Thrombin-induced shedding of tumour endothelial marker 5 and exposure of its RGD motif are regulated by cell-surface protein disulfide-isomerase

Mario VALLON*1, Philipp AUBELE*2, Klaus-Peter JANSSEN† and Markus ESSLER*
*Department of Nuclear Medicine, Technische Universität München, Ismaninger Strasse 22, 81675 Munich, Germany, and †Department of Surgery, Technische Universität München, Ismaninger Strasse 22, 81675 Munich, Germany

INTRODUCTION

Angiogenesis, the sprouting of new blood vessels from pre-existing vessels, and haemostasis, the coagulation of blood, are tightly linked processes [1–3]. At the onset of angiogenesis, endothelial cells lining the inner surface of blood vessels are activated, lose cell–cell contacts and degrade the basement membrane, leading to extravasation of plasma proteins [1]. Tissue factor expressed by subendothelial and activated endothelial cells then initiates the coagulation cascade, resulting in activation of the serine protease thrombin (Factor IIa) [2]. Thrombin cleaves fibrinogen, leading to the formation of a fibrin clot, which provides a provisional pro-angiogenic ECM (extracellular matrix) for endothelial cells [1]. Thrombin also directly stimulates endothelial cell proliferation and migration by activating PARs (protease-activated receptors) [4]. Furthermore, it has been shown that thrombin mediates shedding of the heparan sulfate proteoglycans syndecan-1 and -4 from endothelial cells [5,6].

TEM5 (tumour endothelial marker 5; also known as GPR124) is an adhesion G-protein-coupled receptor containing a cryptic RGD motif in its extracellular domain. TEM5 is expressed in endothelial cells and pericytes during angiogenesis. In the present paper, we report that thrombin mediates shedding of an N-terminal TEM5 fragment of 60 kDa (termed N60) containing the RGD motif in an open conformation. Thrombin directly cleaved rsTEM5 (recombinant soluble TEM5) 5 and 34 residues downstream of the RGD motif, resulting in formation of N60 and its C-terminal counterpart (termed C50). Interestingly, N60 derived from thrombin cleavage of rsTEM5 was covalently linked to C50 by disulfide bonds, whereas N60 shed from thrombin-treated cells was not associated with its membrane-bound C-terminal counterpart. Inhibition of the reducing function of cell-surface PDI (protein disulfide-isomerase) abrogated thrombin-induced N60 shedding. Conversely, addition of reduced PDI enhanced N60 shedding. Furthermore, thrombin cleavage of rsTEM5 was increased by reduced PDI and resulted in dissociation of the N60–C50 heterodimer. We conclude that PDI regulates thrombin-induced shedding of N60 and exposure of the TEM5 RGD motif by catalysing the reduction of crucial disulfide bonds of TEM5 on the cell surface. Binding of N60 to RGD-dependent integrins may modulate cellular functions such as adhesion and migration during angiogenesis.

Key words: cell-surface protein disulfide-isomerase (PDI), cryptic RGD motif, redox regulation, shedding, thrombin, tumour endothelial marker 5 (TEM5).
Recently it was shown that TEM5-knockout mice are embryonically lethal because of a complete lack of vascularization and haemorrhage in the forebrain and neural tube [10, 17, 18]. Moreover, it was reported that TEM5 regulates developmental expression of the blood–brain barrier marker GLUT1 (glucose transporter 1) [10, 17]. Brain endothelial cells overexpressing TEM5 migrate towards CM (conditioned medium) derived from embryonic forebrain cells [10]. Similarly, endothelial cells overexpressing TEM5 migrate faster towards FBS (fetal bovine serum) than untransfected cells, indicating a role for TEM5 in directed cell migration [17].

