Transient Suppression of Ligand-mediated Activation of Epidermal Growth Factor Receptor by Tumor Necrosis Factor-α through the TAK1-p38 Signaling Pathway*

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Epidermal growth factor receptor (EGFR) has been shown to be activated by specific ligands as well as other cellular stimuli including tumor necrosis factor-α (TNF-α). In the present study, we found that cellular stress suppressed ligand-mediated EGFR activity. Both TNF-α and osmotic stress rapidly induced phosphorylation of EGFR. This phosphorylation of EGFR and the activation of mitogen-activated protein kinases and NF-κB occurred independently of the shedding of extracellular membrane-bound EGFR ligands and intracellular EGFR tyrosine kinase activity. Transforming growth factor-β-activated kinase 1 (TAK1) was involved in the TNF-α-induced signaling pathway to EGFR. In addition, experiments using chemical inhibitors and small interfering RNA demonstrated that p38α is a common mediator for the cellular stress-induced phosphorylation of EGFR. Surprisingly, the modified EGFR was not able to respond to its extracellular ligand due to transient internalization through the clathrin-mediated mechanism. Furthermore, turnover of p38 activation led to dephosphorylation and recycling back to the cell surface of EGFR. These results demonstrated that TNF-α has opposite bifunctional activities in modulating the function of the EGFR.

Epidermal growth factor receptor (EGFR)2 is a member of the receptor tyrosine kinase family and plays a critical role in a wide variety of cellular functions, including proliferation, differentiation, and apoptosis (1–4). EGFR has recently been a focus in the molecular target therapy of cancer, because overexpression, amplification, and mutations are involved in carcinogenesis and the progression of several types of cancer (5–7). Chemical inhibitors and neutralizing monoclonal antibodies for EGFR have been developed and show potential anti-cancer activity (8–11). The mutations in the kinase domain are involved in the enhanced carcinogenic activity and sensitivity of EGFR-tyrosine kinase activity to the inhibitors (12–15).

EGF, transforming growth factor-α, heparin-binding EGF, amphiregulin, and betacellulin are known ligands of EGFR (16–19). In addition to these ligands, EGFR is trans-activated by other extracellular stimuli, including agonists for G protein-coupled receptors, ion channels, and integrins (20–24). It has been well documented that the transactivation of EGFR is critical to a complex network of signaling pathways (25). Recently obtained evidence has demonstrated that membrane-bound ligands of EGFR, such as transforming growth factor-α and heparin-binding EGF, are released and bind to the receptor (26). Several members of the disintegrin and metalloprotease (ADAM) family are needed for shedding of the extracellular domain of EGFR ligands (27–30). Cellular stress, for example, from UV light and high osmolar stress also initiates the transactivation program (31–33). Furthermore, it has recently been demonstrated that EGFR plays a role in the tumor necrosis factor-α (TNF-α)-induced proliferation and motility of hepatocytes and mammary epithelial cells (34, 35). However, little is known about the intracellular signaling pathways leading to ADAM-mediated transactivation of the EGFR.

Cellular stress stimulates several intracellular signaling pathways leading to activation of the transcription factors activator protein-1 and NF-κB (36). Activator protein-1 is regulated by cascades of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 pathways (37, 38). The transcriptional activity of NF-κB is regulated by the IκB kinase (IKK)-mediated phosphorylation of IκBα and p65/RelA (39). The protein kinase TAK1 is activated by various cellular stresses, including TNF-α (40). It has recently been demonstrated that TAK1 participates as an upstream kinase of the JNK, p38, and IKK signaling pathways in many cellular functions, including innate immune signaling, the differentiation of lymphocytes, and cancer metastasis (41–44).

