

## Specific Aims

Force generation is essential during the cell migration process. It involves the linkage between matrix molecules (like fibronectin), adhesion receptors (integrins) and the actin cytoskeleton. The centripetal movement of actin filaments powered by myosin generates the contraction force to pull the cell forward and/or the matrix rearward. The linkage between integrin and actin cytoskeleton can be reinforced by the matrix rigidity. This reinforcement process may involve more accumulation of cytoskeletal proteins or local polymerization of actin filaments. An assay of the amount of force exerted on a single integrin-cytoskeleton linkage would provide information about the mechanism of this reinforcement process and could help to define the functions of the molecules involved.

### 1. The Measurement of the Strength of Single Integrin-Cytoskeleton Linkage

Binding of trimeric and pentameric fibronectin fragments (FNIII7-10) with integrins ( $\alpha 5\beta 1$ ) has been shown to activate FAK (focal adhesion kinase) kinase and cause  $\alpha 5\beta 1$  attachment to the cytoskeleton (Coussen et al., in revision). In contrast, binding of monomeric and dimeric FNIII7-10 does not activate FAK. Thus, the smallest cluster of liganded integrins that activates cytoskeleton attachment and kinase is a trimer. Analysis of the force needed to break the cytoskeleton linkages to single (double) trimer bound beads will enable us to determine the strength of the linkages per integrin and will show how the strength scales with integrin numbers.

### 2. The Effects of Interaction between Talin and $\beta 1$ Integrin on the Strength of Integrin-Cytoskeleton Linkage

Each talin subunit has two integrin-binding sites on its head and rod domain, while the talin binding sites in  $\alpha 5\beta 1$  integrin are located at two NPXY motifs in the  $\beta 1$  integrin cytoplasmic domain. We will transfect cells with a series of  $\beta 1$  integrin cytoplasmic domain mutants or talin mutants and measure the strength of integrin-cytoskeleton linkage. The force value will provide information about how the

interaction between talin and  $\beta 1$  integrin cytoplasmic domain affects the strength of integrin-cytoskeleton linkage.

### 3. The Effects of Vinculin Recruitment in the Reinforcement Process

Vinculin has been proposed to be a regulatory instead of a structural protein in focal contacts assembly. Talin (dimer) has six binding sites for vinculin and talin may be responsible for recruiting vinculin to the integrin-cytoskeleton linkage in the reinforcement process. We will transfect talin knock-out (-/-) cells with talins mutated at vinculin-binding sites and measure the strength of integrin-cytoskeleton linkage. This assay will tell us if vinculin accumulation can contribute to the reinforcement process.

## Background

Cell migration is an essential part of many biological phenomena (Lauffenburger and Horwitz 1996). It requires the coordination of a series of biophysical processes including membrane extension and retraction, formation of new attachments at the cell front, generation of contraction and traction force, and release of old attachments at the cell rear (Sheetz et al. 1998). Most of the above steps are found in lamellipodia, where the cell first contacts matrix molecules (Sheetz et al. 1998). When matrix molecules bind to integrins on the cell surface, the integrins will be activated and linked to the actin cytoskeleton (Kucik et al. 1991). The centripetal transport of actin filaments believed to be powered by myosin II will generate rearward force on the matrix molecules, then pull the cell forward (Galbraith and Sheetz 1998).

Obviously, the linkage of matrix (fibronectin)-integrin ( $\alpha5\beta1$ )-cytoskeleton plays an important role in the above process. It provides an anchoring point against which traction/contraction forces can be generated to power the forward migration of the cell. Integrins are heterodimeric, transmembrane proteins composed of  $\alpha$  and  $\beta$  subunits (Howe et al. 1998). Previous experiments (Schmidt et al. 1993) have shown that the cytoplasmic domain of  $\beta1$  integrin is essential for attaching integrin to the underlying cytoskeletal network. Mutational analysis of the 47-amino acid  $\beta1$  cytoplasmic domain has identified two NPXY (NPKY 797-800, NPIY 785-788) motifs important for integrin binding with talin (Pfaff et al. 1998), one component of the cytoskeleton underlying the fibronectin-integrin linkage (Giancotti 2000). In v-src-transformed cells, the NPXY motifs in the  $\beta1$  tail is phosphorylated on tyrosine and talin binding is inhibited (Tapley et al. 1989). This suggests that phosphorylation at these sites may negatively regulate integrin interactions with talin or the cytoskeleton.

