REGULATION OF THE INCORPORATION OF TISSUE FACTOR INTO MICROPARTICLES BY SERINE PHOSPHORYLATION OF THE CYTOPLASMIC DOMAIN OF TISSUE FACTOR

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The mechanisms that regulate the incorporation and release of tissue factor (TF) 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 the serine residues within the cytoplasmic domain of TF. Wild-type and mutant forms of TF, containing alanine and aspartate substitutions at Ser253 and Ser258 were overexpressed in coronary artery and dermal microvascular 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 also the cells was monitored over 150 min. The release of wild-type TF as procoagulant microparticles peaked at 90 min and declined thereafter in both cell types. The TF within these microparticles was phosphorylated at Ser253 but not at Ser258. Aspartate-substitution of Ser253 resulted in rapid release of TF antigen but not activity, whereas TF release was reduced and delayed by alanine-substitution of Ser253 or aspartate-substitution of Ser258. Alanine-substitution of Ser258 prolonged the release of TF following PAR2-AP activation. The release of TF was concurrent with phosphorylation of Ser253 and was followed by dephosphorylation at 120 min, and phosphorylation of Ser258. We propose a sequential mechanism in which the phosphorylation of Ser253 through PAR2 activation results in the incorporation of TF into microparticles, simultaneously inducing Ser258 phosphorylation. Phosphorylation of Ser258 in turn promotes the dephosphorylation of Ser253 and suppresses the release of TF.

Increased levels of circulating endothelial-derived microparticles are recognised as a cause and prognostic marker for vascular disease and injury (1-3). Moreover, these microparticles have been shown to be able to support 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 protein kinase-C (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 PKCα to the membrane (7). The release of TF into membrane-derived vesicles on incubation of cells with trypsin has been demonstrated (8). Furthermore, PKCα is known to phosphorylate the cytoplasmic domain of TF at Ser253 (7,9), while Ser258 is part of a proline-directed kinase (PDK) consensus sequence (10) which becomes phosphorylated separately (11), initiated through the phosphorylation of Ser253 (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 Ser253 and Ser258 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 alternatively 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 since unlike monocytes or tumour cells, these cells do not either constitutively express TF and/or spontaneously release microparticles. 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 signalling 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 48h (supplemental material). The expression of TF was confirmed by measuring TF mRNA and total and cell-surface antigen levels (Supplemental Figure I). In some experiments, the cells were then transfected with peptides corresponding to the last 18-amino acids within the cytoplasmic domain of TF (RKAGVGQSKENWSPLNVS) synthesised in unphosphorylated form (Ser253/Ser258), phosphorylated on the first serine (pSer253/Ser258), the second serine (Ser253/pSer258), double-phosphorylated (pSer253/pSer258) and a random-peptide. The peptides were prepared and confirmed using the Merrifield procedure as before (18), and transfected using the Chariot reagent (Active Motif) as previously described (19). All cells, including untransfected samples, were then transferred to serum-free medium (SFM) (1 ml) for 1.5h, stimulated with either PAR1 agonist peptide (TFLLR; 20 µM), PAR2 agonist peptide (PAR2-AP; SLIGRL; (20 µM) (Sigma Chemical Company), a synthetic scrambled-PAR2 agonist peptide (IRLSGL) (20 µM), 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 monocuclear cells (PBMC) or human coronary artery smooth muscle cells (HCASMC) prepared as before (17), were analysed alongside. Furthermore, in order 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 Zymuphen 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 before (19). Activities of TF-containing and control microparticles were measured using a chromogenic assay based on quantifying the activity of the generated thrombin, as previously described (20). Additionally, aliquots (1 ml) of the wild-type, the mutant forms of TF-containing- and control microparticles were freeze-dried and the phosphatidylserine (PS):phosphatidylcholine (PC) ratios 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 phosphosafe-lysis buffer (Active Motif) and the concentrations of total protein and TF antigen determined using Bradford protein-estimation assay and ELISA respectively. Microparticle lysates were immunoprecipitated using anti-TF antibody-conjugated agarose beads as before (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 (5×10^4), transfected to overexpress the wild-type or mutant forms of TF, were stimulated with PAR2-AP as before and cellular apoptosis was measured at 90 and 150 min and 6h using the DeadEnd fluorescence-based TUNEL assay (Promega). Caspase-3/7 activation was measured by flow cytometry as previously described (22).

