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Mechanism of epidermal growth factor receptor autophosphorylation and high-affinity binding

(receptor-tyrosine kinases/ligand-induced receptor dimerization)

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ABSTRACT **Epidermal growth factor (EGF) receptor monomers and noncovalently associated dimers were isolated by sucrose density gradient centrifugation, and their respective binding and autophosphorylation activities were determined. We find that monomers are low-affinity receptors and dimers are high-affinity receptors. In the absence of EGF, dimers exhibit a 4-fold higher autophosphorylation activity than do monomers. Addition of EGF increases autophosphorylation on monomers an average of 4.8-fold but has a minimal effect on autophosphorylation of dimers. Furthermore, EGF binding shifts the receptor monomer–dimer equilibrium to the dimer form. We conclude that EGF stimulates *in vitro* receptor autophosphorylation by inducing kinase-inactive receptor monomers to associate and form receptor dimers, in which conformation the autophosphorylation activity is enhanced.**

Epidermal growth factor (EGF) exerts growth-promoting activities in a wide range of epithelial and fibroblastic cells. It is widely accepted that EGF induces its biological effects through interactions with a specific cell membrane receptor, the EGF receptor, which is a single-chain, membrane-spanning protein of M_r 170,000 (1). The biochemical details of this interaction can be summarized as first binding of EGF to the extracellular portion of the receptor and then rapid phosphorylation of the cytoplasmic domain of the receptor, which is the result of the receptor's intrinsic tyrosine kinase activity (1–5).

EGF binding sites appear to be functionally heterogeneous because a small portion of the EGF receptor binds ligand with high affinity and a large portion binds ligand with low affinity (6, 7). The underlying structural difference between high- and low-affinity binding sites has not been described. The analysis of the protein sequence deduced from cDNA cloning predicts that the extracellular binding domain and the cytoplasmic kinase domain are connected by a single membrane-spanning sequence (8). This structural feature imposes apparent mechanistic constraints on transmembrane kinase activation and on kinase activation in purified preparations of detergent-solubilized receptors.

A possible mechanism could involve an association of two or more EGF receptor molecules such that protein–protein interaction could lead to the conformational changes required for kinase activation. The potential role of association between EGF receptor molecules was suggested previously: macroaggregation of EGF receptors was correlated with stimulation of mitogenesis by EGF (9). Also, anti-receptor antibodies that stimulate EGF receptor kinase function lose their activity when their monovalent Fab' fragments are used (10). Furthermore, the existence of the EGF receptor dimers in membranes was demonstrated by chemical crosslinking (11).

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In the present study, we have isolated native EGF receptor monomers and dimers from A431 cell membranes, and we have examined their potential to bind EGF and to undergo autophosphorylation, the latter being an assay for the signal transfer from the ligand-binding domain to the kinase domain. Furthermore, we examined the effect of EGF on the monomer–dimer equilibrium. The results strongly support a mechanism for *in vitro* kinase activation in which the key regulatory step is the EGF-dependent conversion of receptor monomers to dimers. These noncovalently associated receptor dimers exhibit a higher autophosphorylation activity than monomers and also have a higher affinity for EGF, and we postulate that a similar mechanism may also work in cells.

MATERIALS AND METHODS

Preparation of Soluble EGF Receptor. Membranes were isolated by shedding vesicles from A431 cells, and receptors were solubilized as described (1). The glycoprotein fraction was isolated by binding and elution to wheat germ agarose (WGA; E-Y Laboratories, San Mateo, CA) (12) in 30 mM Hepes/0.1% Triton X-100/0.02% azide, pH 7.6. Glycoproteins (100 μg) were fractionated on sucrose density gradients as described (13) with the following changes. The gradient was supplemented with 10% (vol/vol) glycerol, the centrifugation time was increased to 18 hr, and the applied *g* force was 200,000. During all purification steps, the buffers were supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 1 μM pepstatin A, 0.05 trypsin inhibitor units of aprotinin per ml, and 1 mM EDTA. Based on tracer EGF binding and autophosphorylation, we recovered 95% of the applied sample from sucrose gradients.

