ABSTRACT The interaction of the α5β1 integrin and its ligand, fibronectin (FN), plays a crucial role in the adhesion of cells to the extracellular matrix. An important intrinsic property of the α5β1/FN interaction is the dynamic response of the complex to a pulling force. We have carried out atomic force microscopy measurements of the interaction between α5β1 and a fibronectin fragment derived from the seventh through tenth type III repeats of FN (i.e., FN7-10) containing both the arg-gly-aspart (RGD) sequence and the synergy site. Direct force measurements obtained from an experimental system consisting of an α5β1 expressing K562 cell attached to the atomic force microscopy cantilever and FN7-10 adsorbed on a substrate were used to determine the dynamic response of the α5β1/FN7-10 complex to a pulling force. The experiments were carried out over a three-orders-of-magnitude change in loading rate and under conditions that allowed for detection of individual α5β1/FN7-10 interactions. The dynamic rupture force of the α5β1/FN7-10 complex revealed two regimes of loading: a fast loading regime (>10,000 pN/s) and a slow loading regime (<1,000 pN/s) that characterize the inner and outer activation barriers of the complex, respectively. Activation by TS2/16 antibody increased both the frequency of adhesion and elevated the rupture force of the α5β1/wild type FN7-10 complex to higher values in the slow loading regime. In experiments carried out with a FN7-10 RGD deleted mutant, the force measurements revealed that both inner and outer activation barriers were suppressed by the mutation. Mutations to the synergy site of FN, however, suppressed only the outer barrier activation of the complex. For both the RGD and synergy deletions, the frequency of adhesion was less than that of the wild type FN7-10, but was increased by integrin activation. The rupture force of these mutants was only slightly less than that of the wild type, and was not increased by activation. These results suggest that integrin activation involved a cooperative interaction with both the RGD and synergy sites.

INTRODUCTION

The interaction between α5β1 integrin and fibronectin (FN) plays an important role in cell differentiation, proliferation (Garcia et al., 1993; Molla and Block 2000) and migration (Pierini et al., 2000) by serving as a bridge between the cell and the extracellular matrix that mediates bidirectional signaling events through inside-out and outside-in pathways (Yamada and Miyamoto 1995; Fernandez et al., 1998). Altered expression of the α5β1 integrin and fibronectin has been correlated with both physiological and pathological processes including neural development, wound healing, tumor metastasis, and atherosclerosis (Baird et al., 2001; Greiling and Clark, 1997; Taverna and Hynes, 2001; Goh et al., 1997). In animal models, mouse embryos lacking fibronectin showed defects in the mesoderm, the neural tube, and in vascular development, and died by embryonic day 9 (George et al., 1993).

The integrin α5β1 is one of 24 known members of the integrin family of adhesion molecules, formed by the noncovalent assembly of an α-subunit (i.e., α5) and a β-subunit (i.e., β1) (Hynes, 1992). Both subunits consist of large extracellular domains of more than 940 (α5) and 630 residues (β1), a transmembrane domain, and a short cytoplasmic domain (Coe et al., 2001; Mould et al., 1997). The current model for the structural organization of α5β1 is largely based on the crystal structure of the αvβ3 integrin (Xiong et al., 2001). The ligand-binding “head” region of the α5β1 is formed by the N-terminal seven bladed β-propeller domain of α5 and the I-like domain of β1 (Mould et al., 2000; Springer 1997). Structural elements in the “head” region implicated in FN binding include the metal ion dependent adhesion site (MIDAS) of β1, and the two to four repeats of the β-propeller of α5 (Coe et al., 2001; Xiong et al., 2001; 2002).

