Direct Activation of TACE-Mediated Ectodomain Shedding by p38 MAP Kinase Regulates EGF Receptor-Dependent Cell Proliferation

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SUMMARY

Inflammatory stimuli activate ectodomain shedding of TNF-α, L-selectin, and other transmembrane proteins. We show that p38 MAP kinase, which is activated in response to inflammatory or stress signals, directly activates TACE, a membrane-associated metalloprotease that is also known as ADAM17 and effects shedding in response to growth factors and Erk MAP kinase activation. p38 MAP kinase interacts with the cytoplasmic domain of TACE and phosphorylates it on Thr357, which is required for TACE-mediated ectodomain shedding. Activation of TACE by p38 MAP kinase results in the release of TGF-α family ligands, which activate EGF receptor signaling, leading to enhanced cell proliferation. Conversely, depletion of p38 MAP kinase activity suppresses EGF receptor signaling and downstream Erk MAP kinase signaling, as well as autocrine EGF receptor-dependent proliferation. Autocrine EGF receptor activation through TACE-mediated ectodomain shedding intimately links inflammation and cancer progression and may play a role in stress and conditions that relate to p38 MAP kinase activation.

INTRODUCTION

Ectodomain shedding results in the release of extracellular domains of transmembrane proteins, including cytokines, growth factors, and cell surface receptors. This process is mediated by membrane-anchored metalloproteases and often regulates the activities of the substrate proteins. Thus, shedding results in the release of soluble cytokines and growth factors that activate receptors on cells at a distance, regulating cell proliferation and migration. (Blobel, 2005; Seals and Courtneidge, 2003). Ectodomain shedding plays roles in inflammation by controlling the cleavage of inflammatory regulators, such as TNF-α and the adhesion protein L-selectin (Seals and Courtneidge, 2003). Shedding of L-selectin disrupts leukocyte binding to endothelial cells, preventing their recruitment at sites of inflammation, and systemic release of TNF-α greatly contributes to cachexia (Beutler et al., 1986).

Inflammation has also been implicated in cancer. Chronic inflammation due to infection or irritation enhances initiation of tumor development (Mantovani et al., 2008). Conversely, cell-intrinsic changes that contribute to carcinogenesis are often accompanied by inflammatory signaling or production of inflammatory mediators that contribute to tumor promotion and progression (Cousens and Werb, 2002; Greten et al., 2004; Mantovani et al., 2008; Pikarsky et al., 2004). Inflammatory cytokines, pathogenic stimuli, and cell stress activate signaling leading to p38 MAP kinase (MAPK) through MKK3, MKK4, or MKK6, which, in turn, are activated by MAPK kinase kinases (Ono and Han, 2000). p38 MAPK contributes to inflammation, e.g., by activating cytokine expression and modulating their signaling (Dong et al., 2002). Among the four p38 MAPK isoforms, p38α is best characterized and crucial for inflammatory cytokine production. The p38 MAPK pathway can contribute to cancer progression through effects on cell proliferation (Dolado et al., 2007; Ono and Han, 2000; Ventura et al., 2007) and its roles in inflammation, e.g., in inducing VEGF and metalloprotease expression (Cousens and Werb, 2002; Mantovani et al., 2008). Further, inflammatory mediators that induce p38 MAPK activation can stimulate cell proliferation and contribute to malignant progression (Moore et al., 1999; Vidal-Vanaclocha et al., 2000). Yet, the p38 MAPK pathway can also exert a tumor suppressor effect by modulating cell-cycle regulation, senescence, and DNA damage responses (Han and Sun, 2007) and triggering apoptosis (Dolado et al., 2007).

In carcinomas, EGF receptor (EGFR) activation aids the progression by promoting cell proliferation and cell survival (Yarden and Sliwkowski, 2001), and interference with EGFR activation is the basis for therapies (Zhang et al., 2007). Autocrine EGFR signaling is enhanced by increased receptor and/or TGF-α family ligand expression, as observed in cancers of neuroectodermal origin (Hynes and Lane, 2005). Accordingly, increased release of soluble ligands, such as TGF-α and amphiregulin, through ectodomain shedding (Blobel, 2005; Sahin et al., 2004) enhances the EGFR activity and cancer progression (Borrell-Pagès et al., 2003; Kenny and Bissell, 2007; Zhou et al., 2006).

Among the metalloproteases, TACE, also known as ADAM17, mediates ectodomain shedding of inflammatory cytokines and TGF-α family ligands (Seals and Courtneidge, 2003). TACE expression is often enhanced in inflammation (Patel et al., 1998) and in carcinomas, especially in breast cancers (Mochizuki and Okada, 2007). The release of TGF-α proteins by TACE stimulates cell proliferation and cancer progression (Borrell-Pagès...
Ectodomain Shedding

Extracellular Cues that Activate p38 MAP Kinase Induce osmotic shock or ionomycin. Transmembrane TGF-α at the cell surface

soluble TGF-α lysates was visualized by western blotting (Figure 1B), and (Figure 1A). This scenario of TGF-α membrane TGF-

tion of ectodomain shedding based on the decrease of trans-

membrane TGF-α shedding, the responsible metalloproteases that are activated

by this pathway have remained unclear. In fact, it has been proposed that stimuli that activate p38 MAPK do not act through TACE (Diaz-Rodriguez et al., 2002; Hinkle et al., 2003; Takenobu et al., 2003). Using transmembrane TGF-α as model, we show that p38 MAPK directly activates TACE through targeted phosphorylation of its cytoplasmic domain. Activation of TACE by p38 MAPK in breast carcinoma cells leads to a release of TGF-α ligands, resulting in increased EGF receptor activation and proliferation. Our results establish a mechanism for the regulation of EGF receptor and Erk MAPK signaling, as well as cancer cell proliferation, by the p38 MAPK pathway.

RESULTS

Transmembrane TGF-α as Reporter of Ectodomain Shedding

We compared the ectodomain shedding responses to extracellular inducers of Erk or p38 MAPK activation using C2 cells, a CHO cell derivative that expresses TGF-α. TGF-α undergoes N-glycosylation and cleavage of the prodomain, giving rise to three transmembrane forms: (1) an ~22 kD form I that corresponds to the precursor with its N-terminal unglycosylated prodomain and is primarily located in the endoplasmic reticulum, (2) an ~28 kD form II with N-glycosylation of the prodomain, which occurs primarily in the Golgi, and (3) an ~16 kD form III with the prodomain proteolytically removed (Bringman et al., 1987). Ectodomain shedding releases soluble TGF-α and thereby generates a truncated transmembrane protein that is not recognized by anti-TGF-α antibodies. Cell surface protein biotinylation revealed that transmembrane TGF-α forms II and III are at the cell surface (Figure 1A). This scenario of TGF-α maturation allows quantification of ectodomain shedding based on the decrease of transmembrane TGF-α levels at the cell surface or release of soluble TGF-α in the medium.

Extracellular Cues that Activate p38 MAP Kinase Induce Ectodomain Shedding

C2 cells were treated with PMA, serum, anisomycin, UV light, osmotic shock or ionomycin. Transmembrane TGF-α in cell lysates was visualized by western blotting (Figure 1B), and soluble TGF-α was measured in the media by ELISA (Figure 1C). All treatments resulted in a decrease of the TGF-α forms II and III at the cell surface and release of soluble TGF-α, indicating ectodomain shedding. We correlated these results with activation of Erk or p38 MAPK signaling. PMA and serum activated Erk MAPK, and anisomycin, UV light, and osmotic shock activated p38 MAPK with only a minimal effect on Erk MAPK (Figure 1B). These data suggested that p38 MAPK activation, like Erk MAPK signaling, induces TGF-α shedding in C2 cells.

