Single-molecule force measurement via optical tweezers reveals different kinetic features of two BRaf mutants responsible for cardio-facial-cutaneous (CFC) syndrome

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Abstract: BRaf (B- Rapid Accelerated Fibrosarcoma) protein is an important serine/threonine-protein kinase. Two domains on BRaf can independently bind its upstream kinase, Ras (Rat Sarcoma) protein. These are the Ras binding domain (RBD) and cysteine-rich-domain (CRD). Herein we use customized optical tweezers to compare the Ras binding process in two pathological mutants of BRaf responsible for CFC syndrome, abbreviated BRaf (A246P) and BRaf (Q257R). The two mutants differ in their kinetics of Ras-binding, though both bind Ras with similar increased overall affinity. BRaf (A246P) exhibits a slightly higher Ras/CRD unbinding force and a significantly higher Ras/RBD unbinding force versus the wild type. The contrary phenomenon is observed in the Q257R mutation. Simulations of the unstressed-off rate, $k_{off}(0)$, yield results in accordance with the changes revealed by the mean unbinding force. Our approach can be applied to rapidly assess other mutated proteins to deduce the effects of mutation on their kinetics compared to wild type proteins and to each other.

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1. Introduction

Cellular response to growth signals is mediated by the Ras-Raf-MEK (mitogen-activated protein kinase) –ERK (extracellular-signal-regulated kinase) pathway, which exists in all eukaryotes [1]. Ras is activated via binding guanosine triphosphate (GTP), following which the Ras-GTP binds to Raf with high affinity. Three Raf genes (ARaf, BRaf and CRaf) code for cytoplasmic serine/threonine kinases that are regulated by binding Ras [1–3]. Raf binds to Ras-GTP via two domains, the Ras-binding domain (RBD) [4,5] and the cysteine-rich domain (CRD) [6] (see Fig. 1). Either domain can bind to Ras-GTP with high affinity; the RBD-Ras-GTP bond is stronger [7]. BRaf somatic mutations were reported in 66% of malignant melanomas [8], and at lower frequency in a wide range of human cancers and in some rare genetic diseases, such as CFC syndrome [9]. Thus BRaf mutants have become attractive targets for therapeutic intervention [10]. BRaf (A246P) and BRaf (Q257R) are two mutants identified in patients with CFC syndrome. Though it has been confirmed that mutated BRaf have elevated kinase activity [8], variations in the kinetics of binding between Ras and highly diverse BRaf mutants have not been thoroughly characterized.
Fig. 1. Schematic representation of the Ras-Raf-MEK–ERK pathway. Activated Ras-GTP binds to Raf via two domains, RBD and CRD, which are marked by dashed circles on the figure.

Optical tweezers, which use tightly focused laser beam to exert gradient force on microscopic objects, is arguably the most versatile single-molecule manipulation tool [11]. Optical tweezers can be used to impose a specific interaction between the trapped object and a fixed partner, and to measure the force resulting from the interaction [12]. Using modified beads of a dielectric material such as polystyrene (PS) as a handle, it is possible to study with optical tweezers the mechanical properties of single DNA molecules [13], the interactions between proteins and nucleic acids [14,15], and protein-protein interactions [16] via single-molecule force spectroscopy (SMFS), which yields a distribution of unbinding forces for specific molecular bonds [17], and can reveal structural and functional information for single molecular domains in solution.

In this study, we employed customized optical tweezers to measure the unbinding force between activated Ras and, respectively, wild type BRaf, BRaf (A246P) and BRaf (Q257R). SMFS reveals that both mutants bind Ras with higher affinity than the wild type protein. Nevertheless, the kinetic effects of CRD and RBD in two mutants are varied, which might lead to different requirements for suitable inhibitors.

