Real-Time Measurements of Kinetics Of EGF Binding to Soluble EGF Receptor Monomers and Dimers Support the Dimerization Model for Receptor Activation†

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ABSTRACT: We have tested one aspect of the allosteric dimerization model for the activation of EGF receptor (EGFR) by EGF: whether EGF binding favors dimerization of the receptor. For this to be true, EGF molecules must bind with higher affinity to dimeric receptors than to monomeric receptors. We have tested this directly in a defined system using the soluble, extracellular ligand binding domain of EGFR monomers (sEGFR) and sEGFR dimers stabilized by treatment with a covalent cross-linking agent. We describe real-time kinetic measurements of EGF binding to receptor monomers and dimers employing the method of total internal reflection (surface plasmon resonance). Our data show that sEGFR dimers bound EGF with 30–40-fold higher affinity [K_D = (2–3) × 10^{-8} M] than did sEGFR monomers. The enhanced binding affinity of sEGFR dimers resulted mainly from a reduced off-rate with k_{off} = 0.001 s^{-1} for sEGFR dimers as compared to k_{off} = 0.06 s^{-1} for sEGFR monomers. These measurements indicate that dimerization of sEGFR increases its affinity for EGF by prolonging the amount of time that EGF remains bound to the receptor. This provides evidence that EGF binding stabilizes receptor dimerization and provides further support for the allosteric dimerization model as a mechanism for ligand induced receptor activation.

Growth factor induced receptor dimerization is a general phenomenon among growth factor receptors with tyrosine kinase activity (Schlessinger, 1988). Receptor dimerization is thought to play an important role in receptor activation, tyrosine autophosphorylation, and signal transmission across the plasma membrane (Schlessinger, 1988; Ullrich & Schlessinger, 1990). Recent studies provide evidence that additional families of surface receptors, such as growth hormone receptor (HGHR) (Cunningham & Wells, 1989; Cunningham et al., 1991) and the erythropoetin receptor (EPOR) (Watowich et al., 1992), undergo ligand-dependent dimerization and that receptor dimerization leads to activation of biological function. A plausible hypothesis, therefore, is that receptor dimerization may represent a general mechanism for the activation of most, if not all, signaling membrane receptors containing one transmembrane domain connecting the extracellular ligand binding domain to the cytoplasmic domain. If this is true, then receptor dimerization could be the mechanism underlying the regulation of the activity of many types of surface receptors, including receptor tyrosine kinases (Schlessinger, 1988; Ullrich & Schlessinger, 1990), receptor tyrosine phosphatases (Fischer et al., 1991), receptors with serine/threonine kinase activity such as the TGFβ receptor (Lin et al., 1992), receptors with guanylyl cyclase activity such as the NPR receptor (Koller et al., 1992; Lowe et al., 1989; Schulz et al., 1989), and various cytokine and hormone receptors which contain a relatively short cytoplasmic domain and appear to mediate their biological functions by activating cytoplasmic tyrosine kinases. The elucidation of the molecular mechanism of receptor dimerization is therefore an important question of general interest.

It is already clear that different hormones or growth factors are able to induce receptor dimerization by different mechanisms. For example, growth factors such as PDGF or CSF1 are dimeric structures, and the dimerization of PDGFR or CSF1R is mediated at least in part, by ligand-induced cross-linking of two neighboring PDGF or CSF1 receptors (Bishayee et al., 1989; Hart et al., 1988; Heldin et al., 1988, 1989; Li & Stanley, 1991). Interestingly, analysis of the three-dimensional structure of growth hormone bound to growth hormone receptor shows that dimerization of growth hormone receptors (HGHR) is mediated by a single growth hormone molecule able to bind to two growth hormone receptors (Cunningham et al., 1991). Yet another variation on the same theme is represented by the insulin receptor, which is displayed on the cell surface as a disulfide-bridged heterotetramer of α and β insulin receptor subunits. In this particular case, insulin probably activates its surface receptor by inducing an allosteric transition within an already preexisting dimeric structure (Ullrich & Schlessinger, 1990).

