Ligand-Binding Enhances the Affinity of Dimerization of the Extracellular Domain of the Epidermal Growth Factor Receptor[1]

Masafumi Odaka,*[,2] Daisuke Kohda,*[,3] Irit Lax,[†] Joseph Schlessinger,[†] and Fuyuhiko Inagaki*[,4]

*Department of Molecular Physiology, The Tokyo Metropolitan Institute of Medical Science, Honkomagome 3-chome, Bunkyo-ku, Tokyo 113; and [†]Department of Pharmacology, New York University Medical Center, New York, NY 10016, USA

Received for publication, February 12, 1997

We studied the dimerization of the recombinant soluble extracellular domain of the epidermal growth factor receptor (sEGFR) in response to EGF-binding using multi-angle laser light scattering with size exclusion chromatography (SEC-MALLS). In the absence of EGF, sEGFR behaved as a monomer. However, upon EGF-binding, sEGFR formed a dimer with the stoichiometry of two EGF molecules bound to two sEGFR molecules [$(EGF)_2$-$(sEGFR)_2$]. We analyzed the chemical equilibrium of the dimer formation by SEC-MALLS using a dissociation constant of 0.25 μM for the binding of EGF to sEGFR. The calculated dissociation constant for EGF-induced sEGFR dimerization was found to be 2.4±0.9 μM. These experiments demonstrated that EGF induces receptor dimerization and that two EGF molecules are bound to an EGF-receptor dimer.

Key words: epidermal growth factor, epidermal growth factor receptor, ligand-induced dimerization, ligand-receptor binding stoichiometry, laser light scattering.

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 2, 2012

Ligand-induced dimerization is a universal phenomenon among receptor tyrosine kinases as well as many receptors for hormones and cytokines. Receptor dimerization plays an important role in transmembrane signaling by promoting interactions between the cytoplasmic domains of the dimerized receptors leading to stimulation of catalytic activity (*1, 2*). Moreover, specific tyrosine residues in the cytoplasmic domains are phosphorylated through an intermolecular mechanism. The resulting phosphorylated tyrosine residues serve as binding sites for molecules containing Src homology 2 domains (SH2), that transduce various intracellular signals (*3, 4*).

Epidermal growth factor (EGF) receptor was the first protein tyrosine kinase that was shown to undergo dimerization upon binding to its ligands, EGF and transforming growth factor α (*5*). EGF receptor is a 170-kDa glycoprotein that consists of two major functional domains, extracellular and cytoplasmic domains, connected by a single transmembrane region (*5, 6*). Ligand-induced dimerization of EGF receptor was studied in detail. Oligomerization in living cells and membrane preparations was revealed by means of rotational diffusion measurements (*7, 8*), the use of chemical cross-linking agents followed by SDS-polyacrylamide gel electrophoresis (PAGE) analysis (*9, 10*). Sucrose density gradient ultracentrifugation and non-denaturing PAGE experiments showed that detergent-solubilized EGF receptors partially formed non-covalent dimers in the absence of EGF and that the amounts of the dimers increased in response to binding of EGF (*11, 12*). However, the role of the extracellular domain of EGF receptor remains somewhat controversial. Two groups did not detect dimerization of the recombinant soluble extracellular domain of EGF receptor (sEGFR) on sucrose density gradient ultracentrifugation (*13, 14*), whereas others detected it on chemical cross-linking (*15, 16*) or sedimentation equilibrium analysis (*17*). We therefore examined the dimerization process of sEGFR in the presence of EGF using laser light scattering and determined the EGF/sEGFR binding stoichiometry in the dimer complex after EGF-binding. Furthermore, by means of a combination of laser light scattering with gel filtration chromatography, we determined the dissociation constant for ligand-induced dimerization of the soluble extracellular domain of EGF receptor.

MATERIALS AND METHODS

EGF and sEGFR—Recombinant human epidermal growth factor (EGF) was a gift from Wakunaga Pharmaceutical, Tokyo. Large scale preparation of the recombinant soluble extracellular domain of human epidermal growth factor receptor (sEGFR) was achieved in transformed CHO cells, and it was purified as described (*18*). The purified

[1] This work was supported by a grant from the Human Frontier Science Program.

Present addresses: [2]Biochemical Systems Laboratory, The Institute of Physical and Chemical Research (RIKEN), Hirosawa 2-1, Wako, Saitama 351-01; [3] Nuclear Magnetic Resonance Group, Biomolecular Engineering Research Institute, Furuedai 6-chome, Suita, Osaka 565.
[4] To whom correspondence should be addressed. Fax: +81-3-3823-1247, E-mail: inagaki@rinshoken.or.jp

Abbreviations: SH2, Src homology 2 domain; EGF, epidermal growth factor; sEGFR, the recombinant soluble extracellular domain of the epidermal growth factor receptor; SEC-MALLS, multi-angle laser light scattering with size exclusion chromatography; M_{app}, apparent molecular weight; M_w, weight-average molecular weight; GH, growth hormone; dn/dc, differential refractive index increment.

protein was stored at 4°C in 10 mM sodium phosphate buffer (pH 7.5) containing 0.05% sodium azide. A few days before the light scattering experiments, sEGFR was further purified by size exclusion chromatography (Superose 12, Pharmacia). The column was equilibrated with 50 mM sodium phosphate buffer (pH 6.8) containing 100 mM NaCl and 0.05% sodium azide (buffer A). The purified sEGFR was concentrated to 9.0 mg/ml with a Centriprep30 (Amicon), and stored at 4°C. The concentrations of EGF and sEGFR were determined on the basis of the following absorption coefficients: EGF, A_{280} (1.0 mg/ml, 1.0 cm) = 3.1; sEGFR, 0.74 (*13*). The molecular weight of sEGFR was estimated to be 85 kDa on the basis of the results in Fig. 3B.

