

Activation of Insulin–Epidermal Growth Factor (EGF) Receptor Chimerae Regulates EGF Receptor Binding Affinity

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Abstract. Cell surface tyrosine kinase receptors are subject to a rapid activation by their ligand, which is followed by secondary regulatory processes. The IHE2 cell line is a unique model system to study the regulation of EGF binding to EGF receptors after activation of the EGF receptor kinase. IHE2 cells express both a chimeric insulin-EGF receptor kinase (IER) and a kinase-deficient EGF receptor (HER K721A). We have previously reported that IER is an insulin-responsive EGF receptor tyrosine kinase that activates one or several serine/threonine kinases, which in turn phosphorylate(s) the unoccupied HER K721A. In this article we show that insulin through IER activation induces a decrease in ¹²⁵I-EGF binding to IHE2 cells. Scatchard analysis indicates that, as for TPA, the effect of insulin can be accounted for by a loss of the high affinity bind-

ing of EGF to HER K721A. Since this receptor transmodulation persists in protein kinase C downregulated IHE2 cells, it is likely to be due to a mechanism independent of protein kinase C activation. Using an in vitro system of ¹²⁵I-EGF binding to transmodulated IHE2 membranes, we illustrate that the inhibition of EGF binding induced by IER activation is related to the phosphorylation state of HER K721A. Further, studies with phosphatase 2A, or at a temperature (4°C) where only IER is functional, strongly suggest that the loss of high affinity EGF binding is related to the serine/threonine phosphorylation of HER K721A after IER activation. Our results provide evidence for a “homologous desensitization” of EGF receptor binding after activation of the EGF receptor kinase of the IER receptor.

THE EGF receptor is a 170-kD transmembrane glycoprotein. The extracellular region contains the growth factor binding domain, whereas the intracellular region carries the tyrosine kinase catalytic domain (33, 38). These two functions of the EGF receptor, i.e., EGF binding and tyrosine kinase activity, play cardinal roles in the induction of the EGF biological effects and are subject to fine regulatory mechanisms. Receptor tyrosine kinase activity is positively regulated by EGF binding, which leads to tyrosine phosphorylation of the receptor (15, 20, 21, 28) and of cellular substrates (38). Recent data have indicated that tyrosine phosphorylation of the carboxy-terminal tail of the EGF receptor promotes the interaction with signaling molecules such as phospholipase C- γ and GTP-ase activating protein (GAP)¹ (29). In addition to these positive regulations, both the binding function and the kinase activity of the EGF receptor are subject to negative controls, which are mainly mediated by activation of protein kinase C. Thus, activators of protein kinase C, such as tetradecanoyl phorbol acetate (TPA) or PDGF, decrease the intrinsic tyrosine kinase ac-

tivity of the EGF receptor (7, 16, 17, 23). The major EGF receptor site phosphorylated by protein kinase C appears to be threonine 654 (12, 22). Studies using site-directed mutagenesis have shown that phosphorylation of the EGF receptor at threonine 654 is responsible for protein kinase C-induced inhibition of the receptor tyrosine kinase (11). EGF binding is also altered by activation of protein kinase C (25, 34, 41), but the mechanism by which protein kinase C induces a loss of high affinity binding sites on EGF receptor is not entirely elucidated. In fact, replacement of threonine 654 with an alanine residue (9, 11) or a tyrosine residue (27) does not affect regulation of EGF binding caused by TPA-induced activation of protein kinase C. However, Lin et al. have reported that the phosphorylation of threonine 654 was required for the inhibition of EGF binding by TPA (26). A protein kinase C-independent mechanism has also been implicated in the regulation of the high affinity binding sites of the EGF receptor. Indeed, PDGF, interleukin 1, and tumor necrosis factor are able to induce a loss of the high affinity binding sites in cells depleted of protein kinase C after a long-term TPA treatment (4, 9, 31). This effect seems to be linked to the phosphorylation state of the EGF receptor (5, 13). This heterologous receptor transmodulation provides a negative regulation of EGF-induced biological effects (33).

1. *Abbreviations used in this paper:* GAP, GTP-ase activating protein; HER K721A, human kinase-deficient EGF receptor; IER, insulin-EGF receptor chimerae; TPA, tetradecanoyl phorbol acetate.

In a previous report, using a cell line expressing both a chimeric insulin-EGF tyrosine kinase receptor (IER) and a kinase-deficient EGF receptor (HER K721A), we have shown that the insulin-activated IER chimeric receptor phosphorylates the HER K721A on tyrosine residues (37). In addition to this tyrosine transphosphorylation, insulin enhances the phosphorylation of HER K721A on serine/threonine residues. This implies that IER activates one or several serine/threonine kinases, which in turn phosphorylates the unoccupied HER K721A. Those observations demonstrate the existence of cross-talk between an unoccupied EGF receptor and a ligand-activated EGF receptor kinase (37).

In this work, taking advantage of the same receptor model system, we investigated the regulation of EGF binding to the HER K721A after insulin-induced stimulation of the IER. We show that insulin through IER activation induces a decrease in EGF binding to IHE2 cells and, as for activation of protein kinase C, this effect can be accounted for by an inhibition of the high affinity binding of EGF to cell surface kinase-deficient EGF receptors. Since this receptor transmodulation persisted in IHE2 cells depleted of protein kinase C, it appears to be due to a mechanism independent of protein kinase C. Studies with phosphatase 2A, or at a temperature where virtually only tyrosine kinases are functional, strongly suggest that this inhibition of high affinity EGF binding is related to the serine/threonine phosphorylation of HER K721A after IER activation. In conclusion, we describe here an original "homologous desensitization" situation of the EGF receptor binding function after activation of the EGF receptor kinase of the IER receptor.

