Caveolin-1 Enhances Tissue Factor Pathway Inhibitor Exposure and Function on the Cell Surface*§

Cristina Lupu‡§, Xiaohong Hu‡, and Florea Lupu‡¶

From the ¶Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, and the §Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104

Tissue factor pathway inhibitor (TFPI) blocks tissue factor-factor VIIa (TF-FVIIa) activation of factors X and IX through the formation of the TF-FVIIa-FXa-TFPI complex. Most TFPI in vivo associates with caveolae in endothelial cells (EC). The mechanism of this association and the anticoagulant role of caveolar TFPI are not yet known. Here we show that expression of caveolin-1 (Cav-1) in 293 cells keeps TFPI exposed on the plasma membrane surface, decreases the membrane lateral mobility of TFPI, and increases the TFPI-dependent inhibition of TF-FVIIa. Caveolae-associated TFPI supports the co-localization of the quaternary complex with caveolae. To investigate the significance of these observations for EC we used RNA interference to deplete the cells of Cav-1. Functional assays and fluorescence microscopy revealed that the inhibitory properties of TFPI were diminished in EC lacking Cav-1, apparently through deficient assembly of the quaternary complex. These findings demonstrate that caveolae regulate the inhibition by cell-bound TFPI of the active protease production by the extrinsic pathway of coagulation.

Tissue factor (TF) is a transmembrane protein that triggers blood coagulation in vivo. Assembly of TF with factor VIIa (FVIIa) on cell surfaces initiates limited proteolysis of factors IX and X (FX), leading to thrombin generation. TF elicits thrombogenic responses in sepsis, cancer, and atherosclerosis (1–4), promotes metastasis, angiogenesis, and intima hyperplasia after arterial injury (5, 6), and acts as signaling events, such as membrane trafficking and cellular signal transduction (reviewed in Ref. 22).

TFPI in vivo is mainly produced by endothelial cells (EC), has an intact C terminus, and associates with the plasma membrane through mechanisms that are not fully identified (3, 10–13). Heparin releases a portion of the full-length, functionally active cell-associated TFPI, either from cell surface-binding sites or from intracellular stores (14, 15). However, the bulk of heparin-resistant cellular TFPI is released by phosphatidylinositol-phospholipase C in vitro, which indicates that most cellular TFPI associates with the cell surface via a glycosphingolipid (16, 17). Furthermore, endogenous TFPI in resting endothelium (10), monocytes (18), and the ECV304 cell line (11, 16) partitions in low-density fractions insoluble in cold detergent (lipid rafts). In monocytes and ECV304 endogenous TFPI inhibits efficiently FVIIa-TF activity through translocation of the TF-FVIIa-FXa complex in lipid rafts (11). These findings suggest that cell-bound TFPI, particularly the lipid raft-associated pool, plays a critical role in regulating cell surface FVIIa-TF and FXa activity.

Lipid rafts are domains rich in cholesterol and sphingolipids that can exist by themselves or as caveolae. Caveolae are small (50–80 nm) plasma membrane invaginations that have a protein “coat” composed of caveolin family members. Caveolin-1 (Cav-1) is an integral membrane protein and the principal component of caveolae (19). The role of Cav-1 in caveolae formation was confirmed in Cav-1-deficient cells, which lack morphologically identifiable caveolae (20). Expression of Cav-1 in these cells induces caveolae formation (21). Caveolae are multifunctional organelles in which Cav-1 plays a direct role in various events, such as membrane trafficking and cellular signal transduction (reviewed in Ref. 22).

We reported previously that TFPI is localized in caveolae in EC both in vitro and in vivo (3, 10). The mechanism of TFPI association with caveolae and the anticoagulant role of caveolar TFPI are not yet known. We report here that Cav-1 regulates the distribution and function of TFPI in HEK293, a cell system where we controlled the expression of Cav-1 and TFPI by transfection. We show for the first time that caveolae keep TFPI associated with the cell surface and enhance the anticoagulant activity of the inhibitor. Furthermore, using RNA interference (RNAi) to deplete HUVEC and EA.hy926 cells of Cav-1, we show that EC which lacks Cav-1 displayed several-fold enhanced procoagulant activity. In conclusion, we identified Cav-1 as an active regulator of TFPI-dependent inhibition.
Caveolin-1 Modulates TFPI Function

22309

of TF-FVIIa activity, which adds the hemostatic function as a novel dimension to the biological significance of caveolae.