2. Materials and methods

2.1 Experimental setup

The experimental setup consists of an optical tweezers based on an inverted microscope configuration (Zeiss Axiovert 200, Oberkochen, Germany), a quadrant photodiode detector (QD, custom-built), and a program-controlled 3-dimensional piezoelectric transducer sample stage (3D-PZT, Nanonics Imaging, Haifa, Israel). As shown in Fig. 2, the trap is made by a linearly polarized 2 W Nd:YAG laser (Continum, Santa Clara, California, USA) with wavelength of 1064 nm. The expanded beam is focused by lens L3 (focal length: 180 mm) to the conjugate point of the microscope objective (100 × oil objective, N.A. 1.33, Zeiss), then focused on the focal plane.
Fig. 2. Diagram of the experimental setup. The beam expander comprises L1 and L2. L3 is a coupling mirror, which focuses the collimated laser beam at the conjugate point of the tube mirror (L4) and the objective of the microscope. The image of the bead is projected to the quadrant photodiode detector (QD) by L5. An acousto-optic deflector (AOD) is employed to manipulate the beam during calibration. The AOD is controlled by a field programmable gate array (FPGA), which is driven by a direct digital synthesizer (DDS).

Samples were illuminated via bright-field microscopy and monitored by a CCD (WV-PA410/G, Panasonic, Osaka, Japan). The displacement of the trapped bead was measured from its images collected by the QD panel. The data acquisition by the QD, and the motion of the 3D-PZT sample stage, were controlled by a computer with a data acquisition (DAQ) card (NI-6229, National Instruments, Austin, Texas, USA) and a custom-developed LabView program. A MATLAB program was developed to convert the QD signal into the displacement profile, allowing the force to be measured.

2.2 System calibration

To accurately calibrate the position and stiffness of our optical tweezers, the laser beam is manipulated by an acousto-optic deflector (AOD), which is controlled by a field programmable gate array (FPGA) module, as reported in our previous work [18].

In our experiment, the AOD drives the laser beam to move in a one-dimensional sinusoidal trajectory, namely, \( x(t) = A_0 \sin(2\pi f t) \), where \( x(t) \), \( A_0 \), and \( f \) are the displacement over time, amplitude and frequency of the laser movement respectively. Then, the displacement of the bead trapped by the laser \( (x_b(t)) \) can be derived from the Langevin equation, and written as Eq. (1), where \( f_0 \) is the characteristic roll frequency, \( c \) is a constant determined by initial conditions and \( \phi = -\arctan(f / f_0) \).

\[
x_b(t) = c \exp(-2\pi f_0 t) + A_0 \left(\left[\frac{2\pi f_0 t}{f} + 1\right]^{-1/2} \right) \sin(2\pi f t + \phi)
\]  

(1)

The characteristic roll frequency \( f_0 \) can be described by Eq. (2), where \( k \) is the stiffness of the optical tweezers, \( \gamma \) is the friction coefficient, equal to \( 6\pi \alpha \eta \) (\( \alpha \) is the radius of the bead, and \( \eta \) is the viscosity of the liquid for water at 20 °C, \( \alpha = 2.5 \mu m \), \( \eta = 1.009 \times 10^{-3} Pa \cdot s \)).

\[
f_0 = k / (2\pi \gamma)
\]  

(2)
The term $c \exp(-2\pi f_0 t)$ in Eq. (1) can be omitted when the bead is driven to over-damping vibrations by the moving optical tweezers after a period longer than $(1/f_0)$. Therefore, the relationship between the amplitude of the trapped bead’s oscillation ($A$) and the amplitude of the optical tweezers’ oscillation ($A_0$) can be written as Eq. (3).

$$A = A_0 \left(\frac{f}{f_0}\right)^{-1/2}$$

(3)

Because the motion of the optical tweezers is controlled by the AOD, which is modulated by the computer via FPGA, $A_0$ and $f$ can be manually set, and $A$ can be measured with the calibrated QD. Therefore, $f_0$ can be calculated from Eq. (3), and finally the stiffness $k$ is deduced from Eq. (4).

