Multiple-state reactions between the epidermal growth factor receptor and Grb2 as observed by using single-molecule analysis

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Phosphorylation of the cytoplasmic tyrosine residues of the epidermal growth factor receptor (EGFR) upon binding of EGF induces recognition of various intracellular signaling molecules, including Grb2. Here, the reaction kinetics between EGFR and Grb2 was analyzed by visualizing single molecules of Grb2 conjugated to the fluorophore Cy3 (Cy3–Grb2). The plasma membrane fraction was purified from human epithelial carcinoma A431 cells after stimulation with EGF and attached to coverslips. Unitary events of association and dissociation of Cy3–Grb2 on the EGFR in the membrane fraction were observed at different concentrations of Grb2 (0.1–100 nM). The dissociation kinetics could be explained by using a multiple-exponential function with a major (>90%) dissociation rate of 8 s⁻¹ and a few minor components, suggesting the presence of multiple bound states. In contrast, the association kinetics could be described by a stretched exponential function, suggesting the presence of multiple reaction channels from many unbound substates. Transitions between the unbound substates were also suggested. Unexpectedly, the rate of association was not proportional to the Grb2 concentration: an increase in Cy3–Grb2 concentration by a factor of 10 induced an increase in the reaction frequency approximately by a factor of three. This effect can compensate for fluctuation of the signal transduction from EGFR to Grb2 caused by variations in the expression level of Grb2 in living cells.

EGFR | signal transduction | tyrosine phosphorylation

The epidermal growth factor receptor (EGFR) is a transmembrane receptor protein embedded in the plasma membrane of many types of cells and is responsible for cell proliferation, movement, and carcinogenesis (1, 2). Binding of the extracellular ligand, epidermal growth factor (EGF), activates EGFR to induce mutual phosphorylation of the tyrosine residues in the cytoplasmic domain (3–5). The phosphotyrosine residues are then recognized by various cytoplasmic proteins, including Grb2 (6), Shc (7, 8), and PLC-γ (3–5). The phosphotyrosine residues are then recognized by various intracellular signaling molecules, including Grb2. Here, the reaction kinetics between EGFR and Grb2 was analyzed by visualizing single molecules of Grb2 conjugated to the fluorophore Cy3 (Cy3–Grb2). The plasma membrane fraction was purified from human epithelial carcinoma A431 cells after stimulation with EGF and attached to coverslips. Unitary events of association and dissociation of Cy3–Grb2 on the EGFR in the membrane fraction were observed at different concentrations of Grb2 (0.1–100 nM). The dissociation kinetics could be explained by using a multiple-exponential function with a major (>90%) dissociation rate of 8 s⁻¹ and a few minor components, suggesting the presence of multiple bound states. In contrast, the association kinetics could be described by a stretched exponential function, suggesting the presence of multiple reaction channels from many unbound substates. Transitions between the unbound substates were also suggested. Unexpectedly, the rate of association was not proportional to the Grb2 concentration: an increase in Cy3–Grb2 concentration by a factor of 10 induced an increase in the reaction frequency approximately by a factor of three. This effect can compensate for fluctuation of the signal transduction from EGFR to Grb2 caused by variations in the expression level of Grb2 in living cells.

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The authors declare no conflict of interest. Abbreviation: EGFR, epidermal growth factor receptor.

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Single-molecule observation of interactions between EGFR and Cy3–Grb2. (A and B) Cy3–Grb2 bound to the plasma membrane fraction with (A) and without (B) EGF stimulation. The fluorescence intensity of each spot represents the frequency of Cy3–Grb2 binding because images were accumulated during 1 min. (Scale bar: 5 μm.) (C) Repeated binding and release of Cy3–Grb2 were observed at a single site on the stimulated membrane fraction (see SI Movie 1). (D) Changes in the fluorescence intensity at single binding sites plotted against time. A histogram of the fluorescence intensity is shown on the right of the plot at 10 nM Cy3–Grb2. The averages and σ value are used to determine thresholds for the on and off states (see Materials and Methods). (E) The on- and off-time for each event were determined from the fluorescence intensity.

