Analysis of Ligand-Receptor Interaction on the Surface of Living Cells by Fluorescence Correlation Spectroscopy

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Abstract: Ligand-receptor interaction on the cell surface has traditionally been analyzed by using radioisotope-labeled ligands. The reaction is carried out at low temperatures such as 4°C or on ice in order to prevent endocytosis of the ligand-receptor complex, followed by extensive washing to remove unbound ligands from cells. To overcome these drawbacks, in the present study we have employed fluorescence correlation spectroscopy (FCS) with single-molecule sensitivity for the analysis of the interaction of epidermal growth factor (EGF) with its receptor, EGFR, on the surface of living cells, and have successfully determined their dissociation constants. Moreover, FCS has also been used to determine the diffusion coefficient of EGFR in the membrane as well as to quantify the number of EGFR molecules expressed on the cell surface.

Keywords: Affinity, Diffusion coefficient, Dissociation constant, Epidermal growth factor receptor, FCS, Scatchard plot.

INTRODUCTION

Radioisotope-labeled peptide/hormone ligands have widely been used to analyze ligand-receptor interaction on the surface of living cells. This approach includes the binding reaction step at low temperatures such as 4°C or on ice to prevent endocytosis of the ligand-receptor complex, and requires several extensive washing steps at the low temperatures to remove unbound ligands. In certain cases, the half-life of the ligand-receptor complex may be often shorter or similar to the time required for the separation of free and bound ligands. Furthermore, the binding reaction at the low temperatures may not reflect physiological binding profiles at 37°C. Using fluorescently labeled ligands, FCS with single-molecule sensitivity has also been used to quantitatively analyze ligand-receptor interaction on the living cell surface by measuring fluorescence intensity fluctuations in the small confocal volume (~0.2 fL) [1-3]. Indeed, FCS has previously been applied for the determination of dissociation constants for EGF with EGFR with limited success [4].

EGFR regulates cell migration, proliferation and survival, and its aberrant activation is frequently implicated in many types of human cancers. The receptor molecule consists of an extracellular ligand binding domain, a single pass transmembrane α-helix and an intracellular tyrosine kinase domain followed by multiple auto-phosphorylation sites. The receptors on the cell surface have biphasic affinities for the ligand EGF; 80-98% with low (*Kd* = ~1-32 nM) and 2-20% with high (*Kd* = ~0.1-0.7 nM) affinities, depending upon experimental conditions and cell lines used [5-9]. The precise nature of the origin of the two affinities has yet to be determined [5, 6]. Ligand binding induces the receptor clustering and endocytosis in clathrin-coated pits on the cell surface, followed by either recycling back to the cell surface or moving through a series of endosomes to lysosome for degradation [10]. In the present study, we have successfully applied FCS for the determination of biphasic affinities, which are comparable to those determined using radioisotope-labeled ligands, for EGF with EGFR expressed on the living cell surface at 37°C without removing unbound ligands from cells. Using FCS, furthermore, we have also quantified the number of EGFR molecules expressed on the cell surface, and successfully determined the diffusion coefficient of EGFR in the membrane of living cells at 37°C.

MATERIALS AND METHODS

Chemicals

Rhodamine [tetramethylrhodamine-5-(and-6)-isothiocyanate]-labeled epidermal growth factor (Rh-EGF) was purchased from Invitrogen (Carlsbad, CA, USA). Unlabeled EGF was purchased from Austral biologicals (San Ramon, CA, USA). All other reagents are of standard grade.

Cell Culture

The human epidermoid carcinoma cell line A431 and Chinese hamster ovary (CHO-K1) cell line were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA), and cultivated in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Kyoto, Japan) and F-12 nutrient mixture (Ham's F12; Invitrogen), respectively. Both of the media were supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 100 U/mL of penicillin-streptomycin (Nacalai Tesque).

