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Engineered Cystine-Knot Peptides That Bind $\alpha\beta3$ Integrin With Antibody-Like Affinities

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Abstract

The $\alpha\beta3$ integrin receptor is an important cancer target due to its overexpression on many solid tumors and the tumor neovasculature, and its role in metastasis and angiogenesis. We used a truncated form of the Agouti-related protein (AgRP), a 4 kDa cystine-knot peptide with four disulfide bonds and four solvent-exposed loops, as a scaffold for engineering peptides that bound to $\alpha\beta3$ integrins with high affinity and specificity. A yeast-displayed cystine-knot peptide library was generated by substituting a 6-amino acid loop of AgRP with a 9-amino acid loop containing the Arg-Gly-Asp (RGD) integrin recognition motif and randomized flanking residues. Mutant cystine-knot peptides were screened in a high-throughput manner by fluorescence-activated cell sorting (FACS) to identify clones with high affinity to detergent-solubilized $\alpha\beta3$ integrin receptor. Select integrin-binding peptides were expressed recombinantly in *Pichia pastoris* and were tested for their ability to bind to human cancer cells expressing various integrin receptors. These studies showed that the engineered AgRP peptides bound to cells expressing $\alpha\beta3$ integrins with affinities ranging from 15 nM to 780 pM. Furthermore, the engineered peptides were shown bind specifically to $\alpha\beta3$ integrins, and had only minimal or no binding to $\alpha\beta5$, $\alpha5\beta1$, and $\alpha1\beta3$ integrins. The engineered AgRP peptides were also shown to inhibit cell adhesion to the extracellular matrix protein vitronectin, which is a naturally-occurring ligand for $\alpha\beta3$ and other integrins. Next, to evaluate whether the other three loops of AgRP could modulate integrin specificity, we made second generation libraries by individually randomizing these loops in one of the high affinity integrin-binding variants. Screening of these loop-randomized libraries against $\alpha\beta3$ integrins resulted in peptides that retained high affinities for $\alpha\beta3$ and had increased specificities for $\alpha\beta3$ over $\alpha1\beta3$ integrins. Collectively, these data validate AgRP as a scaffold for protein engineering and demonstrate that modification of a single loop can lead to AgRP-based peptides with antibody-like affinities for their target.

Keywords

protein engineering; yeast display; RGD; integrin; cystine-knot

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Introduction

The $\alpha v\beta 3$ integrin receptor has generated much clinical interest as a cancer target because of a correlation between $\alpha v\beta 3$ integrin expression and the invasiveness of tumors, particularly melanomas and liver cancer.¹⁻³ Integrins are a class of cell surface adhesion receptors consisting of α and β subunits that form heterodimers in various $\alpha\beta$ configurations, each one of which has its own ligands and cell signaling properties.⁴ Integrins bind to extracellular matrix (ECM) proteins such as vitronectin, fibronectin, collagens, and laminins, which promotes cell adhesion to the ECM and activation of signaling pathways involved in cell cycle progression.⁵ Consequentially, certain integrins have been implicated in tumor cell growth, invasion, and metastasis, as well as angiogenesis. In particular, $\alpha v\beta 3$ integrins are highly expressed on activated endothelial cells in the tumor neovasculature, but are weakly expressed in resting endothelial cells and most normal tissues and organs.^{1,6} Therefore, there is a critical need for compounds that will target $\alpha v\beta 3$ integrins with high binding affinity and specificity for cancer diagnosis and therapy.⁷⁻⁹ Roles for $\alpha v\beta 5$ and $\alpha 5\beta 1$ integrins in tumor growth and metastasis have also been proposed, but these integrins are expressed on a wider range of non-cancerous cell types.²

Many integrins, including those containing the αv subunit, as well as $\alpha 5\beta 1$ and $\alpha iib\beta 3$ integrins, recognize an Arg-Gly-Asp (RGD) tripeptide motif found in ECM ligands, including fibronectin, vitronectin, fibrinogen, and osteopontin.¹⁰ The linear RGD peptide sequence has a much lower affinity for integrins than the proteins from which it is derived, due to conformational specificity afforded by folded protein domains not present in linear peptides.¹¹ Although the RGD sequence can be promiscuously recognized by various integrins, the sequence and structural contexts in which it is presented are critical in determining the molecular properties of a given ligand-integrin interaction. In natural ligands, the RGD motif is typically found in flexible solvent-exposed loops; however, the amino acid residues flanking the RGD motif and hence the three-dimensional orientation of these loops varies substantially across integrin ligands. Consequentially, the myriad RGD-containing ligands are differentially recognized by the various integrin receptors. For example, the RGD-ligand osteopontin binds only to $\alpha v\beta 3$ on platelets even though $\alpha iib\beta 3$ integrins are present on these cells at 100-fold greater concentration than $\alpha v\beta 3$ integrins.¹²

The development of peptides and proteins that bind to integrins has been facilitated by use of the RGD sequence. While it has been fairly straightforward to insert RGD motifs into linear or cyclic peptide libraries and screen for integrin binders with micromolar affinities,¹³ generation of peptides that bind with therapeutically relevant concentrations (low nanomolar) or high specificities to particular integrins requires that the RGD sequence is appropriately positioned for binding the integrin of interest.¹⁴ Like natural integrin ligands, the affinities and specificities of these RGD-containing peptides and proteins are largely dependent on the orientation of the Arg and Asp residues, as well as the conformation of the RGD loop, which is dictated by the amino acids flanking the RGD sequence. Rigidifying the RGD motif by backbone cyclization or placing it within a disulfide-bonded loop can improve integrin binding affinities;^{15,16} however, development of peptides and proteins that are selective for a particular integrin has been difficult because sequences or structural motifs that dictate integrin binding specificity are unclear or unknown.