To study the roles of the Erk and p38 MAPK pathways in the induction of ectodomain shedding, we treated C2 cells with the MEK inhibitor U0126, preventing activation of Erk MAPKs, or with the p38 MAPK inhibitor SB203580. Because anisomycin, UV light, and osmotic shock also activate JNK MAPK, we also tested the effect of a JNK MAPK pathway inhibitor. Further, because PMA activates protein kinase C, we examined the effect of the protein kinase C inhibitor BIM. Figure 1D shows that TGF-α shedding in response to anisomycin or UV light was unaffected by inhibitors of the Erk or JNK MAPK pathways or protein kinase C. In contrast, inhibition of p38 MAPK blocked shedding and increased the cell surface levels of TGF-α forms III and II. PMA-induced shedding was blocked by U0126, as reported (Fan and Derynck, 2000; Gechtman et al., 1999), but not by the p38 MAPK inhibitor. These results indicate that p38 MAPK activates ectodomain shedding induced by anisomycin or UV light and that Erk MAPK or protein kinase C signaling is not involved.

We evaluated whether ectodomain shedding responded to the same signals in HeLa cells ectopically expressing TGF-α (Figures S1A and S1B available online) and T4-2 breast carcinoma cells (Kenny and Bissell, 2007) (Figure 1E). PMA, anisomycin, and UV light induced TGF-α shedding in HeLa cells. We could not detect the low endogenous levels of transmembrane TGF-α in T4-2 cell lysates, but ELISA revealed the release of TGF-α in the medium in response to PMA or anisomycin. As in C2 cells, PMA-induced shedding in HeLa or T4-2 cells was inhibited by U0126, and anisomycin-induced shedding was inhibited by SB203580 (Figures 1E, S1A, and S1B). The cytokine interleukin (IL)-1β, which activates p38 MAPK signaling (Ono and Han, 2000) and is frequently expressed in human breast cancers (Nicolini et al., 2006), also induced TGF-α shedding in T4-2 cells, albeit at a lower level than anisomycin, and this induction was similarly inhibited by SB203580 (Figure 1E). These data using C2, HeLa, and T4-2 cells indicate that p38 MAPK activation results in ectodomain shedding.

p38α MAP Kinase Activates Anisomycin- and UV Light-Induced Ectodomain Shedding

All four p38 MAPKs—p38α, p38β, p38γ, and p38δ—are activated by MKK3 or MKK6 (Ono and Han, 2000). Two of these, p38α and p38β, are inhibited by SB203580. Activation of p38 MAPK and TGF-α ectodomain shedding in response to anisomycin or UV light were inhibited by SB203580 with similar sensitivities (Figure 2A). The nearly complete inhibition at 625 nM SB203580 was close to the reported IC50 of 600 nM SB203580 for p38 MAPK inhibition (Cuenda et al., 1995), suggesting that p38α or p38β MAPK mediates anisomycin- and UV light-induced ectodomain shedding.

We isolated p38α MAPK cDNAs from CHO cell mRNA and designed siRNA based on this sequence. Transfection of the siRNA downregulated p38α MAPK expression in C2 cells and inhibited p38 MAPK activation in response to anisomycin or UV light, as apparent from the minimal p38 MAPK phosphorylation (Figure 2B). Downregulation of p38α MAPK expression also prevented the induction of ectodomain shedding and release of TGF-α into the medium in response to anisomycin or UV light,
Figure 1. Activation of the p38 MAP Kinase Pathway Induces Ectodomain Shedding of TGF-α

(A) Cx cells express transmembrane TGF-α forms I, II, and III, as shown by immunoblotting of cell lysates. Forms II and III are visualized by cell surface protein biotinylation.

(B and C) Cx cells were treated as indicated, and ectodomain shedding of TGF-α was examined by immunoblotting of cell lysates for transmembrane TGF-α (B, top) or quantifying TGF-α released into the medium by ELISA (C). In (B), the middle and bottom panels show activation of Erk (anti-pErk) or p38 MAPK (anti-pp38) versus total Erk or p38 MAPK. *p < 0.01, compared with control.

(D) Effects of pathway inhibitors on TGF-α shedding. Cx cells were treated with PMA, anisomycin, or UV light without or with the indicated inhibitor. Immunoblotting of cell lysates shows transmembrane TGF-α and activated Erk (anti-pErk) or p38 MAPK (anti-pp38) versus total Erk or p38 MAPK.

(E) Ectodomain shedding of TGF-α in T4-2 cells treated with PMA, anisomycin, or IL-1β without or with U0126 or SB203580. TGF-α released into the medium was quantified by ELISA. *p < 0.001, compared with control. **p < 0.01, compared with corresponding PMA, anisomycin, or IL-1β stimulation. Error bars show the standard deviations based on triplicate values of each data set.
Figure 2. p38α MAP Kinase Mediates Anisomycin- and UV Light-Induced Ectodomain Shedding
(A) Ca cells were treated with anisomycin, UV light, or PMA and increasing doses of SB203580, and shedding was visualized by immunoblotting for transmembrane TGF-α. The bottom panels show p38 MAPK activation using antiphospho-p38 MAPK immunoblotting.
(B and C) Ca cells were transfected with p38α MAPK siRNA or control siRNA. Ectodomain shedding of TGF-α was induced by PMA, anisomycin, or UV light and evaluated by immunoblotting of cell lysates for transmembrane TGF-α (B) or ELISA of TGF-α released in the medium (C). Immunoblotting for p38 MAPK or phospho-p38 MAPK revealed the p38α MAPK activation. *p < 0.01, compared with control. **p < 0.01, compared with corresponding control siRNA transfection.
(D) Ca cells were treated with PMA, anisomycin, or UV light without or with cycloheximide or brefeldin A. Shedding was assessed by immunoblotting of cell lysates for transmembrane TGF-α.
assessed in Cx (Figures 2B and 2C) and HeLa cells (Figure S2A). Downregulation of p38α MAPK expression did not affect shedding in response to PMA, as apparent by western blotting of transmembrane TGF-α (Figure 2B) and ELISA of TGF-α in the medium (Figures 2C and S2A). These results indicate that p38α MAPK activates anisomycin- or UV light-induced ectodomain shedding.

p38 MAP Kinase-Activated Shedding Does Not Require Protein Synthesis or Reactive Oxygen Species

Cycloheximide did not inhibit PMA-induced shedding but slightly inhibited ectodomain shedding in response to anisomycin or UV light (Figure 2D, lanes 5–8). Brefeldin A, which disrupts transport through the Golgi apparatus, elevated the level of immature TGF-α form I, consistent with the disruption of protein transport through the Golgi, which occurs prior to ectodomain shedding of cell surface proteins. However, it did not affect shedding of the cell surface TGF-α form III in response to PMA and slightly affected shedding induced by anisomycin or UV light (Figure 2D, lanes 8–12). These data indicate that new protein synthesis is not required for activation of ectodomain shedding by the Erk or p38 MAPK pathways.