Fibronectin, a major element of the extracellular matrix in many tissues, is a dimeric glycoprotein, and serves as a ligand for α5β1. Each subunit is composed of multiple homologous domains termed FN1, FNII, and FNIII. The arg-gly-asp (RGD) sequence in FNII domain 10 (FN10) is the crucial attachment site for FN receptors, including the integrin α5β1 (Pierschbacher and Ruoslahti, 1984; Yamada and Kennedy, 1984). A synergy site that is important for binding α5β1 is located in FN9 (Aota et al., 1994; Kimizuka et al., 1991; Obara et al., 1998; Danen et al., 1999). The sequence PHSRN was earlier reported to be important for the synergy effect (Bowditch et al., 1994, Aota et al., 1994) but more recently the synergy site has been identified as a more extended surface of FN9 (Redick et al., 2000; Kauf et al., 2001).
In recent years extensive studies have been focused on the molecular basis of integrin–ligand binding. The binding pocket of the \(\alpha_5\beta_1\)/fibronectin interaction appears to be more complex and involves both \(\alpha\)– and \(\beta\)-subunits of the integrin and FN9 and FN10 of fibronectin as determined by antibody mapping, mutagenesis, and structural studies. Crystallographic analysis of the \(\alpha_5\beta_1\) integrin complexed to a cyclic RGD peptide suggests that the RGD loop in FN10 is recognized by both \(\alpha_5\) and \(\beta_1\) subunits (Xiong et al., 2002). The aspartate residue coordinates with the metal ion in the MIDAS of the \(\beta A\) domain and the arginine and glycine residues make contact with the \(\beta\)-propeller of the \(\alpha_5\) subunit (Xiong et al., 2002). The synergy site in FN9 comprises half a dozen surface amino acids on the side of the domain facing the direction of the RGD (Redick et al., 2000; K auf et al., 2001) and is recognized primarily by the third and fourth repeats in the \(\beta\)-propeller of the \(\alpha_5\) subunit (Burrows et al., 1999; Mould et al., 1997; Mould et al., 1998).

Recent models predict that the \(\alpha_5\beta_1\) integrin can exist in multiple conformational states (inactivated, intermediate activated, and fully activated) that have different affinities for FN (Garcia et al., 1999b). The \(\alpha_5\beta_1\) integrin is activated by inside-out signaling that appears to act on the cytoplasmic tails, which releases the constraint between the two subunits and subsequently exposes a high affinity “open” conformation for ligand binding (Takagi et al., 2001). \(\alpha_5\beta_1\) can also be activated by nonphysiological stimuli such as activating monoclonal antibodies (e.g., T52/16 and AG89; Arroyo et al., 1993; Tsuchida et al., 1998) and high concentrations of extracellular Mg\(^{2+}\) or Mn\(^{2+}\) (Mould et al., 1995). Structural analysis of the \(\alpha_5\beta_1\) integrin has lent support to the idea that \(\alpha_5\beta_1\) activation may involve conformational change in the \(\beta A\) domain and a reorientation of the extracellular domains of the \(\alpha\)– and \(\beta\)-subunits (Xiong et al., 2002).

Cell–matrix interactions usually occur in the context of a complex process where either an external or internal force acts on the cell. For example, when cells migrate, the traction force generated inside the cell is applied to the integrin/FN complex. It is, therefore, important to understand how a pulling force affects the dynamics of the integrin–ligand complex.

A number of advanced techniques have been developed to measure the rupture force of molecular adhesion. Commonly used techniques employed in single molecule force measurements include the biomembrane force probe (Evans et al., 1995), the atomic force microscopy (AFM) (Moy et al., 1994), optical tweezers (Litvinov et al., 2002; Thoumine et al., 2000; Thoumine and Meister, 2000), and the parallel-plate flow chamber (Chen and Springer 2001). The dynamics of selectin-mediated adhesion was characterized by biomembrane force probe and AFM measurements (Evans et al., 2001; Fritz et al., 1998), and in the parallel-plate flow chamber (Chen and Springer 2001). The AFM technique was also used to measure the adhesion force between osteoblast and several RGD-containing ligands (Lehenkari and Horton 1999). In a recent study closely related to ours, Litvinov et al. (2002) used laser tweezers to measure the rupture force of \(\alpha_{IIb}\beta_3\) integrin to fibrinogen, with the integrin both on living platelets and as purified protein (Litvinov et al., 2002).

In this report, we present direct force measurements, acquired by AFM, of the interaction between the integrin \(\alpha_5\beta_1\) and fibronectin. In these experiments, we used the K562 cell line, which expresses the \(\alpha_5\beta_1\) integrin, but no other FN receptors (Hemler et al., 1987). Our measurements were carried out using the fibronectin fragment, FN7-10, which consists of FN type III repeats 7–10, rather than whole plasma fibronectin to avoid misinterpretation of measurements acquired from a system that has multiple binding sites (Hocking et al., 1998). The \(\alpha_5\beta_1\) integrins of K562 cells are constitutively inactive, but can be activated by various monoclonal antibodies against \(\beta_1\) (e.g., T52/16). The acquired data were analyzed in the framework of the dynamic force model of Evans and Ritchie (1997). Studies were also carried out with FN fragments that contain either a deletion of RGD sequence or mutations of the synergy site to identify the contributions of these sites to the interactions of the \(\alpha_5\beta_1\)/FN complex.

