Regulation of the Incorporation of Tissue Factor into Microparticles by Serine Phosphorylation of the Cytoplasmic Domain of Tissue Factor*\[1\]

The mechanisms that regulate the incorporation and release of tissue factors (TFs) into cell-derived microparticles are as yet unidentified. In this study, we have explored the regulation of TF release into microparticles by the phosphorylation of serine residues within the cytoplasmic domain of TF. Wild-type and mutant forms of TF, containing alanine and aspartate substitutions at Ser\(^{253}\) and Ser\(^{258}\), were overexpressed in coronary artery endothelial cells and microparticle release stimulated with PAR2 agonist peptide (PAR2-AP). The release of TF antigen and activity was then monitored. In addition, the phosphorylation state of the two serine residues within the released microparticles and the cells was monitored for 150 min. The release of wild-type TF as procoagulant microparticles peaked at 90 min and declined thereafter in both cell types. The phosphorylation state of Ser\(^{258}\) was found to be reduced and delayed by alanine substitution of Ser\(^{258}\) or aspartate substitution of Ser\(^{258}\). Alani substitution of Ser\(^{258}\) prolonged the release of TF following PAR2-AP activation. The release of TF was concurrent with phosphorylation of Ser\(^{253}\) and was followed by dephosphorylation at 120 min and phosphorylation of Ser\(^{258}\). We propose a sequential mechanism in which phosphorylation of Ser\(^{253}\) through PAR2 activation results in the incorporation of TF into microparticles, simultaneouly inducing Ser\(^{258}\) phosphorylation. Phosphorylation of Ser\(^{258}\) in turn promotes the dephosphorylation of Ser\(^{253}\) and suppresses the release of TF.

Increased levels of circulating endothelium cell-derived microparticles are recognized as a cause and prognostic marker for vascular disease and injury (1–3). Moreover, these microparticles have been shown to be capable of supporting the induction of thrombosis through a TF-dependent mechanism (3, 4). The mechanisms that regulate the incorporation and release of TF into cell-derived microparticles are as yet unidentified. Furthermore, it appears that cellular activation (5) for example through activation of PKC (6) can induce microparticle release although this is not completely understood. The proteolytic activation of PAR2 by enzymes such as factor Xa and trypsin is known to result in the translocation of PKC\(\alpha\) to the membrane (7). It has previously been shown that the incubation of cells with trypsin can induce the release of TF into membrane-derived vesicles (8). Furthermore, PKC\(\alpha\) is known to phosphorylate the cytoplasmic domain of TF at Ser\(^{253}\) (7, 9), whereas Ser\(^{258}\) is part of a proline-directed kinase consensus sequence (10), which becomes phosphorylated separately (11) initiated through the phosphorylation of Ser\(^{253}\) (9).

The cytoplasmic domain of TF is not required for the procoagulant activity of TF or de-encryption of this protein (12, 13). Moreover, it has been reported that substitution of the cytoplasmic domain of TF with the cytoplasmic domain of decay-accelerating factor does not alter the release of TF in cells that are capable of releasing TF-containing microparticles without activation (14). However, it is known that cells spontaneously release decay-accelerating factor under standard culture conditions (15). In this study, by aspartate substitution (to mimic phosphoserine) (16) or alanine substitution (to prevent phosphorylation) of two of the serine residues within the cytoplasmic domain of TF, we investigated the contribution of these residues to the regulation of TF incorporation and release into cell-derived microparticles.

**EXPERIMENTAL PROCEDURES**

**Plasmid Vectors**—The pCMV-XL5-TF plasmid for the expression of full-length human TF was obtained from OriGene. Aspartate and alanine substitutions at Ser\(^{253}\) and Ser\(^{258}\) were carried out to produce mutated constructs (pCMV-XL5-TF\(^{Asp253}\) pCMV-XL5-TF\(^{Ala253}\) pCMV-XL5-TF\(^{Asp258}\) and pCMV-XL5-TF\(^{Ala258}\)) or alternative combinations of these mutants (see supplemental material).

