FAK MODULATES CELL ADHESION STRENGTHENING VIA TWO DISTINCT MECHANISMS: INTEGRIN BINDING AND VINCULIN LOCALIZATION

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FAK MODULATES CELL ADHESION STRENGTHENING VIA TWO DISTINCT MECHANISMS: VINCULIN LOCALIZATION AND INTEGRIN BINDING KINETICS

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To my family for your love and support
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SUMMARY

Cell adhesion to the extracellular matrix (ECM) provides tissue structure and integrity as well as triggers signals that regulate complex biological processes such as cell cycle progression and tissue-specific cell differentiation. Hence, cell adhesion is critical to numerous physiological and pathological processes, including embryonic development, cancer metastasis, and wound healing, as well as biotechnological applications, such as host responses to implanted devices and integration of tissue-engineered constructs. During the adhesion process, integrin surface receptors bind ECM proteins, cluster, and associate with the actin cytoskeleton. Subsequent strengthening of the integrin/actin cytoskeleton interaction occurs via complexes of proteins known as focal adhesions. Due to the close association between biochemical and biophysical processes within adhesion complexes, mechanical analyses can provide important new insights into structure-function relationships involved in regulating the adhesion process. The objective of this project was to investigate the role of the protein tyrosine kinase FAK in cell adhesion strengthening. Our central hypothesis was that FAK regulates adhesion strengthening by modulating interactions between integrins and FA structural components. Using a novel combination of genetically engineered cells to control the interactions of FAK, a spinning disk adhesion assay with micropatterned substrates to obtain reproducible and sensitive measurements of adhesion strength, and quantitative biochemical assays for analyzing changes in adhesive complexes, we demonstrate that FAK modulates adhesion strengthening via two distinct mechanisms: (1) FAK expression results in elevated integrin activation leading to regulation of strengthening rate and (2) FAK regulates steady-state adhesion strength via vinculin recruitment to focal adhesion. We also show that the autophosphorylation and catalytic sites of FAK are critical to this regulation of adhesion strengthening. This work is significant because it both identifies functional mechanisms of FAK and provides the first evidence that focal adhesion signaling regulates the adhesion strengthening process. Furthermore, this research demonstrates that the dependency of migration on adhesion strength is highly complex and establishes a need for adhesion strengthening metrics in analyzing the functional mechanisms of molecules within adhesion complexes.
CHAPTER 1

INTRODUCTION

Specific Aims

The goal of this project was to analyze the role of the protein tyrosine kinase focal adhesion kinase (FAK) in cell adhesion strengthening. Cell adhesion to the extracellular matrix (ECM) provides tissue structure and integrity as well as triggers signals that regulate complex biological processes such as cell cycle progression and tissue-specific cell differentiation. Hence, cell adhesion is critical to numerous physiological and pathological processes, including embryonic development, cancer metastasis, and wound healing, as well as biotechnological applications, such as host responses to implanted devices and integration of tissue-engineered constructs. During adhesion, receptors bind to extracellular matrix proteins, cluster, and associate with the cytoskeleton forming specialized, discrete complexes called focal adhesions. Many proteins interact within these focal adhesions to provide both mechanical linkage to the cytoskeleton and trigger signaling pathways that regulate cell survival, growth, and differentiation. Previous work from our lab, by Dr. Nathan Gallant, outlined a mechanism by which structural molecules contribute to adhesion strength. This work takes the analysis further to create a framework by which proteins known as signaling molecules also contribute to adhesion strengthening.

FAK is a widely expressed non-receptor protein tyrosine kinase central to focal adhesion function. FAK is essential to development as shown by early lethality in FAK null embryos. Upon integrin ligation and/or clustering, FAK becomes activated through tyrosine phosphorylation, notably by releasing autoinhibition at the Y397
autophosphorylation site, and interacts with multiple targets such as paxillin, PI3 kinase, Src, and Cas. These interactions provide signals for pathways such as the ERK and JNK mitogen activated pathways, and FAK has been implicated in cellular responses such as cell spreading, proliferation, migration, and apoptosis. While the roles of FAK in migration and signaling are well established, the contributions of FAK to cell adhesion strengthening remain poorly understood.

The objective of this project was to investigate the role of the protein tyrosine kinase FAK in cell adhesion strengthening. Our central hypothesis was that FAK regulates adhesion strengthening by modulating interactions between integrins and focal adhesion structural components. Using a novel combination of genetically engineered cells to control the interactions of FAK, a spinning disk adhesion assay with micropatterned substrates to obtain reproducible and sensitive measurements of adhesion strength, and quantitative biochemical assays for analyzing changes in focal adhesion structure, we have addressed the following specific aims:

1. Analyze the contributions of FAK expression to cell adhesion strengthening.

FAK expression modulates cell migratory behavior suggesting that FAK plays a role in focal adhesion turnover. A kinetic study of cell adhesion strength with and without FAK expression was completed using our spinning disk assay resolving differences in both steady-state adhesion strength and the rate of strengthening. An investigation into the factors that lead to changes in adhesion strength was made by biochemically quantifying changes in overall expression of integrins and focal adhesion proteins, integrin binding, and the area covered by focal adhesion proteins within the focal adhesion area.
Hypothesis: Using a tetracycline-inducible FAK expression system in FAK null cells, we expected to see differences in cell adhesion strengthening dependent on the presence of FAK.

2. Investigate the role of FAK activation sites in these adhesion differences.

Using wild-type tet-FAK (WT) cells, deactivated autophosphorylation site tet-FAK (Y397F) cells, and deactivated catalytic site tet–FAK (Y576F/Y577F) cells, critical phosphorylation sites on the FAK molecule that contribute to adhesion strengthening were analyzed. Changes in steady-state adhesion strength as well as rate of strengthening due to expression of mutated FAK were quantified. Both the composition and area of focal adhesions and the integrin binding kinetics were quantified to expose factors leading to this modulation of adhesion strength.

Hypothesis: Phosphorylation sites in the FAK catalytic domain act as recruiting sites for other focal adhesion signaling molecules leading to the transduction of signals for focal adhesion assembly and cell adhesion strengthening.

3. To investigate the functional mechanism by which FAK modulates adhesion strengthening.

The results from specific aims 1 and 2 suggested that FAK modulates adhesion strengthening via two distinct mechanisms: rate of integrin binding and localization of vinculin to focal adhesions. Results from this aim clarified that these processes are critical to FAK modulation of strengthening and identify the biological mechanism by which FAK is able to modulate these processes. Short-term adhesion assays to analyze integrin contributions to strength and integrin activation state were combined
with analysis of integrin activation in suspended cells in order to uncover the mechanism by which FAK is able to modulate integrin binding and strengthening rate. This analysis of the contributions of FAK to adhesion strengthening has provided new insights into the functional role of this molecule as well as established a basis for the study of signaling molecules in adhesion strengthening. Furthermore, comparing these adhesion strengthening results with migration data reveals a complex relationship between these processes that was not previously known.

**Thesis Outline**

Chapter 2 provides detailed background information and describes the significance of studying cell adhesion strengthening and the signaling molecules involved. Chapter 3 describes the framework by which cell adhesion strengthening and the components of the adhesion process are analyzed. Chapter 4 addresses the role of FAK in the cell adhesion strengthening process and the biological mechanisms by which FAK is able to modulate adhesion strengthening (AIM 1 and AIM 3). Chapter 5 addresses the functional significance of both the autophosphorylation and catalytic sites in adhesion strengthening (AIM 2) and correlates adhesion strengthening with migration. Chapter 6 concludes with the importance of this thesis research and future studies based on this work.
CHAPTER 2

BACKGROUND AND SIGNIFICANCE

Cell adhesion to the extracellular matrix (ECM) provides tissue structure and integrity as well as triggers signals that regulate complex biological processes such as cell cycle progression and tissue-specific cell differentiation (Danen et al., 2003; Wehrle-Haller et al., 2003; Walker et al., 2005; Garcia et al., 2005). Hence, cell adhesion is critical to numerous physiological and pathological processes well as biotechnological applications (Jin et al., 2004; Garcia, 2005; Hubbell, 1999; Wilson et al., 2005). The cell adhesion process involves binding of integrin surface receptors to ECM proteins, clustering of these bound integrins, association with the actin cytoskeleton, and subsequent strengthening of the integrin/actin cytoskeleton interaction via complexes of proteins and lipids known as focal adhesions (FAs) (Hynes, 2002; Zamir et al., 2001; Zimerman et al., 2004). Due to the close association between biochemical and biophysical processes within adhesion complexes, mechanical analyses can provide new insights into structure-function relationships involved in regulating the adhesion process.

Cell Adhesion and Physiology

Cells adhere to each other or ECM to provide the basic structure of tissues. In vertebrates, the major tissue types are epithelial, nerve, muscle, blood, lymphoid, and connective tissues (for more information see (Alberts et al., 2002a)). Each type of adhesion, cell-cell adhesion and cell-matrix adhesion, provides a unique structure to tissues leading to differences in function. For example, epithelial tissues consist
predominantly of cell-cell adhesions and provide selective permeable barriers which separate fluids of different chemical compositions. Whereas connective tissues consist predominantly of ECM with sparsely distributed cells and provide a supportive structure and resistance to mechanical loading. Tissue layers come together to form organs, each of which has a specific function leading to the culmination of highly complex processes which bring about life. Without cell adhesion, most of the basic functions necessary for life in multicellular organisms would not occur.

Because cell adhesion to ECM provides structure as well as triggers signals that regulate complex biological processes (Danen et al., 2003; Wehrle-Haller et al., 2003; Walker et al., 2005; Garcia et al., 2005), cell adhesion is critical to numerous physiological and pathological processes, such as embryonic development, wound healing, and cancer metastasis, as well as biotechnological applications, such as host responses to implanted devices and integration of tissue-engineered constructs (Jin et al., 2004; Garcia, 2005; Hubbell, 1999; Wilson et al., 2005).

Embryonic Development

Embryogenesis is the process of cell division and differentiation that results in development of an embryo (for more information see (Alberts et al., 2002b)). This process comprises zygote formation (due to fertilization), cleavage (rapid proliferation), blastulation (implantation of the blastocyst into the uterine lining, differentiation of the inner cell mass, and secretion of ECM), gastrulation (migration and differentiation into three germ layers), and organogenesis and patterning of appendages. During embryonic development, cells migrate along ECM and cell-cell adhesion components within embryonic tissue until reaching an activating stimulus which signals the differentiation of
those cells into a particular tissue-specific cell type (De Arcangelis et al., 2000; Wehrle-Haller et al., 2003). Genetic deletion of adhesion components, including ECM ligands (including fibronectin and laminin), integrin receptors (including integrin subunits $\alpha_5$ and $\beta_1$), and focal adhesion proteins (including FAK and vinculin), results in lethality at early embryonic stages commonly due to failure to implant or inability to migrate and differentiate properly during gastrulation (George et al., 1993; Stephens et al., 1995; Furuta et al., 1995; Xu et al., 1998; Fassler et al., 1995). These knockout studies clearly demonstrate that cell-ECM adhesion is essential to development.

**Wound Healing**

Cell adhesion is also critical to wound healing, the repair and regeneration of damaged tissues. The best known example of wound healing occurs in dermal tissues. This wound healing response occurs in four overlapping phases: inflammation, granulation tissue formation, wound contraction, and maturation (Mitchell et al., 1997). During inflammation, platelets aggregate and secrete inflammatory factors while cells such as neutrophils and macrophages identify and phagocytose bacteria and debris (Anderson, 1996). These inflammatory factors stimulate migration of other cells into the wounded site to repair the tissue in the granulation tissue phase. The granulation tissue phase involves angiogenesis (vascularization of injured tissue), granulation tissue formation (fibroblast secretion of preliminary ECM), epithelialization (migration of epithelial cells to enclose the new tissue). Wound contraction occurs as myofibroblasts contract in order to shrink the size of the wound. During maturation, fibroblasts and endothelial cells remodel and align the ECM (primarily collagen) according to the
required anisotropic properties of the tissue. Cell adhesion is a basic process critical to wound healing in all phases including:

1. $\alpha_{IIb}\beta_3$ mediates platelet adhesion in the clotting response
2. $\beta_2$ integrins (e.g. $\alpha_M\beta_2$) promote leukocyte adhesion and differentiation
3. $\beta_1$ integrins mediate fibroblast, endothelial, and epithelial cell adhesion.

Additionally, selectins are involved in the homing and recruitment of blood-borne leukocytes to sites of inflammation.

Altered adhesive events can lead to pathological blood clotting and wound healing (Danen et al., 2003). Mice lacking $\beta_2$ integrins, which are exclusively expressed in cells of the immune system, suffer from bacterial infections and impaired wound healing (Mizgerd et al., 1997). This phenotype is consistent in patients with type I leukocyte adhesion deficiency (LAD-1) due to lack of $\beta_2$ integrins. Another form of LAD has been reported in patients presenting features of LAD-1 and a bleeding disorder known as Glanzmann thrombasthenia. In this form of LAD, patients have normal $\beta_1$, $\beta_2$, and $\beta_3$ integrin levels but the activation state of their integrins is not properly regulated (Hogg et al., 2000). In mice and human patients with Glanzmann thrombasthenia, deficient platelet aggregation and clot retraction occurs due to null mutations in the genes encoding either $\alpha_{IIb}$ or $\beta_3$ integrin subunits (McDowall et al., 2003; Hodivala-Dilke et al., 1999).

**Cancer Metastasis**

Metastatic tumors, containing cells able to migrate and invade other tissues, are the primary cause of death in cancer patients (Hood et al., 2002). Metastasis is a complex process that requires the coordination of altered signal transduction pathways in cancer cells which allows proliferation, matrix remodeling, invasion and migration through
various tissues, differentiation, and angiogenesis in the new tumor (Hood et al., 2002).

Metastasis is initially mediated by changes in the expression of integrins, secretion of proteases to remodel the ECM, and deposition of new ECM macromolecules, thereby activating signaling cascades that regulate gene expression, cytoskeletal organization, cell adhesion, and cell survival. Integrins also modulate abnormal growth factor signaling critical to cancer metastasis, as evidenced by targeted deletion of $\beta_4$ integrin suppression of experimental mammary tumor onset and invasive growth due to EGF/ErbB2 signaling (Guo et al., 2006). Therefore, cell adhesion to ECM plays a vital role in metastatic events and has become a target for drug therapies (Hood et al., 2002; Carmeliet et al., 2000; Jin et al., 2004).

Host Response to Implanted Devices

Integrin-ECM interactions play central roles in host responses to implanted devices (Garcia, 2005; Anderson, 2001). Implantation of a biomaterial begins with injury to the tissue and blood-interactions with the material which initiate an inflammatory response. During the initial inflammatory response known as acute inflammation, leukocytes adhere to proteins on the implant surface, and neutrophils and macrophages begin releasing enzymes and phagocytosing any microorganisms or small foreign materials. In chronic inflammation, monocytes and macrophages initiate a healing response where in fibroblasts and endothelial cells proliferate and deposit matrix forming granulation tissue. The foreign body reaction, composed of foreign body giant cells and granulation tissue components, then generates a fibrous encapsulation surrounding the biomaterial thereby isolating the implant from the local tissue. Isolation of implanted
devices can lead to destruction of the surrounding tissue and eventual need for implant replacement (Anderson, 2001).

Integrins mediate cellular responses to biomaterial surfaces by binding ECM ligands that can be adsorbed from solution, engineered at the surface, or deposited by cells (Garcia, 2005). For instance, integrin $\alpha_{\text{IIb}}\beta_3$ binds several ligands involved in platelet aggregation, including fibrinogen, von Willebrand factor, and fibronectin (Hynes, 2002; Wehrle-Haller et al., 2003). This integrin also participates in the initiation of blood activation cascade upon contact with synthetic surfaces (Broberg et al., 2002; Gorbet et al., 2003). Also, leukocyte $\beta_2$ integrins, in particular $\alpha_M\beta_2$, mediate monocyte and macrophage adhesion to ligands, such as fibrinogen, fibronectin, IgG, and complement fragment iC3b (Tang et al., 1996; Flick et al., 2004). Current strategies to circumvent the inflammatory response involve design of bioactive materials to direct cellular function and promote integration with surrounding tissues through integrin interactions (Garcia, 2005; Hubbell, 1999).

**Tissue Engineered Constructs**

Tissue engineering combines cells, biomaterials, and suitable biochemical factors to develop biological substitutes that restore, maintain, or improve tissue function (Nerem, 2006; Langer et al., 1993). Successful tissue engineering strategies require design of constructs, scaffolds and/or delivery systems, that promote the coordinated growth and differentiation of cells into a three-dimensional tissue which integrates with surrounding tissues (Polak et al., 2006; Nerem, 2006; Atala, 2006; Seliktar, 2005). Construct strategies often involve materials that are degradable and can be replaced by secreted, native ECM. Cellular integration with constructs through the integrin-mediated cell
adhesion not only promotes tissue integrity but also guides differentiation and proliferation of cells as well as secretion of native ECM in order to create a self-renewing engineered tissue.

**Cell-Matrix Adhesion Components**

The ECM is an intricate network of macromolecules, predominantly proteins and polysaccharides, which is secreted by cells in the surrounding tissue (for more information see (Haralson *et al.*., 1995) and (Comper, 1996)). Initially, ECM was thought to primarily provide a supporting framework holding cells and tissues together. However, the ECM is much more than a structural component of tissues. ECM also provides signals to surrounding cells guiding many cellular functions including migration, proliferation, differentiation, and apoptosis. Variations in the relative amounts of matrix macromolecules and organization of these molecules do provide structure yielding the properties of particular tissues. For example, the matrix in bone becomes calcified making bone tissue harder and resistant to compressive loads whereas the matrix in tendons is organized in rope-like structures giving them tensile strength (Hayes *et al.*, 1997; Mow *et al.*, 1997a). Organization of glycosaminoglycans in proteoglycans, such as aggrecan, within the matrix can also provide important structural significance, by drawing in water to the ECM and creating swelling pressure thereby giving shock absorption properties to cartilage (Mow *et al.*, 1997b). The composition, orientation, and amount of matrix molecules also results in variations in cellular responses, such as modified differentiation or proliferation (Haralson *et al.*, 1995; Keselowsky *et al.*, 2005).
Figure 2.1. Key players in cell adhesion: Cell adhesion involves binding integrin receptors to extracellular matrix proteins, clustering of integrins, and association of these integrins with the cytoskeleton forming complexes called focal adhesions. FAs provide mechanical linkage to the cytoskeleton and trigger signaling pathways that regulate cell survival, growth, and differentiation.

Cell adhesion to ECM components, such as collagen, laminin, and fibronectin, is primarily mediated by integrin receptors. Integrins are heterodimeric, transmembrane receptors consisting of non-covalently associated α and β subunits (Hynes, 2002). 18 known α subunits and 8 known β subunits selectively dimerize yielding 24 distinct integrins with specific binding to various ECM ligands (Hynes, 2002). Recognition of matrix proteins by multiple integrins provides redundancy as well as specificity. For example, integrin αvβ3 binds fibronectin as well as other RGD containing proteins such as vitronectin and thrombospondin whereas α5β1 integrin solely binds fibronectin with an added requirement of binding the PHSRN synergy site (Aota et al., 1994; Garcia et al., 2002). Cells are capable of receiving signals from the ECM through outside-in signaling and regulating the activity of their integrins through inside-out signaling (Takagi et al., 2002; Hughes et al., 1998; Hynes, 2002). Inside-out signaling allows cells to rapidly respond to an appropriate stimulus rather than requiring de novo
synthesis. This feature of integrin activation is especially important in leukocytes which circulate until activated by the appropriate stimulus, allowing rapid adhesion to the blood vessel wall and repair of a pathological state (Takagi et al., 2002; Hynes, 2002).

Following activation via changes in conformation, integrins bind their target ligands and cluster together. The cytoplasmic domains of bound and clustered integrins form supramolecular complexes with FA proteins, such as talin, FAK, vinculin, alpha actinin, Src, paxillin, ILK, and zyxin, and couple to the actin cytoskeleton (Figure 2.1) (Zamir et al., 2001; Zimerman et al., 2004; Galbraith et al., 2002; Beningo et al., 2001). Through these initial adhesive complexes, the cell generates myosin-mediated contractile forces resulting in enhanced, more mature FA complexes and a strengthened cell-ECM interaction (Galbraith et al., 2002). Most FA proteins contain multiple binding sites for other adhesion components. Thus, FA complexes can assemble in several structural configurations, suggesting that the regulation of interactions between these components is central to the function of these complexes in cell adhesion (Zamir et al., 2001).

**Focal Adhesion Kinase (FAK)**

FAK is a widely expressed non-receptor protein tyrosine kinase central to the regulation of FA assembly. FAK interacts with many other FA proteins including Src, Cas, paxillin, and talin (Hanks et al., 1992; Schaller et al., 1992; Schaller et al., 1994; Schaller et al., 1999; Vuori et al., 1996). The expression of FAK is important not only to development but also cell migration, proliferation, and survival (Owen et al., 1999; Sieg et al., 1999; Cary et al., 1996; Richardson et al., 1997; Sieg et al., 2000; Wang et al., 2001; Oktay et al., 1999; Zhao et al., 1998; Frisch et al., 1996; Ilic et al., 1998).
Moreover, overexpression of FAK has been shown in preinvasive, invasive, and noninvasive tumors (Canel et al., 2006), and FAK inhibition reduces experimental metastatic tumor formation (van Nimwegen et al., 2005). Although the role of FAK in migration and focal adhesion turnover has been established (Owen et al., 1999; Sieg et al., 1999), there is still a gap in the current knowledge as to if and how the expression of FAK regulates cell adhesion strengthening. The results of this study are significant because they

1. provide a rigorous, quantitative analysis of the role of FAK in cell adhesion strengthening
2. uncover a novel functional mechanism by which FAK modulates adhesion strengthening
3. analyze critical activation states of FAK in the adhesion process
4. correlate migration and adhesion
5. provide an experimental framework by signaling molecules are able to contribute structurally to the cell adhesion strengthening process.

Upon integrin ligation and/or clustering, FAK becomes activated through Tyr phosphorylation and interacts with multiple targets such as paxillin, PI3 kinase, Src, and Cas (Burridge et al., 1992; Guan et al., 1992; Hanks et al., 1992; Kornberg et al., 1992). These interactions provide signals for pathways such as ERK and JNK, and FAK has been implicated in cell spreading, proliferation, growth factor signaling, migration, oncogenesis, and apoptosis (Owen et al., 1999; Sieg et al., 1999; Cary et al., 1996; Richardson et al., 1997; Sieg et al., 2000; Wang et al., 2001; Oktay et al., 1999; Zhao et al., 1998; Frisch et al., 1996; Ilic et al., 1998; Sieg et al., 2000).
FAK is comprised of a central catalytic domain and large amine-terminal and carboxyl-terminal non-catalytic domains (Figure 2.2). The amine-terminal domain of FAK binds the cytoplasmic domain of β-integrin subunits, thereby providing anchorage of FAK to FA sites (Schaller et al., 1995). This domain allows anchorage of FAK to sites of integrin or growth factor receptor clustering and plays a role in regulating the interaction of FAK with other activating proteins (Dunty et al., 2002; Sieg et al., 2000). The carboxyl-terminal domain of FAK consists of protein-protein interaction sites (Tyr and PXXP) and includes the focal adhesion targeting (FAT) domain that directs FAK to newly formed and existing FA complexes and has been shown to bind the FA component paxillin (Martin et al., 2002; Hildebrand et al., 1993; Sieg et al., 1999; Thomas et al., 1999; Arold et al., 2002; Hayashi et al., 2002; Liu et al., 2002).

FAK activation occurs through phosphorylation of several Tyr residues located in the catalytic domain of FAK as well as sites in the carboxyl-terminal domain (Figure 2.2). The residue most critical to FAK activation is Tyr397, the FAK autophosphorylation site (Schaller et al., 1994). Tyr397 is a binding site for SH2 domains of Src-family kinases (SFKs) (Schaller et al., 1994; Xing et al., 1994; Polte et al., 1995). The interaction between Tyr397 and SFKs has been shown to recruit SFKs to FA sites and activate catalytic activity of FAK by release of the autoinhibition due to SH2/SH3 interactions (Schaller et al., 1999). Tyr397 has also been shown to mediate several other SH2 signaling effectors including PI3 kinase, Grb7, Shc, and phospholipase C-γ1, thereby implicating FAK in cell survival, cell migration during embryogenesis, Grb-2 mediated interactions for ERK2 signaling, and protein kinase C regulation of intracellular Ca\(^{2+}\), respectively (Chen et al., 1996; Han et al., 1999; Schlaepfer et al., 1998; Zhang et al., 1999).
Phosphorylation of Tyr397 is critical for the phosphorylation of other FAK Tyr residues and binding of other signaling factors, including the SFK Src under normal physiological conditions (Polte et al., 1995; Schaller et al., 1994; Xing et al., 1994). This requirement is overridden by the expression of the oncogene v-Src (Roy et al., 2002). Other FAK Tyr residues have also been identified as activation sites and characterized. Tyr576/Tyr577 are located in the kinase domain activation loop of FAK (Calalb et al., 1995). Mutation of these residues reduces FAK autophosphorylation activity (Owen et al., 1999). Phosphorylation of Tyr925 in the carboxyl-terminal domain by c-Src activates an interaction with the adaptor protein Grb2, which contributes to adhesion-stimulated activation of ERK2 mitogen-activated protein kinase (MAPK) (Schlaepfer et al., 1994; Schlaepfer et al., 1998). Still more Tyr residues have been identified within the FAK molecule including Tyr407 of the catalytic domain and Tyr861 of the carboxyl-terminal domain; however, their functional role in FAK activity is unclear (Calalb et al., 1995; Calalb et al., 1996). A mutual activation of FAK and SFKs has been suggested to create a positive feedback loop which leads to a fully activated Tyr kinase complex. Also, FAK has been suggested to serve as a docking protein to recruit SFKs for phosphorylation of FA substrates, based on the observation that SFK binding to FAK Tyr397 is important for phosphorylation of FAK associated substrates Cas and paxillin (Ruest et al., 2001; Klinghoffer et al., 1999; Felsenfeld et al., 1999).
Figure 2.2. FAK has many phosphorylation and binding sites available for protein-protein interactions giving the molecule the ability to act as a scaffolding protein.

