THE EMBO JOURNAL

Single-molecule analysis of epidermal growth factor binding on the surface of living cells

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Global cellular responses induced by epidermal growth factor (EGF) receptor (EGFR) occur immediately with a less than 1% occupancy among tens of thousands of EGFR molecules on single cell surface. Activation of EGFR requires the formation of a signaling dimer of EGFR bound with a single ligand to each molecule. How sufficient numbers of signaling dimers are formed at such low occupancy rate is still not known. Here, we have analyzed the kinetics of EGF binding and the formation of the signaling dimer using single-molecule imaging and mathematical modeling. A small number of EGFR on the cell surface formed dimeric binding sites, which bound EGF two orders of magnitude faster than the monomeric binding sites. There was a positive cooperative binding of EGF to the dimeric binding sites through a newly discovered kinetic intermediate. These two mechanisms facilitate the formation of signaling dimers of EGF/EGFR complexes.

Introduction

Epidermal growth factor (EGF) receptor (EGFR) belongs to the ErbB family of receptor tyrosine kinases responsible for cell proliferation, differentiation and migration in embryogenesis and carcinogenesis (Carpenter, 1987; Schlessinger, 2000). Binding of the ligand and formation of the signaling dimer are the initial and critical events in the EGF signaling pathway. In the signaling dimer of EGFR composed of two ligand and two receptor molecules, the intrinsic tyrosine kinase activity of EGFR is enhanced and induces the phosphorylation of tyrosine residues in the carboxy-terminal domain of EGFR, providing scaffolds for various cytoplasmic signaling proteins (Schlessinger, 2002).

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The process of formation of EGF/EGFR complexes is not fully understood. Cells sensitive to EGF typically express several tens of thousands of EGFRs on the plasma membrane. EGF-induced global responses in cells, like the calcium response or loss of affinity to the ligand, were observed within 1 min after application of EGF at less than 1% occupancy of EGFRs (Wiley *et al*, 1989; Uyemura *et al*, 2005). Recently, it was demonstrated that the EGFR has the ability to form dimers independently of ligand binding (predimer) on the cell surface (Moriki *et al*, 2001; Yu *et al*, 2002). Predimers appear to be responsible for the effective formation of signaling dimers. However, assuming simple association reactions, the probability of signaling dimer formation with an occupancy rate of less than 1% would be too low to induce global cellular responses. Under simple association, even if all EGFR molecules present as predimers, only a few signaling dimers will be formed on cells expressing 50 000 EGFR molecules with a 1% occupancy rate.

In fact, the association between EGF and EGFR is not simple. Many studies have demonstrated that EGFRs on the cell surface exhibit two apparently different affinities to EGF in the Scatchard plot analysis; the majority have low affinity, whereas only a small number exhibit high affinity (Shoyab *et al*, 1979; King and Cuatrecasas, 1982; Krupp *et al*, 1982; Boonstra *et al*, 1985). Recent crystallographic studies on the extracellular domain of EGFR have shown the presence of two configurations (Cho and Leahy, 2002; Garrett *et al*, 2002; Ogiso *et al*, 2002; Ferguson *et al*, 2003): the tethered conformation is thought to be a low-affinity binding state to EGF and cannot form dimers, whereas the extended conformation is thought to form the signaling dimers. However, there appears to be no direct or static correlation between the affinity difference in the Scatchard plot analysis and the conformational differences in the crystallographic studies. Some mathematical models for the dimerization and activation of the EGFRs, where the different affinity sites represent receptors in different conformations, could not explain the experimental results obtained (Wofsy *et al*, 1992; Chung *et al*, 1997; Klein *et al*, 2004). Therefore, to understand the mechanism of EGF signaling, kinetic analysis of the association between EGF and EGFR is important, in which the dynamic changes in the affinity between EGF and EGFR should be correlated with the formation of preformed and signaling dimers.

In this study, the formation of EGF/EGFR complex and its dimerization, especially, in the early stages of EGF signaling on the living cell surface, was quantitatively analyzed using single-molecule imaging. Single-molecule analyses in living cells have been effectively used to analyze intracellular reaction kinetics and protein dynamics (Sako and Yanagida, 2004). Examples include kinetic analysis of receptor–ligand interaction (Ueda *et al*, 2001; Uyemura *et al*, 2005), measurement of protein dynamics and reactions in the nucleus (Yang *et al*, 2004) or plasma membrane (Sako *et al*, 2000; Schütz *et al*, 2000; Iino *et al*, 2001; Hibino *et al*, 2003; Ichinose *et al*,

2004; Douglass and Vale, 2005). Here, analysis of single-molecule process of EGF binding to the cell surface revealed two association rate constants, the presence of a binding intermediate not previously identified and the rate constant for the formation of this intermediate. Global fitting of the binding curves to a simple model containing the kinetic intermediate with experimentally obtained rate constants provides new insight into the formation of signaling dimers of EGF/EGFR complexes.

