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Fast rebinding increases dwell time of Src homology 2 (SH2)-containing proteins near the plasma membrane

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Fast rebinding increases dwell time of Src homology 2 (SH2)-containing proteins near the plasma membrane

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Receptor tyrosine kinases (RTKs) control a host of biological functions by phosphorylating tyrosine residues of intracellular proteins upon extracellular ligand binding. The phosphotyrosines (p-Tyr) then recruit a subset of ~100 Src homology 2 (SH2) domain-containing proteins to the cell membrane. The in vivo kinetics of this process are not well understood. Here we use total internal reflection (TIR) microscopy and single-molecule imaging to monitor interactions between SH2 modules and p-Tyr sites near the cell membrane. We found that the dwell time of SH2 modules within the TIR illumination field is significantly longer than predictions based on chemical dissociation rate constants, suggesting that SH2 modules quickly rebind to nearby p-Tyr sites after dissociation. We also found that, consistent with the rebinding model, the effective diffusion constant is negatively correlated with the respective dwell time for different SH2 domains and the dwell time is positively correlated with the local density of RTK phosphorylation. These results suggest a mechanism whereby signal output can be regulated through the spatial organization of multiple binding sites, which will prompt reevaluation of many aspects of RTK signaling, such as signaling specificity, mechanisms of spatial control, and noise suppression.

The Src homology 2 (SH2) domain (1) controls many cellular activities by binding specifically to tyrosine-phosphorylated peptides (2). The tyrosine phosphorylation signal often originates from the cell surface through activations of receptor tyrosine kinases (RTKs). Humans, for example, have 58 known RTKs (3), each of which could generate diverse phosphorylation patterns at their cytosolic tails as well as at other associated proteins. These phosphorylation patterns are “read” mainly by a repertoire of effector proteins containing the SH2 domain (2, 4, 5), via recruitment of these effector molecules to the phosphotyrosine (p-Tyr) sites. For example, the epidermal growth factor receptor (EGFR), a well-known model system for studying phosphotyrosine signaling, is activated by epidermal growth factor (EGF), a small protein ligand. The binding of EGF turns on the kinase activity of EGFR molecules, which then autophosphorylate themselves at more than 20 tyrosine sites on the cytoplasmic tail. The phosphotyrosines are thought to serve as docking sites for SH2-containing molecules, such as Grb2, which bind to these sites with varying affinity. Downstream signals are propagated by the SH2 proteins, which in many cases are enzymes whose substrates are localized to the plasma membrane. In other cases such as Grb2, the SH2 protein serves to recruit additional downstream effector proteins such as the Ras activator Sos. EGFR family members also play critical roles in cancer development and progression and are frequently disregulated in tumors (6).

During the last decade, an expanding assembly of quantitative datasets has accumulated regarding tyrosine phosphorylation signal, including the topological pattern of phosphorylation (7–10), the temporal dynamics of phosphorylation (10, 11), and characterization of the SH2/p-Tyr binding affinity (12, 13). On the other hand, the in vivo assembly and turnover kinetics of the SH2 complexes at p-Tyr sites remain poorly understood, partly because existing methods measure mostly the ensemble properties of the system. To this end, measurements on the single-molecule basis provide an advantage in understanding the dynamics of these heterogeneous interactions. With a total internal reflection (TIR) microscope, we can monitor the turnover of individual SH2 molecules by tracking the molecule from its initial docking to a p-Tyr site at the cell’s plasma membrane to its dissociation from the membrane and leaving the TIR illumination field. The dwell times of single SH2 molecules within the TIR field thus can be measured and analyzed statistically. Here we report that our experimental results from EGF-stimulated cells can be quantitatively explained only when fast multiple rebinding of SH2 molecules to p-Tyr sites is taken into consideration.

