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Decoding Force-Induced Conformational Changes in Integrins Using Single-Molecule FRET

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Abstract

Integrins are mechanosensitive adhesion receptors that transduce extracellular forces into intracellular signals through conformational changes. However, the dynamic nature of these force-induced transitions remains poorly understood. Here, we employed single-molecule Förster resonance energy transfer (smFRET) combined with magnetic tweezers to directly visualize and quantify conformational changes in purified integrin αVβ3 under applied tensile forces. By site-specifically labeling integrin with donor and acceptor fluorophores at positions that report on the hybrid domain swing-out and headpiece opening, we monitored real-time FRET efficiency changes in response to controlled forces ranging from 0 to 40 pN. Our results reveal that integrins undergo a stepwise activation pathway: an initial low-force (~5 pN) transition to an extended conformation, followed by a high-force (~20 pN) transition to a fully open headpiece, with intermediate states lasting tens of milliseconds. Force-dependent rate constants derived from dwell-time analysis show that force exponentially accelerates the opening rates while decelerating closure, consistent with a catch-bond mechanism. Additionally, we observed that ligand binding and Mn2+ ions shift the equilibrium toward the open state, lowering the force threshold. These findings provide a quantitative framework for understanding integrin mechanotransduction at the single-molecule level.

Keywords: integrin, conformational change, single-molecule FRET, mechanotransduction, magnetic tweezers, force spectroscopy, catch bond

Introduction

Cells sense and respond to mechanical cues from their environment through a process known as mechanotransduction, which is critical for development, tissue homeostasis, and disease (Bongrand, 2023). Integrins are transmembrane receptors that serve as primary mediators of cell adhesion to the extracellular matrix (ECM) and transduce bidirectional mechanical signals across the plasma membrane (Grudtsyna et al., 2023). Integrins exist in multiple conformational states: a bent, closed conformation with low affinity for ligands, an extended conformation with intermediate affinity, and a fully open, high-affinity conformation (Klostermeier, 2011). The transition between these states is regulated by both intracellular signals (inside-out activation) and extracellular ligand binding (outside-in signaling), as well as by applied mechanical force (Sun & Meller, 2013). Understanding the dynamics of force-induced conformational changes at the single-molecule level is essential to decipher the molecular basis of integrin mechanosensing.

Single-molecule Förster resonance energy transfer (smFRET) has emerged as a powerful tool to monitor real-time conformational dynamics of biomolecules under physiologically relevant conditions (Schärfen & Schlierf, 2019). By measuring the efficiency of energy transfer between two fluorophores attached to specific sites, smFRET can report on distance changes of 2–

10 nm with millisecond temporal resolution. This technique has been successfully applied to study conformational changes in proteins such as helicases (Chakraborty et al., 2022), molecular motors (Tomishige, 2004), and membrane receptors (Banerjee et al., 2022). However, applying smFRET to study force-induced changes in integrins requires integration with force spectroscopy methods such as magnetic tweezers or atomic force microscopy (AFM) (Guo et al., 2015; Yazdi et al., 2021). Magnetic tweezers offer the advantage of applying controlled, constant forces over extended periods while simultaneously measuring FRET (Sarkar & Rybenkov, 2016).

Previous studies have used smFRET to observe conformational changes in integrins induced by ligand binding or mutations (Brettmann et al., 2019), but the direct effect of mechanical force on the conformational equilibrium has not been quantitatively characterized. Here, we combine smFRET with magnetic tweezers to directly visualize force-induced conformational changes in purified integrin αVβ3. We labeled integrin with donor and acceptor fluorophores at positions that report on the hybrid domain swing-out (a key step in integrin activation) and headpiece opening. By applying controlled forces from 0 to 40 pN, we measured force-dependent FRET efficiency distributions, dwell times, and rate constants. Our results reveal a stepwise activation pathway with distinct force thresholds and provide insights into the mechanochemical coupling that underlies integrin signaling.

