The Synergy Peptide PHSRN and the Adhesion Peptide RGD Mediate Cell Adhesion through a Common Mechanism†

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ABSTRACT: This work reports on the role of the synergy peptide PHSRN in mediating the adhesion of cells. The attachment of baby hamster kidney cells and 3T3 Swiss fibroblasts to model substrates presenting either GRGDS or PHSRN was evaluated using self-assembled monolayers of alkanethiolates on gold presenting the peptide ligands mixed with tri(ethylene glycol) groups. These substrates permit rigorous control over the structures and densities of peptide ligands and at the same time prevent nonspecific interactions with adherent cells. Both cell types attached efficiently to monolayers presenting either the RGD or the PHSRN peptide but not to monolayers presenting scrambled peptide GRDGS or HRPSN. Cell attachment was comparable on substrates presenting either peptide ligand but less efficient than on substrates presenting the protein fibronectin. The degree of cell spreading, however, was substantially higher on substrates presenting RGD relative to PHSRN. Staining of 3T3 fibroblasts with anti-vinculin and phalloidin revealed clear cytoskeletal filaments and focal adhesions for cells attached by way of either RGD or PHSRN. Inhibition experiments showed that the attachment of 3T3 fibroblasts to monolayers presenting RGD could be inhibited completely by a soluble RGD peptide and partially by a soluble PHSRN peptide. IMR 90 fibroblast attachment to monolayers presenting PHSRN could be inhibited with anti-integrin α_5 or anti-integrin β_1 antibody. This work demonstrates unambiguously that PHSRN alone can support the attachment of cells and that the RGD and PHSRN bind competitively to the integrin receptors.

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The extracellular matrix (ECM)[1] is an insoluble aggregate of large proteins and glycosaminoglycans that plays a vital role in the maintenance and organization of cells in tissue (*1*−*4*). The ECM provides cells with a physical scaffold that structurally organizes tissue and with numerous ligands that mediate adhesion and influence the behaviors of cells, including differentiation, proliferation, and apoptosis. Fibronectin (FN) is a predominant ECM protein that mediates the adhesion and spreading of many cell types (*5*−*7*). The seminal discovery by Pierschbacher and Ruoslahti that the tripeptide Arg-Gly-Asp (RGD) is the principal adhesive ligand that binds integrin receptors, including the $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ receptors (*8*, *9*), was followed by the identification of several additional ligands that influence cell adhesion. But in many cases an understanding of the roles for these ligands is incomplete. In this paper we employ model substrates that serve as mimics of ECM to investigate the mechanism by which the peptide Pro-His-Ser-Arg-Asn (PHSRN) from FN mediates cell adhesion. We show that PHSRN mediates cell attachment in a manner analogous to RGD and that the two peptides bind competitively to cell surface integrin receptors.

The peptide PHSRN is found in the 9th type III domain of FN, adjacent to the 10th domain that contains the RGD peptide (*10*−*14*). PHSRN has been identified as a synergy ligand that enhances the spreading of cells that are attached to substrates that present the RGD peptide (*15*−*25*). Yamada and co-workers first identified this peptide by comparing the attachment and spreading of baby hamster kidney (BHK) cells to plastic tissue culture plates that were coated with either the 10th domain of fibronectin or a recombinant fragment that contained both the 9th and 10th domains. The number of cells that attached to the plates and the degree to which these adherent cells spread were greater on substrates that presented both domains than on substrates that presented only the 10th domain. Substrates that presented only the 9th domain, by contrast, were inactive and gave no cell attachment (*15*). Mardon and Grant reported similar results but also found that cells were less spread on substrates coated with a mixture of separate 9th and 10th domains than on substrates coated with a single recombinant protein containing both domains (*16*). A combination of site-directed mutagenesis and homology scanning identified the PHSRN peptide as the synergy sequence that gave rise to enhanced adhesion (*15*, *17*).

There is now wide consensus that the PHSRN peptide plays an important role in cell adhesion, but there is not yet agreement as to the molecular mechanism by which the synergy peptide increases adhesive activity. A crystal structure of a fragment of FN that comprises domains 7 through 10 revealed that the PHSRN and RGD peptides are on the same face of the protein and separated by ap-

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[1] Abbreviations: ECM, extracellular matrix; FN, fibronectin; GRGDS, Gly-Arg-Gly-Asp-Ser; PHSRN, Pro-His-Ser-Arg-Asn; BHK cells, baby hamster kidney cells; SAMs, self-assembled monolayers; FAK, focal adhesion kinase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry.

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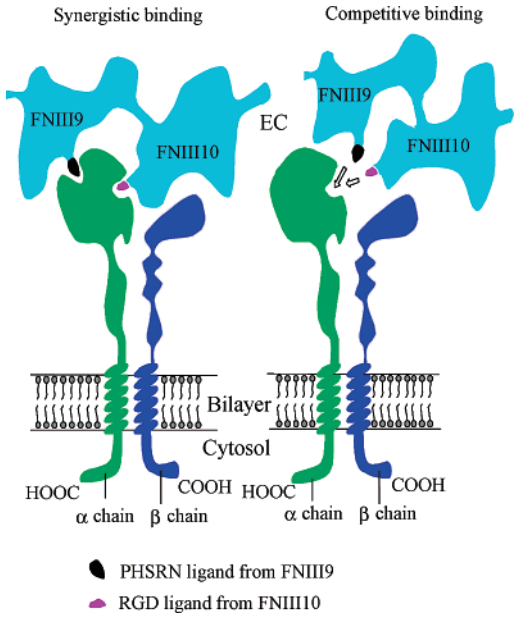

FIGURE 1: Two models by which the PHSRN peptide can act in synergy with RGD to enhance the degree of spreading of adherent cells. In the first model (left) the two peptides, RGD from the 10th domain and PHSRN from the 9th domain, bind to nonoverlapping sites on a single integrin receptor and hence increase the binding affinity for the integrin receptor. In the second model (right) the peptides bind competitively to a common site (or to allosterically related sites).

proximately 35 Å. On the basis of this structural information, Leahy and co-workers proposed that the two peptides could bind opposite sides of a single integrin receptor and act in synergy to increase the binding affinity of integrin for ECM (Figure 1) (*18*). Grant and co-workers provided further evidence in support of this model by showing that the insertion of flexible polypeptides between domains 9 and 10 disrupted the synergistic activity of the two peptide ligands (*19*). They concluded that a strict spatial disposition of the two peptides is required for simultaneous binding to a common integrin receptor. A separate study advanced a similar model but suggested that the binding sites for the two peptides were separated by a smaller distance (*20*, *21*). Inhibitory antibodies suggested that the synergy region is recognized by the α_5 subunit while the β_1 subunit plays the major role in binding of the RGD sequence. Ginsburg, Yamada, and co-workers, however, presented evidence in support of an alternative model, by showing that the two peptides are not able to bind simultaneously to a single $\alpha_{IIb}\beta_3$ integrin receptor. This study found that the attachment of cells to substrates coated with either the 9th or 10th domain of FN could be inhibited by either of the two soluble peptides, RGD or PHSRN (*22*). The degree of inhibition was complete in all cases. These results, of course, cannot distinguish between a competition of the two peptides for a common site or for nonoverlapping sites that are linked allosterically.

