Two EGF molecules contribute additively to stabilization of the EGFR dimer

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Receptor dimerization is generally considered to be the primary signaling event upon binding of a growth factor to its receptor at the cell surface. Little, however, is known about the precise molecular details of ligand-induced receptor dimerization, except for studies of the human growth hormone (hGH) receptor. We have analyzed the binding of epidermal growth factor (EGF) to the extracellular domain of its receptor (sEGFR) using titration calorimetry, and the resulting dimerization of sEGFR using small-angle X-ray scattering. EGF induces the quantitative formation of sEGFR dimers that contain two EGF molecules. The data obtained from the two approaches suggest a model in which one EGF monomer binds to one sEGFR monomer, and that receptor dimerization involves subsequent association of two monomeric (1:1) EGF–sEGFR complexes. Dimerization may result from bivalent binding of both EGF molecules in the dimer and/or receptor–receptor interactions. The requirement for two (possibly bivalent) EGF monomers distinguishes EGF-induced sEGFR dimerization from the hGH and interferon-γ receptors, where multivalent binding of a single ligand species (either monomeric or dimeric) drives receptor oligomerization. The proposed model of EGF-induced sEGFR dimerization suggests possible mechanisms for both ligand-induced homodimerization of the EGFR (or erbB) family of receptors.

Keywords: EGF receptor/epidermal growth factor/receptor dimerization/titration calorimetry/X-ray scattering

Introduction

Induction of receptor oligomerization upon ligand binding is the first step in the activation of growth factor receptors and other cytokine receptors that contain a single transmembrane α-helix (Kishimoto et al., 1994; Lemmon and Schlessinger, 1994; Heldin, 1995). Receptor tyrosine kinases are activated upon growth factor-induced receptor dimerization (Canals, 1992; Schlessinger and Ullrich, 1992), which brings the cytoplasmic tyrosine kinase domains of the two receptors into close proximity. Autophosphorylation of tyrosine, considered to be an intermolecular process (Honegger et al., 1990), leads to the activation of the kinase domains for phosphorylation of other substrates. Crystallographic studies of tyrosine kinase domains from the insulin and fibroblast growth factor receptors suggest possible mechanisms for the control of this activation event (Hubbard et al., 1994; Mohammadi et al., 1996).

Relatively little is known about the precise molecular details of ligand-induced receptor dimerization. An exception is the case of human growth hormone (hGH) binding to its receptor (hGH-R). The hGH ligand is monomeric, yet forms a 1:2 (ligand:receptor) complex with its receptor (Cunningham et al., 1991). Crystallographic studies of the complex between hGH and the hGH-R extracellular domain have shown that a single molecule of hGH binds simultaneously to two receptor molecules (de Vos et al., 1992). A sequential binding model has been proposed, in which hGH binds first to one receptor molecule to form a 1:1 complex. This complex then binds to a second, unliganded, receptor through a second binding site on hGH plus receptor–receptor contacts (Cunningham et al., 1991; Fuh et al., 1992; Kossiakoff et al., 1994; Wells, 1996). The mechanism of hGH-induced hGH-R dimerization is thought to represent a paradigm for receptor activation by other monomeric cytokines (Sprang and Bazan, 1993). Erythropoietin (EPO), for example, utilizes a broadly similar mechanism (Philo et al., 1996a), although granulocyte colony-stimulating factor (G-CSF) is an exception, apparently being a monomeric monovalent ligand (Horan et al., 1996).

In addition to the results with hGH, crystallographic views of ligand-induced receptor oligomerization have been obtained for the dimeric cytokine interferon-γ (IFN-γ) bound to the α-chain of its receptor (Walter et al., 1995), and for the tumor necrosis factor (TNF-β) trimer bound to the extracellular domain of its receptor (Banner et al., 1993). In these cases, the dimeric or trimeric ligand is bi- or tri-valent, and multivalence is the key for ligand-induced receptor oligomerization, as seen with hGH. Similarly, the ligands for several receptor tyrosine kinases have also been shown to induce receptor dimerization by virtue of their bivalence. Platelet-derived growth factor (PDGF) is a covalently linked dimer that binds simultaneously to two receptor molecules (Heldin et al., 1989; Fretto et al., 1993). The neurotrophins are also dimeric, with a single dimer binding to two receptors (Philo et al.,...
1994), and stem cell factor (SCF) is a non-covalent dimer of four-helix bundle protomers that binds simultaneously to two molecules of its receptor, Kit, thus inducing Kit dimerization (Lev et al., 1992; Philo et al., 1996b; Lemmon et al., 1997). In a variation on this theme, acidic fibroblast growth factor (aFGF) is monomeric when free, but oligomerizes when several molecules of aFGF bind to a single heparan sulfate proteoglycan (HSPG) molecule (Spivak-Kroizman et al., 1994). The resulting (FGF)₃–HSPG complex is multivalent in its binding to the FGF receptor, thus causing receptor oligomerization and activation (Spivak-Kroizman et al., 1994; Schlessinger et al., 1995).

The mechanism of epidermal growth factor (EGF) receptor activation by its ligands, including EGF, is less clear despite being the first receptor tyrosine kinase for which dimerization was shown to be the key activating step (Yarden and Schlessinger, 1987a,b). EGF binds to, and can be activated by, a number of different ligands of the EGF family, including EGF, transforming growth factor-α (TGF-α), heparin binding EGF-like growth factor (HB-EGF) (Higashiyama et al., 1991), betacellulin (Shing et al., 1993), amphiregulin (Plowman et al., 1990) and epiregulin (Toyoda et al., 1995). Binding and activation of the receptor by EGF has been most thoroughly studied. EGF is presumed to be monomeric, and has been reported to bind to its receptor in a 1:1 complex (Weber et al., 1984; Günther et al., 1990). These observations indicate that the mode of EGF-induced receptor dimerization may be different from that seen with the other receptors mentioned here. Certainly, ligand bivalence cannot necessarily be assumed given the reported stoichiometry. Here we report studies of EGF binding to the EGF receptor extracellular domain (sEGFR), as well as the resulting quantitative dimerization of this domain, using a variety of biophysical techniques. The results obtained from the different experimental approaches suggest a model that can explain previous, apparently conflicting, results reported for this system. The best model differs from that for the induction of receptor dimerization by hGH (Wells, 1996) in that sEGFR dimerization requires the participation of two molecules of monomeric EGF (in a 2:2 dimer), and involves the dimerization of a stable intermediate 1:1 EGF–sEGFR complex. No direct evidence was obtained for formation of a 1:2 EGF–sEGFR complex. The dimerization model that we propose provides a context for understanding the ability of different EGF-like ligands to induce heterodimerization of the EGFR family of receptor tyrosine kinases (Lemmon and Schlessinger, 1994).

Results and discussion

The extracellular domain of the EGF receptor (sEGFR) was produced by secretion from CHO cells, and was purified from conditioned medium as described (Lax et al., 1991a). EGF binding to sEGFR, as well as to fragments of this domain, was studied by isothermal titration calorimetry (ITC). sEGFR dimerization upon EGF binding was also analyzed using small-angle X-ray scattering (SAXS) and chemical cross-linking approaches. Quantitative dimerization of sEGFR was observed upon stoichiometric binding of EGF, and the data obtained were used to develop an equilibrium model of this event that initiates EGFR signaling.