Results and discussion

Visualization of single molecules of EGF bound to the surface of living cells

Rh-EGF was applied to the medium of HeLa cells at a final concentration of 0.1–0.5 nM under an oblique illumination microscope (Figure 1A and B). After the application of Rh-EGF, fluorescence spots appeared on the cell surface. Individual spots emitted almost constant fluorescence, then suddenly disappeared (Figure 1C, red). The disappearance was most likely caused by the photobleaching of Rh and not by the dissociation of EGF molecules, as dissociation of EGF from EGFR is known to be slow. Single-step photobleaching indicated that a single molecule of Rh-EGF had bound to the EGFR (Sako et al, 2000). Spots that disappeared in two steps were also observed (Figure 1C, black), indicating that these spots contained two molecules of Rh-EGF.

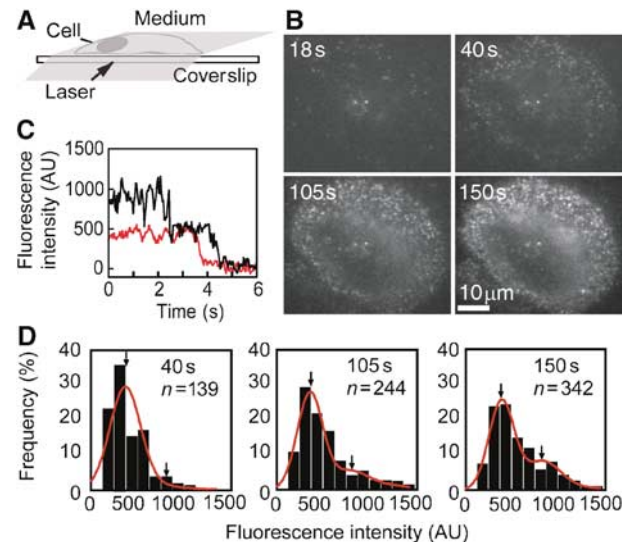The distribution of the fluorescence intensity of Rh-EGF spots bound to the cell surface was obtained at different time points after the addition of Rh-EGF (Figure 1D). The distributions were fitted to the sum of two Gaussian functions, which allowed the ratio of the Rh-EGF monomer and the dimer to be calculated. As the mean of the first component was the same as that for the photobleaching step, it is most likely that this component contained a single Rh-EGF molecule. The second component, which emitted a signal twice as intense as the first component, should represent spots containing two Rh-EGF molecules. This component possibly represents two Rh-EGF molecules bound to a single EGFR dimer or to two EGFR monomers positioned closer than the spatial resolution of the microscope. The latter case is less likely to occur because of the low density of fluorescent spots and the synchronized lateral diffusion movements of two Rh-EGF molecules forming a single spot (Supplementary data). Fractions that contained more than three Rh-EGF molecules were small.

The total number of bound molecules of Rh-EGF per cell was subdivided into monomers and dimers according to the distribution of fluorescence intensity at each time point (Figure 1D), and plotted as a function of time (Figure 2). As shown here, using single-molecule visualization, it is possible to separately analyze the formation of monomeric and dimeric binding sites of EGF. Significant internalization of Rh-EGF did not take place until 180 s after the application of Rh-EGF to the medium (Supplementary data). Data during this period were used in the mathematical modeling of EGFR dimerization in the later sections.

Single-molecule analysis of the association of the first EGF molecule

The fluorescent spots that appeared on the cell surface after the addition of Rh-EGF to the cell culture medium (Figure 3A) were due to the binding of single Rh-EGF molecules to EGFRs in the plasma membrane. Molecules in solution cannot be observed as spots owing to the rapid Brownian movement in solution. The time taken for the appearance of individual spots was measured immediately after the addition of Rh-EGF to the medium (Figure 3B).

Figure 1 Single-molecule visualization of Rh-EGF bound on the living cell surface. (**A**) Oblique illumination with a thin laser beam through the objective lens of a microscope allows single-molecule visualization on the apical cell surface. (**B**) Once the apical surface near the edge of a cell was brought into focus, Rh-EGF was added to the extracellular medium. Pictures taken 18, 40, 105 and 150 s after the addition of Rh-EGF (0.5 nM final concentration) are shown. (**C**) A typical fluorescent spot on the cell was photobleached in a single step (red) or double steps (black), indicating that the spot contained a single molecule or two molecules of Rh-EGF, respectively. (**D**) Distribution of the fluorescence intensity of Rh-EGF spots bound on the cell shown in (B) analyzed 40, 105 and 150 s after the addition of Rh-EGF. The distributions were fitted to a sum of two Gaussian functions (red line). Arrows indicate the mean of the fractions containing one and two Rh-EGF molecules. n: total number of spots; AU: arbitrary unit.
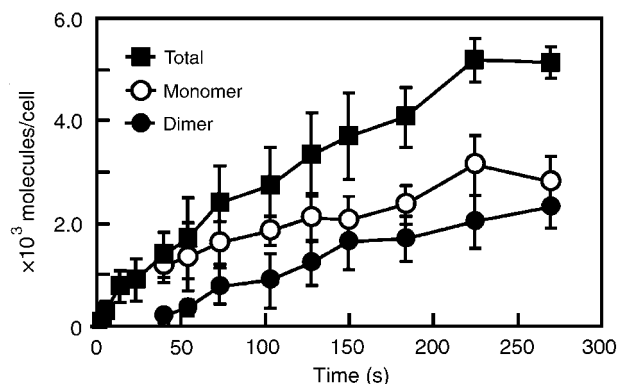

