TECHNICAL NOTES

Probing for Integrin $\alpha_v\beta_3$ Binding of RGD Peptides Using Fluorescence Polarization

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Integrin $\alpha_v\beta_3$ is an adhesion molecule involved in tumor invasion, angiogenesis, and metastasis. There is substantial interest in developing novel agents that bind to integrin $\alpha_v\beta_3$. Here we report the synthesis and characterization of a fluorescent integrin $\alpha_v\beta_3$ probe and its use in a nonradioactive, simple, sensitive fluorescence polarization (FP) assay to quantify binding to integrin $\alpha_v\beta_3$. For assay validation, the FP assay was compared to a cell adhesion assay. In the two assays, probe binding to integrin $\alpha_v\beta_3$ showed a similar dependence on probe concentration. The FP assay was successfully applied to measure the binding affinity to integrin $\alpha_v\beta_3$ of several cyclic peptides containing the Arg-Gly-Asp (RGD) motif. The FP assay we describe here may be appropriate for high-throughput screening for integrin $\alpha_v\beta_3$-binding ligands used for anti-integrin therapy or noninvasive imaging of integrin expression.

INTRODUCTION

Integrins are heterodimeric transmembrane proteins that function principally as receptors for spatially restricted extracellular ligands (*1*). The α_v-integrins ($\alpha_v\beta_3$, $\alpha_v\beta_5$) play a critical role in tumor angiogenesis and are associated with tumor progression and metastasis (*2*−*4*). They are overexpressed on brain and melanoma tumor cells and newly formed tumor microvessels but not on normal cells and quiescent endothelial cells (*5*−*7*). Antibody, peptide, and peptidomimetic integrin $\alpha_v\beta_3$ antagonists have been shown to impair angiogenesis, growth, and metastasis of solid tumors (*8*−*10*). Furthermore, imaging the expression of α_v-integrins may allow determination of the optimal biological dose and schedule of integrin-targeted therapeutics and may allow monitoring of tumor responses to such therapeutics. Thus, there is substantial interest in developing novel agents that bind to α_v-integrins for both therapeutic and diagnostic purposes.

Existing in vitro methods for detecting integrin $\alpha_v\beta_3$ binding include a binding assay that uses immobilized integrin and a radiolabeled tracer (*11*, *12*), a competitive enzyme-linker immunosorbent assay (*13*), and surface plasma resonance (*14*). All of these methods, however, require immobilization of integrins on a solid surface.

A fluorescence polarization (FP) assay would be a more suitable assay for detecting integrin $\alpha_v\beta_3$ binding because FP is a homogeneous technology requiring only simple mix reagents and read format that can be easily automated (*15*). The technique has recently been introduced for high-throughput screening in the drug discovery process (*16*). FP depends on the excitation of a fluorescent molecule with plane-polarized light. This results in the emission of photons in the planes parallel and perpendicular to the excitation plane and confers information about the local environment of the fluorophore. Smaller molecules rotate faster and hence have smaller polarization values. Conversely, larger molecules (e.g., fluorescent probe bound to target) rotate more slowly and thus have bigger polarization values. Thus, the observed polarization value gives a direct measure of the molecular size of the analyte and the extent of tracer binding to macromolecules at constant temperature and viscosity. FP assays work well in colored and cloudy solutions. There is no need for radioisotopes, and there are no solid-phase artifacts. The technique does not require washing steps, and the system is easily automated.

To our knowledge, no fluorescence-labeled integrin $\alpha_v\beta_3$ ligands that could be used in an FP assay have previously been validated. Here we report the synthesis and characterization of a fluorescent derivative of the cyclic pentapeptide cyclo(Lys-Arg-Gly-Asp-phe) [c(KRGDf)]. We show that this probe tightly bound integrin $\alpha_v\beta_3$ and that its interaction with integrin $\alpha_v\beta_3$ was competed with by integrin ligands containing the Arg-Gly-Asp (RGD) motif.