Recent studies with the recombinant soluble, extracellular ligand binding domain of EGF receptor (sEGFR) have shown that EGF induces a conformational change (Greenfield et al., 1989) and dimerization of the soluble ligand binding domain (Hurwitz et al., 1991; Lax et al., 1991). In this report, we explore the mechanism of receptor dimerization by comparing the kinetic parameters and equilibrium dissociation constants of the binding of EGF to sEGFR monomers and dimers. We describe real-time kinetic measurements of EGF binding toward its receptor, employing the method of total internal reflection (Fägerstam, 1991; Johnsson et al., 1991; Felder et al., 1993) (surface plasmon resonance) to detect specific interactions between EGF and sEGFR. Our data show that the binding affinity of EGF toward covalently cross-linked sEGFR dimers [K_D = (2–3) × 10^{-8} M) calculated directly from the kinetic parameters (k_{off}/k_{on}) or from Scatchard analysis of steady-state binding values is 30–40-fold higher than the affinity of EGF toward sEGFR monomers. More-

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over, the enhanced binding affinity of dimers versus monomers is determined by the off-rate (k_{off}). These results are consistent with a model in which an equilibrium exists between EGF receptor monomers and dimers and EGF binding stabilizes the formation of EGF receptor dimers, thus promoting stimulation of protein tyrosine kinase activity by an intermolecular mechanism (Schlessinger, 1988; Ullrich & Schlessinger, 1990).

MATERIALS AND METHODS

Purification of sEGFR. Recombinant sEGFR was purified from the conditioned medium of CHO cells transfected with a construct designated HERXCD which expresses the extracellular ligand binding domain of the EGF receptor (Greenfield et al., 1989; Lax et al., 1991; Livneh et al., 1986). The sEGFR was purified in two purification steps: first by affinity chromatography using 108 monoclonal antibody directed against the extracellular domain of EGFR, and subsequently by Mono Q chromatography and elution with 10 mM HEPES (pH 7.5) and 100 mM NaCl as described by Lax et al. (1991).

Generation of sEGFR Dimers. Dimers of soluble receptor were generated using 10 μM of pure sEGFR which were incubated with 20 μM mouse EGF (Toyoba, Japan) in 20 mM HEPES (pH 7.4) and 150 mM NaCl for 1 h at room temperature, to which disuccinimidyl suberate (DSS) was added to a final concentration of 0.25 mM for an additional 30 min. The reaction was terminated by adding Tris-HCl buffer at a final concentration of 10 mM. The sample was then applied to a Suparose 12 column, which was preequilibrated with 10 mM HEPES (pH 7.5) and 150 mM NaCl, and eluted fractions were analyzed on 4–12% SDS–PAGE Tris/glycine gradient gel (Novex, Encinitas, CA). Fractions containing purified dimers or monomers of sEGFR were pooled and concentrated using Amicon Centricon 30. The purified material was stored with 0.2% sodium azide at 4 °C. The dimers prepared in this way were called "E-dimers", and the monomers were called "monomers". Receptor treated in the same way in the absence of EGF formed stabilized dimers at a much lower efficiency. Dimers prepared in the absence of EGF were simply referred to as "Dimers".

Immobilization of EGF on BIAcore Matrix. Human EGF was cross-linked to the hydrogel matrix of the biosensor. The hydrogel matrix was activated with *N*-hydroxysuccinimide (NHS) and *N*-ethyl *N'*-[3-(diethylamino)propyl]carbodiimide (EDC). EGF at 50 μg/mL was injected for 5 min in 10 mM sodium acetate (pH 4), and then non-cross-linked EGF was removed and unreacted sites on the matrix were blocked with 1 M ethanolamine hydrochloride (pH 8.5). The resonance signal increased following this procedure by roughly 400 resonance units (RU), suggesting that 0.4 ng/mm^2 (in a gel thickness of roughly 0.1 μm) of EGF was cross-linked to the hydrogel surface (corresponding to 700 nM EGF within the matrix). The procedure and estimation of protein cross-linked are according to the manufacturer (Johnsson et al., 1991). Running buffer [10 mM HEPES (pH 7.4) 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20 (HBS)] was flowed through the system at a rate of 8 or 2 μL/min (affinity measurements were not dependent on the flow rates). Background nonspecific binding and bulk refractive index changes were determined by injection of receptor onto a hydrogel matrix activated and blocked as above but without EGF. The background ranged from 10 to 50 RU for lowest and highest protein concentrations and was subtracted from each curve before analysis.

