when examined in cell adhesion assays [18,19]. Unlike other 3fn or Ig module pairs, the relative rotation, extent of buried surface area, and degree of tilt between the 3fn9 and 3fn10 modules are all substantially smaller [6]. Analysis of the solution structures and dynamics of the 3fn9–10 module pair has shown that only a few nonspecific interactions exist between these two modules, allowing for substantial flexibility between 3fn9 and 3fn10 [19,20]. Taken together, a balance must exist between 3fn9–3fn10 flexibility and the correct spatial orientation of the contact residues required for integrin binding.

The process of in vitro evolution, which combines random mutagenesis, recombinant expression, and selection, has successfully been used to improve the binding affinity of antibodies for specific antigens (reviewed in [21]). In this study, in vitro evolution was used to determine whether the integrin binding properties of Fn 3fn modules could be enhanced. We describe a positive selection technique and the substitution mutants identi-
fied that exhibited an increased avidity for the integrins αIIbβ3 and α5β1. Gain-of-function mutations would be expected to arise via improvement of steric complementarity, creation of novel contacts between 3fn9–10 and the integrin, or stabilization of a preferred binding conformation. Thus, further insight into the molecular aspects of Fn–integrin binding beyond that provided by site-directed mutagenesis approaches might be attained by analysis of the isolated gain-of-function mutants. The results presented here demonstrate that substitution of Asp118 with valine, tyrosine, or glycine significantly augmented binding of the specificity. resulting in a conformation more favourable to integrin binding.

via alteration of the module–module interactions in solution modules, suggesting that the effects of these substitutions may be via alteration of the module–module interactions in solution resulting in a conformation more favourable to integrin binding. The success of this strategy provides a starting point for alteration of other aspects of integrin 3fn9–10 binding such as integrin specificity.

EXPERIMENTAL

Materials

Human Fn was purified as described previously [22]. Rabbit anti-phage specific antibodies were generated by standard methods using purified fUSE5 virions, without an insert in the cloning site, as the antigen [23]. The anti-Fn monoclonal antibodies (mAbs) fnI-11 and fnI-16 which recognize epitopes in the 3fn9 module have been described previously [11]. The rat anti-α5 mAb 2G12 was kindly provided by Dr V. Woods (Department of Medicine, The University of California, San Diego, U.S.A.). The hybridoma cell line expressing the anti-human IgG, Fc-specific HB43 and the cell line K562 were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Chinese hamster ovary (CHO) cells expressing human αIIbβ3(D118Y) have been described previously [24]. The peptide KTGRGDSP was synthesized by American Peptide Company (Sunnyvale, CA, U.S.A.). All chemical reagents were obtained from Sigma (St. Louis, MO, U.S.A.) unless otherwise stated.

Construction of fUSE5 libraries

Manipulation of recombinant DNA was by standard techniques [25]. Restriction and modification enzymes were purchased from Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.). Oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer. Dr Jamie K. Scott (Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, Canada) kindly provided the fUSE5 virions and Escherichia coli K91Kan [26,27]. DNA encoding the human 3fn9–10 module pair (residues G11924–T3159) was amplified by PCR from the plasmid pIH910 [12] with the oligonucleotide primers F5FNF (5′GCTCTAGAGCCGACGGGCTGATACCTGTCTTGATTCCAACTGG3′) and F5FNR (5′GGATCGA-TAGCCCCAGCGCCAGATCTTGTTCGTATTAATGGAAT3′). The PCR product was digested with BglII, subcloned into the fUSE5 phage vector digested with SfiI, and electroporated into E. coli MC1061. Phage particles produced by the transformed E. coli MC1061 grown in Luria–Bertani broth (LB) containing tetracycline (12.5 μg/ml) were then used to infect E. coli K91Kan to enrich for phage harbouring the 3fn9–10 insert. Infected E. coli K91Kan were plated for single colonies on LB containing kanamycin (50 μg/ml) and tetracycline (12.5 μg/ml). Single-stranded DNA was purified from phage particles isolated from individual clones and sequenced to verify the construct.

