

Aggregation-Induced Activation of the Epidermal Growth Factor Receptor Protein Tyrosine Kinase

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ABSTRACT: Various agents are able to stimulate the EGF receptor protein tyrosine kinase in the absence of ligand binding. To characterize their mechanism of action, we investigated their effects on the kinase activity of the intracellular domain of the EGF receptor (EGFR-IC). EGFR-IC (67 kDa) lacking the extracellular domain and transmembrane segment of the EGF receptor, but retaining kinase and autophosphorylation domains, was produced and purified as a soluble, cytoplasmic protein from Sf9 insect cells infected with a recombinant baculovirus. EGFR-IC was able to undergo autophosphorylation in a manner similar to full-length EGFR. Synthetic substrate peptides showed similar affinity to EGFR-IC as to the full-length receptor. The activity of the EGFR-IC was found to be dependent on divalent cations, Mn^{2+} being a more potent activator than Mg^{2+} . Agents capable of aggregating the kinase by direct interaction (cross-linking antibodies, polycations) or through altering the surrounding solvent structure and thereby decreasing protein solubility [ammonium sulfate, poly(ethylene glycol), 2-methyl-2,4-pentanediol] activated the kinase in a manner which correlated with their ability to precipitate the EGFR intracellular domain. The widely different chemical nature of these agents suggests that they do not act by direct interaction with specific allosteric regulatory sites, but rather by facilitating the interactions between kinase molecules. These results support the hypothesis that full-length receptor aggregation itself, induced by ligand binding to the extracellular domain, results in intracellular domain interactions and the activation of kinase activity.

The EGF receptor (EGFR), a 180-kDa transmembrane glycoprotein with intrinsic protein tyrosine kinase activity, responds to EGF stimulation by activation of its kinase. Numerous cellular substrates are phosphorylated, including the EGFR itself (Carpenter & Cohen, 1990; Ullrich & Schlessinger, 1990). The kinase activity is absolutely required for EGF receptor mediated biological responses: a point mutant of the EGFR (K721A) devoid of kinase activity is incapable of eliciting a mitotic response (Chen *et al.*, 1987; Honegger *et al.*, 1987a,b; Moolenaar *et al.*, 1988).

Receptor dimerization has been proposed to be involved in transmitting the activating signal across the plasma membrane to the intracellular kinase domain (Yarden & Schlessinger, 1987a). EGF binding to full-length EGF receptor leads to the dimerization of the receptor both *in vitro* and in living cells (Yarden & Schlessinger, 1987b; Cochet *et al.*, 1988). The intracellular domain is not essential for ligand-induced aggregation. Soluble EGFR extracellular domains lacking transmembrane and intracellular domains display ligand-induced dimerization (Hurwitz *et al.*, 1991; Lax *et al.*, 1991). Within a full-length receptor, dimerization of extracellular domains brings the kinase domains into contact, allowing them to interact. Dimerization of the kinase domains then provides the energy for a conformational change resulting in the activation of the kinase. Ligand-induced activation predominantly affects the V_{max} of the kinase, while the affinities for ATP and peptide substrate are not significantly altered (Erneaux *et al.*, 1983).

Chimeras consisting of the extracellular domain of one member of the protein tyrosine kinase receptor family and the kinase domain of a second (e.g., EGFR/neu, insulin receptor/EGFR) demonstrate that different members share the same mechanism of signal transmission. The extracellular domains determine the ligand specificity, while the kinase domains determine the specificity of the response, but the activation signal is transmitted even if the ligand binding domain and the intracellular domain of a chimeric receptor originate from different members of the family (Riedel *et al.*, 1986, 1989; Lammers *et al.*, 1989; Lee *et al.*, 1989).

While EGF-dependent receptor dimerization has been demonstrated by a variety of methods (Fanger *et al.*, 1986; Böni-Schnetzler & Pilch, 1987; Yarden & Schlessinger, 1987a,b; Cochet *et al.*, 1988), it has been more difficult to prove that aggregation is indeed both necessary and sufficient for receptor activation under physiological conditions. Multivalent antibodies aggregating the extracellular domains can mimic ligand activation and stimulate both kinase activity and biological responses (Schreiber *et al.*, 1981, 1983; Yarden & Schlessinger, 1987a). However, reports of *in vitro* studies on the correlation between receptor aggregation and kinase activity present conflicting results. Frequently, receptor autophosphorylation was used as a measure of receptor activity. Since autophosphorylation of the EGFR can occur by an intermolecular mechanism, both *in vitro* and in living cells (Honegger *et al.*, 1989, 1990), aggregation might increase autophosphorylation even if the specific activity of the kinase is not altered.

