A Cautionary Tail: Changes in Integrin Behavior with Labeling

Catherine G. Galbraith[1,], Michael W. Davidson[2], and James A. Galbraith[1,*]*

[1]Oregon Center for Spatial Systems Biomedicine, Biomedical Engineering, Oregon Health Science University, Portland, OR

[2]National High Magnet Laboratory, Florida State University, Tallahassee, FL

* These authors contributed equally to this work

Correspondence to galbrcat@ohsu.edu or galbrjam@ohsu.edu

ABSTRACT

Integrins are heterodimer transmembrane proteins essential for cell adhesion and signaling during development, immunological responses, wound healing, and metastasis. Critical to their function is their unique ability to dynamically modulate their adhesive and signaling properties through changes in conformation, protein-protein interactions, and cellular distribution. While molecular, biochemical, and structural studies have uncovered ligand and regulatory protein interactions, unraveling the dynamic modulation of integrin adhesivity and signaling has relied on live-cell imaging. Fluorescence microscopy and specialized applications (FRET, FRAP, FLIP) have been critical in advancing our understanding of integrin behaviors. However, recent progress in super-resolution live cell imaging reveals integrin behaviors visible at the single molecule level that challenge our current knowledge of integrin behaviors derived from fluorescence microscopy. We found that the adhesivity and signaling of integrin heterodimers depends upon the whether the alpha or the beta subunit of the heterodimer is labeled. While imaging with conventional microscopy did not reveal any differences in unbound integrin behavior or the ability to localize to adhesions, single molecule super-resolution imaging uncovered significant differences in dynamic behavior of unbound integrins. These differences are manifested at the cellular level by alterations in protrusive activity, adhesion size, and cell spreading. Comparison with behaviors of untagged, integrins in normal and elevated affinity states indicates that fluorescently tagging the beta, but not the alpha, subunit elevates the integrin affinity state. Thus, our dense-field, live-cell single molecule super-resolution microscopy approach resolves molecular behaviors and interactions that are not visible with ensemble imaging but are functionally significant for prescribing cell behavior.

INTRODUCTION

Integrins are heterodimers composed of an alpha and a beta subunit. There are bidirectional signaling molecules that mediate interactions with other cells, matrix, and pathogens outside of the cell and with the cytoskeleton and signaling molecules within the cell. The overall adhesiveness or avidity of the cell is determined by the affinity of the individual integrins and the number of integrins-ligand bonds on the cell surface. In vertebrates there are 18 α subunits and 8 β subunits, forming 24 $\alpha\beta$ pairs. Both the alpha and beta subunits are type I transmembrane glycoproteins with large extracellular domains, single spanning transmembrane domains, and, with the exception of β_4, short cytoplasmic domains. The extracellular domain is a large globular N-terminal binding head domain, and the transmembrane and cytoplasmic domains are two legs or stalks. The legs are severely bent at the knee in the inactive conformation and fully extended when they are in the active conformation that is bound to ligand. Integrins transition from inactive to active conformation to become primed, a high affinity state of the receptor that is not yet bound to ligand at the leading edge of protruding regions of migrating cells[1].

The initial increase in affinity begins with priming begins by separating the alpha and beta integrin cytoplasmic and transmembrane domains. As a direct consequence, the extracellular interface between the alpha and beta subunits in the tailpiece destabilizes, facilitating the straightening of the legs[2]. For $\alpha_v\beta_3$ integrin, electron microscopy studies and hydrodynamic and surface plasmon resonance ligand-binding studies, revealed that the integrins were bent in the resting state[3,4]. Activation by Mn^{2+} induced extension of

the integrin with a mixture of open and closed headpieces, while ligand binding exclusively produced extended legs with open headpieces[3,4].

Live-cell studies of GFP tagged integrins have shown that primed integrins redistribute on the cell surface in response to ligand. Irrespective of whether the GFP tag is on the alpha or the beta subunit, integrins express on the cell surface, and they redistribute in response to stimuli, indicating that they are functional[5]. However, these studies were limited to ensemble measures of populations rather than individual molecules. Since reproducible molecular behaviors that are indicative of cell function can be undetectable by ensemble measures[6], we applied dense-field single molecule super-resolution analysis to integrins with different tagged subunits[7]. To our surprise, we found that integrin molecular behavior was dependent upon whether the alpha or the beta integrin subunit was labeled. We then asked the specific question of whether this difference in molecular behavior was indicative of a change in integrin affinity. While integrins with beta labeled subunits were still functional in response to ligand and organized into adhesions, the cells have distinctly different patterns of dynamic integrin mobility at the single molecule level. These differences in molecular behavior resulted in increased cell protrusive activity, increased spreading, and elongated adhesions compared to alpha subunit tagged integrin. Comparison of cellular behaviors between untagged, alpha-tagged, beta-tagged, and Mn^{2+} activated indicated that alpha-tagged integrins behaved similarly to untagged, but beta-tagged had a higher affinity for ligand but the affinity was less than Mn^{2+} activation. Taken together, these results point to a cautionary tale – labeling the beta subunit can elevate affinity to partially prime the integrin.