**METHODS**

**Cells and reagents**

The \(\alpha_5\beta_1\)-expressing K562 (ATCC, CCL-243) cell lines was maintained in continuous culture in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Irvine Scientific, Santa Ana, CA), penicillin (50 U/ml, Gibco BRL, Grand Island, NY) and streptomycin (50 \(\mu\)g/ml, GIBCO BRL). The K562 cells were expanded on a 3-day cycle.

The T52/16 monoclonal antibody, which activates \(\beta_1\) integrins, was derived from supernatants of hybridoma cells (ATCC, HB243) maintained in culture. The K562 cells were activated by adding 1/10 volume (v/v) of T52/16 culture supernatant for 10 min at room temperature. The anti-\(\beta_1\) integrin monoclonal antibody, PS2D, which inhibits the binding of \(\beta_1\) integrins to FN, was a generous gift from Dr. J. Li (Dept. of Dermatology, University of Miami) (Caixia et al., 1991). JBS5 is a mouse IgG against \(\alpha_5\) integrins and was purchased from Serotec (Raleigh, NC) (Wayner et al., 1993). Polyclonal mouse IgG was purchased from Sigma (St. Louis, MO).

A plasmid containing FN7-10 in the pET11b vector was previously described (Aukhil et al., 1993; Leahy et al., 1996). A mutant deleting the RGD (FN7-10(\DeltaRGDS)), and a triple mutant that inactivates the synergy site (FN7-10(R1374A/P1376A/R1379A)), referred to as FN7-10(\DeltaSyn), were described in Redick et al. (2000). Proteins were expressed in BL21 cells and purified by (NH4)2SO4 precipitation followed by chromatography on a mono Q and crystallization in low pH sodium formate (Redick et al., 2000). Human plasma fibronectin (pFN) was purchased from Sigma and used without further purification.

**Attachment of cell to AFM cantilever**

K562 cells were attached to the AFM cantilever by concanavalin A (Con A)-mediated linkages. The cantilevers were soaked in acetone for 5 min, UV irradiated for 30 min, and incubated in biotinamidocaproyl-labeled bovine serum albumin (biotin-BSA, 0.5 mg/ml; Sigma) overnight at 37°C. The cantilevers were then rinsed three times with phosphate buffer and incubated in 0.5 mg/ml streptavidin (Pierce; Rockford, IL) for 10 min at room temperature. After the removal of unbound streptavidin, the cantilevers were used without further purification.
incubated in 0.5 mg/ml biotinylated Con A (Sigma) and then rinsed. To attach the cell to the cantilever, the tip of the Con A-functionalized cantilever was positioned above the cell and lowered onto the cell for ~1 s. The attached cell was positioned behind the tip of the cantilever as shown in Fig. 1. To obtain an estimate of the strength of the cell-cantilever linkage, we allowed the attached cell to interact with a petri dish coated with Con A. Upon retraction of the cantilever, separation always (N > 20) occurred between the cell and the Con A-coated petri dish. In these measurements, the average force needed to induce separation was ~2 nN, much larger than the forces found for an individual α5β1/FN bond. This observation is important inasmuch as it ensures that the cell remained bound to the cantilever during the single molecule α5β1/FN force measurements.

### Immobilized protein

Whole human plasma fibronectin, FN7-10, or FN7-10 with deleted RGD or synergy site mutants (100 μg/ml in 0.1M NaHCO3) were adsorbed overnight at 4°C on 35-mm tissue culture dishes. Unbound FNS were removed and the dish was incubated with 1% bovine albumin for 1 h at 37°C to block the exposed surface of the tissue culture dish.

### AFM force measurements

The AFM force measurements were performed on an apparatus designed to be operated in the force spectroscopy mode (Heinz and Hoh, 1999). A K562 cell was attached to the end of the AFM cantilever as described above. A piezoelectric translator was used to lower the cantilever/cell onto the FN-coated dish. Contact between the attached K562 cell and the sample was indicated by deflection of the cantilever, which was measured by reflecting a laser beam off the cantilever into a position sensitive two-segment photodiode detector.

