Murine tissue factor coagulant activity is critically dependent on the presence of an intact allosteric disulfide

Lisa G. van den Hengel,[1] Susanne Osanto,[1,2] Pieter H. Reitsma,[1] and Henri H. Versteeg[1]

[1]Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, the Netherlands and [2]Department of Clinical Oncology, Leiden University Medical Center, Leiden, the Netherlands

ABSTRACT

Tissue factor activation (decryption) has been proposed to be dependent on the cysteine 186-cysteine 209 allosteric disulfide in the tissue factor extracellular domain. Tissue factor procoagulant activity is under the control of protein disulfide isomerase-dependent modulation and nitrosylation of this disulfide. Human tissue factor disulfide mutants have been proposed as a model for encrypted tissue factor, but poor expression of these mutants hampers research into tissue factor decryption. We, therefore, investigated whether mouse tissue factor cysteine 186-cysteine 209 disulfide bond mutants form a better suited model for tissue factor decryption. Stable mouse wild-type tissue factor, tissue factor^{C190A}, tissue factor^{C213A} and tissue factor$^{C190/213A}$ disulfide mutant-expressing baby hamster kidney cells with equal levels of surface tissue factor were established. Tissue factor coagulant activity on these cells was determined using an active factor Xa-dependent chromogenic assay. The effect of nitrosylation on tissue factor function was also assessed. A tissue factor$^{C190/213A}$ mutant exerted marginal procoagulant activity, also after addition of supraphysiological concentration of factor VIIa. Tissue factor^{C190A} and tissue factor^{C213A} mutants showed reduced activity and the presence of tissue factor dimers. Nitrosylation of wild-type tissue factor cells decreased procoagulant function, an effect which was reversed by incubation with bacitracin, an inhibitor of protein disulfide isomerase, suggesting that this isomerase promotes de-nitrosylation of tissue factor. Mouse tissue factor procoagulant function is dependent on the Cys190-Cys213 disulfide bond and is modulated by nitrosylation. The murine model of disulfide-mutated tissue factor is more suitable for studying tissue factor decryption than are human tissue factor mutants.

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Introduction

Tissue factor (TF), a 47 kDa transmembrane protein, is the primary initiator of the coagulation cascade. Following vascular injury, TF expressed on subendothelial cells becomes exposed to the blood stream and forms a high-affinity complex with circulating factor VII (FVII). Subsequently, FVII is converted into activated FVII (FVIIa) followed by TF:FVIIa-dependent proteolytic activation of factor X (FX) and thrombin, finally resulting in the formation of a fibrin clot. Most TF resides on the cell surface in an inactive or cryptic state, but cellular stimulation rapidly reverts TF to a procoagulant state (decryption). The phospholipid content of cell membranes has been proposed to be an important determinant in the regulation of TF procoagulant activity.[1] Negatively-charged phosphatidylserine is actively kept at the inner leaflet of the plasma membrane, but cellular apoptosis and calcium ionophore stimulation disrupt this phosphatidylserine asymmetry, thereby providing a procoagulant surface for membrane-anchored TF.[2] The mechanisms underlying TF decryption do, however, remain elusive as TF decryption cannot be fully explained by increases in phosphatidylserine exposure.[3,4] Thus, phosphatidylserine-independent events have also been proposed to play pivotal roles in decryption.

The extracellular domain of human TF consists of N-terminal and C-terminal fibronectin-like modules, each containing a disulfide bond. Only the disulfide bond between cysteine residues at position 186 and 209 (Cys186-Cys209) is allosteric, which has been suggested to control TF function in a redox-dependent manner.[5,6] Oxidized TF with an intact Cys186-Cys209 disulfide bond is able to bind FVII with high affinity. However, reduction of TF, thus breaking the disulfide bond, renders TF coagulant inactive because of a low affinity for FVII.[7] Identification of protein disulfide isomerase (PDI) as a regulator of TF disulfide switching and TF-dependent fibrin generation *in vivo* strengthens the hypothesis that TF decryption is dependent on its redox status.[6,8,9] Additionally, PDI catalyzes nitrosylation of TF free cysteine residues, keeping TF in a cryptic state[6]

