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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 homo- and heterodimerization 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 $(FGF)_n$–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). EGFR 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.

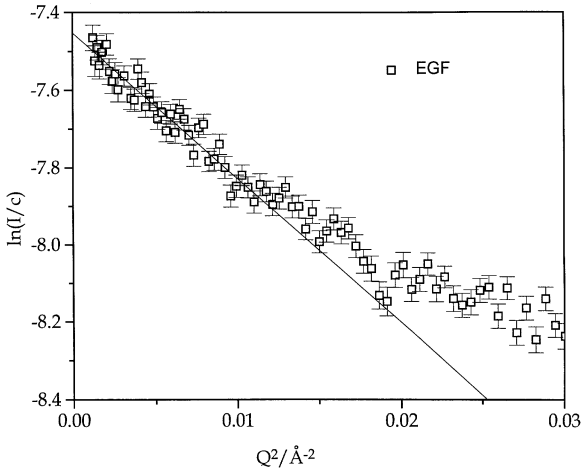

Fig. 1. SAXS data obtained for EGF at 8.7 mg/ml (1.36 mM), represented in the form of a Guinier plot. The intensity of scattered radiation (I) was normalized using the mass concentration of EGF (c). Ln (I/c) is plotted against Q^2, where $Q = 4\pi \sin\theta/\lambda$; λ is the wavelength of the X-ray 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 $(R_G)^2/3$, giving an R_G 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 K_D values for EGF binding, which range from 100 to 500 nM. To determine directly the stoichiometry of this

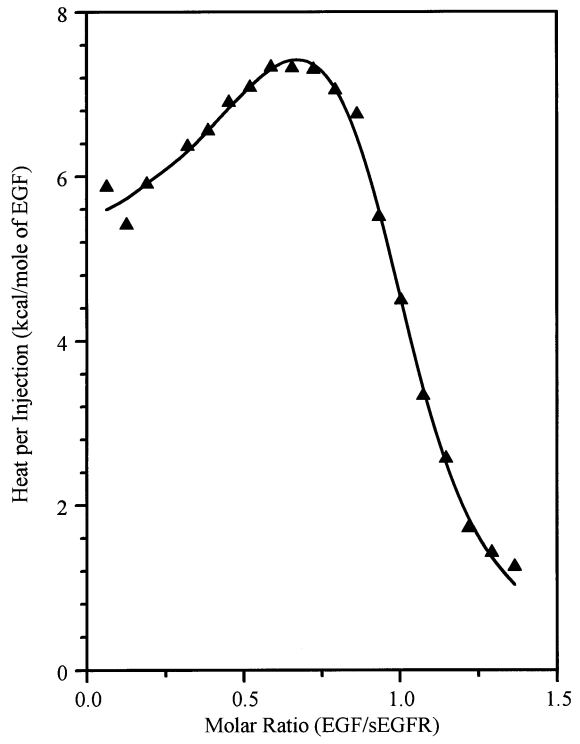

Fig. 2. Representative ITC data for binding of EGF to sEGFR. Aliquots (20×10 μM) of EGF (130 μM) were injected into a solution of sEGFR (12 μM) present in the calorimeter cell (volume 1.39 ml) at 25°C, as described in Materials and methods. Each point represents the integrated heat absorbed (since the reaction is endothermic) for an individual injection, normalized by the amount of ligand added (kcal/mol of EGF). The first point has been neglected. The solid line represents the simple initial fit assuming two independent classes of site, that subsequently was found to be incorrect, as described in the text.

binding, and to analyze the thermodynamics of EGF binding to sEGFR, we employed ITC. The titrations (Figure 2) allow several clear statements to be made about EGF binding to sEGFR. Since heat is absorbed throughout the titrations, the overall enthalpy of binding (ΔH) at 25°C is positive and hence unfavorable. The net free energy of binding in this endothermic reaction is therefore derived from the entropy of binding. This contrasts with receptor binding by hGH (Cunningham *et al.*, 1991), EPO (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 $(FGF)_n$–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 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

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

Ligand	Oligomeric state	Stoichiometry of receptor binding[a]	Reference
Multivalent			
hGH	monomer	1:2	Cunningham *et al.* (1991)
EPO	monomer	1:2	Philo *et al.* (1996a)
IFN-γ	dimer	1:1	Walter *et al.* (1995)
aFGF	oligomer with HSPGs	1:1	Spivak-Kroizman *et al.* (1994)
PDGF	dimer	1:1	Fretto *et al.* (1993)
Neurotrophins	dimer	1:1	Philo *et al.* (1994)
TNF-β	trimer	1:1	Banner *et al.* (1993)
Valence unclear			
G-CSF	monomer	1:1	Horan *et al.* (1996)
EGF	monomer	1:1	this work

