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TAK1-Mediated Serine/Threonine Phosphorylation of Epidermal Growth Factor Receptor via p38/Extracellular Signal-Regulated Kinase: NF-κB-Independent Survival Pathways in Tumor Necrosis Factor Alpha Signaling[▽]

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The kinase TAK1, a mitogen-activated protein kinase kinase kinase (MAP3K), has been widely accepted as a key kinase activating NF-κB and MAPKs in tumor necrosis factor alpha (TNF-α) signaling. We have recently reported that TAK1 regulates the transient phosphorylation and endocytosis of epidermal growth factor receptor (EGFR) in a tyrosine kinase activity-independent manner. In the present study, we found that Thr-669 in the juxtamembrane domain and Ser-1046/1047 in the carboxyl-terminal regulatory domain were transiently phosphorylated in response to TNF-α. Experiments using chemical inhibitors and small interfering RNA demonstrated that TNF-α-mediated phosphorylation of Thr-669 and Ser-1046/7 were differently regulated via TAK1-extracellular signal-regulated kinase (ERK) and TAK1-p38 pathways, respectively. In addition, p38, but not ERK, was involved in the endocytosis of EGFR. Surprisingly, modified EGFR was essential to prevent apoptotic cellular responses; however, the EGFR pathway was independent of the NF-κB antiapoptotic pathway. These results demonstrated that TAK1 controls two different signaling pathways, IκB kinase–NF-κB and MAPK-EGFR, leading to the survival of cells exposed to the death signal from the TNF-α receptor.

Epidermal growth factor receptor (EGFR) is a member of the receptor tyrosine kinase (TK) family and plays a critical role in a wide variety of cellular functions, including proliferation, differentiation, and apoptosis (33–35, 38, 42). EGFR has recently been a focus of molecular targeted cancer therapy, because overexpression, amplification, and mutations are involved in carcinogenesis and the progression of several types of cancer (19, 21, 27, 34, 51). Multiple tyrosine residues of the EGFR intracellular domain are autophosphorylated upon dimerization with ligands such as EGF and, thereafter, recruit a variety of downstream substrates, including Grb2, Shc, and phospholipase C-γ, which trigger signaling waves to mitogen-activated protein kinases (MAPKs) and Akt (22, 23). In addition, c-Cbl and Cbl-b, E3 ubiquitin ligases, bind phosphorylated Tyr-1045, which induces the ubiquitination of EGFR (16). Modified EGFR internalizes with the ligand via clathrin-coated pits and is subsequently sorted to late endosomes/lysosomes where it is degraded to terminate signal transduction (9, 46).

Tumor necrosis factor alpha (TNF-α), a proinflammatory and apoptosis-inducing cytokine, stimulates several intracellular signaling pathways, leading to the activation of transcription factors AP-1 and NF-κB (3, 44). AP-1 is regulated by cascades of MAPKs, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 pathways (15, 43). The transcriptional activity of NF-κB is regulated by the IκB kinase (IKK)-mediated phosphorylation of IκBα and p65/RelA (10, 11). It has been demonstrated that kinase TAK1, a member of the MAP3K family, participates as an upstream kinase of the MAPK and IKK signaling pathways (2, 25, 29, 31, 47). We have reported that TAK1 promotes TNF-α-induced metastasis of colon cancer cells (7). On the other hand, TNF-α triggers apoptosis via formation of the death-inducing signaling complex (5, 13, 14). This complex consists of trimerized receptors, the death domain-containing adaptor protein FADD (Fas-associated death domain protein), and caspase-8. Activation of caspase-8 leads to the direct activation of downstream caspases, such as caspase-3, and subsequently the cleavage of poly(ADP-ribose) polymerase (PARP), a nuclear enzyme activated by binding to DNA breaks (41). Cells deficient in TAK1, IKKβ, or p65 are sensitive to TNF-α-induced apoptosis, indicating that the TAK1-NF-κB signaling pathway functions as a survival signal (4, 18, 26, 36).

It has recently been demonstrated that cellular stress conditions, including exposure to TNF-α, UV irradiation, genotoxic agents, and high osmolarity, induce the phosphorylation and clathrin-dependent endocytosis of EGFR (20, 40, 45, 49, 53). Unlike ligand stimulation, this event is completely independent of EGFR TK activity and Cbl ubiquitin ligase. p38 MAPK has been shown to be a common regulator of EGFR modification. We have also shown that the TAK1-p38 pathway is involved in TNF-α-induced phosphorylation of EGFR (40). After internalization in cytokine-stimulated cells, EGFR is dephosphorylated and then recycles back to the cell surface, whereas in irradiated cells it arrests in Rab5-containing endosomes (53). During intracellular trafficking, cells are not able to respond to extracellular EGFR ligands (20, 40). These observations sug-

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gest critical functions of EGFR in cells under stress; however, the molecular mechanisms and physiological functions of EGFR phosphorylation are not fully understood.

In the present study, we have tried to identify TAK1-mediated phosphorylation sites and signaling pathways to the EGFR. We found that Thr-669 and Ser-1046/1047 are target residues, which were separately phosphorylated via ERK and p38 pathways. Moreover, EGFR was essential for protection from TNF-α death signals in an NF-κB-independent manner.

MATERIALS AND METHODS

Antibodies and reagents. An anti-phospho-TAK1 (Thr-187) antibody was generated as described previously (39). Other phospho-specific antibodies against p38 (Thr-180, Tyr-182), JNK (Thr-183, Tyr-185), ERK (Thr-202, Tyr-204), p65 (Ser-536), Akt (Ser-473), EGFR (Tyr-845, Tyr-974, Tyr-992, Tyr-1045, Tyr-1068, Tyr-1173, Thr-669, and Ser-1046/1047), and PRAP and caspase-3 were purchased from Cell Signaling Technology. Antibodies against TAK1 (M-579), TAB1 (C-20), TAB2 (K-20), p38 (C-20-G), JNK (FL), ERK1 (C-16), ERK2 (C-14), p65 (C-20-G), EGFR (1005), phosphotyrosine (PY20), c-Cbl (C-15), Cbl-b (C-20), clathrin heavy chain (CHC; C-20), PCNA (PC10), α-tubulin (B-7), and actin (C-11) were obtained from Santa Cruz Biotechnologies. Recombinant human TNF-α and EGF were obtained from R&D Systems; SB203580, SP600125, U0126, and PD153035 were from Merck Biosciences; and 5Z-7-oxozeaenol, a selective TAK1 inhibitor, was a gift from Chugai Pharmaceutical Co., Ltd. (24). All chemical inhibitors were dissolved in dimethyl sulfoxide, and the final concentration of dimethyl sulfoxide was less than 0.1%.

Cell cultures. HeLa and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (high-glucose conditions) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2. A549 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2.

Establishment of stable cell lines. HeLa cells were transfected with pSUPER-gfp/neo vectors containing short hairpin RNAs (shRNAs) against human TAK1 and firefly luciferase GL2 (Luc) using Lipofectamine reagent. The target sequence for TAK1 is 5′-ATGACGATTCATGAGTGTTAG-3′, which is located in the 3′ untranslated region. Stable cell lines were selected by G418 at 1 mg/ml, and green fluorescent protein (GFP) expression was confirmed by fluorescence-activated cell sorter (FACS) analysis. The cells were maintained in medium containing G418.

Transfection of plasmid DNAs. Human EGFR cDNA was amplified by reverse transcription-PCR and inserted into pcDNA3.1 vector. Deletion and substitution mutants were generated by a QuikChange site-directed mutagenesis kit (Stratagene). Expression vectors for TAK1, TAB1, and TAB2 were previously described. HEK293 cells were transfected using a Lipofectamine 2000 reagent.