Fig. 1. SAXS data obtained for EGF at 8.7 mg/ml (1.36 mM), represented in the form of a Guinier plot. The intensity of scattered radiation (I) was normalized using the mass concentration of EGF (c). Ln (I/c) is plotted against Q², where Q = 4π sinθ/λ, λ is the wavelength of the X-ray radiation and 2θ is the scattering angle. The scattered intensity at zero angle, I(0, which is proportional to the molecular mass of the protein, is obtained from the y-intercept (when Q = 0). By comparison with I(0)/c values measured for chymotrypsin, this experiment gave a molecular mass of 6.1 (± 0.6) kDa for EGF, in good agreement with the value predicted for monomeric EGF from its amino acid composition (6.2 kDa). The slope of the linear region of the Guinier plot is equal to (Rg)²/3, giving an Rg for EGF of 11.5 (± 0.44) Å, consistent with its known elongated structure. SAXS experiments performed at two other lower EGF concentrations gave similar results, showing that intermolecular interaction effects were not apparent at the EGF concentrations studied.

Oligomeric state of EGF

A key initial question in considering the mechanism of ligand-induced growth factor receptor dimerization concerns the oligomeric state of the ligand itself. Although it is generally assumed that EGF is monomeric in solution, quantitative demonstration of this has not, to our knowledge, been reported under conditions applicable to biophysical analysis of ligand-induced receptor dimerization. We therefore used SAXS to determine the oligomeric state of EGF in solution at several different concentrations. The concentration-normalized intensity of forward scatter, I(0), estimated in a SAXS experiment is proportional to the weight-averaged molecular mass of molecules in a solution scattering sample. Using well-characterized proteins as standards, SAXS can be used to determine molecular mass. EGF was thus found to occur in solution as a monomeric species of 6.1 (± 0.6) kDa (Figure 1)—in good agreement with its predicted monomeric molecular mass (6.2 kDa)—at three different concentrations up to 8.7 mg/ml (1.36 mM). Intermolecular interaction effects were not evident, showing that EGF is monomeric in all of the experiments reported here.

Binding of EGF to sEGFR

A number of studies of EGF binding to sEGFR have been reported (Greenfield et al., 1989; Günther et al., 1990; Hurwitz et al., 1991; Lax et al., 1991a; Zhou et al., 1993; Brown et al., 1994). These reports differ in their conclusions regarding the ability of EGF to induce sEGFR dimerization, but agree relatively closely in the measured Kd values for EGF binding, which range from 100 to 500 nM. To determine directly the stoichiometry of this
Dimerization of EGF receptor extracellular domain

Dimerization of EGF receptor extracellular domain (Spivak-Kroizman et al., 1994; Philo et al., 1996a), aFGF (Spivak-Kroizman et al., 1994) and SCF (Philo et al., 1996b; Lemmon et al., 1997), where ligand binding is enthalpy driven.

The titration in Figure 2 also shows that the final stoichiometry of EGF binding to sEGFR is 1:1. Such 1:1 complexes are also formed by aFGF (Spivak-Kroizman et al., 1994) and SCF (Philo et al., 1996b; Lemmon et al., 1997) with their respective receptors, while hGH and EPO both form 1:2 (ligand:receptor) complexes (Cunningham et al., 1991; Philo et al., 1996a). Table I compares the stoichiometries of receptor binding and oligomeric state for each of these ligands. This comparison suggests that EGF must differ from hGH, EPO, aFGF and SCF in binding to its receptor. Unlike other monomeric ligands, EGF does not bind with a 1:2 stoichiometry. Unlike other ligands that bind with 1:1 stoichiometry, EGF is not dimeric in solution [considering the oligomeric (FGF)–HSPG complex as the effective ligand in that case].

A further feature specific to EGF is seen in the shape of the titrations (Figure 2). ITC studies of receptor binding by hGH (Cunningham et al., 1991), aFGF (Spivak-Kroizman et al., 1994) and SCF (Philo et al., 1996b; Lemmon et al., 1997) all indicate a single binding mode, giving simple sigmoidal titrations. By contrast, EGF–sEGFR titrations show two clear phases, suggesting two or more different binding events (Figure 2). Similar curves were obtained both for titrations of EGF into sEGFR and for titrations of sEGFR into EGF. Without additional data, these curves can only be fit by assuming multiple independent binding sites, which we show below to be inappropriate for this case. The best independent site model predicts that a 1:2 EGF–sEGFR complex would form with high affinity, and that a second EGF would bind to this complex. If this were correct, maximal sEGFR dimerization would occur at an EGF:sEGFR ratio of 1:2. However, the SAXS experiments described below show this prediction to be wrong, arguing that the ITC data reflect multiple interacting (rather than independent) sites. By combining the results from both our SAXS and ITC analyses, we develop below an EGF binding model that is consistent with all of these experimental observations.

**EGF-dependent dimerization of sEGFR**

The simplest (two independent sites) model suggested by ITC studies of EGF binding to sEGFR predicted that

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**Table I. Oligomeric state and binding stoichiometry of selected growth factors and cytokines that induce oligomerization of their receptor extracellular domains**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Oligomeric state</th>
<th>Stoichiometry of receptor binding</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hGH</td>
<td>monomer</td>
<td>1:2</td>
<td>Cunningham et al. (1991)</td>
</tr>
<tr>
<td>EPO</td>
<td>monomer</td>
<td>1:2</td>
<td>Philo et al. (1996a)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>dimer</td>
<td>1:1</td>
<td>Walter et al. (1995)</td>
</tr>
<tr>
<td>aFGF</td>
<td>oligomer with HSPGs</td>
<td>1:1</td>
<td>Spivak-Kroizman et al. (1994)</td>
</tr>
<tr>
<td>PDGF</td>
<td>dimer</td>
<td>1:1</td>
<td>Fretto et al. (1993)</td>
</tr>
<tr>
<td>Neurotrophins</td>
<td>dimer</td>
<td>1:1</td>
<td>Philo et al. (1994)</td>
</tr>
<tr>
<td>TNF-β</td>
<td>trimer</td>
<td>1:1</td>
<td>Banner et al. (1993)</td>
</tr>
</tbody>
</table>

Valence unclear

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Oligomeric state</th>
<th>Stoichiometry of receptor binding</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF</td>
<td>monomer</td>
<td>1:1</td>
<td>Horan et al. (1996)</td>
</tr>
<tr>
<td>EGF</td>
<td>monomer</td>
<td>1:1</td>
<td>this work</td>
</tr>
</tbody>
</table>

