Characterization of Growth Factor-induced Serine Phosphorylation of Tumor Necrosis Factor-α Converting Enzyme and of an Alternatively Translated Polypeptide*

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Tumor necrosis factor-α converting enzyme (TACE) is a prototype member of the adamalysin family of transmembrane metalloproteases that affects ectodomain cleavage and release of many transmembrane proteins, including transforming growth factor-α. Growth factors that act through tyrosine kinase receptors, as well as other stimuli, induce shedding through activation of the Erk mitogen-activated protein (MAP) kinase pathway without the need of new protein synthesis. How MAP kinase regulates shedding by TACE is not known. We now report that the cytoplasmic domain of TACE is phosphorylated in response to growth factor stimulation. We also identified a naturally expressed smaller polypeptide corresponding to most of the cytoplasmic domain of TACE. This protein, which we named SPRACT, is derived through alternative translation of the TACE-coding sequence and is, similarly to TACE, phosphorylated in response to growth factor and phorbol 12-myristate 13-acetate stimulation. Phosphoamino acid analysis revealed that growth factor-induced phosphorylation of TACE occurs only on serine and not on threonine or tyrosine. Transgenic mapping experiments coupled with site-directed mutagenesis identified Ser791 as the major target of growth factor-induced phosphorylation, whereas Ser791 undergoes dephosphorylation in response to growth factor stimulation. The phosphorylation of Ser791, but not the dephosphorylation of Ser791, depends on activation of the Erk MAP kinase pathway. Increased SPRACT expression or mutation of the TACE cytoplasmic domain to inactive growth factor-induced phosphorylation did not detectably affect growth factor-induced shedding of transmembrane transforming growth factor-α by TACE. The roles of SPRACT and the cytoplasmic phosphorylation of TACE remain to be defined.

Various cell surface proteins with diverse functions undergo regulated proteolytic cleavage of their extracellular domains, a process that results in ectodomain shedding (for review see Refs. 1–4). The subsequent release of the polypeptide from the cell can have profound consequences not only at the cellular physiological level but also at a more tissue-wide or even systemic level. For example, transmembrane L-selectin is an adhesion protein for leukocytes, and ectodomain cleavage of L-selectin may therefore down-regulate inflammation by limiting neutrophil accumulation and lymphocyte activation (5–8). Because shedding plays such an important role in the regulation of cellular functions, its dysregulation can contribute to disease conditions. Thus, ectodomain cleavage of transmembrane tumor necrosis factor-α (TNF-α) and consequent release of soluble TNF-α is thought to contribute to cachexia and arthritis, which cannot be induced by transmembrane TNF-α (9, 10). In addition, families carrying a non-cleavable TNF-α receptor are presented with severe autoimmune reactions (11). However, despite its importance, the molecular mechanisms underlying ectodomain processes are poorly understood.

Ectodomain shedding also regulates the activities and roles of transmembrane growth factors such as transforming growth factor-α (TGF-α) and its family members. These growth factors are expressed at the cell surface as transmembrane proteins, yet can also be released as soluble growth factors as a result of their ectodomain shedding (12–17). Both the soluble and the transmembrane forms of these growth factors can activate the receptors, i.e. the EGF/TGF-α receptor or related transmembrane tyrosine kinases (13, 18–21). Thus, transmembrane TGF-α or related growth factors only exert autocrine activity and stimulate receptors on adjacent cells and are most likely unable to induce receptor internalization. In contrast, release of soluble ligand following ectodomain shedding allows the cell to induce biological effects, e.g. TGF-α-mediated proliferation, on non-adjacent cells and allows for ligand-induced internalization of the receptors. In addition to these two modes of ligand signaling depending on ectodomain shedding of the transmembrane growth factor, their receptors can also be subject to ectodomain cleavage, thereby down-regulating ligand-induced receptor activation (22–24).

Diverse stimuli are known to activate ectodomain shedding

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† The abbreviations used are: TNF-α, tumor necrosis factor-α; PMA, phorbol 12-myristate 13-acetate; TACE, TNF-α converting enzyme; MAP, mitogen-activated protein; TGF-α, transforming growth factor-α; Erk, extracellular signal-regulated kinase; ADAM, adamalysin; CHO, Chinese hamster ovary; DMEM, Dulbecco’s modified minimal essential medium; FBS, dialyzed fetal bovine serum; PVDF, polyvinylidene difluoride; EGF, epidermal growth factor; HB-EGF, heparin-binding-EGF-like growth factor; FGF, fibroblast growth factor; MEK, MAP kinase/Erk kinase.
Phosphorylation plays a critical role in intracellular signaling. It has been reported that PMA, an artificial shedding activator, can induce TACE phosphorylation (45, 50). Interestingly, MDC9, which shares sequence similarity with TACE in its cytoplasmic domain, is phosphorylated by protein kinase C (39). In one study this phosphorylation was shown to regulate the induction of HB-EGF shedding by PMA (51), whereas other findings indicate that MAP kinase activation (via MAPKAP kinase 2) and TACE shedding through the Erk MAP kinase signaling pathway instead (9, 35). We now show that growth factor or PMA stimulation leads to enhanced phosphorylation of TACE through activation of the Erk MAP kinase signaling pathway. We also identified an alternative translation product of TACE that we name SPRACT. SPRACT corresponds to most of the cytoplasmic domain of TACE and similarly to TACE undergoes regulated phosphorylation. We show that TACE is phosphorylated on serines upon growth factor or serum stimulation. We identified the major site of phosphorylation in response to growth factor stimulation, as well as another serine, which shows decreased phosphorylation upon stimulation. The function of these phosphorylation sites and the role of SPRACT remain to be characterized.

