Single-Molecule Analysis of Epidermal Growth Factor Signaling that Leads to Ultrasensitive Calcium Response

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ABSTRACT Quantitative relationships between inputs and outputs of signaling systems are fundamental information for the understanding of the mechanism of signal transduction. Here we report the correlation between the number of epidermal growth factor (EGF) bindings and the response probability of intracellular calcium elevation. Binding of EGF molecules and changes of intracellular calcium concentration were measured for identical HeLa human epithelial cells. It was found that 300 molecules of EGF were enough to induce calcium response in half of the cells. This number is quite small compared to the number of EGF receptors (EGFR) expressed on the cell surface (50,000). There was a sigmoidal correlation between the response probability and the number of EGF bindings, meaning an ultrasensitive reaction. Analysis of the cluster size distribution of EGF demonstrated that dimerization of EGFR contributes to this switch-like ultrasensitive response. Single-molecule analysis revealed that EGF bound faster to clusters of EGFR than to monomers. This property should be important for effective formation of signaling dimers of EGFR under very small numbers of EGF bindings and suggests that the expression of excess amounts of EGFR on the cell surface is required to prepare predimers of EGFR with a large association rate constant to EGF.

INTRODUCTION

Research in molecular biology has identified many intracellular signaling molecules and traced signal transduction pathways routing these molecules. One of the next steps in understanding the mechanism of intracellular signal transduction is to investigate the quantitative relationships between inputs and outputs of signaling systems in living cells. At the beginning, it should be determined how many molecules of extracellular ligand bound to cell surface receptors induce a certain cellular response. The purpose of this study is to clarify how many epidermal growth factor (EGF) molecules are needed to induce intracellular calcium response in a human epithelial cell, HeLa. EGF is a polypeptide whose main biological function is to lead various types of cells to proliferation (Carpenter and Cohen, 1979). Mammalian cells express only a single species of the receptor protein for EGF (EGFR) that has a single binding site on EGF. However, binding of EGF to EGFR is known to be a complicated process with multiple components of the dissociation constant and association and dissociation rate constants (Bellot et al., 1990; Berkers et al., 1991; Karunagaran et al., 1996; Wilkinson et al., 2001). In addition, cellular reactions against EGF signaling, including calcium response, start much faster than the binding of EGF to EGFR reaches the equilibrium state. Therefore it is not easy to determine the number of EGF molecules required to induce cellular reactions.

EGFR has an intracellular portion containing a tyrosine kinase domain (Carpenter, 1987) followed by a carboxyl terminal domain containing tyrosine residues that are phosphorylated by the tyrosine kinase domain of other EGFR molecules after stimulation with EGF (Schlessinger, 2000). The phosphotyrosine residues of EGFR are recognized by various cytoplasmic signaling molecules, for example, Grb2 (Lowenstein et al., 1992) and Shc (Sakaguchi et al., 1998), that relay EGF signals to Ras and the MAP kinase cascade, and phospholipase Cγ (PLCγ) for calcium response (Wahl et al., 1990). PLCγ recognizes the phosphotyrosine residues in activated EGFR, and is activated through phosphorylation by EGFR (Wahl et al., 1990). Then, PLCγ hydrolyzes phosphatidylinositol 4,5-bisphosphate in the plasma membrane to produce the second messengers diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) (Berridge et al., 1984; Nishizuka, 1984; Majerus et al., 1986). On the surface of the endoplasmic reticulum (ER), cells have IP_3 receptors (IP_3R) that are IP_3-gated Ca^{2+} channels (Michikawa et al., 1996). The ER is an intracellular Ca^{2+} store and binding of IP_3 to IP_3R liberates Ca^{2+} to increase intracellular Ca^{2+} concentration. Thus, the signal transduction pathway from the binding of EGF to the intracellular calcium response has been clarified in detail. However, it is not known in detail how many molecules of EGF are required to induce calcium response and how the calcium response function depends on the amount of EGF.

Measurement of the calcium concentration in living cells using fluorescent calcium indicators is an established technique (Thomas and Delaville, 1991). Furthermore, in recent years, methods for detecting single fluorescent molecules in living cells have been developed (Sako et al., 2000; Schütz et al., 2000). EGF can be conjugated with

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a fluorophore with a ratio of exactly 1:1 at the amino terminus without affecting its biological activity (Chatelier et al., 1986; Sako et al., 2000). Therefore, single-molecule visualization of fluorescent EGF molecules can be used for counting the number of molecules on the cells. Total internal reflection fluorescence microscopy (TIRFM) has been widely used to detect single fluorescent dyes in living cells (Sako and Uyemura, 2002; Sako and Yanagida, 2003). The evanescent field, which has a typical decay length of only a few hundred nanometers, is used for excitation in TIRFM, providing very good contrast to allow single-molecule detection. Using TIRFM, however, the region that can be observed is usually limited to the vicinity of the reflection surface and the excitation power depends on the distance from the surface.

To quantify the number of fluorescent EGF molecules on the entire surface of a cell, a technique is desired that allows quantitative single-molecule detection independent of the focal height. We adopted low-angle oblique laser illumination fluorescence microscopy (Tokunaga and Imamoto, 2002) modified to use two excitation laser beams, and successfully observed the fluorescently labeled EGF molecules from the bottom to top of the cell surface with single-molecule sensitivity (Sako and Uyemura, 2002). We made a hybrid of this type of microscope and a conventional epifluorescent microscope for the measurement of the intracellular calcium concentration using a fluorescent indicator dye (Sako et al., 2003). Applying this technique, we first determined the exact number of EGF molecules required to induce intracellular calcium response and obtained the response probability curve. In addition, we analyzed the relationship between association rate of EGF and cluster size of EGFR quantitatively by direct observation.

EXPERIMENTAL PROCEDURE

Cells and materials

A human cervix carcinoma cell line, HeLa, was cultured on glass coverslips using a culture medium (Dulbecco's modified Eagle medium, Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum. The medium was exchanged for Eagle's minimum essential medium (MEM, Nissui Pharmaceutical) without serum and phenol red 24 h before the experiments.