Figure 2 Time course of Rh-EGF binding on the cell surface. The total number (closed square) of Rh-EGF molecules bound on individual cells was calculated at the indicated times. The numbers were then subdivided into number of monomers (open circle) and dimers (closed circle) of Rh-EGF according to fluorescence intensity distributions (Figure 1D). The average and standard deviation of 10 cells are shown. In the initial stages of EGF binding (~ 20 s), the total numbers of EGF molecules bound to the cell surface were quite small (< 70), so they were not subdivided into groups. These results were obtained in the presence of 0.5 nM Rh-EGF. The average of the cell surface areas was 4500 μm^2.
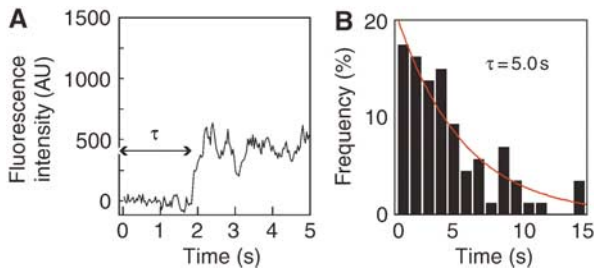
Figure 3 Kinetic analysis of the first molecule of Rh-EGF binding. (**A**) A typical fluorescence intensity change in the binding site of a single Rh-EGF molecule. Sudden step-like increases in fluorescence intensity indicate the binding of single Rh-EGF molecules. (**B**) Histogram of the duration (τ) of each single-molecule binding event after the addition of 0.5 nM Rh-EGF to the medium bathing the cells. The total number of events was 102. The histogram was fitted to the single exponential function (red lines) as described in equation (1), where $k_{on} = 4.0 \times 10^8\,M^{-1}\,s^{-1}$. AU: arbitrary unit.

The association reaction between the ligand (L) and the receptor oligomer (R_x) forming a complex (LR_x) can be written as

$$L + R_x \xrightarrow{k_{on}} LR_x \qquad (Scheme\ 1)$$

where k_{on} is the second-order association rate constant and x is the number of receptor molecules contained in a single binding site. In our experiments, the number of EGFR molecules in each individual binding site should be one (monomer) or two (predimer) in most cases as a functional binding site (Figure 2). Scheme 1 represents the association of the first Rh-EGF molecule in the formation of a signaling dimer. The histogram of the duration until the first binding event could be fitted to the following function (Supplementary Scheme 1):

$$f(t) = C \exp(-k'_{on}t) \qquad (1)$$

Here, k'_{on} is the first-order association rate constant ($k'_{on} = k_{on}[L]$; [L] is the concentration of L). The histogram obtained (Figure 3B) was fitted with this function, where $k_{on} = 4.0 \times 10^8\,M^{-1}\,s^{-1}$.

Single-molecule analysis of the association of the second EGF molecule

Occasionally, the fluorescence intensity of single spots increased suddenly by a factor of approximately two (Figure 4A). After the increase of fluorescence intensity, these spots moved as a unit along the cell membrane. These events are most likely the result of the association of a second Rh-EGF molecule to the complexes, which already contained single Rh-EGF and more than one vacant EGFR molecule. Figure 4B shows the histograms of the durations between the first and second EGF binding to the same site. The histograms show a peak at around 1 s (Figure 4B). The presence of the peak suggests that the process did not arise from a simple ligand–receptor association reaction but had two sequential transitions. We propose that a kinetic intermediate exists during EGFR dimerization, which has not been identified in previous studies.