EXPERIMENTAL PROCEDURES

Materials. All N^{α}-Fmoc-amino acids, 1-hydroxybenzotriazole (HOBt), benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), and solid support linker [4-(4-hydroxymethyl-3-methoxyphenoxy)-acetic acid] (HMPA) were purchased from Novabiochem (San Diego, CA). Trifluoroacetic acid (TFA) was purchased from Chem-Impex International, Inc. (Wood Dale, IL). PL-DMA resin was purchased from Polymer Labo-

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ratories (Amherst, MA). *N,N*-Diisopropylethylamine (DIPEA), triethylsilane (TES), 5(6)-carboxyfluorescein, tetrakis(triphenylphosphine)palladium ($Pd^0[P(C_6H_5)_3]_4$), *N*-methylmorpholine (NMP), and 1,3-diisopropylcarbodiimide (DIPCDI) were purchased from Aldrich Chemical Co. (St. Louis, MO). Integrin $\alpha_v\beta_3$ was purchased from Chemicon International, Inc. (Temecula, CA). All solvents were purchased from VWR (San Dimas, CA). A 96-well flat-bottom assay plate (black polystyrene) was purchased from Corning, Inc. (Corning, NY).

Analysis. Products were purified by reverse-phase high-performance liquid chromatography (HPLC), eluted with H_2O/acetonitrile containing 0.1% TFA, and validated by analytical HPLC and matrix-assisted laser desorption ionization (MALDI) mass spectrometry. Analytical HPLC was carried out on an Agilent 1100 system (Wilmington, DE) equipped with a Vydac peptide and protein analytical C-18 column (Anaheim, CA). Preparative HPLC was carried out on a Rainin Rabbit HP system (Walnut Creek, CA) equipped with a 25 × 2.5 cm Vydac C-18 column. MALDI was performed in the proteomics laboratory in the Department of Molecular Pathology at The University of Texas M. D. Anderson Cancer Center (Houston, TX).

Synthesis of Cyclic Peptides. RGD peptides were synthesized on a solid support. Briefly, PL-DMA resin (1 g) was treated with ethylenediamine (30 mL) overnight. HMPA linker (3 equiv) was attached to resin in the presence of DIPCDI (3 equiv) and HOBt (3 equiv). Peptides Fmoc-phe-X-Arg(Pbf)-Gly-Asp(resin)OAll, where X represents Lys(Boc), Glu(OBut), or Lys(Mtt), were synthesized using Fmoc solid-phase chemistry. The protecting groups of the amino acid side chains were *N-tert*-butoxycarbonyl (Boc) or 4-methyltrityl (Mtt) for Lys, *tert*-butyl (But) for Glu, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg, and allyl (All) for Asp. Amino acids (3 equiv) were coupled stepwise in the presence of DIPCDI (3 equiv) and HOBt (3 equiv) coupling reagents. After removal of All and Fmoc protecting groups using $Pd^0[P(C_6H_5)_3]_4$ (3 equiv) in dichloromethane(DCM)/NMP/AcOH (37/1/2) and 20% piperidine in dimethylformamide (DMF), respectively, the cyclization was carried out on the solid support using PyBOP (3 equiv), HOBt (3 equiv), and DIPEA (6 equiv) as coupling agents to afford cyclo-[Lys(Boc)-Arg(Pbf)-Gly-Asp(resin)-phe], cyclo-[Glu(OBut)-Arg(Pbf)-Gly-Asp(resin)-phe), and cyclo-[(Lys(Mtt)-Arg(Pbf)-Gly-Asp(resin)-phe]. Mtt protecting group was removed by treatment with TFA/TES/DCM (1/2/97). To synthesize dimeric RGD peptide, Lys(Boc), Glu(OAll), phe, Asp(OBut), Gly, and Arg(Pbf) were successively attached to cyclo-[Lys-Arg-(Pbf)-Gly-Asp(resin)-phe] to form an undecapeptide Fmoc-Arg(Pbf)-Gly-Asp(OBut)-phe-Glu(OAll)-Lys(Boc)-cyclo-[Lys-Arg(Pbf)-Gly-Asp(resin)-phe]. After removal of All and Fmoc protecting groups, the second cyclization was carrier out on the solid support. Deprotection and cleavage of all these compounds from the solid support were carried out simultaneously by treatment with TFA/H_2O/TES (95/1/4) to afford c(KRGDf), cyclo(Glu-Arg-Gly-Asp-phe) [c(ERGDf)], or c(RGDfE)-K-c(KRGDf). All products were purified by reverse-phase HPLC and validated by MALDI mass spectrometry.