Transfection of siRNAs. Duplex small interfering RNAs (siRNAs) were synthesized at Hokkaido System Science Co., Ltd. or Invitrogen (Stealth RNA interference [RNAi]). The target sequences were as follows: 5′-TAATCCAAT TCGAAGACCAAT-3′ (CHC), 5′-TCACACAGGGTTCCTGACAGAATA T-3′ (ERK2), 5′-GCAUUACAACCAGACAGUUGAUAUU-3′ (p38α), 5′-TG CTGGGTGCGGAAGAGAAAGAATA-3′ (EGFR 1), 5′-CCUAUGCCUUAG CAGUCUUAUCUAA-3′ (EGFR 2), 5′-GCCCTATCCCTTTACGTCATT-3′ (p65/RelA), and 5′-CGUACGCGGAAUACUUCGA-3′ (firefly luciferase GL2). HeLa cells were transfected with siRNAs in a final concentration of 20 to 100 nM using Lipofectamine. At 72 h posttransfection, the cells were stimulated.

Immunoblotting. After stimulation, whole-cell lysates were prepared as described previously (30). Cell lysates were resolved by 7.5%, 10%, or 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis 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 immunoglobulin G (Dako) and visualized with the enhanced chemiluminescence system (Amersham Biosciences). Some antibody reactions were carried out in Can Get Signal solution (Toyobo).

Fluorescence microscopy. To establish HeLa cells stably expressing enhanced GFP (EGFP)-fused EGFR, human EGFR cDNA was inserted into the pEGFP-N1 vector. Stably transfected cells were selected by G418 as described above. Cells were seeded in a glass-bottom culture dish (Fastgene) and stimulated by ligand and inhibitor. After methanol fixation, fluorescence was analyzed by TCS-SP5 confocal microscopy (Leica).

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. 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 (clone LA1; Upstate) and incubated on ice for 30 min. After being washed with FACS buffer, cells were incubated with fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G antibody (Dako) on ice for 30 min and analyzed by the FACSCalibur system (BD).

RESULTS

Different mechanisms of phosphorylation and endocytosis of EGFR by TNF-α and EGF. Stimulation of HeLa cells with EGF strongly induced the phosphorylation of tyrosine residues in the intracellular domain of EGFR, which was observed in parallel to a shift in the mobility of EGFR on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1A). Similarly, TNF-α caused a mobility shift within 10 min; however, no tyrosine phosphorylation was observed (Fig. 1A). We previously reported that the shift is canceled by treatment with λ-phosphatase in vitro, suggesting that TNF-α-induced phosphorylation induced a conformational change of EGFR (40). To confirm the role of TAK1 in the modification of EGFR, we established a stable cell line expressing TAK1 shRNA vector (HeLa-shTAK1). TAK1 protein expression was severely reduced in HeLa-shTAK1 cells compared to that in control HeLa-shLuc cells (Fig. 1B). The mobility shift of EGFR was also suppressed in HeLa-shTAK1 cells (Fig. 1B). In addition, a TAK1 selective inhibitor, 5Z-7-oxozeaenol, blocked TNF-α-induced modification of EGFR, whereas an EGFR TK inhibitor, PD153035, showed no inhibitory activity (Fig. 1C). In contrast, EGF-induced modification of EGFR was almost completely inhibited by PD153035 but not by 5Z-7-oxozeaenol (Fig. 1D).

We have previously shown by flow cytometry that TNF-α-induced phosphorylation of EGFR triggers its rapid internalization (40). Here we confirmed the cellular event using a HeLa cell line stably expressing C-terminally GFP-fused EGFR. Similar to the phosphorylation status, TNF-α-induced endocytosis of EGFR was dependent on TAK1, but not its TK activity (Fig. 1E). In sharp contrast, EGF-induced internalization was dependent on TK activity, but not TAK1 (Fig. 1F). These results indicate that endocytosis by TNF-α and EGF was controlled by different mechanisms.

Identification of Thr-669 and Ser-1046/1047 as phosphorylation sites of EGFR. Figure 2A shows the structure and typical phosphorylation sites in the juxtamembrane (JM), TK, and C-terminal regulatory (CR) domains of EGFR. To identify the phosphorylation sites, we tried to reconstitute TNF-α-induced EGFR phosphorylation in HEK293 cells ectopically expressing EGFR and TAK1. Overexpression of EGFR induced ligand-independent TK activation, which caused autophosphorylation of the tyrosine residues (Fig. 2B). It is interesting that TAK1, which was activated by its adaptor proteins TAB1 and TAB2, completely suppressed TK activation (Fig. 2B). Doublet bands of EGFR were detected in immunoblotting; however, the ratio of the upper band was slightly increased when TAK1 was overexpressed with EGFR (Fig. 2B). This result suggests that TAK1 induced additional modifications of EGFR. To explore

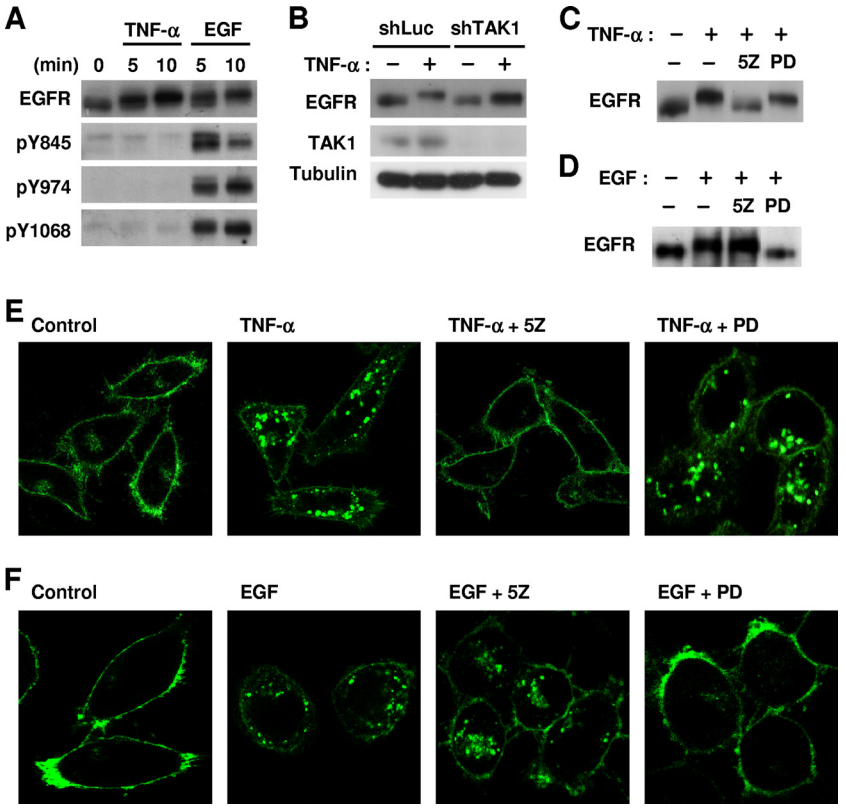

FIG. 1. Role of TAK1 and EGFR TK activity in TNF-α- and EGF-induced phosphorylation and endocytosis of EGFR. (A) HeLa cells were treated with 20 ng/ml TNF-α or 10 ng/ml EGF for 5 or 10 min. Whole-cell lysates were immunoblotted with phospho-EGFR (Tyr-845, Tyr-974, and Tyr-1068) and EGFR antibodies. (B) HeLa-shLuc and HeLa-shTAK1 cells were stimulated with TNF-α for 10 min. Whole-cell lysates were immunoblotted with EGFR, TAK1, and PCNA antibodies. (C and D) HeLa cells were pretreated with 5Z-7-oxozeaenol (5Z) (0.3 μM) and PD153035 (PD) (1 μM) for 30 min and then stimulated with 20 ng/ml TNF-α (C) or 10 ng/ml EGF (D) for another 10 min. Whole-cell lysates were immunoblotted with EGFR antibody. (E and F) HeLa cells stably expressing EGFR-GFP were pretreated with 5Z-7-oxozeaenol (0.3 μM) and PD153035 (1 μM) for 30 min and then stimulated with TNF-α (C) or EGF (D) for another 15 min. Subcellular localization of EGFR-GFP was examined by confocal fluorescent microscopy.

