Evidence for Activation of Tissue Factor by an Allosteric Disulfide Bond†

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ABSTRACT: Tissue Factor (TF) is the mammalian plasma membrane cofactor responsible for initiation of blood coagulation. Binding of blood coagulation factor VIIa to TF activates the serine proteinase zymogens factors IX and X by limited proteolysis leading to the formation of a thrombin and fibrin meshwork that stabilizes the thrombus. TF on the plasma membrane of cells resides mostly in a cryptic configuration, which rapidly transforms into an active configuration in response to certain stimuli. The extracellular part of TF consists of two fibronectin type III domains. The disulfide bond in the membrane proximal domain (Cys186−Cys209) is atypical for domains of this type in that it links adjacent strands in the same β sheet, what we have called an allosteric bond. Ablation of the allosteric disulfide by mutating both cysteine residues severely impairs procoagulant activity. The thiol-alkylating agents *N*-ethylmaleimide and methyl methanethiolsulfonate block TF activation by ionomycin, while the thiol-oxidizing agent $HgCl_2$ and dithiol cross-linkers promote activation. TF activation could not be explained by exposure of phosphatidylserine on the outer leaflet of the plasma membrane. Cryptic TF contained unpaired cysteine thiols that were depleted upon activation, and de-encryption was associated with a change in the conformation of the membrane-proximal domain. These findings imply that the Cys186−Cys209 disulfide bond is reduced in the cryptic form of TF and that activation involves formation of the disulfide. It is likely that formation of this disulfide bond changes the conformation of the domain that facilitates productive binding of factors IX and X.

Tissure Factor (TF)[1] is a 45kD glycoprotein with structural homology to the class II cytokine receptor family. It consists of extracellular (residues 1−219), transmembrane (residues 220−242) and cytoplasmic (residues 243−263) domains. It is constitutively expressed at biological boundaries such as skin, organ surfaces, vascular adventitia, and epithelial−mesenchymal surfaces (*1*). TF is not typically expressed in vascular endothelial cells and monocytes, but expression and surface localization can be induced in these cells by cytokines. Binding of factor VIIa to TF switches on the VIIa active site, which is reflected as an increase in the catalytic rate constant. The rate of factors IX and X activation increases by about 4 orders of magnitude, which is due to both the enhanced catalytic constant and the membrane binding of the substrates (*2*).

From studies of TF on different cell types, it is clear that two populations of the cofactor exist on the cell surface: a minor population of active TF that binds factor VIIa and cleaves both a peptidyl substrate and factor X and a major population of cryptic TF that also binds factor VIIa and cleaves a peptidyl substrate but not factor X (*3*−*5*). A characteristic feature of cryptic TF is the slow rate of binding of factor VII/VIIa. Equilibrium binding of VII/VIIa to active TF is established within 1 min, while binding to cryptic TF takes 1−2 h to reach equilibrium. Cryptic TF is activated upon exposure of the cell to a calcium ionophore or by physical disruption of the cell without increasing TF message or protein expression. Notably, de-encryption of TF involves the extracellular part of the protein as a mutant TF lacking the cytoplasmic domain is activated the same as the full-length protein (*6*).

A clue to the molecular trigger for TF activation came from studies of a type of disulfide bond that we have called the allosteric disulfide (*7*). Many allosteric disulfides link cysteines in adjacent strands in the same antiparallel β sheet and are often found straddling a β hairpin structure. The bonds have a high potential energy, which arises from the torsional energy of the linkage as well as the deformation energy in the sheet itself (*8*, *9*). The botulinum neurotoxins (*9*, *10*), the immune receptor CD4 (*11*, *12*), and the HIV envelope glycoprotein gp120 (*9*, *13*, *14*) are examples of proteins that are controlled by allosteric disulfides. One of the two TF disulfides has the features of an allosteric bond.

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[1] Abbreviations: BHK, baby hamster kidney; BMH, bismaleimidohexane; BMOE, bismaleimidoethane; MMTS, methyl methanethiolsulfonate; MPB, 3-(*N*-maleimidylpropionyl)biocytin; NEM, *N*-ethylmaleimide; PBS, phosphate buffered saline; TF, Tissue Factor.

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The extracellular part of TF consists of two fibronectin type III (Ig-like) domains, or a single class II cytokine-binding module. The backbone of fibronectin type III domains are defined by two antiparallel β sheets, each containing seven β strands, arranged in a sandwich-like structure. One β sheet contains strands A, B, and E, and the other sheet has strands C, C′, F, and G. The disulfide bond in the membrane proximal domain of TF is the potential allosteric bond. Cys186 in the F strand links with Cys209 in the adjacent G strand of the β-sheet. The disulfide bond in the N-terminal domain is a conventional disulfide bond, linking Cys49 in the C′ strand and Cys57 in the E strand.

The Cys186−Cys209 TF disulfide is exposed to solvent in the crystal structure (*15*, *16*), which in itself is most unusual for a fibronectin type III disulfide. Importantly, ablation of the allosteric bond by mutating both cysteines to serines severely impairs procoagulant activity (*17*). Mutating both cysteines to alanines similarly impairs procoagulant activity (unpublished observations). We hypothesized that this disulfide bond mutant was, in fact, a mimic of the cryptic form of TF. Here, we provide evidence that the allosteric bond is reduced in the cryptic form of TF and that activation involves formation of the disulfide.

EXPERIMENTAL PROCEDURES

Cell Culture. Human myeloid (M2) leukemia HL-60 cells (ATCC, Bethesda, MD) were cultured in Iscove's modified Dulbecco's medium with 20% fetal bovine serum and 600 μg mL^{-1} gentamicin. Baby hamster kidney (BHK) cells stably transfected with human TF (*18*) were cultured in Dulbecco's modified Eagles medium with 10% fetal bovine serum, 2 mM glutamine, 100 units mL^{-1} penicillin, and 100 units mL^{-1} streptomycin with positive selection using 1 μM methotrexate. Human HaCaT keratinocytes were cultured in Dulbecco's modified Eagles medium with 10% fetal bovine serum and 2 mM glutamine. Human umbilical vein endothelial cells were maintained and transduced as described (*19*).