Experimental Procedures

Reagents and Cells—Antibodies and suppliers used were: monoclonal antibodies (mAb) TFPiK-9 against human r-TFPI (gift from Dr. T. Hamuro, KAKETSUKEN, Kumamoto, Japan); rabbit anti-human r-TFPI (1–240) IgG (23); mAb JLI-8 anti-green fluorescent protein (GFP, BD Biosciences Clontech); goat anti-human TF IgG (American Diagnostica); mAb 10H10 and 9-5B7 against human r-TF (kind gifts from Dr. J. Morrissey, University of Illinois, Urbana-Champaign, IL); rabbit anti-Cav-1 IgG (sc-894, Santa Cruz Biotechnology); mAb anti-Cav-1 (BD Transduction); mAb HPC4, recognizes a calcium-dependent epitope in activated protein C (24) (gift from Dr. C. Esmon in our institute); mAb anti-human lamin A/C (Serotec); horse anti-mouse IgG/FTTC (Vector Laboratories); donkey anti-goat IgC5y, donkey anti-rabbit IgG-Cy3, and donkey anti-rabbit IgG-Cy3 (Jackson ImmunoResearch Laboratories). Human coagulation factors VIIa, X, and Xa were from Enzyme Research Laboratories. Chromogenic substrate S-2765 specific for FXa was from DiaPharma. Human tumor necrosis factor-α was from R&D Systems, ionomycin was from EMD Biosciences, and human TNFα (0.22 μg of protein) was from Dade Behring. Restriction enzymes were from Promega. Cell culture media and supplements were from Invitrogen and HyClone. Rabbit brain thromboplastin and all other reagents were from Sigma unless otherwise stated. Human embryonic kidney epithelial cells HEK293 were grown in Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin, 1 mg/ml streptomycin, and 10% fetal calf serum (FCS) and incubated in 5% CO2, 95% humidified air at 37 °C. The immortalized hybrid EC line EA.hy926 (gift from Dr. Cora-Jean Edgell, University of North Carolina, Chapel Hill, NC) was maintained in Dulbecco’s modified Eagle’s medium supplemented with 4 mM glucose, 15 mM HEPES, 10% FCS, and antibiotics, in 10% CO2, 90% humidified air at 37 °C. Human umbilical vein EC (HUVEC) were used at passages 1–5, and were maintained in antibiotic-free medium 199 with Earles salts, supplements, 1% fetal bovine serum (FBS), and 1.100 v/v% penicillin and streptomycin. Mouse brain was homogenized in sodium phosphate buffer (pH 8.1), followed by centrifugation and phase separation was induced for 5 min at 37 °C, followed by brief centrifugation to separate detergent and aqueous phases. The same buffer lacking detergent was added and the extraction repeated once before the aqueous phase was collected. A further aqueous extraction was performed and the remaining experiments were treated with 1% Triton X-100, washed with 0.3% bovine serum albumin and 0.1% Triton X-200 in PBS, incubated for 1 h with primary antibody diluted in PBS containing 1% bovine serum albumin and 0.1% Triton X-200, and then incubated for 1 h with appropriate secondary antibodies conjugated with alkaline phosphatase or horseradish peroxidase. The blots were developed with the DAB substrate kit for horseradish peroxidase or Vector Red AP substrate kit 1 (Vector Labs).

Transfection of Cav-1 X11 Cell Extracellular Membrane Monolayers on ice were scraped in ice-cold 0.1 M TBS, pH 8.1, plus intermediates, and lysed in pre-condensed 1% Triton X-114 in the same buffer by incubating for 1 h on ice with repeated mixing. Debris was removed by centrifugation and phase separation was induced for 5 min at 37 °C, followed by brief centrifugation to separate detergent and aqueous phases. The same buffer lacking detergent was added and the extraction repeated once before the aqueous phase was collected. A further aqueous extraction was performed and the remaining experiments were treated with 1% Triton X-100, washed with 0.3% bovine serum albumin and 0.1% Triton X-200 in PBS, incubated for 1 h with primary antibody diluted in PBS containing 1% bovine serum albumin and 0.1% Triton X-200, and then incubated for 1 h with appropriate secondary antibodies conjugated with alkaline phosphatase or horseradish peroxidase. The blots were developed with the DAB substrate kit for horseradish peroxidase or Vector Red AP substrate kit 1 (Vector Labs).

Immunofluorescence—Immunofluorescence was carried out as described (10). The effect produced by FVIIa/FX on the distribution of TF, TFPI, and Cav-1 was studied in cells fixed with 3% paraformaldehyde in PBS at room temperature at the end of the FX activation assay. Intracellular immunostaining was achieved on cells permeabilized with 0.1% Triton X-100.