As well as the specific ligands of the receptors, a number of other substances have been reported to stimulate the *in vitro* activity of protein tyrosine kinases in the absence of ligand. In addition to its role in facilitating ATP binding,

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Mn²⁺ significantly increases the basal activity of EGFR compared to Mg²⁺ (Koland & Cerione, 1988; Wedegaertner & Gill, 1989). Ammonium sulfate stimulates the kinase activity of both the EGFR intracellular domain and the full-length receptor, while sodium sulfate or ammonium chloride at equivalent concentrations do not stimulate activity (Koland & Cerione, 1988; Wedegaertner & Gill, 1989). Poly(lysine) and poly(ornithine), random copolymers of lysine with serine or alanine, peptides with a high lysine content, and histone H2 were found to increase the tyrosine kinase activity in membranes from *Xenopus* oocytes as well as from various cultured mammalian cell lines (Gatica *et al.*, 1987). Activation by cationic polypeptides has been especially well studied for the insulin receptor and its kinase domain (Rosen & Lebowitz, 1988; Fujita-Yamaguchi *et al.*, 1989; Kohanski, 1989; Morrison *et al.*, 1989), and these agents have also been shown to stimulate the kinase activity of immunoaffinity-purified full-length EGF receptor (Hubler *et al.*, 1992). While the charged random polymer of Glu, Lys, Ala, Tyr was shown not to be a substrate of the EGF receptor kinase, it does nevertheless significantly activate receptor autophosphorylation (McGlynn *et al.*, 1992). The polyamine protamine activates a number of protein tyrosine kinases, including the EGF receptor (Hubler *et al.*, 1992) and the insulin receptor (Rosen & Lebowitz, 1988), as does the lipid sphingosine (Northwood & Davis, 1988; Davis *et al.*, 1988; Wedegaertner & Gill, 1989).

The mechanism(s) by which these substances activate the different protein tyrosine kinases is (are) not known. It has been speculated that they function as allosteric regulators, substituting for yet unknown physiological regulators of kinase activity (Rosen & Lebowitz, 1988; Wedegaertner & Gill, 1989). However, the varied chemical nature of the different activating agents points toward an indirect mechanism of activation rather than the specific binding of these agents to allosteric regulatory sites on the kinase.

In this study, we used the purified, soluble intracellular domain of the EGF receptor (EGFR-IC) (67 kDa) to investigate the regulation of kinase activity by these agents. The protein was produced in Sf9 insect cells infected with recombinant baculovirus (Hsu *et al.*, 1990) and purified to 80–90% homogeneity. We found that the activation of EGFR-IC by the agents tested correlated directly with their ability to cause aggregation and precipitation of the kinase. They appear to activate the kinase by facilitating kinase–kinase interactions resulting in an increase in the specific activity of the kinase. Since these agents are able to stimulate the kinase activity of the purified EGFR-IC, their interaction with the EGFR does not require an involvement with the extracellular or transmembrane domains, nor do they require the involvement of other cellular proteins in their mechanism of action.

MATERIALS AND METHODS

Production and Purification of EGFR-IC. The complete intracellular domain of the human EGF receptor (EGFR-IC), comprised of its kinase region as well as its autophosphorylation sites, was produced in *Spodoptera frugiperda* (Sf9) cells infected by a recombinant baculovirus (Hsu *et al.*, 1990). It lacks the extracellular and transmembrane sequences of the full-length receptor and is therefore unable to bind EGF. Soluble, active EGFR-IC was purified by ion-exchange chromatography, hydrophobic interaction chromatography, and ammonium sulfate precipitation as previously described (Hsu *et al.*, 1990).

Sucrose Gradient Centrifugation. Purified EGFR-IC (10 µg/sample) was preincubated with 15 mM Mg²⁺ or 5 mM

Mn²⁺ in the presence or absence of ATP (100 µM), in a volume of 100 µL, for 15 min on ice and subsequently layered on a 7–14% sucrose density gradient (12 mL) in 50 mM HEPES, pH 7.4, containing Mg²⁺ or Mn²⁺ and ATP at the same concentration as the preincubation mixture. Corresponding gradients were calibrated with bovine serum albumin as a molecular mass marker (66 kDa, dimer 132 kDa). The gradients were centrifuged for 12 h at 10⁵g. Gradient fractions were dot-blotted onto nitrocellulose, and receptor was detected using an anti-EGFR antiserum and ¹²⁵I-protein A. Autoradiographs were densitometrically scanned to determine relative quantities of EGFR-IC in the different fractions.

Aggregation of EGFR-IC. Purified EGFR-IC was centrifuged for 15 min at 10⁴g to remove preexisting precipitate and diluted in 50 mM HEPES, pH 7.4, to the concentration used in kinase activation assay. Aliquots (60 ng) were incubated for 15 min on ice with different concentrations of ammonium sulfate (0–30% v/v of a saturated solution), poly(ethylene glycol) (PEG 1000 or PEG 8000, 0–35% w/v), 2-methyl-2,4-pentanediol (MPD, 0–35% v/v), poly(D-lysine) (average mass 45 kDa, 0–7.4 µM), protamine (0–3.3 mg/mL), or sphingosine (0–1.1 mM) in a volume of 40 µL. The samples were centrifuged for 5 min at 10⁴g, and the supernatants were discarded. The precipitates were dissolved in SDS sample buffer, subjected to SDS-PAGE, and transferred to nitrocellulose. The blots were developed with an anti-peptide antiserum recognizing the C-terminal sequence of the EGFR (anti-C-term) and with ¹²⁵I-protein A. Following autoradiography, the relative amounts of EGFR-IC in each sample were quantitated by cutting out the radioactive bands and counting in a γ-counter.