Measurements of unitary α5β1/FN rupture forces were obtained under conditions that minimized contact between the K562 cell and the FN-coated dish. An adhesion frequency of <30% in the force measurements ensured that there is a >83% probability that the adhesion event is mediated by a single α5β1/FN bond (Merkel et al., 1999; Tees et al., 2001). We were able to acquire measurements with loading rates between 10 and 50,000 pN/s by adjusting the retraction speed of the cantilever (1-15 μm/s) and by variations in the local elasticity of the cell (0.01-5.0 mN/m) that allowed for the system spring constant (the cell-cantilever combination) to vary between 0.01 and 3.33 mN/m. To satisfy the condition of constant loading rate required for our analysis, measurements were selected for our analysis only if there was a sustained linear increase in force with respect to time before bond dissociation. At fast cantilever retraction speeds (~1 μm/s), the hydrodynamic drag on the cantilever resulted in smaller forces recorded than were actually applied to rupture the complex. To correct for the hydrodynamic force exerted on the cantilever, we determined the damping coefficient of the cantilever ξ (~2 pN-s/μm) in the culture medium by measuring the deflection of cantilever at different retraction speeds. The rupture force plotted in Figs. 4-8 is the sum of the measured force and the hydrodynamic force. All AFM force measurements were carried out at 25°C with fresh culture medium supplemented with 10 mM HEPES buffer.

The AFM cantilevers were purchased from TMD microscopes (Sunnyvale, CA). The largest triangular cantilever (320 μm long and 22 μm wide) from a set of five on the cantilever chip was used in our measurements. These cantilevers were calibrated by analysis of their thermally induced fluctuation to determine their spring constant (Hutter and Bichhoefer, 1993). The experimentally determined spring constants were consistent with the nominal value of 10 nN/m given by the manufacturer.

### RESULTS

#### Measurements of α5β1/FN interactions by AFM

Direct force measurements by AFM were employed to characterize the adhesive interaction between immobilized FN and α5β1 integrin expressed on the surface of the human chronic myelogenous leukemia cell line, K562. The cell adhesion studies were carried out with a K562 cell coupled to the AFM cantilever and FN adsorbed to a tissue culture dish (Fig. 1). In these measurements, the K562 cell was lowered onto the dish until contact was made. The interaction between the cell and the dish was regulated by the applied force of 100–500 pN exerted by the cantilever. After a given contact duration, the K562 cell was withdrawn from the dish at a separation rate of 5 μm/s while the force versus piezo displacement trace of the process was recorded (Fig. 2A). The adhesive interactions between the cell and protein coating on the dish were detected as downward deflections of the cantilever. We and others have found that this experimental design allows investigators to study the dynamics of cell adhesion involving many adhesion molecules, as well as the properties of individual molecular complexes (Benoit et al., 2000; Zhang et al., 2002).

Fig. 2 A presents measurements acquired under conditions where cell adhesion was mediated by multiple α5β1/FN7-10 complexes. These complexes did not necessarily rupture simultaneously during detachment as revealed by the "sawtooth" profile in the AFM traces. Each of the force jumps as indicated by the arrows in Fig. 2 A is interpreted to correspond to the breakage of one or more α5β1/FN7-10 complexes. It is unlikely that these force jumps corresponded to the breakage of other molecular linkages and/or the unfolding of proteins on the basis of single molecule measurement analysis as discussed below. Adhesion between the K562 cell and FN7-10 increased after the addition of TS2/16, an activating monoclonal antibody against β1 integrins (Aroyo et al., 1993). Activation of α5β1 by TS2/16 was dose-dependent. 10% culture supernatant of TS2/16 (~1 μg/ml antibody in final concentration) was sufficient to fully activate α5β1. Higher concentrations (up to 20 μg/ml)
ruptured. The shaded area in the middle trace is the detachment energy. Higher detachment energies are due to both a larger number of adhesions and larger forces of detachment. The error bar is the standard deviation.

