Intrapeptide Autophosphorylation of the Epidermal Growth Factor Receptor: Regulation of Kinase Catalytic Function by Receptor Dimerization†

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Received July 12, 1984

ABSTRACT: The epidermal growth factor (EGF) receptor is a transmembrane polypeptide of 170 000 daltons (Da) with a cytoplasmically facing protein kinase domain. The regulation of the tyrosine kinase activity of the EGF receptor by added EGF and by receptor association state was studied in an in vitro system. The rate of autophosphorylation of the solubilized and purified EGF receptor was found to be independent of receptor concentration. To determine whether the zero-order kinetics observed point to intrapeptide phosphorylation, we measured the sedimentation characteristics of the undenatured solubilized receptor. The receptor was found to exist in two association–dissociation states—a monomeric 7.7S form and a dimeric 12S form. The 7.7S form is an active tyrosine kinase; it has high basal activity, and the activity is not further stimulated by EGF; it appears to be an EGF-independent form of the receptor kinase. The 12S form is devoid of catalytic activity, but in the presence of EGF it dissociates into the active monomeric form. Freshly purified receptor preparations contain mainly the monomeric receptor, have high basal kinase activity, and show low EGF stimulatability (<1.3-fold). Aging of the receptor results in progressive dimerization and decay of EGF-independent kinase activity (and increase in EGF stimulatability). All of these processes are reversed in the presence of EGF or dithiothreitol. These results suggest that (a) the solubilized EGF receptor is catalytically active only in its monomeric 170 000-Da form, (b) autophosphorylation of the solubilized receptor is an intrapeptide function of the monomeric form, (c) bimolecular receptor–receptor interaction leads to reversible inactivation of the protein kinase site, and (d) EGF appears to stimulate the kinase activity of the receptor by shifting the association equilibrium toward monomer formation. The pertinence of these results, obtained with solubilized and purified receptor, to the in vivo situation remains to be established.

The experiments described here relate to possible modes of regulation of the epidermal growth factor (EGF) receptor system. The demonstration in recent years of structural and functional homologies between normal growth control molecules and those responsible for cell transformation (Downward et al., 1984; Waterfield et al., 1983) adds considerable interest to these studies.

The EGF receptor is a single-chain transmembrane polypeptide of 170 000 daltons (Da) (Das & Fox, 1978; Cohen et al., 1982), with the EGF-binding site outside the cell and a tyrosine-specific protein kinase site on the inside (Cohen et al., 1980; Linsley et al., 1981; Das et al., 1984a). Normally, in whole cells or isolated membranes, the protein kinase site of the receptor exists in a dormant state, but the binding of growth factor to the extracellular ligand-binding site activates the intracellular kinase domain. The ligand-induced regulatory interactions experienced by the growth factor receptor in the complicated whole cell/plasma membrane environment are too complex for proper analysis at the present time. As an initial step we have investigated regulatory interactions in an in vitro system. The results suggest a role for receptor–receptor interaction and ligand-induced modulation of this interaction in the regulation of the kinase catalytic site.

Materials and Methods

EGF. EGF was purified from mouse submaxillary gland according to the method of Savage & Cohen (1972) and radioiodinated by the chloramine-T procedure (Das et al., 1977) to a specific radioactivity of 150 000 cpm/ng. EGF–Affi-Gel was prepared as described (Das et al., 1984b) with Affi-Gel 10 from Bio-Rad Laboratories.

EGF Receptor Isolation. The EGF receptor used in this study was purified from the plasma membrane fraction of human epidermoid carcinoma cell line A431 by EGF affinity chromatography after solubilization with Triton X-100 (Cohen et al., 1982; Das et al., 1984b). A431 cells were grown in Dulbecco's modified Eagle's medium containing 7% fetal bovine serum and 10 μg/mL gentamicin. Plasma membranes were isolated from A431 cells by using Ca^{2+}- and Mg^{2+}-free solutions as described (Das et al., 1984b). The membranes (2 mg of protein) were stirred at 20 °C for 30 min with 1 mL of a solution containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.4, 10% glycerol, 1% Triton X-100, and 10 μg/mL leupeptin (Sigma Chemical Co.). After centrifugation at 100000*g* for 60 min, the supernatant was added to 0.5 mL of packed EGF–Affi-Gel (EGF–agarose containing 0.4 mg of covalently bound EGF). After being stirred at 20 °C for 30 min, the gel pellet was washed 4 times at 4 °C with 2 mL of a solution containing 10% glycerol and 0.2% Triton X-100 and the receptor eluted by stirring the washed gel 3 times (10 min at 4 °C each time) with 1 mL of 5 mM ethanolamine solution, pH 10, containing 10% glycerol and 0.2% Triton X-100, and the solution was immediately brought to pH 7.0 with 0.05 M HCl. The solution was concentrated by ultrafiltration using PM-30 filters. During the concentration, the buffer was adjusted to 20 mM Hepes, pH 7.4, 10% glycerol, and 0.2% Triton X-100. The amount of receptor present was estimated by Scatchard analysis of ^{125}I-EGF binding data, assuming a 1:1 complex of EGF and receptor. Recoveries varied from 40% to 60% of the input solubilized activity.

[γ-^{32}P]ATP. Labeled ATP was prepared with inorganic [^{32}P]phosphate (ICN) and Gamma-Prep A kit (Promega

† This work was supported by NIH Grants AM-25819 and HD-17896 and Research Career Development Award AM-00693 awarded to M.D.

0006-2960/85/0424-3795$01.50/0

Biotech, Madison, WI) following the manufacturer's instructions.

^{125}I-EGF Binding Assay. The receptor (50–500 fmol) was incubated at 20 °C for 45 min with ^{125}I-EGF (2–200 nM) in 50 μL of a solution containing 20 mM Hepes, pH 7.4, 0.2% Triton X-100, 2% glycerol, and 1 mg/mL bovine serum albumin (Cohen et al., 1980). The following ice-cold solutions were then added sequentially: (a) 50 μL of 20 mM Hepes, pH 7.4; (b) 0.25 mL of 1 mg/mL bovine γ-globulin in 0.1 M sodium phosphate buffer, pH 7.0; (c) 0.25 mL of 20.5% poly(ethylene glycol) (M_r 8000). The mixture was passed through a Millipore EHWP 0.5 μm pore filter. The filters were washed 3 times with 8.5% poly(ethylene glycol) in 0.1 M sodium phosphate buffer, pH 7.0, and assayed for radioactivity in a γ counter. Nonspecific binding was determined in the presence of 5 μM unlabeled EGF.

