TACE-mediated ectodomain shedding of the type I TGF-β receptor downregulates TGF-β signaling

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Summary
Regulating TGF-β receptor presentation provides an avenue to alter a cell’s responsiveness to TGF-β. We report that activation of the Erk MAP kinase pathway decreases the TGF-β-induced Smad3 activation, due to decreased cell surface levels of the type I receptor TβRI, but not the type II receptor. Inhibition of TACE activity or expression enhanced the cell surface TβRI levels and TGF-β-induced Smad3 and Akt activation. Accordingly, silencing TACE expression in cancer cells enhanced the TβRI presentation and TGF-β responsiveness, including the antiproliferative effect of TGF-β, and epithelial to mesenchymal transition. These results establish a mechanism for downregulating TGF-β signaling through TACE activation by the Erk MAP kinase pathway, and a strategy for evasion of tumor suppression and modulation of epithelial to mesenchymal transition, during cancer progression. The decreased growth inhibition by TGF-β, due to elevated TACE activity, complements the growth stimulation resulting from increased release of TGF-α family ligands.

Introduction
In epithelial cells, transforming growth factor-β (TGF-β) inhibits cell proliferation. The transition of normal epithelial cells into carcinoma cells is accompanied by downregulation of the growth inhibition response to TGF-β, allowing the cells to escape the autocrine tumor suppressor activities of TGF-β signaling (Grady and Markowitz, 2007; Siegel and Massagué, 2003). In addition, the increased TGF-β production by carcinoma cells contributes to the invasive and metastatic behavior of cancer cells. Notably, TGF-β can induce an epithelial to mesenchymal transition (EMT) that allows the cells to become migratory and invasive (Derynck et al., 2001; Massagué, 2008; Xu et al., 2008). Finally, the increased TGF-β production exerts effects on stromal and immune cells to provide a favorable microenvironment for cancer progression (Bierie and Moses, 2006; Derynck et al., 2001; Siegel and Massagué, 2003; Massagué, 2008).

TGF-β signals through receptor complexes of type II and type I dual specificity kinases (Feng and Derynck, 2005; Lee et al., 2007; Shi and Massagué, 2003). In response to ligand, the TβRII receptors phosphorylate and activate the TβRI receptors, which then activate Smad2 and Smad3 through C-terminally phosphorylation. These then form complexes with Smad4, translocate into the nucleus, and regulate the transcription of TGF-β-responsive genes. TGF-β signaling also activates signal transducers other than Smads, such as Erk MAP kinases, PI-3 kinase, Rho-like GTPases, protein phosphatase 2A, and Par6. These signaling events lead to...
responses that do not involve Smad-mediated transcription, although Erk MAP kinases also regulate Smad signaling (Derynck and Zhang, 2003; Ozdamar et al., 2005; Zhang, 2007).

Carcinoma cells have strategies to attenuate or inactivate the tumor suppressor activities of TGF-β signaling. Inactivating or attenuating mutations in the TGFBR2 and TGFBR1 genes, encoding TβRII or TβRI, are found in a variety of cancers (Akhurst and Derynck, 2001; Grady and Markowitz, 2007). Epigenetic silencing due to aberrant DNA methylation or histone modification of the TGFBR2 or TGFBR1 promoters, and aberrant transcription regulation also attenuate the growth inhibitory effect of TGF-β signaling in carcinomas (Hinshelwood et al., 2007; Kang et al., 1999; Lee et al., 2001). Impaired TGF-β responses in carcinomas also associate with mutations in Smad genes or altered Smad expression (Akhurst and Derynck, 2001; Grady and Markowitz, 2007), or increased expression of the inhibitory Smad7 (Grady and Markowitz, 2007; Kleeff et al., 1999). Finally, repression of Smad2/3 activity by a Smad corepressor, such as Evi-1, c-Ski and SnoN, whose genes were identified as oncogenes, provides another mechanism for inhibition of TGF-β signaling (Feng and Derynck, 2005; Massagué et al., 2005).

Following activation, the activities of Smad2 and Smad3 are regulated by other kinases (Derynck and Zhang, 2003; Luo, 2007). The best studied pathway to regulate Smad signaling is the Erk MAP kinase pathway, which is potently activated by growth factor receptors and Ras, and is upregulated in about a third of all cancers (Dhillon et al., 2007). Phosphorylation of the linker regions of Smad2 and Smad3 by Erk MAP kinases can inhibit the TGF-β-induced nuclear translocation of these Smads and the antiproliferative effect of TGF-β (Kretzschmar et al., 1999), although other reports found that Erk MAP kinase activation enhances or does not affect the Smad activities (Funaba et al., 2002; Hayashida et al., 2003; Janda et al., 2002). The effects of Erk MAP kinase on Smad signaling may depend on the context, possibly on Smad phosphorylation by other kinases (Wrighton and Feng, 2008). Erk MAP kinase activation is not known to affect the TGF-β-induced C-terminal phosphorylation, and thus the TβRI-mediated activation, of Smad2 or Smad3.

Ectodomain shedding, mediated by membrane-anchored metalloproteases, releases ectodomains of cell surface proteins that act as ligands or receptors in inflammation, growth control and other processes. TACE (TNF-α converting enzyme), also known as ADAM17, mediates shedding of cytokines, growth factors, receptors and adhesion proteins (Huovila et al., 2005). TACE is highly expressed in cancers (Mochizuki and Okada, 2007), and has been implicated in the shedding of TGF-α family growth factors, resulting in increased EGF/TGF-α receptor (EGFR) signaling (Borrell-Pages et al., 2003; Kenny and Bissell, 2007; Zhou et al., 2006). Since activation of Erk MAP kinase confers TACE activation (Diaz-Rodriguez et al., 2002; Fan and Derynck, 1999), the increased Erk MAP kinase activity observed in carcinomas is expected to increase ectodomain shedding. Increased release of TGF-α or amphiregulin by TACE has been linked to increased EGFR-mediated cell proliferation and transformation, as observed in breast and non-small cell lung carcinoma cells (Kenny and Bissell, 2007; Zhou et al., 2006).