EGF Binding, Scatchard Analysis, and Receptor Autophosphorylation. ^{125}I-labeled EGF (New England Nuclear) binding was performed with a polyethylene glycol precipitation assay essentially as described for the insulin receptor (13, 14). Briefly, receptor was incubated for 2 hr at room temperature (binding reached equilibrium; data not shown) with 80 pM ^{125}I-labeled EGF. Nonspecific tracer binding was determined in the presence of 0.2 μM unlabeled EGF (Calbiochem) and was 10–13% of the total binding. Samples for Scatchard analysis were obtained by pooling receptor monomers and dimers from three gradients. The fractions of the monomer and dimer peaks were subjected to a competition binding in triplicate, and the data were analyzed by the LIGAND program (15).

For autophosphorylation, receptor preparations were incubated for 30 min at room temperature in the presence or absence of 0.2 μM EGF and were chilled on ice; phosphorylations were initiated by the addition of 15 μCi (1 μCi = 37 kBq) of [γ-^{32}P]ATP {50 μM ATP total; prepared from [^{32}P]orthophosphate (New England Nuclear) with a γ prep kit from Promega Biotec, Madison, WI}, 10 mM $MgCl_2$, and 4 mM

Abbreviation: EGF, epidermal growth factor.

$MnCl_2$ (final concentrations). After 8 min of incubation on ice, the autophosphorylation reaction was stopped either by adding 50 mM EDTA (final concentration) or by adding Laemmli sample buffer. Autophosphorylation reaches a plateau for all receptor species in less than 8 min.

Polyacrylamide Gel Electrophoresis and Immunoblotting. Samples were electrophoresed on 3–10% $NaDodSO_4$/polyacrylamide gels as described by Laemmli (16). Gels were transferred to nitrocellulose (Schleicher & Schuell) and immunoblotted as described (17) except that Carnation dry milk was used instead of fetal calf serum. The monoclonal anti-EGF receptor antibody used to blot receptor was obtained from ICN ImmunoBiologicals, and the second antibody, ^{125}I-labeled goat anti-mouse IgG, was purchased from New England Nuclear. Autoradiography was performed as described (13). Quantitation of EGF receptor by immunoblotting was achieved by cutting and assaying bands from blots. A standard curve of EGF receptor was constructed and was linear over the range of receptor concentrations we used. All experiments were performed at least three times, and the standard deviations are given in *Results*.

RESULTS

Isolation of Noncovalent EGF Receptor Dimers from A431 Membranes. We reasoned that noncovalently associated EGF receptor dimers would be more likely to form when the receptor concentration was high. Therefore, we partially purified and concentrated EGF receptors by wheat germ agarose affinity chromatography and subsequently fractionated monomeric and dimeric EGF receptors by sucrose density gradients under conditions similar to those used for separating monomeric and dimeric insulin receptor species (13). Because A431 cells are rich in EGF receptors (*ca.* 2×10^6 receptors per cell), this protein is the major component seen in silver-stained $NaDodSO_4$ gels of gradient fractions.

When tracer ^{125}I-labeled EGF binding to sucrose density gradient fractions was measured, two peaks of binding activity were observed (Fig. 1 *Lower*), corresponding to EGF receptor dimers (fractions 17–19) and to EGF receptor monomers (fractions 22–26). The gradients were calibrated with native insulin receptor $\alpha_2\beta_2$ (360 kDa), with the reduced insulin receptor $\alpha\beta$ half (180 kDa), and with ^{14}C-labeled marker proteins with known S values (data not shown; see ref. 13). In contrast to tracer binding, little, if any, receptor dimer was detected by immunoblotting at the exposure used (Fig. 1 *Upper*). In six different receptor preparations analyzed by sucrose gradients and immunoblotting, 24 ± 8% of the receptor sediments as dimers (see also Fig. 5). In all of these experiments, we noted that the relative amount of EGF receptor dimer detected by tracer EGF binding exceeded the relative amount of receptor dimer detected by immunoblotting. These data suggest that EGF receptor dimers might have a higher affinity for EGF than receptor monomers and, hence might bind more tracer EGF. Therefore, we determined the affinity of ligand for the two EGF receptor species.