[a]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, incompletely resolved, 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 EGF-induced 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 ($M_{\bar{w}}$) 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 μM, 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 $M_{\bar{w}}$) increased significantly (Figure 3). This increase in $M_{\bar{w}}$ was maximal (2.2-fold) at an $[EGF]_{Tot}$:$[sEGFR]_{Tot}$ 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-α binding (data not shown). Since the molecular mass of sEGFR is ~110 kDa, while that of EGF or TGF-α is just 6.2 kDa, doubling of $M_{\bar{w}}$ 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 $[EGF]_{Tot}$:$[sEGFR]_{Tot}$ 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-α therefore differ from hGH in their mode of ligand-induced receptor dimerization.

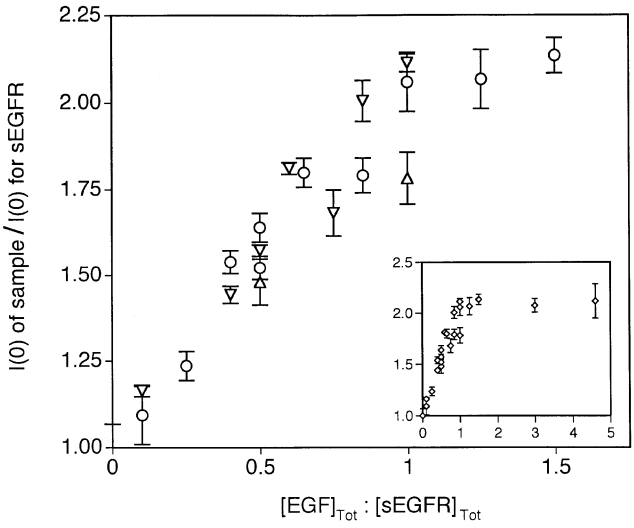

Fig. 3. SAXS analysis of sEGFR dimerization upon addition of EGF. The I(0) was measured, as described in Materials and methods, for samples of sEGFR to which EGF had been added at the $[EGF]_{Tot}$:$[sEGFR]_{Tot}$ ratios noted. I(0) was normalized using the mass concentration of sEGFR Each point represents a single experiment, corresponding to a 10 000 s measurement period for that sample. Normalized I(0) values were divided by the normalized I(0) measured for free sEGFR, to give a fold-increase [I(0) of sample/I(0) for sEGFR] that corresponds to the fold-increase in weight-averaged molecular mass $M_{\bar{w}}$ (see text). The fold-increase in I(0) is given for three series of experiments performed at sEGFR concentrations of 45 (▽), 64 (△) and 82 μM (○) respectively. The inset is an expanded version of the main graph, showing data for the complete range of $[EGF]_{Tot}$:$[sEGFR]_{Tot}$ ratios studied. It is clear that EGF-induced sEGFR dimerization is complete for $[EGF]_{Tot}$:$[sEGFR]_{Tot}$ = 1:1, and no higher order oligomerization occurs.

