Threonine Phosphorylation Diverts Internalized Epidermal Growth Factor Receptors from a Degradative Pathway to the Recycling Endosome*

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Transregulation of the epidermal growth factor receptor (EGFR) by protein kinase C (PKC) serves as a model for heterologous desensitization of receptor tyrosine kinases, but the underlying mechanism remained unknown. By using c-Cbl-induced ubiquitination of EGFR as a marker for transfer from early to late endosomes, we provide evidence that PKC can inhibit this process. In parallel, receptor down-regulation and degradation are significantly reduced. The inhibitory effects of PKC are mediated by a single threonine residue (threonine 654) of EGFR, which serves as a major PKC phosphorylation site. Biochemical and morphological analyses indicate that threonine-phosphorylated EGFR molecules undergo normal internalization, but instead of sorting to lysosomal degradation, they recycle back to the cell surface. In conclusion, by sorting EGFR to the recycling endosome, heterologous desensitization restrains ligand-induced down-regulation of EGFR.

Activation of growth factor receptors by their ligands is followed by the desensitization processes, which can be grouped into homologous and heterologous types (reviewed in Ref. 1). Homologous desensitization is initiated by ligand binding, and it entails endocytic removal of the activated receptors from the cell surface ("down-regulation"). Ligand-receptor complexes are rapidly recruited to clathrin-coated regions of the plasma membrane, which rapidly invaginate to form coated vesicles. Within minutes or less the coated vesicle delivers its content to the sorting early endosome, a peripheral vesicular compartment, whose internal pH is moderately acidic (2). Sorting of incoming epidermal growth factor receptors (EGFRs) to the endosomal carrier vesicle (also called the multivesicular body) depends on the intrinsic tyrosine kinase activity of EGFR, and its default pathway appears to be delivery to the recycling endosome (reviewed in Ref. 3). The mechanism underlying endosomal sorting has been recently attributed to trans-phosphorylation of the c-Cbl adaptor protein by EGFRs (4). Upon its phosphorylation, the c-Cbl ubiquitin ligase (5, 6) elevates receptor ubiquitination and thereby targets EGFR to proteosomal/lysosomal degradation in late endocytic compartments (4, 7).

Whereas homologous desensitization is driven by the intrinsic kinase activity of EGFR, other protein kinases play a role in heterologous desensitization by a wide variety of stimuli. These include heterologous growth factors such as the platelet-derived growth factor (8), calcium (9), and 4β-phorbol 12-myristate 13-acetate (PMA; Ref. 10 and references therein). Both calcium and PMA stimulate protein kinase C (PKC), whose major phosphorylation site on the EGFR is threonine 654 (11). Of the multiple effects of PKC on EGFR, both inhibition of tyrosine kinase activity and deceleration of receptor down-regulation have been attributed to threonine 654, but the disappearance of high affinity EGF binding sites appears independent of this residue (10). Although trans-regulation by PKC significantly alters signaling downstream to EGFR, the exact mechanism remains unknown. For example, PKC activation causes translocation and stimulation of certain tyrosine phosphatases (12), which may explain why the surface EGFR is desensitized, but upon endocytosis its phosphorylation is significantly enhanced (13). According to an alternative model, PKC affects internalization of EGFR through a mechanism distinct from the one stimulated by EGF binding (14). Further, on the basis of the observation that PMA inhibits internalization and significantly reduces tyrosine kinase activity, it has been concluded that the juxtamembrane domain is involved in the transmission of conformational information from the extracellular ligand binding site to the cytoplasmic kinase domain (10). One such mechanism may involve inhibition of receptor dimerization (15), but a recent study proposed that phosphorylation at the juxtamembrane domain stabilizes ligand-induced receptor dimers (16). Thus, despite a consensus that PKC affects several receptor functions, a unifying model is still unavailable.

Because earlier works showed that activation of PKC leads to a disappearance of the unoccupied EGFR from the cell surface, but this is not accompanied by receptor degradation (14, 17), we suspected that an endocytic mechanism may provide an explanation. To test this model we utilized receptor ubiquitination as a biochemical indication for EGFR sorting to the late endosome (4). The results we present indicate that phosphorylation at threonine 654 diverts internalized EGFR molecules from a degradative fate to a recycling pathway. The emerging
regulatory role of PKC in vesicular trafficking of a growth factor receptor may be relevant to other surface molecules whose endocytosis is ligand-mediated.