EGF Receptor Dimers Are High-Affinity Binding Sites and Monomers Are Low-Affinity Binding Sites. We added up to 25% sucrose and observed no effect on tracer ligand binding (data not shown), thus ruling out the possibility that the higher sucrose concentration present in the dimer fractions is responsible for the higher ^{125}I-labeled EGF binding to this species. We then performed a binding isotherm to EGF receptor monomers and dimers and subsequently determined their affinities for ligand by Scatchard analyses. A representative Scatchard plot is shown in Fig. 2, where the dimer shows a higher affinity for EGF than does the monomer. In three separate experiments, the K_d for monomers averaged $1.9 \pm 0.4 \times 10^{-8}$ M, and the average K_d was $4.9 \pm 0.4 \times 10^{-9}$ M for the dimers. This result also rules out the possibility that

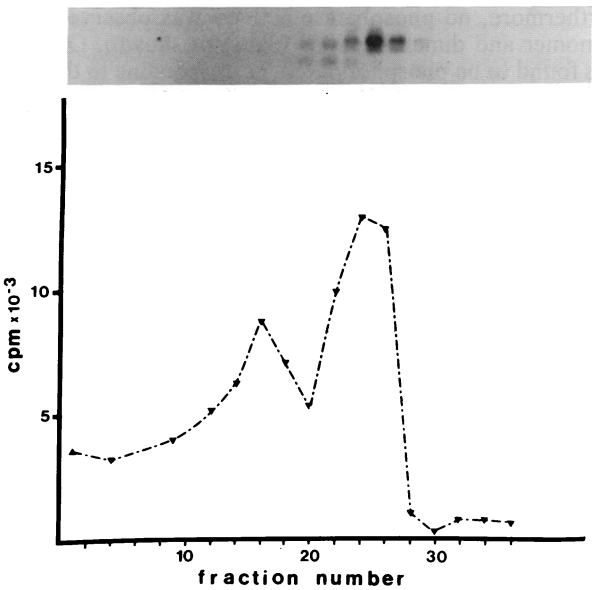

FIG. 1. EGF receptor monomers and dimers can be separated on sucrose density gradients. Soluble EGF receptor was fractionated on sucrose density gradients as described. Gradient fractions were subjected to EGF binding at tracer ligand concentration (80 pM), and the specific binding was plotted against the gradient fraction number (*Lower*). The smaller numbers correspond to the bottom of the gradient and the bigger numbers to the top. The autoradiogram (*Upper*) shows a 2-hr exposure of an immunoblot of gradient fractions with a monoclonal anti-EGF receptor antibody.

the polyethylene glycol assay preferentially precipitates dimers (thus, enhancing tracer binding) because this possibility would affect the measurable number of binding sites but not the K_d. Note that we made no attempt to equalize the amount of monomer and dimer in these Scatchard plots; thus, these results only apply to the differences in affinity of monomers and dimers for EGF.

Autophosphorylation of Dimers and Monomers. We separated EGF receptor monomers and dimers on sucrose gradients and subjected the peak fractions to autophosphorylation in the absence or the presence of saturating EGF concentrations. Again, control experiments showed that sucrose does not affect the autophosphorylation reaction.

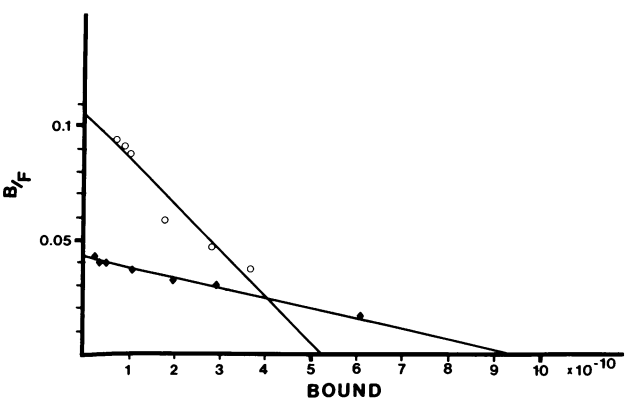

FIG. 2. EGF receptor dimers are high-affinity binding receptors and monomers are low-affinity receptors. EGF receptor monomers (♦) and dimers (○) isolated by preparative sucrose density gradient centrifugation were subjected to a competition binding assay with ^{125}I-labeled EGF at a final concentration of 80 pM as a tracer and with addition of increasing concentrations of unlabeled EGF. The resulting competition binding curves were transformed to Scatchard plots, and the affinities for EGF were determined with the aid of the LIGAND analysis (15).