EXPERIMENTAL PROCEDURES

Reagents and Expression Vectors—Recombinant acid fibroblast growth factor (FGF) and EGF were purchased from Calbiochem. Diallyl-folate bovine serum (FBS) was purchased from Invitrogen. The MEK1 inhibitor U0126 and sequencing grade modified trypsin were purchased from Promega (San Luis Obispo, CA). TAPI-1, an inhibitor of metalloproteases, including TACE, was a gift from Dr. T. K. Kishimoto (Boehringer Ingelheim) (30, 39). Phenanthroline, another metalloprotease/TACE inhibitor, was purchased from Sigma (47).

Four polyclonal anti-TACE antibodies were used throughout the course of this study. A rabbit antiserum from QED Bioscience, Inc. (San Diego, CA), and a goat antiserum from Santa Cruz Biotechnology (Santa Cruz, CA) both recognize a carboxyl-terminal sequence. The antibody from QED Bioscience was found preferable for analyzing endogenous TACE because of a higher affinity to protein A-agarose beads in immunoprecipitation and a cleaner background in Western blotting as compared with the antibody from Santa Cruz Biotechnology. The blocking peptide for the antiserum as well as normal rabbit IgG was purchased from Santa Cruz Biotechnology. A rabbit polyclonal antibody raised against the recombinant TACE cytoplasmic domain fused to glutathione S-transferase, and a polyclonal antiserum against the extracellular domain of TACE were generous gifts from Dr. Roy Black (Immunex, Seattle, WA). The expression vector for the wild type TACE was constructed by PCR amplification of the human TACE cDNA cloned in a bacteriophage λ vector that was provided by Dr. Roy Black (Immunex, Seattle, WA). The PCR-amplified cDNA was inserted into the pRK5 vector, which uses the human cytomegalovirus promoter to drive expression of an inserted gene in mammalian cells. Deletion and single or multiple amino acid substitution mutants of the TACE coding sequence were constructed using PCR-based approaches. The sequences of wild type and mutant TACE coding sequences in all expression plasmids were confirmed by automated DNA sequencing performed at the University of California, San Francisco, Biomolecular Resource Center. Information on the plasmid design and construction will be provided upon request. Expression plasmids for constitutively activated MEK1 (ΔN-S218E-S222D) (54) and constitutively activated Erk2 (MCMV5-Erk2-MEK1 LA) (55) were provided by Dr. Nathalie G. Ahn (University of Colorado) and Dr. Melanie H. Cobb (University of Texas Southwestern Medical Center), respectively.

Lines, Culture Conditions, and Transfection—The HeLa S3 cell line and HEK293 cell line were maintained in Dulbecco’s modified minimal essential medium (DMEM) supplemented with 7% fetal bovine serum. CHO cells were cultured in DMEM supplemented with 2 mM proline and 10% fetal bovine serum. Transfection of CHO and HEK293 cells was achieved using the LipofectAMINE and the Plus reagent (Invitrogen). K562,
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THP-1, and U937 cell lines were obtained from the University of California San Francisco Tissue Culture Facility and cultured in RPMI medium supplement with 10% FBS.

In Vivo $^{32}$P and $^{32}$P Labeling of TACE—CHO cells, grown in 6-well plates, were transfected with expression vectors for wild type or mutant TACE and cultured in serum-free medium overnight (32). Metabolic labeling of TACE proteins with $^{32}$Pcysteine/methionine was performed as described previously (32). To detect phosphorylation of TACE in vivo, HeLa S3 cells at 90% confluency in 6- or 10-cm dishes or CHO cells in 6-well plates transiently transfected with a TACE expression vector were subjected to overnight serum starvation before $^{32}$P-labeling, washed with phosphate-free DMEM, and cultured with the DMEM supplemented with 10% (v/v) $^{[32P]}$orthophosphate (PerkinElmer Life Sciences). Transiently transfected 10-cm plates, cells grown on 6-well plates, cells were dissolved by adding SDS-PAGE sample buffer (500 μM/well), removed by scraping, and sonicated for 10 s to shear the genomic DNA. Samples of 20 μl were heated to 100 °C for 5 min and subjected to gel electrophoresis. Proteins were transferred onto PVDF membrane with 0.2-μm pore size (Bio-Rad). We found that TACE is relatively poorly retained by PVDF membranes, especially with those with 0.45-μm pore size, and that the retention was sensitive to the amounts of the detergent Tween 20 in the washing buffer and the duration of washes. The final conditions we adopted for the detection of TACE and SPRACT are as follows. The PVDF membrane was blocked with 5% bovine serum albumin for 2 h, reacted with polyclonal antibodies to the cytoplasmic domain of TACE or its carboxy-terminal sequence at the dilution of 1:1000 for 2 h, washed three times each for 10 min with Tris-buffered saline containing 0.05% Tween 20 (TBST), and then reacted to horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) or mouse anti-goat IgG (Santa Cruz Biotechnology) for 1 h. After three washes as described above, visualization of TACE and SPRACT was achieved by chemiluminescence with the ECL kit (Amersham Biosciences).