Molecular Weight Determination by Multi-Angle Laser Light Scattering Measurement (SEC-MALLS)—The theoretical and detailed calculations used for determining molecular weights by light scattering were as described by Wyatt (*19*). Measurements were made with a system composed of a multi-angle laser light scattering photometer (DAWN-F; Wyatt Technology, USA) and a differential refractive index detector (Shodex RI SE-61; Showa Denko, Tokyo) connected to a gel filtration HPLC column (Shodex KW-803; Showa Denko) equilibrated with buffer A. The flow rate was 1.0 ml/min. Samples (0.2 ml) were applied to the column. The solute flowed into the cell of the light scattering detector, in which light scattering was measured almost simultaneously at 10 angles, and subsequently into the refractive index detector. Data collection and processing were generally under the control of a computer driven by the software program, ASTRA (Wyatt Technology, USA). Data were collected at one-second intervals, corresponding to a slice volume of 16.7 μl. The differential refractive index increment (dn/dc) of sEGFR was assumed to coincide with that of bovine serum albumin (0.180) (Wyatt, P.J., personal communication), and to be identical in both the presence and absence of EGF. The protein concentration in each slice was calculated from the refractive index. The apparent molecular weight (M^i_{app}) of the solute in each slice was calculated by ASTRA from the scattered light intensity data and the refractive index. The weight-average molecular weight (M_W) of a peak was calculated by use of the following equation,

$$\text{Weight-average molecular weight: } M_W = \sum_i (c_i M^i_{app}) / \sum_i c_i$$

where M_W is the weight average molecular weight, and M^i_{app} and c_i are the apparent molecular weight and concentration of the solute at slice i, respectively. The solute concentration and M_W of the plateau region in Fig. 3 were determined by the same procedure as described above.

Size Exclusion HPLC—sEGFR (44 μM) was mixed with EGF in a ratio of 1:0.6 or 1:1. After incubation for over 10 min at room temperature, 0.2 ml of the mixture was applied to a gel filtration column (Shodex KW-803), connected to a Tosoh SC8010 chromatograph. The column was equilibrated with buffer A, the flow rate being 1.0 ml/min. Elution was monitored at 298 nm. From 8.0 to 9.4 ml, the eluant was collected in 0.2 ml fractions.

Reversed-Phase HPLC—The amounts of EGF and sEGFR in the fractions were determined on a reversed-phase HPLC column, Senshupak VP-304-1251 (4.6 mm × 250 mm; Senshu, Tokyo), connected to a Tosoh SC8010 chromatograph. Solvent A comprised 0.1% trifluoroacetic acid containing 1% acetonitrile, and solvent B comprised 0.1% trifluoroacetic acid containing 80% acetonitrile. A linear gradient of 0 to 100% of solvent B was run over a period of 20 min at the flow rate of 1.0 ml/min. Fifty microliters of each fraction was diluted with 950 μl of solvent A. After more than 20 min incubation at room temperature, it was applied to the column. EGF was eluted at 13.8 min, while sEGFR was eluted as three peaks at 18.5, 18.8, and 19.3 min. This would be because of the heterogeneity of the oligosaccarides bound to sEGFR. The amounts of EGF and sEGFR were determined from the respective calibration curves.

RESULTS

Determination of the EGF/sEGFR Binding Stoichiometry—Figure 1 shows the results of analyses of EGF-induced sEGFR dimerization by multi-angle laser light scattering combined with size exclusion HPLC (SEC-MALLS). When sEGFR (44 μM) was subjected to SEC-MALLS, it was eluted as a single peak at 8.4 ml. The apparent molecular weight (M_{app}) of the solute was similar and the weight-average molecular weight (M_W) of the peak was about 82 kDa (Fig. 1A), indicating that in the absence of EGF, almost all sEGFR molecules behave as monomers.