Kinetics Analysis. The kinetic data for the association and dissociation phases of the curves were fit with simple bimolecular interaction assumptions, using the RU signal as a function of time as a relative measure of the concentration of receptor:EGF complex. Fitting was by nonlinear least-squares with iterative numeric integration for the full time course of the data, with the following equations.

association:

$$d[ER]/dt = k_{on}[E][R] - k_{off}[ER]$$

or

$$d[ER]/dt = k_{on}[R]\{[ER]_{max} - [ER]\} - k_{off}[ER]$$

which becomes

$$dRU/dt = k_{on}[R]\{RU_{max} - RU(t)\} - k_{off}RU(t)$$

dissociation:

$$d[ER]/dt = -k_{off}[ER]$$

which becomes

$$dRU/dt = -k_{off}RU(t)$$

where k_{on} is the second-order association rate constant, k_{off} is the first-order rate constant of dissocation, [ER] is the concentration of EGFR:EGF complex on the matrix, $[ER]_{max}$ is the maximum occupancy at saturation, [E] is the concentration of unbound EGF on the matrix (= $[ER]_{max}$ – [ER]), and [R] is the concentration of receptor, which is assumed to be a constant during injection and zero after injection. The fitted parameters were RU_{max}, k_{on}, and k_{off}.

We note that some of the data fit by these equations would have been fit more optimally by assuming a more complex model—for example, more than one rate of association or dissociation. However, for the sake of making as few assumptions as possible in data analysis, this simple model was used. When two populations were assumed, the general conclusions were very similar. In particular, the E-dimer data were best fit with two populations, with the higher affinity population accounting for 90% of the total, and the data for the untreated receptor were best fit with two populations having 80% of receptor in the low-affinity population.

RESULTS AND DISCUSSION

Our goal in this study is to compare the kinetic parameters and equilibrium dissociation constants of EGF binding toward EGFR monomers and dimers. In order to measure kinetics and affinity, we have used an optical method recently developed that monitors the index of refraction of a solution very near a surface. The method has been called the biospecific interaction analysis system or BIAcore (Fägerstam, 1991; Johnsson et al., 1991). To a gold-coated glass surface is attached a (carboxymethyl)dextran matrix. The matrix is positioned within a flow chamber, and the phenomenon of surface plasmon resonance is monitored: light incident on the surface below the angle of total internal reflection is absorbed at one particular angle that depends upon the index of refraction near the surface. For the experiment, one component of a binding pair is covalently cross-linked to the matrix. When a solution containing the second component is introduced, the refractive index within the matrix changes slightly due to small changes in the refractive index of the bulk solution and changes more slowly due to the binding interaction and,