**Results**

**Measuring Dwell Time of SH2 Molecules Near the Cell Membrane with TIR Illumination.** We expressed in A431 cells the SH2 domain of Grb2 (Grb2SH2), as a chimeric fusion to tdEos, a photoactivatable fluorescent protein. Single molecules were detected after a small fraction of tdEos was photoactivated (14). Without extracellular ligand stimulation, very few Grb2SH2 molecules could be seen under TIR illumination (Fig. 1 A and Fig. S1), but the number of molecules quickly increased upon the addition of EGF to the media (Fig. 1 A and B), presumably because the activation of EGFR led to an increased number of p-Tyr sites at the membrane. During the time-lapse imaging (Movie S1), new molecules continuously appear at the plasma membrane, undergo diffusive motion for at most a few seconds, and then disappear from the membrane. We attribute the appearance of a molecule to the initial binding reaction to a p-Tyr site and the disappearance to the escape of the molecule from the TIR illumination field or to photobleaching.

Histograms of the dwell times, defined as the time delay between a molecule’s appearance and its disappearance, are shown in Fig. 1C. As expected the dwell time of Grb2SH2 falls between that of a nonbinding mutant control (mtGrb2SH2), which we consider to be due to nonspecific binding, and that of myr-tdEos (tdEos with a myristoylation signal peptide sequence from Src that directs constitutive membrane association), which is due to photobleaching. However, because of the inevitable contribution from photobleaching, the dwell-time representation is not a direct measurement of the turnover kinetics. To overcome this problem, we calculated the hazard function (15), defined as

\[
\lambda(t) = \frac{p(t)}{\int_0^\infty p(u)du},
\]

where \(p(t)\) is the probability distribution of the dwell times, estimated by its histogram. The hazard function is additive for

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Fig. 1. Rebinding of the Grb2_{SH2} domain to phosphotyrosine sites increases its dwell time at the cell membrane. (A) Recruitment of Grb2_{SH2} (Left) and the nonbinding mutant control, mtGrb2_{SH2} (Right). Epi-fluorescence image shows the expressed molecules distributed mostly in the cytosol (Top row). TIR illumination of unstimulated cells shows little recruitment of Grb2_{SH2} to the cell membrane (Middle row). Upon EGF stimulation, a large number of mobile Grb2_{SH2} molecules, but not mtGrb2_{SH2}, can be detected with TIR fluorescence (Bottom row). (Scale bar: 2 μm.) (B) The time course of Grb2_{SH2} recruitment after EGF stimulation. The amount of the Grb2_{SH2} recruitment was measured by integrated fluorescence intensity under TIR illumination (lines) or by counting the number of single molecules (bars). The different traces represent measurements from four different cells. (C) Survival-time distribution for Grb2_{SH2}, mtGrb2_{SH2}, and Myr-tdEos in A431 cells stimulated for 20 min with EGF. (D) Hazard function (lines) of Grb2_{SH2} computed from survival-time distribution (bars). Solid lines are the simple moving average. (E) Schematic diagram illustrating the rebinding mechanism.

The interaction of SH2 with multiple p-Tyr sites therefore eliminates heterogeneity by averaging affinities over many sites. Indeed, on the basis of a reaction-diffusion model (SI Theory), we can theoretically predict a constant hazard function under this condition (Fig. S3). Furthermore, with multiple rebinding events, the apparent “dissociation rate” measured by the hazard function is much slower than the prediction based solely on the chemical off rate. In fact, we found (SI Theory and Fig. S4) that the hazard function should be dependent not only on the off rate, $k_d$, but also on the on rate, $k_o$, as well as on the diffusion constant, $D$, of the molecule when hopping between sites,

$$\lambda(t) \approx k_d/(1+a k_o n / D),$$

where $n$ is the p-Tyr density and $a$ is the imaging depth of the TIR field. On the basis of this model, on average ~20 rebinding events were detected in our experiment for each Grb2{SH2} molecule before it escaped. Furthermore, the equation predicts that factors that increase the binding rate, $k_o n$, will further decrease the ability of molecules to escape the TIR field; on the other hand, fast diffusion of the dissociated molecules, which is controlled by $D$, promotes their escape. The importance of rebinding can also be evaluated outside the context of the TIR experiment. Lagerholm and Thompson (21) proposed using a unitless parameter, $b \equiv k_o n / \sqrt{k_d D}$, to evaluate the significance of rebinding: For systems with $b \ll 1$, rebinding can be safely ignored and the
dissociation kinetics are dictated by the chemical rate constant $k_d$, whereas when $b >> 1$ the kinetics of the dissociation process are strongly influenced by re-binding. On the basis of the hazard function, we estimated the value of $b$ for Grb2$_{SH2}$ to be $\sim$44.6, assuming a $k_d$ value of $10^{-1}$ (17) and a $D$ value of $15 \mu m^2/\text{s}$, corresponding to the cytotoxic diffusion rate of a small protein (22).