We have recently reported that gefitinib, an EGFR tyrosine kinase inhibitor, abrogated the intrahepatic metastasis of
murine hepatocellular carcinoma (27). TNF-α-induced cellular responses, such as the activation of MAPK, expression of integrin, and invasion are also regulated by the ADAM-mediated transactivation of EGFR. In the present study, we found that TNF-α actually suppressed the ligand-mediated activation of EGFR via TAK1-p38-mediated signaling in HeLa cells.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—An anti-phospho-TAK1 (Thr-187) antibody was generated as described previously (40). Other phosphospecific antibodies against p38, JNK, ERK, p65, S6K, and EGFR (Tyr-845, -1045, -1068, and -1173) were purchased from Cell Signaling Technology. Antibodies against TAK1, p38, JNK, ERK1, and ERK2 were obtained from Santa Cruz Biotechnology. A neutralizing anti-EGFR monoclonal antibody (clone LA1, mouse IgG1) and isotype control IgG1 were purchased from Upstate Biotechnology and R & D Systems, respectively. Recombinant human TNF-α and EGF were obtained from R & D Systems, SB203580, SP600125, U0126, SC-514, GM6001, PD153035, and AG825 from Merck Biosciences, and recombinant human p38α, a TAK1-TAB1 fusion protein, and λ-phosphatase from Upstate Biotechnology. 5Z-7-oxozeaenol, a selective TAK1 inhibitor, was a gift from Chugai Pharmaceutical Co., Ltd. All of the chemical inhibitors were dissolved in Me2SO, and the final concentration of Me2SO was <0.1%.

**Cell Cultures**—HeLa and A549 cells were maintained in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO2.

**Transfection of Small Interfering RNAs**—Duplex small interfering RNAs (siRNAs) with two nucleotides overhanging at the 3’ end of the sequence were designed at iGENE Therapeutics and synthesized at Hokkaido System Science Co., Ltd. The target sequences for TAK1, p38α, clathrin heavy chain, c-Cbl, Cbl-b, and firefly luciferase (GL2) were reported previously (14, 40, 46). HeLa cells were transfected with the siRNAs in a final concentration of 25–50 nM using Lipofectamine reagents. At 72 h post-transfection, the cells were stimulated.

**Phosphatase and Kinase Reactions**—EGFR immunoprecipitated from untreated or TNF-α-stimulated HeLa cells were incubated with λ-phosphatase at 30 °C for 30 min or with recombinant p38α and TAK1-TAB1 fusion protein in the presence of [32P]ATP. Phosphatase activity was analyzed as a shift in mobility on immunoblotting. The kinase activity was visualized by autoradiography.

**Immunoblotting**—After the stimulation, whole cell lysates were prepared as described previously. Cell lysates were resolved by 7.5, 10, or 12.5% SDS-PAGE and transferred to an Immobilon-P nylon membrane (Millipore). The membrane was treated with BlockAce (Dainippon Pharmaceutical Co., Ltd., Suita, Japan) and probed with primary antibodies. The antibodies were detected using horseradish peroxidase-conjugated anti-rabbit, anti-mouse, and anti-goat IgG (DAKO) and visualized with the ECL system (Amersham Biosciences). Some antibody reactions were carried out in the Can Get Signal solution (TOYOBO).
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FACS Analysis—After the stimulation, HeLa cells were harvested in phosphate-buffered saline. Cells were fixed with 2% paraformaldehyde for 20 min at room temperature. Where indicated, cells were permeabilized in phosphate-buffered saline containing 0.1% Triton X-100. The cells were resuspended in 100 μl of FACS buffer (phosphate-buffered saline containing 0.5% bovine serum albumin and 0.05% NaN3) containing 1 μg of anti-EGFR monoclonal antibody (LA1) and incubated on ice for 30 min. After washing with FACS buffer, the cells were incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG antibody (DAKO) on ice for 30 min and analyzed by the FACSCalibur system (BD Biosciences). The ratio of internalization of EGFR was calculated using the median values of fluorescence.

Statistical Analysis—The significance of differences between groups was determined by applying Student’s two-tailed t test. Values of p < 0.05 were considered significant.

RESULTS

Stress-induced Modifications of EGFR—HeLa cells have commonly been used for characterization of the TNF-α- and EGF-induced signaling pathways. We first confirmed activation of the downstream signaling pathways. Fig. 1A shows that both TNF-α and EGF rapidly activated MAPKs (ERK, JNK, and p38) at similar time points within 10 min. In contrast, TAK1 and NF-κB p65 were only activated by TNF-α. The total expression level of these proteins was comparable (data not shown).