the region of these modifications, we constructed deletion mutants of EGFR. Downregulation of EGFR tyrosine phosphorylation by TAK1 was still observed in EGFR-Δ1043, a mutant lacking amino acids from 1044 to the C-terminal end (Fig. 2C). Moreover, although a TAK1-induced shift in the mobility of EGFR-Δ1113 was clearly detected, it disappeared in EGFR-Δ1043 (Fig. 2C). Based on these overexpression results, we speculated that there are at least two TAK1-mediated modification sites (probably Ser/Thr) on EGFR among amino acids 1043 to 1113 and within 1043. To screen for the possible phosphorylation sites, we searched the PhosphoSitePlus database on the website (http://www.phosphosite.org/homeAction.do). We focused on the following two sites, Thr-669 in the JM domain and Ser-1046/1047 in the CR domain, and then monitored the phosphorylation status of these residues using their phospho-specific antibodies. As shown in Fig. 2D, all Ser/Thr/Tyr sites were phosphorylated in wild-type EGFR. Interestingly, the phosphorylation of Thr-669 and Ser-1046/1047 was increased by coexpression of activated TAK1, whereas Tyr phosphorylation (PY20) completely disappeared. Moreover, the increase in Ser/Thr phosphorylation was also observed in a TK-dead mutant, EGFR-KK/AA, suggesting that TAK1-induced Ser/Thr phosphorylation was independent of the TK activity of EGFR. Substitution of the Ser/Thr residues with Ala disrupted the specific bands, indicating that the antibodies recognized the phosphorylated residues (Fig. 2E and F). This result was consistent with the observation that PD153035 did not inhibit the TNF-α-induced phosphorylation of EGFR in HeLa cells (Fig. 1C). Collectively, these results indicate that Thr-669 and Ser-1046/1047 are the candidate sites for TNF-α-induced phosphorylation of endogenous EGFR.

Phosphorylation of Thr-669 and Ser-1046/1047 in endogenous EGFR. We next investigated the phosphorylation of endogenous EGFR in HeLa cells. As shown in Fig. 3A, multiple tyrosine residues in EGFR were phosphorylated by EGF, but not by TNF-α. The TNF-α-induced mobility shift was transient, and there was a return to the control level at 60 min. Similar to this mobility shift, Thr-669 and Ser-1046/1047 were significantly phosphorylated at 10 and 30 min and then dephosphorylated at 60 min. EGF also induced Ser/Thr phosphorylation; however, the duration and frequency were significantly different from TNF-α stimulation. Thr-669 phosphorylation was prolonged until 60 min, and only weak phosphorylation of Ser-1046/1047 was detected within 10 min. On the other hand, high levels of osmotic stress caused a rapid and sustained shift in the mobility and Ser/Thr phosphorylation of EGFR, in

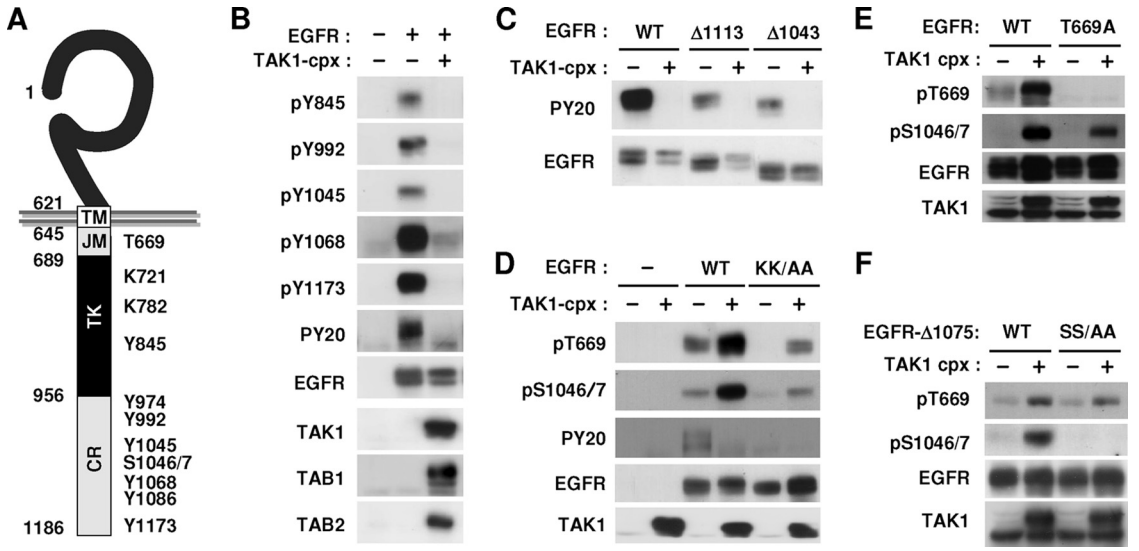

FIG. 2. Identification of phosphorylation sites on EGFR by overexpression experiments in HEK293 cells. (A) Structure of EGFR. EGFR is composed by extracellular, transmembrane (TM), JM, TK, and CR domains. Numbers represent the number of amino acids of EGFR that the N-terminal signal peptide (24 amino acids) processed. (B) HEK293 cells were transfected with expression vectors for EGFR and the TAK1/TAB1/TAB2 complex (TAK1-cpx). Twenty-four hours after transfection, cells were harvested and whole-cell lysates were immunoblotted with phospho-EGFR (Tyr-845, Tyr-992, Tyr-1045, Tyr-1068, and Tyr-1173), phosphotyrosine (PY20), EGFR, and TAK1, TAB1, and TAB2 antibodies. (C) Cells were transfected with expression vectors for the wild type (WT) or deletion mutants (Δ1113 and Δ1043) of EGFR and the TAK1 complex. Whole-cell lysates were immunoblotted with PY20 and EGFR antibodies. (D) Cells were transfected with expression vectors for the wild type or a kinase dead mutant (KK/AA) of EGFR and the TAK1 complex. Whole-cell lysates were immunoblotted with phospho-EGFR (Thr-669 and Ser-1046/1047), PY20, EGFR, and TAK1 antibodies. (E and F) Cells were transfected with expression vectors for the wild type and substitution mutants (T669A or SS1046/1047AA) of full-length or deleted (Δ1075) EGFR and the TAK1 complex. Whole-cell lysates were immunoblotted with phospho-EGFR (Thr-669 and Ser-1046/1047), EGFR, and TAK1 antibodies.

which the known tyrosine residues were not phosphorylated (Fig. 3B). In addition, TNF-α-induced Ser/Thr phosphorylation was observed in A549 lung adenocarcinoma cells (Fig. 3C) and DU145 prostate cancer cells (data not shown). Interleukin 1β (IL-1β) also induced the phosphorylation in A549 cells (Fig. 3C). Moreover, TNF-α induced the phosphorylation of Thr-669 and Ser-1046/1047 of ectopically expressed wild-type and kinase-dead EGFR-KK/AA in HEK293 cells (Fig. 3D), which was consistent with the observation that TNF-α-induced phosphorylation of endogenous EGFR was not inhibited by PD153035 in HeLa cells (Fig. 1C). These results indicate that Thr-669 and Ser-1046/1047 are commonly phosphorylated under cellular stress conditions in an EGFR TK-independent manner.

