Tissue Factor Coagulant Activity is Regulated by the Plasma Membrane Microenvironment

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Abstract

Background Tissue factor (TF) can be present in a non-coagulant and coagulant form. Whether the coagulant activity is affected by the plasma membrane microenvironment is unexplored.

Objective This article studies the presence and coagulant activity of human TF in plasma membrane micro-domains.

Methods Plasma membranes were isolated from human MIA PaCa2 cells, MDA-MB-231 cells and human vascular smooth muscle cells by Percoll gradient ultracentrifugation after cell disruption. Plasma membranes were fractionated by OptiPrep gradient ultracentrifugation, and the presence of TF, flotillin, caveolin, clathrin, protein disulphide isomerase (PDI), TF pathway inhibitor (TFPI) and phosphatidylserine (PS) were determined.

Results Plasma membranes contain two detergent-resistant membrane (DRM) compartments differing in density and biochemical composition. High-density DRMs (DRM-H) have a density (ρ) of 1.15 to 1.20 g/mL and contain clathrin, whereas low-density DRMs (DRM-L) have a density between 1.09 and 1.13 g/mL and do not contain clathrin. Both DRMs contain TF, flotillin and caveolin. PDI is detectable in DRM-H, TFPI is not detectable in either DMR-H or DRM-L and PS is detectable in DRM-L. The DRM-H-associated TF ($>$ 95% of the TF antigen) lacks detectable coagulant activity, whereas the DRM-L-associated TF triggers coagulation. This coagulant activity is inhibited by lactadherin and thus PS-dependent, but seemed insensitive to 16F16, an inhibitor of PDI.

Conclusion Non-coagulant and coagulant TF are present within different types of DRMs in the plasma membrane, and the composition of these DRMs may affect the TF coagulant activity.

Keywords
- tissue factor
- coagulant activity
- phosphatidylserine
- plasma membrane
- detergent-resistant membrane

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Introduction

Plasma membranes contain two forms of detergent-resistant membrane (DRM) compartments, low-density DRMs (ρ 1.09–1.13 g/mL; DRM-L) and high-density DRMs (ρ 1.15–1.18 g/mL; DRM-H).[1] Such DRMs differ not only in their biophysical properties but also in their biochemical composition. For example, DRM-H contain clathrin,[1] whereas the transmembrane receptors glycoprotein 41 (GP41)[2] and glycosyl-phosphatidylinositol (GPI)-linked receptors[3,4] are present in both DRM-H and DRM-L.

Tissue factor (TF) is associated with membrane 'lipid rafts'.[5–12] Because lipid rafts and DRM-L both (1) are defined as DRM compartments, (2) compartmentalize cellular functions such as cell signalling, (3) are enriched in cholesterol and sphingolipids, (4) are resistant to non-ionic detergents, (5) contain flotillin and caveolin (caveolar rafts) and (6) have the same density,[9,12] we will use the term DRM-L throughout this manuscript instead of lipid rafts.

Our current knowledge regarding the presence and distribution of TF antigen and coagulant activity in the plasma membrane is limited for several reasons. First, whether TF is associated with DRM-H in plasma membranes is unknown because the density range of most earlier applied gradients was insufficient to separate DRM-H from detergent soluble compartments (summarized in ►**Table 1**, see also ►**Fig. 1B**). Second, most investigators studied the distribution of TF not in purified plasma membranes but in whole cell lysates (►**Table 1**), but only a fraction of the total cellular TF is present within the plasma membrane.[13] Third, thus far the *relative* distribution of TF has been studied (►**Table 1**). In the relative distribution, fractions are normalized per µg protein to show the relative enrichment of a protein such as TF, but the real and quantitative distribution remains unknown. Even when the before-mentioned limitations are set aside, there is also no consensus about the extent of association between TF and/or TF coagulant activity and DRM-L, making the contribution of DRM-L to regulate the TF coagulant activity questionable.[5,7–10,12,14,15]

In summary, the distribution of TF and TF coagulant activity in plasma membranes is virtually unexplored. Therefore, we investigated the quantitative distribution of TF antigen and coagulant activity in both DRM-L and DRM-H in purified plasma membranes.

Methods

Cloning of Human TF from HUVEC

Human Umbilical Vein Endothelial Cells (HUVECs) were isolated and cultured as described previously,[16] but human serum (10%) was replaced by fetal calf serum (FCS; 20%). HUVEC were activated with interleukin-1α (IL-1α; 5 ng/mL; Sigma, St. Louis, Missouri, United States) for 1 hour. Subsequently, cells were harvested and ribonucleic acid (RNA) was isolated (RNeasy Mini Kit; Qiagen, Hilden, Germany) and used for complementary deoxyribonucleic acid (cDNA) preparation (1st Strand cDNA synthesis kit; Roche, Basel, Switzerland). The TF open reading frame was amplified using a 5′primer: GGGGACAAGTTTGTACAAAAAAGCAGGCTCCACCATGGAGACC CCTGCCTGG, and a 3′primer: GGGGACCACTTTGTACAAGAAAG CTGGGTTTTATGAAACATTCAGTGGGGAGT.

Using the Gateway technology (Invitrogen, Carlsbad, California, United States), the resulting polymerase chain reaction (PCR) fragment was shuttled via pDON/Zeo (Invitrogen), into pEGFP-N3 (Clontech, Mountain View, California, United States). The resulting vector expresses the TF mRNA/protein under control of the cytomegalovirus (CMV) promoter.

Transfection of MIA PaCa-2 Cells with TF

MIA PaCa-2 cells were a gift from the Department of Neurogenetics (Academic Medical Center, Amsterdam, The Netherlands). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% FCS

Table 1 Overview of TF distribution in cells, investigated in various studies

Starting material	Relative or absolute distribution	Range of density gradient sufficient to isolate DRM-H[a]	TF antigen in DRM-L	TF coagulant activity in DRM-L	Ref.
Membranes	Relative	No	+/−	+	10
Whole cell lysate	Relative	No	+/−	ND	5
Whole cell lysate	Insufficiently documented	No	+	ND	8
Whole cell lysate	Relative	No	+	ND	6
Membranes	Insufficiently documented	Yes	+	+	9
Whole cell lysate	Relative	No	+	ND	11
Whole cell lysate	Insufficiently documented	No	Below detection limit	ND	7
Whole cell lysate	Relative	No	+/−	Below detection limit	12

Abbreviations: DRM-H, high-density detergent-resistant membrane; DRM-L, low-density detergent-resistant membrane; ND, not determined; TF, tissue factor.

Note: This table summarizes the available literature on distribution of TF in whole cell lysates and membrane fractions. +/ − = partially; + = unclear.

