Protease-activated Receptor 2-dependent Phosphorylation of the Tissue Factor Cytoplasmic Domain*

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Tissue factor (TF) is the physiological activator of the coagulation cascade that plays pathophysiological roles in metastasis, angiogenesis, and inflammation. Downstream in coagulation, thrombin is the central protease that signals through G protein-coupled, protease-activated receptors (PARs). However, the TF-VIIa-Xa complex upstream in coagulation also activates PAR1 and PAR2. Here, we address the question of whether signaling of the TF initiation complex is a relevant pathway that leads to TF cytoplasmic domain phosphorylation. In heterologous expression systems and primary endothelial cells, we demonstrate that the ternary TF-VIIa-Xa complex induces TF phosphorylation specifically by activating PAR2 but not through PAR1 signaling. In addition, TF cytoplasmic domain phosphorylation is induced only by TF-dependent signaling but not by other coagulation factors in endothelial cells. Phosphorylation of the Pro-directed kinase target site Ser258 is dependent on prior phosphorylation of Ser253 by protein kinase C (PKC) α. TF phosphorylation is somewhat delayed and coincides with sustained PKCα activation downstream of PAR2 but not PAR1 signaling. Phosphatidylcholine-dependent phospholipase C is the major pathway that leads to prolonged PKCα recruitment downstream of PAR2. Thus, PAR2 signaling specifically phosphorylates TF in a receptor cross-talk that distinguishes upstream from downstream coagulation protease signaling.

Cell signaling through G protein-coupled protease-activated receptors (PARs) is closely linked to the activation of the coagulation protease cascade (1). Genetic evidence established an essential in vivo role of PAR signaling for platelet activation and the hemostatic response (2, 3). However, PAR signaling also has important nonhemostatic functions in development (4), inflammation (5), and tumor biology (6). All PARs, except for PAR2, are directly activated by thrombin, and PAR signaling is typically attributed to the action of thrombin in vivo. However, in vitro evidence indicates that thrombin receptors are also targets for other proteases at meaningful physiological concentrations (7–10). Cellular receptors can concentrate serine proteases at the cell surface for PAR cleavage (11). More importantly, protease co-factors, such as tissue factor (TF) and endothelial cell protein C receptor, couple the initiation of the pro- and anti-coagulant pathways to PAR signaling by retaining transiently the initial protease products Xa and activated protein C on the cell surface (9, 10).

Different PARs produce only partially redundant signaling responses in vascular cells. In the case of PAR1 and PAR2, the predominant endothelial cell expressed PARs, several immediate early gene induction events are overlapping, but only PAR1 signaling induces the mRNA for monocyte chemoattractant protein (MCP) 1 (10). PAR signaling induces distinct cytoskeletal effects. PAR1 is a potent activator of the small GTPase Rho, which leads to the disruption of intercellular junctions and to increased permeability of the endothelium (12, 13). In contrast, PAR2 activation promotes cell motility by activation of the Rac/p21-activated kinase pathway and by forming a scaffolding complex containing β-arrestin and extracellular signal-regulated kinase (ERK). This complex is implicated in pseudopodia extension and chemotaxis (14, 15). PAR selectivity is corroborated by in vivo studies demonstrating that agonist peptides specific for PAR1 and PAR2 elicit selective responses in vascular tone (16, 17) and that developmental early embryonic lethality is specific for genetic deletion of PAR1 (4). Thus, protease signaling specificity is in part dependent on the signal transducing PAR, but additional complexity possibly arises from the co-factors/protease receptors that assist PAR cleavage.