PDI (protein disulfide-isomerase) catalyses thiol–disulfide interchanges, which can result in formation, reduction or isomerization of protein disulfide bonds. Although PDI contains the ER (endoplasmic reticulum) retention signal KDEL (Lys-Asp-Glu-Leu) and catalyses the formation and isomerization of disulfide bonds in the ER [19], it is also expressed on the cell surface, where it mediates the reduction of disulfide bonds [20–22]. The reductive function of cell-surface PDI has been implicated in cleavage of the disulfide-linked diphtheria toxin heterodimer [22], entry of HIV into cells [23], shedding of the thyrotropin receptor ectodomain [24] and exposure of the cryptic RGD motif of the ECM protein thrombospondin-1 [25].

In the present paper we report that thrombin mediates shedding of the TEM5-N60 fragment from cells. Thrombin-induced shedding of N60 was tightly regulated by cell-surface PDI and resulted in exposure of the TEM5 RGD motif.

**EXPERIMENTAL**

**Materials**

A peroxidase-conjugated monoclonal anti-FLAG antibody (clone M2), thrombin, hirudin, PDI and GSH were purchased from Sigma–Aldrich. A function-blocking monoclonal anti-PDI antibody (clone RL90) was from Novus Biologicals. Vitronectin was from R&D Systems. A monoclonal anti-His tag antibody (clone ADI.I.I0) was from AbD Serotec. A peroxidase-conjugated polyclonal anti-Myc tag antibody and peroxidase-conjugated monoclonal anti-β-actin antibody (clone 8226) were from Abcam. GM6001, bacitracin, MMP1 and MMP9 were from Merck Chemicals. Cyclic RGD (H-GPenGRGDSPCA-OH) and TFLLR-NH2 peptides were from Bachem. NEM (N-ethylmaleimide) was from Thermo Scientific.

**Cell culture**

HEK (human embryonic kidney)-293 cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium; Biochrom) supplemented with 10% FBS (Biochrom). Stably transfected HEK-293 cells were maintained in culture medium supplemented with 0.6 mg/ml genetin (Life Technologies). Pooled HUVECs (human umbilical vein endothelial cells) were purchased from PromoCell and cultured in endothelial cell growth medium (PromoCell). Cells were maintained at 37°C and 5% CO₂.

**Expression constructs and transfection**

Full-length TEM5 and sTEM5 were cloned into the expression vector p3XFLAG-CMV-9 (Sigma–Aldrich) as described previously [16]. Point mutations at the indicated positions (referring to GenBank® accession number NP_116166) were introduced using the QuikChange® Lightning site-directed mutagenesis kit (Agilent Technologies) and specific primers (Eurofins MWG Operon). HEK-293 cells were transfected using the calcium phosphate co-precipitation method, as described by Graham and van der Eb [26], and stably transfected cells were selected with 0.6 mg/ml genetin. HUVECs were transfected using an optimized calcium phosphate co-precipitation method as described by Segura et al. [27].

**Generation of recombinant proteins and protein fragments**

Recombinant wild-type sTEM5 and sTEM5 point mutants were purified from CM of HEK-293 cells stably expressing the corresponding construct, as described previously [16]. The N60–SS–C50 heterodimer was generated by incubating sTEM5 (10 μM) with thrombin (5 μM) in PBS for 16 h at 37°C. Free N60 was generated by reducing N60–SS–C50 for 45 min with TCEP [tris-(2-carboxyethyl)phosphine]–agarose (Thermo Scientific). N80 was generated by incubating sTEM5 (10 μM) with MMP1 (0.23 μM) in 50 mM Tris/HCl (pH 7.6), 300 mM NaCl, 5 mM CaCl₂, 1 μM ZnCl₂ and 0.05% Brij-35 for 16 h at 37°C. CM-derived N60 was generated by incubating HEK-293 TEM5 for 72 h with 270 nM thrombin in serum-free medium. All TEM5 fragments were purified from digests, reduced digests and CM using anti-FLAG M2–agarose (Sigma–Aldrich).

**Co-immunoprecipitation**

N60–SS–C50 Recombinant sTEM5 (2 μM) was incubated with thrombin (0.25 μM) in PBS for 48 h at 37°C. The digest was diluted in PBST (PBS containing 0.05% Tween 20) and incubated with a monoclonal anti-His tag antibody or an irrelevant monoclonal antibody and Protein G plus-agarose beads (Merck Chemicals) overnight at 4°C under constant rotation. Beads were washed three times with PBST and bound proteins were eluted with reducing Laemmli sample buffer for 5 min at 95°C.