Reactive oxygen species (ROS) and nitrogen oxide (NO) have been proposed to play a role in activating TACE by inducing dissociation of the inhibitory prodomain from active TACE (Zhang et al., 2001) or activating p38 MAPK signaling (Dolado et al., 2007; Fischer et al., 2004). However, the antioxidant MNTmPyP did not inhibit anisomycin- or PMA-induced ectodomain shedding of TGF-α, nor did the NO scavenger PTIO and iNOS inhibitor L-NIL (Figure 2E). Our data suggest that ROS and NO do not mediate p38 MAPK- or Erk MAPK-activated ectodomain shedding.

TACE Mediates TGF-α Release in Response to Activation of p38 MAP Kinase

To address the role of TACE, we transfected siRNAs, based on the Chinese hamster sequence of the catalytic domain of TACE, in Cx cells. Silencing TACE expression blocked shedding of transmembrane TGF-α (Figure 3A) and decreased the release of soluble TGF-α (Figure 3B) in response to anisomycin or UV light, similarly to its effect on PMA-induced shedding. A similar inhibition of TGF-α release was seen in HeLa cells expressing tagged TACE (Figure S2B) or T4-2 cells expressing endogenous TGF-α (Figure 3C), using siRNA targeting the human TACE sequence.

CHO-derived M2 cells lack induction of ectodomain shedding in response to PMA or serum due to an inactivating Cys600 mutation in TACE (Li and Fan, 2004). Anisomycin and UV light were, similarly to PMA or serum, unable to induce shedding. However, introduction of TACE conferred TGF-α shedding in response to PMA, as well as anisomycin or UV light, and this rescue was not seen with the catalytically inactive Glu406Ala TACE (Figure 3D). These results indicate that TACE mediates shedding in response to p38 MAPK activation.

We also incubated T4-2 cells with anisomycin, IL-1β, or lipopolysaccharide (LPS)—which is also known to activate p38 MAPK (Ono and Han, 2000)—immunoprecipitated TACE, and assessed its activity in vitro by cleavage of a substrate peptide spanning a known TACE cleavage site. As shown in Figure 3E, anisomycin, LPS, and IL-1β increased the activity of TACE, and those increases were abolished by incubating the cells with p38 MAPK inhibitor SB203580 prior to isolation of TACE. We conclude that p38 MAPK activation results in increased enzymatic activity of TACE. In these assays, all TACE was immunoprecipitated, and not only the TACE at the cell surface that accounts for only a small fraction of TACE in the cell lysate and is likely the population that is activated by p38 MAP kinase.

TACE Is Directly Phosphorylated by p38α MAP Kinase

Because activation of p38α MAPK resulted in ectodomain shedding by TACE, we explored whether p38 MAPK interacts with TACE. Using Cx cells expressing tagged TACE and p38α MAPK, we showed that p38α MAPK associated with TACE. In contrast, the kinase-inactive AF mutant of p38α MAPK did not associate. Also, p38β MAP kinase, but not its kinase-inactive mutant, associated with TACE (Figure 4A).

Using purified tagged proteins translated in vitro, we phosphorylated wild-type and kinase-inactive p38α MAPK with MKK6. These were then immunopurified and incubated with γ32P-ATP and purified tagged TACE, which, due to lack of glycosylation, was smaller than TACE made in mammalian cells. TACE was phosphorylated by activated wild-type p38α MAPK, but not its inactive mutant (Figure 4B). Similar results were obtained using proteins that were expressed in Cx cells. Thus, p38α MAPK or its kinase-inactive mutant were coexpressed with activated MKK6, and immunopurified p38α MAPK was incubated with tagged TACE that was also immunopurified from transfected cells. Figure 4C shows phosphorylation of TACE by p38α MAPK, which was inhibited by SB203580. These data illustrate that TACE is a kinase substrate for p38α MAP kinase.

We evaluated whether p38 MAPK phosphorylates TACE in response to anisomycin using in vitro kinase assays with tagged p38α or p38β MAPK and TACE that were coexpressed in Cx cells and then purified. Anisomycin induced TACE phosphorylation to a level similar to coexpression of activated MKK6 (Figure 4D). Kinase-inactive p38α MAPK did not phosphorylate coexpressed TACE, and p38β MAPK only weakly phosphorylated TACE when compared with p38α MAP kinase, either in response to anisomycin or when coexpressed with activated MKK6 (Figure 4D). Thus, even though p38α and p38β MAPK associated similarly with TACE in vitro (Figure 4A), TACE was the preferred substrate of p38α MAP kinase.

In Cx cells, TACE was phosphorylated in vivo in response to anisomycin by endogenous p38α MAPK, as apparent by the inhibition by SB203580 (Figure 4E). We also examined the phosphorylation of endogenous TACE in T4-2 cells. Because p38 MAPK is a proline-directed Ser/Thr kinase, we used anti-phosphoThr-Pro to detect phospho-TACE by immunoblotting.

(E) Ectodomain shedding was induced with PMA or anisomycin without or with MNTmPyP, PTIO, or L-NIL and was evaluated by immunoblotting of cell lysates for TGF-α.

Error bars show the standard deviations based on triplicate values of each data set.
Figure 3. TACE Effects TGF-α Release in Response to p38 MAPK Activation

(A and B) Cα cells were transfected with TACE siRNA or control siRNA and were stimulated with PMA, anisomycin, or UV light, and shedding of TGF-α was evaluated by immunoblotting cell lysates for transmembrane TGF-α (A) or by ELISA of TGF-α in the medium (B). Immunoblotting cell lysates with anti-TACE or anti-phospho-p38 MAPK antibodies showed silencing of TACE expression or activation of p38 MAPK in response to anisomycin or UV light. *p < 0.05, compared with corresponding transfection with control siRNA.

(C) T4-2 cells, transfected with TACE siRNA or control siRNA, were treated with PMA, anisomycin, or UV light, and TGF-α released in the medium was quantified by ELISA. Silencing of TACE expression was apparent by immunoblotting of cell lysates for TACE (bottom). *p < 0.05, compared with corresponding transfection with control siRNA.

(D) M2 cells lacking active TACE were transfected to express TGF-α and TACE or inactive E406A TACE mutant. Shedding of TGF-α was visualized by immunoblotting of cell lysates for transmembrane TGF-α. The bottom panel shows p38 MAPK activation, assessed by immunoblotting for phospho-p38 MAPK. *p < 0.05, compared with DMSO control.

(E) In vitro proteolytic activity of TACE immunoprecipitated from T4-2 cells that were treated with anisomycin, LPS, or IL-1β in the absence or presence of SB203580. *p < 0.05, compared with DMSO control.

Error bars show the standard deviations based on triplicate values of each data set.
TACE Mediates p38 MAPK-Induced Cell Proliferation

(Figure 4F), SB203580 inhibited the low basal level of phosphorylation, and anisomycin and IL-1β enhanced the immunoreactivity of TACE. The lower level of immunoreactivity of TACE upon IL-1β stimulation, when compared to anisomycin, is consistent with the lower level of p38 MAPK activation (Figure 4G) and shedding (Figure 1E). The enhanced phosphorylation of endogenous TACE upon stimulation was abolished by SB203580 (Figure S3) or when cells were transfected with siRNA to p38α MAPK (Figure 4F). Finally, the ability of p38α MAPK to associate with TACE and increased phosphorylation of TACE in response to anisomycin or IL-1β suggest that p38 MAPK interacts with TACE upon stimulation. Even though kinase-substrate interactions are notoriously difficult to detect by communoprecipitation, we detected a low-level interaction of endogenous p38 MAPK and TACE in T4-2 cells in response to anisomycin or IL-1β; again, the effect of anisomycin was stronger than that of IL-1β. These data show that TACE is a substrate for p38α MAPK kinase and that p38α MAPK phosphorylates TACE on Thr in response to anisomycin and IL-1β.