TF Activity Assays. The TF procoagulant activity of intact HL-60 cells was measured by a one-stage clotting assay using a ST Art mechanical clot detection coagulometer (Diagnostica Stago, France). HL60 cells were resuspended in fresh media at 10^6 cells per milliliter the day before assaying. On the day of assay, cells were stimulated for 6 h in 1 μM phorbol myristate acetate (PMA, Sigma, St Louis, MO) to induce TF expression, then washed 3 times with 10 mM Hepes, pH 7.4 buffer containing 137 mM NaCl, 5.38 mM KCl, 5.55 mM glucose, and 12.5 mM $CaCl_2$ (Hepes buffered saline), and resuspended to 10^6 cells per milliliter. Aliquots (200 μL) of the cells were equilibrated at 37 °C for 3 min and then incubated with ionomycin, $HgCl_2$, or the dithiol cross-linkers bismaleimidohexane (BMH) and bismaleimidoethane (BMOE) (Pierce, Rockford, Il) at varying concentrations and for different times. On some occasions, the cells were incubated with annexin V (Alexis, San Diego, CA), *N*-ethylmaleimide (NEM) (Sigma, St. Louis, MO), or methyl methanethiolsulfonate (MMTS) (Sigma, St. Louis, MO) prior to addition of ionomycin or $HgCl_2$. Clot formation was initiated by the addition of 100 μL of a 1:1 mix of pooled normal human plasma and PBS. Control reactions with vehicle only (DMSO) were run in parallel. On some occasions, the supernatant of HL60 cells post-ionomycin or $HgCl_2$ treatment was separated by centrifugation at 800*g* for 5 min, and the clotting times of supernatant was compared with that of the cellular fraction resuspended in Hepes buffered saline containing $CaCl_2$. The plasma was pooled from 20 donors in 3.2% sodium citrate mixed with an equal volume of sterile PBS (*20*). On occasion, factor VIIa (NovoNordisk, Copenhagen, Denmark) was added to the diluted plasma to a final concentration of 5 or 50 nM. Clotting times were converted to arbitrary units of TF procoagulant activity from a standard curve constructed using recombinant human TF reconstituted into phospholipid vesicles (Innovin, Dade Behring, Deerfield, IL). Results are expressed as change in TF activity.

BHK cells were plated into 96 well plates and grown to 70−80% confluence. Media was changed the day before assay. On the day of assay, cells were washed 3 times in 50 mM Hepes, pH 7.4 buffer containing 0.11 M NaCl, 1 mg mL^{-1} PEG 6000, and 5 mM $CaCl_2$. Plates were mixed on a shaker in the presence of 100 nM factor X (Calbiochem, San Diego CA) and 100 μM chromogenic substrate Spectrozyme fXa (American Diagnostica, Greenwich, CT) and then equilibrated at 37 °C for 3 min. Ionomycin or $HgCl_2$ was added 30 s before the addition of 10 nM Factor VIIa (Novo Nordisk, Copenhagen), and the rate of factor Xa generation was continuously monitored by detection of Spectrozyme Xa cleavage at 405 nM absorbance using a Molecular Devices Thermomax Plus (Palo Alto, CA) microplate reader. The initial velocities of factor Xa generation were calculated for each sample.

The parabolic progress curves were fit to eq 1 (*21*) by nonlinear least squares regression using GraphPad Software (San Diego, CA).

$$[\mathrm{pNA}] = k_1 k_2 t^2/2 \quad (1)$$

[pNA] is the concentration of liberated *p*-nitroanaline, t is time in s, k_1 is a constant for hydrolysis of Spectrozyme Xa by factor Xa, equal to k_{cat}[spectrozyme fXa]/(K_m + [spectrozyme fXa]), and k_2 is the initial rate of factor Xa formation in M s^{-1}. The kinetic parameters for hydrolysis of Spectrozyme fXa by factor Xa were determined from measurements of the initial velocity of amidolysis of the chromogenic substrate as a function of Spectrozyme fXa concentration at a fixed concentration of factor Xa (2 nM). The initial velocity data were fitted to the Michaelis−Menton equation by nonlinear least squares regression. The k_{cat} and K_m were calculated to be 548 s^{-1} and 137 μM, respectively. Therefore, k_1 in the system was 233 s^{-1}.

Labeling of Free Thiols in TF. BHK-TF cells, 1 mL of 5 × 106 cells mL^{-1} in 10 mM Hepes, pH 7.5 buffer containing 137 mM NaCl, 5.38 mM KCl, 5.55 mM glucose, and 12.5 mM $CaCl_2$ (Hepes buffered saline), were incubated with vehicle DMSO, 10 μM ionomycin (Sigma), or 100 μM $HgCl_2$ (Sigma, St. Louis, MO) for 30 s or 2 min at room temperature and then labeled with 100 μM 3-(*N*-maleimidylpropionyl)-biocytin (MPB) (Molecular Probes, Eugene, OR) for 2 min. The cells were washed 3 times with Hepes buffered saline, and residual unreacted MPB was quenched with 200 μM glutathione for 30 min at room temperature on a rotating wheel. Control samples were incubated with 2 mM sulfosuccinimidobiotin (SSB) (Pierce, Rockford, IL), and unreacted label was quenched with 100 mM glycine. The labeled cells