[a]5–30% sucrose (ρ 1.0179–1.1270 g/mL),[5] 5–35% sucrose (ρ 1.0179–1.1513 g/mL),[10] 5–40% sucrose (ρ 1.0179–1.1764 g/mL),[6–8,12] 5–45% sucrose (ρ 1.0179–1.2025 g/mL) [11] and OptiPrep 10–50% (ρ 1.0179–1.266 g/mL).[9]

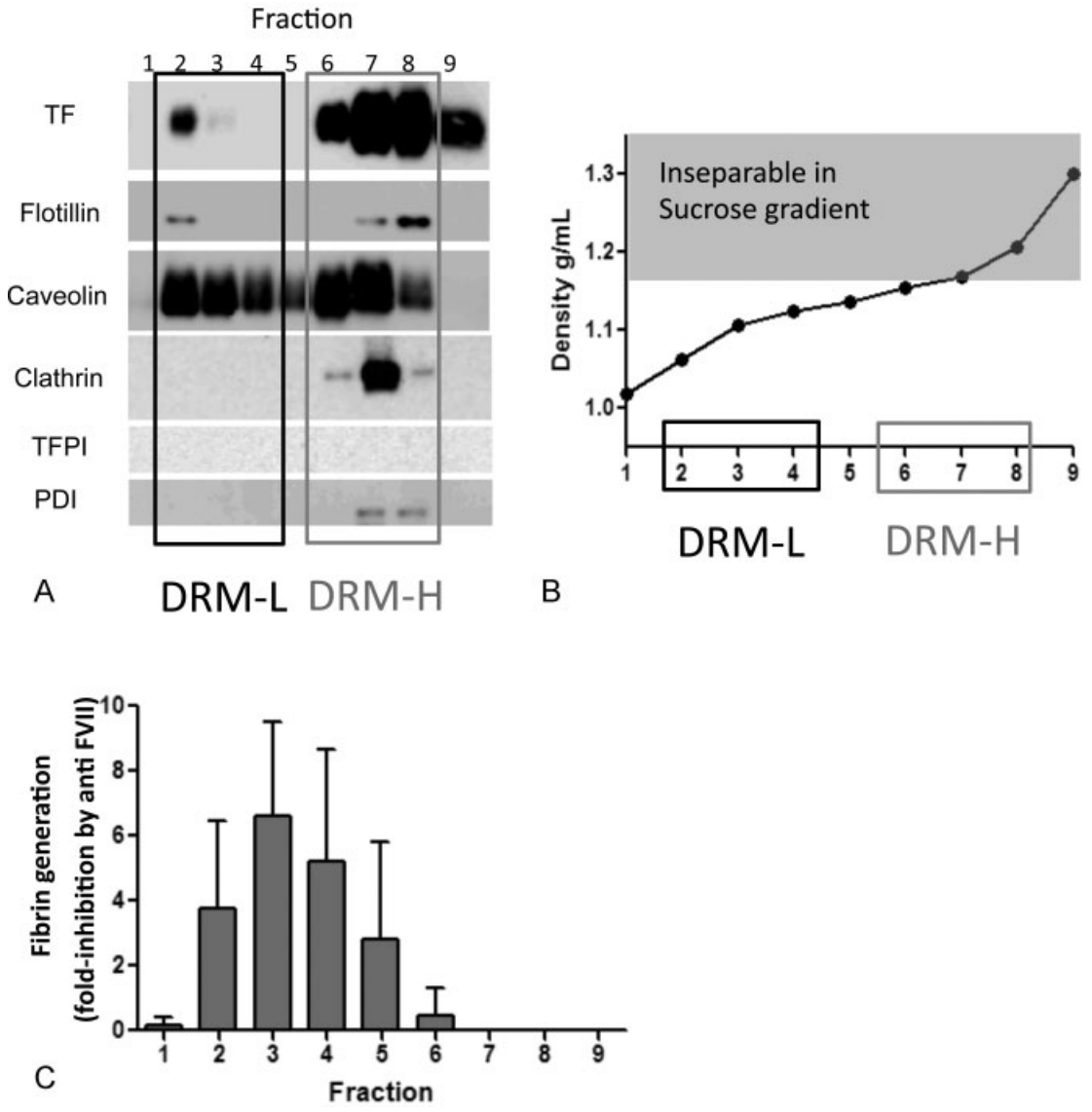

Fig. 1 Absolute distribution of tissue factor (TF) in plasma membranes of MDA-MB-231 cells. (**A**) Absolute distribution of TF in purified plasma membranes of MDA-MB-231 cells in two forms of detergent-resistant membranes (DRMs). Western blots incubated with anti-TF, anti-flotillin and anti-caveolin, anti-clathrin (DRM-H marker), anti-tissue factor pathway inhibitor (TFPI) and anti-protein disulphide isomerase (PDI), showing their *absolute* distribution in Triton X-100 OptiPrep gradient fractions of plasma membranes. Fractions are normalized per volume. Blue box indicates DRM-L, red box indicates DRM-H. (**B**) Density of each Triton X-100 OptiPrep gradient fraction to determine the fractions containing DRM-L and DRM-H. Blue box indicates DRM-L, red box indicates DRM-H, grey box indicates densities inseparable in sucrose gradients. (**C**) TF coagulant activity in Triton X-100 OptiPrep gradient fractions of plasma membranes as measured by capacity to induce fibrin formation ($n = 3$, mean ± standard deviation [SD]). Of each fraction, fibrin formation is expressed as the factor of inhibition of ½ V_{max} by anti-FVII.

(PAA, Pasching, Austria), penicillin and streptomycin (10 units/mL and 10 µg/mL, respectively; Invitrogen). Before transfection, cells were cultured in 75 cm^2 culture flask for 3 days. Thereafter, 7 µg DNA and 70 µL Lipofectin (Invitrogen) were added to the cells, and after 24 hours, the culture supernatant was removed and replaced by fresh culture medium. After 48 hours, cells were harvested.

Culture of MDA-MB-231

The breast cancer cell line MDA-MB-231 was kindly provided by B. Kocaturk (Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, The Netherlands) and cultured in DMEM F12 (Invitrogen) supplemented with 10% FCS (PAA), penicillin and streptomycin (10 units/mL and 10 µg/mL, respectively; Invitrogen).

Isolation and Culture of (Human) (Vascular) Smooth Muscle Cells

Human smooth muscle cells (SMCs) were isolated from human umbilical arteries. First, the artery was isolated and divided in sections. Then, the artery sections were placed on a gelatin (Invitrogen, 0.1% in phosphate-buffered saline [PBS])-coated 6-wells plate supplemented with culture medium (M199 (Invitrogen), 10% FCS (PAA) and penicillin and streptomycin (10 units/mL and 10 µg/mL, respectively; Invitrogen). After 3 weeks, the SMC had migrated out of the

artery sections. Then, SMC were further cultured on T75 flasks and passage 3 was used for experiments.

DRM Isolation from (Total) Cell Lysates

Cells (a single T75 flask of TF-transfected MIA-PaCa [48 hours]) were washed twice with ice cold PBS. Then, 1.0 mL lysis buffer, containing 10 µL protease inhibitors and 1% (v/v) Triton X-100 (both caveolae/rafts isolation kit, Sigma-Aldrich, Zwijndrecht, The Netherlands) was added and cells were harvested by scraping. Cells were incubated on ice for 1 hour followed by centrifugation (5 minutes, 2,000 × *g*, 4°C). The cell lysate (supernatant) was diluted 2.4-fold in 60% OptiPrep (Caveolae/rafts isolation kit, Sigma-Aldrich) and placed on the bottom of a polyallomer tube. Then, a 8-mL OptiPrep gradient (5–35%; Caveolae/rafts isolation kit, Sigma-Aldrich), was loaded on top of the cell lysate. Thereafter, the gradient was centrifuged for 16 hours at 137,000 × *g* (Beckman; SW41Ti; Beckman XL90 ultracentrifuge). After centrifugation, nine fractions of 1 mL were collected by carefully pipetting from the top.

