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Abstract:	Factor VIIa/tissue factor (FVIIa/TF) initiates blood coagulation by promoting FXa generation (extrinsic-Xa). Subsequent generation of intrinsic FXa (intrinsic-Xa) amplifies thrombin formation. Previous studies suggested that FVIIa/TF activates FVIII rapidly in immediate coagulation-reactions, and FVIIa/TF/FXa activates FVIII prior to thrombin-dependent feedback. We investigated FVIII/FVIIa/TF/FXa relationships in early coagulation mechanisms. Total FXa generated by FVIIa/TF and FVIIa/TF-activated FVIII (FVIIIaVIIa/TF) was 22.6±1.7 nM (1 min); total FXa with FVIIa-inhibitor was 3.4±0.7 nM; whereas FXa generated by FVIIa/TF or FVIII/TF was 10.4±1.1 or 0.74±0.14 nM, respectively. Little Xa was generated by FVIII alone, suggesting that intrinsic-Xa mechanisms were mediated by FVIIIaVIIa/TF and FVIII/TF in the initiation-phase. Intrinsic-Xa was delayed somewhat by von Willebrand factor (VWF). FVIII activation by FXa with FVIIa/TF was comparable to activation with Glu-Gly-Arg-inactivated-FVIIa/TF. TF counteracted the inhibitory effects of VWF on FXa-induced FVIII activation mediated by Arg372 cleavage. The FVIII-C2 domain bound to cytoplasmic domain-deleted TF (TF1-243), and VWF blocked this binding by >80%, indicating an overlap between VWF- and TF1-243-binding site(s) on C2. Overall, these data suggest that FVIII-associated intrinsic-Xa, governed by both FVIIa/TF-induced and FXa-induced FVIII activation mediated by FVIII-TF interactions, together with FVIIa-dependent extrinsic-Xa mechanisms, may be central to the initiation-phase of coagulation.
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Potential role of activated factor VIII (FVIIIa) in FVIIa/tissue factor-dependent FXa generation in initiation phase of blood coagulation

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Summary

Factor VIIa/tissue factor (FVIIa/TF) initiates blood coagulation by promoting FXa generation (extrinsic-Xa). Subsequent generation of intrinsic FXa (intrinsic-Xa) amplifies thrombin formation. Previous studies suggested that FVIIa/TF activates FVIII rapidly in immediate coagulation-reactions, and FVIIa/TF/FXa activates FVIII prior to thrombin-dependent feedback. We investigated FVIII/FVIIa/TF/FXa relationships in early coagulation mechanisms. Total FXa generated by FVIIa/TF and FVIIa/TF-activated FVIII ($FVIIIa_{VIIa/TF}$) was 22.6±1.7 nM (1 min); total FXa with FVIIa-inhibitor was 3.4±0.7 nM; whereas FXa generated by FVIIa/TF or FVIII/TF was 10.4±1.1 or 0.74±0.14 nM, respectively. Little Xa was generated by FVIII alone, suggesting that intrinsic-Xa mechanisms were mediated by $FVIIIa_{VIIa/TF}$ and FVIII/TF in the initiation-phase. Intrinsic-Xa was delayed somewhat by von Willebrand factor (VWF). FVIII activation by FXa with FVIIa/TF was comparable to activation with Glu-Gly-Arg-inactivated-FVIIa/TF. TF counteracted the inhibitory effects of VWF on FXa-induced FVIII activation mediated by Arg^{372} cleavage. The FVIII-C2 domain bound to cytoplasmic domain-deleted TF (TF^{1-243}), and VWF blocked this binding by >80%, indicating an overlap between VWF- and TF^{1-243}-binding site(s) on C2. Overall, these data suggest that FVIII-associated intrinsic-Xa, governed by both FVIIa/TF-induced and FXa-induced FVIII activation mediated by FVIII-TF interactions, together with FVIIa-dependent extrinsic-Xa mechanisms, may be central to the initiation-phase of coagulation.

Key words; FVIIa, tissue factor, FVIII, FXa generation, initiation phase

Introduction

Factor (F)VIII functions as a cofactor in the tenase complex responsible for phospholipid (PL) surface-dependent conversion of FX to FXa by FIXa [1]. FVIII is synthesized as a single chain molecule, arranged into six domains (A1-A2-B-A3-C1-C2), consisting of 2,332 amino acid residues with a molecular mass of ~300 kDa. It is processed into a series of metal ion-dependent heterodimers of a heavy chain (HCh) consisting of the A1-A2 domains, together with heterogeneous fragments of the B domain, linked to a light chain (LCh) consisting of the A3-C1-C2 domains [2,3]. FVIII circulates as a complex with von Willebrand factor (VWF), and VWF protects and stabilizes the FVIII cofactor activity [4]. The catalytic efficiency of FVIII in the intrinsic tenase complex is enhanced over 10^5-fold by conversion into an active form, FVIIIa [5]. Cleavage of the HCh by thrombin and/or FXa occurs at Arg372 and Arg740, and produces 50-kDa A1 and 40-kDa A2 subunits [6]. The 80-kDa LCh is cleaved at Arg1689, generating a 70-kDa subunit. Cleavage at the latter site liberates VWF [4], contributing to the overall specific activity of the cofactor [5,7].

The active form of FVII (FVIIa) forms a complex with tissue factor (TF), to generate a potent enzyme responsible for initiating and propagating the blood coagulation process in normal hemostasis [8]. The primary role of FVIIa/TF complex is to activate FIX and FX [9]. Following injury to the blood vessel wall, TF is exposed to circulating blood and forms a complex with FVIIa. FVIIa/TF initiates hemostasis by generating FIXa and FXa and promoting the formation of the extrinsic tenase (FVIIa/TF/FX) (Figure 1A). This early response leads to the generation trace amounts of thrombin that dissociates FVIII from VWF and promotes platelet activation. Subsequently, thrombin generation is amplified in the propagation phase by the formation of intrinsic tenase (FVIIIa/FIXa/FX) and prothrombinase (FVa/FXa/FII) on negatively-charged PL exposed on platelet membranes and other cell surfaces [10,11]. The overall process reflected in these interactions has become widely accepted as the cell-based coagulation model.

Berntorp and colleagues reported that the *in vitro* addition of FVIII to plasma from hemophilia A patients with high responding inhibitors significantly enhanced FVIIa/TF-induced thrombin generation [12]. Thrombin is widely recognized as a potent activator of FVIII *in vivo*. In addition, FVIIa/TF has been shown to activate FVIII proteolytically, and to inactivate thrombin-activated FVIII [13]. In this context, we previously described a mechanism in which

TF/PL-dependent FVIIa activated FVIII more rapidly than thrombin in early coagulation phases by proteolytic cleavages at Arg372 and Arg740, irrespective of the presence of VWF [14]. In addition, our series of studies on hemostatic potential in the co-presence of FVIII and bypassing agents demonstrated that pre-activation of FVIII in the presence of TF together with FVII/FVIIa contained in the bypassing agents, contributed to enhanced FVIII coagulation activity, even in the presence of anti-FVIII inhibitor antibodies (pathway-I, Figure 1B) [15-17]. Furthermore, a recent investigation by Ruggeri and colleagues using an animal model focused on the possible contribution of FVIII activation by FVII/TF/FXa complexes prior to thrombin-dependent feedback-loop in thrombogenesis (pathway-II) [18]. The full potential of these interactions in the initiation of coagulation remained to be fully determined, however. The present study was designed, therefore, to further investigate FVIIa/TF/FVIII-associated mechanisms of intrinsic FXa generation (pathway-I and II) in the primary phase of coagulation.

