Published in final edited form as:

J Cell Physiol. 2008 November ; 217(2): 450–458. doi:10.1002/jcp.21512.

Mixed Extracellular Matrix Ligands Synergistically Modulate Integrin Adhesion and Signaling

Catherine D. Reyes¹, Timothy A. Petrie², and Andrés J García^{*,1,2}

¹Woodruff School of Mechanical Engineering, Georgia Institute of Technology, Atlanta, Georgia, 30332

²Coulter Department of Biomedical Engineering, Georgia Institute of Technology, Atlanta, Georgia, 30332

Abstract

Cell adhesion to extracellular matrix (ECM) components through cell-surface integrin receptors is essential to the formation, maintenance and repair of numerous tissues, and therefore represents a central theme in the design of bioactive materials that successfully interface with the body. While the adhesive responses associated with a single ligand have been extensively analyzed, the effects of multiple integrin subtypes binding to multivalent ECM signals remain poorly understood. In the present study, we generated a high throughput platform of non-adhesive surfaces presenting well-defined, independent densities of two integrin-specific engineered ligands for the type I collagen (COL-I) receptor $\alpha_2\beta_1$ and the fibronectin (FN) receptor $\alpha_5\beta_1$ to evaluate the effects of integrin cross-talk on adhesive responses. Engineered surfaces displayed ligand density-dependent adhesive effects, and mixed ligand surfaces significantly enhanced cell adhesion strength and focal adhesion assembly compared to single FN and COL-I ligand surfaces. Moreover, surfaces presenting mixed COL-I/FN ligands synergistically enhanced FAK activation compared to the single ligand substrates. The enhanced adhesive activities of the mixed ligand surfaces also promoted elevated proliferation rates. Our results demonstrate interplay between multivalent ECM ligands in adhesive responses and downstream cellular signaling.

Keywords

collagen; fibronectin; cell adhesion; focal adhesion; integrin

Introduction

Extracellular matrices (ECMs) play critical roles in tissue morphogenesis, homeostasis, and repair by providing structural and signaling scaffolds that organize, coordinate, and regulate cellular activities. Many of these matrix effects are mediated by the integrin family of cell surface receptors, which consist of non-covalently associated α and β subunits with large extracellular domains that bind to the ECM and short cytoplasmic domains that interact with cytoskeletal elements (Hynes, 2002). Upon ligand binding, integrins cluster to form focal adhesions, transmembrane complexes enriched in specific cytoskeletal structural and signaling proteins, including vinculin, FAK, α -actinin, and talin. In addition to anchoring cells by linking the ECM to the cytoskeleton, integrins mediate the bidirectional transfer of biochemical signals across the plasma membrane (Dedhar and Hannigan, 1996; Hynes,

*Address correspondence to: Andrés J García, Woodruff School of Mechanical Engineering, Georgia Institute of Technology, 315 Ferst Drive, Atlanta, Georgia 30332-0363, Tel. 404-894-9384; Fax. 404-385-1397; E-Mail: andres.garcia@me.gatech.edu.

2002) to control a wide variety of cellular processes, including cell cycle progression (Dike and Ingber, 1996; Zhu et al., 1996), differentiation (Gronthos et al., 1997; Suzawa et al., 2002; Takeuchi et al., 1997; Tamura et al., 2001; Xiao et al., 2002a), and apoptosis (Boudreau et al., 1995; Frisch and Ruoslahti, 1997).

Cross-talk between integrins and growth factor receptors often leads to enhanced intracellular signaling and specific patterns of gene expression (Kiely et al., 2005; Miyamoto et al., 1995; Reginato et al., 2003; Sieg et al., 2000). Moreover, interactions among integrin receptor types modulate adhesive interactions, often via intracellular components such as talin, paxillin, and FAK (Calderwood et al., 2004; Ly et al., 2003; Rose et al., 2003). However, little is known about the effects of multiple integrin signals converging on a particular downstream cellular response, which occurs in cells that adhere to complex, multivalent extracellular matrices via multiple integrin receptors. Although integrins can independently propagate intracellular signals, integration of multiple signals from the extracellular matrix may provide specificity and regulation of complex cellular processes. For instance, interactions between integrin $\alpha_5\beta_1$ and fibronectin (FN) and integrin $\alpha_2\beta_1$ and type I collagen (COL-I) have both been implicated in the proliferation and differentiation of osteoblasts (Globus et al., 1998; Gronthos et al., 1997; Jikko et al., 1999; Mizuno et al., 2000; Mizuno and Kuboki, 2001; Moursi et al., 1996; Moursi et al., 1997; Suzawa et al., 2002; Takeuchi et al., 1997; Xiao et al., 2002b). Analyses of integrin-mediated adhesion to combinations of ligands would provide insights into the convergence of diverse matrix signals into tissue-specific patterns of gene expression and cellular behavior during normal development and pathological conditions. Nevertheless, these studies have been limited by (i) the inability to generate well-defined substrates that independently control the presentation of multiple adhesive ligands and (ii) the presentation of multiple integrin binding domains and/or ECM interactions sites within a particular ECM ligand.

The objective of this study was to elucidate the combined downstream effects of two separate integrin binding interactions, $\alpha_5\beta_1$ -FN and $\alpha_2\beta_1$ -COL-I, using biointerfaces presenting engineered ligands that recapitulate the primary, secondary, and tertiary structure of the native matrix protein in order to reconstitute full biological activity as well as integrin binding specificity. Our strategy uses mixed biotinylated ligands on avidin substrates, providing a simple and easily controlled approach to efficiently screen a large number of mixed surface compositions using short term assays. These surfaces were examined for cell adhesion, integrin binding, and integrin-mediated signaling responses.

Materials and Methods

Cells and Reagents

HT1080 human fibrosarcoma cells (CCL-121, American Type Culture Collection, Manassas, VA) were grown in Dulbecco's Modified Eagle medium containing 10% fetal bovine serum and 1% penicillin-streptomycin and subcultured every two days using standard techniques.

NHS-fluorescein, biotin-LC-PEO-amine reagent, and Slide-A-Lyzer Dialysis Cassettes (3,500 MWCO) were purchased from Pierce (Rockland, IL). Anti-FN (HFN7.1) was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Rabbit polyclonal anti-FAK antibody was obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-FAK [pY397] phospho-specific antibody was purchased from BioSource International (Camarillo, CA). Biotin-conjugated donkey anti-rabbit IgG was obtained from Jackson ImmunoResearch (West Grove, PA). ECF substrate for spot blotting was acquired from Amersham Pharmacia Biotech (Piscataway, NJ). DH5 α and JM109 bacterial cells used for cloning and fragment production were obtained from Invitrogen and

Promega (Madison, WI), respectively. The XA3 Pinpoint Vector biotinylation expression system was obtained from Promega. The APC BrdU Flow Kit was purchased from BD Biosciences (San Jose, CA). Fetal bovine serum was purchased from Hyclone (Logan, UT). Additional bacterial and mammalian cell culture reagents and spot blotting supplies were obtained from Invitrogen (Carlsbad, CA). All other chemical and biological reagents were purchased from Sigma Chemical (St. Louis, MO).