**AFM measurements of individual \( \alpha_5\beta_1/FN \) interactions**

To assess the bond strength of an individual \( \alpha_5\beta_1/FN7-10 \) interaction, contact between the cell and the dish was minimized by reducing both contact duration (<50 ms) and compression force (100 pN). Examples of unfiltered force measurements acquired under these conditions are shown in Fig. 3A. Under these conditions ~30% of measurements resulted in adhesion (i.e., a 30% frequency of adhesion). Traces that registered a sharp transition of more than 30 pN were counted as an adhesion event. Forces of less than 30 pN were excluded inasmuch as the observed fluctuations of the free cantilever were frequently 20 pN. The rupture force of the \( \alpha_5\beta_1/FN7-10 \) complex was derived from the magnitude of the force transition after correction for hydrodynamic drag. It should be noted that the majority of the measurements acquired under these conditions registered a single transition in force. This transition stemmed from the forced unbinding of a single \( \alpha_5\beta_1/FN7-10 \) complex. Moreover, such observations also revealed that the dissociation of the \( \alpha_5\beta_1/FN7-10 \) complex does not involve the initial unfolding of the proteins as observed in the forced unfolding of FN domains (Oberhauser et al., 1998).

To assess the contributions of the RGD loop and of the synergy site residues of FN to the binding force of the \( \alpha_5\beta_1/FN \) interaction, we have obtained measurements of the mean rupture forces of untreated and activated \( \alpha_5\beta_1 \) complexed to FN7-10, FN7-10(Δsyn), and FN7-10(ΔRGD). The force values presented in Fig. 3B were from measurements acquired at loading rates of 1800–2000 pN/s. Under these conditions, the mean rupture force of the \( \alpha_5\beta_1/FN7-10 \) complex was elevated from 69 pN ± 1.5 (mean ± SE) to 93 pN ± 1.5 after integrin activation by the TS2/16 mAb.
However, there was no enhancement in the bond strength of either α5β1/FN7-10(Δsyn) or α5β1/FN7-10(ΔRGD) complexes after antibody activation. The force measurements also revealed a slightly lower rupture force for both α5β1/FN7-10(Δsyn) and α5β1/FN7-10(ΔRGD) complexes relative to the wild type complex.

The specificity of these measurements was verified by a significant reduction in the frequency of adhesion after the addition of the function-blocking anti-α5 antibody, JBS5 (Fig. 3 C). The frequency of adhesion did not change when polyclonal murine IgG (50 μg/ml) was added (data not shown). Interestingly, the frequency of adhesion for α5β1/FN7-10 interactions is only doubled when the integrin is activated. However, it should be emphasized that the adhesion frequency is dependent on the association rate of the α5β1/FN interaction and the lateral diffusion of α5β1. Inasmuch as the force measurements were carried out with surface contact time of less than 50 ms, there was insufficient time for a complete lateral redistribution of membrane proteins. Hence, under these conditions, differences in the frequency of adhesion of the different α5β1/FN pairs reflected differences in the association rate (k\textsubscript{on}) of the different pairs. Our observation that the number of adhesions is only doubled for FN7-10 when the integrin is activated revealed that the k\textsubscript{on} of activated α5β1 is slightly faster than the k\textsubscript{on} of the low affinity α5β1. This suggests that the enhanced cell adhesion stemmed primarily from changes in the dissociation rate of the complex.

Fig. 4, A–D present histograms of the rupture force of the α5β1/FN7-10 complex. A shift in the force histogram toward higher forces was observed with increasing force loading rates for both untreated (compare Fig. 4, A and B) and TS2/16 activated (compare Fig. 4, C and D) K562 cells. There was also a shift toward higher forces (compare Fig. 4, A and C) upon cell activation at a slow loading rate (230–240 pN/s), but not at the fast loading rate of 13,000–13,500 pN/s (compare Fig. 4, B and D). Fig. 4 E presents the force spectrum (i.e., force versus loading rate relation) of the α5β1/FN7-10 interaction for loading rates of 20 pN/s to 50,000 pN/s. The force spectrum revealed that the rupture force of the α5β1/FN7-10 complex increased gradually over three orders of magnitude in loading rate. After α5β1 activation with the TS2/16 antibody, the rupture forces of the complex were elevated over the range of loading rates between 20 to 10,000 pN/s, but did not change the dynamic response of the system spring constant and is used to determine the loading rate of the measurement. The cantilever retraction rate of the measurements was 5 μm/s. (A) Mean rupture forces of individual α5β1 integrin/FN bond, k\textsubscript{5} is the system spring constant and is used to determine the loading rate of the measurement. The cantilever retraction rate of the measurements was 5 μm/s. (B) Mean rupture forces of individual α5β1 integrin/FN bond, k\textsubscript{5} is the system spring constant and is used to determine the loading rate of the measurement. The cantilever retraction rate of the measurements was 5 μm/s. (C) Adhesion frequency of K562/FN binding under conditions of limited tip–substrate contact. Open and gray bars correspond to adhesion frequencies before and after the addition of the function-blocking antibody, JBS5 (20 μg/ml), respectively.
complex at loading rates greater than 10,000 pN/s. Similar results were obtained in force measurements between K562 cells and plasma fibronectin immobilized on a solid surface (Fig. 5).