Activation of the EGFR-IC Kinase. EGFR-IC (60 ng/sample in 50 mM HEPES, pH 7.4, in a volume of 40 µL) was preincubated for 15 min on ice with 15 mM Mg²⁺ or 5 mM Mn²⁺ and different concentrations of the activating agent. A synthetic peptide (K1) representing the major autophosphorylation site (Tyr-1173) of the EGFR was added as a kinase substrate (300 µM final concentration). The kinase reaction was started by addition of [³²P]ATP (5 µM final concentration, approximately 10⁴ cpm/pmol). After 1 min at room temperature, the reaction was stopped by addition of SDS sample buffer and heating to 95 °C. Under the conditions used in each of the kinase assays, we had established that each reaction represented initial rate kinetics. To separate the phosphorylated peptide from autophosphorylated EGFR-IC and excess [³²P]ATP, the samples were analyzed by SDS-PAGE on gels consisting of a 5–15% polyacrylamide gradient overlaid on a 20% polyacrylamide gel. EGFR-IC autophosphorylation and substrate phosphorylation were quantitated separately by measuring the Cherenkov radiation emitted by the excised radioactive bands.

Purification of Antibodies and Production of Fab Fragments. IgGs from rabbit antiserum raised against a synthetic peptide representing the C-terminal sequence (amino acids 1176–1186) of the human EGFR (anti-C-term) were purified by affinity chromatography on protein A-Sepharose. The monovalent fragments of the anti-C-IgG antibody were prepared by papain digestion using the Immupure Fab preparation kit (Pierce). Fab fragments were purified by protein A affinity. SDS-PAGE analysis demonstrated that the 48-kDa Fab-anti-C was purified without traces of either the intact antibody or the Fc fragment. The monovalent Fab fragment coupled to goat anti-rabbit antibodies conjugated to protein A-Sepharose beads was able to immunoprecipitate the EGFR-IC, demonstrating that it retained its antigen

binding capacity. In addition, following immunoblotting of the EGFR-IC with the Fab fragment, we were able to detect the EGFR-IC with goat anti-rabbit antibodies conjugated to alkaline phosphatase.

A second antiserum was generated in rabbits against a synthetic peptide representing the juxtamembrane region (amino acids 650–661) of the human EGFR (VR-1). VR-1 (IgG) was purified by affinity chromatography using peptide coupled to Sepharyl S-100 (Sorokin *et al.*, 1993).

RESULTS

Activation of EGFR-IC Kinase by Manganese. Previously, we reported the kinetics of autophosphorylation and the phosphorylation of exogenous substrates by purified, soluble EGFR intracellular domain (Hsu *et al.*, 1990). The enzymatic properties displayed by this region were essentially the same as those of solubilized full-length EGF receptor in the absence of ligand. A number of synthetic peptides comprising the phosphorylation site of known EGFR substrates were tested as exogenous substrates. In this study, we used a 13 amino acid peptide representing the sequence of the main autophosphorylation site of the EGFR receptor, including and flanking Tyr-1173. This peptide was phosphorylated with a K_m of approximately 0.1 mM both by full-length EGF receptor (Honegger *et al.*, 1988) and by the EGFR intracellular domain (Hsu *et al.*, 1990). We have previously shown (Hsu *et al.*, 1990) that at a concentration of 1 pmol/30 μ L (approximately 30 nM) the EGFR-IC kinase exhibited a basal specific activity (V_{max}) of approximately 0.1–0.2 pmol of phosphate incorporated min^{-1} (pmol of kinase) $^{-1}$ in 50 mM HEPES, pH 7.4, 15 mM Mg^{2+} , and 5 μ M ATP. Substitution of Mg^{2+} by Mn^{2+} increased the V_{max} of the phosphorylation reaction 5–10 times to 1–2 pmol of phosphate min^{-1} (pmol of kinase) $^{-1}$. The K_m of different peptide substrates was not significantly affected. We employed sucrose gradient centrifugation to evaluate the effect of Mn^{2+} on EGFR-IC aggregation (Figure 1). Substituting Mn^{2+} for Mg^{2+} increased EGFR-IC aggregation. This effect was enhanced by ATP. Mn^{2+} -mediated aggregation was highly pH-dependent. Below pH 7.0, almost no dimers were detectable on sucrose gradients. At pH 7.4, 10–20% of the EGFR-IC was in the form of dimers, and at pH 8.0, approximately 50% of the EGFR-IC was in dimeric form (data not shown). However, the aggregation was not limited to dimerization, but lead to precipitation of the kinase. Therefore, determination of dimer concentration by sucrose gradient centrifugation or gel filtration was not a reproducible measure of aggregation. However, the higher aggregates could be harvested by simple centrifugation in an Eppendorf centrifuge and then quantitated by immunoblotting. We therefore utilized the centrifugation and immunoblot method in subsequent experiments.