RESULTS

Single-Molecule Distribution of Integrins Depends on which Subunit is Fluorescently Tagged

Although the affinity of integrin heterodimers depends upon the spacing between the alpha and the beta subunits, the effect of adding a protein tag on the function of the heterodimer has not been characterized. Since our single molecule super-resolution analyses have proven to be sufficiently sensitive to detect differences in molecular behavior with single point mutations in the integrin cytoplasmic tail[7], we sought to test whether labeling one subunit versus the other led to differences in the mobility of the integrin heterodimer. We expressed either mEos2b alpha V and untagged beta 3 or untagged alpha V and mEos2b beta 3. We stochastically photo-converted the tagged subunits to localize the integrins in live cells and imaged cells with 25 ms exposure at a frame rate of 40Hz (Supplemental movies S1 and S2). Photo-converted integrin molecules were localized and their movement tracked. We calculated the diffusion coefficient and classified molecular mobility behavior as confined, free, or directed diffusion[8,9]. We discovered that for all cells analyzed, the mobility of freely diffusing integrins was statistically lower if the expressed integrins had tagged beta subunits rather than tagged alpha subunits (Fig 1a-c). Integrins classified as confined or directed had similar mobilities with either tag (Fig 1d). These data indicate that tagging the beta subunit changes the molecular mobility of the expressed integrin.

Protrusive Activity Changes When the Beta Subunit is Tagged

We then sought to determine if these decreases in integrin mobility that occurred when the beta subunit was tagged also led to a decrease in the protrusive activity of the entire

cell edge. CHO-K1 cells transfected with either mEmerald alpha V and untagged beta 3 or untagged alpha V and mEmerald beta 3 were trypsinized and plated on fibronectin coated-coverslips approximately 24 hrs after transfection. Between 2 and 3 hrs later, timelapse images of cells were collected every second. We found that the leading edges of cells transfected with the tagged alpha construct are consistently more motile than the leading edges of cells transfected with the tagged beta construct (Fig 2a). The quiescence of the leading edge of the cells transfected with the beta subunit tagged is consistent with the notion that integrins with the tagged beta subunit are at least partially activated.

Adhesion Size Increases When Beta Subunit is Tagged

We next investigated whether tagging the beta subunit would result in an increase in adhesion size as would be expected if the integrin were activated[10]. CHO-K1 cells were transfected with either: 1) untagged alpha V and beta 3, 2) mEmerald tagged alpha V and untagged beta 3, or 3) untagged alpha V and mEmerald beta3. In addition, cells transfected with untagged alpha V and beta 3 integrins were treated with 0.5 mM Mn^{2+}. Cells were plated on fibronectin-coated coverslips for 3-5 hrs then fixed, and cells transfected with both the alpha and beta subunit untagged were labeled with the human alpha V beta 3 specific LM609 antibody and Alexa 488 (Fig 2b). We discovered that adhesions were statistically the same when cells were transfected with untagged subunits or with tagged alpha V (Fig 3). In contrast, adhesions were consistently larger when cells were transfected with tagged beta3 or when cells expressing untagged integrins were treated with Mn2+. This data, as well as the similarity in size between

the tagged beta 3 integrins and the untagged integrins treated with Mn^{2+}, further support the interpretation that tagging the beta subunit is activating the integrin.

Cell Response to Ligand Increases when Beta Subunit is Tagged

Finally, we analyzed the spreading ability of cells transfected with both untagged subunits, tagged alpha, or tagged beta, and either untreated or treated with Mn^{2+}. The cells transfected with untagged integrins were also transfected with an empty EGFP vector to visualize the cell contour. Approximately 24 hrs post transfection, cells were trypsinized and separated into two groups. One group was treated with Mn^{2+}, and both groups were allowed to spread on fibronectin for 30, 60, or 90 min prior to fixation. Treating cells expressing untagged integrins or tagged alpha V with Mn^{2+} increased cell spreading, indicating, as expected, that integrin activation increased cell spreading. In contrast, treating cells expressing tagged beta 3 integrin with Mn^{2+} did not produce any additional increase cell spreading (Fig 4). Together these results indicate that tagging the alpha subunit does not activate the integrins, but tagging the beta integrin does activate the integrin to a level that is similar to Mn^{2+} treatment.