Autophosphorylation Assay. Receptor (0.01–1 pmol) was either treated (at 4 °C for 15 min) or not treated with 0.2–0.4 μM EGF (unlabeled) and then incubated at 20 °C (unless otherwise indicated) with 20 μM [γ-^{32}P]ATP (70–100 cpm/fmol) in 20 μL of a solution containing 1 mM $MnCl_2$, 20 mM Hepes, pH 7.4, 5% glycerol and 0.1% Triton X-100. The reaction was terminated by rapid successive addition of 1 μL of 20 mg/mL bovine serum albumin and 1 mL of 10% trichloroacetic acid. The precipitate was subjected to sodium dodecyl sulfate–polyacrylamide gradient (5–20%) gel electrophoresis (Das et al., 1977). After electrophoresis the gel slabs were dried and subjected to autoradiography using Kodak X-Omat AR films and Du Pont Cronex Lightning Plus intensifying screens. The extent of receptor phosphorylation was determined by measuring the radioactivity in the region of the dried gel containing the receptor band (in a toluene-based scintillation fluid). Dried gel strips of similar dimensions from adjacent regions were also counted to correct for background radioactivity.

Assay of Phosphorylation of a Synthetic Substrate. The receptor was incubated at 20 °C with 20 μM [γ-^{32}P]ATP (100 cpm/fmol), 1 mM $MnCl_2$, 20 mM Hepes, pH 7.4, 5% glycerol, 0.1% Triton X-100, and 1 mM peptide substrate (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly, from Peninsula Labs) in a total volume of 20 μL (Pike et al., 1982). The reaction was terminated by rapid successive additions of 1 μL of bovine serum albumin (1 mg/mL) and 30 μL of 5% trichloroacetic acid. After 15 min at 4 °C, the mixture was centrifuged. Thirty-microliter aliquots of the acid supernatants were spotted on phosphocellulose filter paper squares (Whatman, 2 × 2 cm). The filter papers were washed for 15 min in 30% acetic acid, followed by 15 min in 10% acetic acid (3 times) and finally in acetone, and then counted in a toluene-based scintillation fluid. The values were corrected for blank values determined in the absence of peptide. ^{32}P incorporation into the synthetic peptide with 20–100 fmol of receptor was linear up to about 20 min under the conditions used.

Sedimentation Analysis by Sucrose Gradient Centrifugation. The method described by Martin & Ames (1961) was used to estimate molecular weights and sedimentation rates. The equation used was

$$R = S1/S2 = (M_r1/M_r2)^{2/3}$$

where R = (distance traveled from meniscus by test protein)/(distance traveled from meniscus by standard protein), $S1$ = sedimentation rate of the test protein, $S2$ = sedimentation rate of the standard protein, M_r1 = molecular weight of the test protein, and M_r2 = molecular weight of the standard protein. We have tested the sedimentation relationship proposed by Martin & Ames (1961) for four standard proteins in 0.2% Triton X-100 containing gradient buffers. The standard proteins used were the following: bovine serum albumin, 4.6 S; human immunoglobulin G, 7 S; horse catalase, 11.2 S; horse apoferritin, 15.9 S. We found that a linear relationship exists between the distance traveled from the meniscus and the known sedimentation rates of these proteins. It should be noted that these standard proteins are not known to bind Triton X-100, whereas the receptor (being an integral membrane protein) is likely to bind Triton X-100.

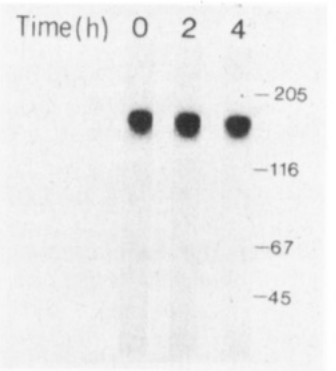

FIGURE 1: Autoradiographic visualization of ^{32}P-labeled receptor and the absence of dephosphorylating activity. Receptor (10 fmol) was incubated at 4 °C for 1 h with 15 μM [γ-^{32}P]ATP (10^4 cpm/pmol) in 20 μL of 0.2 μM EGF, 1 mM $MnCl_2$, 20 mM Hepes, pH 7.4, 5% glycerol, and 0.1% Triton X-100. At the end of incubation, 1 μL of unlabeled 20 mM ATP was added, and at the indicated times the reaction was terminated with trichloroacetic acid as described under Materials and Methods. The acid-precipitable radioactivity was subjected to electrophoresis and autoradiography. Densitometric analysis showed that the amounts of control (0-h) radioactivity present in the 2- and 4-h samples were 97% and 105%, respectively.

In comparison with the four standard proteins, the EGF receptor sedimented as 7.7S (170–180-kDa) and 12S (330–340-kDa) species in 0.2% Triton X-100 containing gradient buffers. It was assumed that the receptor molecule has a roughly spherical shape and a partial specific volume similar to that of the standards. The distance traveled from the meniscus was independent of receptor amount in the range of 1–40 pmol and independent of Triton X-100 concentratioion in the range of 0.1–0.5% Triton X-100. In the absence of Triton X-100 (or other detergents) the receptor aggregated near the bottom of the tube.

Results

The purified receptor bound ^{125}I-EGF with a K_d of 20–30 nM. [The corresponding K_d values for the receptor in A431 plasma membranes and in crude Triton X-100 extracts were 10 nM and 25 nM, respectively (Das et al., 1984b; Kawamoto et al., 1983).] The affinity-purified receptor was stable with respect to EGF binding for at least 7 days, but the kinase activity was extremely labile. Typically, the freshly purified receptor incorporated 1.3 mol of phosphate (mol of receptor)$^{-1}$ min^{-1} when incubated at 20 °C with 15 μM [γ-^{32}P]ATP. However, within 30 h at 4 °C this activity decays to less than 0.2 mol of phosphate incorporation (mol of receptor)$^{-1}$ (min)$^{-1}$. Therefore, unless otherwise mentioned, all phosphorylation experiments described here were performed within 2 h of receptor purification.