The stoichiometry (ligand:receptor) refers to the ligand monomer: for example, one IFNγ dimer binds to two receptors.
sEGFR dimerization would be maximal at an EGF:sEGFR molar ratio of 1:2. To test this prediction, we monitored sEGFR dimerization directly as a function of the EGF:sEGFR ratio, using SAXS. This experiment also addresses an important, yet experimentally untested, question for EGFR—does the isolated extracellular domain dimerize quantitatively upon EGF binding? Previous chemical cross-linking (Hurwitz et al., 1991; Lax et al., 1991a) and sedimentation equilibrium centrifugation experiments (Brown et al., 1994) indicated only a modest degree of sEGFR dimerization upon ligand binding, with some formation of higher order oligomers (Lax et al., 1991a). Density gradient centrifugation studies, performed at the significantly lower protein concentrations commonly used for EGF binding studies, showed no EGF-induced dimerization of sEGFR (Weber et al., 1984; Greenfield et al., 1989). Furthermore, we could not detect EGFinduced sEGFR dimerization using size-exclusion chromatography (which may simply reflect a small difference in hydrodynamic radius between monomeric and dimeric sEGFR). We have therefore used SAXS to monitor EGF-induced sEGFR dimerization directly. SAXS provides a method for analyzing molecular mass changes in a shape-independent manner. To achieve this, the X-ray scattering curves obtained in a SAXS experiment are extrapolated to zero-angle to give the intensity I(0) of forward (or zero-angle) scatter (as seen in Figure 1). The magnitude of I(0), normalized by the mass concentration of the sample, reflects the volume of the scattering particles, and, through the partial specific volume, is directly proportional to the weight-averaged molecular mass \( M_w \) of the particles in the sample solution (regardless of their shape). SAXS analysis of sEGFR alone showed that it does not self-aggregate significantly at concentrations up to 100 \( \mu \)M, and the measured I(0) was consistent with its expected monomeric molecular mass. However, as EGF was titrated into a solution of sEGFR, I(0) (and therefore \( M_w \)) increased significantly (Figure 3). This increase in \( M_w \) was maximal (2.2-fold) at a \([\text{EGF}]_{\text{Ttot}}:[\text{sEGFR}]_{\text{Ttot}}\) ratio of 1:1, beyond which no further increase was observed with additional EGF (up to a 5-fold molar excess). Similar results were obtained in more limited studies of sEGFR dimerization induced by TGF-\( \alpha \)-binding (data not shown). Since the molecular mass of sEGFR is \( \approx 110 \) kDa, while that of EGF or TGF-\( \alpha \) is just \( 6.2 \) kDa, doubling of \( M_w \) can only occur if the EGF–sEGFR complex involves an sEGFR dimer. Dimerization is complete under the conditions of this experiment, and there is no evidence for the formation of higher order oligomers. The I(0) values reported here were normalized only by the mass of sEGFR, which was constant: the fact that I(0) does not increase further at \([\text{EGF}]_{\text{Ttot}}:[\text{sEGFR}]_{\text{Ttot}}\) ratios greater than 1:1 shows that excess EGF remains free in solution. These experiments, therefore, provide additional support for the final 1:1 stoichiometry determined in the ITC studies. They also demonstrate that sEGFR dimerizes quantitatively in an EGF-dependent manner. The monotonic increase of I(0) to a maximum at a stoichiometry of 1:1 suggests a model for EGF-induced sEGFR dimerization in which one EGF molecule must bind to each molecule of sEGFR in order to induce dimerization (Figure 3). This is clearly inconsistent with the simple multiple independent site interpretation of the ITC results outlined above, which requires that maximal sEGFR dimerization occurs at an EGF:sEGFR ratio of 0.5. EGF and TGF-\( \alpha \) therefore differ from hGH in their mode of ligand-induced receptor dimerization.

The SAXS analysis shows that the simplest interpretation of the ITC data presented above is inadequate, so multiple interacting binding events must be considered. Conversely, the most straightforward interpretation of the SAXS analysis alone would predict simple sigmoidal titrations in our ITC studies, similar to those seen for SCF binding to the Kit extracellular domain (Philo et al., 1996b; Lemmon et al., 1997). Figure 2 shows that this is not the case. By analyzing the data in more detail, we therefore sought to develop a straightforward model for EGF-induced sEGFR dimerization that is consistent with the results from both our ITC and SAXS experiments, as well as results previously reported for this system by others. Development of this model was aided by additional experiments in which we have analyzed EGF binding to an isolated subdomain from sEGFR.

**Binding of EGF to an isolated subdomain from sEGFR**

Previous studies suggest that EGF can bind to sEGFR in the absence of sEGFR dimerization, with a \( K_D \) in the range 100–500 nM (Greenfield et al., 1989; Günther et al., 1990; Hurwitz et al., 1991; Lax et al., 1991a; Zhou et al., 1993; Brown et al., 1994). As a starting point in developing a model for EGF-induced sEGFR dimerization, we assumed a \( K_D \) in this range for the formation of a
and stoichiometry is 1.10:1 (EGF:sEGFRd3); solid line represents the best fit to this particular set of data, for which monomeric sEGFR. It can be argued, therefore, that EGF

Fig. 4. Representative ITC data for binding of EGF to sEGFRd3 (see Materials and methods). Aliquots (18 μl) of EGF (175 μM) were injected into sEGFRd3 (17 μM) present in the calorimeter cell at 25°C. Eight points represent the integrated heat per mole of injectant binding studies, EGF-induced sEGFR dimerization is not detectable (Greenfield et al., 1989; Günther et al., 1990; Hurwitz et al., 1991; Lax et al., 1991a; Zhou et al., 1993; Brown et al., 1994). At the low (nanomolar range) concentrations of sEGFR employed for these reported binding studies, EGF-induced sEGFR dimerization is not detectable (Greenfield et al., 1989; Günther et al., 1990), suggesting that the measured KD reflects EGF binding to monomeric sEGFR. It can be argued, therefore, that EGF binding to monomeric sEGFR has approximately the same KD as EGF binding to isolated domain 3. This agreement supports the finding that domain 3 is the primary site of interaction between EGFR and EGF (Lax et al., 1989) and argues that interactions with EGF that involve other portions of sEGFR, if they occur, are weak. In developing a model for EGF-induced sEGFR dimerization, we will therefore assume that this KD (≈400 nM) is valid for EGF binding to the sEGFR monomer to form a 1:1 complex. We have not been able to generate isolated domain 1 from sEGFR to determine its independent EGF binding characteristics. However, while previous studies suggest that domain 1 does interact with EGF, it appears to do so much less strongly than domain 3 (Lax et al., 1990, 1991b; Woljtj et al., 1992).

How does EGF induce sEGFR dimerization?

Using the facts that neither EGF nor sEGFR dimerize independently, that EGF forms a 1:1 monomeric complex with domain 3 of sEGFR (KD ≈400 nM) and that EGF binding can induce complete sEGFR dimerization under appropriate conditions, we have developed an equilibrium thermodynamic model for EGF-induced sEGFR dimerization. As will be described, this model is consistent with all of our studies, as well as those presented elsewhere in the literature. Following the approach of Levitzki and Schlessinger (1974) and Wofisy et al. (1992), the equilibria describing each possible two-species binding event can be written (see Table II) for a case in which the ligand

### Table II. Equilibria describing a dimerization coupled ligand binding event

<table>
<thead>
<tr>
<th>Event</th>
<th>EGF binding</th>
<th>sEGFR dimerization</th>
</tr>
</thead>
<tbody>
<tr>
<td>R + L</td>
<td>K1</td>
<td>RL ⇔ R2L2</td>
</tr>
<tr>
<td>R2 + L</td>
<td>2K2</td>
<td>R2L ⇔ R2L2</td>
</tr>
<tr>
<td>R2L + L</td>
<td>K2/2</td>
<td>RL + R ⇔ R2L2</td>
</tr>
</tbody>
</table>