Phage libraries expressing 3fn9–10(D118Y) modules randomly mutated within designated 60 bp windows were constructed by subcloning the 3fn9–10(D118Y) encoding cDNA produced by the ‘mega-primer’ method into the fUSE5 vector [28]. First, individual primary PCR products were generated from the plasmid pQE9-10(D118Y)A encoding each of ten internally ‘spiked’ oligonucleotides and the F5FNR oligonucleotide [29]. The 60 base spiked oligonucleotides used for mutagenesis were synthesized using phosphoramidites deliberately contaminated with 1% of the other three phosphoramidites. The gel-isolated primary PCR products were used as primers with the F5FNF oligonucleotide to generate a set of secondary full-length PCR products from the plasmid pQE9-10(D118Y)A template. Each secondary PCR product was digested with BglI and subcloned into the fUSE5 vector digested with SfiI. The fUSE5 vector ligated with the randomly mutated 3fn9–10(D118Y) encoding cDNA was then electroplated into E. coli MC1061 and phage purified by polyethylene glycol (PEG) precipitation. The number of independent clones from each library was determined by plating an aliquot of electroplated E. coli MC1061 directly on LB plates containing 12.5 μg/ml tetracycline.

Purified integrin microtiter well assay

The purified integrin binding assay was performed in modified Tyrode’s buffer (2.5 mM Hepes, 150 mM NaCl, 2.5 mM KCl, 12 mM NaHCO3, and 1 mg/ml BSA, pH 7.4) containing 1 mM MgCl2, as described previously [11]. Phage binding to purified integrin was performed as described for biotinylated Fn, except that bound phage were disclosed by addition of rabbit anti-phage antisera, and subsequent incubation with biotinylated donkey anti-rabbit IgG specific antibody (Chemicon International, Inc., Temecula, CA, U.S.A.). Human αIIbβ3 was purified from normal platelets by mAb PMI-1 affinity chromatography [15]. Human αIIbβ3(D118Y) was purified from transfected CHO cells by PMI-1 affinity chromatography. Human α5β1 was purified from K562 cells by KTGRGDSP-Sepharose affinity chromatography as described previously [30], except that the cells were lysed in 10 mM Hepes, pH 7.4, 150 mM NaCl, 50 mM n-octyl-β-d-glucopyranoside, 1 mM Pefobloc (Roche Molecular Biochemicals, Laval, Quebec, Canada), 10 μg/ml leupeptin, 1 mg/ml n-ethylmaleimide, and 1 mM MnCl2. Purified human αvβ3 was purchased from Chemicon International (Temecula, CA, U.S.A.).

Selection of gain-of-function mutants

Each phage library was individually subjected to four rounds of selection on a purified integrin. Immulon 2 (Dynatech Laboratories, Inc., Chantilly, VA, U.S.A.) 96-well plates were coated for 16 h at 4 °C with 1 μg/ml purified integrin diluted in Hepes/Saline/MgCl2 (10 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM MgCl2). The wells were then blocked for 1 h at 22 °C with either 3% BSA (rounds one and three) or 3% blotto (rounds two and four) diluted in Hepes/Saline/MgCl2. One hundred representations of a phage library diluted into 100 μl of modified Tyrode’s buffer was added to each of ten microtiter wells and incubated for 16 h at 4 °C. The wells were then washed ten times with modified Tyrode’s buffer and eluted with 20 μl/well of 100 mM glycine, pH 2.0, for 20 min at 22 °C. The eluted phage from the library were combined and immediately neutralized with 20 μl of 1 M Tris, pH 10. The neutralized phage suspension was used to infect E. coli K91Kans for amplification and PEG purification. The amplified phage were then diluted 1:5 in modified Tyrode’s
buffer and subjected to another round of selection on the purified integrin. Individual colonies were isolated by infecting E. coli K91Kans with various dilutions of phage and plating on LB agar containing kanamycin (50 μg/ml) and tetracycline (12.5 μg/ml). The sequence of the entire 3fn9–10 coding region was determined by nucleotide sequencing of isolated single-stranded DNA from purified phage.