Mouse EGF conjugated with tetramethylrhodamine (Rh-EGF) was purchased from Molecular Probes (Eugene, OR). We examined the dye/protein ratio of Rh-EGF as follows. After reconstruction, as recommended by the manufacturer, Rh-EGF solution containing 1% bovine serum albumen was applied to a μRPC reversed phase column (SMART system, PharmaciaBiotech, Uppsala, Sweden). The bound fraction was eluted by a 0–100% gradient of acetonitrile with 0.1% trifluoroacetic acid. The Rh-EGF peak eluted at 31–34% acetonitrile was collected, and the concentrations of EGF ($A_{280} = 1.6 \times 10^4$) and tetramethylrhodamine ($A_{280} = 2.6 \times 10^4$, $A_{555} = 8.5 \times 10^4$) were determined from optical absorbance. The dye/protein ration was 0.98. No peak was detected at 27% acetonitrile where free EGF should be eluted. Bovine serum albumen was eluted after 36%. Since mouse EGF has no reactive amino residue other than the N-terminus, almost all Rh-EGF molecules were conjugated with a single rhodamine dye molecule at the specific position.

Loading calcium indicator

Cells were incubated with 2 μM Fluo-4 acetoxymethyl ester (Molecular Probes) in the perfusion medium (MEM with 5 mM PIPES without serum and phenol red, pH 7.4) for 30 min at 25°C, washed with the perfusion medium, and incubated for 30 min for deesterification.

Measurements of calcium response and EGF binding

All experiments were carried out at 25°C. Cells loaded with Fluo-4 on a coverslip were set in a handmade perfusion chamber whose volume was 10 μl and put on the stage of a double-beam low-angle fluorescence microscope (Fig. 1 *A*; Sako and Uyemura, 2002) equipped with an oil-immersion objective lens (PlanApo 60×, NA 1.4, Olympus, Tokyo, Japan). In this microscope, the low-angle oblique illumination reduced the background to allow single-molecule detection and two thin laser beams from opposite direction were used for homogeneous illumination of the observation field.

Changes in intracellular calcium concentration ($[Ca^{2+}]_{in}$) and the number of EGF molecules on the cell surface were measured as follows (Fig. 2 *A*): First, images of Fluo-4 were acquired to measure intracellular calcium concentration ($[Ca^{2+}]_{in}$) using a Xe-arc lamp with a 470- to 490-nm excitation filter and a 515- to 550-nm emission filter. The culture medium in the chamber was perfused continuously at a flow rate of 5 μl/s during the observation. This flow did not stimulate mechanical calcium response. Spontaneous fluctuation of $[Ca^{2+}]_{in}$ was monitored for 1 min at a sampling rate of 0.5 Hz. At 60 s after the start of measurement, various concentrations (final concentration 0.2–2.0 nM) of Rh-EGF were added to the flow for 20 s. Then, Rh-EGF was removed and the perfusion was continued. $[Ca^{2+}]_{in}$ was measured until 180 s after the start of the observation. After the measurement of $[Ca^{2+}]_{in}$, perfusion was stopped and Rh-EGF molecules bound to the cell surface were observed using the 568-nm line of an Ar-Kr laser (model 643 RYB-AO2, Melles Griot, Carlsbad, CA) and a 580- to 625-nm emission filter. The focus of the image was changed at intervals of 0.3 μm using a piezo stage (model P-721.10, Phisik Instrumente, Waldbronn, Germany) to scan the entire cell surface. The typical number of images acquired during a scan over the height of single cells was 25. Exposure time was 0.3 s for each image in a set of vertical slices. At the end of a measurement series, a $[Ca^{2+}]_{in}$ calibration was carried out. A saturated concentration of nonlabeled mouse recombinant EGF (100 ng/ml; Higeta, Tokyo, Japan) was added to the cells for positive control of calcium response. This treatment induced calcium response in almost all cells, and the small number of cells without calcium response were eliminated from the measurements. At the end of experiments, cells were treated with 10 μM final concentration of ionomycin (Sigma, St. Louis, MO) for calibration of $[Ca^{2+}]_{in}$ (Thomas and Delaville, 1991).

Images of both Fluo-4 and Rh-EGF were acquired using a back-illuminated cooled CCD camera (Micro Max 512BFT, Roper Scientific, Trenton, NJ). The camera, piezo stage for the objective and shutters to exchange the illumination light sources were controlled by MetaMorph software (Universal Imaging Corporation, Downingtown, PA). Image analysis was carried out using IPLab software (Scanalytics, Fairfax, VA).

RESULTS

Single-molecule detection of Rh-EGF

HeLa cells were perfused with a medium containing EGF conjugated with the fluorescent dye tetramethlyrhodamine (Rh-EGF) for 20 s at 25°C. Here, single EGF molecules were

