Epidermal Growth Factor Receptor Activation under Oxidative Stress Fails to Promote c-Cbl Mediated Down-regulation*

Received for publication, May 13, 2002, and in revised form, June 11, 2002
Published, JBC Papers in Press, June 12, 2002, DOI 10.1074/jbc.M204677200

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Activation of the epidermal growth factor (EGF) receptor by its ligand, EGF, rapidly enhances receptor internalization and degradation, which desensitizes receptor signaling. In contrast, we have shown previously that exposure to oxidative stress in the form of hydrogen peroxide (H2O2) activated the EGF receptor but that the levels of activated receptors did not decline, which resulted in prolonged receptor signaling. This study provides mechanistic insights into these different modes of EGF receptor activation. Here we demonstrate that the pattern of receptor tyrosine phosphorylation induced by H2O2 differs from that induced by its ligand, EGF. Importantly, H2O2 generates a receptor with negligible phosphorylation at tyrosine 1045, the major docking site for c-Cbl. As a result, H2O2-activated receptors fail to recruit c-Cbl and do not undergo ubiquitination and endocytosis. In summary, H2O2-stimulation results in an activated receptor uncoupled from normal down-regulation, a process that may contribute to oxidant-mediated tumorigenesis.

Down-regulation of activated receptors is crucial for normal cell development and maintenance. Activation of the epidermal growth factor (EGF) receptor by its ligands is followed by desensitization processes initiated by ligand binding, which entails removal of the activated receptors from the cell surface (“down-regulation”) (1). Interaction of the receptor with the ubiquitin ligase c-Cbl has been shown to be essential for efficient receptor down-regulation (2). Recently this interaction has also been shown to depend upon receptor phosphorylation at tyrosine 1045, the major docking site for c-Cbl (3). Following interaction, c-Cbl is phosphorylated by the EGF receptor, resulting in the activation of the ubiquitin ligase activity of c-Cbl. The latter of these events promote the recruitment of the ubiquitin-conjugating enzyme UbcH7 (4) and the subsequent receptor ubiquitination, which targets the receptor to proteasomal/lysosomal degradation (2–5). Recent studies (6, 7) have shown that c-Cbl additionally regulates EGF receptor endocytosis by engaging the formation of multiprotein “endocytotic complex” with activated EGF receptors at the plasma membrane, thus controlling receptor internalization. Hence, the recruitment of c-Cbl to the EGF receptor has surfaced as a critical step in receptor down-regulation. By mediating receptor endocytosis and “tagging” it with ubiquitin molecules, c-Cbl increases the affinity of the receptor for the lysosomal and/or proteasomal degradation.

Several studies indicate that the oncogenic action of the EGF receptor correlates with its overexpression at the plasma membrane (8, 9). Therefore, removal of activated receptor molecules from the cell surface by sorting for degradation is expected to inhibit their oncogenic potential. We and others have shown previously (10, 11) that exposure to oxidative stress in the form of H2O2 preferentially enhances tyrosine phosphorylation of the EGF receptor. However, this aberrant EGF receptor phosphorylation does not enhance receptor turnover rate (11). Moreover, activation of EGF receptor by oxidants was shown to coincide with enhanced cell proliferation (12) and to facilitate tumor promotion processes (13). Thus, the accumulation of activated EGF receptor during oxidative stress may provide the trigger for cell hyperplasia. Yet, the molecular mechanisms that link oxidative stress, EGF receptor signaling, and tumorigenic responses are poorly understood.

We hypothesized that H2O2 could affect signaling pathways involved in cell proliferation by enhancing receptor phosphorylation without subsequent down-regulation. We suggested, as a possible mechanism, that activation of the EGF receptor under oxidative stress might fail to stimulate c-Cbl-mediated down-regulation. To test this hypothesis, we utilized an enzymatic reaction, namely the conversion of glucose to gluconolactone and H2O2 by glucose oxidase (GO), as a source for H2O2 generation, and we examined EGF receptor stability, ubiquitination, and the association with c-Cbl as determinants for receptor down-regulation. The results we present indicate that although the EGF receptor is tyrosine-phosphorylated under oxidative stress, phosphorylation at tyrosine residue 1045 is insignificant and therefore fails to initiate c-Cbl-mediated receptor internalization and degradation. This emerging role of H2O2 in EGF receptor signaling may also be relevant to other receptor tyrosine kinases that similarly undergo c-Cbl-mediated down-regulation, and it is currently under investigation.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—A549 cells were from ATCC and maintained in F12K (knight’s modification; Invitrogen) medium, supplemented with 10% fetal bovine serum and penicillin/streptomycin. Prior to experiments, cells at 50–60% confluence were serum-starved overnight in F12K medium containing 0.5% dialyzed fetal bovine serum. H2O2 was generated by adding glucose oxidase (type II from Aspergillus niger, 15,500 units/g; Sigma) to serum-free Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) containing 25 mM glucose and 0.5% BSA.