Antibody-Mediated Activation of the EGFR-IC Kinase. When we immunoprecipitated intact EGFR from cell lysates using protein A–Sepharose-bound antibodies (RK2, C-term) recognizing various epitopes within the intracellular domain, we observed a relatively high basal activity of the receptor kinase compared to that of EGFR immunoprecipitated with antibodies against epitopes in the extracellular domain (data not shown). To test whether this activation was a direct effect of the interaction between the EGFR and the antibodies or due to increased interaction between two EGFR molecules bound to the divalent IgG, we compared the effects of intact antibodies with those of monovalent Fab fragments of the same antibodies on the specific activity of the EGFR-IC. IgGs

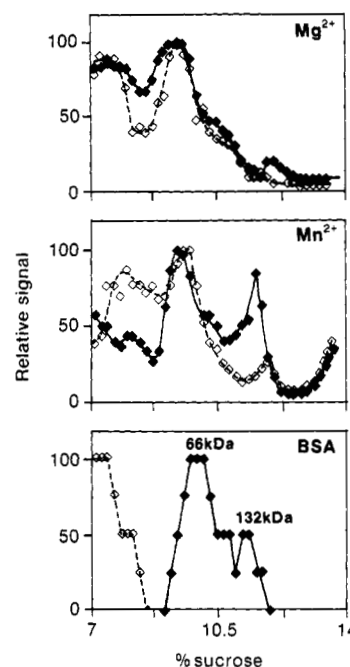


FIGURE 1: Stimulation of EGFR-IC aggregation by Mn^{2+} and ATP. EGFR-IC was incubated for 15 min on ice with either 15 mM Mg^{2+} or 5 mM Mn^{2+} in the presence (●) or absence (○) of 100 μ M ATP. A sucrose density gradient (7–14%) was used to separate monomeric from aggregated EGFR-IC. Fractions of the gradient were dot-blotted onto nitrocellulose and the blots developed with anti-EGFR antiserum and with ^{125}I -protein A. Autoradiographs were scanned in a densitometer and the relative signals plotted against the expected gradient. A gradient profile of BSA markers showing relative positions of monomers (66 kDa) and dimers (132 kDa) is shown.

from rabbit antiserum raised against a synthetic peptide representing the C-terminal sequence of the human EGF receptor (anti-C-term) were purified. Fab fragments of anti-C-term IgG were generated by digestion with papain as described under Materials and Methods.

When EGFR-IC was preincubated with intact anti-C-term antibodies, a 2–4-fold stimulation of the kinase autophosphorylation activity was achieved (Figure 2B,C). As one would expect for activation by dimerization, the activation curve showed a narrow concentration optimum (approximately 0.5–1.5 μ M). Excess antibody inhibited the kinase. This effect was seen in the presence of either Mg^{2+} or Mn^{2+} (Figure 2C). In contrast, Fab fragments of the same antibodies did not have any significant effect on the kinase activity (Figure 2A). The increased kinase activity in the presence of divalent antibodies was not restricted to autophosphorylation. Phosphorylation of a synthetic substrate peptide (K1, 300 μ M) was stimulated to an equal or higher degree than receptor autophosphorylation (Figure 2D). The stimulation of phosphorylation of peptides was greater in the presence of Mg^{2+} than Mn^{2+} . Since saturating concentrations of both peptide substrate and ATP were used throughout the experiment, the stimulation of the kinase activity represented an increase of V_{max} rather than an increase of substrate affinity (decrease of K_m). This was confirmed by a detailed analysis of the kinetics of peptide phosphorylation (data not shown).

In order to demonstrate that this antibody effect was not restricted to the anti-C-term antibody, a second antibody, VR-1, directed against the juxtamembrane portion of the receptor was evaluated. As was seen with the anti-C-term antibodies, intact VR-1 antibodies stimulated EGFR-IC autophosphorylation (Figure 2E). Optimal stimulation was again seen at approximately 0.5 μ M.