**Cell Culture, Transfection, and Microparticle Isolation**—Human coronary artery endothelial cells (HCAEC), dermal microvascular endothelial cells (HDMEC), and umbilical vein endothelial cells (HUVEC) (PromoCell) were used throughout the investigation because in contrast to monocytes or tumor cells, dermal microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; SFM, serum-free medium; PBMC, peripheral blood mononuclear cells; pSer, phosphoserine.
these cells do not either constitutively express TF and/or release microparticles spontaneously. HCAEC have been shown to express PAR2 on the cell surface (17), whereas HUVEC are devoid of this receptor and can therefore be used without signaling contribution from this receptor. Cells were transiently transfected with the wild-type or mutant forms of pCMV-XL5-TF plasmid (1 µg) and allowed to express TF for 48 h (supplemental material). The expression of TF was confirmed by measuring TF mRNA and total and cell-surface antigen levels (supplemental Fig. I). In some experiments, the cells were then transfected with peptides corresponding to the last 18-amino acids within the cytoplasmic domain of TF (RKAGVQSKENGWLPLNVS) synthesized in the unphosphorylated form (Ser253/Ser258), phosphorylated on the first serine (pSer253/Ser258), the second serine (Ser253/pSer258), double-phosphorylated form (pSer253/pSer258), and a random peptide. The peptides were prepared and confirmed using the Merrifield procedure as before (18) and were transfected using the Chariot reagent (Active Motif) as described previously (19). All cell samples, including the untransfected cells, were then transferred to serum-free medium (SFM) (1 ml) for 1.5 h, stimulated with either PAR1 agonist peptide (TFLLR; 20 µM), PAR2 agonist peptide (PAR2-AP); SLIGRL (20 µM; Sigma), a synthetic scrambled PAR2 agonist peptide (IRLGL; 20 µM), and a random peptide or recombinant human factor Xa (10 nM; American Diagnostica). For comparison, HUVEC transiently transfected to overexpress TF were activated with TNFα (10 ng/ml) or IL-1β (10 ng/ml). Released cell-derived microparticles were isolated from media according to published procedures (17, 20). In some experiments, calyculin A (1 nM) was added to the cells 30 min prior to activation, to inhibit cellular phosphatase 1/2A activities. The pellet was resuspended in 1 ml of PBS, divided into batches, and frozen at −80 °C or used immediately. For comparison, in some experiments, microparticles from THP-1 cells, isolated peripheral blood mononuclear cells (PBMC), or human coronary artery smooth muscle cells prepared as before (17) were analyzed in parallel. Furthermore, to compare and contrast the TF content and phosphorylation state of TF within the endothelial cell-derived microparticles, to those found naturally within the circulation in healthy and diseased conditions, microparticles were also isolated from the plasma of cancer and cardiovascular patients or normal subjects (Innovative Research).

Analysis of Microparticle Density, Phospholipid Content, and TF Antigen and Activity—The concentration of microparticles in each sample was determined using the Zymaphen Microparticle Assay kit (Hyphen BioMed) and by flow cytometry using FITC-conjugated anti-TF antibody (American Diagnostica). Total TF antigen within each isolated sample was measured using a TF ELISA kit (Affinity Biologicals) as described previously (19). Activities of TF-containing and control microparticles were measured using a chromogenic assay based on quantifying the activity of the generated thrombin as described previously (20). Additionally, aliquots (1 ml) of the wild-type and the mutant forms of TF-containing and control microparticles were freeze-dried, and the phosphatidylserine:phosphatidylcholine ratios were determined by thin layer chromatography as described in the supplemental material.

Western Blot Analysis of Ser253 and Ser258 Phosphorylation within TF—HCAEC or microparticles were lysed in phospho-safe lysis buffer (Active Motif), and the concentrations of total protein and TF antigen were determined using Bradford protein estimation assay and ELISA, respectively. Microparticle lysates were immunoprecipitated using anti-TF antibody-conjugated agarose beads as described previously (21) prior to Western blot analysis. To assess the ratios of phosphorylated: total TF in the cell lysates, equivalent amounts of TF protein were examined for phosphoserine (PKC substrate consensus), phospho-Ser258 TF, and for total TF by Western blot as described in the supplemental material.