Materials and Methods

Mouse EGF "receptor grade" was from Sigma Chemical Co. (St. Louis, MO) and insulin was from Novo Nordisk Biolabs, Copenhagen, Denmark). EGF was iodinated as described using the chloramine T method to a specific activity of 150 $\mu\text{Ci}/\mu\text{g}$ (19). [^{32}P]Orthophosphate was obtained from Amersham Corp. (Bucks, UK). TPA, Triton X-100, protein A-Sepharose, and BSA (A7030) were from Sigma Chemical Co. All reagents for SDS-PAGE were from Bio-Rad Laboratories (Richmond, CA). Antiphosphotyrosine antibodies were obtained by immunization of a sheep with phosphotyrosine coupled to bovine IgG and affinity-purified on a phosphotyrosine-agarose column (2). mAb108 is a monoclonal antibody directed to the extracellular domain of the human EGF receptor (19). Protein phosphatase 2A was a gift of P. Cohen (Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee, UK).

Cell Lines

Details concerning the generation and the culture of the IHE2 cell lines have been previously described (37). In brief, IHE2 cells express both a kinase-deficient human EGF receptor, HER K721A, and a chimeric receptor IER composed of the extracellular domain of the human insulin receptor linked to the transmembrane and the cytosolic domain of the human EGF receptor. IHE2 cells expressed 2×10^5 IER receptors and 2×10^5 HER K721A receptors per cell. IER cells are mouse NIH3T3 fibroblasts transfected with the expression plasmid encoding for the chimeric IER receptor (32). The parental cell line, NIH3T3, expresses 2×10^4 and 1.6×10^4 endogenous mouse insulin and EGF receptors per cell, respectively.

^{125}I -EGF Binding to IHE2 Cells

Confluent IHE2 cells in 12-well culture dishes were incubated for 2 h at 37°C in serum-free DME containing 0.2% BSA. After treatment with 0.1 μM insulin or 0.1 μM TPA at 37°C, cells were rapidly cooled on ice. The binding of subsaturating concentrations of ^{125}I -EGF (0.1 nM) to cell surface receptors was measured by incubation of the cells at 4°C for 3 h in DME containing 0.2% BSA and 30 mM Hepes (pH 7.4). For Scatchard

analyses, the cells were incubated for 3 h at 4°C with increasing concentrations of ^{125}I -EGF (from 10 to 3,000 pM) in the same binding medium. Nonspecific binding was estimated in incubations containing a 100-fold excess of unlabeled EGF. Cells were rinsed two times with ice-cold PBS and solubilized in 0.2 N NaOH. The radioactivity was determined in a gamma-radiation counter.

Preparation of IHE2 Membranes

After being starved for 2 h in DME containing 0.2% BSA, IHE2 cells in 100-mm culture dishes were treated without and with insulin for 15 min at 37°C. The medium was aspirated and the cells were collected by scraping either in ice-cold buffer A (50 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EGTA, and protease inhibitors [100 U/ml aprotinin and 1 mM PMSF]) or in ice-cold buffer B, which contains in addition to buffer A, phosphatase inhibitors (50 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EGTA, 10 mM EDTA, 100 mM NaF, 2 mM vanadate, 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, and protease inhibitors [100 U/ml aprotinin and 1 mM PMSF]). Thereafter, the cells were homogenized by 15 passages through a 26-gauge needle. The crude membrane preparation was obtained by centrifugation at 15,000 g for 15 min. Binding of ^{125}I -EGF to IHE2 membranes was measured by incubation of 10–15 μg of membranes in 0.3 ml of 50 mM Hepes, pH 7.4, 150 mM NaCl, and 0.2% BSA in the presence of 0.1 nM ^{125}I -EGF and unlabeled EGF for the determination of the nonspecific binding. After 3 h of incubation at 4°C, the membranes were washed two times with PBS and counted.

^{32}P -Labeling of Cells and Immunoblotting with Antiphosphotyrosine Antibodies

IHE2 cells were labeled with [^{32}P]orthophosphate and HER K721A receptors isolated by immunoprecipitation with mAb108 and SDS-PAGE as described (37). Immunoblotting analysis using anti-phosphotyrosine antibodies was performed as previously described (37).

Protein Phosphatase 2A Treatment

Membranes from IHE2 cells exposed to insulin for 15 min were prepared in buffer B as described above. After two washes to remove phosphatase inhibitors, 10–15 μg of membranes were incubated in buffer C (150 mM NaCl, 5 mM Tris, pH 7.4, 1 mM EGTA, and protease inhibitors) in the presence of protein phosphatase 2A (10 U/ml final) at 30°C for 20 min. Control incubations were performed using protein phosphatase 2A inactivated with inhibitors (100 mM NaF and 10 mM $\text{Na}_4\text{P}_2\text{O}_7$). At the end of the treatment, the reaction was stopped by the addition of buffer B and the binding of ^{125}I -EGF to membranes was measured. In parallel to the binding studies, membranes from IHE2 cells or ^{32}P -labeled IHE2 cells were processed and treated with protein phosphatase 2A as described above. Thereafter, membranes were solubilized for 15 min in buffer B containing 1% Triton X-100 and receptors were obtained by immunoprecipitation with mAb108 prebound on protein A-Sepharose. Finally, receptors were subjected to SDS-PAGE. Unlabeled receptors were revealed by immunoblotting with antibodies to phosphotyrosine and ^{125}I -protein A, whereas ^{32}P -labeled receptors were visualized by autoradiography.