In our AFM measurements, it was assumed that the measured rupture force stemmed from the unbinding of the $\alpha\beta_5/FN$ complex, although there are other linkages that can break during the measurement. For example, it is conceivable that $\alpha\beta_5$ could be extracted from the cell membrane or that FN could come unbound from the surface of the petri dish. However, inasmuch as the interaction of $\alpha\beta_5$ with plasma FN and FN7-10 displayed similar force spectra (Fig. 5), it is unlikely that the measured rupture force stemmed from the detachment of FN. An earlier study (Garcia et al., 1998a), using fluid shear to detach cells, showed that when the $\alpha\beta_5/FN$ bond was stabilized by covalent cross-linking, the force for detachment doubled. This implies that this bond is weaker than that holding the integrin in the membrane, or that FN could come unbound from the surface of the petri dish.

FIGURE 5 Single molecule force measurements between human plasma fibronectin and unactivated K562 cells (open square) or K562 cells (solid square) activated by TS2/16. The mode rupture force is plotted as a function of loading rates. Measurements of the $\alpha\beta_5/FN7-10$ interaction are plotted in open and closed circles.

FIGURE 6 Single molecule force measurements of the interactions between live K562 (open circle) or fixed K562 cells (solid circle) and FN7-10. The mode rupture force is plotted as a function of loading rates.

The force spectrum obtained using live cells, an observation that is consistent with $\alpha\beta_5$ remaining attached to the cell. Considered together, these observations are all consistent with the measured breakage occurring at the $\alpha\beta_5/FN$ junction. Moreover, the measurements acquired with the fixed cells also served to demonstrate that the viscoelastic properties of the K562 cell did not significantly alter the force measurements.

Deletion of the RGD loop from FN7-10 suppressed the rupture force at fast loading rates

To assess the role of the RGD loop of FN10 in the interactions of $\alpha\beta_5$ with FN, we have also carried out AFM measurements between individual $\alpha\beta_5$ integrin and a fibronectin fragment with the RGD loop deleted. Although the interaction between $\alpha\beta_5$ and FN7-10($\Delta$RGD) was weak, we were able to demonstrate specific binding by measuring the adhesion frequency before and after the addition of the inhibitory anti-$\alpha_5$ monoclonal antibody JBS5. Under conditions that resulted in an initial 10% adhesion frequency, the addition of the JBS5 lowered the adhesion frequency to <3%, a 70% reduction (Fig. 3 C). The addition of polyclonal murine IgG (50 $\mu$g/ml) did not affect adhesion (data not shown).

Fig. 7 presents the force spectra of the interaction between the FN7-10 RGD deletion mutant and $\alpha\beta_5$ of untreated and TS2/16 activated K562 cells. There were no striking differences between FN7-10($\Delta$RGD) and wild type FN7-10. First, the TS2/16 antibody showed no elevation of the rupture force. The force at lower loading rates was comparable to that for FN7-10, but there was no increase upon activation. Second, the binding to FN7-10($\Delta$RGD) did not show the increase in slope at high loading rates. Thus, it appears that the inner activation barrier of the $\alpha\beta_5/FN$ interaction was suppressed by the deletion of the RGD sequence from FN.
Mutations to the synergy site of fibronectin suppressed the rupture force at slow loading rates

The binding of FN to αβ₁ integrin is significantly enhanced by interactions of the synergy site residues within FN9 with αβ₁ integrin (Aota et al., 1994; Kimizuka et al., 1991; Ibata et al., 1988; Redick et al., 2000; Kau et al., 2001). To assess the contribution of the synergy site residues in the αβ₁/FN interaction, we have carried out AFM force measurements with a FN fragment with triple mutations in the synergy site (R1374A/P1376A/R1379A) (Fig. 8). The interaction between αβ₁ and FN7-10(Δsyn), the synergy site mutant, was weak, but detectable. As with the RGD deletion mutant, the binding to FN7-10(Δsyn) was not enhanced when the integrin was activated by TS2/16. However, in contrast to the RGD mutant, binding to FN7-10(Δsyn) showed the increased force slope at high loading rates, essentially the same as wild type FN7-10.