We next investigated the effects of TNF-α on the phosphorylation of EGFR. Interestingly, TNF-α rapidly induced a shift in the mobility of EGFR on SDS-PAGE within 10 min (Fig. 1A). In contrast, the mobility of other EGFR family members (ErbB2–4) was not changed (data not shown) in HeLa cells. EGF also caused a mobility shift within 10 min (Fig. 1A). It should be noted that, although EGF induced strong phosphorylation at Tyr-845, -1045, -1068, and -1173 in the intracellular tyrosine kinase domain of EGFR, only a faint tyrosine phosphorylation was detected in response to TNF-α (Fig. 1A). These results indicated that the TNF-α-induced mobility shift of EGFR is independent of the tyrosine phosphorylation.

Once EGFR is activated by a specific ligand, it has been shown to rapidly enter a program of degradation through the phosphorylation of Tyr-1045 and subsequent c-Cbl-mediated ubiquitination. In fact, the form shifted by EGF largely disappeared within 2 h (Fig. 1B). In contrast, the TNF-α-induced mobility shift was transient, and there was a return to the control level at 60 min (Fig. 1B). A similar mobility shift was observed in A549 human lung adenocarcinoma cells (Fig. 1B). High osmotic stress with additional 300 mM NaCl also caused a rapid shift in the mobility of EGFR without the tyrosine phosphorylation (Fig. 1C); however, it was sustained for at least 2 h. Osmotic stress also induced prolonged activation of MAPKs but not TAK1 and NF-κB (Fig. 1C). Interestingly, no obvious degradation of EGFR was observed in cells treated with TNF-α and osmotic stress.

Stress-induced Modification of EGFR Is Independent of ADAM-mediated Shedding of EGFR Ligand—G-protein-coupled receptor-mediated transactivation of EGFR has been shown to be mediated by the ADAM-dependent release of membrane-bound extracellular EGFR ligands. To explore involvement of this mechanism in stress-induced modification of EGFR, we first examined the effects of PD153035, a potent EGFR tyrosine kinase inhibitor. Fig. 2A shows that PD153035 at 0.1 μM completely inhibited the EGF-induced mobility shift as well as the tyrosine phosphorylation of EGFR, whereas AG825, a selective ErbB2 tyrosine kinase inhibitor, had no inhibitory effect. In contrast, although a higher concentration (10 μM) of PD153035 blocked the TNF-α-induced mobility shift of EGFR, the TNF-α-induced modification was not influenced by PD153035 at the concentration having an effect on the EGFR tyrosine kinase (1 μM). These results indicate that the tyrosine kinase activity of EGFR was not involved in the mobility shift (Fig. 2B).

GM6001, a broad spectrum inhibitor for metalloproteases, has widely been used as an ADAM inhibitor that blocks the transactivation of EGFR. However, GM6001 was not able to block the TNF-α and osmotic stress-induced mobility shift (Fig. 2, C and D). Furthermore, a neutralizing monoclonal anti-EGFR antibody did not inhibit the TNF-α- and osmotic stress-induced modifications of EGFR, although it effectively abrogated the EGF-induced activation of EGFR (Fig. 2E). Collectively, these results clearly demonstrated that the stress-
induced modification of EGFR occurs independent of the ADAM-mediated shedding of the ligands.

Effects on the Downstream Signaling Pathways—We and others have demonstrated that TNF-α-induced signaling pathways are partly dependent on the tyrosine kinase activity of EGFR in several types of cells (27, 34, 35). Therefore, we next confirmed the effects of EGFR blockers on stress-induced signaling pathways in HeLa cells. Although PD153035 clearly blocked EGF-induced signaling pathways in a concentration-dependent manner (Fig. 3A), TNF-α induced activation of p38, JNK, ERK, TAK1, and NF-κB was resistant at up to 10 μM (Fig. 3A). Similarly, osmotic stress-induced signaling pathways were not affected (Fig. 3B). In addition, neither GM6001 nor the EGFR neutralizing antibody blocked the TNF-α signaling pathways (Fig. 3, D and E). These results clearly demonstrated that the modification of EGFR in response to TNF-α and osmotic stress is not a process of EGFR transactivation.