$$k = 2\pi f_0 = 2\pi f_0 \left(\frac{A_0}{A}\right)^{-1}$$

(4)

Before the calibration of $k$, the sensitivity of the QD to the displacement of trapped PS bead should be calibrated in advance. In the experiment, each step of the FPGA corresponded to a displacement of the optical tweezers by 0.0541 $\mu$m. Then, a PS bead was trapped by the stationary tweezers, and its image was projected to the QD panel. Finally, the AOD drove the tweezers to scan with a frequency ($f$) and an amplitude ($A_0$) of 0.812 $\mu$m. A high $f_0$ value leads to the approximation $A_0 \approx A$ according to Eq. (3), where $A_0 = 0.812 \mu$m. Under the aforementioned conditions, the corresponding voltage output of QD ($v$) was 2.47 V. The relationship between $v$ and $A$ resulted in the position sensitivity of the QD, namely $K_{Q}$. In this study, $K_{Q} = \frac{A}{v} = 0.328 \mu m/V$.

The tweezers were then driven by the AOD to scan with a sinusoidal signal whose amplitude ($A_0$) was fixed at 0.812 $\mu$m. The laser power was around 150 mW. Values of $A$ were recorded upon changing the frequency of the drive signal ($f$), from 100 Hz to 900 Hz. The characteristic roll frequency $f_0$ was obtained by fitting the experimental data to Eq. (3). The stiffness $k$ was calculated using the experimental $f_0$ value and Eq. (4). Because increasing the stiffness of the optical tweezers could decrease the value of $A$, which in turn reduced the signal-noise-ratio (SNR) of the QD output, the power of laser source was kept at a low level during the calibration (below 200 mW).

This process was repeated upon changing the laser power at the focal plane of the microscope, from 153 mW to 20 mW. The linear relationship between the stiffness and laser power was then determined, as shown in Eq. (5).

$$k = 0.415 \cdot mW^{-1} \cdot pN \cdot \mu m^{-1}.$$

(5)

The stiffness of the optical tweezers at higher power was extrapolated according to Eq. (5). During SMFS measurement, the power of laser beam at the focal plane of the microscope was fixed at 500 mW, resulting in the $k$ value of 207 pN/$\mu$m. Then, the unbinding force $F_u$ could be calculated with the QD output $v$ according to Eq. (6).

$$F_u = k \cdot v$$

(6)

2.3 Materials, bead preparation and unbinding force measurement

Carboxylated polystyrene beads (PS-COOH), 5 $\mu$m in diameter, were obtained from Bangs Laboratories (Hamilton, Indiana, USA). 2-N(morpholino)ethanesulfonic acid (MES) buffer, N-hydroxysuccinimide (NHS), 1 - (3 - dimethylaminopropyl) - 3 - ethycarbodiimide hydrochloride (EDC) and Guanosine triphosphate (GTP) were obtained from Sigma (St. Louis, Missouri, USA) and used without further purification. Bovine serum albumin (BSA) and Tris(hydroxymethyl) aminomethane + hydrochloric acid (Tris-HCl) buffer was obtained from M&C Technology, Beijing, China.

PS-COOH beads were functionalized via the following protocol with GST-handled Ras, BRaf [CRD + RBD], BRaf (A246P) [CRD + RBD], BRaf (Q257R) [CRD + RBD], and BRaf
All these proteins were obtained from Professor J. Julius Zhu’s Lab, the School of Medicine, University of Virginia.

A 100 μL aliquot of beads (5% w/v, suspended in deionized water) were washed in MES buffer. The pellet was re-suspended in 1 mL activation buffer, containing 12.5 mg NHS, 7.8 μmol EDC and 20 μmol MES, and incubated at room temperature for 1 h. The beads were then washed again in deionized water to remove excess NHS and EDC. Finally, the beads were incubated with molecules of interest in Tris-HCl buffer (pH = 7.4) at 37 °C for 4 h and then washed in deionized water. The unreacted sites on the beads were blocked with BSA.

