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Investigating complexity of protein-protein interactions in focal adhesions

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Abstract

The formation of focal adhesions governs cell shape and function; however, there are few measurements of the binding kinetics of focal adhesion proteins in living cells. Here, we used the fluorescence recovery after photobleaching (FRAP) technique, combined with mathematical modeling and scaling analysis to quantify dissociation kinetics of focal adhesion proteins in capillary endothelial cells. Novel experimental protocols based on mathematical analysis were developed to discern the rate-limiting step during FRAP. Values for the dissociation rate constant k_{OFF} ranged over an order of magnitude from $0.009 \pm 0.001/s$ for talin to $0.102 \pm 0.010/s$ for FAK, indicating that talin is bound more strongly than other proteins in focal adhesions. Comparisons with *in vitro* measurements reveal that multiple focal adhesion proteins form a network of bonds, rather than binding in a pair-wise manner in these anchoring structures in living cells.

INTRODUCTION

The focal adhesion (FA) is a transient structure that enables cell adhesion, spreading and migration. The formation of the FA is triggered by the binding of transmembrane integrin receptors to immobilized extracellular matrix (ECM) molecules, such as fibronectin and vitronectin [1]. Integrin ligation and clustering promote recruitment of various intracellular proteins to the cytoplasmic tails of integrins that mechanically link them to the actin cytoskeleton [2]. Cytoskeletal anchoring molecules recruited to FAs include proteins, such as talin, vinculin, α -actinin, paxillin, zyxin, and focal adhesion kinase (FAK)[3].

FAs are dynamically assembled and disassembled by cells. Continuous remodeling of FAs is critical for cell movement[4] and dynamic responses to mechanical forces [5] and thus, there is considerable interest in understanding how FA assembly and disassembly is coordinated by its various molecular components [6]. The dynamic assembly of the FA is presumably governed by the modulation of transient interactions between its constituent proteins. However, due to the lack of methods to quantify the kinetics of these interactions in living cells, our understanding of the FA remains poor.

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To shed light on how FA proteins may interact with one another *in situ*, we developed an approach to quantify the dissociation kinetics of proteins inside intact FAs within living cells. By combining mathematical modeling, scaling analysis and novel experimental measurements, we identified the rate-limiting step as being the dissociation of bound molecules. Comparison of the measured parameters with previously reported values from *in vitro* studies revealed that some FA proteins may bind to more than one binding partner simultaneously inside living cells. By providing insight into the nature of this inter-molecular wiring, these results represent a first step towards development of a microscopic model of protein binding networks inside FAs.

MATERIALS AND METHODS

Cell Culture, transfection and immunofluorescence

Bovine capillary endothelial cells (passage 10 to 15) were maintained at 37°C in 10% CO₂ on tissue culture dishes in growth medium as described previously[7;8]. Cells were transfected with focal adhesion protein expression plasmids tagged to GFP using Effectene (Qiagen, Chatsworth, CA). The plasmids used were GFP-Talin [9], GFP-paxillin [10], GFP-β3 integrin [11], GFP-FAK [12] and GFP-α-actinin [13]. GFP-tagged proteins used here have been rigorously characterized and shown to be functionally identical to corresponding endogenous proteins [11;12;13]. To confirm that our cells expressed functional forms of these fusion proteins, we performed immunostaining of fixed cells expressing each GFP-FA protein using specific antibodies and an Alexa594-labelled secondary antibody (see supplementary figure S1).

FRAP experiments

Live cell imaging studies were carried out in optically clear medium containing Hank's balanced salts as described previously. [14] FRAP analysis was performed on the Zeiss LSM 510 META/NLO microscope using a 63×0.95 NA IR corrected water immersion lens as described previously[14]. The 488 nm line of an Argon/2 multiple-lined single-photon laser source (10% of full power) was used for GFP excitation; 100% of the 488 nm line was used for photobleaching with 10 iterations corresponding to less than a millisecond. Measured recovery curves were normalized to the amount of photobleached protein and fit to $1 - e^{-k_{OFF}t}$ with the method of least squares (MATLAB function lsqcurvefit) to calculate k_{OFF} . The sample size for all k_{OFF} measurements was more than 20. Images were collected using the Zeiss LSM 510 software (version 3.2). All experiments on the microscope were performed at 37°C using a temperature-controlled stage.