GFOGER Peptide and Fibronectin Fragment

The peptide GGYGGGPC(GPP)₅GFOGER(GPP)₅GPC [O=hydroxyproline] was synthesized by the Emory University Microchemical Facility as previously described (Reyes and García, 2003a). This peptide selectively binds the $\alpha_2\beta_1$ integrin and exhibits equivalent biological activity as type I collagen (Reyes and García, 2003a; Reyes and García, 2004). Peptide was supplied in the purified form as a trifluoroacetic acid (TFA) salt and reconstituted at a stock concentration of 10 mg/ml in 0.1% TFA. In all experiments, the GFOGER peptide was diluted to working concentrations in Dulbecco's phosphate-buffered saline (DPBS). For surface immobilization, the GFOGER peptide was biotinylated using a biotin-LC-PEO-amine reagent (Reyes and García, 2003a). The terminal primary amine of this molecule selectively labels the terminal carboxyl group of the GFOGER peptide. For antibody detection, biotinylated GFOGER peptide was labeled with fluorescein using an NHS-ester labeling reagent (NHS-fluorescein, Pierce) targeting the terminal primary amine. For the coupling reaction, 0.5 mg of NHS-fluorescein was dissolved in 500 μ l of DMSO to form a stock reagent solution. The stock reagent was slowly added to the GFOGER peptide stock solution (1 part NHS-fluorescein to 10 parts GFOGER peptide solution), while vortexing. The sample was then placed on ice for 2 h. Unreacted NHS-fluorescein was removed by overnight dialysis in DPBS. The final concentration of fluorescein-labeled GFOGER peptide was determined by monitoring the 280 nm absorbance.

A monobiotinylated fibronectin fragment spanning the 7-10th type III repeats of FN, FNIII7-10, was produced using standard recombinant DNA techniques (Petrie et al., 2006). We previously showed that this recombinant fragment exhibits equivalent adhesive activity as human plasma fibronectin and displays significantly higher selectivity for $\alpha_5\beta_1$ integrin compared to linear RGDS peptide (Petrie et al., 2006).

Mixed Ligand Surface Preparation

Tissue culture treated polystyrene surfaces were incubated with avidin (NeutrAvidin, 100 μ g/ml, Pierce) for 1 h at 22°C. The surface was then blocked with 1% heat denatured bovine serum albumin (BSA) for 1 h to prevent non-specific protein adsorption. After washing with DPBS, varying concentrations of FNIII7-10 (0-5 μ g/ml in DPBS) were introduced to the avidin support layers for 1 h at 22°C. Surfaces were washed with DPBS and incubated with varying concentrations of GFOGER peptide (0-5 μ g/ml in DPBS) for 1 h at 22°C.

Surface Density Measurements

Relative surface density measurements were obtained using a standard enzyme-linked immunosorbent assay (ELISA). Mixed ligand surfaces were generated in a 96-well plate as previously described and blocked with 5% FBS in DPBS (blocking buffer) for 1 h. The surfaces were then incubated with an anti-FITC alkaline phosphatase-conjugated antibody (1:1000 dilution in blocking buffer) for GFOGER peptide detection or a mouse anti-FN antibody (HFN7.1, 1:4000 dilution) for FNIII7-10 detection for 1 h at 37°C. After washing, the wells that were incubated with the mouse anti-FN antibody were then incubated with an anti-mouse alkaline phosphatase-conjugated antibody (1:1000 dilution) for 1 h at 37°C. After rinsing all wells, substrate (4-methyl-umbelliferyl-phosphate, 60 μ g/ml) was added for 1 h at 37°C. Reaction product fluorescence was measured in a microwell plate reader (360-

nm excitation, 465-nm emission). Absolute surface density measurements were obtained by surface plasmon resonance using a Biacore X instrument (Petrie et al., 2006).

Centrifugation Cell Adhesion Assay

Mixed ligand surfaces were generated in a 96-well plate and blocked with 5% non-fat dry milk in DPBS for 1 h to prevent non-specific protein adsorption and cell adhesion. Cell adhesion to these surfaces under serum-free conditions was measured using a centrifugation assay as previously described (Reyes and García, 2003b). Briefly, near-confluent HT1080 cells were loaded with 2 $\mu\text{g}/\text{ml}$ calcein-AM and resuspended serum-free in DPBS + 2 mM dextrose. Cells were seeded onto the substrates (10,000 cells/well) and allowed to attach for 1 h at 37°C. For blocking experiments, cells were incubated in suspension for 15 minutes in the presence of 20 $\mu\text{g}/\text{ml}$ anti-human VLA-2 ($\alpha_2\beta_1$) integrin monoclonal antibody (MAB1998Z) or anti-human CD49e (α_5) (CBL497) antibody and then seeded onto the mixed ligand surfaces for 1 h at 37°C. The surfaces were then inverted and centrifuged at the specified speed for 5 min on a Beckman Allegra 6 centrifuge (GH 6.8 rotor) to detach the cells. The post-spin fluorescence data was normalized by the pre-spin data and plotted against ligand density to obtain adhesion profiles (fraction of adherent cells vs. coating concentration).

Immunostaining for Focal Adhesions

Mixed ligand surfaces were prepared in 35 mm dishes and blocked with 5% non-fat dry milk in DPBS for 1 h to prevent non-specific protein adsorption and cell adhesion. HT1080 cells were seeded at a density of 225 cells/ mm^2 in 10% serum for 6 h. Cells were then permeabilized in ice-cold buffer (50 mM NaCl, 150 mM sucrose, 3 mM MgCl_2 , 50 mM Tris, pH 6.8) supplemented with 0.5% Triton X-100 and protease inhibitors (20 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ leupeptin, 2 mM phenylmethylsulfonyl fluoride) for 5 min to remove membranes and soluble non-cytoskeletal cytoplasmic components. Detergent-extracted cells were fixed in cold formaldehyde (3.7% in DPBS) for 5 min, blocked in blocking buffer (5% FBS in DPBS) for 1 h, and incubated with anti-vinculin (1:500 dilution in blocking buffer) 1 h at 37°C. Primary antibody was visualized using an AlexaFluor 488-conjugated secondary antibody (anti-mouse IgG; 1:200 dilution) for 1-h incubation. Images were captured using a Nikon Eclipse E400 fluorescence microscope with a 100X objective and ImagePro Plus image acquisition software.