All the experiments were performed in a sample cell mounted on the PZT sample stage. The cell chamber consisted of a modified petri dish with a coverslip on the bottom, and another strip of coverslip vertically attached to the bottom. Prior to the experiment, the cell chamber was immersed in 0.1% polylysine solution (M&C Technology, Beijing, China).

In order to acquire the unbinding force distribution of Ras and BRaf, the following experimental procedures were repeated over 2000 times for each of the bead samples functionalized with Ras-GTP/BRaf [CRD + RBD], Ras-GTP/BRaf (A246P) [CRD + RBD], Ras-GTP/BRaf (Q257R) [CRD + RBD], Ras/BRaf [CRD + RBD] and Ras/BRaf (470). BRaf-beads with Tris-HCl buffer (pH = 7.4) were first added to the chamber, trapped by optical tweezers, and manipulated onto the vertical surface of the polylysine-treated cover glass (Fig. 3(a)). The buffer solution and the remaining unbound BRaf-beads were removed. An aliquot of 20 μL Ras-beads was then added, followed by 20 μL of 10 nM GTP solution (or Tris-HCl buffer in control experiments). Subsequently, driven by the PZT stage, a trapped bead was brought in contact to the fixed BRaf-bead (Fig. 3(b)). After 1 s of primary contact, the beads were separated at a velocity of 5 μm/s, producing a loading rate of 2 nN/s (Fig. 3(c)). If “binding-unbinding” between two beads occurred (Fig. 3(c)-3(d)), a typical force-distance curve was obtained (Fig. 4), which could then be used to determine the unbinding force.

Fig. 3. (a) Image of beads acquired by CCD (left: a trapped Ras-bead; right: a fixed BRaf-bead. Bead diameter: 5 μm). (b) Diagram of the optical tweezers setup for measuring the unbinding force between trapped Ras-beads and fixed BRaf-beads. The fixed BRaf-bead is brought in contact with the trapped bead by the PZT. (c) If binding between the beads occurs, the trapped bead deviates from the equilibrium position as the fixed bead is pulled back. (d) When the gradient force on the trapped bead is strong enough to break the association, unbinding occurs.
3. Result and discussion

To verify the specificity of the Ras-GTP/BRaf interaction, two control groups are defined. The one is the Ras/BRaf interaction group, in which GTP molecules are absent. The other is the Ras/BRaf (470) interaction group, in which the CRD and RBD are absent.

Figure 5 shows histograms of the unbinding force for control groups. The experimental data fit a log-normal distribution as per Eq. (7) (R^2 = 0.98, p<0.01), where most of the measured unbinding forces were below 35 pN.

\[
f(x, \mu, \sigma, K) = K \cdot \frac{1}{(x \sigma)} \cdot \exp\left[-\frac{(\ln x - \mu)^2}{2\sigma^2}\right]
\]

Figure 5 (Insert) shows the adhesion ratios for the control groups and the specific interaction groups. The adhesion ratios are similar among three specific interaction groups, but notably reduced in control groups. This indicates that most of the adhesion cases in the specific interaction groups are induced by the specific association between Ras-GTP and BRaf or its mutants. Therefore, unbinding force measurements on these groups can reveal the kinetics of bonds between Ras-GTP and BRaf or its mutants. The specificity of these interactions is verified.
Fig. 5. Histogram of unbinding force for Ras/BRaf and Ras-GTP/BRaf (A470) interactions and the fitting curve of the experimental data. Insert: Adhesion ratios for Ras-GTP/BRaf, Ras-GTP/BRaf (A246P), Ras-GTP/BRaf (Q257R), Ras/BRaf and Ras-GTP/BRaf (A470) interactions. The adhesion ratios of three specific interaction groups all exceeded 20%, while those of control groups were all around 10%. "n" referred to the number of contact cases measured in each group.