Measurement of Cell Apoptosis—Sets of HCAEC (5 × 10⁴), transfected to overexpress the wild-type or mutant forms of TF, were stimulated with PAR2-AP as described previously, and cellular apoptosis was measured at 90 min, 150 min, and 6 h using the DeadEnd fluorescence-based TUNEL assay (Promega). Caspase-3/7 activation was measured by flow cytometry as described previously (22).

Statistical Analysis—All data represent the calculated mean values from the number of experiments stated in each figure legend ± the calculated S.E. Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS, Inc.). One-way analysis of variance was used for the analysis of variance of data against the control with Tukey’s honestly significant difference test to highlight statistically significant differences.

RESULTS

Plasmid transfection efficiency of the cells was consistently >35 and 32% in HCAEC and HDMEC, respectively. Following transient transfection of the cells, the expression of TF protein increased within 24 h and remained constant for up to 3 days. Furthermore, levels of cellular TF mRNA and total cell-surface TF protein expression were consistent between wild-type TF and mutant forms of TF (supplemental Fig. I). Transfection efficiencies of >75% were obtained for the peptides, tested using a fluorescent-labeled TF-peptide, which also appeared to be evenly distributed throughout the cell.

Time Course of TF Release as Microparticles upon PAR2 Activation—Stimulation of HCAEC or HDMEC overexpressing TFWT with either PAR2-AP (20 µM) or FXa (10 nM) resulted in the transient incorporation and release of TF antigen into microparticles, which peaked at 90 min post-stimulation (Fig. 1A) but not in the untransfected (but PAR2-AP-activated) cells, PAR1-activated cells, or PAR2-AP-activated HUVEC (data not shown). Moreover, incubation of cells with the scrambled PAR2-AP or random peptide did not result in the release of microparticles. Furthermore, incubation of TF-overexpressing HUVEC with either TNFα (10 ng/ml) or IL-1β (10 ng/ml) did not induce TF release at 90 min, but significant amounts of TF were released by HUVEC at 6 h post-activation (supplemental Table I A). The release of microparticles by HCAEC was also measured by flow cytometry and reflected the increased proportion of TF-containing microparticles compared with control microparticles at 90 min (supplemental Fig. II). However, because microparticles are readily taken up by endothelial cells in culture (supplemental Fig. III) (17), it has to be noted that the
FIGURE 1. Analysis of the release of wild-type and mutant forms of TF in microparticles. HCAEC and HDMEC (2 × 10⁵) overexpressing TFWT or untransfected cells were placed in SFM and activated with either PAR2-AP, scrambled-PAR2-AP (20 µM), or recombinant FXa (10 nM), and microparticles were isolated from the media. TF antigen concentrations were determined by ELISA (n = 7) (A), and TF activities of microparticles were measured using a chromogenic assay (n = 7) (B). HCAEC and HDMEC (2 × 10⁵) overexpressing TFAsp253, TFAala253, TFAsp258, or TFAala258 in SFM, were activated with PAR2-AP, and the microparticles were isolated. TF antigen concentrations (n = 6; C) and TF activities of microparticles (n = 6; D) were determined. *, p < 0.05 versus respective time 0 sample. U, unit.
observed changes in TF concentrations in the media represent the net flux of the TF-containing microparticles. Increases in released TF antigen concentrations were concurrent with increased TF activity as measured using the chromogenic assay (Fig. 1B). In contrast, neither the total protein concentration nor the released microparticle densities were significantly altered between the samples (supplemental Table 2). The values for endothelial cell-derived microparticle densities, TF\textsubscript{WT} concentrations and TF\textsubscript{WT}:microparticle ratios were in line with those isolated from similar volumes of patient plasma or media from activated THP-1, PBMC, and human coronary artery smooth muscle cells. Moreover, the phosphatidylserine to phosphatidylserine:phosphatidylcholine percentage in the TF-containing microparticles remained constant between the various samples. Finally, we have demonstrated previously the lack of Tsg101 and, therefore, the absence of exosomes from our preparations of HCAEC-derived microparticles (17).