To synthesize the cyclic pentapeptide Ac-Ala-c(Cys-Asn-Gly-Arg-Cys)-Gly [Ac-A-c(CNGRC)-G], linear peptide Ac-ACNGRCG was first prepared using Fmoc solid-phase peptide synthesis. After removal of the peptide from the solid support by TFA/H_2O/TES (95/1/4), cyclization was achieved in NH_4OH aqueous solution (pH 9). The product was purified by reverse-phase HPLC and validated by MALDI mass spectrometry.

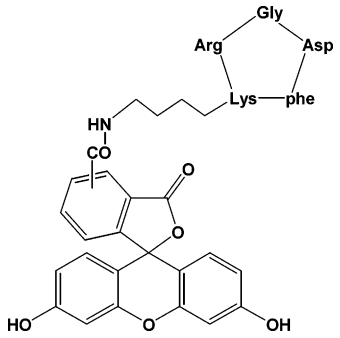


Figure 1. Structure of 5(6)-carboxyfluorescein−c(KRGDf) conjugate.

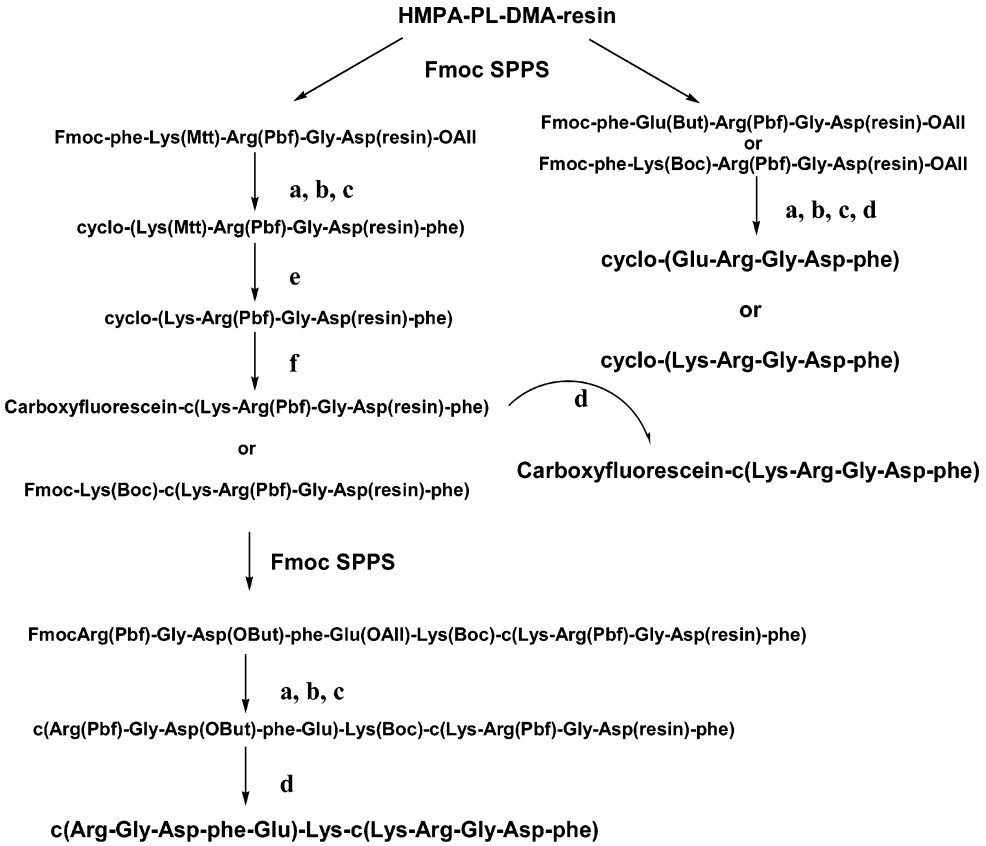
Conjugation of Fluorescein to c(KRGDf). Carboxyfluorescein (3 equiv) was coupled to c[Lys-Arg(Pbf)-Gly-Asp(resin)-phe] using DIPCDI (3 equiv) and HOBt (3 equiv) as the coupling agents. Deprotection and cleavage from the solid support were carried out simultaneously by treatment with TFA/H_2O/TES (95/1/4) to afford the target compound carboxyfluorescein-c(KRGDf) (FL-RGD) (Figure 1). The product was purified by reverse-phase HPLC and validated by MALDI mass spectrometry.