Endogenous TACE and SPRACT could also be detected in a similar manner as described above using larger amounts of cell extracts and the more sensitive ECL Plus kit for visualization. However, the increased total cell extracts distorted the gel leading to poor resolution of the protein bands. We found that in HeLa S3 and HEK293 cells this problem could be circumvented by modifying the sample preparation. Confluent cells grown on 6-well plates were extracted with the lysis buffer used for immunoprecipitation (200 μM/well). The lysates were mixed with 0.2 volume of the 5× concentrated sample buffer, and 50 μl of the mixtures were subjected to gel electrophoresis. The remaining steps of the Western blotting to detect the endogenous proteins were as described for transfected CHO cells, except for the use of the ECL Plus kit (Amersham Biosciences).

Evaluation of TGF-α Ectodomain Shedding—TGF-α ectodomain shedding was assessed using a pulse-chase assay with transiently transfected CHO cells, as described (32, 58). The same assay was also adapted for the use of EC2 cells that lack the catalytically active TACE.

RESULTS

TACE Is Phosphorylated in Response to Growth Factor and PMA—Growth factor stimulation induces ectodomain shedding of transmembrane proteins, including TGF-α, TNF-α, and L-selectin, through activation of the Erk MAP kinase signaling pathway (32). Because the transmembrane metalloprotease TACE has been implicated in the cleavage of these transmembrane proteins, we assessed the phosphorylation state of TACE. In extracts prepared from $^{32}$Porthophosphate-labeled HeLa S3 cells, two radioactive TACE bands were detected, a 128- and a 100-kDa band (Fig. 1A). Both TACE bands were also apparent in parallel Western blots of these lysates (Fig. 1B). According to previous studies (47), the 100-kDa band is the mature TACE that is derived from the 128-kDa glycosylated TACE band after the removal of the 196-amino acid prodomain (Fig. 1A).

Treatment of serum-starved cells with FGF, EGF, fetal bovine serum, or PMA for 15 min significantly increased the level of the total TACE protein (Fig. 1A). Although some non-specific bands also underwent increased phosphorylation (Fig. 1A), they were also present in the IgG control immunoprecipitations and were not detected in Western blots using the TACE

CNP mapping. $^{32}$P-Labeled TACE and SPRACT were immunoprecipitated from transiently transfected 10-cm plates of CHO cells, resolved by discontinuous SDS-PAGE as described above, and transferred onto nitrocellulose membranes. The radioactive bands were cut out and incubated with 5 μCi CNBr in 70% formic acid (56). The resulting peptides were resolved by 18% SDS-PAGE, and their phosphorylation was revealed by autoradiography.

Tryptic Peptide Mapping—$^{32}$P-Labeled TACE was prepared as for phosphoamino acid analysis, and phosphorylated TACE was visualized by direct autoradiography of the wet gels. Gel bands containing radio- labeled TACE and SPRACT were excised, cut into 6-8 pieces, and subjected to two 45-min washes at 37 °C with a solution containing equal volumes of acetonitrile and 20 mM ammonium carbonate, followed by overnight in-gel digestion with modified trypsin. The resulting peptides were separated by thin layer electrophoresis followed by thin layer chromatography (56) and visualized by autoradiography.

Western Blotting—For detection of TACE and SPRACT from transfected CHO cells, cells grown on 6-well plates, cells were dissolved by adding SDS-PAGE sample buffer (500 μM/well), removed by scraping, and sonicated for 10 s to shear the genomic DNA. Samples of 20 μl were heated to 100 °C for 5 min and subjected to gel electrophoresis. Proteins were transferred onto PVDF membrane with 0.2-μm pore size (Bio-Rad). We found that TACE is relatively poorly retained by PVDF membranes, especially by those with 0.45-μm pore size, and that the retention was sensitive to the amounts of the detergent Tween 20 in the washing buffer and the duration of washes. The final conditions we adopted for the detection of TACE and SPRACT are as follows. The PVDF membrane was blocked with 5% bovine serum albumin for 2 h, reacted with polyclonal antibodies to the cytoplasmic domain of TACE or its carboxy-terminal sequence at the dilution of 1:1000 for 2 h, washed three times each for 10 min with Tris-buffered saline containing 0.05% Tween 20 (TBST), and then reacted to horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) or mouse anti-goat IgG (Santa Cruz Biotechnology) for 1 h. After three washes as described above, visualization of TACE and SPRACT was achieved by chemiluminescence with the ECL kit (Amersham Biosciences).
serum-starved, labeled with $^{32}$P orthophosphate, and treated with the indicated stimuli. Cells were then lysed, and $^{32}$P-labeled TACE was immunoprecipitated using an antibody recognizing the cytoplasmic domain of TACE obtained from QED Bioscience or normal rabbit IgG. The 128-kDa larger TACE form is the glycosylated TACE zymogen with its prodomain, whereas the 100-kDa band corresponds to the mature form without the prodomain. These TACE forms are marked as $T$ in all figures. The 20-kDa protein is SPRACT, which is marked as $S$ in all figures. $B$, stimulation with growth factors and PMA did not lead to significant changes of the level of TACE protein. HeLa S3 cells were serum-starved, treated with indicated stimuli, and lysed. Western blotting analysis was performed with the same antibody or control rabbit IgG as in A, C, the blocking peptide specifically interferes with the detection of phosphorylated TACE and SPRACT. $^{32}$P labeling of HeLa S3 cells and immunoprecipitation of TACE were performed as described in A. For peptide competition, the estimated molar ratio of peptide to antibody is 1000 to 1, based on the average molecular mass of IgG as 180 kDa. Note only the TACE and SPRACT bands but not the nonspecific bands were blocked by the peptide in the cell lysate prepared from PMA-stimulated cells.