In the initiation phase of coagulation, TF is involved in two signaling complexes: the TF-VIIa complex that activates PAR2 and the ternary TF-VIIa-Xa complex in which Xa cleaves PAR1 or PAR2 (8, 9, 18, 19). Because the ternary complex activates PAR1 and PAR2, it is an unresolved question whether immediate or delayed responses distinguish between TF-dependent signaling through PAR1 or PAR2. In this study we further characterize how the TF cytoplasmic domain becomes phosphorylated in endothelial cells. Although TF phosphorylation can be induced by phorbol 12-myristate 13-acetate (PMA) stimulation (20–22), to date no agonist pathway of potential physiological relevance has been identified that leads to TF cytoplasmic domain phosphorylation. Here, we show that PAR2 but not PAR1 signaling induces TF cytoplasmic domain phosphorylation. PMA-induced TF phosphorylation in endothelial cells was found to involve PKCα-mediated phosphorylation of Ser253 (22) and subsequent phosphorylation of Ser258 by a potential Pro-directed kinase (20–22). Whereas PKCα is activated downstream of PAR1 and PAR2 signaling, sustained membrane recruitment of PKCα is specific for PAR2 signaling and coincides with the onset of TF cytoplasmic domain phosphorylation. TF phosphorylation is a characteristic response of the initiation
EXPERIMENTAL PROCEDURES

Materials—VIIa, inactive Ser253 to Ala TF, generated by PCR mutagenesis, and PAR1 prototype vectors for full-length and C245S-mutated TF were described in detail (22). Ser253 to Ala TF, generated by PCR mutagenesis, and PAR1 prototype vectors for full-length and C245S-mutated TF were described in detail (22). Sequencing grade HEPES combined with 20 mM Tris-HCl, pH 7.6, 1 mM CaCl2 and PKCα antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and phospho- and non-phospho-ERK1/2 antibodies (Cell Signaling, Beverly, MA) were obtained commercially. Hirudin, conventional PKC inhibitors Go6983 and Go6976, PKCα inhibitor Safingol, and PKCβ inhibitor Hipsedin were purchased from Calbiochem (San Diego, CA). Anti-PAR1 antibodies ATAP2 and WEDE15 were kindly provided by Dr. L. F. Brass (24). Recombinant mastectomy anticogulant proteins 5 (NAP5) and 2 (NAP2) (25, 26) were kindly provided by Dr. G. Vlasuk (Corvas International, San Diego, CA). Wild-type and kinase inactive PKCα (R388N) constructs were gifts from Dr. Alex Toker (Boston, MA).

Cell Culture and Transient Transfection—Chinese hamster ovary (CHO) cells expressing full-length TF (CHO/TF cells) (27) were transiently transfected by electroporation. Cells (2 × 10^7) in 200 μl of Dulbecco’s modified Eagle’s medium, 2% fetal bovine serum, 1 mM HEPES combined with 20 μg of PAR1 or PAR2 cDNA constructs received a single pulse (250 V, 500 microfarads) in a gene pulser (Bio-Rad). The experiments were performed 48 h after transfection.

Adenoviral Vectors and Transduction of Endothelial Cells—Ad5 se- rotype vectors for full-length and C245S-mutated TF were described in detail (22). Ser253 to Ala TF, generated by PCR mutagenesis, and PAR1 and PAR2 cDNA were similarly subcloned into adenoviral vectors. The virus was plaque-purified twice, expanded for large scale production, and purified on CsCl gradient. Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (San Diego, CA) or Cascade Biologics (Portland, OR) and cultured in EGM medium containing 2–5% fetal bovine serum, 1 mg/ml hydrocortisone, 10 μg/ml human epidermal growth factor, and 3 mg/ml bovine brain extract. HUVECs were transduced for 4–6 h, and the experiments were performed 48 h later. Typically, the cells were transferred to serum-free medium for 2–4 h with the fresh medium, before the agonists were added. To monitor the levels of Xa, an aliquot of culture supernatant was quenched in buffer containing EDTA, and the Xa concentrations were determined by hydrolysis of the chromogenic substrate Spectrozyme FXa, based on calibration curves of purified Xa.

