A Cautionary Tail: Changes in Integrin Behavior with Labeling

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Fluorescence microscopy and specialized applications (FRET, FRAP, FLIP) are critical tools for understanding how integral membrane proteins regulate cell signaling during development, immunological responses, and diseases such as cancer metastasis. Although molecular, biochemical, and structural studies can uncover specific protein-protein interactions, unraveling the dynamic modulation of proteins relies on live-cell imaging of fluorescently tagged proteins, which are validated by correct localization and response to stimuli. Here we examined the effects of tagging on the molecular behavior of integrins, transmembrane heterodimers that modulate their adhesive and signaling properties through changes in conformation and cellular distribution. Extensive ensemble-based measurements report that tagging does not alter integrin behavior. However, using live-cell, dense-field, single molecule super-resolution microscopy[1], we identified differences in the mobility of integrins that depend on which subunit is tagged. Moreover, direct measures of integrin affinity further reveal that tagging the tail of one subunit, but not the other, elevates the affinity of integrins molecules.

Both the alpha and beta integrin subunits have large globular extracellular domain, single transmembrane domains, and short cytoplasmic tails. The transmembrane and cytoplasmic domains are bent in the inactive conformation and fully extended when the integrin is activated. We expressed $\alpha_v\beta_3$ integrins in the null background of CHO-K1 cells[2], and regardless of which subunit was tagged with a fluorophore, the expressed integrins inserted into the cell membrane and responded to ligand by organizing into adhesions (Supplementary Fig 1). Although integrins confined into adhesions had similar mobilities (Supplementary Fig 2), the mobility of freely diffusing integrins was significantly lower if the beta rather than the alpha subunits were untagged (Fig 1a, Supplementary Movies 1-2)[3]. Similarly, cells expressing tagged beta subunits had substantially less protrusive activity than cells expressing labeled alpha subunits (Fig 1b), suggesting that integrins with tagged beta subunits are more likely to interact with the extracellular matrix (ECM) ligand. Consistent with this notion, labeling the beta subunit created

adhesions that morphologically appear similar, but were significantly larger than adhesions assembled from untagged integrins or integrins with only the alpha subunit labeled (Fig 1d, Supplementary Fig 2). These larger adhesions were similar in size to those formed when neither subunit was tagged but the integrins were forced into a high affinity state by Mn^{2+} treatment[4].

We measured the effects of integrin activation on the ability of the cell to interact with ligand by quantifying cell spreading[5]. Mn^{2+} increased the spreading of cells expressing untagged or alpha tagged integrins, but it did not increase the spreading of cells expressing beta tagged integrin (Supplementary Fig 3). These data suggest that tagging the beta integrin subunit activates the integrin, similar to Mn^{2+} treatment. This interpretation is consistent with an earlier report that labeling the beta subunit caused CHO-K1 cells expressing alpha IIb beta 3 integrin to spontaneously aggregate in the presence of soluble ligand[6]. However, that study concluded that GFP fusion to the cytoplasmic tail of either subunit allows for normal surface expression of a functional receptor since tagging either subunit led to the formation of adhesions in the presence of ligand[6]. Our single molecule results, as well as our careful comparison of focal adhesion size and cell spreading all suggest that labeling the beta subunit changes the affinity of the expressed integrin for its ligand.

To test whether subunit dependent changes in integrin functionality were independent of the specific integrin heterodimer expressed, we expressed untagged, alpha tagged, and beta tagged human $\alpha_5\beta_1$ integrins in CHO-B2 cells, which lack endogenous alpha 5 integrin[7]. We then directly measured the affinity of the expressed integrin by quantifying the labeling of 9EG7, a commercially available conformation specific β1 integrin antibody[4]. Activation of integrins was not significantly different when both subunits were untagged or only the alpha subunit was tagged. However, integrins with tagged beta subunits and untagged integrins treated with Mn^{2+} were both activated compared to untagged integrins and were not statistically different from

each other (Fig 1e). These data indicate that integrins tagging the beta subunit elevates the integrin affinity state by the same mechanism as Mn^{2+}, and exposing the ligand-induced binding site (LIBS)[8].

The present study suggests a need for re-evaluating expression and localization assays as a standard for expressed protein functionality. In the example presented here, molecular behaviors that create subtle but significant differences in cellular function were typically missed by standard assays but easily detected by single molecule imaging. This cautionary tale -- labeling the beta subunit can elevate affinity to partially prime the integrin – demonstrates that to ensure molecular functionality conventional ensemble level imaging needs to be validated by dynamic molecular imaging assays.

AUTHOR CONTRIBUTIONS: C.G.G. and J. A.G. designed experiments. C.G.G. performed experiments and J.A.G. performed analysis. M. W. D. designed and created the labeled constructs. All authors discussed results. C.G.G. and J.A.G. wrote the manuscript.