Incubation of the receptor with [γ-^{32}P]ATP resulted in the labeling of only a 170-kDa band (Figure 1). Occasionally a minor band of 150 kDa was seen. As shown below, phospho amino acid analysis of the ^{32}P-labeled receptor revealed the presence of only phosphotyrosine and no phosphoserine or phosphothreonine, suggesting the absence of other contami-

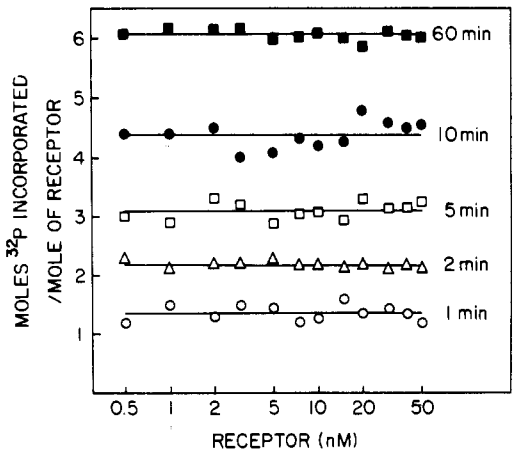

FIGURE 2: Time and receptor concentration dependence of autophosphorylation. Receptor was incubated at 20 °C for 10 min with 0.27 μM EGF in 15 μL of 27 mM Hepes, pH 7.4, 7% glycerol, 0.13% Triton X-100, and 1.3 mM $MnCl_2$. Phosphorylation was initiated by the addition of 5 μL of 80 μM [γ-^{32}P]ATP (10^4 cpm/pmol). (The final receptor concentrations are indicated.) After incubation at 20 °C for the indicated time periods, the extent of receptor phosphorylation was determined as described under Materials and Methods.

nating kinases (see Figure 4). The autophosphorylation reaction plateaued at ATP concentrations of 10–12 μM (Das et al., 1984b). The ^{32}P-labeled receptor did not dephosphorylate to any noticeable extent over a period of 4 h at 20 °C, suggesting the absence of contaminating phosphatases (Figure 1).

With the freshly purified receptor, the EGF stimulatability of the phosphorylation reaction was small (1.1–1.3-fold) and somewhat variable. The small stimulation is due to high basal activity. The variability is due to variations in the relative proportions of monomer to dimer in the receptor preparation (see below).

Kinetics of Autophosphorylation. The time and receptor concentration dependence of autophosphorylation (with 0.2 μM EGF and 20 μM [γ-^{32}P]ATP) is shown in Figure 2. Receptor phosphorylation was quantified by counting the gel slices containing the receptor band and by densitometric analysis of autoradiograms. For the wide range of receptor concentrations tested, the incorporation achieved in 1 min of incubation with [γ-^{32}P]ATP was always about 1.3 mol of phosphate/mol of receptor, the incorporation was 3 mol in 5 min, and it reached a saturation value of approximately 6 mol of phosphate/mol of receptor after 1 h of incubation (Figure 2) or 2 h (not shown); i.e., the time course was independent of receptor concentration. The fractional extent of phosphorylation for any given period of time was identical for all the receptor concentrations tested.

Thus for the various incubation times used (1–120 min) and the range of receptor concentrations tested (0.5–50 nM), autophosphorylation was found to be zero order in receptor concentration. Autophosphorylation in the absence of EGF was also a zero-order reaction (not shown). The extent of molar phosphorylation in the absence of EGF varied between receptor batches. It is shown below that this correlates with variations in the relative proportions of monomer and dimer in the receptor preparation.

Sedimentation Characteristics of the Receptor Kinase. The zero-order kinetics of autophosphorylation indicate that the kinase reaction is intramolecular, is not dependent upon the rate of intermolecular collisions, and is consistent with either of the two following possibilities. (a) Intrapeptide autophosphorylation: the 170-kDa receptor polypeptide exists as a monomer, within which intramolecular interactions occur

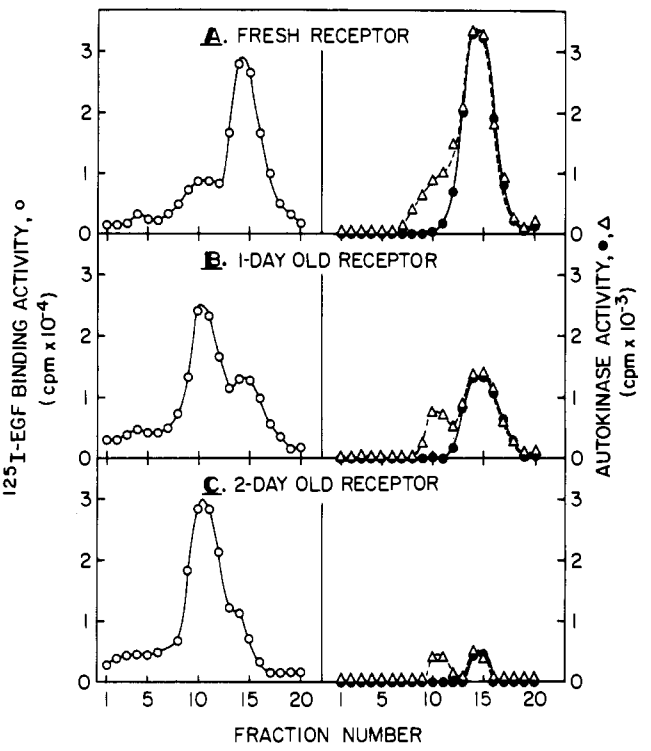

FIGURE 3: Centrifugal analysis of kinase and EGF-binding activity in fresh (A), 1-day-old (B), and 2-day-old (C) receptors. Approximately 20 pmol of receptor (in 100 μL of 20 mM Hepes, pH 7.4, 0.2% Triton X-100, and 3% glycerol) was centrifuged at 4 °C for 6 h at 45 000 rpm in a SW5091 swinging bucket rotor through a 5–20% sucrose gradient (5 mL) in 20 mM Hepes, pH 7.4, 0.15 M NaCl, and 0.2% Triton X-100. At the end of the run, the tubes were punctured at the bottom, and 250-μL fractions were collected. Ten microliters of each fraction was used for EGF-binding and autokinase assays, as described under Materials and Methods. The EGF-binding values plotted (○) are specific binding data with 150 nM ^{125}I-EGF that were obtained after subtraction of nonspecifically bound radioactivity (about 5000 cpm). The autophosphorylation values plotted (Δ, with 0.2 μM EGF; ●, without EGF) have been corrected for any background radioactivity present in adjacent regions of the dried gel.

between the kinase catalytic site and the phosphate acceptor tyrosine sites. (b) Interpeptide autophosphorylation: the receptor exists as a dimer or an oligomer, in which the kinase catalytic site of one subunit 170-kDa peptide interacts with the paired phosphate acceptor tyrosines of another subunit 170-kDa peptide.

To distinguish between intrapeptide and interpepetide phosphorylation, the sedimentation behavior of freshly purified receptor was studied under nondenaturing conditions in sucrose density gradients (Figure 3A). Two forms of EGF-binding activity were seen. The molecular weights of the slow (7.7S) and fast (12S) sedimenting EGF receptors were estimated to be 170K–180K and 330K–340K, respectively (see the appropriate section under Mateials and Methods). The 170K–180K molecular weight of the 7.7S form is consistent with the known 170K–190K molecular weight of the EGF receptor polypeptide (Das et al., 1977; Cohen et al., 1982). Regarding the estimated molecular weight (330K–340K) of the fast sedimenting receptor form, it can be noted that a similar integral membrane protein, the insulin receptor (M_r 350K), sediments at 11–12S in 0.2% or 1% Triton X-100 (Cuatrecasas, 1972; Siegal et al., 1981).