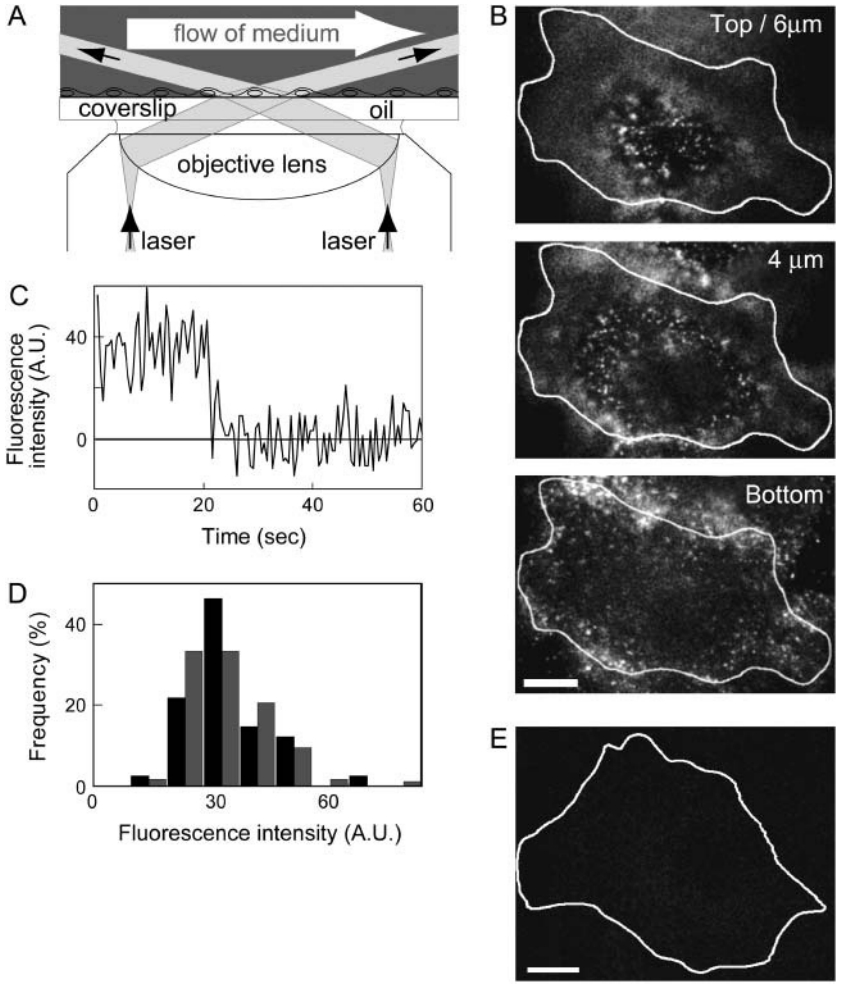

FIGURE 1 Observation of Rh-EGF molecules binding on the cell surface. (*A*) Double-beam low-angle fluorescent microscope. Two laser beams obliquely illuminate the specimen from opposite directions to excite fluorophores. Cells were placed in a flow chamber under the microscope. (*B*) Images of a HeLa cell bound with Rh-EGF. Images were obtained by changing the focal plane from the bottom to the top of a single cell at intervals of 0.3 μm. The exposure time was 0.3 s for each image. Three representative images (*Bottom*, *4 μm*, and *Top*) are shown. The white line indicates the boundary of the cell. Scale bar, 10 μm. (*C*) A typical fluorescence intensity change of a single Rh-EGF spot on the cell surface. The spot was photobleached in a single step, suggesting a single molecule. (*D*) Distributions of the photobleaching step sizes. In multistep step decays, the step size of the last decay (*black*) and others (*shaded*) show a single peak at the same fluorescence intensity. The step size (average $\pm$ SE in A.U.) was 28 $\pm$ 11 (*black*) and 26 $\pm$ 11 (*shaded*). (*E*) Images of cells perfused for 20 s with 2 nM Rh-EGF in the presence of 200 nM nonlabeled EGF. The white line indicates the boundary of a cell. Scale bar, 10 μm.

conjugated to single dye molecules with a ratio of exactly 1:1 (Sako et al., 2000). After washing with medium, Rh-EGF molecules on the cell surface were observed using a double-beam low-angle fluorescence microscope (Fig. 1 *A*). By changing focus from the bottom to the top of the cells, fluorescent spots were observed over the entire cell surface from basal to apical membrane (Fig. 1 *B*). Fluorescent spots of Rh-EGF were sparsely bound on the cell surface with a maximum density of $0.2/\mu m^2$, which was small enough to distinguish individual spots using fluorescence microscopy.

Under continuous laser illumination, individual fluorescence spots photobleached in single or plural step-like decay rather than a gradual decay (Fig. 1 *C*). The step size distribution of the first decay in the multistep decays was the same as that of the second or later decay, regardless of the location and focal depth of the fluorescent spots on the cell surface (Fig. 1 *D*). These properties of photobleaching indicate that the step size of decay represented the fluorescent intensity of single Rh-EGF molecules, and that the fluorescent intensity of each spot before photobleaching was proportional to the number of Rh-EGF molecules contained in each binding site. Thus, Rh-EGF molecules were successfully observed with single-molecular sensitivity everywhere on the cell surface, allowing determination of the absolute number and cluster size distribution of molecules for individual cells. Binding of Rh-EGF was completely blocked by the presence of excess amount of nonlabeled EGF (Fig. 1 *E*). Also, Rh-EGF did not bind at all to CHO-K1 cells, which are EGFR-null (data not shown). These results indicate specific binding of Rh-EGF to cell-surface EGFR.

Detection of EGF-binding and calcium response of identical cells

EGF induces intracellular calcium response. Changes in intracellular calcium concentration ($[Ca^{2+}]_{in}$) and the number of EGF molecules on the cell surface were measured as described above in Experimental Procedures (Fig. 2 *A*). Cells were loaded with a fluorescent calcium indicator, Fluo-4, and set in the perfusion chamber. First, spontaneous fluctuation of $[Ca^{2+}]_{in}$ was monitored for 1 min. Various concentrations of Rh-EGF (final concentration 0.2–2.0 nM) were added to the flow at 60 s after the initiation of $[Ca^{2+}]_{in}$ measurements for 20 s. Fig. 2 *B* shows successive images of