More recently, Garcia and co-workers used a method that directly measures the binding force that mediates cell adhesion to fibronectin (*26*). This study found that human K562 erythroleukemia cells, following activation of the $\alpha_5\beta_1$ integrin, required both RGD and PHSRN for efficient adhesion, with PHSRN serving as the ligand that gives strong mechanical coupling to the integrins. The authors proposed a model wherein the $\alpha_5\beta_1$ integrin first binds the RGD peptide, which then serves to orient and activate (through a possible conformational change) the integrin for binding to the PHSRN peptide.

Limitations of Current Approaches. It is important to understand the limitations that are intrinsic to the methods that are commonly used to decipher the roles of peptide ligands on cell adhesion because these limitations provide a strong motivation for the use of model substrates described below. Substrates are typically prepared by allowing purified ECM or recombinant proteins containing selected domains from ECM to adsorb to glass or polystyrene tissue culture-ware. Peptide ligands within the proteins can then mediate the attachment of cells. The adsorption of protein, however, is a complex and heterogeneous process which results in a distribution in orientation and conformation of the adsorbed protein. It is therefore difficult (in practice, it is often not possible) to determine the fraction of peptide ligands that are properly oriented and *accessible* for binding to cellular receptors. It is also improper to assume that two similar proteins that have modest changes in sequence, as is common in mutagenesis experiments, will adsorb to give films that are otherwise similar. Ramsden and co-workers, for example, showed that the initial rates of adsorption onto a silicon oxide surface of two cytochrome 5 proteins (that differed by switching two residues in the primary sequence) varied by 20-fold (*27*). Finally, because cells can efficiently remodel their matrices, it is difficult to control the ligands presented on the substrate over longer periods in culture (*28*).

In this work we use self-assembled monolayers (SAMs) of alkanethiolates on gold that present peptide ligands as model substrates for integrin-mediated adhesion of baby hamster kidney (BHK), 3T3 Swiss fibroblasts, and IMR 90 cells. These monolayers are well suited for evaluating the adhesive properties of peptide ligands because they permit ligands to be immobilized at controlled densities and in a homogeneous environment and at the same time prevent nonspecific protein adsorption and cell adhesion. Hence, adhesion of cells to these model substrates is mediated *entirely* by the interactions of cellular receptors with the immobilized peptides. The synthesis of the alkanethiols terminated with peptide ligands is straightforward (Figure 2), and the immobilized peptide ligands are readily characterized with MALDI-TOF mass spectrometry (*29*, *30*). In this paper, we show that BHK and 3T3 Swiss cells attach to monolayers presenting either RGD or the PHSRN peptide and that 3T3 fibroblast adhesion on either monolayer can be inhibited by soluble peptides containing either sequence. With a second, independent model system which immobilizes peptide ligands by Diels−Alder chemistry, we show that 3T3 Swiss and IMR 90 fibroblasts adhere to and spread on monolayers presenting RGD and PHSRN but not on monolayers presenting scrambled ligands RDG and HRPSN and that IMR 90 fibroblast attachment to PHSRN can be inhibited by either anti-integrin α_5 or anti-integrin β_1 antibody.

MATERIALS AND METHODS

Antibodies and Reagents. Rabbit anti-mouse IgG antibody and goat anti-rabbit IgG rhodamine antibody were purchased from Chemicon (Temecula, CA). Anti-integrin antibodies LM609 (anti-$\alpha_v\beta_3$), P1F6 (anti-$\alpha_v\beta_5$), AV1 (anti-α_v), P1D6

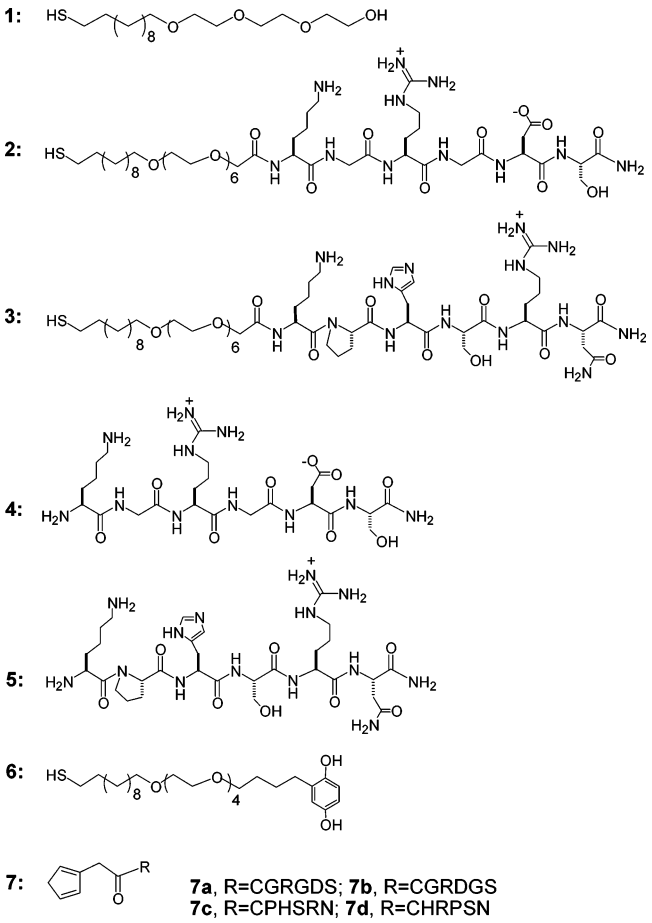

FIGURE 2: Structures of the substituted alkanethiols, hydroquinone conjugate, and peptides used in this work. Compound **1** is the tri(ethylene glycol) terminated alkanethiol. Compounds **2** and **3** are alkanethiols substituted with peptides containing the ligands RGD and PHSRN, respectively. Compounds **4** and **5** are the soluble analogues of the two peptides. Compound **6** is the hydroquinone conjugate used on SAMs to immobilize cyclopentadiene−peptide conjugates **7**.

(anti-α_5), and 6S6 (anti-β_1) were obtained from Chemicon. Soluble peptides CGRGDS, CGRDGS, CPHSRN, and CHRPSN were purchased from BioSynthesis (Lewisville, TX). Monoclonal anti-human vinculin antibody was obtained from Sigma. Fluorescent goat anti-mouse IgG conjugate antibody and Alexa-564 phalloidin were obtained from Molecular Probes (Eugene, OR). Bovine plasma fibronectin and all cell culture media and reagents were purchased from Gibco BRL (Gaithersburg, MD).

Cell Culture. Baby hamster kidney (BHK) cells and 3T3 Swiss fibroblasts, both purchased from ATCC (Rockville, MD), were cultured in Dulbecco's modified Eagle's medium (DMEM) with 7% and 10% fetal bovine serum (FBS), respectively, and 0.5% gentamicin. IMR 90 human lung fibroblasts, obtained from the laboratory of Dr. Alex O. Morla (The University of Chicago), were maintained in DMEM supplemented with 10% fetal bovine serum and penicillin. All cell cultures were incubated with 7.5% CO_2 at 37 °C.