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During experiments, cells were incubated for 15 min with medium that was pre-conditioned with GO for 15 min. For incubation periods greater than 15 min, GO-containing media was replaced every 15 min with pre-conditioned medium. For EGF treatments, cells were incubated in the same media supplemented with 100 ng/ml EGF (Upstate Biotechnology, Inc.; Waltham, MA). Similar to GO treatments, during prolonged incubations, EGF-containing media was replaced every 15 min. 

Lyse Preparation, Immunoprecipitation, and Western Blotting—
Lysate preparation and protein immunoprecipitation were performed as described by Bao et al. (14). After treatments, cells were extracted in solubilization buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM EDTA, protease inhibitor mixture (Sigma), and phosphatase inhibitor mixture (Sigma). Lysates were cleared by centrifugation, and protein concentrations were measured. Proteins were immunoprecipitated by overnight incubation with the indicated antibodies at 4°C, followed by protein A (Repligen Corp. Needham, MA) precipitation for 2 h. Precipitating antibodies used are as follows: anti-EGF receptor clone C225 (a generous gift from ImClone System Inc.), and anti-c-Cbl clone C-15 from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoprecipitates were washed three times with HNTG buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol, resolved by gel electrophoresis, and transferred to a nitrocellulose membrane. Membranes were blocked for 1 h in Tris-buffered saline, pH 7.5, containing 0.5% Tween 20, 1% BSA, and 1% milk and then blotted overnight with primary antibodies followed by secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) linked to horseradish peroxidase. Immunoreactive protein bands were detected with an enhanced chemiluminescence reagent (Pierce). Blotting antibodies used are as follows: anti-EGF receptor R22 (kindly provided by Dr. J. Schlessinger, New York University Medical School; New York, NY); anti-phosphotyrosine residues 845, 1045, and 1068 from Cell Signaling Technology (Beverly, MA); anti-phosphotyrosine residue 1173; anti-phosphotyrosine PY-20, and anti-c-Cbl C-15 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); and anti-ubiquitin antibody PAG7 from Covance (Princeton, NJ).

H₂O₂ Determination—Quantification of H₂O₂ generated by 1 unit/ml GO was performed by the method of Thurman et al. (15). DMEM containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 10% FBS, supplemented with 100 ng/ml EGF (Upstate Biotechnology, Inc.; Waltham, MA) were added to the supernatant. Absorption of the ferrithiocyanate complex was measured using a GENios Multi-Detection Reader (Tecan; Männedorf, Switzerland) at 480 nm and compared with standard curves obtained from dilutions of a standard H₂O₂ solution.

Receptor Down-regulation—Serum-starved A549 cells were washed with ice-cold PBS and incubated with 0.5 mg/ml of Biotin-X-NHS (Calbiochem) dissolved in a borate buffer (10 mM boric acid, 150 mM NaCl, pH 8.0) for 45 min at 4°C. Biotin coupling was terminated by washing the plates with ice-cold PBS containing 15 mM glycine. Cells were then treated as indicated, after washing with room temperature PBS. Following treatments, proteins were lysed and immunoprecipitated using anti-EGF receptor C225 antibody. Protein biotinylation was detected by horseradish peroxidase-conjugated streptavidin (Calbiochem). The optical intensity of the corresponding bands was quantified using Image-Quant software (Amersham Biosciences).

