

Substrates for Cell Adhesion Prepared via Active Site-Directed Immobilization of a Protein Domain

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Received September 16, 2003. In Final Form: December 5, 2003

Recent studies in basic cell biology and bioengineering call for model substrates that present active proteins, with control over protein density, pattern, and orientation, to more directly mimic the natural extracellular matrix. Herein we demonstrate a strategy for controlled, irreversible immobilization of a cell adhesion protein domain onto an otherwise bioinert substrate with well-defined protein orientation and density. Our approach uses a tri(ethylene glycol)-terminated self-assembled monolayer presenting a phosphonate ligand that is used to immobilize an engineered fusion protein. One component of the fusion protein, the 22 kDa serine esterase cutinase, reacts with the surface-bound ligand to form a site-specific covalent adduct, and the second component of the fusion protein is therefore immobilized on the surface. Here we use this approach to immobilize an engineered version of the 12 kDa 10th domain of fibronectin III (FnIII₁₀Eng) to direct cell adhesion. Substrates presenting this protein mediated rapid attachment and spreading of Swiss 3T3 fibroblasts, while substrates presenting cutinase or the phosphonate ligand alone did not support cell attachment. In addition, we used Chinese hamster ovary cells engineered to express specific integrin receptors to show that FnIII₁₀Eng interacts with multiple integrin cell surface receptors, including human α_v - and α_5 -containing integrins. This general approach, in principle, can be used to immobilize any protein with an available gene sequence, providing an enabling technique for fundamental cell biology and tissue engineering.

Introduction

An important theme in biosurface chemistry has aimed to develop model biological substrates that directly mimic properties of the protein extracellular matrix (ECM). The ECM plays a central role in organizing cells into tissue and operates by presenting ligands that bind cell surface receptors. This work has led to substrates that present short, bioactive peptides on highly bioinert substrates in lieu of nonspecifically adsorbed ECM proteins to examine cell adhesion, migration, and differentiation.^{1–5} For example, Hubbell and co-workers utilized an Arg-Gly-Asp (RGD)-terminated glass substrate to study the influence of ligand spacing on $\alpha_v\beta_3$ integrin-mediated cell adhesion and focal contact formation,^{2,6} and Mooney and co-workers used bioinert alginate hydrogels that presented

RGD to study the relative influence of substrate mechanics and RGD ligand spacing on myoblast phenotype.⁴ These studies have achieved control over ligand–receptor interactions, making it routine to probe cell–substrate interactions while avoiding complicating factors associated with adsorbed ECM protein layers, such as cell-mediated surface remodeling and limited ligand availability.^{7–12} A key limitation of the model substrates that present peptide ligands is that they do not approach the complexity of the natural ECM. There is a need to develop model substrates that present active proteins, with control over protein density, pattern, and orientation, to more directly mimic the ECM. Herein we demonstrate a strategy for controlled immobilization of a cell adhesion protein domain onto an otherwise bioinert substrate with well-defined protein orientation and density, using a covalent immobilization approach that we developed in a previous study.¹³ The resulting system more closely mimics the natural ECM and represents a general protein immobilization strategy that can be used to create functional ECM model systems.

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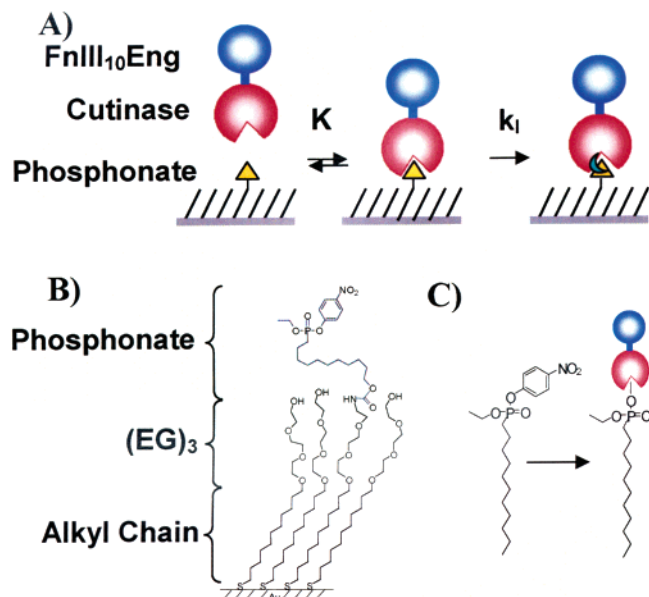


Figure 1. Schematic representation of (A) the enzyme-directed protein immobilization approach, (B) the monolayer presenting phosphonate groups for immobilization of cutinase fusions, and (C) the reaction of a serine active-site residue with the phosphonate ligand to give covalent immobilization of the protein.