In recent years, the functional role of FAK in cell spreading and migration has been established. (Cary et al., 1996; Richardson et al., 1997; Sieg et al., 1999; Owen et al., 1999; Sieg et al., 2000; Wang et al., 2001) Using FAK-null embryonic fibroblasts that express WT-FAK and FAK phosphorylation site mutants under an inducible promoter, Owen and colleagues have shown that FAK re-expression increases cell motility, based on Boyden chamber cell migration analysis, and reduces focal adhesion size, based on immunostaining in spread cells (Owen et al., 1999). Furthermore, mutation of the Tyr397 autophosphorylation site and the Tyr576/Tyr577 catalytic site show that these sites are critical to cell spreading and migration. These results suggest that FAK is important to FA turnover. In addition, Wang et al. demonstrated that FAK-null cells have reduced migratory speed and did not change migratory path in response to applied force whereas the migratory path of FAK expressing cells changed due to the application of force (Wang et al., 2001). Moreover, Chen and others showed that FAK-null cells treated with Y27632, a Rho kinase inhibitor, regain fibroblast morphology and increased motility suggesting that the lack of FA turnover in FAK null cells may be due to a change in regulation of Rho-related contractility (Chen et al., 2002). Cell migration and
spreading are complex processes that involve multiple steps and are highly regulated. Although changes in cell spreading and migration are typically studied to give insight into FA function, obtaining a fundamental understanding of how FA components regulate these processes is difficult.

**Thesis Overview**

By integrating our adhesion experimental framework (see Chapter 3) with an inducible expression system for controlled expression of proteins in cells, we can rigorously analyze the contributions of these proteins to adhesion strengthening and the functional mechanisms of adhesion components as outlined in this study and Chapter 3. Here, FAK-null cells were genetically engineered to express WTFAK, F397-FAK, or F576/577-FAK to systematically analyze the regulation of adhesion strengthening due to FAK expression and uncover the functional mechanisms by which FAK modulates adhesion strengthening.

**References**


Cell adhesion to the extracellular matrix (ECM) provides tissue structure and integrity as well as triggers signals that regulate complex biological processes such as cell cycle progression and tissue-specific cell differentiation (Danen et al., 2003; Wehrle-Haller et al., 2003; Walker et al., 2005; Garcia et al., 2005). Hence, cell adhesion is critical to numerous physiological and pathological processes, including embryonic development, cancer metastasis, and wound healing, as well as biotechnological applications, such as host responses to implanted devices and integration of tissue-engineered constructs (Jin et al., 2004; Garcia, 2005; Hubbell, 1999; Wilson et al., 2005). For example, genetic deletion of adhesion components, including ECM proteins, integrin receptors, and focal adhesion proteins, results in lethality at early embryonic stages (George et al., 1993; Stephens et al., 1995; Furuta et al., 1995; Xu et al., 1998). These deletion studies clearly demonstrate that cell-ECM adhesion is essential to development. The cell adhesion process involves binding of integrin surface receptors to ECM proteins, clustering of these bound integrins, association with the actin cytoskeleton, and subsequent strengthening of the integrin/actin cytoskeleton interaction via complexes of proteins known as focal adhesions (Fas) (Hynes, 2002; Zamir et al., 2001; Zimerman et al., 2004). Due to the close association between biochemical and biophysical processes within adhesion complexes, mechanical analyses can provide important new insights into structure-function relationships involved in regulating the adhesion process.

Cell spreading and migration are often used as indirect indicators of adhesion strength. However, these multistep, dynamic processes exhibit complex dependencies on adhesion strength, and therefore do not provide sufficiently direct or sensitive metrics of adhesion (Palecek et al., 1997). This lack of quantitative understanding of the regulation of adhesion strength limits the interpretation of functional studies of structural and signaling adhesive components. Furthermore, it is increasingly evident that mechanotransduction between cells and their environment regulates gene expression and cell fate (Wozniak et al., 2003; Engler et al., 2004; McBeath et al., 2004; Mammoto et al., 2004; Polte et al., 2004). Direct measurements of cell–ECM adhesion strength are therefore necessary for understanding these mechanosensory functions.

**Cell Adhesion**

Cell adhesion to ECM components, such as collagen, laminin, and fibronectin, is primarily mediated by integrin receptors. Integrins are heterodimeric, transmembrane receptors consisting of non-covalently associated α and β subunits (Hynes, 2002). Following activation, via changes in conformation, integrins bind their target ligands and cluster together. The cytoplasmic domains of bound and clustered integrins form supramolecular complexes with FA proteins, such as talin, FAK, vinculin and paxillin, and become associated with the actin cytoskeleton (see Figure 1) (Zamir et al., 2001; Zimerman et al., 2004; Galbraith et al., 2002; Beningo et al., 2001). Through these initial adhesive complexes, the cell generates myosin-mediated contractile forces resulting in enhanced, more mature FA complexes and a strengthened cell-ECM interaction (Galbraith et al., 2002). Most FA proteins contain multiple binding sites for
other adhesion components. Thus, FA complexes can assemble in multiple structural configurations and the regulation of such complex interactions among FA components is central to the function of these complexes in cell adhesion (Zamir et al., 2001).

Figure 3.1. Cell adhesion involves binding integrin receptors to extracellular matrix proteins, clustering of integrins, and association of these integrins with the cytoskeleton forming complexes called focal adhesions.

Cytoskeletal tension and focal adhesion assembly also drive changes in cell shape and spreading. In addition, coordinated adhesive interactions and force generation are central to cell migration (Lauffenburger et al., 1996; Sheetz et al., 1998). During migration, cellular processes extend and attach to ECM at the leading edge, allowing actomyosin contractile forces to be transmitted to the ECM as “traction forces” (see the chapters by Dembo and Lee). As the cell pulls itself forward with these traction forces, it extends more processes to sustain the forward motion. Once the cell has moved sufficiently forward relative to the initial attachment point, the attachment at the rear is released and adhesion components are recycled for reuse at the front of the cell (Palecek et al., 1996), or else proteolytically cleaved. Therefore, migration involves coordinated regulation of adhesive interactions along the cell-ECM interface, and the resulting
distribution of traction forces is highly non-uniform and position dependent (Dembo et al., 1999).

Measurement Systems for Adhesion Characterization

Cell migration and spreading are complex processes that involve multiple steps and are highly regulated (Lauffenburger et al., 1996). Although changes in cell spreading and migration are commonly studied to gain insights into adhesive interactions and FA function, obtaining a fundamental, mechanistic understanding of how FA components regulate these processes is difficult. Moreover, the highly complex dependency of migration and spreading on adhesion strength, down to the position of an adhesive complex within a migrating cell, makes the interpretation of these results with regard to adhesion challenging (Palecek et al., 1997; Palecek et al., 1999; DiMilla et al., 1991). Due to the close association between biochemical and biophysical processes within adhesion complexes, mechanical analyses can provide insights into structure-function relationships in the regulation of the adhesion process. Mechanical analyses have identified two stages of cell adhesion, consisting of initial integrin-ligand binding followed by rapid strengthening (Lotz et al., 1989; McClay et al., 1981; Choquet et al., 1997; Garcia et al., 1998b). This strengthening response arises from i) cell spreading, which increases in cell–substrate contact area, ii) clustering, which entails receptor recruitment to anchoring sites, and iii) focal adhesion assembly, which involves integrative interactions with cytoskeletal elements that lead to enhanced force distribution among bound receptors through local membrane stiffening (Lotz et al., 1989; Gallant et al., 2005).
Several mechanical methods have been developed to quantify adhesion strength, including hydrodynamic shear force, centrifugation, and micromanipulation assays (Mohandas et al., 1974; Doroszewski et al., 1977; McClay et al., 1981; Lawrence et al., 1987; Lawrence et al., 1987; Garcia et al., 1997; Garcia et al., 2003). Wash assays provide an easy method to apply hydrodynamic shear force to detach cells and compare the number of attached cells after force application. But, the amount of force applied in wash assays is non-uniform and uncontrolled, limiting the reproducibility and interpretation of these results. Centrifugation and micromanipulation measurements do apply controlled forces; however, these assays apply a single force at one time, making them tedious to extend over a range of forces, and are often limited in the overall range of forces than can be applied. Hydrodynamic techniques, based on systems such as parallel plate, rotating disk(s), and radial flow between parallel disks, can be configured to apply a controlled, wide range of forces and provide consistent, reliable measurements of adhesion strength. Based on characterized flow patterns in a specialized flow chamber, fluid flow over adherent cells generates detachment shear forces. The adhesion strength is typically reported as the shear stress (force/area) at the flow chamber wall ($\tau_w$) that produces a prescribed level of cell detachment (e.g. 50% detachment). Although these assays provide sensitive and reproducible measurements of adhesion strength, they are often limited by the ability to apply sufficient forces for detachment of cells with strengthened adhesion. Furthermore, complex detachment mechanics are associated with these hydrodynamic assays due to variability in cellular morphology (spread versus rounded cells) as well as non-uniform distributions of adhesive structures thus limiting the interpretation of results.
Sophisticated mechanical experimental systems have recently been developed to further elaborate structure-function relationships in cell adhesion. Using laser tweezers to trap ECM coated beads and apply small forces (nN) in a short time period (sec), Sheetz and colleagues have shown that mechanical force itself can be converted into a signal to strengthen initial integrin-ECM adhesions in advancing lamella (Galbraith et al., 2002; Giannone et al., 2003). These studies elegantly demonstrate that applied force can enhance maturation of initial FA complexes and provide insights into mechanical mechanisms at the leading edge of migrating cells. Similarly, cantilever based techniques analogous to atomic force microscopy have been developed to study cellular force application or generation (Micoulet et al., 2005; Chen et al., 2000). These methods resolve forces at localized points on the cell during short time periods thereby demonstrating maturation of initial FAs induced by externally applied or internally generated forces. Extending our understanding of force generation at edges of adherent cells, traction force microscopy techniques incorporate embedded beads in or extended posts on compliant substrates to measure forces applied by the cell to the substrate, based on the mechanical theory of material deformation or beam deflection (Dembo et al., 1999; Reinhart-King et al., 2005; Balaban et al., 2001; Tan et al., 2003). Notably, Balaban, Geiger, and colleagues discovered a direct correlation between local generated force and the orientation, intensity, and area of focal adhesions, suggesting that internally generated force and extent of focal adhesion maturation combine to a constant net stress of approximately 5.5 nNmm$^{-2}$ at adhesive complexes (Balaban et al., 2001). Although traction force microscopy techniques provide an excellent means for studying force generated by cells, these techniques are presently limited to 2-D measurements and are
therefore only able to resolve forces in the tangential (parallel) plane to the surface. This limitation obscures the interpretation of results at the center of contact. In addition, traction force measurements, while highly relevant to migration, do not provide direct measurements of adhesion strength. In all, these recent studies have enhanced our understanding of various adhesion-related functions; however, all of these methods examine single cell interactions with substrates and focus on a small population of FA complexes at a specific time in FA development, and none of these methods provide direct information on the strength of the adhesive interaction.

We have developed a robust approach for studying adhesion strengthening which incorporates a hydrodynamic adhesion strength assay and quantitative biochemical assays to analyze the mechanisms of the strengthening process (Garcia et al., 1997; Garcia et al., 1998a; Gallant et al., 2005). Using population based assays, we can obtain reproducible measurements of adhesion strength and focal adhesion assembly. These measurements may be performed over multiple time points to extend our characterization of strengthening from initial adhesion to steady-state adhesion. By studying detachment of cells, our analysis focuses on the maximum force that a cell can resist before failure of attachment, a common parameter used in mechanics. This experimental failure strength measurement can then be extended to theoretical mechanical analysis to enhance our understanding of the strength of adhesion complexes under various conditions and time points.
Hydrodynamic Assay for Quantifying Adhesion Strength

Cell adhesion strength is quantified using a submerged spinning disk device which applies a well-defined range of hydrodynamic forces to adherent cells on the disk surface (Garcia et al., 1998). Using this device, a large range of forces sufficient to detach cells can be applied in the laminar flow regime, before turbulence obscures the generation of regular, controlled hydrodynamic forces as commonly occurs in other devices such as parallel plate flow chambers. These large forces are often necessary to detach cells with strengthened adhesions (e.g., spread fibroblasts).

Experimental Design

Cells are seeded on a 25 mm diameter coverslip/disk and maintained under the appropriate conditions at 37°C until reaching the desired experimental point (see micropatterning paragraph below for details). Seeded coverslips are mounted on the spinning device which imparts detachment forces due to fluid shear stress. The flow patterns in the spinning disk device approximate the flow around an infinite disk spinning in an infinite fluid (Garcia et al., 1997). Due to the “no-slip” condition between the fluid and the solid surface, a velocity gradient is generated which imparts a shear stress at the surface of the disk. This shear stress $\tau$ (force/area) increases linearly with radial position $r$ along the surface of the disk as given by Equation 1. Fluid density $\rho$, viscosity $\mu$, and rotational velocity $\omega$ all remain constant for each disk spun.

$$\tau = 0.8r \sqrt{\rho \mu \omega^3}$$  \hspace{1cm} (Eqn. 1)
Therefore, the device generates a range of detachment forces such that no force is imparted at the center of the disk ($r = 0$), whereas large forces are applied at the edge of the disk.

![Image of spinning disk device]

**Figure 3.2.** Our adhesion strength assay incorporates a spinning disk device, which applies a well-defined range of hydrodynamic shear stress to cells. The surface for cell adhesion is micropatterned to restrict contact area and generate uniform cellular geometries. This technique provides reproducible measurements of the mean adhesion strength for a population of cells.

Uniformly distributed, adherent cells are subject to spinning for 5 min in serum-free media (PBS + 2mM dextrose) at room temperature and subsequently fixed and stained. Cells are counted in 60 fields across four axes of radial position. Radial position is then converted to shear stress based on the experimental values of the parameters in Equation 1, and the resulting fraction of adherent cells post spin ($f$) versus shear stress is fit to a sigmoidal curve (Equation 2).

$$f(\tau) = \frac{1}{1+e^{b(\tau - \tau_{50})}}$$  \hspace{1cm} (Eqn. 2)

$\tau_{50}$ is the shear stress for 50% cellular detachment (see Figure 3) and represents the mean adhesion strength for a population of cells. In order to achieve appropriate values of $\tau_{50}$ for a given population of cells, appropriate input values for rotational velocity $\omega$ must be determined to yield a range of shear stresses such that cells remain attached at the center.
of the disk and less than 10% of the cells remain attached at the edge of the disk. Shifts in the resulting adhesion profile represent changes in the adhesion strength, and the force applied to the cells by the experimental shear stress can be determined as illustrated in the modeling section of this text (VI).

Because the geometry of an object in flow is critical to the amount of force applied to that object, we use micropatterning techniques to maintain uniform cell geometry across all conditions of interest (Munson et al., 1998). Under normal plating conditions, cells spread across the surface over time, and various cellular conditions can modulate the extent of cell spreading. This modulation of cell spreading thereby modulates the amount of force applied, thus making interpretation of strengthening results difficult. By confining the amount of area available to cells for spreading, we can maintain uniform cellular geometry across various conditions and time points. Moreover, the micropatterned substrates control the size and position of the adhesive area, an important parameter in interpreting contributions to adhesion strength (Gallant et al., 2005).

We are able to generate adhesive islands for attachment of rounded, single cells using photolithography techniques in conjunction with micro-contact printing of alkanethiol self-assembled monolayers on gold (see Figure 2 and chapter by Ingber; Gallant et al., 2002). Alkanethiols consist of a long-chain carbon backbone with a thiol group at one terminus and an interchangeable group at the other terminus. These molecules self-assemble from solution onto gold surfaces (through strong coordination of S to Au) to form stable, well packed and ordered monolayers presenting the end-group of interest (Ulman et al., 1989; Bain et al., 1989). In our assay, well-define arrays of CH$_3$-
terminated alkanethiol (HS-(CH\(_2\))\(_{11}\)-CH\(_3\)) circles or “islands” are stamped onto a gold-coated glass coverslips using a PDMS stamp (see Chapter by Ingber). The remaining exposed gold is then filled in with a tri-ethylene glycol-terminated alkanethiol (HS-(CH\(_2\))\(_{11}\)-(EG)\(_3\)OH), which resists protein adsorption and cell attachment. The patterned coverslip is coated with human plasma fibronectin (pFN) which adsorbs to the CH\(_3\)-terminated alkanethiol through hydrophobic interactions, blocked with 1% heat denatured BSA to prevent non-specific cellular binding, and incubated in PBS to elute proteins that are weakly bound to the tri-ethylene glycol surface. This process results in patterned areas of adsorbed pFN (Figure 2) in an array of circular islands varying in diameter, currently from 2 to 20 \(\mu\)m, that are spaced 75\(\mu\)m apart to promote single cell attachment to each island. On 2, 5, and 10 \(\mu\)m islands, the island size is much smaller than the diameter of cell allowing cells to remain rounded. On 20 \(\mu\)m islands, the cells form a hemisphere over the island due to the circular pattern of FN. Cells are seeded on these patterned coverslips in complete media and uniformly adhere across the disk surface such that one cell attaches to one island and remains rounded. The uniform, rounded shape of the cell allows for consistent application of force across all cells on the surface and is used to determine the force applied at the cell-ECM bond as shown in Section VI. With an empirically-derived cell density of \(~200\) cells/mm\(^2\), cells uniformly distribute on patterned substrates placed in a tissue-culture dish. Presently, we have patterned several cell types using this technique, including NIH3T3, MC3T3-E1, and C2C12 cell lines and primary fibroblasts.

Measurements of cell adhesion strength at various time points after plating yield a kinetic adhesion profile that shows an exponential rise to maximum (Equation 3) and fits
the solution of a simple first-order differential equation which characterizes the biological strengthening process.

\[
\tau_{50}(t) = \tau_{\infty} \left(1 - e^{-k_s t}\right)
\]  

(Eqn. 3)

This curve fitting generates two parameters: \(\tau_{\infty}\) is equal to the maximum adhesion strength achieved at steady-state and has units of shear stress, and \(k_s\) characterizes the strengthening rate and is given by the time required to reach 67% of the maximum strength (see Figure 3). Using these two parameters, we can determine if the experimental treatment modulates the steady-state adhesion strength or the rate to reach this steady-state strength.

**Figure 3.3.** The adhesion assay yields cell counts from 60 fields subject to a range of applied shear stresses. The adherent cell fraction profile is fit with a sigmoidal curve (left), and the shear stress at which 50% of the cells remain attached is considered the mean adhesion strength for the population of cells. Measurements of adhesion strength over time can be regressed using an exponential rise to maximum curve, to yield the steady-state adhesion strength and the strengthening rate (right).

**Interpretation of Adhesion Strength Results**

Changes in either steady-state strength or strengthening rate provide information on the functional mechanism of a particular component or process in adhesion.
strengthening (Figure 4). For instance, if the localization of a protein were to modulate preferentially the steady-state strength, then this protein must in some way act to enhance the overall mechanical coupling of the ECM to the cell. This enhancement could in turn be due to an increased number of bonds, enhanced position/distribution of bonds, or enhanced coupling of bonds to the cytoskeleton (see modeling section VI). Coupling of FA proteins and integrins has also been shown to modulate the affinity of the integrin/ECM bond. This modulation of affinity is likely to cause adhesion strengthening through an increase in the number of bonds, rather than an increase in the force required to disrupt individual bonds, because the enhanced affinity increases the probability of bond formation rather than the strength of each bond. However, proteins that modulate preferentially the strengthening rate likely do so by enhancing the rate of mechanical coupling. This enhancement could be due to an enhanced recruitment of strengthening molecules to the site of interaction or altered localization of bond positions for a more optimal distribution of load. It should be noted that enhancements of bond strength and formation rate are not mutually exclusive, as some adhesion components may be able to modulate both parameters. As an example of this type of analysis, increasing the number of bound integrins via an increase in FN surface density preferentially modulates steady-state adhesion strength rather than the strengthening rate (Gallant et al., 2005). This preferential modulation suggests that an increased density of FN-integrin bonds increases the overall adhesion strength by increasing the number of bonds rather than increasing the rate of bond formation or optimizing the position of the bonds. How an adhesive component modulates the strength of adhesion may in turn be determined by
investigating changes in the size, position, and composition of adhesive complexes following the activity of that component.

![Figure 3.4](image.png)

**Figure 3.4.** Different responses of adhesion strengthening to molecular activity. Preferential modulation of either steady-state strength or strengthening rate can give insight into the functional mechanism of a particular protein in adhesion strengthening.

**Quantitative Biochemical Methods for Adhesion Analysis**

Within adhesion complexes, we have identified two key factors that contribute significantly to mechanical strength: integrin binding and FA complexes (Gallant *et al.*, 2005). These factors contribute to strengthening through both increasing the number of molecules bound to the FA complex and changing the position and distribution of localized molecules. We have therefore developed biochemical techniques to quantify both of these contributing factors.

**Quantification of Bound Integrin**

We quantify the number of bound integrins using a cross-linking, extraction, and reversal technique (Garcia *et al.*, 1999). This technique employs a membrane-impermeable, homobifunctional cross-linker to couple primary amine groups in the
integrin and ECM ligand. Equivalent results have been obtained for sulfo-BSOCHOES (13 Å spacer arm) and DTSSP (12 Å spacer arm) cross-linkers. Taking advantage of the fact that most ECM proteins are detergent insoluble, the bulk of the cellular components, including unbound receptors, are then extracted using an ionic detergent (SDS). After washing, DTT is used to cleave the disulfide bonds in the remaining cross-linkers thus releasing the bound integrins, which can be collected and quantified by Western blotting (Figure 5).

**Figure 3.5.** Quantification of bound integrins using a cross-linking, extraction, and reversal procedure. (A) This method involves addition of a reversible, cell-impermeable cross-linker to capture integrins bound to matrix proteins. The cellular milieu is then removed with an ionic detergent. The bound fraction is collected after reversal of the cross-linker, and analyzed by Western blotting. (B) Quantification of Western blotting results is achieved using image intensity analysis. Representative data for cells patterned on different island sizes (●) and unpatterned surfaces (○) reveal that integrin binding increases nonlinearly with adhesive area. The relationship is accurately described by a hyperbolic curve (Gallant et al., 2005).
Wet-cleaving Assay for Localized FA Protein Quantification

We have modified the wet-cleaving technique developed by Brands and Feltkamp for quantification of the amount of specific FA proteins, for example vinculin and talin, localized to FA complexes (Figure 6; (Brands et al., 1988; Keselowsky et al., 2005). This wet-cleaving assay mechanically disrupts the cellular membrane to release and remove cytosolic elements that are not associated with adhesion complexes. It involves placing a piece of nitrocellulose membrane on top of cells, allowing non-specific interactions between the nitrocellulose membrane and the dorsal cell membrane. The nitrocellulose membrane is then ripped from the substrate, thereby rupturing the cellular membrane and releasing cytosolic proteins and cellular milieu into a protease inhibiting solution. The released cellular fraction is washed away and the materials that remained on the substrate are collected with a detergent-containing solution for quantification by Western blotting. By controlling the cleaving conditions (overlay time and volume of liquid at the cell/nitrocellulose interface), cells can be ruptured at different planes relative to the underlying surface. With an overlay time of 1 minute, most cells rupture close to the cell/ECM interface.
Figure 3.6. Quantification of adhesive membrane associated FA proteins using a wet-cleaning assay. (A) Using non-specific interactions between nitrocellulose membrane and the dorsal cell membrane, the cell is mechanically disrupted, unbound cytosolic molecules are washed away, and substrate bound molecules are removed by detergent conditions and collected for quantification by Western blotting. (B) Quantification of Western blotting results is completed using image intensity analysis. The representative data for cells patterned on different island sizes (●) revealed that vinculin localization also increases nonlinearly with the adhesive area. The relationship is accurately described by a hyperbolic curve (Gallant et al., 2005). Vinculin localization on unpatterened surfaces (○) is given as a reference.
Immunofluorescence Staining and Quantification

The distribution of bound integrins and localization of FA proteins to the adhesion area is visualized and quantified by immunofluorescence staining. Analysis of such images is particularly useful for quantifying the area occupied by adhesion components in cells on micropatterned substrates. Bound integrins are visualized by modifying the cross-linking and extraction technique such that after washing away the unbound cellular components the remaining surface is blocked and labeled with primary and secondary antibodies, without reversing the cross-linker. FA proteins are visualized using standard immunofluorescence staining techniques, including permeabilization of the membrane, fixation, blocking, and labeling with primary and secondary antibodies. Once the images have been collected using a fluorescence microscope, the amount of adhesion area occupied by FA molecules can be quantified using image analysis software. This technique provides a visual depiction of differences in protein localization in relation to variations in cellular systems. Immunofluorescence staining and biochemical quantification techniques provide complementary information, such as the total amount of adhesive components versus component localization and area of occupation, for mechanistic analysis of adhesion strengthening.

Simple Mathematical Modeling of Adhesion Strengthening Mechanics

Mathematical modeling of adhesion strengthening mechanics can help us interpret our experimental results and expand our understanding of the process through inferring functional mechanisms that may generate these results. Our analysis is based on earlier models which predict the effects of focal contact formation on adhesion strength (Ward et
However, our analysis uses experimentally derived parameters, including adhesion strength, number of bonds, geometries of cell-substrate contacts, and FA areas, to allow direct comparisons between theoretical and experimental results. By modeling the density and distribution of bound integrins (both uncoupled and coupled to the cytoskeleton) within the contact area, we can calculate the overall adhesive forces that resist the applied hydrodynamic forces, in order to analyze systematically the contributions of different adhesive parameters to overall adhesion strength (Gallant et al., 2006).