**Cell-surface TEM5–SS–PDI**

HEK293–TEM5 cells were seeded in a six-well plate (1 × 10⁶ cells per well) and incubated for 24 h. Cells were washed three times with ice-cold PBS ± 20 mM NEM. Cells were incubated for 30 min on ice in PBS containing 0.4 mM sulfo-NHS-LC-biotin [sulfosuccinimidyl-6-(biotinamido) hexanoate; Thermo Scientific] ± 20 mM NEM. After detachment by gentle rinsing, cells were washed three times with ice-cold PBS. Cells were lysed on ice for 30 min in PBS ± 20 mM NEM containing 2% Triton X-100, 2 mM EDTA and protease inhibitor cocktail (Sigma–Aldrich). Lysates were cleared by centrifugation (16000 g for 10 min at 4°C) and incubated with anti-FLAG M2–agarose beads for 1 h under constant rotation. Beads were washed three times with ice-cold buffer A [20 mM Tris/HCl (pH 7.6), 137 mM NaCl, 2% Triton X-100 and 2 mM EDTA] and bound proteins were eluted with non-reducing Laemmli sample buffer for 5 min at 95°C. Eluates were diluted 1:50 with buffer A supplemented with protease inhibitor cocktail and incubated with NeutraAvidin agarose beads (Thermo Scientific) for 1 h under constant rotation. Beads were washed three times with ice-cold buffer A and bound proteins were eluted with reducing Laemmli sample buffer for 5 min at 95°C.

**Western blotting**

Cells were washed once with PBS and lysed using reducing Laemmli sample buffer. CM were cleared by centrifugation (16000 g for 10 min at room temperature) and TEM5 fragments or total protein were precipitated using anti-FLAG M2–agarose
or TCA-DOC (trichloroacetic acid and sodium deoxycholate) respectively. Immuno-TCA-DOC precipitates were dissolved in Laemmli sample buffer. Unless otherwise indicated reducing Laemmli sample buffer was used. Samples were incubated for 5 min at 95°C and Western blotting was performed as described previously [9].

Protein sequencing

Thrombin digests were separated by reducing SDS/PAGE and transferred on to PVDF membranes using Tris/borate transfer buffer. Proteins were stained using Coomassie Brilliant Blue and relevant bands were excised and sequenced by N-terminal Edman degradation (Proteome Factory).

Cell-adhesion assay

ELISA plates were coated overnight at 4°C with 10 μg/ml of the indicated protein in PBS. Adsorption of indicated proteins to the plate was essentially equal as determined by ELISA and the indicated protein in PBS. Adsorption of indicated proteins basal medium (PromoCell) containing 0.1% BSA. Cells (4 x 10⁴ cells per well) were seeded in the prepared plate. Cells were allowed to adhere for 1 h at 37°C, non-adhering cells were washed away with PBS, and adhering cells were quantified using the AlamarBlue assay (AbD Serotec) according to the manufacturer’s instructions.

RESULTS

Thrombin cleavage mediates exposure of the TEM5-RGD motif

We previously reported that proteolytic processing of recombinant sTEM5 with MMP9 in a cell-free system results in formation of two N-terminal fragments of 60 and 80 kDa (N60/N80) and exposure of the cryptic TEM5-RGD motif in one or both fragments [16]. By screening different proteases involved in angiogenesis for sTEM5 cleavage, we identified thrombin and MMP1 as proteases that merely mediate formation of N60 and N80 respectively (Figures 1A and 1B). Furthermore, thrombin and MMP1 cleaved sTEM5 more efficiently than MMP9. To determine in which fragment the RGD motif is exposed, N60 and N80 were purified from quantitative thrombin and MMP1 digests of sTEM5. N60-RGE derived from thrombin cleavage of the sTEM5 point mutant D364E (RGD→RGE) served as a negative control. Purified proteins were immobilized on polystyrene and HUVECs were allowed to adhere for 1 h. Non-adhering cells were washed away and cell adhesion was quantified using the AlamarBlue assay (AbD Serotec) according to the manufacturer’s instructions.