Signaling Pathways Leading to EGFR—As shown in Fig. 1, TNF-α and osmotic stress rapidly induces intracellular signaling pathways. To identify the signaling pathway leading to the modification of EGFR, we first tried to examine the effects of chemical inhibitors for downstream kinases. The TNF-α-induced modification of EGFR was completely inhibited by pretreatment with 5Z-7-oxozeaenol and SB203580, inhibitors for TAK1 and p38, respectively (Fig. 4A). SB203580 (but not 5Z-7-oxozeaenol) also abrogated the osmotic stress-induced modification (Fig. 4A). In contrast, inhibitors for IKK, JNK, and MEK (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase) (SC-514, SP600125 and U0126, respectively) did not block modification of EGFR (Fig. 4A). Similar results were obtained in experiments using siRNA against TAK1 and p38α (Fig. 4, B and C). In contrast, these siRNAs were not effective in blocking the shift in the mobility of EGFR caused by EGF (Fig. 4, B and C).

TNF-α-induced Phosphorylation of EGFR—To identify the kinds of modifications that occurred on EGFR, we first investigated the possibility of phosphorylation. EGFR was immunoprecipitated from cells untreated or treated with TNF-α for 10 min, and the immunoprecipitates were incubated with λ-phosphatase in vitro. Fig. 4D shows that the reduced mobility was completely restored, indicating that phosphorylation at unknown Ser/Thr residues is involved, at least in part, in the TNF-α-induced modification of EGFR. As shown above, p38α and TAK1 are possible candidates for Ser/Thr kinases. We therefore performed in vitro kinase assays using recombinant kinases. Whole cell lysates prepared from unstimulated HeLa cells were immunoprecipitated with normal IgG or anti-EGFR antibody. The immunoprecipitates were then incubated with recombinant p38α or TAK1-TAB1 fusion protein (an active TAK1 protein) in the presence of 32P-labeled ATP. In the absence of recombinant kinases, EGFR had an autophosphorylation activity. Both p38α and TAK1 induced the incorporation of radioactivity into the EGFR (Fig. 4E); however, the activity was not as strong as that against the known substrates ATF2 and MKK6, respectively (data not shown).

Stress-induced Phosphorylation Suppressed Ligand-mediated Activation of EGFR—We investigated the role of the transient modification of EGFR in ligand-mediated activation. HeLa cells were pretreated with TNF-α for 10 or 60 min and then stimulated with EGF for another 2 or 10 min in the presence of TNF-α. The 10-min pretreatment induced a complete shift in the mobility of EGFR (Fig. 5A). The stimulation with EGF did not induce any additional mobility shift. Surprisingly, the EGF-induced tyrosine phosphorylation of EGFR was impaired by the pretreatment with TNF-α for 10 min (Fig. 5A). Interestingly, with the disappearance of the mobility shift at 60 min after pre-TNF-α stimulation, the ability of EGFR to...
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To investigate whether the suppression of EGFR is mediated through the TAK1-p38 pathway, we tried to test the effect of a TAK1 inhibitor. HeLa cells were pretreated with the inhibitor before being treated with TNF-α and EGF. In the absence of TNF-α pretreatment, the inhibitor did not block the EGFR-induced mobility shift and tyrosine phosphorylation of EGFR and subsequent activation of p38 (Fig. 5C, lanes 2 and 4). In contrast, it abolished the TNF-α-induced activation of p38 and subsequent modification of EGFR in the absence of EGF stimulation (Fig. 5C, lanes 5 and 7), which clearly indicates its selectivity toward the TNF-α signaling pathway. Blockade of the TNF-α-induced activation of the TAK1-p38 pathway canceled the TNF-α-induced suppression of ligand-induced tyrosine phosphorylation of EGFR (Fig. 5, C [lanes 6 and 8] and D). These results demonstrated that the TAK1-p38 pathway is the main route for suppression of the EGFR by TNF-α.