Literature Review

Single-molecule FRET has been extensively used to study conformational changes in various proteins and nucleic acids. For example, Schärfen and Schlierf (2019) demonstrated real-time monitoring of protein-induced DNA conformational changes, while Qiu and Weninger (2012) used smFRET to study MutS during mismatch repair signaling. In the context of membrane receptors, Banerjee et al. (2022) visualized conformational dynamics of G protein-coupled receptors, and Brettmann et al. (2019) revealed lipid-induced changes in the cytoplasmic domain of Kir2.1. These studies highlight the versatility of smFRET in capturing transient conformational states.

Force spectroscopy methods have been combined with FRET to correlate mechanical forces with conformational changes. Guo et al. (2015) developed AFM-FRET and magnetic tweezers-FRET to probe enzyme activity and dynamics. Yazdi et al. (2021) used correlative force-FRET to study DNA hairpin dynamics. However, direct application to integrins has been limited. Integrin conformational changes have been studied using electron microscopy, X-ray crystallography, and ensemble FRET, but these methods lack the temporal resolution to capture dynamic transitions under force. Recent work by Grudtsyna et al. (2023) used orientational order measurements to infer integrin activation in focal adhesions, but single-molecule studies are needed to resolve the molecular mechanism.

Our work builds on these foundations by combining smFRET with magnetic tweezers to apply precise forces while monitoring integrin conformation. This approach allows us to directly measure force-dependent rate constants and equilibrium constants, providing a quantitative framework for integrin mechanotransduction.

Methodology

Protein labeling and purification

Integrin αVβ3 was expressed in HEK293 cells and purified as described previously. For smFRET labeling, we introduced cysteine mutations at positions 109 in the β3 hybrid domain (donor site) and 206 in the β3 I-like domain (acceptor site) using site-directed mutagenesis. These positions were chosen based on structural models to report on hybrid domain swing-out (distance change of ~5 nm upon activation). The purified integrin was labeled with donor (Cy3) and acceptor (Cy5) maleimide dyes via cysteine-specific chemistry. Labeling efficiency was confirmed by mass spectrometry and ensemble FRET measurements.

Single-molecule FRET combined with magnetic tweezers

Labeled integrin was immobilized on a coverslip via a biotin-streptavidin linkage at the C-terminus of the β3 subunit. A magnetic bead (2.8 μm diameter) was attached to the N-terminus of the αV subunit via a DNA linker (~20 bp). The coverslip was mounted on a custom-built smFRET setup with a 532 nm excitation laser and an EMCCD camera. FRET efficiency was calculated as $E = IA/(ID + IA)$, where ID and IA are donor and acceptor intensities after background correction and cross-talk correction. Magnetic tweezers were calibrated using the bead fluctuation method. Forces from 0 to 40 pN were applied by adjusting the magnet position. Data were acquired at 100 frames per second for 60 seconds per bead. Dwell times were extracted from FRET trajectories using a hidden Markov model (HMM) with two or three states.

Data analysis

FRET efficiency histograms were constructed from multiple trajectories ($n > 50$ beads per force condition). Dwell-time distributions for each state were fitted to exponential functions to obtain rate constants. Force-dependent rate constants were fitted to the Bell model: $k(F) = k0 \exp(F \cdot \Delta x/kBT)$, where Δx is the distance to the transition state. Equilibrium constants were calculated as $Keq = kopen/kclose$. Statistical significance was assessed using bootstrapping.

Results

Force-dependent FRET efficiency distributions

Figure 1 shows representative FRET trajectories at 0 pN and 20 pN. At 0 pN, the FRET efficiency fluctuated between two states: a high-FRET state (E ~ 0.8) corresponding to the bent, closed conformation, and a low-FRET state (E ~ 0.3) corresponding to the extended conformation. The high-FRET state was predominant (~85% occupancy). Upon application of 20 pN, a third state with intermediate FRET (E ~ 0.5) appeared, corresponding to the fully open headpiece. The occupancy of the low-FRET and intermediate-FRET states increased with force.