TF disulfide mutant cells provide a useful tool for studying the function of the allosteric disulfide bond in regulating TF coagulant activity. A number of mutagenesis studies found that elimination of the Cys186-Cys209 disulfide bond abolished procoagulant function.[5-7] In contrast, others found that coagulant activity normalized per TF molecule at supraphysiological FVIIa concentrations is indistinguishable between these mutants and wild-type TF (TF^{WT}), arguing that the disulfide mutants do not mimic cryptic TF.[10] However, in these

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Correspondence: Henri H. Versteeg. E-mail: h.h.versteeg@lumc.nl

studies the TF disulfide mutants were expressed at much lower levels than TF^{WT}, and excessive membrane surface may have led to an overestimation of the procoagulant activity of the mutants. Furthermore, single thiol mutants showed a tendency to dimerize and we have recently shown that TF dimerization also leads to overestimated procoagulant activity.[11] To overcome these problems, a model with similar expression of TF^{WT} and its disulfide mutants is needed. While human TF disulfide mutants are in general poorly expressed (human TF^{C186A} mutant is almost completely retained intracellularly[6]), TF decryption models in other species may be more successful.

Sequence alignment analysis shows complete conservation of cysteine 186 and cysteine 209 of TF among several other species, including murine TF;[12] however, direct evidence for functional conservation of this allosteric disulfide bond in TF decryption is lacking. We, therefore, investigated whether mouse TF disulfide mutants are a suitable model for TF decryption and whether modulation of the Cys190 -Cys213 allosteric disulfide bond in murine TF is involved in the procoagulant function of TF.

Design and Methods

Reagents

pcDNA3.1 vector, pTrcHis vector, zeocin and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA, USA). Basal medium and supplements were acquired from PAA Laboratories (Cölbe, Germany). NHS-LC-LC-biotin was acquired from Pierce (Rockford, IL, USA). Reduced glutathione and n-ethyl maleimide were from Sigma Aldrich (St. Louis, MO, USA). Ascorbic acid was purchased from Merck (Darmstadt, Germany) and sodium nitroprusside was from Enzo Life Sciences (Lausen, Switzerland). n-Octyl-beta-D-glucopyranoside (OG) was obtained from Calbiochem (Darmstadt, Germany). 3-(N-maleimidopropionyl)-biocytin (MPB), streptavidin- and protein G-coupled Dynabeads and horseradish peroxidase-streptavidin antibody were purchased from Invitrogen (Carlsbad, CA, USA). FVIIa was a kind gift from Novo Nordisk (Bagsvaerd, Denmark) and FX was from Kordia (Leiden, the Netherlands). Spectrozyme FXa was obtained from American Diagnostica (Stamford, CT, USA). Bacitracin was acquired from Sigma Aldrich. Rabbit anti-mouse TF antibody was kindly provided by Dr. James H. Morrissey (University of Illinois, Urbana, USA).

Generation of tissue factor-expressing cell lines

To create cells expressing wild-type murine TF or TF disulfide mutants, the mouse TF gene was cloned into pcDNA3.1 and subsequently mutated, resulting in Cys-to-Ala conversions at positions 190 and/or 213 by site-directed mutagenesis, according to the manufacturer's protocol (Stratagene, La Jolla, CA, USA). Stable transfections of baby hamster kidney (BHK) cells were performed with either mouse TF^{WT} or one of the disulfide mutants (TF^{C190A}, TF^{C213A} and $TF^{C190/213A}$) by using Lipofectamine 2000. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 1% penicillin. Clonal selection was performed after addition of 100 μg/mL zeocin. Clones were screened for TF protein expression by western blotting and selected on the basis of similar TF expression at the cell surface.