TACE Phosphorylation at Thr735 Is Required for Activation of Shedding by p38 MAP Kinase

A previous study reported that Erk MAPK phosphorylates Thr735 in the TACE cytoplasmic domain (Diaz-Rodriguez et al., 2002). In addition, Ser819 is phosphorylated and Ser791 is dephosphorylated in response to growth factor stimulation (Fan et al., 2003). To assess whether these sites play roles in TACE phosphorylation by p38 MAPK, we expressed wild-type TACE, TACE mutants with either site replaced by alanine, and two double mutants in transfected cells. Immunopurified wild-type and mutant TACE were subjected to in vitro phosphorylation by p38α MAPK that was activated through coexpression with activated MKK6 (Figures 5A and S4). The Thr735P labeling of TACE correlated with proTACE phosphorylation (Figure S3). Mutation of Thr735 strongly decreased the TACE phosphorylation by p38α MAPK and abolished the immunoreactivity, whereas mutation of Ser819 mildly decreased its phosphorylation. In contrast, the Ser791 mutation conferred increased phosphorylation. Because only Pro-Gln-Thr735-Pro in the TACE cytoplasmic domain has Thr in a Thr-Pro dimer and the Thr735Ala mutant is not phosphorylated by p38α MAPK kinase, we conclude that the phosphoPro-Thr antibody recognizes phosphoThr735 and that activation of p38 MAPK results in TACE phosphorylation at Thr735. This is consistent with Pro-Gln-Thr735-Pro as a predicted p38 MAPK target sequence (http://scansite.mit.edu). Accordingly, Thr735Ala TACE had a much lower level of phosphorylation than wild-type TACE, both in vitro using purified proteins (Figure 4B) and in Cx cells in response to anisomycin (Figure 4E).

To examine the role of TACE phosphorylation in response to p38 MAPK activation, we coexpressed each TACE mutant individually with TGF-α in M2 cells that lack active TACE expression and measured ectodomain shedding by the amount of TGF-α released into the medium (Figure 5B). Without TACE coexpression, very little TGF-α was detected in the medium, and no increase was observed upon p38 MAPK activation. TACE expression enhanced the level of TGF-α in the medium, consistent with a basal shedding that is apparent when TACE is overexpressed. The TGF-α release increased in response to anisomycin and decreased when the cells were treated with SB203580 or expressed the dominant-negative AF mutant of p38α MAPK, suggesting a role of p38α MAPK in the basal shedding activity. The Thr735Ala mutant of TACE had a much lower level of basal shedding, similar to the level when p38 MAPK was inhibited, and its activity was only marginally enhanced in response to anisomycin. The Ser819Ala mutation conferred a lower overall activity when compared to wild-type TACE, whereas TACE Ser791Ala had an activity profile similar to that of wild-type TACE.

We also generated derivatives of Cx cells expressing wild-type or mutant TACE at levels comparable to endogenous TACE (Figure 5C). Because of the endogenous TACE activity, anisomycin and PMA stimulated shedding in cells expressing wild-type or Thr735Ala TACE, as apparent by the decrease in the TGF-α forms II and III (Figure 5C). Silencing endogenous TACE expression with siRNA that did not affect the transfected human TACE expression blocked TGF-α shedding (Figure 5C). In CxTACE cells that express wild-type human TACE, the endogenous TACE silencing did not affect the TGF-α shedding by human TACE in response to Erk or p38 MAPK activation. In contrast, the shedding in response to anisomycin was severely impaired in Cx cells expressing Thr735Ala TACE. This was most apparent from the minimal decrease in TGF-α form III, which was comparable to the level in Cx cells that had endogenous TACE expression silenced and did not express human TACE. The shedding in response to PMA was much less affected by the Thr735Ala mutation.

The induction of TGF-α shedding in these cells was confirmed by ELISA for TGF-α in the medium (Figure 5D). When silencing endogenous TACE expression, the strong increase of soluble TGF-α in response to anisomycin was only observed when wild-type human TACE was expressed, whereas its Thr735Ala mutant conferred a limited response to p38 MAPK activation. TGF-α shedding in response to Erk MAPK signaling in these cells was largely unaffected by the Thr735Ala mutation (Figure 5D).

Together, these data illustrate that Thr735 phosphorylation is required for TACE activation by p38 MAPK, but not by the Erk MAPK pathway.

p38 MAP Kinase Signaling Increases the Presentation of TACE at the Cell Surface

The molecular mechanism of TACE activation has largely remained elusive but has been suggested to affect the secretory transport (Sood et al., 2005). We therefore examined the effect of p38 MAPK activation on the cell surface levels of TACE in Cx cells (Figure 6A). As reported (Schlöndorff et al., 2000), TACE appeared as two forms at the cell surface: pro-TACE that has an N-terminal prodomain and corresponds to the bulk of TACE that resides inside cells and active TACE (mTACE) that has this prodomain removed (Figure 6A). Because cells in culture have basal p38 MAPK signaling, we compared cells in which p38 MAPK signaling was inhibited by SB203580 with cells treated with anisomycin to activate p38 MAPK. p38 MAPK activation resulted in an increased cell surface level of mTACE that was also seen in the presence of the TACE inhibitor TAPI-1 and therefore does not depend on its catalytic activity. The increase in cell surface
**Figure 4. p38 MAPK Kinase Interacts with and Phosphorylates TACE**

(A) Ca cells were transfected to express Myc-tagged TACE with Flag-tagged p38α or p38β MAPK or inactive p38α-AF or p38β-AF. Cell lysates were subjected to anti-Myc immunoprecipitation and anti-Flag immunoblotting. TACE and p38 MAPK expression were shown by immunoblotting of cell lysates.

(B) TACE, TACE (T735A) mutant, p38α MAPK, p38α-AF, and activated MKK6 (MKK6-E) were generated by in vitro transcription and translation. p38α MAPK was then activated through phosphorylation by MKK6-E. Phosphorylated p38α MAPK and TACE were purified using anti-Flag beads and were incubated with 32P-γ-ATP in a kinase assay. The bottom panels show immunoblotting of the proteins in the kinase assays. TACE is smaller than in vivo expressed TACE due to lack of glycosylation.

(C) In vitro kinase assays as in (B) were performed with purified proteins from transfected Ca cells with SB203580 added to inhibit p38α MAPK.
Molecular Cell
TACE Mediates p38 MAPK-Induced Cell Proliferation

presentation of mTACE in response to anisomycin correlated with ectodomain shedding of TGF-α, as apparent from the strong decrease in cell surface levels of TGF-α forms II and III (Figure 6A).