Statistical analysis- All data represent the calculated mean values from the number of experiments stated in each figure legend ± the calculated standard error of the mean. Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS Inc.). One-Way ANOVA procedure 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 above 35% and 32% in HCAEC and HDMEC respectively. Following transient transfection of the cells, the expression of TF protein increased within 24h and remained constant for up to 3 days. Furthermore, levels of cellular TF mRNA and total and cell-surface TF protein expression were consistent between wild-type TF and mutant forms of TF (Supplemental Figure I). Transfection efficiencies of greater than 75% were obtained for the peptides, tested using a fluorescent-labelled 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 TF_{Wt} 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 (Figure 1A) but not in the untransfected (but PAR2-AP activated) cells, PAR1-activated cells or PAR2-AP-activated HUVEC (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 6h post-activation (Supplemental Table IA). The release of microparticles by HCAEC was also measured by flow cytometry and reflected the increased proportion of TF-containing microparticles compared to control microparticles at 90 min (Supplemental Figure II). However, since microparticles are readily taken up by endothelial cells in culture [17] (and Supplemental Figure III), it has to be noted that the 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 (Figure 1B). In contrast, neither the total protein concentration nor the released microparticle densities were significantly altered between the samples (Supplemental Table II). The values for endothelial cell-derived microparticle densities, TF_{wt} concentrations and TF_{wt}:microparticle ratios were in line with those isolated from similar volumes of patient plasma or media from activated THP-1, PBMC and HCASMC. Moreover, the PS to PS+PC percentage in the TF–containing microparticles remained constant between the various samples. Finally, we have previously demonstrated the lack of Tsg101 and therefore, the absence of exosomes from our preparations of HCAEC-derived microparticles (17).

The influence of Ser253 and Ser258 mutations on the release of TF. Substitution of Ser253 with aspartate (TF_{Asp253}), to mimic phosphoserine resulted in the accelerated release of TF antigen peaking at 45 min (Figure 1C) but was not reflected in the released TF activity which peaked at 90 min (Figure 1D). This may due to the lower proportions of incorporated PS as seen at 45 min (Supplemental Table II), which is pre-requisite for the procoagulant activity of TF and is in agreement with previous reports (23). In contrast, alanine-substitution of Ser253 (TF_{Ala253}) suppressed the release of TF antigen and activity. Aspartate-substitution of Ser258 (TF_{Asp258}) resulted in both reduction, and delay of TF release. Furthermore, alanine-substitution of this residue (TF_{Ala258}) resulted in the release of TF which was dependent on PAR2 activation but did not subside after 90 min. Furthermore, replacement of microparticle-containing conditioned medium with fresh medium at 90, 120 and 150 min, did not result in the depletion of TF release which reappeared within 30 min (Supplemental Figure III). Moreover, incubation of microparticles with cells resulted in uptake.

Finally, double-aspartate substitution ($TF_{Asp253/Asp258}$) resulted in a pattern of TF release that was similar to that of TF_{Asp253} alone, while $TF_{Ala253/Ala258}$ or $TF_{Ala253/Asp258}$ mutants did not induce the release of TF (not shown).

The influence of TF Ser253 and Ser258 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 III). Activation of HCAEC or HDMEC transfected with either TF_{Wt} or TF_{Asp253} reduced the rate of cellular apoptosis compared to the control cells at 6h, small increases in cell apoptosis were observed on activation of cells overexpressing TF_{Ala253} (Supplemental Figure IV) which was preceded by enhanced caspase-3/7 activity in these cells at 5h (not shown). Activation of untransfected cells with PAR2-AP did not induce cellular apoptosis. Incubation of HUVEC with TNFα or IL-1β resulted high levels of cell apoptosis in all treated samples at 6h but not at 90 min (Supplemental Table IB and Supplemental Figure V).

Analysis of the phosphorylation state of Ser253 and Ser258 within microparticle-associated TF, The phosphorylation state of Ser253 within TF_{Wt}-microparticles was evaluated following immunoprecipitation of TF from HCAEC and HDMEC-derived microparticles isolated at 90 min. 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, HCASMC and from TNFα and IL-1β-treated HUVEC at 6h, 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 II). TF_{Wt} was recognised by an anti-phospho-PKC-substrate antibody in all TF-containing microparticles. However, except in TNFα and IL-1β-treated HUVEC, none of the cell-derived microparticles were recognised by the anti-phospho-Ser258 TF antibody (Figure 2) suggesting that the released TF_{Wt} in microparticles is phosphorylated at Ser253 alone. No microparticles could be isolated from cells following activation with the scrambled-PAR2-AP.

Time-course of the phosphorylation of Ser253 and Ser258 within TF on activation with PAR2-AP. Incubation of TF_{wt}-overexpressing HCAEC with PAR2-AP resulted in peak phosphorylation ratio of Ser253 (to total TF), at 90 min (Figure 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 Ser253 was observed in the cells expressing TF_{Asp258} and no phosphorylation was detected on incubation of HCAEC overexpressing TF_{Wt} with the scrambled-PAR2-AP. Analysis of Ser258 phosphorylation following stimulation with PAR2-AP showed increased phosphorylation of Ser258 at 120 min in TF_{Wt} and TF_{Asp253} (Figure 3B) but no phosphorylation in the cells expressing TF_{Ala253} (not shown). Moreover, a second earlier Ser258 phosphorylation peak was detected at 45 min in cells expressing TF_{Asp253}. No apoptosis was observed in any of the cell samples for the duration of the assays (Supplemental Table III).