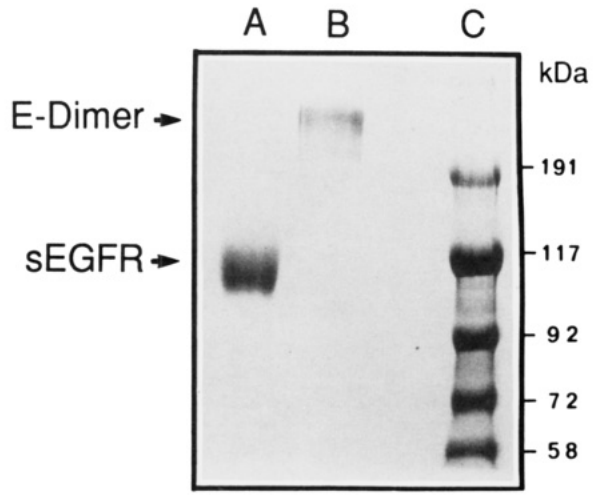

FIGURE 1: Generation of sEGFR dimers. Purified sEGFR (10 μM) was incubated with EGF (20 μM) for 1 h at room temperature in 20 mM HEPES buffer (pH 7.4) and 150 mM NaCl and subsequently with disuccinimidyl suberate (DSS) (0.25 mM) for 30 min. Cross-linking was terminated by the addition of Tris-HCl buffer (10 mM), and the sample was resolved by size exclusion chromatography on a Suparose 12 column. The column was preequilibrated and eluted with 10 mM HEPES and 150mM NaCl (pH 7.5) at a flow rate of 0.5 mL/min. Figure 1 is an SDS–PAGE analysis of the peaks eluted (5 μg): (A) the untreated sEGFR monomer fraction (sEGFR); (B) the EGF-treated sEGFR dimer fraction (E-dimer); (C) molecular weight standards.

hence, the concentration of the second component within the matrix. The time course of binding is observed directly. The time course of dissociation is followed by monitoring the signal during elution of the second component.

In order to analyze the kinetics of sEGFR binding, we have immobilized either murine or human EGF to the hydrogel–gold matrix using the covalent cross-linking agent *N*-ethyl-*N*′-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC). As large quantities of pure native EGFR are unavailable, we have used a soluble, recombinant, extracellular ligand binding domain of EGFR (sEGFR) which has been generated for structural and biophysical studies (Hurwitz et al., 1991; Lax et al., 1991). We have previously demonstrated that sEGFR binds EGF with $K_D \approx 15 \times 10^{-8}$ M, which is 2–3-fold higher than the dissociation constant of the detergent-solubilized native EGFR (Hurwitz et al., 1991; Lax et al., 1991). Moreover, like wild-type receptor, sEGFR is endowed with the capacity to undergo EGF-dependent dimerization (Hurwitz et al., 1991; Lax et al., 1991). We have compared the kinetic parameters and the dissociation constants of the following forms of sEGFR: untreated sEGFR monomers (sEGFR), control sEGFR monomers which were treated with the cross-linker but did not form dimers (monomers), purified covalently cross-linked sEGFR dimers (dimers), and purified EGF-treated covalently cross-linked sEGFR dimers (E-dimers). In experiments using sEGFR and [^{125}I]EGF, we estimate that approximately 90% of EGF binding sites in the E-dimers are unoccupied and available for EGF binding (data not shown). Apparently the murine EGF used was covalently cross-linked to the receptor with very low efficiency, consistent with the fact that there is only one amino group on the amino terminus of EGF available for cross-linking. We have previously described detailed analysis of sEGFR produced either in CHO cells (Lax et al., 1991) or in insect cells (Hurwitz et al., 1991). Figure 1 shows sEGFR and E-dimers purified by size exclusion chromatography and analyzed by SDS gel electrophoresis followed by Coomassie brilliant blue staining.

The various monomeric and dimeric forms of sEGFR were injected into the Biosensor flow system, and association with the immobilized EGF was followed for 3–15 min. Buffer alone was then injected, and dissociation from the matrix was followed. Figure 2 shows the real-time measurements of the association and dissociation phases of interaction of sEGFR (Figure 2A), monomers (Figure 2B), dimers (Figure 2C), and E-dimers (Figure 2D) with immobilized human EGF, performed at several different concentrations of receptor. The rates of association and dissociation were noticeably different for monomeric and dimeric sEGFR. These data were fit to kinetic expressions for the association and dissociation phases of the curves, respectively, and the best fit kinetic parameters are listed in Table I, along with the estimated equilibrium dissociation constants calculated from the kinetic measurements. Further, the association phases of the curves of Figure 2 approached steady-state values, approximating the binding at equilibrium for each concentration of sEGFR.