Antiserum (Fig. 1B). Furthermore, they were not competed out in immunoprecipitations in the presence of excess peptide immunogen (Fig. 1C). These results indicate that both the glycosylated pro-TACE and mature TACE are phosphorylated and that EGF, FGF, serum, and PMA, which are known to activate ectodomain shedding, rapidly induce phosphorylation of the TACE cytoplasmic domain.

Growth factor- or PMA-induced phosphorylation of TACE was also examined using CHO cells, transfected to express the cloned human TACE cDNA. CHO cells express a very low level of TACE, but transfection of TACE readily allowed detection of TACE by immunoprecipitation of $^{35}$S-labeled (Fig. 2A) or $^{32}$P-labeled (Fig. 2B) proteins. Besides the 126-kDa precursor form of TACE and the 100-kDa mature TACE, we also detected a 112-kDa form (Fig. 2A). Pulse-chase experiments suggested that the intermediate 112-kDa band is likely to be the unglycosylated TACE precursor (data not shown). Previous studies (47) suggest that TACE matures rather inefficiently. This may explain why the major TACE form detected in transfected CHO cells is the glycosylated full-length TACE precursor. FGF treatment for 15 min significantly increased the TACE phosphorylation level (Fig. 2B), without an effect on the level of TACE protein (Fig. 2A). In these experiments, the phosphorylation was restricted to the larger, glycosylated TACE form (Fig. 2B). Fetal bovine serum and PMA, which also induce ectodomain shedding, also stimulated the level of TACE phosphorylation after 15 min (Fig. 2C). These results indicate that TACE, encoded by the characterized cDNA, is phosphorylated in response to FGF, serum, or PMA as observed with endogenous TACE.

SPRACT, a Polypeptide That Corresponds to the TACE Cytoplasmic Domain—Immunoprecipitation and Western blot analyses of HeLa S3 cell lysates using an antibody against the carboxy-terminal sequence of TACE detected not only the two TACE forms mentioned above but also a 20-kDa protein, which we named SPRACT for “small protein reactive with antibody against the cytoplasmic domain of TACE.” Fig. 1). SPRACT was also detected in 293 human embryonic kidney cells and the U937, THP-1, and K562 hematopoietic cell lines by direct Western blotting or immunoprecipitation followed by Western blotting, using two additional antibodies against the cytoplasmic domain of TACE (data not shown). Additionally, SPRACT was expressed from the cloned TACE cDNA in transfected CHO cells (Fig. 2). Similarly to the TACE forms, SPRACT showed rapidly increased phosphorylation in response to EGF, FGF, serum, or PMA stimulation (Fig. 1A and Fig. 2, B and C), whereas its protein levels remained constant (Figs. 1B and 2A). The detection of SPRACT by antibodies against the cytoplasmic domain of TACE in Western blot analyses rules out the possibility that SPRACT is a TACE-binding protein and suggests that SPRACT may correspond to a cytoplasmic segment of TACE. Consistent with this interpretation, SPRACT was not detected by an antibody that was raised against the extracellular domain of TACE (47) (data not shown). Furthermore, excess peptide to which the anti-TACE antiserum was raised competed out the signals of $^{32}$P-labeled TACE and SPRACT in the extracts of PMA-stimulated HeLa S3 cells (Fig. 1C). It should be noted that SPRACT is poorly retained by the PVDF membrane. Up to 90% of SPRACT can be lost depending on the stringency of the wash condition (data not shown), and therefore the level of SPRACT, detected by Western blotting, may be underestimated.