Talin is a homodimeric protein, composed of two ~270kDa subunits arranged in antiparallel. Each subunit has an N-terminal head domain and an elongated C-

terminal rod domain (Critchley 2000). Talin has binding sites for integrin  $\beta 1$  cytoplasmic domain (Calderwood et al. 1999), cytoskeletal proteins (vinculin and F-actin) (Bass et al. 1999) (Hemmings et al. 1996) and the focal adhesion kinase (FAK) (Borowsky and Hynes 1998). For talin knock-out (-/-) cells, they couldn't assemble focal adhesions and actin stress fibers. This indicated that talin may play a key role in linking liganded integrins to F-actin (Priddle et al. 1998) (Critchley 2000).

As an important mediator of FN-integrin-talin-actin cytoskeleton linkage, talin can also recruit more cytoskeletal proteins like vinculin to stabilize/strengthen the linkage. There are potentially six vinculin binding sites in the antiparallel talin dimer (Bass et al. 1999; Critchley 2000). Vinculin has binding sites for talin,  $\alpha$ -actinin, F-actin, PIP2 and VASP (Critchley 2000). Thus, it has been proposed that vinculin may be able to crosslink and therefore stabilize the interactions both between talin and F-actin and between talin and the lipid bilayer (Critchley 2000). It has been shown that reduced levels of vinculin decreased the mechanical stiffness of the integrin-cytoskeleton linkage and increased cell motility (Xu et al. 1998). However, in vinculin knock-out (-/-) cells, focal adhesions could still be assembled although they are less numerous and have an altered morphology (Volberg et al. 1995). These findings support the idea that vinculin is not an essential factor for focal adhesion assembly and it may play a regulatory instead of a structural role in focal contacts.

Choquet et al (1997) demonstrated that matrix rigidity can cause the strengthening of integrin-cytoskeleton linkage, which results in a greater traction force. This phenomenon was defined as a "reinforcement" process (Choquet et al. 1997), which indicated that cells could sense and respond to the physical nature of their extracellular contacts. As the linkage becomes stronger, it will reach a point which the maximum strength of the linkage is achieved. If a bead bound to the cell surface is restricted by the laser tweezer, a greater trap force more than the maximum strength of the linkage will cause the breaking of this linkage. Although

phosphorylation may involve in the regulation of the reinforcement process (Choquet et al. 1997), its definite mechanism is not known. A simple proposed model is that additional cytoskeletal proteins such as vinculin can be recruited to the attaching point, while the linkage number between integrin and cytoskeleton is kept discrete (Figure 1, Model1). Another model may involve the local stimulation of actin filament polymerization by the force-dependent signal and those new filaments will reinforce the integrin-cytoskeleton linkage (Figure 1. Model2).