Plasmid Construction

The expression plasmid pEGFR-eGFP was constructed by inserting a PCR fragment encoding the full-length EGFR,

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which was amplified from the plasmid pNUT-EGFR [8], into the expression vector pEGFP-N1 (Clontech Laboratories, Mountain View, CA, USA), which was designed to produce an enhanced green fluorescent protein (eGFP) fusion protein at the carboxyl terminus of a partner protein. This construct was transfected into the CHO cell line, which is known not to express endogenous EGFR, and its stable cell line was established by selecting a single colony that emits eGFP fluorescence under a fluorescence stereomicroscope, and then by cultivating the cells in the presence of 0.8 mg/mL G418 (Nacalai Tesque).

Sample Preparation

A431 cells were cultivated in DMEM supplemented with 10% FBS in glass-bottom dishes (Lab-tek chambered coverglass, 8-well; Nalge Nunc International K.K., Tokyo, Japan). To inhibit endocytosis of EGFR upon EGF stimulation, the cells were incubated in culture media containing ATP synthesis inhibitors (10 mM NaN_3, 2 mM NaF and 5 mM 2-deoxy-D-glucose) at 37°C for 45 min as previously described [8, 11]. The cells were washed four times with 0.4 mL of Dulbecco's phosphate-buffered saline (DPBS; Invitrogen) and then once with 0.4 mL of Hanks' balanced salt solution (HBSS; Invitrogen). Then, the cells were incubated with Rh-EGF at various concentrations, which range from 3.29 nM to 224 nM at final concentrations, in HBSS containing ATP synthesis inhibitors for 15 min at room temperature.

Fluorescence Microscopy

Live cells were observed using an LSM 510 META inverted confocal laser microscope (Carl Zeiss, Jena, Germany) with a water immersion objective (C-Apochromat, 40 x, NA 1.2; Carl Zeiss) as previously described [12]. Rh-EGF was excited at 514 nm with an argon laser, and emission signals were detected through a long path filter that detects longer wavelength than 575 nm. eGFP was excited at 488 nm with an argon laser, and its emission at 505 nm was detected through a band path filter, BP505-550.

Fluorescence Correlation Spectroscopy

FCS measurements were carried out by using a ConfoCor 3 FCS microscope (Carl Zeiss) with a water immersion objective (C-Apochromat, 40 x, NA 1.2; Carl Zeiss). Confocal pinhole diameter was adjusted to 75 μm. Rh-EGF was excited at 514 nm with 1-3% laser power and its emission signals were detected through a dichroic mirror, HFT 405/514/633, and a long pass filter, LP580. eGFP was excited at 488 nm with 1% laser power and its emission signals were detected through a dichroic mirror, HFT 405/488/633, and a band pass filter, BP 505-540.

Data Analysis

Fluorescent correlation function, $G(\tau)$, was acquired online with Zeiss FCS software by using the following equation (1) based on one- or two-component model:

$$G(\tau)=\frac{\langle I(t)\cdot I(t+\tau)\rangle}{\langle I(t)\rangle^2}=1+\frac{1}{N}\sum_i F_i\left(1+\frac{\tau}{\tau_i}\right)^{-1}\left(1+\frac{\tau}{s^2\tau_i}\right)^{-\frac{1}{2}}\left(1+\frac{T_t e^{-\frac{\tau}{\tau_t}}}{1-T_t}\right) \quad (1)$$

where F_i and τ_i are the fraction and diffusion time of component i, respectively. N is the number of fluorescent molecules in the confocal volume defined by radius ω_0 and length $2z_0$. The structural parameter 's' and diffusion time 'τ_0' were determined by fitting the autocorrelation function of rhodamine 6G (Rh6G) based on one component model. T_t is the average fraction of triplet, and τ_t is the decay time of the triplet.