While there are a number of examples in the literature of linear^{13,17} or cyclic peptides,^{18,19} as well as antibodies,^{20,21} that target integrins, there has been limited work in developing small, structured peptide scaffolds as high affinity integrin-binding agents. Non-antibody protein scaffolds have attracted attention recently because of the limitations of antibodies, which include expensive recombinant production in mammalian cells, poor penetration of solid tumors, inability to site-specifically incorporate chemical modalities, and large

intellectual property barriers for development.²² Members of the cystine-knot family attracted our interest as scaffolds for engineering integrin-binding peptides because they overcome these limitations and have several additional advantages. Cystine-knots (also known as knottins), are small, compact peptides (typically 20-60 amino acids) that consist of a core of at least 3 disulfide bonds that are interwoven into a “knot” conformation.²³ As a result, they possess high thermal and proteolytic stability, and they are amenable to both chemical synthesis and recombinant expression.²⁴ There is great sequence diversity among cystine-knot family members as only the disulfide-bonded core is conserved; consequently, the loops connecting the cysteine residues are highly tolerant to substitution or incorporation of additional amino acids.²⁵

In this work, we engineered a cystine-knot peptide, the 34-residue C-terminal domain of the human Agouti-related protein (AgRP), to bind to $\alpha\text{v}\beta 3$ integrin with high binding affinity and specificity. We substituted a 6-amino acid loop in AgRP with a 9-amino acid loop containing an RGD motif, created a library of mutants by randomizing the residues flanking the RGD sequence, and identified clones with high affinity for $\alpha\text{v}\beta 3$ integrins using yeast surface display. We found that the engineered AgRP peptides bound to $\alpha\text{v}\beta 3$ integrins with antibody-like affinities and were highly specific for $\alpha\text{v}\beta 3$ over other integrins.

Results

Engineering AgRP to bind to $\alpha\text{v}\beta 3$ integrins using yeast surface display

Our initial protein engineering efforts were based on the idea that one of the AgRP loops could be substituted with a loop containing an RGD sequence, and a library of mutants with randomized residues flanking the RGD motif could be then screened for $\alpha\text{v}\beta 3$ integrin binders. We chose to engineer loop 4 of the AgRP peptide (Figure 1a) because it is the most solvent-exposed of the AgRP loops and could presumably offer the greatest surface area and flexibility for interaction with an integrin target receptor. In addition, there were several similarities between AgRP loop 4 and an RGD-containing loop within the 10th domain of fibronectin: both loops are flanked by anti-parallel beta sheets and are relatively similar in size and shape (Figure 1a-b). We modeled sequences from the fibronectin RGD loop into loop 4 of AgRP using ICM-Pro (Molsoft) and found that substitution of the sequence TGRGDSPAS showed the greatest structural similarity to the conformation of the original fibronectin loop. Consequently, we used the relative position of the RGD motif in this sequence to design our initial AgRP mutant library. Using degenerate codons (NNS; where N = A, C, G, or T; S = G or C), we constructed a library of AgRP genes by overlap extension PCR, replacing loop 4 (RFFNAF) with the sequence XXRGDXXXX, where X is any amino acid (Figure 1C). The library DNA was electroporated with digested (linear) yeast-display plasmid pCT into the *S. cerevisiae* strain EBY100²⁶ as described.^{27,28} Homologous recombination afforded a library of approximately 5×10^6 yeast transformants. In our experience, cystine knot libraries created in this manner contain roughly 40-60% frame-shift mutations and internal stop codons; however, DNA sequencing indicated that these aberrant clones were removed after three rounds of library screening by flow cytometric sorting.

The yeast library was induced in selective media containing galactose to promote expression of AgRP mutants as fusions to the cell-surface Aga2p protein. A schematic of the yeast display system and the cell surface labeling/detection strategies are shown in Figure 2a. The yeast display construct contains a C-terminal cMyc epitope tag (EQKLISEEDL) (Figure 2a), which allows AgRP expression levels to be monitored using an anti-cMyc antibody. The library was subjected to seven rounds of screening by fluorescence-activated cell sorting (FACS) to identify AgRP mutants that were well expressed on the yeast cell surface and that bound high levels of $\alpha\text{v}\beta 3$ integrin. For each round of screening, yeast were incubated at

room temperature in the presence of detergent-solubilized $\alpha\beta 3$ integrin and a chicken anti-cMyc antibody for 2 h, followed by fluorescein- and phycoerythrin-labeled secondary antibodies, respectively, as shown in Figure 2a. To increase the sorting stringency, the concentrations of $\alpha\beta 3$ integrin were decreased sequentially over sorting rounds, from 500 nM in round one to 25 nM in round seven, as indicated in Figures 2b-e. After two rounds of FACS using a square sort gate, cells were collected along a diagonal sort gate to select for clones that bound $\alpha\beta 3$ integrin most tightly for a given expression level (Figure 2b-e). We sequenced 10-12 clones after each sort round; unique sequences from sort rounds 6 and 7 are shown in Table 1. All of the clones isolated in sort round 7 had previously appeared in sort rounds 5 and/or 6, indicating enrichment of these mutants in the final sorting rounds. The sequences obtained did not show any obvious consensus; however, some trends were observable: a glycine residue immediately preceded the RGD sequence, and several hydrophilic or charged residues followed the RGD sequence.

We randomly chose several AgRP clones isolated from sort rounds 6 and 7 and measured their affinities for $\alpha\beta 3$ integrins using a direct binding assay on the surface of yeast. As a negative control, we also displayed an AgRP mutant on the yeast surface with the same sequence as clone 7C, except that the RGD sequence was scrambled as RDG. All of the AgRP mutants tested from sort rounds 6 and 7 bound to 50 nM $\alpha\beta 3$ integrin. In contrast, the RDG negative control did not bind $\alpha\beta 3$ integrin, as expected (data not shown). We selected five mutants that possessed high affinities to $\alpha\beta 3$ integrin when tethered to the yeast surface for further study: 6C, 7A, 7C, 7E, and 7J (Table 1).

