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Phorbol Ester-induced Shedding of the Prostate Cancer Marker Transmembrane Protein with Epidermal Growth Factor and Two Follistatin Motifs 2 Is Mediated by the Disintegrin and Metalloproteinase-17*[S]

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The transmembrane protein with epidermal growth factor and two follistatin motifs 2 (TMEFF2) is expressed in prostate and brain and shed from the cell surface in a metalloproteinase-dependent fashion. Neither the sheddase(s) responsible for TMEFF2 shedding nor the physiological significance or activity of the soluble TMEFF2 ectodomain (TMEFF2-ECD) has been identified. In the present study we present new evidence that a disintegrin and metalloproteinase-17 (ADAM17) is responsible for phorbol 12-myristate 13-acetate-induced release of TMEFF2-ECD using small interfering RNA to ablate ADAM17 expression or by inhibiting enzymatic activity. A single well shedding assay monitoring the release of alkaline phosphatase-tagged TMEFF2-ECD into medium and the generation of 22- and 14-kDa C-terminal fragments in lysates were dependent on ADAM17 activity. A γ-secretase inhibitor prevented the formation of a 10-kDa fragment in cell lysates, thus establishing TMEFF2 as a novel substrate for regulated intramembrane proteolysis. We assigned proliferation-inducing activity to TMEFF2. Inhibition of TMEFF2 shedding using synthetic metalloproteinase inhibitors or small interfering RNA targeting TMEFF2 expression yielded a statistically significant reduction of cell proliferation in the lymph node-derived prostate cancer cells (LNCaPs) and a human embryonic kidney (HEK293) cell line overexpressing TMEFF2. The TMEFF2-ECD was able to induce ERK1/2 phosphorylation in an epidermal growth factor receptor (or ErbB1)-dependent manner in HEK293 cells. Our data suggest that TMEFF2 contributes to cell proliferation in an ADAM17-dependent autocrine fashion in cells expressing this protein.

The transmembrane protein with epidermal growth factor and two follistatin motifs 2 (TMEFF2)[2] gene encodes a modular protein consisting of two follistatin modules, an epidermal growth factor (EGF)-like repeat, and a transmembrane domain connected to a short cytoplasmic tail with a potential G-protein activation motif. TMEFF2 is highly up-regulated in 74% of primary prostate cancer (PCa) and 42% of metastatic lesions from lymph nodes and bone irrespective of hormonal disease status (1). TMEFF2 expression correlates with onset of cellular proliferation after castration in the CWR22 PCa mouse model (2) as well as in the TEN12 xenograft model (3), suggesting that it is not a tumor suppressor (1). However, in a different LNCaP human PCa progression model opposing results were obtained where TMEFF2 expression was highest in low grade disease and down-regulated in aggressive metastatic C4-2 xenografts (4). TMEFF2 expression is absent in androgen-independent PCa cell lines PC3 and DU145 (5). When TMEFF2 expression was re-established in PC3 and DU145 cell lines by stably transfecting these cell lines with TMEFF2 cDNA a 50% decrease in cell proliferation was observed (5), but this finding could not be corroborated by Afar *et al.* (1).

The biological function of TMEFF2 in PCa is unknown, but it has been implicated in cell signaling (6), neuronal cell survival (7), tumor suppression (5, 8, 9), and Alzheimer disease (10). Uchida *et al.* (6) showed that the TMEFF2-ECD induced tyrosine phosphorylation of ErbB4, an epidermal growth factor receptor/ErbB family member in MKN28 gastric cancer cells. However, it is unlikely that the TMEFF2-ECD is able to induce ErbB4 phosphorylation in PCa as this receptor tyrosine kinase is not expressed in PCa cell lines (11, 12).

TMEFF2 ectodomain shedding is induced by proinflammatory cytokines such as interleukin-1 and tumor necrosis factor-α (TNF-α) in A172 glioma cells (13) and is mediated by unknown metalloproteinase(s). This finding implied to us that TMEFF2 release may be the result of proteolytic processing by membrane-anchored metalloproteinases of the ADAM (a dis-

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[2] The abbreviations used are: TMEFF2, transmembrane epidermal growth factor repeat protein with two follistatin motifs 2; TMEFF2-ECD, TMEFF2 ectodomain; ADAM, a disintegrin and metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; siRNA, small interfering RNA; PMA, phorbol 12-myristate 13-acetate; EGF, epidermal growth factor; PCa, prostate cancer; TNF, tumor necrosis factor; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AP, alkaline phosphatase; AP-TMEFF2, AP-tagged TMEFF2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ANOVA, analysis of variance; HA, hemagglutinin; MBP, maltose-binding protein; ST14, suppressor of tumorigenicity 14; LNCaPs, lymphnode-derived prostate cancer cell line; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine-*t*-butyl ester.

integrin and metalloproteinase) family as these enzymes are major regulators of protein ectodomain shedding (for a review, see Ref. 14). An increasing number of transmembrane proteins are released from the cell surface by ectodomain shedding, which is mainly but not exclusively mediated by members of the ADAM family of metalloproteinases (14). The substrate spectrum of the ADAM family reaches from syndecans, cadherins, the hyaluronan receptor CD44, the tumor necrosis factor receptor, fractalkines, mucins, FAS ligand, the receptor activator of NFκB ligand (RANKL), Notch, the amyloid β precursor protein, ephrins to EGF-like growth factors, their receptors ErbB2 and ErbB4 to receptor tyrosine phosphatases, vascular adhesion molecule, and other proteins (15–31). Therefore the biological spectrum of ADAM function is currently rapidly expanding as these enzymes play important roles in regulating signaling, cell adhesion or repulsion, migration, proliferation, and cell death. ADAMs play fundamental roles in regulating development, morphogenesis, and diseases such as arthritis and cancer. The proteolytic activity of ADAMs is regulated in part by the tissue inhibitors of metalloproteinases (TIMPs) with TIMP3 displaying the broadest range of inhibitory capability toward various ADAM family members, such as ADAM10, ADAM17, or the soluble ADAM12 variant (32–34), whereas others are not regulated by TIMPs. These initial *in vitro* findings are now increasingly corroborated by interesting phenotypes from the TIMP3-null mice demonstrating a broad range of activities linking TIMP3 loss with increased ADAM or ADAM with thrombospondin motifs (ADAMTS) activity in affected tissues, such as heart, joints, and the immune system among others (35–39).

In cancer, a major function of ADAMs is to regulate the bioavailability of the ErbB ligands, thereby triggering signal transmission through the ErbB receptor tyrosine kinase network (14, 26, 40–42). Some ligands are able to convey signals in juxtacrine mode when still attached to the cell membrane (43), but there is now ample evidence that ectodomain release from the cell surface is a prerequisite for most ligands to signal via their appropriate ErbB receptor tyrosine kinases (44–46). The corresponding membrane-associated ligands therefore mostly represent inactive precursors. Aberrant ErbB signaling has been identified as a contributor to malignant transformation where these proteins contribute to aggressive cancer phenotypes (47). Therapeutic approaches now target breast cancer with anti-ErbB2 antibodies in overexpressing tumors, and small synthetic ErbB1 receptor tyrosine kinase inhibitors are used to block aggressive growth of lung cancer (40, 48, 49).

We hypothesized that the release of the TMEFF2-ECD would result in downstream signaling events that may drive proliferation in cells expressing this protein and possibly activate ErbB signaling due to the amount of homology between TMEFF2 and the EGF-like/neuregulin family of growth factors. Here we present evidence that TMEFF2 is a new ADAM17 and γ-secretase substrate and demonstrate for the first time that the TMEFF2-ECD has growth promoting activity in an HEK293 TMEFF2 overexpression model system by activating ERK1/2 phosphorylation in an ErbB1-dependent manner. Our work implies that ADAM17 and TMEFF2 targeting would benefit PCa sufferers.