Design Rationale

Our approach to prepare model substrates that present protein domains is illustrated in Figure 1. The strategy employs a self-assembled monolayer (SAM) of alkanethiolates on gold that presents a phosphonate ligand among tri(ethylene glycol) [(EG)₃] groups. The (EG)₃ groups serve to prevent nonspecific interactions of proteins with the surface, and the phosphonate group serves to immobilize a fusion protein. One component of the fusion protein, the 22 kDa serine esterase cutinase, interacts with the phosphonate ligand, which mimics the tetrahedral transition state of an ester hydrolysis and is attacked by the catalytic serine residue (Ser-120) of cutinase to form a site-specific covalent adduct.¹⁴ The other component, the 12 kDa 10th domain of fibronectin III (FnIII₁₀Eng), is an ECM protein that contains the RGD cell adhesion sequence. The FnIII₁₀Eng domain was previously engineered to bind optimally to human $\alpha_v\beta_3$ integrin receptors¹⁵ by systematically varying the amino acids adjacent to the RGD sequence, resulting in the optimal RGDWXE sequence. Therefore, presentation of this immobilized protein domain was expected to result in strong binding of $\alpha_v\beta_3$ integrin receptors to mediate cell adhesion.

Results and Discussion

Protein Synthesis and Characterization. To construct the cutinase-FnIII₁₀Eng fusion protein, we first amplified the cutinase gene from *Fusarium solani* genomic DNA and ligated it into a pET-22b(+) vector (Novagen) to yield the pCut22b plasmid, as described previously.¹³ The plasmid encoding FnIII₁₀Eng was isolated from the FNfn10-3JCLI4 clone, which was identified via phage

display in a previous study.¹⁵ We amplified the FnIII₁₀Eng gene from this plasmid¹⁶ and cloned it into the pCut-(22b) plasmid.¹⁷ The resulting plasmid (pCut-FnIII₁₀Eng) encoded a gene for FnIII₁₀Eng fused to the C-terminus of cutinase. We expressed cutinase-FnIII₁₀Eng in *Escherichia coli* [BL21(DE3), Novagen] harboring pCutFnIII₁₀Eng with a T7 expression system.¹⁸ We then purified the fusion protein via size-exclusion chromatography using a Sephadex G-75 column (75 cm × 2 cm, 1 mL/min) equilibrated with phosphate-buffered saline (PBS) (15 mg active protein from a 500 mL culture at 95% purity). We also expressed, purified, and characterized cutinase alone to use as a control.¹³ We confirmed cutinase activity of the expressed proteins (alone and in the cutinase-FnIII₁₀Eng fusion protein) with an established colorimetric assay based on the hydrolysis of *p*-nitrophenyl butyrate. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry ($m/z_{\text{exp}} = 33\,531$; $m/z_{\text{calc}} = 33\,483$) confirmed the appropriate molecular weight of cutinase-FnIII₁₀Eng.