To identify the thrombin cleavage site in sTEM5, the C-terminal counterpart of N60 (C50) was sequenced by N-terminal Edman degradation (Figure 2). Sequencing revealed the sequence ASRRC found C-terminal to Arg³⁶⁹, indicating that thrombin cleaves TEM5 at the peptide bond between Arg³⁶⁹ and Ala³⁷⁰ (Figure 2B). C50 migrated as a double band on SDS/PAGE; however, both bands had the same N-terminal sequence, indicating that they represent alternatively glycosylated forms of recombinant sTEM5. To confirm the result from the N-terminal protein sequencing, we cloned a point mutant of sTEM5 where Arg³⁶⁹ was mutated to glycine (R398G). Interestingly, sTEM5-R398G was still cleaved by thrombin; however, the C50 band shifted up ~5 kDa (C55), indicating that a second cleavage site exists upstream of Arg³⁶⁹. N-terminal Edman sequencing of C55 yielded the sequence TLAGI which is located C-terminal to Arg³⁶⁹, five residues downstream of the RGD motif. As expected, mutating Arg³⁶⁹ to alanine (R369A) did not inhibit thrombin cleavage, but resulted in a shift of the N60 band to ~65 kDa (N65), indicating that cleavage only occurred at the C-terminal site in that mutant. Introducing both point mutations into sTEM5 (R369A/R389G) completely abrogated thrombin cleavage. The identified cleavage sites are consistent with the fact that thrombin only cleaves peptide bonds C-terminal to arginine and lysine residues [28].

Thrombin mediates shedding of N60 from cells

To determine whether thrombin also mediates shedding of N60 from cells, HEK-293-TEM5 cells were treated with thrombin at different concentrations and CM as well as cell lysates were analysed by Western blotting (Figure 3B). Indeed, thrombin at concentrations of 10 nM and higher induced N60 shedding. N60 and its membrane-bound C-terminal counterpart C130 were detectable in the CM and cell lysates respectively after 4 h of thrombin (100 nM) treatment (Figure 3C). N60 shedding increased continually until at least 16 h of incubation, whereas formation of the C130 fragment reached a steady-state level after ~7 h. Thrombin also induced shedding of N60 in primary endothelial cells (HUVECs) transfected with TEM5 (Figure 3D). We have shown previously that HUVECs transfected with TEM5

Figure 1 Thrombin cleavage mediates exposure of the TEM5-RGD motif

(A) Domain structure of the sTEM5 expression construct. FLAG, FLAG epitope tag; LRR, leucine-rich repeat domain; CT, C-terminal domain; IG, Ig domain; HormR, hormone receptor domain; GPS, GPCR proteolysis site domain; His, His tag; N60/N80, N-terminal 60/80 kDa fragment. (B) Recombinant sTEM5 (2 μM) was incubated with or without (control) the indicated proteases (10 μg/ml) for 20 h at 37°C. Digests were analysed by Western blotting using an anti-FLAG tag antibody. The molecular mass in kDa is indicated on the left-hand side. (C) The indicated TEM5 fragments were derived from recombinant sTEM5 or the point mutant sTEM5-D364E (RGE) and generated as described in the Experimental section. Vitronectin (control), purified TEM5 fragments and BSA were immobilized on polystyrene and HUVECs were allowed to adhere for 1 h. Non-adhering cells were washed away and cell adhesion was quantified using the AlamarBlue assay. Values are means ± S.D., n = 3, *P < 0.001 compared with BSA.
Thrombin-induced shedding of N60 is independent of metalloproteinase and PAR1 activation

