TMEFF2 shedding is regulated by oxidative stress and mediated by ADAMs and transmembrane serine proteases implicated in prostate cancer†

Running title: ADAMs 9, 12 and TTSPs matriptase-1 and hepsin cleave TMEFF2

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Abstract

TMEFF2 is a type I transmembrane protein with two follistatin (FS) and one EGF-like domain over-expressed in prostate cancer, however its biological role in prostate cancer development and progression remains unclear, which may, at least in part, be explained by its proteolytic processing. The extracellular part of TMEFF2 (TMEFF2-ECD) is cleaved by ADAM17 and the membrane-retained fragment is further processed by the gamma-secretase complex. TMEFF2 shedding is increased with cell crowding, a condition associated with the tumour microenvironment, which was mediated by oxidative stress signalling, requiring jun-kinase (JNK) activation. Moreover, we have identified that TMEFF2 is also a novel substrate for other proteases implicated in prostate cancer, including two ADAMs (ADAM9 and ADAM12) and the type II transmembrane serine proteinases (TTSPs) matriptase-1 and hepsin. Whereas cleavage by ADAM9 and ADAM12 generates previously identified TMEFF2-ECD, proteolytic processing by matriptase-1 and hepsin produced TMEFF2 fragments, composed of TMEFF2-ECD or FS and/or EGF-like domains as well as novel membrane retained fragments. Differential TMEFF2 processing from a single transmembrane protein may be a general mechanism to modulate transmembrane protein levels and domains, dependent on the repertoire of ADAMs or TTSPs expressed by the target cell.

Keywords: TMEFF2, matriptase-1, hepsin, oxidative stress, ADAM

Abbreviations: TMEFF2, transmembrane protein with EGF like and two follistatin like domains 2; ADAM, a disintegrin and metalloproteinase; TTSP, type II transmembrane serine protease
1. Introduction

TMEFF2, a type I transmembrane protein with two follistatin and epidermal growth factor (EGF) domains is expressed selectively in the adult brain and prostate (Horie et al. 2000; Liang et al. 2000), with elevated TMEFF2 expression in prostate cancer (PCa) cell lines and clinical samples (Afar et al. 2004; Glynne-Jones et al. 2001; Gery et al. 2002). However, the role of TMEFF2 in PCa development and progression remains unclear, and TMEFF2 activity may depend on disease stage and/or post-transcriptional regulation. The ectodomain of TMEFF2 (TMEFF2-ECD), comprised of the two follistatin and EGF domains, is cleaved from the cell surface by ADAM17 and the membrane-retained fragment undergoes further processing by the gamma-secretase complex (Ali and Knäuper 2007). Shedding of TMEFF2-ECD is induced by pro-inflammatory cytokines TNFα and IL-1β (Lin et al. 2003) or phorbol esters (Ali and Knäuper 2007), which are known to upregulate ADAM-mediated protein shedding (Brose and Rosenmund 2002).

Ectodomain shedding may, at least partially, be responsible for pro- or anti-proliferative TMEFF2 functions in PCa. Over-expression of full length, transmembrane TMEFF2 in PCa cells impairs proliferation due to an interaction between the cytoplasmic domain of TMEFF2 and sarcosine dehydrogenase (SARDH). This interaction results in decreased levels of sarcosine (Chen et al. 2011a; Green et al. 2013), an amino acid associated with PCa progression (Sreekumar et al. 2009). Full length TMEFF2 also attenuates the migratory properties of PCa cells (Chen et al. 2014), indicating a tumour suppressor function. However, the TMEFF2-ECD released, due to shedding may act as a soluble growth factor. Indeed, treatment of HEK293 cells with purified recombinant TMEFF2-ECD stimulated ERK activation and increased their proliferation rate (Ali and Knäuper 2007; Chen et al. 2011a). On the other hand, conditioned medium of cells expressing TMEFF2-ECD reduced p-ERK levels in RWPE1 cells in response to PDGF-AA treatment (Chen and Ruiz-Echevarría 2013) which was also reported to suppress PDGF-AA stimulated growth of NR6 fibroblasts (Lin et al. 2011). While this points to TMEFF2 possessing opposing biological roles the molecular mechanism underlying this dual functionality is
unclear. We hypothesise that TMEFF2’s biological functions may be regulated by differential proteolysis, which generates not only TMEFF2-ECD but additional protein fragments, which may modulate mitogenic signalling. Indeed, additional soluble TMEFF2 forms can arise from alternative splicing, generating a soluble protein composed of the FS-I module and a truncated FS-II module (Quayle and Sadar 2006).