Release of TF in the presence of peptides corresponding to TF cytoplasmic domain. Transfection of TF_{Wt}-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 (Figure 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 (Figure 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 (Figure 4B) and untransfected cells (Figure 3A). In addition to measuring the release of TF, the phosphorylation state of Ser258 in the above cells was analysed. Transfection of cells with either pSer253/Ser258 or pSer253/pSer258 induced the phosphorylation of Ser258 which peaked at 90 min (Figure 4C) upon activation with PAR2-AP only. No cellular apoptosis was observed in any of the cell samples during the assays (Supplemental Table IV).

Finally, in the absence of Calyculin-A, transfection of cells with Ser253/pSer258 resulted in decreases in TF_{Ala258} release at 90 min (Figure 4D) and was concurrent with the reduction in Ser253 phosphorylation in these cells (Figure 4E). However, the inclusion of Calyculin-A partially restored TF_{Ala258} release (Figure 4D) and was reflected in the maintained

phosphorylated state of Ser253 (Figure 4E). In agreement, the release of TF_{Asp258} at 90 min (Figure 4F) and the phosphorylation of Ser253 (Figure 4G) were partially restored in the presence of Calyculin-A.

DISCUSSION

The function of endothelial-derived microparticles as carriers of TF and their procoagulant properties, during disease has recently become established and discussed before (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 (Figure 5). 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 24h (27). Moreover, the presence of phosphorylation at Ser253 but not Ser258 in endothelial-derived TF-microparticles, isolated PBMC and THP-1 leukocytes, and smooth muscle cells was in agreement with the complex sets of microparticles isolated from patients' plasma, 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 6 out of the 9 species (Figure 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 also smooth muscle cells spontaneously release microparticles. 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 PDK (9) which phosphorylates Ser258 (Figure 3). In cells expressing TF_{Asp253}, 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 (Figure 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 TF_{Asp258} 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 PDK-consensus sequence, in all the species indicated in figure 6, further emphasises a crucial function for this residue and the participation of a specific PDK 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 signalling mechanisms which then feedback on TF. 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 synthesised 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 further supports the role of Ser253 as the on-switch. Furthermore, the phosphorylated form of Ser253 appears to be capable of inducing TF release regardless of the phosphorylation state of Ser258. Intriguingly, while the rate of TF release was accelerated in these cells, the rate of Ser253 phosphorylation was not significantly altered during these time-points (Figure 4B). This finding indicates that although Ser253 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 signalling mechanisms (Ettelaie et al unpublished data). 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 II). Interestingly, despite the accelerated rate of Ser258 phosphorylation by these peptides in HCAEC (Figure 4C), no suppression of TF release was observed until 90 min (Figure 1A). Therefore, we suggest that as well as Ser258 phosphorylation, dephosphorylation of Ser253 also requires the induction of signalling mechanisms arising from PAR2 activation and may take up to 90 minutes 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_{Ala258} in HCAEC did not lead to the suppression of TF release (Figure 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 stabilisation of the microparticles within the medium (Supplemental Figure III). However, subsequent transfection of these cells with the Ser253/pSer258 peptide did suppress TF release (Figure 4D), through a mechanism probably involving the induction of phosphatase activation (Figure 4E). Moreover, the ability of Asp258 to suppress TF release was partially reversed by the inhibition of phosphatase 1/2A activities (Figure 4F). However, the level of Ser253 phosphorylation (Figure 4G) was comparable to that observed with TF_{Ala258} + Ser253/pSer258 peptide (Figure 4E). Therefore, these data indicate that the suppression of TF release by Ser258 phosphorylation involves the co-ordination of signalling mechanisms by TF itself, although we cannot dismiss possible additional contributions from conformational changes within the TF protein (28).

In conclusion, we hypothesise that Ser253 phosphorylation induces the incorporation and release of TF, while the phosphorylation of Ser258 acts to regulate the incorporation of TF into released microparticles through dephosphorylation of Ser253. 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.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Analysis of the release of wild-type and mutant forms of TF in microparticles. HCAEC and HDMEC (2×10^5) overexpressing TF_{Wt} or untransfected, 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. (A) TF antigen concentrations were determined by ELISA (n=7) and (B) TF activities of microparticles were measured using a chromogenic assay (n=7). HCAEC and HDMEC (2×10^5) overexpressing TF_{Asp253}, TF_{Ala253}, TF_{Asp258} or TF_{Ala258} in SFM, were activated with PAR2-AP and the microparticles isolated. **(**C) TF antigen concentrations (n=6) and (D) TF activities of microparticles (n=6) were determined. (*=$p<0.05$ vs. respective time zero sample).

Fig. 2. Analysis of the phosphorylation of Ser253 and Ser258 in microparticles. Microparticles were isolated at 90 min from HCAEC and HDMEC (2×10^5) overexpressing TF_{Wt} or control cells, plasma of three patients, one healthy individual and media of THP-1, isolated PBMC and HCASMC and HUVEC treated with TNFα or IL-1β. TF was immunoprecipitated and (A) phosphorylation of Ser253 measured using an anti-phospho-PKC-substrate antibody. (B) Phosphorylation of Ser258 was measured using the anti-phospho-Ser258 TF antibody. Cell-derived microparticles micrographs represent 5 preparations; patient plasma-microparticles represent duplicates. The quantity of TF in the isolated microparticles prior to immunoprecipitation is outlined in Supplemental Table II.