Simple sigmoidal titrations were obtained, showing that a single class of sites exists. EGF forms a 1:1 complex with sEGFRd3, in an exothermic reaction (ΔH = −2 ± 0.8 kcal/mol), with an average KD of 480 ± 186 nM. Since the small ΔH of this interaction made it difficult to measure a precise KD, surface plasmon resonance studies were also performed, which gave a similar value for KD of 440 nM (data not shown). We were not able to detect sEGFRd3 dimerization upon EGF binding either in gel filtration or chemical cross-linking experiments (data not shown), suggesting that the KD value measured here reflects interaction of EGF with an sEGFRd3 monomer. The KD value is very similar to that reported for EGF binding to sEGFR in several studies (Greenfield et al., 1989; Günther et al., 1990; Hurwitz et al., 1991; Lax et al., 1991a; Zhou et al., 1993; Brown et al., 1994). At the low (nanomolar range) concentrations of sEGFR employed for these reported binding studies, EGF-induced sEGFR dimerization is not detectable (Greenfield et al., 1989; Günther et al., 1990), suggesting that the measured KD reflects EGF binding to monomeric sEGFR. It can be argued, therefore, that EGF binding to monomeric sEGFR has approximately the same KD as EGF binding to isolated domain 3. This agreement supports the finding that domain 3 is the primary site of interaction between EGFR and EGF (Lax et al., 1989) and argues that interactions with EGF that involve other portions of sEGFR, if they occur, are weak. In developing a model for EGF-induced sEGFR dimerization, we will therefore assume that this KD (≈400 nM) is valid for EGF binding to the sEGFR monomer to form a 1:1 complex. We have not been able to generate isolated domain 1 from sEGFR to determine its independent EGF binding characteristics. However, while previous studies suggest that domain 1 does interact with EGF, it appears to do so much less strongly than domain 3 (Lax et al., 1990, 1991b; Woljtj et al., 1992).

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1:1 EGF–sEGFR complex. The particular value that we employed was measured in studies of EGF binding to an isolated subdomain of sEGFR (domain 3) that is incapable of ligand-induced dimerization.

The 621 amino acid extracellular domain of EGFR can be divided into four subdomains (1–4 from the N- to C-termini) on the basis of amino acid sequence homology (Lax et al., 1988a,b). Subdomains 2 (residues 160–310) and 4 (residues 475–621) are cysteine rich, with a pattern of conserved cysteines that resembles the structural motif found in the TNF receptor extracellular domain (Ward et al., 1995). Subdomains 1 (residues 1–160) and 3 (residues 310–475) share 37% sequence identity (Lax et al., 1988a), and have both been implicated in EGF binding in experiments involving domain deletion, interspecies domain swapping and affinity cross-linking (Lax et al., 1989, 1991b; Wu et al., 1990; Woljtj et al., 1992). Domain 3 itself has also been isolated from sEGFR using limited proteolysis (Kohda et al., 1993), arguing that it is an independently folded domain, which may also be true for the other subdomains. The domain 3 fragment (sEGFRd3), containing amino acids 302–503 of EGFR, was shown to bind TGF-α with a KD of ~1 μM, but not to dimerize upon TGF-α binding (Kohda et al., 1993). Using ITC, we analyzed EGF binding to sEGFRd3, for which a representative titration is shown in Figure 4.
(L) does not self-associate (as demonstrated for EGF). A subset of these equilibria can describe completely any model for dimerization of sEGFR (R) upon binding of EGF (L).

The intrinsic binding and dimerization constants (equal to \(1/K_\alpha\)) that describe these events are interdependent, and only four of the six described in Table II are required to describe the system completely (if the nature of the product is assumed to be independent of the way it is formed). The four equilibria that we consider can be chosen, based upon experimental accessibility, to minimize the number of variables. SAXS analysis showed that sEGFR at concentrations up to 0.1 mM does not dimerize significantly (<5%) in the absence of EGF. Therefore \(K_j\) is \(<500 \text{ M}^{-1}\). In addition, following the arguments outlined above, we will assume that the dissociation constant \((K_\alpha)\) for EGF binding to monomeric sEGFR (without resulting dimerization) is \(<400\) nM, corresponding to a binding constant \(K_j\) of \(10^5 \text{ M}^{-1}\). Using these starting values for \(K_\alpha\) and \(K_j\), the two remaining variables are \(K_\beta\) and \(K_\gamma\) which will attempt to fit. We can write expressions for the concentration of each species in the system:

\[
[R_2] = K_\alpha [R]^2
\]

\[
[RL] = K_\gamma [R][L]
\]

\[
[R2L] = 2K_\beta [R][L] = 2K_\gamma K_\beta [R]^2 [L]
\]

\[
\]

where \([R]\) = concentration of free sEGFR and \([L]\) = concentration of free EGF. It should be noted that the values of \([R]\) and \([L]\) are significantly smaller than those of \([R_2]\) and \([L_2]\) under the conditions of the experiments reported here and, therefore, must be treated explicitly. It follows from Equations 1–4 that:

\[
[R_2] = [L] + K_\alpha [R] + 2K_1 K_\beta [R][L] + 2K_\gamma K_1^2 [R]^2 [L]^2
\]

\[
[RL] = [R] + 2K_\beta [R][L] + K_\gamma [R][L]
\]

\[
[R2L] = 4K_\beta [R][L] + 2K_\gamma K_1^2 [R]^2 [L]^2
\]

\[
[R2L2] = 2(K_\alpha + 2K_\beta K_\gamma [L] + 2K_\gamma K_1^2 [L]^2) [R]^2 + (1 + K_1 [L])[R]
\]

Using the quadratic formula to solve Equation 7 for \([R]\), Equation 8 is generated (see foot of page), which expresses \([R]\) in terms of \([L]\), \([R_2]\), and the relevant binding constants.

Likewise, Equation 5 can be solved for \([L]\). Using Equation 8, curves describing the relationship between \([R]\) and \([L]\) can be generated for any set of values for \([R_2]\), \(K_\gamma\), \(K_\beta\) and \(K_\gamma\). From the values of \([L]\) and \([R]\) defined by this relationship, the concentration of each species of interest can be calculated for any condition using Equations 1–6.

The SAXS experiments described above provide a monitor of changes in the weight-averaged molecular mass (\(M_w\)) of the species in solution as the [EGF]_tot:[sEGFR]_tot ratio is increased. To assess the agreement between the experimental SAXS data and any model proposed for EGF-induced sEGFR dimerization (defined by \(K_\beta\) and \(K_\gamma\), with \(K_\alpha\) and \(K_1\) fixed as described above), the above equations can be used to calculate the change in \(M_w\) predicted by the model as EGF is added. The molecular mass of sEGFR (\(M_\alpha\)) is 62 kDa, and that of EGF (\(M_\beta\)) is 6.2 kDa. The absolute value of I(0)_abs measured in a SAXS experiment is proportional to \(\Sigma n_i M_i^2\), for all values of \(i\), where there are \(i\) species that have molar concentration \(n_i\) and molecular mass \(M_i\). I(0) values determined in this study were normalized using only the mass concentration of sEGFR (= [R]_tot \times M_\alpha); the added EGF was neglected. Therefore, the normalized I(0) for any EGF/sEGFR mixture with known \([L]_\text{tot}\) [I(0)_[L]_tot] is proportional to \(\Sigma n_i M_i^2 / [R]_\text{tot} M_\alpha\), and the normalized I(0) for sEGFR alone is proportional to \(M_\alpha\) and can be written:

\[
\frac{[L]_\text{tot}}{I(0)_{\text{EGF}}} = \frac{\Sigma n_i M_i^2}{[R]_\text{tot} M_\alpha} = \frac{[L]_\text{tot}}{I(0)_{\text{EGF}}} = \frac{\Sigma n_i M_i^2}{[R]_\text{tot} M_\alpha}
\]