The molar concentrations of N_{free}, free ligands in solution, and N_{bound}, ligands bound to the cell-surface receptors, were calculated from the following equations (2) and (3), respectively:

$$N_{free}=\frac{N}{\pi\omega_0^2 2z_0 N_A} \quad (2)$$

$$N_{bound}=\frac{NF_{bound}n_{cell}}{\pi\omega_0^2 V_{well}N_A}S \quad (3)$$

where N_A is Avogadro's number, F_{bound} is the fraction of ligands bound to the cell-surface receptors. Autocorrelation functions are fitted by assuming two components, free ligands in solution and ligands bound to the cell-surface receptors, with two distinct, fast and slow, diffusion times. The S value is the average surface area, 588.8 μm^2 (n = 20) or 1206.4 μm^2 (n = 20), of CHO or A431 cells, respectively. n_{cell} is the average number of cells in a culture well, 6.20 x 10^5 cells (n = 3) or 2.89 x 10^5 cells (n = 3) , of CHO or A431 cells, respectively. V_{well} is the volume of HBSS buffer, 400 μL, in a culture well.

Dissociation constants for EGF with EGFR were determined from Scatchard plots of their binding profiles by using KELL programs (Biosoft, Cambridge, UK). Data are shown as mean ± SD.

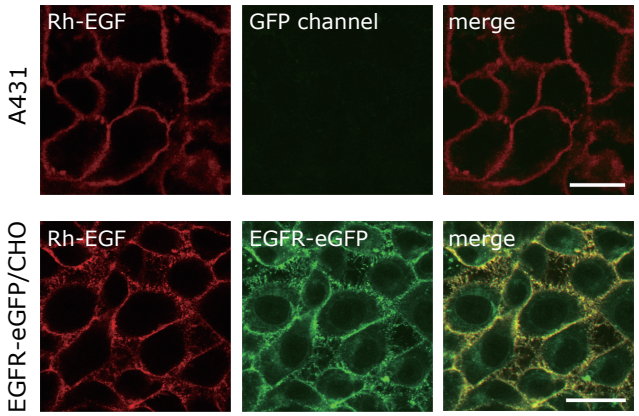

Fig. (1). Cell surface expression of EGFR. (Top) After incubating A431 cells with ATP synthesis inhibitors, Rh-EGF (16.5 nM, final concentration) was added to the culture. After being incubated for 15 min at room temperature, the cells were observed under a confocal microscope. (Bottom) After preincubation with ATP synthesis inhibitors, CHO cells expressing EGFR-eGFP were incubated with Rh-EGF, and were observed as described above.

RESULTS AND DISCUSSION

The human epidermoid carcinoma cell line A431 expresses EGFR at exceptionally high levels (>2 million molecules per cell), and has frequently been used for biochemical and biophysical analyses of EGFR. In the present

study, we also used A431 cells to analyze EGF-EGFR interaction by FCS. Confluent cultures of A431 cells were incubated with Rh-EGF, and were observed by scanning laser fluorescent confocal microscopy. As expected, Rh-EGF fluorescence was only detected on the cell surface (Fig. **1**). When Rh-EGF was incubated with the cultured cells in the presence of a large, 1,000-fold, excess of unlabeled EGF, this cell-surface fluorescence was not observed (data not shown), indicating the binding was specific. By FCS, we then observed free Rh-EGF in solution and Rh-EGF bound to EGFR on the surface of A431 cells. Autocorrelation functions of Rh-EGF in solution and bound to the A431 cell surface demonstrated that the diffusion time and coefficient of free Rh-EGF were 78.8 $\pm$ 4.1 μs and 94.3 $\pm$ 4.7 $\mu m^2/s$ (n = 14), respectively, and that those of receptor-bound Rh-EGF on the A431 cell surface were 41.9 $\pm$ 15.2 ms (n = 21) and 0.19 $\pm$ 0.06 $\mu m^2/s$ (n = 21), respectively (Fig. **2A**). From autocorrelation functions of Rh-EGF bound to CHO cells expressing EGFR-eGFP (Fig. **2B**), similarly, it was measured that the diffusion time and coefficient of EGFR-bound Rh-EGF were 61.2 $\pm$ 15.0 ms (n = 14) and 0.13 $\pm$ 0.03 $\mu m^2/s$ (n = 14), respectively. These diffusion time and coefficient of receptor-bound Rh-EGF are comparable to those of membrane proteins, which were previously published [11, 13-15].