**Expression of recombinant 3fn modules**

DNA encoding the human 3fn9–10 module pair (residues G1526–T1539) was PCR amplified from the plasmid pHi910 with the oligonucleotides BGL2910F (5’GAAGATCTGGATTCGCGCT-CTGGCATG3’) and MalR2 (5’tcgcTATTACGCGCTG-CGCGT3’), digested with BglII and HindIII, subcloned into the hexa-histidine expression vector pQE30 (Qiagen Inc., Valencia, CA, U.S.A.) with the restriction enzymes BamHI and HindIII, and transformed into E. coli M15[pREP4]. Oligonucleotide-directed mutagenesis was performed using PCR overlap extensions with the pHi910 plasmid as the template [31,32]. Oligonucleotides were synthesized by the Molecular Biology Resource Facility at the University of Oklahoma Health Sciences Centre. DNA encoding a mutant form of the 3fn9–10 module pair was digested with the restriction enzymes BglII and HindIII, then subcloned into the plasmid pQE30 as described above. The correct coding sequences for the native and mutant forms of recombinant 3fn9–10 modules were verified by nucleotide sequencing of the insert in the plasmid.

**Expression of hexa-histidine fusion proteins**

Cells harbouring the various pQE30-3fn9–10 recombinant plasmids were grown in SB media (30 g/l tryptone, 20 g/l yeast extract, 10 g/l 13-(N-morpholino)propane-sulphonic acid, pH 7.0) plus 100 μg/ml ampicillin and 50 μg/ml kanamycin at 37 °C to an A600 of 0.5–0.7. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 2 mM and the cells were further incubated at 37 °C for 2 h. The cell culture was centrifuged at 4000 g for 10 min, the pellet resuspended in sonication buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl), and stored at −80 °C. The thawed cell suspension was sonicated, centrifuged at 11000 g for 20 min at 4 °C, the supernatant removed and diluted 1:3 in sonication buffer, and affinity purified on Ni-nitrilotriacetate agarose (Qiagen, Chatsworth, CA, U.S.A.). Material bound to the Ni-nitrilotriacetate agarose was washed with 50 mM sodium phosphate, pH 6.0, 300 mM NaCl, 10 % (v/v) glycerol and then eluted with the same buffer at pH 4.0. The purified fusion protein was dialysed in PBS and examined on a 12 % (w/v) polyacrylamide gel. The correct amino acid composition was verified by matrix-assisted laser desorption/ionizing time-of-flight MS.

**Biochemical procedures**

Protein concentrations were determined by the bicinchoninic acid assay (Pierce Chemical Company, Rockford, IL, U.S.A.). ELISAs were performed by standard procedures in Immulon 2 96-well plates [33]. The 3fn9–10 module pair was 125I-labelled by the Iodogen procedure (Pierce Chemical Company). Fn was biotinylated as previously described [34]. Biotinylated proteins were revealed by incubation with avidin–biotin-horseradish peroxidase (Vector Laboratories, Inc., Burlingame, CA, U.S.A.), and development with o-phenylenediamine.