To address the hypothesis that TMEFF2 may undergo differential proteolysis we focussed our investigation on proteases contributing to the pathogenesis of PCa. These include members of the disintegrin and metalloproteinase family, ADAM9 (Peduto et al. 2005; Fritzsche et al. 2008), ADAM12 (Peduto et al. 2006) and ADAM15 (Lucas and Day 2009) as well as membrane associated serine proteases implicated in PCa, such as type II transmembrane serine proteases (TTSPs) (Webb et al. 2011) or the GPI-anchored prostasin (Chen et al. 2004). Prominent TTSPs involved in PCa progression include matriptase-1, matriptase-2 and hepsin. Matriptase-1 overexpression correlates with Gleason score (Riddick et al. 2005) promoting cell invasion, metastasis and prostate tumor growth (Sanders et al. 2006; Ko et al. 2015) by regulating MET signaling in PCa. Interestingly, matriptase-1 interacts with a close relative of TMEFF2, TMEFF1, where the EGF-like domain of TMEFF1 binds to the matriptase-1 CUB domain (Ge et al. 2006). Matriptase-2 also contains CUB domains and is implicated in PCa cell behaviour (Sanders et al. 2010). Significant over-expression of hepsin is common in 90% of PCa tumours, correlating with Gleason score, serum PSA levels as well as early relapse following radical prostatectomy (Dhanasekaran et al. 2001; Goel et al. 2011). In contrast, prostasin levels are high in normal prostate epithelial cells and decrease in PCa (Takahashi et al. 2003). We therefore tested the hypothesis that TMEFF2 is cleaved at different sites by ADAMs and TTSPs and we provide evidence of complex TMEFF2 proteolysis by these proteases that may impact the biological function of TMEFF2 reported in the literature.
2. Materials and methods

2.1 Reagents

PMA and the NADPH oxidase inhibitor apocynin (APOC) were from Sigma Aldrich. N-acetylcysteine (NAC), p38 inhibitor (SB203580) and JNK inhibitor (SP600125) and the broad spectrum metalloproteinase inhibitor GM6001 were from Calbiochem. ADAM10 and ADAM17 inhibitors GI254023X and GW280264X were a gift from Dr. Augustin Amour and GlaxoSmithKline. DMEM and Ham’s F12 cell were from Lonza, FBS and hygromycin B from Invitrogen. FuGENE 6 Transfection Reagent was from Roche.

2.2 Expression constructs, cell culture, transient transfection and Western blotting

ADAM9 and ADAM12 expression constructs were a kind gift from Dr. Carl Blobel. The cloning of ADAM15 A, B, C isoforms and ADAM15 B E/A inactive mutant into pcDNA4-V5/His vector were described previously (Zhong et al. 2008; Maretzky et al. 2009). The corresponding coding sequences were sub-cloned into pcDNA5/FRT/Flag-His plasmid using HindIII and XhoI. Matriptase-2 expression plasmid was described in (Folgueras et al. 2008). Matriptase-1, hepsin and prostasin plasmids were described in (Gray et al. 2014) and used to generate constructs expressing inactive S-A mutants by QuikChange mutagenesis (Agilent). Generation of HEK293 cells expressing AP/V5 TMEFF2 and AP/V5 Δ303-320 TMEFF2 was described previously (Ali and Knäuper 2007) and were maintained in DMEM with 10% FBS and 100 µg/ml hygromycin B at 37°C in a humidified incubator with 5% CO₂. For shedding experiments 1x10⁵ HEK293 cells, expressing alkaline phosphatase tagged wild type or mutant TMEFF2, were plated per well into a 24 well plate and grown overnight in antibiotic free medium. 0.5 µg of expression plasmid encoding the active and inactive proteases in question were mixed with 1.5 µl of FuGENE 6 Transfection Reagent added to each well and grown for two days prior to shedding experiments in the presence or absence of ADAM 10, ADAM17 or general metalloproteinase inhibitor GM6001 described previously (Ali and Knäuper 2007). Additional experiments were performed using DU145 cells transiently transfected with AP-TMEFF2. Here cell lysates were
analyzed for total AP-activity to normalize the release of soluble AP-TMEFF2 ectodomain into the medium following cell crowding experiments. The data are displayed as percentage of shed TMEFF2.