Furthermore, no phosphatase activity was observed in the monomer and dimer fractions (data not shown). One band was found to be phosphorylated, corresponding to the intact 170-kDa form of the EGF receptor (Fig. 3 *Upper*). Note that the monomer fraction was more responsive than the dimer fraction to EGF. We examined this effect in more detail in another experiment (Fig. 3 *Lower*) by subjecting every fraction of the gradient to autophosphorylation and then quantitating the extent of phosphate incorporation. EGF significantly increased the autophosphorylation of the monomer fraction (fractions 22–27) but did not appreciably stimulate the autophosphorylation of the dimers in this preparation (fractions 17–21). In four different experiments, we found that the stimulation of autophosphorylation by EGF was an average of 1.4-fold in the dimer fraction and 4.8-fold in the monomer fraction (see also Fig. 4). In the case of autophosphorylation in the absence of EGF, monomers and dimers showed the same level of autophosphorylation (Fig. 3 *Lower*), whereas in immunoblotting experiments 76% of the receptors sedimented as monomers and 24% as dimers. These data suggest that the autophosphorylation capacity of receptor dimers is higher than that of monomers in the absence of EGF.

To test this possibility, we normalized the extent of autophosphorylation for the amount of intact 170-kDa species in each gradient fraction as determined by immunoblotting. This method is advantageous in comparison to other assays because it allows us to distinguish between the kinase-active 170-kDa form and the proteolytically derived lower molecular weight receptor forms that showed no autophosphorylation activity. Also, the different affinities of receptor monomers and dimers did not allow the use of tracer EGF binding to normalize the receptor amount. The normalized autophosphorylation data in each fraction were expressed as arbitrary units (Fig. 4). A comparison of the phosphorylation in the absence of EGF between monomers and dimers revealed that the dimers have a much higher autophosphorylation activity than the monomers. The average difference from three preparations was 4.1-fold. The EGF-stimulated autophosphorylation of the monomers corresponded to that obtained with the dimers in the absence of EGF. In some experiments, the dimers could not be further stimulated by the addition of EGF (see Fig. 3), whereas in other experiments, at most a 2-fold stimulation was observed (Fig. 4). The average stimulation was 1.4-fold. The increased kinase activity of dimers in the absence of added EGF was intrinsic to the dimers and was not the result of the presence of EGF, which remained bound to the receptor during the receptor isolation. EGF was efficiently removed during membrane solubilization in Triton X-100 (1.5%) and the subsequent steps of washing wheat germ agarose-bound receptor and of centrifugation. Labeled EGF, added to receptors prior to purification, was undetectable in sucrose gradient fractions (data not shown). Furthermore, the resulting dimer preparations were fully capable of binding tracer concentrations of EGF (see Fig. 2) with a high affinity. We conclude from these data (*i*) that the monomeric form is the reactive species with regard to EGF-stimulated autophosphorylation and (*ii*) that the dimeric form represents an already activated receptor form.

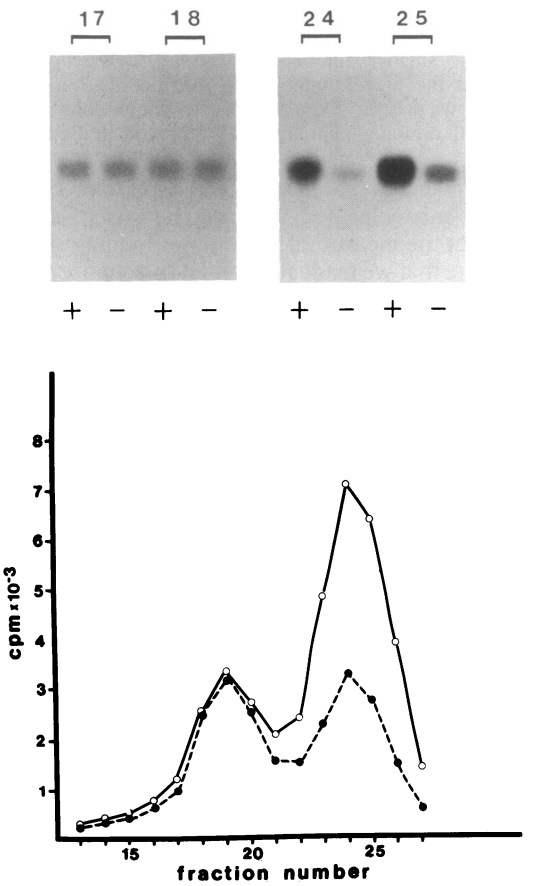

FIG. 3. Autophosphorylation of monomers and dimers in the absence or presence of EGF. (*Upper*) Monomer (lanes 24/25) and dimer fractions (lanes 17/18) with the highest binding activity were subjected to autophosphorylation in the presence (lanes +) or absence (lanes −) of EGF as described. The samples were separated in a 3–10% $NaDodSO_4$/polyacrylamide gel, and the bands were visualized by autoradiography. (*Lower*) In a separate experiment, each fraction of the gradient was subjected to autophosphorylation in the presence (——) or absence (----) of EGF, and separated in $NaDodSO_4$/polyacrylamide gels as described in *A*. The ^{32}P incorporation into receptor bands was quantitated by excising the bands and assaying the Cerenkov radiation.