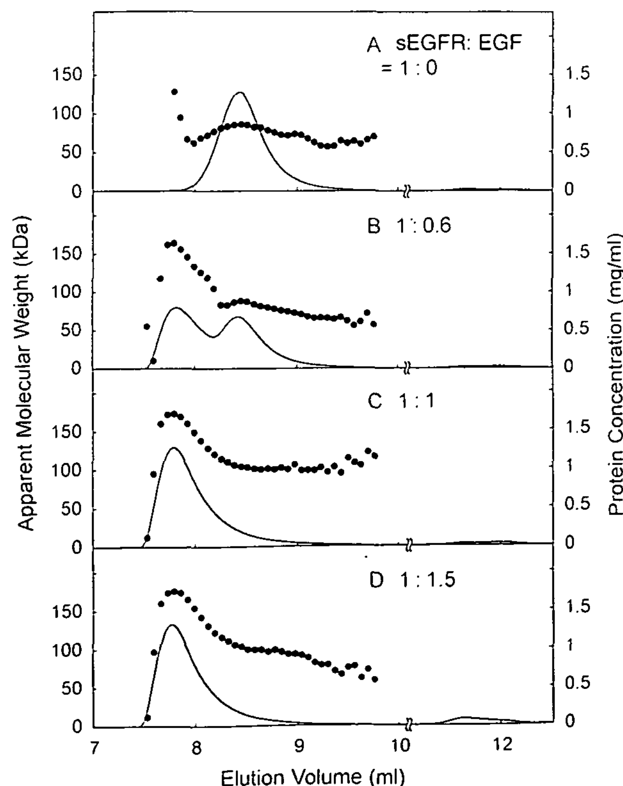


Fig. 1. **Analysis of sEGFR dimerization in the absence or presence of EGF by SEC-MALLS.** sEGFR (44 μM) was mixed with EGF at ratios of: A, 1:0; B, 1:0.6; C, 1:1; and D, 1:1.5. After incubation for over 10 min at room temperature, 0.2 ml of each mixture was subjected to SEC-MALLS. The solid lines are the elution profiles recorded with a differential refractive index detector (right scale). The closed circles show the M^i_{app} profile (left scale). To match the data from the two detectors, the elution volumes were corrected with the software program, ASTRA, to omit the dead volume.

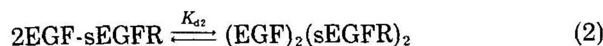
Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 2, 2012

This value is 15-20% smaller than M_{app} estimated on SDS-PAGE (*15, 18*). This lower value could be due to the differential refractive index increment (*dn/dc*) of bovine serum albumin (0.180) that was used for the calculation. This value would be larger than that of sEGFR because the latter is highly glycosylated (*15*), and because the *dn/dc* value of the carbohydrate moiety of sEGFR may be smaller than that of the protein moiety (*20*). We next added EGF to sEGFR (44 μM) in molar ratios of 1:0.6, 1:1, and 1:1.5 for 10 min, and then analyzed samples by SEC-MALLS (Fig. 1, B-D). It was previously reported that the dissociation constant (K_d) of EGF binding to sEGFR is 0.25 μM (*15*). Therefore, under these conditions more than 98% of the added ligand is bound to sEGFR. At a ratio of sEGFR to EGF of 1:0.6, the mixture was eluted as two peaks at 7.8 and 8.4 ml. M_{app} of the solute at the 7.8 ml peak was about 165 kDa, followed by a gradual decrease with a shoulder around 8.1 ml of about 120 kDa, finally being reached about 83 kDa (Fig. 1B). The decrease in M_{app} from 165 to 83 kDa is due to a change in the monomer-dimer stoichiometry. The area under the dimer peak was about 50% of the total area, suggesting that the sEGFR bound with EGF formed the dimer, while most of the ligand-free sEGFR was eluted as the monomer. Similar results were obtained when the incubation with EGF was extended for 30 min (data not shown), demonstrating that the equilibrium was already achieved after 10 min incubation. At a ratio of sEGFR to EGF of 1:1, most sEGFR was eluted as a single peak at 7.8 ml, followed by a tail in the curve (Fig. 1C). The elution profile of sEGFR incubated with EGF at a ratio of 1:1.5 was identical to that of a 1:1 sEGFR:EGF mixture, except that the excess EGF was eluted in the total column volume (11.7 ml) in addition to a small amount of impurities (Fig. 1D). Under the conditions in Fig. 1, C and D, M_{app} of the 7.8 ml peak was about 170 kDa, and then it decreased gradually to 90-100 kDa, indicating that most sEGFR was eluted as the dimer and that a small fraction of sEGFR was dissociated to form monomers, probably because of dilution during chromatography. Taken together, these results indicate that EGF-induced dimerization is mediated through the association of the EGF-sEGFR complexes.

To confirm the stoichiometry of sEGFR to EGF in the dimer peak, sEGFR (44 μM) incubated with EGF at ratios of 1:0.6 and 1:1 was applied to the same column equilibrated under the same conditions. Fractions of 0.2 ml were collected and then analyzed by reversed-phase HPLC. Figure 2 shows the ratio of EGF/sEGFR in each fraction. For the 1:0.6 (sEGFR:EGF) mixture, the molar ratio of EGF to sEGFR in the dimer peak (from 8.0 to 8.6 ml) was about 0.97. Thereafter, it gradually decreased, reaching 0.2 in the monomer peak (from 8.8 to 9.6 ml) (Fig. 2A). When sEGFR was incubated with an equivalent molar concentration of EGF, the molar ratio in the dimer peak was about 1.0 (Fig. 2B). For the 1:1 (sEGFR:EGF) mixture, almost all the EGF was eluted with sEGFR over the whole peak including the tail region at a concentration as low as 1.3 μM (Fig. 2B). These results indicate that the EGF/sEGFR binding stoichiometry in the peak corresponding to the dimer is 1:1. We, therefore, conclude that sEGFR forms a dimer only when occupied by EGF.