Resolving Forces for a Cell Under Hydrodynamic Shear

The force applied at the cell-ECM interface can be resolved using a force balance approach, which considers the static equilibrium of a cell attached to a micropatterned substrate under shear flow (Figure 7A). Based on Newton’s Laws, forces applied to a stationary object must be balanced by counter forces applied by that object, such that the sum of the forces and the sum of the moments must equal zero. Therefore, before a cell under shear flow detaches, a mechanical equilibrium exists where the applied hydrodynamic shear force \( F_s \) and torque \( T_s \) are balanced by horizontal (resultant \( F_{\text{tan}} \)) and vertical tensile (resultant \( F_T \)) bond forces and compressive forces (resultant \( F_c \)). Using the solution for \( F_s \) and \( T_s \) for a sphere in shear flow (Goldman et al., 1967), \( F_T \) is determined to be the dominant force resisting the applied hydrodynamic loading (Figure 7A). Assuming a peeling detachment mechanism in which bonds at the edge of attachment resist most of the detachment force relative to the center of the cell-ECM contact, \( F_T \) acts through a point at the periphery of the cell-substrate contact area.
Therefore, the distance from the center of the contact area (C) to the point of application of \( F_T \) is approximately equal to the radius of the contact area. Based on these approximations, \( F_T \) is reduced to the expression in Equation 4 and is directly proportional to the applied wall shear stress \( \tau \), which we use for the experimental measurement of adhesion strength.

\[
F_T = 32 R^2 \tau \sqrt{1 + (0.8 R / a)^2} \quad \text{(Eqn. 4)}
\]

where \( R \) is the radius of the spherical adhesive object and \( a \) is the radius of the contact area.
Figure 3.7. Modeling of adhesive forces. The forces applied at the cell-ECM interface can be resolved using a free body diagram of a rounded cell under shear flow and simple mechanics. (A) Based on mechanical equilibrium, the applied shear stress is directly proportional to the dominant force resisting detachment, $F_T$. (B) and (C), this vertical tensile force can then be subject to a mathematical analysis of adhesion strengthening, to systematically dissect the contributions of different adhesive parameters to overall adhesion strength. As the bond number increases, adhesive patch force increases linearly for uniformly distributed bonds (□) and non-linearly for clustered (Δ) and FA-associated (○) bonds, with FA-association enhancing adhesive force by 30% over clustering. This 30% enhancement has been confirmed experimentally in adhesion strength measurements (Gallant et al., 2006).

Mathematical Analysis of Adhesion Strengthening Mechanics

The macroscopic force balance analysis yields values of the net force at the cell-ECM interface, which can then be integrated with a microscopic model to relate the applied force to the number, position, and state of the bound receptors that provide the forces to resist detachment. Due to the peeling detachment mechanism, the adhesive
force resisting the applied hydrodynamic force, $F_T$, is generated by a small area of the total cell-ECM interface localized at the leading edge of the contact area. In our model, this “adhesive patch” is divided into segments (see Figure 7B), which contain the load-bearing, bound receptors. Three cases of bound state are considered:

1. uniformly distributed bonds – bonds are equally distributed among patch segments;
2. clustered bonds – bonds localized to the outermost segment until a saturation number is reached ($B_{\text{max}}$) and then the next segment is filled;
3. FA-associated bonds – a fraction of clustered bonds ($\chi$) which must fail simultaneously (as one rigid unit).

The force produced by each segment ($F_i$) is calculated using Equation 4.

$$F_i = fB_i\left[\chi + (1 - \chi)\kappa \exp(1 - i)\right]$$

(Eqn. 5)

where $f$ is the individual integrin-ligand bond strength, $B_i$ is the number of bonds in segment $i$, $\kappa$ modulates the exponential dependence of segment loading, and $\chi$ is the fraction of bonds associated with FA elements. The force and moment for all segments are added to calculate $F_T$, the adhesive patch force. Due to the exponential dependence of the adhesive patch force ($\Sigma F_i$) on the number of segments ($i$), the first five segments from the edge bear most of the load and the remaining segments contribute negligible resistant force. Therefore, the adhesive patch is located a distance $d$ from the center of the cell (C) (see Figure 2B).

Using experimentally determined values for the parameters in Equation 4, we have been able to derive quantitative relationships among adhesion strength, integrin binding, and FA size and position, as well as perform parametric simulations of the variables in the model, in order to understand the dependence of adhesion strength on
these parameters (Gallant et al., 2006). For instance, our analysis predicts that the
coupling of FAs to bound integrins provides approximately 30% of the overall adhesion
strength, with integrin binding over other segments providing the remaining balance
(Figure 7C) (Gallant et al., 2005; Gallant et al., 2006). Furthermore, this analysis shows
that increasing the cell-ECM contact area (increasing d) increases adhesion strength
proportionally and that the force required to detach an adhesive patch is consistently 200
nN, independent of the patch size (width of segments 1-5).

Discussion

Incorporating experimental results with mechanical analysis provides unique
insights into the adhesion strengthening process. This work provides a robust framework
for the systematic and rigorous analysis of structure-function relationships in the
regulation of cell adhesion strength. Based on this analysis, we have identified dominant
mechanisms that regulate adhesion strength: (i) number of bound integrins, (ii) position
and distribution of bonds, (iii) individual bond strength, and (iv) coupling of integrins to
cytoskeletal elements. We anticipate that this framework will be useful in elucidating
mechanisms that regulate adhesive interactions in normal and pathological processes.
Using this unique, integrative experimental framework and genetically engineered cells
to control the signaling interactions, we demonstrate in this thesis work that FAK
regulates cell adhesion strengthening via two distinct mechanisms: integrin binding and
vinculin localization.
References


CHAPTER 4*

FAK MODULATES ADHESION STRENGTHENING VIA TWO DISTINCT MECHANISMS: INTEGRIN ACTIVATION AND VINCULIN LOCALIZATION

Abstract

Integrin-mediated cell adhesion strengthening is a complex, bi-directional process involving numerous molecular components. Focal adhesion kinase (FAK) plays a central role in integrin-mediated adhesion, regulating focal adhesion turnover, cell spreading, and cell migration. Here, we show that FAK expression modulates both the kinetic and equilibrium states of adhesion strengthening. During early stages of adhesion strengthening, FAK expression in FAK-null cells significantly increased the adhesive force strengthening rate, bound integrin rate, and integrin activation. In fact, antibody-mediated stabilization of the active integrin conformation enhanced short-term adhesion strength in FAK+ cells but did not modify the strength of FAK- cells. At long-term stages of adhesion strengthening, steady-state adhesion strength and vinculin localization to focal adhesions decreased due to FAK expression. These results indicate that FAK regulation of adhesion strengthening occurs through two distinct mechanisms, integrin activation and vinculin localization, dependent on the stage of adhesion strengthening. Moreover, these findings reveal the first evidence that FAK is involved in integrin activation and show that complex mechanisms lead to the regulation of adhesion strengthening.

Introduction

Cell adhesion to the extracellular matrix (ECM) provides tissue structure and integrity as well as triggers signals that regulate complex biological processes such as cell cycle progression and tissue-specific cell differentiation (Danen et al., 2003; Wehrle-Haller et al., 2003; Walker et al., 2005; Garcia et al., 2005). Hence, cell adhesion is critical to numerous physiological and pathological processes as well as biotechnological applications (Jin et al., 2004; Garcia, 2005; Hubbell, 1999; Wilson et al., 2005). The cell adhesion process involves binding of integrin surface receptors to ECM proteins, clustering of these bound integrins, association with the actin cytoskeleton, and subsequent strengthening of the integrin/actin cytoskeleton interaction via complexes of proteins termed focal adhesions (Hynes, 2002; Zamir et al., 2001; Zimerman et al., 2004; Geiger et al., 2001).

Focal adhesion kinase (FAK) is a widely expressed non-receptor protein tyrosine kinase central to the regulation of focal adhesion assembly that interacts with many other focal adhesion proteins including Src, Cas, and paxillin (Hanks et al., 1992; Schaller et al., 1992; Schaller et al., 1994; Schaller et al., 1999; Vuori et al., 1996). Deletion of the FAK gene in mice results in early embryonic lethality (Furuta et al., 1995). FAK expression is not only critical to development but also cell migration, spreading, proliferation, and survival, and FAK acts as a scaffolding protein with multiple protein-protein interaction sites central to the activation of various signaling pathways (Owen et al., 1999; Sieg et al., 1999; Cary et al., 1996; Richardson et al., 1997; Sieg et al., 2000; Wang et al., 2001; Oktay et al., 1999; Zhao et al., 1998; Frisch et al., 1996; Ilic et al., 1998; Renshaw et al., 1999; Ridley et al., 2003). Moreover, overexpression of FAK has
been shown in benign, preinvasive, and invasive tumors (Gabarra-Niecko et al., 2003; Hecker et al., 2003; Kornberg et al., 2003), and inhibition of FAK reduces experimental metastatic tumor formation (van Nimwegen et al., 2005).

Migration studies and live-cell microscopy analyses have demonstrated that FAK regulates cell migration via modulation of focal adhesion turnover (Ilic et al., 1995; Owen et al., 1999; Wang et al., 2001). Fibroblasts from FAK-null mice exhibit decreased migration and spreading but increased number of focal adhesions (Ilic et al., 1995). In particular, FAK signaling regulates focal adhesion disassembly and is critical to turnover at the cell front, correlating with focal adhesion disassembly rates for controlled expression or inhibition of Src, paxillin, ERK, and MLCK (Webb et al., 2004). Nevertheless, cell migration and spreading are multi-step, dynamic processes that exhibit complex dependencies on adhesion strength and focal adhesion and cytoskeletal dynamics (Palecek et al., 1997; Gupton et al., 2006). These outcome measures therefore do not provide sufficiently direct or sensitive metrics of adhesion to interpret functional mechanisms involved. We have developed a robust approach for studying adhesion strengthening which incorporates a hydrodynamic adhesion strength assay and quantitative biochemical assays to analyze the mechanisms of the strengthening process (Garcia et al., 1998; Gallant et al., 2005). Here, we show a novel role for FAK in the generation and regulation of adhesive forces. FAK regulates cell adhesion strengthening via two distinct mechanisms: (i) modulation of the rate of strengthening via integrin activation and binding and (ii) regulation of steady state levels of adhesion strength by controlling vinculin localization to focal adhesion complexes. This work both identifies functional mechanisms of FAK and provides the first evidence that focal adhesion
signaling regulates the adhesion strengthening process.

Results

Our experimental framework provides sensitive and direct, population-based measurements of adhesion strength, focal adhesion assembly, and integrin binding and allows interpretation into the functional mechanisms of adhesion complex molecules (Garcia et al., 1998; Gallant et al., 2005; Michael et al., 2006). These adhesion strength analyses relate the maximum force that a cell can resist before detachment to the structural composition of adhesion complexes. Failure occurs at the integrin-ECM bond, indicating that the measurement is associated with cell-substrate force interactions rather than cell rupture or substrate detachment. Measurements may be performed over multiple time points to extend our characterization of adhesion strengthening from initial, kinetic adhesion to steady-state adhesion at equilibrium. In this study, we incorporate FAK-null cells engineered for tetracycline-inducible FAK expression with micropatterned fibronectin substrates to elicit functional mechanisms of FAK in regulating cell adhesion strengthening. This cellular model provides tight control over the intracellular environment, allowing clear interpretation of experimental results.

We analyzed cell adhesion strengthening over time to determine whether FAK is involved in the kinetics of generating cellular force. Adhesion strength was measured at discrete time points and the results were fit to the solution of a first-order kinetic equation:

$$\tau(t) = \tau_\infty(1 - e^{-k_s t})$$

This analysis yields two parameters describing the strengthening response: (i) $k_s$ represents the strengthening rate, i.e. the time required to reach 67% of the steady-state strength, and (ii) $\tau_\infty$ is equal to the steady-state adhesion
strength. Expression of FAK in FAK-null cells resulted in significant changes in both the strengthening rate and steady-state adhesion strength (Figure 1). Expression of FAK increased the strengthening rate 2.6 fold over cells without FAK expression (p<0.001). Interestingly, steady-state adhesion strength decreased by 27% from that of cells without FAK expression (p<0.0001). These adhesive responses were observed in two separate clones. These results show that FAK increases the initial strength of cell-ECM interactions; however, in long-term, steady-state cell-ECM interactions, the presence of FAK decreases the adhesion strength. Differences in both strengthening parameters indicate that FAK modulates adhesion strength in two different ways.

Figure 4.1 FAK Modulates Adhesion Strengthening. (A) FAK-/- cells were engineered to express FAK with a tetracycline-off inducible promoter. (B) Analysis of adhesion strengthening over time for FAK+ (●) and FAK-(○) cells revealed that FAK expression changes both the strengthening rate (k_s) and the steady state adhesion strength (τ_∞).
FAK Modulates Strengthening Rate

The enhancement in the strengthening rate indicates an increase in the rate of mechanical coupling of the cell to the ECM. Modulation of the strengthening rate could be due to enhanced recruitment of strengthening molecules to the site of interaction, modulation of the activation state of the integrin thereby increasing the number of bonds, or optimization of bond positions for a more optimal distribution of load. The kinetics of bound $\alpha_5\beta_1$ integrins revealed that the bound integrin rate was 3.1 fold higher in cells with FAK expression (Figure 2B), which correlates with the 2.6 fold strengthening rate increase. However, a kinetic study of focal adhesion area occupied by vinculin revealed no detectable differences in area at 1 hour but a consistent reduction in area occupation over 2 (17%), 4 (21%), and 24 (25%) hours (Figure 2C). To analyze the contributions of integrin binding to adhesion strengthening, we examined the effects of inhibiting integrin binding via blocking antibodies on adhesive force at early time points. Blocking $\alpha_5\beta_1$ binding to fibronectin decreased adhesion strength at 15 min in both FAK expressing (85%) and non-expressing cells (79%) (Figure 3A), indicating that integrin $\alpha_5\beta_1$ is the primary adhesion mechanism in this system. Moreover, the blocking antibody eliminated the differences in adhesion strength between FAK-expressing and FAK-null cells, suggesting that the differences in adhesive force arise from differences in integrin binding. Previous work in our lab suggests that integrin binding to the ECM is the primary strength contributing component (~ 70%) of adhesive complexes (Gallant et al., 2005).

FAK-directed modulation of integrin binding has not been demonstrated previously, and the possible mechanism by which FAK expression modulates integrin binding thereby enhancing strengthening rate was unknown. Surface expression of
integrin $\alpha_5\beta_1$ was equal for both FAK+ and FAK- cells (Figure 3B), indicating that FAK modulates binding of integrins already expressed on the cell membrane. Short-term differences in adhesion strength (15 min) were also observed under serum-free conditions (see supplementary information), indicating that these differences do not arise from secondary, serum-dependent mechanisms such as growth factor signaling. A simple explanation for the differences in integrin binding in the absence and presence of FAK is that FAK modulates integrin activation. Indeed, flow cytometry experiments with a conformation-dependent antibody (9EG7) revealed that $\alpha_5\beta_1$ integrins in suspended cells had a higher frequency of being in an active conformation in FAK+ cells than in FAK- cells (Figure 3C). Moreover, stabilizing this active state using the antibody 9EG7 promoted increased adhesion strength in FAK+ cells whereas no differences in adhesion strength due to antibody binding were detected in FAK- cells. These results indicate that FAK modulates integrin binding by regulating integrin activation state.
Figure 4.2 FAK modulated kinetics of strengthening correlates with the bound integrin rate. Western blots of bound integrins over time (A, representative blots) were quantified by intensity readings. (B) Statistical regression of the data revealed differences in the bound integrin rate \( k_b \) due to FAK expression, but no differences were found in the number of steady-state bound integrins \( b_\infty \). (C) Reduction in vinculin localization to focal adhesion area due to FAK expression was consistent over time (average 21%).
Figure 4.3 FAK modulates short-term adhesion strength through integrin activation.

(A) Blocking α5β1 integrin binding to fibronectin reduced adhesion strength at 15 min for both FAK+ and FAK- cells and eliminated strength differences between FAK- and FAK-cells. (B) Integrin activity in suspended cells were analyzed by flow cytometry. (i) α5β1 surface expression (AB1950) was equal for both FAK+ and FAK- cells. (ii) The amount of activated α5β1 (9EG7) on the surface revealed more active integrins in cells expressing FAK. (B) Stabilizing integrins in the active state (9EG7) revealed enhanced short-term strength in FAK+ cells; however, stabilization of active integrins did not enhance adhesion strength in FAK- cells.
FAK Modulates Steady-state Adhesion Strength

In contrast to the strengthening rate results, FAK reduces steady-state adhesion strength, indicating that FAK decreases the equilibrium mechanical coupling of the ECM to the cell at equilibrium. The steady-state strength reduction could be due to a decrease in the number of integrin/ECM bonds, modified position/distribution of bonds, or reduction in the coupling of bonds to the cytoskeleton (steady state focal adhesion assembly). No differences were found in focal adhesion component expression levels (supplementary information), indicating that the differences in adhesion strengthening were not due to overall expression of structural molecules. Furthermore, integrin binding analyses revealed no differences in the numbers of bound integrin (\(\alpha_5\beta_1\) and \(\alpha_v\beta_3\)) for FAK+ and FAK- cells (Figure 4A) and no gross differences in integrin localization within the adhesive interface were observed. Based on these results, we postulated that the differences in steady-state adhesive force arise from differences in focal adhesion assembly. We previously demonstrate that focal adhesion assembly, independently from integrin binding, contributes significantly to adhesion strength (Gallant et al., 2005; Gallant et al., 2006). Immunofluorescence staining of focal adhesion proteins vinculin and talin at steady-state revealed a reduction (44%) in the focal adhesion area occupied by vinculin due to FAK expression (Figure 4B). However, no differences were detected in focal adhesion area occupied by talin. Focal-adhesion assembly mechanically couples integrins together increasing the strength of interaction because several integrin/ECM bonds break as one bond rather than as individual bonds (Gallant et al., 2006). Our previous work showed that vinculin contributes a significant portion of the focal adhesion-enhanced adhesion strength (Gallant et al., 2005). Taken together, these results
demonstrate that FAK modulates vinculin, but not talin, localization to focal adhesions
and that these differences serve to reduce steady-state adhesion strength of cells
expressing FAK.

Figure 4.4 FAK modulates steady-state adhesion strength through vinculin localization.
(A) Steady-state levels of bound integrin (α5β1 and α5β3) were equal for FAK+ and FAK-
cells. (B) The amount of area in the focal adhesion plane occupied vinculin was reduced
due to FAK expression (44%). But, no statistical differences in the amount of area
occupied by talin were found.
Discussion

Cell-ECM interactions through integrin receptors provide mechanical coupling and adhesion strength (Figure 5). The major force-bearing component of adhesion strength is the integrin/ECM bond (Gallant et al., 2005; Gallant et al., 2006). Clustering of integrin/ECM bonds increases the strength of interaction due to an increase in the number of force bearing members within a small area. Subsequent focal adhesion association further couples clustered integrins to the cytoskeleton creating one force bearing structure which distributes load across numerous bonds. This distribution of load enhances the strength of interaction further. Focal adhesion components can act as structural components, mechanically coupling integrin/ECM bonds, and/or signaling components, which recruit structural components or disassemble mechanical structures (Michael et al., 2006). Here, we show that FAK mediates cell adhesion strengthening over time by two mechanisms. Cells expressing FAK have more active integrin receptors prior to adhesion. This increased frequency of activation increases the rate at which integrin/ECM bonds form thereby enhancing the adhesion strengthening rate. Secondly, cells expressing FAK have less vinculin localized within focal adhesions reducing the mechanical coupling of integrin/ECM bonds. These results indicate that FAK plays a multifaceted role in adhesion complexes and strengthening.
Cell adhesion through integrin receptors can occur by outside-in signaling, receiving signals from the ECM, or inside-out signaling, regulating the activity of integrins (Takagi et al., 2002; Hughes et al., 1998; Hynes, 2002). Inside-out signaling allows cells to rapidly respond to an appropriate stimulus rather than requiring de novo synthesis. This feature of integrin activation is especially important in leukocytes which circulate until activated by the appropriate stimulus, allowing rapid adhesion to the blood vessel wall and repair of a pathological state (Takagi et al., 2002; Hynes, 2002). FAK modulation of integrin binding has not previously been demonstrated. By promoting a higher frequency of integrins in an active state, integrins are more probable to bind ECM.
ligands thereby increasing the adhesion strengthening rate.

FAK-mediated regulation of integrin activity in this system could occur through a few different mechanisms (Figure 5A). FAK binds peptides mimicking β integrin subunits (Schaller et al., 1995), suggesting FAK may bind directly to β₁. And, phosphorylation of Y397 has been shown to release autoinhibition of FAK, exposing protein-protein interaction sites (Schaller et al., 1999). Although studies suggest that integrins must be clustered and tethered to ECM substrates to generate FAK activity (Shi et al., 2003; Kornberg et al., 1992), perhaps minimal levels of FAK activity promote interim binding of FAK to β₁ integrin. This transitory yet direct binding of these molecules could increase the frequency of integrin activation. Also, the cellular system used for this study is clonal, allowing great control over the cellular environment, with inducible expression of FAK. In the absence of FAK (FAK-), the same amount of several adhesion components are available for binding as in FAK+; however, FAK is not present. This modification could suggest that another integrin binding partner is inhibiting the frequency of integrin activity in the absence of FAK. For example, structural studies suggest that talin competes with filamin for β₁ binding sites (Kiema et al., 2006). In talin1-deficient cells, the activation of Src family kinases and FAK are normal but reinforcement of integrin–actin connections at early times due to external force application does not occur (Giannone et al., 2003). In filamin-A deficient cells, reduction in reinforcement of integrin-actin connections also occurs to a lesser extent, indicating that talin plays a more significant role in early integrin-actin connections. Neither talin-/- or filamin-A deficient cells displayed differences in spreading. However, integrin-linked kinase also binds the β₁ integrin cytoplasmic tail and deletion of this gene
leads to differences in cell spreading (Hannigan et al., 1996; Boulter et al., 2006). Any of these molecules or others which compete for β1 integrin binding sites could reduce the frequency of integrin activation when FAK is not present.

The mechanism by which FAK regulates the steady-state levels of vinculin localization remains unknown. Immunoprecipitation of vinexin β, a binding partner of activated vinculin (Chen et al., 2005), with FAK showed no interaction, indicating that vinexin β is not a direct link between FAK and vinculin activation (data not shown). It is possible that effectors responsible for focal adhesion disassembly are involved in steady-state adhesion strength reduction. FAK mediation of vinculin localization and adhesion strength could be due to the same FAK-Src signaling regulation of focal adhesion disassembly previously shown to occur through paxillin, ERK, and MLCK (Webb et al., 2004). However, steady-state results involve the net interactions of focal adhesion assembly, and an increase in the rate of disassembly may not correlate with steady-state adhesion strength reduction. A deeper investigation into the interactions of FAK and vinculin could enhance our understanding of FAK-mediated adhesion strengthening and other mechanosensitive processes.

All together, these results indicate that FAK regulation of adhesion strengthening is a complex process involving two major mechanisms: integrin activation and vinculin localization. It is unclear how these results relate to FAK-medicated focal adhesion turnover and migration; however, these results identify two functional mechanisms that could be involved. This study is innovative because it identifies functional mechanisms of FAK that could be involved in numerous cellular processes and provides the first evidence that focal adhesion signaling regulates the adhesion strengthening process.
**Methods**

**Cells/Reagents**

Mouse embryo fibroblast cells with tetracycline-inducible FAK expression were developed as previously described (Owen *et al.*, 1999). Briefly, FAK-/- cells were stably transfected with tetracycline-off expression system plasmids (pTet-tTAK, Invitrogen/Gibco) encoding full length FAK. Cells were maintained at 37°C and 5% CO₂ in supplemented DMEM (Invitrogen) containing 4,500 mg/ml D-glucose and 584 mg/li L-glutamine (+ 10% FBS, Fisher Scientific + 1 mM sodium pyruvate, Invitrogen + 1 mM nonessential amino acids, Invitrogen + 100 mg/ml streptomycin/penicillin, Invitrogen + 0.25 mg/ml amphotericin B, Invitrogen + 1 mg/ml tetracycline, Calbiochem). FAK expression was typically induced 2 days prior to all experiments to achieve maximal expression levels. All reagents were purchased from Sigma unless otherwise stated. DPBS and DPBS without Ca²⁺ and Mg²⁺ were purchased from Invitrogen/Gibco.

**Adhesion strength assay**

Adhesion strength was measured using our spinning disk experimental system. (Garcia *et al.*, 1998; Garcia *et al.*, 1997) Micropatterned substrates with adherent cells were mounted on the spinning disk device and spun in 2 mM dextrose (DPBS) at room temperature for 5 min at a constant rotational speed. The spinning disk device imparted a detaching shear stress (τ) dependent on radial position (r), such that \( \tau = 0.8r(\rho\mu\omega^3)^{1/2} \). After spinning, cells were fixed in 3.7% formaldehyde, permeabilized in 1% Triton X-100, stained with ethidium homodimer (Molecular Probes), and counted at specific radial positions using a Nikon TE300 equipped with a Ludl motorized stage,
Spot-RT camera, and Image-Pro analysis system. Sixty-one fields (80–100 cells/field before spinning, 10X) were analyzed and cell counts were normalized to the number of cells present at the center of the disk. The fraction of adherent cells (f) was then fit to a sigmoid curve $f = 1/(1 + \exp[b(\tau - \tau_{50})])$, where $\tau_{50}$ is the shear stress for 50% detachment and b is the inflection slope. $\tau_{50}$ characterized the mean adhesion strength for a population of cells because this applied shear stress results in detachment of 50% of the cell population. Investigating cell adhesion strengthening over time resulted in a kinetic adhesion strength profile as described in the text.

**Micropatterning**

Adhesive islands for attachment of rounded, single cells were generated as described previously using photolithography techniques in conjunction with micro-contact printing of alkanethiol self-assembled monolayers on gold as previously described (Gallant et al., 2002). Briefly, well-define arrays of $\text{CH}_3$-terminated alkanethiol (HS-(CH$_2$)$_{11}$-CH$_3$) circles or “islands” were stamped onto a gold-coated glass coverslips using a PDMS stamp (Sylgard 184/186 Elastomer kit). The remaining exposed gold was then filled in with a tri-ethylene glycol-terminated alkanethiol (HS-(CH$_2$)$_{11}$-(EG)$_3$OH, Prochimia), which resists protein adsorption and cell attachment. The patterned coverslip was coated with human plasma fibronectin, blocked with 1% heat denatured BSA to prevent non-specific cellular binding, and incubated in DPBS to elute proteins that are weakly bound to the tri-ethylene glycol surface. This process results in patterned areas of adsorbed fibronectin in an array of circular islands 5 $\mu$m in diameter and spaced 75$\mu$m apart to promote single cell attachment to each island.
Western blotting

Following SDS-PAGE and transfer to nitrocellulose, membranes were blocked in 1% non-fat dry milk + 0.1% Tween 20 + 0.02% NaN₃ (DPBS –Ca²⁺, Mg²⁺) overnight, incubated in primary antibody for 1 hr, washed, incubated in biotin-conjugated secondary antibody (1:10000, Jackson Immunoresearch) for 1 hr, washed, incubated in alkaline phosphatase conjugated tertiary antibody against biotin (1:10,000, Sigma) for 1 hr, washed, and exposed to ECF substrate (Pierce) for visualization. Primary antibodies include: Rb anti-FAK (1:1000, Upstate), Ms IgG anti-vinculin (Upstate), Ms IgG anti-talin (Sigma), Rb anti-alpha5 integrin (1:5000, Chemicon, AB1921). Images were taken on a Fuji Phosphoimager and quantified by intensity readings normalized to background intensity using Adobe Photoshop.