Fig. 3. Analysis of the phosphorylation of Ser253 and Ser258 in cells. (A) HCAEC (2×10^5) overexpressing TF_{Wt} or TF_{Asp258}, in SFM were activated with PAR2-AP (20 μM). The cells were lysed and examined for phospho-Ser253 and total TF. The percentage changes in the phospho-Ser253:total TF ratios were calculated against a scrambled-PAR2-AP treated sample. (n=3, *=$p<0.05$ vs. untreated sample, micrographs are representative of 3 experiments). (B) HCAEC (2×10^5) overexpressing TF_{Wt}, TF_{Asp253} or TF_{Ala253}, in SFM were activated with PAR2-AP. The cells were lysed and examined for phospho-Ser258 and total TF. The percentage changes in the phosphorylated:total TF were calculated against a scrambled-PAR2-AP treated sample. (n=4, *=$p<0.05$ vs. untreated sample, micrographs are representative of 4 experiments).

Fig. 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, *=$p<0.05$ vs. scrambled-PAR2-AP-activated samples). (B) HCAEC expressing TF_{Wt} were transfected with the peptide RKAGVGQpSKENWSPLNVS (pS/S) and adapted to SFM. Cells were activated with PAR2-AP (20 μM) and microparticles removed and TF antigen concentration measured, up to 150 min (n=3, *=$p<0.05$ vs. untreated sample). The cells were also lysed and examined for phospho-Ser253 and total TF. (micrographs are representative of 3 experiments). (C) HCAEC (2×10^5) overexpressing TF_{Wt} 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, *=$p<0.05$ vs. untransfected sample, micrographs are representative of 4 experiments collected at 90 min). (D) HCAEC (2×10^5) overexpressing TF_{Ala258} were transfected with the peptide RKAGVGQSKENWpSPLNVS (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, *=$p<0.05$ vs. the control sample #=$p<0.05$ vs. the sample transfected with S/pS peptide but without Calyculin-A). (E) The ratios of phospho-Ser253 to total TF were determined at 90 min and percentage phosphorylation calculated against an untreated sample. (n=4, *=$p<0.05$ vs. the control sample, micrographs are representative of 4 experiments). (F) HCAEC (2×10^5) overexpressing TF_{Asp258} 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, #=$p<0.05$ vs. the sample without Calyculin-A). (G) The ratios of phospho-Ser253 to total TF were determined at 90 min and percentage phosphorylation calculated against an untreated sample. (micrographs are representative of 4 experiments).

Fig. 5. Putative sequential mechanism of TF incorporation into microparticles. TF = tissue factor; PKC = protein kinase C; PS = phosphatidylserine; MP = microparticle; PDK = proline-directed kinase; ? = undetermined.

Fig. 6. Comparison of the protein sequences of the cytoplasmic domain of TF from nine mammalian species.