To examine whether TACE phosphorylation at Thr735 by p38 MAPK affects the cell surface presentation of TACE, we stably transfected Cx cells to express a C-terminally Flag-tagged form of human wild-type or T735A mutant TACE (Figure 6B). Anisomycin induced increased cell surface levels of wild-type mTACE when compared with cells in the presence of SB203580 (Figure 6B), consistent with what we observed for endogenous TACE (Figure 6A). However, anisomycin did not significantly affect the cell surface levels of T735A mTACE. Cells expressing T735A TACE also expressed two smaller Flag-tagged proteins at the cell surface (Figure 6B). Their size and reactivity with antibody to the C-terminal Flag epitope suggest that they derive through proteolytic removal of the catalytic and disintegrin domains. This process is likely to occur primarily at the cell surface because total TACE in the cell lysate showed only low levels of these fragments. We conclude that phosphorylation of Thr735 by p38 MAPK regulates the cell surface levels and processing of TACE.

p38 MAP Kinase Regulates Cell Proliferation by Shedding of EGF Receptor Ligands by TACE
TACE expression is often increased in carcinomas, especially breast carcinomas, and has been implicated in EGFR-mediated cancer progression by TGF-α family growth factors (Borrell-Pagès et al., 2003; Kenny and Bissell, 2007; Zhou et al., 2006). Because p38 MAPK activates TACE and induces the release of TGF-α, we studied the role of p38 MAPK in the proliferation of T4-2 breast carcinoma cells. Like many carcinomas, the proliferation and tumor formation of T4-2 cells depend, in part, on autocrine EGFR-dependent stimulation, resulting from ectodomain shedding of TGF-α and amphiregulin (Kenny and Bissell, 2007).

In cell culture, the proliferation of T4-2 cells was inhibited by TAPI-1, an inhibitor of metalloproteases including TACE, the EGFR inhibitor AG1478, or the MEK inhibitor PD98059, which blocks activation of the Erk MAPK pathway downstream from the EGFR (Figures 7A and 7B). These results indicate that T4-2 cell proliferation depends on TACE activity and EGFR signaling, as reported (Kenny and Bissell, 2007). In addition, the proliferation was inhibited by SB203580, but not its negative control SB202474, indicating an important role of p38 MAPK.

IL-1β enhanced the cell proliferation (Figures 7C and 7D), which was inhibited by silencing p38α MAPK or TACE expression. Further, neutralizing antibodies to TGF-α and amphiregulin or antibodies that prevent ligand binding to the EGFR inhibited the basal and IL-1-induced proliferation (Figures 7C and 7D). Like IL-1β, LPS also enhanced cell proliferation, which was inhibited by SB203580, as well as by TAPI-1 or the EGFR inhibitor (Figure S5). Long-term treatment of cultured cells with anisomycin was toxic, and its effect was therefore not examined in these assays. Together, these results indicate that the activation level of p38 MAPK, which is enhanced in response to inflammatory mediators, is a key determinant of the proliferation of T4-2 cells, mediated at least in part by its effect on TACE activity and acting through the EGFR.

We next studied whether p38 MAPK controls EGFR activation as a consequence of the release of soluble EGFR ligand by TACE. Although T4-2 cells express more amphiregulin than TGF-α (Kenny and Bissell, 2007), we elected to score the TGF-α release using an ELISA, considering that both ligands are released through cleavage by TACE (Sahin et al., 2004). Consistent with previous results (Figure 1E), IL-1β induced the release of TGF-α, albeit to a lower extent than anisomycin (Figure 7E). Silencing the expression of p38α MAPK or TACE repressed its release. LPS similarly induced TGF-α release, and the basal and LPS-induced TGF-α release were inhibited by SB203580 or TAPI-1 (Figure S5C).

We examined the autocrine EGFR activation under the same conditions of IL-1 treatment as in Figure 7E. The EGFR activation, assessed by its level of Tyr phosphorylation (Figure 7F), correlated with the secreted TGF-α levels (Figure 7E). IL-1β or anisomycin induced EGFR activation, which was prevented by silencing the p38α MAPK or TACE expression. As expected, neutralizing TGF-α and amphiregulin antibodies or EGFR antibodies prevented the autocrine EGFR activation (Figure 7F). Consistent with these results, LPS induced EGFR activation, and the p38 MAPK inhibitor SB203580 and TACE inhibitor TAPI-1 inhibited the basal and LPS-induced EGFR activation (Figures S5C and S5D). Whereas the EGFR kinase inhibitor AG1478 inhibited the autocrine EGFR activation rapidly, SB203580 and TAPI-1 required a longer time to do so (Figures S5C and S5D), as they act on the release of ligands that activate the EGF receptor rather than on the EGFR itself. These data suggest that the regulation of the ectodomain shedding by TACE through p38 MAPK signaling defines the EGFR activity. To further define the role of p38α MAPK in EGFR activation, we silenced the TACE or p38α MAPK expression (Figure 7G, left). As...
Silencing TACE or p38α MAPK expression decreased the basal EGFR activation, resulting in decreased Erk MAPK activation (Figure 7G, middle), a downstream effect of EGFR signaling that regulates proliferation. These results illustrate that TACE and p38α MAPK regulate the activation of Erk MAPK by EGFR signaling.
Molecular Cell
TACE Mediates p38 MAPK-Induced Cell Proliferation

We next compared the cell proliferation using BrdU uptake (Figure 7G, right). The decrease in TACE or p38α MAPK expression inhibited cell proliferation by 50% and 36%, respectively, consistent with the effects of TAPI-1 or SB203580 on cell proliferation (Figures 7B and S5E). The stronger inhibition by interfering with p38α MAPK activity, compared with TACE inactivation, may suggest that p38 MAPK signaling involves other aspects of cell proliferation besides the activation of the EGFR through TACE-mediated EGFR ligand shedding. The inhibition of cell proliferation by interfering with p38α MAPK or TACE activity was largely rescued by adding EGFR ligands (Figures 7G, right, and S5E).

Together, these results indicate that p38 MAPK activation, e.g., in response to inflammatory mediators or stress, critically regulates the proliferation of these cells. The induction of proliferation by p38 MAPK signaling results, at least in part, from the activation of TACE and the release through ectodomain shedding of EGFR ligands and increased EGFR signaling.

DISCUSSION

Activation of Shedding in Response to p38 MAP Kinase Signaling Is Mediated by TACE
We have shown that inducers of p38 MAPK signaling induce ectodomain shedding and that inhibition of p38 MAPK blocks shedding in response to these inducers. Our results complement reports that p38 MAPK inhibitors prevent shedding in different contexts (Fan and Derynck, 1999; Fischer et al., 2004; Takenobu et al., 2003). Thus, in addition to the Erk MAPK pathway, p38 MAPK activates ectodomain shedding. Among the p38 MAPKs, shedding was specifically activated by p38δ MAPK, which has been linked to the activities of inflammatory cytokines (Dong et al., 2002; Ono and Han, 2000). Thus, siRNA specific for p38δ MAPK prevented shedding in response to p38MAPK inducers, and no response by other p38 MAPKs was apparent.

TACE was shown to mediate shedding in response to Erk MAPK pathway activation. Which metalloprotease effects shedding in response to p38 MAPK activators has remained unclear, and it has been proposed that shedding in response to such inducers is mediated by other metallopeptidase(s) (Díaz-Rodríguez et al., 2002; Hinkle et al., 2003; Takenobu et al., 2003). Our use of siRNA to silence TACE expression, as well as rescue experiments using wild-type or mutant TACE, show that TACE effects shedding in response to p38 MAPK activation. Further, introduction of wild-type TACE in M2 cells that do not express active TACE (Li and Fan, 2004) rescued ectodomain shedding in response to Erk or p38 MAPK pathway activation. We conclude that TACE is activated by either of these pathways that are, in turn, activated by diverse stimuli.