Bound integrin quantification

The number of bound integrins were quantified using a previously described cross-linking, extraction, and reversal technique(Garcia et al., 1999). By employing a membrane-impermeable, homobifunctional cross-linker (DTSSP, 4°C, 30 min, Pierce), primary amine groups in the integrin and ECM ligand were coupled. The reaction was then quenched (50mM Tris, pH 7.6). Taking advantage of the fact that most ECM proteins are detergent insoluble, the bulk of the cellular components (including unbound receptors) were removed via SDS extraction (0.1% SDS, mM PMSF, mM aprotinin, mM leupeptin in complete DPBS, 5 min). After stringent washing, disulfide bonds in the remaining cross-linkers were cleaved (DTT in 0.1% SDS, 37°C, 30 min), thus releasing
the bound integrins which were collected, concentrated, and quantified by Western blotting.

**Immunofluorescence staining**

For visualization of focal adhesions, cells were permeabilized in cystoskeleton stabilizing buffer (0.5% Triton X-100 + 50 mM NaCl + 150 mM sucrose + 3 mM MgCl₂ + 20 µg/ml aprotinin + 1 µg/ml leupeptin + 1 mM PMSF + 50 mM Tris, pH 6) for 10 min, fixed in 3.7% formaldehyde (DPBS) for 5 min, blocked in 5% FBS (DPBS), and incubated with primary antibodies against focal adhesion components followed by alexafluor-labeled secondary antibodies (1:200) or rhodamine-phalloidin (1:50) and counterstained with Hoechst dye (1:10,000, all from Molecular Probes). Primary mouse IgG anti-vinculin (1:75) and mouse IgG anti-talin (1:200) were obtained from Sigma. Images were captured using a Nikon 100X objective and Spot RT Camera/Software. FA area fractions were quantified using calibrated image analysis software (ImagePro 4.5).

**Integrin Activity and Blocking**

Measurement of integrin surface expression (primary antibody AB1950, 10 µg/mL, Chemicon) and activation state through binding of an activation associated epitope (primary antibody 9EG7, 5 µg/mL, BD Pharmingen) were quantified by flow cytometry analysis (BDLSR). Cells were incubated in primary antibodies for 1 hour, washed 3X in DPBS, incubated in Cy2-labeled secondary anti-goat or FITC-labeled secondary anti-rat IgG antibodies (Jackson ImmunoResearch), and washed again.
Isotype controls (rat IgG2aK, 5 µg/mL, Chemicon / goat AB758, 10 µg/ml, Chemicon) and secondary-only antibody controls were also performed.

Blocking and stabilization of integrin activation adhesion strength measurements were achieved using AB1950 (10 µg/mL) and 9EG7 (5 µg/mL), respectively. Cells were counted, incubated (rocking, 37ºC, 15 min) in either experimental antibody or isotype control and plated on FN coated CH₃ substrates. After 15 min, cells were prepared for adhesion strength analysis.

Statistical Analyses

Non-linear regression analysis was performed using SigmaPlot 2001 software. Analysis of variance (ANOVA) statistical analyses were performed using SYSTAT 11 software. Since each data point of adhesion strength is a separate experimental measurement, hypothesis testing comparing non-linear regression parameters of adhesion strength was performed as a t-test of parameters with known variance (Daniel, 1983). Comparisons of integrin binding parameters were completed by collecting profiles of binding from each Western blot membrane and analyzing variance using ANOVA and SYSTAT 11 software.

Acknowledgements

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References


CHAPTER 5

PERSISTENCE TIME CORRELATES WITH ADHESION STRENGTHENING BASED ON FAK TYROSINE ACTIVATION STATE ANALYSIS

Abstract

Cell migration is critical to numerous processes including embryogenesis and wound healing and pathological tumor metastasis. Focal adhesion kinase (FAK) plays a central role in migration by regulating focal adhesion turnover. We have recently shown that FAK modulates cell adhesion strengthening through integrin activation and vinculin localization. Here, we analyze the role of activation sites (Y397, Y576/Y577) within FAK in cell adhesion strengthening and correlate these effects to migration speed and directional persistence. We show that these activation sites are critical to FAK-mediated adhesion strengthening and the mechanisms behind this process. Using the adhesion strengthening parameters derived from our adhesion analysis, we compare the extent of strengthening rate and steady-state adhesion to migration speed and persistence time. This analysis revealed that migration persistence time directly correlates with strengthening rate but inversely correlates with steady-state adhesion strength. In contrast, migration speed does not correlate with either strengthening rate or steady-state adhesion strength. These results indicate that persistence time, and not migration speed, is modulated by FAK-mediated adhesion strengthening.

*To be submitted for journal publication.
Introduction

Cell adhesion to the extracellular matrix (ECM) provides tissue structure and integrity as well as triggers signals that regulate complex biological processes (Danen et al., 2003; Wehrle-Haller et al., 2003; Walker et al., 2005; Garcia et al., 2005). Adhesion to ECM proteins is primarily mediated by integrin receptors. Following activation, integrins bind their target ligands and cluster together. The cytoplasmic domains of bound and clustered integrins form supramolecular complexes with focal adhesion proteins, such as talin, FAK, vinculin, and paxillin, and become associated with the actin cytoskeleton (Zamir et al., 2001; Zimerman et al., 2004; Galbraith et al., 2002; Beningo et al., 2001). Through these initial adhesive complexes, the cell generates myosin-mediated contractile forces resulting in enhanced, more mature focal adhesion complexes and a strengthened cell-ECM interaction (Galbraith et al., 2002). Coordinated regulation of these discrete interactions allows generation of traction forces and cell migration.

Cell migration is a complex, highly regulated process consisting of (i) protrusion, (ii) adhesion at the leading edge, (iii) generation of contractile forces to translate the cell body, and (iv) release/retraction of the cell rear (Lauffenburger et al., 1996; Ridley et al., 2003). Although changes in migration are commonly studied to gain insights into adhesive interactions and focal adhesion function, obtaining a fundamental, mechanistic understanding of how focal adhesion components regulate these processes is difficult due to the spatiotemporal nature of these adhesive interactions. Furthermore, the highly complex dependency of migration on adhesion strength, down to the position of an adhesive complex within a migrating cell, makes the interpretation of these results with regard to adhesion challenging (Palecek et al., 1997; Palecek et al., 1999a; DiMilla et al.,
This lack of quantitative understanding of adhesion strength regulation limits the interpretation of functional studies of structural and signaling adhesive components.

Focal adhesion kinase (FAK) is a widely expressed non-receptor protein tyrosine kinase central to the regulation of focal adhesion assembly. FAK is essential to development, cell migration, spreading, proliferation, and survival (Owen et al., 1999; Sieg et al., 1999; Cary et al., 1996; Richardson et al., 1997; Sieg et al., 2000; Wang et al., 2001; Oktay et al., 1999; Zhao et al., 1998; Frisch et al., 1996; Ilic et al., 1998). Notably, overexpression of FAK has been shown in benign, preinvasive, and invasive tumors (Canel et al., 2006; Gabarra-Niecko et al., 2003; Hecker et al., 2003; Kornberg et al., 2003), and FAK inhibition reduces experimental metastatic tumor formation (van Nimwegen et al., 2005). Deletion of the FAK gene in mice also reduces cell migration (Ilic et al., 1995) and results in embryonic lethality at day 8.5 due to an improperly formed mesoderm (Furuta et al., 1995). Embryonic fibroblasts from FAK-null mice exhibit increased number of focal adhesions, suggesting that FAK plays a significant role in adhesion turnover (Ilic et al., 1995).

FAK contains multiple sites that interact with multiple targets, including Src, Cas, and paxillin (Hanks et al., 1992; Schaller et al., 1992; Schaller et al., 1994; Schaller et al., 1999; Vuori et al., 1996). In particular, Y397, the autophosphorylation site, is a binding site for SH2 domains of Src-family kinases (SFKs) (Schaller et al., 1994; Xing et al., 1994; Polte et al., 1995). SFK binding releases autoinhibition of FAK exposing many protein-protein interactions sites on the molecule (Schaller et al., 1999). Y576/Y577, the catalytic site, is also critical to FAK phosphorylation activity (Calalb et al., 1995; Owen
et al., 1999). Using a model cellular system with inducible FAK (WT) and inactive FAK (F397 and F576/577) expression, we show that both the autophosphorylation site (Y397) and the catalytic site (Y576/577) in FAK are critical to adhesive process. In addition, we demonstrate a correlation between adhesive strength parameters and migration persistence time but not migration speed.

**Results and Discussion**

We have developed an integrated experimental framework which incorporates an adhesion strength assay and quantitative biochemical analyses to uncover functional mechanisms of adhesion components in adhesion strengthening. Using population based assays, we can obtain reproducible measurements of adhesion strength and analyze the mechanisms by which adhesion components contribute strength. Our spinning disk adhesion assay applies a large range of hydrodynamic detachment forces in a single experiment (the applied shear stress $\tau$ (force/area) varies linearly with radia position $r$, $\tau = 0.8r(\rho\mu\omega^3)^{1/2}$, where $\omega$ is the rotational speed, and $\rho$ and $\mu$ are the fluid density and viscosity, respectively). These measurements can also be taken over a large range of time points (min to hr). This analysis yields two parameters (see Figure 1): (i) the strengthening rate $k_s$, the time required to reach 67% of the maximum adhesion strength and (ii) the steady-state adhesion strength $\tau_x$. The strengthening rate characterizes the dynamics of adhesion strengthening whereas the steady-state strength yields information about the cell-ECM adhesive force at equilibrium. Using a biochemical assay to quantify bound integrin receptors, the kinetics of integrin binding can be similarly analyze to yield a binding rate $k_b$ and steady-state bound level $b_x$. 

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Both the autophosphorylation site and the catalytic site are critical to FAK modulation of strengthening rate through changes in integrin binding. WTFAK expression enhanced strengthening rate over that of FAK-null cells through changes in integrin binding (Chapter 4). Mutating tyrosine residues to phenylalanine prevents phosphorylation of the molecule at that particular tyrosine residue, while maintaining the overall molecular structure, thereby inactivating the residue of interest. Expression of a FAK mutant with an inactive autophosphorylation site (F397FAK) in FAK-null cells

**Figure 5.1.** Representative kinetic profiles of adhesion strengthening and integrin binding. Both profiles are fit with an exponential rise to maximum curve revealing two parameters per profile. Adhesion strength initially rises with strengthening rate ($k_s$) and plateaus at steady-state strength ($\tau_s$). Integrin binding also rises with a binding rate ($k_b$) and saturates at total bound integrin ($b_s$).

\[
\tau_s(t) = \tau_s \left(1 - e^{-k_s t}\right)
\]

\[
B(t) = b_s \left(1 - e^{-k_b t}\right)
\]
reduced the adhesion strengthening rate below or to the same the level as FAK-null cells (Figure 2). Two separate F397FAK clones (18 and 21) are shown. Both the strengthening rate and steady-state adhesion strength for F397FAK clone 18 are statistically different from their FAK-null state, similar to differences in adhesion strengthening parameters with WTFAK expression. Although these discrepancies confound our results, the analytic outcomes relating integrin binding, adhesion strengthening, and migration of these clones remain consistent. The integrin binding rate of cells expressing F397FAK clone 18 was reduced to the same level as FAK-null cells (Figure 2). Expression of a FAK mutant with inactivated residues in the catalytic site (F576/577FAK) reduced both the adhesion strengthening rate and integrin binding rate to the same level as FAK-null cells. Taken together, these results demonstrate that mutation of FAK at either the phosphorylation site or catalytic site slows down the adhesion strengthening process to a similar extent as in the absence of FAK. Therefore, these sites are critical to the dynamic function of FAK.
steady-state adhesion strength below that of FAK-null cells and reduced the area

Figure 5.2. FAK autophosphorylation (Y397) and catalytic (Y576/577) sites are critical to modulation of strengthening rate through integrin binding. (A) Strengthening rate increases in cells expressing WTFAK over FAK null. However, strengthening rate is decreased in cells expressing inactive autophosphorylation FAK (F397FAK) below that of the corresponding FAK-null cells and to the same level as FAK-null for cells expressing inactive catalytic FAK (F576/577FAK). (B) Integrin binding rates in cells expressing both inactive autophosphorylation and catalytic sites were also decreased to the same level as FAK-null cells although the rate is increased in cells expressing WTFAK.

The autophosphorylation and catalytic sites are also critical to FAK modulation of steady-state strength and changes in vinculin localization. WTFAK expression decreased steady-state adhesion strength below that of FAK-null cells and reduced the area
occupied by vinculin in the focal adhesion plane. Expression of F397FAK and F576/577FAK eliminated the decreases in steady-state strength and vinculin localization area (Figure 3). Mutation of either the phosphorylation site or the catalytic site brought the steady state adhesion strength back to the level of FAK-null cells and prevented the reduction in size of vinculin-containing focal adhesions. In all, these results indicate that FAK autophosphorylation and catalytic activity are critical to FAK-modulated adhesion strength regulation and the mechanisms by which this regulation occurs.
Figure 5.3. FAK autophosphorylation (Y397) and catalytic (Y576/577) sites are critical to modulation of steady-state strength through vinculin localization. (A) Steady-state strength decreases in cells expressing WTFAK over FAK null. However, strengthening rate in cells expressing inactive autophosphorylation FAK (F397FAK) and inactive catalytic FAK (F576/577FAK) were equal to their FAK-null counterpart. (B) Total bound integrin at steady-state were not different in all cell types. (C) Area of vinculin localization in cells expressing both inactive sites were also the same level as FAK-null cells although the area is decreased in cells expressing WTFAK.
Previous studies have analyzed the migratory behavior of FAK-null cells versus cells expressing WTFAK, F397FAK, and F576/577FAK (Table 1) (Owen et al., 1999; Wang et al., 2001). Using Boyden chamber assays, Hanks and colleagues demonstrated that expression of WTFAK in FAK-null cells increases migration 50-fold over cells without FAK (Owen et al., 1999). Expression of F397FAK reduced migration 40-fold below that of FAK-null cells. Moreover, F576/577FAK expression resulted in the similar numbers of migratory cells as FAK-null cells. These results demonstrate that FAK is critical to migration and underscore the importance of both the autophosphorylation and the catalytic sites in this process. Using single cell tracking techniques, Wang and colleagues also showed that the migratory defects of FAK-null and F397FAK-expressing cells compared to WTFAK cells were not only manifested in the speed of migration but also directional persistence, as FAK-null and F397FAK cells tend to migrate in random directions rather than maintain a steady path (Wang et al., 2001). We are currently analyzing speed and persistence of these cells on fibronectin coated CH₃-substrates (Au – (S-(CH₂)₁₁-CH₃)) for direct comparative analysis with our adhesion strengthening results.

<table>
<thead>
<tr>
<th></th>
<th>Migration normalized by null</th>
<th>Speed normalized by null</th>
<th>Persistence normalized by null</th>
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<tbody>
<tr>
<td><strong>WTFAK</strong></td>
<td>1.5 fold ↑</td>
<td>2.3 fold ↑</td>
<td>1.5 fold ↑</td>
</tr>
<tr>
<td><strong>F397FAK</strong></td>
<td>40%↓</td>
<td>1.6 fold ↑</td>
<td>23%↓</td>
</tr>
<tr>
<td><strong>F576/577FAK</strong></td>
<td>no difference</td>
<td></td>
<td></td>
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</table>

**Table 5.1.** Collection of published migration results for tet-FAK cells. (A) Hanks and colleagues calculated overall migration of these cells using Boyden chamber analyses (Owen et al., 1999). (B) Speed and persistence measurements were calculated by Wang and colleagues using random walk analysis (Wang et al., 2001). Although total migration and persistence time results correlate with our strengthening rate parameters, migration speed exhibited a more complex dependency on adhesion strengthening.
Comparison of results from integrin binding, adhesion strengthening and migration studies reveals a complex relationship between these processes. We previously found that FAK regulates adhesion strengthening rate through integrin activation (Chapter 4). In fact, plotting strengthening rate versus integrin binding rate yields a strong relationship for all three cell types (Figure 4). Comparing published migration data with our adhesion strengthening rate results revealed that both the percentage of migrating cells and the persistence time were related to the adhesion strengthening rate; however, the speed of migration is not related to strengthening rate (Figure 5). Also, both the percentage of migrating cells and the persistence time related inversely with the steady-state adhesion strength; however, the speed of migration is not related to the steady-state adhesion strength (Figure 6). This analysis suggests that FAK-mediated adhesion strengthening plays a role in how long a cell persists in one direction but does not directly regulate migration speed. This results contradicts previous analyses (Palecek et al., 1999b). In addition, both strengthening parameters relate to migratory behavior. Increasing the adhesion strengthening rate enhances the interactions between the cell and the substrate thereby enhancing persistence time. However, increasing the steady-state strength decreases the persistence time suggesting that the enhanced strength in adhesions at equilibrium counteract the ability of the cell to persist along a set direction. Taken together, this information suggests that the turnover of focal adhesions is critical to the directional persistence of the cell, but an unknown mechanism may be driving changes in migration speed, e.g. differences in force generation due to actin/myosin contractility or cellular detachment due to enzymatic activity.
Figure 5.4. Strengthening rate correlates linearly with integrin binding rate ($r^2 = 0.86$), indicating that integrin binding leads to strengthening. Experimental results for WTFAK, F397FAK, and F576/577 cells were normalized by their FAK-null counterparts and these parameters were fit with a linear regression.
Figure 5.5. Migration speed and persistence time exhibit complex relationships with adhesion strengthening rate. Although the number of migrating cells with respect to their null state (A) correlates with strengthening rate, (B) migration speed does not correlate with strengthening rate. (C) Persistence time does correlate with strengthening rate such that a decreased strengthening rate results in a decreased persistence time (F397FAK clone 21) and an increased strengthening rate results in an increased persistence time. Migration results shown here have been adapted from Hanks (Owen et al., 1999) and Wang (Wang et al., 2001) using the same cell lines. We are currently analyzing our own migration data to corroborate these findings.
Figure 5.6. Migration speed and persistence time also exhibit complex relationships with steady-state adhesion strength. (A) The number of migrating cells with respect to their null state inversely correlated with steady-state adhesion strength. (B) Migration speed does not correlate with steady-state adhesion strength. (C) Persistence time does inversely correlate with steady-state adhesion strength. Migration results shown here have been adapted from Hanks (Owen et al., 1999) and Wang (Wang et al., 2001) using the same cell lines. We are currently analyzing our own migration data to corroborate these findings.
Methods

Cells/Reagents

Mouse embryo fibroblast cells with tetracycline-inducible FAK expression were developed as previously described (Owen et al., 1999). Briefly, FAK-/- cells were stably transfected with tetracycline-off expression system plasmids (pTet-tTAk, Invitrogen/Gibco) encoding full length WTFAK. Cells were maintained at 37°C and 5% CO₂ in supplemented DMEM (Invitrogen) containing 4,500 mg/ml D-glucose and 584 mg/li L-glutamine (+ 10% FBS, Fisher Scientific + 1 mM sodium pyruvate, Invitrogen + 1 mM nonessential amino acids, Invitrogen + 100 mg/ml streptomycin/ penicillin, Invitrogen + 0.25 mg/ml amphotericin B, Invitrogen + 1 mg/ml tetracycline, Calbiochem). FAK expression was typically induced 2 days prior to all experiments to achieve maximal expression levels. All reagents were purchased from Sigma unless otherwise stated. DPBS and DPBS without Ca²⁺ and Mg²⁺ were purchased from Invitrogen/Gibco.

Adhesion strength assay

Adhesion strength was measured using our spinning disk experimental system.(Garcia et al., 1998; Garcia et al., 1997) Micropatterned substrates with adherent cells were mounted on the spinning disk device and spun in 2 mM dextrose (DPBS) at room temperature for 5 min at a constant rotational speed. After spinning, cells were fixed in 3.7% formaldehyde, permeabilized in 1% Triton X-100, stained with ethidium homodimer (Molecular Probes), and counted at specific radial positions using a Nikon TE300 equipped with a Ludl motorized stage, Spot-RT camera, and Image-Pro analysis system. Sixty-one fields (80–100 cells/field before spinning, 10X) were analyzed and
cell counts were normalized to the number of cells present at the center of the disk. The fraction of adherent cells (f) was then fit to a sigmoid curve \( f = 1/(1 + \exp[b(\tau - \tau_{50})]) \), where \( \tau_{50} \) is the shear stress for 50% detachment and b is the inflection slope. \( \tau_{50} \) characterizes the mean adhesion strength for a population of cells because this applied shear stress results in detachment of 50% of the cell population. Investigating cell adhesion strengthening over time results in a kinetic adhesion strength profile as described in the text.

**Micropatterning**

Adhesive islands for attachment of round, single cells are generated as described previously using photolithography techniques in conjunction with micro-contact printing of alkanethiol self-assembled monolayers on gold as previously described (Gallant et al., 2002). Briefly, well-defined arrays of CH\(_3\)-terminated alkanethiol (HS-(CH\(_2\))\(_{11}\)-CH\(_3\)) circles or “islands” are stamped onto a gold-coated glass coverslips using a PDMS stamp (Sylgard 184/186 Elastomer kit). The remaining exposed gold is then filled in with a triethylene glycol-terminated alkanethiol (HS-(CH\(_2\))\(_{11}\)-(EG)\(_3\)OH, Prochimia), which resists protein adsorption and cell attachment. The patterned coverslip is coated with human plasma fibronectin (pFN), blocked with 1% heat denatured BSA to prevent non-specific cellular binding, and incubated in DPBS to elute proteins that are weakly bound to the triethylene glycol surface. This process results in patterned areas of adsorbed pFN in an array of circular islands 5 \( \mu \)m in diameter and spaced 75\( \mu \)m apart to promote single cell attachment to each island.
Bound Integrin Quantification

The number of bound integrins were quantified using a previously described cross-linking, extraction, and reversal technique (Garcia et al., 1999; Keselowsky et al., 2005). By employing a membrane-impermeable, homobifunctional cross-linker (DTSSP, 4°C, 30 min, Pierce), primary amine groups in the integrin and ECM ligand were coupled. The reaction was then quenched (50mM Tris, pH 7.6). Taking advantage of the fact that most ECM proteins are detergent insoluble, the bulk of the cellular components (including unbound receptors) were removed via SDS extraction (0.1% SDS, mM PMSF, mM aprotinin, mM leupeptin in complete DPBS, 5 min). After stringent washing, disulfide bonds in the remaining cross-linkers were cleaved (DTT in 0.1% SDS, 37°C, 30 min), thus releasing the bound integrins which were collected, concentrated, and quantified by Western blotting using a primary anti-α5 integrin antibody (AB1921, Chemicon) and biotin-conjugated secondary antibody (Jackson Immunoresearch), and an anti-biotin alkaline phosphatase-conjugated tertiary antibody (Sigma).

Immunofluorescence Staining

For visualization of focal adhesions, cells were permeabilized in cytoskeleton stabilizing buffer (0.5% Triton X-100 + 50 mM NaCl + 150 mM sucrose + 3 mM MgCl₂ + 20 μg/ml aprotinin + 1 μg/ml leupeptin + 1 mM PMSF + 50 mM Tris, pH 6) for 10 min, fixed in 3.7% formaldehyde (DPBS) for 5 min, blocked in 5% FBS (DPBS), and incubated with primary antibodies against focal adhesion components followed by alexafluor-labeled secondary antibodies (1:200) or rhodamine-phalloidin (1:50) and counterstained with Hoechst dye (1:10,000, all from Molecular Probes). Primary mouse
IgG anti-vinculin (1:75) and mouse IgG anti-talin (1:200) were obtained from Sigma. Images were captured using a Nikon 100X objective and Spot RT Camera/Software. FA area fractions were quantified using calibrated image analysis software (ImagePro 4.5).

Statistical Analyses

Non-linear regression analysis was performed using SigmaPlot 2001 software. Hypothesis testing of regression parameter for adhesion strength and integrin binding were analyzed as a t-test of parameters with known variance (Daniel, 1983).

Acknowledgements

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References


The objective of this project was to investigate the role of the protein tyrosine kinase FAK in cell adhesion strengthening. Our central hypothesis was that FAK regulates adhesion strengthening by modulating interactions between integrins and FA structural components. We formulated this hypothesis based on preliminary findings which indicated that FAK expression modulates steady-state adhesion strength and leads to preferential differences in steady-state focal adhesion structure. Using a novel combination of genetically engineered cells to control the interactions of FAK, a spinning disk adhesion assay with micropatterned substrates to obtain reproducible and sensitive measurements of adhesion strength, and quantitative biochemical assays for analyzing changes in adhesive complexes, we demonstrate that FAK modulates adhesion strengthening via two distinct mechanisms: (1) FAK expression results in elevated integrin activation leading to regulation of strengthening rate and (2) FAK regulates steady state adhesion strength via vinculin recruitment to focal adhesions. We also show that the autophosphorylation and catalytic sites of FAK are critical to this regulation of adhesion strengthening. This work is innovative because it both identifies functional mechanisms of FAK and provides the first evidence that focal adhesion signaling regulates the adhesion strengthening process. Furthermore, this research demonstrates that the dependency of migration on adhesion strength is highly complex and establishes a need for adhesion strengthening metrics in analyzing the functional mechanisms of molecules within adhesion complexes. Both the experimental framework and the
analytical methods (Chapter 2) used in this work also establish a means for future research endeavors investigating the functional mechanisms of adhesive complex components in cell-ECM and cell-cell adhesion.