Stress-induced Internalization of EGFR—To clarify the mechanism of TNF-α-induced suppression of EGFR, cell surface receptor expression was investigated by FACS analysis using the LA1 clone of the anti-EGFR antibody. The receptor expression was significantly reduced when EGF was internalized and degraded by treatment with EGF for 10 or 60 min, indicating the specific binding of LA1 to the EGFR (Fig. 6A). Treatment with TNF-α for 10 min also decreased EGF expression (Fig. 6B). Interestingly, the expression returned to the control level by continuous exposure for 60 min (Fig. 6B). On the other hand, high osmotic stress caused gradual down-regulation of EGFR until 60 min (Fig. 6C). These observations are consistent with the kinetics of phosphorylation and suppression of EGFR by TNF-α or osmotic stress (Figs. 1 and 5A). We next investigated the effect of permeabilization of cytoplasmic membrane in the staining procedure. Fig. 6D shows that permeabilization restored the antibody binding to the TNF-α-treated cells, demonstrating that TNF-α-induced transient down-regulation of EGFR is mediated by its internalization.

To identify the signaling pathways controlling the internalization, the effects of chemical inhibitors were examined. TNF-α-induced internalization of EGFR was completely inhibited by 5Z-7-oxoozaenol and SB203580 (Fig. 6E) but not by PD153035 and GM6001 (Fig. 6F). In addition, osmotic stress-induced internalization was inhibited by only SB203580 (Fig. 6, G and H). These results are in agreement with the effects of these inhibitors on stress-induced modification of EGFR (Figs. 2 and 4A). Furthermore, siRNAs against TAK1 and p38α significantly abrogated the TNF-α-induced internalization.
chain expression abrogated the TNF-α-induced internalization of EGFR without affecting its phosphorylation (Fig. 7, A and B). In contrast, co-transfection of siRNAs against c-Cbl and Cbl-b did not inhibit both events evoked by TNF-α (Fig. 7, A and C). This is consistent with the observation that phosphorylation at Tyr-1045, a critical signal for the binding of Cbl proteins to EGFR, was not induced by TNF-α (Fig. 1). These results demonstrated that the TNF-α-induced internalization of EGFR was preceded by phosphorylation of EGFR and that clathrin is a common mediator for EGFR- and TNF-α-induced internalization of EGFR.

Recycling of EGFR by Turnover of p38 Activation—In Fig. 1, we demonstrated that phosphorylation of EGFR evoked by TNF-α is transient; however, osmotic stress induced a sustained phosphorylation of EGFR. If p38 is a common mediator for this phosphorylation, the duration of which p38 remains active may vary in cells treated with different types of stress. As expected, the transient activation of p38 induced by TNF-α was maintained for 30 min, whereas that induced by osmotic stress was prolonged for at least 60 min (Fig. 8A). These were consistent with the duration of intracellular localization of EGFR as shown in Fig. 6.

We further investigated the mechanism for sequestering EGFR inside the cells and for recycling back to the cell surface. After cells were stimulated with TNF-α for 10 or 30 min, calyculin-A, a protein phosphatase inhibitor, was added to the culture medium. The inhibi-

(fig. 6, I and J). These results demonstrated that the TAK1-p38 pathway plays a critical role in TNF-α-induced internalization of EGFR.

Clathrin-mediated Internalization of EGFR—Upon ligand activation, EGFR has been shown to undergo the clathrin-mediated internalization and subsequent lysosomal protein degradation through ubiquitination by E3 ubiquitin ligases, c-Cbl, and Cbl-b. We therefore examined the effects of siRNAs against clathrin heavy chain, c-Cbl, and Cbl-b on the TNF-α-induced internalization of EGFR. The knockdown of clathrin heavy chain expression abrogated the TNF-α-induced internalization of EGFR without affecting its phosphorylation (Fig. 7, A and B). In contrast, co-transfection of siRNAs against c-Cbl and Cbl-b did not inhibit both events evoked by TNF-α (Fig. 7, A and C). This is consistent with the observation that phosphorylation at Tyr-1045, a critical signal for the binding of Cbl proteins to EGFR, was not induced by TNF-α (Fig. 1). These results demonstrated that the TNF-α-induced internalization of EGFR was preceded by phosphorylation of EGFR and that clathrin is a common mediator for EGFR- and TNF-α-induced internalization of EGFR.