**SPRACT Is an Alternative Translation Product**—It has been suggested that the active form, but not the full-length precursor form, of TACE can release its cytoplasmic domain through autolysis (47). We therefore included phenanthroline (10 mM), which has been demonstrated to inhibit TACE autolysis (47) and yet also inhibits other metalloproteases, in the cell lysis buffer and throughout the subsequent immunoprecipitation and washes used to generate the results in Fig. 1. The detection of both the mature 100-kDa TACE and 20-kDa SPRACT under these conditions suggests that SPRACT is unlikely to be generated by autolysis during sample preparation. Previous studies (30, 39) have shown that the metalloprotease inhibitor TAPI-I inhibits the cleavage activity of TACE. Inclusion of TAPI-I (10 mM) and phenanthroline (10 mM) in the cell lysis buffer and throughout subsequent washes in immunoprecipitation did not decrease the level of SPRACT in such experiments (data not shown). Additionally, transfection with an expression vector encoding a catalytically inactive TACE mutant, due to replacement of Glu406 with alanine, generated the same level of SPRACT as the expression vector for the wild type catalytically active TACE (Fig. 3A). Furthermore, inclusion of numerous other types of protease inhibitors in the cell
Transfected CHO cells were labeled with [32P]orthophosphate and treated with FGF, and 32P-labeled TACE was immunoprecipitated as described in A. Phosphorylation by dialyzed FBS and PMA or PMA for 15 min. FGF was added into the labeling medium for 15 min. Cells were then lysed, and 35S-labeled TACE was immunoprecipitated using a goat antibody recognizing a carboxyl-terminal sequence of TACE. The 128-kDa larger TACE form is the glycosylated TACE zymogen with its prodomain, whereas the 100-kDa band corresponds to the mature form without the prodomain. The intermediate 112-kDa band is likely to be the unglycosylated TACE precursor. pRK5 is the control vector without cDNA insert. B, stimulation of in vivo TACE phosphorylation by FGF. Serum-starved, TACE-transfected CHO cells were labeled with [35S]methionine/cysteine and treated with FGF, and 35S-labeled TACE was immunoprecipitated as described in A. Only the 128-kDa TACE zymogen among the three TACE forms and SPRACT were visibly phosphorylated. C, stimulation of in vivo TACE phosphorylation by dialyzed FBS and PMA. The experiments were carried out as in B, except that cells were treated with medium only (M) or FBS or PMA for 15 min.

Collectively, these data indicate that SPRACT is an alternative translation product that can be initiated at either juxtamembrane ATG codon in the sequence encoding TACE cytoplasmic domain.

Correlation of TACE and SPRACT Phosphorylation with the Erk MAP Kinase Signaling Pathway—We have shown previously that activation of the Erk MAP kinase pathway mediates growth factor- and PMA-induced ectodomain shedding of TGF-α, TNF-α, and L-selectin. Accordingly, ectodomain shedding in response to these inducers is inhibited by U0126, an inhibitor of MEK1/2 that consequently prevents activation of Erk MAP kinase (32). Our data now show that U0126 also inhibits growth factor-induced phosphorylation of TACE and SPRACT in HeLa S3 cells to a level that is similar to the basal phosphorylation level in the absence of stimulation (Fig. 4A). A similar inhibition of phosphorylation by U0126 was also apparent in CHO cells transfected to express TACE (data not shown).

Thus, growth factor-induced phosphorylation of TACE and SPRACT is mediated through activation of the Erk MAP kinase pathway. U0126 also inhibited PMA-induced phosphorylation of TACE, but only minimally decreased the phosphorylation of SPRACT (Fig. 4A). This suggests that PMA-induced phosphorylation of SPRACT, and possibly TACE, utilizes an additional kinase pathway(s) different from the MEK/Erk MAP kinase signaling pathway.

The effect of U0126 on growth factor-induced phosphorylation also led us to evaluate the effects of constitutively activated forms of MEK1 and ERK2. For this, we cotransfected the TACE expression plasmid together with an expression plasmid for a constitutively active form of MEK1 or Erk2 into CHO cells. As shown in Fig. 4B, overexpression of either enzyme resulted in increased phosphorylation of TACE and SPRACT.

Erk is a proline-directed Ser/Thr kinase that can directly phosphorylate Thr-Pro and Ser-Pro with increased preference when a proline is located at the −2 position (60, 61). Interestingly, Thr735–Pro736 is found as part of a Pro-Gln-Thr735-Pro sequence in the cytoplasmic domain of TACE, raising the possibility that TACE might be a direct target for Erk MAP kinase. To test this, we transfected CHO cells with an expression vector for wild type TACE or a mutant in which the Thr735 was substituted by alanine, and we assessed the phosphorylation of TACE and SPRACT by gel electrophoresis and subsequent quantitation (Fig. 4C). Replacement of Thr735 by alanine did...
not abolish the growth factor- and PMA-induced phosphorylation of TACE and SPRACT. This induction of phosphorylation of the T735A mutant TACE was blocked by the MEK inhibitor U0126 (Fig. 4C), similarly to wild type TACE. This suggests that the phosphorylation of TACE and SPRACT does not result from a direct phosphorylation of TACE by Erk MAP kinase but is more likely mediated by another protein kinase downstream from the Erk MAP kinase. This result does not exclude the
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Possibility that Erk MAP kinase has the ability to directly phosphorylate this or another sequence in TACE. Phosphorylation of Serines in the TACE Cytoplasmic Domain—The cytoplasmic domain of TACE contains 16 serines, 7 threonines, including Thr725, mentioned above, and 1 tyrosine residue (Fig. 5A). Phosphoamino acid analysis of hydrolyzed protein showed that the endogenous forms of TACE isolated from EGF-treated HeLa S3 cells were phosphorylated on serine but not on threonine or tyrosine (Fig. 5B). In addition, only phosphorylated serine was detected in phosphoamino acid analyses of TACE in FBS-stimulated, transfected CHO cells (Fig. 5C). The absence of threonine phosphorylation further supports our conclusion that growth factor-induced phosphorylation does not result from direct phosphorylation of Thr725 by Erk1/2 MAP kinase.