Fig. 1. Single-molecule observation of interactions between EGFR and Cy3–Grb2. (A and B) Cy3–Grb2 bound to the plasma membrane fraction with (A) and without (B) EGF stimulation. The fluorescence intensity of each spot represents the frequency of Cy3–Grb2 binding because images were accumulated during 1 min. (Scale bar: 5 μm.) (C) Repeated binding and release of Cy3–Grb2 were observed at a single site on the stimulated membrane fraction (see SI Movie 1). (D) Changes in the fluorescence intensity at single binding sites plotted against time. A histogram of the fluorescence intensity is shown on the right of the plot at 10 nM Cy3–Grb2. The averages and σ value are used to determine thresholds for the on and off states (see Materials and Methods). (E) The on- and off-time for each event were determined from the fluorescence intensity.

the fluorophore Cy3 at the amino terminus (Cy3–Grb2), repeated binding and release of fluorescent Cy3 were observed at the same positions on the glass surface (Fig. 1A and C and SI Movie 1). In the nonstimulated plasma membrane fraction, such repeated binding of Cy3–Grb2 was hardly observed (Fig. 1B), and the total binding duration represented only 2.8% of that for the stimulated membrane fraction. These results suggest specific binding of Cy3–Grb2 to activated EGFR molecules.

Changes in the fluorescence intensity at individual binding sites were measured for 18 min at various concentrations of Cy3–Grb2 (Fig. 1D). The fluorescence intensity of the Cy3–Grb2 spot varied for each binding event, but its distribution was similar to that of single molecules of Cy3 observed under the same conditions. Simultaneous binding of multiple Cy3–Grb2 molecules at the same binding site was rarely observed. The frequency of the binding events increased as the concentration of Grb2 in the solution increased. Thus, recognition between EGFR and Grb2 depending on the activation (tyrosine phosphorylation) of EGFR was observed in single molecules. The durations from the onset of association to the dissociation of single Cy3–Grb2 molecules (on-times) and from the dissociation of a Cy3–Grb2 molecule to the association of the next Cy3–Grb2 molecule (off-times) were measured for further analysis (Fig. 1E). The effects of bleaching and blinking of Cy3 on the on- and off-times were minimal because the decay time for bleaching was 15 s and blinking occurred only once in 147 s under the same excitation conditions. These time constants were much longer than the on-times.

Dissociation Kinetics Between EGFR and Grb2. Dissociation between EGFR and Grb2 can be described by conventional exponential kinetics, independent of the Grb2 concentration. Cumulative histograms of the on-times were fitted by multiple exponential functions. The fittings were significantly improved with an increasing number of exponential components up to three, but the use of four components did not result in further improvement (SI Table 3). Therefore, there seem to be at least three binding states between EGFR and Grb2 (Fig. 2A). The major component (>89%) showed the fastest dissociation rate constant, which was 8.1–7.5 s⁻¹ (Table 1). The second component accounted for 2–11%, for which the rate constant was 1.6–2.6 s⁻¹. The third component was minor but should not be neglected because slow dissociations were the most distinct events in the observation (SI Fig. 7). The results were essentially the same for all Grb2 concentrations. These results
suggest that EGFR and Grb2 have multiple binding states with different dissociation rate constants, but most of the dissociations took place from the major binding state.

The distribution of the dissociation rate for individual binding sites (static disorder) was examined (Fig. 2B). Owing to the small numbers of binding events at single binding sites, especially for low concentrations of Grb2, analysis using multiple exponential fitting was impossible for single binding sites. Therefore, the inverse of the average on-time was calculated for each site as an indication of the dissociation rate. The distributions of this value were similar for all Grb2 concentrations examined: at every concentration, the average was ~3.4 s⁻¹ and the width of the distribution was smaller than one order of magnitude. Thus, large differences in the dissociation rate (by a factor of 20–110) observed from multiple exponential fitting of the on-time distributions (Table 1) cannot be explained in terms of static disorder at the individual binding sites.