Images were collected using a Nikon Eclipse inverted microscope equipped with a confocal Nikon C1 system, using a computer-controlled 488-nm argon laser to excite EGFP, and a helium-neon laser whose green line (544-nm) excites Cy3 and red line (633-nm) excites Cy5 or TO-PRO (nuclear stain). The images were collected using MetaMorph (Universal Imaging). The overlay was quantified using Adobe Photoshop (Adobe Systems), by determining the % of protein A that co-localizes with protein B (29). A-channel minus B-channel gave the non-overlapping A element intensity measured from the background. The resulting overlap signal was expressed as mean % of the total signal for each channel. Triple co-localization was analyzed similarly. Fluorescence intensity measurements were performed on a minimum of 50 cells/group, randomly chosen from at least five pictures for each experimental condition. Experiments were repeated three to four times. Statistical significance of the differences between groups was determined by t-test (Microsoft Excel), and the differences were considered significant when p < 0.05. Descriptive statistics (median values and range) and correlation analysis were performed with InStat (Macintosh).

Fluorescence Recovery after Photobleaching (FRAP)—For live cell recordings, 293 cells stably expressing EGFP-TFPI, Cav1-HPC4ep, or both, were grown on coverslips and mounted in a perfusion chamber at 37 °C (Biophtech FC520). The chamber was installed on the stage of the inverted microscope equipped with a temperature controlled ×83 oil immersion objective. The chamber was perfused with phenol red-free culture medium at 37 °C. Bleaching in 3–5 spots in regions of interest was performed at 37 °C with the 488-nm laser line at full power and full transmission for 2 s. Observation of fluorescence recovery over time was done at full laser power and 1% transmission, to avoid significant photobleaching. Image J software was used to measure pixel intensities in regions of interest, and to correct for overall bleaching by comparison with areas not exposed to the full power of the laser. The % recovery (recovery values × mean of pre-bleach values × 100) shows how much fluorescence returns to the area out of the amount of fluorescence before photobleaching.

Depletion of Cav-1 in EAhy926 and HUVEC by RNAi—Post-transcriptional silencing of the Cav-1 expression was achieved with two

Downloaded from http://www.jbc.org/ by guest on July 24, 2018
duplex RNA oligonucleotides (Dharmacon Research), each composed of 21 bases of sequence that is conserved in Cav-1 from several mammalian species (30). The sense strands of the duplexes were 5'-CCUgAUUGAgAgAAgGACgCgACgUgAcCdTdT-3' and 5'-CgAgAAgGACgCgACgUgAcCdTdT-3' (31). EC were seeded on coverslips to reach ~60% confluence on the following day. Silencing (si) RNA duplexes (25 nM final concentration) mixed with TransIT-TKO (Mirus) in Opti-MEM I were added to the cells in complete medium without antibiotics. Cells were incubated for 2–3 days and analyzed by confocal microscopy after immunostaining. Controls were as described under supplemental materials.

**Supplemental Data**—Details of the following protocols are available: construction and expression of pEGFP-C2/TFPI(C) and pSVZeo-Cav1-HPC4ep, primers, and conditions for reverse transcriptase-PCR, characterization, and selection of 293 cell clones, and RNAi experimental controls.

Fig. S1 demonstrates that EGFP-TFPI has distribution and function similar to native TFPI, therefore is a reliable marker for endogenous TFPI. Fig. S2 displays immunofluorescence characterization of the chimeras expressed in 293 cells. Fig. S3 details the testing and selection of 293 clones. Results of the RNAi controls are illustrated in Figs. S4 and S5 and demonstrate that the effects induced by Cav-1 siRNA transfection of EC on TFPI distribution and activity are because of the silencing of Cav-1 expression.

**RESULTS**

**EGFP-TFPI Is a Reliable Marker for Endogenous TFPI**—Using EA.hy926, we compared the expression and activity of EGFP-TFPI (a chimera with EGFP fused at the N terminus of TFPI) with the properties of native TFPI. Western blotting showed a protein with molecular mass ~65,000 Da immunoreactive with both anti-GFP and anti-TFPI IgGs, which were absent from native (N) and cells expressing EGFP only (GFP) (Fig. S1, A). The distribution and properties of native and chimerical TFPI were similar. Fluorescence imaging showed that 98% of EGFP-TFPI overlapped the anti-TFPI IgG staining (Fig. S1, B, a, d, f, and e), indicating that the EGFP signal originated from the chimera and not from EGFP alone. Both EGFP-TFPI and native TFPI co-localized with endogenous Cav-1 (b, c, and f).