The reaction containing a kinetic intermediate can be generally written as
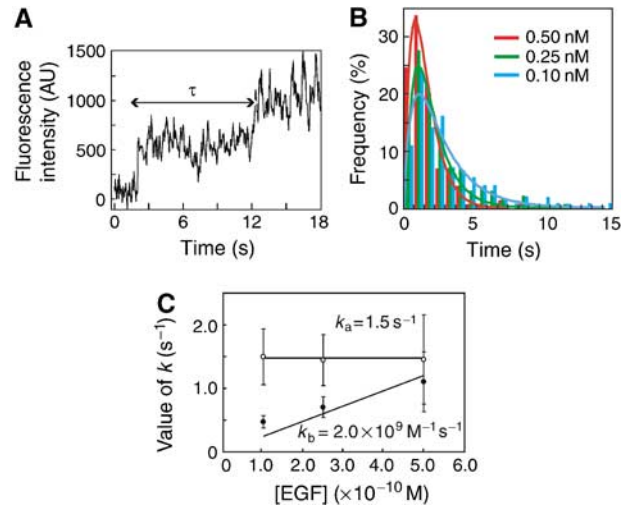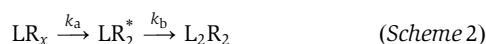
$$LR_x \xrightarrow{k_a} LR_2^* \xrightarrow{k_b} L_2R_2 \qquad (Scheme\ 2)$$

Figure 4 Kinetic analysis of the second Rh-EGF binding event. (**A**) A typical change in the fluorescence intensity at the site of the formation of an EGFR dimer. Each step represents the first and the second binding events of Rh-EGF to an EGFR dimer. (**B**) Histograms of the duration (τ) between the binding of the first and second Rh-EGF molecules to the same binding sites in the presence of 0.10 nM (blue bars), 0.25 nM (green bars) and 0.50 nM (red bars) of Rh-EGF (total number of events was 163, 147 and 157, respectively). These histograms were fitted to equation (2) representing a tandem reaction. The results of the fitting are shown as lines. (**C**) Kinetic parameters k_a (open circles) and k_b' ($= [Rh\text{-}EGF]k_b$; filled circles) in equation (2) obtained by the fitting are plotted as a function of the concentration of Rh-EGF.

Here, LR_x ($x = 1$ or 2) can contain either one (LR) or two (LR_2) receptor molecule(s). LR_2^* was the kinetic intermediate. The reaction in Scheme 2 could be expressed as (Supplementary Scheme 2)

$$g(t) = \frac{Ck_ak_b'}{k_b' - k_a}[\exp(-k_at) - \exp(-k_b't)] \qquad (2)$$

where $k'_b = k_b[L]$ considering the EGF binding as a pseudo-first-order reaction. The dissociation reaction in the second step is not the subject of this analysis, as only binding events have been observed. The reverse reaction of the first step in Scheme 2 has been ignored (Supplementary data).

The histograms shown in Figure 4B were fitted with equation (2). The reaction was analyzed under three different concentrations of Rh-EGF (0.10, 0.25 and 0.50 nM; Figure 4C). One parameter k_a was independent of the concentration of Rh-EGF, indicating that it is for the first and EGF-independent step. In contrast, parameter k_b' increased in proportion to the concentration of Rh-EGF, indicating that it was for the second and EGF-dependent process, that is, the association of the second EGF molecule. The values for k_a and k_b were $1.5\,s^{-1}$ and $2.0 \times 10^9\,M^{-1}\,s^{-1}$, respectively. Thus, single-molecule analysis revealed the rate constants of the association between EGF and EGFR and the existence of a kinetic intermediate, which could not be identified using conventional methods.

Models for the formation of signaling dimers of EGF/EGFR complexes

A simple model has been considered to explain the formation of the signaling dimer of EGF/EGFR complexes including a kinetic intermediate (Model A in Figure 5A). This model was
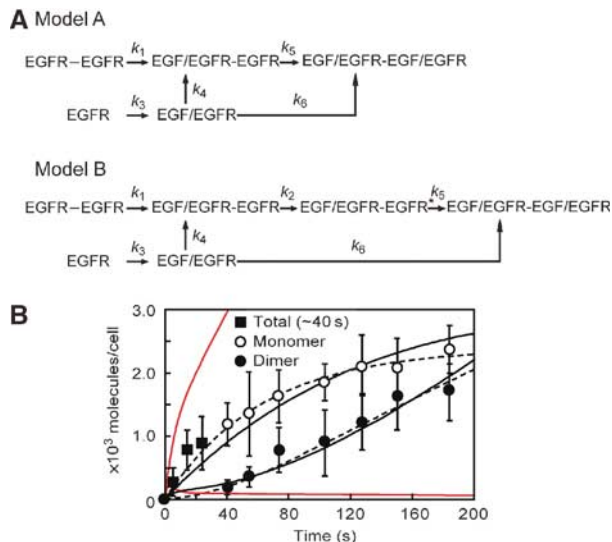
A Model A

Model B

B

Figure 5 Fitting of the time course of Rh-EGF binding to the models for the formation of signaling dimers. (**A**) Two simple schemes for the formation of a signaling dimer (EGF/EGFR–EGF/EGFR). Model A includes the intermediate EGF/EGFR–EGFR formed by the association of EGF/EGFR and EGFR. In Model B, the intermediate is EGF/EGFR–EGFR*. (**B**) Changes in the number of Rh-EGF monomers (open circles) and dimers (filled circles) bound to the cell surface (the same plots shown in Figure 2) have been fitted using functions obtained form Models A (red lines) and B (solid black lines). In fitting, three of the rate constants were fixed to the experimentally obtained values. Model A could not explain the experiments. Results of fitting using Model B floating every parameter are also shown (black broken lines). The total number of bound Rh-EGF molecules before the 40 s time period is shown as closed squares for reference.