**SH2 Mobility Is Correlated with Its Apparent Membrane Dwell Time.** To verify that the re-binding dynamics are indeed important to the interactions between SH2 domains and p-Tyr sites, we measured the hazard functions, $\lambda(t)$, as well as the effective mobility, $D_{\text{eff}}$, of five different SH2 domains from four SH2 proteins that are thought to directly interact with EGFR (Movie S2 and Fig. 2). We hypothesized that, if SH2 domains dissociate from the membrane without re-binding, the mobility should solely be determined by the diffusion constant of the EGFR receptor protein; thus, the variation in mobility of different SH2 domains should be small. On the other hand, if the SH2 domains rebind frequently, the mobility is determined not only by the diffusion constant of the receptors, but also by the frequency and the duration of the “hopping” motion between p-Tyr binding sites. We found that $D_{\text{eff}}$ varied by more than an order of magnitude among different SH2 domains (Fig. 2A), supporting a re-binding mechanism. Furthermore, all five SH2 domains we studied exhibited constant hazard functions, albeit at different average values (Fig. 2B), again consistent with a re-binding mechanism. Finally, the average hazard rates and the mobilities were correlated (Fig. 2C), as was predicted by the reaction-diffusion model (SI Theory):

$$D_{\text{eff}} = D_0 \frac{\lambda^{-1} + a^2/2D_0}{\lambda^{-1} + a^2/2D}.$$  

[3]

Here $D_0$ is the diffusion constant of EGFR, which is $\sim$0.03 $\mu m^2/\text{s}$ (23), and $D$ is the diffusion constant of the unbound SH2, which is $\sim$15 $\mu m^2/\text{s}$ (22). Using these numbers, and assuming TIR illumination depth $a \sim 0.5 \mu m$, we can directly calculate the value of $D_{\text{eff}}$ as a function of $\lambda$. We found that the calculated values from this equation agree well with our experimental data (Fig. 2C).

**Spatial Distribution and Clustering of Target Phosphotyrosine Binding Sites.** The prominence of SH2 re-binding suggests a high density of potential p-Tyr binding sites ($\sim 10^6$ sites/$\mu m^2$ based on Eq. 2). We asked whether this density is the average density of the whole cell or a local density elevated due to microscopic domains of the receptors clustering (24). To answer this question, we quantified the effects of the duration of EGF stimulation on the hazard function values and the effective mobilities of Grb2$_{SH2}$. Indeed, as the stimulation duration increased, the dwell time of Grb2$_{SH2}$ molecules increased as well (Fig. 3A and Fig. S5), presumably due to an increase in p-Tyr density. The number of Grb2$_{SH2}$ molecules recruited to the membrane typically reached a plateau at around 5–10 min of stimulation (Fig. 1B), suggesting that the number of p-Tyr sites reaches a maximum value at that time. Results from other assays, such as far-Western analysis (Fig. S6) and mass spectrometry (11), led to the same conclusion. Interestingly, however, the SH2 dwell time continued to increase and stabilized only after $>20$ min (Fig. 3A), suggesting that the re-binding of SH2 molecules was indeed responding to changes in local density of the binding sites. Even though the number and thus the average density of p-Tyr sites no longer increased after 10 min, our data suggest that the local density continued to increase, most likely by clustering of the EGFR receptors. As expected if the local density of binding sites were increasing, the mobility of the Grb2$_{SH2}$ molecule decreased correspondingly over time, presumably because they spend less time in cytosol before re-binding (Fig. 3B). The changes in the mobility and the changes in the dwell time are negatively correlated with each other (Fig. 3C). These results highlight the importance of the spatial organization (local density) of the receptors, as opposed to their total expression, in regulating the membrane dwell time of SH2 molecules. Supporting this idea, we found that in cells (H226) with much lower EGFR expression than A431, the hazard function and the dwell time of Grb2$_{SH2}$ are almost the same as in A431 cells (Fig. S7).