Soluble expression of engineered AgRP peptides

P. pastoris was chosen for recombinant expression of the engineered AgRP peptides, as it has been successfully used to express proteins with disulfide bonds and significant secondary structure.²⁹ The eukaryotic quality control machinery in the secretory pathway of yeast should help ensure proper folding and high levels of soluble expression of the AgRP peptides, which have four disulfide bonds and complex folds. Using conditions and procedures described in the *P. pastoris* expression kit (Invitrogen K1750-01), AgRP clones 6C, 7A, 7C, 7E, and 7J were produced in yields of 3-10 mg/L culture. The engineered AgRP peptides were expressed with N-terminal FLAG epitope tags (DYKDDDDK) and C-terminal hexahistidine tags (HHHHHH) for use as handles in purification and cell binding assays through antibody detection. The expressed peptides were purified by Ni-affinity chromatography and determined to be >90% pure by reversed-phase HPLC and gel-filtration chromatography. SDS-PAGE analysis was performed on reduced and non-reduced peptides (Supporting Information, Fig. SI-1). Peptide composition was confirmed and exact concentrations were determined by amino acid analysis (data not shown), and masses were obtained by MALDI-TOF mass spectrometry (Supporting Information, Table SI-1).

To determine whether the FLAG and hexahistidine tags would interfere with $\alpha\beta 3$ integrin binding, we prepared one of the AgRP clones, 7C, without epitope tags by solid-phase peptide synthesis using standard Fmoc chemistry. The crude, reduced peptide was purified by reversed-phase HPLC, then oxidized with glutathione and DMSO, as previously described.³⁰ The fully oxidized peptide was purified from unfolded and misfolded states by reversed-phase HPLC, and the mass was confirmed by mass spectrometry (Supporting Information, Table SI-1).

To compare the $\alpha\beta 3$ integrin binding affinities of synthetic and recombinant AgRP peptides, we performed a competition binding assay using U87MG glioblastoma cells, which express approximately 10^5 $\alpha\beta 3$ receptors per cell, as well as $\alpha\beta 5$ and $\alpha 5\beta 1$ integrins.³¹ Recombinant AgRP peptide 7C (20 nM) was pre-incubated with 10^5 cells and binding was then competed off using synthetic AgRP peptide 7C at concentrations ranging

from 1 nM to 500 nM. After washing, the cells were stained with a fluorescein-conjugated anti-FLAG antibody and then analyzed by flow cytometry. Competition of synthetic peptide by recombinant peptide was performed analogously. These experiments gave essentially identical half-maximal inhibitory concentration (IC_{50}) values (22 ± 3 nM and 23 ± 6 nM, respectively; Supporting Information, Fig. SI-2), suggesting that both recombinant and synthetic AgRP peptides are correctly folded and that the FLAG and His epitope tags on the recombinant peptide do not interfere with integrin binding.

Integrin binding affinity and specificity of engineered AgRP peptides

We next performed direct equilibrium binding titrations of the recombinant engineered AgRP peptides on U87MG glioblastoma cells. Peptides were incubated with cells for 3 h at 4 °C, followed by staining with a fluorescein-conjugated anti-His antibody and analysis by flow cytometry. Equilibrium binding constant (K_D) values were obtained by fitting plots of concentration versus mean fluorescence intensity to a sigmoidal curve using KaleidaGraph software (Figure 3a and Table 2). All five engineered AgRP peptides tested bound with low nanomolar to high picomolar affinity, and the tightest binder, 7A, showed 17-fold improvement over the worst binder, 6C, isolated from sort round 6. We note that the saturation levels for the different clones vary roughly with affinity. This suggests that the mutants have different binding off-rates that dictate their K_D values, with weaker binding clones having faster off-rates. Alternatively, the differences in saturation levels could be due to integrin receptor clustering that is differentially elicited or stabilized by clones with varying affinities.

To determine the binding specificities of the AgRP peptides for $\alpha v \beta 3$ integrin versus the $\alpha v \beta 5$ and $\alpha 5 \beta 1$ integrins also expressed on U87MG cells, we obtained K562 leukemia cells that had been stably transfected with individual α and β integrin subunits.³² We first tested to see whether the engineered AgRP peptides could bind to untransfected K562 cells, which intrinsically express $\alpha 5 \beta 1$ integrin. Equilibrium binding assays were performed on the untransfected K562 cells as described above for the U87MG cells. We tested 3 peptide concentrations, and observed negligible signal over background levels (cells stained with fluorescein-conjugated anti-His antibody alone) even at 500 nM, the highest concentration tested (Supporting Information, Fig. SI-3). This demonstrated that the engineered AgRP peptides do not appreciably bind to $\alpha 5 \beta 1$ integrin, and that the K562 cells transfected to express other integrins would be useful in determining integrin binding specificity.