EXPERIMENTAL PROCEDURES

Materials, Buffers, and Antibodies—Iodogen, EGF, monensin, and PMA were purchased from Sigma. GF109203X was from Biomol (Plymouth Meeting, PA). Radioactive materials were from Amersham Pharmacia Biotech. The SG565 monoclonal antibody to EGFR was generated in our laboratory. The anti-hemagglutinin (HA) monoclonal antibody was purchased from Roche Molecular Biochemicals and antibodies to PKC isoforms, phosphotyrosine, and c-Cbl were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The active, doubly phosphorylated form of extracellular signal-regulated kinase 1 and extracellular signal-regulated kinase 2 (mitogen-activated protein kinase) was detected by using a previously described antibody (18). Binding buffer contained RPMI 1640 medium supplemented with 0.5% bovine serum albumin and 20 mM HEPES. Solubilization buffer contained 50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 10 µg/ml pepstatin A, 10 µg/ml aprotinin, and 10 µg/ml leupeptin. HNTG buffer contained 20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol. EGF was labeled with 125I by using the Iodogen reagent. Mouse monoclonal antibody to phospho-tyrosine (4G10) or with PMA (100 nM) at 37 °C were washed with binding buffer (CHO) cells were exactly as described (4). The total amount of DNA in expression vector was a gift from Dirk Bohmann (EMBL, Heidelberg, Germany). The ubiquitin-hemagglutinin A (HA-Ub) mutagenesis kit (Stratagene). The ubiquitin-hemagglutinin A (HA-Ub) expression vector was a gift from Dirk Bohmann (EMBL, Heidelberg, Germany). The protocol for transfection of Chinese hamster ovary (CHO) cells were exactly as described (4). The total amount of DNA in each transfection was normalized with an empty pcDNA3 plasmid.

Construction and Transfection of Expression Vectors—We have previously described the construction of c-Cbl in the pcDNA3 expression vector (Invitrogen) containing the HA sequence tag (4). To generate the T654A and K721A mutants of EGFR (threonine 654 mutated to an alanine or lysine 721 mutated to an alanine), we used the Quick-change mutagenesis kit (Stratagene). The ubiquitin-hemagglutinin A (HA-Ub) expression vector was a gift from Dirk Bohmann (EMBL, Heidelberg, Germany). The protocol for transfection of Chinese hamster ovary (CHO) cells were exactly as described (4). The total amount of DNA in each transfection was normalized with an empty pcDNA3 plasmid.

Ligand Internalization Assay—Cells pretreated with solvent (ethanol) or with PMA (100 nM) at 37 °C were washed with binding buffer and then incubated for up to 8 min in the presence of a radiolabeled EGF (5 ng/ml). The cells were then put on ice and washed twice with binding buffer, and cellular distribution of the radiolabeled ligand was determined by using a 7-min-long incubation in 0.5 ml of solution of 0.2 M sodium acetate (pH 4.5) containing 0.5 µM NaCl. The released radioactivity was considered as cell surface-associated ligand. The remaining radioactivity was solubilized in 100 mM NaOH solution containing 0.1% SDS and considered as internalized ligand.

Receptor Down-regulation Assay—CHO cells were transiently transfected with plasmids encoding a wild type or a mutant EGFR. Forty-eight hours post-transfection cells were treated without or with PMA for 20 min at 37 °C. EGF was then added, and incubation was continued for various time intervals. The medium was then removed, and the cells were washed, and then incubated with binding buffer and twice with an acidic buffer (2.5 mM KCl, 135 mM NaCl, and 50 mM acetic acid) at room temperature to remove surface-bound ligand (19). The cells were then washed twice in cold binding buffer and incubated with 0.5 mM 125I-EGF for 4 h at 4 °C. The cells were then washed twice and solubilized in 100 mM NaOH solution containing 0.1% SDS, and radioactivity was determined in a γ-counter.

Ligand Binding Assays—CHO cells grown in 48-well plates were transfected with expression vectors encoding a WT or T654A receptor. Eighteen hours later cells were pretreated at 37 °C with PMA, and 15 min later they were incubated at 4 °C with increasing concentrations of a radiolabeled EGF. Specific binding of the ligand was determined in duplicates after 2 h and analyzed by using the Scatchard method (20).