**RESULTS**

Integrin recognition of filamentous phage expressing 3fn9–10

Random mutagenesis and in vitro selection techniques were used in this study to identify substitution mutations that augment the binding of the Fn 3fn9–10 modules to the integrins αIIb/3 and α5/β1. First, filamentous phage expressing the human 3fn9–10 modules as an amino-terminal fusion with the bacteriophage gene III product were tested for the ability specifically to bind purified αIIb/3 and α5/β1 (Figure 1). The 3fn9–10 phage bound both integrins in a dose dependent and saturable manner (Figure 2a and c). The fUSE5-3fn9–10 phage binding was also shown to be specific and require the 3fn9–10 domains (Figure 2b and d). In addition, both functions blocking anti-Fn mAbs, fn1-11 and fn1-16, and an RGDS containing peptide, inhibited binding of fUSE5-3fn9–10 to these integrins. Phage expressing the phage gene III product without a 3fn9–10 fusion or with a 3fn9–10(D159S,A) substitution mutation did not bind to either integrin at the concentrations examined (Figure 2a). These results demonstrate that the phage expressing the 3fn9–10 module pair was specifically recognized by the integrins αIIb/3 and α5/β1 in a manner similar to Fn.

In vitro evolution of Fn

Recovery of function from a mutant phenotype by pseudoreversion is well-documented in bacterial genetics [35,36]. We applied this approach to a single molecule and selected for pseudorevertants of an alanine substitution mutation (D159S,A) in the RGD
Various colony-forming units (CFU/ml) of fUSE5-3fn9–10 phage (●) were added to microtiter wells coated with purified αIIbβ3 (a) and α5β1 (c). Binding of fUSE5 phage expressing no pil fusion (■) and fUSE5-3fn9–10(D1495A) (○) to purified αllbβ3 are also shown (a). Integrin-bound phage were discarded with anti-phage polyclonal sera and developed with biotinylated donkey anti-rabbit-IgG specific antibody, streptavidin conjugated peroxidase, and o-phenylenediamine. Background was determined by binding in the presence of 5 mM EDTA and subtracted to show specific binding. FUSE5-3fn9–10 Phage (1 × 10^6 CFU/ml) were added to microtiter wells coated with purified αIIbβ3 (b) and α5β1 (d) in the presence of no inhibitor, an inhibitory anti-αIIbβ3 mAb (2G12), an irrelevant mAb (HB43), an inhibitory anti-α5 mAb (16), the peptide KTGRGDSP (RGD), and two inhibitory anti-Fn mAbs (fnI-11) and (fnI-16). Bound phage were quantified as above.

Binding (%) = (phage bound(EDTA) – phage bound(EDTA))/phage bound(no inhibitor) × 100. The mean and S.D. of triplicate determinations are shown.

Figure 2 Specific recognition of fUSE5-3fn9–10 phage by the integrins αIIbβ3 and α5β1

Various colony-forming units (CFU/ml) of fUSE5-3fn9–10 phage (●) were added to microtiter wells coated with purified αIIbβ3 (a) and α5β1 (c). Binding of fUSE5 phage expressing no pil fusion (■) and fUSE5-3fn9–10(D1495A) (○) to purified αllbβ3 are also shown (a). Integrin-bound phage were discarded with anti-phage polyclonal sera and developed with biotinylated donkey anti-rabbit-IgG specific antibody, streptavidin conjugated peroxidase, and o-phenylenediamine. Background was determined by binding in the presence of 5 mM EDTA and subtracted to show specific binding. FUSE5-3fn9–10 Phage (1 × 10^6 CFU/ml) were added to microtiter wells coated with purified αIIbβ3 (b) and α5β1 (d) in the presence of no inhibitor, an inhibitory anti-αIIbβ3 mAb (2G12), an irrelevant mAb (HB43), an inhibitory anti-α5 mAb (16), the peptide KTGRGDSP (RGD), and two inhibitory anti-Fn mAbs (fnI-11) and (fnI-16). Bound phage were quantified as above.