Surface biotinylation and biotin-switch

Cell surface biotinylation was performed on stably transfected BHK cells to determine surface-exposed TF expression. In brief, cells were washed with ice-cold Hepes-buffered saline (HBS; 10 mM Hepes, 1.5 mM $CaCl_2$, 137 mM NaCl, 5.3 mM KCl, pH 7.4) followed by incubation with 1 mM NHS-LC-LC-biotin in HBS for 20 min. The reaction was quenched by incubation with a 100 mM Tris/glycine solution and cells were then extensively washed with Tris-buffered saline (100 mM Tris/137 mM NaCl with the addition of 1.5 mM $CaCl_2$, 5 mM KCl and 0.5 mM $MgCl_2$) in order to remove residual NHS-biotin.

The biotin-switch method, based on a previously described protocol, was used to detect nitrosylated proteins.[13] In brief, cells were incubated with 1 mM n-ethyl maleimide to block free thiols, followed by addition of ascorbic acid to release protein-bound nitric oxide (NO). MPB was then reacted with the liberated free thiols. MPB-incorporation was detected by western blot after immunoprecipitation of TF. Biotin-switching was also performed after nitrosylation of TF^{WT} induced by incubation of 1 mM reduced glutathione in combination with 1 mM of the NO donor, sodium nitroprusside.

Immunoprecipitation

Immunoprecipitation was performed to assess surface-exposed TF and nitrosylated TF in cell lysates. For this purpose, cells were lysed in OG lysis buffer (50 mM OG in HBS) and crude lysates were centrifuged. Biotinylated proteins in clear lysates were immune-precipitated using streptavidin-coupled Dynabeads or anti-mouse TF coupled to protein G-conjugated beads.

Purification of murine tissue factor protein

BHK cells were transfected with pTrcHis vector to express recombinant His-tagged murine TF^{WT}, TF^{C190A} or TF^{C213A} proteins. After cell lysis with 50 mM OG buffer, the cell lysates were subjected to NTA-Nickel-based purification of His-tagged proteins. Proteins were eluted in low-pH OG buffer (pH 2) and taken up in sample buffer for western blotting.

Western blotting

Total TF protein levels were assessed after cell lysis in sample buffer (0.625 M Tris, 20% glycerol, 4% sodium dodecylsulfate, bromophenol blue, pH 6.8) and reduced total TF antigen levels was determined in cell lysates after the addition of 100 mM DTT. The levels of both total and cell surface TF proteins of transfected BHK cells were determined by western blotting with rabbit anti-mouse TF. Nitrosylated mouse TF protein was detected on western blot with biotin/streptavidin-horseradish peroxidase and, as a loading control, mouse TF in total lysates was determined using anti-mouse TF.

Tissue factor procoagulant activity assay

FXa generation assays were performed to measure TF procoagulant activity on stably transfected BHK cells. The cells were washed with warm HBS and FXa was generated by addition of 1 nM or increasing concentrations of mouse FVIIa combined with 100 nM human FX. The reaction was then quenched in 50 mM Tris solution (pH 7.4) containing 100 mM EDTA. Finally, FX activity was kinetically determined by measuring the absorbance at 405 nm after addition of the chromogenic substrate Spectrozyme Xa. We also determined the effects of incubation with reduced glutathione/sodium nitroprusside in the presence/absence of 1 mM bacitracin on TF activity on cell surfaces.

Statistical analysis

All experiments were performed in triplicate. Results are presented as the mean±SD. An independent t-test was performed to compare means between two experimental conditions. For

