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p38α phosphorylates serine 258 within the cytoplasmic domain of tissue factor and prevents its incorporation into cell-derived microparticles

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ABSTRACT

We previously showed that the phosphorylation of Ser253 within the cytoplasmic domain of human tissue factor (TF) initiates the incorporation and release of this protein into cell-derived microparticles. Furthermore, subsequent phosphorylation of Ser258 terminates this process. However, the identity of the kinase responsible for the phosphorylation of Ser258 and mode of action of this enzyme remain unknown. In this study, p38α was identified as the proline-directed kinase capable of phosphorylating Ser258 specifically, and without any detectable activity towards Ser253. Furthermore, using synthetic peptides, it was shown that the Km for the reaction decreased by approximately 10 fold on substitution of Ser253 with phospho-Ser253. Either inhibition of p38 using SB202190 or knockdown of p38α expression in coronary artery endothelial cells overexpressing wild-type TF, resulted in decreased phosphorylation of Ser258, following activation of cells with PAR2-agonist peptide (PAR2-AP). In agreement with our previous data, inhibition of phosphorylation of this residue maintained the release of TF. Activation of PAR2 in cells transfected to overexpress TF, resulted in two separate peaks of p38 activity at approximately 40 and 120 min post-activation. Furthermore, overexpression of Ala253-substituted TF enhanced the second p38 activation peak. However, the second peak was absent in cells devoid of TF or in cells overexpressing the Asp253-substituted TF. Our data clearly identifies p38α as a kinase capable of phosphorylating Ser258 within the cytoplasmic domain of TF. Moreover, it appears that the presence of TF within the cells regulates the late activation of p38 and consequently the termination of TF release into microparticles.

1. Introduction

Within the sequence of human tissue factor (TF), there are two cytoplasmic serine residues (Ser253 and Ser258) that may be phosphorylated. The phosphorylation of TF has been demonstrated both *in vitro* and *in vivo* and the influence of TF phosphorylation on cellular signalling [1,2], angiogenesis [3–5], tumour development [5–8] and cell migration [9–11] has been demonstrated. Previously, we demonstrated the regulation of the incorporation and release of TF through the phosphorylation of Ser253 and Ser258 [12]. We showed that the phosphorylation of Ser258 within the cytoplasmic domain of TF terminates the incorporation of TF into microparticles. Furthermore, the phosphorylation of Ser258 is enhanced by, and ensues the phosphorylation of Ser253 [12,13]. The phosphorylation of Ser253 is known to be mediated by protein kinase Cα [13,14]. In contrast, the kinase capable of phosphorylating Ser258 has not been identified. The primary sequence of the region encompassing this amino acid corresponds to the consensus substrate site for proline-directed kinases or the nuclear protein $p34^{cdc2}$ [15,16]. Furthermore, the primary sequence of this phosphorylation site is conserved between a number of species [12,17]. In this study we have identified the kinase responsible for the phosphorylation of Ser258 as p38α-MAPK and implicated this in the termination of the release of TF into microparticles.

2. Material and methods

2.1. Cell culture, DNA transfection and microparticle isolation

The pCMV-XL5-TF plasmid for the expression of full-length human TF was obtained from OriGene (Rockville, USA). Mutant plasmids containing aspartate and alanine substitutions at Ser253 or Ser258 were as described before [12]. Human coronary artery endothelial cells (HCAEC) were cultured and adapted to serum-free medium (SFM) and used throughout as before [12,18]. These cells do not either constitutively express TF or spontaneously release microparticles but may be activated using PAR2-agonist peptide (PAR2-AP) to release microparticles [12,18] and previously shown to be similar to activation with factor Xa [12,14]. HCAEC were transfected to overexpress TF or in some experiments were pre-incubated with TNFα (10 ng/ml) for 24 h to induce TF expression. The expression of TF was confirmed by

Abbreviations: TF, tissue factor; HCAEC, human coronary artery endothelial cells; PAR2(-AP), protease activated receptor 2(-activating peptide)

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measuring TF mRNA and total and cell-surface antigen levels as before [12,19].

2.2. Analysis of microparticle density and TF antigen and activity

Total TF antigen within each isolated sample was measured using a TF-ELISA kit (Enzyme Research Laboratories, Swansea, UK) as described before [12,18–21]. In some experiments, the phosphorylated form of TF was detected using a rabbit anti-phosphoserine258-TF antibody (Abcam, Cambridge, UK). To confirm TF release, the activities of TF-containing and control microparticles were measured using a chromogenic assay based on quantifying the activity of the generated thrombin and also, verified by measuring the activity in the presence of an inhibitory anti-TF antibody, as previously described [8,19]. Microparticle release from cells was verified as described previously [12,18,20].

2.3. Western blot analysis

HCAEC were lysed in phosphosafe-lysis buffer (100 μl; Active Motif, Rixensart, Belgium) and the concentrations of total protein and TF antigen determined using Bradford protein-estimation assay and a TF-specific ELISA respectively. The ratios of phosphorylated:total TF in the cell lysates, equivalent amounts of TF protein were assessed as previously described [12]. Briefly, the phosphorylation of Ser258 in cells was examined using a rabbit anti-phosphoserine258-TF antibody (Abcam) specific for phospho-Ser258, diluted 1:2000 (v/v) in TBST which was incubated for 2 h. The specificity and accuracy of the antibody was previously determined [12]. The membranes were then probed with a goat anti-rabbit HRP-conjugated antibody diluted 1:1000 (v/v). All visible bands were recorded using the GeneSnap Program (SynGene, Cambridge, UK). Total TF antigen was also measured as above and the ratios of phospho-Ser258 to total TF antigen determined using a monoclonal antibody to TF (10H10) (Santa Cruz Biotechnology, Heidelberg, Germany). The membranes were then washed and probed with a goat anti-mouse alkaline phosphatase-conjugated antibody (Santa Cruz Technologies) diluted 1:1000 (v/v), incubated for 90 min. The TF bands were then visualised using the Western Blue stabilised alkaline phosphatase-substrate (Promega Corp. Southampton, UK) and recorded as above. The ratio of the phosphorylated to total TF was then determined using the GeneTool program (SynGene). To assess the ratios of phosphorylated:total p38 or ERK1/2 in the cell lysates, equivalent proteins quantities were analysed for phosphorylated and total p38 and ERK1/2 using paired-antibody sets, obtained from Cell Signalling Technology (New England Biolabs, Hitchin, UK), diluted 1:2000 (v/v) in TBST. All samples were probed with goat anti-rabbit HRP-conjugated antibody diluted 1:1000 (v/v), developed and recorded as above.