Binding (%) = (phage bound(EDTA) – phage bound(EDTA))/phage bound(no inhibitor) × 100. The mean and S.D. of triplicate determinations are shown.

site of 3fn9–10 to identify gain-of-function mutations that have enhanced avidity for the integrins αIIbβ3 and α5β1. Random substitution mutations were introduced into cDNA encoding 3fn9–10(D1495A) by spiked oligonucleotide mutagenesis [29]. The advantage of this method over others is that mutational ‘hot spots’ within the template are virtually eliminated and there is a defined probability of two adjacent base changes which allows substitution of all 19 residues at a particular codon. Ten spiked oligonucleotides were designed to span the entire 3fn9–10 coding sequence, except codon 1495. Each of the spiked oligonucleotides were then used to generate one of ten individual fUSE5-3fn9–10(D1495A) libraries mutated within a defined 20 residue window (Table 1). Each library contained between 9.5 × 10^9 to 6.6 × 10^9 independent clones. The extent of mutagenesis was characterized by sequencing twenty randomly selected clones from the 1416–1435 library. These sequences were found to have 25% with no nucleotide changes, 10% with one, 40% with two, and 25% with three nucleotide changes within the 60 bp region. In addition, it was observed that 31% of sequences had transitions and 69% had transversions as would be expected if the mutagenesis were indeed random.

We initially attempted to select for 3fn9–10(D1495A) pseudorevertants that were able to bind αIIbβ3 purified from normal platelets. However these experiments resulted in the isolation of only true D1495 revertants (RGA to RGD) which presumably arose from spontaneous mutagenesis during PCR or phage replication. To circumvent the selection of revertants, purified αIIbβ3 with a D1495 substitution in the β3 subunit was used to select for pseudoreversions of 3fn9–10(D1495A). Previous studies have shown that αIIbβ3(D1495Y) does not bind to RGD-containing peptides [24]. We reasoned that using the αIIbβ3(D1495Y) integrin would prevent the selection of 3fn9–10(D1495A) revertants, but permit the selection of pseudorevertants that effect binding to other regions of the integrin. Purified αIIbβ3(D1495Y) did not bind 125I-labeled Fn in vitro (Figure 3a). However, purified αIIbβ3(D1495Y) was recognized by the heterodimer specific mAb 2G12 (Figure 3b). Each of the randomly mutated phage libraries was individually subjected to four rounds of selection on microtiter wells coated with either αIIbβ3(D1495Y) or native α5β1 purified from the human erythroleukaemic cell line K562. No true revertants of the 3fn9–10(D1495A) substitution were identified using native α5β1. After the fourth round of selection, the complete nucleotide sequence of the 3fn9–10 coding region was determined for at least 12 isolated phage from each library. The substitutions found in each library after selection on αIIbβ3(D1495Y) and α5β1 are shown in Table 1. Most
of the substitutions were localized to the same molecular face of 3fn9–10 shown to interact with αIIbβ3 and α5β1 by alanine scanning mutagenesis [14,15].

### Table 1  Isolated pseudorevertants

The "Mutagenesis window" column identifies the Fn residues spanned by the spiked oligonucleotides (regions spanned in Fn cDNA are in parentheses). The substitutions associated with selection with integrins αⅡbβ3 and α5β1 are described in the two right-hand columns (number of sequences found with each substitution/total number sequenced in parenthesis). N.S., no substitutions.