Protein Immobilization. We used surface plasmon resonance (SPR) spectroscopy to characterize the immobilization of the cutinase-FnIII₁₀Eng fusion protein to a monolayer presenting the phosphonate ligand at 0.5% density.¹⁹ The main objective of these experiments was to establish that protein immobilization is mediated by the selective and covalent reaction of cutinase with the phosphonate ligand. We prepared monolayers as described previously.¹³ In the SPR experiment, PBS was first flowed over the surface for 3 min to establish a baseline refractive index level, followed by a solution of protein in the same buffer for 30 min to observe binding. Finally, we replaced the protein solution with PBS for 3 min to quantify the amount of irreversibly bound protein. Both the cutinase and the cutinase-FnIII₁₀Eng fusion proteins bound irreversibly to monolayers presenting the phosphonate ligand (Figure 2). Application of 0.1% (w/v) sodium dodecyl sulfate (SDS) to the surface after protein immobilization did not remove either protein from the surface (data not shown). This result indicated that the immobilization was covalent, as SDS is a detergent that rapidly removes noncovalently immobilized molecules from a surface.²⁰ (EG)₃-terminated monolayers that did not contain the phosphonate ligand did not support immobilization of protein to the surface, demonstrating that the monolayer was inert to nonspecific protein adsorption. In addition, cutinase-FnIII₁₀Eng fusion proteins did not bind to monolayers presenting the phosphonate ligand in the presence of soluble *p*-nitrophenylphosphonate, demonstrating the protein immobilization was directed by the active site of cutinase.

Cell Adhesion. We prepared a series of monolayers to characterize cell adhesion to substrates presenting cutinase-FnIII₁₀Eng on a bioinert background. Monolayers

(16) PCR primers: 5'-CTAGGATCCATGCAGGTTTCTGATGTTCCG-3' and 5'-TCGATCCTCGAGTTACTATTACCTTTTTTACC-3', from Genosys.

(17) Cloning was via the BamHI and XhoI restriction sites, at the 3' end (C-terminus) of the cutinase gene; restriction enzymes were from New England Biolabs.

(18) We confirmed the presence of the correct inserts by bidirectional DNA sequencing. Cells were grown at 37 °C to A₆₀₀ = 0.5 before induction with 0.5 mM isopropyl β -D-thiogalactopyranoside at 25 °C for 18 h. Cells were collected by centrifugation at 5000g for 15 min and resuspended in 20 mL of PBS. Expressed protein was then liberated via sonication, and the resulting solution was clarified by centrifugation.

(19) All SPR experiments were performed with a Biacore 1000 instrument; all experiments used a flow rate of 1 μ L/min at 25 °C in PBS (pH = 7.4).

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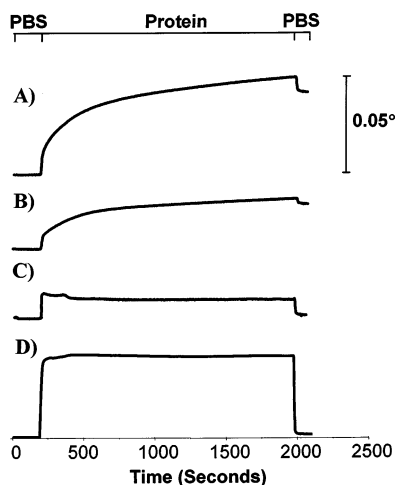


Figure 2. Data from surface plasmon resonance spectroscopy for the interaction of (A) the cutinase-FnIII₁₀Eng fusion with a phosphonate-presenting monolayer, (B) cutinase with a phosphonate-presenting monolayer, (C) cutinase-FnIII₁₀Eng with a tri(ethylene glycol)-terminated monolayer without phosphonate ligand, and (D) cutinase-FnIII₁₀Eng with a phosphonate-presenting monolayer in the presence of soluble *p*-nitrophenylphosphonate inhibitor for cutinase.

presenting cutinase-FnIII₁₀Eng mediated rapid adhesion of Swiss 3T3 fibroblasts (Figure 3a).²¹ The attached cells were spread on the substrate, and immuno-staining of fixed cells showed that cells had mature focal adhesions and cytoskeletal elements (data not shown). In contrast, the monolayers presenting only cutinase or only the phosphonate ligand did not mediate cell adhesion, even in a serum-containing medium (Figure 3b,c). This result indicated that the substrates without covalently immobilized proteins were inert to nonspecific protein adsorption and that covalently immobilized cutinase did not mediate cell adhesion.