2.4. Inhibition of p38 activity and siRNA-mediated knockdown of p38α

In some experiments, the cells were pre-incubated for 30 min with a range of concentrations of the p38 inhibitor SB202190 (30–100 nM) to inhibit the activity of p38. The inhibition of p38 was confirmed beforehand by measuring the phosphorylation of ATF2 by western blot analysis (Supplemental Data A). Also in some cases, the activity of Mek1 was inhibited using PD98059 (0–50 μM) and confirmed by analysing ERK1/2 phosphorylation (Supplemental Data B). To suppress the expression of p38 HCAEC were transfected with p38α-specific siRNA or a control siRNA (Santa Cruz Biotechnology) using Lipofectin (Life Technologies, Paisley, UK) and the expression of p38 monitored using western blot analysis. The optimal amount of siRNA (Supplemental Data C) and the time-point of silencing were determined prior to analysis. In some experiments, cells were co-transfected with pCMV-XL5-TF and p38- or control-siRNA.

2.5. Measurement of the kinase activity of p38α

The activity of recombinant p38α (50 ng/ml) and ERK (83 ng/ml) (ProQinase GmbH, Freiburg, Germany) towards recombinant TF (0–3 μM, American Diagnostica Inc., Stamford, USA) or synthetic peptides (0–40 μM) was measured using the Kinase-Glo assay kit (Promega). All protocols were according to the manufacturer's instructions and the relative light units (RLU) measurements were converted to concentrations of ADP generated according to the standard curve described in the kit. Briefly, the following reagents were assembled at the final dilutions shown. p38 enzyme (50 ng/ml) was reconstituted in the kinase buffer (50 mM HEPES-NaOH pH 7.5, PEG_{2000} (0.25 mg/ml), 1 mM DTT). The substrates, recombinant full-length TF (0–3 μM), or synthetic cytoplasmic domain peptides (0–40 μM), were reconstituted in HEPES buffer-NaOH pH 7.5 (50 mM) beforehand. The assay buffer reagent was assembled to a volume of 10 μl, containing HEPES-NaOH buffer pH 7.5 (50 mM), $MgCl_2$ (3 mM), $MnCl_2$ (3 mM), Na-orthovanadate (3 μM), DTT (1 mM) and DMSO (1% w/v). 5 μl of ultrapure ATP (10 mM), 5 μl of enzyme and 5 μl of the substrate were added and incubated at 30 °C for 40 min. The reaction was then stopped by adding the ADP-Glo reagent (25 μl) and incubated for 40 min. Finally, the Kinase-Detection reagent (50 μl) was added, incubated at room temperature for 60 min and the relative light units (RLU) recorded using a luminometer (Berthold Technologies GmbH & Co., Bad Wildbad, Germany). The assays were carried out at the substrate concentrations stated above and the Km and Vmax values were then calculated. Peptides corresponding to the last 18-amino acids within the cytoplasmic domain of TF were synthesised as wild-type, or with alanine, aspartate or phospho-serine substitutions in either of the two serine residues corresponding to residues 253 and 258 (underlined), as described before [12,22]. The sequences of the synthetic peptides were as shown below.

Ser253/Ser258: RKAGVGQ**S**KENW**S**PLNVS,
Ala253/Ser258: RKAGVGQ**A**KENW**S**PLNVS,
Asp253/Ser258: RKAGVGQ**D**KENW**S**PLNVS,
Ser253/Ala258: RKAGVGQ**S**KENW**A**PLNVS,
pSer253/Ser258: RKAGVGQ**pS**KENW**S**PLNVS,
scrambled: KGSAKESNGPWSNVLQRV.

2.6. 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. Chicago, USA). 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.

3. Results

Transfection efficiencies, TF protein expression and cell viability values were as previously determined and reported [12]. The effectiveness of SB202190 to inhibit p38 activity was confirmed by measuring the phosphorylation of ATF2 by western blot analysis (Supplemental Data A). In addition, the activity of Mek1 was inhibited using PD98059 (0–50 μM) and confirmed by analysing ERK1/2 phosphorylation (Supplemental Data B). Transfection efficiency of the siRNA, determined using FITC-labelled siRNA, was more than 80% and the optimal concentration of siRNA for p38α knockdown was determined to be 0.46 μg (Supplemental Data C). The expression of ERK1/2 and the housekeeping gene GAPDH remained unaltered by either siRNA transfection (Supplemental Data C and D). Additionally, no alteration in cell numbers subsequent to transfection of siRNA or overexpression of TF was observed (not shown). Finally, we have previously demonstrated the

lack of Tsg101 and therefore, the absence of exosomes from our preparations of HCAEC-derived microparticles [18].

3.1. Time-course of p38 and ERK1/2 phosphorylation in TF-overexpressing cells following PAR2 activation

Stimulation of HCAEC overexpressing TF_{Wt} with PAR2-AP (20 μM) induced the transient phosphorylation of p38 which peaked at two distinct time-points of 40 and 120 min post-activation (Fig. 1A). The earlier peak was concurrent with the phosphorylation of ERK1/2 (Fig. 1B). In contrast, only the earlier p38 peak, together with the activation of ERK1/2, was detected in cells devoid of TF. Overexpression of the TF mutant with Ala-substitution of residue 253 (TF_{Ala253}) resulted in the augmentation of the second p38 peak at 120 min (Fig. 1C) without affecting the earlier p38 peak (Fig. 1D) or ERK1/2 activity (not shown). The profiles of p38 activation (Fig. 1C and D) and ERK1/2 (not shown) phosphorylations, in HCAEC overexpressing Asp253-substituted TF (TF_{Asp253}), exhibited a pattern similar to untransfected cells. Finally, overexpression of Ala258-substituted TF (TF_{Ala258}) did not alter the phosphorylation pattern of p38, from that of wild type TF.