Receptor Recycling Assay—To follow receptor recycling we used transfected CHO cells and a previously described protocol (21). Cells were rinsed with ice-cold binding buffer and incubated with 1 ng/ml 125I-EGF at 4 °C for 1 h. Cells were then rinsed twice with cold Dulbecco's modified Eagle's medium and allowed to internalize the ligand for 10 min at 37 °C. Next, cells were rinsed with cold Dulbecco's modified Eagle's medium, and 125I-EGF remaining on the cell surface was removed by using a 2.5-min-long wash with a mild acidic buffer (2.5 mM KCl, 135 mM NaCl, and 50 mM acetic acid). 125I-EGF-loaded cells were incubated at 1 h at 4 °C with a nonradioactive EGF (100 ng/ml) to saturate surface receptors and then switched to 37 °C for different time intervals to allow for receptor trafficking. At the end of each incubation period, cells were placed on ice, and media were collected to determine the amount of degraded and intact 125I-EGF. This was followed by a 2.5 min-long acid wash (pH 2.8) to determine the amount of surface-bound 125I-EGF. Cells were then solubilized with 1 N NaOH to determine the amount of intracellular 125I-EGF. To separate intact from degraded 125I-EGF products, trichloroacetic acid and phosphotungstic acid were added to the collected medium to final concentrations of 3 and 0.3%, respectively. The mixture was incubated at 4 °C for 30 min and centrifuged to collect precipitates. These were solubilized with 1 N NaOH before counting in a γ-counter, and the supernatants were used to calculate the amount of degraded 125I-EGF. For the effect of PMA treatment, the cells were similarly treated except that preincubation for 20 min was performed with PMA. The amount of recycled 125I-EGF was calculated as a fraction of the total cell-associated radioactivity (22).

Immunofluorescence—Twenty-four hours post-transfection cells were grown on coverslips (Molecular Probes) and then transferred to 37 °C for 15 min. Fixation was performed using 3% paraformaldehyde in PBS for 20 min at 37 °C with 3% paraformaldehyde in PBS. For immunofluorescent labeling, cells were permeabilized for 10 min at 22 °C with PBS containing 1% albumin and 0.2% Triton X-100. Coverslips were then incubated for 1 h at room temperature with a monoclonal antibody to EGFR (10 µg/ml) followed by a secondary antibody (0.5 µg/ml) linked to horseradish peroxidase. Immuno-reactive protein bands were detected with an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

Membrane Trafficking and Western Blotting—Cells were exposed to the indicated treatments in serum-free Dulbecco's modified Eagle's medium. After treatment, cells were extracted in solubilization buffer and mixed harshly, and lysates were cleared by centrifugation. The EGFR in the lysate supernatants was immunoprecipitated for 2 h at 4 °C. Immunoprecipitates were washed three times with HNTG, resolved by gel electrophoresis, and transferred to a nitrocellulose membrane. Membranes were blocked for 1 h in PBS containing 0.5% Tween-20 and 1% milk and blotted for 2 h with a primary antibody (1 µg/ml) followed by a secondary antibody (0.5 µg/ml) linked to horseradish peroxidase. Immunoreactive protein bands were detected with an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

RESULTS

PMA Inhibits Ligand-induced Down-regulation and Ubiquitination of EGFR—CHO cells were selected as a cellular system because these cells express no endogenous EGFR. The receptor was transiently expressed in living cells by using transfection, and cells were treated with EGF subsequent to their exposure to either PMA or to the solvent. Approximately a 50% reduction in the level of the cell surface localized receptor was observed after a 60-min-long exposure to EGF, but pretreatment with PMA decreased the rate of ligand-induced down-regulation of EGFR (Fig. 1A). PMA alone partially down-regulated EGFR, in agreement with previous reports (17, 14). Because down-regulation is the net result of receptor degradation and recycling back to the cell surface, we tested the ability of PMA to affect the ubiquitin-mediated tagging of EGF to lysosomal degradation (4). Co-expression of a peptide-tagged ubiquitin together with EGF allowed sensitive detection of ubiquitin adducts, and further enhancement of this process was achieved by simultaneous overexpression of c-Cbl. As expected, EGF-induced ubiquitination of EGFR was significantly
enhanced by an overexpressed c-Cbl. However, pretreatment of living cells with PMA remarkably reduced the level of receptor ubiquitination, even in the presence of an overexpressed c-Cbl (Fig. IB). Ubiquitination levels correlated with the degradation of EGFR; maximal degradation was observed upon EGF treatment of c-Cbl-overexpressing cells. These characteristics were better reflected in an experiment that tested increasing concentrations of PMA, up to 1 μM; at high concentrations PMA alone exerted only a small inhibitory effect on EGFR degradation, but when tested together with EGF we observed remarkable inhibition of both receptor degradation and ubiquitination (Fig. 2A). Concomitant with these extensive inhibitory effects, PMA also moderately reduced receptor phosphorylation on tyrosine residues (Fig. 2B). Control experiments that tested mitogen-activated protein kinase activation indicated that both EGF and PMA stimulated this kinase cascade (Fig. 2B, lower panel). Taken together, the results presented in Figs. 1 and 2 indicate that the inhibitory effects of PMA on ubiquitination and degradation of EGFR are functionally linked, suggesting a common mechanism that limits homologous desensitization of EGFR.