We next tested to see whether the engineered AgRP peptides could bind to K562 cells expressing $\alpha v \beta 5$, $\alpha i i b \beta 3$, or $\alpha v \beta 3$ integrins. We initially tested the peptides at three concentrations and found very little signal over background for cells expressing $\alpha v \beta 5$ integrin, even at 500 nM, the highest concentration tested (Supporting Information, Fig. SI-3). The peptides bound weakly to the K562 cells expressing $\alpha i i b \beta 3$ integrins and, as expected, strongly to the K562 cells expressing $\alpha v \beta 3$ integrins. In order to determine K_D values for the engineered AgRP peptides against K562- $\alpha i i b \beta 3$ and K562- $\alpha v \beta 3$ cells, we performed binding titrations over a larger range of concentrations (Figure 3b and 3c). We were able to obtain K_D values for AgRP peptide binding to K562- $\alpha v \beta 3$ cells; these values were essentially identical to the K_D values obtained for the U87MG cells, indicating that binding of engineered AgRP peptides to U87MG cells is mediated by $\alpha v \beta 3$ integrin (Table 2). K_D values for peptide binding to K562- $\alpha i i b \beta 3$ cells could not be determined as we were unable to fit the data to sigmoidal curves because binding was still increasing at the highest concentration (5 μ M) of peptides tested (Figure 3c). However, from the data we estimate that the K_D values for AgRP peptide binding to the K562- $\alpha i i b \beta 3$ cells are much greater than 100 nM.

Inhibition of vitronectin-mediated cell adhesion by engineered AgRP peptides

We determined whether the engineered AgRP peptides could inhibit cell adhesion mediated by vitronectin, the primary ligand for $\alpha\beta3$ integrin.⁸ We incubated K562- $\alpha\beta3$ cells with varying concentrations of peptides in microtiter wells coated with vitronectin to determine the ability of the peptides to inhibit cell adhesion. The engineered AgRP peptides were able to block vitronectin-mediated adhesion of the K562- $\alpha\beta3$ cells with IC_{50} values ranging from 9.9 to 650 nM (Figure 4a and Table 2). The IC_{50} values for inhibition of cell adhesion were 6- to 67-fold greater than the K_D values against the K562- $\alpha\beta3$ cells. This difference may be a result of multivalent interactions between the cell surface $\alpha\beta3$ integrins and the immobilized vitronectin, thereby making it more difficult for the peptides to compete.

We also tested whether the engineered AgRP peptides could block vitronectin-mediated adhesion to the U87MG cells using an analogous assay. However, adhesion of the U87MG cells was only partially blocked by the peptides, even at concentrations up to 1 μ M. In contrast, the RGD-containing disintegrin echistatin, which binds strongly to $\alpha\beta3$, $\alpha\beta5$, $\alpha5\beta1$, and $\alpha i i b \beta 3$ integrins,³³ blocked U87MG cell adhesion to vitronectin with an IC_{50} of 5.8 nM (Figure 4b). The AgRP peptides may not effectively block U87MG adhesion compared to the K562- $\alpha\beta3$ cells because the $\alpha\beta5$ integrins co-expressed on the surface of the U87MG cells could also contribute to vitronectin-mediated adhesion and compensate for the loss of $\alpha\beta3$ integrin function. These data provide further evidence that the engineered AgRP peptides bind to $\alpha\beta3$ but not to $\alpha\beta5$ integrins.

Mutagenesis of AgRP loops 1, 2 and 3

We next determined whether modification of the remaining AgRP loops (loops 1, 2, or 3) could alter integrin binding affinity or specificity. We also expected these experiments would provide information about the tolerance of the other AgRP loops to mutagenesis for further protein engineering studies. We prepared yeast-displayed libraries using clone 7C as a starting point, with loops 1, 2, or 3 individually substituted with randomized sequences using degenerate codons (Figure 5a). We subjected these randomized loop libraries to four rounds of screening by FACS to ascertain whether it would be possible to select mutants that retained binding to $\alpha\beta3$ integrin. In each screening round the yeast were labeled for peptide expression using the cMyc epitope and incubated with 50 nM $\alpha\beta3$ integrin, followed by staining with fluorescently-labeled secondary antibodies. Although the initial libraries showed significantly diminished binding to $\alpha\beta3$ integrin compared to the parent clone 7C, mutants that retained affinity for $\alpha\beta3$ integrin were enriched after four rounds of screening (data not shown).

After sort round four, six yeast-displayed clones were chosen at random from each loop-mutagenized library (Table 3), and were tested for their ability to bind integrins. Yeast displaying each mutant were treated with 50 nM $\alpha\beta3$, 50 nM $\alpha i i b \beta 3$, or 50 nM $\alpha\beta5$ integrin, then stained with an appropriate fluorescein-conjugated anti-integrin antibody and analyzed by flow cytometry. None of the clones bound to $\alpha\beta5$ integrin (data not shown). In contrast, all of the clones bound $\alpha\beta3$ integrin at levels close to that of the parent clone 7C (Figure 5b). The clones also weakly bound to $\alpha i i b \beta 3$ integrin, albeit at lower levels compared to the parent clone 7C (Figure 5c). The ratio of $\alpha\beta3$ binding to $\alpha i i b \beta 3$ binding was increased for all of the clones over the parent clone 7C. These data suggest that AgRP loops 1, 2, and 3 are in principle tolerant to mutagenesis and they may contribute to binding specificity through either direct integrin contacts or through structural changes in the engineered AgRP peptides.

Discussion

In this study, we engineered AgRP, a small compact cystine-knot peptide with no intrinsic binding affinity for integrins, to bind to $\alpha v\beta 3$ integrins with low nanomolar affinity and high specificity over other RGD-recognizing integrins. AgRP was selected as a protein engineering scaffold for several reasons: (i) it is small (4 kDa) and rigid, yet contains four solvent-accessible loops that could be used for mutagenesis; (ii) it is likely to be non-immunogenic because it is of human origin and its high thermal and proteolytic stability; (iii) loop 4 of AgRP resembled the RGD-containing loop of the 10th domain of fibronectin in that it is flanked by two anti-parallel beta sheets; (iv) its small size makes it amenable to both recombinant and synthetic production, which will allow site-specific incorporation of labels or chemical functionality in future studies.^{30,34} The natural AgRP loop 4 is made up of six residues flanked at either end by cysteines that form a disulfide bond which is not part of the core cystine-knot fold. We replaced the native RFFNAF sequence from AgRP loop 4 with a 9-amino acid loop containing the RGD motif, and randomized the flanking residues in the orientation XXRGDXXXX. The size of this loop and the register of the RGD motif were derived from gross molecular modeling studies of the RGD-containing loop of fibronectin.