Materials and Methods

Reagents - Lipidated full-length recombinant (r)TF (lipidated TF; Innovin®; Dade Behring, Marburg, Germany), non-lipidated full-length rTF (TF^{1-263}), the cytoplasmic domain-deleted non-lipidated rTF comprised of all region of extracellular domain and a trans-membrane domain (TF^{1-243}; Altor BioScience, FL) were purchased from the indicated vendors. The rFVIII (Kogenate FS®) and rFVIIa (Novoseven®) were provided by Bayer Corp. Japan (Osaka, Japan) and Novo Nordisk (Bagsværd, Denmark). An anti-FVIII monoclonal antibody (mAb)C5, recognizing the C-terminal acidic region (resides 351-365) of the A1 domain [19], was a generous gift from Dr. Carol Fulcher. VWF was purified from FVIII/VWF concentrates (Confact®; KAKETSUKEN, Kumamoto, Japan) using gel filtration on a Sepharose CL-4B column and immune-beads coated with immobilized anti-FVIII mAb [20]. FVIII-deficient patients' plasma (George-King Inc., Overland Park, KS), human FIXa, FX, FXa, (Hematologic Technologies, Inc., Burlington, VT), α-thrombin, recombinant hirudin, Glu-Gly-Arg-chloromethylketone (EGR-ck), Pro-Pro-Arg-chloromethylketone (PPA-ck) (Calbiochem, San Diego, CA), a FVIIa-specific inhibitor peptide E-76 (Ac-ALCDDPRVDRWYCQFVEG-NH_2; Bachem, Bubendorf, Switzerland), recombinant tissue factor pathway inhibitor (TFPI; American Diagnostica GmbH, Pfungstadt, Germany), and FXa substrate S-2222 (Chromogenix, Milano, Italy) were purchased from the indicated vendors. An anti-FVIII C2 antibody was obtained from a severe hemophilia A patient with inhibitor as previously described [21]. Anti-A2 mAb (mAbJR8) and anti-C2 mAb (mAbESH8) were obtained from JR Scientific Inc. (Woodland, CA) and American Diagnostica

Inc. (Greenwich, CT), respectively. Two mAbs against the extracellular domain of TF were purchased from American Diagnostica Inc. and R&D Systems (Minneapolis, MN). EGR-modified FVIIa was obtained as previously reported [14]. Peroxidase-conjugated IgG was prepared using Peroxidase Labeling Kit (Dojindo, Kumamoto, Japan). PL vesicles containing phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine (10:60:30%) were prepared as previously reported [22].

Preparation of FVIII/FVIIIa subunits - FVIII subunits were isolated from rFVIII. FVIII (1.5 μM) was treated overnight at 4°C in buffer containing 10 mM MES, pH 6.0, 0.25 M NaCl, 50 mM EDTA, and the intact LCh (80-kDa) and intact HCh (90-210 kDa) were isolated following chromatography on SP-Sepharose and Q-Sepharose columns, respectively [23]. The purified HCh was cleaved by thrombin, and the A2 and A1 subunits were purified by high performance liquid chromatography (HPLC) using Hi-Trap Heparin column and SP-Sepharose column [23]. The 70-kDa LCh was purified from thrombin-cleaved 80-kDa LCh, following SP-Sepharose chromatography [24]. A cDNA coding the C2 domain sequence of human FVIII was constructed, transformed into *Pichia pastoris* cells and expressed in yeast secretion systems [25]. The C2 protein was purified by ammonium sulfate fractionation and cation-exchange HPLC. SDS-PAGE of the isolated subunits followed by staining with GelCode Blue Reagent (Pierce, Rockford, IL) showed >95% purity. Protein concentrations were determined by the Bradford method [26].

FXa generation assay - The rate of conversion of FX to FXa was monitored in a purified system at 22°C [27]. FVIII was reacted with FVIIa/TF (1 nM/0.1 nM) with PL vesicles (20 μM). FVIIa activity was terminated after 30 sec by the addition of E-76 (2.5 U/ml), and FXa generation was initiated by the addition of FIXa (1 nM) and FX (150 nM). Aliquots were removed at appropriate times to assess the initial rates of product formation and were added to EDTA-containing tubes to quench the reactions. Rates of FXa generation were determined by the addition of S-2222 (0.46 mM). Reactions were read at 405 nm using a Labsystems Multiskan Multisoft microplate reader (Labsystems, Helsinki, Finland). Control experiments demonstrated that the FX preparation used in this study was not contaminated with FXa (data not shown).

FVIII activation by FXa in the co-presence of FVIIa - FVIII activity was measured in a one-stage clotting assay using FVIII-deficient plasma. All reactions were performed at 37°C.

FVIII (10 nM) and PL (20 μM) were incubated with either FVIIa/TF (0.2 nM/0.2 nM), FXa (0.2 nM), or both in 20 mM Hepes, 140 mM NaCl, pH 7.2 (HBS) containing 5 mM $CaCl_2$. Samples were removed from the mixtures at indicated times, and FXa/FVIIa/TF reaction was immediately terminated by the addition of E-76 (2.5 μM), PPA-ck (2.5 μM), and hirudin (0.25 nM), and 500-dilution. FVIII activity was calculated using a standard curve of FVIII in FVIII-deficient plasmas. The presence of FXa, FVIIa/TF, and protease inhibitors in the diluted samples did not affect FVIII activity (<0.5%) in this assay.

FVIII cleavage by FXa - FVIII (50 nM) preincubated with VWF (50 μg/ml) was mixed with FXa (1 nM), PL (100 μM), and TF (1 nM) in HBS-buffer containing 5 mM $CaCl_2$ at 37°C. Aliquots were removed at the indicated times and the reactions were immediately terminated and prepared for SDS-PAGE by adding SDS and boiling for 3 min.

Electrophoresis and Western blotting - SDS-PAGE was performed using 8% gels at 150 V for 1 hr, followed by Western blotting analysis. Protein bands were probed using anti-FVIII mAbs, followed by goat anti-mouse peroxidase-linked secondary mAb. Signals were detected using enhanced chemiluminescence (PerkinElmer Life Science, Boston, MA). Densitometric scans were quantitated using Image J 1.38 (National Institute of Health, USA).

ELISA for the FVIII-TF binding - Microtiter wells were coated with FVIII (10 nM) in 20 mM Tris, 140 mM NaCl, pH 7.4 (TBS) overnight at 4°C. The wells were washed with HBS and were blocked with HBS containing 10% skimmed milk for 2 h at 37°C. Various amounts of TF were then added in HBS containing 5 mM $CaCl_2$ and 0.01% bovine serum albumin and were incubated for 2 h at 37°C. Peroxidase-conjugated anti-TF mAb IgG (2 μg/ml) was added, and bound IgG was quantified by the addition of TMB reagent (KPL, Gaithersburg, MD). Reactions were terminated by the addition of 2 M H_2SO_4, and absorbances were measured at 450 nm. The amount of nonspecific binding of anti-TF mAb IgG without TF was <3% of total signal. Specific binding was recorded after subtracting the nonspecific binding.

Surface-plasmon resonance (SPR)-based assay - Kinetics of TF interaction with FVIII or its subunits were determined by SPR-based assays at 37°C using Biacore T200 instrument (GE Healthcare). Soluble TF was covalently coupled to the surfaces of a CM5 chip at a coupling density of 22 ng/mm^2. Ligand binding was monitored in running buffer (20 mM Hepes, 1 mM

$CaCl_2$, 0.005% polysorbate20) for 2 min at a flow rate 20 μl/min. The dissociation of bound ligand was recorded over a 2-min period by replacing the ligand-containing buffer with buffer alone. The level of nonspecific binding, corresponding to ligand binding to the uncoated chip, was subtracted from the signal. Washing buffer (50 mM NaOH) was used for regenerations of the sensor chips. The rate constants for association (k_{asso}) and dissociation (k_{diss}) were determined by nonlinear regression analysis using evaluation software provided. Dissociation constants (K_d) were calculated as k_{diss}/k_{asso}.

Data analyses - All experiments were performed on three separate occasions, and the average values and standard deviations were determined. Nonlinear least squares regression analysis was performed using Kaleidagraph (Synergy Software, Reading, PA). Analysis of interaction between the FVIII subunits and TF forms in ELISAs were performed using a single-site binding model.

Results

FVIIa/TF-induced FVIII-dependent FXa generation - We first examined FVIIa/TF-dependent FXa generation in the presence of FVIII to clarify the possible role of FVIII in FVIIa/TF-induced initiation of coagulation. FVIII (1 nM) and PL (20 μM) were preincubated with FVIIa (1 nM) and lipidated TF (0.1 nM) for 30 sec. FXa generation was then initiated by the addition of FIXa (1 nM) and FX (150 nM), followed by reaction for 4 min (Figure 2A). The *inset* shows the reaction after the initial 1 min. In these circumstances, the reactant mixtures contained both intrinsic tenase mediated by FVIIa/TF-catalyzed FVIIIa and extrinsic tenase facilitated by FVIIa/TF. The amounts of generated FXa increased time-dependently after the addition of native FX, up to 22.6±1.7 nM at 1 min and 60.4±4.2 nM at 4 min (*open circles*). In the absence of FVIII, however, FVIIa/TF-initiated extrinsic FXa generation (10.4±1.1 nM at 1 min, 38.2±1.8 nM at 4 min; *open triangles*) were about half of those in its presence. As expected, very little FXa generation was evident in the presence of FVIII alone (0.05±0.03 nM at 1 min, 7.9±0.7 nM at 4 min; *open squares*). These results indicated that FXa generation by FVIIa/TF was significantly enhanced by FVIII activated by FVIIa/TF together with gradually generated FXa.