Figure 1

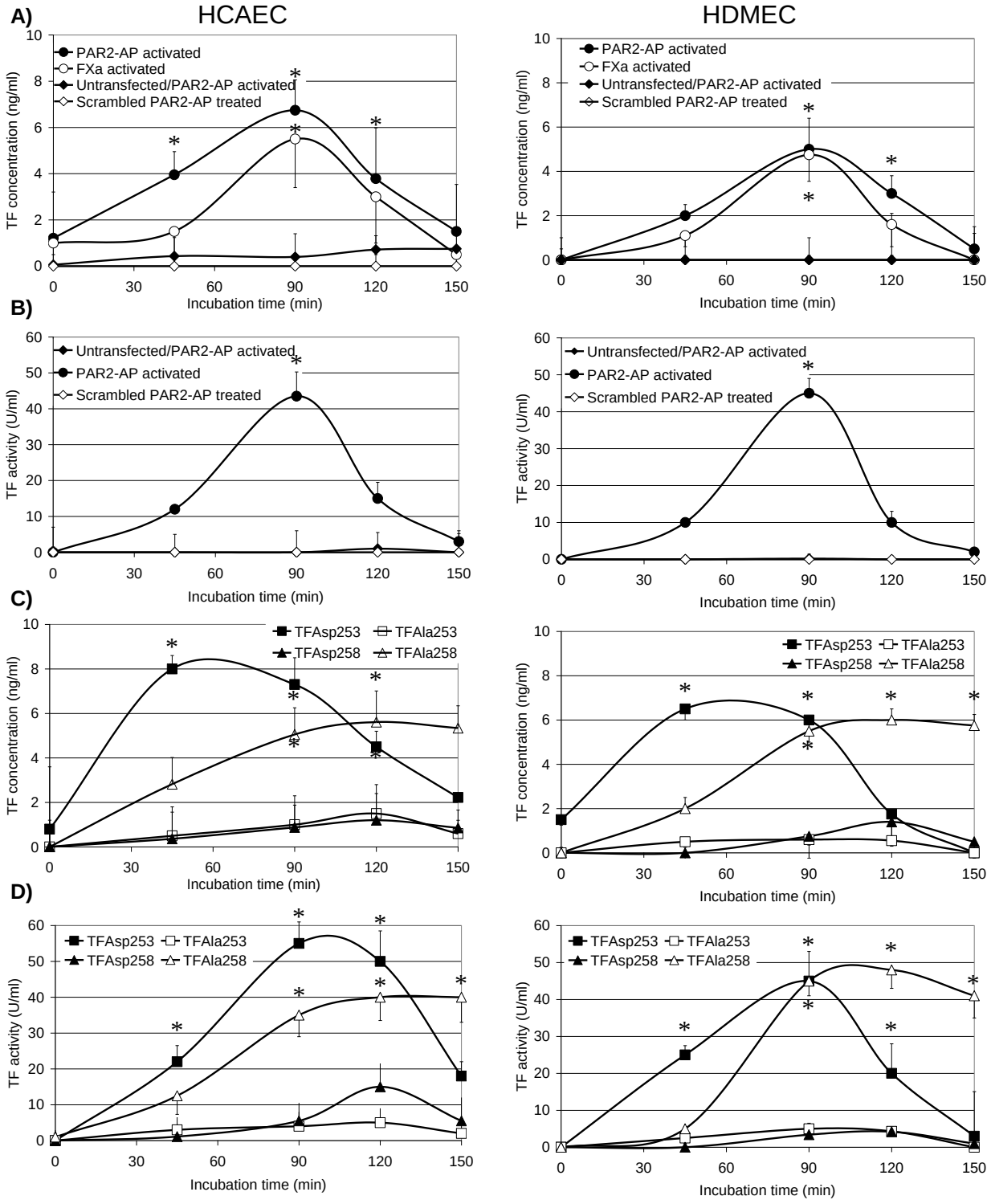

A)
HCAEC
HDMEC
PAR2-AP activated
FXa activated
Untransfected/PAR2-AP activated
Scrambled PAR2-AP treated
TF concentration (ng/ml)
Incubation time (min)
B)
TF activity (U/ml)
C)
TFAsp253
TFAla253
TFAsp258
TFAla258
D)

Figure 2

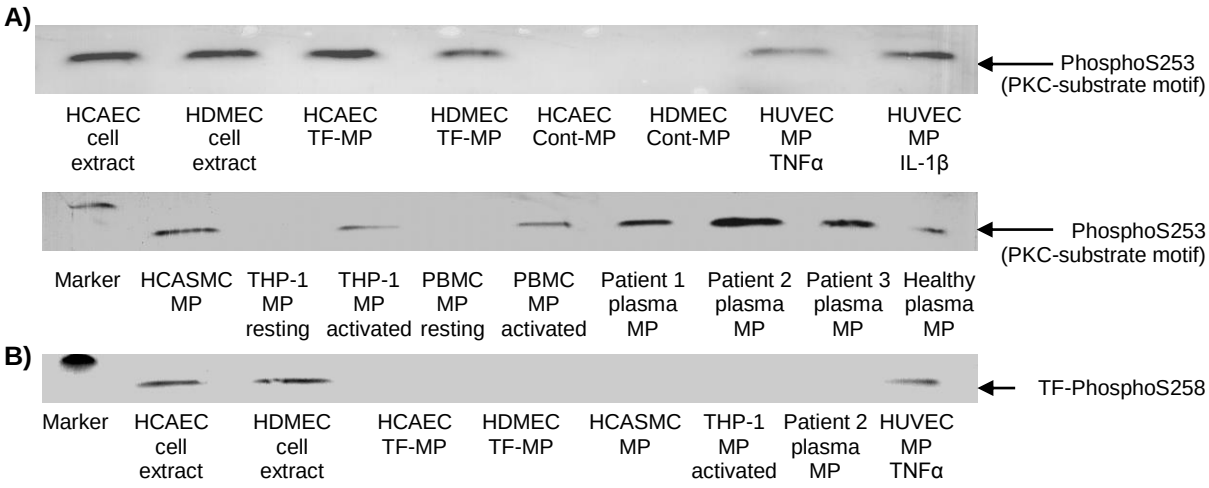

A)
PhosphoS253
(PKC-substrate motif)
HCAEC cell extract
HDMEC cell extract
HCAEC TF-MP
HDMEC TF-MP
HCAEC Cont-MP
HDMEC Cont-MP
HUVEC MP TNFα
HUVEC MP IL-1β
PhosphoS253
(PKC-substrate motif)
Marker
HCASMC MP
THP-1 MP resting
THP-1 MP activated
PBMC MP resting
PBMC MP activated
Patient 1 plasma MP
Patient 2 plasma MP
Patient 3 plasma MP
Healthy plasma MP
B)
TF-PhosphoS258
Marker
HCAEC cell extract
HDMEC cell extract
HCAEC TF-MP
HDMEC TF-MP
HCASMC MP
THP-1 MP activated
Patient 2 plasma MP
HUVEC MP TNFα