3.2. The influence of p38 inhibition/suppression on the phosphorylation of Ser258 within the cytoplasmic domain of TF, following PAR2 activation

HCAEC were transfected to overexpress TF_{Wt} and the cells were incubated with a range of concentrations of SB202190 to inhibit p38 prior to activation of PAR2. Since when phosphorylated at serine 258, TF is retained by the cells and is not released into microparticles [12], the ratio of Ser258-phosphorylated TF, to total remaining TF may then be determined by western blot analysis (Fig. 2A). Inclusion of SB202190 reduced the phosphorylation of TF at Ser258, in activated cells, at concentrations as low as 30 nM (Fig. 2B). The reduction in the level of the phosphorylation of Ser258 within TF was also achieved on suppression of p38α expression in cells using a p38α-specific siRNA, but not with the control siRNA (Fig. 2C). This reduction in Ser258 phosphorylation was observed in HCAEC transfected to overexpress TF, or

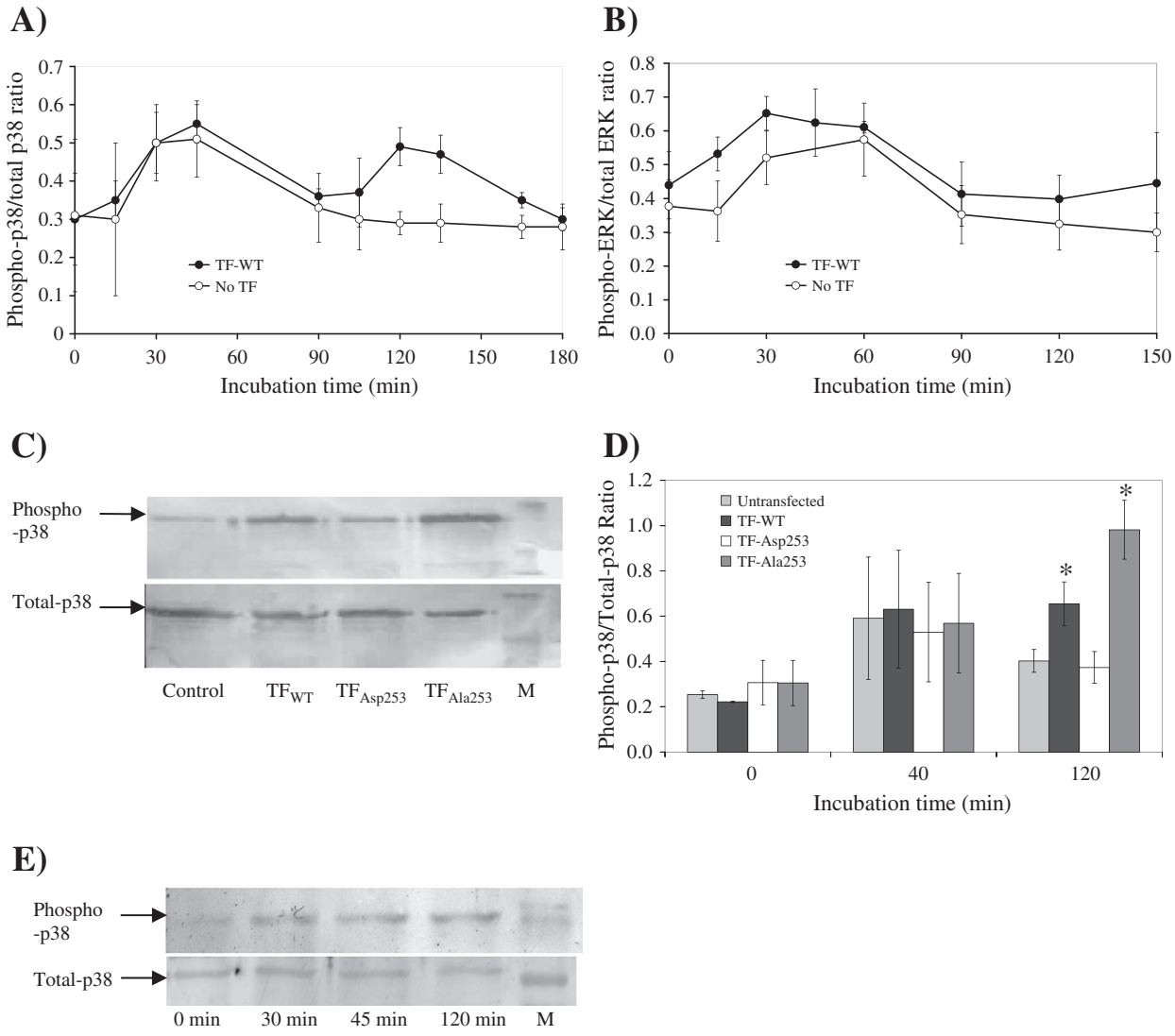

Fig. 1. Time-course of p38 and ERK1/2 phosphorylation following PAR2 activation. HCAEC (2×10^5 in SFM) overexpressing TF_{Wt} or untransfected, were activated with PAR2-AP (20 μM). Cells were then lysed at intervals and analysed for total and phospho-p38 and for total and phspho-ERK1/2, by western blot. The ratios of p38 (A) and ERK1/2 (B) phosphorylation were then determined. ($n=5$). In addition, HCAEC (2×10^5 in SFM) overexpressing TF_{Wt}, TF_{Asp253}, TF_{Ala253} or untransfected were activated with PAR2-AP (20 μM). Cells were then lysed at 0, 40 and 120 min post-activation and analysed for total and phospho-p38 by western blot; (C) the blot at 120 min is illustrated. (D) Band intensities were quantified and the ratios against total TF protein determined. ($n=3$, $^*=p<0.05$ vs. untreated sample). (E) HCAEC (2×10^5 in SFM) overexpressing TF_{Ala258} were activated with PAR2-AP (20 μM). Cells were then lysed at 0, 30, 45 and 120 min post-activation and analysed for total and phospho-p38 by western blot. (Data is representative of 3 separate experiments).