Integrin-binding AgRP mutants were engineered using yeast surface display, which has been shown previously to be a robust platform for the expression and screening of combinatorial libraries of proteins with disulfide bonds and complex folds such as antibodies,³⁵ T-cell receptors,³⁶ and epidermal growth factor.³⁷ Yeast display offers several advantages over other display platforms; in particular, the use of dual-color FACS for library screening affords quantitative discrimination between AgRP mutants based on normalization of yeast expression levels with $\alpha v\beta 3$ integrin binding. In addition, the secretory pathway of yeast acts as a quality control mechanism, ensuring that correctly folded peptides are displayed on the cell surface,²⁷ which is presumably critical for the expression of AgRP mutants that contain the cystine-knot structure and that will retain their function when untethered from the yeast surface.

After 7 rounds of screening the randomized AgRP loop 4 library by FACS, all of the clones selected bound $\alpha v\beta 3$ integrins with high affinity. The presence of the RGD sequence was necessary for $\alpha v\beta 3$ binding, as a high-affinity mutant with a scrambled RDG sequence in place of RGD did not exhibit binding to $\alpha v\beta 3$ integrin. The flanking residues were also important in positioning the RGD motif for $\alpha v\beta 3$ recognition, as most expressing clones in the initial randomized library did not bind $\alpha v\beta 3$ integrin (Figure 2b). Engineered AgRP peptides were expressed and purified from *P. pastoris*, and competition binding experiments against a synthetic AgRP mutant showed that neither the N-terminal FLAG tag nor the C-terminal His tag interfered with binding (Supporting Information, Fig. SI-2). The recombinant peptides bound to $\alpha v\beta 3$ integrins on the surface of U87MG glioblastoma and K562- $\alpha v\beta 3$ cells with affinities ranging from 780 pM to 15 nM. Furthermore, we showed that other loops of AgRP could be mutated with minimal effects on $\alpha v\beta 3$ integrin binding affinity, but with improved specificity for $\alpha v\beta 3$ over $\alpha iib\beta 3$ integrins (Figure 5). In future studies it would be interesting to screen these AgRP-7C loop 1, 2, and/or 3 libraries to identify mutants with enhanced integrin binding affinity or altered integrin binding specificity.

In comparison to previous examples of integrin-binding peptides, linear and cyclic RGD peptides isolated from phage libraries typically have shown affinities in the high nanomolar to micromolar range.^{17,38} Similarly, RGD motifs have been substituted into lysozyme³⁹ and tissue plasminogen activator (tPa)⁴⁰ but only modest affinities for $\alpha v\beta 3$ integrin were achieved. Reiss, et al. grafted an RGD-containing $\alpha iib\beta 3$ integrin-binding peptide

recognition motif from the disintegrin obtustatin into AgRP, but found that it was less effective in inhibiting platelet aggregation than natural obtustatin.⁴¹ These protein engineering studies failed to generate integrin binders with therapeutically relevant affinities, presumably because the sequence and hence, structural context, of the RGD-containing loop was not optimized. In contrast, in cases where RGD was inserted into a protein scaffold such as T lymphocyte-associated antigen 4 (CTLA-4)⁴² or tendamistat¹⁴ and libraries with randomized flanking residues were screened for integrin binding, proteins with low nanomolar binding constants could be isolated. In a related approach, Richards et al. engineered the 10th domain of fibronectin for enhanced affinity and specificity for $\alpha v \beta 3$ integrin by randomizing the residues flanking RGD.⁴³ In comparison, our work provides a unique example where a small, constrained peptide scaffold has been engineered to target $\alpha v \beta 3$ integrins with high affinity and specificity, and is one of the first examples where yeast surface display has been used to engineer new recognition properties into a scaffold with a non-antibody (Ig)-like fold.

Our binding studies demonstrate that the engineered AgRP peptides are selective for $\alpha v \beta 3$ over several other integrins that also recognize RGD sequences, namely $\alpha v \beta 5$, $\alpha 5 \beta 1$, and $\alpha i i b \beta 3$. The engineered peptides did not bind at all to $\alpha v \beta 5$ or $\alpha 5 \beta 1$, whereas weak binding was observed against $\alpha i i b \beta 3$ integrin. This result was somewhat serendipitous considering negative selections were not performed against these related integrins. It has been a challenge in the past to select peptides that are selective for $\alpha v \beta 3$ over $\alpha i i b \beta 3$ and vice versa.¹⁴ RGD ligands bind to $\alpha v \beta 3$ and $\alpha i i b \beta 3$ near β -propeller loops of the α subunit that form a cap subdomain and a so-called specificity-determining loop in the $\beta 3$ subunit.⁴⁴⁻⁴⁶ Structural differences between αv and $\alpha i i b$ are responsible for variations in the cap subdomain and the $\beta 3$ specificity-determining loop, while the remainder of the αv and $\alpha i i b$ β -propeller structures are conserved.⁴⁶ Consequently, specificity of RGD-containing peptides for $\alpha v \beta 3$ versus $\alpha i i b \beta 3$ is controlled by the deeper β -propeller pocket of $\alpha i i b$. The Arg residue in RGD must be in an extended conformation to reach into the $\alpha i i b$ pocket to hydrogen bond with $\alpha i i b$ -Asp224, while αv -Asp150 and αv -Asp218 residues are found in a shallower pocket. Furthermore, $\alpha i i b \beta 3$ shows a preference for aliphatic residues flanking RGD, as αv -Asp218 is replaced by a hydrophobic Phe231 in $\alpha i i b$.⁴⁶ The engineered AgRP peptides we tested have predominantly hydrophilic or charged residues flanking RGD, which may clash with $\alpha i i b$ -Phe231.