were washed 3 times with phosphate buffered saline (PBS) and then lysed with 25 mM octylglucopyranoside in the presence of a protease inhibitor cocktail (Roche Diagnostics, Australia) at room temperature for 5 min, and the lysate was clarified by centrifugation. The MPB or SSB labeled proteins were collected on streptavidin coated Dynabeads (Dynal, Melbourne, Australia) at 4 °C on a rotating wheel overnight. The beads were washed 5 times with 25 mM octylglucopyranoside in the presence of the protease inhibitor cocktail, and the biotinylated proteins eluted from the beads with SDS−Laemmli buffer containing 20 mM dithiothreitol. The samples were boiled for 5 min, and the reduced proteins were alkylated with 40 mM iodoacetamide. Samples were resolved on 8−16% SDS−PAGE and transferred to a PVDF membrane. Biotin labeled TF was immuno-blotted with polyclonal goat anti-human anti-TF antibody (1 μg mL^{-1}) and a horseradish peroxidase−rabbit anti-goat antibody (Dako, Carpinteria, CA) at a 1:1000 dilution and detected using chemiluminescence (Pierce, Rockford, Il). Band intensity was measured using Quantity One v 4.5.2 software (BioRad, Hercules, CA). Membranes were stripped and then reprobed with streptavidin peroxidase (Dako, Carpinteria, CA).

TF Immunoprecipitation. HaCaT cells were incubated with 100 μM BMH or $HgCl_2$ in Hepes buffered saline for 15 min at room temperature. The cells were washed, and the TF immunoprecipitated from 50 mM *n*-octyl-β-D-glucopyranoside lysates used monoclonal antibodies coupled to Dynabeads. The samples were Western blotted for TF as described previously.

Flow Cytometry. A TF9-9C3 monoclonal mouse anti-human TF antibody was conjugated to an Alexafluor 488 succinimidyl ester (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. FITC conjugated mouse−anti-human TF antibody recognizing an epitope within amino acids 181−214 (V1C7) was from America Diagnostica, Greenwich, CT. PMA stimulated HL60 cells were washed 3 times in cold Hepes buffered saline containing 12.5 mM $CaCl_2$ and resuspended to 10^6 cells per milliliter. Cells were incubated with 10 μM ionomycin or 100 μM $HgCl_2$ for 30 s and then fixed in 1% paraformaldehyde. Fixed cells were washed 3 times in ice cold PBS containing 1% bovine serum albumin and labeled with 20 μg mL^{-1} 9C3 or V1C7 antibodies, or the antibodies were preincubated with a 250 molar excess of recombinant soluble TF (1−219) for 30 min on ice. Excess antibody was removed by washing 3 times with PBS, the cells were subjected to flow cytometry (Becton Dickinson LSR FACS, San Jose, CA), and the data were analyzed using FCS Express V2 (De Novo Software, Thornhill, Ontario).

Statistical Analyses. Results are presented as means ± SD. All tests of statistical significance were two-sided, and *p* values <0.05 were considered statistically significant.

RESULTS

Thiol-Oxidizing Reagent, $HgCl_2$, Activated TF. If the TF allosteric disulfide was reduced in the cryptic form of the protein, then thiol-oxidizing agents might be expected to promote activation. $HgCl_2$ oxidizes dithiols to disulfides and has been shown to activate TF on the cell surface (*22*). The thiolate anion of one of the cysteine residues undertakes a nucleophilic attack on the mercuric chloride bond, displacing a chloride anion and forming a sulfur−mercuric chloride intermediate. The other cysteine thiolate then attacks its neighboring sulfur−mercury bond, resulting in displacement of the chloromercuri group and formation of the disulfide. $HgCl_2$ can also bridge unpaired cysteine thiols forming a Cys−S−Hg−S−Cys adduct. Either mechanism results in approximation of the cysteine sulfur atoms.

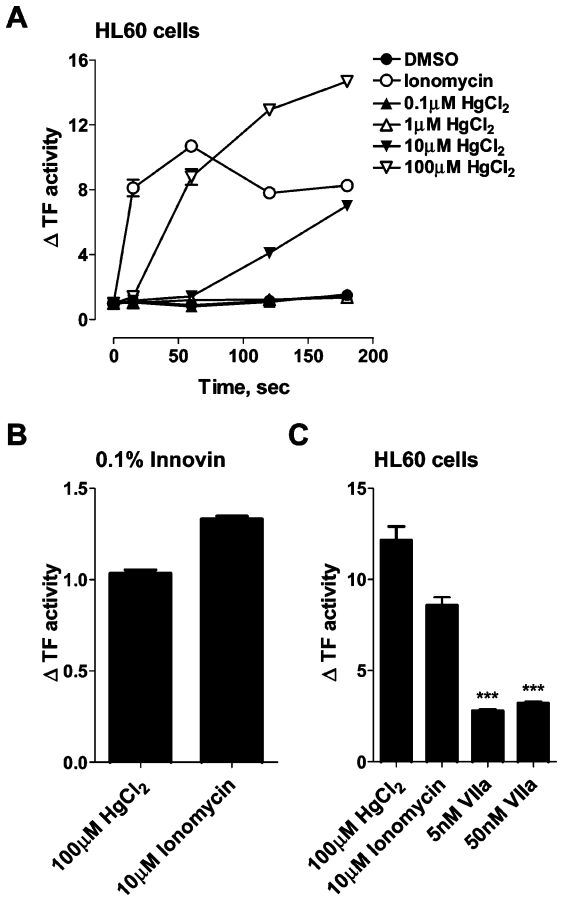

FIGURE 1: Thiol-oxidizing reagent, $HgCl_2$, activated TF. (A) HL-60 cells were treated with PMA to induce expression of TF and then incubated with 10 μM ionomycin, vehicle DMSO, or $HgCl_2$ at the indicated concentrations. The procoagulant activity of the cells was measured at discreet times up to 2 min. The data points and error bars are the mean ± SD of four determinations. (B) Ionomycin or $HgCl_2$ do not appreciably change TF-mediated coagulation. Recombinant relipidated TF (0.1% Innovin) was incubated with 10 μM ionomycin or 100 μM $HgCl_2$ for 30 s, and coagulation was initiated by the addition of pooled normal human plasma. The results are expressed as change in TF coagulant activity. The data points and error bars are the mean ± SD of 20−24 determinations. (C) $HgCl_2$ activation of HL60 TF is not a result of enhanced conversion of factor VII to VIIa. PMA-stimulated HL-60 cells were incubated with 100 μM $HgCl_2$, 10 μM ionomycin, or 5 or 50 nM VIIa for 30 s, and the procoagulant activity of the cells was measured. The data points and error bars are the mean ± SD of eight to 12 determinations. The *p* values (***, $p < 0.001$) are the significant difference from $HgCl_2$ activation.