Western Blotting and Fluorescence-activated Cell Sorter Analysis—From cells treated with inhibitors and agonists, TF was immunoprecipi- citated in the case of CHO cells and PAR1-deficient fibroblasts. MDA-MB-231 breast cancer cells or endothelial cells were directly lysed in reducing sample buffer for Western blot analysis with phosphorylation (αT-FTCD) or pan-specific (α-TFCD) antibody to the TF-cytosolic domain (23). To assess surface expression of TF, PAR1, or PAR2, the cells were harvested with EDTA and resuspended in serum-free medium, 2 mM CaCl2. To determine PAR internalization, the cells were stimulated with ternary complex-mediated ERK phosphorylation (Fig. 1C). These data are consistent with similar efficiency of PAR1 or PAR2 activation by ternary complex signaling. However, only PAR2 expression resulted in ternary complex-induced TF cytoplasmic domain phosphorylation (Fig. 1D), and PAR2, ternary complex phosphorylation caused TF cytoplasmic domain phosphorylation specifically through PAR2 activation.

PAR2 Signaling Is Sufficient to Support TF Cytosolic Domain Phosphorylation—Because endogenous PAR1 was co-expressed in PAR2-transfected CHO cells, it remained unclear whether PAR1 is required, albeit not sufficient to trigger TF phosphorylation. To address this question, fibroblasts from PAR1-deficient mice that also lack other PARs (9, 19) were used. PMA-stimulated TF cytosolic domain phosphorylation is negatively regulated by palmitoylation of the cytoplasmic Cys245 residue in endothelial cells (22), and mutation of Cys245 greatly facilitates the detection of TF cytosolic domain phosphorylation. PAR1-deficient fibroblasts were co-transduced with adenovirus to express human Cys245 to Ser mutated TF together with PAR2 or PAR1, achieving expression levels shown in Fig. 2A. The cells were stimulated with PAR1 or PAR2 agonist peptides, and with thrombin, trypsin, or ternary complex as relevant proteases that activate PARs. No TF phosphorylation was observed in PAR1-expressing cells, but TF cytosolic domain phosphorylation was observed in PAR2-transduced cells stimulated with appropriate PAR2 agonists (Fig. 2B). These data demonstrate that endogenous PAR1 is not required in the signaling pathway that phosphorylates TF. Note the more quickly migrating form of TF in the loading
control that was performed by reprobing with pan-specific anti-TF cytoplasmic domain antibody. We had previously found in PMA-stimulated cells that certain N-linked carbohydrate isoforms of TF cannot be phosphorylated (22). The lack of phosphorylation of the more quickly migrating forms of TF in this and other experiments shown below indicates that Golgi-dependent carbohydrate modification, and thus presumably a certain subcellular protein trafficking route, is also important for phosphorylation of the TF cytoplasmic domain in response to PAR2 activation.

**TF Cytoplasmic Domain Phosphorylation Is Specific for Upstream Coagulation Signaling in Primary Cells**—The relative importance of PAR1 and PAR2 signaling in TF cytoplasmic domain phosphorylation was analyzed in primary HUVECs transduced with adenovirus to express TF and variable levels of PAR1 and PAR2. HUVECs express endogenous PAR1 (Fig. 3A, UT) and very little PAR2 (data not shown). HUVECs were adenovirus transduced to achieve comparable expression levels of PAR1 or PAR2. PAR activation was confirmed by measuring internalization by flow cytometry and stimulation with the NAPc2 stabilized ternary complex similarly induced internalization of both receptors (Fig. 3B). With both PAR1 and PAR2, internalization by TF-dependent signaling was somewhat less efficient when compared with the cleavage by thrombin and trypsin, respectively. Consistent with data in the CHO cell model, activation of agonist peptide is remarkable, considering the fairly low levels of cell surface-expressed PAR2 relative to PAR1. Thus, in cells that express endogenous TF, PAR1, and PAR2, TF cytoplasmic domain phosphorylation occurs specifically downstream of PAR2 signaling.