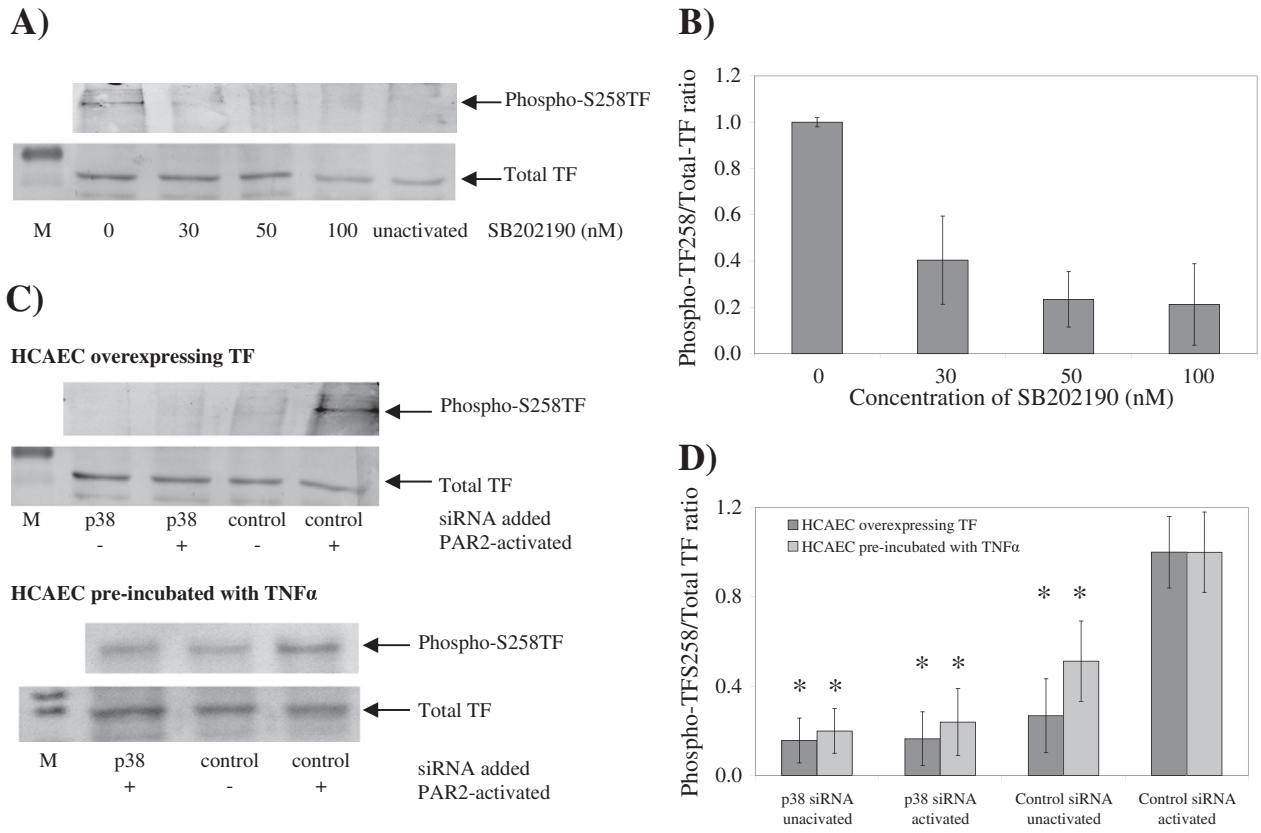

Fig. 2. The influence of p38 inhibition/suppression on the phosphorylation of Ser258 within the cytoplasmic domain of TF. (A) HCAEC (2×10^5 in SFM) overexpressing TF_{Wt} were pre-incubated with SB202190 (0–100 nM), activated with PAR2-AP and the phosphorylation of TF at Ser258 was analysed at 120 min post-activation, by western blot and (B) quantified against the total TF protein and finally, normalised against the control sample. ($n=3$, $^*=p<0.05$ vs. sample devoid of the inhibitor). (C) Samples of cells were co-transfected to overexpress TF, together with either p38-siRNA or control siRNA. Other sets of cells were transfected with p38-siRNA or control siRNA and were pre-incubated with TNFα for 24 h prior to activation. The cell samples were activated with PAR2-AP. An unactivated negative control set was included alongside. The phosphorylation of Ser258 within TF was analysed at 120 min by western blot, and (D) quantified against the total TF protein and finally, normalised against the control sample. ($n=3$, $^*=p<0.05$ vs. the sample containing control siRNA following PAR2 activation). (M = markers).

cells pre-incubated with TNFα to express TF. However, complete suppression of Ser258 phosphorylation was not achieved (Fig. 2D).

3.3. p38 phosphorylates recombinant TF in vitro

To confirm the ability of p38 to phosphorylate TF *in vitro*, an attempt was made to analyse the phosphorylation of non-phosphorylated recombinant full-length TF (0–3 μM) in a luminescence-based kinase assay employing a recombinant-active p38α protein (50 ng/ml). The Km value for the reaction was calculated to be 1.74 ± 0.25 μM and the Vmax for the reaction was 12.8 ± 1.5 nmol/s/mg enzyme and the calculated Kcat value was 256 s^{-1} (Table 1). The calculated p38α Vmax value, with non-phosphorylated full-length TF as substrate was similar to that provided by the supplier (ProQinase) for the p38-substrate ATF2 (14.9 nmol/s/mg) and the Km value is in line with published value [23]. To confirm the phosphorylation of the recombinant TF (1.5 μM), the reaction was also carried out in the presence and absence of ATP and phosphorylation state of TF determined by western blot and ELISA analysis, probed with a rabbit anti-phosphoserine258-TF antibody. The product from the reaction containing ATP, but not from the reaction devoid of ATP was recognised by the anti-phospho-TF antibody when examined by western blot or by ELISA (Fig. 3) and confirming the phosphorylation of TF. In addition, conducting the kinase assay using ERK instead of p38 resulted in minimal phosphorylation of TF but only at high concentrations of the substrate protein.

3.4. Demonstration of the specificity of p38α activity towards Ser258 within TF

In order to demonstrate that p38 specifically phosphorylated Ser258 within TF, a number of peptides corresponding to the last 18 amino acids of the cytoplasmic domain of human TF were synthesised and tested using the p38α kinase assay. The peptides included the wild-type or containing alanine substitutions in the positions corresponding to residues 253 and 258. Significant p38 kinase activity was detected towards all of the peptides that contained serine at position 258 (Fig. 4) while no measurable activity was detectable when this residue was substituted with alanine or when using a scrambled peptide.