Isolation of DRM from Purified Plasma Membranes

Cells (two T75 flasks of TF-transfected MIA-PaCa [48 hours], MDA-MB-231 or SMC) were washed twice with ice cold PBS. Then, cells were harvested by scraping in the presence of 5 mL PBS containing protease inhibitors. Thereafter, plasma membranes were isolated as described before.[17] In short, cells were disrupted in a nitrogen bomb (1,030 Psi, 20 minutes, on melting ice). After disruption, the total cell lysate was centrifuged to remove cellular debris (15 minutes at 1,500 × *g* at 4°C). The supernatant was collected (5 mL) and mixed with ice cold PBS (10 mL), Percoll (8.6 mL; GE Healthcare, Uppsala, Sweden) and aquadest (1.4 mL) at pH 7.4, and centrifuged for 23 minutes at 79,000 × *g* at 4°C (polycarbonate tubes, Beckman; 70Ti rotor; Beckman XL90 ultracentrifuge). After ultracentrifugation, the upper part of the fluid (11 mL), containing both plasma membranes and intracellular membranes, was collected and mixed with ice cold PBS (22 mL) and Percoll (18.9 mL), and the pH was carefully adjusted to 9.6. Subsequently, the mixture was again centrifuged for 23 minutes at 79,000 × *g* at 4°C. After ultracentrifugation, the upper part (11 mL), containing the purified plasma membranes, was collected. Finally, plasma membranes were pelleted by ultracentrifugation for 68 minutes at 200,000 × *g* at 4°C. After isolation, plasma membranes (500 µL) were diluted with lysis buffer (500 µL; supplemented with Triton X-100 v/v 2%, protease inhibitors 10 µL), incubated for 60 minutes and DRMs were isolated as described for cell lysates. Contamination of the plasma membranes by intracellular membranes was determined by the presence of coatomer sub-unit β (β-COP), a marker of Golgi membranes, which was below the detection limit in all fractions, indicating that contamination with intracellular membranes is negligible (data not shown).

TF Enzyme-Linked Immunosorbent Assay (ELISA)

After ultracentrifugation, 100 µL of each fraction was frozen to determine the TF antigen by TF ELISA (Sekisui Diagnostics, Lexington, Massachusetts, United States). The ELISA was performed as described by the manufacturer.

Density of Fractions

The density of the nine fractions of the Triton X-100 OptiPrep gradient was determined by refractometry as described previously.[18]

Western Blot

Proteins of each fraction were precipitated with trichloroacetic acid (20% final concentration; Sigma-Aldrich) and the protein concentration was determined by a Coomassie blue protein detection assay (Thermo Scientific, Rockford, Illinois, United States). From each fraction, equal amounts of protein or equal amounts of volume were dissolved in reducing sample buffer, loaded on 8 to 16% gradient polyacrylamide gel electrophoresis (PAGE) gels (Biorad, Hercules, California, United States) and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, Massachusetts, United States). Blots were incubated with anti-TF (4503, Sekisui Diagnostics), anti-caveolin-1 (marker for caveolar DRMs, caveolae/rafts isolation kit, Sigma-Aldrich), anti-flotillin-1 (marker for DRMs, BD Transduction Laboratories, San Jose, California, United States), anti-clathrin-HC (BD Transduction Laboratories), anti-TF pathway inhibitor (TFPI; LifeSpan Biosciences, Seattle, Washington, United States) and anti-protein disulphide isomerase (PDI, Thermo Scientific). As secondary antibodies, goat-anti-mouse (GAM)-horseradish peroxidase (HRP) (Dako, Glostrup, Denmark) or goat-anti-rabbit (GAR)-HRP (Dako, only used for anti-caveolin) were used. To visualize the bands, membranes were incubated with a fivefold diluted peroxidase substrate (LumiLight, Roche Diagnostics, Almere, The Netherlands) for 5 minutes, followed by analysis of luminescence using a LAS3000 luminescence image analyser (Fuji, Valhalla, New York, United States).

Fibrin Generation Test

Of each gradient fraction, 20 µL was added to vesicle-depleted normal pool plasma (70 µL) to measure the clotting time (CT) as described previously,[19] in the absence or presence of anti-FVII (Sanquin, Amsterdam, The Netherlands), or in the presence of lactadherin (HTI, Essex Junction, Vermont, United States). In earlier control experiments,[19] we compared the activity and specificity of anti-FVIIa and anti-TF in vesicle-depleted normal plasma to inhibit thromborel- and kaolin-induced fibrin generation. Both anti-FVIIa and anti-TF (4502, Sekisui Diagnostics) completely inhibited thromborel-induced fibrin generation but did not inhibit kaolin-induced fibrin generation. In the experiments described herein, we used anti-FVIIa because this antibody can be used at a lower concentration (1.0 µg/mL) than anti-TF (7.8 µg/mL) to completely inhibit TF-initiated coagulation. The extent of inhibition was calculated as follows: [(CT at ½ V_{max} of fraction X in the presence of anti-FVII) – (CT at ½ V_{max} of fraction X in the absence of anti-FVII)] / CT at ½ V_{max} of fraction X in the absence of anti-FVII; V_{max}: the maximum optical density measured in the test. TF activity in plasma membrane fractions was calculated using Innovin Reagent (Siemens Healthcare, Erlangen, Germany). Innovin, a mixture of recombinant TF and phosphatidylserine (PS), was diluted to make a calibration curve as described earlier (►**Supplementary Fig. S1B**, available in the online version).[20,21] Because both TF

and PS will be diluted, and PS may be a rate-limiting factor at low concentrations of TF, the real TF activity may be slightly underestimated at the lower TF activity range.

Inhibition of PDI

MDA-MB-231 cells were cultured in the presence of the PDI inhibitor 16F16 (50 µM; Sigma-Aldrich) or vehicle, dimethyl sulfoxide (DMSO), as control. Then, plasma membranes were isolated and fractionated in the absence (DMSO cultured cells) or presence (16F16 cultured cells) of 16F16 (50 µM). In addition, in an extra sample of DMSO cultured cells, 16F16 was added only during fractionation of isolated plasma membranes. 16F16 inhibited PDI by 61% ($n = 5$; data not shown), as determined in the PROTEOSTAT PDI Assay (Enzo, Farmingdale, New York, United States). After fractionation of the plasma membranes, the coagulant activity of TF was analysed in the nine fractions by fibrin generation test in the absence or presence of anti-FVII as described.