Cell lysates were harvested at the end of shedding experiments and analyzed using 10% SDS-PAGE followed by Western blotting using PVDF membranes.

2.2 Statistical analysis

Mean values ±SD from three independent experiments with 4 internal replicates were analyzed using GraphPad Prism 5.0 and One-way ANOVA with Tukey’s test (** p < 0.01; * p < 0.05). P-values below 0.05 were considered significant.

3. Results and discussion

Our first aim was to identify whether TMEFF2 could be targeted by other proteases accounting for soluble TMEFF2 (Quayle and Sadar 2006). We assessed a panel of ADAMs with ADAM9 and ADAM12 over-expression leading to increased shedding of TMEFF2, while the expression of ADAM15 isoforms did not increase AP-activity in media when compared to co-expression of an inactive ADAM15 EA mutant used as a transfection control (Fig. 1A). C-terminal TMEFF2 fragments were indistinguishable from previously described ADAM17 fragments (Ali and Knäuper 2007) (not shown).

We then hypothesized that TTSPs could cleave TMEFF2, as matriptase-1 is known to interact with TMEFF1 (Ge et al. 2006). Over-expression of matriptase-1 or hepsin increased AP-TMEFF2 fragment release into medium by 2-3.5 fold, compared to inactive matriptase-1/hepsin S-A mutant co-expression. Matriptase-2 over-expression was less efficient and increased AP-activity in media 1.5 fold, while prostasin had no effect (Fig. 1B). Western blot analysis for remaining membrane associated fragments following TTSP cleavage, showed novel, C-terminal TMEFF2 fragments in matriptase-1 (~24 and ~28 kDa) and hepsin (~20 kDa) expressing cells (Fig. 1C). These fragments were
absent upon expression of their inactive S-A mutant counterparts, in addition to the ~17 kDa fragment which is due to background ADAM activity previously described (Ali and Knäuper 2007). Therefore, matriptase-1 and hepsin cleave TMEFF2 in different positions than ADAMs, generating novel transmembrane retained fragments. No additional C-terminal fragments were detected in cells expressing matriptase-2 (Fig. 1C), despite increased AP-activity levels in medium (Fig. 1B). Thus matriptase-2 either cleaves TMEFF2 close to the ADAM cleavage site or alternatively activates ADAMs to induce proteolysis. To address this question shedding experiments were performed in the presence of selective ADAM10 and ADAM17 inhibitors, GW280264X and GI254023X or the broad spectrum metalloproteinase inhibitor GM6001. The data in Fig. 1D showed that matriptase-2 dependent release of AP-TMEFF2-ECD required ADAM activity, whereas matriptase-1-dependent release did not. Additional experiments were performed to exclude contribution from ADAM9 and other metalloproteinases such as MMPs using the broad spectrum metalloproteinase inhibitor GM6001 (Maretzky et al. 2017) and cells overexpressing matriptase-1 or hepsin, as well as their inactive counterparts. This analysis indicated that matriptase-1 and hepsin were genuine TMEFF2 sheddases as inhibitor treatment was ineffective (Fig. 1E).