We report that induction of the Erk MAP kinase pathway decreases the TβRI-mediated Smad3 activation, due to a decrease in cell surface level of TβRI, but not TβRII. Depletion of TACE activity or expression blocked the decrease in cell surface level of TβRII and TGF-β signaling. Accordingly, silencing TACE expression in HaCaT keratinocytes or T4-2 breast cancer cells enhances the TβRI cell surface presentation and antiproliferative effect of TGF-β signaling, and TGF-β’s ability to elaborate an epithelial to mesenchymal transition. Our results establish a mechanism for downregulation of TGF-β signaling through activation of TACE by the Erk MAP kinase pathway.
Results

Erk MAP kinase pathway activation inhibits TGF-β-induced Smad3 phosphorylation

To evaluate the effect of the Erk MAP kinase pathway on TGF-β-induced Smad activation, we treated CHO cells with PMA, a potent activator of Erk MAP kinase signaling (Amaral et al., 1993). As shown in Fig 1A, treatment with PMA inhibited the TGF-β-induced phosphorylation of Smad3. This inhibition was reversed by treating the cells with U0126, an inhibitor of the MEK1 and MEK2 kinases that activate Erk MAP kinases 1 and 2 (DeSilva et al., 1998). PMA is known to activate protein kinase C, and Erk MAP kinase pathway activation by PMA depends epistatically on protein kinase C activation (Qiu and Leslie, 1994). Accordingly, the decrease in TGF-β-induced phosphorylation of Smad3 by PMA treatment was comparably reversed by BMI, a protein kinase C inhibitor, and U0126. In contrast, the PI3-kinase inhibitor LY294002 had no effect (Fig. 1B).

Erk MAP kinase pathway activation downregulates the cell surface presentation of TβRI, but not TβRII

The inhibition of TGF-β-induced Smad3 phosphorylation raises the possibility that Erk MAP kinase pathway activation affects the levels or activities of TβRII and/or TβRI. We therefore tested whether PMA induced alterations in the cell surface levels of TβRII or TβRI, detected by cell surface protein biotinylation (Fig. 1C). PMA induced a time-dependent decrease of cell surface TβRI, but not of TβRII, accompanied by the appearance of a smaller form of TβRI. This form was poorly labeled by cell surface biotinylation, had the size of an extracellularly truncated TβRI and appeared to undergo degradation. In the presence of the proteasomal degradation inhibitor MG-132, the level of truncated TβRI at the cell surface increased and correlated inversely with the reduction of full length TβRI at the cell surface (Fig. 1D). The decrease of TβRI at the cell surface and appearance of truncated TβRI were inhibited by the MEK1/2 inhibitor U0126. Consistent with the epistatic dependence of PMA-induced Erk MAP kinase pathway activation on protein kinase C, the protein kinase C inhibitor BMI and MEK1/2 inhibitors U0126 or PD98059 prevented the decrease of cell surface TβRI and the appearance of truncated TβRI in response to PMA, while the PI3-kinase inhibitor LY294002 had no effect (Fig. 1E). Similarly to PMA, serum also induced a decrease in cell surface levels of TβRI but not TβRII, resulting from activation of the Erk MAP kinase pathway. The effect of serum was less pronounced than that of PMA, consistent with the stronger Erk MAP kinase activation by PMA (Fig. 1F).

The reduction in cell surface TβRI, resulting from Erk MAP kinase pathway activation, is mediated by TACE

To test whether the decrease in cell surface presentation of TβRI resulted from ectodomain shedding, we examined the effect of TAPI-1, a metalloprotease inhibitor. As shown in Fig. 2A, B, TAPI-1 inhibited the decrease in cell surface TβRI levels in response to PMA or serum, indicating that this decrease resulted from metalloprotease-mediated shedding of TβRI at the cell surface.

We have shown that activation of the Erk MAP kinase pathway, e.g. in response to PMA or serum, results in increased ectodomain shedding by TACE (Fan and Derynck, 1999; Fan et al., 2003). Since TAPI-1 inhibited the decrease in TβRII levels at the cell surface, and TAPI-1 inhibits TACE (Slack et al., 2001), we examined the effect of knocking down TACE expression using transfected siRNA. As shown in Fig. 2C, D, silencing TACE expression blocked the decrease in TβRI cell surface levels and TβRII shedding in response to PMA or serum. These data indicate that TACE is required for TβRII ectodomain shedding, and strongly suggest that TACE may be the effector of TβRII shedding in response to PMA or serum.
TACE cleaves the type I receptor TβRI, but not the type II receptor TβRII

To examine whether TACE can serve as effector of TβRII ectodomain shedding, we co-expressed TACE with C-terminally Flag-tagged TβRI or TβRII in CHO cells. Under these conditions of overexpression, TACE has proteolytic activity, not requiring activation in response to e.g. PMA (Fan et al., 2003). TACE co-expression resulted in the appearance of a 40 kd TβRI band, about the size of extracellularly truncated TβRI, concomitantly with a decrease in the level of full size TβRI (Fig. 3A). The 40 kd band was expressed at much lower level, and full size TβRI level was at much higher level, in cells without TACE co-expression. Equal expression of the catalytically inactive E406A TACE mutant did not confer TβRII ectodomain shedding. Further, TACE did not affect the TβRII levels or generate a truncated TβRII, suggesting that TβRII is not a substrate of TACE (Fig. 3A).