<table>
<thead>
<tr>
<th>Complexity of library (no. of independent clones)</th>
<th>Mutagenesis window</th>
<th>Integrin</th>
<th>αⅡbβ3</th>
<th>α5β1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.3 x 10⁶</td>
<td>G1326-L1345 (1326–1345)</td>
<td>PM-1 affinity purified</td>
<td>N.S. (8/12), P1347L (2/12)</td>
<td>I133T (6/12), S1350T (2/12)</td>
</tr>
<tr>
<td>9.2 x 10⁶</td>
<td>T1334L-P1336 (1344–1363)</td>
<td></td>
<td>T133S (1/12), T1344P (1/12)</td>
<td>T1335p (2/12), S1342A (2/12)</td>
</tr>
<tr>
<td>1.2 x 10⁶</td>
<td>N1342-A1399 (1362–1391)</td>
<td>H134P (11/12)</td>
<td>P1345Q (4/12), H1348p (4/12)</td>
<td>A1359G/Y1360S (2/12)</td>
</tr>
<tr>
<td>1.5 x 10⁶</td>
<td>(1380–1399)</td>
<td>W1358L/Y1359F (1/12)</td>
<td>V1360L (2/12)</td>
<td>P1370R (12/12)</td>
</tr>
<tr>
<td>2.0 x 10⁶</td>
<td>G1338–S1417 (1398–1417)</td>
<td>R13398H (10/12)</td>
<td>N1358K/L1359F/V1360S (8/12)</td>
<td>S1361T/P1362R (2/12)</td>
</tr>
<tr>
<td>6.6 x 10⁶</td>
<td>V1366–I1363 (1416–1435)</td>
<td></td>
<td>T1382S (2/12)</td>
<td>N.S. (8/12), G1402R (2/12), I1416S (2/12)</td>
</tr>
<tr>
<td>1.2 x 10⁶</td>
<td>L1401-E1453 (1434–1453)</td>
<td></td>
<td>N.S. (8/12), G1402R (2/12), I1416S (2/12)</td>
<td></td>
</tr>
<tr>
<td>3.5 x 10⁶</td>
<td>G1412-T1471 (1452–1471)</td>
<td></td>
<td>N.S. (8/12), G1402R (2/12), I1416S (2/12)</td>
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</tr>
<tr>
<td>2.1 x 10⁶</td>
<td>S1478-R1493 (1470–1493)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2.0 x 10⁶</td>
<td>S1496–T1509 (1496–1509)</td>
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</tr>
</tbody>
</table>

Figure 3  Heterodimer formation of purified αⅡbβ3(D119Y)

To demonstrate that purified αⅡbβ3(D119Y) formed a heterodimer and was dysfunctional for Fn binding, PM-1 affinity purified αⅡbβ3 and αⅡbβ3(D119Y) were coated onto microtiter wells at a concentration of 2.0 μg/ml. After blocking the plate in 2% BSA the integrins were examined by incubation with strepavidin-conjugate-peroxidase and development with o-phenylenediamine. The mean and S.D. of triplicate determinations are shown.

Substitutions at Fn residue 1418 augment αⅡbβ3 and α5β1 integrin binding

Interestingly, substitutions obtained within the 3fn9–3fn10 linker region (Y1416, T1419) appeared to specifically target the Asp1188 residue when selected on αⅡbβ3(D1395Y) or α5β1 (Figure 4). Extension of the linker region has been previously shown to decrease α5β1 dependent cell adhesion [18], suggesting that the spatial relationship between the 3fn9 and 3fn10 modules affects integrin-Fn binding. Phage encoding 3fn9–10(D1490A) with each of the identified Asp1418 substitutions were amplified, purified, and various concentrations of each phage were assayed for the ability to bind purified αⅡbβ3, α5β1, and αvβ3. All the Asp1418 substitutions except D1418A demonstrated increased binding to αⅡbβ3 and α5β1 compared with the parental D1418A phage (Figure 5). As found with αⅡbβ3 and α5β1, the SEU5 phage expressing the native 3fn9–10 module pair bound purified αvβ3. However αvβ3 did not bind any of the Asp1418 substitution phage significantly better than the SEU5–3fn9–10(D1490A) phage, indicating that these substitutions were integrin specific in their effect.

To determine if the Asp1418 substitution mutations augmented binding of native 3fn9–10 in monomeric form, each of the Asp1418 substitutions were introduced into the native 3fn9–10 coding region by site-directed mutagenesis and expressed recombinantly in E. coli. The recombinant (r)3fn9–10 modules were tested for the ability to inhibit 10 nM biotinylated Fn binding to purified αⅡbβ3 (Figure 6). Each of the Asp1418 mutations identified by in vitro selection demonstrated increased inhibitory potency compared to the native r3fn9–10 module pair. A hierarchy for activity was observed with V(IC50 = 4 ± 2 nM) > G (IC50 = 21 ± 4 nM) > Y (IC50 = 33 ± 5 nM) > A (IC50 = 138 ±
Figure 4  Location of residue D1418 in the linker region between the 3fn9 and 3fn10 modules
(a) The α-carbon backbone and (b) a space-fill (stereo view) representation of the 3fn9–10 modules. The spatial location and side groups of Asp1418, as well as Fn residues required for optimal binding to both αIIbβ3 and α5β1 (1369, 1371, 1379, 1445, 1493, and 1495), are shown.