Fluorescence Polarization Assay. A solution of 200 μM FL-RGD or fluorescein in phosphate-buffered saline was diluted with Tris buffer (50 mM Tris, pH 7.4, 1 mM $CaCl_2$, 10 μM $MnCl_2$, 1 mM $MgCl_2$, 100 mM NaCl) to give a 100 nM stock solution. The reaction mixture (100 μL) in each well was composed of 10 nM FL-RGD probe (10 μL of 100 nM stock solution) and different concentrations of integrin $\alpha_v\beta_3$ (0 to 650 nM) in Tris buffer. The microwell assay plate was incubated at 29 °C for 0.5 h. Fluorescein alone (10 nM) and FL-RGD alone (10 nM) served as controls. FP was measured with a Tecan Polarion reader (Research Triangle Park, NC). The polarization value was expressed in millipolarization units (mP). The integrin titration data were analyzed using Graph-Pad PRISM software (GraphPad Software, Inc., San Diego, CA) with the one-site binding equation $FP = FP_{max}C/(K_d + C)$, where FP_{max} is the maximal FP value, C is the concentration of the protein, and K_d represents the dissociation constant.

Competitive displacement studies were performed with the integrin ligands c(KRGDf), c(ERGDf), and c(RGDfE)-K-c(KRGDf). The cyclic pentapeptide containing the Asn-Gly-Arg (NGR) motif, Ac-A-c(CNGRC)-G, was used as a negative control. Stocks of these compounds were made in phosphate-buffered saline at a concentration of 500 μM. The ligands were serially diluted in the assay buffer to concentrations ranging from 0.01 to 5 μM. The FL-RGD probe and $\alpha_v\beta_3$ were added at concentrations of 10 nM and 290 nM, respectively. The reaction mixture was shaken, and the FP values in mP were recorded. The mP values were plotted against the ligand concentration. The Polarion data were analyzed using Graph-Pad PRISM software with linear regression to obtain the concentration at which 50% inhibition of FL-RGD's binding to integrin $\alpha_v\beta_3$ was achieved (IC_{50} values).

Cell Adhesion Assay. Human melanoma M21 cells were obtained from Dr. David Cheresh (Scripps Research Institute, San Diego, CA). The cells were seeded in Dulbecco's modified Eagle's medium/F12 culture medium supplemented with 10% fetal bovine serum for 24 h. Cells (1×10^5) with different concentrations of c(KRGDf), c(ERGDf), c(RGDfE)-K-c(KRGDf), FL-RGD, or Ac-A-c(CNGRC)-G were added to vitronectin-coated microtiter wells under serum-free conditions and incubated at 37

Scheme 1. Synthesis of RGD Derivatives and RGD Fluorescent Probe[a]



[a] Reagents and conditions: (a) $Pd^0[P(C_6H_5)_3]_4$ (3 equiv), $CHCl_3$/AcOH/NMM (37/2/1, v); (b) 20% piperidine, DMF; (c) PyBOP/HOBt/DIPEA (3/3/6 equiv) in DMF; (d) TFA/H_2O/TES (95/1/4, v); (e) TFA/DCM/TES (1/97/2, v); (f) 5(6)-carboxyfluorescein (3 equiv) or Fmoc-Lys(Boc) (3 equiv), DIPCDI (3 equiv), HOBt (3 equiv) in DMF and DCM (1/1, v); (g) *p*-succinicamido-benzyl-DTPA penta-*tert*-butyl ester (2 equiv), PyBOP (3 equiv), HOBt (3 equiv), DIPEA (6 equiv) in DMF. SPPS, solid-phase peptide synthesis.

°C for 1 h. After washing steps, the bound cells were stained with 5% crystal violet, and then 0.1 M HCl was added to each well. The concentrations of crystal violet were determined by UV/Vis absorption at 627 nm. IC_{50} values were estimated from the dose−activity curves.