Analysis of the [γ-^{32}P]ATP-reacted gradient fractions by electrophoresis and autoradiography revealed the presence of only a 170-kDa band. No other ^{32}P-labeled band of higher or lower molecular weight was visible in any of the gradient fractions even after prolonged autoradiographic exposure.

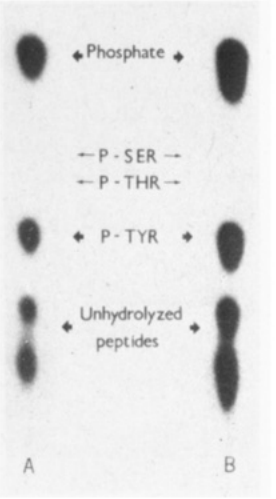

FIGURE 4: Phospho amino acid analysis of the ^{32}P-labeled 7.7S receptor (A) and the unfractionated receptor (B). Receptor (1 pmol) was treated with [γ-^{32}P]ATP as described in the legend to Figure 1. After electrophoresis, staining, and destaining, the region of the gel slab corresponding to the receptor band was excised and subjected to Pronase digestion (25 μg of Pronase in 1.5 mL of water at 37 °C for 12 h in a shaking bath). Samples were lyophilized, and the dry mass was subjected to 6 N HCl hydrolysis at 110 °C for 100 min. The hydrolysate was thoroughly lyophilized and then dissolved in 50 μL of pyridine/acetic acid/water (2:20:78 by volume), pH 3.5. Aliquots (10 μL) were subjected to electrophoresis on cellulose thin-layer plates using the same pH 3.5 buffer. The samples were run at 500 V for 90 min. At the completion of the run, the thin layers were dried, stained with ninhydrin, and then subjected to autoradiography.

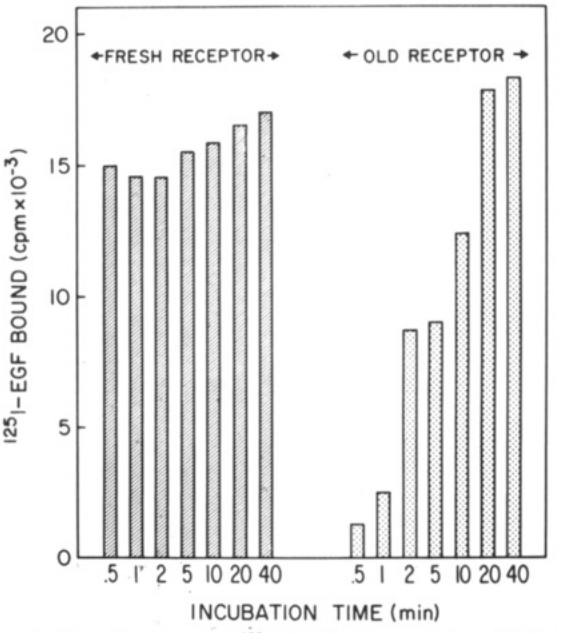

FIGURE 5: Time dependence of ^{125}I-EGF binding to fresh and 2-day-old receptors. Thirty-five femtomoles of freshly prepared receptor (hatched bars) or 2-day-old receptor (dotted bars) was incubated at 20 °C with 20 nM ^{125}I-EGF (150 000 cpm/ng) in 50 μL of 20 mM Hepes, pH 7.4, 0.1% Triton X-100, 5% glycerol, and 1 mg/mL bovine serum albumin. After incubation for the indicated time periods, poly(ethylene glycol) precipitable radioactivity was determined as described under Materials and Methods. The values plotted are specific binding data that were obtained after subtraction of nonspecifically bound radioactivity (about 1100 cpm).

There was a cosedimentation of ^{125}I-EGF-binding activity and autokinase activity at the 7.7S peak (Figure 3A). The kinase activity at 7.7 S was EGF independent and was not stimulated by EGF. Phospho amino acid analysis of the ^{32}P-labeled 7.7S peak revealed the presence of only phosphotyrosine (Figure 4).

A second minor EGF-binding peak at 12 S did not have protein kinase activity, but preincubation with EGF resulted in the expression of autokinase activity in this fraction (Figure 3A). Thus the 12S activity, presumably a dimer, is totally EGF dependent for its kinase function. As indicated below, EGF activation of the kinase site in the 12S receptor is accompanied by its dissociation into the 7.7S form.

The 7.7S sedimentation rate is consistent with a monomeric structure for the catalytically active receptor kinase and indicates an intramolecular mechanism of autophosphorylation.

Time-Dependent Conversion of the 7.7S EGF Receptor to the 12S Form. To determine the relationship between the two EGF-binding forms (7.7S and 12S) and to study their link to the decay of kinase function, we examined the sedimentation behavior of the receptor at 20 and 44 h after purification (the receptor was stored at 4 °C during this time). As shown in Figure 3B,C, there was a time-dependent decrease in the 7.7S form, accompanied by a concomitant increase in the 12S form. The summed amount of EGF-binding activity in the two peaks (7.7S and 12S) did not decrease with time; i.e., there was no decay of total EGF-binding activity. However, the EGF-independent protein kinase activity of the receptor decayed, and the decay was proportional to the decrease in the 7.7S form. The results shown above suggest that the 7.7S receptor is the catalytically active, EGF-independent form of the EGF receptor. The 12S form, which increased with time, did not display protein kinase activity without prior activation by EGF.

Note that the concentration of receptor used in protein kinase assays is lower than the concentration applied over gradient tubes in these sedimentation experiments. This could have led to dissociation of the 12S form to the active 7.7S form. However, in the EGF-independent kinase assays, activity was not seen in the 12S gradient fractions, suggesting that the dilutions during assay may not have been sufficient to cause a detectable shift in the association equilibrium.

It is significant that the time-dependent increase in the 12S EGF receptor is not associated with a proportional increase in EGF-dependent kinase activity (compare panels A, B, and C in Figure 3). The lack of proportionality suggests irreversible inactivation of the kinase site subsequent to the dimerization event (see Discussion).