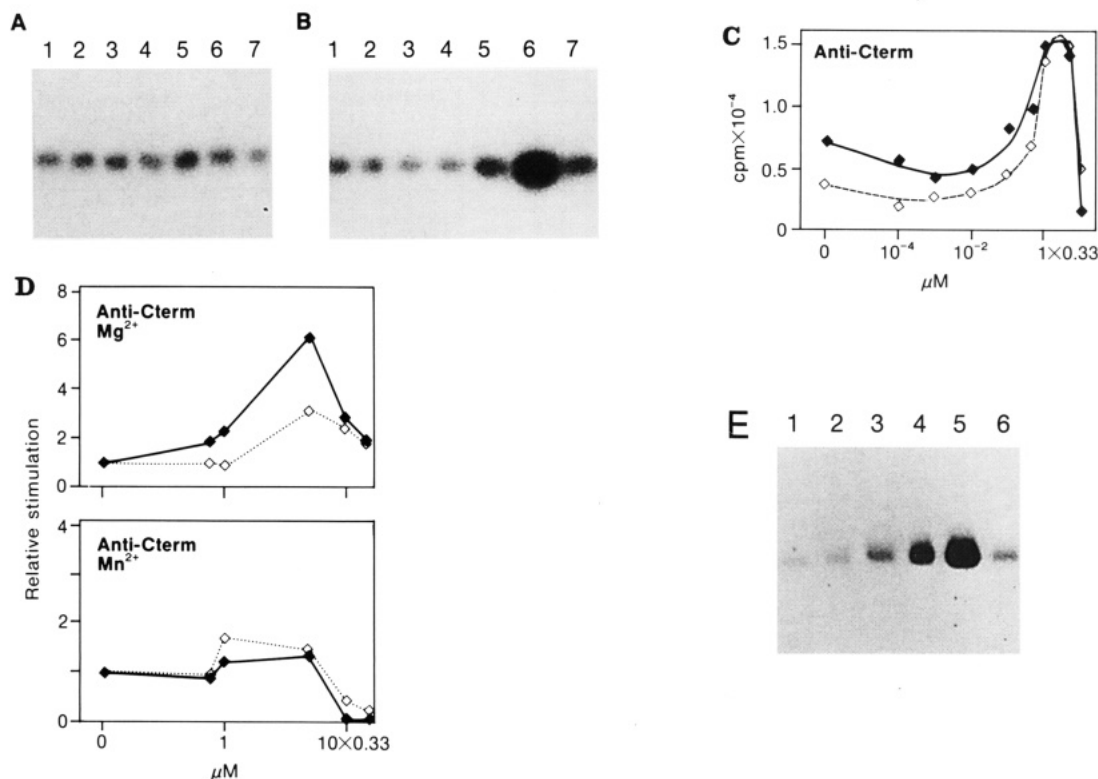


FIGURE 2: Activation of EGFR-IC by anti-EGFR antibodies. EGFR-IC (20 nM final concentration) was preincubated with different concentrations of IgG (0–5 μ M) purified from rabbit antisera raised against synthetic peptides representing the C-terminal sequence or the juxtamembrane sequence of EGFR. Alternatively, EGFR-IC was incubated with Fab fragments prepared from the former antibodies. For substrate phosphorylation, a synthetic peptide (K1) was added to a final concentration of 300 μ M. The phosphorylation reaction was started by addition of 5 μ M [³²P]ATP (approximately 10⁴ cpm/pmol) and 15 mM Mg²⁺ or 5 mM Mn²⁺. After 1 min, the reaction was stopped by addition of SDS sample buffer and heating to 95 °C. The reaction products were separated from free [³²P]ATP by SDS-PAGE, and ³²P incorporation into peptide substrate and EGFR-IC was quantitated by Cherenkov counting of the radioactive bands. (A) Autoradiograph of EGFR-IC autophosphorylated in the presence of Fab fragments of anti-C-term IgG in the presence of Mg²⁺. Lane 1 is in the absence of antibodies. Lanes 2–7 are in the presence of 0.033, 0.17, 0.25, 0.33, 1.67, and 3.3 μ M antibody, respectively. (B) Autoradiograph of EGFR-IC autophosphorylated in the presence of anti-C-term IgG in the presence of Mg²⁺. Lanes are as described in (A). (C) Dose-dependent activation of EGFR-IC autophosphorylation in the presence of Mg²⁺ (\diamond) or Mn²⁺ (\blacklozenge). (D) Dose-dependent activation of EGFR-IC autophosphorylation (\diamond) and the phosphorylation of peptide K1 substrate (\blacklozenge) by anti-C-term IgG in the presence of 15 mM Mg²⁺ or 5 mM Mn²⁺. (E) Autoradiograph of EGFR-IC autophosphorylated in the presence VR-1 antibodies and Mg²⁺. Lane 1 is in the absence of antibody. Lanes 2–6 are in the presence of 0.0003–3.3 μ M antibody in 10-fold steps.

Activation of the EGFR-IC Kinase by Protein Precipitants.

To test whether the reported kinase-stimulating effect of ammonium sulfate is linked to its well-known effect on protein solubility, we compared the level of kinase activation and precipitation at increasing concentrations of ammonium sulfate. The two effects turned out to be closely correlated, as determined by autophosphorylation (Figure 3A) and phosphorylation of the substrate peptide (data not shown). We therefore tested other agents commonly used to modulate protein solubility. At low concentrations, both poly(ethylene glycol) (PEG) and 2-methyl-2,4-pentanediol (MPD) activated the EGFR-IC kinase in direct proportion to their ability to precipitate the kinase (Figure 3A). In the presence of Mg²⁺, PEG at higher concentrations was also a strong activator of kinase activity. However, PEG at higher concentrations interfered with kinase activity. We have no explanation for this except to suggest that PEG at higher concentrations may have formed complexes with the Mn²⁺ ions.

Effects of Polycations on the EGFR-IC Kinase Activity.