Recycling of EGFR by Turnover of p38 Activation—In Fig. 1, we demonstrated that phosphorylation of EGFR evoked by TNF-α is transient; however, osmotic stress induced a sustained phosphorylation of EGFR. If p38 is a common mediator for this phosphorylation, the duration of which p38 remains active may vary in cells treated with different types of stress. As expected, the transient activation of p38 induced by TNF-α was maintained for 30 min, whereas that induced by osmotic stress was prolonged for at least 60 min (Fig. 8A). These were consistent with the duration of intracellular localization of EGFR as shown in Fig. 6.

We further investigated the mechanism for sequestering EGFR inside the cells and for recycling back to the cell surface. After cells were stimulated with TNF-α for 10 or 30 min, calyculin-A, a protein phosphatase inhibitor, was added to the culture medium. The inhibitor blocked dephosphorylation of EGFR at 60 min (Fig. 8B). In contrast, p38 was largely dephosphoryl-
Suppression of EGFR by p38

In 1996, Rosette and Karin (47) reported that UV light and osmotic stress activates the JNK pathway through multiple growth factor receptors including EGFR. In the past ten years, the mechanisms underlying the transactivation of EGFR have been extensively investigated. Latent membrane-bound ligands, such as transforming growth factor-α and heparin-binding EGF, are released by proteolysis and bind to EGFR via an extracellular mechanism (48). Alternatively, EGFR is activated by soluble intracellular tyrosine kinases such as Src (49). However, further study is essential to understanding the roles of EGFR in cellular communication networks.

FIGURE 7. Clathrin-mediated internalization of EGFR. HeLa cells were transfected with siRNAs against clathrin heavy chain (CHC) (50 nM), c-Cbl (25 nM), and luciferase (Luc) (50 nM). At 72 h post-transfection, cells were stimulated with TNF-α for 10 min. Phosphorylation of EGFR (A) or cell surface expression of EGFR (B and C) were analyzed by immunoblotting or FACS, respectively. Cont, control.

FIGURE 8. Recycling of EGFR to the cell surface. A, HeLa cells were treated with TNF-α or NaCl for the indicated periods. Whole cell lysates were immunoblotted with anti-EGFR or phosphospecific or control p38 antibodies. B, cells were stimulated with TNF-α. At 10 or 30 min post-stimulation, 10 ng/ml calyculin A (Caly-A) was added to the culture medium. Phosphorylation of EGFR and p38 at 10, 30, and 60 min was analyzed by immunoblotting. C, cells were stimulated with TNF-α. At 10 min post-stimulation, calyculin-A was added to the culture medium. Cell surface expression of EGFR was analyzed by FACS at 10 or 60 min. D and E, cells were stimulated with 300 mM NaCl (Osmo). At 15 min post-stimulation, the high osmotic culture medium was replaced with that containing 300 mM NaCl (+ → +) or normal medium (+ → −). Phosphorylation of EGFR and p38 at the indicated time points (D) or cell surface expression of EGFR (E) were analyzed by immunoblotting or FACS, respectively. Cont, control.

TFN-α has also been shown to trigger EGFR-mediated signaling that culminates in cellular functions. Chen et al. (34) report that the proliferation and motility of human mammary epithelial cells induced by TNF-α is prevented by inhibiting membrane protein shedding with a metalloprotease inhibitor, by blocking EGFR kinase activity, or by limiting ligand-receptor interactions with an antagonistic anti-EGFR antibody. Similar mechanisms play roles in the replication and metastatic properties of TNF-α-treated hepatocyte cell lines (27, 35). In addition, Janes et al. (50) recently performed a large experimental and computational analysis of cross-talk between TNF-α and EGFR in HT-29 human colonic adenocarcinoma cells, the results of which also support a model of TNF-α-induced extracellular cross-talk with EGFR. Collectively, these results indicate a TNF-α-induced successive autocrine pathway to EGFR. But, in the present study, we demonstrated that TNF-α triggers suppressive signals for tyrosine kinase activity of EGFR in HeLa cells.