3.5. Phosphorylation of Ser253 enhances the phosphorylation of Ser258 by p38

In order to assess the impact of the phosphorylation state of Ser253, on the p38 kinase activity towards Ser258, a set of peptides, each 18 amino acids long were prepared, but containing serine, alanine, aspartate and phospho-serine residues at position corresponding to Ser253 and the kinase activity of p38α assessed. The data show that either aspartate or phospho-serine substitution at Ser253 enhanced the rate of p38α activity towards Ser258, compared to peptides containing serine or alanine at the position corresponding to Ser253 (Fig. 4). The enhanced rate of reaction for p38α was reflected in lower Km values in the presence of aspartate or phospho-serine at position 253 and indicates a higher affinity of p38α (up to 10-fold) for the Ser253-phosphorylated

Table 1
Calculated kinetics values for the activity of p38α. The activity of recombinant p38α (50 ng/ml) towards recombinant TF (0–3 μM) or synthetic peptides (0–40 μM) corresponding to the last 18-amino acids within the cytoplasmic domain of TF was measured using the Kinase-Glo assay kit and the relative light units (RLU) measurements converted to concentrations of ADP generated according to the standard curve described in the kit. The Km and Vmax values were then calculated.

Substrate designation	Sequence	Km (μM)	Vmax (nmol/s/mg)
SS	RKAGVGQ**S**KENWSPLNVS	2.21 ± 0.35	10.0 ± 0.7
DS	RKAGVGQ**D**KENWSPLNVS	0.78 ± 0.23	13.3 ± 1.8
pSS	RKAGVGQ**pS**KENWSPLNVS	0.22 ± 0.09	10.2 ± 1.1
-	Recombinant TF protein (unphosphorylated)	1.74 ± 0.25	12.8 ± 1.5

substrate (Table 1). Moreover, the Vmax values for the kinase reactions did not alter significantly indicating no change in the enzyme activity of p38α.

3.6. The influence of p38 inhibition/suppression on TF release as microparticles, following PAR2 activation

HCAEC were transfected to overexpress TF_{Wt} and the cells were incubated with a range of concentrations of SB202190 to inhibit p38, or PD98059 to prevent the activation of ERK1/2 by Mek1, prior to activation of PAR2. In agreement with our previous data [12], stimulation of HCAEC overexpressing TF_{Wt} with PAR2-AP (20 μM) resulted in the transient incorporation and release of TF antigen into microparticles at 90 min but declined by 120 min (Fig. 5A). However, we have previously shown that the microparticles are readily taken up by endothelial cells in culture [12,18]. Therefore, the observed changes in TF concentrations in the media represent the net flux of the TF within the released microparticles. In agreement with our previous data, at the point of maximal release, the relative amount of cellular TF was marginally less in the cells overexpressing Asp253-substituted TF compared to wild-type TF, and was higher in the sample overexpressing Ala253-substitued TF (Fig. 5B). In addition, no TF release was observed in either untransfected PAR2-AP activated cells, or in cells that overexpressed TF but were incubated with a scrambled PAR2-AP peptide (Fig. 5A). Pre-incubation of cells with SB202190 marginally reduced the release of TF at 90 min (Fig. 5C) which could be explained by a modest reduction in microparticle density (Fig. 5D) and is in agreement with previous reports [24]. In contrast, the level of released TF was increased at 120 min (Fig. 5C). A greater alteration in the level of the incorporation of TF into microparticles was also achieved on suppression of p38 expression in cells, with a p38-specific siRNA but not with the control siRNA (Fig. 5E) while the alteration in microparticle density was not significant (Fig. 5D). Incubation of cells with higher concentrations of PD98059 marginally increased the level of TF released at 90 min (Fig. 5F) but not 120 min (not shown).

4. Discussion

We previously demonstrated the regulation of the incorporation of TF into cell-derived microparticles through the phosphorylation of the cytoplasmic domain of this protein [12]. In this study, the ability TF to induce p38 MAPK pathway activation was demonstrated (Fig. 1). The induction of p38 MAPK signalling following the activation of PAR2 has been established [11]. However, unlike the initial p38 activation peak, initiated by PAR2 and observed at 40 min, the TF-mediated induction of p38 activity, observed at 120 min was distinct from that initiated by PAR2 alone. This ability arose from the presence of TF within the cells, following activation of PAR2. The direct induction of p38 activation by TF has been suggested [11] but a mechanism not demonstrated

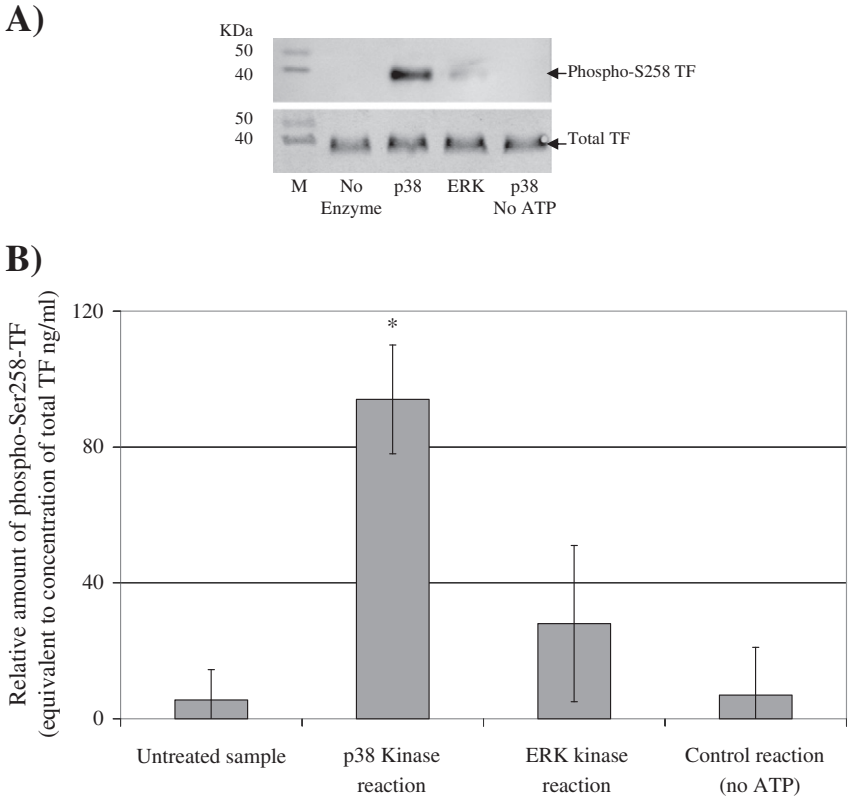

Fig. 3. Recognition of TF following kinase reaction by anti-phosphoserine258-TF antibody. p38α kinase reactions were carried out using recombinant human TF (1.5 μM) in the presence or absence of ATP. Similar kinase assays were also performed in the presence of recombinant ERK or devoid of any kinase. (A) Following the reaction, the products were analysed by western blot analysis using an anti-phosphoserine258-TF antibody (Representative of 3 separate experiments). (B) The relative amount of TF phosphorylation was also determined by ELISA. Following the kinase reaction, TF was captured using anti-TF antibody and probed for using an anti-phosphoserine258-TF antibody. ($n = 3$, $^* = p < 0.05$ vs. untreated sample).