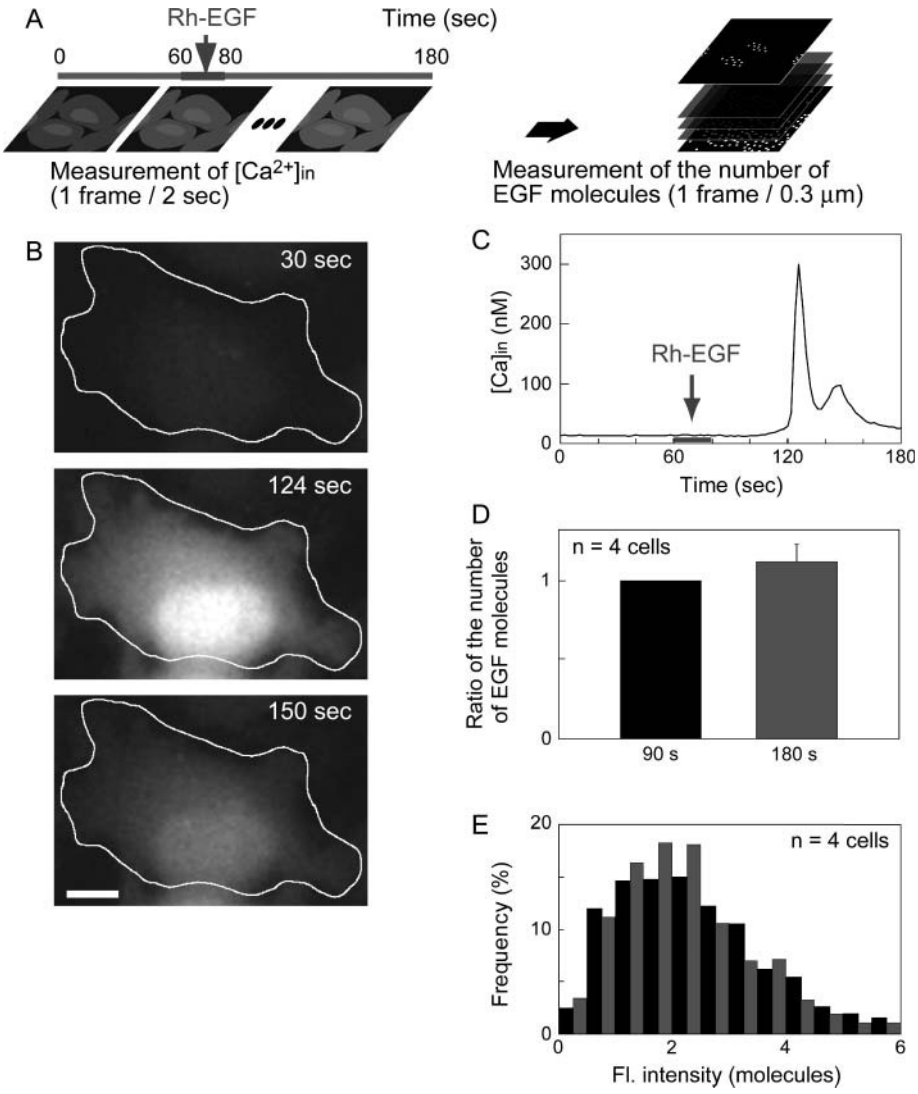

FIGURE 2 Detection of calcium response and EGF binding in the identical cells. (*A*) Measurement procedure. Under continuous perfusion of the culture medium, $[Ca^{2+}]_{in}$ was monitored for 3 min using a fluorescent indicator (Fluo-4). Rh-EGF was added to the flow 60 s after the beginning of the experiment for the next 20 s. After the $[Ca^{2+}]_{in}$ measurement, Rh-EGF molecules bound to the cell surfaces were observed by scanning the focus of the microscope. (*B*) Time-lapse images of the Ca^{2+} response in the cell shown in Fig. 1 *B*. Times from the start of measurement are indicated. The white line shows the boundary of the cell. Scale bar, 10 μm. (*C*) The change of $[Ca^{2+}]_{in}$ in the response to Rh-EGF as shown in Fig. 2 *B*. The $[Ca^{2+}]_{in}$ before stimulus was 13 nM for this case, and was 27 $\pm$ 14 nM (average $\pm$ SD) for all cases examined. (*D* and *E*) The number of molecules (*D*) and the intensity distribution of fluorescence spots (*E*) of Rh-EGF bound to the cells measured immediately after the Rh-EGF perfusion (90 s, *black*) and at the end of the $[Ca^{2+}]_{in}$ measurement (180 s, *shaded*). The number of molecules and the intensity distribution remained unchanged. In *D*, the number at 90 s was normalized to unity. Bar shows SE. (*E*) The accumulation of four cells is examined.

a typical calcium response caused by the addition of Rh-EGF. $[Ca^{2+}]_{in}$ was measured until 180 s after the start of the observation. Immediately after the end of the $[Ca^{2+}]_{in}$ measurement, Rh-EGF molecules bound to the cell surface were observed by changing the height of the focal plane from the bottom to the top of the cell.

For individual cells, changes of $[Ca^{2+}]_{in}$ were calculated and plotted as a function of time to determine the peak intensity and average elevation of the calcium response (Fig. 2 *C*). The peak intensity or average elevation was defined as the peak or average $[Ca^{2+}]_{in}$, respectively, during 60–180 s after subtraction of the average $[Ca^{2+}]_{in}$ during 0–60 s.

Since the fluctuation of single-molecule fluorescence intensity was rather large (Fig. 1 *D*), the fluorescent intensities of all Rh-EGF spots on individual cells were summed up and divided by the average fluorescent intensity of a single Rh-EGF molecule determined from the photobleaching step size (Fig. 1, *C* and *D*) to count the number of EGF molecules bound to each cell. To estimate dissociation and photobleaching of Rh-EGF during the calcium measurements and the scanning of different focal planes for counting, a control experiment was carried out, in which 2 nM final concentration of Rh-EGF was added to cells during a 20-s period starting 60 s after the initiation of medium perfusion. At 80 s, the remaining Rh-EGF in solution was washed out by continuous perfusion with a medium without Rh-EGF. The numbers of Rh-EGF were repeatedly measured at 90 s (immediately after the perfusion of Rh-EGF) and 180 s (at the end of Ca^{2+} measurements in other experiments). In this experiment, $[Ca^{2+}]_{in}$ was not measured. Obtained numbers did not change significantly in all cells examined, indicating that dissociation and photobleaching of Rh-EGF were negligible (Fig. 2 *D*). This result is consistent with the slow dissociation rate constants of EGF from EGFR (Berkers et al., 1991; Karunagaran et al., 1996). In addition, fluorescence intensity distributions of Rh-EGF spots at 90 s and 180 s were almost the same (Fig. 2 *E*). Therefore, the number and intensity distribution of Rh-EGF spots observed at 180 s

faithfully reflected those of Rh-EGF at the time of calcium response stimulation. Aggregation of EGFR on the cell surface has been reported after EGF binding (Carraway et al., 1989; Gadella and Jovin, 1995). In our experiments, aggregation did not occur, which must be due to very sparse binding of EGF. Also, internalization of Rh-EGF was not observed at 90 s and <8% of bound ligand was internalized at 180 s. As shown in Fig. 2, *D* and *E*, the number and intensity distributions of Rh-EGF spots were not changed by the internalization.

Number of EGF molecules required to induce the calcium response

Imaging of bound Rh-EGF and measurement of $[Ca^{2+}]_{in}$ were carried out for identical cells after a perfusion with 0.2–2 nM Rh-EGF for 20 s. Although the peak intensity (Fig. 3 *A*) and average elevation (Fig. 3 *B*) of $[Ca^{2+}]_{in}$ in individual cells generally increased with the number of EGF molecules, cell-to-cell fluctuations were very large for these values. Here, to focus on the early event of EGF-induced calcium signaling, that is from the binding of EGF and EGFR to the beginning of calcium elevation throughout cells, properties of the calcium response in individual cells were neglected and cellular response was evaluated only by the occurrence of calcium response. To achieve this, the peak intensity was adopted for the judgment of occurrence, because the peak intensity was usually recorded at the beginning of the response and should reflect the early events of calcium signaling more appropriately than the average elevation. When perfusion medium was applied instead of Rh-EGF, the difference between average $[Ca^{2+}]_{in}$ before and the maximum $[Ca^{2+}]_{in}$ after addition of the medium was <30 nM for >95% of cells (156 cells were examined). Therefore, peak intensities >30 nM were judged to be positive reactions induced by EGF. The results scarcely changed if the average elevation was adopted for the judgment. The threshold of positive reaction in this case was determined to be 10 nM using the same method as in the case of the peak intensity. Disagreement of the two judgments was found only for a single case out of 67. Cells were classified into four groups according to the number of EGF. The percentages of cells that showed a positive response were calculated for each group (Fig. 3 *C*).

Binding of 293 ± 9 EGF molecules induced the calcium response in half of the cells. When the average elevation was adopted as the judgment, the number was 314 ± 7. The number of EGF was quite small compared to the number of EGFR expressed on HeLa cells, 50,000/cell (Berkers et al., 1991).