Characterization of Receptor–Ligand Interactions. To gain insight into which particular integrin receptors on the cell surface were mediating cell adhesion to FnIII₁₀Eng, we compared the adhesion of three types of engineered Chinese hamster ovary (CHO) cells.²² CHO-B2 cells, which do not express α_5 integrin subunits and are defined by their inability to bind to fibronectin,²³ did not attach to monolayers presenting cutinase-FnIII₁₀Eng (Figure 3d). CHO-B2/v7 cells, which stably express the human α_v subunit,²⁴ and CHO-B2/ α_27 cells, which stably

express the human α_5 subunit,²⁵ efficiently attached and spread on monolayers presenting cutinase-FnIII₁₀Eng (Figure 3e,f). These data suggest that adhesion to FnIII₁₀Eng is mediated by both the human α_v and α_5 integrin subunits. We also qualitatively observed that adhesion of these engineered CHO cells occurred more rapidly to FnIII₁₀Eng-presenting substrates when compared with RGD-peptide-presenting substrates that we have used previously.^{1,3,5} As expected, none of these cell types adhered to monolayers presenting cutinase (Figure 3g–i) or the phosphonate ligand only (data not shown), again confirming the biospecificity that is intrinsic to monolayers that present ligands against a background of oligo(ethylene glycol) groups.^{3,5,13} We also examined Swiss 3T3 fibroblast attachment to surfaces presenting cutinase-FnIII₁₀Eng after preincubation of the cells in solutions containing soluble cutinase-FnIII₁₀Eng or fibronectin (10 μ g/mL) to preoccupy receptors for fibronectin. No cell attachment was observed in these conditions (Figure 3j,k), indicating that cell adhesion to the cutinase-FnIII₁₀Eng-presenting substrates is indeed mediated by FnIII₁₀Eng.

We performed immunoblocking experiments with the engineered CHO cells and IMR-90 human fibroblasts to further characterize the role for human α_v and α_5 integrin subunits in cell adhesion to our engineered 10th domain of fibronectin III. Cells were preincubated in their respective media^{21,24} containing antibodies against human α_5 integrin (P1D6, Invitrogen), human $\alpha_v\beta_3$ integrin (LM609, Chemicon), or both anti- α_5 and anti- $\alpha_v\beta_3$, prior to cell seeding onto each surface type. We then counted the number of adherent cells 1 h after cell seeding.²⁶ There was a 3-fold decrease in binding of CHO-B2/v7 cells to monolayers presenting FnIII₁₀Eng in the presence of an antibody to $\alpha_v\beta_3$ integrin (Figure 4), indicating that cell adhesion is partially mediated by the human α_v integrin subunit. This partial inhibition was expected because the FnIII₁₀Eng and the anti- $\alpha_v\beta_3$ antibody used here have comparable affinity (sub-nM) to human $\alpha_v\beta_3$.¹⁵ There was also a 3-fold decrease in binding of CHO-B2/ α_27 cells in the presence of an antibody to the α_5 integrin subunit (Figure 4), indicating that cell adhesion is also partially mediated by integrins containing the α_5 subunit (e.g., $\alpha_5\beta_1$). However, in each case the presence of the antibody did not completely inhibit cell adhesion to FnIII₁₀Eng. In addition, IMR-90 human fibroblasts were adherent to substrates presenting cutinase-FnIII₁₀Eng in the presence and absence of anti- α_5 , anti- $\alpha_v\beta_3$, and both antibodies together, but these cells did not adhere and spread as readily in the presence of each individual antibody or both antibodies together (data not shown). Taken together, these results indicate that both human α_v and human α_5 integrin subunits are important for cell attachment to FnIII₁₀Eng but that they are likely not the only integrin receptors capable of binding to this cell adhesion domain.

These results reveal that the engineered 10th domain of type III fibronectin, developed to specifically bind to the human $\alpha_v\beta_3$ integrin in a monovalent interaction,¹⁵ can mediate cell adhesion through at least two types of human integrin receptor subunits, α_5 and α_v . This is perhaps not surprising, as previous studies have shown that CHO-B2/v7 cells and CHO-B2/ α_27 cells are capable of interacting with adsorbed fibronectin, and the interac-

(21) [Albino] Swiss 3T3 fibroblasts and IMR-90 human lung fibroblasts (obtained from ATCC, Manassas, VA) were cultured in DMEM + 10% fetal bovine serum (FBS) + antibiotics. For all cell adhesion assays on SAMs, cells were harvested by trypsin/EDTA treatment, collected, and then washed and resuspended in their respective growth medium. Suspensions of 150 000 cells/mL were added to each substrate and incubated at 37 °C for 1 h. Substrates were then washed 3 \times with PBS, fixed in 3.7% paraformaldehyde at 37 °C for 20 min, mounted with 1:1 glycerol/PBS, and visualized with a Zeiss Axiovert 135 microscope.