Preparation of Monolayers. The tri(ethylene glycol)-terminated alkanethiol **1**, the peptide-terminated alkanethiols **2** and **3**, hydroquinone conjugate **6**, and peptide conjugates **7** (Figure 2) were synthesized as reported previously (*31*, *32*). The soluble peptides **4** and **5** (Figure 2) were prepared using standard Fmoc solid-phase synthesis and purified with reverse-phase HPLC. Substrates were prepared by evaporating titanium (1 nm) and then gold (12 nm) onto glass coverslips. These substrates were cut into 1 cm^2 pieces and immersed in 0.3 mL of an ethanolic solution containing 1-mercaptoundec-11-yltri(ethylene glycol) (**1**) and one of the peptide-terminated alkanethiols (**2** or **3**) (1 mM total thiol) for 6 h to give a final peptide density of 0.01%, 0.05%, or 1%. The final densities were related to the relative concentrations of the alkanethiols in the solutions used to prepare for the monolayers. The substrates were removed from solution, rinsed with absolute ethanol, dried under a stream of nitrogen, and then used immediately in cell culture.

To prepare SAMs presenting Diels−Alder immobilized peptide ligands, gold substrates were immersed in a methanolic solution containing hydroquinone conjugate **6** and tri(ethylene glycol) conjugate **1** (10 μM in compound **6**, 1 mM in total thiol) for 6 h. The substrates were rinsed with absolute ethanol and dried under a stream of nitrogen. Substrates were treated with a saturated aqueous solution of 1,4-benzoquinone for 5 min to oxidize the immobilized hydroquinone group to the corresponding quinone. The substrates were washed with water and dried under a stream of nitrogen. To immobilize peptide conjugates **7**, each substrate was inverted onto 50 μL of an aqueous peptide solution (2 mM) on parafilm. The substrates were kept in a humidified chamber at 37 °C for 2 h, washed extensively with water, dried under a stream of nitrogen, and then immediately used in cell culture.

Fibronectin-coated substrates were prepared by adsorbing protein to monolayers of hexadecanethiolate (*33*). A drop of PBS (100 μL) was applied to each substrate, and then 2.5 μL of a concentrated solution of FN was added to the drop with a micropipet. The final concentration of FN in the contacting solution was 25 μg/mL. After 1 h, the substrates were rinsed with PBS and placed in a 12-well culture dish for cell culture. This procedure was used because it avoids the direct application of protein-containing solutions to slides, which often gives irregular deposition of protein (*33*).

Assay for Cell Adhesion. For cell adhesion assays with the alkanethiolate SAMs, confluent layers of BHK or 3T3 cells were removed from culture plates with a 0.25% trypsin/0.5 mM EDTA solution, rinsed with fresh serum-free DMEM, and then resuspended in DMEM with fetal bovine serum containing 0.5% gentamicin. Cells (200000/mL) were added to monolayer substrates in 12-well culture dishes. After 4 h the serum medium was exchanged for serum-free DMEM. Cultures were photographed after 10 h, and the number of cells that attached per field was counted. For cell adhesion assays with SAMs presenting Diels−Alder immobilized peptides, 3T3 or IMR 90 fibroblasts were added in the absence of serum, and the adherent cells were photographed after 4 h.

Assay for Inhibition of Cell Adhesion. Suspensions of 3T3 fibroblasts in serum-free DMEM (700 μL, 200000 cells/mL) were incubated with a soluble peptide (either **4** or **5**) at concentrations ranging from 10 nM to 1 mM for 5 min at room temperature. The cells were then added to monolayers presenting either GRGDS or PHSRN and incubated at 37 °C. After 30 min, the substrates were rinsed gently with PBS and transferred to a second culture dish containing serum-free DMEM. Cells were counted in four fields for each of

the substrates. The degree of inhibition is reported as a percentage of cells that attached relative to control experiments that used no soluble peptide.

Inhibition of IMR 90 fibroblast adhesion with anti-integrin antibodies was carried out by a similar procedure. The concentration of antibodies (anti-integrin α_5, β_1, α_v, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ antibodies) was 50 μg/mL, and IMR 90 fibroblasts were preincubated with antibodies for 15 min, then added to monolayers presenting Diels−Alder immobilized PHSRN, and incubated for 30 min at 37 °C. The degree of inhibition is reported as a percentage of cells that attached relative to control experiments that used no antibody.

Immunostaining. BHK or 3T3 cells were allowed to attach to and spread on model substrates as described above. Media were removed, and the substrates were rinsed with PBS. Cells were fixed with 4% paraformaldehyde in Ca^{2+}/Mg^{2+}-free PBS (pH 7.4) for 20 min at room temperature. PBS containing 50 mM NH_4Cl was added, and cells were permeabilized with 0.3% Triton X-100 in PBS for 5 min. Cells were then rinsed twice with 0.01% saponin/0.25% gelatin/0.02% NaN_3 in PBS. Cells were stained with monoclonal anti-human vinculin IgG to visualize focal adhesions and with phalloidin to visualize actin filaments (*34*). Substrates were rinsed thoroughly and mounted on microscope slides with a 90% glycerol/5% DABCO PBS solution and visualized with a Zeiss 63X Plan Apo objective.

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS). Substrates for MALDI-MS study were prepared in the same way as described above for the cell adhesion assays. The MALDI-MS measurements were performed on a Voyager-DE PRO MALDI-TOF mass spectrometer (PerSeptive Biosystems) with 2,5-dihydroxybenzoic acid as matrix.

RESULTS

Self-Assembled Monolayers as Model Substrates. We used self-assembled monolayers presenting oligo(ethylene glycol) groups and peptide ligands because these monolayers are currently the most effective model substrates for controlling the ligand−receptor interactions between cell and substrate (Figure 3A) (*35*). Most importantly, the glycol groups make the surface inert and therefore prevent the nonspecific adsorption of protein. The immobilized ligands then engage in selective binding with cell surface receptors. A further benefit with monolayer substrates is that matrix-assisted laser desorption/ionization and time-of-flight (MALDI-TOF) mass spectrometry can be used to confirm the presence of peptide on the substrate. Analysis of a SAM presenting the PHSRN peptide showed the peaks for the sodium adducts of the symmetric glycol-substituted disulfide ($C_{34}H_{70}O_8S_2Na$, m/z = 693.7), for PHSRN-terminated alkanethiol **3** ($C_{55}H_{100}N_{14}O_{16}$-SNa, m/z = 1267.9), and for the mixed disulfide containing one glycol group and one peptide-terminated alkanethiol ($C_{72}H_{133}N_{14}O_{20}S_2Na$, m/z = 1601.8) (Figure 3B). Disulfides were the dominant species as observed in other MS studies of thiolate SAMs (*29*, *36*). Extensive previous work with patterned substrates establishes that the surfaces remain inert to cell spreading for as long as 1 week in serum-containing media (*37*−*40*). In previous work, we have validated the use of these model substrates for studies of cell adhesion by demonstrating the selective attachment of cells to monolayers