Enzyme-linked immunosorbent assay (ELISA) for TFPI antigen confirmed that EC overexpressing EGFP-TFPI synthesized and secreted two times more TFPI than native cells (Fig. S1, C, a). TFPI-mediated inhibition of exogenously added TF-FVIIa was similarly increased (Fig. S1, C, b). EGFP-TFPI matched the Triton X-114 partition properties of native TFPI, a finding that suggests direct or indirect glycosylphosphatidylinositol anchoring. Enzymatic cleavage of the glycosylphosphatidylinositol anchor with phosphatidylinositol-phospholipase C (10, 11, 32) substantially reduced TFPI partitioning into the detergent phase of Triton X-114 (Fig. S1, C, c).

Taken together, these data establish that EGFP-TFPI is a valid fluorescent indicator of native TFPI, and can be used to study the distribution and function of TFPI in live as well as fixed cells.

**Analysis of the Expression of EGFP-TFPI and Cav1-HPC4ep in 293 Cells**—In HEK293 transiently expressing Cav1-HPC4ep (a chimera where the small HPC4 epitope was fused to the C terminus of Cav-1), immunostaining with mAb HPC4 (24) revealed the typical distribution of native Cav-1 (Fig. S2, a–c). In cells expressing EGFP-TFPI 99% of the chimeras overlapped the immunolabeled TFPI, showing that no cleaved EGFP was present (Fig. S2, d–f). Co-localization of each protein with its tag exceeded 90% (Fig. S2).

Five stable cell clones were selected for each experimental condition. The cell clones were re-named: “TFPI” (express EGFP-TFPI only); “Cav” (cells express Cav1-HPC4ep alone); and “TFPI + Cav” (express both proteins). As detailed in Fig. S3, the total level of TFPI was not significantly different between TFPI cells and TFPI + Cav cells (panel A). Although the constitutive secretion of TFPI was significantly decreased in the presence of Cav-1. The capability of TFPI cells to prevent endogenous TF-dependent FX activation was significantly diminished as compared with TFPI + Cav cells (Fig. S3, B).

Native (wild type, wt) cells were also tested, either quiescent or after transfection with pEGFP-C2. There was no significant variation in TFPI distribution or function between these two conditions; therefore we used wt cells in all subsequent experiments.

Wt and Cav cells expressed very low levels of TFPI mRNA, whereas TFPI cells and TFPI + Cav cells expressed mRNA for both TFPI and EGFP-TFPI (Fig. 1A, semi-quantitative reverse transcriptase-PCR). Lysates of TFPI cells and TFPI + Cav cells displayed a protein band of ~65 kDa that was immunoreactive with both anti-GFP and anti-TFPI IgGs, but was absent from wt cells (Fig. 1B). Cav cells and TFPI + Cav cell lysates contain a protein that was immunoreactive with anti-Cav-1 IgG (band

![Fig. 1. Analysis of the expression of EGFP-TFPI and Cav1-HPC4ep in HEK293.](http://www.jbc.org/)}
at ~20 kDa), present only as trace in lanes “wt” and “T.” The equivalent band appeared in EA.hy926 lysates used as positive control (lane EA). Immunofluorescence confirmed that wt cells contained very low levels of TFPI and Cav-1 (Fig. 1C, a and b), and that EGFP-TFPI largely co-localized with Cav-1 (j-i).

Similar to TFPI in EC, EGFP-TFPI in 293 cells partitioned predominantly in the detergent phase of Triton X-114 (Fig. 1D). The equivalent of the 1:3 ratio between the water-soluble and detergent-soluble TFPI normally found in EC was achieved in 293 cells only in the presence of Cav-1 (Fig. 1D). The total amount of TFPI was not significantly different among the TFPI clones. Cav-1 expression in 293 cells induces formation of caveolae—We used freeze-fracture EM to analyze the formation of invaginations/caveolae in wt and Cav cells. In wt cells (Fig. 2a) the external leaflet of the membrane displayed a smooth aspect, hence lack of caveolae. Cav cells exhibited pits with dimensions and aspect typical of caveolae (Fig. 2b), thus confirming that expression of Cav-1 in deficient cells induced invagination of caveolae.

Kinetics of Fluorescent TFPI Were Different in the Presence and Absence of Cav-1—We used FRAP to quantify the kinetic properties of EGFP-TFPI in the plasma membrane of living cells. The fluorophore was bleached in “regions of interest” at the peripheral EGFP-TFPI-labeled rim of each cell and the fluorescence recovery was followed for 10 min. Non-bleached molecules diffused into the bleached areas according to their membrane mobility.