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

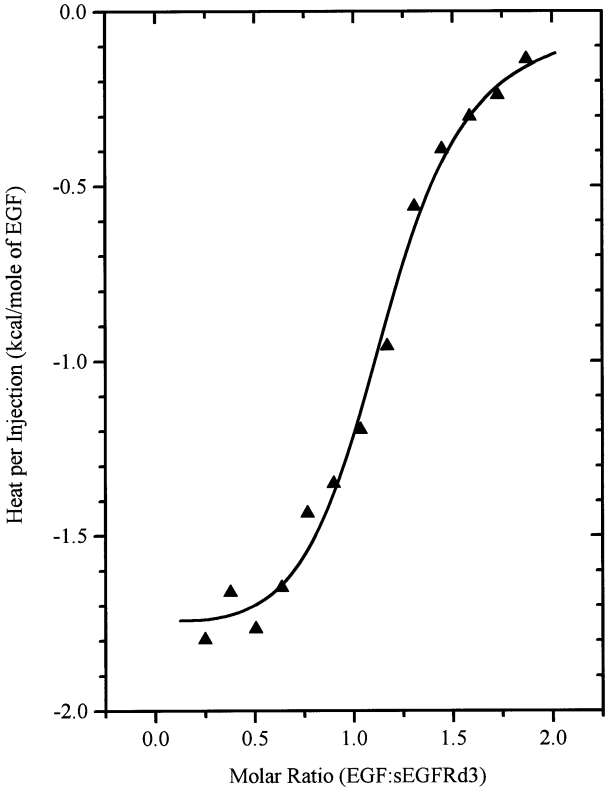

Fig. 4. Representative ITC data for binding of EGF to sEGFRd3 (see Materials and methods). Aliquots (18 μl) of EGF (175 μM) were injected into sEGFRd3 (17 μM) present in the calorimeter cell at 25°C. Each point represents the integrated heat per mole of injectant (EGF) for that particular injection. Note that, in contrast to the EGF–sEGFR titration (Figure 2), the heats are negative (although very small) in this case, since EGF binding to sEGFRd3 is exothermic. The solid line represents the best fit to this particular set of data, for which stoichiometry is 1.10:1 (EGF:sEGFRd3); K_β = 1.9 (±0.8)×10^6 M^{-1}; and ΔH = –1.8 (±0.1) kcal/mol.

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; Woltjer *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 K_D 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.

Table II. Equilibria describing a dimerization coupled ligand binding event

EGF binding			sEGFR dimerization		
R + L	$\overset{K_1}{\Leftrightarrow}$	RL	R + R	$\overset{K_\alpha}{\Leftrightarrow}$	R_2
R_2 + L	$\overset{2K_2}{\Leftrightarrow}$	R_2L	RL + R	$\overset{2K_\beta}{\Leftrightarrow}$	R_2L
R_2L + L	$\overset{K_3/2}{\Leftrightarrow}$	R_2L_2	RL + RL	$\overset{K_\gamma}{\Leftrightarrow}$	R_2L_2

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 K_D of 480 ± 186 nM. Since the small ΔH of this interaction made it difficult to measure a precise K_D, surface plasmon resonance studies were also performed, which gave a similar value for K_D, 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 K_D value measured here reflects interaction of EGF with an sEGFRd3 monomer. The K_D 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 K_D reflects EGF binding to monomeric sEGFR. It can be argued, therefore, that EGF binding to monomeric sEGFR has approximately the same K_D 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 K_D (≈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; Woltjer *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 (K_D ≈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 Wofsy *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

(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 $1/K_D$) 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_α is $\leqslant 500\ M^{-1}$. In addition, following the arguments outlined above, we will assume that the dissociation constant (K_D) for EGF binding to monomeric sEGFR (without resulting dimerization) is ≈400 nM, corresponding to a binding constant K_1 (= $1/K_D$) $\approx 2.5\times10^6\ M^{-1}$. Using these starting values for K_α and K_1, the two remaining variables are K_β and K_γ, which we will attempt to fit. We can write expressions for the concentration of each species in the system:

$$[R_2] = K_\alpha [R]^2 \quad (1)$$

$$[RL] = K_1 [R] [L] \quad (2)$$

$$[R_2L] = 2K_\beta [RL] [R] = 2K_1 K_\beta [R]^2 [L] \quad (3)$$

$$[R_2L_2] = K_\gamma [RL]^2 = K_\gamma K_1^2 [R]^2 [L]^2 \quad (4)$$

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 $[R]_{Tot}$ and $[L]_{Tot}$ under the conditions of the experiments reported here and, therefore, must be treated explicitly. It follows from Equations 1–4 that:

$$[L]_{Tot} = [L] + K_1 [R] [L] + 2K_1 K_\beta [R]^2 [L] + 2K_\gamma K_1^2 [R]^2 [L]^2 \quad (5)$$

$$[R]_{Tot} = [R] + 2K_\alpha [R]^2 + K_1 [R] [L] + 4K_1 K_\beta [R]^2 [L] + 2K_\gamma K_1^2 [R]^2 [L]^2 \quad (6)$$

$$= 2(K_\alpha + 2K_1 K_\beta [L] + 2K_\gamma K_1^2 [L]^2)[R]^2 + (1+K_1 [L])[R] \quad (7)$$

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_{Tot}, 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 $[R]_{Tot}$, K_1, K_α, K_β and K_γ. 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 ($M_{\bar{w}}$) 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_β and K_γ, with K_α and K_1 fixed as described above), the above equations can be used to calculate the change in $M_{\bar{w}}$ predicted by the model as EGF is added. The molecular mass of sEGFR (M_R) is ~110 kDa, and that of EGF (M_L) 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 (= $[R]_{Tot}\times M_R$): the added EGF was neglected. Therefore, the normalized I(0) for any EGF/sEGFR mixture with known $[L]_{Tot}$ [$I(0)_{LTot}$] is proportional to $\Sigma n_i M_i^2/[R]_{Tot}M_R$, and the normalized I(0) for sEGFR alone is proportional to M_R. We can therefore write:

$$\frac{I(0)_{L_{Tot}}}{I(0)_{sEGFR}} = \frac{\Sigma n_i M_i^2}{[R]_{Tot} M_R^2} \quad (9)$$