Comparison of EGF Binding to Freshly Prepared and Aged Receptors. Under equilibrium binding conditions (45 min at 20 °C) the freshly prepared receptor (>80% 7.7S) and the 2-day-old receptor (>80% 12S) displayed identical characteristics of maximal binding capacity and affinity for EGF ($K_d \simeq 25$ nM). However, the fresh and aged receptors were markedly different in their time dependence of EGF binding (Figure 5). EGF binding to the fresh receptor reached 80% of equilibrium value within 1 min. In contrast, binding to the aged receptor showed a distinct lag, but by 20–40 min it reached an equilibrium value similar to that for the fresh receptor. The results are consistent with an interconversion between receptor forms that is influenced by EGF in a linked-function sense (Wyman, 1964). In this view the 7.7S receptor binds EGF more tightly than the 12S form and shifts the equilibrium toward the 7.7S form.

Parallel Decay of EGF-Independent Autophosphorylating Activity and EGF-Independent Ability To Phosphorylate a Synthetic Substrate. The time-dependent decay of EGF-independent autokinase activity associated with 7.7S → 12S conversion could be due to inactivation of either the catalytic site or the phosphate acceptor tyrosine sites. To distinguish between these alternatives, decay of the protein kinase activity of the receptor at 4 °C was studied by using both the auto-

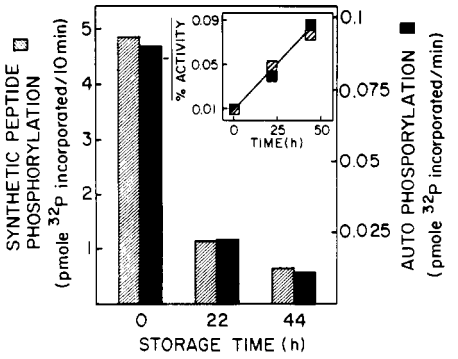

FIGURE 6: Concomitant decay of EGF-independent autokinase activity (hatched bars) and EGF-independent ability to phosphorylate an exogenous synthetic substrate (solid bars). Receptor (70 fmol) was incubated at 4 °C for the indicated time periods in 10 μL of 20 mM Hepes, pH 7.4, 10% glycerol, and 0.2% Triton X-100 and then assayed for autokinase activity (1-min incubation at 20 °C) or synthetic peptide phosphorylating activity (10-min incubation at 20 °C) as described under Materials and Methods.

phosphorylation assay and an assay for phosphorylation of an exogenous synthetic substrate. The synthetic peptide, Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly (Pike et al., 1982), contains no serine or threonine and therefore can be a substrate for only tyrosine kinases. The results (Figure 6) exhibit parallel decay of both autophosphorylating activity and synthetic substrate phosphorylating activity at 4 °C (Figure 6). Thus the decay of EGF-independent activity is due to blockage of the kinase site.

Second-Order Decay Kinetics. The sedimentation rates of the two forms of the EGF receptor in Figure 3 suggest a monomer–dimer relationship. If so, the decay/disappearance of EGF-independent kinase (the 7.7S form) should be a bimolecular process involving receptor–receptor interaction. To test this, the kinetics of decay of EGF-independent kinase at 4 °C was investigated in detail (Figure 7). A semilog plot of the data shows that the decay is not first order (Figure 7B). However, a plot of the inverse of the activity remaining against time is well fit by a straight line, indicating a second-order, bimolecular process consistent with a receptor–receptor association reaction (Figure 7C and Figure 6, inset). This finding is also consistent with the results of sedimentation experiments (Figure 3), which suggested that a doubling in apparent molecular weight is associated with loss of kinase function.

The results in Figure 7 indicate that the biomolecular rate constant at 4 °C, pH 7.4, is 10^3–10^4 M^{-1} s^{-1}. The decay rate was temperature sensitive. Storage of the receptor at −20 or −70 °C instead of at 4 °C drastically slowed the decay process (▲ and ■ in Figure 7).

EGF Effect on Reversal of Decay and Conversion of the 12S Receptor to the 7.7S Form. In the presence of 0.2 μM EGF or 0.1 mM dithiothreitol, or a combination of both, the decay of EGF-independent kinase was considerably slowed (Figure 8). EGF was found to reactivate decayed receptor kinase activity (data not shown). As mentioned earlier, the freshly purified receptor kinase showed very little EGF stimulatability. However, with aged, partially decayed receptor preparations there was a noticeable rise in EGF stimulatability. In contrast, receptor kinase preparations that were stored in the presence of dithiothreitol, a stabilizer, showed only insignificant EGF stimulation. It is shown in Figure 9 that receptors stored in the presence of dithiothreitol tended to maintain the 7.7S form.

To test unequivocally whether the 12S receptor generates the 7.7S form upon EGF incubation, the 12S receptor isolated by centrifugation was incubated with EGF–Affi-Gel, reacted

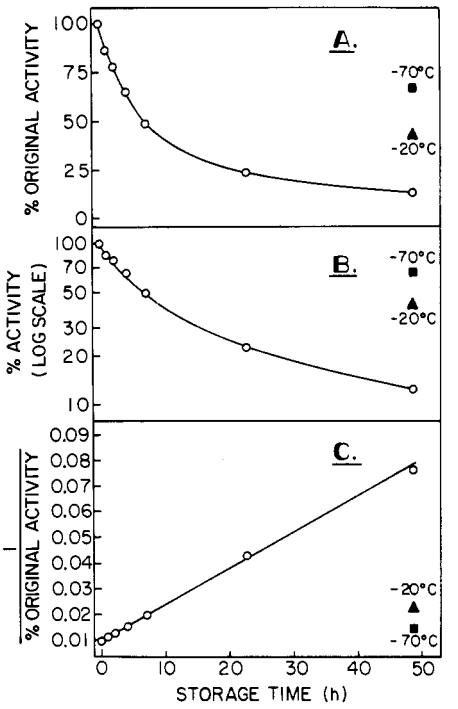

FIGURE 7: Kinetics of decay of EGF-independent autokinase activity. (A) Plot of activity remaining against time; (B) semilog plot of activity remaining against time; (C) plot of inverse of activity against time. Receptor (70 fmol) was stored at 4 °C for the indicated time periods and then assayed for autokinase activity (○) as described in the legend to Figure 6 and under Materials and Methods. One hundred percent activity represents the incorporation of 90 fmol of ^{32}P in 1 min. In other experiments, receptor was stored at either −20 (▲) or −70 °C (■) for 48.5 h and then assayed for activity.