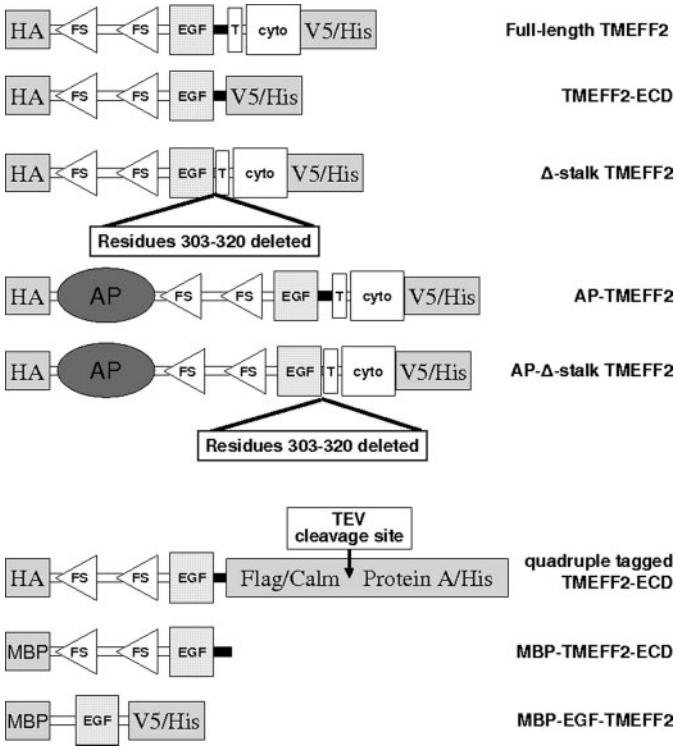

FIGURE 1. **Schematic representation of mammalian TMEFF2 expression constructs and maltose-binding fusion proteins of the complete or the EGF-like domain of TMEFF2.** *FS*, follistatin module; *EGF*, EGF-like repeat; *T*, transmembrane domain, *cyto*, cytoplasmic tail; *HA*, HA epitope tag; *V5*, V5 epitope tag; Flag, FLAG epitope tag; *Calm*, calmodulin binding domain; *His*, His_6 tag; *black box*, juxtamembrane stalk sequence motif (residues 303–320).

EXPERIMENTAL PROCEDURES

Reagents—Synthetic metalloproteinase inhibitors, the γ-secretase inhibitor DAPT were purchased from Calbiochem. Anti-TMEFF2 antibody was from R&D Systems, and anti-V5 epitope antibody was from Invitrogen. Anti-mouse or anti-rabbit secondary peroxidase-conjugated antibodies were from Jackson ImmunoResearch Laboratories. The protease inhibitor cocktail and the ErbB1 inhibitor tyrphostin (AG1478) were from Sigma. The generation of TMEFF2 expression constructs, overexpression cell lines, and shedding conditions are detailed in the supplemental information. Expression constructs are schematically shown in Fig. 1.

Analysis of TMEFF2 Shedding by Western Blotting—Cells were seeded at the indicated cell densities in 6-well plates (see figure legends) and grown overnight. Serum-containing growth medium was removed, and monolayers were washed with phosphate-buffered saline prior to treatment with various compounds in serum-free medium for variable periods of time as indicated in the respective figure legends. Medium was removed, cells were washed twice with phosphate-buffered saline, and lysed in RIPA buffer (20 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1% Triton X-100 supplemented with proteinase inhibitor mixture (Sigma) and 250 μg/ml sodium vanadate). Cell debris were removed by centrifugation, and protein content was determined using the DC protein assay (Bio-Rad) prior to equal loading of lysates for 12.5 or 10% SDS-PAGE and Western blotting. Proteins were detected using anti-TMEFF2 (1:1,000), or anti-V5 (1:5,000) primary antibodies with appro-

priate secondary antibodies conjugated to horseradish peroxidase (1:5,000 or 1:10,000) allowing visualization using enhanced chemiluminescence (Pierce). Equal loading was verified using a mouse monoclonal GAPDH antibody (at 1:20,000; Advanced Immunochemical).

Quantifiable Shedding of Alkaline Phosphatase (AP)-tagged TMEFF2 (AP-TMEFF2)—To be able to sensitively follow TMEFF2 shedding we used the AP-TMEFF2-expressing cell line to detect release of AP activity into the medium during PMA-induced shedding. The assay was performed as described by Sahin *et al.* (26, 50) for AP-tagged ErbB ligands.

Knockdown of ADAMs by siRNA—The following siRNA oligonucleotides were used to ablate ADAM-9, -10, and -17 gene expression: control, UAGCGACUAAACACAUCAAUU; ADAM-17, GAGGAAGCAUCUAAAGUUUUU; ADAM-10, GGACAAACUUAACAACAAUUU; and ADAM-9, AAUGUACAAGAGAUACCUGUA.

The siRNA transfections were performed according to Dharmacon's protocol. Briefly cells were seeded at 6×10^5 cells/well in polylysine-coated 6-well dishes and grown overnight. 4 μl of Dharmafect transfection reagent was diluted in 200 μl of serum-free medium prior to addition of prediluted 100 nM siRNA and incubation for 20 min. Spent growth medium was removed and replaced with 1.6 ml of fresh medium and 400 μl of the siRNA transfection mixture. Transfections were repeated 48 h later prior to induction of TMEFF2 shedding following a total of 4 days of siRNA treatment. Results shown are representative for two independent experiments performed in duplicate.

Knockdown of TMEFF2 in LNCaPs and TMEFF2-overexpressing HEK293 Cells Using siRNA and Analysis of Cell Proliferation—TMEFF2-overexpressing HEK293 cells and LNCaP cells were seeded at 1×10^4 cells/well using a 96-well plate. The next day siRNA transfections were carried out using Dharmacon SmartPool siRNA (M-010654-00-0020). The SmartPool siRNA targets the coding sequences for the extracellular domain of TMEFF2. A dose of 100 nM was used. For each cell line two independent experiments with 15 repeats for each treatment were analyzed using the MTT assay 4 days later. Alternatively TNF-α-induced shedding was analyzed in siRNA-treated LNCaP and HEK293 cells expressing TMEFF2 by Western blot analysis of conditioned medium.

Cell Proliferation Assays—Cells overexpressing full-length TMEFF2 or TMEFF2-ECD were seeded at 2,500 cells/well in a 96-well plate and grown overnight. The next day the medium was removed and replaced with 1% serum-containing medium supplemented with solvent or TNF-α protease inhibitor (TAPI-1) to prevent shedding in case of full-length TMEFF2. Following a 4-day growth period, 30 μl of MTT solution (5 mg/ml; Sigma) was added to each well prior to addition of 100 μl of lysis solution 4 h later. Following a 1-h incubation period, plates were read at 570 nm. Alternatively experiments were performed using non-transfected HEK293 cells and purified recombinant TMEFF2-ECD.

Analysis of ErbB1-dependent ERK1/2 Phosphorylation by TMEFF2-ECD and EGF-TMEFF2—Cells grown in 24-well plates were serum-starved overnight prior to stimulation with the indicated amounts of TMEFF2-ECD in the appropriate growth medium in the presence or absence of AG1478. Cells were harvested into SDS sample buffer (1% SDS, 1% dithiothreitol, 62.5 mM Tris/HCl, pH 6.8, 10% glycerol, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 0.01% bromphenol blue), heated to 100 °C for 5 min, and analyzed by SDS-PAGE and Western blotting as described above but using 1% Blocking Agent (Roche Applied Science) in 50 mM Tris/HCl, pH 7.5, 150 mM NaCl containing 0.05% Tween 20. The membranes were probed overnight with mouse antibodies against phosphorylated ERK1/2 (Thr^{202}/Tyr^{204}; 1:5,000; Cell Signaling Technologies) in Blocking Agent (Roche Applied Science). Washed membranes were incubated for 1 h with secondary anti-mouse IgG-peroxidase conjugate in Blocking Agent and developed as described above. To test for uniform gel loading and transfer to the membrane, the blots were then reprobed with an anti-GAPDH antibody diluted 1:20,000 in Blocking Agent for 1 h and detected as above.