based on the reports that EGFR molecules present on the cell surface either as monomeric or dimeric binding sites before application of EGF to cells (Moriki *et al*, 2001; Yu *et al*, 2002). In this model, there are three possible routes for the formation of the signaling dimer. First, two EGF molecules bind sequentially to a preformed dimeric binding site. Second, an EGF binds to a monomeric binding site and couples with a vacant binding site by lateral diffusion and collision, then the second EGF binds to the vacant binding site. Third, two EGF/EGFR complexes laterally diffuse and collide to form a signaling dimer. The intermediate in Figure 4 was found between successive bindings of the first and the second ligands from the solution. In Model A, the intermediate is LR_2.

The changes in the number of monomeric and dimeric EGF molecules bound to the cell surface by 180 s after the addition of Rh-EGF (Figure 2) were fitted with the functions obtained by solving the coupled differential equations according to Model A (Supplementary data) using nonlinear least-squares method (Figure 5B). In the fitting, the rate constants obtained from the single-molecule experiments (Figures 3 and 4) were fixed as $k_1 = k_{on}$ ($4.0 \times 10^8 \, M^{-1} s^{-1}$), $k_4 = k_a$ ($1.5 \, s^{-1}$) and $k_5 = k_b$ ($2.0 \times 10^9 \, M^{-1} s^{-1}$). Other parameters ($k_3$, k_6, $R(0)$ and $R_2(0)$) were floated. $R(0)$ and $R_2(0)$ are the numbers of monomers and dimers of EGFR per cell before the addition of EGF. The best-fit results according to Model A could not explain the experiments (Figure 5B; Supplementary data). There is a possibility that k_{on} represents k_3 (and not k_1), that is the association rate constant between EGF and monomeric EGFR. However, substitution of 4.0×10^8 ($M^{-1} s^{-1}$) for k_3

instead of k_1 also did not agree with the experimental data (data not shown). Thus, Model A cannot explain EGF binding on the cell surface.

Model A was extended to contain a separated intermediate state (LR_2^*) between LR_2 and L_2R_2 (Model B in Figure 5A). Model B assumes a conformational change of the dimeric binding sites bound with a single EGF molecule. The experimental results shown in Figure 2 were fitted with the functions obtained from Model B (Supplementary data). In the fitting, the rate constants obtained from the single-molecule experiments (Figures 3 and 4) were fixed as $k_1 = k_{on}$ ($4.0 \times 10^8 \, M^{-1} s^{-1}$), $k_2 = k_a$ ($1.5 \, s^{-1}$) and $k_5 = k_b$ ($2.0 \times 10^9 \, M^{-1} s^{-1}$). Other parameters ($k_3$, k_4, k_6, $R(0)$ and $R_2(0)$) were floated. The result of fitting agreed with the experiment within the range of experimental errors (Figure 5B). The best-fit parameters are shown in Table I. Parameters predicted from the fitting but not determined directly in this study (k_3, the association rate constant of EGFR monomer; $R(0) + 2R_2(0)$, the total number of receptor molecules) were consistent with the values reported in previous studies. Substitution of 4.0×10^8 ($M^{-1} s^{-1}$) for k_3 instead of k_1 resulted in physically meaningless fitting (a negative value for k_4). If k_4 was constrained to be positive, the best-fit results did not agree with the experiments at all (data not shown).

Fittings resulted in fundamentally the same conclusion when all the parameters were floated (Figure 5B and Table I). The parameters $k_1 = 2.0 \times 10^8 \, M^{-1} s^{-1}$, $k_2 = 1.0 \, s^{-1}$ and $k_5 = 2.0 \times 10^9 \, M^{-1} s^{-1}$ obtained from this fitting were consistent with experimentally obtained values. Probably, some of the dimeric spots of Rh-EGF were pseudo-dimers constructed by two monomers accidentally presented nearby. However, the analysis of movements of dimeric spots suggests that almost all (98%) of the molecules in dimeric spots were moving synchronously (Supplementary data) and the presence of pseudo-dimers up to 50% of the observed dimeric fraction does not essentially change the results of fitting (Supplementary Table II). Fitting to Model A could not explain the experiments even when all of the parameters were floated (Supplementary data). Models containing the reverse reactions are too complex to be solved analytically. However, numerical analysis (Sasagawa *et al*, 2005) of the models confirmed that all reverse reactions can be ignored (Supplementary data).