Ectodomain shedding of membrane-bound proteins releases extracellular domains into the medium. Accordingly, the extracellular domain of TβRI, HA-tagged between amino acids 27 and 28, was detected in the medium of cells expressing wild-type TACE, but not inactive TACE (Fig. 3B). Further evidence that TβRI can serve as TACE substrate was provided by the cleavage of TβRI by TACE in vitro, using immunopurified proteins (Fig. 3C). The low cleavage efficiency was not surprising since we do not know how to activate TACE in vitro. The 40 kd in vitro cleavage product had the same size as the extracellularly truncated TβRI in Fig. 3A.

These results, together with the results using TAPI-1 and TACE siRNA, indicate that shedding of TβRI by TACE mediates the decrease in cell surface TβRII levels in response to Erk MAP kinase activation. Cleavage of a TGF-β family type I receptor by TACE was specific for TβRI, since it was not observed with other type I receptors (Fig. 3D). Accordingly, BMP-2-induced Smad1 activation was not affected when TACE expression was silenced (data not shown).

TACE expression defines the level of Smad activation by TGF-β

We next evaluated whether the decrease in TGF-β-induced Smad3 activation upon Erk MAP kinase pathway activation was due to the activity of TACE. As shown in Fig. 4A, TAPI-1 inhibited the PMA-induced decrease of Smad3 phosphorylation, indicating that inhibition of metalloprotease activity restored Smad3 activation by TGF-β to a level similar to that in the absence of Erk MAP kinase activation. To specifically address whether TACE mediated the decrease in Smad3 activation, cells were transfected with TACE siRNA or control siRNA. Silencing TACE expression reversed the inhibition of TGF-β-induced Smad3 phosphorylation by Erk MAP kinase pathway activation (Fig. 4B). In addition, the long-term activation of Smad3 in the absence of PMA was enhanced when TACE expression was silenced (Fig. 4C). Finally, the effect of TACE on TGF-β signaling extended to non-Smad signaling. Indeed, also the level of TGF-β-induced Akt activation was enhanced when TACE expression was silenced (Fig. 4D).

These data indicate that activation of the Erk MAP kinase pathway inhibits the TGF-β-induced Smad3 activation through TACE, and that TACE expression and activity define the Smad and non-Smad signaling activity in response to TGF-β.

TACE expression defines the cell surface TβRI levels and TGF-β responses in HaCaT epithelial cells

We next evaluated whether TACE regulates the cell surface TβRI levels and TGF-β responsiveness in other epithelial cell systems. As shown in Fig. 5A, silencing TACE expression enhanced the TβRI cell surface level in HaCaT epithelial cells, T4-2 and MDA-MB-468 breast cancer cells, Hela squamous carcinoma cells, A549 lung carcinoma cells and HepG2 hepatoma cells. The cell surface TβRI levels were not increased in MCF-7 and...
MCF-10A breast cancer cells upon transfection with TACE siRNA (data not shown). These data indicate that TACE expression regulates the cell surface level of TβRI in a variety of cells.

HaCaT cells are seen as a model for how non-transformed, epithelial cells respond to autocrine and paracrine TGF-β signaling, but the role of TACE in these cells has not been explored. To evaluate the regulation of TGF-β signaling by TACE, we compared the TGF-β responses in HaCaT cells, in which TACE expression was silenced, with HaCaT cells transfected with a control siRNA. As shown in Fig. 5B, silencing TACE expression increased the cell surface levels of TβRI, without affecting the total TβRI levels, and did not affect the cell surface or total TβRII levels. Further, these cells showed a higher level of Smad3 activation in response to TGF-β, which was still apparent after long exposure to TGF-β (Fig. 5C). We also examined the effect of TACE downregulation on the TGF-β target genes encoding Smad7 and PAI-1. As shown in Fig 5D, cells with silenced TACE expression showed increased Smad7 and PAI-1 mRNA levels in response to TGF-β, when compared to control cells. The basal level of Smad7 mRNA in the absence of added TGF-β was also increased, reflecting autocrine TGF-β signaling. These data demonstrate that TACE negatively regulates TGF-β-induced Smad activation and transcription.

**TACE regulates the control of cell proliferation by TGF-β**

Considering the effect of TACE on TGF-β-induced transcription, we compared the proliferation of HaCaT cells with downregulated TACE expression and control HaCaT cells, in the absence or presence of TGF-β. Cell proliferation was assessed by BrdU incorporation, and examined after 12 or 36 h in the presence of TGF-β. As expected, the antiproliferative effect of TGF-β was more pronounced after 36 h (Fig. 5E).

Treatment of HaCaT cells with TGF-β reduced proliferation with ~60 % after 36 h. In cells with silenced TACE expression, the proliferation of the TGF-β-treated cells was only 8% of that of the untreated cells. In the absence of added TGF-β, their proliferation rate was only half of that of the control cells, reflecting a high sensitivity to autocrine control of proliferation by TGF-β, as confirmed by the increased proliferation in the presence of the TβRI inhibitor SB431542 (Fig. 5E). These data illustrate that the activity of TACE is a determinant of the antiproliferative effect of TGF-β and of cell proliferation, which is under autocrine control of TGF-β. Thus, increased TACE activity decreases the growth inhibition by autocrine or paracrine TGF-β, and may in this way contribute to cancer progression.