(22) Chinese hamster ovary (CHO)-B2, -B2/v7, and -B2/ α_27 cells (a generous gift from Dr. Erkki Ruoslahti, Burnham Institute, La Jolla, CA) were cultured in α -MEM + 10% FBS + antibiotics. G418 (400 μ g/mL) was added to the medium of the B2/v7 and B2/ α_27 cells. CHO cells expressing GFP-labeled paxillin (a generous gift from Dr. Alan F. Horwitz, University of Virginia) were cultured as previously described: Laukaitis, C. M.; Webb, D. J.; Donais, K.; Horwitz, A. F. *J. Cell Biol.* **2001**, *153*, 1427–1440.

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(26) The number of attached cells was quantified manually by counting the number of cells in four separate fields of view per sample, and four samples were analyzed per condition. Student's *t*-tests were used to identify statistically significant differences in attached cell number.

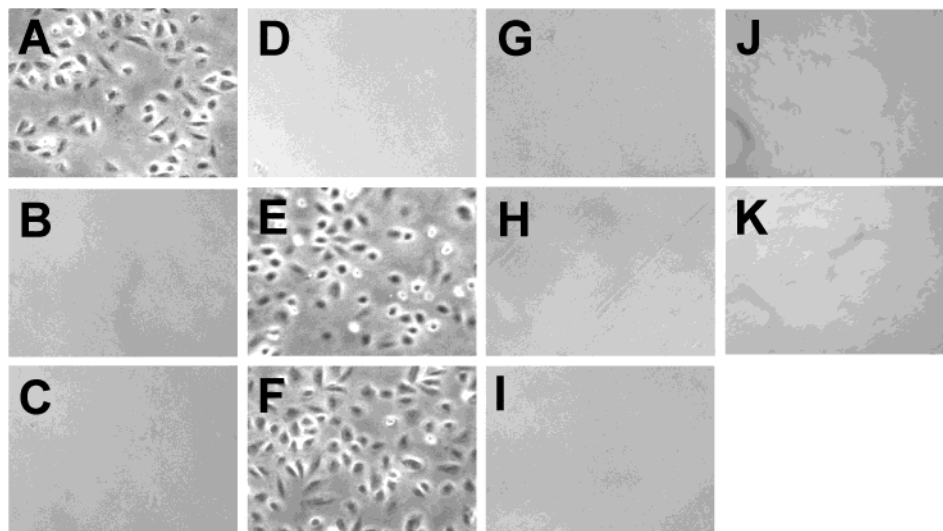


Figure 3. Swiss 3T3 cells were cultured on SAMs presenting the cutinase-FnIII₁₀Eng fusion (A), the phosphonate ligand only (B), or cutinase (C). Three CHO mutants were cultured on SAMs presenting either cutinase or the cutinase-FnIII₁₀Eng fusion protein. CHO-B2 cells, which lack α_5 integrin subunits, did not attach to surfaces presenting cutinase-FnIII₁₀Eng or cutinase (D,G). Human α_v -expressing CHO cells (E) and human α_5 -expressing CHO cells (F) attached to and spread on the cutinase-FnIII₁₀Eng surface but did not attach to surfaces presenting cutinase (H,I). Swiss 3T3 cells that were preincubated with soluble cutinase-FnIII₁₀Eng (J) or fibronectin (K) did not attach to surfaces presenting the cutinase-FnIII₁₀Eng construct.