Results

Effect of Insulin and TPA on ^{125}I -EGF Binding to IHE2 Cells

IHE2 cells expressing both the IER and the HER K721A were used to study the effect of insulin-activated IER on ^{125}I -EGF binding to HER K721A receptors. Confluent IHE2 cells were incubated at 37°C with insulin for increasing periods of time, and thereafter the binding of ^{125}I -EGF to cell surface receptors was measured at 4°C. Fig. 1 A shows that exposure of IHE2 cells to insulin caused a rapid decrease in the binding of ^{125}I -EGF to kinase-deficient EGF receptors. Maximal inhibition (50% of the specific initial binding) was observed after 10 min of insulin treatment, and it remained constant up to 20 min of insulin stimulation. The time course of insulin-induced inhibition of EGF binding to

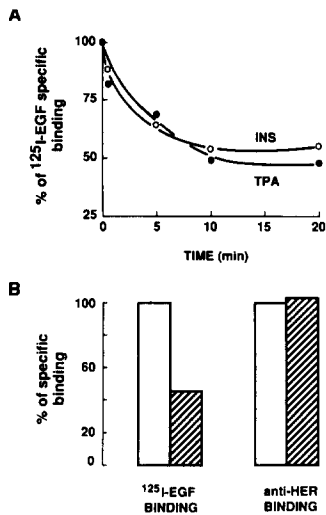


Figure 1. (A) Effect of insulin and TPA on ^{125}I -EGF binding to IHE2 cells. IHE2 cells in 12-well dishes were incubated with $0.1 \mu\text{M}$ insulin (\circ) or $0.1 \mu\text{M}$ TPA (\bullet) for the indicated times at 37°C . Specific ^{125}I -EGF binding to cell surface receptors was determined at 4°C as described in Materials and Methods. (B) Effect of insulin on HER K721A number in IHE2 cells. IHE2 cells in 12-well dishes were incubated with $0.1 \mu\text{M}$ insulin (hatched bars) for 15 min at 37°C . Thereafter, the binding of ^{125}I -EGF or antibody to HER K721A (anti-HER) was measured at 4°C . Antibody

binding was determined by incubation with ^{125}I -protein A. Data shown are the mean of triplicate determinations expressed as a percentage of specific binding observed in untreated cells. Similar results were obtained in three separate experiments. *INS*, insulin.

IHE2 cells was compared with that induced by TPA, which has been shown to decrease the high affinity EGF binding (25, 34). No significant difference between the action of TPA and insulin on ^{125}I -EGF binding to IHE2 cells was observed (Fig. 1 A).

In Fig. 1 B we examined the number of cell surface kinase-deficient EGF receptors after 15 min of exposure to insulin. The number of receptors was estimated using a monoclonal antibody against the extracellular domain of the EGF receptor. We found that insulin did not change the number of immunoreactive HER K721A. These data suggest that the insulin-induced decrease in EGF binding to IHE2 cells cannot be accounted for by a decrease in the number of cell surface kinase-deficient EGF receptors.

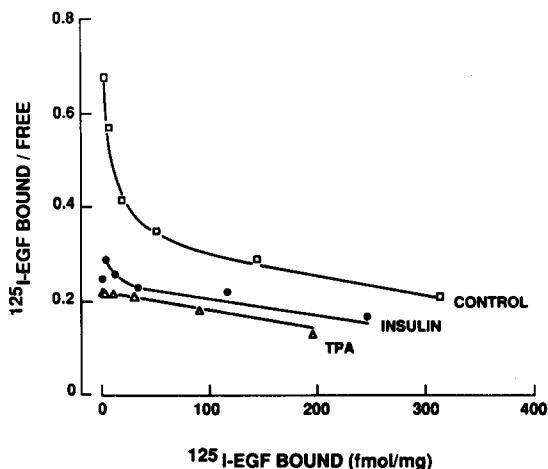


Figure 2. Scatchard analysis of ^{125}I -EGF binding to IHE2 cells after treatment with insulin and TPA. IHE2 cells in 12-well dishes were treated with buffer (\square), $0.1 \mu\text{M}$ insulin (\bullet), or $0.1 \mu\text{M}$ TPA (Δ) for 15 min at 37°C . Specific ^{125}I -EGF binding (from 10 to 3,000 pM) to cell surface receptors was determined at 4°C . Binding data were analyzed according to Scatchard. Similar results were obtained in three separate experiments.

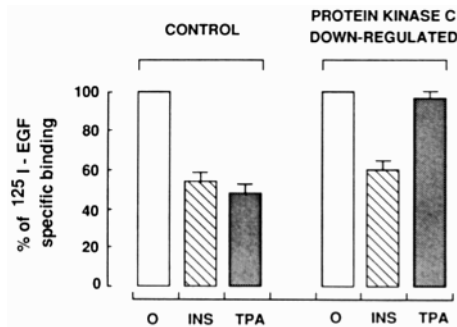


Figure 3. Effect of insulin on ^{125}I -EGF binding in protein kinase C downregulated cells. IHE2 cells in 12-well dishes were exposed to $1 \mu\text{M}$ TPA for 24 h at 37°C in order to downregulate protein kinase C. Thereafter, control and downregulated IHE2 cells were incubated for 15 min without (open bars) or with $0.1 \mu\text{M}$ insulin (hatched bars) or $0.1 \mu\text{M}$ TPA (closed bars). Specific ^{125}I -EGF binding was measured at 4°C . The results are presented as the mean \pm SEM of three separate experiments, and are expressed as the percentage of specific binding observed in untreated cells.

Scatchard Analysis of ^{125}I -EGF Binding to IHE2 Cells After Exposure to Insulin or TPA

Given this unchanged receptor number we examined the possibility that the effect of insulin was due to a change in EGF receptor affinity as previously documented for TPA (25, 34). Therefore, Scatchard analysis of ^{125}I -EGF binding to IHE2 cells treated for 15 min with insulin and TPA was performed (Fig. 2). In control cells we obtained a curvilinear Scatchard plot, which has previously been interpreted to reflect the presence of two populations of binding sites (19, 24), one in a high affinity state with a dissociation constant of $\sim 0.1 \text{ nM}$ (5% of the sites) and a second one with a low affinity and a dissociation constant of $\sim 4.9 \text{ nM}$ (95% of the sites). Addition of TPA or insulin led to the virtually complete loss of the high affinity binding of EGF to the cell surface HER K721A. Scatchard analysis of binding experiments with higher competing EGF concentrations demonstrated that control and TPA- or insulin-treated cells have an identical number of low affinity HER K721A receptors (2×10^5 receptors per cell; data not shown). Taken together these data indicate that the effect of insulin on EGF binding in IHE2 cells was due to a decrease in the high affinity binding to cell surface kinase-deficient EGF receptors.