Unfortunately, no crystal structures are available for $\alpha v \beta 5$ or $\alpha 5 \beta 1$ integrins. However, ligand binding to $\alpha 5 \beta 1$ requires a synergy site found on the 9th type III fibronectin domain in addition to the RGD site in the 10th domain, which may explain why the engineered AgRP peptides do not bind $\alpha 5 \beta 1$ integrin.⁴⁷ Without an $\alpha v \beta 5$ crystal structure, it is more difficult to explain the specificity for $\alpha v \beta 3$ over $\alpha v \beta 5$, especially since most engineered proteins and peptides that bind to $\alpha v \beta 3$ also bind $\alpha v \beta 5$ integrin.¹⁴ It appears likely that, like many other ligands with a preference for integrins with the $\beta 3$ subunit, the RGD sequence in the engineered AgRP peptides is oriented by the $\beta 3$ specificity-determining loop for optimal hydrogen bonding to αv subunit residues. Consequentially, the presumably differently structured specificity-determining loop in the $\beta 5$ subunit may not properly direct the RGD sequence for binding to $\alpha v \beta 5$ integrin. Structural information on our engineered peptides and $\alpha v \beta 5$ integrins will be necessary to fully understand their molecular recognition properties.

In summary, this work has shown the feasibility of using AgRP as a scaffold for peptide engineering using yeast surface display. We have demonstrated that AgRP loop 4 can be engineered to bind a target receptor with high affinity, and that other AgRP loops can be further engineered to modulate affinity and specificity. The peptides engineered in this study show promise for specific targeting to $\alpha v \beta 3$ integrins on cancer cells, and in the future we

will evaluate their potential as tumor-imaging and therapeutic agents in mouse models. Finally, this work shows the potential for using short recognition motifs to generate new molecular recognition properties in cystine-knot scaffolds, which may prove useful for targeting other clinically important receptors.

Materials and Methods

Media and reagents

YPD media contained 20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract. Selective SD-CAA media contained 20 g/L glucose, 6.7 g/L yeast nitrogen base without amino acids, 5.4 g/L Na₂HPO₄, 8.6 g/L NaH₂PO₄·H₂O, and 5 g/L Bacto casamino acids; SG-CAA media was identical except glucose was replaced with galactose. Media and plates for *P. pastoris* (RDB, BMMY, BMGY) were prepared as described in the Multi-Copy Pichia Expression Kit (Invitrogen K1750-01). $\alpha\text{v}\beta 3$ and $\alpha\text{v}\beta 5$ integrins were purchased from Millipore as octyl- β -D-gluopyranoside formulations, and $\alpha\text{ii}\beta 3$ integrin was purchased from Enzyme Research Laboratories as a Triton-X100 formulation. Integrin binding buffer (IBB) was composed of 20 mM Tris pH 7.5 with 1 mM MgCl₂, 1 mM MnCl₂, 2 mM CaCl₂, 100 mM NaCl, and 1 mg/mL bovine serum albumin. BPBS buffer was composed of phosphate-buffered saline and 1 mg/mL bovine serum albumin.

AgRP library synthesis

In the initial library, the fourth loop of AgRP (RFFNAF) was substituted with the sequence XXRGDXXXX, where X represents a randomized position. Libraries were prepared by PCR with Taq polymerase (Invitrogen) in the presence of DMSO using overlapping primers. Positions for randomization contained the NNS degenerate codon, which codes for all 20 amino acids and the TAG stop codon. The assembly PCR products were then amplified using primers with 40 bp overlap to the pCT plasmid upstream or downstream of the NheI and BamHI restriction sites, respectively. Amplified PCR products were purified on an agarose gel and electroporated into *S. cerevisiae* strain EBY100,²⁶ along with pCT plasmid that had been linearized with NheI and BamHI restriction enzymes for homologous recombination in yeast. Electroporation reactions contained 0.5-1 μg linearized plasmid and 5-10 μg insert. The yeast were allowed to recover in YPD media at 30 °C for 1 h and were then transferred into selective SD-CAA media.^{27,28} Libraries consisted of $\sim 5 \times 10^6$ transformants as estimated by plating serial dilutions on SD-CAA plates. Yeast cultures were grown to confluence (OD₆₀₀ = 10-12) and then split into new SD-CAA cultures. Cells were induced in 5 mL cultures of SG-CAA media at 30 °C.

AgRP library screening

For library screening, various concentrations of $\alpha\text{v}\beta 3$ integrin were added to yeast (typically $5\text{-}25 \times 10^6$ cells) suspended in IBB for 2 h at room temperature. Without washing, a 1:250 dilution of chicken anti-c-myc IgY (Invitrogen A-21281) was added, and the cells were incubated for an additional hour at room temperature. The yeast were then centrifuged at 4 °C, and the resulting supernatant was removed by aspiration. Next, yeast were resuspended in ice-cold BPBS containing a 1:25 dilution of fluorescein-conjugated anti- αv antibody (Biolegend 327907) and a 1:100 dilution of phycoerythrin-conjugated goat anti-chicken IgY secondary antibody (Santa Cruz Biotechnology sc-3730) for 30 min on ice. Cells were washed as above and FACS was performed to select $\alpha\text{v}\beta 3$ integrin binders using a Vantage SE/DiVa Vantoo instrument (Stanford FACS Core Facility) and CellQuest software. For the first round of sorting, $\sim 2 \times 10^7$ yeast were run through the flow cytometer. Collected cells were cultured in SD-CAA media, induced for expression in SG-CAA media, and subjected to additional rounds of FACS to obtain an enriched population of yeast displaying high-affinity $\alpha\text{v}\beta 3$ integrin binding peptides. For subsequent sort rounds, at least 10 times the

number of yeast collected in the previous round were screened to ensure the remaining library diversity was sampled. Sort stringency was also increased by decreasing the $\alpha\beta 3$ integrin concentration in subsequent rounds as follows: round 1 = 500 nM, round 2 = 250 nM, round 3 = 100 nM, rounds 4-5 = 50 nM, rounds 6-7 = 25 nM. Plasmid DNA was recovered from yeast clones using a Zymoprep kit (Zymo Research) and transformed into XL-1 blue supercompetent *E coli* cells (Stratagene) for plasmid miniprep. DNA sequencing of resulting clones was performed by Elim Biopharmaceuticals (Hayward, CA).