We further carried out the analysis of EGF-EGFR binding on the surface of living A431 cells at different concentrations of EGF in order to assess whether or not the EGF binding to receptors on the cell surface is ligand-concentration dependent and saturable. As can be seen in Fig. (**3A**), increasing concentrations of EGF clearly lead to an increased binding and the binding was saturated at nanomolar concentrations of EGF (Fig. **3A**, insert). From the Scatchard plot of the binding isotherm, two distinct dissociation constants, 31.60 nM (88.7% of total receptors) and 0.48 nM (11.3% of total), were determined (Fig. **3A**). These dissociation constants are comparable to those previously determined by using radioisotope-labeled EGF [5-9].

By FCS, we also determined dissociation constants for EGF with EGFR expressed in CHO cells, which do not express endogenous EGFR. We analyzed interactions between Rh-EGF and EGFR-eGFP expressed on the CHO cell surface after having established stable cell lines. Increasing concentrations of EGF clearly lead to an increased binding and the binding was saturated at nanomolar concentrations of EGF (Fig. **3B**, insert). From the Scatchard plot of the binding isotherm, two dissociation constants, 31.27 nM (88.7% of total receptors) and 0.35 nM (11.3% of total), were determined; these values are comparable with those of EGFR on A431 cells and those previously published [5-9].

By FCS analysis of EGFR-eGFP, we also measured the number of EGFR-eGFP molecules, (1.01 $\pm$ 0.76) x 10^5 (n = 15) molecules per cell, expressed on the surface of CHO cells. This number of EGFR molecules on the cell surface is consistent with that, (1.70 $\pm$ 0.11) x 10^5 molecules per cell, determined by the Scatchard analysis of EGF-EGFR binding using Rh-EGF in the present study. Similarly, the diffusion coefficient, 0.32 $\pm$ 0.21 $\mu m^2/s$ (n = 15), of EGFR-eGFP was also determined to be comparable to those above measured from Rh-EGF-bound EGFR.