Since we are interested only in the fold-increase in $M_{\bar{w}}$ over that for monomeric sEGFR, values for each M_i can be considered simply as multiples of M_R. Thus, $M_{R2} = 2M_R$, $M_{RL} = 1.06M_R$, $M_{R2L} = 2.06M_R$, $M_{R2L2} = 2.11M_R$, and $M_L = 0.06M_R$. The observed fold-increase in the mass concentration-normalized I(0) for a given value of $[L]_{Tot}$ [$I(0)_{LTot}$], as plotted in Figure 5, is then:

$$\frac{I(0)_{L_{Tot}}}{I(0)_{sEGFR}} = \frac{[R] + 4[R_2] + 1.12\ [RL]}{[R]_{Tot}} + \frac{4.23[R_2L] + 4.46[R_2L_2]}{[R]_{Tot}} + \frac{3.2\times10^{-3}[L]}{[R]_{Tot}} \quad (10)$$

With $[R]_{Tot}$ fixed at 65 μM, Equation 10 was used to calculate the expected behavior of $I(0)_{LTot}$ as the ratio of $[L]_{Tot}$ to $[R]_{Tot}$ was increased in the scattering samples. This fitting procedure was first performed for a series of values of K_γ, with $K_\beta = 1$ and $K_1 = 2.5\times10^6\ M^{-1}$ (for reasons described above). The fits were found to be completely insensitive to variations in K_α within the limits ($K_\alpha \leqslant 500\ M^{-1}$) defined above. The value of K_γ that gave the best fit to the experimental data was guided initially by inspection, and then by monitoring χ^2 for the fit to the data. Using the initial estimate of K_γ, a similar approach was used to obtain a best-fit value for K_β. Reasonable fits to the experimental data could only be obtained with K_β in the range 5×10^2–$3\times10^4\ M^{-1}$, and K_γ in the range 1×10^5–$1\times10^6\ M^{-1}$. The best-fit values, after several iterations, were $1\times10^4\ M^{-1}$ and $3\times10^5\ M^{-1}$ for K_β and K_γ respectively. Figure 5 shows the level of agreement between model calculations using these K_β and K_γ values and the SAXS data. χ^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_γ (Figure 5B) and K_β (Figure 5C). Although the SAXS experiment was performed under conditions close to an end-point titration,

$$[R] = \frac{-(1 + K_1[L]) + \sqrt{(1 + K_1[L])^2 - 8R_{Tot}(K_a + 2K_1K_\beta[L] + K_\gamma K_1^2[L]^2)}}{4(K_\alpha + 2K_1K_\beta[L] + K_\gamma K_1^2[L]^2)} \quad (8)$$