Since we are interested only in the fold-increase in \(M_w\) over that for monomeric sEGFR, values for each \(M_i\) can be considered simply as multiples of \(M_\alpha\). Thus, \(M_{R_2} = 2M_\alpha\), \(M_{RL} = 1.06 M_\alpha\), \(M_{R2L} = 2.06 M_\alpha\), \(M_{R2L2} = 2.11 M_\alpha\), and \(M_\alpha = 0.06 M_\alpha\). The observed fold-increase in the mass concentration-normalized I(0) for a given value of \([L]_\text{tot}\) [I(0)_[L]_tot], as plotted in Figure 5, is then:

\[
\frac{RI_\text{tot}}{R_{H/L}} = \frac{[R] + 4[R]_2 + 1.12 [RL]}{[R]_\text{tot}} + 4.23 [R_2 L] + 4.46 [R_2 L_2]
\]

\[
+ 3.2 \times 10^{-3} [L] + [R]_\text{tot}
\]

With \([R]_\text{tot}\) fixed at 65 \(\mu\)M, Equation 10 was used to calculate the expected behavior of I(0)_[L]_tot as the ratio of \([L]_\text{tot}\) to \([R]_\text{tot}\) was increased in the scattering samples. This fitting procedure was first performed for a series of values of \(K_\gamma\) with \(K_\beta = 1\) and \(K_1 = 2.5 \times 10^6 \text{ M}^{-1}\) (for reasons described above). The fits were found to be completely insensitive to variations in \(K_\gamma\) within the limits \((\gamma <500 \text{ M}^{-1})\) defined above. The value of \(K_\gamma\) that gave the best fit to the experimental data was guided initially by inspection, and then by monitoring \(\chi^2\) for the fit to the data. Using the initial estimate of \(K_\beta\), a similar approach was used to obtain a best-fit value for \(K_\beta\). Reasonable fits to the experimental data could only be obtained with \(K_\beta\) in the range \(5 \times 10^2 - 3 \times 10^4 \text{ M}^{-1}\), and \(K_1\) in the range \(1 \times 10^4 - 1 \times 10^6 \text{ M}^{-1}\). The best-fit values, after several iterations, were \(1 \times 10^4 \text{ M}^{-1}\) and \(3 \times 10^5 \text{ M}^{-1}\) for \(K_\beta\) and \(K_1\), respectively. Figure 5 shows the level of agreement between model calculations using these \(K_\beta\) and \(K_1\) values and the SAXS data. \(\chi^2\) for the best fit is 0.0941, with 20 degrees of freedom. Figure 5 also gives a view of the sensitivity of the fit to variations in \(K_\gamma\) (Figure 5B) and \(K_\beta\) (Figure 5C). Although the SAXS experiment was performed under conditions close to an end-point titration,
Fig. 5. Best fits to the SAXS I(0) data. The fold increase in $M_w$ over that for sEGFR $[M_w \text{ (mixture)}/M_w \text{ (sEGFR)}]$, upon addition of EGF, was calculated for different values of $K_\beta$ and $K_\gamma$ as described in the text. Individual points and error bars correspond to the experimental SAXS data presented in Figure 3. (A) The final best fit, with the fit parameters and $\chi^2$ noted. (B) and (C) The degree of sensitivity of the fit to variations in $K_\beta$ and $K_\gamma$ respectively. The unbroken line shows the best fit, while broken lines (as listed in the key) depict the closeness of fit when $K_\gamma$ or $K_\beta$ is increased or reduced by a factor of 5. (B) shows that a 5-fold change in $K_\gamma$ markedly worsens the fit. (C) shows that, while increases in $K_\beta$ worsen the fit considerably, reductions do not. The best-fit value for $K_\beta$ is therefore best considered as a maximum value.

It is clear that a 5-fold increase in either binding constant leads to an inferior fit. Similarly, a 5-fold decrease in $K_\gamma$ results in a poor fit, although reductions in the value of $K_\beta$ used in our model do not make it significantly worse. The best-fit value for $K_\beta$ ($1 \times 10^4$ M$^{-1}$) is therefore best considered as a maximum value. Values for $K_1$–$K_3$ estimated from this fitting procedure are listed in Table III.

To determine whether the best-fit model obtained by analysis of the SAXS data is consistent with the ITC results presented above, attempts were made to reproduce the shape of the ITC titration curves by ascribing heats to the different binding events. During this process, it quickly became clear that the cumulative heat absorbed in the progress of the forward ITC titration (EGF into sEGFR) closely resembles the predicted accumulation

| Table III. Predicted binding constants for EGF-induced sEGFR dimerization obtained from the best-fit model |
|----------------|------------------|
| Binding constant | Best-fit values for $K_a$ (M$^{-1}$) |
| $K_1$ (experimental)$^a$ | $2.5 \times 10^6$ |
| $K_2$ | $5 \times 10^7 = K_2 = 2.5 \times 10^10$ |
| $K_3$ | $7.5 \times 10^9$ |
| $K_\alpha$ | $< 5 \times 10^2$ |
| $K_\beta$ | $= 1 \times 10^4$ |
| $K_\gamma$ | $= 3 \times 10^5$ |

$^a$See text for explanation.
curve for $R_2L_2$. It was then found that the observed ITC results can be reproduced very closely simply by assuming a significant positive enthalpy (10 kcal/mol) for dimerization of RL (the $\Delta H$ component of $K_r$), and a small negative enthalpy ($-2$ kcal/mol) for binding of EGF to monomeric sEGFR (the $\Delta H$ component of $K_1$). These are the only two binding events that occur to a significant extent according to the model (see below). The value for the $\Delta H$ component of $K_1$ that gives the best fit to the ITC data is equal to $\Delta H$ for EGF binding to sEGFRd3, lending further confidence to this fit. The prediction using these parameters is compared with the experimental ITC data in Figure 6A; note that the heat per injection (as opposed to cumulative heat) is plotted against the [sEGFR]<sub>Tot</sub>/<R]<sub>Tot</sub> ratio. As mentioned above, reversed titrations of sEGFR into a solution of EGF gave very similar curves. By solving the quadratic Equation 5 for [L]<sub>Tot</sub>, the results of such a reversed ITC experiment at fixed [L]<sub>Tot</sub> were predicted using the same $\Delta H$ values and other parameters (Figure 6B). Although the precision of the agreement between predicted and experimental data is poorer than that in Figure 6A, the shape of the titration is clearly reproduced. The poorer agreement may result in part from errors in measuring [sEGFR] (>20 mg/ml) in the solution used for this single titration.

**Elements of a model for EGF-induced sEGFR dimerization**

As shown in the previous section, the model represented by the equilibrium constants listed in Table III can predict adequately the results obtained from both our ITC and SAXS experiments. The main features of this model are as follows. (i) sEGFR does not dimerize significantly in the absence of EGF. (ii) EGF binds to monomeric sEGFR with a $K_{D1}$ ($1/K_1$) of 400 nM, to form the RL complex. (iii) RL may interact weakly ($K_D = 1/2K_2 \approx 50$ μM) with another receptor molecule to yield the $R_2L_2$ complex, but this species does not accumulate significantly under the conditions explored here (Figure 7). (iv) RL associates much more readily with a second molecule of RL ($K_D = 1/K_3 \approx 3.3$ μM) to yield the $R_2L_2$ dimer. This species predominates upon EGF binding when [sEGFR] $\geq 1/K_r$, while RL predominates when [sEGFR] $\leq 1/K_r$ (Figure 7).

(v) The RL complex can be considered as the primary intermediate in the formation of $R_2L_2$, which is the only form of sEGFR dimer that occurs to a significant extent under the conditions studied.

