Effects of Protein Kinase C Activation after Epidermal Growth Factor Binding on Epidermal Growth Factor Receptor Phosphorylation

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The possible role of epidermal growth factor (EGF) receptor phosphorylation at threonine 654 in modulating the protein-tyrosine kinase activity of EGF-treated A431 cells has been studied. It has been suggested that EGF could indirectly activate a protein-serine/threonine kinase, protein kinase C, that can phosphorylate the EGF receptor at threonine 654. Protein kinase C is known to be activated, and threonine 654 is phosphorylated, when A431 cells are exposed to 12-O-tetradecanoylphorbol-13-acetate (TPA). The protein-tyrosine kinase activity of EGF receptors is normally evidenced in EGF-treated cells by phosphorylation of the receptor at tyrosine. This is inhibited when TPA-treated cells are exposed to EGF. We now show that receptor phosphorylation at threonine 654 can also be detected in EGF-treated A431 cells, presumably due to indirect stimulation of protein kinase C or a similar kinase. Some receptor molecules are phosphorylated both at threonine 654 and at tyrosine. Since prior phosphorylation at threonine 654 inhibits autophosphorylation, we propose that protein kinase C can phosphorylate the threonine 654 of autophosphorylated receptors. This provides evidence for models in which protein kinase C activation, consequent upon EGF binding, could reduce the protein-tyrosine kinase activity of the EGF receptor. Indeed, we find that 12-O-tetradecanoylphorbol-13-acetate, added 10 min after EGF, further increases threonine 654 phosphorylation and induces the loss of tyrosine phosphate from A431 cell EGF receptors.

The epidermal growth factor (EGF) receptor is a transmembrane glycosylated polypeptide with its N terminus outside the cell and C terminus inside (for review, see Ref. 1). The extracellular region contains an EGF binding domain and the intracellular region includes a 260-residue domain with protein-tyrosine kinase activity. The protein-tyrosine kinase is activated within 1 min of EGF binding to its receptor and may mediate rapid cellular responses to EGF (2-5). The extracellular region contains an EGF binding domain, and the intracellular region includes a 260-residue domain with protein-tyrosine kinase activity. The protein-tyrosine kinase is activated within 1 min of EGF binding to its receptor and may mediate rapid cellular responses to EGF (2-5). The extracellular region contains an EGF binding domain, and the intracellular region includes a 260-residue domain with protein-tyrosine kinase activity. The protein-tyrosine kinase is activated within 1 min of EGF binding to its receptor and may mediate rapid cellular responses to EGF (2-5).

In accord with the above results of in vitro assays, both autologous and heterologous tyrosine phosphorylations seem to be catalyzed by the EGF receptor in intact cells (5, 10, 11). This has been detected in a number of cell types, but is most evident in A431 cells, which are human tumor cells that have unusually large numbers of EGF receptors (12). When intact A431 cells are exposed to EGF, activation of the EGF receptor kinase is detectable as an increase in phosphotyrosine content of the receptor itself and of specific cellular proteins (5). In vivo, autophosphorylation occurs primarily at tyrosine 1173, the other 3 sites being phosphorylated to a lesser degree than in vitro (5, 8, 13, 14).

Protein kinase C is a phospholipid/Ca2+-dependent, diacylglycerol-stimulated protein-serine/threonine kinase (for review, see Ref. 15). Protein kinase C is activated in the cell either by tumor promoters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), or by an increase in the cellular content of diacylglycerols (DAGs), which are products of degradation of phosphoinositides (PIs) (for review, see Ref. 16). Addition of either TPA or DAG to A431 cells inhibits subsequent binding of EGF, apparently by specifically preventing binding to a subpopulation of receptors that have "high affinity" for EGF (17). In some other cell types, a similar decrease in EGF binding (18-23) has been ascribed to internalization of receptors into a compartment from which they can be recycled to the cell surface (24). In A431 cells and human fibroblasts, TPA also causes a decrease in the receptor kinase activity, as shown by a reduction in EGF-induced tyrosine phosphorylation of the receptor and of other cell proteins (17, 25-27). The effects on binding and on tyrosine phosphorylation correlate with, and have been attributed to, phosphorylation of the EGF receptor at a threonine (residue 654) (13, 26, 28, 29). This phosphorylation is probably catalyzed by protein kinase C; protein kinase C can phosphorylate this residue in vitro (13, 29), and the ability of various DAGs to affect EGF receptor function correlates with their ability to activate protein kinase C in vitro (30). This phosphorylation is probably the prime cause of altered EGF binding, since fibroblasts bearing mutant EGF receptors, in which threonine 654 is replaced by alanine, do not exhibit reduced EGF binding after TPA treatment (31). Importantly, purified receptors phosphorylated at threonine 654 in vitro have a reduced binding affinity for EGF, and a reduced EGF-stimulated kinase activ-
tyrosine phosphorylation is not the only modulator of EGF receptor function in cells in which protein kinase C is activated.