These measurements were combined and plotted in Scatchard format (Figure 3) in order to directly determine the equilibrium dissociation constants. Figure 3 shows that linear Scatchard plots were obtained for the steady-state binding of the untreated (sEGFR) and treated monomer and for the dimer forms of the soluble EGFR. However, a nonlinear Scatchard plot was obtained for the binding of E-dimers to the EGF-containing matrix. The reason for the nonlinear Scatchard plot of E-dimer binding to the EGF matrix is not clear. It may reflect heterogeneity in the population of E-dimer molecules caused by the attachment of the covalent cross-linking agent to different free amino groups on the soluble EGFR molecules. The best fit dissociation constants determined from steady-state binding are listed in Table I. The kinetic parameters presented in Table I show that treatment with the cross-linker reduced the rate of receptor binding to sEGFR roughly 5-fold from 15×10^4 to 2.9×10^4 M^{-1} s^{-1}, and the rate of receptor dissociation was reduced roughly 2-fold from 0.062 to 0.029 s^{-1}. This effect may be due to changing the surface charge on the receptor or to attachment of the cross-linker to amino acid residues which play a role in EGF binding.

Overall, control treatment with cross-linker reduced the affinity of sEGFR toward EGF by roughly 2-fold. Hence, treatment with covalent cross-linker alone has a relatively small effect on ligand binding. Both dimers and E-dimers also showed reduced association rates, but more significantly their dissociation rate was up to 60-fold slower as compared to the dissociation rate of either untreated or treated monomeric receptors. Hence, for the soluble extracellular domain of the EGF receptor, the treated dimeric form has a 40-fold higher binding affinity compared with treated monomers, and the relative affinity is determined predominantly by the off-rate (Table I). The same data analyzed by steady-state binding and Scatchard analysis yielded similar results (Table I). Namely, the binding affinity for EGF of the dimeric receptors is approximately 30-fold higher than the binding affinity of the similarly treated monomeric receptors.

It is now well established that EGF and other growth factors induce dimerization of their specific receptors and that dimerization is required for receptor activation and tyrosine autophosphorylation (Schlessinger, 1988; Ullrich & Schlessinger, 1990). It was shown that the dimeric PDGF ligand dimerizes its receptor by binding to and bridging two neighboring PDGF receptor molecules. Yet, the mechanism of EGF-induced receptor dimerization is not defined in molecular terms. It was previously reported that one EGF