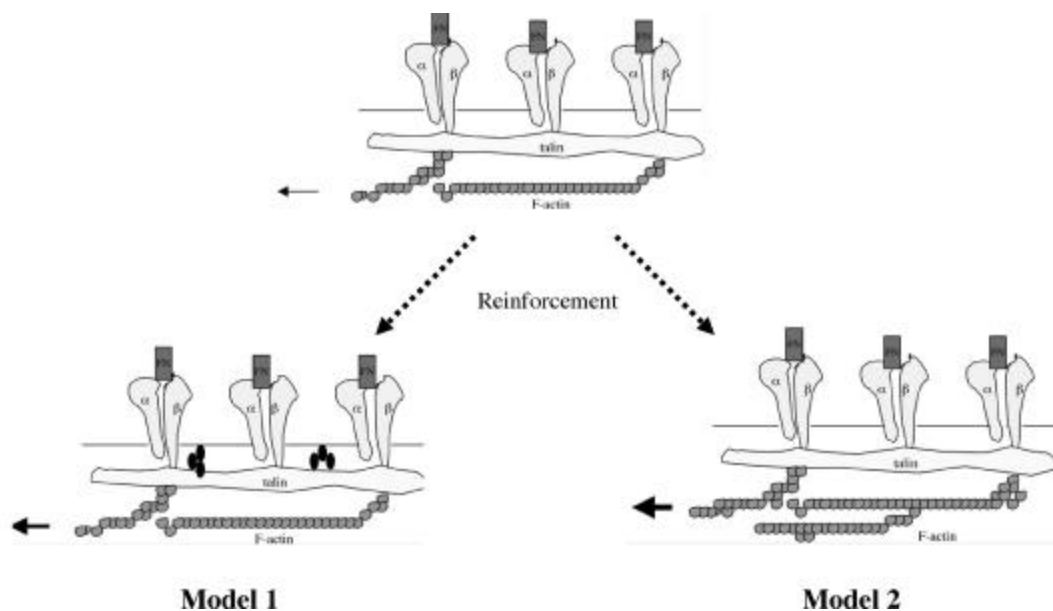


Figure 1. Two proposed models for the reinforcement process. Model 1 may require the accumulation of additional cytoskeletal proteins (like vinculin) and the linkage between integrin and cytoskeleton is kept discrete. Model 2 is involved with the local actin assembly, which may potentially strengthen the the integrin-cytoskeleton linkage.

Both ligand binding and receptor clustering are required for integrins to have full interactions with cytoskeleton (Miyamoto et al. 1995). Coussen et al (in revision) recently demonstrated that the minimum cluster of liganded integrins that will activate cytoskeleton linkage was a trimer, mediated by the trimeric FNIII7-10 fragments. By coating the FNIII7-10 trimer to latex beads, we can measure the force needed to break the cytoskeleton linkages to single (double) trimer with laser

tweezer microscopy (Sheetz 1998). This will then enable us to determine the strength of a single integrin-cytoskeleton linkage and will show how the strength scales with integrin number. If linkage strength increases linearly with the number of fibronectin-integrin bonds, then Model 1 in Figure 1 is correct. If linkage strength increases nonlinearly (quadratic or higher order), Model 2 will be perhaps the correct one. A similar assay in  $\beta 1$  integrin cytoplasmic domain mutants or talin mutants can help to define how the interaction between integrin, talin and vinculin affects the strength of this linkage.

## Experimental Design and Methods

### 1. Measure the Strength of Single Integrin-Cytoskeleton Linkage

NIH 3T3 cells were plated on acid-washed, silane-treated coverslips coated with laminin (Choquet et al. 1997). 0.71 $\mu$ m carboxylated latex beads were activated by carbodiimide, then coupled with biotinylated FNIII7-10 trimer in the presence of biotinylated BSA (10mg/ml). As the ratio of BSA:trimer increases, it can be expected that only one (or) two trimer can be available for binding when a bead is placed on the cell membrane.

Trimer-coated beads were held on the cell surface for 3 seconds, then released by turning off the laser. If the bead bound to the cell membrane, the laser was turned on again. The movement of the bead held by the laser was recorded until the bead finally escaped away from the trap force field.

While following the bead, it was often observed that the bead would move rearward from the trap center for some distance, then popped back to the trap center as if it were held by a “spring”. This kind of “breaking” event could occur several times for a single bead before it completely escaped the trap force field and moved towards the nucleus (Figure 2).

Initially the force of the trap would hold the bead at the trap center. However, since the bead was also linked to the cytoskeleton (fibronectin-integrin-cytoskeleton linkage), the force generated by the cell (centripetal movement of actin filaments powered by myosin) would pull the beads away from the trap center. If the force from the cell was greater than that of the trap, the bead would be pulled away from the center of the trap. Since the force from the trap scales up from the center and the maximum force is at the edge of the trap force field (~400nm from the center), the bead would be brought back to the trap center if the force from the cell

couldn't overcome trap force. Since the integrin-cytoskeleton linkage can be strengthened in response to increased matrix rigidity, which results in greater traction force, the bead could finally move out of the trap force field toward the nucleus.