Immunoprecipitation/Western Blotting of Integrins

Mixed ligand surfaces were prepared in 60 mm dishes as described above and blocked with 5% non-fat dry milk in DPBS for 1 h to prevent non-specific protein adsorption and cell adhesion. HT1080 cells were seeded at a density of 30,000 cells/ cm^2 in 10% serum for 1 h. Cells were then lysed in 250 μl mild lysis buffer (1% NP-40, 0.15 N NaCl, 0.01 M sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM NaF, 0.2 mM $\text{Na}_3(\text{PO}_4)$, 100 U/ml aprotinin). Protein concentration was determined using a Pierce Micro BCA protein assay kit.

For immunoprecipitation, volumes equivalent to 200 μg of sample protein were added to NET gel buffer (50mM tris HCl, pH7.5, 100 mM NaCl, 0.1% NP-40, 1 mM EDTA, pH 8.0, 0.25% gelatin, 0.02% NaN_3) for a total volume of 500 μl . To precipitate α_2 or α_5 integrins, 5 μl of rabbit anti-human integrin α_2 or α_5 polyclonal antibody (AB1936 or AB1928, Chemicon) was added to each sample. These mixtures were then incubated overnight at 4°C to promote antibody binding. Twelve hours later, 40 μl of protein A-agarose beads (Immunopure, Pierce) was added to each sample and incubated for 3 h with agitation. The beads were washed two times with NET gel buffer and one time with 0.1% NP-40 in 10 mM Tris. The beads were then boiled in Laemmli sample buffer (2% SDS, 10% glycerol, 100

mM DTT, 60 mM Tris-HCl pH 6.8, and 0.001% bromophenol blue) for 10 min and separated on a 7% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel.

Proteins were transferred onto nitrocellulose membranes and blocked in Blotto (5% non-fat dry milk, 0.02% sodium azide, 0.2% Tween 20 in DPBS w/o $\text{Ca}^{2+}/\text{Mg}^{2+}$) overnight at 4°C. Membranes were then incubated with primary antibody – rabbit anti-integrin α_5 polyclonal antibody (1:1000 dilution, AB1928, Chemicon) or rabbit anti-human integrin α_2 polyclonal antibody for verification of the IP (1:1000, AB1936, Chemicon) – in Blotto for 1 h at room temperature under gentle rocking. Membranes were washed in TBS-Tween (20 mM Tris HCl pH 7.6, 137 mM NaCl, 0.1% Tween 20) for 30 min and incubated in secondary antibody (biotin-conjugated anti-rabbit IgG, 1:20,000 dilution in Blotto) for 1 h at room temperature under gentle rocking. Membranes were washed again in TBS-Tween for 30 min and incubated in a tertiary detection antibody (alkaline phosphatase-conjugated anti-biotin IgG, 1:10,000 dilution in Blotto) for 1 h at room temperature under gentle rocking. After antibody incubation, membranes were washed in TBS-Tween for 30 min and immunoreactivity was detected using ECF fluorescent substrate. Bands were visualized using a Fuji Image Analyzer. Samples in which cell lysate, immunoprecipitating antibody, or Protein A-beads were omitted served as negative controls to establish the stringency and specificity of the immunoprecipitation/immunoblotting procedure.

Integrin Binding Assays

Integrin binding levels were assessed using a cross-linking/extraction/reversal procedure (Petrie et al., 2006). Mixed ligand surfaces were generated in a 96-well plate as previously described. After 30 min adhesion, bound integrins were cross-linked with DTSSP, and cells were extracted in 0.1% SDS with protease inhibitors. After stringent washing, cross-links were cleaved in DTT at 37°C. Recovered integrins levels were quantified by Western blotting as described above.

Spot Blotting Analysis of FAK Activation

A high-throughput spot blot assay was used to quantify FAK phosphorylation. Mixed ligand surfaces were generated in a 96-well plate and blocked with 5% non-fat dry milk in DPBS for 1 h to prevent non-specific protein adsorption and cell adhesion. HT1080 cells were incubated in serum-free suspension (DMEM + 5% BSA) for 40 min with mild shaking to reduce focal adhesion kinase (FAK) background activation. The cells were then seeded onto the surfaces (10,000 cells/well) and allowed to attach for 2 h at 37°C. For blocking experiments, cells were incubated in suspension for last 15 min in the presence of 20 $\mu\text{g}/\text{ml}$ anti-human VLA-2 ($\alpha_2\beta_1$) integrin monoclonal antibody (MAB1998Z) or anti-human CD49e (α_5) antibody and then seeded onto the surfaces for 2 h at 37°C.

Adherent cells were washed once with DPBS and lysed with 110 μl cold radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 150 mM Tris-HCl pH 7.2, 350 $\mu\text{g}/\text{ml}$ phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, and 1 mM $\text{Na}_3(\text{PO}_4)$) for 20 min on ice. Lysates were then split in half (to probe for total FAK and activated FAK) and added to the wells of a Minifold I Spot-Blot System (Schleicher & Schuell Bioscience) containing a 0.20 μm pore nitrocellulose membrane, prepared according to the instructions. The lysates were incubated for 30 min and then filtered through the membrane with a vacuum for 5 min. Membranes were blocked in Blotto overnight at 4°C.

Membranes were then incubated with primary antibody – anti-FAK (1 $\mu\text{g}/\text{ml}$) or anti-FAK pY397 (0.35 $\mu\text{g}/\text{ml}$) – in Blotto for 1 h at room temperature, rocking. Membranes were washed in TBS-Tween (20 mM Tris HCl pH 7.6, 137 mM NaCl, 0.1% Tween 20) for 30

min and incubated in secondary antibody (biotin-conjugated anti-rabbit IgG, 1:20,000 dilution in Blotto) for 1 h at room temperature, rocking. Membranes were washed again in TBS-Tween for 30 min and incubated in a tertiary detection antibody (alkaline phosphatase-conjugated anti-biotin IgG, 1:10,000 dilution in Blotto) for 1 h at room temperature, rocking. After antibody incubation, membranes were washed in TBS-Tween for 30 min and immunoreactivity was detected using an ECF fluorescent substrate. Bands were visualized using a Fuji Image Analyzer and further quantified and analyzed using Adobe Photoshop software. FAK phosphorylation levels were normalized to the amount of total FAK in each experimental run.

BrdU Analysis of Cell Proliferation

Proliferation was measured using the APC bromodeoxyuridine (BrdU) flow kit from BD Biosciences according to the manufacturer's instructions. Mixed ligand surfaces were generated on 60 mm dishes and blocked with 5% non-fat dry milk in DPBS for 1 h to prevent non-specific protein adsorption and cell adhesion. HT1080 cells were seeded at a density of 5,000 cells/cm² in 10% serum for 24 h. Cells were then exposed to BrdU for 12 h to identify actively cycling populations. Cells were then fixed and permeabilized via the BrdU flow kit reagents. DNase was added to the samples to expose DNA epitopes. An APC-conjugated anti-BrdU antibody was used to stain for incorporated BrdU. Each sample was analyzed for APC-positive staining via flow cytometry.