DISCUSSION

Integrins composed of both untagged subunits, tagged alpha subunits, or tagged beta subunits all properly localized to adhesion complexes. However, subtle differences in the organization of adhesions when visualized with conventional TIRF microscopy suggested underlying phenotypical differences. Measuring the mobility of integrin molecules revealed that integrins with tagged beta subunits had a significantly lower diffusion coefficient than integrins with tagged alpha subunits. These subtle changes at the single molecule level were manifested in cells expressing the tagged beta integrins

having less dynamic leading edges, larger adhesions, and larger surface areas in spreading assays compared to integrins with tagged alpha subunits. Importantly, cells expressing untagged integrins treated with Mn^{2+} had similar size adhesions compared to cells expressing tagged beta subunits. These findings suggested to us that tagging the beta subunit was activating the integrin.

An earlier study noted that CHO-K1 cells expressing GFP-tagged alpha IIb (beta 3) to expression of alpha IIb (GFP-tagged beta 3) were more likely to spontaneously aggregate in the presence of soluble fibrinogen, the ligand for alpha II (beta 3) integrin[5]. However, the study noted the characteristic redistribution of either integrin in the early stages of cell spreading and attachment, concluding that GFP fusion to the cytoplasmic tail of either subunit allows for normal surface expression of a functional receptor[5]. Our single molecule results, as well as our careful comparison of focal adhesion size and cell spreading all indicate that labeling the beta subunit does change the function of the expressed integrin by increasing its affinity for ligand.

Additional conformation for the interpretation that tagging the beta subunit increases integrin affinity comes from the direct comparisons with cells treated with Mn^{2+}, a known integrin activator. Mn^{2+} increases the size of adhesions of cells that express untagged subunits so that they are the same size as integrins with tagged beta subunits. Moreover, Mn^{2+} increases the spreading area of cells expressing integrins with both subunits untagged or with the alpha subunit tagged – it does not increase the spreading area of cells expressing integrins with tagged beta subunits. Taken together, these data indicate that integrins with tagged beta subunits still localize to adhesions and function in cell spreading, but these integrins have an elevated affinity state, likely due to

labeling of the beta subunit separating the alpha and beta cytoplasmic tails. Thus, our data suggests that functionality of molecules needs to be evaluated by molecular behaviors and that localization and reorganization on the cellular level are inadequate metrics for guaranteeing the functionality of tagged molecules.

MATERIALS AND METHODS

Cell culture and transfection

CHO-K1 cells, which do not express endogenous alpha V or beta 3 integrin heterodimers (ATCC) were grown in DMEM-F12 supplemented with 10% FBS. Cells were transfected with human alpha V beta 3 heterodimers using a Nucleofector II (Lonza) and Ingenio (Mirus) transfection reagents following manufacturer's protocols. Untagged integrins were in pcDNA3.1 vectors, and tagged vectors (mEos2 or Emerald) were constructed as previously described[7,11]. The tagged vectors are available through Addgene as mEos2-Alpha-V-integrin-N-25, mEmerald-Alpha-V-integrin-N-25, mEos2-Integrin-Beta3-N-18, and mEmerald-Beta3-N-18. The untagged alpha and beta subunits were gifts from Mark Ginsberg (UCSD). Cells were plated on plasma-etched cover glass that had been coated overnight with 5μg/ml human plasma fibronectin.

Cell Spreading, activation, and immunohistochemistry

Approximately 24 hrs after transfection, cells were trypsinized and plated for 30, 60, or 90 min for spreading assays or 4 hrs for adhesion assays. Cells were then fixed with 2% paraformaldehyde in PHEM[12]. Cells that were transfected with untagged integrins were co-transfected with an empty Emerald vector for visualization of the cell perimeter. For activation, treatment with 0.05mM Mn^{2+} initiated 5 min prior to plating and was

maintained throughout the spreading assay[13]. To detect adhesions in cells transfected with untagged integrins, cells were labeled with LM609 (Millipore) prior to secondary labeling with Alexa 488.

Microscopy

All imaging experiments were performed on an Olympus IX71 with a 60X 1.49 NA objective using TIRF illumination. To create the TIRF beam four laser lines (405, 488, 561, 633 nm) (Coherent) were merged and introduced through free space into the TIRF illumination port of the microscope. Position of the beam in the back aperture of the objective was motorized to ensure repeatability of the penetration depth of the evanescent TIRF wave. For the single molecule experiments a subpopulation of the mEos2 labeled molecules was stochastically excited with a low level of 405 nm activation and 561 nm excitation light (5 μW and 2.5 mW at the back aperture respectively). In these experiments cell edges were identified by collection of an image of unconjugated EGFP. In the case of live cell single-molecule experiments, every 10 sec (400 frames), the excitation light was switched to 488 nm (100 μW at the back aperture) using an acousto-optic tunable filter (AOTF, AA Opto-Electronic). Live cell experiments were imaged at 37° C for a minimum of 5 min and cells did not display any abnormal morphology or decreased motility at the end of this interval. Images were acquired at a final magnification of 111 nm/pixel with an Andor 897 EMCCD camera using an exposure time of 25 ms.