To further clarify the role of FVIIa in these reactions, similar experiments were repeated using a FVIIa-inhibitor peptide (E-76) added 30 sec after mixing FVIIa/TF with FVIII. The amounts of FXa generated in these circumstances were 3.4±0.7 nM at 1 min and 30.9±1.0 nM at 4 min (*closed*

circles). FXa generation was further assessed using mixtures of FVIII and TF without FVIIa. The activity of FXa was low at 1 min (0.74±0.14 nM) (*closed squares*), but was significantly greater than that with FVIII alone. A 4 min, however, measurements of FXa were 30.0±0.5 nM at 4 min, demonstrating a marked amplification of FXa generation. These assays reflected FVIIa/TF-induced FVIIIa-intrinsic tenase, and the data were consistent with the hypothesis, therefore, that the activity of FVIIa/TF-extrinsic tenase contributed predominantly to the rapid FXa generation recorded in the absence of E-76, and that intrinsic tenase induced in the presence of FVIIIa was produced at later time points during the initiation of coagulation. In addition, TF appeared to positively participate in FVIII-dependent FXa generation independently of FVIIa.

Impact of VWF or TFPI on FVIIa/TF-related FVIII-dependent FXa generation - FVIII is complexed with VWF in circulating blood [4]. To examine the impact of FVIII/VWF on tenase generation, therefore, similar experiments were in the presence of VWF (1 μg/ml) (Figure 2B). FVIII binds to VWF with high affinity (K_d: ~0.3 nM) [28], and under our experimental conditions, >90% of FVIII should be present as the complex with VWF. FXa concentrations using FVIII/VWF in combination with FVIIa/TF (reflecting both extrinsic and intrinsic tenase) were 16.7±1.4 nM at 1 min, corresponding to ~75% of that without VWF (*see* Figure 2A). After 4-min, the FXa levels were 60.6±4.6 nM and were similar to that without VWF. Similarly, in the presence of VWF, FXa generated with FVIII/FVIIa/TF/E-76 (reflecting intrinsic tenase) and that with FVIII/TF (reflecting activation by endogenous FXa) were moderately decreased (by 29-59%) compared to those in the absence of VWF. In contrast, as expected, the addition of VWF had little influence on FXa generation by FVIIa/TF in the absence of FVIII (reflecting extrinsic tenase), but significantly decreased that added FVIII (reflecting activation by endogenous FXa). These results were in keeping with the inhibitory potential of VWF on the association between FVIII and FXa, although the FVIII/VWF complex appeared to have a limited effect on FVIIa/TF-related FVIII-dependent FXa generation.

TFPI down-regulates blood coagulation by inhibiting FXa and FVIIa/TF. The physiological concentration of TFPI in plasma is ~1.6 nM, although most of the circulating TFPI binds to lipoproteins (~80%), and the physiological concentration as the 'free-TFPI' form is ~0.5 nM [29]. In the coagulation process, however, TFPI increases rapidly by ~30-fold (~15 nM) following various intravascular mechanisms including shear stress and thrombin generation [30]. Most TFPI binds to FVIIa/TF and FXa, simultaneously inhibiting FXa and FVIIa/TF within 30-60 sec

after FX activation [31]. We examined, therefore, the impact of different conditions of TFPI on FVIIa/TF-related FVIII-dependent FXa generation (Figure 2C). The amounts of FXa generated after 4 min by FVIIa/TF mixed with TFPI at 0.5 nM (*panel* **a**) and 15 nM (*panel* **b**) were 32.9±1.4 nM (86% of control) and 11.5±0.8 nM (30% of control), respectively. Corresponding levels of FXa with FVIII/FVIIa/TF mixed with 0.5 nM TFPI were 49.2±1.7 nM (81% of control), but were only modestly decreased (38.0±3.3 nM, 63% of control) in the presence of 15 nM TFPI. In contrast, FXa generated by FVIII/FVIIa/TF in the presence of E-76 were not affected by TFPI, suggesting that the potential of FVIII/FVIIa/TF-related to FXa generation in early-timed reactions (<30 sec) was not restricted by TF inhibition. Moreover, assays of FXa generation by FVIII/TF also were similar at each concentration of TFPI. Taken together, the enhancing effect of TF in FVIII-dependent FXa generation in the absence of FVIIa, appeared to be evident even in the presence of TFPI.

FVIII activation in the co-presence of FXa and FVIIa/TF - To clarify the mechanisms of FVIII activation by FVIIa/TF and FXa (pathway-II in Figure 1), we investigated FXa-induced FVIII activation in the presence of FVIIa. FVIII (10 nM) and TF/PL (0.1 nM/20 μM) were incubated with FXa (0.2 nM) and/or FVIIa (0.2 nM), prior to measuring FVIII activity in a one-stage clotting assay (Figure 3). Both FXa and FVIIa increased FVIII activity by ~2.2- or ~1.6-fold of initial levels, respectively. Subsequently, somewhat different, time-dependent reductions in activity were recorded as previously reported [14,32]. In the co-presence of FXa and FVIIa, FVIII activity increased by ~2.9-fold of initial, demonstrating an additive effect. Furthermore, FXa together with active-site modified EGR-FVIIa (inactivated form) also promoted similar increases in FVIII activity (~3.1-fold of initial). These results suggested that FXa-induced FVIII activation could be enhanced by the co-presence of FVIIa, or inactivated FVII in primary coagulation reactions.

FXa activation of FVIII/VWF in the presence of TF - Since VWF protects FVIII from FXa-catalyzed proteolysis [33,34], the conversion to FVIIIa is severely depressed. Consequently, enough amounts of FXa, which likely participate in initial physiological coagulation phases (pathway-II; Figure 1), may not be generated. The role of TF in the FVIII/VWF and FXa interactions remains unclear, however. To examine these mechanisms, FVIII (10 nM) was preincubated with VWF (10 μg/ml) and mixed with FXa/PL (0.2 nM/20 μM) in the presence of TF (0.2 nM) prior to measuring FVIII activity (Figure 4A). In the absence of TF, the FVIII peak

activity after FXa-induced activation of the FVIII/VWF complex was significantly decreased (by ~1.3-fold of initial) compared to that of purified FVIII (by ~4.1-fold). In the presence of TF, however, similar FVIII activities (by ~2-fold of initial) were demonstrated in the early phases in the absence and presence of VWF, although a more rapid decline in FVIII activity was observed in the later stages in the presence of VWF. Although the inhibitory mechanism by addition of TF on FXa-catalyzed FVIII activation without VWF was unclear, TF might compete with VWF in interaction with FVIII.

We also investigated the impact of TF on FXa-induced cleavage of FVIII HCh in the FVIII/VWF complex. FVIII (50 nM) was preincubated with VWF (50 μg/ml) and mixed with FXa/PL (1 nM/100 μM) and TF (1 nM), prior to SDS-PAGE/Western blotting analysis. Proteolytic cleavage of the HCh at Arg372 and Arg740 was visualized using an anti-A1 mAb (*panel* **a**) and an anti-A2 mAb (*panel* **b**) (Figure 4B). Acceleration in the conversion from A1-A2(-B) (90-200 kDa) to A1 (50 kDa; *panel* **a**) or A2 (40 kDa; *panel* **b**) was evident in the presence of TF, compared to that in its absence, in keeping with the results of FVIII activation. Cleavage at Arg372 was quantified by densitometry of the intact A1 or A2 band (*panels* **c**). Cleavage at Arg372 (and Arg740) by FXa occurred rapidly in the presence of TF. These findings suggested that TF might compete with VWF in FXa-induced FVIII interactions.

Direct binding of TF to immobilized FVIII - Our results suggested the possibility of a direct interaction between FVIII and TF related to tenase generation. We further investigated these relationships, therefore, using a solid phase-based ELISA. Various concentrations of lipidated TF were incubated with FVIII (10 nM) immobilized on microtiter wells, and bound TF was detected using anti-TF mAb. Control experiments showed that the anti-TF mAb had little effect on substrate binding (date not shown). Lipidated TF bound to FVIII directly with a saturable, dose-dependent binding curve (Figure 5A). This technique did not reflect a true equilibrium-binding assay, however, and the K_d was considered to represent an apparent (K_d^{app}) for the interactions. Nevertheless, the data fitted a single-site binding model, with a low K_d^{app} (3.6±0.7 nM). Similar binding experiments were performed using lipidated TF immobilized on microtiter wells using anti-TF mAb for capture, and a similar K_d^{app} (3.1±0.5 nM) to that obtained with immobilized FVIII was recorded (data not shown). To examine the specificity of the binding reactions, various concentrations of FVIII were preincubated with constant concentrations

of TF (3 nM) in a fluid phase, prior to addition to the immobilized FVIII. The fluid-phase FVIII completely inhibited binding (Figure 5A *inset*), confirming the validity of the assay.