Characterization of signaling pathways leading to Ser/Thr phosphorylation. As shown in Fig. 1B, TNF-α-induced activation of MAPKs and the mobility shift of EGFR were impaired in HeLa-shTAK1 cells. Here we tried to investigate the role of TAK1 in TNF-α- and EGF-induced Ser/Thr phosphorylation of EGFR. TNF-α-induced activation of ERK, JNK, and p38 MAPKs as well as phosphorylation of Thr-669 and Ser-1046/1047 were severely impaired in HeLa-shTAK1 cells (Fig. 4A). In contrast, TAK1 was dispensable in ligand-induced MAPK activation and EGFR phosphorylation at Ser/Thr/Tyr residues (Fig. 4A). To further characterize the signaling pathways downstream of TAK1, HeLa cells were treated with TNF-α in the presence of chemical inhibitors of TAK1, MAPKs, and EGFR TK. Similar to the results in the shRNA experiment, a TAK1 inhibitor, 5Z-oxozeaenol, blocked the phosphorylation of both Thr-669 and Ser-1046/1047 (Fig. 4B). Interestingly, a MEK inhibitor, U0126, and a p38 inhibitor, SB203580, blocked the phosphorylation of Thr-669 and Ser-1046/47, respectively, indicating that Thr-669 and Ser-1046/1047 were phosphorylated independently (Fig. 4B). In contrast, EGFR TK and JNK did not contribute to EGFR phosphorylation (Fig. 4B). The mobility shift of EGFR is canceled by 5Z-oxozeaenol and SB203580, but not by U0126 (Fig. 4B), indicating that TAK1-p38-mediated Ser-1046/1047 phosphorylation induces a conformational change. We next examined the effects of the inhibitors on ligand-induced EGFR phosphorylation (Fig. 4C). EGF-induced phosphorylation of Thr-669, Ser-1046/1047, and Tyr-845 was completely inhibited by PD153035. In addition, U0126 and SB203580 also inhibited EGF-induced phosphorylation of Thr-669 and Ser-1046/1047, respectively. These results suggested that ligand-induced phosphorylation of Thr-669 and Ser-1046/1047 is also dependent on the ERK and p38 pathways, respectively. To address the role of ERK and p38 in TNF-α-induced Thr-669 and Ser-1046/1047 phosphorylation, we performed RNAi experiments using siRNAs against ERK2 and p38α, major MAPKs in HeLa cells. Figure 4D demonstrates that ERK2 was essential for TNF-α-induced phosphorylation of Thr-669, but not Ser-1046/1047. In contrast, p38α was essential for Ser-1046/1047 phosphorylation, but not Thr-669 (Fig. 4D). Moreover, time course analysis showed that p38-mediated Ser phosphorylation preceded ERK-mediated Thr phosphorylation (Fig. 4E). Collectively, these results demonstrated that TAK1 regulates two independent MAPK signaling pathways in TNF-α-induced phosphorylation of EGFR.

Role of p38 and ERK in endocytosis of EGFR. We next addressed the roles of p38 and ERK in TNF-α-induced endo-

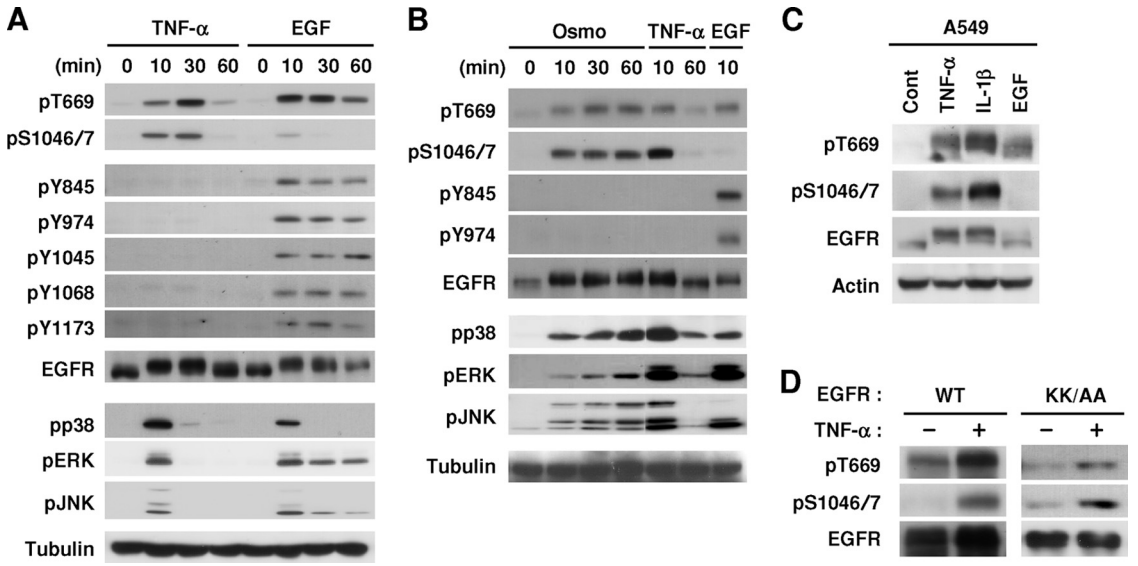

FIG. 3. Phosphorylation of endogenous EGFR at Thr-669 and Ser-1046/1047. (A) HeLa cells were treated with TNF-α or EGF for 10, 30, or 60 min. Whole-cell lysates were immunoblotted with phospho-EGFR (Thr-669, Ser-1046/1047, Tyr-845, Tyr-974, Tyr-1045, Tyr-1068, and Tyr-1173), EGFR, phospho-MAPKs (p38, JNK, and ERK), and tubulin antibodies. (B) Cells were exposed to a high concentration of salt (Osmo) (300 mM NaCl), TNF-α, or EGF for the indicated time periods. Whole-cell lysates were immunoblotted with the indicated antibodies. (C) A549 cells were treated with TNF-α, IL-1β, or EGF for 10 min. Whole-cell lysates were immunoblotted with phospho-EGFR (Thr-669 and Ser-1046/1047), EGFR, and actin antibodies. (D) HEK293 cells were transfected with expression vectors for the wild type (WT) and a kinase dead mutant (KK/AA) of EGFR. Twenty-four hours after transfection, cells were treated with TNF-α for 10 min. Whole-cell lysates were immunoblotted with phospho-EGFR (Thr-669 and Ser-1046/1047) and EGFR antibodies. Cont, control.

cytosis of EGFR. In accordance with our previous experiment using a flow cytometer, pretreatment with SB203580 completely inhibited TNF-α-induced endocytosis of GFP-fused EGFR (Fig. 5A); however, U0126 showed no inhibitory effect (Fig. 5A). In contrast, ligand-induced endocytosis was partially inhibited by SB203580, although U0126 showed no inhibitory activity (Fig. 5B). The partial inhibition by SB203580 was investigated by FACS analysis, in which cell surface expression of endogenous EGFR was able to evaluate quantitatively (40). Although TNF-α-induced internalization of EGFR was completely inhibited by SB203580, the EGF-induced event was partially inhibited (Fig. 5C). These results suggest that a p38-mediated mechanism is also involved, at least in part, in ligand-induced endocytosis of EGFR. These results suggest that p38-mediated Ser-1046/1047 phosphorylation is a major mechanism for stress-induced endocytosis and is a minor mechanism for ligand-induced endocytosis. In contrast, the ERK pathway is dispensable for stress- and ligand-induced internalization. It has been reported that cytokine-induced endocytosis is mediated by the formation of clathrin-coated pits. We have previously reported that knockdown of CHC inhibited endocytosis (40); therefore, we examined the effects of CHC siRNA on TNF-α-induced phosphorylation of EGFR (Fig. 5B). Although TNF-α-induced activation of p38 as well as phosphorylation of Ser-1046/1047 were intact, the activation of ERK and subsequent phosphorylation of Thr-669 were impaired in CHC-knocked down cells (Fig. 5D). This result suggests that Thr-669 phosphorylation is dependent on the formation of clathrin-coated pits or occurs after internalization.