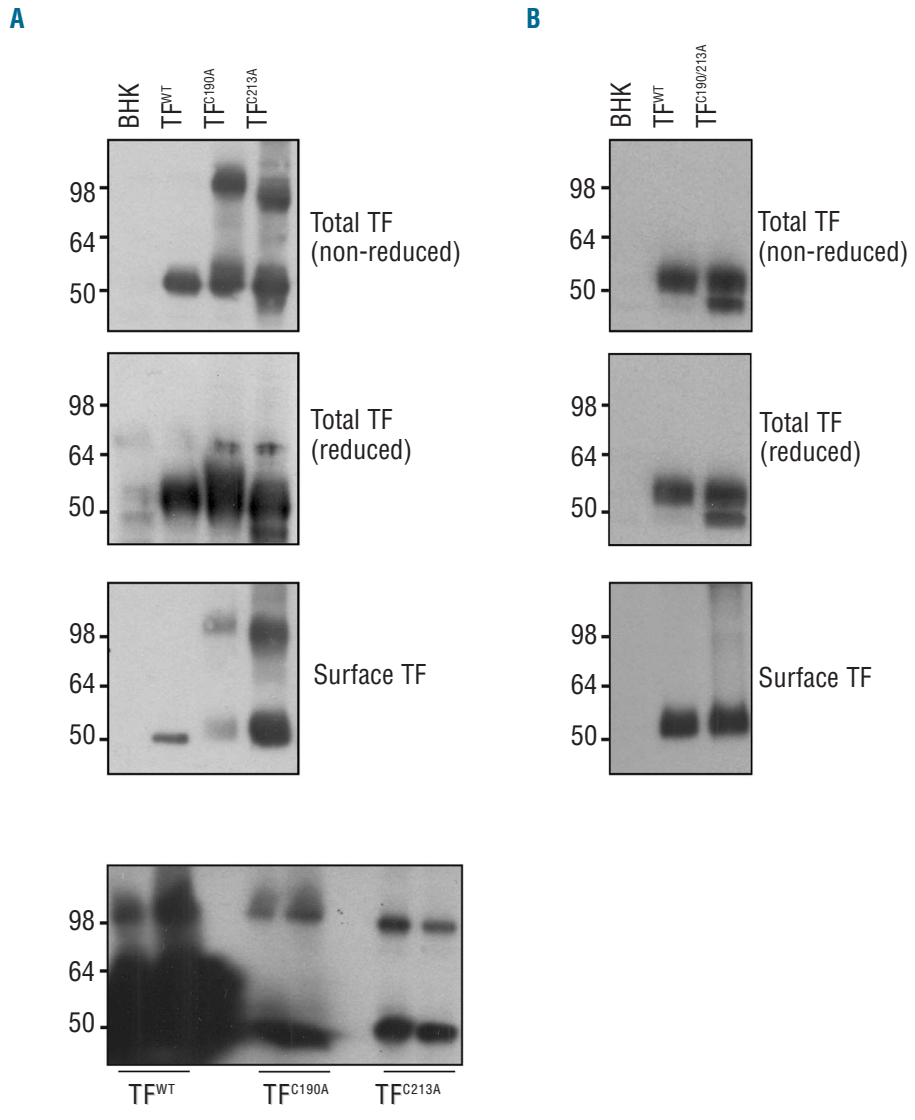

Figure 1. Wild type and disulfide-mutated murine TF are similarly expressed in BHK cells. BHK cells were stably transfected with constructs expressing mouse wild type TF (TF^{WT}) or murine TF containing Cys-to-Ala substitutions at positions 190, 213, or both (TF^{C190A}, TF^{C213A} and $TF^{C190/213A}$, respectively). Total cell lysates were prepared in the presence/absence of the reducing agent DTT and TF was detected by western blotting. Cell surface TF was determined by labeling the cell surface with NHS-biotin followed by precipitation with streptavidin beads and TF detection on a western blot. (A) Non-reduced total TF (upper panel), reduced total TF (middle panel) and TF cell surface expression (lower panel) in BHK cells expressing TF^{WT}, TF^{C190A} and TF^{C213A}. (B) Non-reduced total TF (upper panel), reduced total TF (middle panel) and TF cell surface expression (lower panel) in BHK cells expressing TF^{WT} and $TF^{C190/213A}$. Non-transfected BHK cells were included as a negative control. (C) TF protein was purified from OG lysates of BHK cells transfected with His-tagged TF^{WT}, TF^{C190A} and TF^{C213A} after purification using NTA-Nickel beads followed by TF detection on a western blot. Samples were loaded to show approximately equal amounts of dimers. Consequently, the TF^{WT} monomer is present at higher amounts compared to the TF mutants.

multiple comparison analysis, a one-way ANOVA was performed. *P* values <0.05 were considered statistically significant.