Presence of Phosphatidylserine

The presence of PS on DRMs was studied by flow cytometry. Fractions (20 µL) were stained with lactadherin-fluorescein isothiocyanate (FITC) (2.5 µL; HTI) and incubated for 2 hours at room temperature. Thereafter, 200 µL of filtered (50 nm) PBS/0.32% citrate was added and samples were measured for 1 minute at a flow rate of 3.0 µL/min using an Apogee A60 flow cytometer (Apogee Flow systems, Hemel Hempstead, UK), which has a detection limit of single 100 nm silica beads. The number of PS-containing DRM per fraction was calculated as follows:

$$\frac{N\ (number\ of\ positive\ events)}{t\ (minutes) \times flow\ rate\ (iL/minutes)} \times 1{,}000 \times c$$

= concentration of PS-containing DRM (events/mL).

Results

TF Distribution in Whole Cell Lysates

To develop a model system in which the distribution of TF can be studied in detail, we used a TF-deficient human pancreatic cell line (MIA PaCa-2), which was transfected with full-length human TF. First, we determined the *relative* distribution of TF and the DRM proteins flotillin and caveolin in whole cell lysates in this system by loading equal concentrations of protein from each fraction on the gel. As shown in ►**Fig. 2A**, the low density

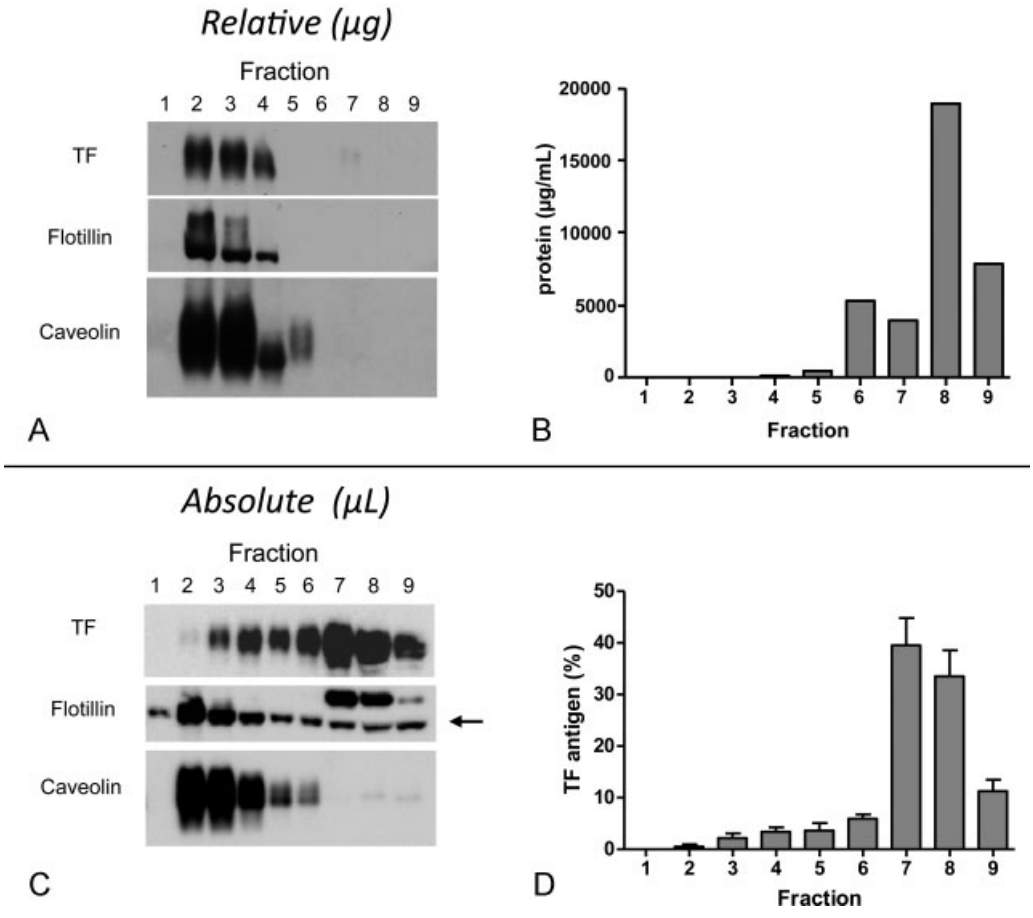

Fig. 2 Tissue factor (TF) distribution in whole cell lysates. (**A**) Relative distribution of TF in cell lysates of TF-transfected MIA PaCa-2 cells. Western blots incubated with anti-TF, anti-caveolin and anti-flotillin (both markers of detergent-resistant membranes [DRMs]), showing their *relative* distribution in Triton X-100 OptiPrep gradient fractions of whole cell lysates. Fractions are normalized per µg protein. (**B**) The protein concentration of each gradient fraction. (**C**) Absolute distribution of TF in cell lysates. Western blots incubated with anti-TF, anti-caveolin and anti-flotillin, showing their *absolute* distribution in Triton X-100 OptiPrep gradient fraction of cell lysates. Fractions are normalized per volume. (**D**) The absolute quantities of TF in Triton X-100 OptiPrep gradient fractions of cell lysates as determined by enzyme-linked immunosorbent assay (ELISA) (fractions normalized per volume, $n = 3$) expressed as percentage (mean ± standard deviation [SD]) of the total amount of TF in cell lysates.

fractions 2 to 4 contain TF, flotillin and caveolin. Because the protein concentration in fractions 1 to 5 is low compared with fractions 6 to 9 (►**Fig. 2B**), almost the entire (volume of) fractions 1 to 5 had to be loaded on gel, whereas only small aliquots of fractions 6 to 9 were used. Although the results of ►**Fig. 2A** suggest a clear co-localization of TF, flotillin and caveolin, these data do not reflect the real (quantitative) distribution of these proteins.

The *absolute* distribution of TF was studied by applying equal volumes from each fraction on gel (►**Fig. 2C**). The bulk of TF is predominantly present in fractions 7 to 8, and to a lesser extent in fractions 3 to 6 and 9. Flotillin is present in all fractions, but mostly in fractions 2 to 3, whereas caveolin is present mainly in fractions 2 to 6. The results of the absolute distribution of TF are confirmed by TF ELISA ($n = 3$, ►**Fig. 2D**). Thus, although TF is *relatively* enriched in fractions 2 to 4 (►**Fig. 2A**), the bulk of TF is mostly present in fractions 6 to 9 (►**Fig. 2C**). Therefore, in the rest of our studies we determined the *absolute* distribution of TF.