Statistics

All experiments were performed at least three times in triplicate unless otherwise noted. Data are reported as mean $\pm$ standard error. Results were analyzed by one-way ANOVA using SYSTAT 8.0 (SPSS). If treatment level differences were determined to be significant, pair-wise comparisons were performed using a Tukey post-hoc test. A 95% confidence level was considered significant.

Results

Mixed Collagen- and Fibronectin-Mimetic Surfaces

We selected the collagen-mimetic peptide (GFOGER) and the fibronectin-mimetic fragment (FNIII7-10) to specifically target the $\alpha_2\beta_1$ and $\alpha_5\beta_1$ receptors, respectively. These receptors play central roles in adhesive functions for several mesenchymal cells, including osteoblasts, fibroblasts, and chondrocytes. In addition, both of these receptors are highly expressed in these cell types, and these cell types interact with both fibronectin and collagen type I in their native environment. The collagen-mimetic GFOGER peptide has the primary sequence GGYGGGPC(GPP)₅GFOGER(GPP)₅GPC (Reyes and García, 2003a; Reyes and García, 2004). This synthetic peptide has been engineered to contain the hexapeptide sequence, GFOGER, from type I collagen that is recognized by the $\alpha_2\beta_1$ integrin. The GPP triplets on either side of the GFOGER recognition site provide cooperative clusters that promote the formation of a stable right-handed triple helical structure at room temperature (Fields and Prockop, 1996; Knight et al., 2000; Nagarajan et al., 1998). This triple-helical conformation is essential for integrin recognition and $\alpha_2\beta_1$ -mediated cell adhesion (Messent et al., 1998; Morton et al., 1997; Morton et al., 1994). The FN-mimetic ligand used in these experiments is FNIII7-10, a recombinant fragment of FN that spans the 7-10th type III repeats of FN and contains the PHSRN and RGD adhesion motifs that cooperatively form the recognition site for the $\alpha_5\beta_1$ integrin (García et al., 2002). This recombinant fragment has equivalent adhesive activity as human plasma FN (Petrie et al., 2006).

To create model mixed ligand surfaces, we exploited the high affinity and specificity of the biotin-avidin interaction. NeutrAvidin biotin-binding protein, a commercially available

deglycosylated avidin derivative with exceptionally low nonspecific binding properties, was passively adsorbed onto tissue culture-treated polystyrene as a non-fouling support layer. FNIII7-10 was monobiotinylated during expression; GFOGER was biotinylated at the C-terminus with biotin-LC-PEO-amine using carbodiimide chemistry. To generate mixed ligand surfaces, the biotinylated ligands were added sequentially to the NeutrAvidin support layer. Based on the significant size differences between the FNIII7-10 fragment and the GFOGER-peptide, biotinylated fragment was introduced to the NeutrAvidin surface first for 1 h. After rinsing, biotinylated GFOGER-peptide was added for an additional hour. Control experiments demonstrated that only biotinylated ligands were immobilized onto the NeutrAvidin surfaces (data not shown). Using this strategy, we generated 32 distinct mixed formulations presenting controlled densities of the adhesive ligands within a non-adhesive background.

Surface Density Measurements

ELISA was used as a high throughput assay to determine the relative ligand densities present on mixed ECM surfaces. For detection purposes, fluorescein was coupled to the terminal amine of the GFOGER peptide using NHS-fluorescein. The immobilized ligands were then detected by ELISA using anti-FN or anti-fluorescein antibodies. Fig. 1 shows the immobilized ligand densities for 4 FNIII7-10 coating concentrations (0, 0.2, 2.5, 5.0 $\mu\text{g/ml}$), each of which were incubated in 2-fold serial dilutions of GFOGER-peptide (from 0 to 5 $\mu\text{g/ml}$). The density of immobilized FNIII7-10 (Fig. 1, triangles, right axis) increases with increasing FNIII7-10 coating concentration and is insensitive to the coating concentration of GFOGER-peptide (horizontal axis). This effect is due to the fact that the FN fragment is allowed to tether before the GFOGER peptide is introduced. The density of tethered GFOGER-peptide (Fig. 1, circles, left axis) increases with peptide coating concentration and is modulated by the density of immobilized FNIII7-10.

The leftmost GFOGER-peptide tethering profile represents a surface that contains no FNIII7-10. As the amount of FN fragment added to the surface increases, the fragment begins to occupy the surface anchoring sites first, leaving less available sites for GFOGER peptide tethering. This decrease in GFOGER immobilization with increasing amounts FNIII7-10 shifts the tethering profiles to the right, until the FN fragment is saturated on the surface.

Absolute surface density measurements were obtained by surface plasmon resonance (SPR) spectroscopy (Table 1). These measurements were correlated with the ELISA data and subsequent figures reflect absolute density values. The wide range of peptide surface densities (30-fold for FNIII7-10, 100-fold for GFOGER) generated by this immobilization process provides for well-defined bioadhesive surfaces with independent control of two specific integrin-targeted ligands.

Cell Adhesion to Mixed Ligand Surfaces

Cell adhesion to these mixed ligand surfaces was examined using a centrifugation assay in which HT1080 cells were seeded for 1 h in serum-free conditions and then centrifuged to detach the cells. An HT1080 human fibrosarcoma cell model was chosen because these cells adhere to type I collagen by a single mechanism involving the integrin $\alpha_2\beta_1$ (Grenz et al., 1993; Tuckwell et al., 1995). These cells also recognize fibronectin through integrin-mediated processes and thus represent a relevant cell model to examine the interplay between these two matrix proteins. The resultant cell adhesion profiles (Fig. 2) correlated well with the relative ligand densities reflected in the ELISA data. The line graph shows that for high FNIII7-10 coating concentrations (Fig. 2a, circles), adhesion is dominated by the FN fragment and the adhesion profiles are insensitive to changes in the GFOGER-peptide

density, especially at low GFOGER-peptide densities. It is only when the amount of GFOGER peptide on the surface is nearly saturated that subsequent increases in adhesion are observed. As FNIII7-10 coating concentration decreases, adhesion drops at the lower GFOGER-peptide densities and the data begins to approach the more familiar profile of density-dependent increases in cell adhesion with increasing GFOGER peptide density (Reyes and Garcia, 2003b) (Fig. 2a, triangles, squares). When FNIII7-10 is completely omitted from the surface, the adhesion profile again shifts to the right demonstrating the reduced adhesive potential of the single-ligand functionalized surfaces compared with the mixed ligands (Fig. 2a, diamonds).