Results

Generation of mouse tissue factor wild-type and disulfide mutant cells

Human TF disulfide mutants are considered a model for TF decryption, but these mutants express the factor poorly. We, therefore, attempted to create a model based on murine TF. BHK cells were stably transfected with TF^{WT}, TF^{C190A}, TF^{C213A} and $TF^{C190/213A}$ DNA constructs and clones were selected based on similar cell surface TF expression. We were able to identify BHK clones expressing cell surface TF^{WT} and disulfide mutants at similar levels by means of surface labeling followed by western blotting, although expression of TF^{C213A} was somewhat higher (Figure 1A,B). Comparable TF protein levels were also found in total lysates of these different BHK clones, showing that murine TF cell surface expression is not substantially hampered by the lack of the allosteric disulfide (Figure 1A,B). Importantly, TF^{C190A} was efficiently expressed, while the human TF^{C186} homologue is retained completely intracellularly[6]. Mutation of a single cysteine in TF resulted in substantial TF dimerization and these dimers were found in total lysates as well as at the surface of single disulfide mutants (Figure 1A). These dimers were sensitive to reduction with DTT confirming that dimerization resulted from disulfide bonding (Figure 1A). Dimers were not detected in lysates of cells expressing TF^{WT} or the $TF^{C190/213A}$ double mutant (Figure 1B). However, purification of His-tagged TF proteins clearly showed dimerization of TF^{WT}, which suggests that mouse TF contains unpaired cysteine thiols (Figure 1C). Strikingly, TF^{WT} and TF^{C190A} dimers, but not TF^{C213A} dimers, showed similar mobility on sodium dodecylsulfate polyacrylamide gel electrophoresis (~95 kDa *versus* ~100 kDa), suggesting that TF^{WT} dimers are in a Cys213-Cys213 disulfide conformation and that one of these dimer fractions consists of a heterodimer (Figure 1A,C). Nevertheless, we did not detect PDI after probing TF-immunoprecipitated lysates of TF^{C190A} and TF^{C213A} clones on a western blot with a PDI antibody (*data not shown*).

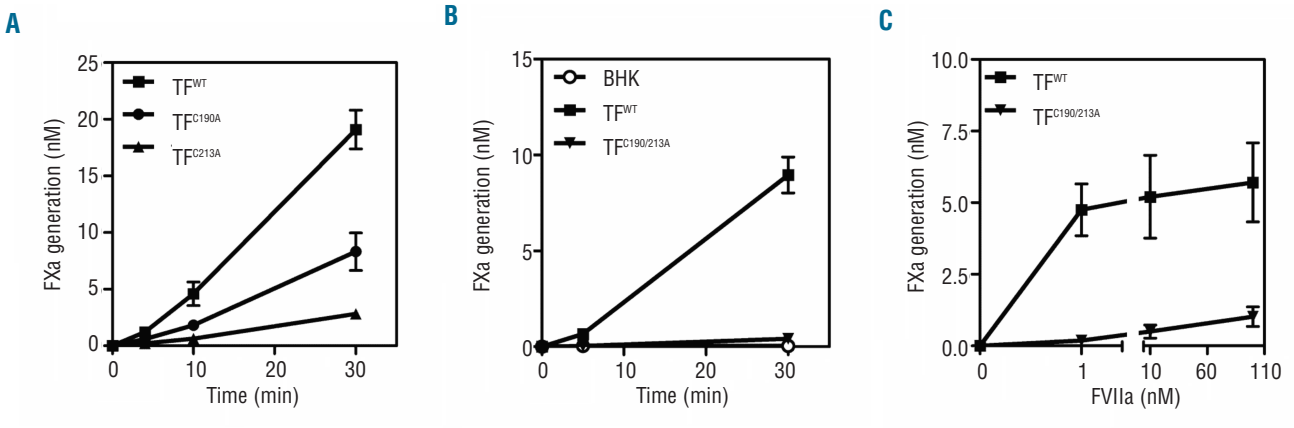

Figure 2. Murine TF procoagulant activity requires an intact Cys190-Cys213 disulfide bond. (A) FXa generation assays were performed to compare TF activity on the surface of transfected BHK cells expressing TF^{WT}, TF^{C190A} or TF^{C213A} after addition of 1 nM mouse FVIIa and 100 nM human FX. (B) The kinetics of FXa were measured on TF^{WT} or TF^{C190A}-expressing cells using 1 nM mouse FVIIa and 100 nM human FX. (C) TF procoagulant activity on BHK expressing $TF^{C190/213A}$ and TF^{WT} was determined after addition of the indicated concentrations of FVIIa. Results represent mean±SD.