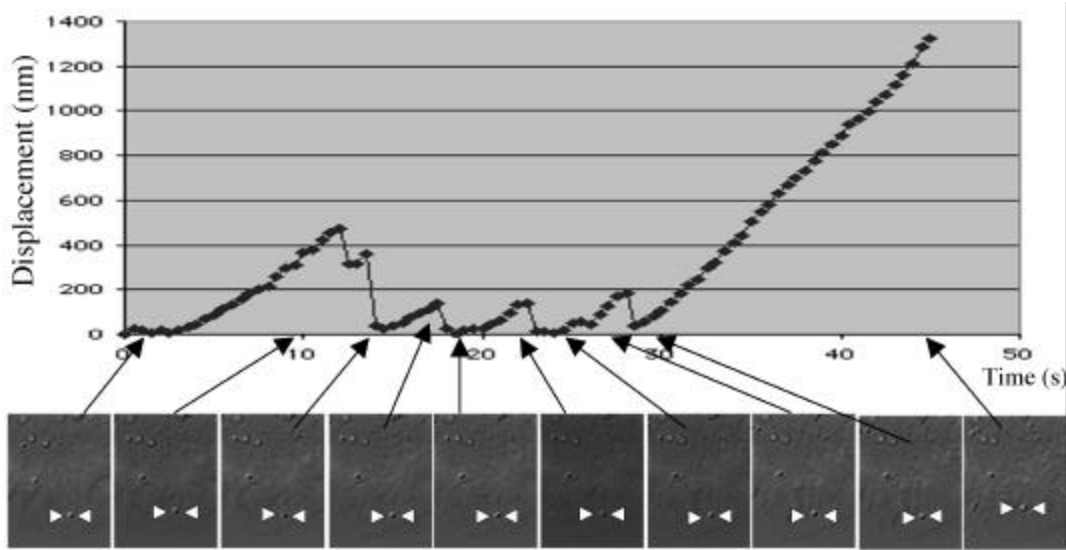


Figure 2 (Top) Plot of bead displacements from the trap center versus time. The trap remained on all the time. (Bottom) Sequential DIC images of the movement of the bead.

By measuring the displacements of the bead for each breaking event (Figure 2), we can estimate the strength of the integrin-cytoskeleton linkage. The calibration of the trap force was performed according to Stokes' Law ( $F=6\pi\eta rv$ ) as described (Simmons et al. 1996; Dai and Sheetz 1999) and the stiffness of present laser is  $0.3\text{pN}/\mu\text{m mW}$  ( $\mu\text{m}$ -displacements of the bead,  $\text{mW}$ -the laser power. The present laser is set at  $75\text{mW}$ ). So if a bead gave a displacement of  $300\text{nm}$  before popping back to the trap center, the strength of the integrin-cytoskeleton linkage is approximately  $7\text{pN}$  ( $0.3\text{ pN}/\mu\text{m.mW} \times 300\text{nm}/1000 \times 75\text{mW}$ ). If we make a histogram with the X-axis as the strength of integrin-cytoskeleton linkage and Y-axis as the number of events, several peaks may be observed. The force value of the first peak may correspond to the strength of a minimum cluster of integrin-cytoskeleton linkage, which should be a trimer mediated by the trimeric FNIII7-10

on the beads. The force value of the second peak may indicate the strength of six integrin-cytoskeleton linkages, which is mediated by two trimeric FNIII7-10. From the force value of the first peak, the strength of single integrin-cytoskeleton linkage can be derived. The force value of other peaks will show how the strength scales up with integrin numbers. These information will then help us differentiate the two models (protein accumulation model and actin polymerization model) in Figure 1. Preliminary data showed that the minimum force generated by the cell was around 2pN (Figure 3). Further experiments need to be done to further refine the histogram.