Protein kinase C may be involved in regulating EGF receptor kinase activity in cells exposed to other agents besides TPA and DAG. In A431 cells, PI degradation (and resynthesis) is promoted by EGF, so protein kinase C activation could follow EGF binding (32-34). If protein kinase C can phosphorylate EGF receptors that have EGF bound, then attenuation of the EGF-induced tyrosine phosphorylation might result, although, ultimately, EGF receptor protein-tyrosine kinase activity is probably terminated by degradation of the receptor. In several fibroblastic cell types, some mitogens, such as platelet-derived growth factor (PDGF), stimulate PI turnover, inhibit EGF binding, and stimulate threonine 654 phosphorylation (35, 36). If protein kinase C could terminate tyrosine phosphorylation by active EGF receptors, then PDGF addition to fibroblasts after EGF could negate any effects of EGF. It is known that PDGF must be added to BALB/c/3T3 cells before, not after, EGF for a maximal mitogenic response (37).

Although experiments in which tumor promoters, growth factors, or DAG were added to cells prior to challenge with EGF have been described, effects of sequential EGF receptor and protein kinase C activation have not been investigated in detail. This is reviewed for two reasons. Firstly, protein kinase C may be unable to phosphorylate EGF receptors that are "active" (i.e. have EGF bound and are phosphorylating themselves and substrate proteins). Such an inability could occur if the EGF receptor can exist in two mutually exclusive states: one that is kinase-active and tyrosine-phosphorylated, and one that is kinase-inactive and threonine 654-phosphorylated. Secondly, it is possible that protein kinase C could phosphorylate active receptors, but that these receptors would continue to be active. In other words, although threonine 654 phosphorylation apparently prevents the EGF receptor switching "on," it may not switch "off" active receptors. In either case, a role for protein kinase C in diminishing an ongoing response to EGF would be in doubt.

We have addressed these questions using A431 cells and find in these cells that: EGF stimulates tyrosine phosphorylation and can stimulate receptor phosphorylation at threonine 654, albeit to a lesser extent than TPA; further activation of protein kinase C by TPA added after EGF induces further phosphorylation of threonine 654 and reduces tyrosine phosphorylation; and tyrosine-phosphorylated receptors can be phosphorylated at threonine 654.

**EXPERIMENTAL PROCEDURES**

**General Procedures**—Procedures for growth and labeling of A431 cells, lysis and immunoprecipitation of EGF receptors, and analysis of tryptic peptide mapping and partial acid hydrolysis and separation of phosphoamino acids were separated. EGF alone stimulated incorporation of phosphate into tyrosine contained in two tryptic peptides (Fig. 1IC). One peptide contains tyrosine 1173, and the peptide containing tyrosine 1068 (residues 1045-1078), as described by Downward et al. (10 pg/10^6 cells) was used to precipitate EGF receptors. The peptide containing phosphotyrosine 1173 and two peptides containing phosphotyrosine 3068 was identified as described before, pretreatment with TPA for 10 min led to phosphorylation of the EGF receptors at the tryptic peptide that contains phosphothreonine 654 and reduces tyrosine phosphorylation; and tyrosine-phosphorylated receptors can be phosphorylated at threonine 654.

**RESULTS AND DISCUSSION**

**Prior Addition of TPA**—A431 cells were labeled with ^32P, for 18 h and exposed to TPA (160 nM) for 10 min before adding EGF (8 nM) for a further 10 min. ^32P-labeled EGF receptors were isolated, and their tryptic peptides and phosphoamino acids were separated. EGF alone stimulated incorporation of phosphate into tyrosine contained in two tryptic peptides (Fig. 1C). One peptide contains tyrosine 1173, and the second probably contains tyrosine 1068. This peptide was poorly resolved from a major phosphothreonine-containing peptide (REF, Fig. 1). As described before, pretreatment with TPA lessened the EGF-induced increase in phosphorylation of the EGF receptors at the tryptic peptide that contains tyrosine 1173 (Fig. 1, C and D). We could not determine whether TPA pretreatment inhibited phosphorylation of the peptide containing tyrosine 1068. Pretreatment with TPA for 10 min gave about 50% inhibition of subsequent EGF-stimulated tyrosine phosphorylation of the receptor (Table 1). Other investigators have observed an almost complete inhibition of tyrosine phosphorylation, assayed by the ability of the receptor to bind to antibodies that recognize phosphotyrosine (17).