Kinetics of the formation of signaling dimer

The formation of signaling dimers of EGF/EGFR complexes could be well explained using Model B. In this model, a conformational change in EGFR dimer bound with a single EGF molecule (EGF/EGFR–EGFR*) was taken as the kinetic intermediate. The result provides some insight into the formation of signaling dimer of EGF/EGFR complexes:

(1) About 1–2% of EGFR molecules ($2R_2(0)/\{R(0) + 2R_2(0)\}$; Table I) on the cell surface formed dimeric binding sites independent of EGF binding (preformed dimeric binding sites). If monomeric and preformed dimeric binding sites on the cell surface were in a state of equilibrium,

$$2R \underset{k_{\text{dissociation}}}{\overset{k_{\text{association}}}{\rightleftarrows}} R_2 \qquad (Scheme\ 5)$$

the dissociation constant would be quite large ($K_D = R^2 / R_2 \sim 4 \times 10^6$ sites/cell). In cells expressing high amounts

Table I Kinetic parameters obtained from Model B and experiments

Parameter	Reaction	Fitting with Model B		Experiments
		Fixed[a]	Floated[b]	
k_1 (M^{-1} s^{-1})	L + R$_2$ → LR$_2$	$4.0 \times 10^{8\,c}$	2.0×10^8	$4.0 \times 10^{8\,c}$
k_2 (s^{-1})	LR$_2$ → LR$_2$*	1.5^c	1.0	1.5^c
k_3 (M^{-1} s^{-1})	L + R → LR	4.0×10^6	2.3×10^6	3.3–$9.8 \times 10^{6\,d}$
k_4 (s^{-1})[e]	LR + R → LR$_2$	3.0×10^{-3}	3.0×10^{-3}	ND
k_5 (M^{-1} s^{-1})	LR$_2^*$ + L → L$_2$R$_2$	$2.0 \times 10^{9\,c}$	2.0×10^9	$2.0 \times 10^{9\,c}$
k_6 (s^{-1})[e]	LR + LR → L$_2$R$_2$	3.3×10^{-3}	3.3×10^{-3}	ND
$R(0)$ (/cell)		25 000	27 000	47 000[f]
$R_2(0)$ (/cell)		100	200	

Kinetic parameters obtained from fitting the time course of Rh-EGF-binding (Figure 2) to Model B in Figure 5 are listed. L and R represent EGF and EGFR, respectively. $R(0)$ and $R_2(0)$ are the number of monomeric and preformed dimeric biding sites of EGFR per cell in the absence of EGF. Averages of the best-fit values of parameters from two sets of experimental data at different concentrations of [Rh-EGF] (0.1 and 0.5 nM) are shown. ND: not determined.
[a]k_1, k_2 and k_5 were fixed to the experimental values determined in this study.
[b]All parameters were floated.
[c]Experimental values determined in this study.
[d]Bellot et al (1990), Berkers et al (1991), Felder et al (1992), Chung et al (1997) and Wilkinson et al (2001).
[e]These are first-order rate constants specific at the receptor density in HeLa cells.
[f]Total number of EGFR molecules (Berkers et al, 1991).

of EGFR, such as A431 (3×10^6 EGFR/cell; Defize et al, 1988), the amount of preformed dimeric binding site should be 7×10^5 sites/cell. This number is similar to that suggested previously (Gadella and Jovin, 1995; Martin-Fernandez et al, 2002).

It is expected that the number of EGFR predimer decreases proportionally to the square of the receptor density. As a result, in cells expressing smaller amounts of EGFR, the formation of signaling dimers will be slow and diffusion and collision between EGF/EGFR monomers will become more important. In such cells, the number of EGF molecules required to induce cellular responses will be increased.

(2) EGF bound to the predimeric binding sites (L + R$_2$ $\xrightarrow{k_1}$ LR$_2$; $k_1 = 2.0$–4.0×10^8 M^{-1} s^{-1}) two orders of magnitude faster than to the monomeric binding sites (L + R $\xrightarrow{k_3}$ LR ; $k_3 = 2.3$–4.0×10^6 M^{-1} s^{-1}). This is most likely important in facilitating the formation of signaling dimers of EGF/EGFR complexes at low concentrations of EGF. The formation of signaling dimers from predimeric sites would be more efficient than association of two EGF/EGFR complexes, which is limited by diffusion along the plasma membrane, as predicted by Mayawala et al (2005) based on our previous results (Sako et al, 2000). The association rate constant to monomeric sites (k_3) is consistent with the association rate constants between EGF and EGFR, as reported previously (Bellot et al, 1990; Berkers et al, 1991; Felder et al, 1992; Chung et al, 1997; Wilkinson et al, 2001). Our calculation suggests that approximately 98–99% of EGFRs exist as monomeric sites on the cell surface. The association rate constant obtained using conventional biochemical techniques should be close to that for monomeric sites.