**Influence of TF Ser\textsuperscript{253} and Ser\textsuperscript{258} Mutations on Cell Survival**—No cell apoptosis was observed at 90 or 150 min following activation of HCAEC or HDMEC with PAR2-AP (supplemental Table 3). Activation of HCAEC or HDMEC transfected with either TF\textsubscript{WT} or TF\textsubscript{Asp253} reduced the rate of cellular apoptosis compared with the control cells at 6 h. Small increases in cell apoptosis were observed on activation of cells overexpressing TF\textsubscript{Ala253} (supplemental Fig. IV), which was preceded by enhanced caspase-3/7 activity in these cells at 5 h (data not shown). Activation of untransfected cells with PAR2-AP did not induce cellular apoptosis. Incubation of HUVEC with TNF\textalpha or IL-1\beta resulted in high levels of cell apoptosis in all treated samples at 6 h but not at 90 min (supplemental Table IB and Supplemental Fig. V).

**Analysis of Phosphorylation State of Ser\textsuperscript{253} and Ser\textsuperscript{258} within Microparticle-associated TF**—The phosphorylation state of Ser\textsuperscript{253} within TF\textsubscript{WT} microparticles was evaluated following immunoprecipitation of TF from HCAEC- and HDMEC-derived microparticles isolated at 90 min. The TF protein was present in the microparticles isolated from the media of TF-transfected HCAEC and HDMEC, the plasma of patients, activated THP-1 and PBMC, human coronary artery smooth muscle cells, and from TNF\textalpha and IL-1\beta-treated HUVEC at 6 h, was lower in the microparticles from the healthy plasma, and there was no detectable TF in the media of untransfected HCAEC or HDMEC (supplemental Table 2). TF\textsubscript{WT} was recognized by an anti-phospho-PKC-substrate antibody in all TF-containing microparticles. However, except in TNF\textalpha and IL-1\beta-treated HUVEC, none of the cell-derived microparticles were recognized by the anti-phospho-Ser\textsuperscript{258} TF antibody (Fig. 2), suggesting that the released TF\textsubscript{WT} in microparticles is phosphorylated at Ser\textsuperscript{253} alone. No microparticles could be isolated from cells following activation with the scrambled PAR2-AP.

**Time Course of Phosphorylation of Ser\textsuperscript{253} and Ser\textsuperscript{258} within TF on Activation with PAR2-AP**—Incubation of TF\textsubscript{WT}-overexpressing HCAEC with PAR2-AP resulted in peak phosphorylation ratio of Ser\textsuperscript{253} (to total TF) at 90 min (Fig. 3A), which correlates with the observed release of TF at 90 min. The level of phosphorylation declined at >120 min, which suggests dephosphorylation of this residue. In contrast, little phosphorylation of

---

**Regulation of Release of TF as Microparticles**

**FIGURE 2. Analysis of the phosphorylation of Ser\textsuperscript{253} and Ser\textsuperscript{258} in microparticles.** Microparticles (MP) were isolated at 90 min from HCAEC and HDMEC (2 × 10\textsuperscript{5}) overexpressing TF\textsubscript{WT} or control cells, plasma of three patients, one healthy individual and media of THP-1, isolated PBMC and human coronary artery smooth muscle cells, and HUVEC treated with TNF\textalpha or IL-1\beta. TF was immunoprecipitated, and phosphorylation of Ser\textsuperscript{258} was measured using the anti-phospho-Ser\textsuperscript{258} TF antibody. Cell-derived microparticles micrographs represent five preparations; patient plasma microparticles represent duplicates. The quantity of TF in the isolated microparticles prior to immunoprecipitation is outlined in supplemental Table 2.
Ser253 was observed in the cells expressing TFAsp258, and no phosphorylation was detected on incubation of HCAEC-overexpressing TFWT with the scrambled PAR2-AP. Analysis of Ser258 phosphorylation following stimulation with PAR2-AP showed increased phosphorylation of Ser 258 at 120 min in TFWT and TFAsp253 (Fig. 3B) but no phosphorylation in the cells expressing TFAla253 (data not shown). Moreover, a second earlier Ser258 phosphorylation peak was detected at 45 min in cells expressing TFAsp253. No apoptosis was observed in any of the cell samples for the duration of the assays (supplemental Table 3).