Effect of Insulin on ^{125}I -EGF Binding in Protein Kinase C Downregulated IHE2 Cells

To further compare the action of insulin and TPA we investigated the effect of insulin on protein kinase C downregulated IHE2 cells. It has previously been documented that incubation of cultured cells with phorbol esters causes the downregulation of protein kinase C (31). Confluent IHE2 cells were exposed to $1 \mu\text{M}$ TPA for 24 h at 37°C in order to decrease protein kinase C activity (Fig. 3). In comparison to control cells, acute TPA addition (15 min) to protein kinase C downregulated IHE2 cells did not lead to an inhibition of the high affinity ^{125}I -EGF binding to HER K721A. In contrast, after 15 min of exposure, insulin decreased ^{125}I -EGF binding to a similar extent in both control and TPA-pretreated cells. These results suggest that the mechanism by

Table I. Effect of Insulin and TPA on EGF Binding to NIH3T3, IHE2, and IER Cells

Cell lines	% specific ¹²⁵ I-EGF binding		
	Control	TPA	Insulin
NIH3T3	100 ± 1	19 ± 3	102 ± 3
IHE2	100 ± 9	45 ± 4	49 ± 8
IER	100 ± 2	23 ± 1	28 ± 1

Cells in six-well dishes were incubated with 0.1 μM of insulin or 0.1 μM of TPA for 20 min at 37°C. Specific ¹²⁵I-EGF binding to cell surface receptors was determined at 4°C. The results are presented as the mean ± SEM of three separate experiments, and are expressed as the percentage of specific binding observed in untreated cells (control). 100% of specific binding (control values) were 3,443 ± 31 cpm, 40,987 ± 3,000 cpm, and 2,738 ± 39 cpm for NIH3T3, IHE2, and IER cells, respectively.

which insulin-induced activation of the chimeric IER transmodulates EGF binding differs from that of TPA, and is therefore very likely to be independent of protein kinase C.

Effect of Insulin and TPA on ¹²⁵I-EGF Binding to Wild-Type EGF Receptors in IER Cells

Next we were interested to see whether insulin-induced activation of the chimeric receptor altered EGF binding to wild-type EGF receptors. To address this question we have studied the binding of ¹²⁵I-EGF to wild-type endogenous EGF receptor in IER cells, which express only the chimeric receptor. The effect of insulin and TPA on ¹²⁵I-EGF binding was examined in parental NIH3T3, IER, and IHE2 cells (Table I). In the three cell lines, addition of TPA resulted in inhibition of ¹²⁵I-EGF binding. Treatment of IER cells by insulin led to a pronounced decrease of ¹²⁵I-EGF binding (72% of the specific initial binding) to wild-type endogenous EGF receptor, whereas in nontransfected cells insulin did not change ¹²⁵I-EGF binding to EGF receptors. These data indicate that IER activation induced a decrease in EGF binding not only to the kinase-deficient EGF receptor, but also to the wild-type EGF receptor.

Role of Receptor Phosphorylation on the Insulin-induced Decrease in ¹²⁵I-EGF Binding to IHE2 Cells

In a previous report we have shown that in IHE2 cells activation of the insulin-EGF chimeric receptor by insulin increases the tyrosine, serine, and threonine phosphorylation of the kinase-deficient EGF receptor (37). To investigate the relationship between the phosphorylation state of HER K721A and its transmodulation by IER, we studied ¹²⁵I-EGF binding to membranes from IHE2 cells exposed for 15 min to insulin. IHE2 membranes were prepared in either the absence or presence of phosphatase inhibitors. When insulin-stimulated IHE2 cells were used to prepare membranes in a buffer containing phosphatase inhibitors, we observed a 40% decrease in high affinity ¹²⁵I-EGF binding to these membranes (Fig. 4). In contrast, the effect of insulin was not found when the membrane fractions were isolated in the absence of phosphatase inhibitors. These data indicate that transmodulation of the kinase-deficient EGF receptor affinity by insulin-evoked IER activation was dependent on the receptor phosphorylation state.

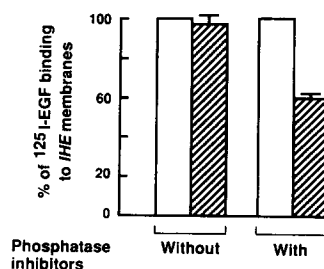


Figure 4. ¹²⁵I-EGF binding to membranes from IHE2 cells exposed to insulin: role of receptor phosphorylation. Membranes from control (open bars) or insulin-treated IHE2 cells (hatched bars) were prepared in the absence or presence of phosphatase inhibitors as described in Materials and Methods. Specific ¹²⁵I-EGF

binding to IHE2 membranes (10–15 μg/point) was measured at 4°C for 3 h. The results are presented as the mean ± SEM of three separate experiments and are expressed as the percentage of specific binding observed in control membranes.

Influence of Temperature on the Insulin-induced Decrease in ¹²⁵I-EGF Binding to IHE2 Cells