HEK-293 cells, as well as HUVECs, express metalloproteinases and PAR1, which are activated by thrombin and might be involved in thrombin-induced N60 shedding [29–31]. While the pan metalloproteinase inhibitor GM6001 did not inhibit N60 shedding from TEM5-transfected HEK-293 cells (Figure 4) and HUVECs (results not shown), it was blocked by the specific thrombin inhibitor hirudin, indicating that proteolytic activity of thrombin, but not of metalloproteinases, is required. Furthermore, the PAR1-activating peptide TFLLR did not induce N60 shedding from HEK-293 cells (Figure 4) or HUVECs (results not shown). Involvement of other PARs in thrombin-induced N60 shedding can be excluded. PAR2 is only activated by PAR1 or by trypsin and tryptases [30]. PARs 3 and 4 are not expressed in HEK-293 cells [29]. Although HUVECs express PARs 1–3, it has been shown that PAR3 is not responsive to thrombin in these cells [30].

N60 and C50 derived from thrombin cleavage of recombinant sTEM5 are covalently linked by disulfide bonds

In an attempt to purify N60 from thrombin digests of sTEM5 using an anti-FLAG affinity resin, we found that C50 co-purifies with N60 (results not shown). Conversely, immunoprecipitation of C50 using an anti-FLAG antibody resulted in co-precipitation of N60 (Figure 5A). The interaction between N60 and C50 was strong and resisted sodium chloride concentrations of up to 1 M (results not shown). Thrombin cleaves TEM5 within its HormR domain. HormR domains contain four highly conserved cysteine residues that form disulfide bonds [32]. Figure 5(B) shows the thrombin cleavage sites of TEM5 in the context of the conserved cysteine residues of the HormR domain. Both thrombin cleavage sites are flanked by cysteine residues that are predicted to form disulfide bonds. To determine whether the interaction between N60 and C50 is mediated by disulfide bonds, sTEM5 quantitatively cleaved by thrombin (+) or incubated alone (−) was analysed by SDS/PAGE under reducing and non-reducing conditions followed by anti-FLAG Western blotting (Figure 5C, cell-free). While untreated sTEM5 migrated at ~110 kDa under reducing and non-reducing conditions, thrombin-cleaved sTEM5 migrated at 60 kDa under reducing conditions (free N60) and at 110 kDa under non-reducing conditions, representing a disulfide-linked heterodimer of N60 and C50 (N60–SS–C50). In contrast, N60 shed from thrombin-treated cells did not shift up under...
Figure 5 N60 and C50 derived from thrombin cleavage of recombinant sTEM5 are covalently linked by disulfide bonds

(A) Recombinant sTEM5 was quantitatively cleaved by thrombin and immunoprecipitated (IP) using an α-FLAG tag antibody (α-FLAG) or an irrelevant antibody (IgG). Immunoprecipitations were analyzed by Western blotting using an anti-FLAG tag antibody (top panel) and an anti-mouse IgG antibody (bottom panel). HC, heavy chain. (B) Sequence of the TEM5 HormR domain. Arrows indicate thrombin cleavage sites. Square brackets above the sequence indicate predicted disulfide bonds between conserved cysteine residues (bold). (C) Cell-free: recombinant sTEM5 (5 μM) was incubated with (+) or without (−) thrombin (3 μM) for 16 h at 37°C. Cell-based: HEK-293-TEM5 cells were treated with 100 nM thrombin (+) for 24 h in serum-free medium. TEM5 fragments were immunoprecipitated from the digest (cell-free) and CM (cell-based) using an anti-FLAG tag antibody and immunoprecipitates were analyzed by SDS/PAGE under reducing (R) and non-reducing (NR) conditions followed by anti-FLAG Western blot analysis. The asterisk indicates the N60–SS–C50 heterodimer. The molecular mass in kDa is indicated on the left-hand side.

non-reducing conditions as it is no longer associated with its membrane-bound C-terminal counterpart (C130) (Figure 5C, cell-based).