Distribution of TF in Plasma Membranes of MIA PaCa-2 Cells

TF is also associated with intracellular membranes. Such TF is inaccessible to FVII and thus cannot initiate coagulation. Therefore, this intracellular TF is a confounder which needs to be removed before one can study the real interaction(s) between the plasma membrane (exposed) TF and coagulation. In the purified plasma membranes of TF-transfected MIA PaCa-2 cells, fractions 6 to 8 contained detectable amounts of TF, flotillin and caveolin (►**Fig. 3A** and **B**). In two independent experiments, the TF-dependent coagulant activity was detectable in fractions 2 to 6 (►**Fig. 3C**; ►**Supplementary Fig. S1A** and **S1B** [available in the online version]), but not in fractions 7 and 8 which contain most of the TF antigen (►**Fig. 3B**). TFPI (►**Fig. 3A**) was below the detection limit in all fractions, but fractions 7 to 8 contain detectable levels of PDI (►**Fig. 3A**), suggesting that PDI may be involved in the regulation of the TF coagulant activity in these fractions. Because the results in ►**Figs. 2** and **3** are based on an overexpression

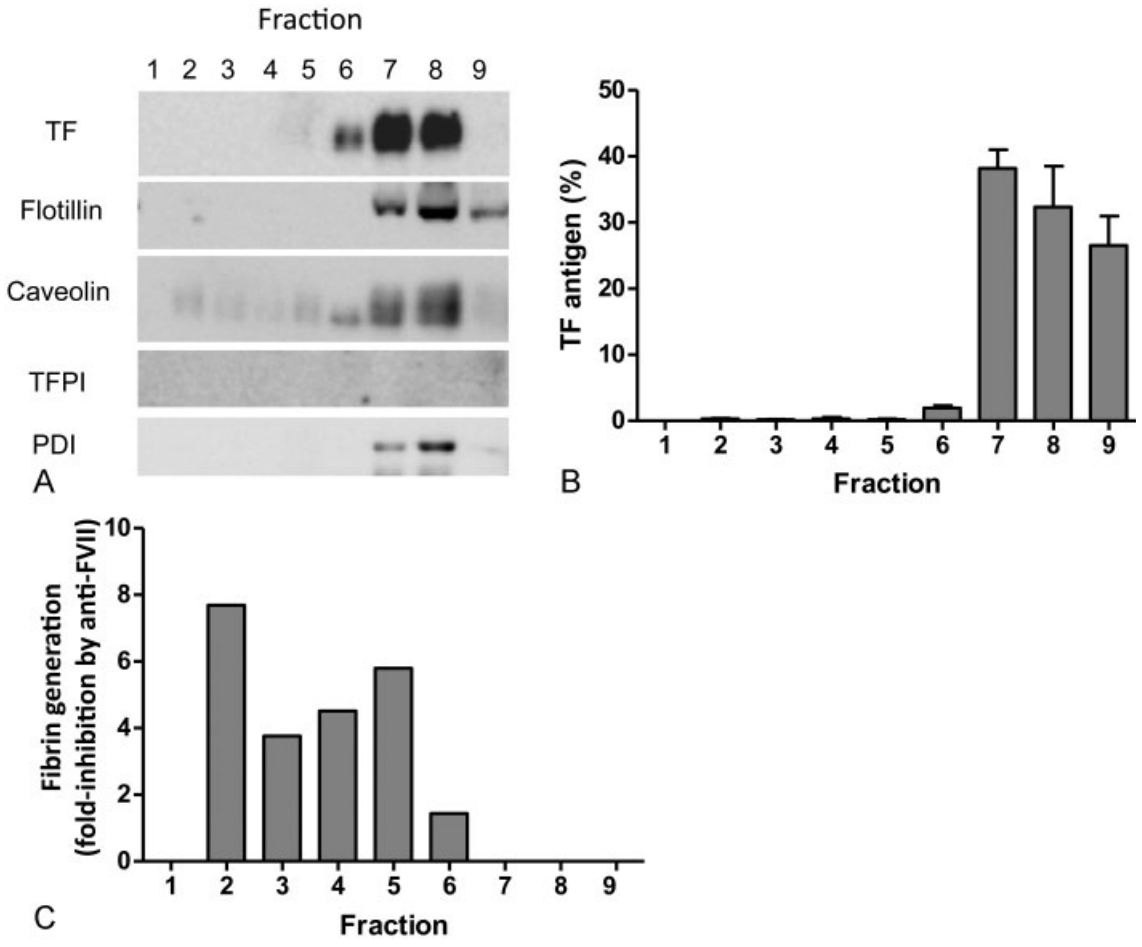

Fig. 3 Absolute distribution of tissue factor (TF) in plasma membranes of MIA PaCa-2 cells. (**A**) Absolute distribution of TF in purified plasma membranes of TF-transfected MIA PaCa-2 cells. Western blots incubated with anti-TF, anti-flotillin and anti-caveolin, anti-tissue factor pathway inhibitor (TFPI) and anti-protein disulphide isomerase (PDI,) showing their *absolute* distribution in Triton X-100 OptiPrep gradient fractions of plasma membranes. Fractions are normalized per volume. (**B**) The absolute quantities of TF in Triton X-100 OptiPrep gradient fractions of plasma membranes as determined by enzyme-linked immunosorbent assay (ELISA) (fractions normalized per volume, $n = 3$) expressed as percentage (mean ± standard deviation [SD]) of the total amount of TF in plasma membranes. (**C**) TF coagulant activity in Triton X-100 OptiPrep gradient fractions of plasma membranes as measured by capacity to induce fibrin formation ($n = 2$, mean). Of each fraction, fibrin formation is expressed as the factor of inhibition of ½ V_{max} by anti-FVII.

system, we also studied the distribution of TF in purified plasma membranes of cells constitutively producing TF.

Distribution of TF in Plasma Membranes of MDA-MB-231 Cells

Plasma membranes of the human breast cancer cell line MDA-MB-231 were purified and analysed (►**Fig. 1**). TF was detectable in fractions 2 to 3 and mostly in fractions 6 to 9 (►**Fig. 1A**). Flotillin was detectable in fractions 2, 7 and 8. Caveolin was abundantly present in fractions 2 to 3, decreased thereafter but increased again in fractions 6 to 7, suggesting that there may be distinct forms of DRMs present. To confirm whether these DRMs are similar to the earlier reported DRM-L and DRM-H,[1] we determined the density of the fractions as described by Ford et al.[18] ►**Fig. 1B** shows the density of each of the nine fractions. The density of fractions 2 to 4 corresponds to the earlier reported density of DRM-L, and the density of fractions 6 to 8 corresponds to DRM-H. Because the distributions of clathrin and calnexin are limited to DRM-H,[1,22] clathrin (►**Fig. 1A**) and calnexin (data not shown) were measured and found to be present in the later fractions, confirming the identification of DRM-H.[1,22]

In 7 out of 8 earlier studies on TF distribution (►**Table 1**), sucrose density gradients were applied with a density range that was insufficient to separate DRM-H from detergent soluble compartments of the plasma membrane (►**Fig. 1B**, grey area). Consequently, the DRM-H-associated proteins, including TF, will appear as detergent sensitive proteins and results may therefore have been misinterpreted.

The coagulant activity of TF was detectable in DRM-L (►**Fig. 1C**, ►**Supplementary Fig. S2A** and **S2B** [available in the online version], fractions 2–4), whereas the DRM-H fractions containing the bulk of TF, i.e. fractions 6 to 9, had a low or undetectable coagulant activity, confirming earlier studies that the presence of TF does not necessarily coincide with a detectable TF coagulant activity.

TFPI was undetectable in all fractions, but PDI was present in the DRM-H fractions 7 to 8 (►**Fig. 1A**). Taken together, the results obtained for plasma membranes from MDA-MB-231 cells are essentially similar to those of TF-transfected MIA PaCa-2 cells (►**Fig. 3**).