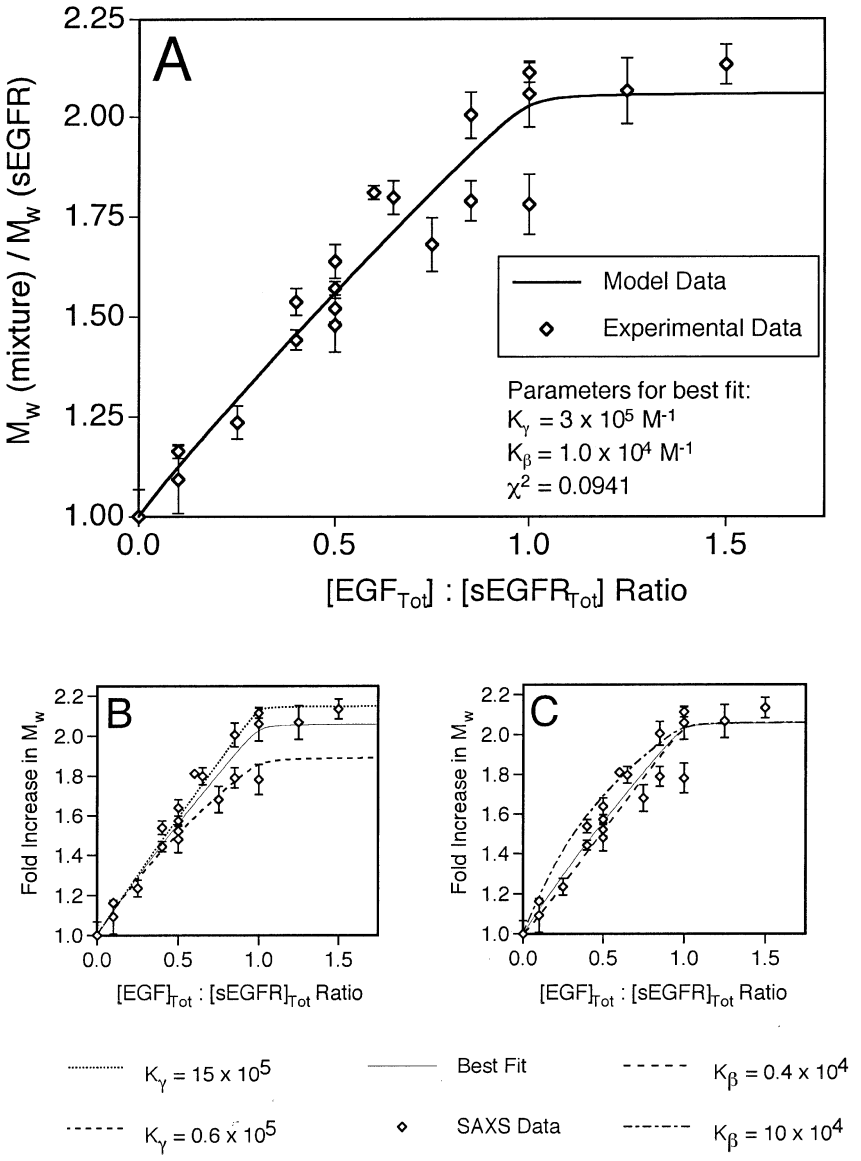

Fig. 5. Best fits to the SAXS I(0) data. The fold increase in $M_{\bar{w}}$ over that for sEGFR [$M_{\bar{w}}$ (mixture)/$M_{\bar{w}}$ (sEGFR)], upon addition of EGF, was calculated for different values of K_β and K_γ as described in the text. Individual points and error bars correspond to the experimental SAXS data presented in Figure 3. (**A**) The final best fit, with the fit parameters and χ^2 noted. (**B**) and (**C**) The degree of sensitivity of the fit to variations in K_γ and K_β respectively. The unbroken line shows the best fit, while broken lines (as listed in the key) depict the closeness of fit when K_γ or K_β is increased or reduced by a factor of 5. (B) shows that a 5-fold change in K_γ markedly worsens the fit. (C) shows that, while increases in K_β worsen the fit considerably, reductions do not. The best-fit value for K_β 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_γ results in a poor fit, although reductions in the value of K_β used in our model do not make it significantly worse. The best-fit value for K_β (1×10^4 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_n (M^{-1})
K_1 (experimental)[a]	2.5×10^6
K_2	$5\times10^7 \leqslant K_2 \leqslant 2.5\times10^{10}$
K_3	$\approx 7.5\times10^7$
K_α	$<5\times10^2$
K_β	$\approx 1\times10^4$
K_γ	$\approx 3\times10^5$

[a]See text for explanation.

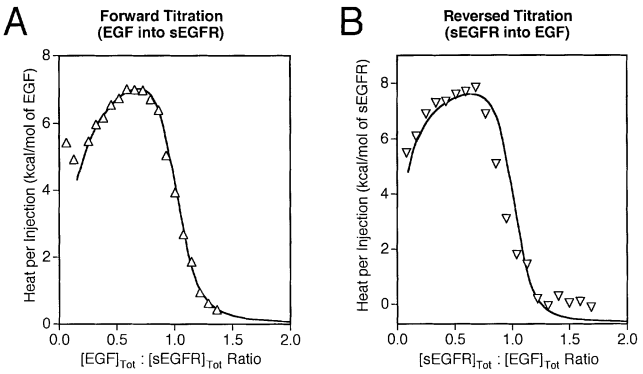

Fig. 6. The model defined by fitting to the SAXS data can adequately account for the shapes of titrations obtained in ITC experiments. ITC data (as shown in Figure 2) were predicted (solid lines) according to the parameters that define the best fit in Figure 5. Assuming only a ΔH value of –2 kcal/mol for EGF binding to monomeric sEGFR (ΔH_1) and a ΔH value of +10 kcal/mol for dimerization of the monomeric sEGFR:EGF (RL) complex (ΔH_γ), good agreement with the experimental data (triangles) for both (**A**) titration of EGF into sEGFR and (**B**) titration of sEGFR into EGF could be obtained.