**TACE regulates epithelial to mesenchymal transition in response to TGF-β**

Epithelial cells can undergo EMT in response to TGF-β. This response combines Smad and non-Smad signaling and results in a loss of epithelial characteristics, such as cortical actin organization and localization of E-cadherin at cell junctions, and acquisition of mesenchymal characteristics, such as reorganization of the actin cytoskeleton, increased fibronectin expression, migration and invasion (Zavadil and Bottinger, 2005). Considering the role of TACE in defining the TGF-β responsiveness, we evaluated whether silencing TACE expression affects the TGF-β-induced EMT response of HaCaT cells (Fig. 6).

HaCaT cells transfected with control siRNA have, in the absence of added TGF-β, an epithelial cobblestone morphology with cortical actin staining, localization of E-cadherin at cell contacts, and lack of fibronectin staining. In the presence of increasing TGF-β levels, starting at 0.25 ng/ml of added TGF-β, the cells lost their epithelial phenotype, reorganized their actin cytoskeleton, lost junctional E-cadherin localization and showed increased fibronectin staining (Fig. 6A). In contrast, cells with silenced TACE expression (Fig. 6B), had already in the absence of added TGF-β a disorganized actin pattern, lacked E-cadherin staining at cell contacts and showed a low level fibronectin staining. Further, the cells acquired a more pronounced...
fibroblast phenotype at much lower concentrations of added TGF-β, compared with HaCaT cells transfected with control siRNA, reflecting an increased sensitivity to TGF-β (Fig. 6A).

The loss of epithelial and acquisition of mesenchymal properties in cells with silenced TACE expression, in the absence of added TGF-β, may result from autocrine TGF-β signaling. As shown in Fig. 6B, adding the TβRI inhibitor SB431542 to HaCaT cells with downregulated TACE expression reversed the cells to an epithelial morphology with cortical actin staining, E-cadherin staining at cell-cell contacts, and lack of fibronectin staining. These results illustrate that the EMT response to endogenous or added TGF-β is regulated by TACE.

**TACE downregulates TGF-β signaling in T4-2 breast cancer cells**

Increased TACE expression has been implicated in the control of signaling by TGF-α family growth factors through the EGFR in breast cancer cells. Indeed, increased TACE activity confers increased TGF-α release, resulting in increased, EGFR-mediated growth stimulation (Borrell-Pages et al., 2003; Kenny and Bissell, 2007; Zhou et al., 2006). We examined the regulation of TGF-β signaling by TACE in T4-2 breast cancer cells, which show autocrine, EGFR-dependent parameters of malignant transformation, resulting from TACE-mediated ectodomain shedding of TGF-α and amphiregulin. Downregulation of TACE in these cells results in decreased EGFR activation and reverses the transformed phenotype (Kenny and Bissell, 2007).

Consistent with the regulation of TACE activity by the Erk MAP kinase pathway (Fan and Derynck, 1999; Diaz-Rodriguez et al., 2002) (Fig. 7A), addition of EGF, which enhances Erk MAP kinase signaling, decreased the level of cell surface TβRI, whereas inhibition of Erk MAP kinase signaling using the MEK inhibitor PD98059 enhanced the TβRI cell surface level. Consequently, EGF signaling decreased, and addition of PD98059 enhanced the level of Smad3 activation (Fig. 7B).

Using TACE siRNA, we generated T4-2 cells with silenced TACE expression and compared these with T4-2 cells transfected with control siRNA (Fig. 7C-E). As in HaCaT cells, silencing TACE expression resulted in increased cell surface presentation of TβRI, without an effect on the cell surface TβRII level (Fig. 7C). Consistent with this difference in cell surface TβRI levels, the decrease in TACE expression resulted in a modestly increased Smad3 activation in response to TGF-β (Fig. 7B).

We also examined the proliferation of these cells in the absence or presence of TGF-β for 24 or 48 h. Since these cells show EGFR-dependent signaling that is regulated by TACE’s effects on EGFR ligand production, the T4-2 cells were cultured in the presence of EGF, thus providing maximal EGFR activation. Under these conditions, TGF-β induced a modest growth inhibition, which was enhanced when TACE expression was silenced. As with HaCaT cells, the antiproliferative effect of TGF-β was more pronounced after 48 h, compared to 24 h (Fig. 7E). Thus, also in these breast cancer cells with maximized EGFR signaling, did TACE regulate the growth inhibition response to TGF-β, with increased TACE levels making the cells less prone to growth inhibition by TGF-β.

**Discussion**

TGF-β signaling is regulated by signaling crosstalk that targets the TGF-β receptors or Smads (Derynck and Zhang, 2003; Luo, 2007). At the receptor level, TGF-β signaling is regulated by TβRI ubiquitylation and degradation (Ebifasawa et al., 2001; Kavsak et al., 2000), and TβRI sumoylation, which enhances Smad2 and 3 recruitment and activation (Kang et al., 2008). At the Smad level, activation of Smad2 and 3 is inhibited by Smad7 binding to TβRI (Shi and Massagué, 2003; Feng and Derynck, 2005), Smads undergo ubiquitylation and degradation.
(Arora and Warrior, 2001), and sumoylation of Smad4 enhances TGF-β-induced transcription (Lee et al., 2003; Lin et al., 2003; Ohshima and Shimotohno, 2003). The Smad activities are also regulated by kinases other than the TGF-β receptor kinases, most notably by Erk MAP kinases, which are activated in response to growth factors and phosphorylate the Smad linker segments (Derynck and Zhang, 2003; Zhang, 2007).