Statistical Analysis—Statistical analysis was performed using ANOVA with post-test Tukey analysis for experiments with more than one variable. Student's *t* test was performed for experiments with one variable only. Proliferation experiments were repeated twice.

RESULTS

Shedding of TMEFF2 Is Induced by TNF-α in LNCaPs and TMEFF2-overexpressing HEK293 Cells

To confirm that the introduction of an IgG secretion sequence, N-terminal HA tag, and C-terminal V5/His tags had no consequences on TNF-α-induced TMEFF2 shedding we compared TMEFF2-ECD release in LNCaPs, which naturally express this protein, with shedding of tagged TMEFF2 from stably transfected HEK293 cells. Cells were grown to 90% confluency prior to TNF-α stimulation of TMEFF2 shedding in serum-free conditions for 12 h. These conditions allowed accumulation of sufficient soluble TMEFF2-ECD into the medium. Concentrated medium was analyzed by Western blotting using a commercial TMEFF2 antibody that recognizes the ectodomain of the protein. As shown in Fig. 2*A* TNF-α treatment of both LNCaPs and HEK293 cells expressing tagged TMEFF2 resulted in a dramatic increase of soluble TMEFF2-ECD in conditioned medium (Fig. 2*A*, *lanes 2* and *4*) when compared with non-stimulated controls (Fig. 2*A*, *lanes 1* and *3*). This is in good agreement with data published previously (3), and we will refer to HA- and V5/His-tagged TMEFF2 as TMEFF2 from now on for simplicity. Additional experiments were performed to analyze shedding in these two cell lines using the phorbol ester PMA. As shown in Fig. 2*B* (*lane 6*) PMA-induced TMEFF2 shedding in the TMEFF2 overexpression HEK293 cell model. LNCaP cells showed similar results but started to undergo apoptotic changes in the presence of PMA (not shown), and thus further PMA shedding experiments were performed with TMEFF2-overexpressing HEK293, Chinese hamster ovary, or PC3 cell model systems.

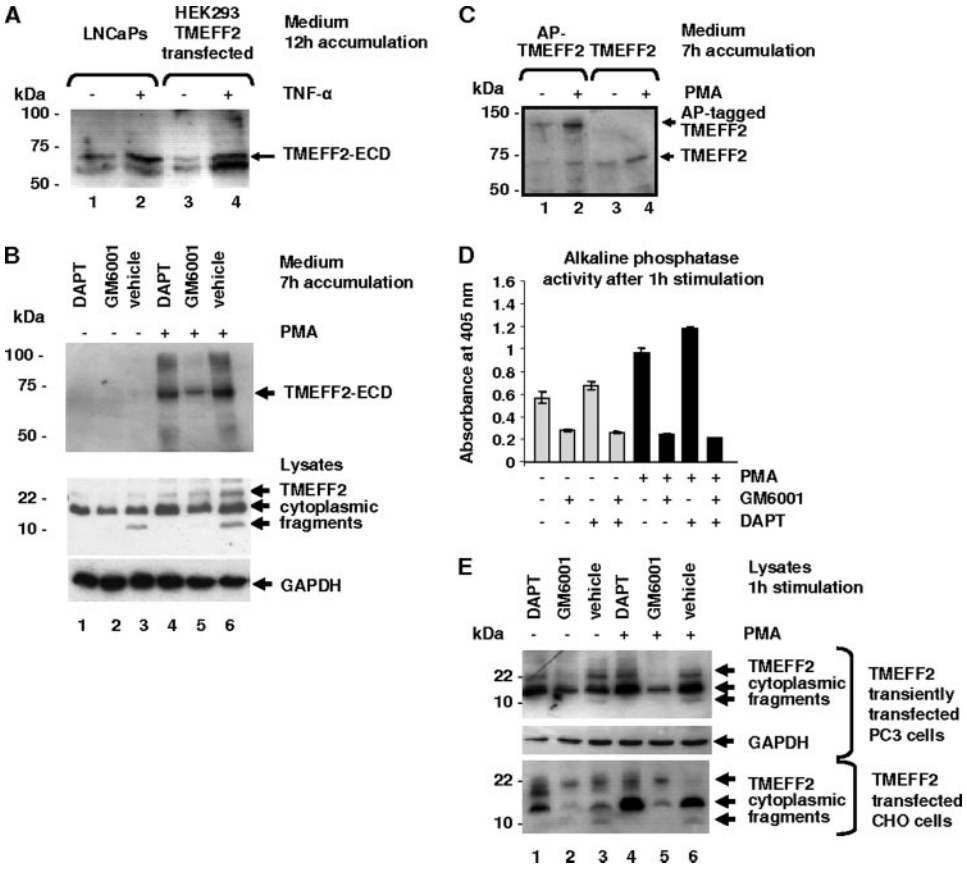

FIGURE 2. *A*, TMEFF2 shedding is induced by TNF-α in LNCaPs or HA-TMEFF2-V5/His-overexpressing HEK293 cells: detection of soluble fragments in medium. *Lanes 1* and *3*, controls; *lanes 2* and *4*, medium following TNF-α treatment for 12 h. Specific bands were identified at 75 and 60 kDa that correspond to the expected molecular mass of the TMEFF2-ECD. *B*, PMA-induced TMEFF2 shedding is dependent on metalloproteinase activity: analysis of medium and lysates from double tagged TMEFF2-expressing HEK293 cells following 7-h accumulation of fragments. *Lanes 1–3*, unstimulated cells treated with 5 μM DAPT (*lane 1*), 50 μM GM6001 (*lane 2*), or Me_2SO vehicle (*lane 3*). *Lanes 4–6*, PMA-treated cells treated with 5 μM DAPT (*lane 4*), 50 μM GM6001 (*lane 5*), or Me_2SO vehicle (*lane 6*). Medium was concentrated 10-fold and analyzed using anti-TMEFF2 antibody (*top panel*). Lysates were analyzed using the V5 epitope antibody to stain for C-terminal fragments (*middle panel*). A GAPDH loading control is shown to demonstrate equal loading of lysates (*bottom panel*). *C*, AP-TMEFF2 shedding is induced by PMA: analysis of medium following 7-h accumulation of fragments. *Lanes 1* and *2*, AP-TMEFF2: *lane 1*, vehicle control; *lane 2*, PMA-stimulated. *Lane 3* and *4*, TMEFF2: *lane 3*, vehicle control; *lane 4*, PMA-stimulated. The Western blot is stained with anti-TMEFF2 antibody. *D*, AP-TMEFF2-ECD release after 1-h induction with PMA is blocked by GM6001. Single well shedding assays were performed using eight replicas for each treatment and a 1-h incubation period for PMA stimulation (50). Alkaline phosphatase activity was monitored by hydrolysis of *p*-nitrophenyl phosphate. Background AP activity from non-transfected cells was subtracted. Data are represented as mean ± S.E. of six replicas, and inhibitor treatment is indicated. *E*, C-terminal TMEFF2 processing depends on both metalloproteinase and γ-secretase activities: analysis of lysates from transiently transfected PC3 and stably transfected Chinese hamster ovary (*CHO*) cells. The inhibitor treatment and staining with V5 epitope antibody was performed as described in *B*, but lysates were prepared following 1-h PMA treatment. *Lanes 1–3*, constitutive TMEFF2 shedding; *lanes 4–6*, PMA-induced processing. *Lanes 1* and *4*, DAPT-treated cells show complete loss of the 10-kDa product; *lanes 2* and *5*, GM6001 inhibition results in reduction in intensity for the 22- and 14-kDa metalloproteinase fragments and complete loss of the 10-kDa γ-secretase product; *lanes 3* and *6*, vehicle controls showing all three fragments. A GAPDH loading control is shown for PC3 cell lysates.