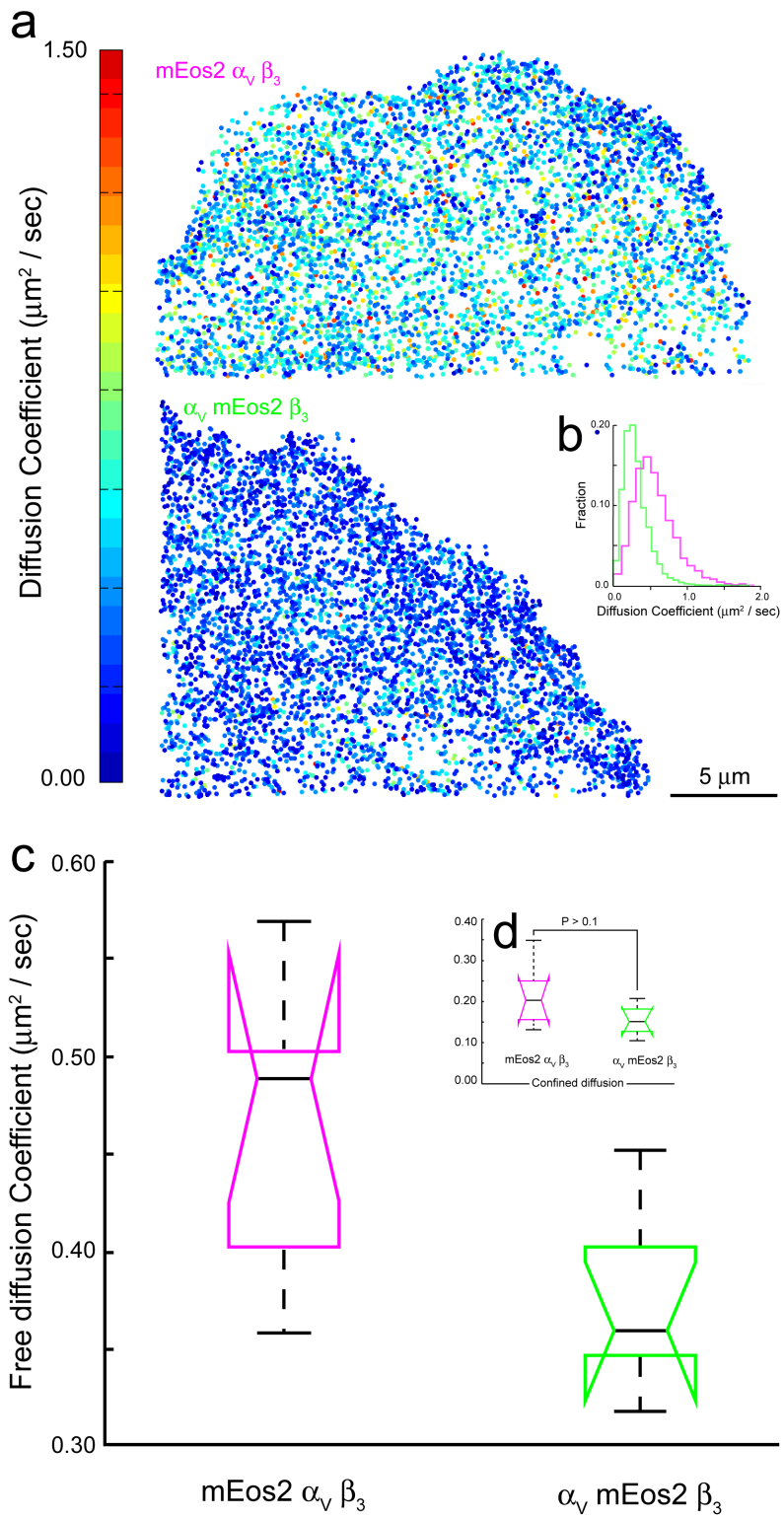
Image processing and single molecule analysis

The cell edge and adhesions were detected by thresholding images in Fiji[14] after smoothing with a 1 pixel Gaussian kernel sigma to reduce noise. Canny edge detection was used on the whole cell images after thresholding to obtain cell contours.

Single molecule analysis was performed using uTrack software[15] to localize and track individual mEos2 integrin molecules. Only molecules localized to better than 25nm precision were used for mobility analysis. Diffusion coefficients for tracks greater than 20 frames were analyzed as previously described and classified as either confined, freely diffusing or undergoing directed movement (i.e. drift)[7,9]. An average of between 6000 and 7000 molecules per cell was analyzed with a minimum of 6 cells per experimental group.