RESULTS

Adhesion protein dynamics in capillary endothelial cells

We used FRAP to measure the dynamic exchange between the bound and cytoplasmic forms of β3 integrin, talin, α-actinin, paxillin and FAK, in bovine capillary endothelial cells. Photobleaching of GFP-tagged forms of these proteins was carried out for a very short time (< 1 msec) within a very small area (< 0.5 μm²) of a single FA. Full fluorescence recovery for β3 integrin occurred within ~ 4 min after photobleaching, and α-actinin and paxillin exchanged on a similar time scale (~2 min); however, talin which binds directly to integrins exchanged much more slowly with complete recovery taking on the order of 10 min (Fig. 1). In contrast, FAK underwent very fast recovery over a period of a few seconds (Fig. 1). We recently showed that vinculin and zyxin recover over time scales of seconds as well [14]. Taken together, these results suggest that each of these FA proteins spend different amounts of time in FAs, and their residence times vary by over an order of magnitude.

Theoretical expressions for FRAP recovery times in focal adhesions

Because our goal was to quantify molecular binding kinetics from the FRAP data, we next modeled the FRAP process in FAs accounting for molecular diffusion in the cytoplasm, as well as exchange between the cytoplasmic molecules and adhesion bound proteins. To do this, we considered the geometry shown in Fig. 2, and allowed the photobleaching of cytoplasmic pools above the FA, in addition to the molecules in the FA itself. The freely diffusing fluorescent protein concentration in the cytoplasm above the FA can be modeled as

$$\frac{\partial C_f}{\partial t} = D \left[\frac{1}{r} \frac{\partial}{\partial r} \left(r \frac{\partial C_f}{\partial r} \right) \right] + \frac{\partial^2 C_f}{\partial y^2} \quad (1)$$

$$\frac{\partial \widehat{C}_f}{\partial t} = k_{ON} C_f (S - \widehat{C}_0) - k_{OFF} \widehat{C}_f \text{ at } y=0, r \in (0, R) \quad (2)$$

where C_f is the concentration of freely diffusing molecules in the cytoplasm, $\widehat{C}_f$ is the concentration of proteins bound in the focal adhesion, D is the diffusion coefficient of molecules in the cytoplasm, k_{ON} , k_{OFF} are binding and dissociation rate constants. S is the total concentration of binding sites, $\widehat{C}_0$ is the concentration of occupied sites at steady state, and C_0 is the concentration of freely diffusing molecules. The boundary conditions are:

$$D \frac{\partial C_f}{\partial y} = k_{ON} C_f (S - \widehat{C}_0) - k_{OFF} \widehat{C}_f \text{ at } y=0, r \in (0, R) \quad (3)$$

$$\frac{\partial C_f}{\partial y} = 0 \text{ at } y=h \quad (4)$$

$$\frac{\partial C_f}{\partial r} = 0 \text{ at } r=0 \quad (5)$$

where h is the vertical height of the cytoplasm above the focal adhesion. The total amount of fluorescent protein in the cytoplasm is orders of magnitude greater than that in the small photobleached spot in one FA. This means that bleaching the small amount of protein in the FA minimally perturbs the total concentration. This enables us to write the final boundary condition:

$$C_f(\infty, y, t) = C_0 \quad (6)$$

The initial conditions are

$$C_f(r \in (0, r_0), y \in (0, \delta), 0) = 0 \quad (7)$$

$$\widehat{C}_f(r \in (0, r_0), 0) = 0 \quad (8)$$

We note that photobleaching does not eliminate the fluorescent concentration in the bleached spot completely. However, as long as the photobleaching is on the same scale as the concentration of bound fluorescent proteins at steady state, this will not affect the scaling analysis. We next performed a scaling analysis of the model and derived expressions for recovery times for two rate-limiting scenarios: effective diffusion or fast binding. The results of our analysis indicate two limiting regimes [see supplementary information and Fig. S2 for details]:

$$\begin{aligned} \text{If } \frac{k_{ON}(S-\widehat{C}_0)\delta}{D} \gg 1, \quad \tau_R = \frac{\widehat{C}_0}{C_0} \frac{r_0}{D} \text{ and,} \\ \text{if } \frac{k_{ON}(S-\widehat{C}_0)\delta}{D} \gg 1, \quad \tau_R = \frac{1}{k_{OFF}} \end{aligned} \quad (9)$$

where τ_R is the characteristic time scale of recovery during a FRAP experiment.

Experimental identification of the rate limiting step

To interpret the FRAP experiments in terms of molecular binding kinetics, it is necessary to identify which regime governs the recovery. If effective diffusion is rate-limiting, τ_R will

depend on the ratio of bound to free concentration $\frac{\widehat{C}_0}{C_0}$ (equation 9). Therefore, we developed an approach to quantify this ratio in cells. Consider a confocal fluorescence image focused at the focal adhesion. We define a measured volume V_m as a spot drawn in the image with bleached spot area A , and height h_1 in nanometers. $\widehat{F}$ is the fluorescent intensity when V_m is within the FA, and F is the fluorescent intensity when V_m is in the cytoplasm. We derived the following inequality (see supplementary information for detailed derivation):

$$\frac{\widehat{C}_0}{C_0} < 0.296 \frac{\widehat{F}}{F} - 0.236 \quad (10)$$

Then we measured the upper bound in equation (10) from confocal fluorescence images of living cells expressing GFP-tagged proteins (Fig. 3A and Suppl Fig. S1). Based on this ratio, and estimated diffusion coefficients (Supplementary Table 1), the largest time scales for recovery predicted by effective diffusion (equation 9) are on the order of several milliseconds (Fig. 3b). These times are several orders of magnitude smaller than the fastest time scale measured for recovery (Fig. 1). Therefore, the only alternative possibility for explaining our results is that the freely diffusing cytoplasmic protein recovers quickly, followed by slow

recovery of the bound proteins over a time scale determined by $\frac{1}{k_{OFF}}$ (equation 9).

While these arguments are valid for cytoplasmic proteins, $\beta 3$ -integrins are membrane proteins and their diffusion coefficient inside the membrane itself may be hindered by clustering of integrins. To measure this diffusion coefficient, we therefore require an experimental model in which $\beta 3$ -integrins are clustered, but not bound to any cytoplasmic proteins. As such an experimental model is unavailable, we report our data in terms of $t_{1/2}$ for $\beta 3$ integrin recovery.

Kinetics of dissociation of proteins in the FA

When the kinetics of dissociation is rate limiting, the model equations (1–2) are considerably simplified, and the solution for the concentration (corrected for initial value, and normalized for final recovery at long times) is simply $1 - e^{-k_{OFF}t}$. We therefore fit our normalized FRAP data to $1 - e^{-k_{OFF}t}$ (the $\beta 3$ integrin data was fit to $1 - e^{-kt}$ where k is a parameter that is not necessarily the same as k_{OFF}). As indicated by the curve fitting (Fig. 4), all FRAP recovery curves were well-described by a single parameter. The estimated rate constants were then used to calculate the residence time as $1/k_{OFF}$ (Table. 1A).

We next compared these *in situ* values of dissociation rate constants to those measured *in vitro* for FA protein binding to F-actin ([15;16;17;18;19] and Table 2B). For α -actinin, the slowest reported k_{OFF} for its *in vitro* binding to F-actin is an order of magnitude smaller than our measured value in FAs (Table 2A). Similarly, the reported *in vitro* value for talin binding to F-actin is nearly four orders of magnitudes greater than the k_{OFF} measured in this study. Conversely, the slowest *in vitro* reported values of vinculin dissociation kinetics from F-actin are remarkably similar to our measured kinetics (Table 2). Binding of α -actinin, vinculin and talin to F-actin is necessary for their localization to focal adhesions [20;21;22;23;24;25;26; 27]. These results suggest that in addition to actin, talin and α -actinin are bound simultaneously to at least one other binding partner, resulting in a lower effective k_{OFF} ; they also imply that vinculin kinetics in FAs may depend strongly on its binding with F-actin.