Release of TF in Presence of Peptides Corresponding to TF Cytoplasmic Domain—Transfection of TFWT-overexpressing HCAEC cells with either pSer253/Ser258 or pSer253/pSer258 peptide, followed by stimulation with PAR2-AP resulted in increased and accelerated (60 min) release of TF (Fig. 4A). Furthermore, the release of TF was reduced on transfection with the Ser253/pSer258 peptide. Time course analysis of the TF content of microparticles isolated from cells transfected with pSer253/Ser258 peptide showed an accelerated rate of wild-type TF release in these cells (Fig. 4B). In contrast, the phosphorylation state of Ser253 in these cells was not significantly increased at 45 min, although there was a significant level of Ser253 phosphorylation in both peptide-transfected (Fig. 4B) and untransfected cells (Fig. 3A). In addition to measuring the release of TF, the phosphorylation state of Ser258 in the above cells was analyzed. Transfection of cells with either pSer253/Ser258 or pSer253/pSer258 induced the phosphorylation of Ser258, which peaked at 90 min (Fig. 4C) upon activation with PAR2-AP only. No cellular apoptosis was observed in any of the cell samples during the assays (supplemental Table 4).

Finally, in the absence of calyculin A, transfection of cells with Ser253/pSer258 resulted in decreases in TFAla258 release at 90 min (Fig. 4D) and was concurrent with the reduction in Ser253 phosphorylation in these cells (Fig. 4E). However, the inclusion of calyculin A partially restored TFAla258 release (Fig. 4D) and was reflected in the maintained phosphorylated state of Ser253 (Fig. 4E). In agreement, the release of TFAsp258 at 90 min (Fig. 4F) and the phosphorylation of Ser253 (Fig. 4G) were partially restored in the presence of calyculin A.

DISCUSSION

The function of endothelial cell-derived microparticles as carriers of TF and their procoagulant properties during disease has recently become established and discussed previously (1–5, 23–27) and is beyond the scope of this study. We have explored the regulation of the incorporation and release of TF into microparticles, and our data suggest that PKC-mediated phosphorylation of Ser253 within TF (7) acts as an “on switch” to initiate the incorporation of TF into microparticles (Fig. 5).
FIGURE 4. Analysis of TF release and phosphorylation in cells transfected with TF cytoplasmic peptides and the influence of calyculin A. Cells were placed in SFM and activated with PAR2-AP (20 μM) or scrambled-PAR2-AP. A, microparticles were isolated at 60 and 90 min and TF concentrations determined (n = 4). *, p < 0.05 versus scrambled PAR2-AP-activated samples. B, HCAEC expressing TFWT were transfected with the peptide RKAGVGQpSKENWSPLNVS (pSer/Ser (pS/S)) and adapted to SFM. Cells were activated with PAR2-AP (20 μM), microparticles were removed, and TF antigen concentration was measured up to 150 min (n = 3). *, p < 0.05 versus untreated sample. The cells were also lysed and examined for phospho-Ser253 and total TF. Micrographs are representative of three experiments. C, HCAEC (2 × 10^6) overexpressing TF_S253 were transfected with the four forms of the TF peptides or a random peptide. The ratios of the phospho-Ser258-TF to total TF in the cells was determined at 60 and 90 min (n = 4). *, p < 0.05 versus untransfected sample. Micrographs are representative of four experiments collected at 90 min. D, HCAEC (2 × 10^6) overexpressing TFAla258 were transfected with the peptide RKAGVGQSKENWpSPLNVS (Ser/phospho-Ser (S/pS)) and adapted to SFM. Calyculin A (1 nM) was added to one set and incubated for a further 30 min prior to activation of all samples with PAR2-AP (20 μM). TF release was determined at 90 min (n = 3). #, p < 0.05 versus the sample without calyculin A. E, the ratios of phospho-Ser253 to total TF were determined at 90 min, and percentage phosphorylation was calculated against an untreated sample (n = 4). *, p < 0.05 versus the control sample. Micrographs are representative of four experiments. F, HCAEC (2 × 10^6) overexpressing TFAsp258 were adapted to SFM and calyculin A (1 nM) was added to one set and incubated for a further 30 min prior to activation of all samples with PAR2-AP (20 μM). TF release was determined at 90 min (n = 3). #, p < 0.05 versus the sample without calyculin A. G, the ratios of phospho-Ser253 to total TF were determined at 90 min, and percentage phosphorylation was calculated against an untreated sample. Micrographs are representative of four experiments.
However, this is unlikely to be a coordinator for the formation and release of microparticles themselves. Therefore, these processes must be induced through other mechanisms that arise from PAR2 activation. In fact, activation of PAR1 by thrombin has been shown to induce the release of microparticles from endothelial cells through activation of Rho kinase II and caspase-2 (24). Furthermore, TNFα has been shown to induce microparticle formation through the p38 MAPK pathway (25). Our data suggest that the rapid short term release of TF on engagement of PAR2 with its agonist peptide occurs as a consequence of the activation of the endothelial cells and is distinct from the prolonged TF microparticle release observed due to cell apoptosis (26) induced by TNFα and IL-1β reported at 24 h (27). Moreover, the presence of phosphorylation at Ser253 but not Ser258 in endothelial cell-derived TF microparticles, isolated PBMC and THP-1 leukocytes, and smooth muscle cells was in agreement with the complex sets of microparticles isolated from the plasma of patients, which contain microparticles from these sources as well as platelets. This observation further supports the presence of a mechanism that regulates the release of TF following cell activation.