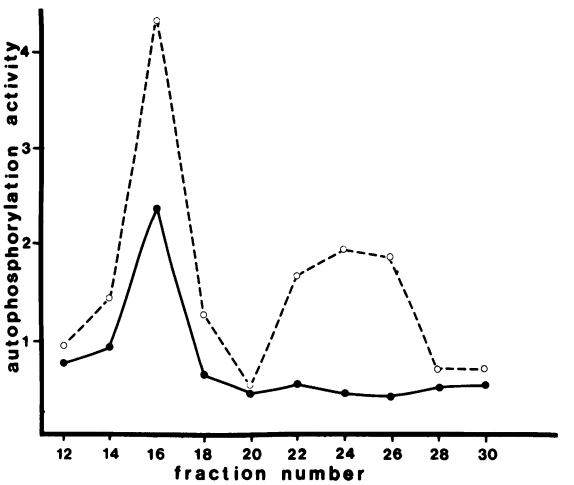

FIG. 4. Normalization of autophosphorylation for receptor amount. EGF receptor monomers and dimers were separated by sucrose density gradients and fractionated into 100-μl fractions. Of each fraction, 20 μl was subjected to autophosphorylation in the absence (——) or the presence (---) of EGF. The amount of ^{32}P incorporation was determined as described in Fig. 3. Of the fractions, 50 μl was subjected to immunoblotting as described, and the relative amounts of the intact 170-kDa receptor form was determined by using a standard curve. The autophosphorylation was then normalized for receptor amount and expressed in arbitrary units. Fractions 14–18 correspond to EGF receptor dimers, and 22–28 correspond to EGF receptor monomers.

EGF Shifts the Receptor Monomer–Dimer Equilibrium to the Dimer Form. The activated state of native dimers could possibly be the result of a receptor modification that occurred by some cellular factors *in vivo*. Alternatively, since we

observe *in vitro* that the monomeric form is most susceptible to kinase activation by EGF, it is also possible that EGF allows enhanced autophosphorylation to occur by inducing an association of receptor monomers to the dimeric species. To examine whether EGF indeed influences the monomer–dimer equilibrium, we treated receptor samples prior to centrifugation with near-saturating concentrations of EGF (0.2 μM) for 30 min at room temperature or with buffer alone. Subsequently, the monomers were separated from dimers by sucrose density gradient centrifugation. The gradient fractions were subjected to immunoblotting (Fig. 5 *Upper*), and the ^{125}I-labeled receptor bands were assayed for radioactivity (Fig. 5 *Lower*). The curve with the solid line shows the migration profile of the untreated receptor. As observed previously, most of the receptors sedimented in fractions 22–25, corresponding to the monomers. Upon addition of EGF, however, most of the receptor was found in fractions 17–19, corresponding to the EGF receptor dimers (dashed line). These data show that EGF binding induces a specific receptor dimerization.

EGF-induced receptor association also could be visualized by chemical crosslinking of receptor dimers (Fig. 6). In this experiment receptor samples incubated in the presence or absence of 0.2 μM EGF were crosslinked by using 2 mM disuccinimidyl suberate and then subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis and immunoblotting. Upon addition of EGF, receptors could be crosslinked and migrated as a covalent receptor dimer (lane 3). The dimeric receptor form was absent when no crosslinker was added (lane 1) and was present in low amounts when no EGF was added prior to crosslinking (lane 4). Apparently because of the poor efficiency of chemical crosslinking, the amount of dimers detected by this method was small. However, the method clearly shows that EGF promotes the dimer formation. This finding is entirely consistent with the results from the density gradient analysis, which allows detection of all dimers formed.