We next set out to define which serines are phosphorylated in response to growth factor stimulation. The cytoplasmic domain of TACE contains 4 methionines including the 2 methionines at positions 715 and 719, which we propose to serve as a dephosphorylation site. TACE phosphorylation. A, amino acid sequence of the carboxy-terminal segment of TACE, composing the transmembrane and cytoplasmic domain. The transmembrane domain is underlined, and the methionine residues whose carboxyl peptide bond is targeted by CNBr are numbered according to their positions in the full-length TACE precursor and shown in boldface. All cytoplasmic serine residues are shown in boldface. In addition, the Ser415 phosphorylation site and the Ser725 dephosphorylation site, identified later, and Thr735 are marked. B, EGF induces only serine but not threonine or tyrosine phosphorylation in both the zymogen (left) and mature (right) forms of TACE. In vivo 32P-labeling and precipitation of endogenous TACE were performed as described in Fig. 2A. TACE forms purified by SDS-PAGE were subjected to HCl hydrolysis. The resulting 32P-phosphoamino acids were resolved by thin layer chromatography using phosphoryserine (PS), phosphothreonine (PT), and phosphotyrosine (PY) as markers. C, serum induces the phosphorylation of overexpressed TACE on serine residue(s) only. In vivo 32P-labeled TACE was immunoprecipitated from serum-stimulated CHO cells transfected to express TACE, purified by SDS-PAGE, and subjected to HCl hydrolysis. The resulting 32P-phosphoamino acids were resolved by thin layer electrophoresis using the phosphoserine, phosphothreonine, and phosphotyrosine markers. D, CNBr mapping of in vivo 32P-phosphorylated TACE and SPRACT. In vivo 32P-phosphorylated TACE was immunoprecipitated from serum-stimulated, transfected CHO cells, purified by SDS-PAGE, and subjected to CNBr hydrolysis. The resulting 32P-peptides were resolved by SDS-PAGE and visualized by autoradiography. Goat anti-TACE polyclonal antibody was used for C and D.

Identification of Phosphorylated Serines in the TACE Cytoplasmic Domain—To identify the phosphorylation sites, we first gel-purified in vivo 32P-labeled, full size TACE, obtained from starved or FGF- or serum-treated, transfected cells, and compared their phosphopeptide maps following trypsin digestion. Comparison of FGF-treated with untreated cells revealed several 32P-labeled peptides, one of which increased and another one decreased in intensity following FGF treatment (Fig. 6A). Serum stimulation resulted in enhanced or decreased 32P-labeled intensities of the same two peptides, whereas some increases in intensities of other minor peptides were noted as well (Fig. 6A). A similar analysis of the corresponding gel segment from mock-transfected cells yielded only one spot, i.e. the one at the start position, as marked in Fig. 6A (data not presented).
shown). Together with phosphoamino acid analysis data shown in Fig. 5, B and C, these results suggest that growth factors induce phosphorylation and dephosphorylation of serine residues.

Because the U0126 inhibitor of MEK1/2 inhibited growth factor-induced shedding (32) and TACE phosphorylation (Fig. 4, A and C), we next assessed its effect on the phosphopeptide distribution. As shown in Fig. 6B, U0126 inhibited the growth factor-induced phosphorylation of the peptide, which normally shows an enhanced phosphorylation level, and did not visibly affect the growth factor-induced dephosphorylation of the other peptides. We therefore conclude that activation of the Erk MAP kinase pathway enhances the phosphorylation of that peptide in response to growth factor stimulation.

We next determined the identity of the two major phosphorylated peptides, i.e. the one with increased and the one with decreased phosphorylation in response to growth factor stimulation. We initially attempted to identify the phosphorylation sites by radioactive microsequencing, but we were unable to generate sufficient in vivo phosphorylated TACE for this purpose. We therefore initiated extensive mutagenesis of the serines following Met715, first in groups of several serine conversions into alanines. The G1 mutation of TACE resulted in replacement of Ser717, Ser718, and Ser723 by alanines, whereas the G2 mutation replaced Ser747 with an alanine. These serines are located within the 4-amino acid Leu716–Met719 and the Asp720–Met730 CNBr digestion fragments, respectively. Phosphoamino acid analyses revealed that these mutations did not affect the ability of serum to enhance or decrease the phosphorylation level of the two peptides, as detected using wild type TACE (data not shown). Two other clustered mutations addressed the phosphorylation states of the serines in the carboxyl-terminal Asp773–Cys824 segment. The G3 mutation replaced Ser785, Ser786, and Ser791, whereas the G4 mutation replaced Ser803, Ser804, and Ser819 by alanines. As shown in Fig. 7A the G3 mutation abolished 32P labeling of the peptide, which normally shows decreased phosphorylation in response to FGF or serum stimulation, and did not affect growth factor-induced phosphorylation of the other peptide. In contrast, the G4 mutation abolished the phosphorylation of the peptide with increased phosphorylation in response to growth factor stimulation (Fig. 7B).

Subsequent analyses using single amino acid mutations allowed the identification of the serines that undergo growth factor-induced phosphorylation or dephosphorylation. In the case of the G3 clustered mutation, single amino acid substitutions in the identified Ser791 as the phosphorylated residue, which shows decreased phosphorylation in response to growth factor stimulation. Thus, Ser791 to alanine mutation abolished phospholabeling of the corresponding peptide in the absence of growth factor stimulation (Fig. 7C; data not shown). Complementary single amino acid mutations led to the identification of Ser819 as the amino acid that shows growth factor-induced phosphorylation. Accordingly, replacement of Ser819 by alanine abolished phospholabeling of the peptide that shows increased phosphorylation in response to growth factor stimulation (Fig. 7D). These results indicate that growth factor stimulation induces the phosphorylation of TACE at Ser819 and dephosphorylation of Ser791. Accordingly, the S819A mutation of TACE showed a low phosphorylation level, when compared with wild type TACE, in the absence of growth factor stimulation, and largely abolished the growth factor-induced phosphorylation of TACE and SPRACT (Fig. 7E).