**EGF-induced dimerization of sEGFR shows a concentration dependence that can account for its occurrence in the cell membrane**

The model presented here provides an explanation for the varying ability of others to detect EGF-induced sEGFR dimerization. Figure 7 shows how RL, $R_2L_2$, and $R_2L_2$ are predicted to accumulate as the [L]<sub>Tot</sub> /[R]<sub>Tot</sub> ratio is increased, for four different values of [R]<sub>Tot</sub> corresponding to alternative experimental regimes. Calculations were performed with [R]<sub>Tot</sub> set at 65 μM to model SAXS experiments; with [R]<sub>Tot</sub> = 20 μM to model ITC experiments; with [R]<sub>Tot</sub> = 5 μM to model a typical sEGFR cross-linking experiment (Hurwitz et al., 1991); and with [R]<sub>Tot</sub> = 6.5 nM to model the interactions at concentrations commonly used in EGF binding assays for sEGFR (Lax et al., 1991a). RL and $R_2L_2$ are the predominant species under all conditions, with a small amount of $R_2L_2$ occurring only under the conditions of SAXS or ITC experiments. When [R]<sub>Tot</sub> approaches 1/$K_r$ (3.3 μM), RL becomes the predominant form and, under the conditions of reported Scatchard analyses ([R]<sub>Tot</sub> in the nM range), $R_2L_2$ formation is negligible. [R]<sub>Tot</sub> must be at least several micromolar for significant sEGFR dimerization to be detected, since the $K_D$ for dissociation of $R_2L_2$ (1/$K_2$) is $\approx 3.3$ μM. Indeed, where significant sEGFR dimerization was reported previously, experiments were performed at concentrations ranging from 2 to 170 μM (Hurwitz et al., 1991; Lax et al., 1991a; Brown et al., 1994), while reports in which sEGFR dimerization was not detected employed density gradient centrifugation with final sEGFR concentrations ranging from 0.1 to 0.5 μM (Greenfield et al., 1989; Günther et al., 1990).

Unlike sEGFR, intact EGF in a cell membrane is restricted to diffusion in two, rather than three, dimensions. EGF also has at least one degree of rotational freedom less than the soluble ligand binding domain. As a result, dimerization of EGF-bound EGF in a cell membrane will be a significantly more favorable reaction than dimerization of the EGF–sEGFR complex studied here. Most cells that respond mitogenically to EGF contain ~10<sup>4</sup>–10<sup>5</sup> receptors per cell. By considering the mean distance between receptor molecules, and translating this from a two-dimensional (membrane) to a three-dimensional case, these numbers correspond to effective receptor concentrations of ~1–10 μM (Schlessinger, 1979). As described above, significant EGF-induced sEGFR dimerization occurs at these concentrations, arguing that our estimated value for $K_r$ is sufficient to account for EGF-induced EGF dimerization at the cell surface. The additional orientational restrictions of EGF receptors in the cell membrane will favor the energetics of EGF-induced dimerization still further. Thus, our model does not require that additional interactions involving the transmembrane and cytoplasmic domains of intact EGFR be invoked in stabilizing the ligand-induced dimer, although it is likely that these regions will contribute, perhaps significantly. If the
The accumulation of the different forms of monomeric and dimeric sEGFR were predicted according to the model obtained from the best-fit in Figure 5 (Table III). The accumulating species were predicted for four different values of $[R]_{\text{Tot}}$, corresponding to (A) a SAXS experiment ($[R]_{\text{Tot}} = 65 \mu M$); (B) an ITC experiment ($[R]_{\text{Tot}} = 20 \mu M$); (C) a chemical cross-linking experiment ($[R]_{\text{Tot}} = 5 \mu M$); and (D) a Scatchard analysis as performed in the literature (Lax et al., 1991). The effective concentration of EGFR in the cell membrane is significantly greater than $1/2K_D$ (50 $\mu M$), then significant occurrence of the 1:2 EGF–EGFR dimeric complex would also be predicted by our model, particularly if transmembrane and cytoplasmic portions of the receptor contribute to dimerization. Thus, although we obtained no direct evidence for the occurrence of a 1:2 dimer, its occurrence is not excluded by our model.

**Cooperativity in EGF binding to sEGFR**

The model defined by the binding constants listed in Table III clearly involves cooperativity in EGF binding to sEGFR under conditions where the $R_2L_2$ dimer is formed. Simulated Scatchard plots are concave-down, indicating positive cooperativity, when $[R]_{\text{Tot}}$ is $>250 \text{ nM}$ (corresponding to the concentrations used for SAXS, ITC and chemical cross-linking experiments). The maximum predicted Hill constant at 50% saturation is 1.5 under the conditions of the SAXS experiments, falling to 1.1 when $[R]_{\text{Tot}} = 250 \text{ nM}$. Simulated Scatchard plots are linear for $[R]_{\text{Tot}}$ values below 250 nM, in agreement with the lack of apparent cooperativity in studies reported in the literature (Greenfield et al., 1989; Günther et al., 1990; Hurwitz et al., 1991; Lax et al., 1991a; Zhou et al., 1993; Brown et al., 1994). All of these studies employed sEGFR concentrations from 5 to 20 nM, where no sEGFR dimerization occurs, and the apparent $K_D$ reflects only $K_1$, since RL is the only species that forms.

Binding of EGF to purified, detergent-solubilized, intact EGFR at low concentration also gives linear Scatchard plots (Yarden et al., 1985; Yarden and Schlessinger, 1987a), and yields $K_D$ values ($=1/K_1$) similar to those obtained in studies of sEGFR. By contrast, Sherrill and Kyte (1996), in a detailed study of EGF binding to EGFR purified from detergent extracts of A431 cells, clearly observed a sigmoidal binding curve characterized by a Hill constant of 1.7 ± 0.5, which agrees closely with the maximum value predicted by our model. We suggest that these differences reflect differences in receptor concentration, and that positive cooperativity will be seen when the effective EGFR concentration is greater than ~250 nM.

There is one observation for EGF binding to EGFR that cannot be explained by our model. Scatchard analysis of EGF binding to cell membranes that contain EGFR usually yields concave-up plots, which are ascribed to heterogeneity in the binding affinities of the receptors (Berkers et al., 1991). In most cases, it is assumed that this Scatchard plot curvature reflects the existence of two (or more) different affinity classes of the receptor (Schlessinger, 1988). It has been difficult to determine the precise origin of this behavior. The degree of curvature seen in the binding curves varies between reports. It has also been found to be altered upon various treatments of the cell with, for example, activators of protein kinase C (Schlessinger, 1988) that may lead to ‘transmodulation’ of the receptor’s binding affinity. Efforts to generate an
equilibrium binding model that can adequately account for the concave-up plots seen for EGF binding to crude cell membranes have not been successful (Wofsy et al., 1992). Rather, additional sources of receptor heterogeneity (or even additional EGF binding sites) must be invoked in order to explain the data. The effects of receptor ‘transmodulation’ by enzymes such as PKC, which may alter EGF binding affinity, would not be accessible to the approaches used in this study. Another possible source of heterogeneity is heterodimerization of EGFR with other erbB receptor family members (see below). Whether interactions between the extracellular domains of these different receptors can explain the observed concave-up Scatchard plots seen for EGF binding to cell membranes is an interesting question that can be addressed using the approaches employed here.

To our knowledge, with the limitation that we cannot explain the Scatchard plots obtained for EGF binding to intact cells (which may reflect heterogeneities in the environment of the cellular receptor), the model that we describe here (Table III) is consistent with all previously reported studies of EGF binding to, and activation of, EGFR.