Dimerization of EGFR is known to be critical for signal transduction (Boni-Schnetzler and Pilch, 1987; Canals, 1992; Sorokin et al., 1994; Sherrill, 1997). To understand the roles of dimerization and clustering of EGFR, the relation between cluster size distribution of EGF and

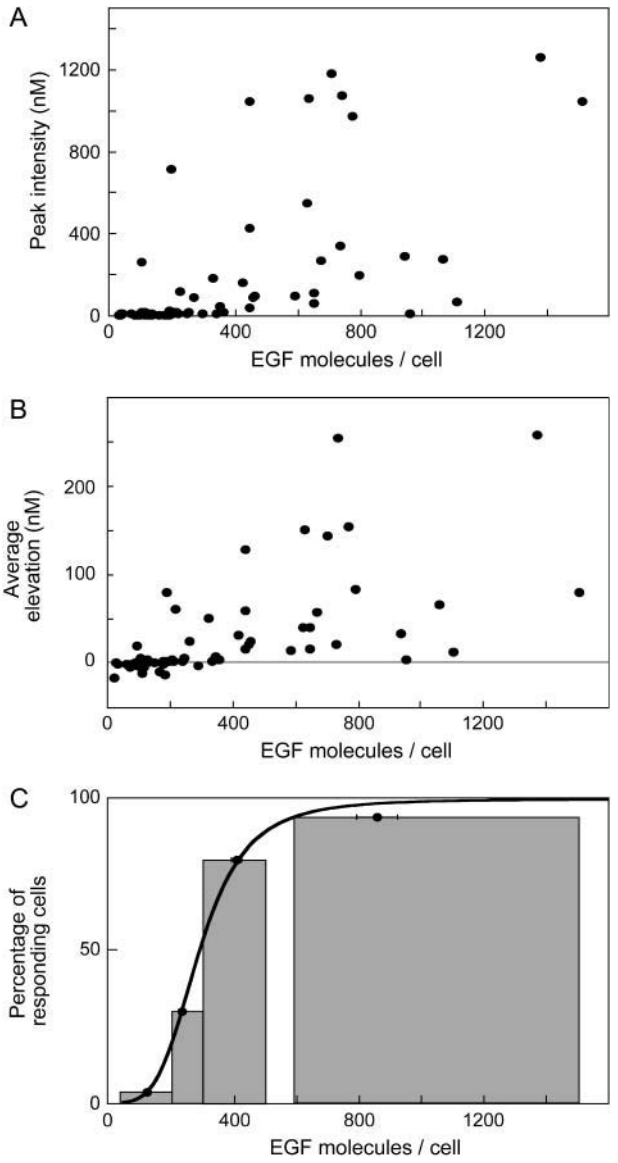

FIGURE 3 Correlation between EGF binding and calcium response. (*A* and *B*) The peak intensity (*A*) and average elevation (*B*) of calcium responses were plotted against the number of EGF on individual cells. Results of cells perfused with 0.2–2 nM Rh-EGF were mixed in these plots. The number of cells examined was 67. Each spot represents a single cell. (*C*) Correlation between the average number of EGF (●) and the percentage of cells responding to EGF. Bars show SE. Cells were classified into four groups according to the number of EGF bonds (10–30 cells in each group). The same data are represented by a bar graph. The width and height of each bar represents the range of bound EGF and response probability, respectively. Binding of 300 EGF molecules induced calcium response in half of the cells. The solid curve is the result of the fit to Hill's equation. The apparent Hill factor was 3.9.

calcium response must be analyzed. Fluorescence intensity distribution of Rh-EGF spots on the cell surface gives information about the number of dimers and clusters of EGFR bound with ligand (Fig. 2 *E*). Cells were classified according to the number of EGF, as in Fig. 3 *C*, and histograms of the fluorescence intensity were made for all

spots bound to each class (Fig. 4 *A*). With increasing percentage of cells responding to EGF (i.e., generally, the number of EGF molecules increased), the fraction of the spots with higher fluorescence intensity increased, as expected from an activation model of EGFR that depends on dimerization. The histograms were fitted to a sum of four Gaussian functions, each with a peak at the fluorescence intensity of one, two, three or four molecule(s) of Rh-EGF. Fitting to more than five Gaussians did not improve the result.

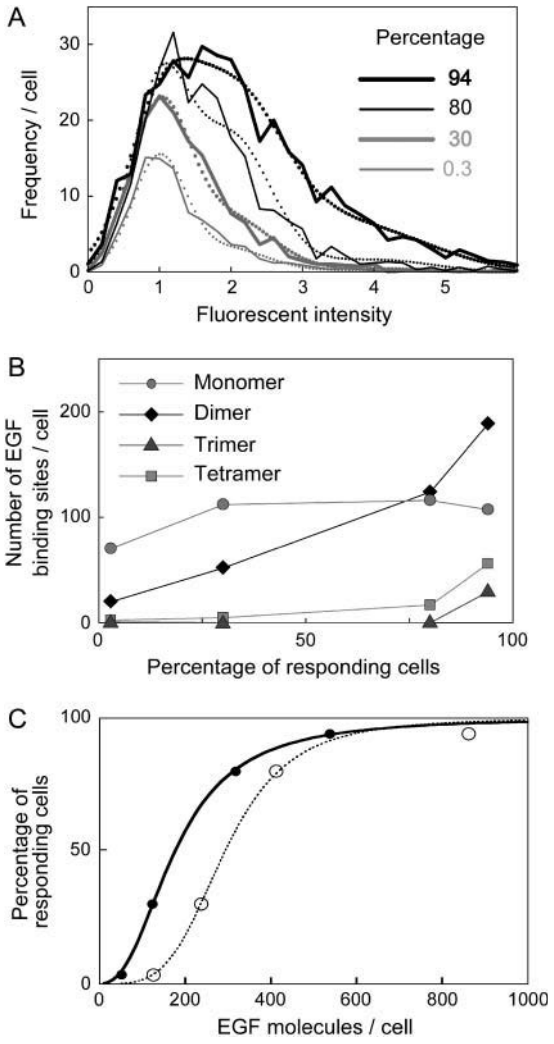

FIGURE 4 The contribution of EGFR dimerization to calcium response. (*A*) Fluorescence intensity distributions of Rh-EGF spots on the cell surface. The cells were classified into four groups as in Fig. 3 *C*. The fluorescence intensity is normalized by the intensity of a single Rh-EGF molecule. The results of fits to a sum of four Gaussian are shown by dotted lines. In the fitting, the peak intensity was fixed and the fractions were determined. The multiple correlation coefficients between data and fits were 0.979–0.995. (*B*) Numbers of EGF molecules contained in one to four EGF clusters estimated from the fits in *A*. The number of molecules in dimers correlated with the percentage of responding cells much better than that in monomers. (*C*) Response probability curves plotted against the total number of Rh-EGF on the cell surfaces (*open circles*; the same plot as in Fig. 3 *C*) and the number included in the multiple-molecule spots (*solid circles*). Each plot was fitted to Hill's equation. The apparent Hill factors were 3.9 (*dotted line*) and 2.4 (*solid line*). The multiple correlation coefficients between the data and fits were 0.998 (*dotted line*) and 0.999 (*solid line*).