Together with the data in Fig. 5B, our results strongly suggest that activation of the Erk MAP kinase pathway mediates the enhanced Ser819 phosphorylation in response to growth factor stimulation but had little effect on the dephosphorylation of Ser791.

**TACE Phosphorylation and SPRACT Do Not Affect Growth Factor-induced TGF-α Ectodomain Cleavage**—The identification of Ser819 as the major growth factor-induced phosphorylation site led us to assess its role in growth factor-induced TGF-α shedding. We used the EC2 cell line, which had been derived from genetically modified mice carrying the ΔZn/ΔZn TACE and lacked functional TACE expression (31, 42). Cells, transfected with a TGF-α expression plasmid, did not show growth factor-induced TGF-α release, as measured using our previously established TGF-α ectodomain shedding assay. In contrast, wild type TACE expression conferred a basal level of TGF-α release, which was further enhanced in response to serum stimulation (Fig. 8A). Similarly, the S819A and the G4 mutants of TACE were also able to confer growth factor-induced TGF-α ectodomain shedding. Furthermore, a TACE mutant with its entire cytoplasmic domain, except for the proximal two amino acids deleted, was also able to confer serum-induced TGF-α cleavage (Fig. 8A). We therefore concluded that Ser819 phosphorylation in response to growth factor stimulation or activation of the Erk MAP kinase pathway was not required for growth factor-induced ectodomain shedding by TACE. Furthermore, ectodomain shedding by TACE did not require its cytoplasmic domain.

To evaluate a possible regulatory role of SPRACT in ectodomain shedding, we assessed the effect of overexpressed...
S819A, G4, and the cytoplasmic domain truncated TACE mutants provide equal shedding of TGF-α into medium was immunoprecipitated and quantitated. B. increased SPRACT expression did not affect TGF-α shedding by CHO cells. The cells were transfected with a TGF-α expression plasmid alone (−SPRACT) or together with a plasmid for the ΔED/ΔT mutant (−SPRACT) (see Fig. 3 for schematic sequence presentation). Pulse-chase analyses were carried out as described in A. C. expression of TACE and SPRACT by wild type and mutated TACE forms. EC2 cells transfected with the indicated expression plasmids were metabolically labeled with [35S]cysteine/methionine, and chased with cysteine and methionine with or without dialyzed FBS as a shedding inducer. Soluble TGF-α released into medium was immunoprecipitated and quantitated. C. TACE was not cotransfected. As shown in Fig. 8 A, the basal and serum-induced TGF-α ectodomain shedding was not affected by SPRACT overexpression.

We also assessed a possible role of SPRACT using transfections of EC2 cells. Cells were transfected with an expression vector for wild type TACE, thus generating full-size TACE and SPRACT or the M715A/M719A mutant of TACE, which only expresses TACE but not SPRACT (Fig. 8C, lanes 2 and 3). As is apparent from Fig. 8D, both versions of the TACE expression vectors conferred an equal ability to induce basal and growth factor-induced ectodomain shedding of TACE. We also cotransfected an expression plasmid for the M715A/M719A mutant of TACE with one that only expresses SPRACT, i.e. the ΔED/ΔT plasmid (Fig. 3, B and C). As is apparent from the 4th lane in Fig. 8C, this cotransfection resulted in high expression of SPRACT and a lower level of the M715A/M719A mutant of TACE, when compared with the expression of TACE in the absence of overexpressed SPRACT. This lower level of M715A/M719A mutant TACE expression in the latter cotransfection experiment may be an artifact, because expression of SPRACT did not affect endogenous TACE expression in HEK293 cells (data not shown). As shown in Fig. 8D, coexpression of SPRACT did not affect the basal and growth factor-induced levels of TGF-α ectodomain shedding, as observed with the M715A/M719A mutant of TACE or wild type TACE. Together, these data do not allow us to conclude that coexpression of SPRACT has an effect on ectodomain shedding by TACE.

DISCUSSION

We demonstrated the growth factor-induced phosphorylation of the TACE cytoplasmic domain on serine. We also identified an alternative translation product, named SPRACT, that corresponds to the majority of the TACE cytoplasmic domain. Similarly to TACE, SPRACT undergoes regulated phosphorylation. Our findings represent a first direct demonstration that a natural inducer of ectodomain shedding induces phosphorylation of an ADAM family sheddase. While this manuscript was in preparation, Diaz-Rodriguez et al. (50) reported that PMA induces phosphorylation of the TACE cytoplasmic domain and proposed that PMA- and growth factor-induced phosphorylation occurs through direct phosphorylation of Thr735 in the TACE cytoplasmic domain by Erk MAP kinase.