Upon exposure of IHE2 cells to insulin, HER K721A was phosphorylated on tyrosine, serine, and threonine residues (37). Generally speaking, receptor tyrosine kinases have been found to retain activity at 4°C, while serine/threonine kinases show no or little activity at this temperature (1, 6). Therefore, we performed binding and phosphorylation experiments at 4°C to determine whether tyrosine phosphorylation of HER K721A by IER was sufficient for induction of the decrease in high affinity EGF binding. The effect of temperature on the ability of insulin to decrease ¹²⁵I-EGF binding to cell surface HER K721A receptors is illustrated in Fig. 5 A. Confluent IHE2 cells were incubated for 2 h either at 37°C or at 4°C, and thereafter exposed for 15 min to insulin. Finally, ¹²⁵I-EGF binding to IHE2 cells was measured at 4°C. As described previously, at 37°C insulin decreased ¹²⁵I-EGF binding to IHE2 cells (50% of inhibition). However, at 4°C this decrease caused by insulin was totally absent. In a parallel experiment we also looked at the phosphorylation state of kinase-deficient EGF receptors. After 15 min of insulin stimulation either at 37 or 4°C, IHE2 cells were solubilized, HER K721A receptors were immunoprecipitated with mAb108 and analyzed by SDS-PAGE. Finally, the tyrosine-phosphorylated proteins were revealed by Western blotting with antibodies to phosphotyrosine. As shown in Fig. 5 B, activation of IER receptors by insulin led to tyrosine transphosphorylation of HER K721A to a comparable extent at both 37 and 4°C. In IHE2 cells labeled with [³²P]orthophosphate, treatment by insulin at 37°C increased phosphorylation of HER K721A, but at 4°C no significant increase in phosphorylation of this receptor was detected. Since we have previously shown that after 15 min of insulin stimulation HER K721A retained some phosphorylation on tyrosine residues with a predominant phosphorylation on serine and threonine residues (37), these data suggest that at 4°C only the tyrosine kinase of the insulin-EGF chimeric receptor was active and able to transphosphorylate the kinase-deficient EGF receptors. As a whole our data indicate that tyrosine transphosphorylation of the cell surface kinase-deficient EGF receptors is not sufficient for the induction of an insulin-mediated decrease in EGF receptor affinity.

¹²⁵I-EGF Binding to IHE2 Membranes Treated with Protein Phosphatase 2A

To determine whether serine/threonine phosphorylation of

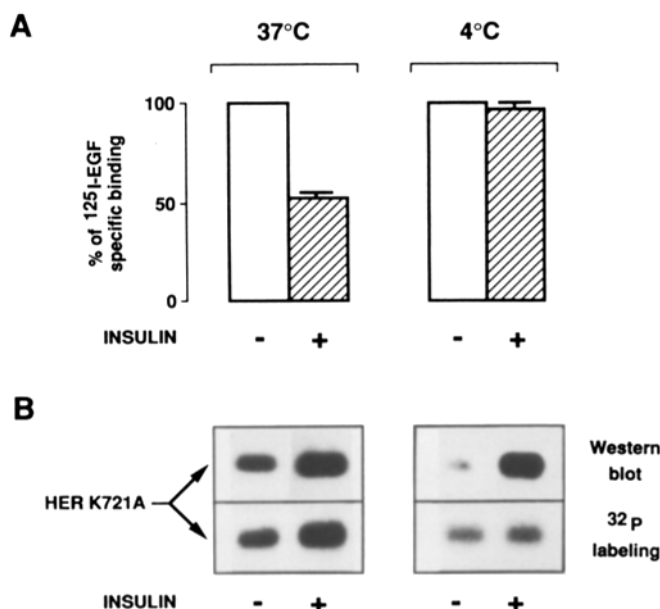


Figure 5. Influence of temperature on the insulin-induced decrease in ¹²⁵I-EGF binding to IHE2 cells. (A) IHE2 cells were incubated at either 37 or 4°C without (open bars) or with 0.1 μM insulin (hatched bars) for 15 min. Specific ¹²⁵I-EGF cell surface binding was measured at 4°C. The results are presented as the mean ± SEM of three separate experiments and are expressed as the percentage of specific binding observed in untreated cells. (B) Western blot: IHE2 cells were incubated at either 37 or 4°C without or with 0.1 μM insulin for 15 min. The cells were solubilized and HER K721A was immunoprecipitated and separated by SDS-PAGE. After transfer to an Immobilon P membrane, the tyrosine-phosphorylated proteins were revealed by antibodies to phosphotyrosine. For in vivo labeling, IHE2 cells were incubated with [³²P]orthophosphate (1 mCi/ml) at 37°C for 2 h followed by 30 min at either 37 or 4°C, and finally for 15 min without or with 0.1 μM insulin. After solubilization, the extracted proteins were precipitated with antibody to HER K721A and analyzed by SDS-PAGE. The gels of the experiment performed at 37 and at 4°C were dried and autoradiographed.

HER K721A was correlated with inhibition of EGF binding, we studied ¹²⁵I-EGF binding to transmodulated IHE2 membranes, which were treated with protein phosphatase 2A, a protein phosphatase considered to be specific for phosphoserine and phosphothreonine (8). Fig. 6 A shows that when membranes from insulin-stimulated IHE2 cells were treated with protein phosphatase 2A, the high affinity EGF binding could be restored. Accordingly, the insulin-induced phosphorylation of HER K721A in ³²P-labeled IHE2 cells disappeared by treatment of membranes with the phosphatase (Fig. 6 B). It has been shown that, in addition to its well-documented phosphatase activity toward phosphoserine and phosphothreonine, protein phosphatase 2A can exert in vitro a phosphotyrosine phosphatase activity (8, 18). To investigate this possibility we probed the protein phosphatase 2A-treated membranes with antibodies to phosphotyrosine. The immunoblot shows that under our experimental conditions the protein phosphatase 2A did not remove phosphate from phosphotyrosine residues (Fig. 6 B). Hence dephosphorylation of phosphoserine and phosphothreonine residues on HER K721A suffices for the restoration of the high affinity EGF binding to IHE2 membranes.