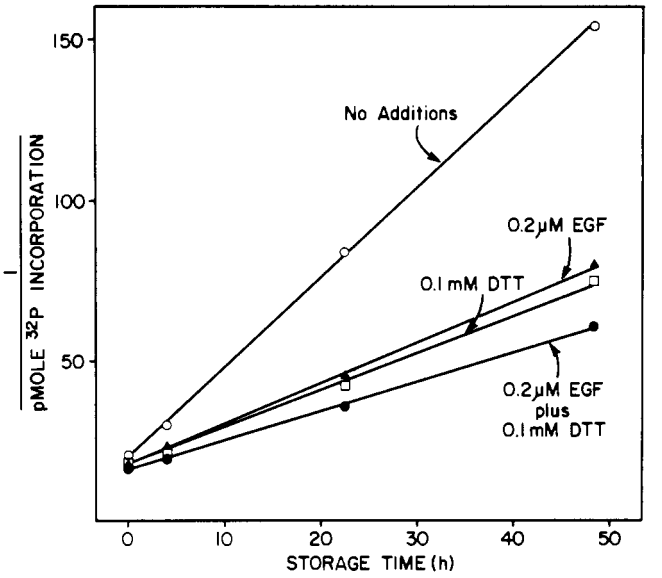

FIGURE 8: Effect of EGF and dithiothreitol on the kinetics of kinase decay. Receptor (70 fmol) was stored at 4 °C for the indicated time periods with or without 0.2 μM EGF or 0.1 mM dithiothreitol as indicated and then assayed for activity as described under Materials and Methods and in the legend to Figure 6. One hundred percent activity represents the incorporation of 0.09 pmol of ^{32}P in 1 min.

or not reacted with [γ-^{32}P]ATP while bound to the solid support, and then eluted and run on a sucrose gradient. Greater than 80% of the input EGF-binding activity (originally 12S) was recovered as the 7.7S entity in the sucrose gradient (● and △ in Figure 10). In the paired tube, all of the ^{32}P receptor radioactivity was recovered in the 7.7S peak (■ in Figure 10). This appears to suggest again a precursor–product relationship between the 12S and the 7.7S receptor forms.

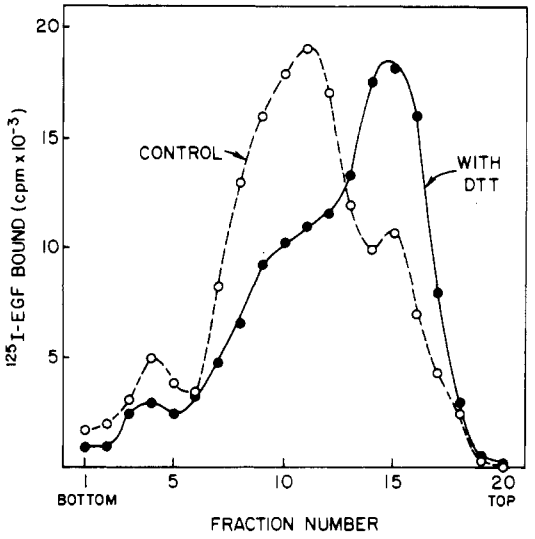

FIGURE 9: Effect of storage with dithiothreitol (DTT) on receptor sedimentation rate. Receptor (10 pmol) was stored at 4 °C for 20 h with or without 0.1 mM DTT in 40 μL of 20 mM Hepes, pH 7.4, 10% glycerol, and 0.2% Triton X-100. The samples were then diluted with 60 μL of 20 mM Hepes, pH 7.4, and 0.2% Triton X-100. The conditions for sucrose gradient centrifugation were the same as those described in the legend to Figure 3, except that 0.1 mM DTT was present in the sucrose gradient for the DTT sample. EGF-binding activity in the fractions was assayed with 150 nM ^{125}I-EGF (150000 cpm/ng) as described in the legend to Figure 3 and under Materials and Methods. Nonspecific binding was approximately 6300 cpm.

Evidently EGF activation of the kinase site in 12S receptor is accompanied by its conversion to the 7.7S form.

Note that ethanolamine per se has no effect on 12S → 7.7S conversion. Incubation of the 12S receptor with 5 mM ethanolamine, pH 10, at 4 °C for 40 min followed by neutralization did not convert it to the 7.7S EGF-binding form (× in Figure 10).

Lack of Interpeptide Disulfide Links in the 12S Receptor. With regard to the nature of the interreceptor bonds in the 12S dimer, one can consider the possibility that the receptor polypeptides are held together by disufide bridges that are dithiothreitol sensitive. To test this, the 12S form (isolated by gradient centrifugation) was labeled with iodine-125 by using the iodogen procedure (Fraker & Speck, 1978; Das et al., 1984b). (In this method, termination of iodination does not require the addition of reducing agents.) The ^{125}I-labeled 12S receptor was subjected to sodium dodecyl sulfate (SDS) gel electrophoresis in the absence and presence of dithiothreitol. Autoradiographic examination of both gels revealed the presence of only the 150K–170K receptor band and no higher molecular weight bands (data not shown). In sucrose gradients, however, the ^{125}I-labeled receptor sedimented as a 12S molecule. This suggests that the dimerization is not due to interpeptide disulfide bonding or any other type of covalent bonding.

EGF Stimulation of the Receptor Kinase in Crude Extracts and Membranes. Some characteristics of the EGF receptor kinase at different stages of isolation are shown in Table I. In the membrane-bound state, phosphorylation of the EGF receptor is strongly stimulated by EGF. After solubilization with Triton X-100 the receptor still remains EGF stimulatable, albeit to a smaller extent, but EGF affinity purification results in an almost total loss of EGF stimulatability. The association state of the membrane-bound receptor is unknown, but in the crude Triton extract, greater than 70% of the receptors exist in dimeric and other associated states (this is in keeping with the observed 3–4-fold EGF stimulation of extract autokinase