We now present evidence for a novel mode of regulation of TGF-β signaling: the cell surface presentation of TβRI, but not TβRII, is downregulated by TACE-mediated ectodomain shedding, which is activated by Erk MAP kinase signaling. This downregulation decreases the Smad activation and TGF-β signaling in response to TGF-β, and thus the TGF-β-induced gene expression and antiproliferative responses. Since TGF-β binding involves primarily TβRII (Feng and Derynck, 2005; Shi and Massagué, 2003), and TβRII is not affected by TACE, this mode of receptor downregulation should not be reflected in the overall level of TGF-β binding to the cell surface. While operative in normal cells, this mechanism may be particularly relevant in cancer cells that are often marked by increased Erk MAP kinase signaling and TACE levels and activity.

**TACE regulates TGF-β signaling**

Our observations correlate TACE activity with the cell surface presentation of TβRI, but not TβRII. Indeed, ectopic expression of TACE, but not an inactive TACE mutant, or activation of endogenous TACE resulted in ectodomain shedding of TβRI, but not TβRII, decreasing the cell surface TβRI levels, and generating a truncated TβRI and a soluble TβRI ectodomain. Conversely, inhibition of TACE with TAPI-1 or silencing TACE expression prevented the decrease in cell surface TβRI and generation of a truncated TβRI, in response to Erk MAP kinase pathway signaling. The enhanced presentation of TβRI, but not TβRII, in HaCaT epithelial and T4-2 carcinoma cells upon silencing TACE expression reflects autocrine activation of TACE.

The decrease in cell surface TβRI level upon TACE activation correlated with decreased TGF-β-induced Smad3 and Akt activation. Accordingly, inhibiting TACE activity or silencing TACE expression enhanced Smad3 and Akt activation by TGF-β. The depletion of TACE expression in HaCaT and T4-2 cells enhanced transcription of TGF-β target genes, in the presence as well as the absence of TGF-β, indicating that TACE also regulates the autocrine TGF-β response. TACE did not affect the cell surface levels of other type I receptors besides TβRI, or BMP-induced Smad1 activation, suggesting that it specifically targets TGF-β signaling through TβRI, and not activin and BMP signaling.

Consistent with the regulation of TGF-β-induced gene expression, decreasing TACE expression enhanced the growth inhibition by TGF-β, and the basal proliferation that is defined by autocrine TGF-β signaling. TGF-β signaling is also known to induce epithelial cells to transition into mesenchymal cells, and this EMT process is thought to be at the basis of invasion and metastasis of carcinoma cells. Decreasing TACE expression made the cells much more susceptible to TGF-β-induced EMT, even to the extent that autocrine TGF-β signaling caused the HaCaT cells to lose their epithelial nature and acquire mesenchymal characteristics in the absence of added TGF-β.

**Regulation of TACE activity affects TGF-β signaling**

Ectodomain shedding is activated by various stimuli, ranging from UV irradiation to osmotic stress, inflammatory mediators, growth factors and autocrine or cell-intrinsic signaling events. Activation of the Erk MAP kinase or p38 MAP kinase pathways accounts for most, if not all, stimulus-mediated activation of ectodomain shedding (Huovila et al., 2005). Erk MAP kinase pathway activation was shown to induce ectodomain shedding of TGF-α, TNF-α and other
transmembrane proteins by TACE (Díaz-Rodriguez et al., 2002; Fan and Derynck, 1999; Fan et al., 2003). Consistent with the regulation of TACE by the Erk MAP kinase pathway, the cell surface presentation of TβRI and the TGF-β response are downregulated under conditions that activate Erk MAP kinase signaling. Accordingly, enhanced Erk MAP kinase signaling in response to EGF decreased the cell surface TβRI levels and Smad3 activation in response to TGF-β in T4-2 cells. Conversely, blocking the Erk MAP kinase pathway in these cells increased the TβRI cell surface levels and TGF-β-induced Smad3 activation. Since activation of the p38 MAP kinase also leads to TACE activation (Xu and Derynck, unpublished data), we propose that cells may also downregulate the TGF-β response under conditions that activate p38 MAP kinase signaling, including inflammation.

**Multiple levels of regulation of TGF-β/Smad signaling by the Erk MAP kinase pathway**

Activation of the Erk MAP kinase pathway has been shown to regulate TGF-β/Smad signaling. Erk MAP kinase signaling targets various transcription factors that cooperate with Smads, thus regulating TGF-β-responsive gene expression (Feng and Derynck, 2005; Luo, 2007). Erk MAP kinases also phosphorylate the linker regions of Smad2 and Smad3, thus regulating the activities of these Smads. Whereas activation of the Erk MAP kinase pathway by Ras or EGF was shown to inhibit TGF-β-induced nuclear translocation of Smad2 and Smad3, thus decreasing TGF-β/Smad-mediated transcription (Kretzschmar et al., 1999), it was also shown to enhance TGF-β-activated Smad signaling (Funaba et al., 2002; Hayashida et al., 2003; Janda et al., 2002;). We now demonstrate a third level of regulation of TGF-β signaling by the Erk MAP kinase pathway, i.e. through activation of shedding by TACE resulting in decreased cell surface availability of TβRI and decreased TGF-β signaling. The downregulation of TGF-β signaling resulted in a lower level of TGF-β-induced gene expression, and decreased growth inhibition and sensitivity to EMT. The net effect of Erk MAP kinase pathway activation on TGF-β signaling presumably depends on the balance of these regulatory mechanisms, including the expression and activation state of TACE.