To corroborate these findings ADAM-cleavage resistant AP-Δ303-320 TMEFF2 (Ali and Knäuper 2007) was used to confirm that matriptase-1 and hepsin cleaved outside and matriptase-2 within the stalk section containing the ADAM cleavage site. Both matriptase-1 and hepsin cleaved AP-Δ303-320 TMEFF2 to a similar extent to wt TMEFF2 (Fig. 2A), while matriptase-2 was unable to directly cleave AP-Δ303-320 TMEFF2 lacking the ADAM-cleavage site. TMEFF2 C-terminal fragment analysis in Fig. 2B confirmed that matriptase-1 and hepsin cleaved AP-Δ303-320 TMEFF2 by generating novel fragments, showing distinctly different molecular weights, when compared to cleaved wt AP-TMEFF2. Matriptase-1 produced 18 and 20 kDa C-terminal fragments, and hepsin a 25 kDa C-terminal fragment of AP-Δ303-320 TMEFF2. It has to be noted that the AP-Δ303-320 TMEFF2 mutant also lacks two potential TTSPs cleavage site motifs, KKD and VRF (indicated in Fig. 2A), as judged by the preferences of TTSPs for P1 Arginine or P1’ Lysine residues (Barré et al. 2014), although other sites can also be cleaved by TTSPs.
Potentially, hepsin cleavage occurs at KKD in wild type TMEFF2 to produce the 20 kDa fragment and disruption of this site to the artificial sequence CEKLI then leads to an additional minor cleavage event, as seen when the AP-Δ303-320 TMEFF2 mutant was cleaved in response to hepsin overexpression, producing a novel 25 kDa fragment. We predict possible cleavage sites for matriptase-1 and hepsin in TMEFF2 (Fig. 2C), with hepsin cleaving in the stalk sequence, releasing a soluble TMEFF2 fragment composed of the TMEFF2 ectodomain. Matriptase-1 likely cleaves TMEFF2 in two positions, generating soluble proteins containing FS-I or both FS modules, which in conjunction with ADAM dependent cleavage in the stalk region also liberates the EGF-like domain. Thus these cleavage events may be at least partially responsible for the generation of soluble TMEFF2 forms previously identified by others (Uchida et al. 1999; Quayle and Sadar 2006).

Soluble TMEFF2 fragments containing FS-domains generated by proteolysis likely modify PDGF-AA growth factor signalling, where PDGF-AA-TMEFF2 complexes modify signalling through PDGFRα, thus full length TMEFF2 or soluble FS-domain containing TMEFF2 fragments may block PDGF-AA signalling (Lin et al. 2011). The FS domains of TMEFF2 also regulate corticotropine-releasing hormone (CRH) signalling in corticotrope cells, where the production of cAMP, CREB and expression of pro-opiomelanocortin was inhibited, resulting in decreased cell proliferation (Labeur et al. 2015). On the other hand, growth-promoting activity of soluble TMEFF2 has been described (Horie et al. 2000; Ali and Knäuper 2007; Chen et al. 2011a; Chen and Ruiz-Echevarría 2013), suggesting that this may be cell type dependent and potentially regulated by the pattern of ADAM and TTSP expression as well as by the growth factors present in the extra cellular environment.