Mutagenesis and screening of AgRP loop 1, 2, and 3 libraries

Libraries of AgRP clone 7C (Table 1) with loop 1, 2, or 3 substituted with 7, 7, or 6 randomized amino acid residues, respectively, were prepared as described above. For screening, each library was incubated with 50 nM $\alpha\beta 3$ integrin and 2×10^7 cells were sorted by FACS as described above. Three additional rounds of FACS were performed to enrich for a pool of clones that retained binding to $\alpha\beta 3$ integrin. In these subsequent sort rounds the libraries were oversampled at least 10-fold to ensure diversity was maintained, but the integrin concentration was kept at 50 nM. Clones from the fourth round of sorting were isolated and sequenced as described above.

Recombinant and synthetic production of engineered AgRP mutants

Peptides were expressed recombinantly using the Multi-Copy Pichia Expression Kit (Invitrogen K1750-01). The open reading frame encoding the clone of interest was inserted into pPIC9K plasmid between the AvrII and MluI restriction sites. In addition, DNA encoding for a FLAG tag was inserted between SnaBI and AvrII sites, while DNA encoding for a hexahistidine tag was inserted between MluI and NotI restriction sites. $\sim 10 \mu\text{g}$ of plasmid was linearized by cutting with SacI then electroporated into the *P. pastoris* strain GS115. Yeast were allowed to recover on RDB plates and were then transferred to YPD plates containing 4 mg/mL geneticin. Geneticin-resistant colonies were grown in BMGY and then induced in BMMY. Cultures were grown for 3 days with methanol concentration maintained at $\sim 0.5\%$.

AgRP Clone 7C was also prepared without FLAG or His tags using solid-phase peptide synthesis on a CS Bio peptide synthesizer (Menlo Park, CA) using standard Fmoc chemistry. The peptide was purified by reversed-phase HPLC and then folded using 4 M guanidine, 10 mM reduced glutathione, 2 mM oxidized glutathione, and 0.5 M DMSO at pH 7.5. The correctly folded peptide was separated from unfolded and partly folded peptides by reversed-phase HPLC, where it appeared as a single peak with a shorter retention time than unfolded or misfolded precursors. All peptides, recombinant and synthetic, were characterized by amino acid analysis (AAA Service Laboratory, Damascus, OR) and MALDI-TOF mass spectrometry (Stanford Protein and Nucleic Acid Facility), which gave a single peak corresponding to the fully folded protein containing four disulfide bonds.

Cell binding assays

All cell lines were cultured at 37 °C with 5% CO₂. Adherent U87MG cells were obtained from ATCC and cultured in DMEM media (Gibco 11995) supplemented with 10% fetal bovine serum. Untransfected K562 cells ($\alpha 5\beta 1$ -positive) were obtained from ATCC and cultured in suspension in IMDM media (Gibco 12440) supplemented with 10% fetal bovine serum. K562 cells stably transfected with $\alpha\beta 3$, $\alpha\beta 5$, or $\alpha\text{IIB}\beta 3$ integrins were obtained from S. Blystone³² and were grown in media supplemented with 10 $\mu\text{g}/\text{mL}$ geneticin. Equilibrium binding assays were performed with 10^5 cells per reaction. Cells were suspended in IBB with varying amounts of engineered AgRP peptide at 4 °C for 3 h with gentle rocking. The cells were washed and resuspended in BPBS with a 1:40 dilution of fluorescein-conjugated anti-6X-His antibody (Bethyl A190-113F) and incubated on ice for

20 min. After washing, the cells were analyzed by flow cytometry using a BD FACSCalibur instrument and CellQuest software (Becton Dickinson, Franklin Lakes, NJ). Mean fluorescence intensity values for each cell population was plotted against concentration on a log scale. Data was fit to sigmoidal curves to obtain equilibrium dissociation constants using KaleidaGraph (Synergy Software), and is presented as average values with standard deviations. Each assay was performed a minimum of three times.

Vitronectin-mediated cell adhesion assays

Varying concentrations of AgRP peptides were mixed with 10^5 U87MG or K562- $\alpha v \beta 3$ cells and added to human vitronectin-coated 96-well microplates (R&D Systems CWP003). The plates were incubated at 37 °C with 5% CO₂ for 2 h, then the wells were washed 2 times with phosphate buffered saline (PBS). A solution of 0.2% crystal violet in 10% ethanol was added to the wells for 10 min, then the wells were washed 3 times with PBS. Solubilization buffer (a 1:1 mixture of 0.1 M NaH₂PO₄ and ethanol) was added and the plate was gently rocked for 15 min to completely solubilize the crystal violet. Absorbance of the wells was measured at 600 nm using a microtiter plate reader (BioTek Instruments), and data was normalized to a negative control containing no peptide. Half-maximal inhibitory concentration (IC₅₀) values were generated by fitting a sigmoidal curve to plots of log concentration peptide versus percent adhesion. Data is presented as average values with standard deviations. Experiments were performed at least three times.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) Structure of AgRP (PDB entry 1hyk) with loop 4 (sequence RFFNAF) shown in red and disulfide bonds shown in yellow. (a) Structure of fibronectin (PDB entry 1fna) with RGD loop (sequence TGRGDSPAS) shown in red and RGD sequence shown in blue. Note that proteins are not shown to scale. (c) Schematic of our protein engineering strategy.