Thrombin cleavage of TEM5 is regulated by cell-surface PDI

Since N60 was not associated with C130 on the cell surface by disulfide bonds but released into the culture medium, we hypothesized that thrombin-induced shedding of N60 is a two-step process involving primary cleavage by thrombin and secondary reduction of the disulfide bonds within the HormR domain. PDI is known to mediate the reduction of disulfide bonds on the cell surface [22–24]. To test our hypothesis, TEM5-expressing cells were treated with thrombin and different PDI inhibitors (Figure 6A). Indeed, thrombin-induced N60 shedding was strongly reduced by bacitracin, a membrane-impermeant PDI inhibitor, and completely abrogated by a function-blocking monoclonal anti-PDI antibody (RL90), but not by a control antibody. In contrast with our expectations, thrombin treatment in combination with PDI inhibition did not result in formation of an N60–SS–C130 heterodimer on the cell surface, indicating that cell-surface PDI also regulates thrombin cleavage itself. Indeed, thrombin-mediated N60 cleavage and shedding was enhanced by a mild reducing agent (GSH) and even more by GSH and PDI in combination (Figure 6B). Thrombin-induced shedding of N60 was not increased by PDI alone.

PDI-mediated reduction of disulfide bonds in stTEM5 enhances thrombin cleavage and results in dissociation of N60–SS–C50

To determine whether PDI-mediated reduction of disulfide bonds in TEM5 also enhances thrombin cleavage in a cell-free system, recombinant stTEM5 was incubated with thrombin at a concentration that mediates incomplete cleavage. The presence of PDI at a catalytic concentration had no effect on thrombin cleavage and did not result in reduction of the disulfide bonds between N60 and C50 (Figure 6C). In contrast, PDI in combination with GSH markedly enhanced thrombin cleavage and resulted in reduction and dissociation of the N60–SS–C50 heterodimer. GSH alone only had a little enhancing effect on thrombin cleavage and mediated partial reduction of N60–SS–C50. Since thrombin also contains disulfide bonds that might be reduced by PDI and thereby modulate TEM5 cleavage, thrombin and stTEM5 were pretreated with PDI/GSH and subsequently incubated with untreated stTEM5 and thrombin respectively. Figure 6(D) shows that PDI/GSH pretreatment of stTEM5, but not of thrombin, resulted in enhanced cleavage.

PDI transiently forms mixed disulfides with TEM5 on the cell surface

PDI transiently forms mixed disulfides with its substrates [19]. To confirm that cell-surface PDI mediates the reduction of disulfide bonds of TEM5, mixed disulfides of PDI and TEM5 were trapped by alkylating free thiols with NEM. Cell-surface proteins were biotinylated. Cell-surface TEM5 was isolated by a two-step pulldown assay using anti-FLAG and anti-Myc tag antibodies respectively. The asterisk indicates a non-specific band.

Figure 6 Thrombin cleavage of TEM5 is regulated by cell-surface PDI

(A and B) HEK-293-TEM5 cells were treated or not (untreated) for 24 h in serum-free medium with 100 nM thrombin alone (control) or in combination with (A) 25 μg/ml function-blocking anti-PDI antibody clone RL90 (α-PDI), 25 μg/ml irrelevant antibody (IgG) and 3.5 mM bacitracin (Bac), or (B) 100 nM PDI, 1 mM GSH, or 100 nM PDI and 1 mM GSH (PDI/GSH). Anti-FLAG immunoprecipitates of CM as well as cell lysates were analysed by Western blotting using anti-FLAG and anti-Myc tag antibodies respectively. The asterisk indicates a non-specific band. (C) Recombinant sTEM5 (1 μM) was incubated for 2 h at 37°C with thrombin (100 nM), PDI (100 nM) and GSH (1 mM) where indicated. (D) Recombinant sTEM5 (1 μM) and thrombin (100 nM) were pretreated for 1 h at 37°C with 100 nM PDI and 1 mM GSH (PDI/GSH pre) followed by alkylation of free thiols and inactivation of PDI/GSH by 10 mM NEM for 2 h. Pretreated sTEM5 and thrombin were incubated for 2 h at 37°C with untreated thrombin (100 nM) and sTEM5 (1 μM) respectively. (C and D) Digests were analysed by SDS/PAGE under reducing and non-reducing conditions followed by anti-FLAG Western blot analysis. (E) Mixed disulfides of PDI and TEM5 in HEK-293-TEM5 cells were trapped (+) or not (−) by NEM and cell-surface proteins were biotinylated. Cell-surface TEM5 was isolated by a two-step pulldown assay using anti-FLAG and neutravidin–agarose. Pulldowns were analysed by Western blotting using a anti-FLAG tag antibody (top panel) and an anti-PDI antibody (clone RL90, bottom panel). csTEM5, cell-surface TEM5; csPDI, cell-surface PDI. The molecular mass in kDa is indicated on the left-hand side.
showed that immobilized MMP9-processed sTEM5 mediates cleavage. Cell-adhesion assays revealed that N60, but not N80, derived from thrombin cleavage and N80 derived from MMP1 respectively. It is conceivable that cleavage of TEM5 five residues downstream of its RGD motif leads to a conformational change that results in exposure of the RGD binding site. Similarly, the RGD motif of the ECM protein osteopontin is exposed by thrombin cleavage seven residues downstream of its RGD motif [13].