The number of EGF binding sites containing one to four Rh-EGF molecules was plotted against the percentage of responding cells (Fig. 4 *B*). The number of single-molecule spots soon reached a plateau before the percentage of responding cells reached a maximum, whereas when the number of multiple-molecule spots increased, especially that of dimers, the number of responding cells increased as well. The percentage of responding cells was replotted against the number of Rh-EGF included in multiple-molecule spots (Fig. 4 *C*, *solid circles*). Here, the binding of 176 ± 3 EGF molecules (71 ± 2 two-molecule spots, 0 three-molecule spots, and 8.0 ± 0.5 four-molecule spots) induced the calcium response in half of the cells. Thus, it seems likely that the minimal requirement to induce calcium signaling is formation of 88 ± 2 EGFR dimers. If the average elevation is adopted as the judgment of the occurrence of calcium response, 96 ± 7 dimers would be required.

Ultrasensitive calcium response depending on dimerization of EGFR

The response probability correlated sigmoidally with the number of EGF molecules in multiple-molecule spots (Fig. 4 *C*) as well as with the total number of EGF molecules bound (Fig. 3 *C*). The extent of ultrasensitivity was evaluated by fitting the response curves to Hill's equation. Since the ratio of monomers and oligomers was reversed as the response probability increased (Fig. 4 *B*), subtraction of the monomeric binding fraction from the response probability curve nonlinearly deformed the response curve and changed the apparent Hill factor. The apparent Hill factor decreased from 3.9 to 2.4 by plotting the response probability against the number of EGF in multiple-molecule spots instead of the total number of EGF. Again, if the average elevation was adopted as the judgment of the occurrence of response, the apparent Hill factor decreased from 3.1 to 2.1 in the same analysis. Cooperative reaction, such as dimerization-dependent activation of EGFR, is one of the typical reasons for ultrasensitivity (Koshland, 1998). The solid line in Fig. 4 *C* is proportional to the response probability as a function of the number of $(EGF/EGFR)_2$ complexes. Here, considering $(EGF/EGFR)_2$ as the individual unit, the effect of dimerization can be ignored. This reduction of the Hill factor demonstrates that dimerization of EGFR actually contributes to the switch-like response curve.

Binding of EGF to oligomers of EGFR

Even though the total number of EGF on the cell surface generally increased with the response probability, the

number of EGF that bound as monomers soon reached a plateau at ~100 molecules/cell (Fig. 4 *B*). This result can be explained if the binding of EGF to dimers (and oligomers) of EGFR is faster than to monomers. As the binding proceeded, EGFR clusters which had been bound with single Rh-EGF, and were counted in the monomer fraction, became bound with multiple Rh-EGFR molecules and thus were counted as oligomers.

The amount of Rh-EGF bound to cells was examined after a 20-s perfusion with various concentrations of Rh-EGF (Fig. 5 *A*). Since the incubation time and the concentration of ligand contribute equally in the association rate equations, the curve in Fig. 5 *A*, in which time was fixed and the concentration of ligand was varied, indicates the binding rate of EGF to cell-surface receptors. Making histograms of the fluorescence spot intensity of Rh-EGF at each concentration and fitting the histograms to a sum of four Gaussian functions (Fig. 5 *B*), the binding curve was resolved into four curves according to the cluster size of bound EGF (Fig. 5 *C*).

The obtained binding curves were fitted to a simple model including three association rate constants: one for binding to monomers (k_1), and the others for the first (k_2) and the second (k_3) bindings to dimers (Fig. 5 *D*), as follows.

$$L + R \xrightarrow{k_1} LR$$

$$2L + R_2 \xrightarrow{k_2} L + LR_2 \xrightarrow{k_3} L_2R_2.$$

Here L and R are the ligand (EGF) and receptor (EGFR). For simplification, trimers or tetramers of EGFR were thought to be composed of a single momomer and a single dimer or two dimers, respectively. Dissociation of EGF and rearrangement of EGFR clusters were neglected (Fig. 2, *D* and *E*). The binding process was represented by the differential equations

$$\frac{d}{dt}LR = k_1[L] \times R$$

$$\frac{d}{dt}R_2 = -k_2[L] \times R_2$$

$$\frac{d}{dt}LR_2 = k_2[L] \times R_2 - k_3[L] \times LR_2$$

$$\frac{d}{dt}L_2R_2 = k_3[L] \times LR_2.$$

$[L]$ is the concentration of ligand (EGF) and R, R_2, LR_2, and L_2R_2 are the numbers of each component. For this fitting, the total number of EGFR was fixed at 50,000 (Berkers et al., 1991).

The results of fitting gave $k_1 = 7.1 \times 10^5$/M/s for 49,000 molecules of EGFR per cell, $k_2 = 5.5 \times 10^7$/M/s, and $k_3 = 2.1 \times 10^8$/M/s for 890 molecules of EGFR per cell (Table 1). These results suggest that a very small population of EGFR with a large association rate constant to EGF was making predimers or clusters and was responsible for the sensitive calcium response.

It is probable that some of the Rh-EGF dimers were pseudodimers constructed by two monomers accidentally near each other. Although the probability of pseudodimer formation was small under our experimental conditions (<0.2 Rh-EGF/μm^2) and almost all fluorescent spots were moving around as individual spots, suggesting real oligomerization, it is still important to examine robustness of the above conclusions. The association rate constants and number of EGFR predimers were calculated assuming 5–15% of dimers were pseudodimers (Table 1). Most of the thus obtained parameters differed by <20% and never by more than a factor of 2 from the parameters calculated under the initial assumption of 0% puseudodimers, indicating that the conclusions are highly robust.