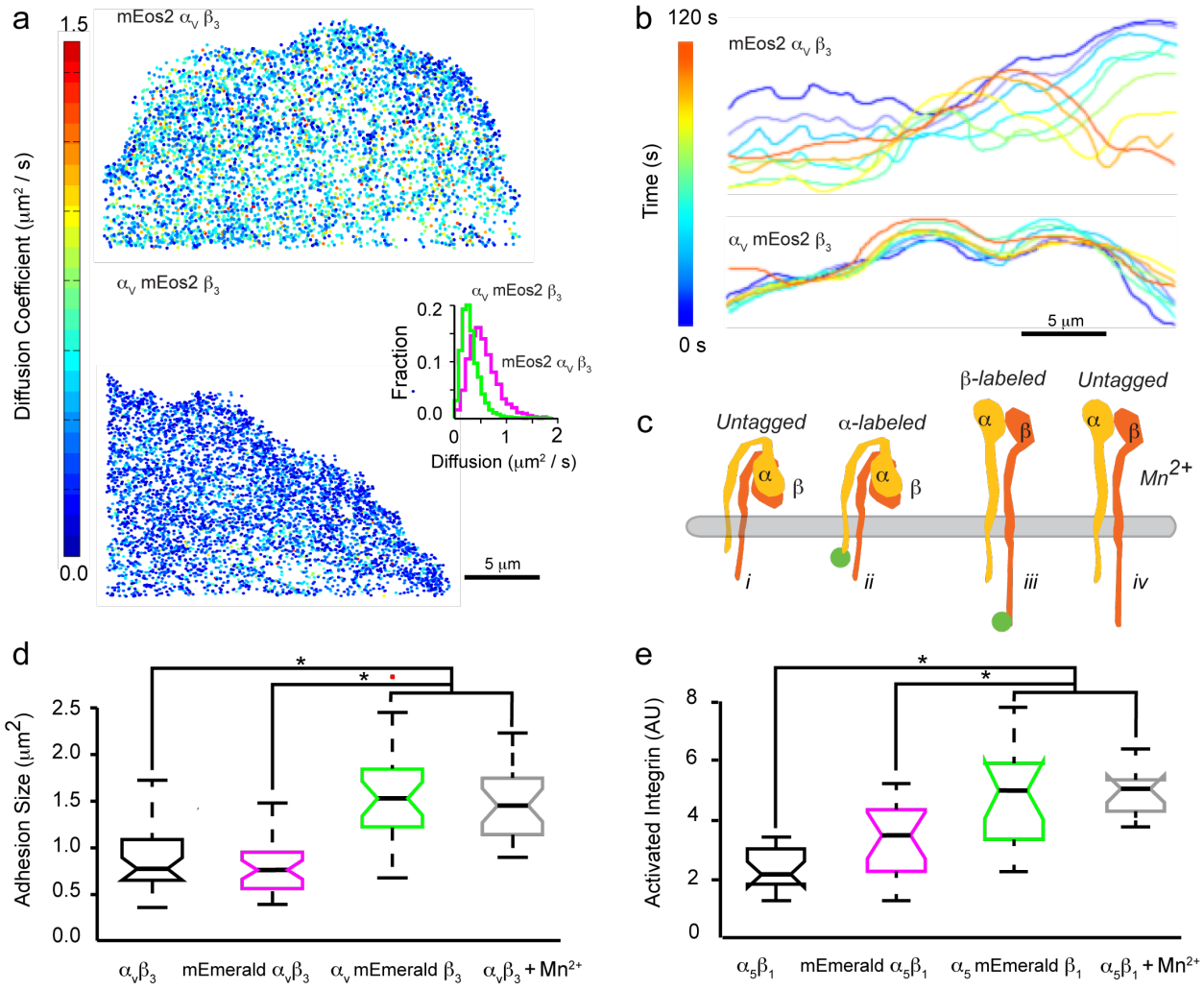

Figure 1: Tagging the Cytoplasmic Tail of the Beta Subunit Activates Integrin Heterodimers. a) Integrins with tagged alpha subunits (top) have higher mobility (larger diffusion coefficients) compared to integrins with tagged beta subunits (bottom). Diffusion coefficient of individual alpha or beta tagged integrin molecules collected over 120 s color-coded and plotted as points whose centroid indicated the mean location of the integrin. Inset: Histogram of diffusion coefficients for the cells in (a). b) Cells expressing integrins with beta tagged subunits show less protrusive activity. Representative cell edge contours plotted every 15 s and color coded for time. c) Cartoon illustrating the four-different experimental conditions: i) untagged integrin, ii) tagged alpha subunit, iii) tagged beta subunit, and iv) untagged integrins exposed to Mn^{2+}. d) Focal adhesions are larger in cells expressing integrins with tagged beta

subunits or untagged subunits and treated with Mn^{2+}, $p<0.0001$*. Adhesions in cells expressing untagged subunits or tagged alpha subunits are similar sized, $p > 0.38$, n=33, 35 adhesions from N=6 and 5 cells, respectively. Adhesions in cells expressing tagged beta subunits or untagged integrins treated Mn^{2+}, have similar sized focal adhesions, $p > 0.35$, n=27 adhesions from N=5 and 4 cells, respectively. e) 9EG7, an antibody that detects the conformational change that occurs when integrins are activated with Mn^{2+}, detects a similar conformational change in CHO-B2 cells expressing tagged beta subunits, but not in cells expressing untagged subunits or tagged alpha subunits. N=18, 17, 17, and 13 cells for untagged, alpha tagged, beta tagged, and untagged treated with Mn^{2+}, respectively.

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