Comparison of the TF protein sequence from a set of mammals shows that the serine residue at the position equivalent to Ser253 in humans is conserved in six of the nine species (Fig. 6). It is possible that the release of TF in species without this phosphorylation site (murine, porcine, and rat) is regulated by microparticle formation alone, and the hydrophilic residue at this position is sufficient for this purpose. It has been reported that substitution of the cytoplasmic domain of TF with that of decay-accelerating factor does not alter TF release by smooth muscle cells (14). However, decay-accelerating factor is spontaneously released by cells under normal culture conditions (15), and smooth muscle cells also release microparticles spontaneously. Therefore, we propose that the cytoplasmic domain of TF controls the incorporation of TF into microparticles in cells that actively regulate TF release, including endothelial cells.

The phosphorylation of Ser253 within TF, together with other PAR2-initiated mechanism(s), initiates the activation of an as yet- unidentified proline-directed kinase (9) that phosphorylates Ser258 (Fig. 3). In cells expressing TFAsp253, the second earlier Ser258 phosphorylation peak at 45 min suggests that the phosphorylation of Ser258 is dependent on, and possibly initiated by, previous phosphorylation of Ser253. The phosphorylation of Ser258 appears to either enhance the activity of phosphatases 1/2A (Fig. 4) or alternatively permit the dephosphorylation of Ser253 through structural alterations within the cytoplasmic domain of TF (28). In support of this hypothesis, the phospho-Ser258-Pro259 motif is a potential recognition site for the peptidylprolyl cis/trans isomerase-1 Pin1 (29), which may be capable of inducing conformational changes within proteins that contain the consensus motif (30). Little phosphorylation of Ser253 was observed in cells expressing TFAsp258, suggesting that Ser258 phosphorylation can accelerate or even induce the dephosphorylation of Ser253 and corroborates the function of Ser258 as an off switch for TF release. The presence of a conserved serine/threonine residue at this position, within a proline-directed kinase consensus sequence, in all the species indicated in Fig. 6, further emphasizes a crucial function for this residue and the participation of a specific proline-directed kinase enzyme, the identity of which we are pursuing. Moreover, although the rate of Ser253 dephosphorylation appears to have been enhanced by the presence of phospho-Ser258, the mechanism also appears to require PAR2 activation.

Phosphorylation of Ser258 may become possible as a consequence of structural alterations in the cytoplasmic domain of TF induced by the phosphorylation of Ser253 (28). Similarly, dephosphorylation of phospho-Ser253 may become permissible as a consequence of structural alterations brought about by Ser258 phosphorylation. Consequently, such changes in phosphorylation would only involve the TF molecule itself. Alternatively, changes in the phosphorylation state of TF may induce signaling mechanisms, which then further modifies the TF molecule. Under these circumstances, every TF molecule can influence other TF molecules. To determine the underlying mechanisms, peptides corresponding to the last 18 amino acids in the C-terminal of TF, including the two serines, were synthesized with different phosphorylation states and transfected into the cells prior to stimulation with PAR2-AP. The ability of phospho-Ser253 peptides to accelerate the rate of TF release...
Regulation of Release of TF as Microparticles