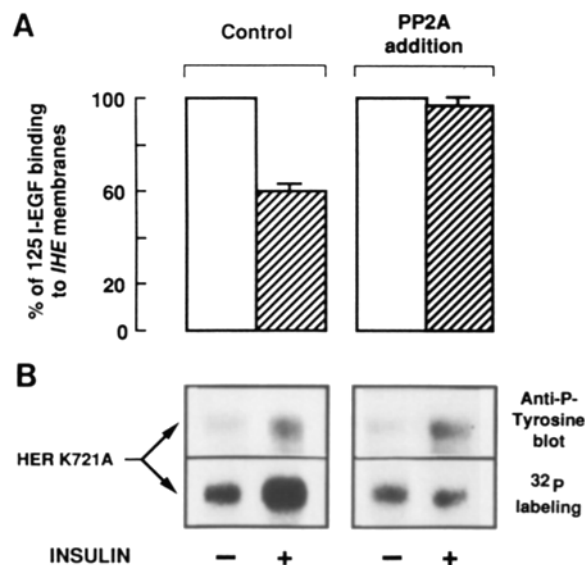


Figure 6. Protein phosphatase 2A treatment of membranes from IHE2 cells exposed to insulin. (A) Membranes from IHE2 cells exposed without (open bars) or with insulin (hatched bars) were incubated with protein phosphatase 2A (10 U/ml) at 30°C for 20 min. Control incubations were performed with protein phosphatase 2A inactivated by 100 mM NaF and 10 mM Na₄P₂O₇. Thereafter, the binding of ¹²⁵I-EGF to membranes was measured as described in the legend to Fig. 4. (B) Membranes from IHE2 cells or ³²P-labeled IHE2 cells were treated with protein phosphatase 2A as described above. Thereafter, membranes were solubilized and HER K721A were obtained by immunoprecipitation with mAb108 and analyzed by SDS-PAGE. Unlabeled receptors were analyzed by immunoblotting with antibodies to phosphotyrosine. The gel with ³²P-labeled receptors was dried and exposed to film. PP2A, protein phosphatase 2A.

Discussion

Taking advantage of IHE2 cells, we have studied the regulation of EGF binding to EGF receptors after activation of a serine/threonine kinase linked to the EGF receptor. IHE2 cells express both a chimeric insulin-EGF receptor kinase (IER) and a kinase-deficient EGF receptor (HER K721A). We have shown in a previous report that IER is an insulin-responsive EGF receptor tyrosine kinase, which phosphorylates the unoccupied HER K721A on tyrosine residues. Further, after IER activation HER K721A is phosphorylated on serine and threonine residues by one or several serine/threonine kinases (37).

Here we demonstrate that treatment of IHE2 cells with insulin results in a reduction in EGF binding to HER K721A. The maximal decrease in EGF binding (50% of initial binding) is seen after 10 min of insulin treatment and is very similar to the one observed with TPA. Scatchard analysis indicates that, as for TPA, the effect of insulin on EGF binding occurs via a reduction in high affinity binding sites of EGF to cell surface HER K721A without detectable change in receptor number. However, in protein kinase C downregulated IHE2 cells, the effect of insulin through IER activation persists on EGF binding. Hence we would like to conclude that the transmodulation of EGF binding caused by insulin is due to a mechanism distinct from that used by TPA, and appears thus to be independent of protein kinase C activation. Further, using IER cells transfected only with the chi-

meric receptor, we show that insulin decreases the binding of EGF to wild-type endogenous EGF receptor, indicating that native EGF receptors are transmodulated upon IER activation.

Two series of observations led us to investigate the role of the EGF receptor phosphorylation state in the transmodulation induced by insulin-activated IER: (a) the EGF receptor phosphorylation on serine and threonine residues appears to play a role in regulation of receptor functions (33, 35), and (b) in IHE2 cells we have previously shown that upon 15 min of insulin stimulation, HER K721A is phosphorylated predominantly on serine and threonine residues (37). Therefore, we studied the relationship between the phosphorylation state of HER K721A and the loss of high affinity binding sites using an in vitro system of EGF binding to transmodulated IHE2 membranes. In these experiments, membranes must be isolated in the presence of phosphatase inhibitors to preserve the decrease in high affinity binding sites induced by insulin. This indicates that inhibition of the high affinity binding sites of HER K721A induced by IER activation is dependent on the receptor phosphorylation state. Studies at 4°C illustrate that tyrosine phosphorylation of the cell surface HER K721A by IER is not a sufficient signal to induce the loss of high affinity binding sites. In fact, at 4°C this transregulation is not detected, while insulin induces tyrosine transphosphorylation of HER K721A to the same extent at both 37 and 4°C. In additional experiments we found that dephosphorylation on phosphoserine and phosphothreonine induced by treatment of transmodulated membranes with protein phosphatase 2A can completely restore the high affinity EGF binding. These data indicate that the inhibition of EGF binding results from the serine/threonine phosphorylation of the EGF receptor.

As a whole, our results strongly suggest that IER activation stimulates at least one serine/threonine kinase distinct from protein kinase C, which in turn phosphorylates the unoccupied HER K721A and consequently alters its affinity for EGF. Two very recent studies have reported that members of the mitogen-activated protein kinase family are able to phosphorylate the EGF receptor on threonine 669 (30, 36). However, we do not favor the idea that a kinase with such a specificity could be involved in the phenomena described in our study, since substitution of threonine 669 by site-directed mutagenesis has shown that the phosphorylation of this residue is not required for the modulation of EGF binding caused by TPA and PDGF (10).

The homologous EGF receptor transmodulation observed in IHE2 cells is, at least in two respects, similar to the transregulation of EGF receptor induced by heterologous stimuli such as PDGF, interleukin 1, and tumor necrosis factor (4, 5, 9, 13, 31). First, both phenomena can occur in protein kinase C-deficient cells, and second, they are related to the serine/threonine phosphorylation state of the receptor.

Whiteley and Glaser previously reported a possible homologous regulation of the EGF receptor (39). However, it appears to be different from the one we observed in our study for the following reasons. The desensitization described by these authors is mediated by activation of protein kinase C, which phosphorylates the EGF receptor at threonine residue 654. Phosphorylation of the EGF receptor on this site is thought to regulate the receptor kinase activity, but not the binding parameters of EGF receptors (11, 27). Additionally,

in the report of Whiteley and Glaser only 40% of the EGF receptors are phosphorylated on threonine 654, whereas in our study we found a complete disappearance of the high affinity binding sites. Since it has been proposed that EGF receptor signal transduction occurs predominantly through this particular population of high affinity EGF receptors (3, 14), we would like to suggest that the homologous desensitization we illustrate here plays a pivotal role in attenuation of EGF signaling.