Distribution of TF in Plasma Membranes of Human Vascular Smooth Muscle Cells

Finally, we studied the distribution of TF in purified plasma membranes of human vascular SMCs which constitutively produce TF. Comparable to MIA PaCa-2 (►**Fig. 3**) and MDA-MB-231 cells (►**Fig. 1**), a modest amount of TF was detectable in DRM-L fraction 2, and the bulk of TF was present in DRM-H fractions 6 to 8 (►**Fig. 4A**). Flotillin was present in DRM-H fraction 7, and caveolin was present again in two clearly distinguishable fractions, revealing the presence of both DRM-L and DRM-H also in plasma membranes of SMCs. Clathrin was again present in DRM-H fractions 7 to 8, TFPI was undetectable and PDI was detectable in DRM-H fractions 7 to 9. Again, the coagulant activity of TF was present mostly in DRM-L fractions 2 to 4 but undetectable in DRM-H fractions (►**Fig. 4B**, ►**Supplementary Fig. S3A** and **S3B** [available in the online version]). These results confirm the results obtained by TF-transfected MIA-PaCa-2 cells (►**Fig. 3**) and breast cancer cells MDA-MB-231 (►**Fig. 1**), suggesting a common TF distribution pattern within purified plasma membranes that may be involved in the regulation of the TF coagulant activity.

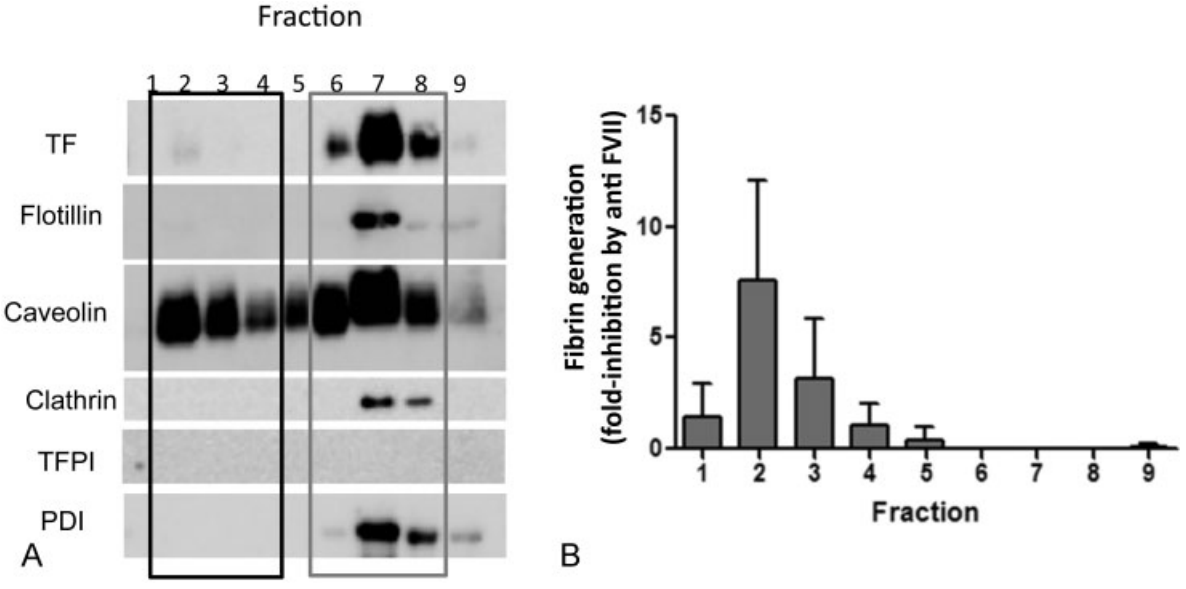

Fig. 4 Absolute distribution of tissue factor (TF) in plasma membranes of human vascular smooth muscle cells. (**A**) Absolute distribution of TF in purified plasma membranes of human smooth muscle cell (SMC) in two forms of detergent-resistant membranes (DRMs). Western blots incubated with anti-TF, anti-flotillin and anti-caveolin, anti-clathrin, anti-tissue factor pathway inhibitor (TFPI) and anti-protein disulphide isomerase (PDI), showing their *absolute* distribution in Triton X-100 OptiPrep gradient fractions of plasma membranes. Fractions are normalized per volume. (**B**) TF coagulant activity in Triton X-100 OptiPrep gradient fractions of plasma membranes as measured by capacity to induce fibrin formation ($n = 3$, mean ± standard deviation [SD]). Of each fraction, fibrin formation is expressed as the factor of inhibition of ½ V_{max} by anti-FVII.

Regulation of TF Coagulant Activity in Detergent-Resistant Membranes of MDA-MB-231

DRM-H fractions contain the bulk of TF but lack coagulant activity (►**Figs. 1**, **2**, **3**). This lack of coagulant activity may be due to the presence of PDI and/or the presence of an unknown inhibitor, or due the composition of the micro-environment itself. Inhibition of PDI by 16F16 during culture and/or isolation did not affect the coagulant activity of TF in DRM-L fractions 3 to 4 (►**Fig. 5A** and **C**), and did not restore the coagulant activity of TF in the DRM-H fractions 6 to 8 (►**Fig. 5B** and **C**). Although it should be noted that the inhibition of PDI was ~60% in an activity assay and thus incomplete, and the experiment was performed only once, our findings suggest that the lack of detectable coagulant activity in the DRM-H fractions seem unlikely to be regulated by PDI.

Because the role of PDI was not observed in our system, we investigated also a possible contribution of PS on the coagulant activity of TF in plasma membrane DRMs of MDA-MB-231. In the presence of the PS-binding protein lactadherin, the coagulant activity of TF in the DRM-L fractions was inhibited (►**Fig. 6A** and **C**), whereas—as expected—no effect was observed on non-coagulant TF in the DRM-H fractions (►**Fig. 6B** and **C**). Analysis of the presence of PS revealed that the DRM-L fractions have an increased concentration of PS compared with DRM-H (►**Fig. 6D**, ►**Supplementary Fig. S4A** and **S4C** [available in the online version]), and contain more PS molecules per DRM (►**Supplementary Fig. S4A** and **S4C**, available in the online version) when compared with DRM-H (►**Supplementary Fig. S4B** and **S4C**, available in the online version). These findings suggest that the lack of PS may explain—at least in part—the lack of a detectable coagulant activity of TF in DRM-H. Taken together, our results show that the presence or absence of PS in DRMs is likely to be the main regulator of the coagulant activity of TF.