Once EGF binds to EGFR, intracellular tyrosine kinase is activated and induces its autophosphorylation to trigger downstream signaling pathways. The Src family of tyrosine kinases is also involved in the ligand-mediated activation of EGFR (49). Signal attenuation from ligand-activated EGFR is mediated in part by receptor endocytosis and trafficking to the lysosomal...
degradative compartment. Phosphorylation at Tyr-1045 is essential for ubiquitination by Cbl proteins, E3 ubiquitin ligases, and subsequent degradation of EGFR (51). However, small amounts of activated EGFR escape from the degradation process and are recycled to the cell surface. In contrast, although the TNF-α-induced phosphorylation of EGFR was associated with down-regulation of EGFR, no obvious degradation was induced (Fig. 1B). In addition, Cbl proteins are not involved in the TNF-α-induced internalization of EGFR (Fig. 7). After stimulation with TNF-α for 45–60 min, almost all of the modified EGFR had returned to its former unstimulated state (Fig. 8A), and the ability to respond to extracellular ligands was restored (Fig. 6A). Osmotic stress also induced a rapid and sustained modification of EGFR without degradation for at least 2 h (Figs. 1C and 8A). These results correlated with the findings that neither TNF-α nor osmotic stress was able to induce phosphorylation at Tyr-1045 (Fig. 1, A and C), a critical phosphorylation site for Cbl-mediated ubiquitination (51). PD153035 as well as PP1, an Src tyrosine kinase inhibitor, did not block the TNF-α-induced phosphorylation of EGFR (Fig. 2B and data not shown), indicating that a novel mechanism participates in the stress-induced modification. In the present study, we demonstrated that the TAK1-p38 signaling pathway is critical for this phosphorylation.

It was recently reported that EGFR is internalized without being degraded in response to several stressors, including aminomycin (an antibiotic that inhibits protein synthesis) and cisplatin (an effective DNA-damaging antitumor agent) through activation of p38 (52, 53). EGFR undergoes a gel mobility shift upon cisplatin treatment, which is mediated by p38-dependent phosphorylation of the receptor at threonine 669 (53). These results suggest that the TNF-α-induced transient suppression (Fig. 5) may be due to the p38-mediated internalization of EGFR. During the preparation of this manuscript, Zwang and Yarden (45) demonstrated that clathrin is involved in UV-mediated internalization of EGFR, and abrogating EGFR internalization reduces the efficacy of chemotherapy-induced cell death. We also confirmed in this study that TNF-α induces clathrin-mediated endocytosis (Fig. 7). They also claimed that phosphorylation of EGFR in a short segment (amino acids 1002–1022) containing multiple Ser and Thr residues is involved in these processes. As shown in Fig. 4, p38α and TAK1 are possible Ser/Thr kinases. However, 10 μM PD153035 largely inhibited the TNF-α-induced modification of EGFR (Fig. 2B) but not activation of TAK1 and p38 (Fig. 3A), suggesting the possibility that unknown kinases are involved in the phosphorylation of EGFR downstream of TAK1-p38. Therefore, the TNF-α-induced transient suppression of the EGF response is regulated by the phosphorylation-mediated internalization of EGFR in a TAK1-p38-dependent and EGFR tyrosine kinase-independent manner. In addition, turnover of p38 activation rapidly induces dephosphorylation and recycling of EGFR (Fig. 8). Identification of the sites of phosphorylation and corresponding kinases and phosphatases is necessary for a full understanding of the interference with EGFR.

Both TNF-α and osmotic stress trigger all three MAPK pathways. Fig. 1A shows that TAK1 and IKK were activated by TNF-α but not by high osmotic stress. In the TNF-α signaling pathways, TAK1 is indispensable for the IKK-NF-κB pathway. Therefore, there is no significant activation of IKK by osmotic stress is due to a lack of TAK1 activation. In addition, we previously demonstrated that TNF-α-induced (but not osmotic stress-induced) p38 activation is largely dependent on TAK1. Genetic findings have also demonstrated the significance of TAK1 in the activation of p38, JNK, and IKK. These results suggest that TAK1 and other MAPK kinase kinases are involved in the TNF-α-induced and osmotic stress-induced activation of p38, respectively, and subsequent modification of EGFR.

In summary, we have identified the molecular mechanism of the stress-induced suppression of EGFR. However, the physiological function of this event is still entirely unknown. In several types of cells, TNF-α induces transactivation of EGFR. Investigation of the different mechanisms underlying these opposite effects of TNF-α on the function of EGFR will provide information for connecting the diverse cellular networks of cytokine and growth factor receptors.

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