This indicates that PDI was not a component of these dimers, which may be because of weak or non-covalent bonding with TF. We also observed an extra band in the reduced and non-reduced total lysates of $TF^{C190/213A}$ double mutant-expressing cells, but not in the cell surface fraction, and we assume that the lower band is non-glycosylated mutant TF (Figure 1B).

In conclusion, in contrast to human TF disulfide mutants, murine TF disulfide mutants are efficiently expressed at the cell surface and may enable better molecular and cellular studies on the involvement of the TF allosteric disulfide in TF decryption.

Disruption of the Cys190-Cys213 disulfide bond diminishes mouse tissue factor procoagulant activity

We next tested the coagulant properties of mouse TF lacking the Cys190-Cys213 disulfide bond. BHK cells expressing TF^{WT}, TF^{C190A}, TF^{C213A} and $TF^{C190/213A}$ were engaged in FXa generation assays using 1 nM murine FVIIa and 100 nM FX. FXa generation was taken as a direct measurement of TF procoagulant activity. Procoagulant activity of TF^{C213A} and TF^{C190A} was decreased by ~85% and ~60% compared to the activity of TF^{WT} (Figure 2A). In addition, $TF^{C190/213A}$ procoagulant activity was completely abolished as the levels of FXa generated on cells expressing this mutant were not different from those generated on untransfected BHK cells. (Figure 2B). The addition of supraphysiological FVIIa concentrations did not restore coagulant function of $TF^{C190A/C213A}$ cells, suggesting that this mutant is impaired in both FVIIa and FX binding (Figure 2C). Taken together, these findings indicate that murine TF procoagulant activity depends on the presence of a disulfide bond between cysteine 190 and cysteine 213 as was observed before using human TF disulfide mutants.[6,7,11]

Nitrosylation of cysteine 190 and cysteine 213 decreases mouse tissue factor activity

Nitrosylation of free cysteine residues down-regulates human TF procoagulant function in a PDI-dependent manner[6]. To assess whether mouse TF is also susceptible to nitrosylation, TF^{WT} cells were subjected to biotin switching. We observed that TF is nitrosylated under basal conditions. Moreover, incubation of TF^{WT} cells with an NO donor (sodium nitroprusside) in a reducing environment (reduced glutathione) resulted in increased nitrosylation of TF (Figure 3A). TF activity was reduced upon nitrosylation of TF^{WT}, whereas $TF^{C190/213A}$-induced activity was not affected by nitrosylation (Figure 3B). This indicates that cysteine 190 and/or cysteine 213 are susceptible to nitrosylation. In addition to TF, nitrosylation also targeted a ~64 kDa protein suggestive of PDI (Figure 3A). We, therefore, explored whether PDI mediates nitrosylation of mouse TF, as was shown before with human TF.[6] Procoagulant activity of TF^{WT} was not affected by blocking PDI with bacitracin. However, nitrosylated TF showed severely reduced procoagulant activity upon PDI inhibition (Figure 3C). This suggests that, in contrast to what is observed in models utilizing human TF, PDI or a PDI homologue inhibits TF nitrosylation.

Discussion

In this study we generated cell lines showing equal cell surface expression of mouse TF^{WT} or TF mutated at the evolutionary conserved cysteine residues at position 190 and/or 213. Using murine TF mutants containing both single and double Cys-to-Ala replacements we observed that the procoagulant activity of murine TF was reduced or even abolished when TF lacks the Cys190-Cys213 disulfide bond. Furthermore, these cysteine residues are susceptible to nitrosylation, leading to a change in mouse TF procoagulant status.