We previously demonstrated that MMP9 cleavage of recombinant sTEM5 results in formation of two N-terminal site [13]. However, the two fragments had not been investigated separately to determine whether one or both contained the RGD motif in an open conformation. In the present study, these two fragments were investigated separately using N60 derived from thrombin cleavage and N80 derived from MMP1 cleavage. Cell-adhesion assays revealed that N60, but not N80, contained an exposed RGD motif. In our previous study we showed that immobilized MMP9-processed sTEM5 mediates endothelial cell survival during angiogenesis by linking GAGs (glycosaminoglycans) to RGD-binding integrins. However, activation of integrins by N60 derived from MMP9 or thrombin cleavage may also mediate other functions during angiogenesis. It may facilitate cell migration by ‘coating’ weakly adhesive GAGs of the ECM. It might also function as a chemoattractant for endothelial cells or other cell types. In fact, it has been reported that soluble CD97, which also belongs to the adhesion family of GPCRs and contains an RGD motif, mediates RGD-dependent chemotaxis of endothelial cells [34]. Recently it has been shown that TEM5 plays an essential role in brain angiogenesis during development and mediates directional migration of endothelial cells [10,17]. Whether regulation of integrin function by the TEM5 RGD motif is involved in these processes remains to be determined. There may be mechanisms by which the RGD motif becomes exposed in full-length TEM5, for example by ligand binding or by proteases that cleave oxidized TEM5 and mediate exposure of the RGD motif in a disulfide-linked heterodimer (e.g. N60–SS–C130).

Thrombin-induced shedding of N60 also results in formation of the membrane-bound C130 fragment of TEM5. It remains to be determined whether that fragment also has a specific function. Thrombin cleavage of TEM5 might lead to receptor activation similar to PAR1 cleavage [30]. However, Western blot analysis of protein tyrosine phosphorylation in thrombin-stimulated HEK-293-TEM5 and control cells did not reveal TEM5-dependent tyrosine phosphorylation (results not shown).

Thrombin activates metalloproteinases on the cell surface that might be involved in thrombin-induced N60 shedding [31]. However, N60 shedding from HEK-293 cells and HUVECs was not inhibited by the pan metalloproteinase inhibitor GM6001. Activation of PAR1 by thrombin might lead to activation of non-metalloproteinases that mediate N60 shedding. However, the PAR1-activating peptide TFLLR did not induce N60 shedding, indicating that thrombin also directly cleaves TEM5 on the cell surface.

Interestingly, N60 and C50 derived from thrombin cleavage of recombinant sTEM5 were covalently associated by disulfide bonds (N60–SS–C50), whereas N60 shed from thrombin-treated cells was not associated with its membrane-bound C-terminal counterpart (C130). The thrombin cleavage sites of TEM5 are located within its HormR domain, which contains four conserved cysteine residues that form disulfide bonds with other HormR domain-containing proteins [32]. Therefore we hypothesized that reduction of the disulfide bonds within the TEM5 HormR domain occurs on the cell surface. PDI is known to catalyse the reduction of disulfide bonds on the cell surface [22–24]. Indeed, we found that PDI mediates the reduction of disulfide bonds of TEM5 on the cell surface, which is crucial for thrombin-induced shedding of N60. The TEM5 extracellular domain contains 24 cysteine residues, but it is unknown how many of them form disulfide bonds. Figure 5(B) shows that reduction of the predicted disulfide bond between Cys153 and Cys203 in the HormR domain would theoretically be sufficient to result in release of the N60 fragment, provided that no other disulfide bonds exist between N60 and C130.