Poly(D-lysine) and protamine both activated the kinase and precipitated EGFR-IC with a relatively narrow concentration optimum. The concentrations required for optimal activation and for optimal precipitation were well correlated (Figure 3B). Higher than optimal concentrations inhibited the kinase. Both poly(L-lysine) and poly(D-lysine) served as a potent

activator, indicating that the interaction between EGFR-IC and poly(lysine) is not stereospecific (data not shown). Sphingosine, a positively charged lipid, also exhibited a correlation between the activation of the soluble kinase and precipitation, although not as closely as with poly(lysine) or with protamine (Figure 3B).

The correlation between the precipitation of EGFR-IC and its stimulation of autophosphorylation activity by protein precipitants, polycations, and sphingolipid was extended to the stimulation of phosphorylation of an external peptide substrate (Figure 4). Similar activation profiles with increasing concentrations of the activating agent were obtained using either autophosphorylation or substrate phosphorylation as criteria for kinase activation except in the case of the phosphorylation of synthetic peptide in the presence of Mn²⁺ due to sphingosine. In that case, no significant activation was seen. In the presence of Mg²⁺, the stimulation of autophosphorylation, in the presence of the external peptide substrate, as well as the phosphorylation of the peptide substrate itself increased as much as 7–8 times over the basal level (Figure 4). Autophosphorylation in the absence of the external peptide substrate was increased even more markedly, up to 45-fold as seen in the case of protamine stimulation (Figure 3B). As expected, the external substrate served as a competitive inhibitor of autophosphorylation, and since it was added at a concentration well above its *K_m*, it reduced the relative

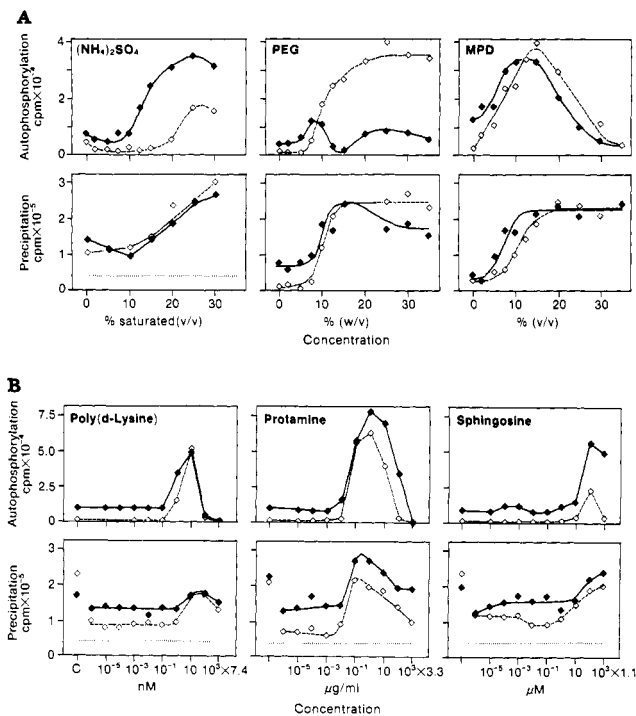


FIGURE 3: Correlation of EGFR-IC kinase activity (top of each set) and aggregation (bottom of each set). (A and B) Aliquots of EGFR-IC were incubated with different concentrations of ammonium sulfate, poly(ethylene glycol), 2-methyl-2,4-pentanediol, poly(d-lysine), protamine, or sphingosine in the presence of 15 mM Mg^{2+} (\diamond) or 5 mM Mn^{2+} (\blacklozenge). Precipitates were harvested by centrifugation in an Eppendorf centrifuge (5 min, 10^4g). The pellet was dissolved in SDS sample buffer and analyzed by SDS-PAGE, and the amount of EGFR-IC in each sample was determined by immunoblotting with anti-C-term antiserum and ^{125}I -protein A. After autoradiography, radioactive bands were cut out and counted in a γ -counter for quantitation. To determine kinase activation, aliquots of EGFR-IC were preincubated with the different activating agents in the presence of 15 mM Mg^{2+} (\diamond) or 5 mM Mn^{2+} (\blacklozenge) and then incubated for 1 min at 22 °C with $[^{32}P]$ ATP. The reaction was stopped by addition of SDS sample buffer and heating to 95 °C, and the reaction products were assayed by SDS-PAGE and Cerenkov counting of radioactive bands.

stimulation of autophosphorylation by the different activating agents.

DISCUSSION

EGF binding to its cell membrane receptor leads to dimerization of its receptors and to the activation of its intrinsic tyrosine kinase. Some antibodies against the extracellular domain of the EGFR can activate the receptor in intact cells (Schreiber *et al.*, 1981) as well as solubilized receptor (Yarden & Schlessinger, 1987a). While multivalent IgMs are capable of activating receptor, monovalent Fab fragments of these antibodies are unable to activate the receptor (Fernandez-Pol, 1985). Similarly, while IgG antibodies directed against the insulin receptor are able to stimulate the receptor kinase activity, monovalent Fab fragments of the same antibody do not possess this stimulatory activity (Heffetz & Zick, 1986). Significantly, cross-linking of the Fab fragments with a secondary antibody returned the Fab's ability to activate the receptor kinase. We have shown in this report that antibodies against the C-terminal sequence and against the juxtamembrane sequence of the EGFR activated purified EGFR-IC with a narrow concentration optimum while monovalent Fab fragments were unable to activate it. This indicates that the activation is not directly due to the interaction of the antibody with its binding site on the kinase, but is aggregation dependent.