A contour plot of the cell adhesion data (Fig. 2b) demonstrates the enhanced adhesion at high binding levels of both integrins, compared to the single ligand surfaces. Adhesion increases with increasing FNIII7-10 until the FN fragment is saturated on the surface. Adding GFOGER-peptide to this saturated level of FNIII7-10, further increases the surface's adhesive potential. Similarly, adhesion also increases with increasing GFOGER peptide. High densities of both ligands results in the highest observed levels of integrin-mediated cell adhesion.

The relative contributions of the $\alpha_2\beta_1$ and $\alpha_5\beta_1$ integrins to cell adhesion were examined by blocking each integrin separately with function-perturbing antibodies. Incubation with a $\alpha_2\beta_1$ integrin blocking antibody (Fig. 3a) completely eliminates the effect of increasing GFOGER peptide density observed in Fig. 2. Adhesion increases with increasing FNIII7-10 density and remains insensitive to any changes in GFOGER peptide density. Conversely, a $\alpha_5\beta_1$ integrin blocking antibody completely eliminates the effect of increasing FNIII7-10 density (Fig. 3b). Cell adhesion increases with increasing GFOGER peptide and the adhesion profiles are identical at two different levels of FNIII7-10 density (Fig. 3b, circles, triangles). As expected, the blocking antibodies also eliminate the enhanced adhesion conferred by high densities of both integrin-targeted ligands compared to the single-ligand surfaces.

Integrin Binding to Mixed Ligand Surfaces

We used a cross-linking/extraction/reversal biochemical technique to quantify the number of bound integrins. We previously demonstrated that this assay specifically measures bound integrins to ECM ligands. Integrin binding was quantified for mixed ECM ligand surfaces presenting low and high densities of FNIII7-10 and low, medium and high densities of GFOGER (Fig. 4). Integrin $\alpha_5\beta_1$ binding increases with FNIII7-10 tethered density and is insensitive to GFOGER density (Fig. 4a). Similarly, integrin $\alpha_2\beta_1$ binding exhibits dose-dependent increases with GFOGER density and is not modulated by FNIII7-10 density (Fig. 4b). These results demonstrate that mixed ECM ligand surfaces exhibit high specificity for the corresponding receptor and minimal cross-reactivity or transactivation between $\alpha_5\beta_1$ and $\alpha_2\beta_1$ integrins.

Focal Adhesions on the Mixed Ligand Surfaces

HT1080 cells were seeded onto mixed ligand surfaces for 6 h in 10% serum to promote the formation of focal adhesions, which are characterized by clustered integrin components and intracellular structural and signaling proteins coupled to the actin cytoskeleton. The cells were then extracted, fixed, and stained for vinculin, an intracellular structural protein that localizes to focal adhesion plaques. Immunofluorescence staining for vinculin (Fig. 5a) reveals that both the single ligand and the mixed ligand surfaces promote the formation of these focal adhesion structures. The two single ligand surfaces are characterized by several relatively small structures diffusely distributed around the periphery of the cell. In contrast, the mixed ligand surfaces consistently promote the formation of larger, more distinct

adhesion plaques localized at the cell edges. The cells were stained concurrently with rhodamine-phalloidin to examine actin organization; however, no differences in actin staining were observed across all conditions (data not shown). The cytoarchitectural effects of the mixed ligand surfaces were limited to focal adhesion structures.

To further understand these adhesion structures, we conducted immunoprecipitation experiments to examine the co-localization of integrin receptors to adhesive clusters. Integrin receptors were immunoprecipitated under mild lysis conditions and then the IP fraction was blotted for the presence of α_2 or α_5 integrin. Elevated staining in the Western blot indicates that the two integrins co-localize to membrane complexes on the mixed surfaces to a much greater extent than on either of the single component surfaces (Fig 5b). The increased co-localization of $\alpha_5\beta_1$ and $\alpha_2\beta_1$ integrins detected by immunoprecipitation is consistent with enhancements in the assembly of vinculin-containing focal adhesions. The results indicate that presentation of mixed ECM ligands promotes co-localization of integrins to adhesive clusters and enhances focal adhesion assembly. Importantly, these differences in focal adhesion assembly among mixed and single ECM ligand surfaces are not dependent on alterations on integrin binding as no differences in bound integrin levels are observed (Fig. 4).

Synergistic Focal Adhesion Kinase Activation

We postulated that the enhanced co-localization of $\alpha_5\beta_1$ and $\alpha_2\beta_1$ integrins to focal adhesions on mixed ECM surfaces would also enhance integrin-mediated signaling. To analyze the effects of the mixed ligand presentation on triggering post-adhesion signaling events, we examined the extent to which these mixed ligand surfaces trigger the activation of focal adhesion kinase (FAK), an intracellular signaling molecule implicated in integrin-mediated signal transduction and downstream differentiation pathways. Using standard spot blotting techniques, we probed the phosphorylation of tyrosine 397, which is the autophosphorylation site of FAK and also binds Src and PI3-kinase. The results clearly demonstrate a synergistic activation of FAK on these mixed surfaces (Fig. 6). While FAK phosphorylation does increase with both increasing GFOGER and FNIII7-10 densities, we observe at least a three-fold increase in FAK activity at high levels of both ligands. At saturating levels of FN fragment alone or GFOGER peptide alone, the FAK activity levels reach ~ 0.5 (rel. units); at saturating levels of both ligands, FAK activity increases four-fold. This effect is clearly evident in the contour plot (Fig. 6b) showing a “hot spot” of FAK activation for high FNIII7-10 and GFOGER densities.

To demonstrate the integrin specificity of this synergistic effect, this assay was repeated in the presence of integrin-blocking antibodies (Fig. 7). The leftmost data set in Fig. 7 shows FAK activation in the absence of any function-blocking antibodies, which recapitulates the same synergistic effect of the mixed ligand presentation demonstrated in Fig. 6. Antibody blocking of the α_2 integrin reduces FAK activation on the GFOGER surfaces to background levels (Fig 7, middle). Blocking also eliminates the synergistic enhancement in signaling on the mixed ligand surfaces. Instead, the signaling is equivalent to that of the single ligand, FNIII7-10 surface. A similar effect is observed upon blocking the α_5 integrin (Fig. 7, right). FAK activation levels on the FNIII7-10 surface fall to background levels and the mixed ligand surface is now equivalent to the single component GFOGER surface. This data verifies that the specific binding of these two separate integrins on the mixed ligand surface is responsible for the synergistic enhancement of this downstream intracellular signal.

Proliferation on Mixed Ligand Surfaces

To determine whether this synergy in intracellular signaling translates to a downstream cellular response, we examined proliferation on mixed and single ligand functionalized

surfaces using BrdU incorporation. Fig. 8 demonstrates enhanced proliferation rate on the mixed surfaces compared to the each of the single ligand-functionalized surfaces. This enhanced proliferation rate on the mixed ligand surface parallels both the cell adhesion and the FAK activation data.