Figure 3

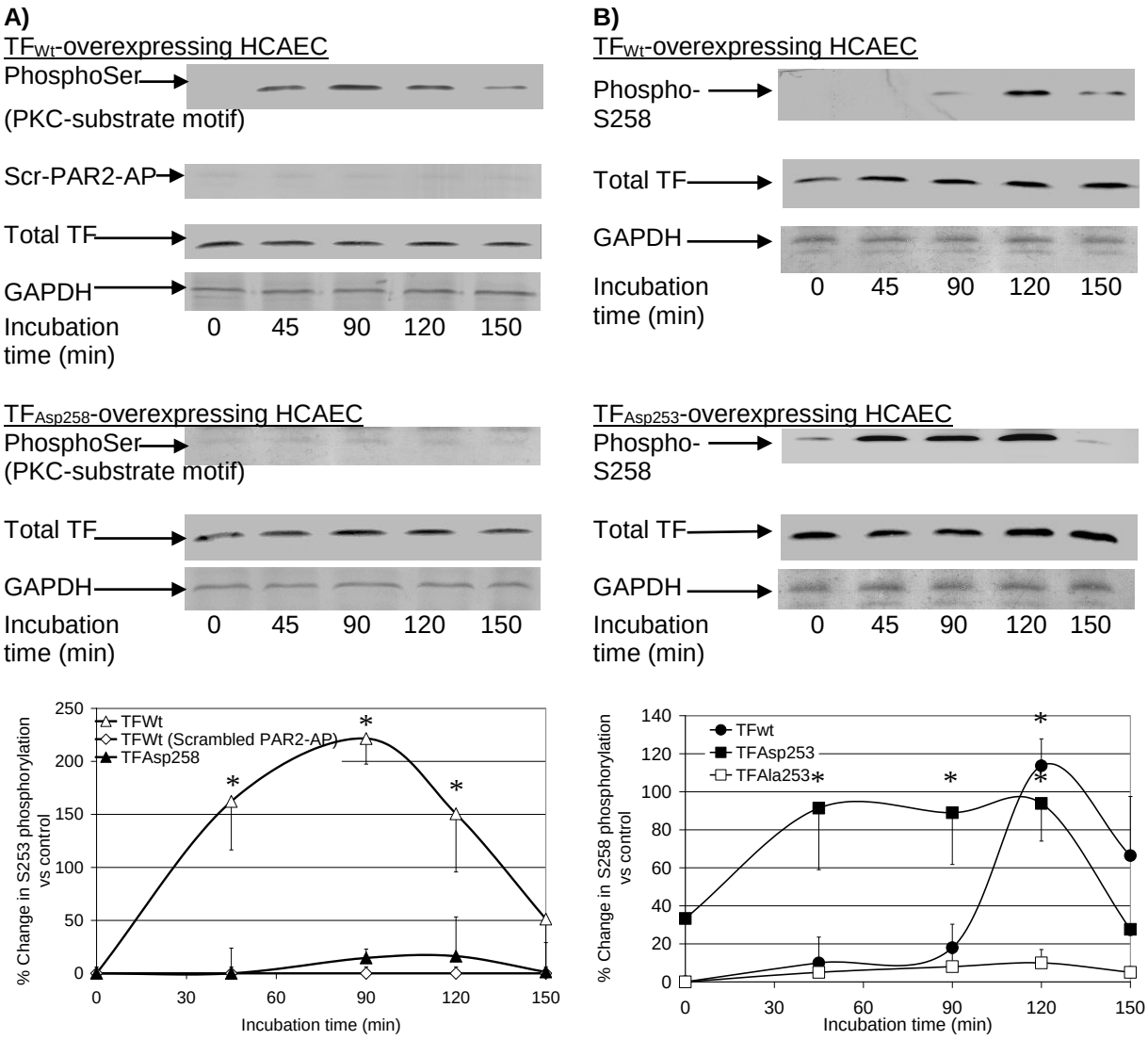
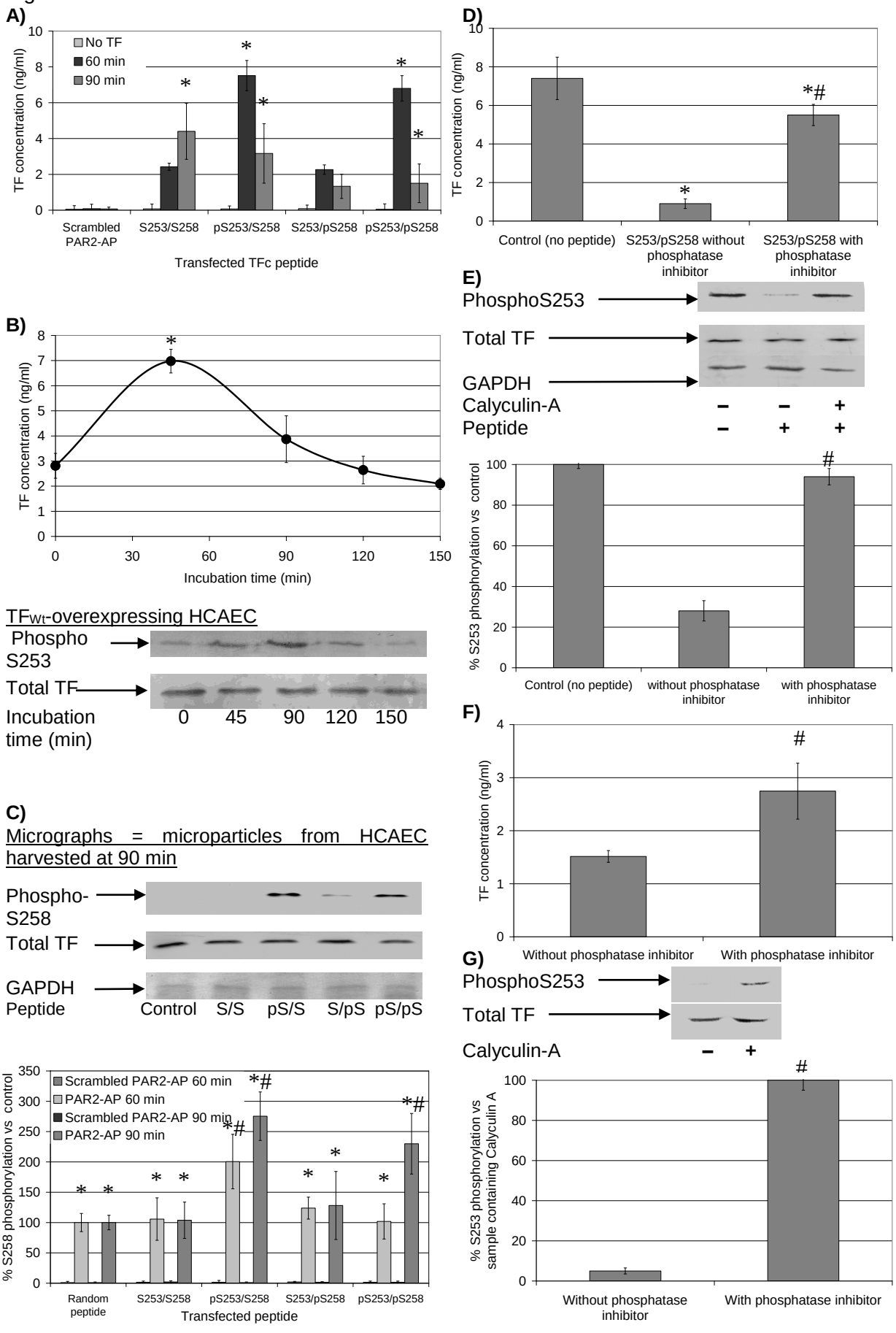