Figure 5  Integrin specific recognition by pseudorevertant phage
1 × 10^10 CFU/ml of FUSE5-3fn9–10 phage containing the designated substitution mutations were added to microtiter wells coated with 1 μg/ml purified αIIbβ3 (white bars), α5β1 (black bars), and αvβ3 (grey bars). The phage were incubated with the integrin for 16 h at 4 °C, non-bound phage were removed by washing, and bound phage quantified as described in Experimental procedures. FUSE5-3fn9–10 phage binding (% mutant phage binding (integrons) / [native 3fn9–10 phage binding (integrons)] × 100. The mean and S.D. of triplicate determinations are shown.

Figure 6  Inhibition of Fn binding to purified αIIbβ3 by D1418 substitutions
Various concentrations of recombinant 3fn9–10 proteins with no substitution ( ), D1418Y (●), D1418V (□), and D1418G (△) substitution were examined for the ability to inhibit 10 nM biotinylated Fn binding to purified αIIbβ3 coated microtitre wells. The amount of bound Fn was determined by incubation with avidin-conjugated peroxidase and developed with o-phenylenediamine. Fn binding (%) = [B(no inhibitor) - B(EDTA)] /[B(inhibitor) - B(EDTA)] × 100. The mean and S.D. of triplicate determinations are shown.
fold lower than that observed for native r3fn9–10 (220 ± 28 nM). These results demonstrate that the gain-of-function mutations isolated by in vitro selection have a similar phenotype when examined as monomeric r3fn9–10 modules containing the native RGD1199 sequence.

**DISCUSSION**

In this study we have used a combination of phage display and random mutagenesis to identify second-site suppressors of the Fn cell binding domain mutation Asp1199Ala (RGA). Introducing the substitution mutations identified at residue Asp1199 into the native recombinant 3fn9–10 sequence demonstrated that the observed gain-of-function was independent of the multivalent character of the phage and the Arg-Gly-Asp site. These results indicate the following: (1) regions of Fn in addition to the RGD loop are in close proximity to zIibβ3 and z5p1 and are capable of participating in the binding to these integrins, (2) the conformational relationship between the 3fn9 and 3fn10 modules may be an important factor in the binding of Fn to these two integrins, and (3) other altered properties of Fn–integrin interactions, such as integrin specificity, may also be selected. This is the first description of Fn mutations selected for the ability to augment binding to integrins. The ability to select for particular phenotypes in vitro and the subsequent characterization of these mutations should further our understanding of the molecular details involved in the association of integrins and their ligands. Additionally, these higher-affinity 3fn9–10 ligands provide a starting point for further in vitro evolution and engineering of integrin-specific modules.