DISCUSSION

The complete lack of information on the kinetics of intermolecular interactions in FAs makes it very challenging to develop microscopic models of FA assembly. In this paper, we developed a rigorous methodology to measure the dissociation kinetics of proteins inside FAs. The dissociation kinetics are proportional to the strength of the bonds that each protein forms with its binding partners in FAs. Therefore, these parameters are indirect read-outs of intermolecular interactions within FAs in living cells. Knowledge of these parameters allows the unambiguous comparison (due to freedom from the effects of diffusion) of intrinsic properties of binding between proteins in different cells, and across different studies. Thus, quantification of dissociation rate constants is invaluable for developing quantitative benchmarks for comparative cell biological studies in the future.

A comparison of our measured values for $t_{1/2}$ (Table 1A) with those published in the literature (Table 2) revealed that talin spends the longest time in FAs among the seven proteins studied here. Others have reported that $\beta 3$ integrin and α -actinin exchange more slowly in melanoma cells [11], fibroblasts [11] and large vessel endothelial cells [28]. Very different values for $t_{1/2}$ have been reported for $\beta 3$ integrins in melanoma cells compared with endothelial cells (Table 2) [11;28]. While α -actinin exchanges with a $t_{1/2}$ of 1 min in endothelial cells [28], a $t_{1/2}$ of 5 min has been reported in fibroblasts [13]. For talin, two studies reported two different $t_{1/2}$ values of 50 sec [29] and 4–7 sec [30], compared to 78 sec that we measured in capillary endothelial cells. Since the reported values for $t_{1/2}$ of the different FA proteins vary greatly with cell type, it is impossible to compare multiple proteins in different cells. Additionally, because of potential diffusion limitations, $t_{1/2}$ is not an ideal metric for comparison of intrinsic binding properties of proteins. Thus, our study is important because it compares a more quantitative measure of molecular binding kinetics (k_{OFF}) for many different key FA proteins in a single cell type.

It has been hypothesized that proteins form an intermolecular network of bonds inside FAs [3] such that each protein may be bound to multiple binding partners. Talin, α -actinin and vinculin are known to be bound to F-actin in FAs [20;21;22;23;24;25;26;27]. Therefore, the fact that talin and α -actinin dissociation is considerably slower than their corresponding binding

kinetics measured with F-actin *in vitro* is consistent with a microscopic model in which these molecules are simultaneously bound to actin and least one other binding partner. Vinculin, surprisingly, has a similar k_{OFF} to vinculin-F-actin binding *in vitro* suggesting that this interaction may dominate vinculin kinetics in FAs formed by living cells.