**TF Cytoplasmic Domain Phosphorylation Is Mediated by PAR2 Signaling in Heterologous Expression Systems**—A, serum-starved CHO/TF cells were stimulated with 10 μM PAR1 agonist, 100 μM PAR2 agonist, or PMA (20 ng/ml) for 1 h. Immunoprecipitated TF was detected by Western blotting with anti-phosphorylated TF cytoplasmic domain antibody (α-TFCD) or pan-specific anti-TF cytoplasmic domain antibody (α-TFCD). B, CHO/TF cells stably expressing a noncleavable mutant of PAR2 (CHO/TF/PAR2/P1) or wild-type PAR2 (CHO/TF/PAR2) were stimulated with PAR2 agonist peptide or NAPc2-stabilized ternary TF-VIIa-Xa complex (iVIIa/Xa/NAPc2 10/20/100 nM) for 1 h, followed by Western blotting for TF phosphorylation. Loading was controlled by reprobing with pan-specific anti-TF cytoplasmic domain antibody. C, transiently transfected CHO/TF cells were analyzed for human PAR expression by flow cytometry using WEDE15 or SAM11 monoclonal antibodies to PAR1 or PAR2, respectively. Controls were stained with isotype matched primary antibody. D, CHO/TF cells transiently expressing human PAR1 or PAR2 were stimulated with ternary complex or PAR agonists for 10 min for ERK1/2 phosphorylation or for 1 h for TF cytoplasmic domain phosphorylation. The loading control in the top panel by reprobing and the anti-ERK1/2 blot were performed on separate transfers. E, left panel, quantitation of receptor internalization after 30 min (37 °C) stimulation by ternary complex or PAR agonist of CHO/TF cells transiently transfected with human PAR1 or PAR2 (means and standard deviations, n = 3). Right panel, the increase in TF cytoplasmic domain phosphorylation, as shown in D, was quantified by laser densitometry (means and standard deviations, n = 3). FITC, fluorescein isothiocyanate.
endogenous PAR1 (not shown) or overexpressed PAR1 did not induce TF phosphorylation (Fig. 3C). In contrast, introducing PAR2 with TF resulted in TF cytoplasmic domain phosphorylation after 10 min of stimulation with ternary complex, PAR2 agonist, or trypsin. Co-expression of PAR2 with Cys245 to Ser mutated TF at similar levels as wild-type TF resulted in significantly enhanced TF cytoplasmic domain phosphorylation after 10 min of stimulation (Fig. 3C) and PAR1 stimulation was also not sufficient to induce phosphorylation of this mutant (data not shown). The differences in TF phosphorylation between wild-type and mutant TF were quantified by densitometry, using normalization for slight differences in expression levels. TF was phosphorylated ~7-fold more efficiently when thioester modification of Cys245 was prevented by mutagenesis (n = 3).

The specificity of TF cytoplasmic domain phosphorylation in response to other coagulation proteases was analyzed in HUVECs expressing PAR2 and Cys245 to Ser mutated TF. The cells were stimulated for 1 h to increase the sensitivity for detecting inefficient responses, but high concentrations of factor IXa and activated protein C as well as thrombin did not induce TF phosphorylation (Fig. 3D). These are important data, because thrombin-cleaved PAR1 has been shown to cross-activate PAR2 (24). Whereas the NAPc2 stabilized TF-VIIa-Xa ternary complex triggered TF cytoplasmic domain phosphorylation with efficiency similar to that of a direct PAR2 agonist, signaling by VIIa (50 nM) alone produced less efficient TF phosphorylation in HUVEC monolayers. In contrast, stimulation with VIIa and X, which mimics activation of coagulation, efficiently induced TF cytoplasmic domain phosphorylation. In these experiments, Xa activity was blocked by adding a Xa inhibitor (NAP5) before free Xa reached levels that could activate PAR2.

The efficiency of ternary complex signaling is further illustrated in the middle panel of Fig. 3D with cells stimulated for only 10 min. The reaction with VIIa and X generated <10 nM Xa in this time period but lead to more efficient TF phosphorylation in comparison with free Xa at concentrations as high as 50 nM. In the right panel, the cells were stimulated with VIIa and X in the presence of the potent Xa inhibitor NAP5. Blocking Xa activity prevented TF cytoplasmic domain phosphorylation, confirming our previous conclusions (9) that in the ternary TF-VIIa-Xa coagulation initiation complex signaling is induced by Xa and not VIIa. Taken together, these experiments provide evidence that phosphorylation of the TF cytoplasmic domain occurs as a result of the activation of a highly specific signaling pathway following the initiation of coagulation.