**Role of TACE-mediated ectodomain shedding of TβRI in cancer cells**

Early during carcinoma development, tumor cells need to overcome the growth inhibition that is normally imposed by autocrine TGF-β signaling in epithelial cells (Derynck et al., 2001; Grady and Markowitz, 2007; Siegel and Massagué, 2003). Carcinoma cells use several strategies to attenuate or inactivate the tumor suppressor activity of TGF-β signaling, including somatic mutations in genes encoding TβRII and Smad4, aberrant expression of inhibitory Smads or Smad corepressors, such as c-Myc, SnoN or Evi-1, and alterations in the expression or activities of cell cycle machinery components (Akhurst and Derynck, 2001; Feng and Derynck, 2005; Grady and Markowitz, 2007; Massagué et al., 2005; Siegel and Massagué, 2003). We now defined a mechanism to downregulate the growth inhibition by TGF-β at the receptor level, i.e. through a TACE-activated decrease of TGF-β presentation, induced by the Erk MAP kinase pathway.

The induction of EMT in epithelial and carcinoma cells by TGF-β activity is thought to play a key role in the invasive and metastatic behavior of carcinomas (Zavadil and Bottinger, 2005; Massagué, 2008). Our results now show that EMT is also regulated by TACE. Increased ectodomain shedding of TβRI, due to increased TACE activity, results in a decreased induction of EMT by TGF-β. This downregulation of the TβRI cell surface levels is expected to result in decreased invasiveness, resembling the phenotypes of tumors with inactivating TGFBR2 mutations (Grady and Markowitz, 2007;). Conversely, inhibition of TACE activity enhances the autocrine signaling to the extent that the epithelial HaCaT cells lose epithelial and acquire mesenchymal characteristics. This enhanced EMT in response to TACE inhibition may contribute to cancer progression and should be of concern in using metalloprotease inhibitors for cancer therapy (Coussens et al., 2002).
Activation of the Erk MAP kinase pathway and elevated TACE expression occur in a substantial fraction of cancers. The effects of elevated TACE activity on cancer progression have been attributed to TACE-mediated ectodomain shedding of membrane-anchored TGF-α family ligands and consequently increased activation of EGFR, which leads to increased cell proliferation, tumor growth and malignant transformation (Borrell-Pages et al., 2003; Kenny and Bissell, 2007; Zhou et al., 2006). Increased EGFR activation also leads to increased Erk MAP kinase signaling, thereby ensuring sustained TACE activation and release of the soluble growth factors. Our results show that TACE-mediated cleavage of TβRI additionally downregulates the growth inhibition by autocrine or exogenous TGF-β signaling, complementing the growth stimulation due to increased EGFR signaling. Accordingly, the TACE activity in T4-2 breast cancer cells not only results in increased cell proliferation resulting from TACE-mediated cleavage of TGF-α ligands (Kenny and Bissell, 2007), but also in decreased growth inhibition by TGF-β under conditions of maximal growth stimulation by EGF. These complimentary effects of TACE activation are operational in cancer cells and may contribute to cancer progression.

Experimental procedures

Expression plasmids and reagents

Expression plasmids encoding C-terminally Flag-tagged versions of TβRII and TβRI (Feng et al., 1995), TACE and TACE E406A mutant (Fan et al., 2003), and TβRI with an HA epitope between amino acids 27 and 28 (Hayes et al., 2002) are described. The latter was provided by Susan Hayes (University of Massachusetts Medical School) and the coding sequence was subcloned into pRK5 (Graycar et al., 1989). TGF-β was from PeproTech and EGF was from Calbiochem. PMA, TAPI-1, PD98059 and U0126 were from Calbiochem. EZ-link Sulfo-NHS-LC-Biotin was from Pierce. The TGF-β type I receptor kinase inhibitor SB431542, the PI-3 kinase inhibitor LY294002 and the proteasome inhibitor MG-132 were from Sigma.

Cell culture, transfections and treatments

CHO cells, HaCaT keratinocytes, MDA-MB-468, Hela and A549 carcinoma cells, HepG2 hepatoma cells and HEK293T cells were cultured in F-12 or DMEM medium with 10% FBS. T4-2 cells, provided by Mina Bissell (Lawrence Berkeley National Laboratory), were cultured as described (Kenny and Bissell, 2007). CHO cells were transfected using Fugene 6 (Roche).

For treatments, CHO cells, but not HaCaT and T4-2 cells, were starved overnight in serum-free medium before treatment. To activate Erk MAP kinase signaling, cells were treated with PMA (50 nM) or 20% FBS for 45 min. Cells were pretreated with U0126 (10 μM) or TAPI-1 (10 μM) for 25 min to inhibit the Erk MAP kinase pathway or TACE, respectively. To inhibit proteasome-mediated degradation, cells were treated with 25 μM MG-132 for 30 min. As needed, cells were treated with TGF-β1 (2 ng/ml) for 30 min, unless otherwise indicated. Cells were treated SB431542 (3 μM) to inhibit TβRI kinase activity.

Cell surface biotinylation

Cells were washed with ice-cold PBS, and incubated with EZ-link Sulfo-NHS-LC-Biotin (0.5 mg/ml in PBS). Biotinylation was stopped with 0.1 M glycine in PBS, and cells were lysed in MLB lysis buffer (20 mM Tris-Cl, 200 mM NaCl, 10 mM NaF, 1 mM NaVO4, 1% NP-40, 2 mM Pefabloc, 0.5 mM Leupeptin, 1 μg/ml Aprotinin, pH 7.5). Biotinylated proteins were precipitated with neutravidin beads (Pierce) and analyzed by immunoblotting using anti-TβRI (V-22) antibody (Santa Cruz) or anti-TβRII (L-21) antibody (Santa Cruz).
RNA interference

TACE siRNA and negative control siRNA were from Qiagen. The target sequence for CHO TACE siRNA was 5'-GAGGAUUUAAAGGUUAUGGAA-3', corresponding to Chinese hamster TACE mRNA nucleotides 900-920 (ref: AY313173). The human TACE siRNA target sequence was 5'-AAGAAACAGAGUGCUAAUUU-3', corresponding to human TACE mRNA nucleotides 2642-2662 (ref: NM_003183.4). Transfections of siRNA were performed using Lipofectamine RNAiMAX (Invitrogen).