Determining a quantitative mapping between the effective k_{OFF} and the binding and unbinding constants for binding to any protein to its partners in the FA remains a fundamental challenge. This challenge is in part due to the many degrees of freedom, such as the stoichiometry, spatial distributions of partners in the FA and allosteric mechanisms. Our measurements of k_{OFF} of many FA proteins in one cell type represent a first step toward this goal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Yamada KM, Pankov R, Cukierman E. Dimensions and dynamics in integrin function. *Braz J Med Biol Res* 2003;36:959–66. [PubMed: 12886449]
2. Wang N, Butler JP, Ingber DE. Mechanotransduction across the cell surface and through the cytoskeleton. *Science* 1993;260:1124–7. [PubMed: 7684161]
3. Zamir E, Geiger B. Molecular complexity and dynamics of cell-matrix adhesions. *J Cell Sci* 2001;114:3583–90. [PubMed: 11707510]
4. Lauffenburger DA, Horwitz AF. Cell migration: a physically integrated molecular process. *Cell* 1996;84:359–69. [PubMed: 8608589]
5. Choquet D, Felsenfeld DP, Sheetz MP. Extracellular matrix rigidity causes strengthening of integrin-cytoskeleton linkages. *Cell* 1997;88:39–48. [PubMed: 9019403]
6. Webb DJ, Donais K, Whitmore LA, Thomas SM, Turner CE, Parsons JT, Horwitz AF. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat Cell Biol* 2004;6:154–61. [PubMed: 14743221]
7. Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. Geometric control of cell life and death. *Science* 1997;276:1425–8. [PubMed: 9162012]
8. Matthews BD, Overby DR, Alenghat FJ, Karavitis J, Numaguchi Y, Allen PG, Ingber DE. Mechanical properties of individual focal adhesions probed with a magnetic microneedle. *Biochem Biophys Res Commun* 2004;313:758–64. [PubMed: 14697256]
9. Partridge MA, David FS, Marcantonio EE. Displacement of the {beta} cytoplasmic domain recovers focal adhesion formation, cytoskeletal organization and motility in swapped integrin chimeras. *J Cell Sci* 2006;119:1175–83. [PubMed: 16507589]
10. Knight B, Laukaitis C, Akhtar N, Hotchin NA, Edlund M, Horwitz AR. Visualizing muscle cell migration in situ. *Curr Biol* 2000;10:576–85. [PubMed: 10837222]
11. Ballestrem C, Hinz B, Imhof BA, Wehrle-Haller B. Marching at the front and dragging behind: differential α V β 3-integrin turnover regulates focal adhesion behavior. *J Cell Biol* 2001;155:1319–32. [PubMed: 11756480]
12. Ezratty EJ, Partridge MA, Gundersen GG. Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. *Nat Cell Biol* 2005;7:581–90. [PubMed: 15895076]