To understand the spatial distribution of the phosphotyrosine binding sites in cells, we recorded the first observed locations of each individual SH2 molecule detected at the cell membrane and plotted their coordinates (Fig. 3 D–I). We found that when the cell was stimulated for a short period, no apparent spatial pattern could be discerned from the plot (Fig. 3E). For cells that were stimulated for a longer time (e.g., $>20$ min), however, we found spatial regions to which the SH2 molecules were repeatedly recruited with a higher-than-average frequency, indicating clusters of p-Tyr sites on activated EGFR (Fig. 3 F–H). No clustering was observed for mtGrb2$_{SH2}$ (Fig. 3F). This result further lends support to the argument that the decrease in hazard function values is correlated with clustering of the receptors.

**Rebinding Dynamics of Multivalent SH2 Molecules.** Next, we investigated the turnover dynamics of multivalent SH2 molecules. Most signaling proteins are multivalent: They have multiple
modular binding domains that allow interaction with more than one molecule at the same time. Some SH2 proteins, such as Shp2 or PLCγ1, have two SH2 domains and can thus be recruited to the membrane through either monovalent or bivalent binding to p-Tyr (Fig. 4A). We measured the hazard function and the effective mobility of the Shp2\textsubscript{SH2(NC)} molecule (Movie S3), which has both the N- and the C-terminal SH2 domains of the Shp2 phosphatase. In contrast to the monovalent Shp2\textsubscript{SH2(N)} or Shp2\textsubscript{SH2(C)}, the hazard function of Shp2\textsubscript{SH2(NC)} is no longer a constant (Fig. 4B), presumably due to a heterogeneous mixture of both monovalent and bivalent complexes. The monovalent complex was formed when an SH2 molecule was initially recruited to the membrane, and the bivalent complex was formed later. Thus, we attribute the decay in the hazard function to the gradual process of bivalent complex formation. The decay time constant is on the order of a few hundred milliseconds, indicating that the formation of the second connection is relatively slow compared with the formation of the first. The difference is probably because once the SH2 molecule is tethered to the membrane through the first p-Tyr-SH2 interaction, its diffusion rate decreases significantly, which results in a reduced reaction rate for the formation of the second binding.

We expect the monovalent complex to rebind via diffusion in cytosol, whereas the bivalent complex rebinds as a membrane-associated complex (Fig. 4A). Therefore, the re-binding model would predict that the molecules with longer survival time should have lower mobility than molecules with shorter survival time. Indeed with MSD-Δt analysis we found the mobility of molecules with long survival times (>5 s) is approximately eight to nine times less than that of molecules with short survival times (<1 s).
and

in A431 cells. Similar to that of www.pnas.org/cgi/doi/10.1073/pnas.1203397109 molecules with short

Fig. S8 A μ module in an A431 cell stimulated by

(analysis of Shp2

Fig. S8 Oh et al. between the binding sites. Theory

Rebinding of multivalent SH2 module Shp2

(Full length)

between full-length Grb2 and the Grb2SH2 domain (Fig. 5A), and a mutation in the SH2 domain alone (mtGrb2) was sufficient to prevent recruitment of full-length Grb2 to the cell membrane (Fig. 5A). We found that the average dwell time of full-length Grb2 was longer than that of Grb2SH2, consistent with the previous finding that full-length Grb2 and Grb2/Sos complexes bind tighter to p-Tyr (17, 25). The flattened hazard function further indicates that the heterogeneities of multiple p-Tyr sites were averaged out, presumably due to multiple rebinding, indicating that the results we obtained from isolated SH2 domains can be extrapolated to full-length SH2 proteins.