Phorbol Ester (PMA)-induced TMEFF2-ECD Release Is Dependent on Metalloproteinase Activity but Not on γ-Secretase Activity

To test the hypothesis that TMEFF2 is shed from the cell surface by members of the metalloproteinase family we analyzed constitutive and PMA-induced processing in the presence and absence of the synthetic metalloproteinase inhibitor GM6001 or the γ-secretase inhibitor DAPT in HEK293 cells expressing TMEFF2 or AP-TMEFF2 (see Fig. 1 for details). Cells were stimulated for 1 h with PMA in serum-free conditions followed by removal of PMA to prevent toxic effects and a further 7-h incubation period in the presence or absence of the inhibitors to allow accumulation of TMEFF2-ECD in the medium. As shown in Fig. 2*B* (*Medium panel*) PMA strongly induced release of TMEFF2-ECD (Fig. 2*B*, *lane 6*), which was inhibited in the presence of GM6001 (Fig. 2*B*, *lane 5*). In contrast, the γ-secretase inhibitor DAPT had no effect on the release of TMEFF2-ECD into the medium (Fig. 2*B*, *Medium panel*, *lane 4*). To be able to quantitate TMEFF2-ECD release we compared AP-TMEFF2 shedding with TMEFF2 release from the cell surface following PMA stimulation. As shown in Fig. 2*C* AP-TMEFF2 was processed in a manner similar to that of TMEFF2 but released a 140-kDa fragment into the medium corresponding to AP-TMEFF2-ECD following a 7-h accumulation period. We then used the AP-TMEFF2-expressing cells to quantify AP-TMEFF2-ECD release in single well shedding assays described by Sahin *et al.* (50) to confirm the effects of inhibitor treatment as described above using only a 1-h PMA stimulation. These experimental conditions were sensitive enough to allow us to monitor constitutive as well as PMA-induced AP-TMEFF2-ECD release, which was not possible by Western blotting. As shown in Fig. 2*D* PMA-induced or constitutive release of AP-TMEFF2-ECD into the medium was inhibited by GM6001 treatment (56% of constitutive release). In contrast, the γ-secretase inhibitor DAPT was unable to block TMEFF2-ECD release as expected. Additional experiments were performed using a combination of GM6001 and DAPT, which also inhibited TMEFF2-ECD cleavage from the cell surface and reached the same value as GM6001 treatment alone (Fig. 2*D*).

Generation of C-terminal TMEFF2 Fragments Is Affected by Both Metalloproteinase and γ-Secretase Inhibitors

To determine whether the metalloproteinase and γ-secretase inhibitors affected the formation of C-terminal TMEFF2 fragments we analyzed cell lysates by Western blotting. Analysis of lysates from non-stimulated and PMA-induced cells following a 7-h accumulation period showed the presence of 22-, 14-, and 10-kDa fragments in solvent control lanes (Fig. 2*B*, *lane*

3 and *6*). Although the metalloproteinase as well as the γ-secretase inhibitor prevented the formation of the smallest, 10-kDa fragment, there were only minor differences between treated and non-treated controls. This suggested to us that shorter incubation periods were required to assess inhibitor efficacy. Therefore 1-h PMA stimulation in the presence and absence of the above inhibitors was used and analyzed by Western blotting for the presence of anti-V5 immunoreactive bands in transfected Chinese hamster ovary and PC3 cells (Fig. 2*E*) or TMEFF2-expressing HEK293 cells (not shown). GM6001 inhibited the generation of the three C-terminal fragments of 22, 14, and 10 kDa in size (Fig. 2*E*, *lanes 2* and *5*) under non-stimulated and PMA-stimulated conditions when compared with each appropriate control (Fig. 2*E*, *lanes 3* and *6*). Furthermore the γ-secretase inhibitor DAPT blocked the formation of the small 10-kDa fragment, whereas it led to the accumulation of the 14-kDa fragment in the 1-h treatment period (Fig. 2*E*, *lanes 1* and *4*) when compared with the solvent control or PMA-treated samples, respectively (Fig. 2*E*, *lanes 3* and *6*). An increase in full-length TMEFF2 in cell lysates was not apparent by Western blot analysis (not shown), which is in good agreement with published data with other proteins such as the amyloid precursor protein-like protein (APLP2) (51). This reflects that only cell surface protein is cleaved from the cell membrane, representing a fraction of the total protein. Our data suggest that both the minor 22- as well as the major 14-kDa fragment represent products formed by metalloproteinase-dependent cleavage of TMEFF2, whereas the 10-kDa fragment is the product of γ-secretase-dependent cleavage. Metalloproteinase cleavage is a prerequisite for γ-secretase-dependent processing within the membrane plane (52).

TIMP inhibition studies revealed that neither TIMP1 nor TIMP2 were able to block the generation of the 14-kDa main metalloproteinase C-terminal TMEFF2 fragment as revealed by Western blot analysis of cell lysates (Table 1). This indicated to us that neither soluble nor membrane-bound matrix metalloproteinases play a role in generating the 14-kDa metalloproteinase fragment. In contrast, TIMP3 and TAPI-1, which are effective inhibitors of ADAMs, prevented the generation of the 14-kDa C-terminal TMEFF2 metalloproteinase fragment indicating that ADAMs may be responsible for its formation (Table 1). In conclusion, TMEFF2 represents a novel substrate for regulated intramembrane proteolysis.

TABLE 1

Inhibition of the generation of the 14-kDa metalloproteinase fragment by synthetic and natural metalloproteinase inhibitors

Inhibitor	Inhibition
	%
TAPI-1	94
TIMP1	No inhibition
TIMP2	No inhibition
TIMP3	90

Identification of the TMEFF2 "Sheddase(s)"

Having established that PMA-induced TMEFF2 shedding is dependent on a TIMP3-sensitive metalloproteinase we set out to identify the proteinase(s) responsible for its processing using siRNA technology. For this purpose siRNA targeting of ADAM9, ADAM10, and ADAM17 was used to ablate expression of these transmembrane metalloproteinases. First we confirmed that ADAM10 and ADAM17 expression was significantly down-regulated by the respective siRNA toward each ADAM. As shown in Fig. 3*A* the signal intensity for ADAM17 protein was dramatically reduced in ADAM17 siRNA-treated cells (Fig. 3*A*, *lane 1*), whereas ADAM17 protein was not affected by either the scrambled control siRNA or ADAM10 siRNA (Fig. 3*A*, *lanes 2* and *3*). ADAM10 protein levels were also specifically down-regulated by siRNA targeting ADAM10 (Fig. 3*A*, *lane 5*), whereas scrambled control siRNA or