13. Edlund M, Lotano MA, Otey CA. Dynamics of alpha-actinin in focal adhesions and stress fibers visualized with alpha-actinin-green fluorescent protein. *Cell Motil Cytoskeleton* 2001;48:190–200. [PubMed: 11223950]
14. Lele TP, Pendse J, Kumar S, Salanga M, Karavitis J, Ingber DE. Mechanical forces alter zyxin unbinding kinetics within focal adhesions of living cells. *J Cell Physiol* 2006;207:187–94. [PubMed: 16288479]
15. Goldmann WH, Guttenberg Z, Tang JX, Kroy K, Isenberg G, Ezzell RM. Analysis of the F-actin binding fragments of vinculin using stopped-flow and dynamic light-scattering measurements. *Eur J Biochem* 1998;254:413–9. [PubMed: 9660199]
16. Goldmann WH, Isenberg G. Kinetic determination of talin-actin binding. *Biochem Biophys Res Commun* 1991;178:718–23. [PubMed: 1907136]
17. Goldmann WH, Isenberg G. Analysis of filamin and alpha-actinin binding to actin by the stopped flow method. *FEBS Lett* 1993;336:408–10. [PubMed: 8282102]
18. Kuhlman PA, Ellis J, Critchley DR, Bagshaw CR. The kinetics of the interaction between the actin-binding domain of alpha-actinin and F-actin. *FEBS Lett* 1994;339:297–301. [PubMed: 8112470]
19. Wachstock DH, Schwarz WH, Pollard TD. Cross-linker dynamics determine the mechanical properties of actin gels. *Biophys J* 1994;66:801–9. [PubMed: 8011912]
20. Burridge K, Nuckolls G, Otey C, Pavalko F, Simon K, Turner C. Actin-membrane interaction in focal adhesions. *Cell Differ Dev* 1990;32:337–42. [PubMed: 2129156]
21. Hemmings L, Rees DJ, Ohanian V, Bolton SJ, Gilmore AP, Patel B, Priddle H, Trevithick JE, Hynes RO, Critchley DR. Talin contains three actin-binding sites each of which is adjacent to a vinculin-binding site. *J Cell Sci* 1996;109(Pt 11):2715–26. [PubMed: 8937989]
22. Humphries JD, Wang P, Streuli C, Geiger B, Humphries MJ, Ballestrem C. Vinculin controls focal adhesion formation by direct interactions with talin and actin. *J Cell Biol* 2007;179:1043–57. [PubMed: 18056416]
23. Menkel AR, Kroemker M, Bubeck P, Ronsiek M, Nikolai G, Jockusch BM. Characterization of an F-actin-binding domain in the cytoskeletal protein vinculin. *J Cell Biol* 1994;126:1231–40. [PubMed: 8063860]
24. Muguruma M, Matsumura S, Fukazawa T. Direct interactions between talin and actin. *Biochem Biophys Res Commun* 1990;171:1217–23. [PubMed: 2121138]
25. Otey CA, Carpen O. Alpha-actinin revisited: a fresh look at an old player. *Cell Motil Cytoskeleton* 2004;58:104–11. [PubMed: 15083532]
26. Pavalko FM, Burridge K. Disruption of the actin cytoskeleton after microinjection of proteolytic fragments of alpha-actinin. *J Cell Biol* 1991;114:481–91. [PubMed: 1907287]
27. Ruhnau K, Wegner A. Evidence for direct binding of vinculin to actin filaments. *FEBS Lett* 1988;228:105–8. [PubMed: 2449360]
28. Tsuruta D, Gonzales M, Hopkinson SB, Otey C, Khuon S, Goldman RD, Jones JC. Microfilament-dependent movement of the beta3 integrin subunit within focal contacts of endothelial cells. *Faseb J* 2002;16:866–8. [PubMed: 11967230]
29. Cohen DM, Kutscher B, Chen H, Murphy DB, Craig SW. A conformational switch in vinculin drives formation and dynamics of a talin-vinculin complex at focal adhesions. *J Biol Chem* 2006;281:16006–15. [PubMed: 16608855]
30. Gupton SL, Waterman-Storer CM. Spatiotemporal feedback between actomyosin and focal-adhesion systems optimizes rapid cell migration. *Cell* 2006;125:1361–74. [PubMed: 16814721]
31. Giannone G, Ronde P, Gaire M, Beaudouin J, Haiech J, Ellenberg J, Takeda K. Calcium rises locally trigger focal adhesion disassembly and enhance residency of focal adhesion kinase at focal adhesions. *J Biol Chem* 2004;279:28715–23. [PubMed: 15102844]
32. Hamadi A, Bouali M, Dontenwill M, Stoeckel H, Takeda K, Ronde P. Regulation of focal adhesion dynamics and disassembly by phosphorylation of FAK at tyrosine 397. *J Cell Sci* 2005;118:4415–25. [PubMed: 16159962]
33. von Wichert G, Haimovich B, Feng GS, Sheetz MP. Force-dependent integrin-cytoskeleton linkage formation requires downregulation of focal complex dynamics by Shp2. *Embo J* 2003;22:5023–35. [PubMed: 14517241]

Fig. 1. FRAP analysis of GFP-tagged FA proteins in living cells

Representative pre- and post-bleach images from a FRAP experiment with cells expressing GFP forms of $\beta 3$ integrin, talin, α -actinin, paxillin, or FAK in capillary endothelial cells. Arrows indicate photobleached spots with an area of less than $0.5 \mu\text{m}^2$ within individual FAs. Note that some experiments had multiple spots bleached simultaneously that exhibited similar recovery dynamics. Talin is the slowest exchanging protein followed by $\beta 3$ integrin, paxillin, α -actinin and FAK (bar, 2 μm).

Fig. 2. Model Geometry

The geometry used for the scaling analysis is shown. Bleaching may occur not only at the FA, but also in the cytoplasm. Therefore, photobleaching is assumed to occur in a region denoted by the shaded cylinder of height δ and radius r_0 . R is the characteristic size of the FA, while h is the height of the cytoplasm above the FA.

Fig. 3. The dynamics of GFP-FA protein exchange is not determined by effective diffusion

A) The upper bound for $\frac{\widehat{C}_0}{C_0}$ was estimated by measuring the fluorescence intensity from confocal fluorescence images focused at the FA. The value ranged from 0.3 to 0.7 for all the proteins studied here. **B)** The estimated times calculated for FRAP recovery if effective diffusion was the governing mechanism. These times are in the range of milliseconds, and therefore cannot explain the time scales observed during our FRAP experiments (error bars indicate standard error of the mean).