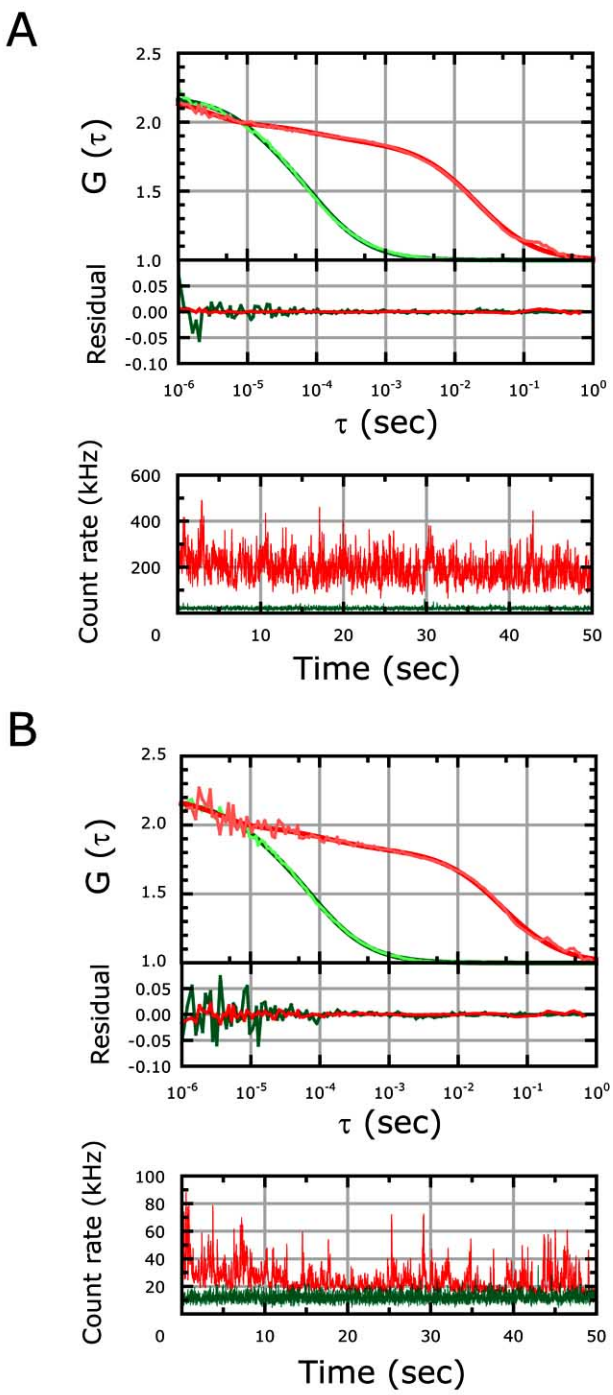

Fig. (2). Autocorrelation curves of bound Rh-EGF to cells and free Rh-EGF in culture media. (**A**) Autocorrelation curves of bound Rh-EGF to A431 cells and free Rh-EGF in culture media are shown in red and green, respectively, with their fits. (**B**) Autocorrelation curves of bound Rh-EGF to CHO cells expressing EGFR-eGFP, and free Rh-EGF in culture media are shown in red and green, respectively, with their fits.

Thus, in the present study we have successfully shown that FCS can be used to determine dissociation constants for EGF with EGFR expressed on the cell surface, and the diffusion coefficient of EGFR in the membrane. By FCS, we

also have successfully quantified the number of EGFR expressed on the cell surface using EGFR fused with eGFP. Experimental conditions developed in this study should be useful for other ligand and receptor interactions on the surface of living cells under physiological conditions without using radioisotope-labeled ligand.

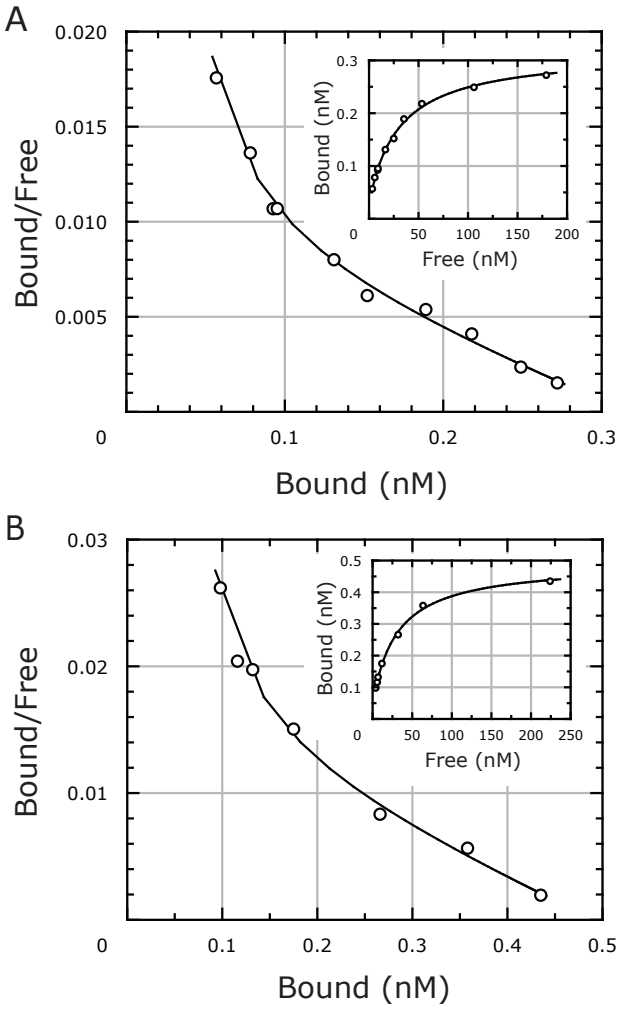

Fig. (3). Scatchard plots of Rh-EGF binding to EGFR expressed on the surface of living A431 cells (**A**) or CHO cells expressing EGFR-eGFP (**B**). After treated with ATP synthesis inhibitors, cells were incubated with Rh-EGF at various concentrations. For the Scatchard analysis, the number of free Rh-EGF in culture media and Rh-EGF bound to the cells was quantified by FCS. Rh-EGF binding curves were also shown in the inserts. The data points are the means of data obtained from at least 10 cells.

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