The research presented here could be extended further to enhance our understanding of FAK-dependent cellular processes. The cellular system used in these studies compares FAK expression with no FAK expression in clonal cell lines. The clonal expression system provides control over the cellular environment allowing dissection of functional outcomes. However, the applicability of these results to the clinical observation that FAK is over-expressed in many tumors (Gabarra-Niecko et al., 2003; Hecker et al., 2003; Kornberg et al., 2003) could be enhanced by over-expressing FAK in a primary cell source and comparing functional measures such as adhesion strength. Controlling the expression level of FAK by time or tetracycline concentration in our inducible cells could also indicate whether the functional outcomes revealed in this study are dependent on the concentration of FAK in the cytosol. In addition, our FAK-dependent integrin activation result has more than one possible mechanism of action. By immunoprecipitating $\alpha_5\beta_1$ integrin, a potential integrin binding partner that may prevent integrin activation could be discovered. Alternatively, protein-protein interaction studies, such as atomic force microscopy of FAK-bound integrin activity, could reveal that direct binding of FAK to integrins enhances the activation state of integrins. By spanning molecular, cellular, and tissue level studies, a more clear understanding of how FAK function manifests in cellular action can help us understand normal physiological processes and pathological conditions.
While pursuing this thesis research, many different avenues beyond what has been presented so far in this thesis were also explored. Some of these ventures could be investigated further in order to extend our understanding of adhesion strengthening and FAK. FAK-null cells exhibit an increase in the size of focal adhesions and a decrease in the number of focal adhesions compared to WTFAK cells, leading to an increase in the adhesion strength as shown in this work. Expressing a F397FAK/Src chimera in FAK-null cells also enhances both the size of steady-state focal adhesions, based on vinculin, paxillin, FAK immunostaining, and steady-state adhesion strength over comparable WTFAK cells (Figure 1) (Siesser et al., 2006). This chimera promotes Src signaling through localized FAK and bypasses other FAK signaling events. Although this correlation suggests that FAK/Src signaling may promote focal adhesion assembly, Horwitz and colleagues have shown that FAK and Src contribute to focal adhesion turnover by enhancing focal adhesion disassembly rate (Webb et al., 2004). These results suggest that FAK regulation of focal adhesion assembly involves a complex net interaction of FAK/Src signaling. Identification of the mechanisms involved in this FAK-dependent regulation of focal adhesion assembly by investigating other FAK binding partners or the force-dependency of molecular interactions of FAK could further enhance our understanding of adhesion strengthening and migration processes.
Figure 6.1. Adhesion strength at 24 hours was determined for cells expressing WTFAK versus cells expressing a FAK/Src/F397 chimera. Expression of this chimera bypasses all FAK signaling pathways other than Src. Adhesion strength increased by 2-fold for FAK/Src/F397 compared to WTFAK (*p = 0.004) (Siesser et al., 2006).

Another FAK binding partner may enhance focal adhesion assembly such that competitive binding of this molecule and Src leads to the regulation of focal adhesion assembly. Once suitable binding partners have been identified, siRNA could be incorporated with the FAK+ and FAK- cells to reduce the expression of these proteins. Adhesion strength measurements and immunofluorescence staining of focal adhesions could then help identify how these proteins contribute functionally to adhesion strengthening. Overexpression of these proteins or dominant negative forms of these proteins in FAK+ and FAK- cells could yield similar results. Also, controlled expression levels of FAK and its binding partner through two separate inducible systems and measuring focal adhesion assembly and disassembly rates with GFP-coupled focal adhesion proteins could give regulation information. However, setting up this system with so many different expression systems could be challenging.
FAK activity could also be force-dependent. Notably, FAK phosphorylation is induced by mechanical loading (Boutahar 2004). FAK phosphorylation leads to activation of extracellular signal-related kinase (ERK) and myosin-light chain kinase (MLCK). MLCK activity leads to an increase in contractility and has been shown to modulate focal adhesion disassembly (Webb et al., 2002). These generated forces could act as a feedback mechanism regulating focal adhesion assembly through FAK activity/binding thereby modulating adhesion strengthening. Our lab has shown that inhibiting Rho-kinase reduces phosphorylation of myosin light chain kinase and reduces serum-induced steady-state adhesion strengthening in fibroblasts (Shin et al., 2006). Using cells with FAK-inducible expression, Rho-kinase inhibition in FAK+ cells also reduced serum-induced adhesion strengthening as well as decreased focal adhesion assembly (Figure 2). However, in the absence of FAK, this inhibition did not alter adhesion strength or focal adhesion assembly. These results indicate that actin-myosin contractility modulates adhesion strengthening via FAK-dependent focal adhesion assembly. The mechanisms driving this process are still unclear.
Figure 6.2. Reduction of serum-induced adhesion strengthening due to Rho-kinase inhibition is FAK-dependent (Shin et al., 2006). (A) Rho inhibition reduces serum-induced steady-state adhesion strength. (B) This reduction occurs in cells expressing FAK, but it does not occur in FAK- cells. (C) and (D) Inhibition of MLCK phosphorylation due to Rho inhibition is FAK also dependent. Reducing MLCK phosphorylation by Rho inhibition to the same level in FAK+ and FAK- cells does not result in reduced adhesion for FAK- cells. These results suggest FAK is a downstream effector of MLCK in adhesion strengthening.

Delving deeper into the molecular mechanics and protein-protein interactions of FAK and mechanotransduction through FAK may increase our understanding of cellular force-responsive elements and help identify the complex force-signaling interactions
involved in mechanosensitive cellular processes such as gene expression. Vogel and colleagues suggest that there may be hidden sites within proteins (e.g. fibronectin) that become exposed due to force application (Vogel et al., 2001). Perhaps force-dependent states of FAK could be found by applying tensile force to individual FAK molecules using atomic force microscopy (AFM) or similar techniques. Each state would have a corresponding deflection plateau. Although this study would be completed in an acellular environment and researchers should keep in mind the magnitude of force generated by a cell, this study could yield a potential force-sensitive mechanism of focal adhesion assembly. Combining this technique in protein lysate/enriched fluid with immunoprecipitation and mass spectrometry could also identify force-dependent binding partners of FAK. Forces have also been applied to distinct positions on cells using AFM or laser tweezers while images of fluorescently tagged proteins are captured to analyze differences in focal adhesion assembly due to applied force and understand mechanotransduction, transduction of mechanical signals into cellular responses (Galbraith et al., 2002; Giannone et al., 2003; Micoulet et al., 2005; Chen et al., 2000). These studies could provide evidence for FAK-dependent focal adhesion assembly due to force application. However, they would be limited based on the position of attachment to the cell since most cells have polarity. Population-based mechanotransduction studies, often assayed as mechanical loading of whole tissue or monolayer with fluid shear stress or deformable substrates, would yield better statistical significance of FAK-dependent force based processes. Mechanotransduction studies could also be completed using our spinning disk device since the device imparts a large range of hydrodynamic shear stress while maintaining supply of nutrients to the cells (Garcia et al., 1997). Furthermore,
micropatterning of cells provides application of known, controlled forces on the cells and separates geometric/shape considerations from force application. Using this device and microscopy techniques, focal adhesion assembly in FAK+ or FAK- cells at different radial positions could implicate FAK in force-induced focal adhesion assembly.

Focal adhesion assembly and adhesion strengthening are processes central to many physiological and pathological processes. Furthermore, many biological processes are mechanosensitive. FAK is a key focal adhesion protein involved in numerous cellular processes including focal adhesion assembly, cell adhesion strengthening, and mechanotransduction of signals. Here, we establish that FAK modulates adhesion strengthening through two different mechanisms and that the activation state of FAK is critical to both of these mechanisms. We also show that the dependency of migration on adhesion strength is highly complex. It is obvious that the molecular mechanisms involved in FAK regulation of cellular function are also highly complex. Further studies into these regulatory mechanisms could enhance our understanding of force-related cellular functions and the normal and abnormal functions of load-bearing tissues.

References


**Figure A.1** Overall expression of adhesion components in WTFAK+ and WTFAK- cells were equal.
Figure A.2 Short-term adhesion strength differences for FAK+ and FAK- were not dependent on serum.
APPENDIX B

Published work of Kristin E. Michael


Adsorption-Induced Conformational Changes in Fibronectin Due to Interactions with Well-Defined Surface Chemistries

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Protein adsorption onto synthetic materials influences cell adhesion and signaling events that direct cell function in numerous biomedical applications. Adsorption of fibronectin (FN) to different surfaces alters protein structure and modulates integrin binding, cell adhesion, cell spreading, and cell migration. In the present study, self-assembled monolayers of alkanethiols on Au were used to analyze the effects of surface chemistry (CH3, OH, NH2, and COOH) on the adsorption of a recombinant fragment of FN, FNIII7-10, that incorporates both the synergy and RGD cell binding motifs. Surface chemistry potentiated differential FNIII7-10 adsorption kinetics and adsorbed structure as determined by surface plasmon resonance spectroscopy and antibody binding assays. FNIII7-10 functional activity, determined by cell adhesion strength, was modulated in a fashion consistent with these structural changes (OH > NH2 > COOH > CH3). However, these changes in protein parameters did not correlate simply to differences in surface hydrophobicity, indicating that additional surface parameters influence protein adsorption. These results demonstrate that surface chemistry modulates adsorbed protein structure and activity and establish a relationship between surface-dependent changes in structural domains of FNIII7-10 and functional activity.

Introduction

Protein adsorption plays a critical role in numerous biomedical and biotechnological applications. Adsorption of proteins onto synthetic surfaces is a thermodynamically driven process.1 Due to the diverse circumstances in which proteins and surfaces come in contact, an understanding of protein adsorption is fundamental to fields as varied as bioseparation, development of biosensors, food processing, and implant technology.1,2 In addition to activating blood clotting and inflammatory responses, adsorbed proteins mediate cell adhesion to synthetic surfaces. Cell adhesion to adsorbed proteins is particularly important in cell function, host responses to implants, and design of tissue engineering substrates.3-5

Protein adsorption is a complex, dynamic process involving noncovalent interactions, including hydrophobic interactions, electrostatic forces, hydrogen bonding, and van der Waals forces.6 Protein parameters including primary structure, size, and structural stability as well as surface properties such as surface energy, roughness, and chemistry have been identified as key factors influencing the adsorption process.6-9 In particular, surface chemistry influences adsorbed protein type, quantity, and conformation.10-12 For example, adsorption of the extra-cellular matrix protein fibronectin (FN) on different surfaces alters protein structure and modulates cell adhesion, spreading, and migration.13-16 Although these adsorption studies provide insights into the relationship...
between surface properties and protein adsorption, many of these experimental systems lack surface homogeneity or have indeterminate surface properties. Recent studies have focused on using model surfaces, such as self-assembled monolayers (SAMs) of alkanethiols on Au, that allow the systematic investigation of the effects of surface chemistry on protein adsorption without altering other surface properties.10,17–20

Current models for protein adsorption indicate that the adsorption process induces a partial unfolding of protein as determined by Fourier transform infrared (FTIR) spectroscopy, NMR, atomic force microscopy (AFM), and total internal reflectance fluorescence (TIRF) spectroscopy.21–28 For example, Raghavachari et al. recently observed structural rearrangements within the repeat units of von Willebrand factor multimers upon adsorption to hydrophobic supports.29 Similarly, Wertz and Santore demonstrated a change in protein footprint area during adsorption to hydrophobic supports.28 Similarly, Wertz and Santore observed structural rearrangements within the repeat

of these experimental systems lack surface homogeneity between surface properties and protein adsorption, many

Surface Preparation and Characterization. SAM surfaces were prepared and characterized as previously described.29 Alkanethiols 1-dodecanethiol (HS–(CH2)11–CH3), 11-mercaptoundecanethiol (HS–(CH2)11–OH), and 11-mercaptoundecanethiol acid (HS–(CH2)10–COOH) were purchased from Aldrich Chemical (Milwaukee, WI) and used as received. The amine-terminated alkanethiol 12-mercapto-1-mercaptodecanodecane (HS–(CH2)12–N2H) was synthesized and characterized by our group.29 The assembled SAMs of their respective alkanethiols are referred to hereafter as CH3OH, COOH, and CH3NH2.

SAMs were assembled on Au-coated glass coverslides (16-well Lab-Tek Chamber Slides, Nalge Nunc International, Naperville, IL) for equilibrium conformation and cell adhesion studies. Au-coated glass coverslides (9 mm square, Belco Glass, Perthville, IL) for equilibrium conformation and cell adhesion experiments. Glass chamber slides were cleaned with 70% H2SO4 and 30% H2O2 at 90 °C for 30 min, washed with DPBS, PBS without Ca2+, and dried under N2. The amine-terminated alkanethiol 12-mercapto-1-mercaptodecanodecane (HS–(CH2)12–N2H) was synthesized and characterized by our group.

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Freshly prepared Au surfaces were immersed in alkanethiol solutions (1 mM in absolute ethanol), and SAMs were allowed to assemble overnight (16 h). SAMs were rinsed in 95% ethanol, dried under N22, and allowed to equilibrate in DPBS for 15 min prior to incubation in FNIII1–10 solutions. Surfaces were validated by contact angle measurements. Ambient air–water substrate contact angle measurements (5 μL of deionized H2O) were taken with a Boe-Haft model no. 100-00 goniometer (Mountain Lakes, N.J.) fitted with a digital camera and analyzed using in-house image analysis software.

Materials and Methods

Reagents. LB agar, LB broth, ampicillin, and IPTG used for bacteria culture and protein production were obtained from Invitrogen (Carlsbad, CA). Chemical reagents, CellYtic B-Clear II, and DNAse I used for bacterial lysis and FNIII1–10 purification were obtained from Sigma Chemical (St. Louis, MO). Hitrap Q Sepharose Fast Flow anion exchange chromatography columns were obtained from Amersham Pharmacia (Piscataway, N.J.). Centrifugal concentration devices were purchased from Gelman Laboratory (Ann Arbor, MI), and Slide-A-Lyzer dialysis cassettes used in protein purification were purchased from Pierce Chemical Co. (Rockford, IL). Bolton-Hunter Reagent for FN III–10 iodination was purchased from NEN Life Science Products (Boston, MA), and Calcein-AM used in cell adhesion detection was acquired from Molecular Probes (Eugene, OR). Cell culture reagents, DPBS, PBS without Ca2+ or Mg2+, and human plasma fibrinectin (pFN) were purchased from Invitrogen. Newborn calf serum was obtained from Hyclone (Logan, UT).

Antibodies and Cells. Several antibodies (Ab′s) were used as structural probes for adsorbed FN in enzyme-linked immuno-sorbent assays (ELISA). Primary monoclonal Ab HFN7.1 (Developmental Hybridoma, Inc., Iowa City, IA) directed against the flexible linker between the 9th and 10th type III repeat, FN11-11 directed against the 9th type III repeat, and mAb1937 (Chemicon, Temecula, CA) directed against the 8th type III repeat were used.30,45,46 An alkaline phosphatase-conjugated donkey anti-mouse IgG Ab (Jackson Immunoresearch, West Grove, PA) was used as a secondary Ab against all three primary antibodies. Murine NIH3T3 fibroblasts (CRL-1658, ATCC, Manassas, VA) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% newborn calf serum and 1% penicillin/streptomycin.

SEM and XPS. The assembled SAM surfaces were imaged with a Hitachi S-5000 scanning electron microscope (SEM) operated at 15 kV. X-ray photoelectron spectroscopy (XPS) was performed with a PHI Quantera spectrometer using Al Kα radiation (1486.6 eV). Survey spectra were collected in the 1000–50 eV range at 0.25 eV pass energy. High resolution spectra were collected in the 280–210 eV range at a pass energy of 10 eV. The maximum takeoff angle was 90° relative to the surface normal.
Recombinant Fibronectin Fragment. FNIII10 was produced and purified as described previously.31 Escherichia coli transformed with cDNA coding for human FNIII10 and ampicillin resistance were streaked onto an LB agar plate containing 100 μg/mL ampicillin and incubated overnight at 37 °C. A single colony was isolated and expanded in 5 mL of LB broth with 50 μg/mL ampicillin at 37 °C. The bacterial culture was then centrifuged at 10 500 g for 5 min and the supernatant discarded. The bacterial pellet was resuspended in 5 mL of LB broth containing 50 μg/mL ampicillin and incubated overnight at 37 °C. The bacterial culture was then centrifuged at 12 000 g for 15 min and the supernatant discarded. The bacterial pellet was resuspended in 10 mL of 0.1 M CAPS buffer (150 mM NaCl, pH 11.0) and flash frozen for storage at -80 °C. Cell pellets were collected at 25 000 g for 10 min and frozen at -80 °C. Upon thawing, bacteria were lysed and cytosolic proteins were exposed by addition of Celllytic B-Clear II (5 mL/g) and DNAse I (5 μg/mL) for 30 min. The lysate was centrifuged at 25 000 g for 15 min. Proteins were precipitated by centrifugation in 40% ammonium sulfate for 25 min at 25 000 g. The pellet was resuspended in 15 mL of start buffer (0.2 M Tris, pH 8.0) and purified by anion exchange chromatography using a BioRad Econo Gradient Pump, UV monitor, and fraction collector (Hercules, CA) with 5 mL HiTrap Q columns. Proteins were bound to the columns, washed with 5 column volumes of start buffer, and eluted via salt gradient (0.2–0.4 M NaCl in 0.5 M Tris, pH 7.7) by stepping up the concentration every 2 column volumes. The desired protein product eluted at approximately 0.27 M NaCl and was verified to be >95% pure FNIII10 by SADS-PAGE. Relevant fractions were concentrated using Microsep 10K Omega centrifugal devices, dialyzed overnight against caps buffer (10 mM CAPS, 150 mM NaCl, pH 11.0), and flash frozen for storage at -80 °C.

Surface Plasmon Resonance Spectroscopy. Protein adsorption kinetics was quantified via surface plasmon resonance (SPR) spectroscopy using a Biacore X instrument (Biacore, Inc., Piscataway, N J). SAMs assembled on Au-coated 9 mm square coverslips were rinsed with ethanol, rinsed with dH2O, dried with N2, and assembled onto a Biacore chip holder (Biacore SLA Au kit, BR-1004-05). The chip was primed twice and equilibrated in the Biacore X by flowing DPBS at 5 mL/min for 3 min. A cleaning injection of 70 μL of detergent (0.3% Triton X-100 in 0.1 M glycine, pH 12) was followed by a rinse with DPBS for 3 min. DPBS (10 μL, 50 μL/min) was injected into the flow channel (50 μL/min) and recorded for comparison to protein injection. FNIII10 (20 μL, 10 μg/mL) in DPBS was added at 50 μL/min, and the adsorption profile was recorded. The desorption profile was recorded for 30 s by flowing pure DPBS following the FNIII10 injection.

Characteristic adsorption parameters based on a mass action adsorption model were obtained by simultaneously solving the governing differential equations and fitting the SPR profile numerically.32 The desired protein product eluted at approximately 0.27 M NaCl and was verified to be >95% pure FNIII10 by SADS-PAGE. Relevant fractions were concentrated using Microsep 10K Omega centrifugal devices, dialyzed overnight against caps buffer (10 mM CAPS, 150 mM NaCl, pH 11.0), and flash frozen for storage at -80 °C.

FNIII10 Radiolabeling and Adsorption Measurements. FNIII10 adsorption onto SAMs was quantified as a function of coating concentration using 125I–FNIII10. FNIII10 was iodinated with the Bolton-Hunter Reagent as described previously.16,18 Briefly, the Bolton-Hunter Reagent benzene solvent was evaporated with a gentle stream of N2, and 100 μg of FNIII10 (10 μg/μL) in 0.1 M sodium borate buffer, pH 8.5 was added and incubated overnight at 4 °C. The coupling reaction was quenched with 50 μL of 0.2 M glycine in 0.1 M sodium borate (pH 8.5). Labeled FNIII10 (125I–FNIII10) was purified by size exclusion chromatography in a Sephadex G-25 column. The column was blocked in 1% heat-denatured bovine serum albumin (dnB-SA) overnight prior to use. The BSA was denatured by heating at 56 °C for 30 min. Fractions containing 125I–FNIII10 were combined and stored at 4 °C. The specific activity (4.39 × 104 cpm/μg of 125I–FNIII10 was determined using a COBRA II Auto Gamma counter (Packard Bioscience, Meriden, CT) and the NanoOrange Protein Quantification Kit (Molecular Probes). To ensure that the labeling of the protein did not alter adsorption behavior, radiolabeled protein was mixed with unlabeled protein at various ratios, and adsorption measurements yielded equivalent results for all dilutions. For adsorption measurements, SAMs were incubated for 30 min in a mixture of 125I–FNIII10 and FNIII10 (1–20 μg/mL) at a coating concentration of 1 nM at 22 °C. Surfaces were then blocked for 30 min in 1% hd-BSA to be consistent with the Ab assays and cell adhesion assays that require blocking of the remaining surface not covered by FN. hd-BSA was used to block nonspecific interactions such as antibody adsorption to the surfaces ensuring detection of the desired specific interaction. Adsorbed 125I–FNIII10 was quantified, and radioactive counts were converted to adsorbed surface densities (ng/cm²).

Ab Assay for FNIII10 Conformation. SAMs were incubated in 2-fold serial dilutions of 125I–FNIII10. Surfaces were then blocked against nonspecific Ab binding using blocking buffer (0.25% hd-BSA, 0.00125% NaN3, 0.1 M EDTA, 2.5% Tween-20 in PBS for 1 h at 37 °C). Blots were incubated in primary Ab (1:4000 for HNF7.1 and maAb1937, 1:10000 for FN11 in blocking buffer) for 1 h at 37 °C. After washing and blocking for 10 min, substrates were incubated in alkaline phosphatase-conjugated anti-mouse IgG (1:1000) for 1 h at 37 °C, washed, blocked, and incubated in 4-methylumbelliferyl phosphate (40 μg/mL in 10 mM diethanolamine, pH 9.5) for 45 min at 37 °C. Reaction products were measured using an HTS 7000 Plus fluorescence microplate reader (Perkin-Elmer, Foster, CA) at 360 nm excitation/465 nm emission.

Cell Adhesion Assay. Cell adhesion to FNIII10-coated SAMs was measured using a centrifugation assay that applies well-controlled detachment forces.29,31 SAMs were coated with a range of FNIII10 concentrations (0–20 μg/mL) for 30 min and blocked in 1% hd-BSA plus 0.1% nonfat dry milk for 30 min to prevent nonspecific adsorption to the substrate. NIH3T3 cells were labeled with 2 μg/mL Calcein-AM and seeded at 200 cells/mm² in 2 mM dextrose-DPBS into reassembled chamber slides for 30 min at 22 °C. The 30 min time point was chosen to investigate initial adhesion and reduce any confounding effects of cell spreading or cellular matrix secretion. The initial fluorescence intensity was measured to quantify the number of adherent cells prior to application of centrifugal force. After the wells were filled with medium and sealed with transparent adhesive, the substrate wells were inversed and spun at a fixed speed in a centrifuge (Beckman Allegra 6, GH 3.8 rotor) to apply a centrifugal force corresponding to 22.4g. After centrifugation, media were exchanged and fluorescence intensity was read to determine remaining adherent cells. For each well, adherent cell fraction was calculated as the ratio of postspin to prespin fluorescence readings.

Nonlinear regression analysis was used to fit experimental data to the appropriate model for radiolabeling, Ab affinity, and cell adhesion assays using SigmaPlot 5.0 (SPSS, Chicago, IL). Results for all experiments were analyzed by one-way ANOVA using SYSTAT 8.0 (SPSS). If treatments were determined to be significant, pairwise comparisons were performed using Tukey’s post hoc test with a 95% confidence level considered significant.

Results

Model Surfaces and Protein. SAMs of alkanethiol on Au were selected to present a wide range of well-defined surface chemistries to examine the effects of surface chemistry on FNIII10 adsorption. Long-chain functionally terminated alkanethiol (HS–(CH2)n–X, n ≥ 10) adsorb from solution onto Au to form stable, well-packed and ordered monolayers.34–36 The functional end groups (X) examined in this study were CH3 (hydrophobic), OH (neutral hydrophilic), NH2 (positively charged at physiological pH), and COOH (negatively charged at physiological pH). These SAM surfaces have been characterized by contact angle measurements as shown in Table 1 as well as by X-ray photoelectron spectroscopy (XPS).29

FNIII<sub>7−10</sub> is a 39 kDa recombinant fragment of FN that encompasses the 7th through 10th type III repeats of the human pFN molecule. FN III<sub>7−10</sub> spans the central cell binding domain which includes the PHSRN synergy site in the 9th type III repeat of FN and the RGD binding motif in the 10th type III repeat. These motifs are required in the 9th type III repeat of FN and the RGD binding motif in the 10th type III repeat. This overshoot observation is consistent with previous work. FN. The full pFN molecule is a large glycoprotein (440 kDa) that contains several functional domains other than cell binding, including sites for collagen and heparin binding as well as self-assembly. We chose to use this recombinant fragment of FN to model full pFN in order to eliminate possible confounding effects from regions outside the central cell binding domain. Recombinant FNIII<sub>7−10</sub> was expressed and purified to high yields (Figure 1). Antibody and cell adhesion assays demonstrated equivalent functional activity between FNIII<sub>7−10</sub> and pFN (data not shown).

**Kinetics of Protein Adsorption.** Kinetics of FNIII<sub>7−10</sub> adsorption onto SAMs were determined by SPR. Kinetic profiles of fragment adsorption revealed an initial rapid association rate that then slowed with time and appeared to approach saturation (Figure 2). The saturating values varied among the surfaces, following the trend NH<sub>2</sub> > CH<sub>3</sub> > COOH = OH. On the CH<sub>3</sub> surface, the response overshoots at approximately 50% saturation and then climbs to a plateau suggesting that the protein adsorbs quickly and then goes through significant reorientation or conformational change. This overshoot observation is consistent with previous work.

The adsorption process was analyzed using a mass action model with two states of the adsorbed protein: (1) reversibly adsorbed state and (2) irreversibly adsorbed state. In the model, the protein first associates with the surface in a reversibly adsorbed state (Figure 3). A fraction of adsorbed molecules then undergo a structural/conformational change to an irreversibly adsorbed state. This model yields the following governing equations:

\[
\frac{dY_1}{dt} = (kc - sY_1)(A_1(1 - f_a Y_1 - f_b Y_2)) - rY_1 \quad (1)
\]

\[
\frac{dY_2}{dt} = sY_1A_1(1 - f_a Y_1 - f_b Y_2) \quad (2)
\]

\[
Y_T = Y_1 + Y_2 \quad (3)
\]

where \( Y_T \) is the total surface density of FNIII<sub>7−10</sub> on the surface (ng/cm<sup>2</sup>), \( Y_1 \) represents the surface density of molecules adsorbed in state 1 (ng/cm<sup>2</sup>), and \( Y_2 \) represents the surface density of molecules adsorbed in state 2 (ng/cm<sup>2</sup>). \( c \) is the concentration of molecules in solution (ng/ml), \( k \) represents the initial association rate of the protein (cm<sup>-1</sup> s<sup>-1</sup>), \( s \) represents the rate of conformational change from state 1 to 2 (cm<sup>2</sup> s<sup>-1</sup>), and \( r \) represents the reversible rate of molecules in state 1 desorbed from the surface (s<sup>-1</sup>). \( A_1 \) is the total surface area (0.75 mm<sup>2</sup>), \( A_2 \) represents the area occupied by one molecule in state 1 (cm<sup>2</sup>/molecule = 10<sup>15</sup>), \( b \) represents the ratio of the area occupied by a molecule in state 2 with respect to the area occupied by a molecule in state 1, and \( f \) is Avogadro's number divided by the molecular weight of the fragment (molecules/ng). The resulting SPR data is related to the total surface density of FNIII<sub>7−10</sub> such that it is the sum of the surface density of molecules in both states (eq 3).