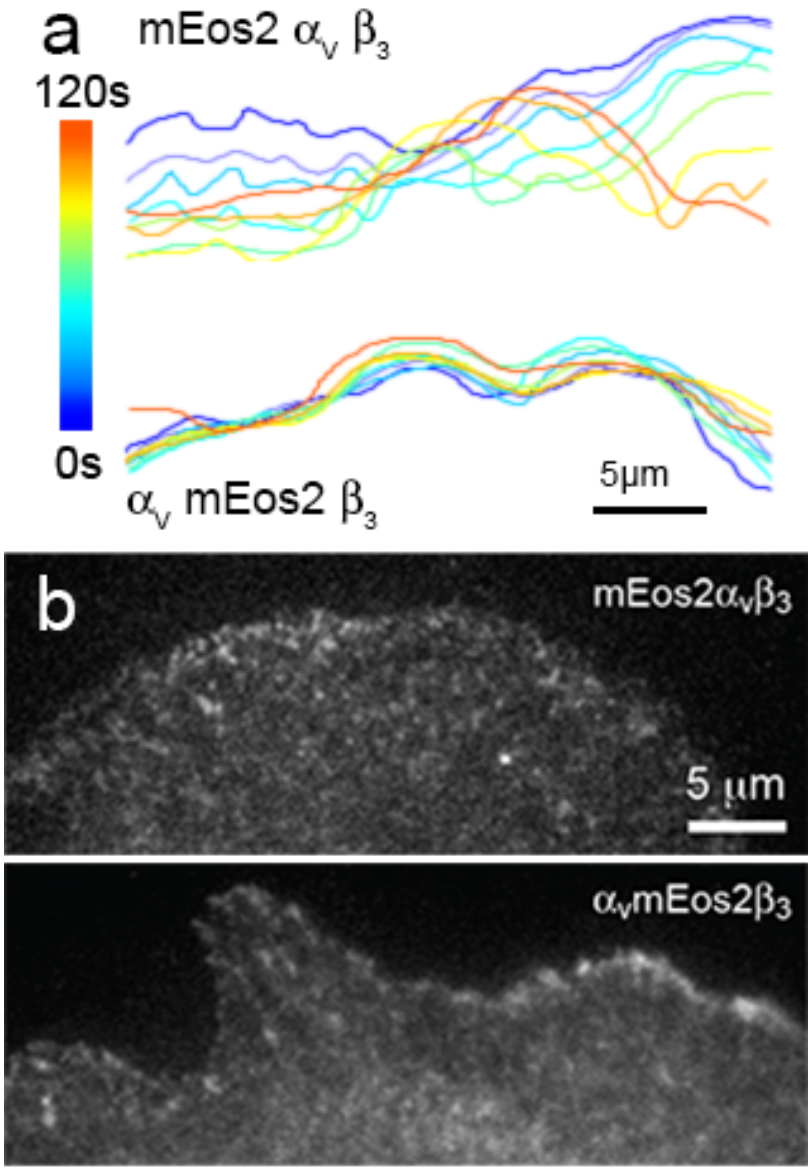
FIGURES AND FIGURE CAPTIONS

Figure 1.