DISCUSSION AND CONCLUSIONS

A theoretical framework for understanding how force can affect the adhesion complex was proposed by Bell (Bell et al., 1984), and later expanded on by other researchers (Evans and Ritchie, 1997; Merkel et al., 1999). In this model, an applied force f distorts the energy landscape of the αβ₁FN complex resulting in a lowering of the activation barrier(s), and consequently increases the dissociation rate constant k(t) as follows: k(t) = k₀ exp(γf/kₐT) where k₀ is the dissociation rate constant in the absence of the applied force, γ is the position of the transition state, T is temperature, and kₐ is Boltzmann’s constant. Under conditions of constant loading r₁, the probability density function for the forced unbinding of the adhesion complex is given by:

\[ P(f) = k₀ \exp\left(\frac{\gamma f}{kₐT}\right) \exp\left\{\frac{kₐk₉T}{\gamma f} \left(1 - \exp\left(\frac{\gamma f}{kₐT}\right)\right)\right\} \]  

(1)

Moreover, the mode of the rupture force distribution f_m can be expressed as a linear function of the ln(r₁),

\[ f_m = \frac{kₐT}{\gamma} \ln\left(\frac{\gamma}{kₐk₉T}\right) + \frac{kₐT}{\gamma} \ln(r₁), \]  

(2)

where the y-intercept is

\[ y₀ = \frac{kₐT}{\gamma} \ln\left(\frac{\gamma}{kₐk₉T}\right) \]  

and the slope

\[ m = \frac{kₐT}{\gamma}. \]  

(see Tees et al., 2001). The force-induced dissociation of a ligand-receptor complex may involve overcoming multiple activation energy barriers. In the case where the system must overcome a series of increasingly higher activation barriers before final dissociation, the dissociation kinetics of the complex at low pulling forces is governed by the properties of the outermost barrier. With increasing pulling forces the outermost barriers are suppressed and the dissociation kinetics of the system is then governed by the properties of an inner activation barrier. Similarly, the force spectrum of the system is divided into multiple loading regimes that characterize the individual activation energy barriers. The dynamic strength (i.e., rupture forces) of the complex measured in slow loading regimes characterizes the outermost activation energy barriers, whereas the force measurements obtained in the fast loading regimes characterize the innermost barriers (Evans and Ritchie, 1997).

An examination of the force spectra of the αβ₁ FN7-10 interaction revealed that our measurements of the αβ₁FN7-10 interaction are not compatible with the single barrier Bell model. However, the acquired force spectra is consistent with
an intermolecular potential that consisted of two activation energy barriers. These energy barriers were characterized by fitting Eq. 2 to the acquired force measurements. The fitted curves are overlaid on the measurements in Fig. 4A and the best-fit parameters, \( k \) and \( \gamma \), are tabulated in Table 1. In Table 1, \( \text{h} \alpha \beta_2 \) and \( \text{h} \alpha \beta_3 \) correspond to the low and high affinity form of \( \alpha \beta_2 \), respectively. This analysis revealed that the forced unbinding of \( \alpha \beta_2/\text{FN} \) complex involved overcoming at least two activation barriers. The positions of the transition states of the inner and outer barriers from equilibrium are \(-0.9\) Å and \(-4.2\) Å, respectively, for both the low and high affinity forms of the \( \alpha \beta_2/\text{FN} \) complex. Moreover, our analysis revealed that the observed increase in rupture force after integrin activation in the low loading regime stemmed from an elevation of the outer activation energy barrier, which is manifested in a lowering of the dissociation rate constant from 0.13/s to 0.012/s. These values are consistent with the dissociation rates obtained in other studies (Akiyama and Yamada, 1985; Thoumine et al., 2000). The inner activation barrier remained unaffected by the integrin activation via TS2/16 inasmuch as there was no change in the dynamic strength of the complex in the fast loading regime after activation. To determine more precisely the change in activation energy of the complex after TS2/16 binding, we estimated that the energy differences \( (\Delta \Delta G^1) \) between transition state energies of high and low affinity complexes \( (\Delta G^1) \) and \( (\Delta G^2) \) to be \(-0.14\) \( k_B T \) for the inner barrier and \(-2.38\) \( k_B T \) for the outer barrier (see Fig. 9). From this analysis, we concluded that the high affinity state of the \( \alpha \beta_2/\text{FN} \) complex stemmed from the elevation of the outer activation energy barrier of the complex.