**Association Kinetics Between EGFR and Grb2.** Different from the average on-times, the average off-times for individual binding sites showed a curious dependence on the Grb2 concentration. The inverse of the average off-time after multiplication by the Grb2 concentration, which has the same dimension as the second-order association rate constant, varied with the Grb2 concentration (Fig. 3A); as the Grb2 concentration decreased, association occurred relatively faster. The distributions of this indication suggest that the association rate gradually changes with the Grb2 concentration. This phenomenon was not caused by irreversible destruction of the binding sites by high concentrations of Grb2 (SI Fig. 8).

Cumulative histograms of the off-times for each Grb2 concentration can be fitted with the sum of three exponential functions (Fig. 3B). However, the rate constants obtained from the multiple-exponential kinetics were distributed widely over three concentrations of Grb2 (Table 2). In addition, the fractions of each component were irregular. Thus, it is hard to obtain a unified view of the reaction based on the exponential kinetics. In general, the (apparent) second-order rate constant decreases with increasing Grb2 concentration. The results of global fitting indicate that four or more components were required for multiple-exponential kinetics to describe the association reaction (SI Fig. 9).

The association reaction was analyzed by using exponential kinetics for individual sites that showed >60 binding events at 10 nM Cy3–Grb2 (29 reaction sites). One-third (9/29) of the histograms were fitted to a single-exponential function (SI Fig. 10.4 left) and the others were fitted to a sum of two exponential functions (SI Fig. 10.4 right). The second-order association rate constants obtained from fitting were widely distributed without evidence of multiple peaks (SI Fig. 10B). Thus, it is highly probable that the association rate was different for every reaction site and could change with time, even at single reaction sites. This result negates the possibility that there were a few defined pathways for association and supports the model of association reaction assuming drifting of the system over multiple substates.

A stretched exponential function (see Materials and Methods) is a candidate for describing reaction kinetics with broad time scales.

### Table 1. Parameters for dissociation kinetics between EGFR and Grb2

<table>
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<tr>
<th>[Grb2], nM</th>
<th>(k_1), s⁻¹</th>
<th>Fraction, %</th>
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<th>Fraction, %</th>
<th>(k_3), s⁻¹</th>
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The dissociation rate constants \((k_1-k_3)\) and fractions were obtained by fitting cumulative histograms of the on-times (Fig. 2A) to the three-component multiple-exponential function.

### Table 2. Parameters for association kinetics between EGFR and Grb2

<table>
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<tr>
<th>[Grb2], nM</th>
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<th>Fraction, %</th>
<th>(k_2), (\mu\text{M}^{-1}\text{s}^{-1})</th>
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The association rate constants \((k_1-k_3)\) and fractions were obtained by fitting histograms of the off-times (Fig. 3B) to the three-component multiple-exponential function.

*The best-fit values for \(k_1-k_3\) were divided by 10 to normalize the concentration of Cy3–Grb2.
EGFR and Grb2 for each reaction site was calculated from the ratio of total on- and off-times (Fig. 5). The on-times were independent of the Grb2 concentrations, but the off-times multiplied by the Grb2 concentration increased with increasing Grb2 concentration (Figs. 2B and 3A). As a result, the dissociation constants increased with increasing Grb2 concentration; i.e., the affinity between activated EGFR and Grb2 decreased approximately by a factor of three as the Grb2 concentration increased by a factor of 10.

Discussion

This study reveals precise kinetics for the recognition between activated EGFR in its intact form embedded in the plasma membrane and an adaptor protein, Grb2, in solution. Single-molecule detection allowed us to measure individual on- and off-times for the reaction to clarify the complex behavior.