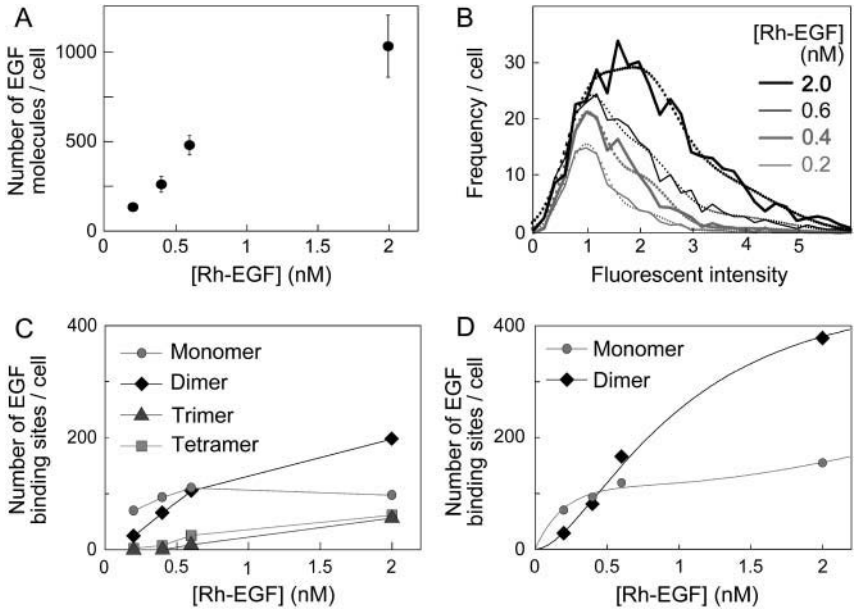

FIGURE 5 Binding of Rh-EGF. (*A*) The number of Rh-EGF on the cells plotted against the concentration in the perfusion medium. Each concentration of Rh-EGF was added to the cells for 20 s. The data are based on the same experiments as in Figs. 3 and 4. (*B*) Fluorescence intensity distributions of Rh-EGF spots on the cells. The cells were reclassified according to the concentration of the Rh-EGF solution, and the histograms for each group are shown. The results of fitting are shown by dotted lines. The multiple correlation coefficients between the data and fits were 0.981–0.993. (*C*) The numbers of EGF binding sites containing one to four Rh-EGF molecules estimated by the fits shown in *B*. Whereas the number of monomers reached a plateau, the number of oligomers increased with the Rh-EGF concentration. (*D*) Fitting to a binding model. Symbols are the same as in *C* but trimers and tetramers are resolved into monomers and dimers for simplification (see text). Lines indicate the results of fitting.

TABLE 1 Estimation of pseudodimer effects

Parameter	Pseudodimer fraction			
	0%	5%	10%	15%
k_1 ($\times 10^5$/M/s)	7.04 (1.00)	8.88 (1.26)	10.1 (1.43)	13.2 (1.88)
k_2 ($\times 10^7$/M/s)	5.48 (1.00)	5.69 (1.04)	5.57 (1.02)	6.56 (1.20)
k_3 ($\times 10^8$/M/s)	2.16 (1.00)	2.01 (0.93)	1.98 (0.92)	1.81 (0.84)
Predimers (per cell)	448 (1.00)	424 (0.95)	406 (0.91)	353 (0.79)

Fitting to the model shown in the text was carried out assuming 0–15% of Rh-EGF dimers in Fig. 5 *D* were pseudodimers constructed by two Rh-EGF accidentally bound to monomeric EGFRs existing nearby. See text for the meaning of each parameter. Numbers in parentheses are ratios with respect to the numbers at 0% pseudodimer.

DISCUSSION

In this study, we first determined the exact number of ligands required to induce cellular response using a single-molecule visualization technique. Binding of 300 or 320 EGF molecules induced the calcium response in half of the cells (Fig. 3 *C*). The number of EGF was quite small compared to the number of EGFR expressed on HeLa cells, 50,000/cell (Berkers et al., 1991). Most of the previous experiments were carried out in the continuous presence of EGF and an extracellular concentration of ~0.1 nM EGF is known to be the threshold to induce cellular responses (Shechter et al., 1978; Gotoh et al., 1995). Considering that the dissociation constant between EGF and EGFR is ~1 nM on average (Berkers et al., 1991), several thousands of EGF molecules bind to single HeLa cells at equilibrium in the presence of 0.1 nM EGF. In our experiment, cells responded to much smaller numbers of EGF than expected from the equilibrium binding. This result suggests the importance of the kinetic approach to study mechanisms of signal transduction. Wiley et al. (1989) calculated occupancy of EGFR from the amount of internalized EGF and the dissociation rate between EGF and EGFR and suggested that global affinity loss of EGFR is triggered by occupancy of <1% of the receptors. Here, we have developed a new direct technique to quantify small numbers of bound EGF molecules and demonstrated that cellular response is actually induced by only a few hundred ligand molecules.

The cell-to-cell fluctuations of the calcium response were very large (Fig. 3, *A* and *B*). This type of calcium response fluctuation has already been observed in several cell signaling systems (Hajjar and Bonventre, 1991; Toescu et al., 1993). However, in the previous experiments, it was not evident whether the fluctuation was caused by the fluctuation of the number of signaling molecules bound to each cell or by the fluctuation of the reaction on intracellular signaling systems. Here, by counting the exact number of the bound signaling molecules, it was clearly demonstrated that there were large cell-to-cell fluctuations of the intracellular signaling systems even at exactly the same amount of extracellular signals. These large fluctuations must include fluctuations of the calcium releasing processes from intracellular calcium stores and the calcium extrusion processes from the cytosol. In this study, to concentrate on the early events of EGF-induced calcium signaling, we mainly analyzed the occurrence of calcium elevation throughout cells.

The response probability of calcium signaling was correlated to the numbers of multiple-molecule spots of EGF better than to the number of spots including a single EGF molecule (Fig. 4). This result reflects that the dimerized EGFR relays the EGF signal to the intracellular signaling network, supporting the model of EGFR activation suggested by many previous authors, in which only dimers of EGFR transduce signals (Boni-Schnetzler and Pilch, 1987; Canals, 1992; Sorokin et al., 1994; Sherrill, 1997). In addition, there are several lines of recent evidence indicating that EGFR makes clusters even in the absence of EGF (Wofsy et al., 1992; Gadella and Jovin, 1995; Sako et al., 2000; Moriki et al., 2001; Yu et al., 2002). Therefore, it is thought that EGF signaling starts by conformational changes of predimers of EGFR or rearrangements of EGFR clusters, forming signaling dimers of EGFR consisting of two EGF molecules, each binding to a single EGFR (Gadella and Jovin, 1995; Moriki et al., 2001; Garrett et al., 2002; Ogiso et al., 2002). According to these models, the number of single-molecule spots should be subtracted from the number of EGF required to induce calcium signaling. In this case, the minimal requirement to induce calcium signaling is formation of 90 or 95 EGFR dimers (Fig. 4 *C*). These numbers are the upper limits because owing to the poor spatial resolution of light microscopy, some monomers in close proximity to each other could be counted as dimers in the above calculation. Such miscounting, however, seems to be rare due to the sparse EGF binding in our experimental conditions. Reorganization of receptor clusters was negligible during the time period of this experiment (Fig. 2 *E*).