Discussion

Transmembrane integrin receptors bind to specific ECM proteins, generating important signals that regulate cell proliferation, differentiation, and migration events (Giancotti and Ruoslahti, 1999). Although there is a wealth of knowledge regarding the convergence of signaling pathways activated by integrin and growth-factor receptors, little is known about how these signals are integrated by cells and whether there are common receptor-proximal control points that synchronize the execution of biological functions such as cell motility.

In the present study, we engineered well-defined surfaces that support two separate integrin interactions, $\alpha_5\beta_1$ and $\alpha_2\beta_1$, using FN- and collagen-mimetic ligands to examine the interplay of two distinct integrin interactions in adhesive responses. Both of these integrin-ligand interactions have been implicated in several signaling and differentiation pathways. The significance of the present study lies on the rigorous, quantitative analyses of cell adhesive responses, including integrin binding, adhesive force, FAK activation, and proliferation, to well-defined surfaces presenting two different ECM ligands. In particular, we demonstrate synergistic enhancements in adhesion strength, signaling (FAK activation), and proliferation for mixed ligand substrates compared to single ligand surfaces. While there is previous evidence that multiple ECM ligands modulate adhesive interactions (Calderwood et al., 2004; Ly et al., 2003; Rose et al., 2003), major strengths of the present work include the use of well-defined ligand densities and rigorous and quantitative analyses of adhesive events, which are absolutely necessary to make conclusions regarding additive, synergistic or antagonistic interactions among ECM ligands. Furthermore, the use of engineered ligands that isolate specific integrin binding sites controls for precise ligand presentation and integrin binding and avoid confounding effects arising from native ECM proteins that contain multiple integrin binding sites and other ECM-interacting domains. We also note that the observed synergistic interactions contrast previous reports of antagonism between integrin receptors (referred to as “transactivation” (Calderwood et al., 2004)). Our study supports a model in which the mixed ligand substrates synergistically enhance adhesive responses by promoting integrin co-localization into adhesive complexes without altering total levels of bound integrins. The results provide new insights into the interplay of ECM ligands and adhesive interactions.

The avidin-biotin immobilization strategy provides a simple and easily controlled platform to efficiently screen a large number of mixed surface compositions using short term assays. In addition, the wide range of mixed ligand densities generated by this process demonstrates independent control over two integrin binding events. We show that high levels of concurrent $\alpha_5\beta_1$ and $\alpha_2\beta_1$ integrin binding result in increased and synergistic cell adhesion and focal adhesion assembly compared to saturating levels of either integrin alone. Since focal adhesions represent junctions of integrin-mediated intracellular signaling, these results suggest an advantage of simultaneously binding two separate integrins in triggering post-adhesion signaling events.

The dual integrin signaling also triggers a synergistic activation of FAK, when compared with single ligand surfaces. FAK is a cytoplasmic tyrosine kinase that plays a key role in integrin-mediated signal transduction through its co-localization with integrins and cytoskeletal proteins in focal adhesion contacts (Hanks and Polte, 1997). The synergistic activation of this signaling molecule at high levels of both $\alpha_5\beta_1$ and $\alpha_2\beta_1$ integrin binding

suggests that these two integrin binding events converge at the level of the focal adhesion to enhance intracellular signal transduction. This explanation is consistent with the presence of more robust focal adhesions on mixed ECM surfaces compared to single ligand substrates. This data underscores the advantage of specifically targeting more than one integrin implicated in a particular signaling pathway and downstream cellular effect.

In a similar manner to integrins and growth factor receptors (Katz et al., 2002; Miyamoto et al., 1996; Schneller et al., 1997), multiple integrin signaling pathways may interact through several mechanisms, from membrane-proximal clustering of the two integrin types to the activation of common downstream signaling pathways. The immunoprecipitation results demonstrate that $\alpha_5\beta_1$ and $\alpha_2\beta_1$ physically co-localize to membrane complexes on the mixed surfaces, suggesting that integrin clustering may contribute to signal enhancement. In addition, other groups have shown that integrin-dependent FAK tyrosine phosphorylation, as well as MAPK signaling, can be induced by integrin aggregation (Katz et al., 2002; Miyamoto et al., 1995), raising the possibility that activating and clustering multiple integrin types may further increase the downstream signaling effect. Cell proliferation results on the mixed ligand surfaces confirm that the enhanced signaling effects observed in the FAK phosphorylation data translate to downstream cellular responses.

We have demonstrated that the presentation of multiple integrin-binding ligands synergize to enhance intracellular signaling and proliferation. This study suggests that, instead of focusing on a single integrin-ligand interaction, in some cases it may be advantageous to consider the interplay of multiple integrins implicated in a desired cell response and their combined effect on downstream cellular signals. Such an approach allows the study of how cells integrate the multiple integrin signals that are triggered due to the complexity of the ECM and the wide array of tissue-specific integrin receptor combinations. Moreover, these analyses provide insights into the rational engineering of optimal biospecific surfaces for implant coatings, wound healing devices, and tissue engineering scaffolds that exploit that complexity of the extracellular matrix and its signaling characteristics.

Acknowledgments

Contract Grant Sponsor: NIH (R01 EB-004496); Georgia Tech/Emory NSF ERC on the Engineering of Living Tissues (EEC-9731643); Arthritis Foundation

References

- Boudreau N, Sympton CJ, Werb Z, Bissell MJ. Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. *Science*. 1995; 267:891–893. [PubMed: 7531366]
- Calderwood DA, Tai V, Di Paolo G, De Camilli P, Ginsberg MH. Competition for talin results in trans-dominant inhibition of integrin activation. *J Biol Chem*. 2004; 279:28889–28895. [PubMed: 15143061]
- Dedhar S, Hannigan GE. Integrin cytoplasmic interactions and bidirectional transmembrane signalling. *Curr Opin Cell Biol*. 1996; 8:657–669. [PubMed: 8939656]
- Dike LE, Ingber DE. Integrin-dependent induction of early growth response genes in capillary endothelial cells. *J Cell Sci*. 1996; 109:2855–2863. [PubMed: 9013333]
- Fields GB, Prockop DJ. Perspectives on the synthesis and application of triple-helical, collagen-model peptides. *Biopolymers*. 1996; 40:345–357. [PubMed: 8765606]
- Frisch SM, Ruoslahti E. Integrins and anoikis. *Curr Opin Cell Biol*. 1997; 9:701–706. [PubMed: 9330874]
- García AJ, Schwarzbauer JE, Boettiger D. Distinct activation states of $\alpha_5\beta_1$ integrin show differential binding to RGD and synergy domains of fibronectin. *Biochemistry*. 2002; 41:9063–9069. [PubMed: 12119020]
- Giancotti FG, Ruoslahti E. Integrin signaling. *Science*. 1999; 285:1028–1032. [PubMed: 10446041]