(3) The association rate constant for the second molecules of EGF to the dimeric binding sites ($k_5 = 2.0 \times 10^9$ M^{-1} s^{-1}) was one order of magnitude higher than that for the first EGF molecule to the preformed dimeric binding sites ($k_1 = 2.0$–4.0×10^8 M^{-1} s^{-1}). This indicates positive cooperativity of EGF binding to the dimeric binding sites, as predicted in our previous study (Uyemura et al, 2005). The first molecules of bound EGF induce a conformational change in

the dimeric sites, which increases the association rate for the second EGF. The positive cooperative binding of EGF guarantees the selective binding of the second ligand to the first binding sites, which further accelerates the formation of signaling dimers at low concentrations of EGF.

(4) The formation of signaling dimer through association of two EGF/EGFR complexes was very slow in the presence of low concentrations of the ligand. EGF has been thought to promote dimerization between EGFRs according to the scheme

$$\text{LR} + \text{LR} \xrightarrow{k6} \text{L}_2\text{R}_2$$
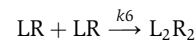
where two EGF/EGFR complexes coupled to each other by lateral diffusion and collision on the cell membrane. According to our model, this process is much slower than the formation of signaling dimer from the predimeric binding sites (k_2 was 1.0–1.5 s^{-1} whereas k_6 was 3.3×10^{-3} s^{-1}) at least under our experimental conditions ([Rh-EGF] = 0.1–0.5 nM). In the initial stages of EGF signaling and/or in the presence of low concentrations of EGF, the association following lateral diffusion may not be important.

Thus, sensitivity of cells to EGF was improved in the initial stages of signaling pathway. Signal amplification has been known in the later stages, for example, secondary phosphorylation of EGFR (Reynolds et al, 2003; Ichinose et al, 2004) and calcium-induced calcium release. However, simple high-gain systems amplify noise at the same time as signal amplification (Shibata and Fujimoto, 2005). Improvement of signals in the earlier stages is desirable to avoid excess signal amplification in the later stages that generates extra noise.

Relation between the kinetics and the structural information

The extracellular EGF-binding domain of EGFR contains four subdomains (subdomains I–IV). Subdomains I and III participate in EGF binding (Lax et al, 1989; Garrett et al, 2002; Ogiso et al, 2002). The crystals of the extracellular region of EGFR prepared in low-affinity conditions have revealed a monomeric form of the EGF/EGFR complex that exists in an

autoinhibited configuration. In this configuration, specific intramolecular interactions between subdomains II and IV tether the structure and EGF interacts with only subdomain I (Cho and Leahy, 2002; Ferguson *et al*, 2003). However, in the dimeric form of EGF/EGFR complex, EGF binds simultaneously to subdomains I and III to induce an extended conformation of EGFR. In this configuration, the dimerization arm in subdomain II is available for mediating interactions with a neighboring extended EGFR molecule (Garrett *et al*, 2002; Ogiso *et al*, 2002). This extended conformation is thought to be a high-affinity conformation. Interactions between two EGF molecules or crosslinking of two receptor molecules by EGF has not been found in the crystals (Garrett *et al*, 2002; Ogiso *et al*, 2002).

Although the conformation of EGFRs in the predimers is not yet known, large increases in the association rate of EGF to the predimeric binding sites suggest that the conformation resembles the extended form. In addition, the positive cooperativity suggests that the association of EGF with one of the EGFR molecules in the dimeric sites induced an allosteric conformational change in the EGF-binding site in the other EGFR molecule. Fluctuations of the vacant receptor molecule may be suppressed and almost fixed to the high-affinity conformation state by binding of EGF to the neighboring molecules in the dimers (Figure 6).

Single-molecule kinetics has revealed that two functionally different populations of binding sites exist on the cell surface to which EGF is associated as a monomer or a dimer. However, the dimerization state of EGFR was not observed directly; thus, it is possible that each binding site is involved in a larger cluster rather than a single site. In addition, there may be EGFR dimers behaving as two individual monomeric binding sites. We cannot exclude the possibility that the dimeric binding sites of Rh-EGF were EGFR molecules confined in small membrane domains and do not interact with each other directly. However, in that case, there must be a mechanism to increase the association rate of EGF to the confined receptors. Such a mechanism has not been proposed as far as we know. The assumption of direct interaction (predimerization of EGFR) is simpler and seems to be preferable at the present time.