Identification of Asp1199Ala (RGA) pseudorevertants that are able to bind zIibβ3(Asp1199Tyr) indicate that residues outside the Arg-Gly-Asp sequence of Fn and the MIDAS motif of the β3-subunit participate in integrin-Fn binding [37,38]. Both zIibβ3 and z5p1 require Fn residues in the 3fn9 and 3fn10 modules in addition to the RGD sequence for optimal binding of this ligand [10–13, 39]. The ability to identify mutations outside the RGD loop of Fn that augment binding to these integrins indicates that other regions of the 3fn9 and 3fn10 modules are in close proximity to the receptors. Although some buried residues were substituted, most substitutions were on the surface and the majority of these surface substitutions were localized to the same face of the 3fn9–10 molecule shown to interact with zIibβ3 and z5p1. It should also be noted that none of the residues previously shown to be involved in Fn binding to either zIibβ3 or z5p1 by site-directed mutagenesis were altered under our selection parameters. Many of the substitutions isolated with zIibβ3(31199Y) were also obtained with z5p1, further suggesting that these two integrins recognize 3fn9–10 by similar mechanisms. Our results are in keeping with a model of Fn–zIibβ3 and Fn–z5p1 interaction involving an extended interface of Fn [14,15] with the 3fn9 module and RGD sequences making contacts with the z-subunit and β-subunit, respectively [40]. Mapping the regions of the z-subunit, upper face of β-propellers 2 and 3, which interact with the synergy site of 3fn9 [41,42], onto the structure of zvβ3 [38] suggests that this region lies 20–40 Å (1Å = 0.1 nm) distant from the MIDAS motif. The distance between the Arg1269 of 3fn9 and Asp1199 of 3fn10 is 37Å [6]. Taken together with previous studies, these measurements and our results support a model for integrin binding involving an extended interface between Fn and the integrins zIibβ3 and z5p1.

Pseudorevertants may arise from an improvement of steric complementarity, the creation of a new contact, or stabilization of a preferred binding conformation. The zIibβ3(D1199Y) receptor was purified as a heterodimer capable of binding the Fn gain-of-function mutations. However, since we were unable to demonstrate that zIibβ3(D1199Y) exists in an ‘active’ conformation when bound to plastic microtiter wells, some substitutions isolated on this integrin may arise from the ability to bind the ‘resting’ form of zIibβ3 with higher avidity. Although the molecular mechanism by which the Asp1199 mutations augment integrin binding is not directly demonstrated, several results support the stabilization of a preferred binding conformation as the mechanism. First, multiple residues resulted in an enhanced binding to zIibβ3 and z5p1, suggesting that the higher affinity binding observed was not via the creation of a novel contact. Secondly, the variation in size of the side groups accommodated suggests that the observed phenotype was not a result of improved steric complementarity. Thirdly, the lack of augmentation for binding the integrin zvβ3 suggests that the effect may be due to enhanced interaction with the 3fn9 module. Both zIibβ3 and z5p1 require residues in the 3fn9 module of Fn for optimal binding. NMR studies of both human and mouse 3fn9–10 module pairs have shown that there is extensive flexibility between the 3fn9 and 3fn10 modules [19,20]. Structure–function studies in which glycine residues were inserted between the 3fn9 and 3fn10 modules demonstrated a decrease in apparent affinity for z5p1, either by disrupting the spacing between the 3fn9 and 3fn10 binding sites or by increasing the flexibility between these modules [18]. We hypothesize that the Asp1199 substitution mutations may bind the integrins zIibβ3 and z5p1 by restricting the flexibility of the two modules, thus providing a better ‘fit’ for binding these integrins. A detailed analysis of the structural dynamics, such as that performed on the native 3fn9–10 module pair, will be required to stringent test this hypothesis.

In vitro evolution has been applied previously to improving antibody–antigen affinity (e.g., [43–47]). The ability to enhance the affinity of the 3fn9–10 module pair for zIibβ3 and z5p1 demonstrates the general utility of in vitro evolution for selection of proteins or structural modules with new binding characteristics. These higher affinity 3fn9–10 ligands should be amenable to similar in vitro evolution approaches that select for preferential binding of one integrin over another. Considering the differential requirement for the 3fn9 module by the related integrins zIibβ3 and zvβ3 [12], the identification of a 3fn9–10 ligand that preferentially binds zIibβ3 may be possible. Mutations that alter the binding specificity between zIibβ3 and z5p1 may be rarer because of the similarities in the mechanisms for Fn recognition used by these two integrins. The resilience of the 3fn module in accepting substitution mutations and its structural similarity to Ig modules may permit more unique altered binding properties, such as engineering non-integrin protein–protein interactions.

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