$HgCl_2$ activated endogenously expressed TF on the surface of human myeloid leukemia HL-60 cells up to 15-fold (Figure 1A). Activation by $HgCl_2$ was concentration and time dependent and maximal at 30 s of $HgCl_2$ exposure. Ionomycin, a calcium ionophore, activated TF ~10-fold. $HgCl_2$ had no effect on prothrombin times with relipidated recombinant tissue factor (Innovin), thus excluding an effect of $HgCl_2$ on the coagulation process downstream from TF/VIIa (Figure 1B). The activating activity of $HgCl_2$ was also not

due to increased conversion of factor VII to VIIa (Figure 1C). Addition of VIIa in concentrations up to 10 times the level of unactivated VII antigen in pooled normal plasma (*23*) did not recapitulate mercury induced TF activation. It has been reported that calcium ionophores induce microparticle formation and that the shed microparticles have TF activity (*24*, *25*). We could not detect TF activity in the supernatant of HL60 cells activated with either ionomycin or $HgCl_2$, which indicates that microparticle release was not a significant factor in de-encryption of TF in our system.

These results supported a direct effect of $HgCl_2$ on de-encryption of TF on the HL60 surface and are consistent with the theory that the TF allosteric disulfide was reduced in the cryptic form and that activation involved formation of the bond.

Activation of TF on HL60 Cells by Ionomycin or $HgCl_2$ Cannot be Explained by Exposure of Phosphatidlyserine on the Plasma Membrane. The prevailing theory for the mechanism of de-encryption of TF relates to increased availability of anionic phospholipid on the cell surface, most probably phosphatidylserine, which is a cofactor for activation of factor IX and X by factor VIIa-TF (*22*, *26*). The contribution of phosphatidylserine exposure to TF activation triggered by ionomycin and $HgCl_2$ was investigated using the phosphatidylserine-binding protein, annexin V. Annexin V blocked the coagulant activity of recombinant relipidated TF at a concentration of 20 nM (Figure 2A). The IC_{50} was ~5 nM, which is the range of the dissociation constant for annexin V binding to cell surface phosphatidylserine (*27*, *28*). A concentration of 100 nM annexin V had little effect on ionomycin-mediated TF activation on HL60 cells (Figure 2B, part ii) and actually enhanced by ~3 times the $HgCl_2$-mediated activation relative to the annexin treated control (Figure 2C, part ii). Annexin V was not limiting in these experiments. The stoichiometry for binding of annexin V to cultured cells is $\sim 7 \times 10^6$ sites per cell (*27*, *28*). The concentration of binding sites on HL60 cells in the experiments shown in Figure 2 was calculated to be ~5 nM, which indicates that annexin V was in excess of HL60-binding sites at all concentrations tested.

Dithiol Cross-Linker Activated TF. We reasoned that chemical cross-linking of the cysteine residues of the reduced disulfide in cryptic TF might simulate formation of the allosteric disulfide and activate TF. The dithiol cross-linkers, BMH and BMOE, activated HL60 TF up to 7- and 5-fold, respectively (Figure 3A). This level of activation was approximately half of that observed with $HgCl_2$. Activation of BMH-treated cells with $HgCl_2$ resulted in a level of activation of TF observed with $HgCl_2$ alone (Figure 3B). This result suggested that the cross-linkers had access to about half of the cryptic HL60 TF. It was possible that the bis-maleimides were activating TF by cross-linking thiols in adjacent TF molecules. TF dimerization has been proposed to play a role in de-encryption (*20*). There was no evidence, however, for formation of TF dimers following incubation of HaCaT cells with BMH or $HgCl_2$ (Figure 3C).

Activation of TF with Ionomycin or $HgCl_2$ Results in Loss of Free Thiols in TF. To test the hypothesis that the cryptic form of TF is defined by a reduced disulfide, or the presence of free cysteine thiols, we compared the labeling of cryptic and active TF with the membrane-impermeable biotin-linked maleimide, 3-(*N*-maleimidylpropionyl)biocytin (MPB). Maleimides specifically alkylate thiols at neutral pH. The efficiency of alkylation of cysteine thiols in proteins is exquisitely dependent on the accessibility of the alkylating agent to the thiol (*29*). MPB is a reasonably bulky compound, so its alkylating efficiency is expected to be severely impaired if the TF thiols are partly buried. For this reason, labeling