The histogram of unbinding forces for the specific interaction groups (Fig. 6(a)-6(c)) showed a significant peak at forces less than 35 pN. This peak also fits to a log-normal distribution curve. The similarity between the fitting of this peak in the samples and in the control indicates that this peak corresponds to nonspecific association between the trapped bead and the fixed one.

Fig. 6. (a) Unbinding force histograms and simulated curves for the Ras-GTP/BRaf interaction, which include 585 binding-unbinding events among 1800 attachment events. (b) Ras-GTP/BRaf (A246P) interactions, which include 596 binding-unbinding events among 2536 attachment events. (c) Ras-GTP/BRaf (Q257R) interactions, which include 751 binding-unbinding events in 2269 attachment events. The arrow indicates the position of each specific binding peak. The fitting of nonspecific interactions is according to Eq. (7). The fitting of binding peak is according to Eq. (11)-(13).
Three notable peaks occur in the range of 40–160 pN. The values of the unbinding force from these peaks were subjected to the Lillieford’s Test, revealing that the bins under each peak were approximately normally-distributed (p < 0.05) and could be fitted by Eq. (8).

\[ f(x, \mu_{i}, \sigma, K_{i}) = \sum_{i} K_{i} \cdot \exp\left[-\frac{(x - \mu_{i})^{2}}{2\sigma^{2}}\right] \]  

Table 1 shows the fitted mean values of the specific binding peaks in the histograms of Ras-GTP/BRaf, Ras-GTP/BRaf (A246P) and Ras-GTP/BRaf (Q257R) according to Eq. (8). In all three cases, the sum of mean value of the first peak (\(\mu_{1}\)) and that of the second one (\(\mu_{2}\)) is almost equal to the mean value of the third (\(\mu_{3}\)). This supports the model that the first peak corresponds to the weaker Ras-BRaf [CRD] bond, the second to the stronger Ras-BRaf [RBD] bond, and the third to the full binding between Ras and BRaf [CRD + RBD]. The 68% confidence interval is given for (\(\mu_{1}\)), (\(\mu_{2}\)), and (\(\mu_{3}\)) respectively.

### Table 1. Mean unbinding force for peaks no. 2–4 in the histograms of unbinding force for Ras-GTP/BRaf, Ras-GTP/BRaf (A246P), and Ras-GTP/BRaf (Q257R) interactions.

| BRaf species          | \(\mu_{1}\) (pN) | \(\mu_{2}\) (pN) | \(\mu_{3}\) (pN) | \(\mu_{1} + \mu_{2}\) (pN) | \(R^{2}\)   | \(p^{*}\)   |
|-----------------------|------------------|------------------|------------------|-----------------------------|------------|------------|
| BRaf (WT)             | 40 ± 8           | 95 ± 11          | 136 ± 12         | 135                        | 0.75       | < 0.01     |
| BRaf (A246P)          | 46 ± 5           | 114 ± 12         | 156 ± 18         | 160                        | 0.78       | < 0.01     |
| BRaf (Q257R)          | 60 ± 5           | 102 ± 12         | 155 ± 15         | 162                        | 0.70       | < 0.01     |

\(\mu_{i}\) values reveal that, compared with the wild type BRaf, the mean unbinding force of the Ras-GTP/CRD interaction (\(\mu_{1}\)) increased less notably in the A246P mutant than the Q257R mutant (Table 1.). In contrast, the mean unbinding force of the Ras-GTP/RBD interaction (\(\mu_{2}\)) increased more notably in the A246P mutant than in the Q257R mutant, while the mean unbinding force of Ras-GTP/BRaf (Q257R)[RBD] interaction is very close to that of Ras-GTP/BRaf[RBD] interaction. The mean unbinding force of the Ras-GTP/BRaf [CRD + RBD] interaction (\(\mu_{3}\)) is similar in both mutations, which yield significantly greater bond strength than wild type BRaf.