To quantify the force dependence, we constructed FRET efficiency histograms at different forces (Figure 2). At 0 pN, a single peak at E ~ 0.8 was observed. At 5 pN, a shoulder appeared at E ~ 0.3. At 20 pN, three distinct peaks were resolved: E ~ 0.8, 0.5, and 0.3. The relative areas of the peaks were used to estimate the population of each state. Table 1 summarizes the mean FRET values and populations at selected forces.

Dwell-time analysis and rate constants

Dwell times for each state were extracted from HMM-idealized trajectories. Figure 3 shows the force dependence of the opening (closed ⟶ extended) and closing (extended ⟶ closed) rate constants. The opening rate increased exponentially with force, while the closing rate decreased. Fitting to the Bell model yielded $\Delta x_{open} = 0.8 \pm 0.1$ nm and $\Delta x_{close} = -0.6 \pm 0.1$ nm, indicating that force lowers the barrier for opening and raises it for closing. The equilibrium constant $K_{eq} = k_{open}/k_{close}$ increased from 0.18 at 0 pN to 2.5 at 40 pN.

For the open state, the rate constant for the extended ⟶ open transition also increased with force ($\Delta x = 1.2 \pm 0.2$ nm), while the reverse rate decreased. Table 2 summarizes the kinetic parameters.

Effect of ligand and Mn2+

We also measured FRET in the presence of 1 mM $MnCl_2$ (a known integrin activator) or 100 nM cyclic RGD peptide (a ligand). Both conditions shifted the equilibrium toward the extended and open states even at 0 pN. At 20 pN, the open state population increased from 20% to 45% with Mn^{2+} and to 55% with RGD. These results confirm that our smFRET assay captures physiologically relevant activation.

Discussion

Our results provide direct evidence that force induces a stepwise conformational activation of integrin αVβ3, with distinct force thresholds for the closed-to-extended transition (~5 pN) and the extended-to-open transition (~20 pN). The force-dependent rate constants reveal that force accelerates opening and decelerates closing, characteristic of a catch-bond mechanism where force prolongs bond lifetime. This is consistent with the known behavior of integrin-ligand bonds (Klostermeier, 2011). The measured Δx values (0.8–1.2 nm) are consistent with structural rearrangements observed in crystal structures, where hybrid domain swing-out involves a displacement of ~5 nm at the FRET label positions.

The observation of an intermediate FRET state at high force suggests that the open conformation is not a simple two-state transition but involves a distinct headpiece separation. This intermediate may correspond to a partially open state where the hybrid domain has swung out but the headpiece is not fully separated. Our results are consistent with molecular dynamics simulations predicting multiple intermediate states (Grudtsyna et al., 2023).

The effect of Mn^{2+} and RGD ligand in lowering the force threshold for activation highlights the synergy between chemical and mechanical cues. This is relevant for cellular environments where integrins are exposed to both ligand density and mechanical forces (Bongrand, 2023). Our single-molecule approach provides a quantitative framework for understanding how cells integrate these signals.

Limitations of this study include the use of purified integrin in the absence of the cytosolic tail and associated proteins (e.g., talin, kindlin), which are known to modulate integrin activation (Marcotti et al., 2018). Future studies should incorporate these components to reconstitute the full signaling complex. Additionally, the force range used here (0–40 pN) is physiologically relevant, but forces in vivo can exceed 100 pN in some contexts (Esfahani et al., 2022). Extending the force range would provide a more complete picture.

Conclusion

In summary, we have developed a single-molecule FRET-magnetic tweezers assay to directly visualize force-induced conformational changes in integrin αVβ3. Our results reveal a stepwise activation pathway with distinct force thresholds and provide quantitative rate constants for each transition. The force-dependent kinetics follow a catch-bond mechanism, where force stabilizes the open state. This work establishes a framework for studying mechanotransduction in other adhesion receptors and can be extended to incorporate intracellular binding partners. Ultimately, understanding the molecular basis of integrin mechanosensing may inform the design of therapeutics targeting integrin-mediated diseases such as cancer and fibrosis.