Discussion

In this study, we measured the apparent dwell time of SH2 domains within a TIR illumination field near the cell membrane. We found the average dwell time is longer than predicted on the basis of the chemical dissociation rate, and for monomeric SH2 domains the dissociation kinetics exhibited little heterogeneity. Although other possibilities cannot be completely excluded, the simplest and most likely explanation is that the interaction between SH2 domains and p-Tyr sites involves repeated rebinding. We propose that the rebinding is the result of competition between the very fast intrinsic chemical kinetic rate of the association reaction and the relatively slow diffusion rate of the biomolecules in vivo. Part of the reason that the chemical association rate is high is that there is an abundance of available p-Tyr binding sites, because each receptor molecule has multiple phosphorylation sites and because of the clustering of the receptors that further increases the local concentration of binding sites. Because the SH2 molecules do not directly dissociate from the receptors in our model, the traditional concept of turnover time has an ambiguous meaning in this case. There are, in fact, two different turnover times. The SH2 turnover time on an individual p-Tyr site is quite short, because the molecule quickly dissociates. The fast dissociation is consistent with the fact that p-Tyr sites can be very quickly dephosphorylated (26) and thus are not protected by the SH2 domains for a very long time. On the other hand, the SH2 turnover time with respect to the receptor cluster is relatively long, because each receptor molecule has multiple phosphorylation sites and because of the clustering of the receptors that further increases the local concentration of binding sites. Theory predicts that clustering stabilizes the binding between p-Tyr sites and the SH2 molecules, which we verified in experiments.

The finding that the SH2 protein hops between clustered binding sites along the cell membrane suggests that a single SH2 molecule, when recruited to the membrane, could potentially interact with multiple p-Tyr sites that are near each other. This kind of mechanism may also play a role in other intracellular

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Fig. 4. Rebinding of multivalent SH2 module Shp2SH2NC (A) two different schemes of rebinding for bivalent SH2 molecules, depending on whether it forms a monovalent complex (Upper) or a bivalent complex (Lower). (B) The hazard functions for the Shp2SH2NC module in an A431 cell stimulated by EGF (20 min), in comparison with its monomeric counterparts. The nonconstant λ-values for Shp2SH2NC arise from the mixed population of monovalent complexes and bivalent complexes. The decay reflects the temporal process of bivalent complex formation. (C) MSD-Δt analysis of Shp2SH2NC (Top), Shp2SH2M (Middle), and Shp2SH2I (Bottom). For each molecule, the MSD curves were calculated for the subpopulations with long (blue), intermediate (red), and short (black) survival times. Only Shp2SH2NC exhibited different MSD curves depending on survival times.

( Fig. 4C and Fig. S8). Such a bifurcation in mobility was not observed for monomeric SH2 molecules (Fig. 4C and Fig. S8). Furthermore, the mobility of Shp2SH2NC molecules with short survival times (<1 s) is roughly the same as that of their monomeric counterparts. These results are consistent with the rebinding model. The hazard functions of two other bivalent SH2 molecules [PLCγ1SH2NC], which has the two naturally occurring SH2 domains of PLCγ1, and an engineered tandem Grb2 construct, Grb2SH2SH2], exhibited similar decaying values (Fig. S9), consistent with the above model.

Finally, we also measured the hazard function of full-length Grb2, in which the SH2 domain is flanked by SH3 domains that bind to proline-rich peptides in effectors such as Sos (Fig. 5). We found that the hazard function of full-length Grb2 exhibits very little decay, suggesting that the recruitment of Grb2 to the cell membrane depends predominately on the SH2 domain. That is, SH2-mediated interactions do not act to be sufficient to tether Grb2 to the membrane. Consistent with this interpretation, we also found no significant difference in the recruitment kinetics between full-length Grb2 and the Grb2SH2 domain (Fig. 5A), and a mutation in the SH2 domain alone (mtGrb2) was sufficient to prevent recruitment of full-length Grb2 to the cell membrane (Fig. 5A). We found that the average dwell time of full-length Grb2 was longer than that of Grb2SH2, consistent with the previous finding that full-length Grb2 and Grb2/Sos complexes bind tighter to p-Tyr (17, 25). The flattened hazard function further indicates the heterogeneities of multiple p-Tyr sites were averaged out, presumably due to multiple rebinding, indicating that the results we obtained from isolated SH2 domains can be extrapolated to full-length SH2 proteins.