PAR2 Signaling Induces Sustained PKCα Activation—The phosphorylation-specific antibody to TF recognizes the conformational change induced by phosphorylation of the cytoplasmic Ser258 residue that probably is the target for a Pro-directed kinase (20–22). However, PKCα-dependent phosphorylation of Ser258 appears to be required for PMA-induced Ser258 phosphorylation in HUVEC (22). Mutation of either the PKC phosphorylation site Ser258 or the PKC consensus recognition residues Lys255 abolished TF phosphorylation induced by PAR2-dependent ternary complex signaling (Fig. 4A). TF cytoplasmic domain phosphorylation was also blocked by inhibitors for conventional PKCs (α, β, and γ), G6976, and G6983, as well as the PKCα-selective inhibitor Safingol but not the PKCβ-inhibitor Hipsidin (Fig. 4B). To further demonstrate PKC specificity, we overexpressed wild-type PKCα or kinase-inactive, Lys68 to Asn mutated PKCα constructs. Ternary complex-induced TF cytoplasmic domain phosphorylation was significantly inhibited in cells expressing dominant negative, inactive PKCα, as compared with vector control or wild-type PKCα transfected cells (Fig. 4C). Thus, PKCα is important for TF cytoplasmic domain phosphorylation downstream of PAR2, presumably by targeting the PKC phosphorylation site at Ser258.

Thrombin-mediated PAR1 signaling activates PKCα in endothelial cells to increase permeability (29, 30), but PAR2-mediated PKCα activation is poorly characterized. TF cytoplas-
repeat experiments, documenting that the prolonged PAR2-dependent translocation of PKCα was highly reproducible. PMA stimulation triggers TF cytoplasmic domain phosphorylation even in the absence of PAR2. PMA stimulation of either untransduced (not shown) or PAR2-transduced endothelial cells also resulted in prolonged PKCα translocation (Fig. 5B). Taken together, these experiments provide evidence that the duration of PKCα activity at the membrane is an important determinant for TF cytoplasmic domain phosphorylation.

A critical question, therefore, is how PAR2 signaling differs from PAR1 activation to induce sustained PKCα translocation. G protein-coupled receptor activation induces phosphatidylinositol (PI) breakdown mediated by PI-specific phospholipase C (PLC) isosforms to generate diacylglycerol required for PKC membrane recruitment. However, diacylglycerol is also generated by phosphatidylinositol-specific PLC (PC-PLC), which is known to promote prolonged membrane recruitment of PKCs (32). To analyze the role of PI- and PC-PLC in PKCα activation downstream of PAR2, the effect of PLC isosform specific inhibitors on TF cytoplasmic domain phosphorylation was analyzed. Whereas the PI-PLC specific inhibitor U73122 was without effect, the PC-PLC-specific inhibitor tricyclohexylamyl xanthogenate (D609) dose-dependently inhibited ternary complex- or PAR2 agonist-induced TF cytoplasmic domain phosphorylation (Fig. 5D and data not shown). To further provide evidence that D609 inhibits the pathway downstream of PAR2 leading to PKC activation, we tested the effect of D609 on TF phosphorylation by PMA, which directly activates PKC. Both D609 and U73122 only marginally reduced PMA-induced TF phosphorylation, demonstrating that pathways downstream of PKC are not influenced by D609.