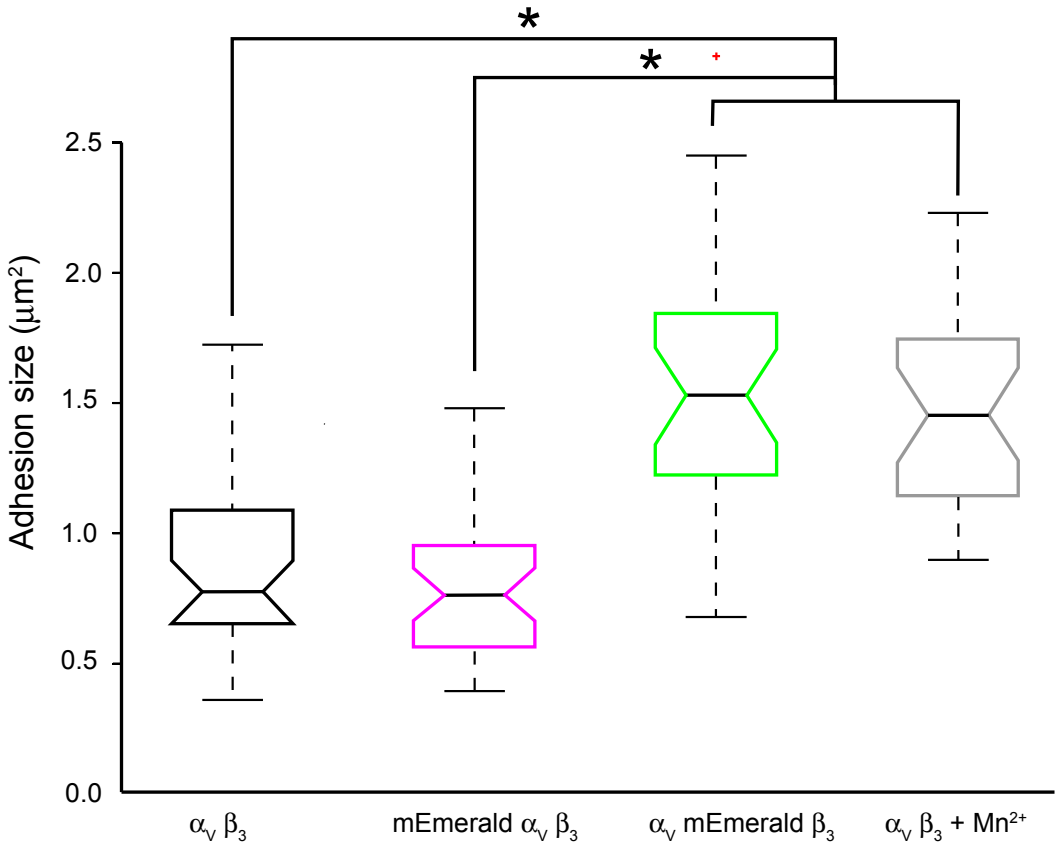
Mobility of Integrins Depends on which Subunit is Fluorescently Tagged. Comparison of mobility of single integrin molecules express as either tagged alpha V-untagged beta 3 or untagged alpha V-tagged beta 3 reveal that tagging the beta subunit slows the mobility of the integrin heterodimer. a) Diffusion coefficient of individual alpha or beta tagged integrin molecules collected over 120 s were color-coded and plotted as points whose centroid indicated the mean location of the integrin. Cells expressing integrins with tagged alpha subunits (top) show populations of integrins have higher mobility (larger diffusion coefficients) compared to populations of integrins with tagged beta subunits (bottom). b) Histogram of diffusion coefficients reveals for the cells in panel a reveal that integrins with tagged beta subunits are slower. c) Diffusion coefficients for unconfined movement are significantly slower, $p < 0.025$, n=5048, 4164 molecules from N = 6 cells for alpha tagged and beta tagged subunits, respectively. d) Diffusion coefficients for integrins showing confined movement (integrins within adhesions) are not statistically different, $p > 0.1$, n=1844, 1323 molecules from N = 6 cells for alpha tagged and beta tagged subunits, respectively.

Figure 2.



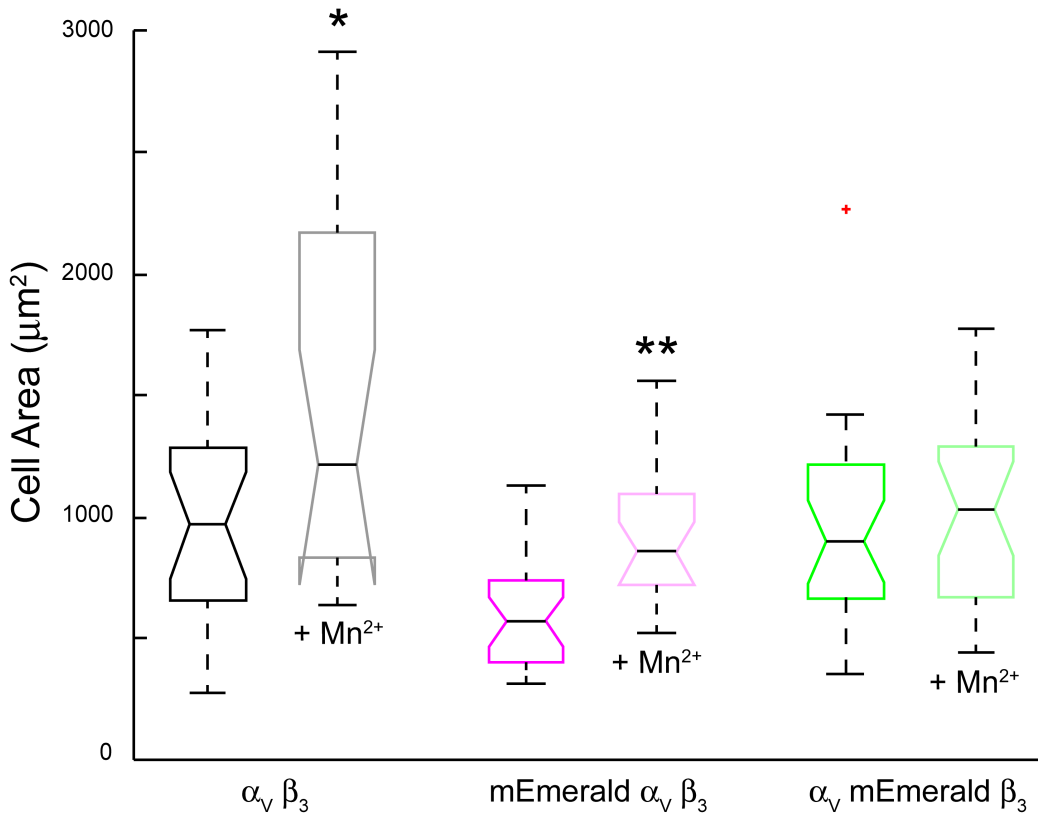
Protrusive Activity Changes When the Beta Subunit is Tagged. Cells expressing integrins with tagged alpha subunits have more protrusive activity and less organized adhesions than cells with tagged beta subunits. a) Representative cell edge contours plotted every 15 s and color coded for time show much more protrusive activity when cells express integrins with tagged alpha subunits in comparison with cells that express integrins with tagged beta subunits. b) Adhesions are larger and more organized at the leading edge when the edge is more stable in cells expressing tagged beta subunits.