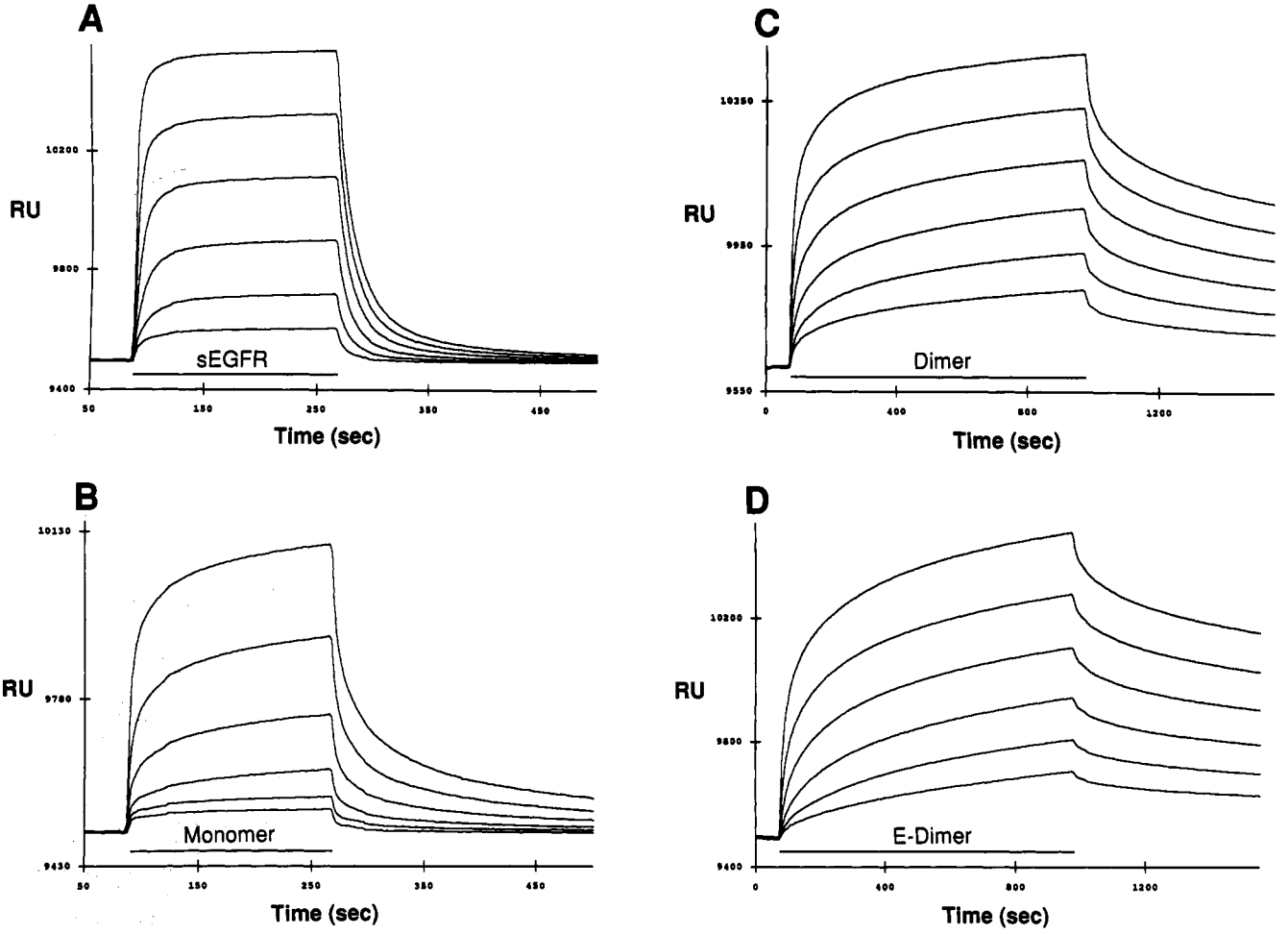

FIGURE 2: Association and dissociation experiments. Plotted is the resonance signal (RU) as a function of time for several concentrations of soluble receptor injected into the system plotted in overlay. The injected receptor was associating specifically with human EGF which had been stably cross-linked to the hydrogel matrix. The bar at the bottom of each panel displays the time during which receptor was injected, and the remainder of the curves shows the traces during elution. Injected were sEGFR (A), monomer (B), dimer, (C), and E-dimer (D). sEGFR and monomer were injected at 31, 63, 125, 250, 500, and 1000 nM, while dimer and E-dimer were injected at 16, 31, 63, 125, 250, and 500 nM. During the injection phase, receptor bound more rapidly and to higher steady-state levels when injected at higher concentrations and then dissociated during the elution phase at rates largely independent of concentration.

Table I: Kinetics Parameters and Calculated Equilibrium Constants[a]

	$k_{on} \times 10^{-3}$ (M^{-1} s^{-1})	k_{off} (s^{-1})	$K_{D,calc}$ (nM)	$K_{D,ss}$ (nM)
sEGFR	153 ± 14	0.062 ± 0.002	410 ± 90	560 ± 100
monomer	29 ± 4	0.029 ● 0.001	980 ± 300	200 ± 200
dimer	81 ± 4	0.0022 ● 0.0006	25 ± 5	62 ± 2
E-dimer	36 ± 5	0.0010 ± 0.0002	25 ± 7	38 ± 5