- Globus RK, Doty SB, Lull JC, Holmuhamedov E, Humphries MJ, Damsky CH. Fibronectin is a survival factor for differentiated osteoblasts. *J Cell Sci.* 1998; 111:1385–1393. [PubMed: 9570756]
- Grenz H, Carbonetto S, Goodman SL. Alpha 3 beta 1 integrin is moved into focal contacts in kidney mesangial cells. *J Cell Sci.* 1993; 105:739–751. [PubMed: 8408301]
- Gronthos S, Stewart K, Graves SE, Hay S, Simmons PJ. Integrin expression and function on human osteoblast-like cells. *J Bone Miner Res.* 1997; 12:1189–1197. [PubMed: 9258748]
- Hanks SK, Polte TR. Signaling through focal adhesion kinase. *BioEssays.* 1997; 19:137–145. [PubMed: 9046243]
- Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell.* 2002; 110:673–687. [PubMed: 12297042]
- Jikko A, Harris SE, Chen D, Mendrick DL, Damsky CH. Collagen integrin receptors regulate early osteoblast differentiation induced by BMP-2. *J Bone Miner Res.* 1999; 14:1075–1083. [PubMed: 10404007]
- Katz BZ, Miyamoto S, Teramoto H, Zohar M, Krylov D, Vinson C, Gutkind JS, Yamada KM. Direct transmembrane clustering and cytoplasmic dimerization of focal adhesion kinase initiates its tyrosine phosphorylation. *Biochim Biophys Acta.* 2002; 1592:141–152. [PubMed: 12379477]
- Kiely PA, Leahy M, O'Gorman D, O'Connor R. RACK1-mediated integration of adhesion and insulin-like growth factor I (IGF-I) signaling and cell migration are defective in cells expressing an IGF-I receptor mutated at tyrosines 1250 and 1251. *J Biol Chem.* 2005; 280:7624–7633. [PubMed: 15611085]
- Knight CG, Morton LF, Peachey AR, Tuckwell DS, Farndale RW, Barnes MJ. The collagen-binding A-domains of integrins alpha(1)beta(1) and alpha(2)beta(1) recognize the same specific amino acid sequence, GFOGER, in native (triple-helical) collagens. *J Biol Chem.* 2000; 275:35–40. [PubMed: 10617582]
- Ly DP, Zazzali KM, Corbett SA. De novo expression of the integrin alpha5beta1 regulates alphavbeta3-mediated adhesion and migration on fibrinogen. *J Biol Chem.* 2003; 278:21878–21885. [PubMed: 12676956]
- Messent AJ, Tuckwell DS, Knauper V, Humphries MJ, Murphy G, Gavrilovic J. Effects of collagenase-cleavage of type I collagen on alpha2beta1 integrin-mediated cell adhesion. *J Cell Sci.* 1998; 111:1127–1135. [PubMed: 9512508]
- Miyamoto S, Teramoto H, Coso OA, Gutkind JS, Burbelo PD, Akiyama SK, Yamada KM. Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. *J Cell Biol.* 1995; 131:791–805. [PubMed: 7593197]
- Miyamoto S, Teramoto H, Gutkind JS, Yamada KM. Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. *J Cell Biol.* 1996; 135:1633–1642. [PubMed: 8978828]
- Mizuno M, Fujisawa R, Kuboki Y. Type I collagen-induced osteoblastic differentiation of bone-marrow cells mediated by collagen-alpha2beta1 integrin interaction. *J Cell Physiol.* 2000; 184:207–213. [PubMed: 10867645]
- Mizuno M, Kuboki Y. Osteoblast-related gene expression of bone marrow cells during the osteoblastic differentiation induced by type I collagen. *J Biochem.* 2001; 129:133–138. [PubMed: 11134967]
- Morton LF, Peachey AR, Knight CG, Farndale RW, Barnes MJ. The platelet reactivity of synthetic peptides based on the collagen III fragment alpha1(III)CB4. Evidence for an integrin alpha2beta1 recognition site involving residues 522-528 of the alpha1(III) collagen chain. *J Biol Chem.* 1997; 272:11044–11048. [PubMed: 9110997]
- Morton LF, Peachey AR, Zijenah LS, Goodall AH, Humphries MJ, Barnes MJ. Conformation-dependent platelet adhesion to collagen involving integrin alpha 2 beta 1-mediated and other mechanisms: multiple alpha 2 beta 1-recognition sites in collagen type I. *Biochem J.* 1994; 299:791–797. [PubMed: 7514871]
- Moursi AM, Damsky CH, Lull J, Zimmerman D, Doty SB, Aota S, Globus RK. Fibronectin regulates calvarial osteoblast differentiation. *J Cell Sci.* 1996; 109:1369–1380. [PubMed: 8799825]
- Moursi AM, Globus RK, Damsky CH. Interactions between integrin receptors and fibronectin are required for calvarial osteoblast differentiation in vitro. *J Cell Sci.* 1997; 110:2187–2196. [PubMed: 9378768]