Dissociation between EGFR and Grb2 could be described by using multiple-exponential kinetics, with the major fraction (≈95%) showing a rate constant of 8 s⁻¹. Rate constants for the minor components were ≈3 s⁻¹ or lower. The kinetics of the association reaction was independent of the Grb2 concentration. EGFR has multiple tyrosine residues that are potentially phosphorylated after ligand binding. Phosphotyrosine (pY) 1068 and pY 1086 in EGFR have been reported to be the primary and secondary phosphorylation sites for each concentration of Grb2 (Fig. 4). There are two typical cases for multiple-state reactions that can be described by using stretched exponential kinetics (24). In the first case, transition between multiple unbound substates takes place before the system reaches one target state for association. In the second case, there are many unbound states, each of which has a channel to the association state. In the former view, the value of α = 0.5 suggests a one-dimensional case of the defect-diffusion model (36). In this model, the binding events of Grb2 and EGFR can be recognized as the first-passage time problem of a one-dimensional random walk along substrate variables to the target state. On the other hand, in single-spot analysis, one-third of the binding sites showed single-exponential behavior with different association rates for each spot, suggesting that the latter view is more suitable for the association between EGFR and Grb2. Other populations of the binding site showed multiple components in single-spot analysis, suggesting that transitions between different unbound substates took place during the observation. Therefore, it is probable that the reality is between these two extreme cases. More detailed information about the correlation between reaction and structural dynamics is required to reach a conclusion on this point.

EGFR and Grb2 are involved in various signaling pathways, including the ErbB receptor family, which is important for cell proliferation, survival, and invasion. The precise molecular dynamics of these interactions are crucial for understanding the mechanisms of these pathways. The results of this study provide new insights into the kinetics of EGFR-Grb2 interactions, which could be further investigated in the context of cell biology and cancer research.

The time constants (τ) for association in the stretched exponential kinetics were greater for the Y1068F mutant of EGFR (SI Fig. 11B). This result is consistent with the report that Y1068 is a potential phosphorylation site in vivo (33). The Y1068F mutant is phosphorylated by only a factor of 2.4 (16). This speculation is supported by the report that phosphorylation of Y1068 and Y1086 induces a conformational change in the EGFR C-terminal tail (19).

Association between EGFR and Grb2 required many reaction parameters to be described by using multiple-exponential kinetics. The distribution of the off-times for each concentration of Grb2 can be fitted with three exponential functions, but the association rates obtained varied, depending on the experimental conditions. In addition, ratios of the fractions of major (50–80%) and minor (20–50%) components were irregular and different from those observed in the dissociation kinetics, meaning that there was no 1:1 relationship between the association and dissociation states in multiple-exponential kinetics. When the association reaction was analyzed for individual binding sites, the rate constants were different for each site, and it was impossible to characterize the reactions at single binding sites by using a few common rate constants (SI Figs. 9 and 10). This result suggests the presence of many unbound substates, leading to the complexity observed in the multiple-exponential kinetic analysis.

Stretched exponential kinetics (35) provide a better representation of the association reaction with an exponent (α) of ≈0.5 for every concentration of Grb2 (Fig. 4). There are two typical cases for multiple-state reactions that can be described by using stretched exponential kinetics (24). In the first case, transition between multiple unbound substates takes place before the system reaches one target state for association. In the second case, there are many unbound states, each of which has a channel to the association state. In the former view, the value of α = 0.5 suggests a one-dimensional case of the defect-diffusion model (36). In this model, the binding events of Grb2 and EGFR can be recognized as the first-passage time problem of a one-dimensional random walk along substrate variables to the target state. On the other hand, in single-spot analysis, one-third of the binding sites showed single-exponential behavior with different association rates for each spot, suggesting that the latter view is more suitable for the association between EGFR and Grb2. Other populations of the binding site showed multiple components in single-spot analysis, suggesting that transitions between different unbound substates took place during the observation. Therefore, it is probable that the reality is between these two extreme cases. More detailed information about the correlation between reaction and structural dynamics is required to reach a conclusion on this point.
contrast to the usual case. Both the average off-time and \( \tau \) decreased more slowly than the increase in Grb2 concentration. This result was not caused by direct competitive or noncompetitive inhibition between Grb2 molecules because simultaneous binding of multiple Cy3-Grb2 molecules at the same binding site was rarely observed, even at the highest concentration of Cy3-Grb2 (10 nM) used in this study. Even at 100 nM Grb2, the average off-time for Grb2 (2 s) expected from the off-times for Cy3-Grb2 was sufficiently longer than the average on-time (0.4 s) to avoid serious effects of simultaneous binding on the association rate. In addition, the effect of high concentrations of Grb2 was reversible (SI Fig. 8), suggesting that dynamic interactions between EGFR and Grb2 caused this phenomenon. The presence of multiple unbound states is highly likely to be responsible for the concentration dependence.