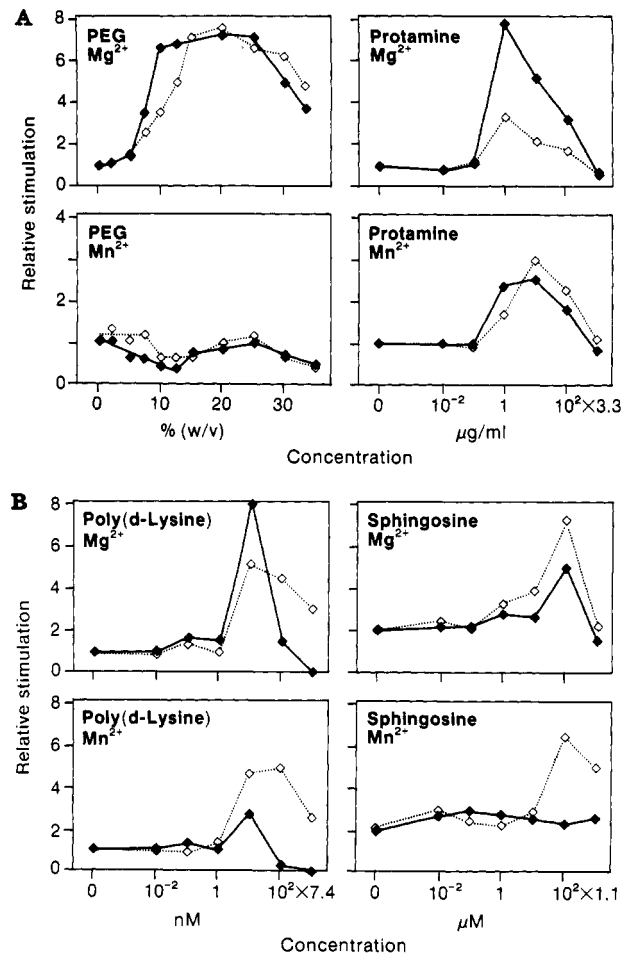


FIGURE 4: Relative stimulation of autophosphorylation and phosphorylation of peptide substrate by different activating agents. (A and B) Aliquots of EGFR-IC were incubated with different concentrations of the various activating agents. After 15-min incubation on ice, $[^{32}P]$ ATP and peptide substrate were added, and the reaction was allowed to proceed for 1 min. The reaction was stopped by the addition of SDS sample buffer, and the reaction products were separated by SDS-PAGE. ^{32}P incorporation into autophosphorylated EGFR-IC (\diamond) and peptide substrate (\blacklozenge) was determined separately.

An excess of antibody resulted in a decrease in kinase activity, since under these stoichiometries each divalent IgG molecule would bind only one molecule of kinase, thereby preventing aggregation.

Receptor dimerization could enhance kinase autophosphorylation by two different mechanisms. Intermolecular autophosphorylation may be facilitated by receptor aggregation through the increase in the local concentration of substrate sites in the vicinity of the kinase active site. Alternatively, dimerization might lead to a conformational change, altering the enzymatic properties of the kinase, either by increasing its affinity to substrate or by increasing the catalytic rate of the enzyme. With an external substrate, whose concentration can be varied independently of the enzyme concentration, the two mechanisms can be distinguished. We have shown that the antibody-mediated dimerization increased the phosphorylation of peptide substrates in addition to autophosphorylation, indicating that the increased autophosphorylation is not simply due to increased accessibility of the autophosphorylation sites, but reflects a change in the specific activity of the kinase.

Various other activating agents were able to increase the kinase activity of EGFR-IC as much as 20-fold over basal activity in the presence of Mg^{2+} . This specific activity is

comparable to that of solubilized full-length receptor in the presence of ligand. In all cases tested, this activation of the EGFR intracellular domain correlated with its increased aggregation. Conditions which optimally stimulated kinase activity led to the formation of large aggregates and to the precipitation of the EGFR-IC. Recently, Canals (1992) demonstrated that the second-order rate constants for the EGF-induced dimerization of solubilized full-length receptor and the activation of the receptor tyrosine kinase by EGF are indistinguishable, and concluded that the dimerization itself is the rate-limiting step in the EGF-induced kinase activation. Consistent with these results are the dominant-negative effects of kinase-defective EGF receptors in heterodimers with wild-type receptors on EGF-induced tyrosine autophosphorylation, the stimulation of DNA synthesis, and cell transformation in living cells (Honegger *et al.*, 1990; Kashles *et al.*, 1991; Redemann *et al.*, 1992).