A)
TFWt-overexpressing HCAEC
PhosphoSer
(PKC-substrate motif)
Scr-PAR2-AP
Total TF
GAPDH
Incubation time (min)
0 45 90 120 150
B)
TFWt-overexpressing HCAEC
Phospho-S258
Total TF
GAPDH
Incubation time (min)
0 45 90 120 150
TFAsp258-overexpressing HCAEC
PhosphoSer
(PKC-substrate motif)
Total TF
GAPDH
Incubation time (min)
0 45 90 120 150
TFAsp253-overexpressing HCAEC
Phospho-S258
Total TF
GAPDH
Incubation time (min)
0 45 90 120 150
TFWt
TFWt (Scrambled PAR2-AP)
TFAsp258
% Change in S253 phosphorylation vs control
Incubation time (min)
TFwt
TFAsp253
TFAla253
% Change in S258 phosphorylation vs control
Incubation time (min)

Figure 4


A)
No TF
60 min
90 min
TF concentration (ng/ml)
Scrambled PAR2-AP
S253/S258
pS253/S258
S253/pS258
pS253/pS258
Transfected TFc peptide
B)
TF concentration (ng/ml)
Incubation time (min)
TFWt-overexpressing HCAEC
Phospho S253
Total TF
Incubation time (min)
0 45 90 120 150
C)
Micrographs = microparticles from HCAEC harvested at 90 min
Phospho-S258
Total TF
GAPDH
Peptide
Control S/S pS/S S/pS pS/pS
Scrambled PAR2-AP 60 min
PAR2-AP 60 min
Scrambled PAR2-AP 90 min
PAR2-AP 90 min
% S258 phosphorylation vs control
Random peptide
S253/S258
pS253/S258
S253/pS258
pS253/pS258
Transfected peptide
D)
TF concentration (ng/ml)
Control (no peptide)
S253/pS258 without phosphatase inhibitor
S253/pS258 with phosphatase inhibitor
E)
PhosphoS253
Total TF
GAPDH
Calyculin-A
Peptide
% S253 phosphorylation vs control
Control (no peptide)
without phosphatase inhibitor
with phosphatase inhibitor
F)
TF concentration (ng/ml)
Without phosphatase inhibitor
With phosphatase inhibitor
G)
PhosphoS253
Total TF
Calyculin-A
% S253 phosphorylation vs sample containing Calyculin A
Without phosphatase inhibitor
With phosphatase inhibitor