Discussion

Our study shows that purified plasma membranes contain both coagulant and non-coagulant TF which is present in

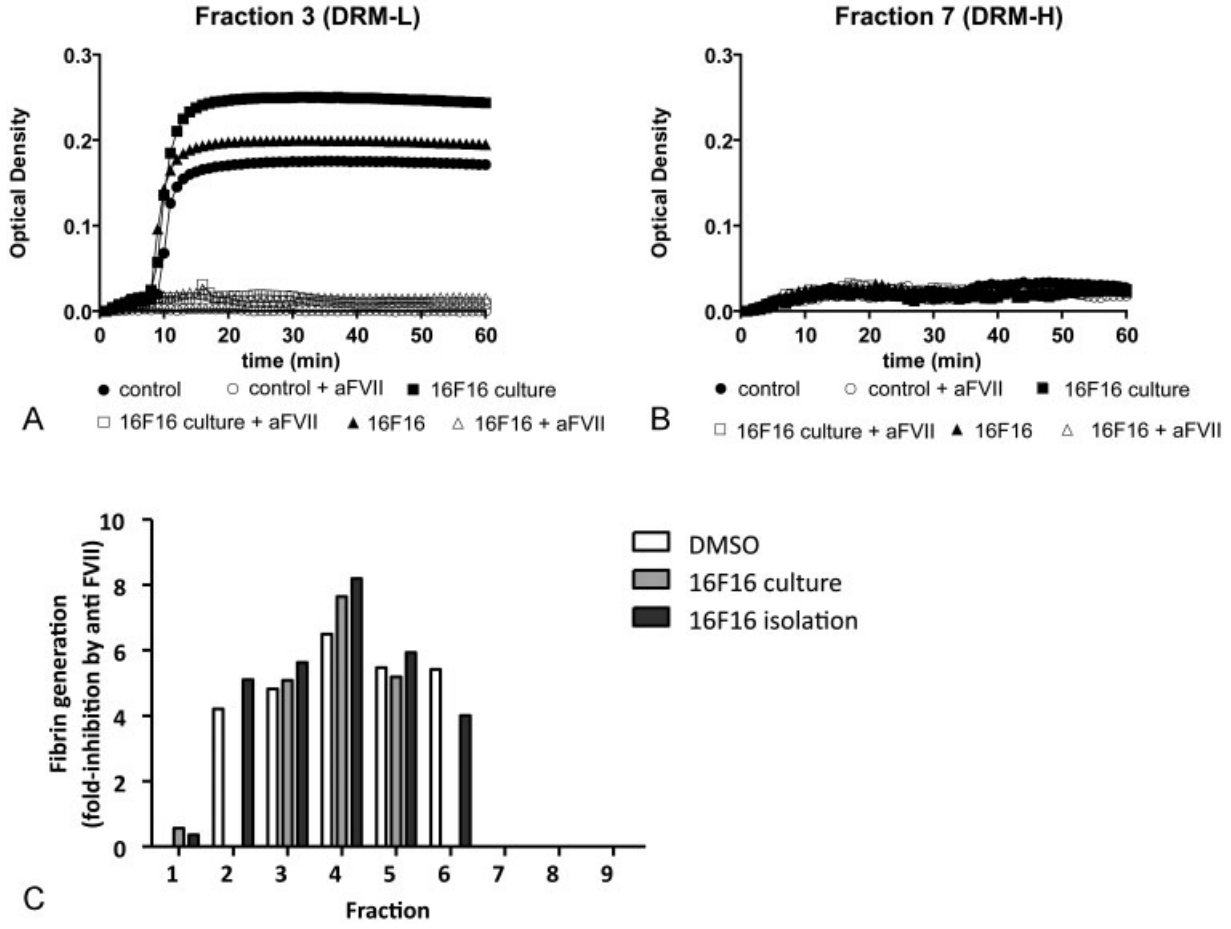

Fig. 5 Influence of protein disulphide isomerase (PDI) on tissue factor (TF) coagulant activity of MDA-MB-231 cells. PDI was inhibited by the addition of 16F16 during cell culture or during isolation of plasma membranes and fractionation of MDA-MB-231 cells. Fractions of isolated plasma membranes of cells incubated with dimethyl sulfoxide (DMSO) were used as control. (**A**) Fibrin generation curves of fractions 3 of PDI inhibited and control plasma membranes in the absence or presence of anti-FVII (aFVII). (**B**) Fibrin generation curves of fractions 7 of PDI inhibited and control plasma membranes in the absence or presence of anti-FVII. (**C**) TF coagulant activity of all nine Triton X-100 OptiPrep gradient fractions of PDI inhibited and control plasma membranes as measured by capacity to induce fibrin formation. Of each fraction, fibrin formation is expressed as the factor of inhibition of ½ V_{max} by anti-FVII.