Unexpectedly, PDI did not only mediate reduction of disulfide bonds of TEM5 and dissociation of N60–SS–C50, but also regulated thrombin cleavage of TEM5 itself. Pretreatment of sTEM5 with reduced PDI mediated enhanced cleavage of sTEM5, whereas pretreatment of thrombin with reduced PDI did not, ruling out an involvement of PDI-mediated reduction of disulfide bonds in thrombin. Thrombin-induced shedding of N60 from cells was increased by the presence of the mild reducing agent GSH and even more by GSH and PDI in combination. Interestingly,
thrombin cleavage of TEM5 was more tightly regulated by PDI on the cell surface than in a cell-free system. While recombinant sTEM5 was cleaved by thrombin without reduced PDI and cleavage was enhanced by the presence of reduced PDI, thrombin-mediated cleavage of TEM5 on the cell surface was completely abrogated by a function-blocking anti-PDI antibody. The difference in PDI-dependence between those two systems may be explained by the better accessibility of sTEM5 in the cell-free system, compared with the restricted accessibility on the cell surface. In the ER, PDI catalyses the formation of disulfide bonds in the TEM5 extracellular domain, possibly in its HormR subdomain, that link the N-terminal part (N60) to the C-terminal part (C50). It seems unlikely that PDI constitutively catalyses the reduction of these disulfide bonds once TEM5 reaches the cell surface. Furthermore, we show in the present study that only a small fraction of cell-surface TEM5 is reduced in HEK-293-TEM5 cells, as addition of reduced PDI markedly enhanced thrombin-induced N60 shedding. Therefore it is likely that PDI-mediated reduction of TEM5 on the cell surface is a regulated process that may be activated by certain stimuli or the cellular microenvironment. In fact, it has been reported that endothelial PDI is redistributed to the plasma membrane and shed upon thrombin stimulation [35]. Angiogenesis is triggered by hypoxia, and under hypoxia the cellular microenvironment becomes less oxidizing, which may also affect PDI-catalysed reactions on the cell surface.

On the basis of the results of the present study we conclude that PDI regulates thrombin-induced shedding of TEM5 and exposure of its RGD motif by catalysing the reduction of crucial disulfide bonds of TEM5 on the cell surface (Figure 8).

FIGURE 8 Model of PDI-regulated thrombin cleavage of TEM5

Oxidized TEM5 (oxTEM5) is resistant to thrombin cleavage on the cell surface. Reduction of certain disulfide bonds in oxidized TEM5 by reduced PDI (redPDI) on the cell surface leads to oxidation of PDI (oxPDI) and exposure of the thrombin cleavage sites in reduced TEM5 (redTEM5). Thrombin cleavage of redTEM5 results in shedding of the N60 fragment containing an exposed RGD motif and in formation of the membrane-bound C130 fragment.

REFERENCES

9 Vallon, M., Rohde, F., Janssen, K.-P. and Essler, M. (2010) Tumor endothelial marker 5 expression in endothelial cells during capillary morphogenesis is induced by the small GTPase Rac and mediates contact inhibition of cell proliferation. Exp. Cell Res. 316, 412–421

AUTHOR CONTRIBUTION

Mario Vallon wrote the manuscript; Mario Vallon and Philipp Aubele planned and performed experiments; Klaus-Peter Janssen and Markus Essler analysed data and edited the paper prior to submission; Markus Essler and Mario Vallon designed the study.

FUNDING

This work was supported by the Wilhelm Sander-Stiftung and the Deutsche Forschungsgemeinschaft (grant number SFB824-B3).