The current TF in a murine system has a major advantage over previously described human TF disulfide mutants as a model for TF decryption.[6,10] There are no limitations in determining functional expression of TF disulfide mutants, as the cell surface expression of mouse TF mutated at cysteine 190, in contrast to the human TF^{C186A} homologue, is not hampered. This feature may make murine TF more suitable for elucidating the role of either

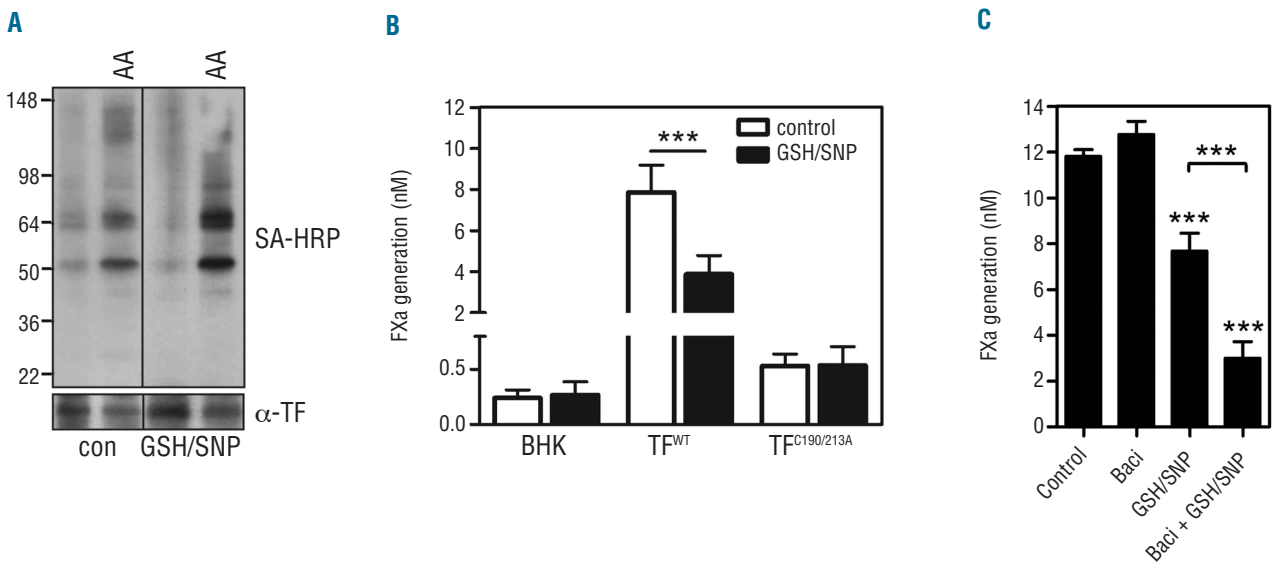

Figure 3. NO-dependent down-regulation of TF procoagulant activity. (A) Biotin switching and TF immunoprecipitation were performed to determine nitrosylation of TF^{WT} in the presence or absence of reduced glutathione (GSH) and the NO-donor sodium nitroprusside (SNP). Addition of ascorbic acid (AA) releases thiol-bound NO to enable MPB labeling. Nitrosylated and total TF were detected by western blot using streptavidin-horseradish peroxidase (SA-HRP) and TF antibody (α-TF), respectively. (B) FXa generation assays were performed on BHK, TF^{WT} and $TF^{C190/213A}$ cells in the presence or absence of GSH and SNP, using 1 nM murine FVIIa and 100 nM FX. TF activity in TF^{WT} and $TF^{C190/213A}$ cells was significantly higher than the activity in BHK cells (not indicated in figure). (C) Procoagulant activity on TF^{WT}-expressing BHK cells was determined after treatment with GSH and SNP in the presence or absence of bacitracin (Baci), using 1 nM FVIIa and 100 nM FX. Results represent mean±SD. $^{**}P<0.01$; $^{***}P<0.001$.

cysteine residue in TF function.