Both the glycosylated pro-TACE as well as mature TACE with its prosegment removed showed enhanced phosphorylation in response to growth factor or PMA stimulation. In transfected cells that overexpressed TACE only the pro-TACE form was visibly phosphorylated, but this may have resulted from the high predominance of pro-TACE and its inefficient maturation (47). It should be noted that phosphorylation of mature TACE was not demonstrated upon PMA stimulation in previous studies (45, 50). The level of TACE phosphorylation correlated with its shedding activity following induction by growth factor or PMA stimulation.

We have shown that enhanced phosphorylation of TACE upon growth factor stimulation required induction of the Erk MAP kinase signaling pathway. Accordingly, U0126, an inhibitor of MEK1/2 which prevents the activation of Erk MAP kinase, inhibits growth factor-induced phosphorylation of TACE and SPRACT to a level similar to that in the absence of stimulation. Our previous results demonstrated that activation of the Erk MAP kinase pathway mediates growth factor-induced shedding of TGF-α, TNF-α, and L-selectin. Therefore, activation of MEK1/2 and the downstream Erk MAP kinase is required for growth factor-induced phosphorylation of the TACE cytoplasmic domain and ectodomain cleavage (32). In contrast, inhibition of the MEK1/2 activity affected only minimally the PMA-induced phosphorylation of SPRACT and possibly TACE, suggesting the involvement of an additional kinase pathway different from the MEK/Erk MAP kinase signaling pathway in PMA-induced phosphorylation. In addition, constitutively active forms of either MEK1 or Erk2 induced enhanced TACE phosphorylation and activated ectodomain shedding.

We demonstrated growth factor-induced phosphorylation of the TACE cytoplasmic domain on serine only but not on threonine or tyrosine. Extensive mutagenesis identified Ser791 as the major target for phosphorylation in response to FGF and serum and Ser791 as the major site for growth factor-induced
Serine Phosphorylation and Alternative Translation of TACE
dephosphorylation. Accordingly, the SS19A mutant has lost the ability to increase the phosphorylation level upon growth factor stimulation. Additionally, the phosphorylation of Ser^{791} is markedly reduced upon the treatment. The dephosphorylation of Ser^{791} is not inhibited by U0126, suggesting that it is independent of the Erk MAP kinase pathway. In contrast, growth factor-induced Ser^{819} phosphorylation is inhibited by U0126 and thus depends on activation of the Erk MAP kinase pathway.

The two phosphorylation sites that we identified in the human TACE can also be found in the mouse, rat, and hamster TACE sequences. Sequence comparison of the cytoplasmic domains of other adamalyses did not reveal conservation of a site similar to the one flanking the phosphorylated Ser^{791} in TACE. Interestingly, the carboxyl-terminal phosphorylated Ser^{819} is near a motif that was identified to interact with PTPH1, which was suggested by overexpression to down-regulate TACE expression and TNF-α shedding (49). It is therefore possible that the phosphorylation of Ser^{819} plays a role in the regulation of TACE processing. In addition, we noticed that the Ser-Lys dimer that composes Ser^{819} exists with unusually high frequencies in the ADAM family. At least 13 of the 30 adamalyses with cytoplasmic domains ranging from 4 (ADAM 26) (62) to 196 amino acids (ADAM 19) (63) have at least one Ser-Lys sequence, with ADAM 30 containing 6 Ser-Lys repeats in its 85 amino acid cytoplasmic domain (64). Therefore, whether this sequence functions as a signaling motif is worth addressing.

While this manuscript was in preparation, Diaz-Rodriguez et al. (50) demonstrated that TACE shows increased phosphorylation on serine and threonine in response to PMA, and that Thr^{735}, located within a favorable MAP kinase consensus site, can be directly phosphorylated by Erk MAP kinase in response to PMA. We did not find phosphorylation on threonine in response to growth factor stimulation, and mutation of Thr^{735} did not affect growth factor- or PMA-induced phosphorylation of TACE and SPRACT. The basis for this discrepancy is unclear but may be related to the use of different cell types and the use of PMA at a 1 μM concentration by Diaz-Rodriguez et al. (50), whereas we used 20 nM PMA as inducer.

Our use of TACE and protease inhibitors, as well as defined experimental conditions and the use of a catalytically inactive TACE, suggested that SPRACT was not derived from autolytic or proteolytic degradation of TACE either in the cell or during sample preparation. Instead, SPRACT appears to be expressed through translational initiation from proximal AGT codons in the TACE cytoplasmic domain. Consequently, mutation of the two proximal ATGs encoding Met^{716} and Met^{719} abolished the expression of SPRACT. Initiation at internal ATGs within an open reading frame has been observed previously (65–68) in several cellular mRNAs and, more commonly, in viral RNAs. The expression of SPRACT in untransfected human cell lines as well as in transfected cells suggests that SPRACT is a physiological endogenous protein. The poor retention of SPRACT by PVDF membrane may explain why it was not recognized previously. Similarly to TACE, SPRACT underwent regulated phosphorylation in response to growth factor or PMA stimulation, and CNBr cleavage yielded the same phosphorylated peptide(s) as TACE. These data suggest that SPRACT may be phosphorylated and dephosphorylated on the same residues as TACE, and raise the possibility of a regulatory role for SPRACT in the function of TACE.

We have been unable to define a function for SPRACT and for the growth factor-induced phosphorylation of TACE and SPRACT. Mutation of Ser^{819} into Ala or replacement of Ser^{803}, Ser^{806}, and Ser^{819} by alanines (the G4 mutation) did not de-