Conformational transition of the binding site can induce multiple reaction kinetics as shown in the allosteric model (37). One hypothesis is that Grb2 repeatedly interacts with EGFR for short times that were outside of our temporal resolution, accelerating substrate transitions in the direction to increase the time constant of the association reaction. This effect seems to depend on specific interactions between EGFR and Grb2 but does not appear to be caused by nonspecific protein–protein interaction because the total protein concentration in the solution, which contained 0.5% casein and 1% BSA, was slightly affected by the different Grb2 concentrations in our experiments. Calculated from the theoretical maximum rate constant for association (38), assuming 2 nM for the interaction radius and 100 \( \mu \)m\(^2\)-s\(^{-1}\) for the diffusion coefficient for the Grb2 molecule, at 10 nM Grb2, one binding site should collide with Grb2 molecules every 70 ms, whereas the average off-time was 9 s in this study. Such partial interactions possibly change the conformation of proteins and thus reduce the on-rates. To realize this mechanism, proteins would require a conformation memory after dissociation. When time constants for conformational transitions of the binding site are comparable to that of association reaction, reaction memory can be induced due to dynamic disorder (20–23). In fact, the existence of reaction memory was suggested by non-Markovian function analysis of the single-molecule reaction trajectories (SI Fig. 12). A much lower reaction memory for the Y1068F mutant of EGFR at 1 nM Grb2 (SI Fig. 12C) supports this hypothesis; i.e., structural relaxation during long off-times would diminish conformational memories, and this could be a reason for the single-exponential kinetics under this condition (SI Fig. 11B).

Because of the Grb2 concentration dependence of the association kinetics, the dissociation equilibrium constant between EGFR and Grb2 changed according to the Grb2 concentration. As the concentration of Grb2 increased by a factor of 10, the affinity between EGFR and Grb2 decreased approximately by a factor of three. This relationship was maintained over a wide range (0.1–100 nM) of Grb2 concentrations. This phenomenon requires intact molecules of EGFR because it has not been observed in previous studies using short phosphopeptides from EGFR. The apparent dissociation constant was calculated from the total on- and off-times for each binding site. The average was 97, 340, and 650 nM at 1, 10, and 100 nM Grb2, respectively (Fig. 5). These values are similar to those reported in previous studies (100–710 nM) between phosphopeptides from EGFR and Grb2 (16–18). Concentration dependence was also observed for the Y1068F mutant (SI Fig. 13). This concentration dependence can compensate for fluctuations in the expression level of Grb2 in living cells to maintain stable signal transduction from activated EGFR to Grb2. The concentration of Grb2 in the cytoplasm has not been quantified precisely yet, but in molecular network simulations of EGFR signal transduction, the concentration of Grb2 was set between 3 nM (39) and 1 \( \mu \)M (40) to reproduce experimental results for the network response. Based on these results, the concentration of Grb2 in this study would not be very far from that in living cells. Therefore, it is highly probable that complex protein dynamics behavior is used to modulate intracellular signal transduction.