RESULTS AND DISCUSSION

Chemistry. Integrins bind extracellular matrix factors, such as vitronectin, that contain the RGD amino acid sequence, which appears to be critical in mediating the ligation signal, allowing endothelial cells to attach to the extracellular matrix and thus support endothelial survival and growth (*5*, *6*, *17*). Integrin antagonists and imaging agents are generally developed on the basis of cyclic peptides composed of five amino acids. One such agent, Cilengitide (c[RGDf(NMe)V]), has entered clinical phase II studies as a potent and selective integrin peptidic antagonist (*18*). Several radiolabeled peptides containing the RGD motif plus various combinations of two additional amino acids have been used for custom-made purposes such as iodination (tyrosine) or linkage of chelators (lysine) (*12*, *19*−*22*). Thus we selected c(KRGDf) peptide as the ligand for FP probe development.

We synthesized fluorescein-conjugated c(KRGDf) to use this agent as a probe for our FP assay. In addition, we synthesized the unconjugated monomeric peptides c(KRGDf) and c(ERGDf) and the dimeric cyclic peptide c(RGDfE)-K-c(KRGDf). All of these compounds were prepared by solid-phase peptide synthesis (Scheme 1). Starting from Asp attached to PL-DMA resin with HMPA linker coupled to the side chain of Asp, linear peptides were synthesized using an Fmoc strategy. The cyclization of pentapeptides was achieved on the solid support after selective removal of the N-terminal Fmoc and C-terminal All protecting groups to afford cyclo[X-Arg(Pbf)-Gly-Asp(resin)-phe], where X represents Lys(Boc), Glu(OBut), or Lys(Mtt). For the preparation of c(KRGDf), Boc was used as a protecting group for Lys. TFA was used to simultaneously cleave the peptide from the resin and remove the side chain protecting groups, yielding cyclic pentapeptides c(KRGDf) and c(ERGDf) (Scheme 1).

For preparation of the dimer c(RGDfE)-K-c(KRGDf) and FL-RGD, Mtt was used as the protecting group for Lys for the construction of cyclo[Lys(Mtt)-Arg(Pbf)-Gly-Asp(resin)-phe]. The side chain protecting group Mtt on Lys was selectively removed in diluted TFA without peptide cleaved from the resin. This allowed further elaboration for the second cyclic peptide or conjugation of carboxyfluorescein through the Lys unit on the solid support (Scheme 1). The physicochemical properties of the peptides synthesized are summarized in Table 1.

Fluorescence Polarization Assay. To demonstrate that the FP signal was a result of binding of FL-RGD with integrin, the FP assay was performed as a function of integrin concentrations with 10 nM probe in the assay. The FP signal rose from 40 mP (background mP value due to intrinsic polarization of the probe) to about 140 mP when the concentrations of integrin increased from 0 to 650 nM (Figure 2). This finding was expected because addition of more integrin would make more integrin available for complexing with the probe. The titration curve showed that the FL-RGD probe bound to integrin $\alpha_v\beta_3$. The dissociation constant (K_d) for FL-RGD−integrin

Table 1. Physicochemical Property of the Fluorescent RGD Probe and Testing Compounds

cyclic peptides	molecular formula	mass spectrometry: calculated MW	mass spectrometry: observed (M + 1)	retention time (min)
c(KRGDf)	$C_{27}H_{41}N_9O_7$	603.313	604.356	10.97[a]
c(ERGDf)	$C_{26}H_{36}N_8O_9$	604.261	605.210	13.93[a]
FL-c(KRGDf)	$C_{48}H_{51}N_9O_{13}$	961.361	962.310	24.94[a]
c(RGDfE)-K−c(KRGDf)	$C_{59}H_{87}N_{19}O_{16}$	1317.658	1318.645	10.24[b]
Ac-A-c(CNGRC)-G	$C_{25}H_{41}N_{11}O_{10}S_2$	719.248	720.225	7.77[a]

[a] Sample was eluted with H_2O and acetonitrile containing 0.1% TFA varying from 0% to 40% over 30 min. [b] Sample was eluted with H_2O and acetonitrile containing 0.1% TFA varying from 10% to 50% over 30 min.