FVIII binds to PL membranes [35], and to exclude the possibility that trace amounts of PL in lipidated TF affected our results, similar experiments were repeated using non-lipidated rTF (TF^{1-263}). TF^{1-263} bound to immobilized FVIII with a saturable and dose-dependent binding curve, although the K_d^{app} appeared to be slightly higher (8.1±0.5 nM) relative to that with lipidated TF (Figure 5B). In addition, TF is trans-membrane protein [36], and FVIII docking to the intracellular portion of TF seems physiologically improbable. Alternative experiments were established, therefore, using cytoplasmic domain-deleted rTF (TF^{1-243}). TF^{1-243} subunit bound directly to immobilized FVIII, although the K_d^{app} was ~3-fold greater (21.9±0.6 nM, Figure 5B) than that with TF^{1-263}. These results were consistent with the direct interaction between FVIII and the extracellular portion of TF, independently of PL.

Contribution of FVIII subunits to binding to TF^{1-243} - To investigate the role of FVIII subunits in the interactions with TF^{1-243} independent of PL, the ELISA method was adapted using isolated FVIII subunit preparations. Various concentrations of TF^{1-243} subunit were incubated with different FVIII subunits (150-200 nM) immobilized on microtiter wells. The TF^{1-243} bound directly to immobilized 80-kDa LCh, 70-kDa LCh, and the C2 domain with fitted dose-dependent binding curves. The estimated K_d^{app} values were 68.7±20.7, 20.2±3.3, and 17.1±1.6 nM, respectively. TF^{1-243} failed to bind to the intact HCh subunit, however (Figure 5C).

These interactions were further assessed using an alternative approach with a fluid-phase SPR-based assay. Table 1 summarizes the binding kinetics corresponding to the association and dissociation of FVIII subunits to TF^{1-243} immobilized on sensor chips. Representative binding curves for 80-kDa LCh and C2 domain are illustrated in Supplemental data 1. The data were comparatively fitted in 1:1 Langmuir binding models. Kinetic constants indicated that FVIII bound to TF^{1-243} with high affinity (K_d: 2.3 nM). The intact 80-kDa LCh and acidic region-deleted 70-kDa LCh bound to TF^{1-243} with similar affinities to FVIII (K_d: 5.8 nM, 11 nM, respectively), and the C2 domain also bound but with a modestly weak affinity (K_d: 63 nM) compared to the 70-kDa LCh. The HCh subunit again failed to bind. The estimated K_d data for FVIII subunit and TF^{1-243} binding in these SPR-based analyses appeared to be somewhat different from those obtained in ELISA-based assays, but nevertheless, the results were mutually supportive.

The results provided complementary evidence that the extracellular portion of TF bound to the LCh (especially C2 domain) in FVIII, independently of PL, although a role for the A3-C1 domain in TF binding could not be excluded.

Binding characteristics of the C2 domain and TF^{1-243} - To examine the binding between the C2 domain and the extracellular portion of TF, the influence of ionic strength on this interaction was analyzed using ELISA. TF^{1-243} (10 nM) binding to immobilized C2 was determined in the presence of various concentrations of NaCl. Incremental NaCl concentrations weakened this interaction (Supplemental data 2). A moderate reduction (~50%) in C2 binding was observed at physiological concentrations of NaCl (~140 mM) compared with lower ionic strength solutions, indicating that this interaction might be sensitive to electrolyte balance. The amount of C2 immobilized onto the microtiter wells was not affected by the ionic strength of wash buffer or the duration of wash and incubation cycles (data not shown).

As described above, TF counteracted the inhibitory effect of VWF in FVIII-FXa mechanisms (*see* Figure 4), and it seemed possible that TF- and VWF-binding sites could overlap on the C2 domain. We examined, therefore, the impact of VWF on TF^{1-243} binding to immobilized C2. The addition of VWF competitively blocked binding by >80% in a dose-dependent manner (Figure 6A). An anti-C2 inhibitor polyAb with type 1 characteristic that competitively blocked VWF and C2 domain interaction [20,21] and an anti-C2 mAb (ESH8) with type 2 behavior (epitope T2272/L2273/V2280/V2282/H2309/Q2311) [37] did not significantly affect C2 and TF^{1-243} binding, however (Figure 6B). These results demonstrated that the TF-binding site on the C2 domain was in proximity to or overlapped the VWF-binding site, but seemed unlikely to be associated with the representative C2 epitopes which the anti-C2 polyAb inhibitor and the specific anti-C2 mAb recognize.

Discussion

FVIIa/TF forms the initial extrinsic tenase enzyme that rapidly generates small amounts of FXa [10,11]. In the present study using a model of the initiation-phase of coagulation, however, the amount of FXa generated in the presence of FVIII together with FVIIa/TF were greater than the additive concentrations of FXa generated by individual preparations of FVIII or FVIIa/TF, suggesting that FVIIa/TF-derived FXa might have activated FVIII. In addition, our data were in keeping with earlier investigations indicating that FVIIa/TF activated FVIII directly [13,14].

Moreover, TF competitively restricted the binding of FVIII to VWF, and FVIII was effectively activated by FXa in the presence of TF even in complex with VWF. Furthermore, FXa-induced FVIII activation was enhanced additively by FVIIa or inactivated FVII. Earlier studies demonstrated that heavy chain cleavage of FVIII by FVIIa/TF produced an inactive FVIII cofactor no longer capable of activation by thrombin [13]. Our findings suggested, therefore, that in addition to the widely accepted FVIIa/TF-mediated extrinsic tenase generation, intrinsic tenase generation, governed activation of FVIII induced by FXa/FVIIa and FVIIa/TF and mediated especially by interactions between TF and the mainly C2 domain of FVIII, could be central to the initiation phase of coagulation, prior to thrombin-dependent feedback. These proposed mechanisms were consistent with studies of FVIII activation by the FVIIa/TF/FXa complex that was recently described [18].

The physiological plasma concentrations of FVIII and TF are 0.2~0.3 nM [35] and ~1.7 pM [38], respectively, and the estimated K_d for FVIII and extracellular TF (TF^{1-243}) interactions in our direct binding assays were in nano molar range. Binding between FVIII and TF in the normal circulation, therefore, appears unlikely. TF is exposed and concentrated on the surface vascular endothelial cells after vascular damage, and FVIIa and FVIII similarly accumulate at these same injury sites. Consequently, it could be anticipated that these pivotal initial phase reactions are promoted locally only after injury, and that FVIIa/TF/FVIII-related FXa generation does not occur in the normal circulating state.

TFPI is an anti-coagulant that inhibits FVIIa/TF-initiated FXa generation. The normal physiological concentration of TFPI in plasma is reported to be ~0.5 nM, and is elevated ~30-fold from baseline (maximum ~15 nM) after the initiation of coagulation [29]. This rise in TFPI concentration, however, appears likely to occur physiologically within 30-60 sec after the initiation of thrombin generation [31]. In addition, peak levels of FVIIa/TF-induced FVIII activation are recorded very rapidly, <15 sec after stimulus [14]. In our studies, neither FVIIa/TF-related nor TF-related FVIII-dependent intrinsic FXa generation was significantly affected by 0.5 nM TFPI, equivalent to that in normal circulation, but was moderately suppressed at 15 nM TFPI, representative of that present after vascular injury. In addition, the addition of a FVIIa-inhibitor peptide (E-76) 30 sec after mixing FVIIa/TF with FVIII enhanced FVIII-induced FXa generation even in the presence of TFPI. The findings suggested, therefore, that TFPI probably does not interfere with the earliest hemostasis reactions but plays a more dominant role in the control of

excessive pro-thrombotic activity at the later stages of not only extrinsic but also intrinsic coagulation cascades.

The effect of the co-presence of FVIIa/TF and FXa on FVIII activation appeared to additive compared to that of the individual enzymes. We initially considered that these findings could be due to different process of FVIII cleavage by FXa or FVIIa. Our data demonstrated, however, that FVIII activation was induced to a similar extent by FXa in the co-presence of the native or inactivated form of FVII/TF. These data were consistent with those of a recent investigation using active-site modified FVIIa mutant [18]. Precise activation mechanisms are unclear, however. A favorable change in the conformational structure of FXa induced by binding to FVIIa/TF on PL might enhance FVIII proteolysis by FXa, and even if FVIIa is immediately inactivated in the initiation phase, FXa bound to the inactivated-FVIIa/TF could enhance activation of FVIII due to the competitive inhibition of FVIII-VWF binding by TF (pathway-II).