Intracellular Distribution of the EGF Receptor during Exposure to Oxidative Stress—As EGF receptor down-regulation is dependent on the activation of its intrinsic tyrosine kinase and the activation of Src family tyrosine kinases, the mechanisms of EGF receptor tyrosine phosphorylation following GO exposure, we compared the response to EGF and to GO in the presence of AG-1478 (Sigma), a specific inhibitor of EGF receptor intrinsic tyrosine kinase activity, and PP1 (Biomol; Plymouth Meeting, PA), a selective inhibitor of Src family tyrosine kinases. Incubation of cells with 2 μM PP1 also resulted in inhibition of EGF receptor tyrosine phosphorylation (Fig. 1C). Although, however, tyrosine phosphorylation by GO was markedly reduced, only minor inhibition was observed during exposure to EGF. These results provide evidence that H₂O₂-induced tyrosine phosphorylation of EGF receptor is due to the activation of its intrinsic tyrosine kinase and the activation of Src family tyrosine kinases.

RESULTS

H₂O₂ Generated by Glucose Oxidase, Targets Tyrosine Phosphorylation of the EGF Receptor—We have shown previously (11) that physiologic concentrations (50–200 μM) of H₂O₂ elevate EGF receptor tyrosine phosphorylation levels in a dose- and time-dependent manner without subsequent receptor down-regulation. Because H₂O₂ is an unstable molecule and EGF is a highly stable one, the actual duration of exposure to these treatments may differ. To address this matter we used another source of H₂O₂, the steady conversion of β-d-glucose to d-gluconolactone and H₂O₂, which is catalyzed by GO (17). As shown in Fig. 1A), EGFR tyrosine phosphorylation was stimulated in a dose-dependent manner after 15 min of treatment with GO. This tyrosine phosphorylation was in direct response to H₂O₂ production because it could be prevented by catalase. Moreover, exposure to 1 unit/ml of GO demonstrates activation of the EGF receptor to the same extent as induced by 100 ng/ml EGF. These observations with GO enable us to expose cells constantly to physiological H₂O₂ levels (see below), which may be more representative of chronic disease states.

To calibrate the concentration of H₂O₂ generated by 1 unit/ml GO with time, H₂O₂ levels were determined, as described (15), and shown to range between ~260 and 330 μM during cells exposures, with a constant production rate of ~4.1 μM H₂O₂/min (Fig. 1B). This confirms that the exposures of the cells to 1 unit/ml GO maintain physiologic concentrations of H₂O₂.

To investigate further the mechanism of EGF receptor tyrosine phosphorylation following GO exposure, we compared the response to EGF and to GO in the presence of AG-1478 (Sigma), a specific inhibitor of EGF receptor intrinsic tyrosine kinase activity, and PP1 (Biomol; Plymouth Meeting, PA), a selective inhibitor of Src family tyrosine kinases. Fig. 1C shows that when A549 cells were cultured in the presence of 1 μM AG-1478, both EGF- and GO-induced tyrosine phosphorylation of the receptor was abolished. Incubation of cells with 2 μM PP1 also resulted in inhibition of EGF receptor tyrosine phosphorylation (Fig. 1C). Although, however, tyrosine phosphorylation by GO was markedly reduced, only minor inhibition was observed during exposure to EGF. These results provide evidence that H₂O₂-induced tyrosine phosphorylation of EGF receptor is due to the activation of its intrinsic tyrosine kinase and the activation of Src family tyrosine kinases.

H₂O₂ Does Not Enhance EGF Receptor Down-regulation—Our previous study (11) demonstrated that although the turnover rate of newly synthesized EGF receptor was enhanced after exposure of A549 cells to EGF, it was reduced after exposure to H₂O₂. To follow the fate of the surface EGF receptors more specifically, the water-soluble, membrane-impermeant agent Biotin-X-NHS, which covalently labels accessible lysine residues via its biotin moiety (18), was used. Biotinylation of surface receptors was followed by immunoprecipitation of the EGF receptor and Western blot analysis as described under "Experimental Procedures." As shown in Fig. 2), biotinylated EGF receptor levels in control cells were kept unchanged with time, whereas in the presence of EGF the levels of the receptor dropped rapidly with a new half-life of ~3.2 h. On the other hand, for cells exposed to GO, only minor changes in biotinylated EGF receptor levels were observed, suggesting that activation of EGF receptor by H₂O₂ has no effect on surface receptor down-regulation.
Therefore, we first examined, by confocal microscopy studies, the intracellular distribution of the receptor. Fig. 3 demonstrates that in A549 cells the EGF receptor is internalized after 15 min of EGF treatment, showing punctate pattern throughout the cytoplasm. On the other hand, the receptor remained on the plasma membrane after 15 min of exposure to H$_2$O$_2$ or to GO, suggesting that in contrast to EGF, H$_2$O$_2$ does not induce rapid EGF receptor internalization. Prolonged exposures to H$_2$O$_2$ and GO up to 2 h similarly showed that the EGF receptor remained at the plasma membrane (data not shown), suggesting that activated receptors under oxidative stress do not undergo endocytosis.