Equilibrium of Dimerization of the EGF-sEGFR Complex—Assuming an EGF/sEGFR binding stoichiometry of 1.0 in the dimer, the equilibria can be represented as:

$$\mathrm{EGF} + \mathrm{sEGFR} \underset{}{\overset{K_{d1}}{\rightleftharpoons}} \mathrm{EGF\text{-}sEGFR} \quad (1)$$



$$2\mathrm{EGF\text{-}sEGFR} \overset{K_{d2}}{\rightleftharpoons} (\mathrm{EGF})_2(\mathrm{sEGFR})_2 \quad (2)$$

$$K_{d1} = [\mathrm{EGF}][\mathrm{sEGFR}]/[\mathrm{EGF\text{-}sEGFR}] = 0.25\ \mu\mathrm{M} \quad (3)$$

$$K_{d2} = [\mathrm{EGF\text{-}sEGFR}]^2/[(\mathrm{EGF})_2(\mathrm{sEGFR})_2] \quad (4)$$

$$[\mathrm{sEGFR}]_{total} = [\mathrm{sEGFR}] + [\mathrm{EGF\text{-}sEGFR}] + 2[(\mathrm{EGF})_2(\mathrm{sEGFR})_2] \quad (5)$$

Therefore, if we determine the total concentration of sEGFR ($[\mathrm{sEGFR}]_{total}$) in the dimer peak, the dissociation constant, K_{d2}, for the dimerization of the EGF-sEGFR complex can be determined from the dependence of M_w of the dimer peak on the receptor concentration. In the SEC-MALLS experiment, sEGFR was diluted when passed through the gel filtration column, and its concentration was therefore constantly changing. The batch method seems to be preferable for determination of the monomer-dimer equilibrium of sEGFR. However, because of the inherent experimental difficulty in light scattering experiments due to the presence of impurities and small bubbles in the sample solution, batch experiments are difficult to perform. In SEC-MALLS, the gel filtration column is essential since it functions not only as a size exclusion device, but also as a filter that reduces impurities and eliminates small bubbles. To overcome the dilution problem, we subjected a large sample volume to SEC-MALLS. When 7.0 ml of sEGFR (4.6 μM) was subjected, it was eluted as a plateau region from 9.0 to 11.5 ml (Fig. 3A). The protein concen-

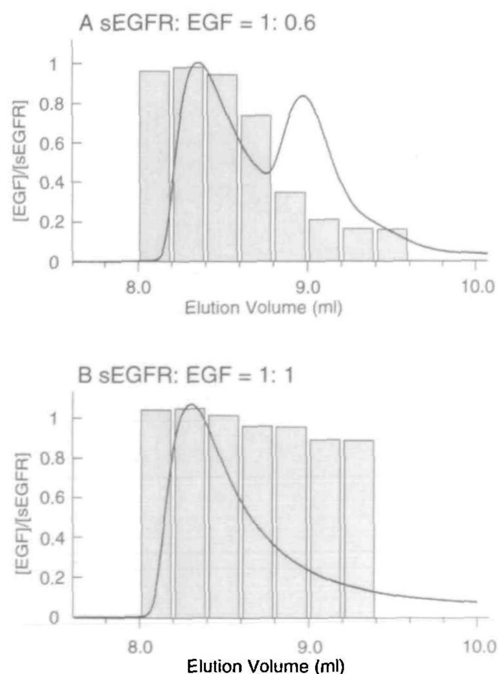


Fig. 2. **Stoichiometry of EGF/sEGFR in the dimer and monomer peaks.** sEGFR (44 μM) was mixed with EGF at ratios of: A, 1: 0.6; and B, 1:1. The solid lines are the elution profiles recorded with a UV monitor at 298 nm and are shown on an arbitrary scale. The bar charts indicate the stoichiometry of EGF/sEGFR in each fraction (left scale). In A, each bar represents the average of three determinations. The details are given under "MATERIALS AND METHODS."