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 ΔH component of K_γ), and a small negative enthalpy (–2 kcal/mol) for binding of EGF to monomeric sEGFR (the ΔH 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 ΔH component of K_1 that gives the best fit to the ITC data is equal to ΔH 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 $[EGF]_{Tot}$:$[sEGFR]_{Tot}$ 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], the results of such a reversed ITC experiment at fixed $[L]_{Tot}$ were predicted using the same ΔH 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_D ($1/K_1$) of 400 nM, to form the RL complex. (iii) RL may interact weakly ($K_D = 1/2K_\beta \approx 50$ μM) with another receptor molecule to yield the R_2L 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 ($K_D = 1/K_\gamma \approx 3.3$ μM) to yield the R_2L_2 dimer. This species predominates upon EGF binding when [sEGFR] $\geqslant 1/K_\gamma$, while RL predominates when [sEGFR] $\leqslant 1/K_\gamma$ (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, R_2L_2 and R_2 are predicted to accumulate as the $[L]_{Tot}$:$[R]_{Tot}$ ratio is increased, for four different values of $[R]_{Tot}$ corresponding to alternative experimental regimes. Calculations were performed with $[R]_{Tot}$ set at 65 μM to model SAXS experiments; with $[R]_{Tot}$ = 20 μM to model ITC experiments; with $[R]_{Tot}$ = 5 μM to model a typical sEGFR cross-linking experiment (Hurwitz *et al.*, 1991); and with $[R]_{Tot}$ = 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 occurring only under the conditions of SAXS or ITC experiments. When $[R]_{Tot}$ approaches $1/K_\gamma$ (3.3 μM), RL becomes the predominant form and, under the conditions of reported Scatchard analyses ($[R]_{Tot}$ in the nM range), R_2L_2 formation is negligible. $[R]_{Tot}$ must be at least several micromolar for significant sEGFR dimerization to be detected, since the K_D for dissociation of R_2L_2 ($1/K_\gamma$) is ≈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 EGFR in a cell membrane is restricted to diffusion in two, rather than three, dimensions. EGFR also has at least one degree of rotational freedom less than the soluble ligand binding domain. As a result, dimerization of EGF-bound EGFR 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 ~10^4–10^5 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_γ is sufficient to account for EGF-induced EGFR dimerization at the cell surface. The additional orientational restrictions of EGFR molecules 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