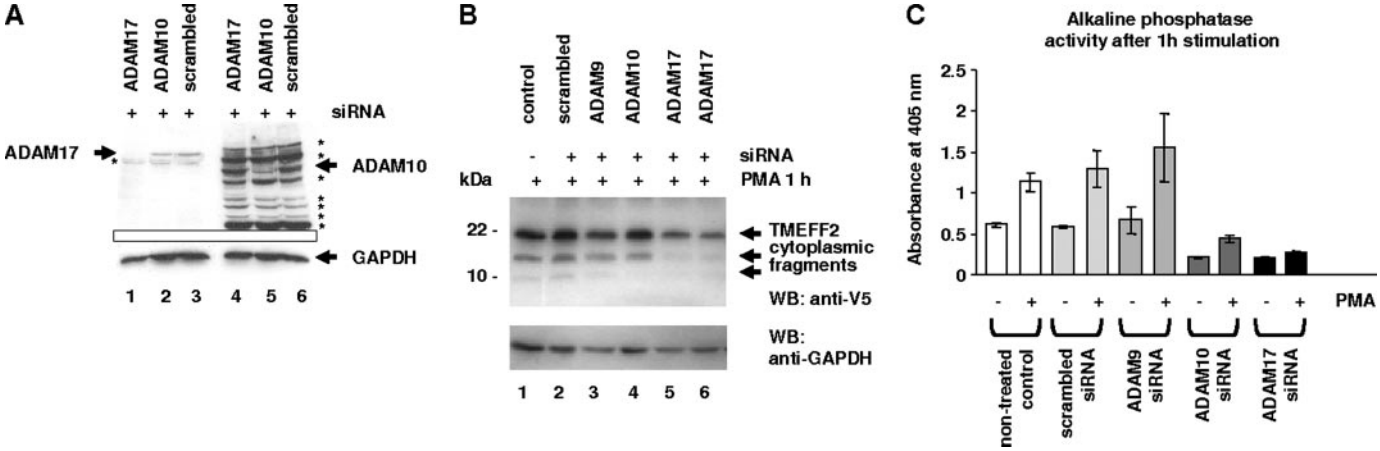

FIGURE 3. **Identification of the TMEFF2 sheddase using siRNA to down-regulate ADAM9, ADAM10, and ADAM17.** *A*, specificity of ADAM10 and ADAM17 siRNA. Lysates from cells treated with siRNA for 4 days were analyzed by Western blotting (*WB*) and stained for either the presence of ADAM17 or ADAM10 using commercial antibodies. *Lanes 1–3*, stained for ADAM17: *lane 1*, ADAM17 siRNA; *lane 2*, ADAM10 siRNA; *lane 3*, scrambled control siRNA. *Lanes 4–6*, stained for ADAM10: *lane 4*, ADAM17 siRNA; *lane 5*, ADAM10 siRNA; *lane 6*, scrambled control siRNA. Nonspecific bands are identified with *stars*, and specific bands are indicated with *arrows*. *B*, TMEFF2 shedding is inhibited by siRNA knockdown of ADAM17 and ADAM10: analysis of lysates from TMEFF2-expressing HEK293 cells treated with siRNA to ablate ADAM9, ADAM10, and ADAM17 expression for 4 days followed by PMA stimulation for 1 h. *Upper panel*, TMEFF2 fragments detected with the V5 epitope antibody: *lane 1*, untreated control; *lane 2*, scrambled control siRNA; *lane 3*, ADAM9 siRNA; *lane 4*, ADAM10 siRNA; *lanes 5* and *6*, ADAM17 siRNA. Note the significant reduction in the signal intensity of the 14-kDa metalloproteinase product in the presence of ADAM17 siRNA. *Lower panel*, GAPDH loading control. *C*, quantification of AP-TMEFF2-ECD release in ADAM siRNA targeted cells: both ADAM10 and ADAM17 contribute to constitutive and induced TMEFF2 shedding. Single well shedding assays (50) demonstrate that AP-TMEFF2-ECD release depends on ADAM17 under PMA-induced conditions (1-h incubation). Background AP activity from non-transfected cells was subtracted. Data are represented as mean ± S.E. of six replicas, and siRNA treatment is indicated.

TABLE 2
PMA response in cells treated with siRNA to ablate specific ADAMs

siRNA treatment	PMA-induced increase
	%
Scrambled	119
ADAM9	133
ADAM10	99
ADAM17	35

ADAM17 siRNA had no effect on the specific bands labeled with an *arrow*. Additional nonspecific bands were recognized by the ADAM10 antibody and are labeled with *stars* (Fig. 3*A*, *lanes 4–6*). We could not confirm a reduction in ADAM9 protein expression levels due to a lack of specific commercial antibodies.

In parallel we analyzed lysates from cells treated for 4 days with siRNA and stimulated for 1 h with PMA for the presence of anti-V5-immunoreactive C-terminal TMEFF2 fragments. As shown in Fig. 3*B* down-regulation of ADAM17 expression reduced the signal intensity of the 14- and 22-kDa metalloproteinase fragments (Fig. 3*B*, *lanes 5* and *6*). A concomitant complete loss of the 10-kDa γ-secretase product was also observed. Densitometric analysis of band intensities was performed and normalized to the loading control GAPDH (not shown). This analysis showed that among the ADAMs tested ADAM17 ablation had a significant effect in PMA-induced conditions. In contrast ADAM9 and ADAM10 siRNA had no effect on the signal intensity of the 14-kDa fragment.

To further validate our results we used the AP-TMEFF2-expressing cells to monitor effects of siRNA treatment to ablate ADAM expression on AP-TMEFF2-ECD release into conditioned medium using the very sensitive single well shedding assay (50). Cells were double transfected with siRNA to ablate the expression of the above ADAMs, and AP-TMEFF2 release was monitored under constitutive as well as PMA-stimulated conditions. We demonstrate in Fig. 3*C* that both ADAM10 and ADAM17 siRNA treatment significantly inhibited AP-TMEFF2-ECD release under constitutive conditions. In contrast, scrambled and ADAM9 siRNA did not affect cleavage of AP-TMEFF2 from the cell surface. Following PMA stimulation only cells treated with siRNA to ADAM17 showed a diminished response, suggesting that indeed ADAM17 plays a major role in TMEFF2 shedding. When calculating the percentage of increase of AP-TMEFF2-ECD release following siRNA treatment only ADAM17 ablation resulted in a significant reduction of 65% in the cellular response to PMA stimulation (Table 2). In contrast, the PMA response in ADAM9, ADAM10, and scrambled siRNA-treated cells was not reduced significantly.

An overall loss of released AP activity in ADAM10 as well as ADAM17 siRNA-treated cells was observed under constitutive conditions. This may indicate that both enzymes contribute to constitutive AP-TMEFF2-ECD release from the cell surface. However, because both ADAM10 and ADAM17 regulate cell proliferation by mediating availability of β-catenin (23) and epidermal growth factor receptor ligands (26), we cannot rule out that the 4 days of siRNA treatment resulted in unequal cell numbers when compared with the scrambled control siRNA- and ADAM9 siRNA-treated cells. Such a scenario would result in an overall reduction of signal intensity. We have, however,

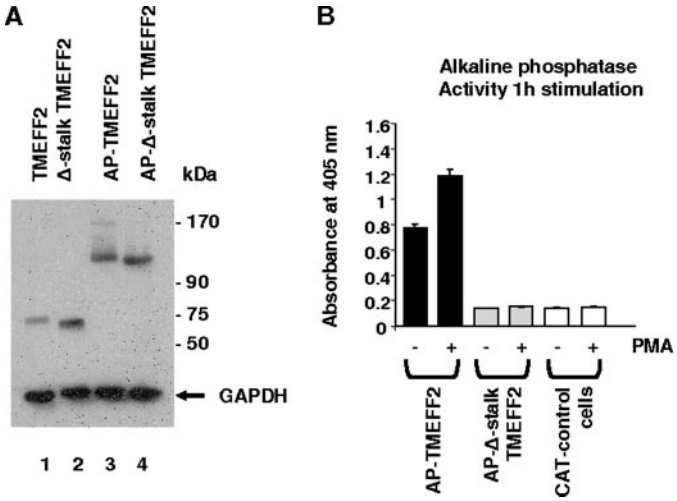

FIGURE 4. **TMEFF2 shedding is dependent on the stalk sequence motif.** *A*, comparison of wild type and mutant TMEFF2 expression levels in isogenic clonal HEK293 cells. Western blot analysis of equally loaded cell lysates stained with the V5 epitope antibody. *Lane 1*, wild type TMEFF2; *lane 2*, Δ-stalk TMEFF2; *lane 3*, AP-TMEFF2; *lane 4*, AP-Δ-stalk TMEFF2. GAPDH loading control is indicated. *B*, comparison of AP-TMEFF2 and AP-Δ-stalk TMEFF2 shedding. Single well shedding assays were performed using six replicas for each treatment and 1-h PMA stimulation (50). Alkaline phosphatase activity was monitored by hydrolysis of *p*-nitrophenyl phosphate. Data represented as mean ± S.E. of six replicas.

additional supporting evidence that ADAM10 is involved in AP-TMEFF2-ECD release as this can be stimulated with the calcium ionophore ionomycin, a strong inducer of ADAM10 activity (not shown) (53).

The Stalk Sequence Motif of TMEFF2 Is Required for Constitutive and Induced Shedding

To investigate whether TMEFF2 shedding depended on the stalk sequence motif we prepared a TMEFF2 mutant lacking the juxtamembrane stalk sequence motif (Δ-stalk TMEFF2 and AP-Δ-stalk TMEFF2; Fig. 1) using overlap extension mutagenesis (for details see the supplemental information).