- Nagarajan V, Kamitori S, Okuyama K. Crystal structure analysis of collagen model peptide (Pro-pro-Gly)₁₀. *J Biochem.* 1998; 124:1117–1123. [PubMed: 9832616]
- Petrie TA, Capadona JR, Reyes CD, García AJ. Integrin specificity and enhanced cellular activities associated with surfaces presenting a recombinant fibronectin fragment compared to RGD supports. *Biomaterials.* 2006; 27:5459–5470. [PubMed: 16846640]
- Reginato MJ, Mills KR, Paulus JK, Lynch DK, Sgroi DC, Debnath J, Muthuswamy SK, Brugge JS. Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. *Nat Cell Biol.* 2003; 5:733–740. [PubMed: 12844146]
- Reyes CD, García AJ. Engineering integrin-specific surfaces with a triple-helical collagen-mimetic peptide. *J Biomed Mater Res.* 2003a; 65A:511–523.
- Reyes CD, García AJ. A centrifugation cell adhesion assay for high-throughput screening of biomaterial surfaces. *J Biomed Mater Res.* 2003b; 67A:328–333.
- Reyes CD, García AJ. Alpha2beta1 integrin-specific collagen-mimetic surfaces supporting osteoblastic differentiation. *J Biomed Mater Res.* 2004; 69A:591–600.
- Rose DM, Liu S, Woodside DG, Han J, Schlaepfer DD, Ginsberg MH. Paxillin binding to the alpha 4 integrin subunit stimulates LFA-1 (integrin alpha L beta 2)-dependent T cell migration by augmenting the activation of focal adhesion kinase/proline-rich tyrosine kinase-2. *J Immunol.* 2003; 170:5912–5918. [PubMed: 12794117]
- Schneller M, Vuori K, Ruoslahti E. Alphavbeta3 integrin associates with activated insulin and PDGFbeta receptors and potentiates the biological activity of PDGF. *EMBO J.* 1997; 16:5600–5607. [PubMed: 9312019]
- Sieg DJ, Hauck CR, Ilic D, Klingbeil CK, Schaefer E, Damsky CH, Schlaepfer DD. FAK integrates growth-factor and integrin signals to promote cell migration. *Nat Cell Biol.* 2000; 2:249–256. [PubMed: 10806474]
- Suzawa M, Tamura Y, Fukumoto S, Miyazono K, Fujita T, Kato S, Takeuchi Y. Stimulation of Smad1 transcriptional activity by Ras-extracellular signal-regulated kinase pathway: a possible mechanism for collagen-dependent osteoblastic differentiation. *J Bone Miner Res.* 2002; 17:240–248. [PubMed: 11811554]
- Takeuchi Y, Suzawa M, Kikuchi T, Nishida E, Fujita T, Matsumoto T. Differentiation and transforming growth factor-beta receptor down-regulation by collagen-alpha2beta1 integrin interaction is mediated by focal adhesion kinase and its downstream signals in murine osteoblastic cells. *J Biol Chem.* 1997; 272:29309–29316. [PubMed: 9361011]
- Tamura Y, Takeuchi Y, Suzawa M, Fukumoto S, Kato M, Miyazono K, Fujita T. Focal adhesion kinase activity is required for bone morphogenetic protein--Smad1 signaling and osteoblastic differentiation in murine MC3T3-E1 cells. *J Bone Miner Res.* 2001; 16:1772–1779. [PubMed: 11585340]
- Tuckwell D, Calderwood DA, Green LJ, Humphries MJ. Integrin alpha 2 I-domain is a binding site for collagens. *J Cell Sci.* 1995; 108:1629–1637. [PubMed: 7615681]
- Xiao G, Gopalakrishnan R, Jiang D, Reith E, Benson MD, Franceschi RT. Bone morphogenetic proteins, extracellular matrix, and mitogen-activated protein kinase signaling pathways are required for osteoblast-specific gene expression and differentiation in MC3T3-E1 cells. *J Bone Miner Res.* 2002a; 17:101–110.
- Xiao G, Jiang D, Gopalakrishnan R, Franceschi RT. Fibroblast growth factor 2 induction of the osteocalcin gene requires MAPK activity and phosphorylation of the osteoblast transcription factor, cbfa1/runx2. *J Biol Chem.* 2002b; 277:36181–36187.
- Zhu X, Ohtsubo M, Bohmer RM, Roberts JM, Assoian RK. Adhesion-dependent cell cycle progression linked to the expression of cyclin D1, activation of cyclin E-cdk2, and phosphorylation of the retinoblastoma protein. *J Cell Biol.* 1996; 133:391–403. [PubMed: 8609171]

Fig 1. Mixed fibronectin- and collagen-mimetic surfaces. Varying concentrations of biotinylated FNIII7-10 and GFOGER-peptide (FITC labeled) were tethered to passively adsorbed NeutrAvidin. Relative surface density was quantified via ELISA using anti-FN antibody (triangles, right axis) or anti-FITC antibody to detect the GFOGER-peptide (circles, left axis).

Fig 2.

Cell adhesion to mixed ECM ligand surfaces **(a)** HT1080 human fibrosarcoma cell adhesion on mixed ligand surfaces (1 h cell adhesion, 12g centrifugation for 5 min). **(b)** Contour plot of cell adhesion on mixed ligand surfaces.

Fig 3.

Effects of integrin blocking on cell adhesion. **(a)** Cell adhesion on mixed ligand surfaces in the presence of an $\alpha_2\beta_1$ integrin blocking antibody. **(b)** Cell adhesion on mixed ligand surfaces in the presence of an $\alpha_5\beta_1$ integrin blocking antibody. Squares represent cells seeded on increasing densities of FNIII7-10 in the absence of GFOGER peptide to verify the action of the $\alpha_5\beta_1$ function-blocking antibody.

Fig 4. Integrin binding analysis on mixed ligand surfaces using a crosslinking/extraction/reversal procedure. Relative binding of **(a)** alpha 5 and **(b)** alpha 2 on mixed ligand surfaces of varying GFOGER and FNIII7-10 ligands. Dashed lines represent the assay background level (average binding values for surfaces with zero FNIII7-10 and GFOGER ligands).

Fig 5. Focal adhesion assembly on mixed ligand surfaces. **(a)** Immunostaining for vinculin in focal adhesions on GFOGER, FNIII7-10, and mixed ligand surfaces. **(b)** Immunoprecipitation and Western blotting showing co-localization of $\alpha_5\beta_1$ and $\alpha_2\beta_1$ integrins in focal adhesions. No cell lysate and no immunoprecipitating antibody negative controls are also shown.

Fig 6. Synergistic activation of focal adhesion kinase (FAK) on mixed ligand surfaces. **(a)** Quantification of spot blotting for phosphorylated tyrosine 397 on FAK. **(b)** Contour plot of FAK activation on mixed ligand surfaces.

Fig 7. Integrin specificity of synergistic FAK activation on mixed ligand surfaces. Cells were plated on surfaces presenting saturating densities of single or mixed ECM ligands. Data shows tyrosine phosphorylation levels on the biomimetic surfaces in the absence and presence of integrin blocking antibodies. * indicates greater than mixed with anti- α_2 or anti- α_5 . † indicates greater than no ligand with anti- α_2 . ‡ indicates greater than no ligand with anti- α_5 .

Fig 8.

Flow cytometry quantification of HT1080 cell proliferation using BrdU incorporation (BrdU added 24 hrs after cell seeding, incorporated for 12 hrs; * indicates different from GFOGER or FN7-10, $p < 0.05$). Cells were plated on surfaces presenting saturating densities of single or mixed ECM ligands.

Table 1

Absolute surface density measurements using Biacore surface plasmon resonance spectroscopy. The italicized values represent extrapolated densities based on the linear portion of the ELISA immobilization curves (Fig. 2). The brackets indicate the standard error of the mean.

GFOGER		FNIII7-10	
Coating Conc. [$\mu\text{g/ml}$]	Surface Density [fmol/cm ²]	Coating Conc. [$\mu\text{g/ml}$]	Surface Density [fmol/cm ²]
5	464 [± 21]	5	352 [± 19]
2.5	240 [± 10]	2.5	177 [± 11]
1.25	185 [± 32]	1.25	88
0.625	58	0.2	11 [± 4]
0.313	36 [± 8]		
0.156	<i>14</i>		
0.078	4 [± 3]		