We have previously shown that internalized EGFR was rapidly dephosphorylated and recycled to the plasma membrane 60 min after TNF-α stimulation (40). As shown in Fig. 4B, we were able to induce a single phosphorylated EGFR at Ser or Thr in the presence of U0126 or SB203580, respectively. We next investigated the effect of the lack of phosphorylation at one site on the phosphorylation (at 15 min) and subsequent dephosphorylation (at 60 min) of the counterpart site. In the presence of U0126, TNF-α-induced phosphorylation and dephosphorylation of Ser-1046/1047 were normally detected (Fig. 5E). On the other hand, inhibition of Ser-1046/1047 phosphorylation and endocytosis by SB203580 resulted in the reduction of Thr-669 phosphorylation at 15 min and normal dephosphorylation at 60 min, strongly suggesting that Thr-669 phosphorylation had occurred at least in part after internalization (Fig. 5F). Collectively, these results indicate that dephosphorylation of Ser and that of Thr are independent of each other.

Antiapoptotic functions of EGFR against the TNF-α death signal. TAK1 has been accepted as an antiapoptotic kinase by activating the NF-κB pathway. We first tested the role of TAK1 in HeLa-shTAK1 cells. Figure 1A shows that TAK1 was essential for early JNK activation within 10 min upon TNF-α stimulation. In contrast, treatment of HeLa-shTAK1 cells with TNF-α for 4 h induced prolonged activation of JNK, but not p38, ERK, and p65 (Fig. 6A), which has recently been demonstrated to be involved in TNF-α-induced cell death (28). This corresponded with the enhanced cleavage of caspase-3 and PARP (Fig. 6A), confirming that TAK1 is essential for cell survival in HeLa cells.

As described above, EGFR is a component of early TNF-α signaling downstream of TAK1-MAPKs. To investigate the physiological functions of TAK1-mediated phosphorylation and endocytosis of EGFR, HeLa cells were transfected with siRNA against EGFR. At 72 h posttransfection, cells showed normal morphology under the microscope, and no cleavage of

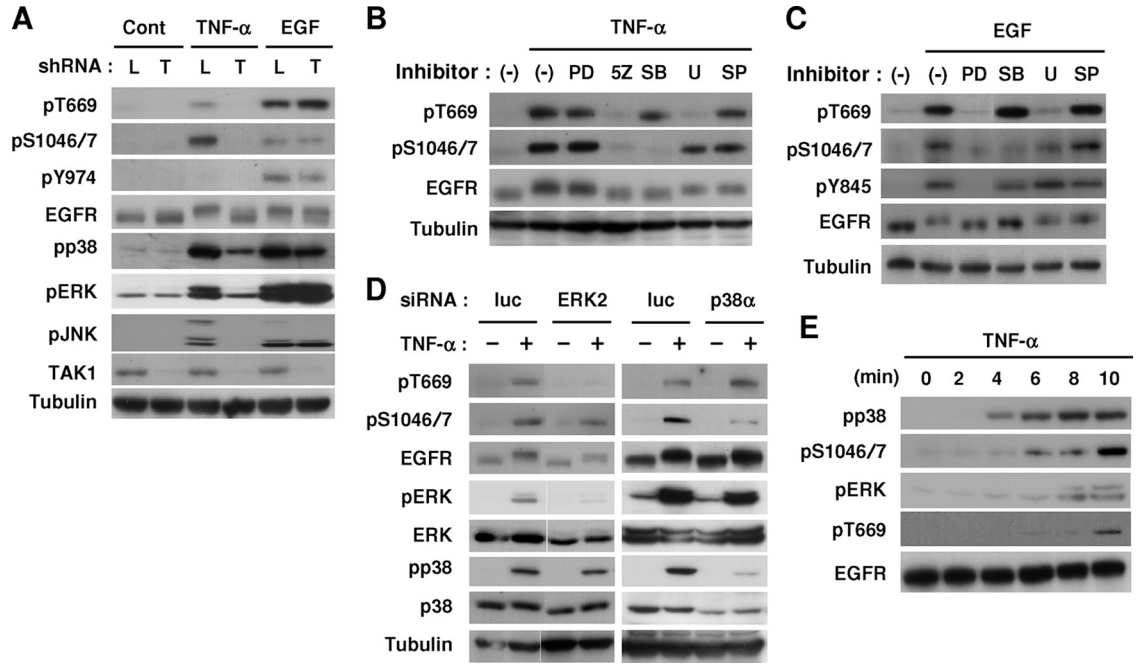

FIG. 4. Signaling pathways leading to Ser/Thr phosphorylation of EGFR. (A) HeLa cells stably expressing shRNA against TAK1 (T) and luciferase (L) were stimulated with TNF-α or EGF for 10 min. Whole-cell lysates were immunoblotted with phospho-EGFR (Thr-669, Ser-1046/1047, Tyr-845, 974, 1045, 1068 and 1173), EGFR, phospho-MAPKs (p38, JNK and ERK), and tubulin antibodies. Cont, control. (B and C) HeLa cells were pretreated with PD153035 (PD) (1 μM), 5Z-7-oxozeaenol (5Z) (0.3 μM), SB203580 (SB) (10 μM), U0126 (U) (5 μM), and SP600125 (SP) (10 μM) for 30 min and then stimulated with TNF-α (B) or EGF (C) for another 10 min. Whole-cell lysates were immunoblotted with phospho-EGFR (Thr-669, Ser-1046/1047, and Tyr845), EGFR, and tubulin antibodies. (D) HeLa cells were transfected with siRNAs against ERK2, p38α, and Luc. At 72 h posttransfection, cells were stimulated with TNF-α for 10 min. Whole-cell lysates were immunoblotted with the indicated antibodies. (E) Cells were stimulated with TNF-α for the indicated time periods (min), and phosphorylation of EGFR, p38, and ERK was detected by immunoblotting.

caspase-3 and PARP was detected (Fig. 6B); however, treatment of knocked down cells with TNF-α for 4 h significantly enhanced the cleavage of caspase-3 and PARP (Fig. 6B). Two independent siRNA sequences against EGFR showed a similar effect on TNF-α-induced proapoptotic events, strongly suggesting a survival function of EGFR (Fig. 6B). We next investigated the role of EGFR TK activity in TNF-α-induced cell death, because it was dispensable for TNF-α-induced Ser/Thr phosphorylation of EGFR (Fig. 4B). HeLa cells were pretreated with PD153035 or 5Z-7-oxozeaenol for 30 min and then stimulated with TNF-α for 4 h. PARP cleavage was not detected in cells treated with PD153035 even in the presence of TNF-α, while 5Z-7-oxozeaenol promoted the effect of TNF-α (Fig. 6C), indicating that EGFR TK activity is not required for protection from cytokine-induced cell death. We further investigated whether ERK and p38 are implicated in TAK1-mediated antiapoptotic reactions using their inhibitors. Figure 6D demonstrates that SB203580, but not U0126, promoted TNF-α-induced cleavage of PARP. In addition, U0126 was not able to enhance the effect of SB203580, suggesting that p38 carry an antiapoptotic signal to the EGFR. Moreover, an RNAi experiment using CHC siRNA demonstrated that the endocytosis of EGFR was not always necessary for its cell survival function (Fig. 6E). Collectively, these observations provide evidence of the role of Ser-1046/1047 phosphorylation of EGFR via the TAK1-p38 pathway in the antiapoptotic signaling pathway from TNF-R1.