Figure 3.



Adhesion Size Increases When Beta Subunit is Tagged. Focal adhesion size is larger in cells expressing integrins with tagged beta subunits and in cells expressing integrins with untagged subunits that have been treated with Mn^{2+}, $p<0.0001$*. Cells expressing integrins with both subunits untagged and cells expressing integrins with tagged alpha subunits have similar sized focal adhesions, $p > 0.38$, n=33, 35 adhesions from N=6 and 5 cells for untagged and alpha tagged subunits, respectively. Cells expressing integrins with the beta subunit tagged and cells expressing integrins with both subunits untagged, but treated with the integrin activator, Mn^{2+}, have similar sized focal adhesions, $p > 0.35$, n=27 adhesions from N=5 and 4 cells for beta tagged and untagged subunits treated with Mn^{2+}, respectively.

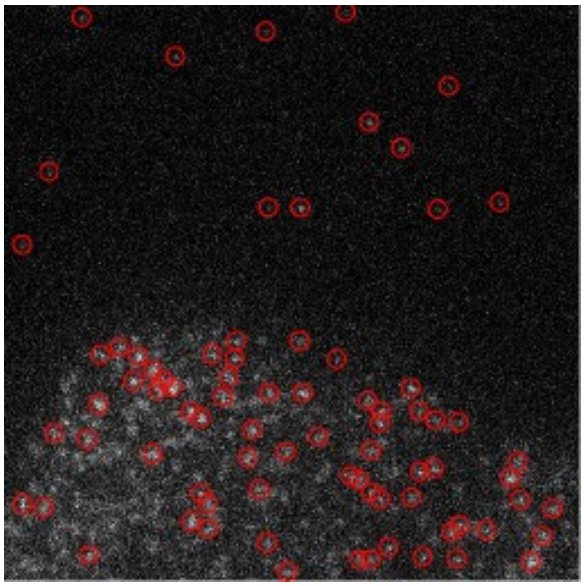
Figure 4.



Cell Response to Ligand Increases when Beta Subunit is Tagged. Cell spreading increases with integrin activation or with expression of integrins with tagged beta subunits. In cells expressing integrins with various combinations of tagged and untagged integrins that were allowed to spread for 90 min, Mn^{2+} treatment yielded the expected increase in cell spreading (untagged: $p<0.03$*, tagged-alpha: $p<0.0005$**), except in cells that were expressing integrins with tagged beta subunits. Cells expressing tagged beta subunits did not increase their area in response to Mn^{2+} treatment ($p>0.5$). N=20, 18, 27, 21, 24, 24 cells for untagged, untagged Mn^{2+}, tagged-alpha, tagged-alpha Mn2+, tagged beta, and tagged-beta Mn^{2+}, respectively.