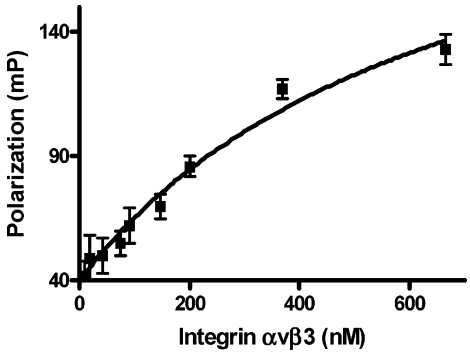


Figure 2. Titration curve for binding of FL-RGD probe to integrin $\alpha_v\beta_3$ as a function of $\alpha v\beta 3$ concentration. FP values (mP) were measured after incubation of FL-RGD (10 nM) and $\alpha_v\beta_3$ (0−650 nM) mixture at 29 °C for 30 min. The mP value obtained in the absence of $\alpha_v\beta_3$ (40 mP) represents the background mP value due to intrinsic polarization of the probe. Data are presented as mean values ± standard deviations from 3 independent experiments.

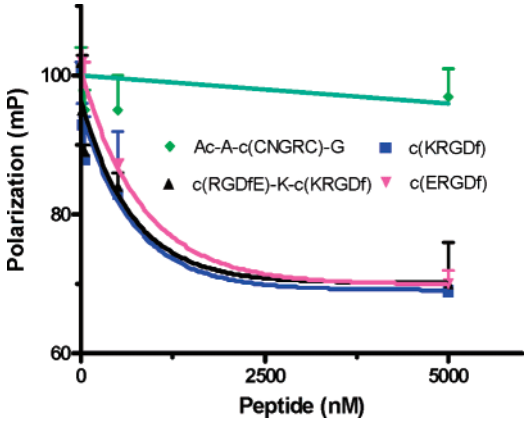


Figure 3. Titration curve for binding of FL-RGD probe (10 nM) to integrin $\alpha_v\beta_3$ (290 nM) in the presence of increasing concentrations of $\alpha_v\beta_3$ inhibitors. The NGR peptide [Ac-A-c(CNGRC)-G] is a negative control that does not bind $\alpha_v\beta_3$.

complex was 0.67 ± 0.15 μM as determined using GraphPad PRISM software with the one-site binding equation $FP = FP_{max}C/(K_d + C)$. The binding was fast; increasing the incubation time beyond 5 min did not have any effect on the FP signal (data not shown). These results showed that the FP assay with FL-RGD as a probe can be used to measure the binding affinity of ligands to integrin $\alpha_v\beta_3$.

To further demonstrate that the FP signal was dependent on formation of the RGD−integrin complex, the FP assay was performed as a function of concentration for several integrin inhibitors containing the RGD motif. Increasing the amounts of RGD peptides at fixed probe and integrin concentrations result in a decreased FP signal (Figure 3). The FP signal fell from about 105 mP to about 70 mP when the concentrations of the RGD peptide competitors increased from 0 to 5 μM. A window

Table 2. Comparison of IC_{50} Values Obtained from FP Assay and Cell Adhesion Assay[a]

cyclic peptides	FP IC_{50} (μM)	cell adhesion IC_{50} (μM)
carboxyfluorescein-c(KRGDf)		4.2 ± 0.27
c(KRGDf)	0.11 ± 0.003	5.0 ± 0.77
c(ERGDf)	0.28 ± 0.004	3.2 ± 0.16
c(RGDfE)-K-c(KRGDf)	0.11 ± 0.003	4.0 ± 0.32
Ac-A-c(CNGRC)-G	not available	not available

[a] Data are presented as mean values ± standard deviations from three independent experiments.

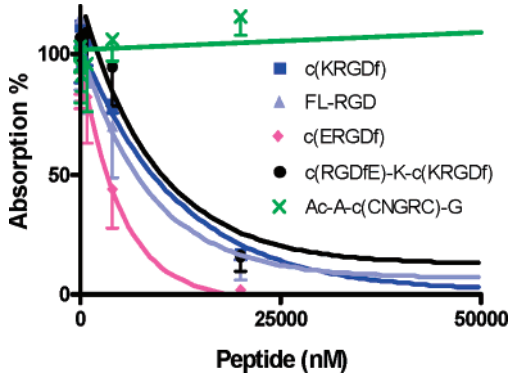


Figure 4. Adhesion of M21 human melanoma cells to vitronectin-coated microtiter wells in the presence of increasing concentrations of $\alpha_v\beta_3$ inhibitors. After washing steps, the bound cells were stained with 5% crystal violet, and then 0.1 M HCl was added to each well. The concentrations of crystal violet were determined by UV/Vis absorption at 627 nm. The percentage absorption values reported on the *y*-axis are relative to the absorption value measured in the absence of RGD peptides.