**Materials and Methods**

**Preparation of the Plasma Membrane Fraction**. Human epithelial carcinoma A431 cells were grown on plastic dishes in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical) supplemented with 10% FBS. The medium was changed before experiments to Eagle's minimum essential medium (MEM) (Nissui Pharmaceutical) containing 1% BSA without phenol red and serum overnight. Cells were stimulated with 100 ng/ml EGF for 5 min in MEM at room temperature to induce EGFR phosphorylation and then washed twice with ice-cold PBS, and the plasma membrane fraction was purified according to Yarden and Schlessinger (3). In brief, cells were treated with a hypotonic buffer and homogenized with a glass homogenizer, and the plasma membrane fraction was purified by using a sucrose density gradient. The buffer used for purification contained 1 mM sodium orthovanadate and 1 mM ATP to avoid dephosphorylation of EGFR, and a protease inhibitor mixture (Sigma–Aldrich). For negative control of EGFR activation, cells were cultured overnight in the presence of 50 nM tyrphostin AG 1478 (Sigma–Aldrich), and the plasma membrane fraction was prepared in the presence of the same concentration of AG 1478. Tyrosine phosphorylation of EGFR in the membrane fraction from EGF-stimulated cells was confirmed by immunoblotting (SI Fig. 6). The membrane fraction was frozen in liquid nitrogen and stored at –85°C.

**Preparation of Cy3-Labeled Grb2**. Preparation and Cy3 labeling of Grb2 were carried out as described by Ichinose et al. (41). Briefly, Grb2 was cloned from HeLa cells, expressed in E. coli, and purified. Monofunctional Cy3 (Amersham Pharmacia) was conjugated to the N terminus of Grb2. The reaction product was separated by using anion exchange column chromatography, and the fraction with a dye/protein ratio of 1 was collected as Cy3–Grb2. The 1:1 labeling was confirmed by single-step photobleaching under a total internal reflection fluorescence microscope (TIR-FM).

**Observation**. To make an observation chamber, a coverslip (18 mm × 12 mm) was attached to a glass slide by using two strips of double-faced adhesive (200 µm thickness) as spacers, leaving two opposite sides open. The plasma membrane fraction was introduced into the chamber and kept on ice for 10 min. By this treatment, membrane fragments as small as the optical resolution (~300 nm) were adsorbed on the coverslip. Then, as a maker to compensate for stage drift during observations, 10 pM Quantum Dot 565 (Quantum Dot Corporation) was introduced into the chamber and kept there for 5 min. After washing the unbound fraction with solution A [20 mM Hepes-KOH (pH 7.3), 110 mM CH3COOK, 5 mM CH3COONa, 2 mM (CH3COO)2Mg, 1 mM EGTA (pH 7.3), 1 mM DTT], the chamber was incubated with blocking solution (solution A containing 0.5% casein, 1% BSA, 0.5% polyethylene glycol). The solution was replaced with Cy3–Grb2 dissolved in blocking solution containing 4.5 mg/ml glucose, 0.036 mg/ml catalase, and 0.22 mg/ml glucose oxidase, and the open sides of the chamber were closed with nail varnish. The concentration of Cy3–Grb2 was varied from 0.1 to 10 nM. Because single-molecule imaging was impossible in the presence of 100 nM Cy3–Grb2, 10 nM Cy3–Grb2 mixed with 90 nM nonlabeled Grb2 was used to analyze reactions at 100 nM Grb2.

The chamber was set on an objective type TIR-FM based on an inverted microscope (IX-70; Olympus) with an oil-immersion objective lens (Plan Apo ×60 N.A. 1.45). A diode-pumped solid-state laser (Millenia; Spectra Physics) with a 532-nm line was used to excite Cy3. Interactions between Cy3–Grb2 and EGFR were observed under the equilibrium condition. Because there were no cytokol and ATP, further phosphorylation and dephosphorylation of EGFR were avoided in the chamber. The image was captured for 18 min by using a monochrome CCD camera (CCD300-RC; Dage-MTI) after passing through an image intensifier (C8600;
stretched exponential function is described as assuming multiple-exponential or stretched exponential kinetics. A fraction were used for analysis. The accumulated numbers of the stimulated membrane fraction to which Cy3–Grb2 bound with frame for the binding duration was checked by eye. Binding sites in measurement. To exclude false binding caused by this effect, every binding site of interest and affected the fluorescence intensity measured as an on- or off-time, respectively.