First we analyzed cell lysates by Western blotting for expression of TMEFF2, Δ-stalk TMEFF2, AP-TMEFF2, and AP-Δ-stalk TMEFF2 to ensure that all proteins were expressed and not degraded. Fig. 4*A* shows expression of all four proteins in cell lysates, suggesting that they are produced in our isogenic clonal cell lines to a similar level.

We then investigated whether TMEFF2-ECD release was impaired in HEK293 cells expressing the N-terminally AP-tagged Δ-stalk TMEFF2 mutant and compared ectodomain release with AP-TMEFF2-expressing cells using the single well shedding assays (50). As demonstrated in Fig. 4*B* constitutive as well as PMA-induced shedding of AP-Δ-stalk TMEFF2 was ablated as these cells showed background AP activity of chloramphenicol transferase-transfected control cells.

Determination of the Biological Activity of TMEFF2

To determine whether TMEFF2 function is dependent on cell surface localization or whether the soluble ectodomain was an active growth factor in analogy to the ErbB ligands we performed additional experiments to answer this important question.

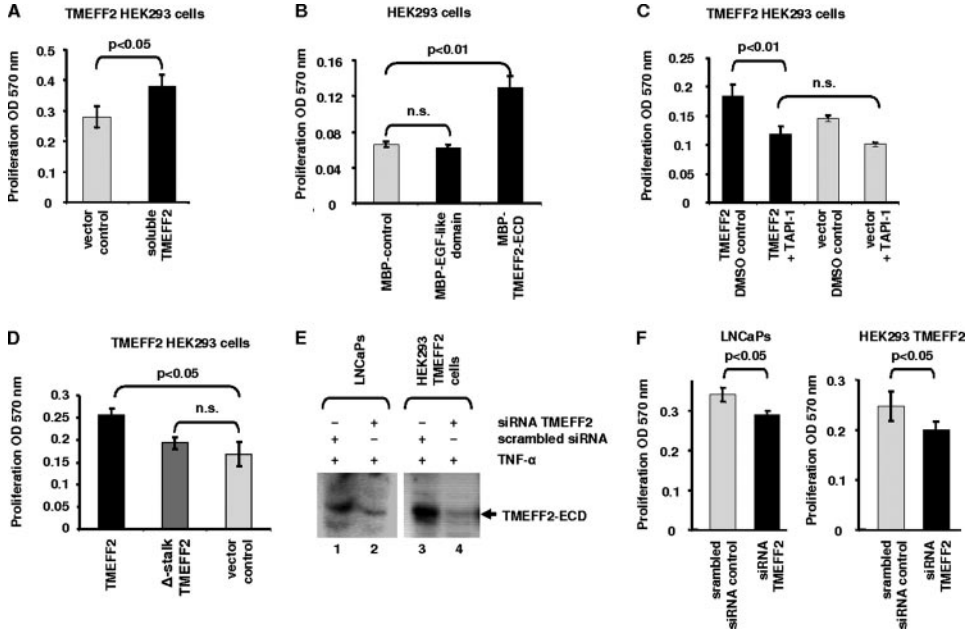

FIGURE 5. **Determination of the biological activity of the soluble TMEFF2-ECD and full-length TMEFF2 using overexpressing HEK293 cells.** Results of MTT proliferation assays are representative for two independent experiments performed with six replicas for each treatment unless otherwise stated. Mean ± S.E. is shown. *A*, overexpression of TMEFF2-ECD increases cell proliferation in HEK293 cells. Statistical analysis was performed using Student's *t* test. *B*, soluble TMEFF2-ECD but not the EGF domain alone increases HEK293 proliferation. MTT proliferation assay using 10 ng/ml maltose-binding protein control (*MBP-control*), MBP-EGF-TMEFF2, or MBP-TMEFF2-ECD to stimulate HEK293 proliferation. Statistical analysis was performed using ANOVA and post-test Tukey analysis. *C*, inhibition of TMEFF2 shedding using TAPI-1 inhibits cell proliferation in HEK293 cells expressing full-length TMEFF2. Statistical analysis was performed using ANOVA and post-test Tukey analysis. Note that inhibition of TMEFF2 shedding using TAPI-1 showed significant reduction of cell proliferation. TAPI-1 treatment of vector cells did not cause significant growth inhibition. *D*, comparison of proliferation rates of wild type TMEFF2-expressing cells with the Δ-stalk TMEFF2 mutant. Statistical analysis was performed using ANOVA and post-test Tukey analysis. Note that there is a reduction in proliferation rates expressing Δ-stalk TMEFF2; however, these are not statistically different to wild type TMEFF2-expressing cells. However, wild type TMEFF2-expressing cells grew significantly faster than vector controls. *E*, knockdown of TMEFF2 expression by siRNA in LNCaP and in TMEFF2-expressing HEK293 cells leads to diminished release of TMEFF2-ECD upon TNF-α stimulation for 12 h. Western blot analysis of concentrated conditioned medium using the anti-TMEFF2 antibody is shown. *F*, knockdown of TMEFF2 expression by siRNA in LNCaP and in TMEFF2-expressing HEK293 cells decreases proliferation in both cell lines. MTT proliferation assay was performed using 15 replicas per treatment and analyzed using Student's *t* test. *n.s.*, not significant.

TMEFF2-ECD Has Growth-promoting Activity That Depends on the Complete Ectodomain

We initially used our HEK293 cells expressing soluble TMEFF2-ECD and vector control cells to investigate whether the soluble TMEFF2-ECD had growth promoting activity. For this purpose we used an MTT assay to determine proliferation rates of these cells and compared these to the rate of vector control cells. Cells expressing soluble TMEFF2-ECD showed increased proliferation rates when compared with vector-transfected controls following statistical analysis using Student's *t* test (Fig. 5*A*).

Our initial experiments with soluble TMEFF2-ECD purified from an overexpressing HEK293 cell line showed that TMEFF2-ECD had growth factor activity (not shown). We could not obtain sufficient purified material using mammalian expression and thus used the maltose-binding fusion proteins encoding MBP-TMEFF2-ECD and MBP-EGF-TMEFF2 for further MTT assays. Purified MBP-TMEFF2-ECD and MBP-EGF-TMEFF2 were expressed in *Escherichia coli*, and a dose response (not shown) for effects on proliferation was established using non-transfected HEK293 cells. This experiment revealed that MBP-TMEFF2-ECD was inducing the highest proliferation rates at an optimal dose of 10 ng/ml, whereas MBP-EGF-TMEFF2 was inactive at concentrations of up to 100 ng/ml (not shown). Further proliferation experiments were performed at an optimal dose of 10 ng/ml MBP-TMEFF2-ECD using non-transfected HEK293 cells. MTT assays were performed 4 days later and revealed that cells treated with soluble MBP-TMEFF2-ECD showed an over 50% increase in cellular proliferation when compared with maltose-binding protein controls or to cells treated with MBP-EGF-TMEFF2, the EGF-like repeat (Fig. 5*B*). These data suggest that the EGF-like repeat alone is unable to promote proliferation under our experimental conditions and that the follistatin modules may play an important role for TMEFF2 function as a growth factor. Attempts to express the two follistatin domains as maltose-binding fusion proteins failed and thus we cannot narrow down domain requirements for function at this point in time.

Inhibition of TMEFF2 Shedding Reduces Cell Proliferation

We hypothesized that TMEFF2 shedding may contribute to cellular proliferation due to constitutive shedding, which occurs in low serum medium as seen in Fig. 2, *B* or *E* (*Lysates panel*, *lane 3*) by the presence of the 14-kDa metalloproteinase product and the increased AP activity in AP-TMEFF2-expressing cells (Fig. 2*D*). To test this hypothesis we performed proliferation assays in the presence of the metalloproteinase inhibitor TAPI-1, which preferentially inhibits ADAMs and previously blocked PMA-induced shedding by 94% (Table 1). TMEFF2 Me_2SO-treated solvent control cells grew significantly faster than TAPI-1-treated TMEFF2-expressing cells (Fig. 5*C*). Me_2SO-treated vector controls showed proliferation rates comparable to those of TAPI-1-treated TMEFF2 expressing cells or TAPI-1-treated controls (Fig. 5*C*). These data reinforce the growth promoting activity observed previously using TMEFF2-ECD-expressing cells and stimulation with recombinant TMEFF2-ECD (Fig. 5, *A* and *B*). Vector control cells also responded to TAPI-1 treatment with a reduction in growth rates, but these changes were not statistically significant using ANOVA and post-test Tukey analysis.