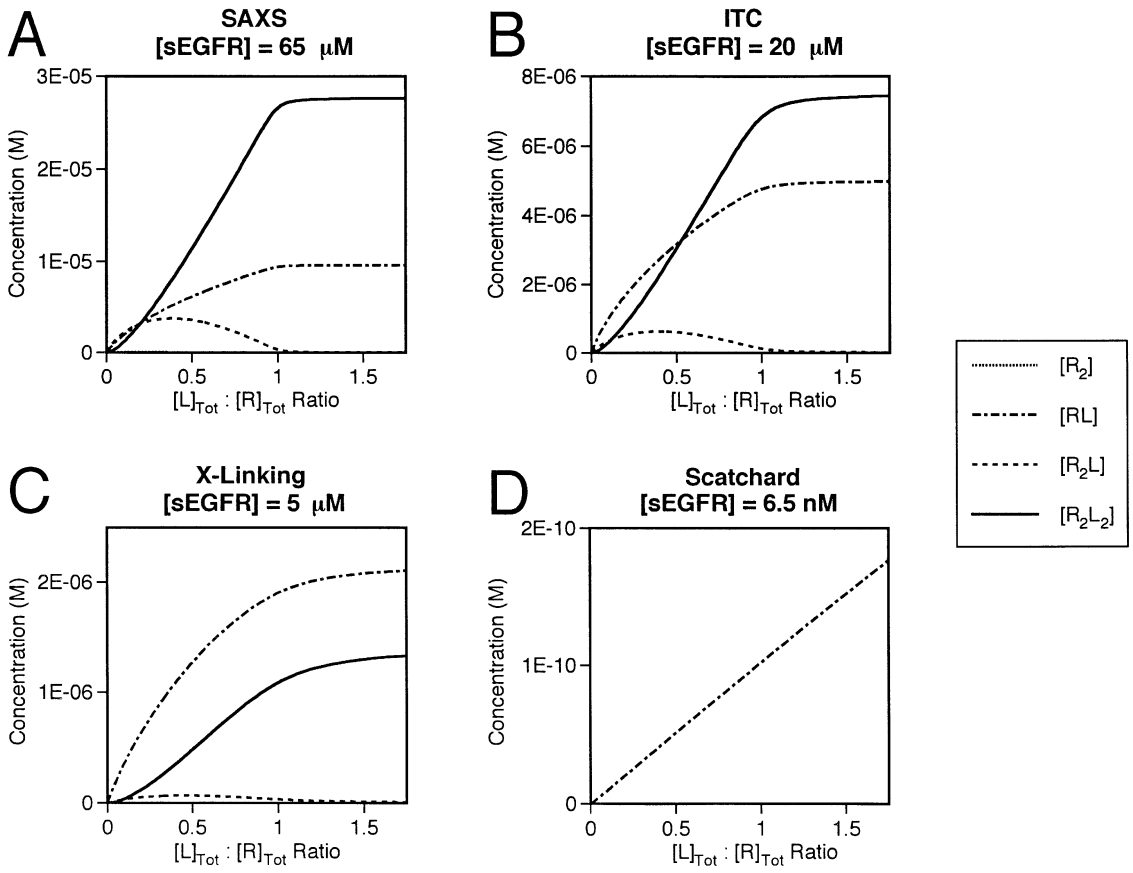

Fig. 7. The accumulation of the different forms of monomeric and dimeric sEGFR were predicted according to the model obtained from the best-fit in Figure 5 (Table III). The accumulating species were predicted for four different values of $[R]_{Tot}$, corresponding to (**A**) a SAXS experiment ($[R]_{Tot}$ = 65 μM); (**B**) an ITC experiment ($[R]_{Tot}$ = 20 μM); (**C**) a chemical cross-linking experiment ($[R]_{Tot}$ = 5 μM); and (**D**) a Scatchard analysis as performed in the literature (Lax *et al.*, 1991a) ($[R]_{Tot}$ = 6.5 nM).

effective concentration of EGFR in the cell membrane is significantly greater than $1/2K_\beta$ (50 μM), 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 $[R]_{Tot}$ is >250 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 $[R]_{Tot}$ = 250 nM. Simulated Scatchard plots are linear for $[R]_{Tot}$ 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 ($\approx 1/K_1$) 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 $[EGF]_{Tot}$:$[sEGFR]_{Tot}$ 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 R_2L_2 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 $[EGF]_{Tot}$:$[sEGFR]_{Tot}$ 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 contraints. The maximum dimension (d_{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 R_2L_2 complex give such a single distribution, indicating that the two sEGFR molecules are intimately associated in the dimeric complex. The value for d_{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 momomeric and dimeric sEGFR in size-exclusion chromatography.