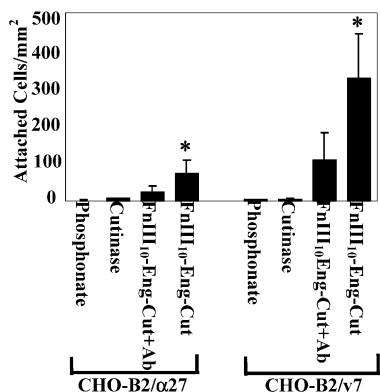


Figure 4. Comparison of the number of CHO cells that attached to SAMs presenting phosphonate (without bound protein), cutinase alone, or the cutinase-FnIII₁₀Eng fusion protein in the presence and absence of antibody. CHO-B2/ $\alpha 27$ cells were cultured in the presence of anti- α_5 , and CHO-B2/v7 cells were cultured in the presence of anti- $\alpha_v\beta_3$. Values represent means and standard deviations, and * denotes statistical significance relative to the other three conditions examined with each cell type.

tions are mediated by complexes of human α_v and human α_5 with the β_1 integrin subunit produced by CHO cells.^{24,25,27} Based on these previous studies using the same engineered CHO cells that we used herein, we can speculate that human α_5 and α_v subunits likely complex with β_1 integrin subunits produced endogenously by CHO cells to form active complexes that bind to immobilized FnIII₁₀Eng.

Previous studies have routinely used flow cytometric analysis and integrin layers adsorbed on tissue culture polystyrene to demonstrate specific binding of integrin receptors to ECM ligands.^{15,23–25} The interaction between cell surface receptors and ECM ligands during cell adhesion is polyvalent in nature, often involving clustering of multiple integrin receptors on the cell surface.²⁸ Integrin clustering is likely to lead to differences in both the

thermodynamics and kinetics of receptor–ligand binding in adherent cells when compared with cells suspended in solution (e.g., during flow cytometry). Therefore, flow cytometric analysis of integrin–receptor interactions may not represent an appropriate analysis of integrin–ligand interactions. In addition, modest changes in ligand density and affinity can have dramatic effects on cell adhesion. Consequently, studies with conventional adsorbed protein layers (e.g., integrin layers) are difficult to interpret due to the ambiguity in the density of adsorbed protein units that are active and able to interact with a ligand.¹ Therefore, it is difficult to make assertions about cell–ECM interactions using flow cytometry or adsorbed integrin monolayer assays. Our study represents a functional evaluation of the interaction of human α_v - or α_5 -containing integrins with FnIII₁₀Eng, which is perhaps a more appropriate analysis of a ligand for cell adhesion. The fundamental contrast between our surface-chemistry-based method and flow cytometry or assays using adsorbed protein layers may have broad implications for studying other types of receptor–ligand interactions, particularly those involving ECM proteins.

This study demonstrates that the enzyme-directed protein immobilization approach is applicable to creating well-defined and more complex substrates for cell adhesion. This system achieves the same level of control over receptor–ligand interactions as peptide presentation approaches developed previously.^{1–5} However, it also addresses limitations in existing peptide immobilization strategies, as it allows for presentation of a 12 kDa cell adhesion protein in a well-defined orientation and with controlled density on a bioinert SAM background. This approach is advantageous over peptide immobilization when the biological activity of a protein cannot be attributed to a specific peptide residue or when the biological activity of a peptide residue is influenced by nonadjacent residues in the protein structure. In addition, our approach allows for control over the density of biologically active protein on a substrate, which is not achievable in the case of protein adsorption onto tissue

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culture substrates. Cutinase-directed protein immobilization is also advantageous when compared with other covalent protein immobilization approaches such as thiol-maleimide and amide bond formation, due to the indirect nature of the protein immobilization (the cutinase active site responsible for immobilization lies on the opposite side of the protein from the C-terminal fusion site, so that the protein to be immobilized has no interaction with the substrate and is not structurally altered by the immobilization procedure) and the specificity of the protein-substrate interaction, which renders protein purification unnecessary. Further, this approach is, in principle, general in the type of protein that can be immobilized, provided that the sequence encoding the protein of interest can be cloned into the cutinase gene. Therefore, enzyme-

directed protein immobilization may serve as an enabling technique in fundamental cell biology and applications in tissue engineering and allow for more direct mimicry of the natural extracellular matrix.

Acknowledgment. This work was funded by the National Science Foundation, the National Institutes of Health, and the Department of Defense (DAMD17-01-1-0385). W.L.M. is supported by a National Research Service Award (F32 GM69045). We are thankful for technical assistance from Young-Sam Lee during protein purification and analysis.

LA035733M