Mechanism of TACE Activation by the p38 MAP Kinase Pathway
The activation of TACE-mediated ectodomain shedding by p38 MAPK was rapid and did not require new protein synthesis, suggesting that TACE activation results from a posttranslational event. Although ROS was proposed to play roles in activation of p38 MAPK or TACE, the antioxidant MNTmPyP did not affect shedding in response to anisomycin.

Wild-type, but not kinase-defective, p38α MAPK associated with and phosphorylated TACE in response to stimuli that activate p38 MAPK, such as anisomycin and IL-1β. The phosphorylation site, Thr735, is located in a predicted p38 MAPK target sequence, i.e., Pro-Gln-Thr735-Pro, and its mutation prevented TACE phosphorylation by p38 MAPK and activation of shedding in response to anisomycin or IL-1β. This finding directly links extracellular cues that activate the p38 MAPK pathway with ectodomain shedding. The targeting of a metalloprotease by p38 MAPK adds to our understanding of the role of p38 MAPK, which hitherto is known to primarily regulate transcription and translation (Ono and Han, 2000), and raises the possibility that p38 MAPK targets other ADAM metalloproteases.

The mechanism by which cell signaling activates TACE has remained elusive. It has been proposed that the TACE prodomain contains a cysteine switch involved in TACE activation,
**TACE Mediates p38 MAPK-Induced Cell Proliferation**

**Molecular Cell**

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**A**

<table>
<thead>
<tr>
<th>Treatment</th>
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<tbody>
<tr>
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<tr>
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</tr>
<tr>
<td>SB203580</td>
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<tr>
<td>TAPI-1</td>
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<tr>
<td>PD98059</td>
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**B**

![Graph showing cell proliferation](image)

**C**

<table>
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<tbody>
<tr>
<td>Control + ctrl siRNA</td>
</tr>
<tr>
<td>IL-1β + ctrl siRNA</td>
</tr>
<tr>
<td>IL-1β + p38α siRNA</td>
</tr>
<tr>
<td>IL-1β + TACE siRNA</td>
</tr>
<tr>
<td>IL-1β + TGF-α/AR Ab</td>
</tr>
<tr>
<td>IL-1β + EGFR Ab</td>
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<td>IL-1β + IgG</td>
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**D**

![Graph showing cell proliferation](image)

**E**

![Graph showing TGF-α in medium](image)

**F**

![Western Blots showing protein expression](image)

**G**

<table>
<thead>
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<tbody>
<tr>
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</tr>
<tr>
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<tr>
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<tr>
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<tr>
<td>pErk</td>
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**Graphical Analysis**

- **A**: Representative images of cell treatments with various drugs.
- **B**: Bar graph showing arbitrary value of cell proliferation for different treatments.
- **C**: Images of cellular conditions under different siRNA treatments.
- **D**: Another bar graph depicting similar data as **B**.
- **E**: Graph illustrating TGF-α in the medium for different conditions.
- **F**: Western blot images showing protein expression for various treatments.
- **G**: Summary of protein expression levels with siRNA treatments.
that TIMP3 acts as a natural inhibitor of TACE, and that TACE maturation and localization are involved in TACE activation. Intriguingly, a phosphomimetic mutation of Thr735 in TACE affects the subcellular localization of TACE toward the secretory pathway (Soond et al., 2005). We now show that p38 MAPK activation enhances the cell surface presentation of active TACE and that this increase correlates with activation of shedding. Replacing Thr735 with alanine that cannot be phosphorylated resulted in high levels of two truncated TACE forms at the cell surface, which are predicted to be nonfunctional. Possibly, Thr735 phosphorylation may regulate proteolysis of TACE at the cell surface, a possibility that needs further exploration.

Erk MAPK has been reported to phosphorylate TACE on Thr735 (Diaz-Rodriguez et al., 2002). However, mutation of Thr735 had little effect on the PMA-induced activation of TACE, which occurs through the Erk MAPK pathway. We have shown that growth factors induce Ser819 phosphorylation and Ser791 dephosphorylation. Ser819 phosphorylation depends on Erk MAPK pathway, whereas Ser791 dephosphorylation does not (Fan et al., 2003). We now show that Ser791 mutation, mimicking dephosphorylation, enhances TACE phosphorylation at Thr735 by p38 MAPK and that mutation of Ser819 decreased Thr735 phosphorylation by p38 MAPK, suggesting a crosstalk between growth factor signaling and p38 MAPK activation of TACE.

Role of Regulation of TACE by p38 MAPK Kinase in EGF Receptor Function and Cancer

Increased autocrine EGFR activation by TGF-α ligands drives carcinoma progression (Hanahan and Weinberg, 2000). Consistent with the notion that soluble TGF-α is more effective than transmembrane TGF-α in carcinoma development (Blobel, 2005; Borrell-Pagés et al., 2003), human cancers show increased TACE expression (Mochizuki and Okada, 2007). In addition, increased TACE-mediated release of TGF-α or amphiregulin enhances EGFR-mediated cell proliferation and transformation, as observed in breast carcinoma cells (Kenny and Bissell, 2007; Zhou et al., 2006). We therefore assume that the increased Erk MAPK activity, observed in many carcinomas (Sebolt-Leopold and Herrera, 2004), increases the TACE-mediated release of TGF-α ligands and, consequently, the EGFR activation. Various studies suggest that the p38 MAPK pathway exerts tumor suppression by modulating aspects of cell behavior. However, inflammatory cytokines that activate p38 MAPK, e.g., IL-1 or TNF-α, have cancer-promoting roles (Moore et al., 1999; Vidal-Vanaclocha et al., 2000), and p38 MAPK promotes the proliferation of cancer and immune cells (Dolado et al., 2007; Ono and Han, 2000). Thus, the overall role of p38 MAPK signaling in cancer is likely to depend on the tumor type and cancer stage. Our observations that TACE is activated by p38 MAPK now link inflammatory signals to increased EGFR signaling. Indeed, p38 MAPK activation, due to the inflammatory cytokine release, activates TACE, resulting in the release of TGF-α family ligands. These, in turn, activate the EGFR, leading to increased Erk MAPK signaling and cell proliferation, possibly contributing to carcinoma progression. Further, p38 MAPK activity in autocrine EGFR-dependent cancer cells is required for maintaining self-sufficient growth, a property acquired in cancer progression (Hanahan and Weinberg, 2000). This scenario allows for crosstalk between Erk MAPK and p38 MAPK signaling that may not only contribute to carcinoma progression, but also be of relevance in inflammation that is not associated with cancer.