The sigmoidal relationship between the number of EGF and the calcium response probability (Fig. 3 *C*) means that this reaction is ultrasensitive. The apparent Hill factor was decreased by plotting the response probability against the number of EGF in multiple-molecule spots instead of the total number of EGF (Fig. 4 *C*). This reduction demonstrates that dimerization of EGFR actually contributes to the switch-like response curve. Being a single-membrane spanning receptor, EGFR is indispensably required to form dimers to transduce signals from the outside to the inside of cells. Single-molecule analysis has added to our understanding of the positive role of the dimerization of EGFR, which causes an ultrasensitive response. The remaining ultrasensitivity (the apparent Hill factor of 2.4 or 2.1) suggests the presence of additional mechanisms to achieve a steep, switch-like response. Recently, we have found an amplification of EGF signaling. In the plasma membrane, unliganded EGFR was secondarily phosphorylated to make clusters of activated EGFR larger than dimers (Ichinose et al., 2004). This process can induce additional ultrasensitivity. Another candidate is the activation process of PLCγ through tyrosine phosphorylation

by EGFR. PLCγ has three tyrosine phosphorylation sites and multiple phosphorylation is needed for full activation (Kim et al., 1991). If the phosphorylation occurs in a multicollision mechanism, it will result in ultrasensitive production of IP_3 (Ferrell, 1996). Another possible mechanism is the Ca^{2+} sensitivity of IP_3R. The open probability of IP_3R is under a positive feedback control at submicromolar Ca^{2+} (Mak et al., 1998). If, by chance, multiple neighboring IP_3Rs open simultaneously, cooperative activation induces an ultrasensitive calcium response. On the other hand, calcium flux from a single IP_3R is thought to be too small to induce whole cell $[Ca^{2+}]_{in}$ elevation.

The ultrasensitive, switch-like property of EGF-induced intracellular calcium response could be important to suppress an erroneous response accidentally induced by very low concentrations of EGF or intrinsic noise of intracellular signaling systems. Otherwise, the positive feedback loop of calcium-induced calcium release will be out of control. On the other hand, at the threshold region of response, fluctuation of both the signal and intrinsic noise are amplified in ultrasensitive reactions (Shibata and Fujimoto, 2005). Though the importance of fluctuation in cell signaling systems is generally unknown, it could be used to generate variations of cell behavior. In fact, cell-to-cell variance of the average elevation of the calcium response normalized against the average at each region was largest at the threshold region (data not shown).

The estimated value of the association rate constant for binding to monomers ($k_1 = 7.1 \times 10^5$/M/s) is comparable to the association rate constant of the slow binding sites reported previously (Bellot et al., 1990; Berkers et al., 1991; Wilkinson et al., 2001). However, the rate constants for dimers ($k_2 = 5.5 \times 10^7$/M/s and $k_3 = 2.1 \times 10^8$/M/s, 890 molecules/cell) were larger than any other reported values for the fast binding sites. Also, though there has been no collective view of the molecular basis for the diversity of rate constants, single-molecule observation suggests that the fast binding site, which must be responsible for the fast and sensitive calcium response, is the oligomer of EGFR. The difference in the first (k_2) and the second (k_3) association rate for dimers suggests cooperative binding of two EGF molecules to EGFR dimers.

EGFR (ErbB1) heterodimerizes with ErbB2, a member of ErbB receptor family. The EGFR-ErbB2 heterodimer has a strong capacity to activate downstream signaling pathways (Goldman et al., 1990). Thus, ErbB2 plays an important role in the development of many types of human cancer (Holbro et al., 2003). ErbB2 is expressed on the surface of Hela cells. However, it is indicated that EGFR-ErbB2 heterodimerization does not alter the affinity between EGFR and EGF, and the number of the EGFR-ErbB2 heterodimers on the surface of HeLa cells is very much smaller than the number of EGFR homodimers (Johannessen et al., 2001). For these reasons, the heterodimer should have a small effect on the EGF binding on HeLa cells. Therefore, we analyzed the experimental results without considering the effect of heterodimers. Of course, it is possible that the EGF signals form a small number ($\sim$300) of receptors was amplified by heterodimers to induce calcium response downstream from the EGF binding events.

The results of this study indicate that EGFR clusters are essential to inducing intracellular calcium response by a small number of EGF molecules. EGF bound much faster to dimers (or clusters) of EGFR compared to monomers. This result is consistent with previous single-molecule observations, in which most of the EGF dimers were made through binding of two EGF molecules at the same binding site (Sako et al., 2000). However, considering the free energy balance of the two pathways that form signaling dimers of EGFR, one being the sequential binding of EGF to the predimers of EGFR, and the other being the diffusion and collision of two liganded receptors, the fast EGF binding to (and slow dissociation from) EGFR predimer should be compensated by the rare formation of predimers (Ichinose and Sako, 2004). Therefore, to prepare enough predimers, cells need to express excess amounts of EGFR. This may be the reason why a much larger number of EGFR (50,000/cell) is expressed on the cell surface than the number of EGF bonds required to induce calcium response (300/cell). Our results suggest that predimerization of EGFR is a feed-forward process of EGF signaling and that expression of excess amount of EGFR on the cell surface is a necessary expense for sensitive signal transduction.

Then, why do cells employ such a seemingly wasteful strategy? From the discussion above, dimerization of liganded EGFR by diffusion and collision should be more effective than the formation of predimer. However, this is not so, because this process depends on diffusion in the viscous plasma membrane and takes a long time at low concentrations of ligand. By preparing predimers or clusters of EGFR during the resting state, cells would achieve a rapid calcium response. One might ask why cells do not prepare a small number of receptors with intermediate rate constants and a much more effective predimer formation as an alternative strategy? In that case, the number of receptor molecules would have to be small, or signaling dimers, which require two EGF molecules, could not be formed at low concentrations of EGF. To regulate the expression of very small amounts of receptor seems to be difficult and causes unstable responses. Another advantage of this strategy is that it can have a wider dynamic range. Slow binding monomers can work at high concentrations of EGF or under prolonged EGF signaling, whereas small numbers of fast binding receptors will saturate immediately. These possibilities should be investigated in the future.

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