[a] Listed are the kinetic and equilibrium parameters for untreated sEGFR (sEGFR), for sEGFR treated with cross-linker (monomer), for cross-linked sEGFR dimers in absence of EGF (dimer), and for sEGFR treated with cross-linker in the presence of EGF (E-dimer). k_{on} and k_{off} were estimated from fitting the kinetics of the association and dissociation phases for the data presented in Figure 2 and for other, replicate data. $K_{D,calc}$ values are the equilibrium dissociation constants calculated directly from the kinetic parameters (k_{off}/k_{on}). $K_{D,ss}$ values were determined by fitting the steady-state binding values directly to the binding equation by Scatchard analysis. Data were collected in duplicate and were repeated for two different batches of sEGFR and its various dimeric forms. Listed are the average values of the kinetics parameters for the two batches. Similar results were obtained in two different experiments for each batch.

molecule binds to a single EGF receptor molecule (Weber et al., 1984). If EGF/EGFR binding stoichiometry is indeed 1:1, then EGF-induced receptor dimerization is probably mediated by a conformational change in the extracellular domain which stabilizes the interaction between two neighboring occupied EGF receptors. However, if the stoichiometry of EGF/EGFR is 1:2 as was shown for the binding of growth hormone to growth hormone receptor (Cunningham et al., 1991), then EGF-induced dimerization of EGF receptor does not necessarily require induction of a large conformational change in the extracellular ligand binding domain.

Biospecific interaction analysis has proved a valuable tool in obtaining reliable, reproducible binding data. Further, not only can affinity measurements be performed but the kinetics of binding interactions can be determined as well. Such an analysis provides additional insights into the physical processes which govern EGFR dimerization. Thus we have been able to show that EGF dissociates much more slowly from dimeric than from monomeric sEGFR. Further, we have shown that dimeric sEGFR formed without EGF shows very much the same kinetics (reduced k_{off} leading to enhanced affinity) as dimeric sEGFR formed by EGF binding. We conclude that EGF serves to stabilize and thereby enhance the formation of receptor dimers, but apparently does not alter the affinity of the dimers. We note that, in this report with the increased accuracy and versatility of the Biosensor system, we have determined that the affinity of dimers is virtually the same as for E-dimers, while using another technique we estimated the affinity of dimers to be lower (Hurwitz et al., 1991). Here we have had the added advantage of kinetics measurements, as well as steady-state measurements, and have found the

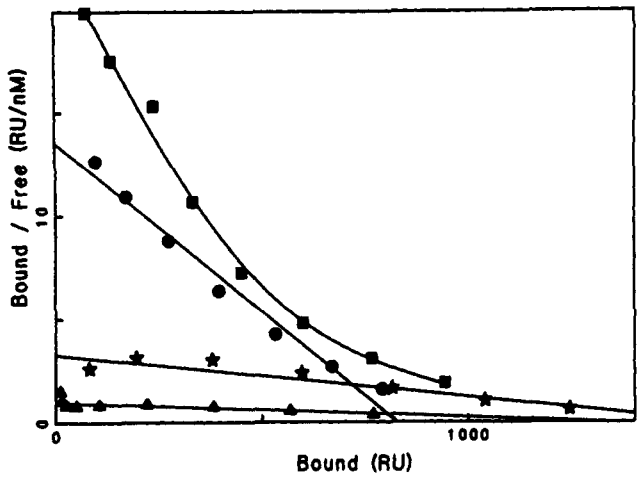

FIGURE 3: Scatchard analysis of binding of various monomeric and dimeric forms of sEGFR to EGF containing matrix. The equilibrium values of the resonance signals shown in Figure 2 for each concentration of EGF receptor were plotted in Scatchard format. Note that the concentration of the dimeric forms is plotted as the concentration of dimeric molecules, not the concentration of monomeric "subunits". The plots represent the best fit for the data. The nonlinear Scatchard plot of E-dimers may be due to heterogeneity in the cross-linking of receptor: sEGFR (▲); monomer (★); dimer (●) and E-dimer (■).