EXPERIMENTAL PROCEDURES

Expression Plasmids, Reagents, and Antibodies

Plasmids encoding C-terminally Flag-tagged p38α or p38β MAPK, or their kinase-inactive forms p38α-ΔF and p38β-ΔF (Huang et al., 1997), or constitutively active MKK6-E (Jiang et al., 1996) were described. Plasmids encoding Flag- or Myc-tagged TACE were made by subcloning the TACE sequence into pRK5 (Graycar et al., 1989). Plasmids encoding the TACE mutants T735A, S791A, S819A, T735A-S791A, and T735A-S819A were made by Quik-Change Site-Directed Mutagenesis (Stratagene) using pRK5-TACE-Flag. The expression plasmids for TGF-α or TACE E406A mutant have been described (Fan et al., 2003). Coding DNA sequences were verified by DNA sequencing. PMA, anisomycin, IL-1β, TAPI-1, SB203580, SB202474, U0126, PD98059, JNK inhibitor II, AG1478, Bisindolylmaleimide I (BIM), brefeldin A, MnTMPyP, PTIO, L-NIL, EG, and TGF-α were from Calbiochem. Cycloheximide, LPS, and anti-Flag (M2) agarose were from Sigma. Protein A Sepharose was from Amersham, ConA-Sepharose was from GE Healthcare, and EZ-link Sulfo-NHS-LC-Biotin and Neutravidin beads were from Pierce. γ-32P ATP and 32P-orthophosphate were from Perkin Elmer.

Goat anti-TGF-α polyclonal antibody (R&D) was used for immunoprecipitation and neutralization, and the monoclonal anti-TGF-α-α3-1 antibody (Calbiochem) was used for immunoblotting. The EGFR was immunoprecipitated or

Figure 7. p38 MAP Kinase Signaling Stimulates T4-2 Cell Proliferation by TACE-Mediated Shedding of EGF Receptor Ligands (A and B) Effects of TAPI-1, the EGFR inhibitor AG1478, MEK inhibitor PD98059, SB203580, or negative control SB202474 on the proliferation of T4-2 cells. BrdU incorporation was measured by ELISA and expressed relative to untreated control. *p < 0.01, compared with DMSO control. (C and D) Effect of IL-1β on proliferation of cells transfected with p38α MAPK, TACE, or control siRNA or treated with neutralizing antibodies to TGF-α and amphiregulin (AR), or the EGFR, or rabbit IgG. BrdU incorporation was measured by ELISA and expressed relative to untreated control. *p < 0.01, compared with control. **p < 0.001, compared with IL-1β-treated cells transfected with control siRNA or treated with IgG. (E and F) T4-2 cells transfected or not with p38α MAPK, TACE, or control siRNA were treated for 60 (E) or 30 min (F) with IL-1α or anisomycin without or with neutralizing antibodies to TGF-α and amphiregulin (AR), or the EGFR, or rabbit IgG. TGF-α released in the medium was quantified by ELISA (E). In (F), anti-EGFR immunoprecipitates were blotted with anti-phosphoTyr (anti-pY) to show EGFR activation or with anti-EGFR for EGFR expression. Endogenous p38 MAPK and TACE were visualized by immunoblotting of cell lysates or Con A-adsorbed proteins. *p < 0.001, compared with control siRNA. **p < 0.001, compared with IL-1α stimulation of cells transfected with control siRNA or treated with IgG. (G) T4-2 cells were transfected with TACE, p38α MAPK, or control siRNA. Immunoblotting of cell lysates showed downregulation of endogenous TACE or p38α MAPK expression in siRNA-transfected T4-2 cells. EGFR receptor activation and expression are shown as in (F). Erk MAPK activation was shown by immunoblotting for phospho-Erk MAPK (pErk) and total Erk MAPK. The right panel shows the proliferation with or without 5 ng/ml TGF-α or EG, *p < 0.01, compared with untreated cells transfected with control siRNA. **p < 0.01, compared with untreated cells transfected with TACE or p38α MAPK siRNA. Error bars show the standard deviations based on triplicate values of each data set.
neutralized using EGFR antibody clone 528 (Calbiochem), its Tyr phosphoryla-
tion was detected by immunoblotting using anti-pY antibody (Zymed Labora-
tories), and EGFR levels were detected by immunoblotting with anti-EGFR
antibody (Santa Cruz Biotechnology). Anti-ampiregulin goat polyclonal anti-
body (R&D) was used for neutralization. Anti-Erk, anti-pErk, anti-p-p38
MAPK, and anti-p-TP antibodies were obtained from Cell Signaling; rabbit anti-p-p38
MAPK polyclonal antibody and anti-p-38α/i I MAPK monoclonal antibody were from
Santa Cruz Biotechnology. Rabbit anti-TACE polyclonal antibody was from QED Biosciences.
Anti-α-tubulin, streptavidin-HRP, anti-Flag (M2), anti-FLAG (rabbit), and anti-Myc (9E10) antibodies were from Sigma.

Cell Culture and Transfections
CHO cells and derivative Ca (Fan and Derynck, 1999) and M2 cells (Li and Fan, 2004) were cultured in F-12 medium with 10% FBS. Ca cells were also supple-
mented with 200 μg/ml Geneticin (Roche) to maintain TGF-α expression. T4-2 cells were provided by Dr. M. Bissell (Lawrence Berkeley National Laboratory) and cultured as described (Kenny and Bissell, 2007). HeLa cells expressing TGF-α (Emoto et al., 2008) were maintained in DMEM, supplemented with 10% FBS and 2 μg/ml puromycin. Transfections of CHO cells were done using Fugene 6 (Roche). Ca cells were stably transfected with plasmids for wild-type TACE or T735A TACE in the presence of pRK7-Hyg, allowing selection with 600 μg/ml hygromycin B (Roche). Colonies were obtained by serial dilution, and immunoblot analysis for TACE expression was performed using anti-
Flag M2 antibody.

Ectodomain Shedding Assay
Cells at 80% confluence were starved overnight, washed, and treated for 60 min with anisomycin (1 μM), LPS (2.5 μg/ml), IL-1β (60 ng/ml), UV light (60 μJ), PMA (25 μM), and 20% FBS in serum-free medium or medium con-
taining 200 mM NaCl (osmotic shock). Effects of an inhibitor or antibody were
be evaluated by incubating the cells for 30 min prior to and during treatment.
T4-2 cells were also incubated with 0.5 μg/ml anti-EGFR neutralizing antibody for 15 min before stimulation to prevent ligand binding to the EGFR. Inhibitors and neutralizing antibodies include the p38 MAPK inhibitor SB203580 (10 μM), its negative control SB202474 (10 μM), MEK1/2 inhibitor U0126 (10 μM), JNK inhibitor II (50 μM), protein kinase C inhibitor Bisindolylmaleimide I (BIM, 1 μM), metalloprotease inhibitor TAPI-1 (10 μM), EGFR kinase inhibitor AG1478 (1 μM), NO scavenger PTO (100 μM), iNOS inhibitor L-NAME (50 μM), anti-oxidant MntTMPyP (60 μM), brefeldin A (10 μM), cycloheximide (10 μM), anti-TGF-α (1 μg/ml), anti-ampiregulin (6 μg/ml) neutralizing antibodies, anti-EGFR neutralizing antibody (1 μg/ml), and rabbit IgG (6 μg/ml). After treat-
ment, cells were lysed in MLB (20 mM Tris-CI, 200 mM NaCl, 10 mM NaF, 1 mM Na2VO4, 1% NP-40, 20 mM β-glycerophosphate, 2 mM L-2,3-bisphosphoglycerate, 0.5 mM leupeptin, and 1 μg/ml aprotinin [pH 7.5]), and lysates were analyzed by 12.5% SDS-PAGE and immunoblotting with anti-TGF-α antibody to visu-
alize transmembrane TGF-α. Released TGF-α was quantified by ELISA (Quan-
tikine Immuno Assay kit; R&D Systems) of medium that was cooled on ice and then cleared at 9000 rpm for 10 min to remove debris.