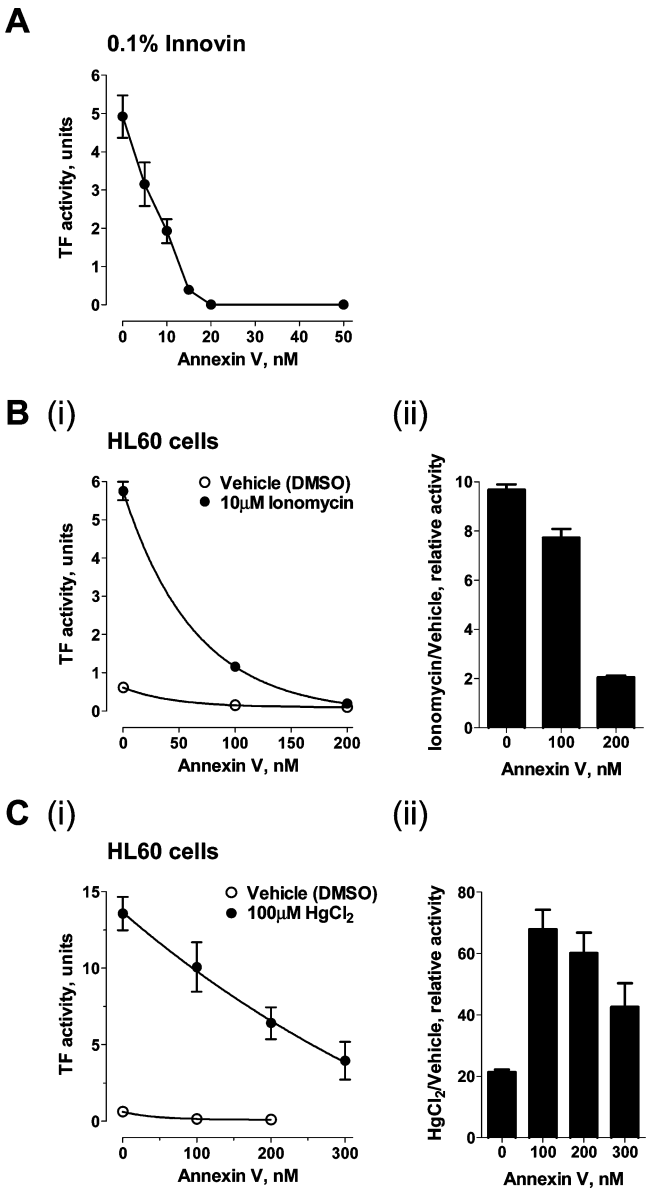

FIGURE 2: Activation of TF on HL60 cells by ionomycin or $HgCl_2$ cannot be explained by exposure of phosphatidylserine on the plasma membrane. (A) Recombinant relipidated TF (0.1% Innovin) was incubated with 0−50 nM annexin V, and coagulation was initiated by the addition of pooled normal human plasma. The results are expressed as TF coagulant activity. The data points and error bars are the mean ± SD of six determinations. (B) PMA-stimulated HL-60 cells were incubated with 10 μM ionomycin or vehicle control and 0−200 nM annexin V for 30 s, and the procoagulant activity of the cells was measured. The results are expressed as TF coagulant activity (i) or the ratio of ionomycin vs control (vehicle) activity (ii). The data points and error bars are the mean ± SD of eight determinations. (C) PMA-stimulated HL-60 cells were incubated with 100 μM $HgCl_2$ or vehicle control and 0−300 nM annexin V for 30 s, and the procoagulant activity of the cells was measured. The results are expressed as TF coagulant activity (i) or the ratio of $HgCl_2$ vs control (vehicle) activity (ii). The data points and error bars are the mean ± SD of eight to 12 determinations.

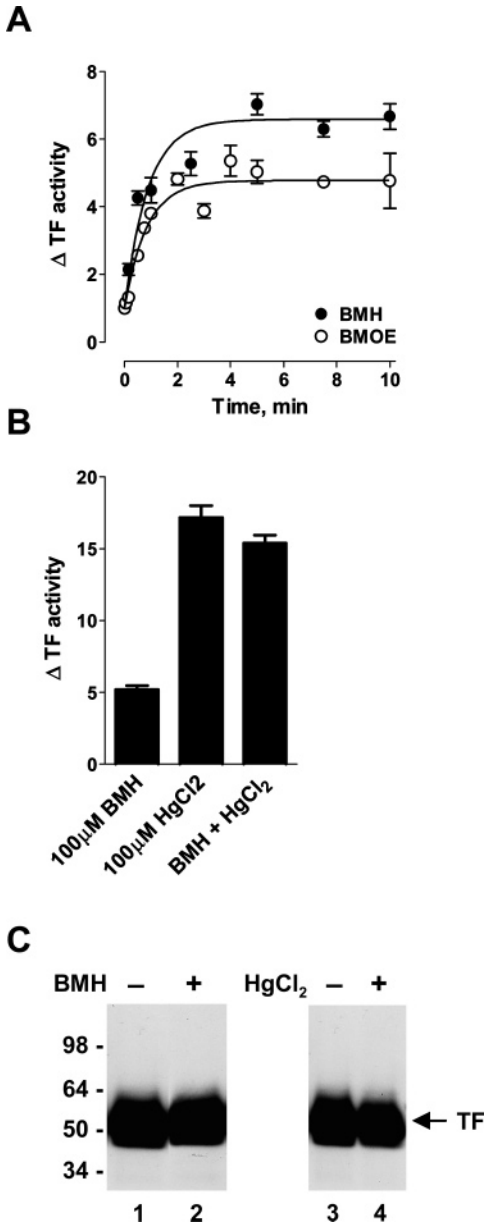

FIGURE 3: Dithiol cross-linker activated TF. (A) PMA-stimulated HL-60 cells were incubated with 100 μM BMH or BMOE, and the procoagulant activity of the cells was measured at discreet times up to 10 min. The data points and error bars are the mean ± SD of four determinations. (B) PMA-stimulated HL-60 cells were incubated with 100 μM BMH for 5 min followed by DMSO control for 30 s, 100 μM $HgCl_2$ for 30 s, or 100 μM BMH or DMSO control for 5 min followed by 100 μM $HgCl_2$ for 30 s, and the procoagulant activity of the cells was measured. The data points and error bars are the mean ± SD of eight determinations. (C) Activation of TF on HaCaT cells by BMH or $HgCl_2$ was not associated with cross-linking or TF. HaCaT cells were incubated with vehicle DMSO (lanes 1 and 3), 100 μM BMH (lane 2), or 100 μM $HgCl_2$ (lane 4) for 2 min. The TF was immunoprecipitated, resolved on SDS−PAGE under nonreducing conditions, and Western blotted using a polyclonal goat anti-human TF antibody. The positions of M_r markers in kDa are shown at left.

studies with MPB are semiquantitative at best. We have not attempted to estimate stoichiometry of MPB labeling because we were not able to ascertain the efficiency of labeling of TF thiols.