Occasionally, Cy3–Grb2 came to the surface very close to the binding site of interest and affected the fluorescence intensity measurement. To exclude false binding caused by this effect, every frame for the binding duration was checked by eye. Binding sites in the stimulated membrane fraction to which Cy3–Grb2 bound with higher frequency than to those in the nonstimulated membrane fraction were used for analysis. The accumulated numbers of association or dissociation events were 1,400–5,200 from 29–38 binding sites for 1–100 nM Grb2. The numbers of association sites and events at 0.1 nM were 16 and 170, respectively.

Kinetic Analysis. Reactions between EGFR and Grb2 were analyzed assuming multiple-exponential or stretched exponential kinetics. A stretched exponential function is described as

\[
\phi(t) = \phi_0 e^{-((t/t)^\alpha)},
\]

where \(\phi(t)\) is the off-time, \(\phi_0\) is a prefactor, \(\alpha\) is the stretching exponent, and \(\tau\) is the time constant. This function is a phenomenological fit that conveniently describes dynamics with broad time scales using only two parameters rather than multiple exponentials. In a wide range of disordered systems, including glasses and polymers, a stretched exponential relaxation phenomenon arises from contributions of a large number of weighted exponentials. In this study, we used cumulative histograms of on- and off-times to avoid the effect of bin-size on the kinetic parameters. The cumulative form of the stretched exponential function is

\[
\Phi_{off}(t) = \frac{\phi_0}{\tau} \int_0^t \phi(t) e^{-((t/t)^\alpha)} dt = \frac{\phi_0}{\alpha} \Gamma(1/\alpha, (t/\tau)^\alpha),
\]

where \(s = (t/\tau)^\alpha\), and \(\Gamma(\cdot, \cdot)\) is the incomplete gamma function. This function can be calculated numerically, and the histograms were fitted to the functions by using the Levenberg–Marquardt method.

Because of the small number of association and dissociation events, the experimental results at 0.1 nM Cy3–Grb2 were not used for the multiple-exponential and stretched exponential analyses, but only the averages of on- and off-times were discussed.

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EGFR Antibody

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$\tau_1 = 16 \ (77)$
$\tau_2 = 35 \ (23)$
A

Number of events

Time (s)

τ₁ = 3.3

τ₁ = 7.2 (38)

τ₂ = 42 (2)

B

Number of events

Log₁₀ k_{on} (µM⁻¹s⁻¹)

k_{on} (µM⁻¹s⁻¹)
A

1nM

$\tau_1 = 0.11 \ (97)$
$\tau_2 = 0.62 \ (2.4)$
$\tau_3 = 1.9 \ (0.67)$

10nM

$\tau_1 = 0.16 \ (94)$
$\tau_2 = 0.59 \ (5.5)$
$\tau_3 = 4.9 \ (0.040)$

100nM

$\tau_1 = 0.09 \ (93)$
$\tau_2 = 0.40 \ (7.3)$
$\tau_3 = 4.0 \ (0.017)$

B

1 nM

$\tau = 29$
$\alpha = 1.0$

10 nM

$\tau = 2.2$
$\alpha = 0.55$

100 nM

$\tau = 0.26$
$\alpha = 0.52$
A

Grb2 concentration

(Average of on-times)$^{-1} (s^{-1})$

B

Grb2 concentration

[Grb2]$^{-1}$(average of off-times)$^{-1}$ ($\mu M^{-1}s^{-1}$)

C

Grb2 concentration

[Grb2] (total off-times) / (total on-times) (nM)

A

3.9

3.3

3.4

B

6.6

9.8

36

C

770

410

110