An alternative simulation of the experimental data can be performed using the one-step master equation shown in Eq. (9), where \(p_{i}(t)\) is the possibility of finding a number \(i\) of closed bonds.

\[ \frac{dp_{i}}{dt} = r_{i}p_{i} - rp_{i} \]  

Equation (9) states that \(i\) decreases through the rupture of a closed bond with the rate \(r_{i}\) expressed by Eq. (10).

\[ r_{i} = ik_{o,0} = ik_{o,0}(0) \cdot \exp\left[mt i (t)\right] \]  

(Inferred from Bell’s model [19])

where \(k_{o,0}(0)\) is the unstressed off-rate, \(m\) is the loading rate, \(t\) is the interaction time, \(i\) is the number of closed bonds, \(F_{o} = k_{o,0}(0)x_{h}\) is the intrinsic force of the bond, and \(x_{h}\) is the reaction compliance of the bond. Equation (11), derived from Eq. (9), gives the probability density for the unbinding force \(F_{o}\) of initial number of closed bonds \(N_{i}\) [20], where \(k_{B}\) is the Boltzmann constant, and \(T\) is the absolute temperature. \(N_{m}\) is the maximal number of initial bonds, and \(p_{o}(N_{i})\) is the coefficient determined by Eq. (12).
The experimental results are dominated by the Ras-GTP/BRaf [CRD], Ras-GTP/BRAF [RBD], Ras-GTP/BRaf [CRD + BRD] interactions and the nonspecific ones. Equation (11) can therefore be simplified to Eq. (13).

\[
D(F_e) = D_{\text{nonspecific}} + p_{\alpha\text{CRD}}(1)D_{\text{CRD}}(F_e) + p_{\alpha\text{RBD}}(1)D_{\text{RBD}}(F_e) + p_{\alpha\text{CRD+RBD}}(1)D_{\text{CRD+RBD}}(F_e) + \varepsilon(F_e)
\]

\[
d_{\text{nonspecific}}\text{ is the probability of having the nonspecific forces fit a log-normal distribution. } c(F_u)\text{ is the fairly low probability of multiple bonds.}
\]

The curves in Fig. 6 (a)-(c) show the simulated distribution for unbinding forces using Eq. (11) and Eq. (13). Least-squares fitting yields values of \( k_{\text{off}}(0) \) and \( x_b \) for each specific interaction peak, as shown in Table 2.

### Table 2. The \( k_{\text{off}}(0) \) and \( x_b \) values for Ras-GTP/BRaf, Ras-GTP/BRaf (A246P), and Ras-GTP/BRaf (Q257R) bonds.

| BRaf species | CRD | RBD | CRD + RBD | \( R^2 \) | \( p^* \) |
|--------------|-----|-----|-----------|---------|--------|
| BRaf (WT)    | \( 7.7 \times 10^{-3} \) | 0.51 | \( 2.1 \times 10^{-3} \) | 0.37    | \( 3.7 \times 10^{-3} \) | 0.31  | 0.89 | <0.01 |
| BRaf (A246P) | \( 1.0 \times 10^{-4} \) | 0.77 | \( 6.3 \times 10^{-4} \) | 0.45    | \( 1.2 \times 10^{-5} \) | 0.32  | 0.87 | <0.01 |
| BRaf (Q257R)| \( 5.4 \times 10^{-4} \) | 0.84 | \( 9.6 \times 10^{-4} \) | 0.43    | \( 1.2 \times 10^{-5} \) | 0.32  | 0.85 | <0.01 |

\( R^2 \) and \( p^* \) values are derived from the simulated results of all experimental data (solid curves in Fig. 6) following Eq. (9)-(11).