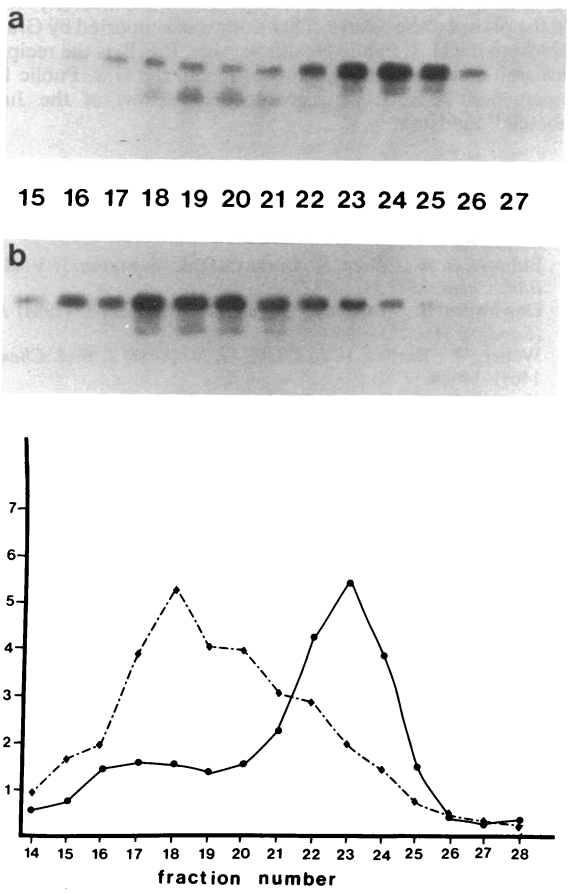

FIG. 5. EGF shifts the monomer–dimer equilibrium to the dimer form. (*Upper*) Soluble receptor that had been bound to and eluted from wheat germ agarose was treated for 30 min at room temperature with 0.2 μM EGF (*Upper b*) or with buffer as control (*Upper a*). The samples were separated on sucrose density gradients; for the EGF-treated sample, EGF was included throughout the gradient. The gradient fractions were then subjected to immunoblotting. Note that only half of fraction 17 was loaded. (*Lower*) The immunoblot was quantitated by excising the bands corresponding to the intact and proteolytically derived receptor forms and by counting the bound ^{125}I with a γ counter (*y*-axis). The label was plotted against the fraction number. ----, Migration profile of samples treated with EGF; ——, untreated control sample.

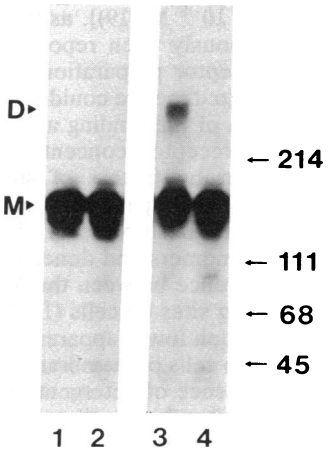

FIG. 6. Chemical crosslinking of EGF receptor dimers. The receptor-containing glycoprotein fraction (20 μg) was treated for 30 min at room temperature with 0.2 μM EGF (lanes 1 and 3) or with buffer (lanes 2 and 4). Subsequently the samples (lanes 3 and 4) were crosslinked on ice with 2 mM disuccinimidyl suberate according to previously published procedures (18). Lanes 1 and 2 were not crosslinked. Receptor bands were visualized by immunoblotting. D, EGF receptor dimer; M, EGF receptor monomer. The numbers denote the position of prestained molecular weight markers from Bethesda Research Laboratories shown × 10^{-3}.

DISCUSSION

In the present study we used an *in vitro* system to examine the potential significance of EGF receptor association for ligand-induced autophosphorylation. Using sucrose density gradient centrifugation, which allows determinations of the S value and hence the molecular weight, we found that an average of 24% of solubilized EGF receptors sediment as noncovalent receptor dimers and the rest as monomers. We do not think that these ratios correspond to the ratios of receptor monomers and dimers in native membranes because the receptor concentration and the environment is changed upon solubilization and purification, and, therefore, the dynamic equilibrium of receptor monomers and dimers is expected to be altered as well. However, the finding of noncovalently associated proteins on sucrose density gradients is indicative for strong and specific protein–protein interactions because reactants with affinities below the nanomolar range typically dissociate during centrifugation. We used this method to separate receptor monomers from receptor dimers and to subsequently characterize their functional properties.