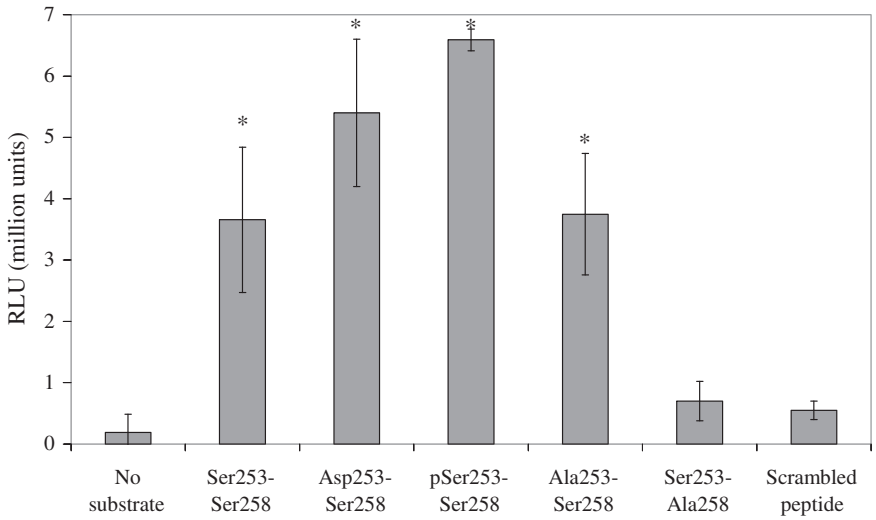

Fig. 4. p38α phosphorylates recombinant TF *in vitro*. Peptides corresponding to the C-terminal 18 amino acids of wild-type TF, and alanine, aspartate and phospho-serine substitutions at positions corresponding to Ser253 or Ser258 were incubated with recombinant p38α, and p38 kinase activity monitored using a luminescence-based kinase assay. ($n=7$, $^*=p<0.05$ vs. sample without substrate).

and remains under investigation. In support of this hypothesis, prevention of TF release through alanine-substitution of residue 253 enhanced the second phase of p38 activity while acceleration of TF release through aspartate–substitution eliminated this peak (Fig. 1C and D). In addition, alanine mutation of Ser258 had no bearing on p38 activation and therefore indicates that the activation of p38 precedes, and is not influenced by the phosphorylation of Ser258. Therefore we hypothesise that the second wave of activation of p38 is dependent on the presence of TF protein itself, but subsequent to induction of PAR2. This fits well with our previous report suggesting that the phosphorylation of Ser258 within human TF is regulated by both TF itself and requires the activation of PAR2 [12]. In addition, it has been shown that the de-palmitoylation of Cys245 is a precursor event to the tandem phosphorylation of the two serine residues [13,15,25]. This event may in effect permit the relocation of TF out of cellular regions, or the mobilisation of TF to specific membrane regions, exposing the cytoplasmic domain to target kinases. Prolonged activation of p38 pathway is thought to be the hallmark of induction of p38-mediated cell apoptosis, while short durations of activity appear to coincide with cell proliferation [26]. Furthermore, the increase in ERK activity coincided with the first p38 activity peak and preceded the release of TF into microparticles. Therefore, it may be suggested that the release of TF in effect has an anti-inflammatory/anti-apoptotic influence on the parent cell, and is mediated through transient phosphorylation of both ERK and p38 pathways. In contrast, it appears that the persistence of TF in cells, following activation, promotes the prolonged activation of p38 which may lead to inflammatory responses and apoptosis [26]. In this study, a model of PAR2-activated endothelial cells, in which TF was overexpressed, was used. This model permits the systematic examination of the regulation of the phosphorylation and release of TF, without any interference from other inflammatory signals. It also allows the study of mutant forms of TF with minimal interference from the wild-type cellular TF. In addition, parallel sets of cells were pre-incubated with TNFα to express the cellular TF. However, the physiological outcomes of these events must also include cellular signals arising from other inflammatory mediators.

The phosphorylation of both p38 and ERK preceded the phosphorylation of Ser258 within TF. Furthermore, both of these proteins may act as proline-directed kinases. Therefore, the possibility that p38 and ERK may be responsible for the phosphorylation of Ser258 within TF was explored. Inhibition of p38 activity using SB202190, prior to activation with PAR2-AP, resulted in reduced phosphorylation of Ser258 within TF (Fig. 2A and B). Moreover, this was concurrent with the maintained level of release of TF within microparticles, at 120 min (Fig. 5C). This is in agreement with our previous data showing that mutation of Ser258 to alanine prevents the curtailment of the release of TF at 120 min, while aspartate-substitution of this residue suppresses the release of TF [12]. Therefore, if p38 was not responsible for the phosphoryaltion of Ser258 within TF, in the presence of p38 inhibitor/siRNA, less total TF ought to be present within the cells and consequently, the ratio of phosphosereine-258-TF/Total TF should be higher than that without the inhibitor/siRNA. However, as may be seen in Fig. 2, this ratio actually dropped as a consequence of p38 inhibition/suppression. Therefore, it may be suggested that once sufficient amounts of TF are released, the process is self-regulated by a mechanism which is itself activated during TF release phase. Interestingly, at a concentration of 30 nM, SB202190 significantly inhibited the phosphorylation of TF by p38. This suggests the involvement of p38α, since p38β activity is not affected by this concentration of SB202190 [27]. Furthermore, knockdown of p38α expression using a specific siRNA, suppressed the phosphorylation of Ser258 within TF at 120 min (Fig. 2C and D) while concurrently prolonging the release of TF at 120 min (Fig. 5D). However, although the phosphorylation of Ser258 was largely inhibited, complete suppression was not achieved. Moreover, low levels of phosphorylation of TF was only achievable using ERK, when using high concentrations of TF (Fig. 3). Consequently, the possibility that other kinases such as ERK may also be capable of phosphorylation, TF under different cellular conditions, cannot be ruled out. In summary, these data support the ability of p38α as the major kinase, responsible for the phosphorylation of Ser258 within TF.