To see whether the non-cleavable Δ-stalk TMEFF2 mutant showed significantly different growth rates compared with those of vector control or TMEFF2-expressing cells we performed additional MTT assays (Fig. 5*D*). The Δ-stalk TMEFF2

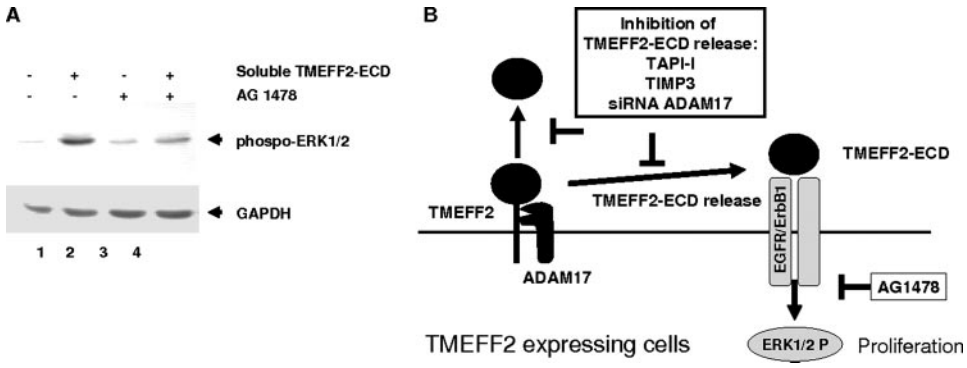

FIGURE 6. **Determination of the TMEFF2-ECD signaling potential and model of TMEFF2 signaling.** *A*, soluble TMEFF2-ECD induces ERK1/2 phosphorylation in an ErbB1-dependent manner in HEK293 cells. Serum-starved non-transfected HEK293 cells were stimulated with 10 ng/ml soluble TMEFF2-ECD in the presence or absence of the ErbB1 tyrosine kinase inhibitor AG1478. Samples were analyzed by Western blotting using an antibody that recognizes phosphorylated ERK1/2, a downstream target of ErbB1 signaling (*upper panel*). *Lane 1*, buffer control; *lane 2*, soluble TMEFF2-ECD; *lane 3*, AG1478 alone; *lane 4*, soluble TMEFF2-ECD plus AG1478. Blots were stained for GAPDH as a loading control (*lower panel*). *B*, schematic representation of TMEFF2 signaling. TMEFF2-dependent proliferation is inhibited by blocking TMEFF2-ECD release. The ErbB1 inhibitor AG1478 blocks TMEFF2-ECD-dependent ERK1/2 phosphorylation. *EGFR*, EGF receptor.

mutant grew at growth rates similar to those of vector controls. In contrast TMEFF2-expressing cells grew significantly faster than vector controls using ANOVA post-test Tukey analysis. The Δ-stalk TMEFF2 mutant-expressing cells grew slower than wild type TMEFF2-expressing cells, which, however, was below statistical significance. We cannot exclude that some shedding of the Δ-stalk TMEFF2 mutant may occur over the 4-day time frame of our proliferation assays that may contribute to cellular growth.

The Role of TMEFF2 in Regulating LNCaP Proliferation

To answer the question whether TMEFF2-ECD functions as a growth factor in PCa, we then stimulated LNCaP cells with TMEFF2-ECD and performed MTT assays using the optimized dose of 10 ng/ml. As shown in supplemental Fig. S1 LNCaP did not respond to TMEFF2-ECD treatment. Although this was surprising we argued that potential TMEFF2 receptors on LNCaPs may be saturated by endogenously released TMEFF2-ECD, thus allowing no further growth promotion under our experimental conditions. To answer this hypothesis siRNA knockdown of TMEFF2 was performed, and effects on proliferation were analyzed.

Knockdown of Endogenous TMEFF2 by siRNA in LNCaP Cells or TMEFF2-expressing HEK293 Cells Results in Down-regulation of TNF-α-induced Shedding

Dharmacon SmartPool siRNA targeting TMEFF2 was transfected using conditions established for the ADAMs followed by analysis of TNF-α-induced release of the TMEFF2-ECD into cell conditioned medium. We used this approach as it was difficult to monitor TMEFF2 levels in lysates of LNCaPs due to high background staining of other proteins with the commercial TMEFF2 antibody (not shown). As demonstrated in Fig. 5*E* siRNA treatment of LNCaPs or TMEFF2-expressing HEK293 cells followed by TNF-α-induced shedding resulted in dramatic reduction of soluble TMEFF2-ECD levels in the medium when compared with scrambled siRNA-treated controls (Fig. 5*E*, compare *lanes 1* and *2* and *lanes 3* and *4*). This result demonstrated that the Dharmacon SmartPool siRNA reduced TMEFF2 levels dramatically.

Knockdown of Endogenous TMEFF2 by siRNA in LNCaP Cells or TMEFF2-expressing HEK293 Cells Results in Down-regulation of Proliferation

To understand the role of TMEFF2 in prostate cancer, we used the LNCaP cell line as a model to address the role of endogenous TMEFF2 in regulating cell proliferation. For this purpose siRNA knockdown of TMEFF2 levels in these cells was performed and compared with scrambled siRNA controls, and proliferation was measured using an MTT assay. The targeting of TMEFF2 in LNCaPs with siRNA resulted in a 15% reduction of proliferation rates when compared with scrambled controls (Fig. 5*F*). Furthermore targeting of TMEFF2 in HEK293 cells with siRNA resulted in a 19% reduction of cell proliferation when compared with scrambled controls (Fig. 5*F*). Although the levels in reduction of cell proliferation are relatively small, our experimental setup is only investigating proliferation effects due to constitutive release of TMEFF2-ECD, which is very low (Fig. 2*B*). Additionally TMEFF2 expression was not completely abolished by the Dharmacon SmartPool siRNA, thus still allowing TMEFF2-ECD release in treated cells albeit at a lower level then scrambled siRNA-treated controls. Nevertheless our results demonstrate that a reduction in TMEFF2 expression levels in LNCaP or our HEK293 model system has antiproliferative effects.

Soluble TMEFF2 Activates ERK1/2 Phosphorylation in an ErbB1-dependent Manner in HEK293 Cells

To obtain some insight into the signaling mechanism that might be activated by soluble TMEFF2-ECD to up-regulate cell proliferation we used non-transfected HEK293 cells as a model system to test the hypothesis that TMEFF2 might regulate phosphorylation of ERK1/2 in an ErbB1-dependent manner. Serum-starved HEK293 cells were stimulated for 5 min with 10 ng/ml TMEFF2-ECD in the presence or absence of AG1478, a specific ErbB1 tyrosine kinase inhibitor. Cell lysates were immediately prepared and analyzed by Western blotting for effects on ERK1/2 phosphorylation. TMEFF2-ECD stimulated ERK1/2 phosphorylation (Fig. 6*A*, *upper panel*, *lane 2*), and this response to TMEFF2-ECD was inhibited, by preincubating cells with the synthetic ErbB1 tyrosine kinase inhibitor AG1478, by ~50% (Fig. 6*A*, *upper panel*, *lane 4*). This result indicated that TMEFF2-ECD was able to stimulate phosphorylation of ERK1/2 in an ErbB1-dependent fashion.