The Inhibitory Effects of PMA on EGFR Ubiquitination and Degradation Are Mediated by PKC—Although tumor-promoting phorbol esters act as specific binders and activators of PKC, they may also modulate other signaling pathways. Two lines of evidence implicate PKC in the reduction of receptor ubiquitination and degradation following treatment with PMA. First, inhibition of PKC by using the highly specific antagonist GF109203X abolished the inhibitory effect of PMA on receptor ubiquitination (Fig. 3A). The antagonistic effect of GF109203X on receptor degradation was apparent especially when ubiquitination and degradation were enhanced by an overexpressed c-Cbl (Fig. 3B), suggesting that PKC action is located upstream to c-Cbl. Because EGF by itself can stimulate PKC in intact cells (24), we asked whether PKC can limit receptor down-regulation following ligand binding to EGFR. Indeed, blocking PKC with the specific antagonist moderately enhanced ligand-induced ubiquitination and degradation of EGFR, especially in the presence of an overexpressed c-Cbl (Fig. 3C). This observation implies that physiological activation of PKC by EGF, probably through the activation of phospholipase C, can reduce the extent of homologous desensitization.

The second line of evidence implicating PKC is presented in Fig. 4; chronic down-regulation of the kinase by using prolonged exposure of cells to PMA enhanced EGF-induced ubiquitination of EGFR, in line with a restrictive role of PKC in EGFR ubiquitination. In addition, the effect of a short treatment with PMA was partially impaired in PKC-depleted cells. Control Western blotting experiments confirmed enhanced degradation of both the α and ε isoforms of PKC upon long exposure to PMA (Fig. 4, lower panels). Conceivably, activation of PKC, either directly (by PMA) or indirectly (by EGF), can reduce the extent of receptor ubiquitination and subsequent degradation.
Threonine 654 of EGFR Mediates the Inhibitory Effect of PKC on Receptor Ubiquitination and Degradation—The results shown in Figs. 1–4 implicate PKC in an escape route from receptor degradation, but they leave open the underlying mechanism and the role played by threonine 654. To address the role of this major PKC phosphorylation site, we replaced the threonine by an alanine (mutant denoted T654A). Initial analyses confirmed that the mutant behaved as predicted by previous studies, namely its ligand-induced phosphorylation on tyrosine residues was not affected by PKA, unlike the wild type receptor, whose phosphorylation was reduced upon treatment with PKA (Figs. 2B and 5B). Likewise, high affinity ligand binding to the wild type receptor was reduced when cells were pretreated with PKA, but this agonist of PKC only minimally affected EGF binding to the T654A mutant (Fig. 6A). Confirmation of the functional characteristics of T654A allowed us to address its ubiquitination and degradation. Evidently, replacement of threonine 654 by an alanine completely abolished the inhibitory effects of PKA on both receptor ubiquitination and receptor degradation (Fig. 5A). First, receptor ubiquitination and degradation were no longer inhibited when cells expressing T654A were exposed to a combination of EGF and PKA, and second, PKA could not abolish degradation of the T654A mutant, as it did in the case of the wild type receptor. Thus, the juxtamembrane domain of EGFR appears to play a major role in the PKC-mediated escape of EGFR from ubiquitination and degradation.

Because tyrosine phosphorylation of c-Cbl is essential for ligand-induced ubiquitination and degradation of EGFR (6), our next series of experiments tested the ability of the T654A mutant to engage c-Cbl. As expected, ligand binding stimulated tyrosine phosphorylation of both c-Cbl and EGFR. However, PKA treatment of cells expressing the wild type receptor, but not the T654A mutant, led to a significant reduction in ligand-induced phosphorylation of both the receptor and the substrate (Fig. 5B). Interestingly, tyrosine phosphorylation of c-Cbl was coupled to its enhanced degradation. Both events were induced by EGF and inhibited by PKA (Fig. 5B). It is noteworthy that according to a recent report, the macrophage growth factor can elevate ubiquitination of c-Cbl, which is followed by de-ubiquitination and no degradation (25), but EGF-induced proteolysis of c-Cbl has not been reported before. Nevertheless, mutagenesis of threonine 654 of EGFR did not protect c-Cbl from EGF-induced degradation. Taken together, the results presented in Fig. 5 imply that PKC-mediated modification of EGFR at threonine 654 impairs the ability of the modified receptor to engage c-Cbl, and therefore subsequent receptor ubiquitination and degradation are reduced.