We were unable to detect incorporation of MPB into HL60 TF (data not shown). This was perhaps due to the low levels of TF expressed by these cells. Baby hamster kidney (BHK) cells stably transfected with human TF (*18*) express at least 1 order of magnitude higher levels of TF on the cell surface (data not shown). A major portion of TF expressed on the surface of BHK-TF cells is constitutively active, so the fold change in activity upon stimulation with ionomycin or $HgCl_2$ is less than that observed with HL60 cells, where the endogenously expressed TF is mostly cryptic. TF activity of the BHK-TF cells was measured from the initial rate of factor X activation because these cells grow as an adherent monolayer. Ionomycin activation of these cells has been reported to result in a 1.5−10-fold increase in TF activity (*6*).

We observed a 1.8 ± 0.1- and 2.1 ± 0.2-fold increase in TF activity with ionomycin or $HgCl_2$ treatment, respectively (Figure 4A). Cryptic TF contained free thiols, and ionomycin or $HgCl_2$ activation resulted in depletion of free thiols to 28 ± 27 and 0% of cryptic TF, respectively, in three separate experiments (Figure 4B). As $HgCl_2$ modified thiols, it blocked reaction of MPB with all cell surface proteins. To test whether TF contains free thiols when expressed in endothelial cells, the protein was introduced into human umbilical vein endothelial cells by adenoviral transduction. The endothelial cell TF contained free thiols, which were lost upon treatment with $HgCl_2$ (Figure 4C). These results provided further support for the hypothesis that the redox state of the TF allosteric disulfide was controlling de-encryption of TF.

Monofunctional Thiol Alkylators Inhibited TF Activation. Blocking of the unpaired cysteine residues in cryptic TF by monofunctional thiol alkylators should prevent activation if formation of the allosteric disulfide is a key event in de-encryption. NEM inhibited TF activation by ionomycin or $HgCl_2$ to 44 ± 0 and 44 ± 16% of control (Figure 5A,B), respectively, while MMTS inhibited TF activation by ionomycin or $HgCl_2$ to 8.0 ± 4.6 or 5.8 ± 0.9% of control (Figure 5D,E). The inhibitory effects of the alkylators were time and concentration dependent, which is consistent with their chemical mechanism. The difference in magnitude of blockade between the two alkylators is likely due to differences in the accessibility of the compounds to the unpaired cysteine thiols in TF.

Evidence for Change in the Structure of the Membrane-Proximal Domain upon TF Activation. Formation of the Cys186−Cys209 allosteric disulfide with TF activation was expected to change the conformation of the domain, particularly in the vicinity of the disulfide bond. A monoclonal antibody that recognizes an epitope within residues 181−214 of human TF (*30*) was used to probe this conformational change. Two populations of unstimulated HL60 cells were defined by the antibody, one that bound the antibody and one that did not (Figure 6). This possibly reflected the pools of cryptic and active TF in the population. Activation of the cells with ionomycin or $HgCl_2$ resulted in a greater proportion (ionomycin) or effectively all cells ($HgCl_2$) binding the antibody.

DISCUSSION

TF is the cell surface cofactor for factor VIIa that initiates blood coagulation in mammals and also plays a fundamental role in blood vessel formation and remodeling (*2*). Moreover, inappropriate TF activity leading to thrombus formation is

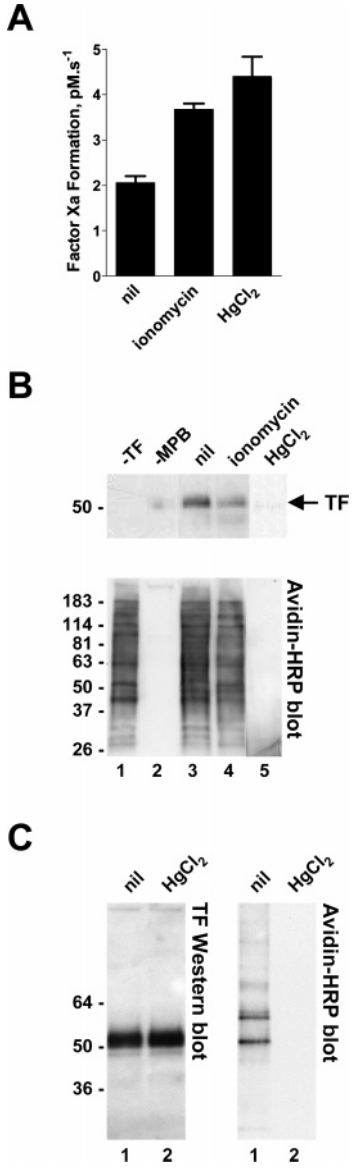

FIGURE 4: Activation of TF with ionomycin or $HgCl_2$ results in loss of free thiols in TF. (A) BHK-TF cells were incubated with DMSO (nil) or 10 μM ionomycin for 30 s or 100 μM $HgCl_2$ for 2 min, and rate of factor Xa generation of the cells was measured. The error is the SD of the best fit of the data to eq 1. (B) BHK-TF cells were incubated with DMSO (nil) or 10 μM ionomycin for 30 s or 100 μM $HgCl_2$ for 2 min and then labeled with 100 μM MPB for 2 min. The biotin labeled proteins from 5 $\times$ 106 cells were collected on streptavidin Dynabeads, resolved on SDS−PAGE, and Western blotted using a polyclonal goat anti-human TF antibody or blotted with streptavidin peroxidase. Lane 1 is BHK cells labeled with MPB, and lane 2 is BHK-TF cells not labeled with MPB. The positions of M_r markers in kDa are shown at left. (C) TF was expressed by adenoviral transduction in human umbilical vein endothelial cells. The cells were incubated with 100 μM $HgCl_2$ for 2 min and then labeled with 100 μM MPB for 20 min. The TF was immunoprecipitated, resolved on SDS−PAGE, and Western blotted using a polyclonal goat anti-human TF antibody or blotted with streptavidin peroxidase. The positions of M_r markers in kDa are shown at left.