EGFR signal is independent of NF-κB cell survival signal. We have shown that TAK1 is required for TNF-α-induced activation of NF-κB in HeLa cells. Here, we confirm the downregulation of TNF-α-induced NF-κB p65 phosphorylation at Ser-536, the IKK phosphorylation site, in HeLa-shTAK1 cells (Fig. 7A). In the present study, we indentified antiapoptotic signals to EGFR downstream of TAK1. To investigate the relationship between NF-κB and EGFR, we tested the effects of each siRNA on the counterpart signaling pathway. First, EGFR expression was downregulated by siRNA, and then the activation of the MAPK and NF-κB signaling pathways was investigated. The signaling pathways were normally activated in EGFR-knocked down cells (Fig. 7B). In addition, phosphorylation of Akt, another survival signal, was also comparable in these cells (Fig. 7B). Similarly, knockdown of RelA/p65, a main subunit of NF-κB, did not affect the TNF-α-induced phosphorylation of EGFR via MAPKs (Fig. 7C). These findings were consistent with the observation that siRNAs against EGFR and p65 synergistically promoted TNF-α-induced PARP cleavage (Fig. 7D). These results demonstrated that two TAK1-regulated signaling pathways, TAK1-NF-κB and TAK1-EGFR, were completely independent.

DISCUSSION

Ligand binding to the extracellular domain of EGFR triggers the autophosphorylation of multiple intracellular tyrosine

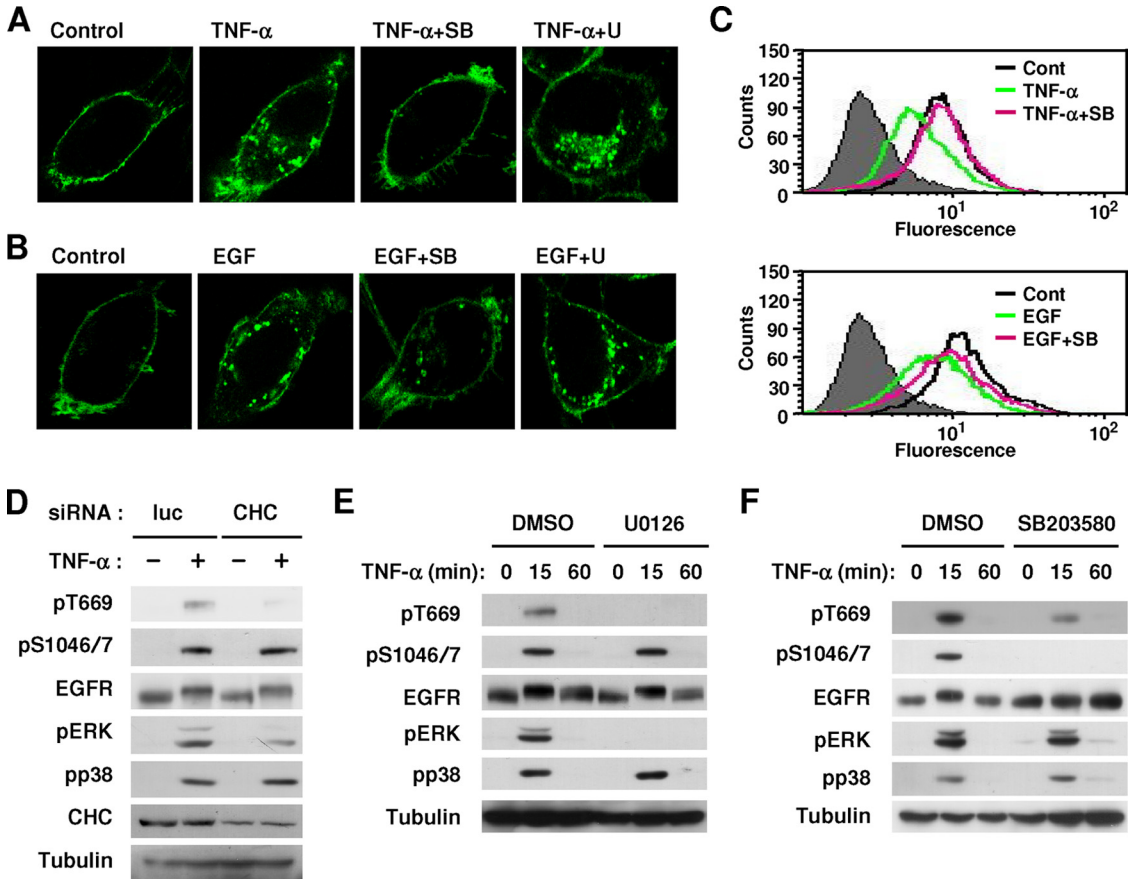

FIG. 5. Phosphorylation-dependent endocytosis and subsequent dephosphorylation of EGFR. (A and B) HeLa-EGFR-GFP cells were pretreated with SB203580 (SB) (10 μM) and U0126 (U) (5 μM) for 30 min and then stimulated with TNF-α (A) or EGF (B) for another 15 min. Subcellular localization of EGFR-GFP was examined by confocal fluorescent microscopy. (C) Cells were pretreated with SB203580 (10 μM) for 30 min and then stimulated with 20 ng/ml TNF-α or 10 ng/ml EGF for 15 min. Cell surface expression of EGFR was investigated by FACS analysis. Cont, control. (D) HeLa cells were transfected with siRNAs against CHC and Luc. At 72 h posttransfection, cells were stimulated with TNF-α for 10 min. Whole-cell lysates were immunoblotted with the indicated antibodies. (E and F) HeLa cells were pretreated with U0126 (5 μM) (E) or SB203580 (10 μM) (F) for 30 min and then stimulated with TNF-α for another 15 and 60 min. Whole-cell lysates were immunoblotted with the indicated antibodies. DMSO, dimethyl sulfoxide.

residues by the formation of an asymmetric dimer, with one kinase domain in the EGF-mediated dimer activating the other through an allosteric mechanism (52). Several adaptor proteins, including Grb2 and Cbl, bind to phosphorylated tyrosines and evoke intracellular signals, including MAPK activation. EGFR has recently been shown to be phosphorylated under cellular stress conditions, including TNF-α, high levels of osmotic stress, anisomycin, UV, and cisplatin through p38. In the present study, we identified two independent signaling pathways from the TNF-α receptor to EGFR through ERK and p38 MAPK pathways, which caused the phosphorylation of EGFR at Thr-669 and Ser-1046/1047, respectively (Fig. 8). Adachi et al. recently reported that anisomycin induces Ser-1046/1047 in colon cancer cells (1). These findings emphasize the functional importance of these residues; however, an effort to search for other unidentified residues is necessary to completely elucidate the role of TNF-α-induced modification of EGFR. Upon EGF stimulation, the serine and threonine were also phosphorylated through the same MAPKs; however, duration and frequency were considerably different. Ligand-induced phosphorylation of Thr-669 was sustained until 60 min, while it had largely disappeared at 60 min in TNF-α-treated cells, although comparable phosphorylation was induced by these treatments at 30 min. It is interesting that TNF-α, but not EGF, induced strong Ser-1046/1047 phosphorylation, although p38 was comparably activated by these stimuli. The marked structural difference of EGFR in cells treated with TNF-α or EGF is whether it is naked (monomer) or phosphorylated (dimer occupied with ligands). Multiple tyrosine residues, including Tyr-1045, the next residue of Ser-1046/1047, are primarily phosphorylated in the process of ligand-induced EGFR activation. Subsequently, MAPKs are activated through Grb2 binding to phosphorylated tyrosines, such as Tyr-1068 and Tyr-1086, and then activated EGFR are targeted by MAPKs for Ser/Thr phosphorylation as a feedback control. This supports the hypothesis that earlier Tyr-1045 phosphorylation causes a conformational change around Ser-1046/1047 that interferes with access of the kinase. In accordance with this hypothesis, it is possible that EGF-induced Ser-1046/1047 phosphorylation occurred only on inactive and monomeric EGFR remaining on the plasma membrane without Tyr-1045 phosphorylation. In contrast, no tyrosine phosphorylation site has been reported