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 2, 2012

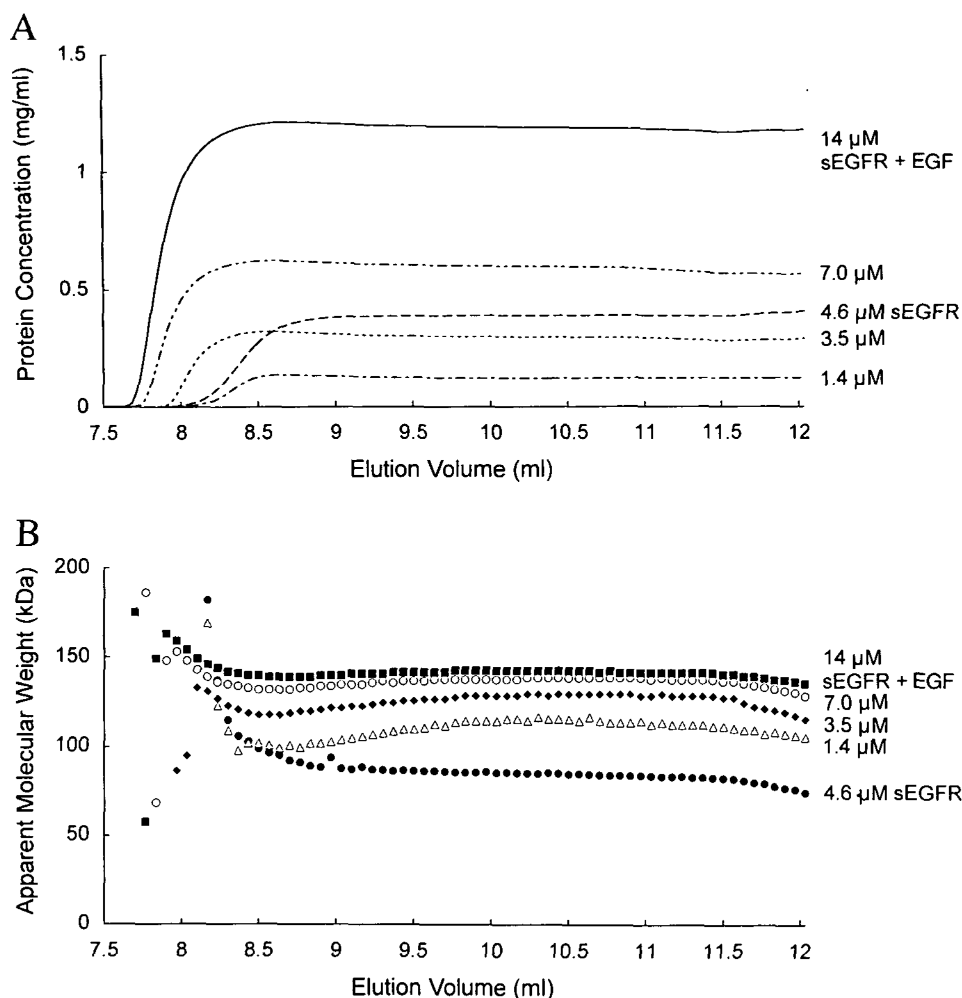


Fig. 3. **Elution profiles and profiles of M^i_{app} of sEGFR incubated with EGF at various concentrations in the plateau region.** A 1:1.1 (sEGFR:EGF) mixture incubated for over 10 min at room temperature was subjected to SEC-MALLS after degassing by sonication. A: Elution profile of sEGFR at each concentration in the plateau region recorded with a differential refractive index detector. The elution profile of 4.6 μM sEGFR in the absence of EGF is indicated as ---, and that of sEGFR incubated with EGF at each $[\text{sEGFR}]_{total}$ is shown as follows; 1.4 μM, —-—; 3.5 μM, ·····; 7.0 μM, ——-; and 14 μM —. B: M^i_{app} profiles in the plateau regions. The closed circles indicate the M^i_{app} values of 4.6 μM sEGFR in the absence of EGF. Open triangles, closed lozenges, open circles, and closed squares indicate those of 1.4, 3.5, 7.0, and 14 μM sEGFR incubated with EGF, respectively.

tration in this region was only <3% lower than that initially applied. M_{app} of ligand-free sEGFR in the plateau region (from 9.0 to 11.5 ml) was almost constant, and M_w in this region was 85 kDa (Fig. 3B). This value is in good agreement with that in Fig. 1A. Regarding the EGF-sEGFR complex, sEGFR was incubated with EGF at a ratio of 1:1.1 prior to the SEC-MALLS. Excess EGF was eluted at 11.7 ml (Fig. 1D) and thus was disregarded on analysis of the plateau regions. When 7.0 ml aliquots of the 1:1.1 (sEGFR:EGF) mixtures of various concentrations were subjected to SEC-MALLS, they gave plateau regions in the range of 8.2-8.5 to 11.5 ml, where their protein concentrations were almost identical to those initially applied (Fig. 3A). When the concentration of sEGFR subjected was 1.4 μM, M_{app} of the solute in the plateau region fluctuated because the lower limit of the protein concentration in MALLS was about 0.1 mg/ml. Along with the increase in $[\text{sEGFR}]_{total}$, M_{app} of the plateau increased (Fig. 3B). When the concentration of sEGFR was below 7.0 μM, M_{app} of the initial phase of the plateau region was slightly lower, but after 9.7 ml, it exhibited a constant value. It is possible that a small amount of sEGFR remains as an unoccupied monomer at the initial phase of the plateau region. Considering the effect of dilution, the average protein concentration and M_w were calculated in the plateau region (from 9.5 to 11.5 ml) for each sample solution. Figure 4 shows a plot of M_w as a

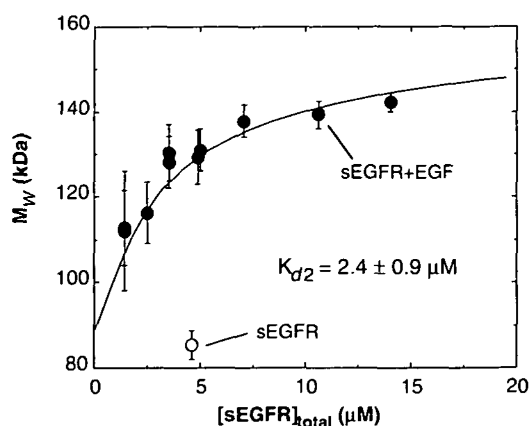


Fig. 4. **M_w of sEGFR incubated with EGF in the plateau region as a function of $[\text{sEGFR}]_{total}$.** ○, sEGFR in the absence of EGF; ●, sEGFR incubated with EGF in a ratio of 1:1.1. From the data in Fig. 3, the average protein concentration and M_w of sEGFR in the plateau region (9.5-11.5 ml) were calculated. The fitting parameters were as follows: $K_{d1}=0.25$ μM; and $K_{d2}=2.4$ μM. The points are experimental and the solid line corresponds to the best-fit curve with the least-squares method. The error bars indicate the M^i_{app} values of slices that differed the most from M_w.