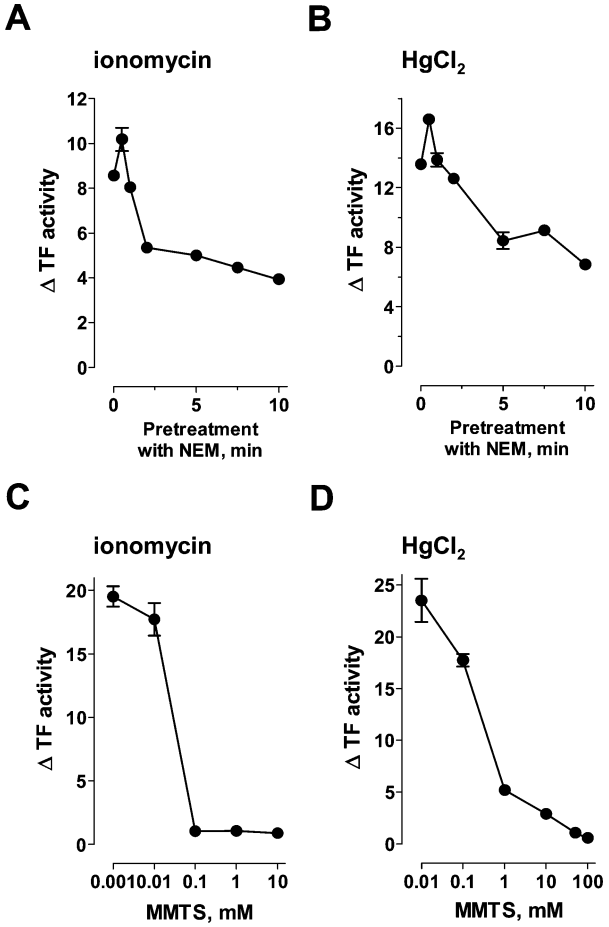

FIGURE 5: Monofunctional thiol alkylators inhibited TF activation. (A and B) PMA-stimulated HL-60 cells were preincubated with 100 μM NEM at discrete times up to 10 min before activation of TF with 10 μM ionomycin (A) or 100 μM $HgCl_2$ (B) for 30 s. The procoagulant activity of the cells was measured immediately. The data points and error bars are the mean ± SD of four determinations. (C and D) PMA-stimulated HL-60 cells were preincubated with 0−100 mM MMTS for 10 min before activation of TF with 10 μM ionomycin (D) or 100 μM $HgCl_2$ (E) for 30 s. The procoagulant activity of the cells was measured immediately. The data points and error bars are the mean ± SD of eight determinations.

the precipitating event in myocardial infarction and stroke. It was recognized soon after TF was discovered some 30 years ago that the cofactor resides in a cryptic configuration and requires activation to initiate coagulation (*31*−*33*). TF activation, therefore, is one of the more important protein transitions in mammalian biology and pathology.

We show herein that thiol-alkylating agents block TF activation by ionomycin and that a thiol-oxidizing agent and dithiol cross-linkers promote activation. In addition, cryptic TF contained free thiols that were depleted upon activation, and de-encryption was associated with a change in the conformation of the membrane-proximal domain. When considered together, these observations imply a role for the Cys186−Cys209 disulfide in de-encryption of TF. More specifically, the results are consistent with a mechanism in which the Cys186−Cys209 disulfide is reduced in the cryptic form of TF and activation involves formation of the bond.

This conclusion is supported by mutagenesis studies. Residues close to the allosteric bond (Tyr157, Lys159, Ser163, Gly164, Lys165, Lys166, and Try185) have been identified as part of the region of TF that interacts with factors IX and X (*34*, *35*). The allosteric bond is also next to a Trp158−Lys159−Ser160 motif that mutation studies have shown to be important for TF activity (*36*). We suggest that formation of the Cys186−Cys209 disulfide repositions the adjacent F and G β strands that reorient nearby residues and enable productive binding of the TF substrates. We have