The mouse TF single cysteine mutants, but not the double mutant, yielded expression of dimers, which indicates that free thiols on both cysteine residues 190 and 213 residues are potentially available for intermolecular disulfide bonding between TF molecules. Notably, we found that the single cysteine mutants showed residual coagulant activity, while the coagulant activity of $TF^{C190/213A}$ was abolished. As previous data on a human TF^{C209S} mutant demonstrated that inhibition of dimerization decreased TF activity, our findings suggest that TF dimers are also involved in the coagulant function of murine TF.[11] Cross-linking studies have suggested the existence of human TF dimers at the cell surface[14,15] and it has been suggested before that TF dimerization plays an important role in the activation of TF procoagulant activity, and possibly decryption. Based on this, it might be assumed that cellular TF exists in a cryptic/reduced configuration and dimerization may skew TF towards an active status. However, observations arguing against this can readily be found in the literature. Human TF dimer formation by chemical or leucine zipper cross-linkage showed reduced or unaffected procoagulant activity.[14-16] Similarly, *E. coli*-expressed human TF^{C209A} dimers are not coagulant-active,[6] but the lack of a membrane environment in this system may lie at the basis of this. The above-described data illustrate that it is still difficult to interpret the role of dimers in procoagulant activity.

Interestingly, the dimers expressed by the mouse TF single mutant cells differed in molecular weight. Mutation at either cysteine residue at position 190 or 213 led to the formation of two homo- or heterodimers with distinct apparent molecular weights. TF^{WT} dimers and TF^{C190A} dimers showed similar mobility on sodium dodecylsulfate polyacrylamide gel electrophoresis, suggesting that TF^{WT} dimers are linked through Cys213 residues. Nevertheless, we cannot exclude the possibility of dimerization through Cys247 in the cytoplasmic domain of murine TF, as a previous human study showed that generation of TF dimers may involve Cys245, the homologous cysteine residue in human TF.[17]

We considered PDI to be a likely candidate as a constituent of one of the dimers formed by the mouse TF single mutants, based on the established function of PDI in TF-dependent coagulation.[6] However, our attempts to detect PDI in association with either TF^{C190A} or TF^{C213A} were unsuccessful (*data not shown*). This does not necessarily exclude other oxidoreductases such as Erp57.[18] It must be considered that murine TF differs from TF in other species in that it does not contain a PSR-N motif.[12] These hydrophilic and polar amino acids form a surface-exposed loop and are thought to function as a binding site for PDI or other oxidoreductases. Alternatively, lack of PDI retrieval in TF dimers may be due to non-covalent PDI binding to TF or due to the weak disulfide bond between TF or PDI. Indeed, on western blot TF/PDI co-precipitates were previously found as two separate bands.[6]

Post-translational modification of mouse TF by nitrosylation of free cysteine residues led to down-regulated TF activity. Although PDI was not found to be associated with mouse TF after TF-immunoprecipitation, PDI or another oxidoreductase appeared to protect mouse TF function from nitrosylation. In this respect, PDI may act as a NO-sequestering protein, or may actively denitrosylate TF. Our data suggest that NO-dependent modulation of TF activity is not species-specific. Moreover altered NO

availability may have a profound impact on TF function in cardiovascular diseases such as atherosclerosis, diabetes and hypertension.[19]

Apart from the Cys186-Cys209 disulfide, the Cys49-Cys57 disulfide bond is also conserved in mice.[12] The N-terminal disulfide has recently been suggested to modulate the redox properties of the Cys186-Cys209 disulfide bond in TF.[20] As the current study shows functional conservation of the Cys186-Cys209 disulfide bond in mice, it would be of interest to determine whether the N-terminal disulfide in murine TF is also indirectly involved in TF procoagulant function.

In conclusion, the current study demonstrates that the TF allosteric disulfide is required for full coagulant activity and that targeting of this disulfide by NO critically regulates TF function. Our results show that modulation of the TF allosteric disulfide and its consequences for coagulant activity are not restricted to human TF. Finally, murine TF-expressing cells may be more appropriate models for studying the role of this disulfide in TF coagulant activity and decryption, and additional research to identify the mechanisms involved in TF nitrosylation and dimerization is warranted.

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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