Conclusion

The mechanisms that guarantee the rapid and sensitive signal transduction of EGF remain to be determined. The model for the formation of the signaling dimer proposed in the present study suggests that a small fraction of EGFR formed predimeric binding sites for EGF, and that the high association rate for the first binding event and the positive cooperativity of the binding of two EGF to the predimeric sites facilitated the formation of signaling dimers. The present model is in good agreement with previous kinetic (Bellot *et al*, 1990; Berkers *et al*, 1991; Felder *et al*, 1992; Chung *et al*, 1997; Wilkinson *et al*, 2001; Uyemura *et al*, 2005) and structural (Cho and Leahy, 2002; Garrett *et al*, 2002; Ogiso *et al*, 2002; Ferguson *et al*, 2003) studies. Formation of the predimeric binding sites of EGFR makes the EGF signaling rapid and very sensitive even when the concentration of EGF is low, and the expression of excess amounts of EGFR on the cell surface is required for the formation of predimeric binding sites of EGFR.
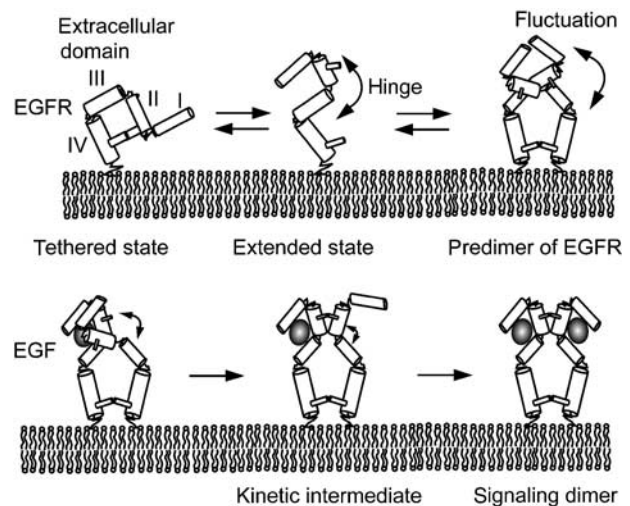

Figure 6 A model for the formation of signaling dimer of EGF/EGFR complexes. The extracellular EGF-binding domain of EGFR contains subdomains I–IV. Subdomains I and III participate in EGF binding (Lax *et al*, 1989). An EGFR molecule exists in the extended low-affinity (low-association rate) state or in the extended high-affinity (high-association rate) state and can switch between the states by dynamic fluctuations mainly at the hinge between subdomains II and III (Cho and Leahy, 2002; Garrett *et al*, 2002; Ogiso *et al*, 2002; Ferguson *et al*, 2003). Monomers of EGFR exist primarily in the tethered state, and the formation of predimers biases the structure of EGFR toward extended state-like conformations with high association rates to EGF. EGF bound to one of the EGFR molecules in a predimer induces an allosteric conformational change in another EGFR molecule to a kinetic intermediate structure with a considerably high association rate to EGF. Thus, a dynamic conformational change in the predimer facilitates the formation of signaling dimer of EGF/EGFR complexes. Only the portion of EGFR outside the cell surface is shown.

Materials and methods

Cell culture
HeLa cells were cultured on glass coverslips in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Before the experiment, the culture medium was changed to that without serum and phenol red overnight.

Optical settings
An objective type total internal reflection fluorescence microscope system (Tokunaga *et al*, 1997; Sako *et al*, 2000) was modified for oblique illumination. The microscope was based on an inverted microscope (IX-70, Olympus, Tokyo, Japan) equipped with an oil-immersion objective (PlanApo $\times$ 100, NA 1.45, Olympus) and an excitation laser (532 nm; Compass 315M, Coherent, Santa Clara, CA). Fluorescence images of the specimen were intensified with an image intensifier (C8600-03; Hamamatsu photonics, Hamamatsu, Japan) and acquired with an electron bombardment CCD camera (C7190; Hamamatsu photonics) at video rate.

Measurement of bound EGF molecules on the cell surface
Tetramethylrhodamine-conjugated mouse EGF (Rh-EGF) was purchased from Molecular Probes (Eugen, OR). In this probe, EGF was conjugated with a single Rh molecule at the N terminus. The ratio of dye to protein of Rh-EGF was 0.98 (Uyemura *et al*, 2005). Rh-EGF was applied to the culture medium of HeLa cells at a final concentration of 0.1–0.5 nM while cells were being observed under the microscope. To study the binding time courses of Rh-EGF onto the cells, images of cells were recorded for a few seconds at each indicated time point after the application of Rh-EGF. Exposure time to acquire images was kept to a minimum to avoid photobleaching. Any slight effect of photobleaching was corrected (Hibino *et al*, 2003). In the single-molecule tracking experiments of EGF binding,

images of cells were recorded continuously. All experiments were performed at 25°C. The apical surface of cells was observed to avoid rate determination of the binding kinetics by the diffusion of Rh-EGF between the basal cell surface and the coverslip. The HeLa cells on the glass coverslip were convex in appearance; thus, only fluorescent spots bound on the apical surface near the edge of cells were observed. The density of bound Rh-EGF molecules in the focus plane was calculated from the area of the plane, the sum of the intensity of every fluorescent spot in the plane and the intensity of single molecules, and then, scaled to the total number of bound molecules per cell with respect to the averaged surface area of the cell ($4500\,\mu m^2$/cell).

Data analysis

Parameter estimation was carried out using a nonlinear least-squares fitting routine program originally developed by our group

(Uyemura *et al*, 2005). This routine program is based on a finite difference, Levenberg–Marquart algorithm. Numerical calculations of coupled differentiation equations were carried out using *GENESIS* simulator (version 2.2) with a *Kinetikit* interface (Sasagawa *et al*, 2005).

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