The effect of PLC isosform-specific inhibitors on PKCα translocation was analyzed to clarify PAR2-mediated PKCα activation. In PAR2 expressing cells, the PC-PLC-specific inhibitor D609 significantly inhibited both initial and sustained PKCα membrane recruitment in response to TF ternary complex signaling. U73122 produced little to no effect on PKCα translocation in PAR2 expressing cells (Fig. 5B). Based on densitometry of three repeat experiments, D609 reduced PKCα translocation at 1 and 10 min by 76 and 77%, whereas the reduction by U73122 was 27 and 22%, respectively. In contrast, TF-dependent PAR1 activation resulted in transient PKCα translocation that was entirely dependent on PI-PLC. Thus, sustained activation of PKCα and TF cytoplasmic domain phosphorylation are predominantly dependent on a PC-PLC pathway downstream of PAR2 signaling.

**DISCUSSION**

In this study, we show that PAR2 signaling specifically targets the TF cytoplasmic domain by inducing TF phosphorylation. Our data demonstrate that the ternary TF-VIIa-Xa complex similarly activates PAR1 or PAR2, based on efficiency of PAR internalization and ERK phosphorylation, but only PAR2 signaling leads to TF phosphorylation. TF cytoplasmic domain phosphorylation is dependent on PKCα activation. The presented mutational data support the concept that phosphorylation of the PKC consensus Ser583 is necessary for subsequent Ser586 phosphorylation, which is recognized by the phosphorylation-specific antibody to TF (22). Rapid PKCα membrane recruitment downstream of PAR1 or PAR2 demonstrates efficient receptor activation by the ternary complex. However, only PAR2 signaling induced sustained PKCα membrane localization, which coincides with the somewhat delayed kinetics of TF phosphorylation. Pharmacological blockade shows that PAR1 and PAR2 activations recruit PKCα through distinct PLC pathways. Sustained activation of PKCα downstream of PAR2 is dependent on PC-PLC, which is known to induce prolonged
PAR2-mediated TF Phosphorylation

Fig. 4. Role of PKCζ in PAR2-dependent TF cytoplasmic domain phosphorylation. A, HUVECs were co-transduced with C245S, K255A, or S253A mutants of TF and PAR2 with the same virus dose as described for Fig. 2. After 48 h, the cells were transferred to serum-free medium for 2 h and stimulated with ternary complex for 1 h. TF cytoplasmic domain phosphorylation was determined by Western blotting and reprobing with αTFCD. B, cells expressing Cys245Ser TF with PAR2 were serum-starved (2 h) and pretreated for 30 min with conventional PKC inhibitors, Go6976 (1 μM), Go6983 (1 μM), or isofom specific inhibitors for PKCζ, Safingol, (50 μM), and PKCβ, Hispidin, (10 μM), followed by stimulation with ternary complex for 1 h at 37 °C. C, cells expressing C245S TF and PAR2 were co-transfected with 2 μg of pcDNA3.1 (vector control), FLAG-tagged PKCs or a catalytically inactive PKCs (K368N) constructs using FuGENE 6. After 48 h, the cells were serum-starved and stimulated with ternary complex for 1 h. Top panel, TF cytoplasmic domain phosphorylation by αTFCD blot. Bottom two panels, PKCζ expression by anti-PKζa and anti-FLAG blot.

Fig. 5. PAR2-dependent TF cytoplasmic domain phosphorylation requires PC-PLC-mediated sustained PKCζ activation. A, time course of ternary complex-induced TF cytoplasmic domain phosphorylation. HUVECs co-expressing C245S TF and PAR2 were transferred to serum-free medium for 2 h and stimulated with ternary complex for the indicated time. TF cytoplasmic domain phosphorylation was determined by Western blotting and reprobing with αTFCD. B, C245S TF and PAR2- or PAR1-expressing cells were serum-starved (4 h) and stimulated with ternary complex for 1 or 10 min followed by separation of membrane and cytosolic fractions. PKCζ distribution was determined by Western blotting of samples with equal protein content. Similar loading of membrane fractions is confirmed by reprobing with α-TF (bottom panel). C, quantitation of PKCζ membrane recruitment by ternary complex signaling in PAR2- or PAR1-expressing cells. Fold inductions of translocation were determined by densitometry (means and standard deviations, n = 3). D, effect of PC- and PI-PLC-specific inhibitors on TF cytoplasmic domain phosphorylation. HUVECs expressing C245S TF and PAR2 were transferred to serum-free medium for 2 h and preincubated with the indicated concentration of the PC-PLC-specific inhibitor D609 or the PI-PLC inhibitor U73122 (10 μM) for 30 min following by stimulation with agonist for 1 h. In the right panel D609 was used at 20 μM. TF cytoplasmic domain phosphorylation was determined by Western blotting, and equal TF expression was confirmed on separate blots. E, HUVECs pretreated with 20 μM D609 or 10 μM U73122 were stimulated, followed by membrane isolation. PKCζ membrane recruitment was determined by Western blotting with α-PKζa and reprobing for confirmation of equal protein loading with α-TF. For all panels ternary complex VIIa/Xa/NAPc2 (10/20/100 nM) and PMA (20 ng/ml) were used.