Comununpreciaptetions and Western Blotting
Ca cells were transfected with 0.2 μg plasmids for Flag-tagged p38α or p38β
MAPK, or their mutants p38α-ΔF or p38β-ΔF, or Myc-tagged TACE. At 48 hr
after transfection, cells were lysed using MLB, and lysates were subjected to immunoprecipitation using anti-Myc monoclonal antibody 9E10 and protein A Sepharose beads for 2 hr. The beads were washed with MLB, and immunoprecipitated proteins were analyzed by 4%–20% SDS-PAGE and immunoblotting using anti-Flag antibody M2.

In Figures 4F and 4G, endogenous TACE and p38 MAPK were commununpreciapteted from T4-2 cells that were incubated for 30 min with SB203580 or were transfected with p38α MAPK siRNA or control siRNA for 48 hr and treated with IL-1β or anisomycin for 15 min. Cells were then lysed using MLB with 150 mM NaCl, and lysates were preincubated with rabbit IgG and protein A Sepharose and subjected to immunoprecipitation using anti-TACE polyclonal antibody (QED) and protein A Sepharose for 2 hr. Immunoprecipi-
tated proteins were analyzed by 4%–20% SDS-PAGE and immunoblotting using anti-p38α/i I MAPK mIgG1 antibody. Immunoprecipitation supernatants were subjected to Con A Sepharose, and the remaining TACE was visualized by immunoblotting using anti-TACE antibody to ensure effective immunopre-
cipitation. Fractions of the cell lysates were subjected to SDS-PAGE and
immunoblotting to control for protein expression.

Cell Surface Protein Biotinylation
Cells were washed with ice-cold PBS and incubated with EZ-link Sulfo-NHS-
LC-Biotin (0.5 mg/ml in PBS) at 4°C for 60 min. Biotinylation reactions were
stopped with glycine (100 mM in PBS), and cells were lysed in MLB. Cell lysates were incubated 2 hr with Neutravidin beads (Pierce). The beads were
washed with MLB, and the adsorbed proteins were analyzed by SDS-PAGE and
blotting with Streptavidin (Sigma) or anti-TGF-α (ab-1), anti-TACE (QED), or anti-Flag antibody (Sigma).

RNA Interference
siRNAs (QiAGEN) to silence TACE expression targeted the Chinese hamster
TACE mRNA sequence 5′-GAGGAUUUAAAGGUAUGGA3′ (nucleotides 900–920; ref: AY313173Z) or the human TACE sequence 5′-AGAACACAGAGU
GCCUUUUUA3′ (hs_ADAM17_8) (nucleotides 2642–2662; ref: NM_003184.4) in
CHO or T4-2 cells, respectively. siRNA to silence p38α MAPK expression in
CHO cells targeted the sequence 5′-UCUGUGAUUGUCAGAAGCUUUA3′
(Rn, Mapk14_4), derived from the CHO cell p38α cDNA (Ref: US731300)
that we generated by RT-PCR using primers for nested PCR. siRNA for human
p38β MAPK targeted the sequence 5′-UCUGAAGUCAAGCAGCCACGA3′-3′
(Hs_MAPK14_7) (nucleotides 1933–1953; ref: NM_001315). Control siRNA
was used to control for possible nonspecific effects of RNA interference.
Transfections of siRNA were done using Lipofectamine RNAiMAX (Invitrogen).
Reverse transfection was used for HeLa and T4-2 cells to reach optimal effi-
ciency. Cells were used at 48 hr after transfection.

TACE Proteolytic Activity Assay
TACE activity was measured using the InnoZyme TACE activity kit (Calbio-
chem). T4-2 cells were seeded in a 12-well plate and treated for 1 hr with ani-
somycin (1 μM), LPS (2.5 μg/ml), or IL-1β (60 ng/ml) without or with SB203580
(10 μM). Cells were lysed with MLB, and a portion of cell lysates was subjected to the TACE activity assay. In brief, TACE in cell lysates was captured by anti-
TACE monoclonal antibody, and its activity was measured using an internally
quenched fluorescent substrate. Cleavage of amide bond of substrate released the fluorophore, resulting in increased fluorescence, which is directly
related to the activity of TACE.

In Vitro Kinase Assay
In vitro kinase assays used proteins generated by in vitro translation or in trans-
fected Ca cells. Plasmids encoding MKK6-E, Flag-tagged p38α MAPK, or
Flag-tagged TACE were subjected to in vitro transcription from the T7 or
SP6 promoter and translation using the TNT Quick-Coupled Transcription/
Translation kit (Promega). p38α MAPK, generated in vitro, was activated by
incubation with in vitro-translated MKK6-E in the presence of 20 μM ATP at
30°C for 30 min. To obtain proteins expressed in transfected cells, Ca cells
were transfected with 0.2 μg of plasmid expressing p38α or p38β MAPK, or
their mutants, MKK6-E, or wild-type or mutant TACE. TACE and p38α MAPK
from in vitro translation or cell lysates were immunoprecipitated using anti-
Flag M2 agarose beads and washed with MLB before in vitro kinase assay.
In vitro kinase assays were done with γ-32P ATP (Perkin Elmer) in 20 mM
HEPES, 10 mM MgCl2, 1 mM DTT, 50 mM NaCl for 20 min at 30°C and
analyzed by 4%–20% SDS-PAGE, transferred to PVDF membrane, and
subjected to autoradiography or western blotting.

In Vivo 32P-Orthophosphate Labeling
Ca, CoTACE, and CoTACE(T735A) cells at confluence were washed with
phosphate-free medium and incubated in phosphate-free medium for 3 hr
and then in phosphate-free medium with 0.5 μCi/ml 32P-orthophosphate (Per-
kin Elmer) for 2 hr. Anisomycin (1 μM) was added for the last 30 min before
cell lysis in MLB. Proteins, immunoprecipitated with anti-Flag agarose, were
analyzed by 4%–20% SDS-PAGE, transferred to PVDF membrane, and
analyzed by autoradiography or immunoblotting with anti-Flag antibody.
Parallel cell lysates without isotope were analyzed by SDS-PAGE and
immunoblotting with anti-α-tubulin antibody.
Cell Proliferation Assay

Cell proliferation was quantified by BrdU incorporation. T4-2 cells, in some cases 24 hr after transfection with siRNA to TACE or p38 MAPK, or control siRNA were seeded in a 48-well plate and treated with SB203580 (10 μM) or its negative control SB202474 (10 μM), TAPi-1 (10 μM), EGFR inhibitor AG1478 (1 μM), MEK inhibitor PD98059 (10 μM), LPS (2.5 μg/ml), or IL-1β (60 ng/ml), anti-TGF-α (1 μg/ml) and anti-angiopressin (5 μg/ml), or anti-EGFR neutralizing antibody (1 μg/ml), or rabbit IgG (6 μg/ml) for 72 hr. Cells were imaged using a LEICA DM4000B microscope, and cells were incubated with BrdU for 3 hr before ELISA. In the rescue analyses, EGF or TGF-α (5 ng/ml) was added. BrdU incorporation assays were done using the BrdU Cell Proliferation Assay kit (Calbiochem).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at doi:10.1016/j.molcel.2010.01.034.

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