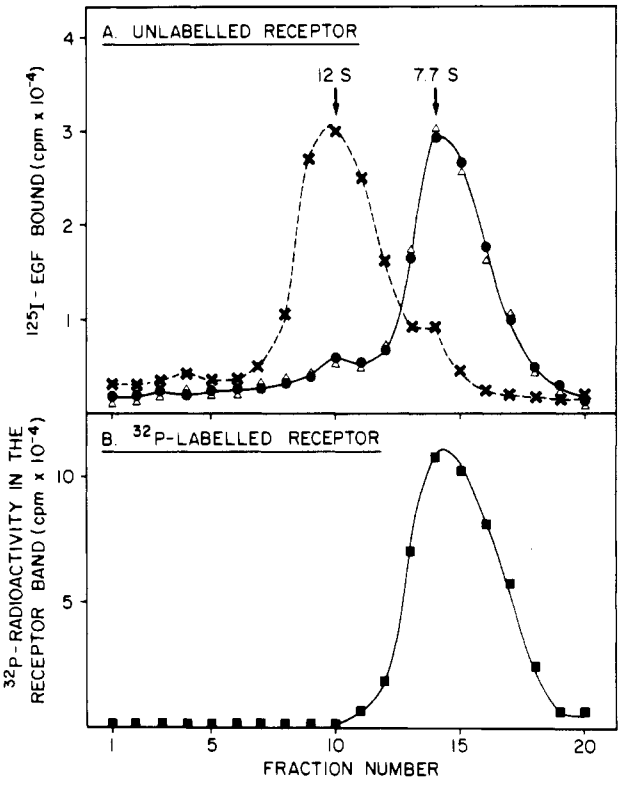

FIGURE 10: EGF-induced conversion of the 12S receptor to the 7.7S form. The 12S form of 6-h-old receptor was isolated by gradient centrifugation as described in Figure 3. The fraction was concentrated and adjusted to 20 mM Hepes, pH 7.4, 10% glycerol, and 0.2% Triton X-100. The concentrated 12S receptor (10 pmol in 200 μL) was stirred with 0.2 mL of packed EGF–agarose at 20 °C for 30 min. (It was deduced by an EGF-binding assay on a small aliquot of the supernatant that approximately 90% of input EGF-binding activity was attached to the EGF–agarose.) The suspension was then incubated at 4 °C for 30 min with either no addition (panel A, ●), 20 μM unlabeled ATP and 1 mM $MnCl_2$ (panel A, △), or 20 μM [γ-^{32}P]ATP and 1 mM $MnCl_2$ (panel B, ■). The suspension was centrifuged to remove unreacted reagents, and the gel was washed 6 times with cold 10% glycerol and 0.2% Triton X-100. The receptor was eluted by stirring the gel 3 times (10 min at 4 °C each time) with 0.5 mL of 5 mM ethanolamine solution, pH 10, containing 0.2% Triton X-100, and the solution was immediately brought to pH 7.0 with 0.05 M HCl. (Greater then 80% of the original input EGF-binding activity was recovered in the eluate.) The eluted receptor was concentrated by ultrafiltration (using PM-30 filters), adjusted to 20 mM Hepes, pH 7.4, 3% glycerol, and 0.2% Triton X-100, and then subjected to sucrose gradient centrifugation as described in the legend to Figure 3. Fractions (250 μL) were collected. The gradient tubes containing no ^{32}P-labeled receptor (panel A, ● and △) were assayed for ^{125}I-EGF-binding activity as described in the legend to Figure 3. In experiments with the ^{32}P-labeled receptor (panel B, ■), the proteins in gradient fractions were precipitated by acid and subjected to electrophoresis and autoradiography. Greater than 80% of the applied EGF-binding activity (in panel A, ● and △) and ^{32}P-labeled receptor radioactivity (in panel B, ■) was recovered in the fractions. In another experiment (panel A, ×), 10 pmol of 12S receptors (obtained as described in the beginning of this legend) was incubated at 4 °C for 40 min with 1 mL of 5 mM ethanolamine, pH 10, 10% glycerol, and 0.2% Triton X-100. The solution was then neutralized to pH 7 with 0.05 M HCl, concentrated, adjusted to 20 mM Hepes, pH 7.4, 3% glycerol, and 0.2% Triton X-100, and then subjected to sucrose gradient centrifugation. Fractions were tested for EGF-binding activity as described in the legend to Figure 3. Nonspecific binding of ^{125}I-EGF was about 5100 cpm.

activity). The low EGF stimulatability, high basal activity, and large proportion of the 7.7S monomeric form found in the purified preparation are probably consequences of receptor interaction with EGF in the affinity column, which shifts the equilibrium toward monomer. The dissociated active state appears to be maintained (at least for a short time period) even

Table I: EGF Stimulatability of Receptor Kinase at Different Stages of Isolation[a]

stage of isolation	stimulation of receptor phosphorylation by 1 μM EGF (x-fold)	association state of unoccupied receptor
plasma membrane fraction	5–10	unknown
crude Triton X-100 extract	3–4	~30% 7.7S form, ~50% 12S form, ~20% higher association states
EGF affinity purified receptor	1.1–1.3	80–90% 7.7S form, 10–20% 12S form

[a] The extent of receptor phosphorylation was determined electrophoretically by counting of the ^{32}P-labeled receptor band. The association state of the receptor in the crude extract was determined by sucrose gradient centrifugation (see legend to Figure 3) and by assaying for specific ^{125}I-EGF-binding activity in the fractions.

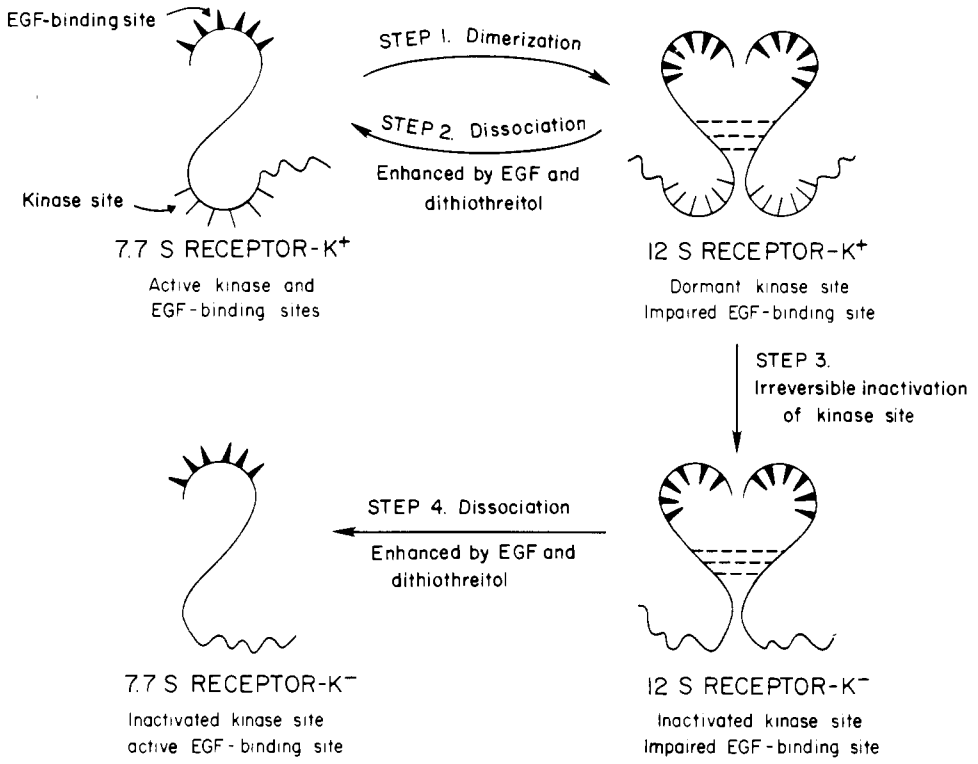

FIGURE 11: Association–dissociation and decay characteristics of the solubilized and purified EGF receptor. K^+ denotes an active or dormant kinase site; K^- denotes an inactivated kinase site. The experimental observations supporting the various steps in the scheme are as follows: step 1, bimolecular decay of EGF-independent kinase (Figure 7) and the precursor–product relationship between 7.7S and 12S receptors at various stages of decay (Figure 3); step 2, EGF-induced generation of the 7.7S receptor kinase from the 12S form of the receptor (Figure 10); step 3, incomplete (EGF-induced) reactivation of the kinase site in the 12S receptor with increasing age of the receptor (Figure 3); step 4, time lag in the binding of EGF to aged receptors, but achievement of the same final equilibrium binding value as observed for the fresh receptors (Figure 5).

after the elution of the receptor from the column.