As reported previously (13, 26, 28, 29), exposure of A431 cells to TPA for 10 min leads to phosphorylation of the EGF receptor at tryptic peptides that contain threonine 654 (Fig. 1B). The stoichiometry of phosphorylation at threonine 654

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1. The peptide containing phosphothreonine 1173 and two peptides containing phosphothreonine 654 have been identified previously (8, 14, 29). The peptide containing phosphothreonine 1068 was identified by its similar mobilities to a peptide previously named "C" detected in digests of receptors phosphorylated in vitro (14). The properties of peptide C are consistent with the predicted phosphopeptide containing tyrosine 1068 (residues 1045-1078), as described by Downward et al. (8).
was about 0.3 mol/mol receptor in TPA-treated cells. The phosphorylation of a significant fraction of receptors by protein kinase C in TPA-treated cells is consistent with this modification being the primary cause of the reduction in tyrosine phosphorylation upon challenge with EGF. In experiments in which antibodies to the external domain of the EGF receptor were added to A431 cells before cell lysis, we have noted that a large fraction (30-70%) of 32Pi-labeled receptors are not exposed on the surface. Since activated protein kinase C becomes associated with cell membranes, specifically the plasma membrane (42), it is probable that the 30% of receptors that are phosphorylated at threonine 654 comprise a large fraction of the receptors that are exposed at the cell surface and able to interact with EGF.

Like TPA, EGF stimulated phosphorylation of the receptor at threonine 654 (Fig. 1C). Threonine 654 phosphorylation

| Experiment | Addition to cells | Phospho-
| Tyrosine | Tyrosine 1173 |
|------------|------------------|------------------|
| 1 | 20 min* | 10 min* | |
| - | - | 5.8 | 0.026 |
| - | EGF | 33.3 | 0.222 |
| TPA | - | 2.5 | 0.022 |
| TPA | EGF | 15.5 | 0.154 |
| 2 | - | - | 2.8 | 0.010 |
| - | EGF | 17.0 | 0.235 |
| EGF | - | 14.4 | 0.207 |
| EGF | TPA | 7.6 | 0.144 |
| EGF | aEGF | 9.6 | 0.147 |

* Time before lysis.

a Appropriate control solution added.

with EGF was about 20-50% of the level with TPA, and TPA followed by EGF gave higher phosphorylation than TPA alone. In the past, we only occasionally detected threonine 654 phosphorylation in EGF-treated A431 cells (25), but now do so routinely. We cannot explain these different observations. Variability in cells or media seem most likely, but we now detect threonine 654 phosphorylation in cells cultured in complete media, in media lacking Ca2+, and in serum concentrations from 0-4%. Ishiwata and Fox (28) observed that EGF and TPA stimulate phosphorylation of the same peptides in the EGF receptor. Most likely, these investigators were observing threonine 654 phosphorylation.

Prior Addition of EGF—To test the effects of protein kinase C activation subsequent to EGF binding, EGF was added to 32P-labeled A431 cells for a total of 20 min. If TPA was added after the first 10 min of exposure to EGF, and incubation continued 10 min longer, there was roughly a 50% decrease in the extent of tyrosine phosphorylation of EGF receptors, and reduced labeling of the peptides containing tyrosines 1173 and 1068 was noted (Fig. 2 and Table I). Phosphorylation of threonine 654, quantified with reference to peptide REF, was enhanced about 50% in cells treated with EGF then TPA, relative to cells treated with EGF alone (data not shown). Thus, further stimulation by TPA of threonine 654 phosphorylation, above the level achieved with EGF alone, induced loss of phosphate from tyrosines 1173 and 1068. A similar magnitude decrease in tyrosine phosphorylation was achieved by adding an excess of an antiserum that binds EGF (Table I). This antiserum did not stimulate threonine 654 phosphorylation (data not shown). It could act by lowering the concentration of free EGF so that rebinding does not occur (the dissociation half-time of EGF is about 2 min), or by inducing a conformation change in bound EGF so that it dissociates.