Figure 2.

Use of yeast surface display to engineer AgRP to bind to $\alpha\beta 3$ integrins. (a) Schematic of the yeast display system. AgRP mutants are displayed as fusions to the yeast cell wall protein Aga2p. Expression of AgRP mutants is measured through detection of a C-terminal cMyc epitope tag using a chicken anti-cMyc primary and a phycoerythrin-labeled goat anti-chicken secondary antibody. $\alpha\beta 3$ integrin binding is detected with a fluorescein-labeled mouse anti-integrin antibody. (b)-(e) Density plots showing sort rounds 1, 3, 5, and 7 for the AgRP loop 4 XXRGDXXXX library. Polygons indicate flow cytometry sort gates used to select yeast cells displaying AgRP peptides that bound to $\alpha\beta 3$ integrin. (b) Round 1, 500 nM $\alpha\beta 3$ integrin (c) Round 3, 100 nM $\alpha\beta 3$ integrin. (d) Round 5, 50 nM $\alpha\beta 3$ integrin. (e) Round 7, 25 nM $\alpha\beta 3$ integrin.

Figure 3. Binding titrations of engineered AgRP peptides to cell-surface integrins. (a) U87MG cells. (b) K562- $\alpha v \beta 3$ cells. (c) K562- $\alpha i i b \beta 3$ cells. Data show mean arbitrary fluorescence units for 10^4 cells bound with varying concentrations of AgRP peptides and stained with fluorescein-labeled anti-6xHis antibody. Symbols are AgRP mutant 6C $\circ$; 7A $\square$; 7C $\blacklozenge$; 7E $\bullet$; 7J $\blacksquare$. Representative data from at least 3 repetitions are shown.

Figure 4.

Inhibition of vitronectin-mediated cell adhesion by engineered AgRP peptides. (a) K562- $\alpha\text{v}\beta\text{3}$ cells. (b) U87MG cells. Vitronectin coated strips were incubated with cells for two hours with the indicated concentrations of AgRP peptides. Adherent cells remaining after several wash steps were quantified with crystal violet staining by absorbance at 600 nm. Values were normalized to a negative control containing no peptide. Symbols are AgRP mutant 6C $\circ$; 7A $\square$; 7C $\blacklozenge$; 7E $\bullet$; 7J $\blacksquare$; echistatin $\blacktriangle$. Representative data from at least 3 repetitions are shown.

Figure 5.

Mutagenesis of AgRP loops 1-3 using degenerate codons. (a) AgRP with loops 1-4 shown in blue, green, cyan, and red, respectively. (b)-(c) Binding of 50 nM integrin to yeast-displayed AgRP peptide clones from sort round 4 of degenerate codon libraries. (b) $\alpha v\beta 3$ integrin; (c) $\alpha iib\beta 3$ integrin. Background, marked "bg" indicates cells stained with fluorescein-conjugated anti-integrin antibodies only.

Table 1

Sequences of AgRP mutants isolated from flow cytometry sort rounds 6 and 7.

Clone ^a	Loop 4 sequence
6C	GCVRLHESCLGQQVPCCDPAATCYCT TGRGDEKL RCYCR
6E	GCVRLHESCLGQQVPCCDPAATCYC VERGDGNRR CYCR
6F	GCVRLHESCLGQQVPCCDPAATCYC ESRGDVVRK CYCR
6P	GCVRLHESCLGQQVPCCDPAATCYC MGRGDVRSQ CYCR
7A	GCVRLHESCLGQQVPCCDPAATCYC SGRGDNDL VCYCR
7B	GCVRLHESCLGQQVPCCDPAATCYC KGRGDARLQ CYCR
7C	GCVRLHESCLGQQVPCCDPAATCYC YGRGDNDL RCYCR
7E	GCVRLHESCLGQQVPCCDPAATCYC VGRGDDNLK CYCR
7J	GCVRLHESCLGQQVPCCDPAATCYC EGRGDRDMK CYCR

^aNumber indicates sort round.

Table 2

Binding and cell adhesion data summary for engineered AgRP peptides.

Clone	binding (K_D ; nM)		adhesion (IC_{50} ; nM)
	U87MG	K562- $\alpha v \beta 3$	K562- $\alpha v \beta 3$
6C	13 ± 2	15 ± 4	650 ± 250
7A	0.78 ± 0.37	0.89 ± 0.36	61 ± 19
7C	1.8 ± 0.6	2.4 ± 0.8	12 ± 6
7E	1.4 ± 0.5	1.6 ± 1.1	9.9 ± 5.8
7J	8.3 ± 1.8	16 ± 8	200 ± 150
Echistatin	nd	nd	3.2 ± 2.7

nd = not determined

Table 3

Sequences of select AgRP clones isolated from randomized loop 1, 2, and 3 libraries

Clone ^a	Modified Loop Sequence
1-1	ASGSGDP
1-2	RPLGDAG
1-3	LAGLSGP
1-4	RSASVGG
1-5	IASGLFG
1-6	DLYGSHD
2-1	GGSVGVE
2-2	DPRVGVR
2-3	ADTLMAA
2-4	EWGRGGD
2-5	GSWGTLA
2-6	WGSILGH
3-1	GTPKPE
3-2	SRSDAH
3-3	SGLGNR
3-4	QGREQS
3-5	TVTNSR
3-6	TSKQHH

^aFirst number indicates mutated loop, second number indicates clone number.