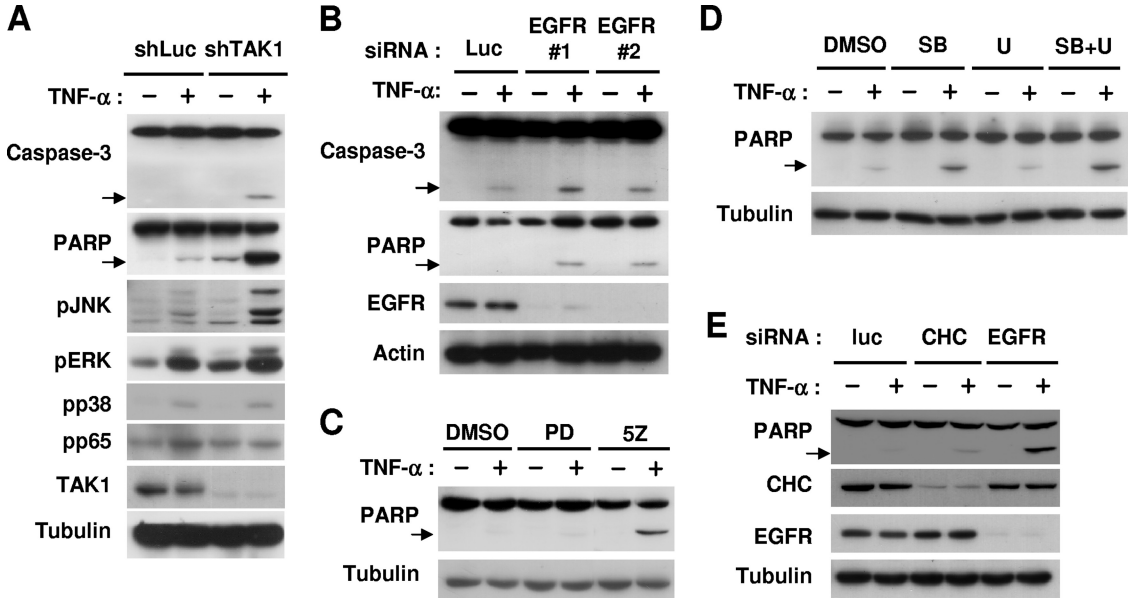

FIG. 6. EGFR is essential for protection from TNF-α-induced proapoptotic signals. (A) HeLa-shLuc or HeLa-shTAK1 cells were treated with TNF-α for 4 h. Whole-cell lysates were immunoblotted with phospho-JNK, JNK, PARP, TAK1, and tubulin antibodies. Arrows show cleaved forms of PARP. (B) HeLa cells were transfected with Luc siRNA or two different siRNAs against EGFR (#1 and #2). At 72 h posttransfection, cells were stimulated with TNF-α for 4 h. Whole-cell lysates were immunoblotted with caspase-3, PARP, EGFR, and actin antibodies. Arrows show cleaved forms of caspase-3 and PARP. (C and D) HeLa cells were pretreated with PD153035 (PD) (1 μM), 5Z-7-oxozeaenol (5Z) (0.3 μM), SB203580 (SB) (10 μM), or U0126 (U) (5 μM) for 30 min and then stimulated with TNF-α for another 4 h. Whole-cell lysates were immunoblotted with the indicated antibodies. DMSO, dimethyl sulfoxide. (E) HeLa cells were transfected with siRNAs against CHC, EGFR (#1), and Luc. At 72 h posttransfection, cells were stimulated with TNF-α for 4 h. Whole-cell lysates were immunoblotted with the indicated antibodies.

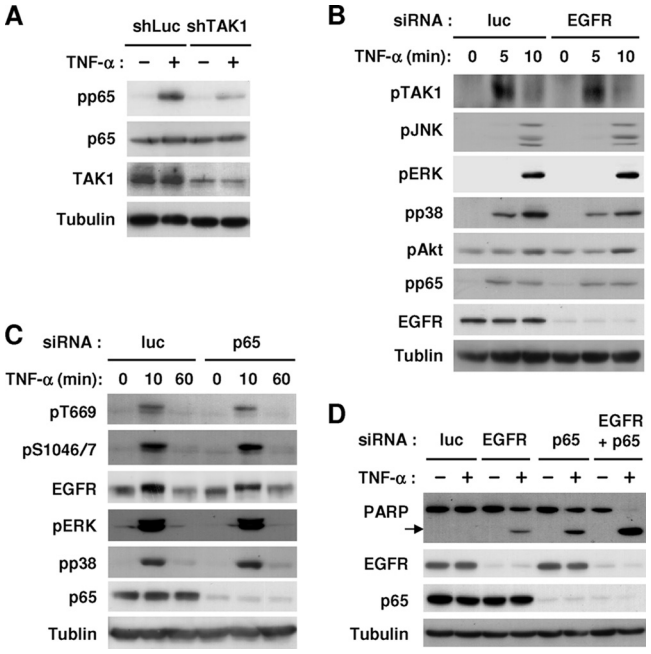

FIG. 7. NF-κB is not involved in EGFR phosphorylation. (A) HeLa-shLuc or HeLa-shTAK1 cells were treated with TNF-α for 5 min. Whole-cell lysates were immunoblotted with phospho-p65, p65, PARP, TAK1, and tubulin antibodies. (B to D) HeLa cells were transfected with siRNAs against p65, EGFR (#1), and Luc. At 72 h posttransfection, cells were stimulated with TNF-α for the indicated time periods (B and C) or 4 h (D). Whole-cell lysates were immunoblotted with the indicated antibodies.

around Thr-669, which may allow later phosphorylation by ERK at this site even in activated EGFR. It has been reported that MEK and ERK are recruited to endosomes to evoke sustained signals from intracellular compartments, suggesting that ligand-induced Thr-669 phosphorylation occurred after internalization. Similarly, TNF-α-induced Thr-669 phosphorylation was delayed by Ser-1046/1047 phosphorylation and inhibited by disruption of clathrin-coated pits. These findings raise the possibility that the different mechanisms of TNF-α- and ligand-induced Ser/Thr phosphorylation of EGFR are reflected by conformational changes, subcellular localization, and signaling complex formation, which influence the access of the corresponding kinases.

Another feature of TNF-α-induced Ser/Thr phosphorylation is that it is rapidly dephosphorylated within 60 min due to the rapid turnover of ERK and p38 activation. In contrast, osmotic stress caused sustained MAPK activation, leading to continuous EGFR phosphorylation. We have previously demonstrated that the rapid turnover of Ser/Thr phosphorylation is associated with EGFR recycling to the cell surface, as discussed below; therefore, identification of Ser/Thr phosphatases is essential to understand the function and postendocytic trafficking of the EGFR.

The molecular mechanisms underlying ligand-mediated intracellular trafficking of EGFR have been extensively studied. Ligand binding induces multiple autophosphorylation and ubiquitination of EGFR. The modified EGFR dimer is rapidly internalized with ligands via clathrin-coated pits and subsequently sorted in early endosomes. In the present study, we found that TNF-α induces EGFR endocytosis through the

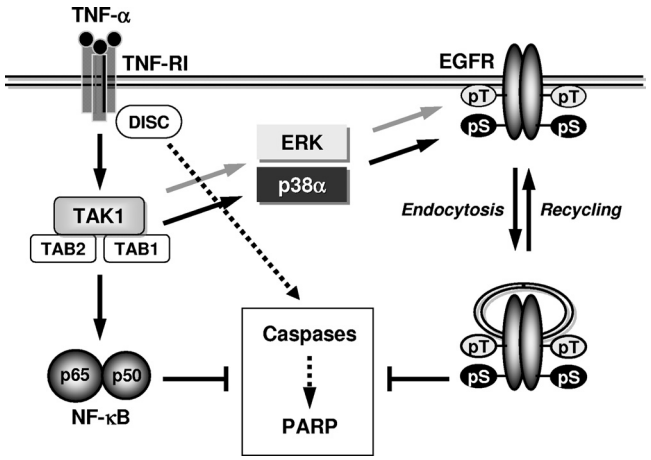

FIG. 8. Schematic diagram of TNF-α-induced antiapoptotic signals. TNF-α binding to TNF-R1 rapidly induces the activation of TAK1. TAK1 induces two independent signaling pathways to EGFR through ERK and p38. TAK1 regulates two independent antiapoptotic pathways, NF-κB and p38-EGFR. These signals coordinately prevent death-inducing signaling complex (DISC)-mediated proapoptotic cleavage of caspase and PARP.