Reactive oxygen species (ROS), influence ADAM expression patterns and activation status (Sung et al. 2006; Willems et al. 2010) and they also regulate TTSP activity, as seen for matriptase-1, which is activated by ROS (Chen et al. 2011b) thus adding additional layers of regulation of shedding events. ROS levels also regulate several signalling pathways in cancer (Hanahan and Weinberg 2000; Liou and Storz 2010), including PCa (Khandrika et al. 2009), which led us to hypothesize that ROS levels
regulate TMEFF2 shedding. To investigate this hypothesis we pre-treated AP-TMEFF2 HEK293 cells with the ROS scavenger NAC or the NADPH oxidase inhibitor APOC prior to stimulation with PMA, a known inducer of ROS generation (Datta et al. 2000) and ADAM17 activator (Brill et al. 2009). Both inhibitor treatments resulted in almost complete inhibition of AP-TMEFF2-ECD release, indicating ROS dependent TMEFF2 processing (Fig. 3A). We hypothesized that ROS-induced TMEFF2 shedding could be mediated by the stress activated protein kinases JNK or p38 to activate ADAM17. The JNK inhibitor completely blocked AP-TMEFF2 release, whereas the p38 inhibitor reduced TMEFF2 shedding by 50% (Fig. 3A) suggesting that TMEFF2 shedding is triggered by oxidative stress signalling. The growth of cells at high cellular density has been reported to be a contributing factor to the increased oxidative stress in cancer (Hanahan and Weinberg 2000). We investigated whether shedding of TMEFF2 was a pathophysiological response triggered by oxidative stress originating from cells growing in high confluency conditions. Equal number of cells were plated onto 24-well or 6-well plates to obtain 95% and 30% confluency respectively and treated with equal volumes of medium containing PMA or control solvent. High cellular density significantly increased the shedding response to PMA activation (Fig. 3B). To confirm that TMEFF2 shedding in response to cell crowding was also relevant in PCa cancer cells, we then transiently transfected DU145 cells with AP-TMEFF2 cDNA and analysed shedding responses. AP-activity in cell lysates was determined and AP-activity in medium calculated as % shed TMEFF2 in cells grown at 30 or 95% confluency respectively (Fig. 3C). Data show significant increase in shedding in response to cell crowding, thus confirming the results obtained using HEK293 cells. Collectively this suggests that cell crowding mimicking conditions frequently found in the tumour microenvironment may trigger TMEFF2-ECD release, and contribute to the high proliferation rate of cancer cells.

In summary, differential TMEFF2 proteolysis producing various soluble fragments may be a general mechanism of changing its biological activity, dependent on the repertoire of ADAMs or TTSP expressed in the target cell, which in this case is regulated by ROS signalling.
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Figure 1: TMEFF2 is a novel substrate for ADAM9, ADAM12 and type II transmembrane serine proteases (TTSPs) – matriptase-1 and hepsin. (A) Schematic representation of AP-tagged TMEFF2 expression construct and ADAM9 and ADAM12 dependent AP-TMEFF2-ECD release into media. (B) Release of AP-TMEFF2-ECD from cells transfected with matriptase-1, matriptase-2, hepsin, prostasin or their inactive S-A mutants. (C) WB analysis for the C-terminal V5-epitope of cell lysates demonstrating generation of distinct novel C-terminal TMEFF2 fragments in matriptase-1 (~25 and 28 kDa) and hepsin (~20 kDa) overexpressing cells. (D) The matriptase-dependent release of TMEFF2-ECD is independent of ADAM activity. (E) Matriptase-1 and hepsin dependent TMEFF2 release is independent of metalloproteinase activity. MP fragment = metalloproteinase fragment

Figure 2: Characterization of TTSPs cleavage sites using the AP-Δ303-320TMEFF2 mutant lacking the ADAM cleavage site. (A) Schematic representation of AP-Δ303-320TMEFF2 expression construct and sequence motif deleted in this mutant, showing potential TTSP cleavage sites. Release of AP-Δ303-320TMEFF2 from cells co-transfected with matriptase-1, matriptase-2, hepsin, prostasin or their inactive S-A mutants. (B) WB analysis of lysates for AP-Δ303-320TMEFF2 C-terminal fragments shows distinct cleavage products for matriptase-1 (~17 & 23 kDa) and hepsin (~25 kDa). (C) Model of predicted TMEFF2 cleavage sites for hepsin and matriptase-1.

Figure 3: Oxidative stress and ROS-activated ADAMs participate in TMEFF2 shedding. (A) PMA-induced shedding from AP-TMEFF2 HEK293 cells pre-treated for 1 hour with NAC, APOC or p38 and
JNK MAPKs inhibitors. (B) PMA-induced shedding of AP-TMEFF2 from 95% and 30% confluent cells.

(C) TMEFF2 shedding in DU145 PCa cells is upregulated by cell crowding.
References:


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Figure 3

A. PMA induced shedding

B. Shedding response to cell crowding

C. % shed TMEPP2 DU145 cells

95% confluence 30% confluence