Immunoprecipitations and western blotting

CHO cells were transfected with expression plasmids. 24 h after transfection, cells were cultured overnight in serum-free medium, lysed using MLB lysis buffer, then subjected to immunoprecipitation using anti-Flag M2 antibody-conjugated agarose beads (Sigma), and adsorbed proteins were eluted using Flag peptide (Sigma). To detect the cleaved extracellular domain of TβRI, culture media were subjected to immunoprecipitation with anti-HA antibody (Covance). Eluted or immunoprecipitated proteins were analyzed by immunoblotting. The antibodies used in western blot analysis are anti-Flag M2 (Sigma), anti-HA and anti-TACE (QEDBio). C-terminal Smad3 phosphorylation was detected using anti-phospho-Smad3 antibody (Cell Signaling) and anti-Smad3 antibody (Zymed). For TGF-β-induced Akt phosphorylation, Anti-Akt and anti-phospho-Thr308 Akt antibodies (Cell Signaling) were used in immunoblotting analyses.

Immunofluorescence microscopy

HaCaT cells were fixed and processed for immunofluorescence with anti-E-cadherin and anti-fibronectin antibodies, or actin staining using rhodamine-conjugated phalloidin, as described (Lamouille and Derynck, 2007). Images were analyzed using NIS-elements (Nikon Laboratories Imaging) and Adobe Photoshop software.

In vitro TACE-mediated cleavage assays

For Fig. 3C, 293T cells, expressing C-terminally Flag-tagged TACE or TβRI, were lysed using MLB lysis buffer, and TACE-Flag and TβRI-Flag were individually purified using anti-Flag M2 antibody-conjugated agarose beads. TβRI was then incubated for 1 h without or with TACE in 25 mM Tris, pH 7.5, 2.5 μM ZnCl2, 0.005% Brij 35 at 37 °C. Reactions were stopped by adding SDS sample buffer and analyzed by immunoblotting.

For Fig. 7A, T4-2 cells were seeded in a 12-well plate and treated with PMA (25 nM) or EGF (20 ng/ml) for 60 min, in the absence or presence of the MEK inhibitor PD98059 (20 μM) or U0126 (10 μM) to block Erk MAP kinase activation. Cells were then lysed with MLB lysis buffer in the absence of protease inhibitors. The enzymatic activity of TACE was quantified using the InnoZyme TACE activity kit (CalBiochem).

RNA preparation and quantitative RT-PCR

RNA was prepared using the RNeasy mini-kit (Qiagen). Reverse transcriptions were performed using M-MLV reverse transcriptase and random hexamer oligodeoxynucleotides (Invitrogen). RT-PCR was done using SYBR Green I Dye (Molecular Probes) with primers for Smad7 and PAI-1. Ribosomal protein rPL19 was used as an internal control. Primer sequences are available upon request.

Cell proliferation assay

Cell proliferation was quantified using a BrdU incorporation assay. HaCaT cells were transfected with TACE siRNA or control siRNA. 36 h after transfection, cells were plated in 48-well plates, incubated with or without 2 ng/ml TGF-β for 12 or 36 h in the presence or
absence of 3 μM SB431542, and treated with BrdU for 12 h. For the proliferation assays in T4-2 cells, 20 ng/ml EGF was added to the medium, and cells were exposed to added TGF-β for 24 h or 48 h, and treated with BrdU for 3 h. BrdU assays were performed using the BrdU Cell Proliferation Assay kit (Calbiochem).

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References


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Figure 1.
Activation of the Erk MAP kinase pathway inhibits TGF-β-induced Smad3 phosphorylation and cell surface presentation of TβRI but not TβRII. (A) CHO cells were treated with TGF-β for 30 min. The Erk MAP kinase pathway was activated with PMA for 45 min before adding TGF-β and then during TGF-β treatment, and inhibited using U0126. Smad3 activation was visualized by western blotting for C-terminal Smad3 phosphorylation. (B) Effects of the protein kinase C inhibitor BMI, the Erk MAP kinase pathway inhibitor U0126 and the PI3-kinase inhibitor LY294002 on the PMA-induced decrease of TGF-β-induced Smad3 activation. (C-F) CHO cells were treated with PMA (C-E) or serum (F) for the indicated times (C, D, F) or for 45 min (E), in the absence or presence of the Erk MAP kinase pathway inhibitor U0126.
(C-F) or PD98059 (E), the protein kinase C inhibitor BMI (E) or the PI3-kinase inhibitor LY294002 (E), or the proteasome inhibitor MG-132 (D). Cell surface TGF-β receptors were detected by biotinylation, neutravidin bead adsorption and immunoblotting. In C and F, the second panel from the top is a longer exposure of the top panel, showing the extracellularly truncated TβRI, marked TβRI(TM+C), which is also visualized in D, E. Other panels show western blots of cell lysates for TβRI, TβRII, pErk1/2, Erk1/2 or α-tubulin (C, F).
Figure 2.
TACE regulates the TβRI cell surface levels. (A, B) TAPI-1 inhibits the downregulation of cell surface TβRI level in response to PMA (A) or serum (B). CHO cells were treated with PMA (A) or serum (B) in the absence or presence of TAPI-1. Cell surface TGF-β receptors were detected by biotinylation, neutravidin bead adsorption and immunoblotting. The second panel from the top is a longer exposure of the top panel, showing the extracellularly truncated TβRI, marked TβRI(TM+C). Other panels show western blots of cell lysates for TβRI, TβRII, pErk1/2, Erk1/2 or α-tubulin (C, F). (C, D) Silencing of TACE expression by transfected TACE siRNA inhibits the decrease in cell surface TβRI induced by Erk MAP kinase activation in
response to PMA (C) or serum (D). CHO cells, transfected with TACE siRNA or control siRNA, were treated with PMA (C) or serum (D) and processed as in A and B.
Figure 3.