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 2, 2012

function of $[\text{sEGFR}]_{\text{total}}$ in the plateau region of the curve. The results demonstrate that the increase in M_w is correlated with the increase in $[\text{sEGFR}]_{\text{total}}$ in the plateau region of the curve. M_w converged at about 170 kDa, confirming that EGF indeed induced sEGFR dimerization and not the formation of higher oligomerization states.

When the data in Fig. 4 were fitted using Eqs. 3 and 4 by the least mean squares method, K_d for EGF-induced dimerization of sEGFR (K_{d2}) was found to be $2.4 \pm 0.9\ \mu\text{M}$. As shown in Fig. 4, the theoretical curve calculated for $K_{d1} = 0.25\ \mu\text{M}$ and for $K_{d2} = 2.4\ \mu\text{M}$ fits the experimental data well.

DISCUSSION

Ligand-induced dimerization is thought to be a key step in the activation of the EGF receptor as well as other receptor tyrosine kinases (*1, 2*). Although the mechanism underlying this process is not fully understood, it was proposed that the EGF receptor exists in equilibrium between monomeric and dimeric forms, and that ligand-binding induces a shift in the equilibrium toward dimer formation (*12, 21, 22*). To evaluate this model, we have studied the dimerization process of sEGFR by means of SEC-MALLS. We have obtained milligram quantities of sEGFR, allowing detailed studies using biophysical approaches (*15*). The dissociation constant, K_{d1}, for EGF binding to sEGFR is $0.25\ \mu\text{M}$, this K_d being about 5-fold higher than that reported for the detergent-solubilized full-length EGF receptor (*15*). The experiments presented in Figs. 3 and 4 clearly demonstrate that sEGFR reversibly oligomerizes to form the dimer but not higher oligomers in response to EGF-binding. The dissociation constant, K_{d2}, for the dimerization of sEGFR in the presence of EGF was found to be $2.4 \pm 0.9\ \mu\text{M}$. However, sEGFR did not dimerize in the absence of EGF under the experimental condition used. Even at a concentration of $44\ \mu\text{M}$, which is 18 times higher than K_{d2}, sEGFR still behaved as a monomer (Fig. 1A). Chemical cross-linking agents showed that in the absence of EGF, a negligibly small amount of sEGFR dimerized at a concentration of $170\ \mu\text{M}$ (*17*). EGF-binding would enhance the affinity for the sEGFR dimerization by over 100-fold. This view is consistent with the results of previous studies performed with sEGFR and the detergent-solubilized EGF receptor (*11, 16, 21, 23-25*).

The K_{d2}, $2.4 \pm 0.9\ \mu\text{M}$, allows a rational explanation for the inconsistencies reported in some of the previous papers on the sEGFR dimerization. Ligand-induced sEGFR dimerization has been demonstrated by chemical covalent cross-linking analysis (*15-17, 23*) and sedimentation equilibrium analyses (*17*) but not by sucrose density gradient ultracentrifugation (*13, 14*). The total concentrations of sEGFR used in these studies were 5-30 μM (*15, 16, 23*), 11 μM (*17*), and 0.1-1 μM (*13, 14*), respectively. Thus, the concentrations of sEGFR in the first two studies were sufficient for sEGFR to dimerize after ligand-binding, whereas those in the latter were not. Sedimentation equilibrium showed that about 40% of sEGFR was present as a dimer at equilibrium when sEGFR (11 μM) was mixed with EGF at a ratio of 1:2 (sEGFR:EGF) (*17*). K_{d2} estimated in this experiment was approximately 1 μM, which is in good agreement with the K_{d2} obtained in this study.

Determination of the EGF/EGF receptor binding stoichiometry is crucial for understanding how EGF induces EGF receptor dimerization and activation. This has been estimated to be one EGF binding site/EGF receptor by means of radioimmunoassaying and immune precipitation (*26*). Non-denaturing PAGE analysis has also shown a binding stoichiometry of 1:1 (*17*). However, the mode of interaction between EGF and the extracellular domain of the EGF receptor within the dimer is not known. Like human growth hormone (hGH), which contains two functionally distinct sites for binding to the hGH receptor to form an $(\text{hGH})(\text{GH receptor})_2$ complex (*27, 28*), the possibility of $(\text{EGF})(\text{sEGFR})_2$ dimer formation cannot be ruled out. Experiments described in this report demonstrated that EGF-induced dimerization of sEGFR is mediated by EGF-sEGFR complexes and that the binding stoichiometry in the dimer is 1:1. Recently, the kinetics of the activation and dimerization of detergent-solubilized EGF receptor by EGF were analyzed in detail (*25*). On comparison of the experimental and theoretical data, it was concluded that the tyrosine kinase active receptor is the dimeric EGF receptor, which is occupied by two EGF molecules.

SEC-MALLS is a useful tool for determination of the molecular weight or stoichiometry of a ligand-receptor complex (*29-32*). However, because of dilution in the gel filtration column, it is impossible to estimate the concentration of the complex using this technique. One way to overcome this problem is to elute the samples at the plateau region of the curve. The self-association of human hemoglobin has been analyzed in this manner (*33*). In the present study, we combined SEC-MALLS with this procedure to analyze the equilibrium of EGF-sEGFR dimerization. Using this approach, we have determined for the first time the affinity of dimerization of sEGFR after EGF-binding. The method presented here is a novel approach for measuring the stoichiometry and equilibrium of other reversible self-associating systems.