Our further studies were designed to focus on the association between TF and FVIII in the critical early stages of the hemostatic response. Direct binding assays demonstrated for the first time that the non-lipidated, extracellular portion TF (rTF^{1-243}) bound to FVIII directly. The results demonstrated, in particular, that the C2 domain contributed significantly to this binding and was sensitive to ion-strength, although the presence of one or more other binding sites within the A3-C1 domain could not be excluded. Moreover, FVIII-dependent intrinsic FXa generation mediated by TF and FVIIa/TF was evident in the presence of VWF. The C2 domain contained a TF-binding site as well as a VWF-binding site [28], and competitive interactions between FVIII-TF and FVIII-VWF were apparent in these mechanisms. The TF-binding site on the C2 domain did not overlap with either the C2 epitope defined by an anti-C2 polyAb inhibitor or that recognized by anti-C2 mAbESH8, however, and the specific TF-binding site(s) on the C2 domain remains unidentified. The C2 domain interacts with PL [35,39], FIXa [40], and FXa [34], and further studies are in progress to determine the precise structural relationship among the C2 domain, TF^{1-243}, and other components on TF-related intrinsic FXa generation.

The coagulation process *in vivo* is accelerated by TF on the surface of intact cells, and the use of a cell free system limits the interpretation of our results. Further investigations are warranted to examine FXa generation mediated by TF on intact cells. Nevertheless, our *in vitro* experiments have provided strong evidence for a model of the initiation phases of coagulation based on three

potential FXa mechanisms; FXa generated by FVIIa/TF in the extrinsic system, FXa induced by FVIIa/TF and FVIIIa in the intrinsic system, and intrinsic FXa mediated by supplementary FXa-FVIIa/TF-FVIII interactions. Actually, our proposed mechanism would support the clinical setting as follows. For example, when the bypassing agents including FVIIa component are administered for hemophilia A patients with inhibitors treated with immune tolerance induction (ITI) therapy, we have often experienced that the hemostatic controls of these patients appear to be better than those untreated with ITI. Even if the inhibitor is present, the presence of a small amount of FVIII could have enhanced the hemostatic potentials through the acceleration of FXa generation via FVIII-FVIIa-TF-FX mechanism in the initial coagulation phase.

Authorship

Contributions

SF; performed experiments, interpreted the data, made the figures, and wrote the paper, KN; designed the research, interpreted the data, wrote the paper, edited the manuscript, and approved the final version to be published, KO; performed experiments and interpreted the data, MS; supervised the studies.

Conflict of interest disclosure

The authors declare that they have no conflicts of interest.

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Figure Legends

Figure 1. The initiation phase of coagulation in the cell-based model of hemostasis - (*Panel* **A)** *Accepted initiation phase of coagulation*; FVIIa/TF/PL initiates hemostasis by activating FIX and FX and promoting the extrinsic tenase complex, resulting in subtle amounts of thrombin generation. **(***Panel* **B)** *Hypothetical initiation process involving both extrinsic and intrinsic tenase generation*; In addition to the extrinsic tenase complex shown in (A), FVIIa/TF converts FVIII to FVIIIa more rapidly than thrombin in early timed-coagulation phases, irrespective of the presence of VWF (pathway-I) (Soeda *et al*, 2010). A further putative mechanism of initial intrinsic tenase generation, mediated by FXa/FVIIa/TF-related FVIII association, has been recently reported (pathway-II) [18].

Figure 2. FVIIa/TF-related FVIII-dependent FXa generation and the effects of VWF and TFPI - FVIII (1 nM) was activated by FVIIa/TF (1 nM/0.1 nM) with PL (20 μM) for 30 sec (*panel* **A**). In parallel experiments, FVIII (1 nM) was mixed with either VWF (1 μg/ml; *panel* **B**) or TFPI (0.5 nM (**a**), 15 nM (**b**); *panel* **C**), followed by incubation with FVIIa/TF (1 nM/0.1 nM) with PL (20 μM) for 30 sec. After no addition (*open circles*) or addition (*closed circles*) of FVIIa-blocking peptide (E-76; 2.5 U/ml) to the samples obtained in (*panel* **A-C**), FXa generation was initiated by the addition of FIXa (1 nM) and FX (150 nM) as described in Methods. FXa generation was measured after the addition of either FVIII alone (*open squares*), FVIII/TF without FVIIa (*closed squares*), or FVIIa/TF without FVIII (*open triangles*). Values of FXa generation during 4 min were plotted as a function of reaction time. The *inset* of *panel* (A) shows an enlarged scale of FXa generation after the initial 1 min. All experiments were performed on three separate occasions, and the average values and standard deviations are shown.

Figure 3. Activation of FVIII by the co-presence of FXa and FVIIa/TF - FVIII (10 nM) was mixed with TF/PL (0.1 nM/20 μM) and incubated with FXa/FVIIa (0.2 nM/0.2 nM; *open circles*), FXa/EGR-FVIIa (0.2 nM/0.2 nM; *closed circles*), FVIIa alone (0.2 nM; *open squares*), or FXa alone (0.2 nM; *closed squares*). The reactions were terminated at the indicated times, followed by measuring FVIII activity as described in Methods. FVIII activity was plotted as a function of incubation time. Initial FVIII activity in the presence or absence of TF was ~16 or ~8 IU/ml, respectively. All experiments were performed on three separate occasions, and average data and standard deviations are shown.

Figure 4. Impact of TF on FXa-catalyzed activation and cleavage of FVIII in FVIII/VWF - (*Panel* **A**) *FVIII activation by FXa*; FVIII (10 nM) in the absence (*open symbols*) or presence (*closed symbols*) of VWF (10 μg/ml) was incubated with FXa (0.2 nM) and PL (20 μM) without TF (*circle symbols*) or with TF (0.2 nM; *square symbols*). Reactions were terminated at the indicated times, followed by measuring the FVIII activity as described in Methods. FVIII activity was plotted as a function of incubation time. The initial activities of FVIII in FVIII alone, FVIII/VWF, FVIII/TF, and FVIII/VWF/TF were ~10, ~6, ~20, and ~15 U/ml, respectively. All experiments were performed on three separate occasions, and average data and standard deviations values are shown. (*Panel* **B**) *FVIII cleavage by FXa*; FVIII (50 nM) with VWF (50 μg/ml) was incubated with FXa (1 nM) and PL (100 μM) in the absence (*left side*) or presence (*right side*) of TF (1 nM) at the indicated times. Samples were analyzed by SDS-PAGE using an 8% gel followed by Western blotting with anti-A1 mAb (*panel* **a**) or anti-A2 mAb (*panel* **b**) for detection as described in Methods. A representative blot and the band densities of intact A1 and A2 (*panel* **c**) are shown. Band densities are represented as arbitrary units. The symbols used are, *open circle*; A1 in TF (−), *closed circle*; A1 in TF (+), *open square*; A2 in TF (−), *closed square*; A2 in TF (+).

Figure 5. Direct binding of TF to FVIII immobilized in ELISA - (*Panel* **A, B**) *FVIII binding to TF forms* - Various concentrations of lipidated TF (A), non-lipidated full-length TF^{1-263} (*open circles*) and non-lipidated extracellular portion TF^{1-243} (*closed circles*) (B) were incubated with intact FVIII (100 nM) immobilized onto microtiter wells. Bound TF was detected using anti-TF mAb (2 μg/ml) as described in Methods. Absorbance was plotted as a function of TF concentration, and data were fitted using a single-site binding model. (*Inset*) The mixtures with various concentrations of FVIII and a constant concentration of lipidated TF (3 nM) were incubated with immobilized FVIII. Bound TF was detected using anti-TF mAb. Absorbance corresponding to FVIII binding to TF in the absence of competitor was defined as 100%. (*Panel* **C**) *FVIII subunits and* TF^{1-243} *binding* - Various concentrations of TF^{1-243} subunit were incubated with 80-kDa LCh (150 nM; *open circles*), 70-kDa LCh (150 nM; *closed circles*), C2 (200 nM; *closed squares*), and HCh (150 nM; *open diamonds*) immobilized onto microtiter wells. Bound TF was detected using anti-TF mAb. Absorbance was plotted as a function of TF concentration, and data were fitted using a single-site binding model. The experiments were performed on three separate occasions, and the average and standard deviations are shown.