**Dimensions of sEGFR monomers and dimers**

In addition to molecular mass information, SAXS also provides information on changes in molecular dimensions that accompany sEGFR dimerization. Figure 8A shows how the radius of gyration \( R_G \) increases as the \([\text{EGF}]_{\text{Tot}} : [\text{sEGFR}]_{\text{Tot}} \) ratio is increased. \( R_G \) for the unliganded sEGFR is 35.7 Å, which increases as EGF is added, following roughly the same trend as seen for I(0). \( R_G \) reaches a maximum value of ~44 Å for the R2L2 complex. Using these \( R_G \) values for monomeric and dimeric sEGFR respectively, we checked that the model defined above can predict adequately the observed increase in \( R_G \) as the \([\text{EGF}]_{\text{Tot}} : [\text{sEGFR}]_{\text{Tot}} \) ratio is increased (calculating the \( z \)-average of the \( R_G \) of monomers and dimers in solution).

As seen in Figure 8A, the fit is reasonably good.

Assuming that ~35% of the mass of the 110 kDa sEGFR monomer is carbohydrate, its volume can be estimated at ~130 000 Å\(^3\). A sphere of this volume would have radius 32 Å, and \( R_G \) ~24.8 Å, significantly smaller than the experimental value of 35.7 Å. Flattening the sphere to an oblate ellipsoid with axial ratio 5.6 (long semiaxes of ~56 Å, short semiaxis of 10 Å) would give approximately the correct \( R_G \) and volume, as well as the correct maximum dimension determined for sEGFR (110 Å). No prolate ellipsoid could simultaneously satisfy these constraints. The maximum dimension \( d_{\text{max}} \) is obtained from the radial Patterson, or pair-distance distribution, function \( P(r) \) that is derived by Fourier inversion of the scattering data (Figure 8B). The \( P(r) \) curve represents the length distribution of interatomic vectors in the molecule of interest, which will be a single distribution for a globular protein. Both sEGFR and the R2L2 complex give such a single distribution, indicating that the two sEGFR molecules are intimately associated in the dimeric complex. The value for \( d_{\text{max}} \) or longest interatomic distance in the distribution, is very similar for both monomeric and dimeric sEGFR: 110 and 120 Å respectively. This result may explain our failure to distinguish between monomeric and dimeric sEGFR in size-exclusion chromatography.

The relative \( d_{\text{max}} \) values for the monomer and dimer suggest that the sEGFR dimer is approximated by a pair of oblate ellipsoids with the dimensions described above, associated with their long axes parallel.

**Implications for EGF-induced EGFR dimerization**

As discussed above, the model for EGF-induced sEGFR dimerization involves formation of a 1:1 EGF:sEGFR (RL) complex \( (K_D = 1/K_I = 400 \text{ nM}) \), followed by dimerization of this complex with a \( K_D (1/K_I) \) of ~3.3 μM. The magnitudes of these equilibrium constants are suffi-
Dimerization of EGF receptor extracellular domain

![Diagram](image)

**Fig. 9.** A scheme depicting the proposed model for EGF-induced dimerization of sEGFR. EGF binds to a monomer of sEGFR (through interactions involving primarily domain 3) with $K_D \approx 400$ nM, to form a 1:1 EGF–sEGFR complex (RL). RL then dimerizes with a $K_D$ of $\approx 3.3 \mu M$ to form the R$_2$L$_2$ dimeric complex. EGF is shaded black and sEGFR gray. Two possibilities for RL dimerization are presented, with a schematic view from the top of the receptor (the membrane would be in the plane of the page). In one possibility (A), RL dimerization is mediated primarily by interactions involving EGF (ligand-mediated). EGF binds to domain 3 on each of the two sEGFR molecules, leaving its putative domain 1-interacting site unoccupied. Dimerization of RL could then be driven by cooperation of two EGF–domain 1 interactions, with a possible additional contribution from direct inter-receptor interactions (shown by contact between the two receptors). In (B), the other possibility (receptor-mediated), EGF binding (to domain 3) results in conformational changes that expose a receptor–receptor interaction site. RL can interact significantly only with another RL complex through this dimerization site, to yield the R$_2$L$_2$ dimer.

A **Ligand-Mediated**

B **Receptor-Mediated**

One appeal of the scheme in Figure 9A is that it does not require major conformational changes in the receptor, likely to lie somewhere between the two extremes. There are several arguments, however, that can be made in favor of the ligand-mediated proposal. The key argument is that the thermodynamics of EGF binding to monomeric sEGFR are very similar to those describing EGF binding to isolated domain 3. If ligand-induced conformational changes were major, a greater difference might be expected when subdomains 1, 2 and 4 are removed by proteolysis. Domain 1 of sEGFR shares 37% amino acid identity with domain 3, and has also been implicated in EGF binding by affinity cross-linking studies (Lax et al., 1988a; Wolter et al., 1992). It is possible that domain 1 contributes weakly to EGF binding to monomeric sEGFR, and we cannot detect its removal since interactions with domain 3 predominate. However, it is equally possible that, as proposed in Figure 9A, an EGF molecule bound to domain 3 of one sEGFR molecule interacts with domain 1 of its partner in the ligand-stabilized dimer. With $K_\gamma = 3 \times 10^5$ M$^{-1}$, the energy stabilizing RL dimerization is $\approx 7.5$ kcal/mol. In the ligand-mediated scheme of Figure 9A, each EGF–domain 1 interaction could contribute 3.75 kcal/mol, equivalent to a $K_D$ for EGF binding to domain 1 alone of $\approx 1.8$ mM. A binary interaction of this strength would not have been detected in any of the studies presented in the literature. Receptor–receptor interactions would probably contribute further to stabilization of the dimer, but the primary driving force would be simultaneous bivalent binding of two EGF molecules. Dimerization of sEGFR by a single EGF would only involve a single EGF–domain 1 interaction, and would occur only at very high effective receptor concentration. The value for $K_D$ in our model (Table III), together with consideration of both the effective concentration of EGF at the cell surface and the rotational restrictions on the membrane-bound receptor, argues that this event is unlikely, but cannot be excluded.

One appeal of the scheme in Figure 9A is that it does not require major conformational changes in the receptor, such as may be required to create the receptor–receptor interaction site depicted in Figure 9B. Studies employing circular dichroism and fluorescence measurements indicate that the conformational alterations elicited by EGF binding are limited in extent (Greenfield et al., 1989). Distinction between the two possibilities presented in Figure 9 will require structural studies of the complex. Determination of the crystal structure of the complex, which has not yielded after a decade of effort by many groups, would be invaluable.