The ability of p38α to phosphorylate Ser258 within TF was confirmed using a luminescence-based kinase assay containing active recombinant p38α. In addition, the product from the reaction, but not from a negative reaction control devoid of ATP, was recognised by a specific antibody against phosphoserine258, within TF (Fig. 3). From the data using peptides corresponding to the cytoplasmic domain of TF (Fig. 4), it may be concluded that the serine residue at position 258 acts as a substrate for p38α. Furthermore, Ser253 does not constitute a viable phosphorylation site for this kinase. Moreover, the phosphorylation of the serine at the position corresponding to residue 258, by p38α, was substantially enhanced by the presence of either aspartate or phospho-serine at the position corresponding to residue 253 (Fig. 4 and Table 1). Therefore, we suggest that the observed reduction of the Km value for the phosphorylation of Ser258 by p38α, following phosphorylation of Ser253 (Table 1) may also arise from the steric exposure of Ser258. It is however important to note the Km value for the

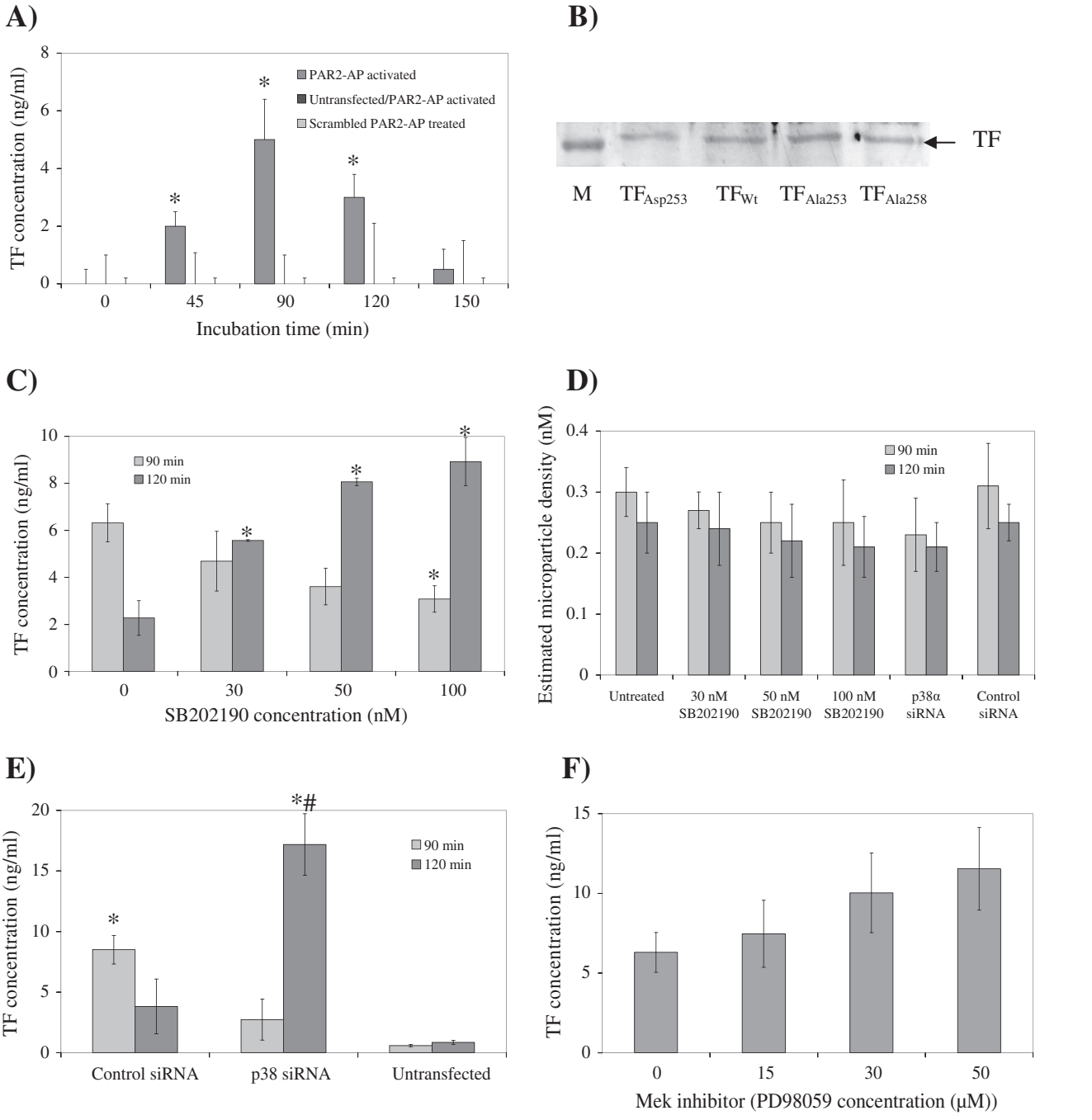

Fig. 5. The influence of p38 inhibition/suppression on TF release as microparticles. (A) Microparticles were isolated from HCAEC (2×10^5 in SFM) overexpressing TF_{Wt} or from untransfected control cells activated with PAR2-AP or a scrambled peptide. TF content of the isolated microparticles was quantified by ELISA. ($n=5$, $^*=p<0.05$ vs. untreated sample). (B) HCAEC (2×10^5 in SFM) overexpressing TF_{Asp253}, TF_{Wt}, TF_{Ala253} or TF_{Ala258} were activated with PAR2-AP (20 μM). Cells were then lysed at 90 min post-activation and analysed for TF by western blot. (Data is representative of 3 separate experiments; M = markers). (C) Samples of cells were pre-incubated with SB202190, activated with PAR2-AP and the release of TF measured at 90 and 120 min post-activation. ($n=3$, $^*=p<0.05$ vs. sample devoid of the inhibitor). (D) HCAEC (2×10^5 in SFM) overexpressing TF_{Wt} were pre-incubated with SB202190 (0–100 nM). In addition, samples of cells were co-transfected to overexpress TF together with either p38-siRNA or control siRNA. Cell were then activated with PAR2-AP (20 μM) and microparticles were isolated from the media by ultracentrifugation. The density of microparticles was estimated and compared using the Zymuphen Microparticle Assay. ($n=3$, $^*=p<0.05$ vs. untreated sample). (E) Samples of cells were co-transfected to overexpress TF, and either p38-siRNA or control siRNA. The cells were activated with PAR2-AP and the release of TF antigen analysed by ELISA. ($n=3$, $^*=p<0.05$ vs. untransfected sample, $\#=p<0.05$ vs. sample containing control siRNA). (F) Samples of cells were pre-incubated with PD98059, activated with PAR2-AP and the release of TF measured at 90 min post-activation.

full-length TF may also be subject to the positioning of this protein within the cell membrane.