We have previously shown that the kinase activity of EGFR-IC is greater in the presence of Mn^{2+} than in the presence of Mg^{2+} (Hsu *et al.*, 1990). In this study, we show that Mn^{2+} , particularly in the presence of ATP, results in a much higher level of EGFR-IC aggregation than does Mg^{2+} . It has been suggested that Mn^{2+} activates the EGF receptor kinase by binding directly to an allosteric binding site on the receptor, thus acting as an effector molecule or a mimic of an effector molecule (Koland & Cerione, 1988). Similarly, the insulin receptor and the insulin receptor kinase domain also exhibit a strong preference for Mn^{2+} over Mg^{2+} . However, direct physicochemical methods could not detect a shift in the electron paramagnetic resonance or intrinsic fluorescence emission spectra of the soluble insulin receptor kinase domain in the presence of Mn^{2+} , demonstrating that Mn^{2+} does not bind tightly to this receptor (Wente *et al.*, 1990).

Ammonium sulfate has been reported to be a potent stimulator of the *in vitro* activity of the EGFR kinase (Wedegaertner & Gill, 1989). In contrast, neither ammonium chloride nor sodium sulfate significantly affects the activity at comparable concentrations, indicating that neither of the two ionic species interacts directly with the kinase to activate it. Ammonium sulfate is known to facilitate protein-protein interactions by organizing the surrounding water structure and thus strengthening hydrophobic interactions. Ammonium sulfate precipitation is a classical method for protein purification. Indeed, it is an efficient purification step for the EGFR-IC, since at pH 7.4, the protein precipitates in 10–20% saturated ammonium sulfate, a concentration at which most other cellular proteins are still soluble (Hsu *et al.*, 1990). Comparing kinase activation and precipitation of the EGFR-IC as a function of ammonium sulfate concentration, we demonstrate in this report that the two effects are closely correlated.

To confirm that the activation of the EGFR kinase by ammonium sulfate is a direct effect of aggregation and not an effect of a direct interaction of either ammonium or sulfate ions with the kinase, other precipitating agents of completely different chemical structure were tested in the same assay system. Like ammonium sulfate, poly(ethylene glycol) (PEG-8000) and 2-methyl-2,4-pentanediol (MPD) are widely used to induce protein crystallization by decreasing protein solubility while at the same time preserving native structure and enzymatic activity. Both PEG and MPD optimally activated the kinase at concentrations which induce precipitation. While PEG was a potent activator in the presence of Mg^{2+} , it interfered with the activation by Mn^{2+} , probably by complexing the metal ion. MPD activated the kinase at low concentrations,

but became inhibitory at high concentrations, where the precipitation of EGFR-IC became irreversible, presumably due to denaturation of the kinase.

A number of polycationic agents, such as poly(L-lysine) and protamine, have been reported to activate the protein tyrosine kinases of insulin receptor and of EGFR. We have shown that these agents also activate the kinase of EGFR-IC. As with previous agents tested, the activator concentrations required to optimally stimulate the kinase corresponded to the concentration optimum for precipitation. At the appropriate ratio of receptor to polycation, binding of negatively charged regions of the kinase to the polycations can lead to the formation of mixed aggregates, while an excess of the polycation would, as in the case of antibodies, be expected to interfere with kinase aggregation and activation. Both poly(lysine) and protamine activate the EGFR-IC with a narrow concentration optimum. The ability of polycations and other basic proteins (K-ras peptide) to stimulate the kinase activity of the insulin receptor has also been correlated with their ability to promote receptor aggregation (Kohanski, 1989; Biener & Zick, 1990; Xu *et al.*, 1991). Significantly, preventing aggregation of the insulin receptor by immobilization eliminated the stimulatory effect of poly(lysine) or receptor kinase activity. The lipid sphingosine might activate the kinase by a similar mechanism, since at the concentration required for stimulation it forms micelles. Their positively charged surface might act as a template for kinase aggregation. The additional negative charges introduced by autophosphorylation might well increase the interaction between the IR intracellular domain and the polycation. In our study, except in one case, EGFR-IC precipitation was measured in the absence of ATP with EGFR-IC containing little or no tyrosine phosphate (Hsu *et al.*, 1990). The exception was the sucrose gradient centrifugation experiments, where Mn^{2+} -induced dimerization seems to be stabilized in the presence of ATP. We measured activation under initial rate conditions where receptor autophosphorylation was low. Thus, it appears that while autophosphorylation may facilitate aggregation of the EGFR-IC by polycations, it is not absolutely required.

The activation of the EGFR-IC by the different agents tested in this study represents an artificial system, but it serves well to demonstrate the direct correlation between kinase activation and dimerization. Both ligand activation of full-length receptor and aggregation of the EGFR-IC predominantly affect the V_{max} of the kinase. *In vivo*, ligand binding to the extracellular domain of the receptor leads to dimerization of the extracellular domain. This brings together the intracellular domains of the dimeric receptors, allowing them to interact. The cell membrane limits dimerization to two dimensions, preventing the formation of aggregates too large to allow efficient substrate exchange. Activation of the soluble intracellular domain by a wide variety of agents able to promote aggregation demonstrates that aggregation is indeed sufficient for kinase activation. These results support the idea that receptor aggregation is indeed the causal link between ligand binding to the extracellular domain of tyrosine kinase receptors and the activation of their intracellular kinases.

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