Because the governing differential equations are nonlinear and an explicit solution is not available, the equations were solved numerically in conjunction with nonlinear regression analysis to yield kinetic parameters for each data curve. The regression results for \( Y_T, Y_1, \) and \( Y_2 \) on the four surface chemistries are shown in Figure 2, and the resulting kinetic parameters are listed in Table 2. The regression analysis yielded a \( y^2 \) value for each curve fit such that \( \alpha > 0.005 \), and pairwise comparisons of model parameters yielded the following statistically significant results:

- \( CH_3 > COOH = OH \)
- \( NH_2 > COOH \)
- \( CH_3 > COOH = NH_2 = OH \)
- \( CH_3 > COOH = NH_2 = OH \)

The modeling implemented here is based on the assumption that the protein adsorbs without mass transport limitations due to the flow of the protein through the flow cell. A calculation for mass transport limitation (MTL) of the system can be made using eq 4, where \( D \) is the diffusion coefficient (cm<sup>2</sup>/s), \( F \) is the flow rate (l/min), \( h \) and \( b \) are the dimensions of the flow cell (mm), and \( l_1 \) and \( l_2 \) are the detection points in the flow cell (mm).

\[
MTL = \frac{L_r}{L_m + L_r} \quad L_m = C_m \left[ \frac{D^2F}{h^2bl_2} \right] \quad C_m = 1.47 \left( \frac{1 - (l_1/l_2)^2}{1 - (l_1/l_2)^3} \right) \quad L_r = kA_1 \quad (4)
\]

MTL varies from 0 when the system is not mass transport limited to 1 when the system is absolutely mass transport limited.
For the \( k \) values estimated in our analysis, the MTL index varies between 0.47 (OH) and 0.77 (CH\(_3\)). This range of MTL values indicates that the experiment is in the transitional regime from reaction rate limited to mass transport limited. Therefore, the fitted values for \( k \) underestimate the true association rate. However, these effective parameters still reveal surface-dependent differences.

**Quasi-Equilibrium FNIII\(_{17-10}\) Adsorption and Structure.** FNIII\(_{17-10}\) structural changes upon adsorption were evaluated as the adsorption process approached quasi-equilibrium. First, adsorbed FNIII\(_{17-10}\) surface density was quantified as a function of coating concentration using radiolabeled protein. For each surface, adsorbed FNIII\(_{17-10}\) density increased linearly until saturation values were approached at high concentrations (Figure 4). The adsorbed protein profiles of adsorbed FN (\( \text{FN}_{\text{ads}} \)) versus FN coating concentration (\( \text{FN} \)) were regressed to a simple hyperbola (eq 5) to obtain estimates of saturation density (\( \text{FN}_{\text{sat}} \)) and half-maximal adsorption (\( [\text{FN}]_{50} \)). FNIII\(_{17-10}\) saturation density followed the trend \( \text{NH}_2 > \text{CH}_3 > \text{COOH} \). This trend in saturation density agrees well with our kinetic observations.

\[
\text{FN}_{\text{ads}} = \frac{\text{FN}_{\text{sat}}}{[\text{FN}] + [\text{FN}]_{50}} \tag{5}
\]

Conformational/structural changes were analyzed by ELISA with a panel of monoclonal Ab's. The use of
monoclonal antibodies as probes for structural or conformational changes in adsorbed proteins is well documented. Adsorption of FNIII\(_{7-10}\) onto synthetic surfaces is a relatively nonspecific process in which molecules are expected to be present in different orientations with respect to the surface. Only a portion of the adsorbed molecules are likely to display any particular domain in a position that is accessible to Ab binding. For molecules in which a particular binding domain is exposed, the average conformation of this domain is influenced by the chemical properties of the underlying surface. Three monoclonal Ab’s directed against sites within the central integrin binding domain of FN were used to detect changes in conformation for both FNIII\(_{7-10}\) and pFN. A sigmoidal binding curve (eq 6) was fit to Ab binding profiles of binding (AB\(_{\text{bound}}\)) versus adsorbed density (FN\(_{\text{ads}}\)) where AB\(_{\text{bkgd}}\) is the background Ab binding, AB\(_{\text{sat}}\) is the saturation level of Ab binding, AB\(_{50}\) is the half-maximal Ab binding, and b is the slope at the inflection point.

\[
AB_{\text{bound}} = AB_{\text{bkgd}} + \frac{AB_{\text{sat}}}{1 + \exp\left(-\left(FN_{\text{ads}} - AB_{50}\right)/b\right)} \quad (6)
\]

Shifts in the Ab binding profiles (Figure 5), characterized by AB\(_{50}\), reflect changes in Ab binding affinity for the protein adsorbed to the different surfaces. These changes in Ab binding affinity reflect adsorption-induced changes in protein conformation/structure and/or the prevention of Ab binding due to adsorbed orientation with respect to the surface. A right shift in the profile corresponds to a lower affinity of the Ab for that particular protein conformation because higher FNIII\(_{7-10}\) densities are necessary to reach comparable amounts of Ab binding. Therefore, the AB\(_{50}\) parameter corresponds to the inverse of the Ab affinity. Similar shifts were detected for FNIII\(_{7-10}\) and pFN (data not shown) using HFN7.1 and FNI-11, indicating that FNIII\(_{7-10}\) is a useful model for the cell binding domain of pFN. Pairwise comparison analysis of HFN7.1 AB\(_{50}\) revealed statistically significant differences among surface chemistries (p < 0.05) such that HFN7.1 affinity followed the order OH > NH\(_2\) > COOH > CH\(_3\) (Table 3). Similarly, analysis of FNI-11 and mAb1937 AB\(_{50}\) demonstrated changes in Ab binding affinity in the order OH = COOH > NH\(_2\) > CH\(_3\).

FN tertiary structure consists of functional \(\beta\)-sheet folded globular domains connected by a flexible linker (Figure 6). HFN7.1 binds to the flexible linker between the 9th and 10th type III repeats. The statistical trend for HFN7.1 binding suggests that the flexible linker undergoes significant structural changes upon adsorption of FNIII\(_{7-10}\) with the OH SAM having the least amount of structural changes and CH\(_3\) having the largest changes. The statistical trend for FNI-11 suggests that the 9th type III repeat undergoes structural changes on the CH\(_3\) surface in comparison to the other three surfaces. Similarly, the loss in mAb1937 affinity for FNII\(_{7-10}\) adsorbed onto CH\(_3\) indicates considerable changes in the structure of the 8th type III repeat.

Cell Adhesion Centrifugation Assay. The activity of adsorbed FNIII\(_{7-10}\) was investigated using a centrifugation adhesion assay that applies controlled, reproducible forces to adherent, fluorescently labeled cells. A sigmoidal adhesion curve was fit to the resulting adherent cell fraction (f) versus adsorbed fragment density (FN\(_{\text{ads}}\)) where f\(_{\text{bkgd}}\) is the background level of adhesion, f\(_{\text{sat}}\) is the maximum adhesion fraction, ADH\(_{50}\) is the half-maximal adhesion, and g is the slope at the inflection point.

\[
f = f_{\text{bkgd}} + \frac{f_{\text{sat}}}{1 + \exp\left(-\left(FN_{\text{ads}} + ADH_{50}\right)/g\right)} \quad (7)
\]

(Figure 5). Ab binding affinity curves for HFN7.1, FNI-11, and mAb1937 Ab’s. The concentration of FNIII\(_{7-10}\) has been normalized by the surface density obtained by radiolabeling. Changes in Ab affinity, which reflect differences in protein conformation, are demonstrated as shifts in the normalized curves.

Table 3. Antibody-Based Assay for FN Conformation

<table>
<thead>
<tr>
<th></th>
<th>HFN7.1</th>
<th>FNI-11</th>
<th>mAb1937</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB(_{50}) (error)</td>
<td>160 (14)</td>
<td>74 (4.1)</td>
<td>59 (5.6)</td>
</tr>
<tr>
<td>OH</td>
<td>31 (2.2)</td>
<td>15 (1.2)</td>
<td>5.2 (0.79)</td>
</tr>
<tr>
<td>NH(_2)</td>
<td>62 (9.2)</td>
<td>22 (2.4)</td>
<td>4.2 (0.76)</td>
</tr>
<tr>
<td>COOH</td>
<td>92 (7.6)</td>
<td>35 (2.7)</td>
<td>8.2 (1.0)</td>
</tr>
<tr>
<td>p</td>
<td>0.0013</td>
<td>0.000029</td>
<td>0.00013</td>
</tr>
</tbody>
</table>

* AB\(_{50}\) represents the surface density of FNIII\(_{7-10}\) required to reach 50% of the total Ab binding. This parameter is inversely proportional to Ab affinity (mean ± standard error).

Changes in strength of cell adhesion to the various surfaces are shown in Figure 7 as shifts in the adhesion curve characterized by half-maximal adhesion (ADH50). A shift in the curve to the right represents a decrease in adhesion strength since more protein is required on the surface for the adhesion of the cells to the substrate; therefore, adhesion strength is inversely related to ADH50. Pairwise comparison testing of ADH50 among the SAMs revealed statistical differences that demonstrate changes in adhesion strength following the trend OH = NH2 > COOH > CH3 (p = 0.00005).

**Structure/Function Relationships.** To provide further insights into FNIII7-10 adsorption, we examined correlations between the structural (AB50) and functional (ADH50) parameters. ADH50 correlated well with AB50 for HFN7.1 (linear, R² = 0.98) and FNI-11 (linear, R² = 0.99). This relationship indicates that structural alterations or adsorbed orientation effects in the 9th and 10th type III repeats of FN significantly modulate protein activity. In contrast, FNIII7-10 adhesive activity correlated poorly with mAb1937 AB50. This low correlation is expected because the epitope for this Ab lies outside the critical integrin binding region of FNIII7-10. Finally, we examined the ability of surface hydrophobicity to predict adhesive activity. Contrary to previous reports,24-28,50 surface hydrophobicity was a poor indicator of functional activity. These results indicate that surface hydrophobicity is not the primary parameter controlling adsorbed protein activity and that other surface properties, including charge, influence adsorbed protein function.

**Discussion**

We analyzed several adsorption parameters, including adsorption kinetics, quasi-equilibrium values, and structural changes (as determined by changes in Ab binding affinity), upon adsorption to well-defined surface chemistries for a FN model fragment. These parameters were correlated to the adhesive activity of the adsorbed protein in order to provide insights into adsorption–function relationships. The 30 min time point for cell adhesion to FNIII7-10 was chosen to restrict interactions to initial adhesion events, ensuring that differences in adhesion could be attributed to FN conformation and thus eliminating confounding effects. Our group has shown that detachment of cells occurs at the integrin–FN bond for these cells.51 In addition, blocking the integrin–FN interaction eliminates cell adhesion to these surfaces, confirming that our adhesion assay detects the functional activity of FN on each surface.29

Recent studies with model surfaces have proposed that surface hydrophobicity is a good predictor of adsorption parameters and that proteins adsorbed onto hydrophobic surfaces undergo greater unfolding/denaturation than on hydrophilic supports.25-28,41,50 In agreement with these studies, our kinetic results indicate faster FNIII7-10 adsorption rates for the CH3 SAM and that proteins adsorbed onto this surface undergo that largest change in protein unfolding as determined by the ratio of area per molecule of irreversibly adsorbed protein (state 2) to area per molecule of reversibly adsorbed protein (state 1). Analysis of protein conformation using antibodies as structural probes revealed drastic reductions in Ab binding affinity for epitopes in the 8th, 9th, and 10th type III repeats of FN for proteins adsorbed onto the CH3 functionality compared to other surfaces. These results suggest gross changes in FN structure throughout the entire molecule and agree well with the conclusions obtained from the kinetic analysis. Finally, FNIII7-10 adsorbed onto the CH3 SAM displayed poor cell adhesion activity. This loss in activity is in good agreement with the significant structural changes observed.

In contrast, kinetic parameters and surface hydrophobicity did not correlate well with FN activity on the OH, NH2, and COOH surfaces. Similarly, no significant differences in the structure of the 8th type III repeat were detected among these surfaces using mAb1937. On the other hand, Ab measurements with two different Ab’s revealed surface-dependent differences in the structure of the central cell binding domain of FN localized to the 9th and 10th type III repeats. These structural changes correlated well with surface-dependent differences in cell adhesive activity (Figure 8). FNIII7-10 adsorption onto the neutral hydrophilic OH SAM exhibited the highest Ab binding affinity for Ab’s localizing to the cell binding domain as well as the highest cell adhesion activity, suggesting that this functionality induces the least amount of protein unfolding or denaturation. The adsorption and functional behavior on the OH surface, as well as the

**References**


hydrophobic CH₃, may be explained by water solvation and restructuring effects at the interface.52,53

This analysis indicates that surface hydrophobicity and adsorption kinetic parameters are partial predictors of adsorbed FN functional activity when comparing hydrophobic and hydrophilic supports. These determinants are likely to be effective for gross/global changes in protein structure. However, these parameters cannot discriminate among neutral, positive, and negative hydrophilic surfaces, even though adsorption onto these functionalities significantly modulates the activity of the adsorbed protein. In contrast, probes for specific structural/functional domains, such as the Ab's used in the present study, provide robust determinants of adsorbed FN activity. Furthermore, the Ab-specific differences among SAMs indicate that local, indicated by each individual Ab, as well as global, indicated by combining the results of all three Ab's, changes in protein structure potentiate protein activity. While the present study provides insights into adsorption-induced changes in structure and function, detailed structure–function analyses are required to elucidate mechanisms involved in surface-dependent modulation of protein activity. As an initial step to this goal, we recently implemented computational molecular modeling approaches to the adsorption of the FN fragment onto the SAMs examined in the present work.54 This computational molecular model predicted adsorption free energy following the trend NH₂ > CH₃ > COOH > OH and structural changes following the trend CH₃ > COOH = NH₂ > OH, both of which are consistent with the experimental results obtained in the present study. Finally, similar structure–function analyses with other proteins are necessary to establish the broad applicability of this approach to analyze protein adsorption to synthetic supports.

Conclusions

Using model surfaces with well-defined surface properties and investigating a range of surface chemistry, we demonstrate significant surface-chemistry-dependent structural changes in FN central cell binding domains and adhesive activity. These findings indicate that adsorption-induced changes in protein structure and activity are not dominated by surface hydrophobicity alone. This study also establishes a relationship between surface-dependent changes in structural domains of FN and FN functional activity.

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Cell Adhesion Strengthening: Contributions of Adhesive Area, Integrin Binding, and Focal Adhesion Assembly

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Mechanical interactions between a cell and its environment regulate migration, contractility, gene expression, and cell fate. We integrated micropatterned substrates to engineer adhesive area and a hydrodynamic assay to analyze fibroblast adhesion strengthening on fibronectin. Independently of cell spreading, integrin binding and focal adhesion assembly resulted in rapid sevenfold increases in adhesion strength to steady-state levels. Adhesive area strongly modulated adhesion strength, integrin binding, and vinculin and talin recruitment, exhibiting linear increases for small areas. However, above a threshold area, adhesion strength and focal adhesion assembly reached a saturation limit, whereas integrin binding transitioned from a uniform distribution to discrete complexes. Adhesion strength exhibited exponential increases with bound integrin numbers as well as vinculin and talin recruitment, and the relationship between adhesion strength and these biochemical events was accurately described by a simple mechanical model. Furthermore, adhesion strength was regulated by the position of an adhesive patch, comprised of bound integrins and cytoskeletal elements, which generated a constant 200-nN adhesive force. Unexpectedly, focal adhesion assembly, in particular vinculin recruitment, contributed only 30% of the adhesion strength. This work elucidates the roles of adhesive complex size and position in the generation of cell–extracellular matrix forces.

INTRODUCTION

Cell adhesion to the extracellular matrix (ECM) is central to development and the organization, maintenance, and repair of tissues by providing anchorage and triggering signals that direct cell survival, migration, cell cycle progression, and expression of differentiated phenotypes (De Arcangelis and Georges-Labouesse, 2000; Danen and Sonnenberg, 2003). Furthermore, abnormalities in adhesive interactions are often associated with pathological states, including blood clotting and wound healing defects as well as malignant tumor formation (Wehrle-Haller and Imhof, 2003; Jin and Varner, 2004). Adhesion to extracellular matrix components, such as fibronectin (FN) and laminin, is primarily mediated by the integrin family of heterodimeric receptors (Hynes, 2002). Integrin-mediated adhesion is a highly regulated process involving receptor activation and mechanical coupling to extracellular ligands (Faull et al., 1993; Choquet et al., 1997; García et al., 1998a). Bound receptors rapidly associate with the actin cytoskeleton and cluster together to form focal adhesions, discrete supramolecular complexes that contain structural proteins, such as vinculin, talin, and α-actinin, and signaling molecules, including FAK, Src, and paxillin (Geiger et al., 2001).

Significant progress has been made in understanding biochemical aspects of integrin-mediated adhesion, particularly in terms of identifying key adhesive components and signaling interactions. This information has been instrumental in deciphering mechanisms regulating cell morphology, migration, and integration of adhesive and growth factor-activated signals that direct high order cellular functions. In contrast, the mechanical aspects of adhesion remain poorly understood due to a lack of robust, quantitative measurement systems and the inherent complexities of the adhesive process. Cell spreading and migration are often used as indirect indicators of adhesion strength, but these multistep, dynamic processes exhibit complex dependencies on adhesion strength (Palecek et al., 1997) and hence do not provide direct or sensitive measurements. This lack of a quantitative understanding of adhesion strengthening limits the interpretation of functional studies of structural and signaling adhesive components. Furthermore, it is increasingly evident that mechanotransduction between cells and their environment regulates gene expression and cell fate (Wozniak et al., 2003; Engler et al., 2004; McBeath et al., 2004; Mammoto et al., 2004; Polle et al., 2004); therefore, it is essential to have direct measurements of cell–matrix adhesion strength to fully analyze these mechanosensory interactions.

The generally accepted model for adhesion strength, proposed by McClay and Erickson, postulates a two-step process consisting of initial integrin-ligand binding followed by rapid strengthening (Lotz et al., 1989). The strengthening response arises from 1) increases in cell–substrate contact area (spreading), 2) receptor recruitment to anchoring sites (clustering), and 3) interactions with cytoskeletal elements that lead to enhanced force distribution among bound receptors via local membrane stiffening (focal adhesion assembly). Subsequent observations from various systems support roles for each of these mechanisms in adhesion strengthening (Choquet et al., 1997; Hato et al., 1998; Stupack et al., 1999; Maheshwari et al., 2000; Balaban et al., 2001; Galbraith et al., 2004).
Glass coverslips (25 mm in diameter) were used as substrates for micropatterned arrays. After cleaning by O₂ plasma etching, coverslips were sequentially coated with optically transparent films of titanium (10 nm) and gold (20 nm) via electron-beam evaporation. For microcontact printing, stamps were cleaned by sonication in 50% ethanol for 15 min and the flat back of the stamp was allowed to self-seal to a glass slide to provide a rigid backing. Gold (Au)-coated samples were rinsed with 95% ethanol and dried under a stream of N₂. The face of the stamp was inked with a 1.0 mM ethanolic solution of tr(ethylene glycol)-terminated alkanethiol for 4 h to create a nonfouling and nonadhesive background around the CH₃-terminated islands. Unpatterned reference substrates, on which cells spread normally, were created by immersion of a gold-coated coverslip in a 1.0 mM ethanolic solution of hexadecanethiol. After rinsing in 95% ethanol and drying, substrates were coated with FN (20 μg/ml in DPBS) for 1 h and then blocked in 1% bovine serum albumin (BSA) for 1 h. Cells were seeded on micropatterned substrates at 225 cells/mm² in DME supplemented with 0.1% FCS. For serum-free experiments, cells were plated in DME supplemented with ITS-A and 1% BSA.

**Cell Adhesion Strength**

Cell adhesion strength was measured with a spinning disk device (Garcia et al., 1997, 1998a). Micropatterned substrates with adherent cells were mounted on the spinning disk device and spun in DPBS + 2 mM glucose at room temperature (18°C) for 5 min at a constant speed. After spinning, cells were fixed in 3.7% formaldehyde + 1% Triton X-100, stained with ethidium homodimer, and counted at specific radial positions using a Nikon TE300 equipped with a Lulid motorized stage, Spot-RT camera, and Image-Pro analysis system. Sixty to eighty cells (80-100 cells/mm²) were counted before spinning. For each cell, cell counts were normalized to the number of cells present at the center of the disk. The fraction of adherent cells (Fₐ) was then fit to a sigmoid curve of the form

\[
F_a = \frac{1}{1 + \exp[b(\tau - \tau_0)]},
\]

where \(\tau_0\) is the shear stress for 50% detachment and \(b\) is the inflection slope.

**Integrin Binding and Focal Adhesion Assembly**

For integrin staining, adherent cells were incubated in 1.0 mM DTSSP in chilled DPBS for 30 min to cross-link bound integrins to the underlying ECM (Garcia et al., 1999). Unreacted cross-linker was quenched for 10 min by the addition of 50 mM Tris. Uncross-linked cellular components were extracted in 0.1% SDS supplemented with protease inhibitors (350 μg/ml PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Samples were blocked in 5% fetal bovine serum (FBS) for 1 h and incubated in integrin subunit-specific antibodies followed by fluorochrome-labeled secondary antibodies. For visualization of focal adhesions, cells were extracted in 0.5% Triton X-100 in 50 mM NaCl, 150 mM sucrose, 5 mM MgCl₂, 20 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF, 50 mM Tris, pH 6, for 10 min to remove membrane and soluble cytoskeletal components. Extracted cells were fixed in 3.7% formaldehyde for 5 min, blocked in 5% FBS, and incubated with primary antibodies against focal adhesion components followed by a fluorochrome-labeled secondary antibodies or rhodamine-phalloidin and counterstained with Hoechst dye.

Bound integrins were quantified using a cross-linking/extraction/reversal method (Garcia et al., 1999; Keselowsky and Garcia, 2005). Adherent cells were incubated in DTSSP (1.0 mM) for 30 min to cross-link integrins to their bound ligand. After quenching unreacted cross-linker with 50 mM Tris, cells were extracted in 0.1% SDS supplemented with protease inhibitors (350 μg/ml PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) to remove uncross-linked cellular components. After washing, proteins cross-linked to the dish were recovered by reversing the cross-linking in 50 mM dithiothreitol (DTT) and 0.1% SDS at 37°C for 30 min. Recovered integrins were quantified by Western blotting. Soluble extracted fractions and whole cell lysates were used as positive controls and reference to normalize for differences in cell number among substrates. In parallel samples, cross-linked integrins were visualized via immunofluorescence staining.

Focal adhesion proteins localized to adhesive complexes were isolated and quantified by a wet cleaving technique (Keselowsky and Garcia, 2005). Cells were washed with DPBS, and a dry nitrocellulose sheet was overlaid onto the cells for 30 s. Cells were then mechanically peeled by rapidly lifting the nitrocellulose sheet with tweezers. Remaining cellular components were rinsed and scraped in Laemmli sample buffer. Recovered proteins were analyzed by Western blotting. Whole cell lysates served as reference for normalization. Parallel samples were analyzed by immunostaining.

**Small Interference RNA (siRNA)**

Inhibition of vinculin expression was performed using vinculin-directed siRNA reagents (mouse vinculin, siGENOME SMARTpool siRNA; Dharmacon, Lafayette, CO). NIH3T3 fibroblasts were transfected with siRNA duplexes using DharmaFECT3 (Dharmacon) according to the manufacturer's
Micropatterned substrates were used to control cell-substrate adhesive area and cell shape. Arrays of circular adhesive islands of varying dimensions (2, 5, 10, 20 μm in diameter) were engineered to examine a 100-fold range in island sizes, available adhesive area can be varied over a 100-fold range. (C) Immunostaining image for FN showing adsorption only to 10-μm-diameter islands (black circles). By using different adhesive island sizes, available adhesive area can be varied over a 100-fold range. (D) Phase contrast image of NIH3T3 fibroblasts localized to 10-μm-diameter islands at 16 h. (E) Immunostaining images showing localization of integrins and talin to 10-μm-diameter adhesive island. Bar, 2 μm.

Statistical Analyses
Experiments were performed in triplicate in at least three independent experiments. Data are reported as mean ± SE of the mean, and statistical comparisons using SYSTAT 8.0 were based on analysis of variance and Tukey’s test for pairwise comparisons, with a p-value < 0.05 considered significant. Curve fits of experimental data to specified functions were conducted using the Marquardt–Levenberg algorithm in SigmaPlot.

RESULTS
Micropatterned Substrates to Control Adhesive Area and Cell Shape
Micropatterned substrates were used to control cell-substrate adhesive area and to eliminate the contributions of changes in cell shape and spreading to cell adhesion strengthening. For example, segregation of adhesion structures to the periphery of spreading cells and changes in cell morphology may influence adhesion strengthening independently from changes in adhesive area. Microcontact printing of self-assembled monolayers of alkanethiols on gold was used to create FN-coated, cell-adhesive domains within a nonfouling/nonadhesive background (Figure 1A). Although this approach has been previously used to control cell spreading (Singhvi et al., 1994; Chen et al., 1997; Lehner et al., 2004), we applied it here to engineer cell adhesive area while maintaining a constant cell shape. Arrays of circular adhesive islands of varying dimensions (2, 5, 10, 20 μm in diameter) were engineered to examine a 100-fold range in cell–substrate-available adhesive area (Figure 1B). The 75-μm interisland spacing eliminated cell–cell interactions and ensured that a cell would only interact with a single adhesive island. FN preferentially adsorbed onto the CH₃-terminated circular islands, whereas the tri(ethylene glycol)-terminated regions remained devoid of adhesive protein (Figure 1C). NIH3T3 fibroblasts adhered to FN-coated micropatterned islands (one cell/island) and remained constrained to the adhesive area (Figure 1D). Unlike endothelial cells that undergo apoptosis when grown on small micropatterns (Chen et al., 1997), NIH3T3 cells on all island sizes remained viable for more than 5 days in culture with no evidence of apoptosis, as determined by annexin V expression or DNA fragmentation (our unpublished data). Furthermore, there were no differences in metabolic activity, as determined by reduction of the tetrazolium salt WST-1, among cells cultured on different adhesive island sizes. More importantly, adhesive structures, including complexes of integrin αvβ₅, vinculin, talin, α-actinin, and paxillin, localized to and remained constrained to the micropatterned island (Figure 1E). These micropatterned adhesive domains also limited cell spreading, constraining cells to a nearly spherical morphology (Figure 1D). This well-defined cell shape allows simple and direct calculation of applied detachment forces and the resultant adhesive forces generated by adhesive complexes. Together, these results demonstrate control of cell adhesive area in order to engineer focal adhesion size and position and decouple integrin clustering and focal adhesion assembly from changes in cell morphology.