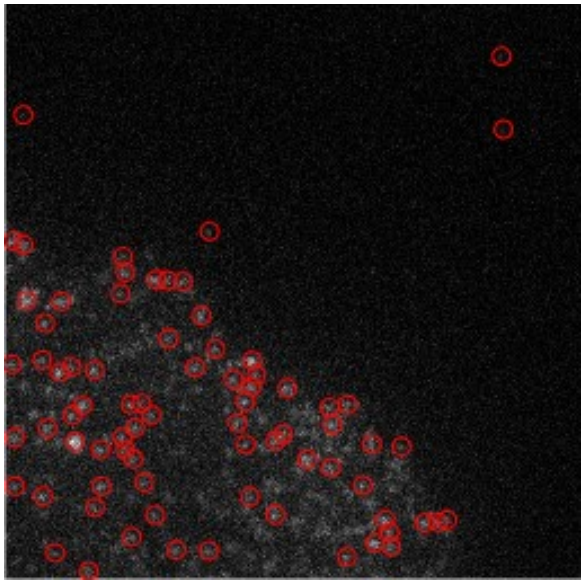
SUPPLEMENTAL MATERIAL

Supplemental Movie S1



Movie S1. Live cell single molecule video of CHO-K1 cell transfected with mEos2 $\alpha_v\beta_3$ imaged at 25 ms per frame for 2 min (4800 frames). Red circles indicate detected molecules. Interspersed every 10s (400 frames) is a GFP fill which allows determination of the cell outline. Field size – 28.4μm x 28.4μm.

Supplemental Movie S2



Movie S2. Live cell single molecule video of CHO-K1 cell transfected with α_v mEos2 β_3 imaged at 25 ms per frame for 2 min (4800 frames). Red circles indicate detected molecules. Interspersed every 10s (400 frames) is a GFP fill which allows determination of the cell outline. Field size – 28.4μm x 28.4μm

REFERENCES

1 Galbraith, C. G., Yamada, K. M. & Galbraith, J. A. Polymerizing actin fibers position integrins primed to probe for adhesion sites. *Science* **315**, 992-995, doi:10.1126/science.1137904 (2007).

2 Carman, C. V. & Springer, T. A. Integrin avidity regulation: are changes in affinity and conformation underemphasized? *Curr Opin Cell Biol* **15**, 547-556 (2003).

3 Takagi, J., Petre, B. M., Walz, T. & Springer, T. A. Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. *Cell* **110**, 599-511 (2002).

4 Takagi, J. & Springer, T. A. Integrin activation and structural rearrangement. *Immunol Rev* **186**, 141-163 (2002).

5 Plancon, S., Morel-Kopp, M. C., Schaffner-Reckinger, E., Chen, P. & Kieffer, N. Green fluorescent protein (GFP) tagged to the cytoplasmic tail of alphaIIb or beta3 allows the expression of a fully functional integrin alphaIIb(beta3): effect of beta3GFP on alphaIIb(beta3) ligand binding. *Biochem J* **357**, 529-536 (2001).

6 Rossier, O. *et al.* Integrins beta1 and beta3 exhibit distinct dynamic nanoscale organizations inside focal adhesions. *Nat Cell Biol* **14**, 1057-1067, doi:10.1038/ncb2588 (2012).

7 Jaqaman, K., Galbraith, J. A., Davidson, M. W. & Galbraith, C. G. Changes in single-molecule integrin dynamics linked to local cellular behavior. *Molecular biology of the cell* **27**, 1561-1569, doi:10.1091/mbc.E16-01-0018 (2016).

8 Jaqaman, K. *et al.* Cytoskeletal control of CD36 diffusion promotes its receptor and signaling function. *Cell* **146**, 593-606, doi:10.1016/j.cell.2011.06.049 (2011).

9 Ewers, H. *et al.* Single-particle tracking of murine polyoma virus-like particles on live cells and artificial membranes. *Proc Natl Acad Sci U S A* **102**, 15110-15115, doi:10.1073/pnas.0504407102 (2005).

10 Ballestrem, C., Hinz, B., Imhof, B. A. & Wehrle-Haller, B. Marching at the front and dragging behind: differential alphaVbeta3-integrin turnover regulates focal adhesion behavior. *J Cell Biol* **155**, 1319-1332, doi:10.1083/jcb.200107107 (2001).

11 McKinney, S. A., Murphy, C. S., Hazelwood, K. L., Davidson, M. W. & Looger, L. L. A bright and photostable photoconvertible fluorescent protein. *Nat Methods* **6**, 131-133, doi:10.1038/nmeth.1296 (2009).

12 Galbraith, C. G., Skalak, R. & Chien, S. Shear stress induces spatial reorganization of the endothelial cell cytoskeleton. *Cell Motil Cytoskeleton* **40**, 317-330, doi:10.1002/(SICI)1097-0169(1998)40:4<317::AID-CM1>3.0.CO;2-8 (1998).

13 Humphries, M. J. Cell-substrate adhesion assays. *Curr Protoc Cell Biol* **Chapter 9**, Unit 9 1, doi:10.1002/0471143030.cb0901s00 (2001).

14 Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**, 676-682, doi:10.1038/nmeth.2019 (2012).

15 Jaqaman, K. *et al.* Robust single-particle tracking in live-cell time-lapse sequences. *Nat Methods* **5**, 695-702, doi:10.1038/nmeth.1237 (2008).