DISCUSSION

High TMEFF2 expression levels have been both associated with growth-promoting and growth-suppressing functions in various PCa xenograft and cell model systems (1–5). However, the biological activity of TMEFF2 has remained elusive. We have here shown that ADAM17-dependent release of the TMEFF2-ECD regulates TMEFF2 activity and function. TMEFF2 ectodomain shedding resulted in increased cellular proliferation that could be suppressed using synthetic metalloproteinase inhibitors (Fig. 6*B*, model of TMEFF2 signaling). Our results could explain the opposing results from the literature as proliferation depended on TMEFF2-ECD release, which had not been studied at the molecular level previously (1–5). Conceivably membrane-associated TMEFF2 could be growth-suppressive, although our work does not support this hypothesis (Fig. 5*D*), which is also in agreement with Afar *et al.* (1).

Analysis of PMA-induced TMEFF2 shedding revealed that TMEFF2 is a novel ADAM17 substrate as assessed by siRNA treatment to ablate expression of this enzyme and quantitative analysis of AP-TMEFF2-ECD release. Deletion of the juxtamembrane stalk sequence in Δ-stalk TMEFF2 showed that cleavage occurs in this region as shown previously for other ADAM substrates, such as angiotensin-converting enzyme, neurotrophin receptor, L-selectin, and the fractalkine CX3CL1, and other substrates (17, 54–56). Therefore TMEFF2 is a typical ADAM17 substrate where cleavage depends on the juxtamembrane stalk sequence.

The proliferative cellular responses toward ADAM-dependent TMEFF2-ECD release were inhibited by the synthetic metalloproteinase inhibitor TAPI-1, suggesting that blocking specific ADAMs could be of therapeutic benefit in PCa. It is important to note in this context that different ADAMs have been assigned critical roles in progression of mouse PCa models by Blobel and co-workers (57, 58) and Kuefer *et al.* (59). The W^{10} mouse prostate cancer model showed well differentiated tumors in an ADAM9-null background when compared with ADAM9-positive litter mates, suggesting that ADAM9 contributed significantly to disease progression (57). This was confirmed by gain of function studies in which ADAM9 overexpression resulted in prostate epithelial hyperplasia and neoplasia within a year. The authors concluded that this process may be due to EGF release by ADAM9 in the prostate epithelial tissue that would activate ErbB1 signaling by an autocrine loop. Although siRNA targeting of ADAM9 did not affect TMEFF2-ECD release under constitutive conditions or following PMA induction we currently cannot exclude that ADAM9 may be able to process TMEFF2; this could be tested by gain of function analysis in ADAM17- or ADAM10-null cells. It would be useful to use individual or multiple ADAM knock-out cell lines to establish a complete picture of TMEFF2 sheddases in the future. However, we have identified that ADAM17 and ADAM10 (not shown) would contribute to the release of TMEFF2-ECD. We have also shown that TMEFF2-ECD is able to induce ErbB1-dependent ERK1/2 phosphorylation albeit in a different cellular context. Conceivably TMEFF2-ECD could contribute toward ErbB1-dependent growth-promoting signals in PCa. In this context it would be interesting to develop ADAM17 or ADAM10 transgenic prostate cancer models and investigate their role in this disease in more detail.

It has recently become clear that ADAMs play important roles in regulating epidermal growth factor receptor/ErbB signaling by releasing active growth factors from the cell surface (26) with subsequent effects on cell proliferation (14, 46), migration, and invasion of cancer cells (Refs. 30, 57, 58, and 60; and for reviews, see Refs. 29 and 61). In PCa ErbB1, ErbB2, ErbB3, and the ligand EGF are expressed by tumor, whereas six ErbB ligands are localized in smooth muscle stroma of the human prostate (62). In mouse PCa models ADAM12 has been found to contribute to tumor progression in the carcinoma-associated stroma by stromal release of growth factors activating ErbB1 signaling in a paracrine manner (58). We cannot exclude that ADAM12 may contribute to TMEFF2-ECD release due to lack of an expression construct to perform gain of function analysis. Recent evidence from both tissue culture models or animal experiments has indicated that epidermal growth factor receptor/ErbB1 signaling is associated with and involved in the development of androgen independence of PCa and metastasis to bone (63–65). Androgen independence of PCa was significantly suppressed by ErbB1 tyrosine kinase inhibitors (63–65). It is important to note in this context that ErbB1 tyrosine kinase inhibition can be overcome by the release of ErbB1 ligands. In breast cancer, inhibition of ADAM17-dependent ligand release has recently been shown to have beneficial effects (66). This may well be true for PCa where ADAM17 and ADAM10 are expressed (60, 67) and would contribute to TMEFF2-ECD release in our LNCaP model. In turn ErbB1 activation has been shown to increase the half-life of ADAM17 in breast tumor cells, which may well be the case in other cell systems and would lead to increased substrate turnover by tumor cells, aiding the evasion of anti-ErbB therapies (68).

Our structure-function analysis revealed that the TMEFF2-ECD was able to induce cell proliferation, whereas the EGF domain alone was inactive when used at the same dose. Among most EGF-like ligands this is uncommon, but recently the *Xenopus laevis* neuregulin1 isoform XeIgNRG1β2 was shown to have similar properties. The immunoglobulin-like N-terminal domain of XeIgNRG1β2 was shown to be involved in the interaction with ErbB4 allowing signaling and binding at low doses, whereas the EGF-like domain had to be used at a 1000-fold higher dose to achieve a biological response (69). Furthermore the proliferation inducing activity of TMEFF2-ECD confirms previous work using hippocampal and mesencephalic neurons, which responded by showing enhanced survival (7), whereas cortical neurons were non-responsive. Therefore TMEFF2-ECD function is cell type-specific, which may explain the opposing results published previously in the context of different PCa cell lines or mouse prostate cancer models (1–5).

Our results support a model of TMEFF2 signaling in which ADAM17 or ADAM10 regulates TMEFF2-ECD release from TMEFF2-expressing cells (Fig. 6*B*) allowing autocrine signaling via ErbB1-dependent ERK1/2 phosphorylation resulting in up-regulation of cell proliferation. This may well be the case in PCa where TMEFF2 is highly expressed. TMEFF2 has attracted considerable attention as a prostate cancer-specific cell surface marker allowing antibody chemotherapy (1, 70). Mice bearing

xenografted LNCaP and CWR22 prostate cancers expressing TMEFF2 were treated with monoclonal antibodies conjugated to auristatin E or with radioactive ^{90}Y, which resulted in significant and sustained inhibition of tumor growth with no display of *in vivo* toxic side effects (1, 70). The soluble TMEFF2 splice variant (71) as well as TMEFF2-ECD release may, however, interfere with efficacy of the antibody therapies described above as these antibodies recognize TMEFF2-ECD. On the other hand clearance of TMEFF2-ECD antibody complexes may well be beneficial in suppressing the growth promoting activity of TMEFF2-ECD.

Our results imply that combinatorial therapeutic approaches targeting both ErbB1 signaling and ligand release via ADAM17-dependent shedding should provide an important step toward preventing tumor growth driven by TMEFF2-ECD released from the tumor cells. Targeting both ErbB1 signaling and ADAM17 activity could help to stop PCa progression to androgen-independent metastatic disease that manifests in bone. Alternatively ADAM17 inhibition in conjunction with existing TMEFF2 antibody therapies could aid this goal. Such combination therapy would both block TMEFF2-ECD release from PCa cells and increase the cell surface expression level of TMEFF2. This would increase the efficacy of the TMEFF2 antibodies by directly targeting the tumor cells (1, 70).

Alternatively TMEFF2 may also interact with membrane-type serine proteinase suppressor of tumorigenicity 14 (ST14) as recently demonstrated for TMEFF1 (72) and influence cell surface proteolysis by this interesting enzyme as it is expressed in human prostate cancer cells (73). However, it is not clear from Ge *et al.* (72) whether TMEFF1 is able to inhibit ST14 activity or whether TMEFF1 represents a novel ST14 substrate. However, their analysis revealed that the interaction between TMEFF1 and ST14 depended on the EGF-like repeat of TMEFF1 and the CUB (for complement C1r/C1s, Uegf, Bmp1) domain of ST14. Clearly further study is needed to fully understand the structure and function of TMEFF2 in prostate cancer.

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