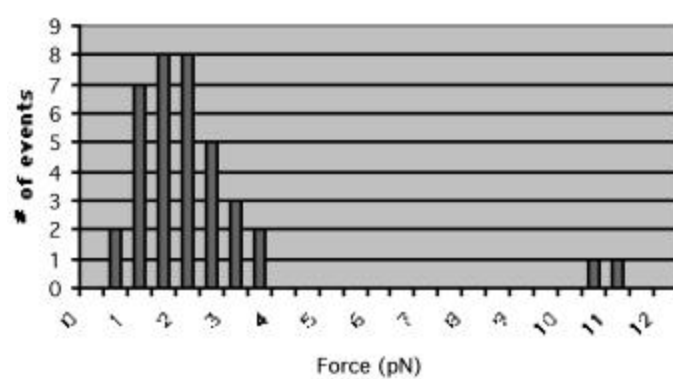


Figure 3 Histogram of cell force measurement in 3T3 cells. X-axis displays the strength of integrin-cytoskeleton linkage for a specific breaking event. Y-axis displays the frequency of breaking events at certain strength value.

In order to see if the strength of single linkage is a unique value for a specific molecular interaction, which is not affected by the nature of extracellular matrix, a similar force assay will also be done with cells plated on vitronectin, collagen or poly-lysine.

## 2. The Effects of Interaction between Talin and $\beta 1$ Integrin Cytoplasmic Domain on the Strength of Integrin-Cytoskeleton Linkage

$\beta 1$  integrin has talin-binding sites in its two NPXY motifs and talin has integrin-binding sites in its head and rod domain. Since talin is the cytoskeletal protein

which directly interact with and link  $\beta 1$  integrin to the actin cytoskeleton, the binding affinity between these two molecules might have effects on the strength of integrin-cytoskeleton linkage.

We have a series of stable cell lines transfected with integrin mutants, which either have a truncated  $\beta 1$  cytoplasmic domain (765t), or a mutation at potential phosphorylation sites (S790D, Y788E, S790M, Y788F, R765I) within the NPXY motifs. The 765t, S790D, Y788E integrins displayed defects in localizing to focal adhesions and talin binding was inhibited, whereas S790M, Y799F integrins localized normally (Schmidt et al. 1993). A force assay described above will be carried out in these cell lines. The force value of single integrin-cytoskeleton linkage in these cell lines will tell us to what extents the binding between talin and  $\beta 1$  integrin affects the linkage strength.

We will also construct a talin mutant with truncation in its rod domain, thus this talin will only have one integrin binding site in the head domain. When this construct is transfected into cells, the linkage between integrin and talin might be compromised. An assay of the strength of integrin-cytoskeleton linkage in these cells will be a second test of the results obtained with  $\beta 1$  integrin mutants.

### 3. The Effects of The Recruitment of Vinculin by Talin on the Strength of Integrin-Cytoskeleton Linkage

It is known that the mechanical stiffness of the integrin-cytoskeleton linkage is reduced in vinculin (-/-) knock-out cells. We will try to find if the recruitment of vinculin by talin contributes to the stiffening of integrin-cytoskeleton linkage.

Each subunit of talin has three binding sites for vinculin, thus, six in the talin dimer. We will mutate the vinculin-binding sites within each talin subunit from three to two or one, thus four or two binding sites in the mutant talin dimer. Then, these talin mutants and wild type talin will be transfected into talin (-/-) knock-out

cells. It can be expected that less vinculin could be recruited by the talin mutants when some of the binding sites are removed. A similar force assay in these cell lines will indicate whether the accumulation of vinculin can contribute to the strengthening of integrin-cytoskeleton linkage or the reinforcement process.

If the strength of single integrin-cytoskeleton linkage is decreased when the vinculin-binding sites in talin is reduced, it will provide strong evidence that the reinforcement process involves the accumulation of more cytoskeletal proteins (Model I, Figure 1). Otherwise, the reinforcement process maybe involve mechanisms other than the simple recruitment of additional proteins.

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