By comparing the NMR-based molecular structures of the cytoplasmic domain of TF, Sen, et. al. [17] suggested that the reduction in the overall positive charge of the peptide may be sufficient to permit the activity of suitable kinase towards Ser258. We have further examined these published structures which also indicate that in the unphosphorylated peptide, Lys255 may be interacting with Ser258 preventing any further interactions (Fig. 6A). The phosphorylation of Ser253 results in the alteration of the φ and ψ torsional angles within Trp254 and Lys255 from extended configuration to almost a loop (Fig. 6B). This in turn appears to reposition both Lys255 and Trp254 away from Ser258, opening a pocket to permit access to Ser258 (Fig. 6A). In addition, examination of the variations in the top ten structures of these peptides [17] suggest

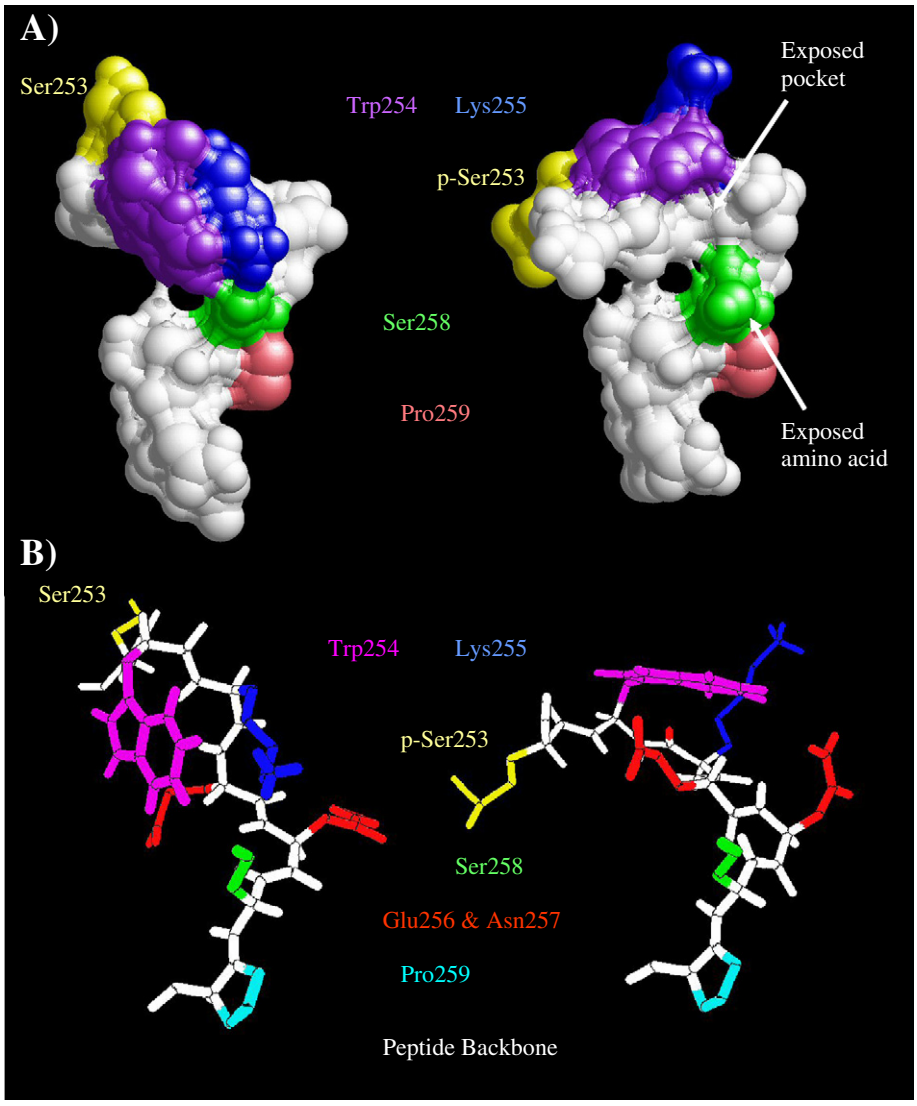

Fig. 6. Proposed molecular mechanism for the induction of Ser258 phosphorylation by p38. The structure of the cytoplasmic domain of TF in the unphosphorylated (2CEH) (left) and phospho-Ser253 (2CEZ) (right) forms (17) were edited to retain the residues 253–263 and (A) then viewed using the Raswin program (version 2.7.5). (B) The sequence of amino acids between residues 253–259 were uploaded onto the Alchemy III program and coloured to highlight the backbone structure.

that the phosphorylation of Ser253 results in greatly reduced flexibility of Lys255 and Trp254 (not shown) and therefore reduced ability of these two amino acids to interfere with the exposed Ser258. Therefore, we suggest that the observed reduction of the Km value for the phosphorylation of Ser258 by p38α, following phosphorylation of Ser253 (Table 1) may also arise from the steric exposure of Ser258.

5. Conclusions

We previously showed an association between the phosphorylation of Ser258 and the termination of TF release within cell-derived microparticles [12]. Our current study has identified p38α as a major kinase, responsible for the phosphorylation of Ser258 within the cytoplasmic domain of TF. Furthermore, the phosphorylation of Ser253 within the cytoplasmic domain of TF increases the affinity of p38α for TF and accelerates the rate of phosphorylation of Ser258. Moreover, the phosphorylation of Ser258 is concurrent with a second activation phase of p38 which itself, appears to depend on the presence of TF in the activated cells. Such a mechanism may constitute a means of monitoring of the amount of TF incorporated into microparticles by the cell, and therefore may also be a means of regulation of the level of cellular TF.

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