Integrin-mediated Adhesion Strength Increases Rapidly until Reaching Steady-State Values
Cell adhesion strength to FN-coated micropatterned islands was quantified using a spinning disk device previously characterized by our group (García et al., 1997, 1998a). This system applies a well-defined range of hydrodynamic forces to adherent cells and provides sensitive measurements of adhesion strength. Substrates containing adherent cells were mounted on the device and spun in buffer at a constant speed. Fluid flow over the cells on the disk produces a detachment force that is proportional to the hydrodynamic wall shear stress τ (force/area). The wall shear stress increases linearly with radial position (r) along the disk surface and is given by

$$\tau = 0.8 r \sqrt{\mu \rho \omega}$$

(1)

where ρ and μ are the fluid density and viscosity and ω is the rotational speed. For this configuration, cells at the center of the sample experience negligible force, whereas cell numbers decrease toward the outside of the disk as the applied force increases. Thus, in a single sample, a linear range of forces is applied to a large cell population (~6000 cells analyzed/sample). After spinning, remaining cells were fixed, stained, and counted at specific radial positions. The fraction of adherent cells (f) was calculated by dividing the number of cells in each field by the number of cells at the center of the array, where negligible forces are applied. The detachment profile (f versus τ) was then fit to a sigmoid curve (f = 1.0/(1.0 + exp[b(τ - τ₀)])) to obtain the shear stress for 50% detachment (τ₀). We define τ₀ as the adhesion strength. Figure 2A shows a typical detachment profile and sigmoid fit (τ₀ = 475 dyne/cm², R² = 0.92). The adhesion strength values obtained with this system have been reproducible over a 2-yr period of analysis.

Blocking antibodies against human FN or integrin αvβ₅ eliminated adhesion strength to these micropatterned surfaces (Figure 2B). This result indicates that adhesion in this
system is mediated by $\alpha_5\beta_1$ binding to preadsorbed FN and excludes significant contributions to adhesion strength from other receptors and/or extracellular ligands. To elucidate the mechanism by which cell detachment occurred under the applied force, cells were spun and stained for FN, integrins, and focal adhesion components. Areas at the periphery of the disk, where cells had detached, displayed complete FN staining and minimal traces of residual integrins or focal adhesion components (Figure 2C). This result indicates that cell detachment took place at the integrin-FN junction, resulting in removal of the entire cell without gross failure. In contrast, cells treated with latrunculin A, an inhibitor of actin polymerization, displayed significant cytoskeletal debris after detachment (Figure 2C). This residual cytoskeletal debris indicates gross cell rupture at points above focal adhesions due to loss of cellular integrity arising from impaired actin polymerization. Together, these results demonstrate that this system provides sensitive and reliable measurements of $\alpha_5\beta_1$ integrin-FN-mediated adhesion strength.

The kinetics of the adhesion strengthening response was analyzed on micropatterned islands coated with subsaturating (20 ng/cm$^2$) or saturating (200 ng/cm$^2$) FN densities. Islands of 5-$\mu$m diameter were selected because this adhesive area corresponds to the cell–substrate contact area observed at early adhesion times (15 min) for unpatterned substrates (Lotz et al., 1989; García et al., 1998a). Adhesion strength increased rapidly at early time points and reached steady-state values by 4 h (Figure 3). There were no differences in adhesion strength between cycloheximide-treated and control cultures, indicating that the increases in adhesion strength do not involve new protein synthesis. The strengthening kinetics is described accurately by a simple exponential fit, which provides two parameters for characterization, the steady-state adhesion strength and rise time. Steady-state adhesion strength was dependent on FN density, consistent with a simple model in which receptor-ligand bond numbers, and hence adhesion strength, increase with ligand density. There were no differences in rise time between FN densities. Furthermore, the use of micropatterned substrates that maintain nearly constant cell morphology and restrict the size and position of adhesive contacts allows analysis of the evolution of adhesion strength independently from changes in cell spreading. Adhesion strength exhibited a sevenfold enhancement from initial binding (15 min) at 65 ± 6.9 to 447 ± 32 dyne/cm$^2$ at steady state. We attribute this strengthening response to integrin recruitment and clustering and focal adhesion assembly, independently from changes in cell spreading. Finally, similar trends in adhesion strengthening have been observed in mouse embryo fibroblasts and other fibroblast lines, indicating that this response is not unique to NIH3T3 cells.

Figure 2. Cell adhesion strength measurements. (A) Detachment profile showing fraction of adherent cells versus applied shear stress for cells adhering to 5-$\mu$m-diameter islands for 16 h. Experimental points were fit to sigmoid to obtain the shear stress for 50% detachment ($\tau_{50}$). (B) Antibodies against human FN or $\alpha_5\beta_1$ integrin completely block adhesion to micropatterned islands. (C) Immunostaining for vinculin after the application of high detachment forces illustrating detachment mechanism. Bar, 5 $\mu$m.
**Adhesion Strength Increases Nonlinearly with Adhesive Area**

We next examined the functional dependence of adhesion strength on available adhesive area by evaluating steady state adhesion strength (16 h) for adhesive islands with different dimensions coated with saturating levels of FN. Adhesive area strongly modulated adhesion strength, resulting in significant increases at small adhesive areas and reaching saturation levels (~600 dyne/cm²) for the 10-µm-diameter islands (Figure 4). The dependence of adhesion strength on adhesive area is accurately described by a hyperbolic curve ($R^2 = 0.94$). This relationship indicates that adhesive area is limiting for small areas, but above a critical value of adhesive area (78.5 µm²), additional increments in adhesive area do not significantly influence adhesion strength. A possible explanation for the saturation limit in adhesive area is that another dominant parameter in the strengthening response, such as availability of receptor and focal adhesion molecules, becomes limiting. Remarkably, the adhesion strength plateau for the micropatterned substrates approximated the adhesion strength for unpatterned cells (average spread area 1575 ± 89 µm²). It is important to note, however, that the effective detachment forces applied to micropatterned and unpatterned cells are different because of differences in cell morphology.

**Adhesive Area Strongly Modulates Integrin Binding and Focal Adhesion Assembly**

The time- and adhesive area-dependent enhancements in adhesion strength can be attributed to integrin recruitment and clustering and/or interactions with cytoskeletal proteins. To derive quantitative relationships between adhesive area and recruitment of integrins and focal adhesion components, immunostaining and biochemical analyses were performed for cells adhering for 16 h to micropatterned islands of various dimensions. Independently of micropattern size, integrins localized to and remained constrained to the adhesive island (Figure 5A). For all island sizes, integrins were preferentially localized to the periphery of the contact area, especially for the smaller islands (~2-5 µm diameter), there was a more uniform distribution of integrins across the adhesive island, whereas for the larger islands integrins were segregated into discrete complexes. These complexes, although smaller, are reminiscent of integrin clusters in spread cells (Figure 5A). This transition from a uniform distribution of receptors to discrete complexes suggests a “set point” or critical number of bound integrins. For the smaller islands, adhesive area is limiting and integrin binding would require dense packing in order to approach the set point. For larger adhesive islands, adhesive area is no longer limiting and integrins can localize to discrete clusters surrounded by regions with lower integrin packing.

As an independent but complimentary approach, bound integrin numbers were quantified using a cross-linking/extraction/reversal method (García et al., 1999; Keselowsky and García, 2005). Bound integrins were covalently cross-linked to FN using the cell-impermeable bifunctional reagent DTSSP. After detergent extraction of cellular components, including unbound receptors, the cross-linker was cleaved in DTT. Recovered integrins were quantified by Western blotting. We previously demonstrated that this approach specifically isolates bound integrins and provides robust measurements of the number of integrin-FN bonds (García et al., 1999; Keselowsky and García, 2005). The number of bound integrins, normalized to the number of bound integrins for unpatterned cells (~20% of the total integrin pool), increased linearly with adhesive area for small islands until reaching saturation values for the larger islands and unpatterned substrates (Figure 5B). The relationship between bound integrin number and adhesive area is accurately described by a simple hyperbola ($R^2 = 0.89$). This
functional dependence indicates that adhesive area limits integrin binding for small adhesive islands, but above a threshold value, integrin binding is independent of adhesive area. Interestingly, the adhesive area for half-maximal binding (77 μm²) is equivalent to the area for the 10-μm-diameter island (78.5 μm²). This adhesive area is also the crossover point from uniformly distributed receptors to discrete clusters (Figure 5A).

Vinculin and talin were selected for analysis of focal adhesion assembly at steady state (16 h) because these proteins are commonly associated with focal adhesions and have been implicated in adhesion strengthening responses (Galbraith et al., 2002; Giannone et al., 2003). In agreement with integrin binding results, vinculin and talin localized to adhesive structures constrained to the micropatterned islands (Figure 6A). These proteins were preferentially localized to the outer rim of the adhesive island, although discrete complexes were evident in the interior of the larger islands. Vinculin and talin recruitment to adhesive structures was quantified using a wet cleaving technique (Keselowsky and Garcia, 2005). A nitrocellulose sheet was overlaid onto cells on micropatterned arrays and rapidly removed to mechanically dissociate cell bodies from basal cell membranes containing adhesive structures. Recovered focal adhesion proteins were analyzed by Western blotting. Levels of recruited vinculin and talin were normalized to recruited levels in unpatterned cells, which corresponded to ~17% of the total cellular pool of these proteins. Both vinculin and talin recruitment increased strongly with adhesive area for the small islands and reached saturation values for the 10- and 20-μm-diameter islands (Figure 6B). Hyperbolic fits described well the relationship between focal adhesion protein recruitment and adhesive area (R² > 0.94). This functional dependence indicates that adhesive area limits focal adhesion assembly for small areas, but above 78.5 μm², recruitment of focal adhesion proteins is independent of available adhesive area. Interestingly, in contrast to integrin binding, saturated levels of recruited vinculin and talin were ~35% lower than levels in unpatterned, spread cells. The reason for this difference is not known, but we hypothesize that this disparity arises from differences in the state of contractility in these cells. Indeed, Chen and colleagues demonstrated that cell shape modulates RhoA activity (McBeath et al., 2004), which regulates contractility and drives focal adhesion assembly (Chrzanowska-Wodnicka and Burridge, 1996).

Correlations between Mechanical and Biochemical Events in Adhesion Strengthening

By combining the quantitative functional relationships presented above, correlations between mechanical (adhesion strength) and biochemical (integrin binding and focal adhesion assembly) events in adhesion strengthening can be derived for the first time (Figure 7). Although these correlations do not provide causal relationships, there are several notable points. First, the fact that the results for micropatterned and unpatterned cells follow the same relationship indicates that micropatterned substrates provide an appropriate model for investigating adhesive interactions. Second,
there is very good correspondence between biochemical events and mechanical outputs, suggesting that these processes are tightly coupled. Therefore, mechanical analyses of adhesion strengthening could provide critical information on structure–function relationships in adhesive interactions. Third, the nonlinear nature of these correlations indicate deviations from simple models in which adhesion strength is directly proportional to the number of integrin–ligand bonds or focal adhesion area and provide insights into the development of new hypotheses or refinement of existing models. For example, simple exponential fits accurately describe the experimental data (except for talin, for which a linear regression performs equally well), in excellent agreement with theoretical analyses modeling nonuniform bond loading within the contact area (Evans, 1985; Dembo et al., 1988).

**Focal Adhesion Assembly Provides Only 30% of the Strengthening Response**

We next examined the relative contribution of focal adhesion assembly to adhesion strengthening. First, the effects of serum stimulation on adhesion strength and focal adhesion assembly were analyzed. Serum stimulation of serum-starved cells drives focal adhesion assembly and stress fiber formation via Rho-mediated contractility (Chrzanowska-Wodnicka and Burridge, 1996; Amano et al., 1997). Cells were cultured under serum-free conditions for 16 h on 5-μm-diameter islands coated with saturating levels of FN. Cells were then switched to media containing 10% serum for 30 min before spinning and biochemical analyses. Serum stimulation enhanced adhesion strength by 30% (Figure 8A, 410 ± 14 dyne/cm² for serum-starved cells versus 542 ± 19 dyne/cm² for serum-stimulated cultures). This increase in

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**Figure 8.** Serum stimulation increases adhesion strength and induces recruitment of vinculin and talin to adhesive area without altering bound integrin levels. (A) Adhesion strength values for cells cultured under serum-free conditions for 16 h and stimulated with 10% serum for 30 min. (B) Immunostaining and biochemical analyses of the effects of serum stimulation on integrin binding. (C) Recruitment of vinculin and talin to adhesive structures as determined by immunostaining and biochemical analyses. Bar, 5 μm.
adhesion strength did not involve changes in integrin binding, as no differences in bound integrin numbers were observed between starved and stimulated cultures (Figure 8B). In contrast, serum stimulation resulted in significant enhancements in vinculin (400%) and talin (90%) recruitment to adhesive structures (Figure 8C). Similar results were observed for LPA-treated samples (our unpublished data). Furthermore, these serum-induced increases in adhesion strength and focal adhesion assembly were completely blocked by inhibitors of contractility, including blebbistatin and Y-27632 Rho-kinase inhibitor (Shin and García, unpublished observations).

To further analyze the contribution of focal adhesion assembly to adhesion strengthening, the adhesion strength of vinculin-deficient cells was evaluated on micropatterned (5-μm-diameter) substrates coated with 200 ng/cm² FN. Vinculin-deficient cells (clone g229) exhibited adhesion strength values that were 20% lower than those for the parental F9 cells (Figure 9, A and B, 292 ± 10.1 versus 362 ± 7.2). Reexpression of full-length vinculin in vinculin-deficient cells (clone M16) rescued the deficits in adhesion strength. Furthermore, deletion of vinculin abolished the serum-induced increases in adhesion strength for serum-starved cells (Figure 9C). The slight but not significant increases in adhesion strength for the vinculin-deficient cells may be attributed to other focal adhesion proteins, including talin and α-actinin (Volberg et al., 1995), which may compensate for the loss of vinculin.

Finally, the contribution of vinculin to the adhesion strength of NIH3T3 cells was examined by inhibiting vinculin expression via siRNA approaches. Transfection with siRNA duplexes reduced vinculin levels by 60–70% compared with control and mock-transfected cells (Figure 10A), whereas no differences were detected in talin expression. More importantly, knockdown of vinculin expression eliminated vinculin localization to focal adhesions (Figure 10B). Knockdown of vinculin expression also reduced adhesion strength by 25% compared with controls (Figure 10C). These results are in excellent agreement with the strengthening analysis for the vinculin-null lines. Together, these results demonstrate that focal adhesion assembly provides ~30% of the strengthening response and suggest that integrin binding and clustering provide the bulk of the enhancements in adhesion strengthening.
Mechanical analysis of adhesion strength. (A) Force balance for a cell (radius R) adhering to micropatterned adhesive island (diameter 2a). Hydrodynamic shear flow results in shear force (F_s) and torque (T_o), which are balanced by tangential (F_{tan}) and normal tensile (F_r) and compressive (F_c) forces at the adhesive interface. For equilibrium, the force produced by an adhesive patch at the leading edge is equal to F_T and is related to the surface shear stress (τ). Table lists predicted F_T for the experimental results (τ_{50}) showing a nearly constant force of 200 nN. (B) Microscopic model of bond loading in the adhesive patch. Adhesive patch is divided into five segments. Bonds in a particular segment can occupy one of three states: 1) uniformly distributed, 2) clustered, and 3) focal adhesion (FA)-associated bonds. Adhesive force produced by segment i (F_i) is given by the force rule shown. Plot depicts resultant adhesive patch force as a function of bond number showing enhancements in adhesive force with integrin clustering and cytoskeletal interactions.

"Adhesive Patch" as Functional Adhesive Unit

A simple engineering analysis was conducted to assist in the interpretation of our experimental data for adhesion strengthening. First, a "macroscopic" model was developed to calculate the forces produced by adhesive structures to resist the applied hydrodynamic forces. The model considers a spherical cell (radius R) adhering to a micropatterned island (diameter 2a) (Figure 11A). For mechanical equilibrium, the applied hydrodynamic shear force (F_s) and torque (T_o) must be balanced by a tangential force (F_{tan}) and tensile (F_r) and compressive (F_c) forces acting normal to the adhesive interface. Because of the use of micropatterned substrates to produce a well-defined cell shape, expressions for F_r and T_o as a function of surface shear stress τ can be easily obtained from the solutions for a sphere in shear flow (Goldman et al., 1967). In line with previous analyses (Evans, 1985; Dembo et al., 1988; Ward and Hammer, 1993), cell detachment is expected to occur via peeling of the leading edge of the cell. For membrane peeling, bond loading is highly nonuniform along the contact area—bond forces are maximal at the periphery and decay rapidly toward the center of the cell. Therefore, we assumed that the tensile force F_r, which represents the resultant of the bond forces in the adhesive area, acts at the leading edge of the adhesive island. By solving the equations of equilibrium, the following expression for the resultant bond force F_T as a function of adhesion strength τ was derived:

\[ F_T = 32 R^2 \tau \left[ 1 + \left( \frac{0.8 R}{a} \right)^2 \right] \] (2)

This expression was used with the experimental values for τ_{50} and geometry to compute the resultant bond force F_T (Figure 11A). Remarkably, the resultant bond force is nearly constant at 200 nN for all island sizes. This result indicates that the force exerted by an adhesive patch at the leading edge of the cell is constant and independent of adhesive area. The increases in adhesion strength (τ_{50}) with adhesive area (Figure 4) can be simply explained by increases in the distance of the adhesive patch from the center of the adhesive area—effectively increases in the lever arm of the resultant bond force. This analysis supports the concept of a functional adhesive unit that provides a maximum adhesive force of ~200 nN.

We next formulated a "microscopic" model to gain insights into the contributions of integrin binding and focal adhesion assembly to adhesive patch function. This analysis is based on a model developed by Ward and Hammer, 1993 to examine the effects of focal contact formation on adhesion strength. The adhesive patch was divided into five segments (i = 1–5), which contained the bonds that provided adhesive forces (Figure 11B). Because the smallest pattern that we analyzed experimentally was 2 μm in diameter, we modeled a 1-μm adhesive patch (200-nm segments) containing a maximum of 3000 bonds (estimated from integrin binding analysis). Three cases were considered: 1) uniformly distributed bonds—bonds were equally distributed among patch segments; 2) clustered bonds—bonds localized to the outermost segment until a saturation number was reached (1000), and then the next segment was filled; 3) focal adhesion-associated bonds—bonds were distributed as in the clustered case but a fraction of them were assigned as "focal adhesion-associated" bonds (see below). The force produced by each segment (F_i) was then calculated using the rule

\[ F_i = f B_i [x + (1 - x) \cdot \exp(1 - i)] \] (3)

where f is the individual bond strength, B_i is the number of bonds in segment i, and x is the fraction of bonds associated with focal adhesions. Based on previous analyses of mem-
brane peeling (Dembo et al., 1988; Ward and Hammer, 1993), bond loading for both uniformly distributed and clustered bonds was assigned an exponential decay with segment number. Focal adhesion-associated bonds were treated as "rigid"—all bonds must break simultaneously. Finally, the forces produced by all segments were added to calculate the overall force for the adhesive patch.

We ran simulations to compute the adhesive patch force as a function of total bond number using published values for the individual bond force (f = 100 pN) (Li et al., 2003) and fraction of bonds coupled to focal adhesions (χ = 0.33) (Coussen et al., 2002) (Figure 11B). For uniformly distributed bonds, adhesive force increases linearly with bond number. This is in excellent agreement with our experimental observations for initial adhesion strength (García et al., 1998a,b).

Integrin clustering alone or in combination with focal adhesion association resulted in exponential increases in adhesion force with bond number, consistent with our experimental results (Figure 7). Integrin clustering alone enhanced adhesion force over uniform bond distribution (1.6-fold enhancement for 3000 bonds) by localizing bonds to the periphery of the adhesive area and enhancing the torque resisting the applied hydrodynamic forces. Focal adhesion association further enhanced the effects of clustering alone (30%) by altering bond loading distribution. Notably, the predicted enhancements in adhesion strength arising from association with focal adhesion components agree well with the experimental results. Furthermore, the predicted values for adhesive patch force are similar to the values derived from the macroscopic model and experiments (100–200 nN). Overall, these simulations agree well with our experimental observations and assist in explaining how adhesive structure components operate as functional mechanical structures.

DISCUSSION

We integrated a robust hydrodynamic adhesion assay, quantitative biochemical approaches, and micropatterned substrates to analyze the adhesion strengthening response and to dissect the contributions of adhesive area, integrin binding, and focal adhesion assembly. By engineering cell–substrate adhesive area, the effects of integrin binding and focal adhesion assembly on adhesion strength were isolated from gross changes in spreading and cell shape. This experimental system provided sensitive and robust measurements of integrin-mediated adhesion strength over a wide range of conditions (time, adhesive area, and ligand density), and generated direct measurements of long-term (steady-state) adhesion strength. These measurements complement and greatly expand results from other approaches, such as laser tweezers, AFM, and centrifugation assays, which are limited to initial adhesion in terms of the magnitude of detachment forces that can be applied. Furthermore, the present work provides new insights on how adhesive area, integrin binding, and focal adhesion are integrated to generate functional mechanical complexes.

Adhesion strength exhibited rapid increases (~170 dyne/cm² h) until reaching steady-state values at 4 h. This fast strengthening response is consistent with previous observations from centrifugation and laser tweezers experiments (Lotz et al., 1989; Choquet et al., 1997). For 5-μm-diameter islands, the strengthening response resulted in a sevenfold enhancement in adhesion strength. This increase in adhesion strength was attributed solely to integrin clustering and focal adhesion assembly. In contrast, cells on unpatterned substrates displayed a 12-fold enhancement in strength, reflecting additional contributions from spreading and segregation of adhesive structures to the periphery of the spread cell. Available adhesive area strongly modulated adhesion strength, exhibiting linear increases for small areas (<75 μm²). Consistent with the strong dependence of adhesion strength on adhesive area, McClay and colleagues showed a positive correlation between initial adhesion strength (<15 min) and areas of cell-substrate close contact (Lotz et al., 1989). Furthermore, several groups have demonstrated a relationship between traction forces and focal adhesion area (Balaban et al., 2001; Galbraith et al., 2002; Tan et al., 2003). Notably, we demonstrate that above a threshold adhesive area (78.5 μm²), adhesion strength and focal adhesion assembly (vinculin and talin recruitment) reach a saturation limit and further increases in adhesive area do not influence these mechanical and biochemical outcomes. In addition, this adhesive area value corresponds to the transition in integrin binding from relatively uniform bond receptors to spatially segregated complexes. This saturation limit does not seem to arise from limiting numbers of FN ligand, integrin receptors or vinculin and talin molecules, as the recruited numbers for these molecules are well below the available surface density or total cellular pool. It is possible that another adhesion molecule becomes limiting above this threshold adhesive area. Alternatively, this critical point could reflect a “set point” for the adhesive interaction. The existence of a set point suggests a higher level of integrated control of the adhesion variables (integrin binding and focal adhesion assembly), different from control at the level of the focal adhesion structure. An important attribute of a set point is that it allows for robust adaptive responses to external stimuli, such as applied forces and soluble factors. For example, the set point could be shifted to increase focal adhesion area and organization to modulate traction forces during mechanical stimulation or growth factor-induced cell migration (Davies et al., 1994; Girard and Nerem, 1995; Greenwood et al., 2000; Riveline et al., 2001).

Adhesion strength exhibited nonlinear increases with bound integrin numbers and vinculin and talin recruitment, and the relationship between adhesion strength and these biochemical events was accurately described by exponential functions. The exponential dependence between adhesion strength and bond clusters is in excellent agreement with theoretical models for cell adhesion (Evans, 1985; Dembo et al., 1988; Ward and Hammer, 1993). These models propose nonuniform bond loading along the adhesive interface, with the adhesive clusters farthest from the center of the cell providing the highest adhesive forces. Indeed, a simple mechanical equilibrium analysis revealed that the increases in adhesion strength with adhesive area could be explained by an adhesive patch localized to the periphery of the adhesive area. This analysis yielded a constant 200-nN force for the adhesive patch, independently of adhesive area. We interpret this value to be an estimate of the maximum adhesive force for a functional adhesive “unit,” which comprises bound integrins and associated cytoskeletal elements. Once this maximum force is exceeded, the adhesive patch breaks and the cell detaches; other adhesive complexes within the adhesive area cannot support the applied load because their effective moment arm is shorter. The 200 nN force is in good agreement with estimates for the peeling force required to detach adherent myotubes (Ra et al., 1999). This value, however, is 10-fold higher than adhesive and propulsive forces measured on elastic substrata (Balaban et al., 2001; Beningo et al., 2001; Tan et al., 2003). This difference suggests that adhesive complexes operate in a force regime well below their adhesion strength and underscores the fact that migra-
tion assays provide measurements of contractile and traction forces and not measures of adhesion strength.

Unexpectedly, focal adhesion assembly contributed only 20–30% of the adhesion strength at steady state as determined in three independent systems. Serum stimulation of quiescent NIH3T3 fibroblasts resulted in significant recruitment of vinculin and talin to adhesive structures and a concomitant 30% increase in adhesion strength, whereas bound integrin levels remained unchanged. Vinculin-deficient F9 cells displayed adhesion strength values that were 20% lower than those for the parental cell line. Furthermore, vinculin was responsible for the adhesion strengthening response to serum stimulation in these cells. Knockdown of vinculin expression in NIH3T3 cells also reduced adhesion strength to levels comparable to those obtained by these other approaches. These results indicate that focal adhesion assembly, in particular vinculin recruitment, contributes only 30% of the adhesion strengthening response. We attribute the bulk of the enhancements in adhesion strengthening to integrin binding and clustering. In fact, a simple force simulation indicates that, compared with the adhesion force produced by uniformly distributed bonds, integrin clustering provides a 60% enhancement in adhesive force, whereas focal adhesion assembly increases adhesion strength above integrin clustering by only 30%. These findings contrast with studies indicating a strong correlation between adhesive forces and focal adhesion area (Balaban et al., 2001; Beningo et al., 2001; Tan et al., 2003). However, the contributions of integrin clustering and focal adhesion assembly were not separated in these analyses. Although this study supports a limited role for focal adhesion assembly on cell adhesion strength, it does not discount a central role for focal adhesions in regulating signaling interactions and establishing the direction/orientation of traction forces. Finally, it is important to point out that the relative contributions of the various steps to adhesion strengthening may vary among cell types or culture conditions due to differences in expression levels of particular adhesive components and/or differences in cytoskeletal and adhesive structures.