Membrane translocation of PKCs (32). The fact that PMA stimulation, independent of PAR2, induces both TF phosphorylation and sustained PKCζ membrane recruitment provides further support for an essential role of prolonged PKCζ signaling in TF cytoplasmic domain phosphorylation.

This study adds to emerging evidence (10, 12, 13) that PAR1 and PAR2 signaling elicit specific and only partially overlapping responses in endothelial cells. More importantly, only TF-dependent signaling, but not the signaling by other coagu-

lant factors, induced cytoplasmic domain phosphorylation. The phosphorylation of the TF cytoplasmic domain thus represents a marker for upstream coagulation signaling through PAR2. Both PAR2 and TF are induced by inflammatory mediators in endothelial cells in tissue culture (33, 34), and TF has been detected in vivo in splenic endothelial cells in severe systemic inflammation (35) or in breast cancer-associated endothelium (36). Moreover, PAR2 is found to be up-regulated upon differentiation of monocytes into macrophages (37), which also express TF. Thus, PAR2-dependent phosphorylation of TF may play regulatory roles in the biology and pathology of vascular cell types that co-express TF and PAR2.

The role of the TF cytoplasmic domain has previously been analyzed in heterologous transfection systems. Deletion of the entire cytoplasmic domain has little influence on the procoagulant function of TF (27, 38, 39) and on TF-VIIa proteolytic signaling, as measured by immediate early gene induction or phosphorylation events downstream of G protein-coupled receptor signaling (40–42). Although the relevant PARs that transmit TF-dependent signaling in these cell models are incompletely defined, these studies are consistent with an interpretation that the TF cytoplasmic domain is not required for PAR activation. Our data show that TF ternary complex signaling in the case of both PAR1 and PAR2 signaling is rapid, leading to PKCζ membrane recruitment within 1 min. However, TF cytoplasmic domain phosphorylation is a delayed response, and it is therefore not surprising that the TF cytoplas-

mic domain played apparently no role in early responses of TF-VIIa signaling. In addition, TF cytoplasmic domain phosphorylation appears to be more efficient by ternary TF-VIIa-Xa in comparison with TF-VIIa stimulation, at least under our experimental conditions, which may indicate that the TF cytoplasmic domain plays a regulatory role in signaling associated
with activation of the coagulation pathways.

TF is important for metastasis (27, 43), cell adhesion, and migration (44–47). Whereas PAR1 signaling potently induces Rho activation (12, 13), PAR2 only transiently activates Rho while activating Rac and recruiting a β-arrestin scaffolding complex for ERK localization (14, 15). These pathways promote cell motility, a cellular function that is possibly supported by PAR2 signaling: PAR2 signaling that targets the TF cytoplasmic domain may operate in this context, and the presented data provide novel insight into the mechanism by which PAR2 signaling feeds back onto the protease-binding receptor TF that supports PAR cleavage by associated proteases. Phosphorylation of the TF cytoplasmic domain may influence the recruitment of adaptors and thus integrate and regulate nonhemostatic roles of TF in cell migration, angiogenesis, and inflammation.

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