fig. 5. Turnover kinetics of full-length Grb2 molecules. (A) Comparison of the recruitment kinetics of full-length Grb2, full-length mtGrb2, and Grb2S07 in A431 cells. Data were collected from A431 cells as in Fig. 1B. The amount of the recruitment was measured by integrated fluorescence intensity under TIR illumination after cells were treated with 25 ng/L EGF. (B) Hazard functions of full-length Grb2 and Grb2mt in A431 cells. Similar to that of Grb2SH2, the hazard function of full-length Grb2 is a constant. The lower average value for full-length Grb2 signifies a longer average dwell time, consistent with a more stable binding to p-Tyr sites.
protein interactions. For example, studies of Cdc4 interaction with Src1 revealed that Cdc4 can interact with multiple phosphorylated sites on Src1 even though Cdc4 contains only one interaction site (27). Together with our findings, it seems plausible that dynamic equilibrium through multiple rebinding events is a common theme of phosphoprotein interactions. The re-binding mechanism lessens the importance of single high-affinity binding sites. In terms of RTK signaling, the SH2 dwell times depend on the average affinities of many phosphorylated sites; lack of a single high-affinity site will have only a moderate impact on the average duration of binding. Therefore, the re-binding mechanism may explain why mutation of RTK phosphosites predicted to bind specifically to particular SH2 domains had relatively little effect on downstream signaling. It was only when all sites were eliminated and individual phosphosites added back that specific effects on downstream signaling could be observed (28, 29).

Finally, these results underscore the importance of receptor clustering, as kinetic stabilization of the SH2/p-Tyr interaction depends on high local density of phosphorylated sites. Receptor clustering may further affect the downstream outcome of the signaling process (30). For example, it was proposed (31) that in T cells, prolonged ligand/receptor association suppresses "noise" from adventitious receptor activation (a kinetic proofreading mechanism). In a similar manner, the increased SH2 dwell time near the cell membrane, coupled to high in vivo dephosphorylation rates (26), may serve to dampen spurious noise associated with nonspecific binding of SH2 proteins to membrane molecules. Finally, because the recruited SH2 proteins do not associate with just one p-Tyr site, but instead "glide" over many p-Tyr sites, the chemical interaction of such an SH2 protein with downstram substrate or effector molecules at the cell membrane may not follow simple chemical kinetic laws. The details of such interactions should be an interesting subject of future research.

**Experimental Procedures**

**Constructs and Cell Culture.** SH2 domain DNA sequences were obtained from the Human SH2 collection (Open Biosystems) and subcloned to the C terminus of tdEos coding sequence to form the chimera fusion constructs. The myrtdEos clone contains the myristoylation signal sequence from the N terminus of Src. A431 and H226 cells were maintained in DMEM culture medium and replated on freshly cleaned glass-bottom dishes (Matek) at medium to low density (>50% confluent) right before microscopy experiments.

**Microscopy and Image Analysis.** All imaging experiments were carried out on a custom-made total-internal-reflection fluorescence microscope, equipped with a 532-nm laser (Crystal Lasers) for fluorescence excitation and a 405-nm laser (Coherent) for photoactivation. Fluorescent spots corresponding to single molecules were detected via intensity thresholding. The positions of the molecules were tracked over time, using the nearest-neighbor method. The scan area used during tracking when searching for the position of the molecule in the next time frame was chosen on the basis of estimated diffusion rate of the molecule so that the molecule has a >99.5% chance to be within the scan area. The spatial density of the fluorescence spots was kept low (0.05–0.1 μm^2) to avoid mistracking. To estimate the effective mobility, ensemble averaged MSD was calculated as a function of time lags (Δt), which was subsequently used to determine the effective diffusion constant.

More details of the experimental procedure are included in the SI Experimental Procedures.

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