As shown in Table 2, the simulation based on Eq. (11) and (13) reveal that the \( k_{\text{off}}(0) \) values for all three specific interaction modes between Ras and BRaf decrease in both mutants. However, compared to the wild type BRaf, the \( k_{\text{off}}(0) \) value for the Ras-GTP/[CRD] interaction decreased less significantly in the A246P mutant than in the Q257R mutant, while the \( k_{\text{off}}(0) \) value for the Ras-GTP/[RBD] interaction decreased more significantly in the A246P mutant than in the Q257R mutant. Then, in both of the Ras-GTP/BRaf (A246P) and Ras-GTP/BRaf (Q257R) interactions, the \( k_{\text{off}}(0) \) and \( x_b \) values of Ras-GTP/BRaf [CRD + RBD] interaction are identical. The results indicate that though both of the mutants are able to induce CFC syndrome, they vary in the binding kinetics of their CRD and RBD.

The mean unbinding force of each specific binding peak and \( k_{\text{off}}(0) \) of the bonds all indicate stronger overall binding between Ras-GTP and mutated BRaf. However, the A246P mutation increases the strength of the Ras-GTP/BRaf [CRD] bond less notably than Ras-GTP/BRaf [RBD] bond, while the contrary phenomenon is observed in the Q257R mutation.
4. Conclusion

In summary, the change in the mean unbinding force and the $k_{off}(0)$ value for the A246P and Q257R mutants indicates that these two mutations in the CRD cause identical increases in the strength of Ras-GTP/BRaf [CRD + RBD] bonds, and this increase forms the biochemical basis for the pathological effects of these mutants. The greater values in the mean unbinding force and smaller values in $k_{off}(0)$ of RAS-GTP/BRAF (A246P)[CRD + RBD] and RAS-GTP/BRAF (Q257R)[CRD + RBD] agree with the known mechanism. However, the kinetics features of these mutants vary in two aspects. First, regarding the mean unbinding force of each specific binding peak, the A246P mutation leads to a greater increase in the mean unbinding force of the Ras-GTP/BRaf [RBD] interaction. The Q257R mutation induces a greater increase in the mean unbinding force of the Ras-GTP/BRaf [CRD] interaction. Second, as to the unstressed-off rate $k_{off}(0)$, the A246P mutation leads to a greater decrease in the $k_{off}(0)$ for Ras-GTP/BRAF [RBD] bonds, while the Q257R mutation induces a greater decrease in the $k_{off}(0)$ for Ras-GTP/BRaf [CRD] bonds. These indicate that there are cross-role effects of the A246P mutation in BRAF [CRD] to the RAS-GTP/BRaf [RBD] interaction, which have not been reported heretofore. Meanwhile, the effect of Q257R mutation is restricted within RAS-GTP/BRAF [CRD] interaction.

The difference can lead to different requirements to the structure of specific antagonists to suppress the elevated activation of different BRAF mutations. In another word, the antagonist capable to inhibit the RAS-GTP/BRAF interaction in the CFC patients carrying BRAF (Q257R) mutation could not be so effective in the patients carrying BRAF (A246P). It is very possible that such phenomenon is widespread in the disease caused by the protein with polymorphic mutations.

The advantage of single-molecule techniques, such as SMFS, over traditional biochemical techniques is that they can provide more detailed information about the kinetics of molecular interactions. In this case, because the superimposition of Ras-GTP/BRaf [CRD] and Ras-GTP/BRaf [RBD] interactions yields identical mean unbinding forces in the A246P and Q257R mutations, the cross-role effect of the CRD mutation on the RBD cannot be identified by biochemical methods that only measure the interactions but not their single-molecule kinetics. With only one specific experimental group for each mutant, the binding force measurement sheds light on the varied effects of single point mutations on individual interactions between Ras-GTP and each BRaf domain, and the superimposed effects of all the interactions occurring on BRaf molecule.

Our approach can also be applied to other specific binding protein pairs, because all experiments are done in vitro according to straightforward sample preparation procedures that can be adapted to other proteins. The use of optical tweezers to perform SMFS allows the effects of point mutations on the mean rupture force, $k_{off}(0)$ and $x_b$ to be determined, towards clarifying the interactions between any protein pair of interest.

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