Figure 6. Binding characteristics on TF^{1-243} subunit and FVIII C2 domain - (*Panel* **A**) *VWF*; TF^{1-243} (10 nM) was mixed with various concentrations of VWF, followed by incubation with immobilized the FVIII C2 domain (200 nM) immobilized onto microtiter wells. (*Panel* **B**) *Anti-FVIII Ab*; TF^{1-243} (10 nM) was mixed with various concentrations of anti-C2 polyAb (*open circles*) or ant-C2 mAbESH8 (*closed circles*), followed by incubation with immobilized C2 (200 nM). Bound TF was detected using anti-TF mAb. Absorbance corresponding to C2 and TF binding in the absence of competitor was defined as 100%. The experiments were performed on three separate occasions, and the average and standard deviations are shown.

Supplemental data 1

SPR-based assay on LCh or C2 binding to TF^{1-243} - Various concentrations of 80-kDa LCh (*panel* **A**) and C2 domain (*panel* **B**) were incubated for 2 min with TF^{1-243} immobilized on the sensor chip, followed by a change of running buffer for 2 min as described in Methods. The experiments were performed on three separate occasions. The lines represent the association and dissociation curves of each subunit.

Supplemental data 2

Effect of ionic strength on TF^{1-243} and C2 domain binding - TF^{1-243} subunit (10 nM) was mixed with various concentrations of NaCl, followed by incubation with the FVIII C2 domain (200 nM) immobilized onto microtiter wells. Bound TF was detected using anti-TF mAb. The experiments were performed on three separate occasions, and the average and standard deviations are shown.

Table 1. Binding parameters for the interaction of FVIII subunits with TF^{1-243} in an SPR-based assay

Assays were performed as described in Methods. All parameters were calculated by nonlinear regression analysis using the evaluation software provided by Biacore™. K_d values were calculated as k_{diss}/k_{ass}.

	TF^{1-243}		
FVIII(a) subunit	k_{asso}	k_{diss}	K_d
	$\times 10^5 M^{-1}s^{-1}$	$\times 10^{-3}s^{-1}$	nM
FVIII	0.092 ± 0.010	0.02 ± 0.004	2.3
Intact HCh	n.d.	n.d.	–
LCh : 80-kDa	14.5 ± 0.54	7.7 ± 0.21	5.8
70-kDa	8.2 ± 0.46	7.7 ± 0.24	11
C2	0.99 ± 0.096	4.6 ± 0.04	63