Recently, Lemmon *et al.* (*34*) analyzed the binding of EGF to sEGFR using isothermal titration calorimetry and small angle X-ray scattering. On the basis of the results of these experiments it was concluded that EGF receptor dimerization results from bivalent binding of two EGF molecules in a complex containing two EGF and two sEGFR molecules. It is noteworthy that this model provides a potential mechanism for understanding how different members of the EGF family induce heterodimerization and activation of the EGF receptor family of receptor tyrosine kinases (*1*). We analyzed the SEC-MALLS data based on a receptor-mediated receptor dimerization model, where dimerization is mediated by receptor-receptor contacts stabilized through ligand-induced conformational changes. Our results are consistent with those reported by Lemmon *et al.* (*34*), since both models cannot be distinguished from each other in a case where the concentration of (EGF) $(\text{sEGFR})_2$ is small.

In conclusion, we studied the EGF-induced dimerization of sEGFR using SEC-MALLS. We have demonstrated that EGF-binding significantly enhances the association between EGF-sEGFR complexes forming a $(\text{EGF})_2(\text{sEGFR})_2$ complex. The association is reversible and the dissociation constant for dimerization was calculated to be $2.4 \pm 0.9\ \mu\text{M}$. Elucidation of the mechanism underlying ligand-induced receptor dimerization requires additional struc-

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 2, 2012

tural analysis. However, this study showed that the extracellular domain plays a critical role in stabilizing the formation of receptor dimers after ligand-binding.

The helpful support and suggestions of Drs. S. Nagashima of RIKEN, and H. Hatanaka of the Tokyo Metropolitan Institute of Medical Science are gratefully acknowledged. We also thank Dr. T. Fuwa of Wakunaga Pharmaceutical Co. for providing the EGF for these experiments. We also thank Mrs. T. Matsuzaki and M. Nakamura of Shoko Co., Ltd. for their skillful advice and technical assistance in the SEC-MALLS experiments.