The mechanism by which EGF receptor phosphorylation affects its binding parameters could be explained at least by the two following hypotheses: (a) phosphorylation on serine/threonine residues of the cytoplasmic domain of the EGF receptor could cause a conformational change that is propagated to the extracellular domain and results in the loss of the high affinity binding sites; and (b) phosphorylation of the EGF receptor on serine/threonine residues could induce association of the receptor with a cellular component, as has previously been described for rhodopsin and arrestin (40). This interaction would lead to the disappearance of the high affinity binding sites. However, we cannot rule out the possibility that the key event in the process leading to the loss of high affinity binding sites is the phosphorylation by a serine/threonine kinase of a cellular component, which in turn interacts with the EGF receptor and decreases the receptor affinity.

In summary, using a model system consisting of a chimeric insulin-EGF receptor and a kinase-deficient EGF receptor we have shown the occurrence of homologous desensitization of the EGF receptor binding function. We would like to suggest that a similar phenomenon also exists among genuine EGF receptors, and provides a newly identified negative feedback loop of EGF receptor regulation. Desensitization of EGF receptor binding in concert with protein kinase C-induced attenuation of the receptor tyrosine kinase could represent an efficient mechanism for limiting the amplitude and duration of the EGF mitogenic response.

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References

- Baccarini, M., D. M. Sabatini, H. App, U. R. Rapp, and E. R. Stanley. 1990. Colony stimulating factor-1 (CSF-1) stimulates temperature dependent phosphorylation and activation of the RAF-1 proto-oncogene product. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3649-3657.
- Ballotti, R., J. C. Scimeca, A. Kowalski, and E. Van Obberghen. 1989. Antiphosphotyrosine antibodies modulate insulin receptor kinase activity and insulin action. *Cell. Signal.* 1:195-204.
- Bellot, F., W. Moolenaar, R. Kris, B. Mirakhor, I. Verlaan, A. Ullrich, J. Schlessinger, and S. Felder. 1990. High affinity epidermal growth factor binding is specifically reduced by a monoclonal antibody, and appears necessary for early responses. *J. Cell Biol.* 110:491-502.
- Bird, T. A., and J. Saklatvala. 1989. IL-1 and TNF transmodulate epidermal growth factor receptors by a protein kinase C-independent mechanism. *J. Immunol.* 142:126-133.
- Bird, T. A., and J. Saklatvala. 1990. Down-modulation of epidermal growth factor receptor affinity in fibroblasts treated with interleukin 1 or