To examine the binding properties of monomers and dimers, respectively, we subjected them to Scatchard analyses and determined that the dimers have an average K_d for EGF binding of 4.9×10^{-9} M, whereas the monomers have a K_d of 1.9×10^{-8} M. Similar affinity constants [namely, 1.33

$\times 10^{-8}$ M (1) and 3×10^{-8} M (19)], as we observe for the monomers, have previously been reported for unfractionated, soluble EGF receptor preparations. The finding that EGF causes receptors to dimerize could potentially compromise the determination of the binding affinity to the monomers. However, the receptor concentration used in the binding assay is lower by factors of 40–50 than in the EGF-induced dimerization experiment, and at this dilution substantial dimerization does not occur. The 4-fold affinity difference between monomers and dimers observed *in vitro* is lower than the difference between the K_d values of high- and low-affinity binding sites on cells (1, 19). However, the EGF receptor has a much lower apparent affinity for ligand in solution compared to cells or membranes (1, 19), probably due in part to the presence of detergent and/or absence of phospholipids. Our *in vitro* finding that the underlying structural difference between high- and low-affinity EGF binding is the receptor association state might apply also to receptors in membranes. This hypothesis is consistent with recent experiments of Dunn *et al.* (20), in which different vesicle pools were isolated, with predominantly high- or low-affinity binding EGF receptors having the same molecular weight and the same tryptic digestion pattern.

From the characterization of the autophosphorylation activity of monomers and dimers, we can draw the following conclusions. First, the monomeric receptor form is most susceptible to stimulation of autophosphorylation by EGF, whereas the dimers show a marginal increase of autophosphorylation in the presence of EGF. Second, when the autophosphorylation activity was normalized for the amount of kinase-active intact receptor, we found that, in the absence of EGF, the dimers exhibit on average a 4.1-fold higher kinase activity than do the monomers. The use of immunoblotting for normalization of the autophosphorylation activity allows us to distinguish between the phosphorylating 170-kDa receptor form and the nonphosphorylated 150-kDa receptor form, which partially cosediments in the monomer fractions. Therefore, we can exclude the possibility that the lower kinase activity of monomers is an artificial result obtained if both the amount of kinase-active and kinase-inactive receptor forms were used for normalization of receptor amount.

Two lines of evidence show that EGF induces a specific association of receptor monomers to dimers. First, if receptor is treated with saturating concentrations of EGF, most of the receptors sediment as noncovalent dimers on sucrose density gradients; second, covalent receptor dimers can be obtained upon treatment with EGF and subsequent chemical cross-linking. The finding that EGF influences the association state of the receptor together with the finding that the dimer represents the activated receptor form with regard to autophosphorylation strongly suggests that EGF induces autophosphorylation principally by promoting the association of monomeric receptor to the activated dimer species. We suggest that this mechanism is also likely to be operative in cells, although this cannot be directly tested at present.

Our data support the model of EGF receptor activation recently proposed by Schlessinger (21) and Yarden and Schlessinger (22, 23). These investigations used nondenaturing electrophoresis to assess EGF receptor properties, whereas we used direct isolation of receptor monomers and dimers by sucrose density gradients. Nevertheless, our conclusions about the functional properties of the EGF receptor are identical to those of Schlessinger and Yarden. In contrast, Biswas *et al.* (24) have used technology similar to our own to reach conclusions opposite from us and from Yarden and Schlessinger. In the study of Biswas *et al.*, freshly isolated receptors were used as a source of monomers and aged receptors as a source of dimers. Considering the low stability of the EGF receptor kinase relative to the higher stability of EGF binding, this approach may be problematic, particularly when binding parameters are used to determine receptor amounts in preparations of different age and to normalize to the assayed kinase activity (24).

We recently have demonstrated the need for a dimeric insulin receptor structure in ligand-induced autophosphorylation (13) and in high-affinity ligand binding (12). The intact insulin receptor covalent structure can be considered analogous to a noncovalent EGF receptor dimer. Isolated insulin receptor halves ($\alpha\beta$) are analogous to EGF receptor monomers and are not able to undergo autophosphorylation if their interaction is prevented. Taken together, these data suggest that the mechanism for activating autophosphorylation by ligand-induced receptor dimerization (as observed for the EGF receptor) or by a ligand-induced conformational change in an intrinsically dimeric structure (as found for the insulin receptor) might be a common feature of the broader class of receptor-tyrosine kinases.

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