TF^{1-243}; the cytoplasmic domain-deleted TF; n.d; no binding detected

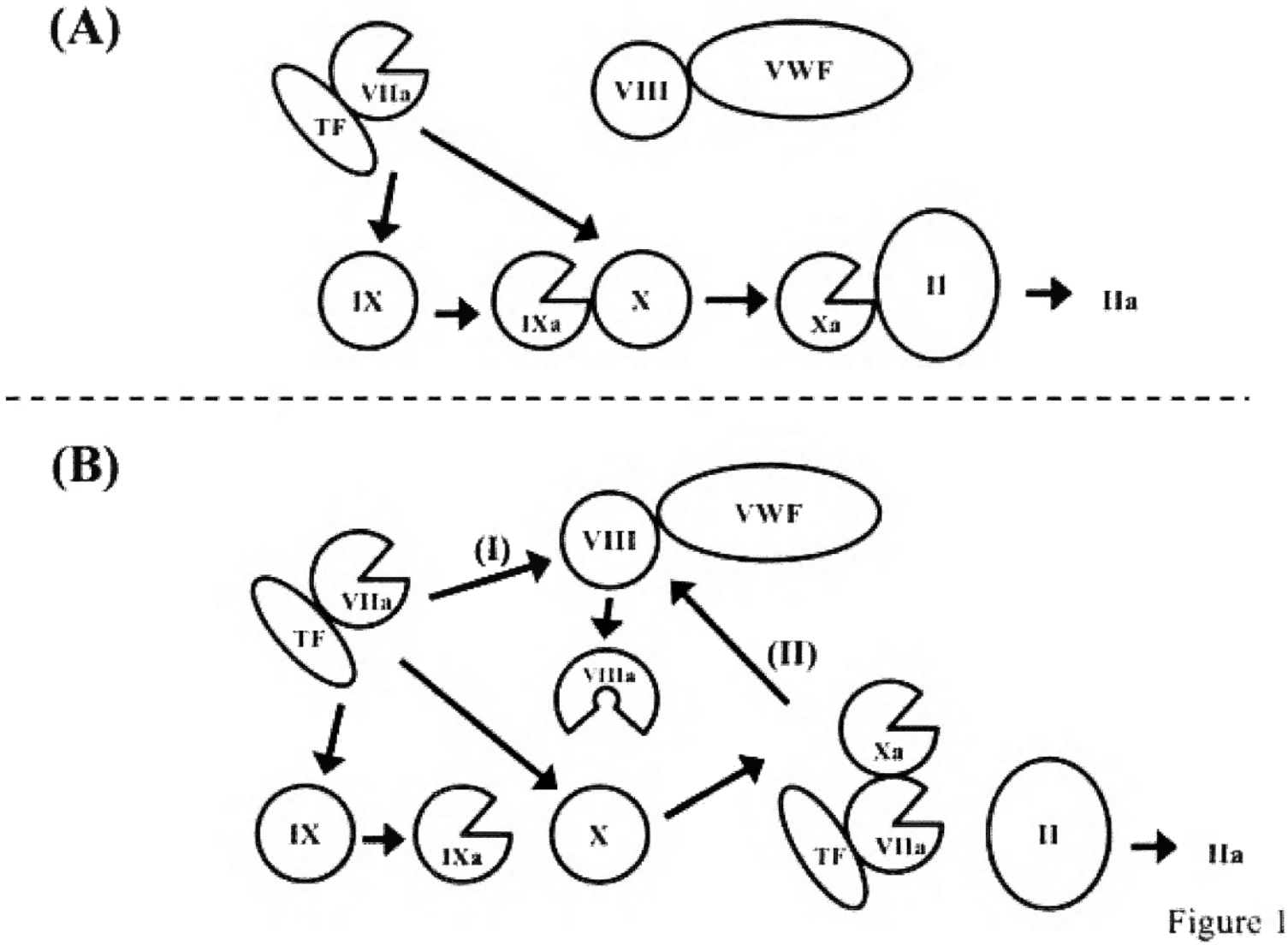


Figure 1

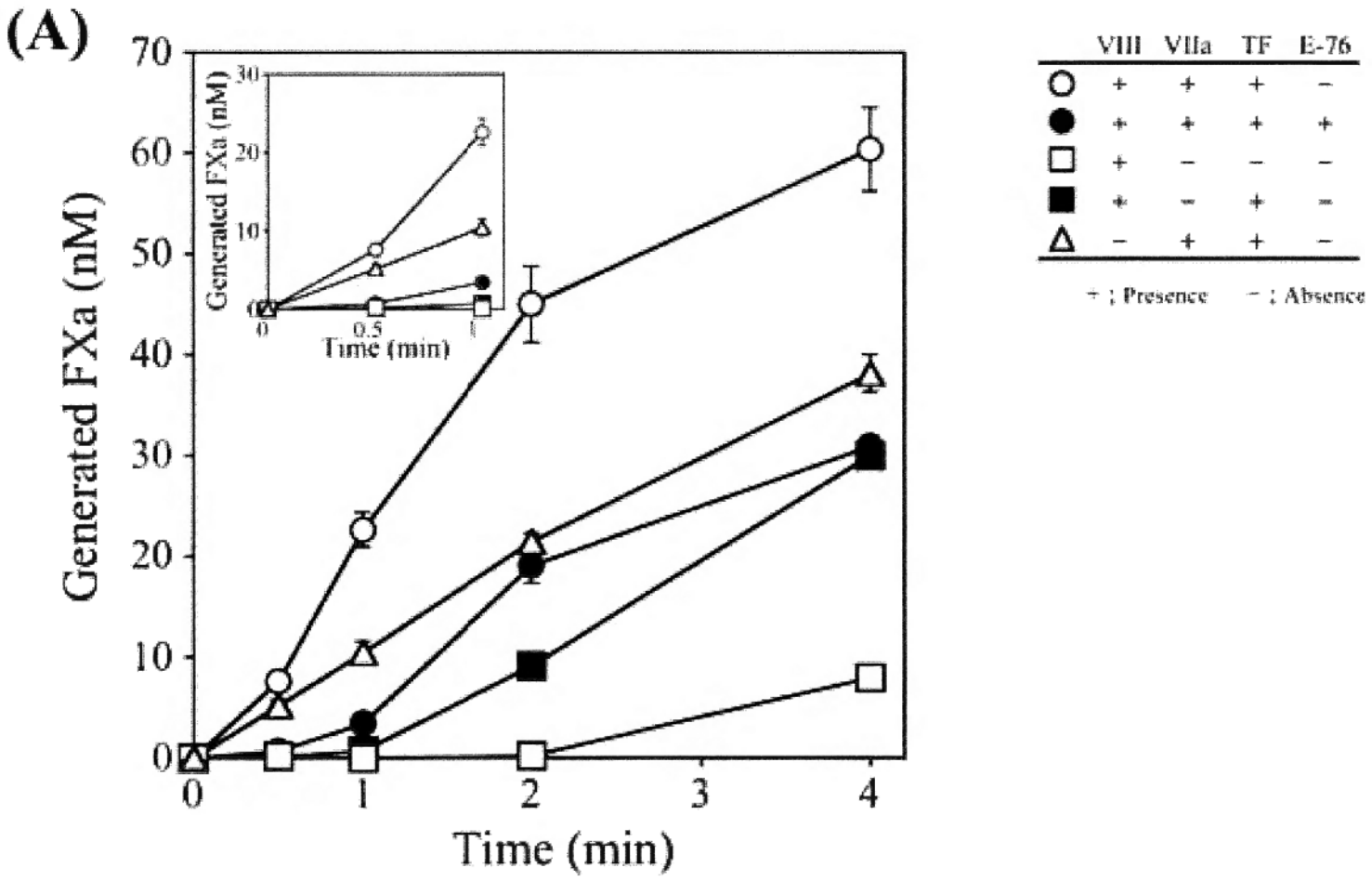
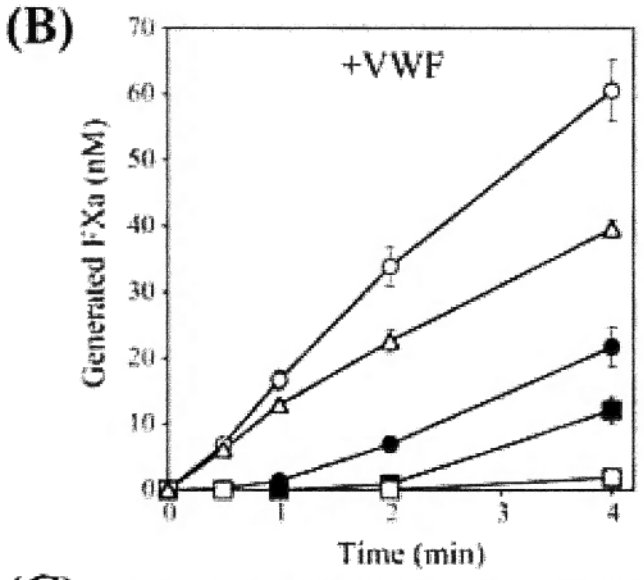