PKC Activation Accelerates Recycling of the EGF-EGFR Complex—Two lines of reasoning led us to suspect that modification
at threonine 654 alters intracellular routing of EGFR. First, it has been reported that PKC-mediated modification of EGFR affects subsequent receptor internalization (17, 14), and second, the engagement of c-Cbl, which is a prerequisite for EGFR ubiquitination, seems to occur within the endosomal compartment (4, 7). Consistent with the possibility that phosphorylation at threonine 654 alters receptor internalization, cell treatment with PMA reduced the extent of down-regulation of the wild type form of EGFR, but it exerted no effect on the behavior of the T654A mutant (Fig. 6B). By analyzing another mutant of EGFR, namely a kinase-defective receptor (K721A), whose down-regulation is minimal because of slow internalization and rapid recycling (28–29), we learned that treatment with PMA reduced down-regulation of the wild type receptor to the minimal extent exhibited by the kinase-defective mutant (Fig. 6B).

Phosphorylation at threonine 654 may slow the rate of EGFR down-regulation because it inhibits internalization, accelerates recycling rate, or simultaneously affects these two processes. To specifically test these different scenarios we compared the rate of internalization of a WT receptor with that of the T654A mutant. For reference we used the kinase-defective mutant of EGFR (K721A), whose rate of internalization is very low (19, 27, 30). Indeed, by expressing the kinase-dead receptor in CHO cells we confirmed its slower internalization rate relative to the wild type receptor (Fig. 6B), but two observations excluded an internalization rate-based mechanism of PMA action. PMA did not significantly affect the rate of EGFR internalization by the WT receptor, and impairment of the major PKC site of phosphorylation at threonine 654 only slightly enhanced the internalization rate (Fig. 6C). Our conclusion that PMA cannot affect the rate of EGFR internalization is consistent with previously reported measurements of receptor internalization rates performed at relatively high ligand concentrations (10).

In the next step we tested the possibility that PKC-modified EGFRs recycle more efficiently than unmodified receptors. Once again we made use of the kinase-defective mutant of EGFR because its recycling rate is relatively high (26). As expected, this mutant recycled an endocytosed radiolabeled EGF to the medium of cells more efficiently than did the WT receptor (Fig. 6D, left panel). However, treatment of cells expressing the wild type EGFR with PMA significantly accelerated recycling, and after 30 min the fraction of recycled ligand reached the level exhibited by the kinase-dead EGFR. Moreover, the capacity of the T654A mutant to recycle EGF was comparable to that of the WT receptor, but this was not altered by PMA (Fig. 6D, right panel). Thus, the ability of PKC to reduce ubiquitination and degradation of EGFR may be attributed to accelerated recycling to the cell surface of EGFR molecules phosphorylated at threonine 654. To further test this model we employed monensin, a carboxylic ionophore that exerts diverse intracellular effects, including disruption of the recycling route of EGFR molecules subsequent to their ligand-induced endocytosis (31). Consistent with limited recycling of ligand-occupied EGFR molecules, monensin exerted an undetectable effect on the rate of down-regulation of EGFR (Fig. 7, left panel). However, the drug significantly enhanced down-regulation of PMA-treated EGFR molecules (Fig. 7). This observation is consistent with the possibility that PKC accelerates recycling of EGFR, and therefore the effect of monensin is detectable only after treatment with PMA.

PKC Activation Inhibits Translocation of EGF-EGFR Complexes to the Late Endosome, but the T654A Mutant Is Resistant to PKC-mediated Sorting—To test the prediction that PKC can alter the normal endocytic trafficking of EGFR by modifying threonine 654, we morphologically analyzed the route of endocytosis of WT and mutant receptors. Two approaches were employed. The first type of experiments used immunofluorescence with anti-EGFR antibodies (Fig. 8A), and the second followed endocytosis of a fluorescent derivative of EGF (Fig. 8B). The immunofluorescence results presented in Fig. 8A show that WT and mutant forms of EGFR are primarily surface-associated in resting cells, but they both undergo rapid uptake into peripheral and perinuclear structures reminiscent of endosomes or lysosomes. The two forms of EGFR differed, however, when stimulation with EGF was preceded by treatment with PMA. Under these conditions the WT receptor remained associated with the plasma membrane and small peripheral structures, which may represent early endosomes, but the T654A mutant reached the relatively large vesicular structures scattered throughout the cytoplasm. Interestingly, cell treatment with PMA affected also unoccupied EGFR molecules, which translocated into small submembranal vesicles without reaching the larger vesicular structures (data not shown). These observations are consistent with previous electron microscopic (17) and biochemical (14) analyses of PMA-treated cells, and they suggest that the juxtamembrane domain of EGFR can regulate endocytic transport of ligand-occupied and nonoccupied receptors.