- tumor necrosis factor is associated with phosphorylation at a site other than threonine 654. *J. Biol. Chem.* 265:235-240.
6. Carpenter, G., L. King, Jr., and S. Cohen. 1979. Rapid enhancement of protein phosphorylation in A-431 cell membranes preparations by epidermal growth factor. *J. Biol. Chem.* 254:4884-4891.
 7. Cochet, C., G. N. Gill, J. Meisenhelder, J. A. Cooper, and T. Hunter. 1984. C-kinase phosphorylates the epidermal growth factor receptor and reduces its epidermal growth factor-stimulated tyrosine protein kinase activity. *J. Biol. Chem.* 259:2553-2558.
 8. Cohen, P. 1989. The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* 58:453-508.
 9. Countaway, J. L., N. Gironès, and R. J. Davis. 1989. Reconstitution of epidermal growth factor receptor transmodulation by platelet-derived growth factor in chinese hamster ovary cells. *J. Biol. Chem.* 264:13642-13647.
 10. Countaway, J. L., P. McQuilkin, and R. J. Davis. 1990. Multisite phosphorylation of the epidermal growth factor receptor. *J. Biol. Chem.* 265:3407-3416.
 11. Davis, R. J. 1988. Independent mechanisms account for the regulation by protein kinase C of the epidermal growth factor receptor affinity and tyrosine-protein kinase activity. *J. Biol. Chem.* 263:9462-9469.
 12. Davis, R. J., and M. P. Czech. 1985. Tumor-promoting phorbol diesters cause the phosphorylation of epidermal growth factor receptors in normal human fibroblasts at threonine-654. *Proc. Natl. Acad. Sci. USA.* 82:1974-1978.
 13. Davis, R. J., and M. P. Czech. 1987. Stimulation of epidermal growth factor receptor threonine 654 phosphorylation by platelet-derived growth factor in protein kinase C-deficient human fibroblasts. *J. Biol. Chem.* 262:6832-6841.
 14. Defize, L. H. K., J. Boonstra, J. Meisenhelder, W. Kruijer, L. G. J. Tewtoolen, B. C. Tilly, T. Hunter, P. M. P. van Bergen en Henegouven, W. H. Moolenaar, and S. W. de Laat. 1989. Signal transduction by epidermal growth factor occurs through the subclass high affinity receptors. *J. Cell Biol.* 109:2495-2507.
 15. Downward, J., P. Parker, and M. D. Waterfield. 1984. Autophosphorylation sites on the epidermal growth factor receptor. *Nature (Lond.)* 311:483-485.
 16. Downward, J., M. D. Waterfield, and P. J. Parker. 1985. Autophosphorylation and protein kinase C phosphorylation of the epidermal growth factor receptor. *J. Biol. Chem.* 260:14538-14546.
 17. Friedman, B., A. R. J. Frackelton, A. H. Ross, J. M. Connors, H. Fujiki, T. Sugimura, and M. R. Rosner. 1984. Tumor promoters block tyrosine-specific phosphorylation of the epidermal growth factor receptor. *Proc. Natl. Acad. Sci. USA.* 81:3034-3038.
 18. Haystead, T. A. J., J. E. Weiel, D. W. Litchfield, Y. Tsukitani, E. D. Fisher, and E. G. Krebs. 1990. Okadaic acid mimics the action of insulin in stimulating protein kinase activity in isolated adipocytes. *J. Biol. Chem.* 265:16571-16580.
 19. Honegger, A. M., T. J. Dull, S. Felder, E. Van Obberghen, F. Bellot, D. Szapary, A. Schmidt, A. Ullrich, and J. Schlessinger. 1987. Point mutation at the ATP binding site of EGF receptor abolishes protein-tyrosine kinase activity and alters cellular routing. *Cell.* 51:199-209.
 20. Honegger, A. M., R. M. Kris, A. Ullrich, and J. Schlessinger. 1989. Evidence that autophosphorylation of solubilized receptors for epidermal growth factor is mediated by intermolecular cross-phosphorylation. *Proc. Natl. Acad. Sci. USA.* 86:925-929.
 21. Honegger, A. M., A. Schmidt, A. Ullrich, and J. Schlessinger. 1990. Evidence for epidermal growth factor (EGF)-induced intermolecular autophosphorylation of the EGF receptors in living cells. *Mol. Cell. Biol.* 10:4035-4044.
 22. Hunter, T., N. Ling, and J. A. Cooper. 1984. Protein kinase C phosphorylation of the EGF receptor at a threonine residue close to the cytoplasmic face of the plasma membrane. *Nature (Lond.)* 311:480-483.
 23. Iwashita, S., and C. F. Fox. 1984. Epidermal growth factor and potent phorbol tumor promoters induce epidermal growth factor receptor phosphorylation in a similar but distinctively different manner in human epidermoid carcinoma A431 cells. *J. Biol. Chem.* 259:2559-2567.
 24. King, A. C., and P. Cuatrecasas. 1982. Resolution of high and low affinity epidermal growth factor receptors. *J. Biol. Chem.* 257:3053-3060.
 25. Lee, L. S., and I. B. Weinstein. 1978. Tumor-promoting phorbol esters inhibit binding of epidermal growth factor to cellular receptors. *Science (Wash. DC)* 202:313-315.
 26. Lin, C. R., W. S. Chen, C. S. Lazar, C. D. Carpenter, G. N. Gill, R. M. Evans, and M. G. Rosenfeld. 1986. Protein kinase C phosphorylation at Thr 654 of the unoccupied EGF receptor and EGF binding regulate functional receptor loss by independent mechanisms. *Cell.* 44:839-848.
 27. Livneh, E., T. J. Dull, E. Berent, R. Prywes, A. Ullrich, and J. Schlessinger. 1988. Release of a phorbol ester-induced mitogenic block by mutation at Thr-654 of the epidermal growth factor receptor. *Mol. Cell. Biol.* 8:2302-2308.
 28. Margolis, B., I. Lax, R. Kris, M. Dombalagian, A. M. Honegger, R. Howk, D. Givel, A. Ullrich, and J. Schlessinger. 1989. All autophosphorylation sites of epidermal growth factor (EGF) receptor and HER2/neu are located in their carboxyl-terminal tails. *J. Biol. Chem.* 264:10667-10671.
 29. Margolis, B., N. Li, A. Koch, M. Mohammadi, D. R. Hurwitz, A. Zilberstein, A. Ullrich, T. Pawson, and J. Schlessinger. 1990. The tyrosine phosphorylated carboxyterminus of the EGF receptor as a binding site for GAP and PLC- γ . *EMBO (Eur. Mol. Biol. Organ.) J.* 9:4375-4380.
 30. Northwood, I. C., F. A. Gonzalez, M. Wartmann, D. L. Raden, and R. J. Davis. 1991. Isolation and characterization of two growth factor-stimulated protein kinases that phosphorylate the epidermal growth factor receptor at threonine 669. *J. Biol. Chem.* 266:15266-15276.
 31. Olashaw, N. E., E. J. O'Keefe, and W. J. Pledger. 1986. Platelet-derived growth factor modulates epidermal growth factor receptors by a mechanism distinct from that of phorbol esters. *Proc. Natl. Acad. Sci. USA.* 83:3834-3838.
 32. Riedel, H., T. J. Dull, A. M. Honegger, J. Schlessinger, and A. Ullrich. 1989. Cytoplasmic domains determine signal specificity, cellular routing characteristics and influence ligand binding of epidermal growth factor and insulin receptors. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:2943-2954.
 33. Schlessinger, J. 1988. The epidermal growth factor receptor as a multifunctional allosteric protein. *Biochemistry.* 27:3119-3123.
 34. Shoyab, M., J. E. Delarco, and G. T. Todaro. 1979. Biologically active phorbol esters specifically alter affinity of epidermal growth factor membrane receptors. *Nature (Lond.)* 279:387-391.
 35. Sibley, D. R., J. L. Benovic, M. G. Caron, and R. J. Lefkowitz. 1987. Regulation of transmembrane signaling by receptor phosphorylation. *Cell.* 48:913-922.
 36. Takishima, K., I. Griswold-Prenner, T. Ingebristen, and M. R. Rosner. 1991. Epidermal growth factor (EGF) receptor T669 peptide kinase from 3T3-L1 cells is an EGF-stimulated "MAP" kinase. *Proc. Natl. Acad. Sci. USA.* 88:2520-2524.
 37. Tartare, S., R. Ballotti, R. Lammers, F. Alengrin, T. Dull, J. Schlessinger, A. Ullrich, and E. Van Obberghen. 1991. Insulin-EGF receptor chimerae mediate tyrosine transphosphorylation and serine/threonine phosphorylation of kinase-deficient EGF receptors. *J. Biol. Chem.* 266:9900-9906.
 38. Ullrich, A., and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. *Cell.* 61:203-212.
 39. Whiteley, B., and L. Glaser. 1986. Epidermal growth factor (EGF) promotes phosphorylation at threonine-654 of the EGF receptor. Possible role of protein kinase C in homologous regulation of the EGF receptor. *J. Cell Biol.* 103:1355-1362.
 40. Wilden, U., S. W. Hall, and H. Kühn. 1986. Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. *Proc. Natl. Acad. Sci. USA.* 83:1174-1178.
 41. Wrann, M., C. F. Fox, and R. Ross. 1980. Modulation of epidermal growth factor receptors on 3T3 cells by platelet-derived growth factor. *Science (Wash. DC)* 210:1363-1364.