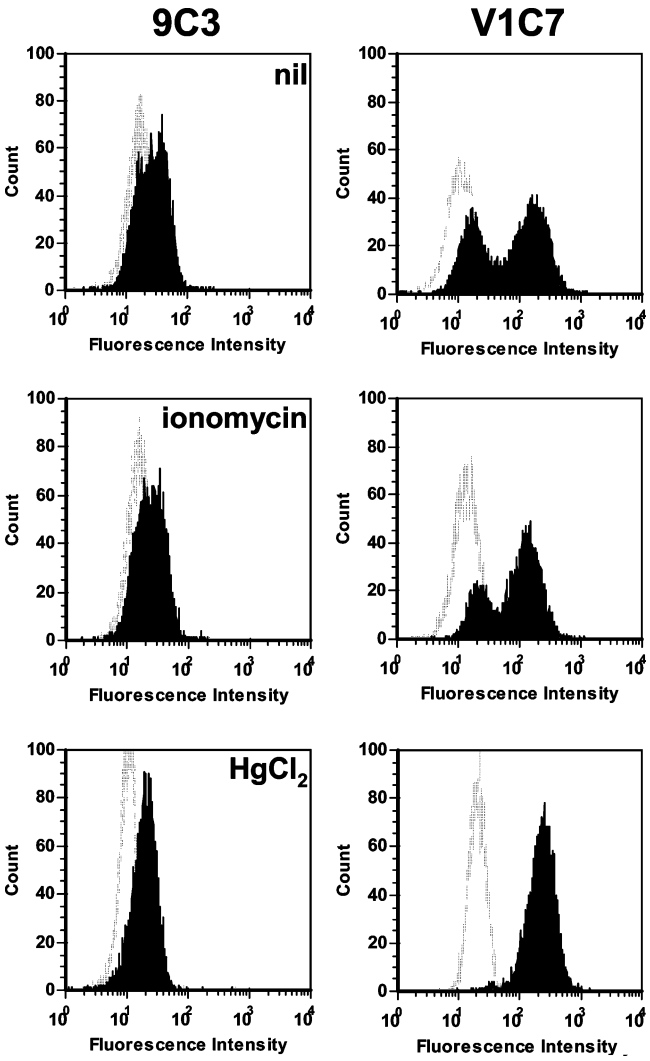

FIGURE 6: Evidence for change in the structure of the membrane-proximal domain upon TF activation. HL-60 cells were treated with PMA to induce expression of TF and then incubated with vehicle DMSO, 10 μM ionomycin, or 100 μM $HgCl_2$ for 30 s and fixed with paraformaldehyde. The fixed cells were incubated with 9C3 or V1C7 antibody (solid histograms) or antibody preincubated with a 250 molar excess of recombinant soluble TF (lined histograms) and analyzed by flow cytometry. In two separate experiments, ionomycin or $HgCl_2$ treatment resulted in a 12.5 ± 0.7 and 46.5 ± 0.7% increase, respectively, in the number of cells binding the V1C7 antibody.

not ruled out a contribution from the N-terminal Cys49−Cys57 disulfide in de-encryption of TF, although it is unlikely to be involved as ablation of this bond by mutating both cysteines to serines does not impair TF coagulant activity (*17*).

It has been proposed that de-encryption of TF relates to increased availability of anionic phospholipid on the cell surface (*22*, *26*). Physical disruption or exposure of cells to calcium ionophore (*26*) or mercury compounds (*22*) leads to activation of TF and increased phosphatidyserine on the outer leaflet of the plasma membrane. Several observations indicate, however, that other factors are involved in de-encryption of TF. First, annexin V, which binds to anionic phospholipid, does not inhibit cleavage of factor X by factor VIIa-TF on ovarian carcinoma cells either before or after cell disruption (*5*). Second, a component to TF activation by calcium ionophores has been reported to be independent of phosphatidylserine exposure (*6*, *26*, *37*). Third, there is a disparity between elevation in intracellular calcium levels induced by $HgCl_2$ and TF activity (*22*). For example, incubation of cells with 5 μM $HgCl_2$ for 30 min resulted in high intracellular calcium levels but only a slight increase in TF activity. Full activation is achieved within 30 s with 50−100 μM $HgCl_2$. In addition, we show that a concentration of 100 nM annexin V, which was predicted to be ~20 times the number of cell-binding sites in the system, actually enhanced by ~3 times the $HgCl_2$-mediated activation of TF on HL60 cells.

There were quantitative differences in the nature of ionomycin versus $HgCl_2$-mediated TF de-encryption. The rate of activation by ionomycin was faster than for $HgCl_2$, annexin V was more effective at blocking activation by ionomyin than $HgCl_2$, thiol alkylators were more efficient at blocking activation by ionomycin than $HgCl_2$, and the extent of the conformational change in TF reported by the V1C7 antibody was less dramatic for ionomycin than for $HgCl_2$ activation. One possibility is that the contribution of phosphatidyserine exposure to de-encryption is different for the different activators. Another possibility is that the differences lie in a third component in the de-encryption process.

Major unanswered questions are what cleaves the allosteric disulfide initially and what mediates formation of the bond? One possibility is that an oxidoreductase cleaves the Cys186−Cys209 bond forming a mixed disulfide between enzyme and TF. Resolution of the complex results in oxidation of the dithiol with release of the oxidoreductase and activation of TF.

A candidate oxidoreductase is protein disulfide isomerase (PDI). PDI is involved in redox control of mammalian cell-surface protein dithiols/disulfides (*13*, *14*, *29*, *38*, *39*). The active sites of oxidoreductases such as PDI contain a reactive dithiol/disulfide in a CysXXCys motif that has a redox potential in the range of −120 to −270 mV. The dithiol/disulfide cycles between reduced and oxidized configurations in coordination with a dithiol or disulfide in a protein substrate (*40*). The outcome can be reduction, formation, or isomerization of a disulfide in the protein substrate. Reduction of a disulfide is achieved when one of the active site thiols attacks the substrate disulfide, cleaving the bond. The mixed disulfide can then decompose via attack by the other active site thiol on the intermolecular disulfide, resulting in a stable disulfide between active site cysteine residues and release of the reduced substrate (*41*).

The activation of TF by bis-maleimides and inhibition of activation by thiol alkylators reported in this study could be explained by such a mechanism. The bis-maleimides, for instance, might dissociate PDI from TF by cross-linking the PDI active site dithiol. Inhibition by thiol alkylators, on the other hand, could be a result of blocking resolution of the PDI-TF complex by alkylating the PDI active site cysteine that is responsible for resolving the mixed disulfide.

Another possible mediator of formation of the Cys186−Cys209 bond is a nonprotein-oxidizing agent. Activation of TF by the thiol-oxidizing agent, $HgCl_2$, supports this scenario. Muller et al. (*42*) showed that stimulated neutrophils promote activation of TF on microvesicles and platelets. Neutrophils make H_2O_2 and HOCl that can oxidize thiols to disulfides

under certain conditions (*43*, *44*). Neither H_2O_2 nor HOCl, however, could activate TF in our systems (data not shown).

It appears, therefore, that de-encryption of TF is another example of a protein controlled by an allosteric disulfide (*7*). Other potential allosteric bonds in thrombosis and thrombolysis proteins are the Cys680−Cys747 and Cys737−Cys765 disulfides in the catalytic domain of plasmin(ogen), the Cys5−Cys9 and Cys21−Cys32 disulfides in the somatomedin B domain of vitronectin, the Cys4−Cys17 disulfide in the N-terminal β finger of GP1bα, the Cys663−Cys687 disulfide in the β tail domain of integrin β_3, and the Cys399−Cys407 disulfide in the fifth EGF-like domain of thrombomodulin.

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