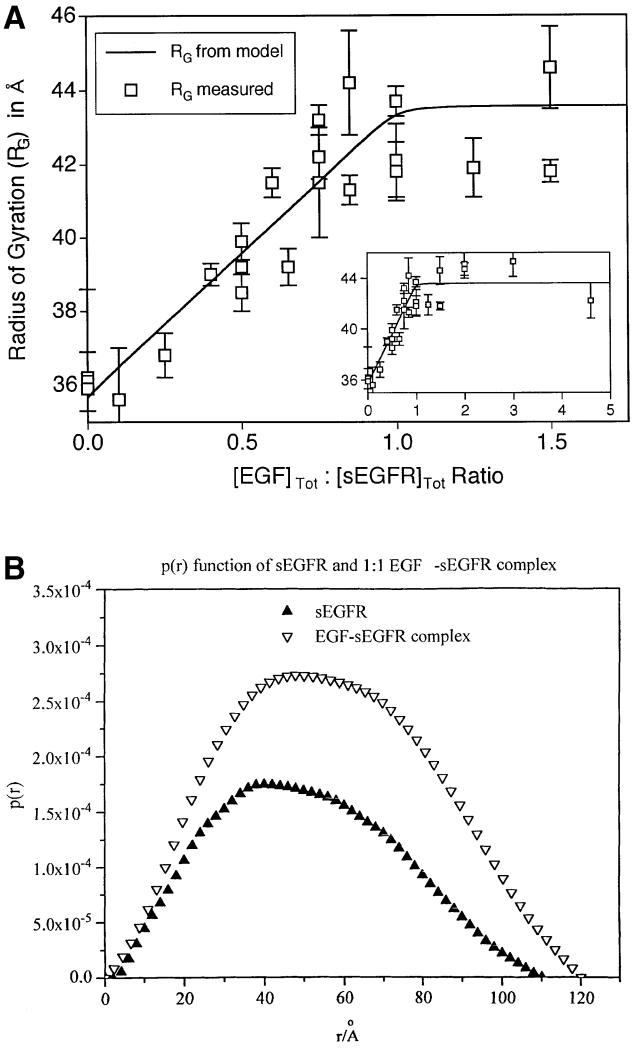

Fig. 8. (**A**) The radius of gyration (R_G), determined from Guinier analysis of the SAXS data that gave the I(0) measurements in Figure 3. Points represent the R_G for a given experiment at a particular $[EGF]_{Tot}$:$[sEGFR]_{Tot}$ ratio, with associated errors from least-squares linear fitting of the Guinier plot. The line through the points represents the predicted R_G according to the model defined by the binding constants in Table III. The concentration of sEGFR monomers and dimers was calculated from the model for each value of $[EGF]_{Tot}$:$[sEGFR]_{Tot}$, and the predicted apparent R_G was calculated as the *z*-weighted average of the R_Gs for monomeric (35.7 Å) and dimeric (44 Å) sEGFR. The inset is an expanded version of the main graph, showing measured and predicted R_G values over the entire range studied. (**B**) The radial Patterson, or pair-distance distribution, function [P(r)] calculated by Fourier inversion of the scattering data for monomeric sEGFR (▲) and the R_2L_2 complex (▽), using the program GNOM (Semeyuk and Svergun, 1991). The estimated d_{max} values are 110 and 120 Å for monomeric and dimeric sEGFR (R_2L_2) respectively.

The relative d_{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 ($K_D = 1/K_1 \approx 400$ nM), followed by dimerization of this complex with a K_D ($1/K_\gamma$) of ~3.3 μM. The magnitudes of these equilibrium constants are suffi-

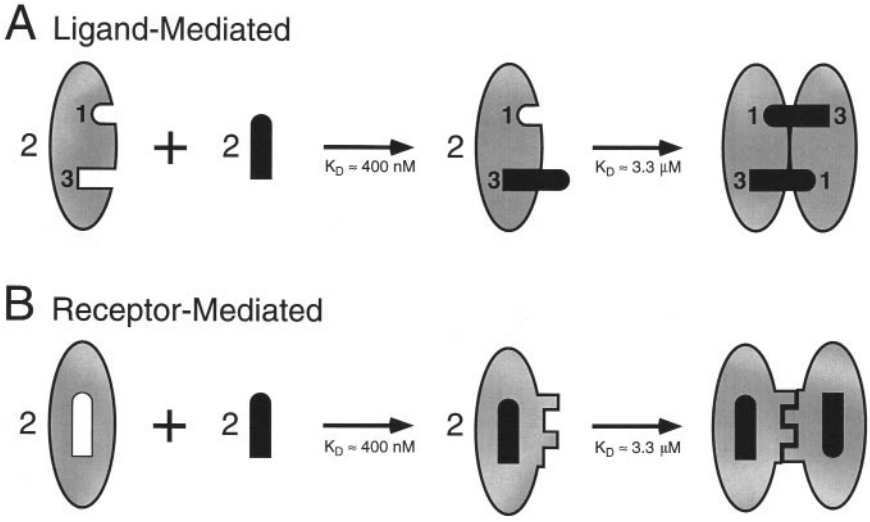

Fig. 9. A scheme depicting the proposed model for EGF-induced dimerization of sEGFR. EGF binds to a monomer of sEGFR (through interactions involving primarily domain 3) with $K_D \approx 400$ nM, to form a 1:1 EGF–sEGFR complex (RL). RL then dimerizes with a K_D of ~3.3 μM to form the R_2L_2 dimeric 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 possibilitiy (**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_2L_2 dimer.

cient to explain EGF-induced EGFR dimerization of the cell surface without requiring a role for other portions of the whole receptor. Formation of the R_2L complex, in which a single ligand molecule stabilizes the sEGFR dimer, is not predicted to be significant except at the very highest sEGFR concentrations (which could occur at the cell surface), and we obtained no direct evidence for its occurrence. As depicted in Figure 9, two possible modes for dimerization of the RL complex can be envisaged. At one extreme (Figure 9A), dimerization is mediated by the bound ligand molecules (ligand-mediated), and at the other by receptor–receptor contacts stabilized through ligand-induced conformational changes (receptor-mediated) (Figure 9B). It is not possible to distinguish between these possibilities from our studies, and the reality is 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; Woltjer *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 $K_\gamma = 3\times10^5\ M^{-1}$, the energy stabilizing RL dimerization is ~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 ~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_β in our model (Table III), together with consideration of both the effective concentration of EGFR 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 (Soltoff *et al.*, 1994), and erbB4 (Cohen *et al.*, 1996) is also thought 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-α, 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_2L complex does occur to a significant extent, it is not activated. This finding further argues that formation of the R_2L_2 complex described here is the key event in EGFR signaling.

Materials and methods

Production of sEGFR and sEGFRd3

sEGFR was produced by overexpression in CHO 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 TGF-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}; TGF-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 pipeted 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 1.5 Å Cu-K_α radiation. The beam was pinhole collimated with an incident beam diameter of 0.6 mm. A two-dimensional multiwire detector with 256×144 pixels, and a sensitive area of 290×288 mm^2 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 (R_G) were obtained by least-squares linear fitting to the Guinier plots in the region where $QR_G \leqslant 1$. The P(r) 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. sEGFR 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 sEGFR (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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