We tested whether the reduction in EGF receptor tyrosine phosphorylation by TPA was an indication of reduced EGF receptor kinase activity by measuring the extent of phosphorylation of total cell proteins at tyrosine (Table II). TPA,
proteins than for the receptor.

Phosphotyrosine level in cells treated with EGF alone was increased of tyrosine phosphorylation of total A431 cell proteins is the same in increase (about 17%) was less pronounced than the decrease in

Turning over tyrosine phosphate. Assuming that threonine phosphorylation catalyzed by protein kinase C is causal, indirectly cause a physical loss of tyrosine phosphate from tyrosine-phosphorylated receptors, then we may conclude that threonine 654 phosphorylation can occur on tyrosine-phosphorylated receptors.

Conclusions—These data imply that activation of protein kinase C with tyrosine-phosphorylated receptors.

Threonine Phosphorylation of Tyrosine-phosphorylated Receptors—We tested whether TPA-induced threonine 654 phosphorylation could occur on EGF receptors already phosphorylated at tyrosine, by physically isolating these receptors using an antiserum that recognizes phosphotyrosine. Receptors that lack phosphotyrosine, from control cells, are not precipitated by this antiserum, whereas receptors from cells exposed to EGF for 20 min are precipitated (Fig. 3, Table III). 80% of the precipitation was prevented by competition with nonradioactive phosphotyrosine (data not shown). A smaller fraction of receptors was precipitated from cells exposed to EGF for 20 min with TPA present for the final 10 min (Fig. 3B and Table III), consistent with their reduced content of phosphotyrosine (Table I). The phosphopeptides containing tyrosine 1068 and tyrosine 1173 were highly labeled relative to a reference peptide (REF) in the receptors purified with anti-phosphotyrosine sera, whereas before purification they were relatively minor (compare Figs. 2A with 3A and 2B with 3B). We conclude that the anti-phosphotyrosine serum specifically precipitates receptors phosphorylated at tyrosine. Some phosphotyrosine was detectable in receptors that were not precipitated, however (data not shown), and the yield of tyrosine-phosphorylated receptors was rather variable (Table II).

The extent to which the tyrosine-phosphorylated receptors were phosphorylated at threonine 654 was estimated from peptide maps (Fig. 3). Peptides containing phosphothreonine 654 were detected in tyrosine-phosphorylated receptors from A431 cells, after exposure to EGF for 20 min, whether or not TPA was added after 10 min (Fig. 3, A and B). In several experiments, the labeling of the most basic threonine 654 phosphopeptide, relative to the reference phosphopeptide (REF), was similar in the phosphotyrosine-containing and total populations (e.g. compare Figs. 2A with 3A and 2B with 3B). Since it appears that threonine 654 phosphorylation prevents tyrosine phosphorylation of EGF receptors, then we may conclude that threonine 654 phosphorylation can occur on tyrosine-phosphorylated receptors.

Conclusions—These data imply that activation of protein kinase C with tyrosine-phosphorylated receptors.

Addition to cells 20 min 10 min

| Addition to cells | Phospho-tyrosine | Phospho-threonine | Phospho-serine |
|------------------|------------------|------------------|----------------|
| EGF              | 0.0787 ± 0.002  | 7.06 ± 0.53      | 92.85 ± 0.54   |
| EGF + TPA        | 0.0678 ± 0.022  | 6.74 ± 0.53      | 93.13 ± 0.58   |
| TPA              | 0.0199 ± 0.038  | 5.97 ± 0.08      | 94.00 ± 0.07   |

* Time before lysis.

** Mean and range of duplicate cell cultures. About 10^6 cpm were analyzed in each case. Relative to cells treated with TPA alone, the phosphotyrosine level in cells treated with EGF alone was increased 290% and, in cells treated with EGF then TPA, was increased 240%.

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tyrosine phosphorylation of the receptor and other proteins. Phosphate from tyrosine in the EGF receptor, but also stimulates that was precipitated in three independent experiments is shown. EGF binding leads to phosphorylation of threonine 654 on         