Fig. 4. Representative FRAP recovery curves for FA molecules

Normalized recovery curves (circles) for GFP-labeled forms of $\beta 3$ integrin, talin, α -actinin, paxillin and FAK; solid lines are curves fit to the data using the method of least squares. A single parameter (k_{OFF} for cytoplasmic proteins, k for integrins) is sufficient to describe the data, suggesting that each protein recovers with a single time scale.

Table 1

Molecular kinetic parameters for FA proteins measured using FRAP. A)

The unbinding rate constant k_{OFF} was calculated by fitting $1 - e^{-k \cdot k_{OFF} t}$ to normalized FRAP data with the method of least squares. $t_{1/2} = -0.5/\log(k_{OFF})$ was computed from measured values of k_{OFF} . Residence times were calculated as $1/k_{OFF}$. Talin spends the most time in FAs, followed by integrins, α -actinin, paxillin and FAK. **B)** Values reported in the literature [15;16;17;18;19] for dissociation kinetics of talin, vinculin and α -actinin from F-actin *in vitro*. The different values for the same interaction are due to studies from different investigators, who employed varying conditions (species, temperature, protein fragments). With the exception of vinculin, the dissociation rate constants *in vitro* are faster than our reported values *in-situ* (compare Table 2A and 2B).

A									
In-situ binding studies	$\beta 3$ -integrin	α -actinin	Paxillin	FAK	Zyxin [14]	Vinculin (slow) [14]	Vinculin (fast) [14]	Talin	
k_{OFF} (1/s)	NA	0.049 ± 0.007	0.039 ± 0.003	0.102 ± 0.01	0.1 ± 0.01	0.060 ± 0.010	0.970 ± 0.100	0.009 ± 0.001	
$t_{1/2}$ (s)	38.5 ± 7	14.146 ± 1.768	17.77 ± 1.269	6.796 ± 0.607	7.0 ± 0.43	11.55 ± 1.650	0.715 ± 0.007	77.016 ± 7.716	
Residence times	NA	20.41 ± 2.55	25.64 ± 1.83	9.800 ± 0.87	10 ± 1.1	16.67 ± 3.3	1.03 ± 0.118	111.11 ± 11.1	

B				
Values reported in the literature from <i>In-vitro</i> binding studies[15–19]				
k_{OFF} (1/s)	α -actinin-F-actin binding		Vinculin –F-actin binding (slow)	Talin-F-actin binding
	0.4		0.035	2.5
	5.2		0.06	
	0.66			
	9.6			
	1.5			

Table 2**Comparison of our results for FA protein dissociation kinetics with those in the literature**

There appear to be large differences in time scales for the same FA proteins across different cell types. These may be indicative of differences in binding partners in different cell types.

	This work (Capillary Endothelial Cells)	Published literature	Cell type
$\beta 3$ integrin	38.51 ± 7.1 s	5.22 ± 0.35 min	Human endothelial cells [28]
		120 s	Mouse B16 melanoma [11]
Talin	77.02 ± 7.72 s	50 s	Mouse F9 carcinoma [29]
		4.51–6.86 s	PtK1 cells [30]
α -actinin	14.14 ± 1.8 s	0.57 min	Human endothelial [28]
		5 min	Mouse fibroblasts [13]
		43 s	Mouse F9 carcinoma [29]
Paxillin	17.8 ± 1.3 s	15.3 s	Mouse embryonic fibroblasts [33]
		41 s	Mouse F9 carcinoma [29]
FAK	6.8 ± 0.7 s	16.3 ± 2.7 s	Human astrocytes [32]
		17.0 ± 4.0 s	Human astrocytes [31]
Vinculin	11.55 ± 0.7 s [14]	8.8 s	Mouse embryonic fibroblasts [33]
		83 s	Mouse F9 carcinoma [29]
		8.92–36.17 s	PtK1 cells [30]
Zyxin	7.1 ± 0.43 s [14]		