of about 35 mP was observed with peptide concentrations from 0 to 5 μM. Because of nonspecific binding, the FP value never dropped to the 40-mP background value observed in the absence of integrin inhibitors. IC_{50} values derived from linear regression of the competition curves for c(KRGDf), c(ERGDf), and c(RGDfE)-K-c(KRGDf) were 0.11, 0.28, and 0.11 μM, respectively. The NGR control peptide could not displace fluorescent probe from binding to integrin even at the maximal tested concentration of 5 μM (Figure 3; Table 2).

Cell Adhesion Assay. To validate the FP-integrin assay procedure, the FP assay was compared to a more standard functional assay that measures the inhibition of M21 tumor cell adhesion to extracellular matrix proteins. FL-RGD and the parent peptide c(KRGDf) gave similar IC_{50} values of 4.2 μM and 5.0 μM, respectively. These values are within the range of 3.2−5.0 μM for other RGD peptides, suggesting that FL-RGD is as efficacious as the parent peptide in inhibiting adhesion of tumor cells to vitronectin-coated microwells (Table 2). The number of attached cells decreased in a dose-dependent manner for all three unconjugated cyclic peptides containing the RGD motif as well as for FL-RGD (Figure 4). No inhibitory activity was observed for the NGR control peptide

(Figure 4). The IC_{50} values obtained from the FP assay paralleled the IC_{50} values obtained from the cell adhesion assay, albeit IC_{50} for integrin binding was approximately 10-times lower than IC_{50} for bioactivity. These data suggest that RGD peptides may possess nonspecific interaction with vitronectin in cell based adhesion assay. Nevertheless, both assays showed no significant differences among the three cyclic peptides containing RGD motif in their ability to bind integrin and inhibit cell adhesion (Table 2). We interpreted these findings as indicating that a similar binding affinity to integrin $\alpha v\beta 3$ resulted in a similar biological activity.

Binding Affinity of Various Cyclic RGD Peptides to Integrin $\alpha_v\beta_3$. The observation of no difference in integrin binding between c(KRGDf) and c(ERGDf) by FP assay confirms a previously reported finding that changing the amino acid X in c(XRGDf) does not significantly affect the binding affinity of cyclic RGD peptide to integrin (*23*). It has been proposed that multimeric constructs based on RGD peptide may lead to ligands with substantially increased binding affinity for integrin $\alpha_v\beta_3$ owing to the multivalency effect. Thumshirn et al. (*24*) observed 2.3- to 22-fold increases in IC_{50} values when monomeric peptides were compared to dimeric peptides linked together through a flexible oligomeric ethylene oxide linker. Janssen et al. (*25*) compared the IC_{50} of the radiolabeled monomer c(RGDfK) and dimeric E-[c(RGDfK)]$_2$ determined in immobilized human $\alpha_v\beta_3$ using an enzyme-linked immunosorbent assay. The affinity of the dimer for $\alpha_v\beta_3$ was 10 times the affinity of the monomer. In our hands, the dimeric peptide c(RGDfE)-K-c(KRGDf) did not show increased integrin binding as compared to the integrin binding of its individual monomers c(ERGDf) and c(KRGDf). It is possible that the distance and orientation between the two cyclic peptide rings could have affected the binding of the dimer to integrin receptors. With the integrin-binding FP assay validated, it is now possible to systematically investigate the effect of linker molecule on the binding of dimeric RGD peptide to integrin $\alpha_v\beta_3$. Work along this line is in progress.

Summary. In summary, the FP assay method should be advantageous compared to other methods reported in the literature for the measurement of integrin binding because the FP assay is a simple, one-step, nonradioactive assay that is performed in solution. We found that a fluorescent integrin $\alpha_v\beta_3$ probe based on the cyclic pentapeptide c(KRGDf) tightly bound integrin $\alpha_v\beta_3$ and that the probe's interaction with integrin $\alpha_v\beta_3$ was competed with by integrin ligands containing the RGD motif. The FP assay we describe here may be appropriate for high-throughput screening for integrin $\alpha_v\beta_3$-binding ligands used for anti-integrin therapy or noninvasive imaging of integrin expression.

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