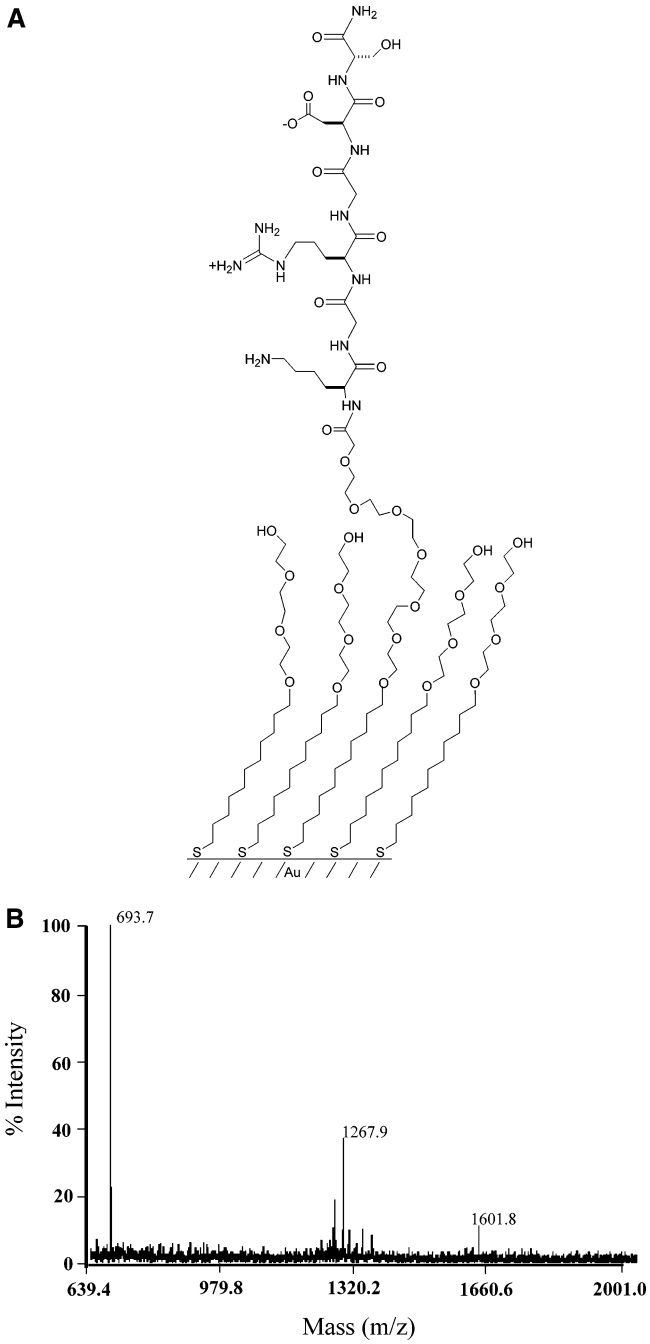

FIGURE 3: Model substrates used in this work. (A) Self-assembled monolayer presenting a peptide ligand among tri(ethylene glycol) groups. The density of the ligand is controlled by adjusting the ratio of the two alkanethiols in the solution from which the monolayer assembles. The glycol groups prevent nonspecific adsorption of protein. (B) MALDI characterization of the SAM presenting peptide ligand PHSRN. Tri(ethylene glycol) disulfide, PHSRN-substituted alkanethiolate **3**, and its disulfide conjugate with ethylene glycol were detected. All were sodium salts.

that present only the RGD peptide and comparing focal adhesion structures in cells attached to monolayers presenting low- and high-affinity ligands (*31*, *41*, *42*).

BHK Cell and 3T3 Fibroblast Attachment to Monolayers Presenting KGRGDS and KPHSRN. We compared the attachment of BHK cells to monolayers presenting either the KGRGDS or KPHSRN peptide ligand. For all monolayer substrates, the peptide-terminated alkanethiol was incorporated at a density of 1% relative to the total alkanethiol. A

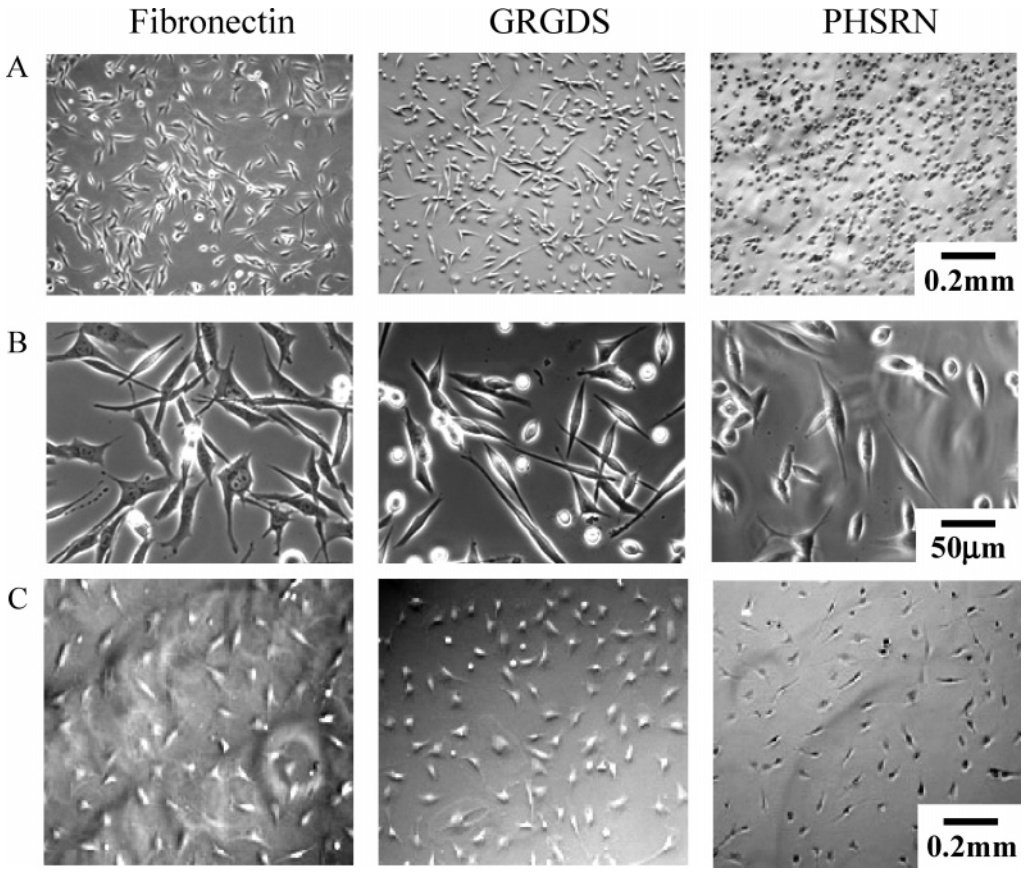

FIGURE 4: BHK cell and 3T3 fibroblast attachment to model substrates. Optical micrographs of BHK cells (A and B) and 3T3 fibroblasts (C) that attached and spread on monolayers presenting either fibronectin (left), KGRGDS (middle), or KPHSRN (right). Images at low magnification show the efficiency of attachment while those at high magnification show the degree of cell spreading. BHK cells and 3T3 fibroblasts attached to all three substrates but showed different degrees of spreading.