King and Cuatrecasas (20) added TPA to KB cells that had surface-bound EGF and noted a rapid dissociation of up to 75% of the EGF with a half-time of about 2 min. The remaining 25% was probably internalized. This suggests that threonine 654 phosphorylation not only promotes the loss of phosphate from tyrosine in the EGF receptor, but also stimulates release of EGF. Our observation that threonine 654 phosphorylation and tyrosine phosphorylation are not mutually exclusive allows us to propose an “active” mechanism for these changes. In this model, EGF receptors that are phosphorylated at tyrosine and are probably functional tyrosine protein kinases are substrates for protein kinase C. Phosphorylation at threonine 654 increases the dissociation constant of EGF and inactivates autophosphorylation, and then phosphatases remove the phosphate from tyrosine. However, our observation that the extent of phosphotyrosine loss is the same following addition of TPA or antiserum to EGF is consistent with a passive model in which threonine 654 phosphorylation has no effect on the dissociation rate of EGF but prevents rebinding, and the rate of loss of tyrosine phosphate is not enhanced beyond the normal turnover rate. Both models are consistent with a decrease in the equilibrium binding affinity of EGF, and the true situation is probably a combination of the two.

The tight linkage between reduced EGF binding and inhibited tyrosine phosphorylation is consistent with a common cause, namely phosphorylation by protein kinase C of the EGF receptor. In fact, the inhibition of kinase activity may be a consequence of reduced EGF binding (13). It is possible, however, that different populations of receptors are affected differently. TPA apparently inhibits EGF binding to only the small fraction (1–5%) of A431 cell receptors that exhibit high affinity binding (17), yet 20–30% of receptors become phosphorylated at threonine 654. 11–24% of A431 cell receptors are phosphorylated on tyrosine after EGF addition (Table III) and are presumably active protein-tyrosine kinases. It is not known whether the tyrosine-phosphorylated receptors subsume, or exclude, the high affinity receptors. It is possible to show that the receptors phosphorylated at tyrosine 1 min after EGF addition are accessible at the cell surface, by adding antibodies to the EGF receptor before cell lysis and immunoprecipitation.7

We have shown that phosphorylation of EGF receptors at threonine 654, an indirect effect of EGF, could play a role in limiting the response of A431 cells to EGF. This could operate within minutes of EGF addition. Ultimately, EGF-induced endocytosis and degradation of receptors probably terminates EGF-stimulated protein-tyrosine phosphorylation. The general principles investigated here may also be important in other cell types, where inhibition of EGF binding by mitogenic agents that stimulate PI turnover has been described.

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7 J. A. Cooper and T. Hunter, unpublished results.

TABLE III

| Addition to cells | % precipitated by antiserum |
|------------------|---------------------------|
|                  | 1            | 2            | 3            |
| 20 min*          | 10 min*      |              |              |
| EGF              | 13           | 16           | 25           |
| EGF + TPA        | 8            | 4            | 7            |
| EGF – TPA        | 2            | 1            | 1            |

*Time before cell lysis.

Appropriate control solution added.

kinase C (or a kinase with identical specificity) subsequent to EGF binding leads to phosphorylation of threonine 654 on active (autophosphorylated), as well as inactive, EGF receptors. This correlates with an attenuation of the EGF-activated tyrosine phosphorylation of the receptor and other proteins. If similar mechanisms pertain in other cells, it would follow that exposure of EGF-treated fibroblasts to agents like PDGF, that activate protein kinase C (or a kinase with identical specificity) subsequent to growth factor addition, could partly negate the effects of EGF on protein tyrosine phosphorylation.

We have studied only the effects of EGF and TPA on receptor phosphorylation, but it is known that TPA also reduces the EGF binding affinity of EGF receptors (17–23). King and Cuatrecasas (20) added TPA to KB cells that had surface-bound EGF and noted a rapid dissociation of up to 75% of the EGF with a half-time of about 2 min. The remaining 25% was probably internalized. This suggests that threonine 654 phosphorylation not only promotes the loss of phosphate from tyrosine in the EGF receptor, but also stimulates release of EGF. Our observation that threonine 654 phosphorylation and tyrosine phosphorylation are not mutually exclusive allows us to propose an “active” mechanism for these changes. In this model, EGF receptors that are phosphorylated at tyrosine and are probably functional tyrosine protein kinases are substrates for protein kinase C. Phosphorylation at threonine 654 increases the dissociation constant of EGF and inactivates autophosphorylation, and then phosphatases remove the phosphate from tyrosine. However, our observation that the extent of phosphotyrosine loss is the same following addition of TPA or antiserum to EGF is consistent with a passive model in which threonine 654 phosphorylation has no effect on the dissociation rate of EGF but prevents rebinding, and the rate of loss of tyrosine phosphate is not enhanced beyond the normal turnover rate. Both models are consistent with a decrease in the equilibrium binding affinity of EGF, and the true situation is probably a combination of the two.

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