**Fig. 1.** H$_2$O$_2$, generated by glucose oxidase, targets tyrosine phosphorylation of the EGF receptor. A, serum-starved A549 cells were exposed for 15 min to elevated concentrations of GO, 2500 units/ml catalase, or 100 ng/ml EGF, as indicated. Cell lysates were prepared in Triton X-100 lysis buffer, subjected to SDS-PAGE electrophoresis, and immunoblotted with either anti-phosphotyrosine antibody PY20 (upper panel) or with anti-EGF receptor antibody RK2 (lower panel). B, DMEM (without phenol red) was incubated at 37°C with 1 unit/ml GO in the presence or absence of 2500 units/ml catalase for the indicated time points. Reactions were stopped by adding trichloroacetic acid to a final concentration of 5%, and H$_2$O$_2$ levels were determined as described under “Experimental Procedures.” C, serum-starved A549 cells were left intact or preincubated with 1 μM AG-1478 or 2 μM PP1 for 1 h. Cells were then exposed for 15 min to 100 ng/ml EGF or 1 unit/ml GO in the presence or absence of 1 μM AG-1478 or 2 μM PP1, as indicated. Cell lysates were detected as in A. Ab, antibody; EGFR, EGF receptor.
min of induction and then it decreased rapidly. In contrast, tyrosine phosphorylation by \( \text{H}_2\text{O}_2 \) was only observed after 15 min, and it remained elevated during all detected time intervals. In addition, tyrosine phosphorylation by EGF was observed concurrently with receptor ubiquitination and with c-Cbl association to the receptor, whereas no such observations were indicated following induction by \( \text{H}_2\text{O}_2 \). In a parallel experiment, EGF receptor was detected in c-Cbl precipitates following exposure to EGF but not to GO (Fig. 4B). Interestingly, tyrosine phosphorylation of c-Cbl was detected not only after EGF treatment but also after exposure to GO, suggesting that c-Cbl does not bind to EGF receptor under oxidative stress exposure even though it is phosphorylated.

**Immunofluorescence Analysis of the Interaction between c-Cbl and the EGF Receptor during Exposure to EGF and Glucose Oxidase**

To explore further where in the signaling pathway the association of c-Cbl and EGF receptor is interrupted, the localization of c-Cbl in respect to the activated EGF receptor was examined by confocal microscopy. Incubation of cells at 37 °C showed that c-Cbl is dispersed throughout the cell in both intact and in GO-treated cells, whereas cells treated with EGF showed colocalization of c-Cbl and EGF receptor in vesicle-like compartments (Fig. 5A, lower panel). Cells were also incubated on ice, under conditions that still allow the recruitment of c-Cbl to the EGF receptor at the plasma membrane but prevent receptor internalization (6). Cells that remained on ice showed EGF receptor staining mainly at the plasma membrane regardless of the treatments (Fig. 5B, upper panel). Staining of c-Cbl (lower panel) shows that this protein is randomly distributed in intact and in GO-treated cells, whereas cells that were incubated with EGF showed an extensive c-Cbl staining at the plasma membrane (Fig. 5B, see arrow), indicating that redistribution of c-Cbl toward the plasma membrane and colocalization with the EGF receptor only occurs when receptors are activated by EGF but not by \( \text{H}_2\text{O}_2 \).