suspension of BHK cells in DMEM was added to monolayer substrates (200000 cells/mL) and kept at 37 °C for 10 h. We added cells in the presence of serum to more closely mimic the physiological setting. The substrates were viewed with optical microscopy to determine the number and morphology of adherent cells (Figure 4A,B). *We found that BHK cells attached to monolayers presenting either the KGRGDS or the KPHSRN peptide ligand.* The number of cells that attached per unit area was comparable on these substrates, but the degree of cell spreading was greater on substrates presenting RGD than on PHSRN. Cells adherent to the former assumed a spindle-shaped morphology whereas cells attached to the latter mostly remained in a rounded morphology and exhibited only partial spreading. For comparison, cells that attached to monolayers having an adsorbed layer of fibronectin assumed a fully spread morphology. Control experiments with monolayers presenting only glycol groups resulted in no cell attachment.

We repeated the experiments described above with 3T3 Swiss fibroblasts to determine whether PHSRN-mediated attachment extended to other cell types. Indeed, we did find that the 3T3 cells attached to monolayers presenting PHSRN and that the morphology of cells on PHSRN, GRGDS, and FN followed a similar trend as was found with the BHK cells (Figure 4C). Cells attached to monolayers presenting either PHSRN or GRGDS with similar efficiency (102 and 105 cells per field, respectively). The fibroblasts in all cases displayed different extents of spreading on the three substrates, with more spreading on GRGDS relative to PHSRN. Again, no cells attached to a monolayer presenting only glycol groups.

Cytoskeletal Structure of Cells Adherent to Model Substrates. We compared the assembly of focal adhesions and stress filaments in BHK cells adherent to monolayers presenting GRGDS, PHSRN, and FN (Figure 5A). Cultures were stopped 4 h after plating, and cells were fixed with 4% paraformaldehyde and probed with anti-vinculin IgG to visualize focal adhesions and with phalloidin−rhodamine to visualize actin stress filaments. Cells adherent to FN-coated substrates were characterized by clear focal adhesions and actin stress filaments. BHK cells on GRGDS monolayers had small focal adhesions, which were primarily found along the cell perimeter, and actin stress filaments that spanned the lengths of cells but that were not as pronounced as those in cells adherent to FN. Cells attached on PHSRN monolayers, by contrast, lacked clear focal adhesions and organized actin stress filaments.

We then characterized the assembly of focal adhesions and actin stress filaments in 3T3 fibroblasts as described above for BHK cells and found that fibroblasts adherent to each of the three substrates had assembled stress filaments and focal adhesions (Figure 5B). Cells adherent to FN-coated monolayers had the most extensive cytoskeletal structure and displayed focal adhesions throughout the area of the cell. The fibroblasts adherent to GRGDS also had actin stress fibers that extended across the entire cell and had numerous focal adhesions, but located mainly toward the perimeter of the cell. Cells adherent to monolayers presenting PHSRN

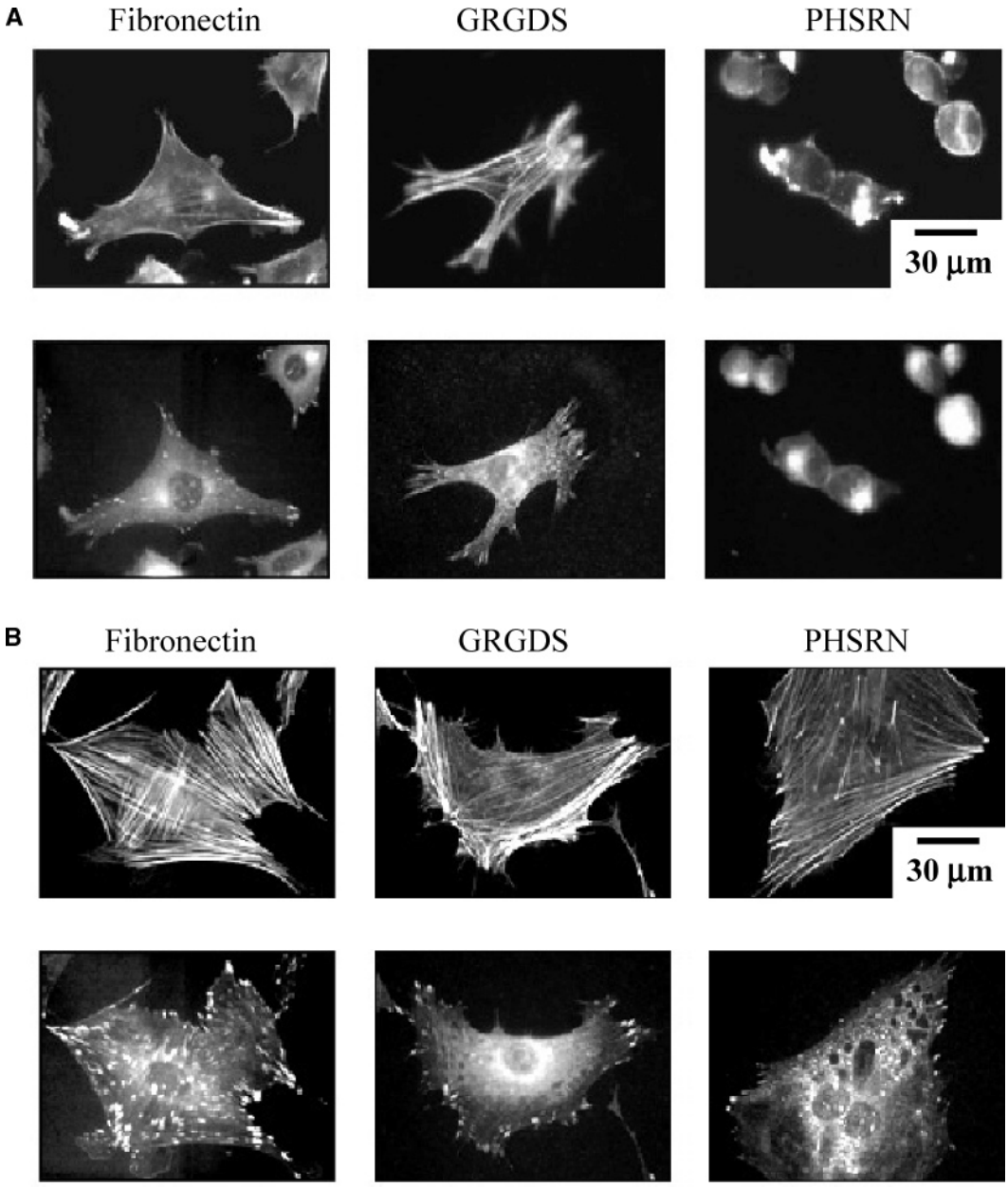

FIGURE 5: Focal adhesions and stress filaments in BHK cells and 3T3 fibroblasts. Immunofluorescence staining reveals focal adhesions and actin stress filaments in BHK cells (A) and 3T3 fibroblasts (B) attached to fibronectin (left), KGRGDS (middle), and KPHSRN (right). Actin stress filaments were visualized with rhodamine phalloidin (upper row), and focal adhesions were visualized with an anti-vinculin antibody (bottom row).

again showed the least developed structures. The actin filaments were apparent and extended across the cell length, but the focal adhesions were less numerous and less apparent than were those of cells on the other substrates.