\[
k_{\text{off}} = 1/\{k_1^{-1} \exp[-f \gamma_1/k_BT] + k_2^{-1} \exp[-f \gamma_2/k_BT]\},
\]

where the subscripts 1 and 2 refer to inner and outer activation energy barriers, respectively (Evans et al., 2001). The kinetic profiles revealed the profound impact of a pulling force on the rate of unbinding of the \( \alpha \beta_2/\text{FN} \) complex (Fig. 10A). As shown, the dissociation rate constant increased exponentially with pulling force from a zero force off rate of \(-0.012/s\) to \(-3/s\) for activated \( \alpha \beta_2 \) at a force of 50 pN. The off rate also increased by about the same amount for low affinity \( \alpha \beta_2 \). The kinetic profiles also revealed that the dissociation rate was less responsive to change in force at higher pulling forces (>~90 pN). It is this ability to resist dissociation at high forces that allowed the \( \alpha \beta_2/\text{FN} \) complex to mediate strong adhesion. It should be noted that the dynamic responses of both low and high affinity forms of the \( \alpha \beta_2/\text{FN} \) interaction are nearly identical at high forces, which would suggest that the molecular determinants of the inner barrier are the same for both high and low affinity \( \alpha \beta_2/\text{FN} \) complexes.
Clues into the molecular determinants of the inner and outer activation barriers were derived from the force measurements obtained with site-directed mutants of FN7-10. The RGD loop in domain 10 of FN is crucial for integrin binding. In the α5β3 integrin, the RGD sequence interacts with both the α- and β-subunits (Xiong et al., 2002). The crucial interaction is the electrostatic interaction between the aspartate residue of the RGD sequence and the chelated metal ion in MIDAS of the βA domain of the β1 subunit. Our studies revealed that the deletion of the RGD loop from FN7-10 resulted in the suppression of rupture force of the α5β3/FN complex in the fast loading regime (>10,000 pN/s; Fig. 7). This region of the force spectrum characterizes the inner activation barrier of the complex. Thus, interactions mediated by the RGD sequence appear to be responsible for the inner activation barrier. However, it should be emphasized that deleting the RGD also affected the low force, outer barrier region, where it eliminated the enhanced binding upon integrin activation. Moreover, the frequency of adhesion was substantially reduced for the inner activation barrier. An outer barrier operates at lower, physiologically relevant forces. The adhesion molecule L-selectin binding to its carbohydrate ligand gave a force spectrum with a shape similar to that of α5β3/FN, but with significantly lower forces (Evans et al., 2001). The most frequent rupture forces were 20 and 70 pN at 200 and 20,000 pN/s. In contrast, the adhesion of LFA-1, a leukocyte integrin, binds to its ligand ICAM-1 with higher forces than that of α5β3/FN (Zhang et al., 2002).

A comparison of kinetic profiles of the α5β3/FN7-10 and LFA-1/ICAM-1 complexes revealed that although the α5β3/FN7-10 interaction is more stable than the LFA-1/ICAM-1 interaction with slow dissociation kinetics in the absence of force, the force dependent dissociation of α5β3/FN7-10 interaction is more sensitive to a pulling force. The dissociation rate of the α5β3/FN7-10 complex quickly exceeds the dissociation of the LFA-1/ICAM-1 complex at ~50 pN (Fig. 10 B). This analysis shows that the LFA-1/ICAM-1 complex is a more stable adhesion system at force >50 pN and suggests that the α5β3/FN7-10 interaction may not be suited for resisting a large pulling force.

In summary, the current study has identified two barriers to the unbinding of the α5β3/FN complex. An inner barrier, which is affected by deletion of RGD, but not by the synergy site, is seen at high forces that may not be physiologically important. An outer barrier operates at lower, physiologically relevant forces. This outer barrier is affected by both RGD and synergy regions, and it is the site of integrin activation. FN lacking either RGD or synergy site binds weakly to α5β3, and there is no enhancement of binding upon integrin activation. In this low force region α5β3 binding to FN is substantially stronger, with an ~10-fold longer lifetime, than the binding of LFA to ICAM-1.

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