further supports the role of Ser\textsuperscript{253} as the on switch. Furthermore, the phosphorylated form of Ser\textsuperscript{253} appears to be capable of inducing TF release regardless of the phosphorylation state of Ser\textsuperscript{258}. Intriguingly, although the rate of TF release was accelerated in these cells, the rate of Ser\textsuperscript{253} phosphorylation was not significantly altered during these time points (Fig. 4B). This finding indicates that although Ser\textsuperscript{253} phosphorylation may directly influence TF release, it also initiates independent events that alter the function of proteins which form the machinery required for the incorporation of TF into microparticles. Such events may include interactions with cytoskeletal proteins (16) or the activation of signaling mechanisms. In contrast, transfection of cells with either peptide in the absence of PAR2 activation or when using scrambled-PAR2-AP failed to initiate the release of TF into microparticles and supports the requirement for separate mechanisms for microparticle formation. Finally, no TF microparticles could be isolated from PAR2-AP treated TF-overexpressing embryonic HUVEC devoid of PAR2 (supplemental Table 2). Interestingly, despite the accelerated rate of Ser\textsuperscript{258} phosphorylation by these peptides in HCAEC (Fig. 4C), no suppression of TF release was observed until 90 min (Fig. 1A). Therefore, we suggest that as well as Ser\textsuperscript{258} phosphorylation, dephosphorylation of Ser\textsuperscript{253} also requires the induction of signaling mechanisms arising from PAR2 activation and may take up to 90 min to become effective. Such mechanisms may also involve alterations in the structure of the cytoplasmic domain of TF by proteins such as Pin1 (28) leading to dephosphorylation events (29) and acting as a time-dependent switch (30). Overexpression of TF\textsubscript{Ala258} in HCAEC did not lead to the suppression of TF release (Fig. 1C). This continuous release of TF after 90 min, was shown to be due to a possible interruption in the “off switch” rather than the stabilization of the microparticles within the medium (supplemental Fig. III). However, subsequent transfection of these cells with the Ser\textsuperscript{253} / pSer\textsuperscript{258} peptide did suppress TF release (Fig. 4D) through a mechanism probably involving the induction of phosphatase activation (Fig. 4F). Moreover, the ability of Asp\textsuperscript{258} to suppress TF release was partially reversed by the inhibition of phosphatase 1/2A activities (Fig. 4F). However, the level of Ser\textsuperscript{253} phosphorylation (Fig. 4G) was comparable with that observed with TF\textsubscript{Ala258} + Ser\textsuperscript{253} / pSer\textsuperscript{258} peptide (Fig. 4E). Therefore, these data indicate that the suppression of TF release by Ser\textsuperscript{258} phosphorylation involves the coordination of signaling mechanisms by TF itself, although we cannot dismiss possible additional contributions from conformational changes within the TF protein (28).

In conclusion, we hypothesize that Ser\textsuperscript{253} phosphorylation induces the incorporation and release of TF, whereas the phosphorylation of Ser\textsuperscript{258} acts to regulate the incorporation of TF into released microparticles through dephosphorylation of Ser\textsuperscript{253}. Therefore, we have identified opposing regulatory roles for the two serine residues within the cytoplasmic domain of TF in the incorporation of TF into microparticles in cells that stringently regulate TF release.