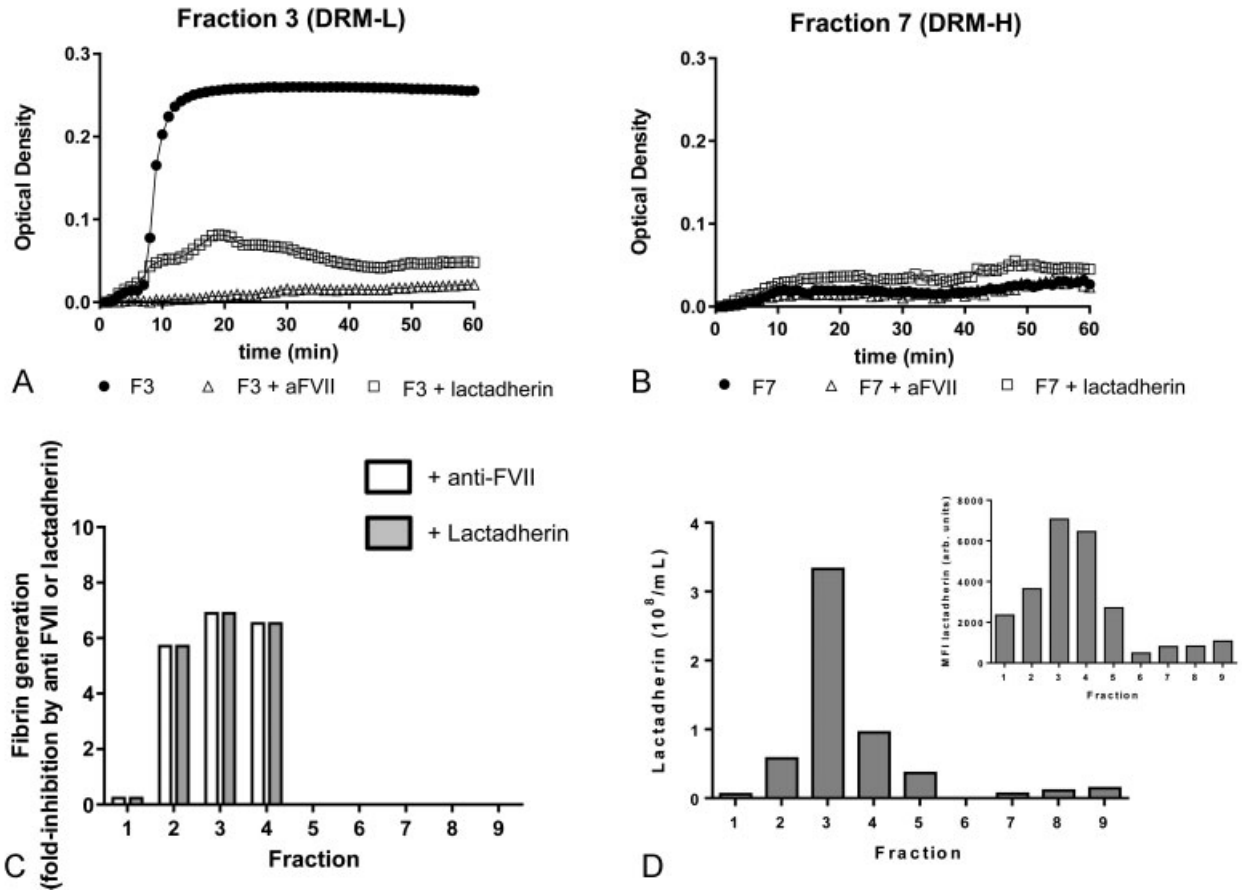

Fig. 6 Influence of phosphatidylserine (PS) on tissue factor (TF) coagulant activity of MDA-MB-231 cells. To investigate the presence and role of PS on the coagulant activity of TF in each fraction, the capacity of each fraction to generate fibrin was studied in the absence or presence of lactadherin to block PS. (**A**) Fibrin generation curves of fraction 3 of in the absence or presence of anti-FVII (aFVII) or lactadherin. (**B**) Fibrin generation curves of fraction 7 of in the absence or presence of anti-FVII (aFVII; open bar) or lactadherin (grey bar). (**C**) TF coagulant activity of all nine Triton X-100 OptiPrep gradient fractions of plasma membranes as measured by capacity to induce fibrin formation. Of each fraction, fibrin formation is expressed as the factor of inhibition of ½ V_{max} by anti-FVII or lactadherin. (**D**) Number of PS exposing detergent-resistant membrane (DRM) in each fraction were studied by flow cytometry by staining with lactadherin-fluorescein isothiocyanate (FITC). Shown is the number of lactadherin positively stained events $\times$ 10^8/mL fraction. Inset: Mean fluorescence intensity of all fractions stained with lactadherin.

physical and biochemical distinct microenvironments. The coagulant form of TF is localized in DRM-L, and the non-coagulant form is present in DRM-H. Our study may facilitate the understanding of several controversies in the available literature regarding TF. ►**Table 1** shows that most investigators studied the *relative* distribution of TF. Although this approach gives insight into the relative enrichment of TF, insight into the real distribution of TF remained unclear. Furthermore, in most studies a sucrose gradient was applied which is unable to separate DRM-H from detergent-sensitive compartments of the plasma membrane.[7] Only by using a gradient capable of separating DRM-H from detergent-sensitive compartments and by measuring the absolute distribution, insight into the real distribution of TF in plasma membranes can be obtained. In addition, within our model, we tried to elucidate the contributions of PDI and PS to regulate the TF coagulant activity.

At present, there is no consensus on the role of PDI in the regulation of the TF coagulant activity. Whereas PDI was reported to play a role in decryption of TF via oxidoreductase activity, initiation of PS exposure and/or by coupling of inflammation and coagulation via a PDI-dependent regulation of a P2 $\times$ 7 receptor-dependent signalling pathway,[23] Krudysz et al showed that the coagulant activity of TF is independent of decryption by PDI since in native TF all cysteines are in the oxidized form,[24] previously shown to be the coagulant active form/conformation of TF.[25] The negligible role of PDI in TF coagulant activity was further strengthened by the fact that PDI was not capable to convert the non-coagulant form of TF, induced by reduction of the four cysteines, into the coagulant form of TF. Also, our study indicates that PDI plays no prominent role in the regulation of the TF coagulation activity. For the three cell types studied, the fractions containing coagulant TF do not contain detectable levels of PDI, and the coagulant activity is unaffected by an inhibitor of PDI. Because the latter was a single experiment performed with only one of the three cell types studied, and because the PDI inhibitor incompletely

blocked the PDI activity, we should be careful with over-interpretation of the results. At present, we cannot explain the results of fractions 2 and 6 (►**Fig. 5C**), but it is clear that to understand the role of PDI in the regulation of the TF coagulant activity in more detail, additional studies are required.

The role of PS on the coagulant activity has been investigated by various research groups.[26–31] Although PS is essential for TF coagulant activity, the precise localization and interaction of TF and PS remain obscure. Shaw et al using artificially generated nano-disks showed that a high local concentration of PS is necessary for the full proteolytic activity of the TF-FVII complex.[31] Our study seems to confirm their finding in native plasma membrane fractions, because the TF coagulant activity exclusively co-localizes with PS in DRM-L. In addition, despite the apparent abundance of TF in DRM-H when compared with DRM-L, DRM-H lacks both PS and TF coagulant activity. Addition of PS to DRM-H was unable to restore the TF coagulant activity (data not shown), indicating that external PS cannot simply replace the PS present within the plasma membrane. Whether the interaction between TF and PS is necessary to de-encrypt the TF coagulant activity has not been determined in our study. Previous studies showed that amino acids in the C-terminus of TF form a 'lipid-binding region' that interacts with PS,[32,33] although also other domains may play a role.[32] Taken together, we demonstrate that the regulation of the TF coagulant activity may be due to the co-localization with PS in micro-domains within the plasma membrane.

Our results show that the coagulant form of TF is DRM-L-associated. Methyl-β-cyclodextrin (MßCD) removes cholesterol and is often used to disrupt DRM-L. The effects of MßCD on TF distribution and coagulant activity, however, are controversial.[5,7,8,10,14,15] In a control experiment, we determined the TF distribution and coagulant activity in purified plasma membranes of MDA-MB-231cells after treatment with MßCD (20 mmol/L; 1 hour, 37°C). Although the distribution of TF was unaffected, a decrease was observed in the presence of caveolin, flotillin in fraction 2 and a decreased in coagulant activity of TF in fractions 2 to 4 (data not shown). These preliminary data confirm earlier results from Dietzen et al, who showed that inhibition of cholesterol synthesis reduced the TF coagulant activity but had no effect on the distribution of TF.[8] We hypothesize that DRM-L is more sensitive than DRM-H to the removal of cholesterol or inhibition of cholesterol synthesis,[8] possibly because DRM-L have a lower concentration of cholesterol than DRM-H.[34] In vivo, statins decreased the hypercoagulability in cardiac transplant recipients,[35] hypercholesterolemia patients[36] and in patients with combined hyperlipemia.[37] Based on our results, one may speculate that part of this effect may be due to disruption of the DRM-L, thereby decreasing the coagulant activity of TF. Clearly, further research is necessary to prove whether this hypothesis is valid. Taken together, our results provide novel insights in a common mechanism in which plasma membrane micro-domains are involved in the regulation of the coagulant activity of TF.

What is known about this topic?

- Both intracellular and plasma membranes contain tissue factor (TF).
- TF in plasma membranes does not always trigger coagulation.
- Regulation of the TF coagulant activity is still poorly understood.

What does this paper add?

- Plasma membrane contains two types of detergent-resistant membranes (DRMs).
- Non-coagulant and coagulant TF are associated with different DRMs within the plasma membrane.
- The DRM composition affects the coagulant activity of TF.

Authors' Contributions

A.N.B., Y.Y. and R.N. designed the study, interpreted results and wrote the manuscript; C.M.H. and N.H. performed experiments; W.R. designed part of the study and interpreted results; A.S. interpreted results and contributed to writing the manuscript; and all authors approved the final version of the manuscript.

Conflict of Interest

None.

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