**Possible implications for heterodimerization of erbB receptors**

The ligand-mediated model (Figure 9A), in which EGF is bivalent, suggests a possible mechanism by which EGF and the other seven (or more) different members of the EGF-like family of growth factors can induce heterodimerization of different erbB receptors (Carraway and Cantley, 1994; Hynes and Stern, 1994; Lemmon and Schlessinger, 1994). EGF itself has been shown to induce
the formation of heterodimers between EGFR and erbB2 (King et al., 1988; Stern and Kamps, 1988; Qian et al., 1992; Spivak-Kroizman et al., 1992). This case has been recapitulated with the extracellular domains alone of erbB2 and EGFR (Spivak-Kroizman et al., 1992). Heterodimerization of EGFR with erbB3 (Solloft et al., 1994), and erbB4 (Cohen et al., 1996) is also thought to be induced by EGF; and the heregulins are thought to induce the formation of other heterodimers involving erbB3 and/or erbB4 (Riese et al., 1995). It has also been found that heregulin and EGF binding are mutually antagonistic to cells that express both EGFR and erbB4, despite the fact that EGF binds only to EGFR and the heregulin binds only to erbB4 (Karunagaran et al., 1995). If EGF and the other EGF family members are bivalent as depicted in Figure 9A, heterodimerization could result from their simultaneous binding to two erbB receptors. EGF, TGF-β, HB-EGF, amphiregulin, betacellulin and epiregulin might all bind similarly to domain 3 of EGFR, but might differ in their proposed domain 1 binding region. Each would then be expected to induce a distinct complement of erbB heterodimers. Indeed, the pattern of responses elicited by each of these ligands, in a given cell type that expresses multiple erbB receptors, has been found to be different in detail (Beerli and Hynes, 1996; Riese et al., 1996). A bivalent mode of ligand interaction is also suggested by the report that substitution of the amino-terminus of EGF by that from heregulin-β generates a bifunctional ligand that binds both EGFR and erbB3/4 (Barbacci et al., 1995). Betacellulin is also bifunctional, binding both EGFR and erbB4 (Riese et al., 1996). Such bifunctional ligands are likely to induce heterodimerization of EGFR with erbB4, and are likely to do so via bivalent interactions. Further detailed studies of ligand binding to, and hetero- and homodimerization of, erbB family extracellular domains are required. One such study has been reported for the extracellular domains of erbB2 and erbB3, using analytical ultracentrifugation (Horan et al., 1995). Neither heregulin-induced homodimerization of erbB3 nor erbB2–erbB3 heterodimerization could be detected. The erbB3 extracellular domain formed a 1:1 monomeric complex with heregulin β2. These studies were performed at significantly lower concentrations (6 μM) than those employed in our studies of sEGFR, and may not adequately account for the difference in diffusional freedom between the two-dimensional (membrane-bound) and three-dimensional (free in solution) cases.

Comparison with ligand-induced dimerization of other receptors

As described in the Introduction, a common theme has emerged from studies of ligand-induced receptor dimerization in which the ligand species is bivalent, and binds simultaneously to two receptor molecules. Studies of hGH-induced dimerization of its receptor initiated this paradigm. The studies presented here for EGF-induced sEGFR dimerization do not fit this paradigm precisely. EGF is a monomeric ligand that induces receptor dimerization by forming a 2.2 complex with its receptor. In the scheme of Figure 9, the receptor-mediated possibility (Figure 9B) would make this a special case. The ligand-mediated proposal (Figure 9A), however, would make it a variation on the theme, requiring the binding of two, rather than one, bivalent ligand moieties for ligand-induced dimerization. Each EGF molecule would bind asymmetrically to the EGFR dimer, contacting one receptor through a high-affinity site (domain 3), and the other through a low-affinity site (domain 1), thus broadly resembling hGH in their mode of association with the complex. Indeed, the model presented in Figure 9A resembles a symmetrical version of the sequential mechanism proposed for hGH-induced dimerization of hGH-R (Wells, 1996). Given this similarity, we favor the ligand-mediated mechanism over the receptor-mediated mechanism for EGF-induced dimerization of sEGFR.

Finally, Sherrill and Kyte (1996) recently described detailed studies of EGF-induced dimerization and activation of intact detergent-solubilized EGFR as a function of both EGF and receptor concentration. From their studies, the model developed for EGF-induced receptor activation agrees remarkably well in its characteristics with the model that we have described here. The actual values estimated for the equilibrium constants are different between the two models. This is expected, since one study was performed with whole EGFR restricted to detergent micelles, while the other (presented here) was performed with sEGFR, which has additional rotational and translational degrees of freedom. One requirement of the model described by Sherrill and Kyte (1996), which was not addressed in our studies, is that, if the R-L complex does occur to a significant extent, it is not activated. This finding further argues that formation of the R-L complex described here is the key event in EGFR signaling.

Materials and methods

Production of sEGFR and sEGFRd3

sEGFR was produced by overexpression inCHO cells, as previously described (Lax et al., 1991b). sEGFR was purified from conditioned medium by immunoaffinity chromatography employing mAb 108, a monoclonal antibody against the extracellular domain of EGFR. sEGFR was eluted from the immunoaffinity column and further purified essentially as described (Lax et al., 1991b). sEGFR contains residues 1–621 of the mature receptor. sEGFRd3 was prepared from purified sEGFR by limited proteolysis with proteinase K, as described (Kohda et al., 1993). The resulting ~35 kDa (glycosylated) fragment includes residues 295–505 of EGFR, including the complete subdomain 3 as originally defined by Lax et al. (1988b), which encompasses residues 310–474. The identity of the products in each case was confirmed by N-terminal sequencing and quantitative amino acid analysis. Purified recombinant EGF (human) was purchased from InterGen (New York, USA). Purified recombinant TG-α was purchased from Bachem (Basel, Switzerland). Molar extinction coefficients (at 278 nm) were determined by quantitative amino acid analysis of aliquots of protein solutions with known absorbance. The values determined were as follows: sEGFR, 58 500 M–1 cm–1; sEGFRd3, 17 100 M–1 cm–1; hEGF, 14 400 M–1 cm–1; TG-α, 1500 M–1 cm–1.

Isothermal titration calorimetry

All ITC studies employed the Omega instrument (MicroCal, Northampton, MA; Wiseman et al., 1989) in the laboratory of Professor Julian Sturtevant (Department of Chemistry, Yale University). For each titration, both the sEGFR variant and EGF were dialyzed into the same reaction buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 3.4 mM EDTA). Each titration was performed at 25°C. A typical titration (Figure 2) involved serial injections of 20×10 μl aliquots of EGF (130 μM) into a solution of sEGFR (12 μM) in the calorimeter cell (volume 1.39 ml). For each case, control experiments were performed to determine the heat of mixing of the components. Heats of mixing were constant throughout the titration, and the measured constant value was subtracted from the heat per injection prior to analysis of the data. Data were analyzed with ORIGIN software (MicroCal), using the fitting algorithms provided.
Small-angle X-ray scattering experiments

Samples of ~20 μl were pipetted into a 2 mm path-length quartz capillary tube for collection of SAXS data (the same capillary was used for each set of measurements). The X-ray source employed was a Rigaku RU-300 rotating anode generator, operating at 50 kV and 180 mA, producing a 1.5 Å Cu-Kα radiation. The beam was pinhole collimated with an incident beam diameter of 0.6 mm. A two-dimensional multidetector with 256×144 pixels, and a sensitive area of 290×288 mm² was placed 1 m from the sample holder. The two-dimensional scattering pattern obtained was both circularly and time averaged. The forward scattering intensity, I(0), and the radius of gyration (Rq) were obtained by least-squares linear fitting to the Guinier plots in the region where QRq < 1. The Pr(0) functions were calculated using the program GNOM (Semenyuk and Svergun, 1991). The data collection time for each protein solution and the buffer blanks was 10 000 s. The buffer blank was collected several times during each set of measurements, and the scattered intensity from the buffer was used to monitor the drift in the beam intensity. sEGF and EGF (or TGF-α) were buffer-exchanged into 50 mM HEPES, pH 7.5, 100 mM NaCl, 3.4 mM EDTA for all experiments, and were present at the concentrations noted in the text.

Chemical cross-linking experiments

Studies of the ability of EGF (20 μM) to enhance covalent cross-linking of the various forms of sEGF (10 μM) were performed using the cross-linking reagent disuccinimidyl suberate (DSS), exactly as described (Lax et al., 1991b). The reaction products were analyzed by SDS–PAGE and stained with Coomassie blue.

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References


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