REFERENCES

1. Lemmon, M.A. and Schlessinger, J. (1994) Regulation of signal transduction and signal diversity by receptor oligomerization. *Trends Biochem. Sci.* **19**, 459-463
2. Heldin, C.H. (1995) Dimerization of cell surface receptors in signal transduction. *Cell* **80**, 213-223
3. Pawson, T. and Schlessinger, J. (1993) SH2 and SH3 domains. *Curr. Biol.* **3**, 434-442
4. Cohen, G.B., Ren, R., and Baltimore, D. (1995) Modular binding domains in signal transduction proteins. *Cell* **80**, 237-248
5. Ullrich, A. and Schlessinger, J. (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203-212
6. Carpenter, G. and Cohen, S. (1990) Epidermal growth factor. *J. Biol. Chem.* **265**, 7709-7712
7. Zidovetzki, R., Yarden, Y., Schlessinger, J., and Jovin, T.M. (1981) Rotational diffusion of epidermal growth factor complexed to cell surface receptors reflects rapid microaggregation and endocytosis of occupied receptors. *Proc. Natl. Acad. Sci. USA* **78**, 6981-6985
8. Zidovetzki, R., Yarden, Y., Schlessinger, J., and Jovin, T.M. (1986) Microaggregation of hormone-occupied epidermal growth factor receptors on plasma membrane preparations. *EMBO J.* **5**, 247-250
9. Fanger, B.O., Austin, K.S., Earp, H.S., and Cidlowski, J.A. (1986) Cross-linking of epidermal growth factor receptors in intact cells: detection of initial stages of receptor clustering and determination of molecular weight of high-affinity receptors. *Biochemistry* **25**, 6414-6420
10. Cochet, C., Kashles, O., Chambaz, E.M., Borrello, I., King, C.R., and Schlessinger, J. (1988) Demonstration of epidermal growth factor-induced receptor dimerization in living cells using a chemical covalent cross-linking agent. *J. Biol. Chem.* **263**, 3290-3295
11. Böni-Schnetzler, M. and Pilch, P.F. (1987) Mechanism of epidermal growth factor receptor autophosphorylation and high-affinity binding. *Proc. Natl. Acad. Sci. USA* **84**, 7832-7836
12. Yarden, Y. and Schlessinger, J. (1987) Epidermal growth factor induces rapid, reversible aggregation of the purified epidermal growth factor receptor. *Biochemistry* **26**, 1443-1451
13. Greenfield, C., Hiles, I., Waterfield, M.D., Federwisch, M., Wollmer, A., Blundell, T.L., and McDonald, N. (1989) Epidermal growth factor binding induces a conformational change in the external domain of its receptor. *EMBO J.* **8**, 4115-4123
14. Günther, N., Betzel, C., and Weber, W. (1990) The secreted form of the epidermal growth factor receptor. Characterization and crystallization of the receptor-ligand complex. *J. Biol. Chem.* **265**, 22082-22085
15. Lax, I., Mitra, A.K., Ravera, C., Hurwitz, D.R., Rubinstein, M., Ullrich, A., Stroud, R.M., and Schlessinger, J. (1991) Epidermal growth factor (EGF) induces oligomerization of soluble, extracellular, ligand-binding domain of EGF receptor. A low resolution projection structure of the ligand-binding domain. *J. Biol. Chem.* **266**, 13828-13833
16. Hurwitz, D.R., Emanuel, S.L., Nathan, M.H., Sarver, N., Ullrich, A., Felder, S., Lax, I., and Schlessinger, J. (1991) EGF induces increased ligand binding affinity and dimerization of soluble epidermal growth factor (EGF) receptor extracellular domain. *J. Biol. Chem.* **266**, 22035-22043
17. Brown, P.M., Debanne, M.T., Grothe, S., Bergsma, D., Caron, M., Kay, C., and O'connor-Mccourt, M.D. (1994) The extracellular domain of the epidermal growth factor receptor. Studies on the affinity and stoichiometry of binding, receptor dimerization and a binding-domain mutant. *Eur. J. Biochem.* **225**, 223-233
18. Kohda, D., Odaka, M., Lax, I., Kawasaki, H., Suzuki, K., Ullrich, A., Schlessinger, J., and Inagaki, F. (1993) A 40-kDa epidermal growth factor/transforming growth factor alpha-binding domain produced by limited proteolysis of the extracellular domain of the epidermal growth factor receptor. *J. Biol. Chem.* **268**, 1976-1981
19. Wyatt, P.J. (1993) Light scattering and the absolute characterization of macromolecules. *Anal. Chim. Acta* **272**, 1-40
20. Arakawa, T., Langley, K.E., Kameyama, K., and Takagi, T. (1992) Molecular weights of glycosylated and nonglycosylated forms of recombinant human stem cell factor determined by low-angle laser light scattering. *Anal. Biochem.* **203**, 53-57
21. Yarden, Y. and Schlessinger, J. (1987) Self-phosphorylation of epidermal growth factor receptor: evidence for a model of intermolecular allosteric activation. *Biochemistry* **26**, 1434-1442
22. Schlessinger, J. (1988) Signal transduction by allosteric receptor oligomerization. *Trends Biochem. Sci.* **13**, 443-447
23. Zhou, M., Felder, S., Rubinstein, M., Hurwitz, D.R., Ullrich, A., Lax, I., and Schlessinger, J. (1993) Real-time measurements of kinetics of EGF binding to soluble EGF receptor monomers and dimers support the dimerization model for receptor activation. *Biochemistry* **32**, 8193-8198
24. Sororkin, A., Lemmon, M.A., Ullrich, A., and Schlessinger, J. (1994) Stabilization of an active dimeric form of the epidermal growth factor receptor by introduction of an inter-receptor disulfide bond. *J. Biol. Chem.* **269**, 9752-9759
25. Sherrill, J.M. and Kyte, J. (1996) Activation of epidermal growth factor receptor by epidermal growth factor. *Biochemistry* **35**, 5705-5718
26. Weber, W., Bertics, P.J., and Gill, G.N. (1984) Immunoaffinity purification of the epidermal growth factor receptor. Stoichiometry of binding and kinetics of self-phosphorylation. *J. Biol. Chem.* **259**, 14631-14636
27. Cunningham, B.C., Ultsch, M., de Vos, A.M., Mulkerrin, M.G., Clauser, K.R., and Wells, J.A. (1991) Dimerization of the extracellular domain of the human growth hormone receptor by a single hormone molecule. *Science* **254**, 821-825
28. de Vos, A.M., Ultsch, M., and Kossiakoff, A.A. (1992) Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. *Science* **255**, 306-312
29. Philo, J., Talvenheimo, J., Wen, J., Rosenfeld, R., Welcher, A., and Arakawa, T. (1994) Interactions of neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF), and the NT-3•BDNF heterodimer with the extracellular domains of the TrkB and TrkC receptors. *J. Biol. Chem.* **269**, 27840-27846
30. Philo, J.S., Aoki, K.H., Arakawa, T., Nahri, L.O., and Wen, J. (1996) Dimerization of the extracellular domain of the erythropoietin (EPO) receptor by EPO: one high-affinity and one low-affinity interaction. *Biochemistry* **35**, 1681-1691
31. Philo, J.S., Wen, J., Wypych, J., Schwartz, M.G., Mendiaz, E.A., and Langley, K.E. (1996) Human stem cell factor dimer forms a complex with two molecules of the extracellular domain of its receptor, Kit. *J. Biol. Chem.* **271**, 6895-6902
32. Horan, T., Wen, J., Narhi, L., Parker, V., Garcia, A., Arakawa, T., and Philo, J. (1996) Dimerization of the extracellular domain of granulocyte-colony stimulating factor receptor by ligand binding: a monovalent ligand induces 2:2 complexes. *Biochemistry* **35**, 4886-4896
33. Ackers, G.K. and Thompson, T.E. (1965) Determination of stoichiometry and equilibrium constants for reversibly associating systems by molecular sieve chromatography. *Proc. Natl. Acad. Sci. USA* **53**, 342-349
34. Lemmon, M.A., Bu, Z., Ladbury, J.E., Zhou, M., Pinchasi, D., Lax, I., Engelman, D.M., and Schlessinger, J. (1997) Two EGF molecules contribute additively to stabilization of the EGF dimer. *EMBO J.* **16**, 281-294

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 2, 2012