Inhibition of Cell Adhesion to Alkanethiolate Monolayers by Soluble Peptides. We next examined the ability of soluble peptides to inhibit the attachment of cells to the monolayers presenting peptide ligands. These experiments are important for two reasons. First, they provide direct evidence that cell attachment is mediated by the immobilized peptide alone and not by nonspecific interactions of the cell with the substrate. Second, these experiments demonstrate that the two peptides are competitive ligands for the integrin receptor. Trypsinized 3T3 fibroblasts were divided into several identical samples, and each was treated with a soluble peptide ligand (GRGDS or PHSRN) at concentrations ranging from 10 nM to 1.0 mM. The suspended cells were incubated with soluble peptide for 5 min to allow the binding of peptide ligands with integrin receptors to reach equilibrium, and then the suspended cells were added to monolayers presenting either the RGD or PHSRN peptide ligand. The cultures were kept at 37 °C for 30 min, and then substrates were inspected with optical microscopy to determine the number of cells that had attached per field of view.

Because monomeric ligands are poor inhibitors of the polyvalent attachment of cells, we used monolayers that presented RGD at a density of 0.05%. We found that the soluble RGD peptide displayed a concentration-dependent inhibition of cell attachment (Figure 6). At a concentration of 50 μM, the soluble RGD reduced the attachment of cells by 50%, while attachment was completely blocked at 500 μM RGD. A parallel experiment with the control peptide GRDGS had no effect on cell attachment, showing that the inhibition is specific. *We found that the soluble PHSRN*

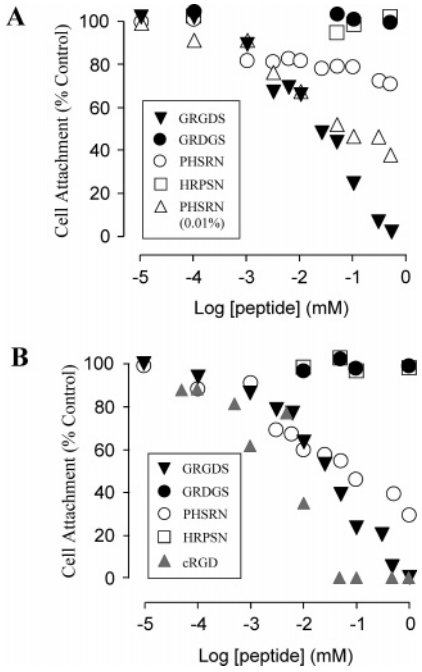

FIGURE 6: Inhibition of 3T3 fibroblast attachment by soluble peptides. Inhibition of 3T3 Swiss fibroblast attachment to monolayers presenting GRGDS (A) and PHSRN (B). Suspended cells were treated with either soluble linear GRGDS (▼), cyclic RGD (▲), or PHSRN (○) at concentrations from 10 nM to 1 mM and then allowed to attach to each of the two monolayers. The plots show the number of cells that had attached for each concentration of soluble peptide, in units that are relative to cell attachment in the absence of soluble inhibitors. The scrambled peptides GRDGS (●) and HRPSN (□) were also used to examine for selective inhibition. Inhibition of cell attachment by the soluble PHSRN was also tested for monolayers presenting GRGDS at a density of 0.01% (△). Greater than 40% of the cells seeded attached to the monolayers in the absence of soluble peptides.

peptide could also inhibit cell attachment to monolayers presenting the RGD peptide, demonstrating that the two peptides are competitive binders for the integrin. The finding that higher concentrations of PHSRN are required to block attachment gives evidence that this peptide has a lower affinity for the integrin receptor than does the RGD peptide (Figure 6). Again, a control experiment with the scrambled peptide HRPSN had no effect on cell attachment. Even at the highest concentrations tested (1 mM), the soluble PHSRN peptide failed to inhibit completely cell attachment. We repeated these experiments with monolayers presenting the RGD peptide at 5-fold lower density (0.01%) and found that inhibition with soluble PHSRN was still incomplete but increased from 30% to 63%. These results are consistent with the observation that monomeric ligands are poor inhibitors of polyvalent attachment (*37*, *38*).

We next repeated the inhibition experiments with monolayers presenting the PHSRN peptide. The soluble RGD peptide was a potent inhibitor of attachment. At a concentration of 25 μM, this peptide reduced by 50% the number of cells that attached and at a concentration of 500 μM prevented entirely the attachment of cells (Figure 6). We again found that the soluble PHSRN peptide showed a concentration-dependent inhibition of attachment but was overall less effective than was the soluble RGD peptide. A concentration of PHSRN at 100 μM prevented 54% of cell attachment, with 70% inhibition observed at a concentration of 1.0 mM. Finally, we found that a cyclic RGD peptide was more effective at inhibiting cell adhesion than either RGD or PHSRN (Figure 6B; but the reverse experiment revealed that the PHSRN ligand has insufficient affinity to block cell attachment to substrates presenting cyclic RGD). Control experiments with the scrambled ligands HRPSN and GRDGS again had no effect on attachment.

Adhesion of IMR 90 Fibroblasts to Monolayers Presenting Diels−Alder Immobilized Peptide Ligands. We also determined whether the observation that cells attach to PHSRN extended to human cell lines. For these studies we used a different strategy to immobilize the peptide ligands to the monolayer. We did so to more vigorously ensure that substrates presenting either peptide did so at a constant density (see, for example, ref *42*). Monolayers presenting hydroquinone were prepared as described in the Materials and Methods section. The hydroquinone was oxidized to quinone with 1,4-benzoquinone, and cyclopentadiene−peptide conjugates were immobilized to the monolayers through Diels−Alder reaction with the interfacial quinone group (Figure 7).

For all monolayer substrates, the peptide density was 1% relative to the total alkanethiol. A suspension of IMR 90 human lung fibroblasts in serum-free DMEM was added to substrates (200000 cells/mL) and incubated at 37 °C for 4 h. Cells were added in the absence of serum to rule out any role for serum-derived ECM proteins in the cell adhesion process. The substrates were viewed with optical microscopy to determine the morphology of adherent cells (Figure 8). We found that IMR 90 cells adhered to and spread on monolayers presenting RGD and PHSRN. Further, the IMR 90 fibroblasts on the RGD substrate had an elongated morphology while these cells only partially spread on a PHSRN substrate. No cells attached to monolayers presenting only glycol groups or 1% hydroquinone group. Further cells did not attach to monolayers presenting scrambled ligands GRDGS and HRPSN.