The use of a fluorescent EGF derivative and confocal microscopy confirmed the immunofluorescence results. Under conditions that allow no internalization, the two ligand-occupied forms of EGFR remained at the cell surface, but at 37 °C they both translocated the ligand to large cytoplasmic vesicles. Differences between the two receptor forms became apparent upon
Mechanism of Receptor Trans-regulation

Fig. 6. Threonine 654 of EGFR mediates the effect of PKC on receptor down-regulation and recycling of the EGFR-EGFR complex. A, CHO cells were transiently transfected with a WT EGFR expression vector or with a plasmid encoding the T654A mutant (right panel). Forty-eight hours post-transfection duplicate cultures were treated for 20 min at 37 °C without (open symbols) or with PMA (100 ng/ml, closed symbols) prior to performing a binding assay with increasing concentrations of a radiolabeled EGF. The results were analyzed using the Scatchard method. B, cells were treated as in A, except that after exposure to PMA, or solvent alone, EGF (100 ng/ml) was added, and incubation was continued for the indicated times at 37 °C. Bound EGF was removed, and the level of surface receptors was determined by incubating the cells for 1 h at 4 °C with a radiolabeled EGF. For control we tested a kinase-defective mutant of EGFR (K721A, triangles). C, CHO cells expressing WT EGFR (left panel) or the T654A mutant (right panel) were pretreated for 20 min at 37 °C without (open symbols) or with PMA (100 ng/ml, closed symbols). Thereafter, cells were incubated for 1 h at 4 °C with a radiolabeled EGF (5 ng/ml) followed by incubation at 37 °C for various time intervals. At the end of incubation, cells were rinsed twice with binding buffer and then treated with a low pH buffer that removes surface-bound ligand. The acid-inaccessible internalized ligand is presented as a fraction of total cell-associated radioactivity prior to cell transfer to 37 °C. For control we tested the behavior of a kinase-defective mutant of EGFR (K721A, triangles). D, CHO cells were preincubated at 4 °C for 1 h with a radiolabeled EGF (5 ng/ml) and then switched to 37 °C for 10 min to allow ligand internalization. Sister cultures were pretreated with PMA prior to exposure to EGF (closed symbols). After removing surface-bound ligand, 125I-EGF-loaded cells were incubated for 1 h at 4 °C with a 100-fold excess of unlabeled EGF. Thereafter, cells were incubated at 37 °C for the indicated periods of time. At the end of incubation, media were collected, and the fraction of degraded 125I-EGF was determined as described under "Experimental Procedures." Surface-bound and internalized ligand fractions were then assayed. The sum of intact radiolabeled EGF (medium and surface-bound) was expressed as the percentage of total radioactivity at each time point. The average and range (bars) of duplicate determinations is shown. For control we tested a kinase-defective mutant of EGFR (K721A, triangles). Each of the experiments shown was repeated three times.

Fig. 7. Monensin inhibits the effect of PKC on receptor down-regulation. CHO cells transiently expressing EGFR and c-Cbl were treated at 37 °C without (left panel) or with PMA (100 ng/ml, right panel), in the absence (squares) or presence (triangles) of monensin (0.1 mM). EGF (100 ng/ml) was added twenty minutes later, and following the indicated time intervals the residual surface level of EGFR was determined in triplicates by using a ligand binding assay. For control we used cells that were not exposed to EGF or monensin (circles).

treatment with PMA; under these conditions the WT receptor could not mediate transfer of the fluorescent ligand molecules to large endocytic vesicles, and they remained primarily at the cell periphery (Fig. 8B). By contrast, treatment with PMA did not affect the endocytic pattern displayed by cells expressing the T654A mutant. Conceivably, the normal endocytic route of EGFR-EGFR complexes is inhibited when threonine 654 is phosphorylated by PKC.