TACE cleaves TβRI but not TβRII. (A) C-terminally Flag-tagged TβRI or TβRII were coexpressed with wild-type TACE or catalytically inactive TACE E406A. Anti-Flag immunoprecipitated proteins were immunoblotted with anti-Flag, and lysates were probed with anti-Flag or anti-TACE. (B) N-terminally HA-tagged TβRI was coexpressed with TACE. Culture media were subjected to immunoprecipitation and western blot with anti-HA antibody. Cell lysates were probed with anti-HA or anti-TACE. (C) Coincubation of immunopurified TβRI and TACE in vitro resulted in cleavage of TβRI by TACE (middle lane). (D) TACE cleaves TβRI, but not the other type I receptors. TACE was coexpressed with the indicated type I receptors. Cell lysates were subjected to anti-Flag immunoprecipitation and immunoblotting with anti-Flag (top panel), or to anti-TACE immunoblotting (lower panel).
Figure 4.
TACE inhibits TGF-β signaling. (A) CHO cells were treated with or without PMA and/or TGF-β for 30 min as in Fig. 1A, in the presence or absence of TAPI-1. (B) CHO cells were transfected with TACE siRNA or control siRNA, 24 h prior to treatments with PMA and/or TGF-β as in A. Cell lysates were assayed by western blotting for phosphoSmad3, Smad3 or TACE. (C) Silencing of TACE expression enhances the long-term TGF-β-induced Smad3 activation. CHO cells, transfected with TACE siRNA or control siRNA, were treated with TGF-β, and lysates were assayed by immunoblotting for phosphoSmad3, Smad3 and TACE. (D) Silencing of TACE expression enhances the TGF-β-induced Akt activation. CHO cells, transfected with TACE siRNA or control siRNA, were treated with TGF-β for 90 min, and Akt activation was visualized by immunoblotting for pT308-Akt. Control blots show Akt and TACE.
Figure 5.
Inhibition of TACE expression enhances the TGF-β response and inhibition of cell proliferation by TGF-β in HaCaT epithelial cells. (A) Role of TACE in TβRI cell surface presentation. HaCaT, T4-2, MDA-MB-468, Hela, A549 and HepG2 cells were transfected with TACE siRNA or control siRNA. Cell surface TβRI and TβRII were visualized by immunoblotting of biotinylated proteins. Control panels show TβRI and α-tubulin in cell lysates, as well as TACE in cell lysates (MDA-MB-468, Hela and A549 cells) or cell lysates enriched for TACE by ConA precipitation (HaCaT, T4-2 and HepG2 cells). (B) Cell surface levels of TβRI and TβRII in HaCaT cells transfected with TACE siRNA or control siRNA. (C) HaCaT cells, transfected with TACE siRNA or control siRNA, were treated with TGF-β. Cell lysates were...
immunoblotted for phospho-Smad3, Smad3 or TACE. (D) Expression of Smad7 and PAI-1 mRNAs in response to TGF-β was quantified using RT-PCR. (E) Cells were cultured in the presence or absence of TGF-β with or without the TβRI inhibitor SB431542 for 12 h or 36 h. BrdU incorporation during a 12 h period is expressed relative to the untreated control. Error bars were generated from triplicate experiments.
Figure 6.
TACE regulates the epithelial to mesenchymal transition of HaCaT cells in response to TGF-β. (A) HaCaT cells were transfected with TACE siRNA or control siRNA, and treated or not with TGF-β. (B) Effect of the TβRI kinase inhibitor SB431542 on HaCaT cells, transfected with TACE siRNA or control siRNA, and treated or not with TGF-β. Top panels shows the actin organization, and middle and lower panels show E-cadherin and fibronectin detected in the same cells using two color immunofluorescence. Bars, 20 μm.
Figure 7.
TACE downregulates TGF-β signaling in T4-2 breast cancer cells. (A) Treatment of T4-2 cells with PMA or EGF enhances the in vitro proteolytic activity of immunoprecipitated TACE, which is inhibited by treating the cells with the MEK inhibitors PD98059 or U0126. (B) T4-2 cells were treated with PD98059 to inhibit, or with EGF to activate the Erk MAP kinase pathway. Biotinylated cell surface proteins and cell lysates were immunoblotted using the indicated antibodies. (C) T4-2 cells were transfected with TACE siRNA or control siRNA. Cell surface TβRI and TβRII were visualized by immunoblotting of biotinylated cell surface proteins. Cell lysates were immunoblotted for TβRI, TβRII, TACE and α-tubulin. (D) T4-2 cells, transfected with TACE siRNA or control siRNA, were treated with TGF-β. Cell lysates were immunoblotted for phospho-Smad3, Smad3 or TACE. (E) Cells were cultured with or without TGF-β for 24 h or 48 h. The relative proliferation inhibition corresponds to the BrdU incorporation in cells with TGF-β treatment divided by BrdU incorporation in cells without TGF-β treatment. Error bars were generated from triplicate experiments.