Figure 2A



	VIII	VIIa	TF	E-76
○	+	+	+	−
●	+	+	+	+
□	+	−	−	−
■	+	−	+	−
△	−	+	+	−

+ ; Presence − ; Absence

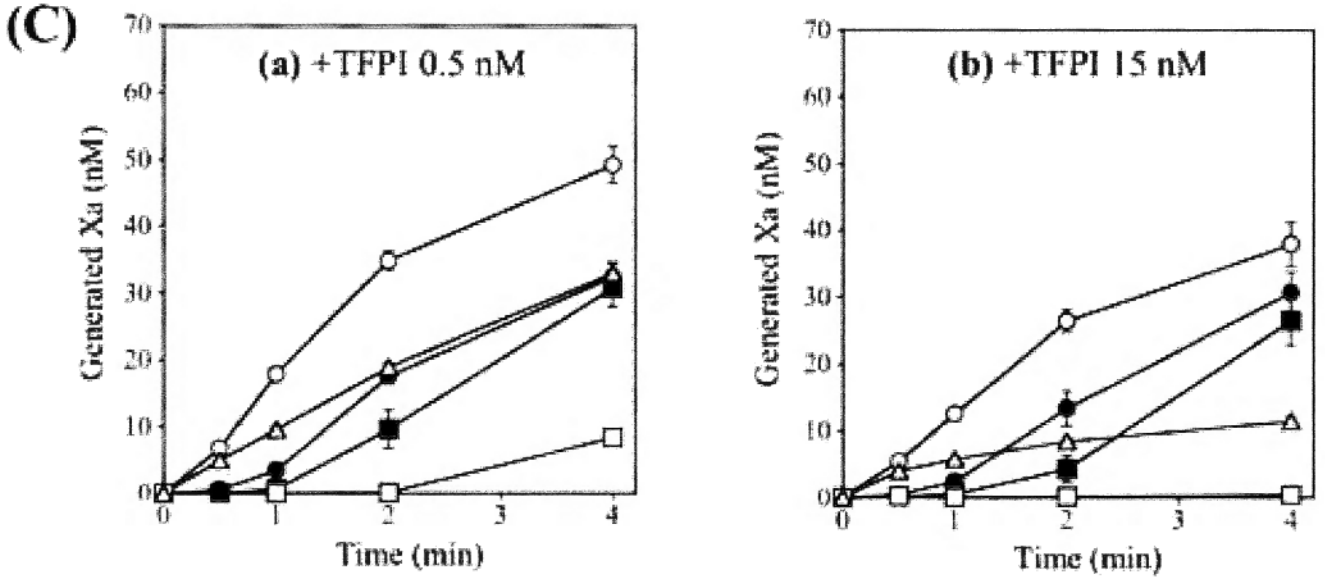


Figure 2B, 2C

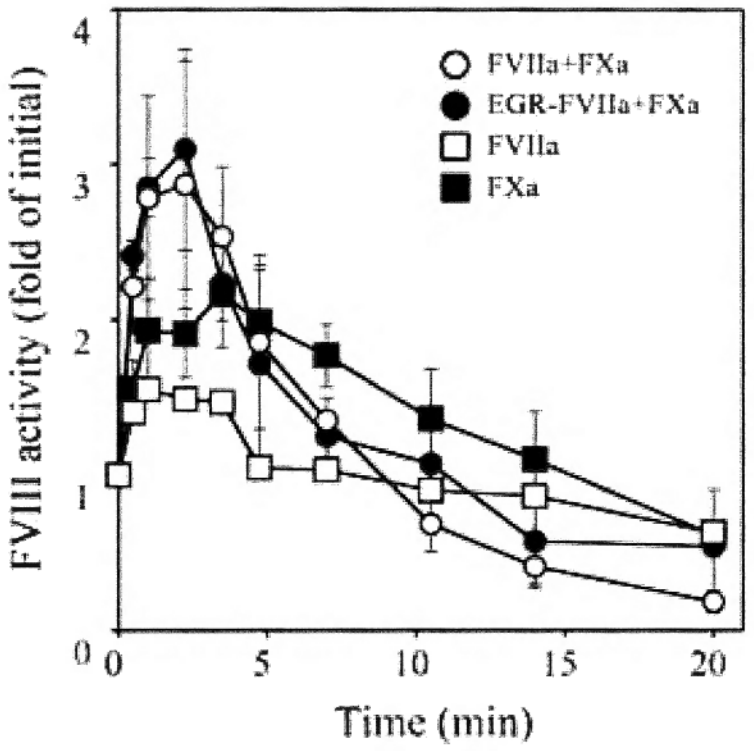


Figure 3

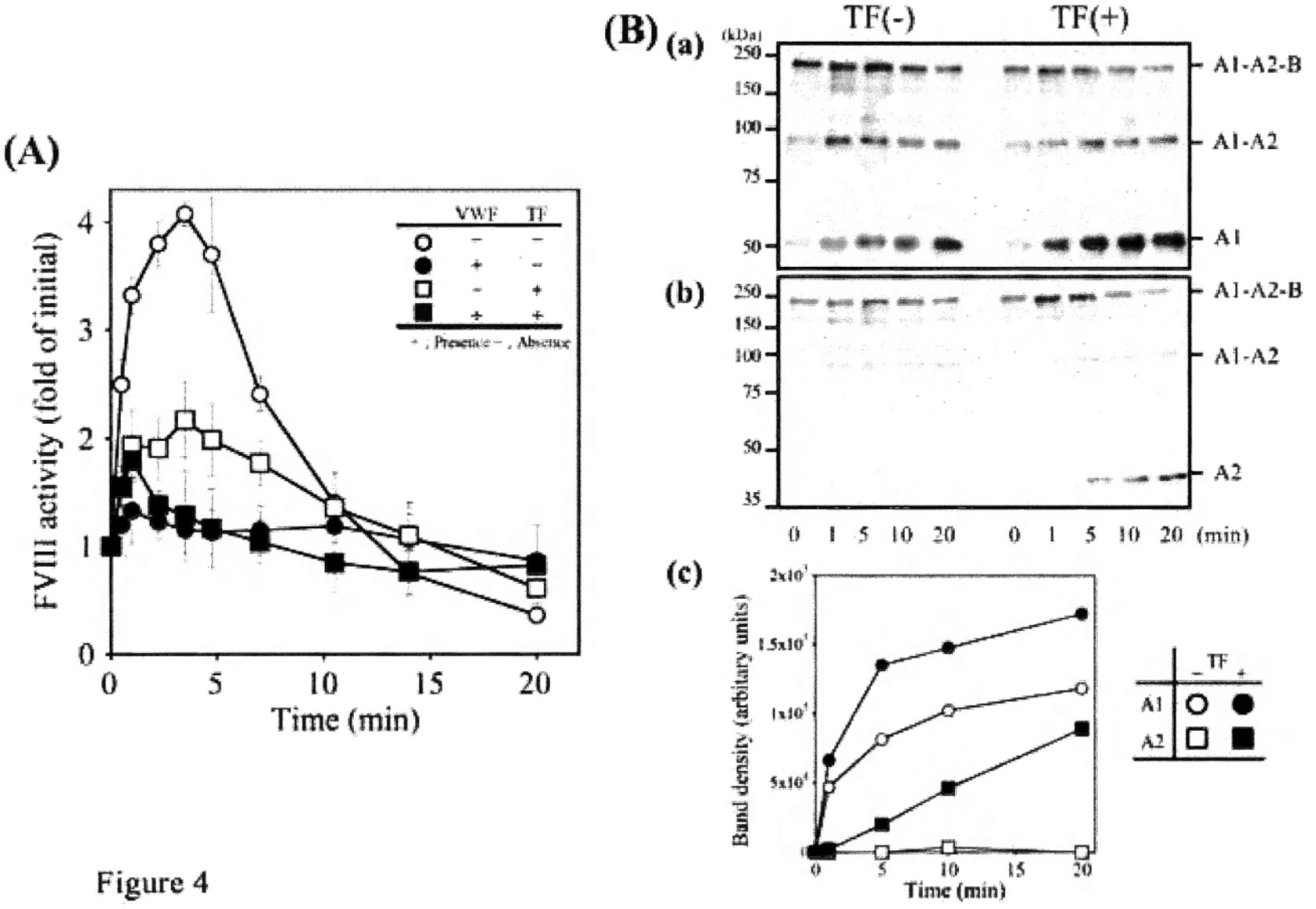


Figure 4

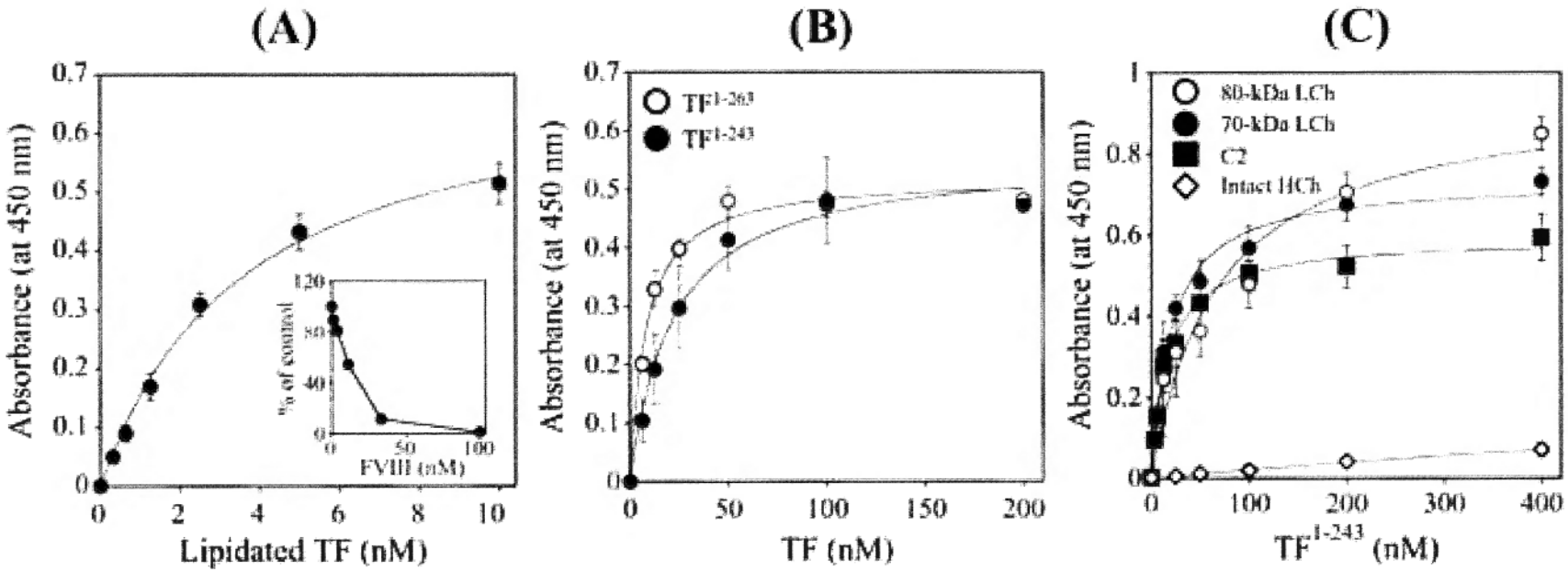


Figure 5

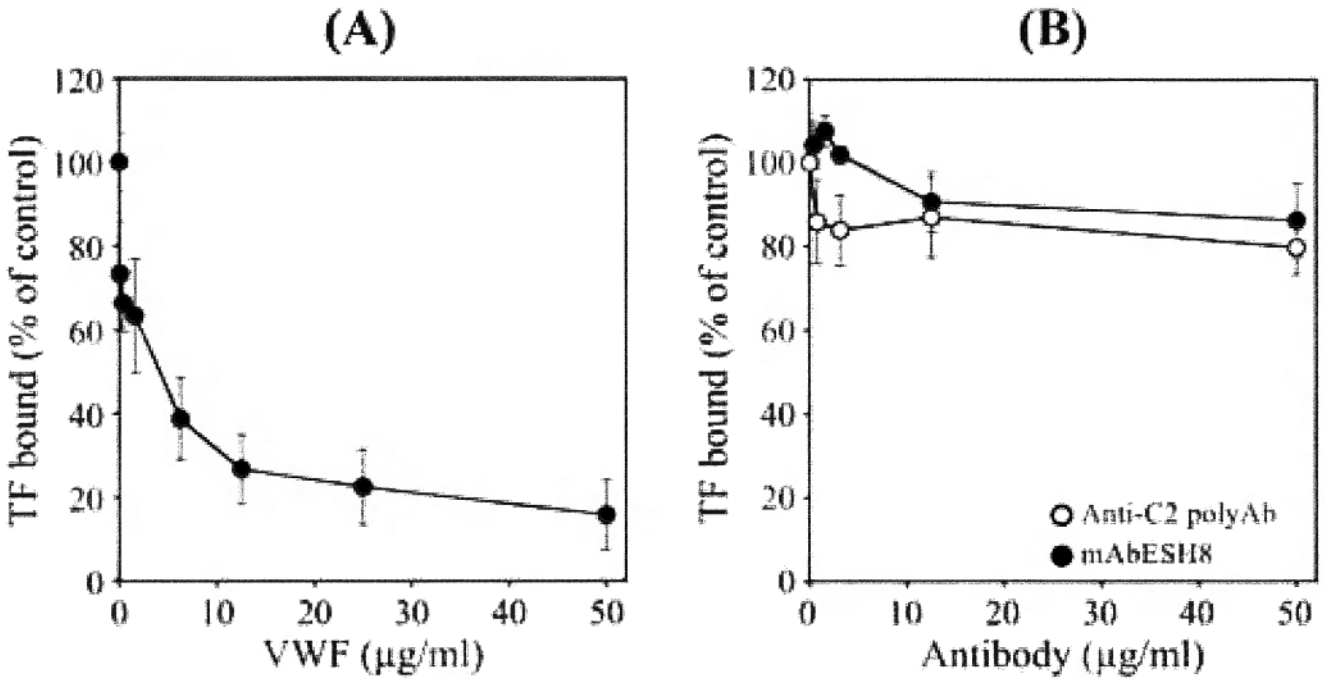


Figure 6