To directly identify the vesicular structures associated with the WT and T654A forms of EGFR we made use of two established markers of endocytic vesicles, transferrin receptor (TfR) and the lysobisphosphatidic acid. Extensive studies of the trafficking of the transferrin-TfR complex (reviewed in Refs. 2 and 32) revealed its initial concentration over coated pits that later internalize into endocytic vesicles, which rapidly fuse with early endosomes scattered throughout the cell periphery. The acidic luminal pH of these sorting endosomes promotes dissociation of Fe3+, and the naked complex now segregates into tubular extensions that bud from the sorting endosome and return to the cell surface. Characteristically, the majority of recycling endosomes containing the Tf/TfR complex cluster in close apposition to the microtubule organizing center. Consistent with this picture, when the WT form of EGFR was transiently expressed in CHO cells and later the cells were allowed to uptake a fluorescent derivative of Tf, we observed fluorescent clusters in all cells (Fig. 9A). Simultaneous staining with an anti-EGFR antibody labeled only the small fraction of sucrose-insoluble EGFR com-plex. A tripartite staining in Fig. 9A, except that a kinase-defective mutant of EGFR was transiently expressed in CHO cells and later the cells were allowed to uptake a fluorescent derivative of Tf, we observed fluorescent clusters in all cells (Fig. 9A). Simultaneous staining with an anti-EGFR antibody labeled only the small fraction of sucrose-insoluble EGFR complex.

Unlike TfR, which recycles between the cell surface and an early endocytic compartment, the lysobisphosphatidic acid is
PKC promotes recycling of ligand-occupied internalized receptors back to the plasma membrane. Importantly, this mechanism may underlie not only trans-regulation of EGF signaling by growth factors that stimulate PKC (e.g. platelet-derived growth factor), but it may act as a feedback loop that limits the extent of homologous desensitization by recycling EGF-bound receptors back to the cell surface (Fig. 3C and (24)). Because intracellular trafficking of endocytosed receptor tyrosine kinases emerges from this study as a common target for both homologous and heterologous ligands, its regulatory role may be more extensive than previously anticipated.

Currently there is no unifying molecular mechanism that satisfactorily explains all of the effects of PKC-mediated phosphorylation at threonine 654 of EGFR. In other words, it is not understood why PMA treatment leads to down-regulation of both tyrosine kinase activity and high affinity EGF binding, and how these effects are coupled to endocytosis, but not to degradation, of EGFR (14). To circumvent effects reflecting differences in high affinity binding of EGF, we used relatively high ligand concentrations in all our experiments. Nevertheless, and in agreement with previous reports, PMA was still able to reduce, albeit moderately, tyrosine phosphorylation of EGFR (Figs. 2B and 5B). Thus, according to one model, PKC can affect ligand-induced dimerization of EGFR (16), thereby inhibiting its tyrosine kinase activity. Consequently, EGFR less efficiently phosphorilates c-Cbl (Fig. 5B), and therefore the phosphorylation-dependent ubiquitin ligase activity of this protein toward EGFR (6) is reduced when cells are treated with both PMA and EGF (Fig. 1B). Although we cannot exclude some contribution by this rather simple model, several observations lead us to implicate receptor endocytosis and a more complex model. First, treatment with high PMA concentrations resulted in complete inhibition of receptor ubiquitination, but this was accompanied by relatively weak reduction in receptor phosphorylation (compare A and B of Fig. 2). Second, unlike the interaction between the macrophage growth factor receptor and c-Cbl, which takes place at the plasma membrane (25), the interactions between c-Cbl and EGFR may not take place before EGFR is translocated to early endosomes (34). Third, treatment with PMA alone leads to a partial disappearance of EGFR from the cell surface (Fig. 1A), and the combination of PMA and EGF restricts down-regulation, two observations that allude to endocytic mechanisms. Several previous studies also implicate the endocytic fate of EGFR in PKC-mediated trans-regulation (9, 10, 17). On the other hand, an unexpected linkage between tyrosine kinase activity, high affinity ligand binding, and endocytosis of EGFR emerged from studies that employed a dominant-negative mutant of dynamin (13, 35); inhibition of receptor endocytosis by this mutant resulted in a disappearance of high affinity binding sites and a significant reduction in receptor phosphorylation.

The model presented in Fig. 10 summarizes our interpretation of the role played by PKC in the internalization of EGFR. Accordingly, when this kinase is active, the otherwise degradation-fated endocytosed receptor is shunted to the recycling pathway. Apparently, phosphorylation at threonine 654 is sufficient to direct incoming receptors to the recycling endosome, whereas phosphorylation at tyrosine residues, through the recruitment of c-Cbl, directs them to the late endosome/prelysosome. Whether threonine phosphorylation overrules the targeting effect of tyrosine phosphorylation or these two modifications occur on distinct EGFR molecules is currently unknown. However, recycling to the plasma membrane may occur also in the absence of tyrosine phosphorylation, in agreement with the effect of PKC on a kinase-defective receptor (10) and in line with observations made by using electron microscopy and

**FIG. 8.** PKC activation inhibits translocation of EGF/EGFR complexes into large endocytic vesicles, but the T654A mutant is resistant to the effect of PKC. A. CHO cells transiently expressing WT EGFR or the T654A mutant were treated for 20 min with solvent (−) or with PMA (100 ng/ml) prior to a 15-min-long incubation in the absence or presence of EGF (100 ng/ml). The cells were then fixed, permeabilized, and treated with a monoclonal antibody to EGFR. The antibody was visualized by using a secondary fluorescent antibody. B. CHO cells expressing the WT or the T654A mutant forms of EGFR were preincubated for 20 min with PMA (100 ng/ml) or solvent only. Then, cells were transferred to 4 °C and incubated for 30 min with Texas red-labeled EGF (0.5 μg/ml). Cells were visualized by using confocal fluorescence microscopy either at this step (upper two panels) or after transfer to 37 °C and further incubation for 20 min.