**Insignificant Phosphorylation of Tyrosine 1045 of the EGF Receptor during Exposure to \( \text{H}_2\text{O}_2 \)**

Our studies demonstrated that \( \text{H}_2\text{O}_2 \), similar to EGF, elevated the total tyrosine phosphorylation of the EGF receptor at tyrosine 1045. In contrast, tyrosine phosphorylation at this site was not observed following exposure to \( \text{H}_2\text{O}_2 \). These findings suggest that the phosphorylation at tyrosine 1045 is not a major contributor to the signal output of the EGF receptor under oxidative stress conditions.
Tyrosine phosphorylation of residue 1045 of the EGF receptor is impaired during exposure to H_2O_2. Serum-starved A549 cells were left intact or incubated with 1 unit/ml GO or 100 ng/ml EGF for the indicated time points. Tyrosine phosphorylation of site-specific tyrosine residues of the EGF receptor (EGFR) was detected from total cell lysates using the indicated anti-phosphotyrosine antibodies. EGF receptor levels were detected using anti-EGF receptor RK2 antibody.

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Tyrosine phosphorylation of residue 1045 of the EGF receptor (Figs. 1 and 4). However, in contrast to EGF, H_2O_2 exposures resulted in a stabilized receptor that did not recruit c-Cbl. Because tyrosine 1045 has been shown to be necessary for c-Cbl recruitment and subsequent receptor ubiquitination and degradation (3), we next analyzed tyrosine phosphorylation of the EGF receptor at this specific site as well as at residues 1068 and 1173, EGF receptor autophosphorylation sites (19), and at residue 845, an established Src phosphorylation site (20). As shown in Fig. 6, both EGF and H_2O_2 enhanced the phosphorylation of tyrosine residues 845, 1068, and 1173 of the EGF receptor, with similar kinetics to those obtained for total tyrosine phosphorylation (Fig. 4). Yet phosphorylation of tyrosine 1045, which was clearly observed following treatment with EGF, was insignificant following GO treatments. Thus, exposure to oxidative stress generates a unique phosphorylation pattern of the EGF receptor, which is uncoupled from c-Cbl-mediated down-regulation.

**DISCUSSION**

We have shown previously (11) that EGF receptor phosphorylation and downstream signaling are targeted by oxidative stress. In the present study we proceed to provide mechanistic insights into these observations. To activate the EGF receptor, we utilized the enzymatic reaction of glucose oxidation by GO, because it has been shown to allow continuous production of H_2O_2 (21). In addition, unlike some studies by others that used high levels of H_2O_2 for the activation of the EGF receptor, tyrosine phosphorylation of the EGF receptor by GO was clearly observed at ~300 μM H_2O_2 or even lower levels (Fig. 1A), which are more relevant physiologically. Our findings that EGF receptor activation by GO is dependent on the intrinsic kinase activity of the receptor and is inhibited by catalase (Fig. 1A, A and C) support our previous conclusions that EGF receptor activation by H_2O_2 is tyrosine kinase-dependent (11) and further substantiates GO treatments as a mode of exposure to H_2O_2. In addition, the inhibition of EGF receptor tyrosine phosphorylation by PP1, observed during exposures to H_2O_2 and EGF (Fig. 1C), implies that similar to EGF, H_2O_2 also has the capability to activate Src family tyrosine kinases.

In the current study we have shown that oxidative stress conferred prolonged EGF receptor tyrosine phosphorylation and suggested that the failure of activated receptors to stimulate c-Cbl-mediated down-regulation accounts for this observation. Several lines of evidence support our model for EGF receptor activation under oxidative stress (Fig. 7). First, we demonstrated by confocal microscopy that, in contrast to EGF-treated cells, the EGF receptor remains at the plasma membrane during exposure to H_2O_2, and it is not translocated to vesicular compartments (Fig. 2). This was complemented by our findings that even though both the EGF receptor and c-Cbl are activated during oxidative stress (Figs. 1 and 4B), c-Cbl is not being translocated to the plasma membrane (Fig. 5). The recruitment of c-Cbl to the EGF receptor at the plasma membrane was recently shown to be a major regulatory step for receptor ubiquitination and internalization. de Melker et al. (6) have demonstrated that c-Cbl associates and ubiquitinates activated EGF receptor at the plasma membrane prior to receptor recruitment into clathrin-coated pits, and they concluded that c-Cbl has a role in clathrin-mediated endocytosis of EGF receptor as well as in intracellular sorting. In addition, a direct role for c-Cbl in endocytosis has been recently shown by Soubeiran et al. (7), who demonstrated that c-Cbl recruits CIN85 (Cbl-interacting protein of 85,000) and endophilins (regulatory components of clathrin-coated vesicles) to form a complex with activated EGF receptors and that inhibition of this interaction is sufficient to block EGF receptor internalization. The results of our present studies are consistent with such a role for c-Cbl in endocytosis and intracellular sorting. Our data imply that the failure of activated EGF receptor to recruit c-Cbl during oxidative stress (Figs. 4 and 5) is the basis for its retention at the plasma membrane (Fig. 2) and for its aberrant down-regulation (Fig. 3), which results in prolonged duration of receptor signaling (Fig. 4).