Discussion

Our finding that the solubilized EGF receptor may exist in two different association–dissociation states (having monomer–dimer relationships) is based on sedimentation experiments and observations on bimolecular decay. The estimated sedimentation rate (7.7 S) and molecular weight (170K–180K) of the slower sedimenting receptor are consistent with the known 170K–190K molecular weight of the EGF receptor polypeptide (Das et al., 1977; Cohen et al., 1982). Regarding the estimated 330K–340K molecular weight of the faster sedimenting (12S) receptor, it can be noted that a similar integral membrane protein, the insulin receptor (M_r 340K), sediments at 11–12 S in 0.2% or 1% Triton X-100 solutions (Cuatrecasas, 1972; Siegel et al., 1981). Thus it appears that the 7.7S and 12S EGF-binding forms may well represent monomeric and dimeric forms of the 170-kDa receptor. With this in mind, the salient points to be noted are as follows:

(*a*) *Intrapeptide Autophosphorylation.* The solubilized EGF receptor appears to be catalytically active only in its monomeric 7.7S (170-kDa) form (Figure 3). Autophosphorylation of the receptor is a zero-order process (Figure 2) and thus an intrapeptide function of the monomeric form. [Note that in the insulin receptor kinase (Kasuga et al., 1983) and the cAMP-dependent kinase (Rangel-Aldao & Rosen, 1976) autophosphorylation is also a zero-order process, but because these kinases are oligomeric, the autophosphorylation could involve either intrapeptide or interpeptide interaction.]

(*b*) *The monomeric 7.7S receptor* has high basal kinase activity, and the activity is not further stimulated by EGF (Figure 3). These results suggest that the 7.7S receptor may be an EGF-independent kinase.

(*c*) *Bimolecular Decay of Kinase.* Interreceptor interaction (that follows second-order kinetics) leads to the demise of EGF-independent kinase activity and the concomitant generation of a 12S receptor form (Figures 3, 6, and 7).

(*d*) *The 12S receptor* (330–340 kDa), presumably a dimer, is apparently devoid of catalytic activity, but it retains a potential for activity (Figure 3). In the presence of EGF it appears to be dissociated into the catalytically active monomeric 7.7S form (Figure 10).

The results summarized in points a–d suggest that EGF is capable of influencing association–dissociation behavior of its

own receptor and it may do so by binding more tightly to the monomeric (7.7S) form than to the dimer (see Figure 5). The results suggest that EGF may influence the kinase activity of its receptor by shifting the equilibrium toward monomer formation. However, it remains to be established that the observed association–dissociation behavior and the EGF effect on this process are not in vitro artifacts of solubilization/purification.

Other points that are worth noting on the behavior of the solubilized and purified receptor are as follows:

(*e*) *EGF Stimulatability of Kinase Is Inversely Related to the Proportion of Receptor Monomers Present.* In freshly purified receptor preparations where the 7.7S monomer predominates, there is less than 1.3-fold stimulation by EGF. Storage of the receptor results in progressive dimer generation, loss of EGF-independent kinase activity, and increased stimulation with EGF. The aging process slows when receptors are stored in the presence of dithiothreitol; they dimerize less, lose less EGF-independent kinase activity, and are less EGF stimulatable.

(*f*) *Irreversible Inactivation of the Kinase Site.* Although the 12S form present in the freshly purified receptor is strongly EGF activatable (Figure 3A), with increasing passage of time there is lower recovery of EGF-activatable kinase in the 12S fraction (Figure 3B,C). This suggests a time-dependent irreversible inactivation (denaturation) of the kinase site in the 12S receptor. In contrast, the EGF-binding site remains stable (as indicated by the results in Figures 3 and 5).

Receptor Association–Dissociation in Vitro and Possible Relationship to in Vivo Situation. The results on physical association and functional activity of the purified EGF receptor kinase, suggested by our in vitro studies, are summarized in Figure 11. While our findings are limited and not directly translatable to the in vivo situation, certain parts of the model in Figure 11—steps 1 and 2—are worth noting. At present, the association state of the unliganded receptor in membranes or whole cells is unknown. In the only study reported on the molecular size of the membrane-bound EGF receptor, the diffusion rate reflected the size of the liganded (EGF-bound) receptor (Hillman & Schlessinger, 1982). It remains to be tested whether the high EGF stimulatability of the receptor in intact cells and membranes is correlated with a low monomer:dimer (oligomer) ratio and whether this ratio is increased upon interaction with EGF. Such EGF-induced monomer generation, if it actually occurs in vivo, would have to be reconciled with the fact that receptors eventually cluster after interaction with the ligand. However, it should be noted that a "cluster" need not be the equivalent of a defined dimer complex; i.e., a cluster may be composed of catalytically active monomeric receptor units.

Nature of the Bond Involved in Dimerization. Present data suggest (see Results) that dithiothreitol-sensitive interpeptide disulfide bridges are not likely to be involved in dimer formation. It is possible that dithiothreitol protection of a sensitive sulfhydryl group at the kinase catalytic site, or at other sensitive sites, leads to the blockage of a crucial noncovalent interaction between receptors. It remains to be seen what region of the receptor is involved in this bonding interaction. We have found that the kinase domain of the EGF receptor (~42 kDa) is proteolytically separable from the EGF-binding domain and the major autophosphorylation locus and can function catalytically even after this detachment (Basu et al., 1984). Our preliminary studies indicate that this 42-kDa kinase does not exhibit any decay (bimolecular or unimolecular) over a period of 10 h at 4 °C. It remains to be seen whether the 42-kDa kinase contains the region of the receptor responsible for intermolecular interaction. Also, the role of autophosphorylation in the control of receptor–receptor bonding needs to be investigated.

ACKNOWLEDGMENTS

We thank Laura Mattes for preparing A431 membranes and Drs. R. Ricker and J. Ray for their help in the phospho amino acid analyses.

Registry No. EGF, 62229-50-9; tyrosine kinase, 80449-02-1.

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