Inhibition of IMR 90 Cell Adhesion to Monolayers with Anti-Integrin Antibodies. We next used anti-integrin antibodies in blocking experiments to determine the identity of the integrin that mediates cell adhesion to PHSRN (Figure 9). IMR 90 human lung fibroblasts were separately preincubated with anti-human integrin $\alpha_v\beta_3$, $\alpha_v\beta_5$, α_v, α_5, and β_1 antibodies for 15 min, and cells were then added to monolayers presenting PHSRN. Function-blocking monoclonal antibodies against β_1 or α_5 integrins nearly completely prevented the adhesion of IMR 90 cells while anti-$\alpha_v\beta_3$ and anti-$\alpha_v\beta_5$ had no significant inhibitory effect. Anti-α_v antibodies partially inhibited IMR fibroblast attachment to substrates presenting PHSRN.

DISCUSSION

The Peptide PHSRN Mediates Cell Attachment. The most important finding in this work is that the peptide PHSRN can mediate the attachment of cells in the absence of other ligands. This conclusion follows from the use of a new class of model substrate that permits the ligand−receptor interactions between an adherent cell and substrate to be controlled entirely. This work investigated three cell types and found that cells were able to attach to monolayers presenting either RGD or PHSRN. Antibody blocking studies showed that the

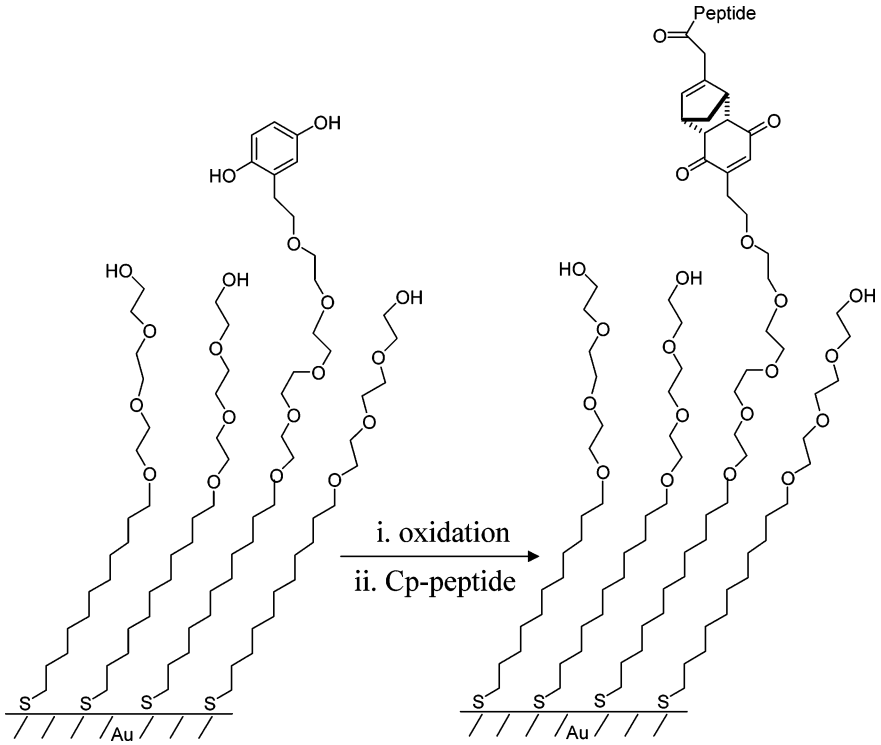

FIGURE 7: Diels−Alder immobilization of cyclopentadiene−peptide conjugates. Monolayers were prepared to present the hydroquinone group. The immobilized hydroquinone group was then oxidized to the corresponding quinone with 1,4-benzoquinine. A Diels−Alder reaction of the cyclopentadiene−peptide conjugate (**7**) with the quinone group yielded a SAM presenting the desired peptide ligand. Reagents: (i) aqueous 1,4-benzoquinone, 5 min; (ii) aqueous peptide conjugate **7** (**7a**, R = CGRGDS; **7b**, R = CGRDGS; **7c**, R = CPHSRN; **7d**, R = CHRPSN), 2 h, 37 °C.

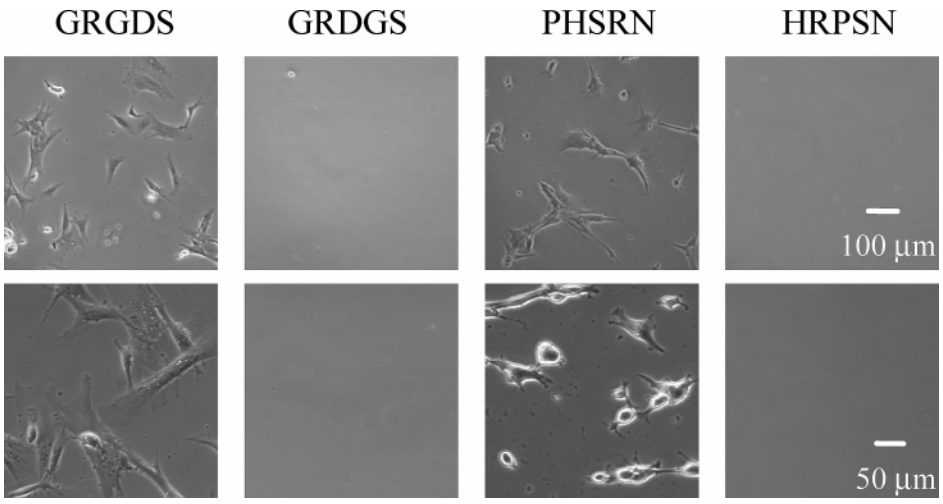

FIGURE 8: IMR 90 fibroblast attachment to monolayers presenting Diels−Alder immobilized peptide ligands. Optical micrographs of IMR 90 fibroblasts that attached and spread on monolayers presenting (from left to right) either GRGDS, GRDGS, PHSRN, or HRPSN. Images at low magnification show the efficiency of attachment while those at high magnification show the degree of cell spreading. Cells only attached to these substrates presenting RGD or PHSRN but not to substrates presenting scrambled peptide ligands.

$\alpha_5\beta_1$ integrin mediates adhesion to the two peptides, in agreement with previous studies (*26*). Further, inhibition studies showed that the two peptides bind competitively to the integrin and the PHSRN ligand binds with lower affinity than does RGD. Finally, experiments with scrambled peptide sequences establish that the binding of RGD and PHSRN for the integrin receptor is highly specific.