REFERENCES

1. Diamant, M., Tushuiizen, M. E., Sturk, A., and Nieuwland, R. (2004) Eur. J. Clin. Invest. 34, 392–401
2. Jimenez, J. J., Jy, W., Mauro, I. M., Horstman, L. L., Bidot, C. J., and Ahn, Y. S. (2005) Adv. Clin. Chem. 39, 131–157
3. Nomura, S., Ozaki, Y., and Ikeda, Y. (2008) Thromb. Res. 123, 8–23
4. Combes, V., Simon, A. C., Grau, G. E., Arnoux, D., Camoin, L., Sabatier, F., Mutin, M., Sanmarco, M., Sampol, J., and Dignat-George, F. (1999) J. Clin. Invest. 104, 93–102
5. Rak, J., Klement, P., and Yu, J. (2006) Vitr. Let. 52, 135–138
6. Pilzer, D., Gasser, O., Moskovich, O., Schifferli, J. A., and Fishelson, Z. (2005) Springer. Semin. Immunopathol. 27, 375–387
7. Ahamed, J., and Ruf, W. (2004) J. Biol. Chem. 279, 23038–23044
8. Maynard, J. B., Beckman, C. A., Pitlick, F. A., and Nemerson, Y. (1975) J. Clin. Invest. 55, 814–824
9. Dorfluehr, A., and Ruf, W. (2003) Blood 102, 3998–4005
10. Zioncheck, T. F., Roy, S., and Vehar, G. A. (1992) J. Biol. Chem. 267, 3561–3564
11. Mody, R. S., and Carsson, S. D. (1997) Biochemistry 36, 7869–7875
12. Mueller, B. M., and Ruf, W. (1998) J. Clin. Invest. 101, 1372–1378
13. Carson, S. D., and Bormberg, M. E. (2000) Thromb. Haemost. 84, 657–663
14. Schecter, A. D., Spinn, B., Rosskchina, M., Giesen, P. L., Bogdanov, V., Fallon, J. T., Fischer, E. A., Schnapp, L. M., Nemerson, Y., and Taubman, M. B. (2000) Circ. Res. 87, 126–132
15. Nasu, J., Mizuno, M., Uesu, T., Takeuchi, K., Inaba, T., Ohya, S., Kawada, M., Shimo, K., Okada, H., Fujita, T., and Tsuji, T. (1998) Clin. Exp. Immunol. 113, 379–385
16. Ott, I., Fischer, E. G., Miyagi, Y., Mueller, B. M., and Ruf, W. (1998) J. Cell Biol. 140, 1241–1253
17. Collier, M. E., and Ettealaie, C. (2010) Arterioscler. Thromb. Vasc. Biol. 30, 1810–1817
18. Ettealaie, C., Adam, J. M., James, N. I., Oke, A. O., Harrison, J. A., Bunce, T. D., and Bruckdorfer, K. R. (1999) FEBS Lett. 463, 341–344
19. Li, C., Collier, M. E., Frentzou, G. A., Greenman, J., and Ettealaie, C. (2008) Cancer Immunol. Immunother. 57, 1347–1355
20. Ettealaie, C., Su, S., Li, C., and Collier, M. E. (2008) Microvasc. Res. 76, 152–160
21. Ettealaie, C., James, N. I., Wilbourn, B., Adam, J. M., Naseem, K. M., and Bruckdorfer, K. R. (1996) Arterioscler. Thromb. Vasc. Biol. 16, 639–647
22. Pradier, A., and Ettealaie, C. (2008) J. Vasc. Res. 45, 19–32
23. Abid Hussein, M. N., Böing, A. N., Biró, É., Hoek, F. J., Vogel, G. M., Meuleman, D. G., Sturk, A., and Nieuwland, R. (2008) Thromb. Res. 121, 865–871
24. Sapet, C., Simoncini, S., Loriöd, B., Puthier, D., Sampol, J., Nguyen, C., Dignat-George, F., and Anfosso, F. (2006) Blood 108, 1868–1876
25. Curtis, A. M., Wilkinson, P. F., Gui, M., Gales, T. L., Hu, E., and Edelberg, J. M. (2009) J. Thromb. Haemost. 7, 701–709
26. VanWijk, M. J., VanBavel, E., Sturk, A., and Nieuwland, R. (2003) Cardiovasc. Res. 59, 277–287
27. Lakota, K., Mrak-Poljsak, K., Rozman, B., and Sodin-Semrl, S. (2009) Mediators Inflamm. 2009, 146872
28. Sen, M., Heritz, M., Craft, J. W., Creath, A. L., Agrawal, S., Ruf, W., and Legge, G. B. (2009) Open. Spectrosc. J. 3, 58–64
29. Zhou, X. Z., Lu, P. J., Wu, G., and Lu, K. P. (1999) Cell. Mol. Life. Sci. 56, 788–806
30. Lu, K. P., Finn, G., Lee, T. H., and Nicholson, L. K. (2007) Nat. Chem. Biol. 3, 619–629

\(^3\) C. Ettealaie, A. M. ElKeeb, and M. E. W. Collier, unpublished data.