![Image](http://www.ncbi.nlm.nih.gov/pubmed/26184)

**Mechanism of Receptor Trans-regulation**

**DISCUSSION**

Desensitization of signaling pathways is ubiquitously coupled to their activation, and it plays an essential role in many physiological processes. Along with the β-adrenergic receptor, desensitization of the EGF receptor is one of the best understood systems (reviewed in Ref. 33). Whereas it is clear by now that the major mechanism underlying homologous desensitization of the EGFR is by means of ligand-dependent endocytosis and degradation of the activated receptors, the mechanism responsible for heterologous desensitization by PKC remained obscure. The results we present identify the pathway underlying this major desensitization process of EGFR. Accordingly,
unoccupied receptors (17). Indeed, PKC seems able to accelerate the rate of endocytosis of nonoccupied EGFR molecules (Fig. 1A), but it is unable to further accelerate the internalization of ligand-occupied receptors (Fig. 6C). Thus, it is possible that PKC accelerates two independent processes, internalization of unoccupied EGFRs and recycling of both occupied and

FIG. 9. Ligand-activated EGFR is localized primarily to recycling endosomes of PMA-treated cells, but the T654A mutant translocates to late endosomes. A, CHO cells were transfected with an EGFR expression plasmid (WT, a–c) or with a vector encoding the T654A mutant (d–f). Forty-eight hours later, cells were treated for 20 min at 37 °C with PMA (100 ng/ml) prior to adding EGF (100 ng/ml) and rhodamine-labeled transferrin (red) and further incubation for 30 min. Cells were then fixed and stained with an anti-EGFR antibody that was detected by using a secondary fluoresceine-conjugated antibody (green). Areas of overlap between EGFR and transferrin appear in yellow (arrows). Note that only a fraction of cells expresses the transfected EGFR. B, cells expressing either WT EGFR (g–i) or the T654A mutant (j–l) were pretreated with PMA and then with EGF as described in A. The cells were then fixed and stained with an anti-EGFR antibody followed by a rhodamine-labeled secondary antibody (red) or with the 6C4 antibody (23) followed by a Cy3-conjugated secondary antibody (green). Areas of co-localization appear in yellow and are marked with arrows.

FIG. 10. PKC activation sorts EGFR to recycling. The model depicts two alternative intracellular pathways of EGFR following endocytosis. Ligand binding to the nonphosphorylated receptor is followed by autophosphorylation at several tyrosine residues (Y). When PKC is active, EGFR is also phosphorylated at threonine 654 (T). According to the proposed model, the two forms of EGFR are similarly recruited into coated pits and subsequently sorted to the early endosome. However, receptors whose juxtamembranial threonine 654 is modified are sorted to the recycling endosome, whereas other receptors are destined to the late endosome through the action of c-Cbl. Targeting of these receptors to lysosomal degradation involves their tagging with ubiquitin (Ub). The pathway taken by PMA-treated unoccupied receptors is not shown. The sorting mechanism responsible for recycling of PKC-modified EGFR molecules is currently unknown.
unoccupied receptors. It is noteworthy, however, that according to previous analyses the recycling pathway serves as the default route to lysosomal degradation (reviewed in Ref. 36). These considerations suggest an alternative model, which is minimal; activation by PMA only accelerates receptor internalization in a ligand-independent manner. According to this model, the internalized receptors are not sorted to the late endosome even when the receptor is activated by a ligand, because c-Cbl is inefficiently recruited to threonine-phosphorylated receptor molecules.

The model presented in Fig. 10 and the variant discussed above raise two interesting questions. The first relates to the mechanism enabling PKC to accelerate internalization of unoccupied EGFRs. It is relevant that internalization of several yeast surface proteins is promoted by their mono-ubiquitination (37), a possibility that has not been addressed in mammalian cells. The second question relates to the post-internalization action of PKC. According to one scenario, PKC enhances directing EGFR to the default pathway of recycling back to the cell surface.

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