These findings contrast with a number of recent reports that have identified PHSRN as a synergy ligand that enhances cell spreading to RGD but is otherwise inactive when presented alone (*15*−*21*). Other published reports, however, have provided evidence consistent with the role of PHSRN as an independent ligand. For example, Ginsberg and co-workers showed that the interaction of fibronectin and integrins could be completely inhibited by either soluble RGD or soluble PHSRN (*22*). Our work is consistent with this latter report and adds to the evidence that PHSRN can mediate adhesion alone. We emphasize that much previous work has used substrates that were prepared by adsorbing FN fragments to tissue cultureware or beads and can be at risk for false negative results. The observation that cells do not attach to substrates coated with proteins that preserve the PHSRN sequence cannot strictly be interpreted to mean that the peptide PHSRN is not a ligand for attachment. It is

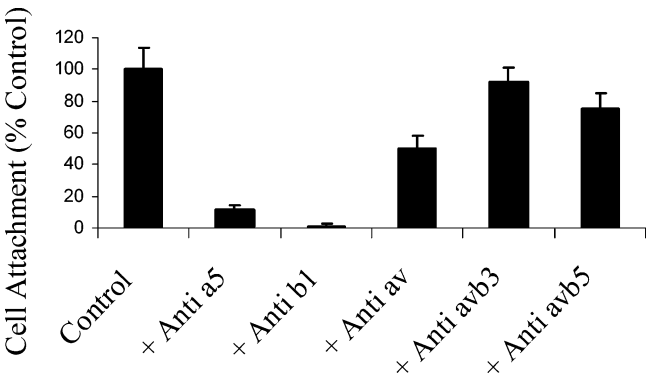

FIGURE 9: Inhibition of IMR 90 fibroblast attachment to monolayers presenting PHSRN with anti-integrin antibodies. Inhibition of IMR 90 fibroblast attachment to monolayers presenting PHSRN. Suspended cells were treated with anti-integrin antibodies (50 μg/mL) and then allowed to attach to the monolayer. The plots show the number of cells that had attached for each antibody, in units that are relative to cell attachment in the absence of any anti-integrin antibody. Greater than 40% of the cells seeded attached to the monolayers in the absence of blocking antibodies.

possible, for example, that the FNIII-9 domain preferentially adsorbs in an orientation that does not make the PHSRN sequence accessible for binding cell surface receptors or that the protein undergoes extensive denaturation at the surface with a corresponding loss in activity. Further, the small quantities of protein adsorbed to the substrate make it intractably difficult to assess these events. These issues illustrate the principle limitation in using protein-coated substrates to identify peptide ligands that mediate cell attachment: a lack of attachment cannot be rigorously assumed to be evidence that the ligand is not active for attachment (or other adhesion-dependent events).

Using a structurally well-defined substrate, we can adjust the density of the ligands on the substrate and present these ligands at a controlled density. Extensive work done in our group and others has established that mixed monolayers presenting mainly ethylene glycol and a very small percentage of peptide ligand ($\leq$1%) maintain the inertness of the surface, preventing any protein adsorption from the medium or adherent cells and thus avoiding any interference by matrix protein such as fibronectin either from medium or, in case of studies in the absence of medium, secreted by adherent cells. It is also worth noting that although the peptide densities used in this study (1% or lower) are relatively high compared to densities in physiological settings, these densities are comparable to the peptide densities in studies using FN-coated substrates.

RGD and PHSRN Bind Competitively to the Integrin Receptor. Our experiments demonstrate clearly that the two peptides bind competitively to the integrin receptor and that α_5 and β_1 integrins mediate cell adhesion to PHSRN. The attachment of fibroblasts to monolayers presenting RGD is completely inhibited by a soluble RGD peptide and partially inhibited by a soluble PHSRN peptide. The incomplete inhibition with the latter is likely due to its lower binding affinity. Control experiments with a soluble GRDGS or HRPSN peptide had no effect on cell attachment, showing that the inhibition is specific. Further, parallel experiments that used monolayers presenting RGD at 5-fold lower density indicated that the soluble PHSRN ligand was a more effective inhibitor of attachment (though still not complete) for substrates having the lower density of RGD, which is consistent with extensive previous work on polyvalent interactions (*43*, *44*). Inhibition experiments with monolayers presenting PHSRN gave similar results in that soluble RGD completely inhibited attachment and soluble PHSRN incompletely inhibited attachment. PHSRN proved more effective at blocking cell attachment to monolayers presenting PHSRN at lower density. In every case, however, RGD was a more effective inhibitor than was PHSRN. Finally, the cyclic RGD peptide, which has approximately 300-fold higher affinity than the linear peptide, was the most effective at blocking cell attachment, and PHSRN was a poor inhibitor of cell attachment to monolayers presenting the cyclic RGD ligand (*42*). These inhibition experiments are valuable because they provide an *equilibrium* method for reducing the number of available integrin receptors on the cell surface. The relationship between the fraction of available integrin receptors and the efficiency of adhesion, however, is very complicated and not understood.

This view that PHSRN is a lower affinity ligand that binds competitively to the integrin with RGD is also consistent with our finding that a fewer number of BHK cells attached to the former and those cells were spread to a lesser extent. For both BHK cells and fibroblasts, the enhanced spreading on RGD was marked by a greater number of focal adhesions and a more pronounced cytoskeletal structure. Importantly, these results show that the adhesive activity of PHSRN is not restricted to BHK cells and that this peptide does support the formation of focal adhesions and stress filaments in other cell types.

Blocking assays with anti-integrin antibodies showed that only anti-β_1 and anti-α_5 antibodies almost completely inhibited IMR 90 fibroblast adhesion to monolayers presenting the PHSRN peptide ligand. Early studies showed that PHSRN binds to the α_5 subunit (*20*, *23*) while RGD binds to both the α_5 and β_1 subunits (*45*−*48*). It was also demonstrated that PHSRN is not required for cell adhesion to FN and FN matrix assembly when integrins are in the activation state (*23*, *49*). But more recently, using a method that directly measures the binding force between the integrin receptors and fibronectin, Garcia and co-workers showed that PHSRN provides the mechanical strength for the binding between fibronectin and $\alpha_5\beta_1$ integrin (*26*). These findings are consistent with our data in that PHSRN is an independent ligand for cell binding through integrin. Our work to date has not addressed the role of the RGD and PHSRN ligand in mediating the mechanical coupling of the integrin to the immobilized peptide or the role for differential activation of the integrin. We are addressing these issues in current work.

Self-Assembled Monolayers as Models of the Extracellular Matrix. This work has a broader importance in that it establishes the value of self-assembled monolayers of alkanethiolates on gold as a model substrate for mechanistic studies of cell adhesion. These substrates are the best available for introducing specific ligand−receptor interactions between the substrate and an adherent cell but at the same time rigorously exclude nonspecific interactions and matrix remodeling by the cell (*35*, *50*). This property stems from the inert properties of the glycol-terminated alkanethiolates. A second benefit with these substrates is the synthetic flexibility available for controlling the structures, densities, and environments of immobilized ligands (*51*, *52*). Finally,

more recent work is developing classes of *dynamic substrates* that can alter, in real time, the ligand−receptor interactions between a cell and substrate. These substrates have generated new strategies for patterning cellular cocultures (*53*), screening molecules that affect cell migration (*54*), and mechanistic studies of matrix regulation of cell behavior.

This work has used a new class of model substrates to determine unambiguously that PHSRN and RGD both support the integrin-mediated attachment of cells and that the two ligands bind competitively, not synergistically. This approach will be important for understanding the properties of other candidate peptide and carbohydrate ligands. We believe that a combination of experimental approaches that use the model substrates described here and protein-coated substrates that are more representative of ECM will provide mechanistic understanding of cell−ECM interactions.

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