Additional support for this model (see Fig. 7) is provided in recent studies by Yarden and co-workers (3, 22), who demonstrated that a mutant EGF receptor, whose tyrosine 1045 was...
changed to phenylalanine (Y1045F), lost its ability to undergo ubiquitination (3) and therefore resides at the plasma membrane upon induction with EGF (22). In an analogy to the tyrosine 1045 mutant, we have shown that activation of EGF receptor by oxidative stress fails to phosphorylate tyrosine 1045, while enhancing the phosphorylation of other tyrosine residues, such as tyrosines 845, 1068, and 1173. Therefore, H$_2$O$_2$ exposure may cause a distribution of tyrosine phosphorylation sites similar to that of the mutant receptor (Y1045F).

In both cases tyrosine 1045 is not phosphorylated, and the receptor is defective in endocytosis and ubiquitination. Thus, upon H$_2$O$_2$ activation, the receptor becomes aberrantly phosphorylated and therefore is incapable of recruiting c-Cbl. This in turn confers longer receptor signaling capacity.

The failure of activated EGF receptors to be degraded in a ubiquitin-mediated fashion is an uncommon response to H$_2$O$_2$, counteracting the general notion that oxidative stress results in rapid removal of oxidized proteins by the ubiquitin-proteasome system (23–25). At the same time, our data cannot exclude the possibility that the ubiquitin machinery by itself may be targeted by H$_2$O$_2$, as suggested by De Wit et al. (26). Yet the short time intervals of exposures to GO, the low doses of H$_2$O$_2$ production, and more importantly, the notion that c-Cbl recruitment to the EGF receptor precedes receptor ubiquitination suggest that the failure of activated receptors to recruit c-Cbl, rather than a general inhibition of protein ubiquitination, is the basis for receptor stabilization at the plasma membrane and for the prolonged duration of EGF receptor signaling.

It has been known for many years that the majority of activated EGF receptors are located in endosomes (27). However, the data presented here suggest that during oxidative stress the majority of activated receptors reside at the plasma membrane. The differences in localization may have implications in both the magnitude of the EGF receptor response and its outcome (28). For example, it has been proposed that short duration of growth factor-mediated signaling will promote proliferation of PC12 cells, whereas prolonged signaling leads to differentiation (29). Furthermore, it has been shown that preventing EGF receptor down-regulation facilitates cell proliferation and transformation (2, 22, 30). This provides strong evidence that receptor down-regulation is important for the mechanism regulating EGF receptor responses. Therefore, our data showing that exposure to H$_2$O$_2$ elicits delayed EGF receptor activation with prolonged duration at the plasma membrane offer additional support to H$_2$O$_2$ oncogenic ability, which had been demonstrated to be EGF receptor-dependent (13).

In summary, the results of this study demonstrate that exposure of cells to H$_2$O$_2$-mediated oxidative stress enhances tyrosine phosphorylation of the EGF receptor without being accompanied by receptor down-regulation (Fig. 7). Such exposures create an irregular EGF receptor activation pattern, the essence of which is a failure of the activated receptors to promote c-Cbl recruitment, due to insufficient tyrosine phosphorylation at residue 1045. These findings fit well with the paradigm suggesting that the oncogenic action of the EGF receptor depends on its accumulation at the plasma membrane and may implicate the phosphorylation of tyrosine 1045 as a target for cancer prevention during exposures to oxidative stress.
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J. Biol. Chem. 2002, 277:31214-31219.
doi: 10.1074/jbc.M204677200 originally published online June 12, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M204677200

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