Regulation of the release of TF as microparticles

Figure 5

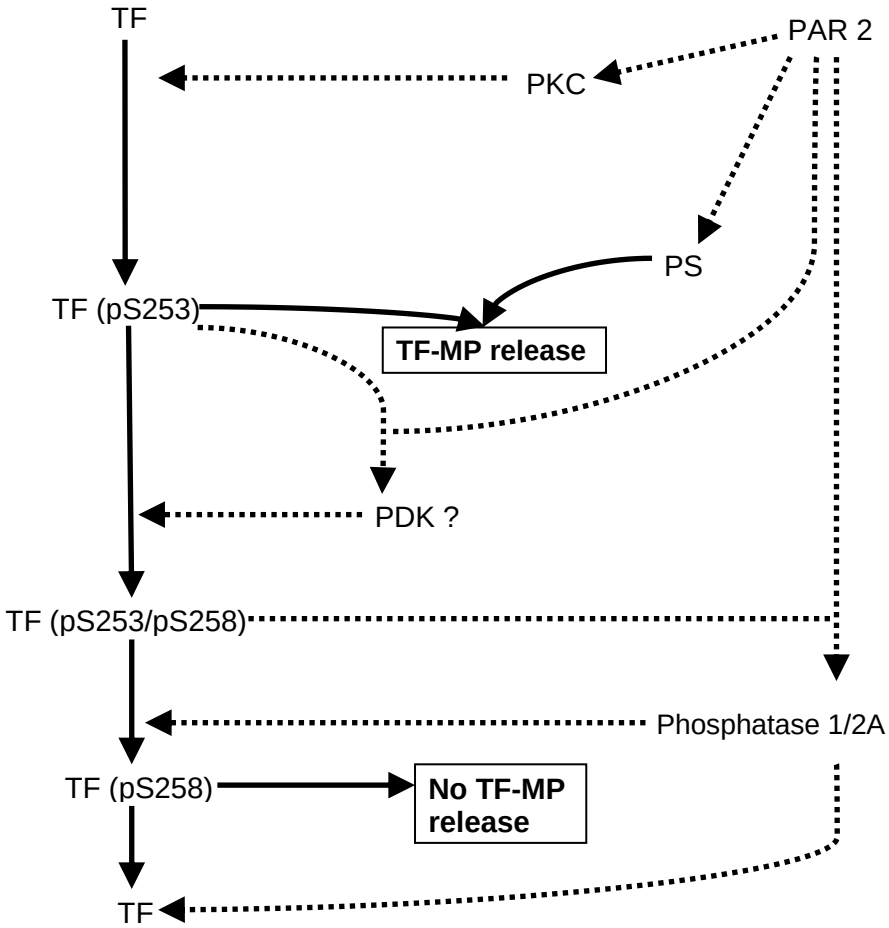
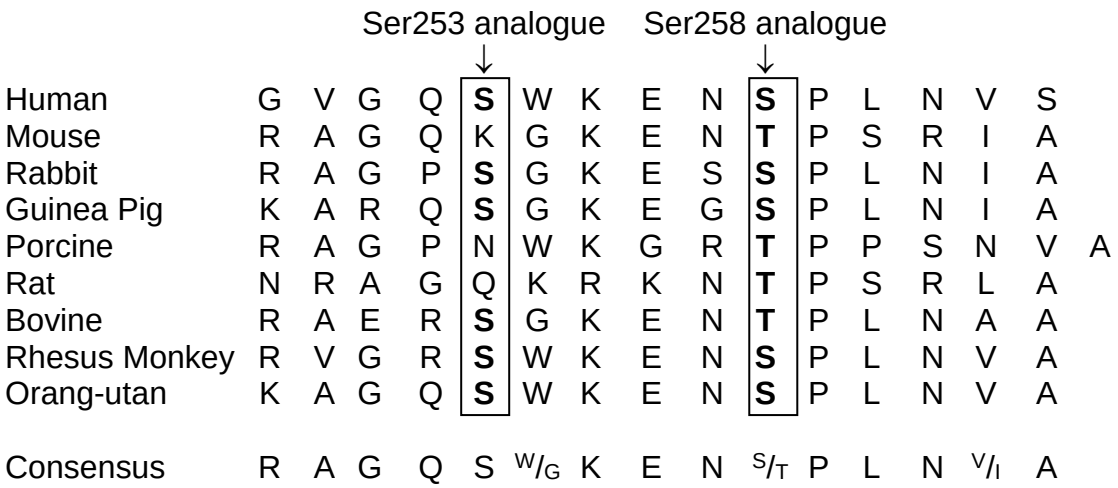


Figure 6


Ser253 analogue
Ser258 analogue
Human G V G Q S W K E N S P L N V S
Mouse R A G Q K G K E N T P S R I A
Rabbit R A G P S G K E S S P L N I A
Guinea Pig K A R Q S G K E G S P L N I A
Porcine R A G P N W K G R T P P S N V A
Rat N R A G Q K R K N T P S R L A
Bovine R A E R S G K E N T P L N A A
Rhesus Monkey R V G R S W K E N S P L N V A
Orang-utan K A G Q S W K E N S P L N V A
Consensus R A G Q S W/G K E N S/T P L N V/I A