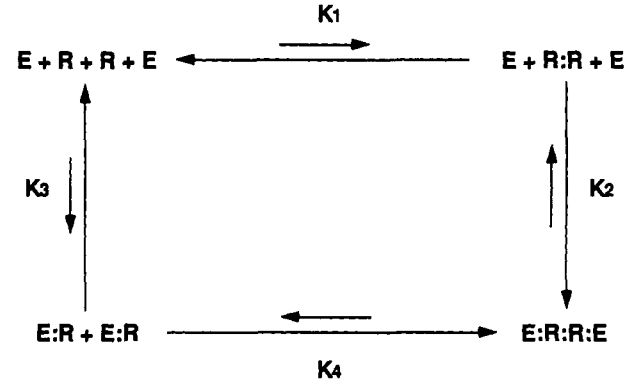

FIGURE 4: Receptor dimerization coupled to ligand binding. This expression is based upon the assumption that one EGF molecule binds one EGFR molecule (Weber et al., 1984). In this expression the equilibrium association constants are as follows: K_1, for dimerization of unoccupied receptor; K_2, for EGF binding to dimeric receptor; K_3, for EGF binding to monomeric receptor; K_4, for dimerization of occupied receptors. The standard free energy change following the pathway of dimerization before ligand binding (reactions 1 and 2) is $dG^\circ_{1,2} = -RT \ln K_1 - 2RT \ln K_2$. This must equal the standard free energy for the alternative pathway, ligand binding before dimerization (reactions 3 and 4): $dG^\circ_{3,4} = -2RT \ln K_3 - RT \ln K_4$. Therefore, $K_4/K_1 = K_2^2/K_3^2$. Hence, if $K_2/K_3 = 30$–40, as suggested by the data presented here, then $K_4/K_1 = 900$–1600; that is, EGF binding serves to drive receptor dimerization. This is a simplified model. For example, it is assumed that the dissociation constant K_1 for binding of EGF to the unoccupied EGF receptor dimer (R:R) is equal to the dissociation constant for binding of EGF to the partially occupied EGF receptor dimer (R:RE). More detailed models for EGF receptor dimerization require unequivocal determination of EGF binding stoichiometry toward EGFR, which is currently unavailable.

kinetics to be virtually the same, especially the dissociation rates which are measured very accurately, and do not rely on the knowledge of the exact concentration of active receptor in the preparations. Further, the previous technique required the use of an anti-EGFR antibody, which may have reacted differently with the different forms of the sEGFR. Here we have tested all forms under identical conditions.

The wild-type EGF receptor is obviously a better experimental system for binding studies than the soluble extracellular ligand binding domain, as significant receptor–receptor interactions could be lost upon truncation of the transmembrane and cytoplasmic domains of EGFR. Unfortunately, large quantities of wild-type, detergent-solubilized EGFR are unavailable. We also note that the addition of covalent cross-linking agent alone causes some effect on the kinetic properties of EGF binding to the soluble receptors. Nevertheless, despite these limitations, it is clear from the steady-state binding and kinetic results that the binding affinity of the dimeric soluble extracellular domain of EGF receptors is approximately 30–40-fold higher than the binding affinity of monomeric EGF receptors. These values have interesting implications concerning the mechanism of EGFR activation by EGF. It has been proposed that monomeric inactive EGF receptors are in equilibrium with dimeric activated EGF receptors (Schlessinger, 1988). If the binding affinity of the dimers toward EGF is higher than the binding affinity of the monomers toward EGF (Figure 4), as shown in this study, then the preferential binding of EGF to EGFR dimers will shift the equilibrium toward the dimeric state which possesses elevated protein tyrosine kinase activity. In other words, receptor occupation will shift the equilibrium to favor the active dimeric state (Figure 4). Moreover, given the rapid rotational and translational diffusion of EGF receptors in the plasma membrane (Schlessinger et al., 1978; Zidovetzki et al., 1981), receptor dimerization is not only thermodynamically favorable but also kinetically feasible on the cell surface. We conclude that the establishment of quantitative kinetic parameters and equilibrium dissociation constants for EGF binding to soluble receptor dimers and monomers provides further support for the allosteric dimerization model (Schlessinger, 1988; Ullrich & Schlessinger, 1990) as a mechanism for ligand-induced receptor activation.

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