In addition, the present analysis presents a “snap shot” in time or average measurement of a highly dynamic process. The experimental framework presented provides a robust system to analyze cell adhesion strengthening responses for a wide range of experimental conditions. The combination of micropatterned substrates to control cell adhesive area size and position and the hydrodynamic adhesion assay provides sensitive and reproducible measurements of mechanochiral events at the adhesive interface. The present work focusing on the effects of adhesive area, integrin binding, and focal adhesion assembly establishes a baseline for the analysis of the function of structural and signaling adhesive components and the regulation of adhesive interactions.

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Myoblast proliferation and differentiation on fibronectin-coated self-assembled monolayers presenting different surface chemistries

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Abstract

Biomaterial surface properties modulate protein adsorption and cell adhesion to elicit diverse cellular responses in biomedical and biotechnological applications. We used alkanethiol self-assembled monolayers presenting well-defined chemistries (OH, CH\textsubscript{3}, NH\textsubscript{2}, and COOH) to analyze the effects of surface chemistry on myoblast proliferation and differentiation. Surfaces were pre-coated with equivalent densities of fibronectin. C2C12 skeletal myoblasts exhibited surface-dependent differences in cell proliferation (COOH > NH\textsubscript{2} > CH\textsubscript{3} = OH). Myogenin and troponin T gene expression levels were up-regulated on CH\textsubscript{3} and OH surfaces compared to other chemistries. Furthermore, immunostaining for sarcomeric myosin revealed surface chemistry-dependent differences in myogenic differentiation following the pattern OH > CH\textsubscript{3} > NH\textsubscript{2} = COOH. Immunostaining analyses of integrin subunits demonstrated surface chemistry-dependent differences in integrin binding to adsorbed fibronectin. OH and CH\textsubscript{3} surfaces supported selective binding of \( \alpha_5 \beta_1 \) integrin while the COOH and NH\textsubscript{2} functionalities displayed binding of both \( \alpha_5 \beta_1 \) and \( \alpha_\nu \beta_3 \). Myogenic differentiation correlated with differences in integrin binding; surface chemistries that supported selective binding of \( \alpha_5 \beta_1 \) displayed enhanced differentiation. Finally, blocking \( \beta_1 \); but not \( \beta_3 \); integrins inhibited differentiation, implicating specific integrins in the differentiation process. These results demonstrate that surface chemistry modulates myoblast proliferation and differentiation via differences in integrin binding to adsorbed fibronectin.

Keywords: Cell adhesion; Integrin; Self-assembled monolayers; Fibronectin; Differentiation; Myoblast

1. Introduction

Cell-material interactions play critical roles in host responses to implanted devices, biointegration of biomaterials and tissue engineering constructs, and the performance of cell arrays and biotechnological culture supports [1–4]. In many instances, cells adhere to synthetic surfaces via proteins adsorbed from physiological fluids and culture media. The physicochemical properties of the underlying substrate, including topography, chemistry, and surface energy, modulate protein adsorption in terms of adsorbed species density and biological activity [5,6]. Substrate-dependent differences in protein adsorption and activity often have profound consequences on cellular functions, including adhesion, spreading, migration, and differentiation [7–11].

Cell adhesion to adsorbed proteins is primarily mediated by integrin receptors [12]. Integrins represent
a widely expressed family of heterodimeric transmembrane receptors that bind to adhesive motifs present in various extracellular matrix proteins, including fibronectin (FN), vitronectin, laminin, and collagen [12,13]. Integrins function in both mechanical and signaling capacities. Following ligand binding, integrins cluster and associate with cytoskeletal elements to form focal adhesions, supramolecular assemblies of structural and signaling proteins that provide anchorage forces and activate signaling cascades regulating cell cycle progression and differentiation [14]. Using self-assembled monolayers (SAMs) to present well-defined chemistries, we demonstrated that surface chemistry modulates the structure and activity of adsorbed FN [15,16]. These differences in FN structure regulated integrin binding and focal adhesion assembly and signaling, resulting in surface chemistry-dependent differences in osteoblast differentiation [17]. In the present work, we investigated the effects of surface chemistry on the proliferation and differentiation of skeletal myoblasts. The rationale for this study was to establish whether surface chemistry modulates higher order cellular functions in other cell types. Because myogenic differentiation is a well-understood process [18], myoblasts represent an excellent cell model to analyze the effects of biomaterials on cell proliferation and differentiation. Following removal of serum growth factors, proliferation is down-regulated while expression of transcriptional myogenic regulatory factors, such as MyoD and myogenin, is up-regulated. These myogenic transcriptional activators coordinate expression of myocyte-specific proteins associated with contractile machinery, including troponin T and sarcomeric myosin, and lead to cell fusion into myotubes. Notably, the switch between proliferation and differentiation in myoblasts is also regulated by integrin binding to extracellular matrix proteins, including FN and laminin [10,19]. We demonstrate that surface chemistry modulates myogenic proliferation and differentiation via alterations in integrin binding to adsorbed FN. These results indicate that cellular activities can be modulated by the properties of the underlying substrate.

2. Materials and methods

2.1. Reagents

Cell culture reagents, human plasma FN, and Dulbecco’s phosphate-buffered saline (DPBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 7H2O, 1.5 mM KH2PO4, 0.9 mM CaCl2·2H2O, 1.0 mM MgCl2·6H2O, pH 7.4) were obtained from Invitrogen (Carlsbad, CA). Fetal bovine and horse sera were purchased from Hyclone (Logan, UT). Monoclonal MF20 (specific for sarcomeric myosin) and HFN 7.1 (specific for human FN) antibodies were purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA). Biotinylated anti-mouse IgG antibody was obtained from Jackson ImmunoResearch (West Grove, PA), while monoclonal anti-BrdU, FITC-conjugated anti-biotin and rabbit polyclonal anti-FN antibodies were obtained from Sigma-Aldrich (St. Louis, MO). Integrin-specific rabbit polyclonal antibodies were purchased from Chemicon (Temecula, CA) while integrin blocking and isotype control monoclonal antibodies were obtained from BD Biosciences Pharmingen (San Diego, CA). Ethidium homodimer-2 and Alexa Fluor 488-conjugated anti-mouse and anti-rabbit IgG and TexasRed-conjugated anti-mouse IgG antibodies were obtained from Molecular Probes (Eugene, OR). Cross-linking reagent DTSSP was obtained from Pierce Chemical (Rockford, IL). Reagents for RNA extraction and purification were obtained from Qiagen (Valencia, CA), while the Superscript First-Strand cDNA Synthesis System for reverse transcription was purchased from Invitrogen. Oligonucleotide primers were designed using Primer Express software (Applied Biosystems, Foster City, CA) and synthesized by Integrated DNA Technologies (Coralville, IA). All other real-time PCR reagents were purchased from Applied Biosystems.

2.2. Self-assembled monolayers

Alkanethiols 1-dodecanethiol (HS-(CH2)11–CH3), 11-mercaptop-1-undecanol (HS-(CH2)12–OH), and 11-mercaptoundecanionic acid (HS-(CH2)10-COOH) were purchased from Aldrich Chemical (Milwaukee, WI) and used as received. The amine-terminated alkanethiol 12-amino-1-mercaptododecan (HS-(CH2)12–NH2) was synthesized and characterized by our group [15]. Assembled SAMs of their respective alkanethiols are referred to hereafter as CH3, OH, COOH, and NH2. Gold-coated substrates were prepared by sequential deposition of optically transparent films of 75 Å Ti and 150 Å Au using an electron beam evaporator (Thermionics, Hayward, CA) at a deposition rate of 2 Å/s and a chamber base-pressure of approximately 2 x 10−6 Torr. Au-coated 35 and 100 mm tissue culture-treated polystyrene dishes were used as substrates for SAM assembly in cell proliferation and differentiation experiments. For integrin binding and FN immunostaining analyses, Au-coated 22-mm square glass coverslips were used, while Au-coated Thermanox plastic coverslips (15 mm diameter) were used for antibody blocking experiments.

For SAM assembly, freshly prepared Au-coated substrates were immersed overnight in alkanethiol solutions (1.0 mM for CH3, COOH, and OH and 0.2 mM for NH2 in absolute ethanol). SAMs were validated by contact angle goniometry and yielded equivalent results to previous goniometry and X-ray photoelectron spectroscopy characterization [15,16].
Following assembly, SAMs were rinsed with 95% ethanol, equilibrated in DPBS for 10 min, coated with human plasma FN (in DPBS) for 30 min, and blocked in 1% heat-denatured bovine serum albumin for 1 h. FN coating concentrations were adjusted to yield equivalent FN surface densities (40 ng/cm²) for all surfaces [15]. This value represents the saturation density for the OH SAM and lowest saturation density of all SAMs.

2.3. Cell model

Murine C2C12 myoblasts (ATCC CRL-1772) were used to examine the effects of surface chemistry on cell proliferation and differentiation. Cells were maintained in Dulbecco’s Modified Eagle Medium supplemented with 15% fetal bovine serum and 1% penicillin-streptomycin in a humidified atmosphere at 37°C and 5% CO₂. Cells were subcultured prior to reaching confluence (approximately every 2 days). For experiments, C2C12 cells were seeded at 1500 cells/cm² on SAMs pre-coated with equal surface densities of FN (40 ng/cm²) in Dulbecco’s Modified Eagle Medium supplemented with 0.1% fetal bovine serum and 1% penicillin-streptomycin to induce myogenic differentiation [10].

2.4. Integrin binding and FN deposition analyses

Integrin binding to FN-coated SAMs was analyzed via immunostaining following cross-linking of bound integrins to FN and extraction of cellular components [20]. Cells were seeded on FN-coated SAMs for 4 h. Cultures were rinsed in DPBS and incubated in ice-cold DTSSP (1.0 mM final concentration in DPBS+2 mM dextrose) for 30 min. Unreacted cross-linker was quenched with 50 mM Tris in DPBS for 15 min and bulk cellular components were extracted in 0.1% SDS+350 μg/mL phenylmethylsulfonyl fluoride in DPBS. After blocking with 5% FBS+0.1% Tween-20 for 1 h, bound integrins were immunostained with anti-α5 or anti-αv polyclonal antibody (1.3 μg/ml) and AlexaFluor488-conjugated secondary antibody (5 μg/ml).

For FN staining, C2C12 cultures (4 h) were fixed in 3.7% formaldehyde in ice-cold DPBS for 10 min, permeabilized in 0.1% Triton X-100 for 5 min, and blocked with 1% serum albumin for 1 h. Cultures were immunostained as described above. Human FN-specific mouse monoclonal antibody HFN7.1 (1.0 μg/ml) and FN rabbit polyclonal antibody (2.5 μg/ml) were used to probe for pre-adsorbed FN only and pre-adsorbed and cell-deposited FN, respectively. Primary antibodies were visualized via AlexaFluor488-conjugated anti-mouse IgG and TexasRed-conjugated anti-rabbit IgG secondary antibodies (5 μg/ml).

2.5. BrdU incorporation

BrdU (10 μg/ml final concentration) was added to cultures at 24 h post-seeding and incubated for 12 h. After washing with DPBS, cultures were fixed in 95% ethanol for 10 min and denatured in 2N HCl for 20 min. Following neutralization in 50 mM NaCl in 100 mM Tris-HCl (pH 7.4) and blocking in 5% FBS+1% serum albumin in DPBS, cultures were sequentially incubated in mouse anti-BrdU antibody (1:1000) and Alexa-Fluor488-conjugated anti-mouse IgG (1:200). Cell nuclei were counter-stained with Hoechst (1:10,000). Cultures were scored by fluorescence microscopy for proliferation as the percentage of cells positive for BrdU incorporation relative to cell nuclei using an in-house image analysis routine. Twenty representative images were analyzed per sample.

2.6. Real-time RT-PCR

Real-time RT-PCR was used to measure gene expression levels of myogenic markers. Cells were plated on FN-coated SAMs and harvested on day 3. Total RNA was purified using the RNasea RNA isolation kit. cDNA synthesis was performed on DNaseI-treated (27 Kunitz units/sample) total RNA (0.5 μg) by oligo(dT) priming using the Superscript™ First Strand Synthesis System. Real-time PCR using SYBR Green intercalating dye was performed with the ABI Prism 7700 Sequence Detection System (Applied Biosystems) (40 cycles, melting: 15 s at 95°C, annealing and extension: 60 s at 60°C) [21]. Oligonucleotide primers were described previously [22]. Primer specificity was confirmed by agarose gel electrophoresis and ABI Prism 7700 Dissociation Curve software. Standards for each gene were amplified from cDNA using oligonucleotide primers, purified using a Qiagen PCR purification kit, and diluted over a functional range of concentrations. Transcript concentration in template cDNA solutions was quantified from a linear standard curve, normalized to 1 μg of total RNA, and expressed as femtomoles of transcripts per microgram of total RNA. Detection limits for each gene were determined by reactions without cDNA and were at least an order of magnitude below the most dilute sample.

2.7. Myogenic differentiation assay

C2C12 cells were cultured on FN-coated SAMs for 3 days under differentiation conditions and immunostained for sarcomeric myosin [10]. Briefly, cultures were fixed in 70% ethanol/37% formaldehyde/glacial acetic acid (20:2:1) and then blocked in 5% horse serum in DPBS for 1 h. Samples were sequentially incubated in MF-20 mouse antibody (2 μg/ml), biotinylated anti-mouse IgG secondary antibody (5 μg/ml), and
FITC-conjugated anti-biotin antibody (10 μg/ml), and cell nuclei were counter-stained with ethidium homodimer-2 (200 nM). Cultures were scored by fluorescence microscopy for differentiation as the percentage of cells positive for sarcomeric myosin using an in-house image analysis routine. For inhibition experiments, integrin blocking or isotype control antibody (7.3 μg/ml final concentration for HFN7.1, 40 μg/ml final concentration for all others) was added to culture media on day 1 in order to avoid altering initial adhesion.

2.8. Statistics

Results represent at least three independent experiments performed in triplicate. Data are reported as mean ± standard error of the mean, and statistical comparisons using SYSTAT 8.0 were based on an analysis of variance (ANOVA) and Tukey’s test for pairwise comparisons with a p-value < 0.05 considered significant. In order to make the variance independent of the mean, statistical analysis of real-time PCR data was performed following logarithmic transformation of the raw data [21].

3. Results

3.1. Surface chemistry modulates myoblast integrin binding and FN reorganization

Alkanethiol SAMs were used as model surfaces to present well-defined functional groups. The CH3 and OH SAMs represent hydrophobic and neutral hydrophilic surfaces, respectively, while the COOH and NH2 chemistries display negatively and positively charged groups, respectively, at the experimental conditions (pH 7.4). We selected C2C12 murine skeletal myoblasts to analyze the effects of surface chemistry on cell proliferation and differentiation in order to extend and generalize our previous observations with osteoblasts [17]. C2C12 cells recapitulate the myogenic differentiation process associated with primary skeletal myoblasts. Furthermore, differentiation in this cell line is modulated by integrin binding to FN and blocking of either α5β1 integrin receptors or FN inhibits differentiation [10].

Integrin binding to SAMs coated with equivalent FN densities (40 ng/cm²) was evaluated using a cross-linking/extraction and immunostaining protocol. This approach specifically isolates integrins ligated to FN [10,20]. We examined binding of α5β1 and αVβ3 integrins to adsorbed FN because these receptors provide the primary adhesion mechanism to FN in this cell model [10]. Immunofluorescence staining using integrin subunit-specific antibodies revealed surface-dependent differences in integrin binding (Fig. 1). Similar staining patterns were observed for α5 and β1 subunits and αV and β3 subunits. FN-coated OH and CH3 SAMs exhibited robust α5β1 integrin binding and assembly into focal plaques and minimal αVβ3 binding. In contrast, cells on FN-coated NH2 surfaces displayed prominent, well-defined adhesive structures containing α5β1 and less intense clusters for αVβ3 integrins. The COOH surface supported binding of both α5β1 and αVβ3 integrins.

These trends in integrin binding are consistent with binding patterns observed for MC3T3-E1 immature osteoblasts adhering to the same FN-coated SAMs except for the CH3 SAM [15]. Binding of α5β1 integrin on the CH3 surface was unexpected because this surface chemistry alters adsorbed FN structure to significantly reduce α5β1 binding [15]. An explanation for these differences in integrin usage between these two cell types is surface chemistry-dependent differences in FN
reorganization. To examine this hypothesis, cultures were immunostained with FN-specific antibodies with different species reactivity. The HFN7.1 monoclonal antibody is specific for human plasma FN and does not cross-react with either bovine (serum derived) or mouse (cell secreted) FN[23] and was therefore used to probe for pre-adsorbed FN. In contrast, the rabbit polyclonal FN antibody recognizes human, mouse, and bovine FN and was used as a marker for total (pre-adsorbed and reorganized) FN. Comparing staining patterns for these antibodies revealed substrate-dependent differences in FN reorganization (Fig. 2). The CH3 SAM exhibited significant removal of pre-adsorbed FN and considerable reassembly/deposition of FN (including reorganized human FN, secreted murine and/or deposited bovine FN). The NH2 and COOH SAMs displayed considerably less FN rearrangements and the OH surface exhibited minimal removal of pre-adsorbed FN and deposition of murine/bovine FN. These results indicate that skeletal muscle cells reorganize pre-adsorbed FN to different extents depending on the properties of the underlying support.

3.2. Surface chemistry alters myogenic cell proliferation and differentiation

C2C12 myoblasts initially adhered to and spread on FN-coated surfaces. There were no gross differences in initial adherent numbers among CH3, NH2, and COOH SAMs, but approximately 20% less cells adhered to the OH surface. After one day in culture, cells became round on OH SAM while they remained spread on the other chemistries. BrdU is an analogue of thymidine that is incorporated into DNA during the synthesis phase of the cell cycle and is commonly used to determine cell proliferation. Significant differences in cell proliferation as determined by BrdU incorporation were observed among surface chemistries (Fig. 3). Greater than 80% of the cells on the COOH and NH2 surfaces were undergoing cell cycle progression over a 12-hr period, while the OH and CH3 SAMs supported significantly lower levels of proliferation.

The expression of myogenic differentiation genes was analyzed at 3 days in culture by quantitative real-time RT-PCR. Both early (myogenin) and late (troponin T) markers of myogenic differentiation exhibited similar surface chemistry-dependent differences in gene expression (Fig. 4). CH3 and OH SAMs exhibited high expression levels of myogenin and troponin T. The NH2 surface exhibited intermediate expression levels and the COOH functionality supported low levels of expression. Gene expression levels for all SAMs were higher than negative control cultures maintained on tissue culture-treated polystyrene under proliferative conditions (15% serum). No differences were observed in MyoD gene expression among SAMs, most likely because these cells committed myoblasts express MyoD at very early stages of differentiation. To further analyze substrate-dependent differences, differentiation was assessed via immunostaining for sarcomeric myosin. Fig. 5 presents fluorescence images showing sarcomeric myosin-positive differentiated cells (green) and nuclei (red). Consistent with trends in the initial adhesion and proliferation results, cell numbers at 3 days followed the
pattern COOH > NH₂ = CH₃ > OH. Myogenic differentiation displayed substrate-dependent differences following the pattern OH > CH₃ > NH₂ = COOH. These results are in good agreement with the gene expression data and follow an inverse trend with proliferation results. Finally, it is noteworthy that sarcomeric myosin-positive cells on the OH SAM were round and did not attain the bipolar morphology exhibited on other SAMs.

3.3. Specific integrins regulate myogenic differentiation on SAMs

The pattern of differentiation on FN-coated SAMs correlates with the ability of SAMs to support specific integrin binding, particularly α₅β₁. To examine the role of specific integrins on C2C12 differentiation on the CH₃ SAM, antibodies that block different integrin subunits were added to the culture media. The CH₃ surface was selected for analysis because it supports high differentiation levels and, in contrast to the OH functionality, retains high numbers of spread cells. Blocking antibodies directed against the β₁ integrin subunit inhibited myogenic differentiation, while β₁-specific antibodies had no effect when compared to IgG isotype controls (Fig. 6). Interestingly, an antibody that blocks human FN, but not murine or bovine FN, had no effects on myogenic differentiation, suggesting that the pre-adsorbed FN to the CH₃ SAM did not contribute to myogenic differentiation. This result is consistent with the observation of enhanced FN reorganization and deposition on this surface (Fig. 2).

4. Discussion

We demonstrate that surface chemistry modulates myoblast proliferation and differentiation. Previous studies have shown surface chemistry-dependent effects in adhesive interactions for other cell types [15,24–28]. Notably, our results demonstrate that the effects of surface chemistry extend beyond influencing cell spreading and adhesion to influence higher order cellular activities, such as proliferation and differentiation. We used SAMs as model substrates to present well-controlled chemistries. Coating concentrations were adjusted to yield equivalent FN densities in order to have controlled experimental conditions with a single adhesive ligand for initial adhesion. This experimental system, however, does not control for subsequent cell-mediated alterations in adhesive ligand density or composition. Myoblast differentiation exhibited an inverse relationship with proliferation, consistent with the molecular mechanisms controlling commitment to differentiation in this cell type [18]. The differences in myogenic differentiation can be attributed to surface chemistry-dependent differences in integrin binding. Surfaces that supported high, selective binding of α₅β₁ displayed enhanced differentiation. Furthermore, blocking β₁, but not β₃, integrins inhibited differentiation. These observations are in excellent agreement with α₅β₁ integrin-dependent myogenic differentiation on other synthetic and natural supports [10]. The variations in integrin binding most likely influence myogenic proliferation and differentiation by differentially modulating signaling pathways. For example, we demonstrated that surface chemistry-dependent differences in integrin binding modulate FAK phosphorylation [17]. This signaling cascade has been implicated in myogenic differentiation [29–31]. While these results demonstrate an important role for specific integrin binding in myogenic differentiation, they do not rule out contributions from other factors. For instance, differences in cell shape/spreading may account for differences in differentiation between the OH and CH₃ surfaces. Consistent with this hypothesis, Rowley and Mooney showed that
substrate compliance in combination with adhesive ligand density regulates differentiation in this cell line [32]. Additionally, other ECM-cell interactions have been shown to regulate myoblast differentiation, such as $\alpha_7\beta_1$ binding to laminin [33].

We previously demonstrated that FN undergoes significant structural changes upon adsorption to these chemistries and these alterations modulate integrin binding and differentiation in MC3T3-E1 osteoblast-like cells [15–17]. These findings are in general agreement with our present observations for C2C12 myoblasts. However, the particular chemistries supporting differentiation were different between these two cell types. For myoblasts, differentiation followed the relationship $\text{OH} > \text{CH}_3 > \text{NH}_2 = \text{COOH}$, while MC3T3-E1 osteoblastic differentiation exhibited the pattern $\text{OH} = \text{NH}_2 > \text{CH}_3 = \text{COOH}$. The OH SAM, which supported selective $\alpha_5\beta_1$ binding for both cell types, exhibited high differentiation for both cell types, and the COOH functionality, which showed little selectivity between $\alpha_5\beta_1$ and $\alpha_V\beta_3$, displayed the lowest differentiation. These results are consistent with the role of $\alpha_5\beta_1$ binding in both myogenic and osteoblastic differentiation [10,34]. In contrast, the CH$_3$ SAM supported high levels of myogenic differentiation while displaying minimal osteoblastic differentiation. These

![Image](image_url)

Fig. 5. Surface chemistry-dependent differences in myogenic differentiation at 3 days. (A) Fluorescence images showing sarcomeric myosin-positive cells (green) and cell nuclei (red) on FN-coated SAMs (bar 30 $\mu$m). (B) Quantification of sarcomeric myosin-positive and total cells. (C) Myogenic differentiation as determined by the percentage of sarcomeric myosin-positive cells showing surface-dependent differences (ANOVA, $p < 2 \times 10^{-11}$). * vs. CH$_3$ ($p < 0.0005$), † vs. OH ($p < 1 \times 10^{-5}$), $\Psi$ vs. COOH ($p < 0.0001$), $\Omega$ vs. NH$_2$ ($p < 0.0005$).

![Image](image_url)

Fig. 6. Myogenic differentiation on CH$_3$ SAM is mediated by $\beta_1$ integrins. Blocking or IgG isotype control antibodies were added to cultures at 1 day and myogenic differentiation was assessed at 3 days by immunostaining. Results are expressed as percent inhibition compared to IgG isotype control. ANOVA revealed surface-dependent differences in inhibition ($p < 0.002$). § vs. IgG control ($p < 0.03$), $*$ vs. HFN7.1 ($p < 0.01$).
differences can be attributed to cell-specific differences in \( \alpha_5\beta_1 \) integrin binding. Myoblasts displayed high levels of \( \alpha_5\beta_1 \) binding and clustering to FN-coated CH3 while osteoblasts had minimal \( \alpha_5\beta_1 \) binding. An explanation for this discrepancy in integrin usage is that myoblasts reorganize and deposit FN on the CH3-terminated SAM to overcome the pre-adsorbed FN, which does not support robust \( \alpha_5\beta_1 \) binding [15]. Immunostaining analyses confirmed enhanced removal of pre-adsorbed FN and deposition on the CH3 SAM compared to other surface chemistries. This model is also consistent with the inability of an anti-human FN antibody to block myogenic differentiation on the CH3 surface while this same antibody efficiently blocks myogenic and osteoblastic differentiation on other substrates [10,17]. Finally, Werner and colleagues have shown substrate-dependent differences in FN reorganization [35], consistent with our observations.

5. Conclusions

We demonstrate that surface chemistry modulates myogenic proliferation and differentiation via alterations in integrin binding. These results extend our previous analyses with osteogenic cells to demonstrate that surface chemistry-dependent differences in integrin binding regulate cell function. Notably, we showed cell type-specific responses in the reorganization of adsorbed FN to overcome deficiencies in adhesive activity due to surface chemistry-dependent changes in protein structure. The findings provide insights into mechanisms controlling cell-material interactions and provide materials-based strategies to modulate cellular activities.

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