TAK1-p38 pathway in a Ser-1046/1047 phosphorylation-dependent manner, in which ERK-mediated Thr-669 phosphorylation and TK activity of EGFR were dispensable. In contrast, EGF slightly induced p38-mediated phosphorylation of Ser-1046/1047, while it was also involved in the endocytosis of EGFR. Since ligand-induced p38 activation is dependent on EGFR TK activation, p38-mediated endocytosis is also dependent on its TK activity. Based on these observations, we proposed the following two different mechanisms of the ligand-mediated endocytosis: the major route in a primary TK-dependent fashion and a secondary p38-mediated feedback mechanism on inactive EGFR proteins which remains on the cell surface as a monomer. The secondary mechanism is similar to the stress-induced phosphorylation and endocytosis of EGFR, raising the possibility that TNF-α amplifies a ligand-induced minor reaction to overcome cellular stress conditions.

In ligand-induced internalization, a large part of EGFR is transported into multivesicular bodies and late endosomes and finally degraded in lysosomes; however, a small number of receptors are incorporated into recycling endosomes. We have shown that internalized EGFR mediated by TNF-α-induced p38 activation is efficiently recycled to the plasma membrane after dephosphorylation, which is similar to the proposed secondary mechanism of ligand-mediated endocytosis; therefore, it is possible that internalized EGFR with no phosphorylated tyrosine via the secondary Ser-1046/1047-dependent mechanism escapes from degradation. This possibility is supported by the finding that phosphorylated Tyr-1045 is a Cbl-binding site for ubiquitination that regulates subsequent degradation in lysosomes.

It should be emphasized that preservation of EGF-receptor association in endosomes is essential for sorting EGFR to the degradation pathway. Some ligands of EGFR other than EGF, including TGF-α, have weaker affinities to the receptor and dissociate from the receptor in the acidic environment of endosomes. For instance, TGF-α is released from the receptor in early endosomes, leading to receptor dephosphorylation and recycling back to the plasma membrane (8); therefore, TGF-α does not cause significant degradation of EGFR. Similarly, it has been shown that EGFR is colocalized with Rab5 and EEA1, early endosomal markers, and internalized EGFR is not occupied by the ligand under stress. These observations point out the significance of comparative analyses of Ser/Thr/Tyr phosphorylation, endocytosis, dephosphorylation, and postendocytic trafficking by some ligands and TNF-α to understand a variety of physiological functions of the EGFR.

Thr-669 is a unique site commonly and efficiently phosphorylated by both ligand and TNF-α stimulation; however, rapid turnover of TNF-α-induced phosphorylation was well contrasted by the sustained phosphorylation by EGF. This shows a good correlation with the duration of ERK activation (Fig. 3A). Figure 5 shows that phosphorylation of this residue was dispensable for ligand- and TNF-α-induced endocytosis. In addition, the phosphorylation status at Thr-669 did not affect the dephosphorylation of Ser-1046/1047 and recycling to the cell surface upon stimulation of TNF-α. Li et al. recently demonstrated that Thr-669 negatively regulates EGF-induced EGFR kinase activity by promoting EGFR degradation in overexpression of the mutated EGFR (17); however, TNF-α-induced Ser/Thr phosphorylation does not direct EGFR toward degradation; rather, these sites are rapidly dephosphorylated during recycling. Moreover, Thr-669 phosphorylation is not required for TNF-α-induced antiapoptotic cellular responses. The role of stress-induced Thr-669 phosphorylation is still unknown; therefore, study of this site will shed light on the common physiological functions of the ERK pathway in EGFR regulation.

TNF receptor 1 (TNF-R1) is a death receptor that transduces both death and survival signals, but the molecular mechanisms via which TNF-R1 mediates these signals are not yet fully understood. NF-κB is a ubiquitously expressed transcription factor that plays a pivotal role in antiapoptotic functions in TNF-R1 signaling pathways. TAK1 is well known as an NF-κB-regulating kinase, and TAK1-deficient mouse embryonic fibroblasts, keratinocytes, and intestinal epithelial cells are sensitive to TNF-α-induced apoptosis (12, 32). In addition, it has been reported that RNAi-mediated knockdown of TAK1 in cancer cells, including HeLa and A549 cells, results in enhanced apoptotic cell death upon stimulation with TNF-α or TRAIL (6). In the present study, we identified the EGFR pathway via TAK1 as a novel survival signal from TNF-R1 in a ligand- and TK-independent manner. This signal is independent of the TAK1-NF-κB pathway. These findings suggest that TAK1 controls two independent survival pathways to protect apoptosis in a stress condition, especially in EGFR-overexpressing epithelial cancer cells. It has recently been demonstrated that the expression of EGFR, a receptor TK associated with cell proliferation and survival, is overactive in many tumors of epithelial origin, and anti-EGFR agents, including neutralizing antibodies and TK inhibitors, have been used for cancer therapy. Investigations into functional interactions between TNF-R1 and the EGFR signaling pathway have recently been extensively studied. These experiments showed that cancer cell resistance to the cytotoxic effects of TNF-α could be induced by EGFR ligands EGF and TGF-α in a TK-dependent manner (50). On the other hand, TK activity was not necessary for the

phosphorylation of EGFR at Ser-1046/1047 to prevent TNF-α-induced proapoptotic signals. A recent report by Weihua et al. adds a new wrinkle to the role of EGFR in cancer: it demonstrated that kinase-inactive EGFR facilitates glucose transport into cells by associating with and stabilizing a sodium/glucose cotransporter (SGLT1) (48). We observed that the sensitivity of EGFR-knocked down cells to TNF-α-induced cell death was significantly enhanced under low-glucose culture conditions (data not shown), raising the possibility that the TAK1-EGFR pathway regulated the nutritional environment. Furthermore, we are also interested in another unknown function of the Ser/Thr phosphorylation of EGFR in the IL-1 signaling pathway, since IL-1 does not control apoptosis as TNF-α. Identification of EGFR-targeted genes in TNF-α and IL-1 signaling pathways will help our understanding of the TK-independent function of the EGFR.

It has been well known that TNF-α and EGF colocalize in tumor microenvironments and inflamed tissues. We have reported that TNF-α suppresses extracellular EGF responses through internalization of the EGFR (40). Similarly, Fig. 2 demonstrated that overexpression of TAK1 inhibited phosphorylation of multiple tyrosine residues on EGFR. We recently reported the pathway of intracellular signaling in the opposite direction. EGFR activation interferes with TNF-α-induced TAK1 activation via p38-mediated phosphorylation of TAB1 (37). Collectively, these results indicate that the TNF-α and EGFR signaling pathways interfere with each other. The cross-interference suggests that a balance between TNF-α and EGF stimuli determines which signal is dominant in cells that face both EGFR and TNFR activation at the same time.

In summary, we identified novel signaling pathways to EGFR via TAK1 and MAPKs. Study of the molecular mechanisms of the novel survival function of EGFR will be an attractive subject to understand functional interactions between cellular stress factors and growth/survival factors in cancer cells. It is also interesting whether cytokine-induced phosphorylation of EGFR occurs in lung adenocarcinoma cells with activating mutations in the TK domain. These analyses will contribute to the establishment of a more effective therapeutic strategy with anti-EGFR agents.

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