In the presence of Cav-1, clusters next to regions of interest maintained their fluorescence after bleaching and did not move into the bleach area, suggesting that only a little lateral diffusion of fluorescent TFPI took place (not shown). Fig. 3 shows the FRAP curves after curve fitting of the means of 10 independent recordings for each cell clone. The recovery of EGFP-TFPI in TFPI + Cav cells was much slower than in TFPI cells. The mobile fraction of EGFP-TFPI was ~35% in the presence of Cav-1 and ~85% in its absence (Fig. 3, p < 0.01).

Expression of Cav-1 in 293 Cells Increases Both Cell Surface TFPI and the Inhibition of FVIIa-TF by TFPI—From the measurement of TFPI and Cav-1 fluorescence intensity after immunostaining (Fig. 4A), it results that expression of Cav-1 in TFPI + Cav cells doubled or even tripled the amount of TFPI exposed on the cell surface (Fig. 4A, compare TFPI + Cav cell clones (TC) 1, 2, 4, and 5, with TFPI cell clones). Only TFPI + Cav cells displayed a significantly positive correlation ($r^2 = 0.92$) between Cav-1 expression and the level of cell surface TFPI. To determine the functional activity of the surface exposed TFPI, we assessed its inhibitory potency, both against exogenously added TF, and toward the endogenous TF, which is normally expressed by HEK293.

The functional activity of cell surface TFPI tested against exogenous TF-FVIIa was significantly different between the clones. We measured the capability of the TFPI exposed on the cell surface to prevent the activation of FX by pre-formed complexes consisting of 10 nM FVIIa and 1:15 diluted thromboplastin added in the overlying cells medium. The activity of TFPI, expressed as arbitrary units, was extrapolated from standard curves made with serial dilutions of normal human plasma, run in parallel in identical conditions. In Fig. 4B we illustrated for each cell clone the activity of cell surface TFPI expressed as milliunits, together with the corresponding TFPI antigen (ng) measured by enzyme-linked immunosorbent assay on cell monolayers (all values were normalized to $10^6$ cells).

TFPI cells displayed a significant increase of both antigen and activity of cell surface TFPI as compared with wt cells (p < 0.01 for all the clones). Nevertheless, there was no positive correlation between the activity and either TFPI or Cav-1 antigen levels (Fig. 4C, $r^2 = 0.31$ and 0.35, respectively). In TFPI + Cav cells, increases of up to five times of cell surface antigen led to an enhancement of TFPI activity of as much as 40 times over the wt cell levels (Fig. 4B, right panel; note the different scale of the y axis). In comparison with TFPI cells, the average 2.5-fold increase of TFPI antigen on the cell surface of TFPI + Cav cells could be translated into as much as 10 times higher inhibitory potency (Fig. 4B). After normalization for TFPI antigen levels it results that TFPI on TFPI + Cav cells were, on average, 3.5 times more potent than TFPI on TFPI cells in

![Freeze-fracture replicas](image)

**Fig. 2.** Expression of Cav-1 in deficient cells induces formation of caveolae. Wi and Cav1-HPC4-expressing 293 cells were processed by freeze-fracture EM. During freeze-fracture, the plasma membrane is split horizontally through the hydrophobic region of the bilayer, exposing the inner fracture faces of the two leaflets. View of the external leaflet of the plasma membrane shows a smooth aspect of the membrane in wt cells (a), and the presence of pits typical of caveolae in Cav cells (b, arrowheads). Bar, 500 nm.
inhibiting exogenous TF-FVIIa activity. Specificity controls included parallel assays run in the presence of 50 μg/ml anti-TFPI IgG, which abrogated almost entirely the activity of TFPI (not shown).

Only in Cav-1-expressing cells did the activity of cell surface TFPI correlate positively with the TFPI antigen, and implicitly with Cav-1 levels (Fig. 4C, $r^2 = 0.95$ and 0.93, respectively). The inhibitory activity of cell surface TFPI was severely impaired in the absence of Cav-1, even for cells that displayed equal or higher amounts of TFPI antigen (Fig. 4C, compare TFPI cell clones 4, 3, 5, and 1, with TFPI + Cav cell clone 3). Conversely, the presence of Cav-1 alone was enough to bring the activity of even very small amounts of TFPI antigen, such as the one present in Cav cells, up to levels that were equivalent with the activities measured on TFPI cells (Fig. 4B, compare Cav cell clones 3, 4, and 5 with TFPI cell clones 2, 4, and 5).

Next, we tested the inhibitory potency of TFPI against endogenous TF on TFPI cell clone 1 and TFPI + Cav cell clone 4. TF mRNA determined by reverse transcriptase-PCR and total TF antigen in cell lysates were comparable (not shown), indicating that the expression of TF was similar among the 293 cell clones. TFPI cells and TFPI + Cav cells displayed similar amounts of cell surface TF antigen (11 and 12.2 pg, respectively). FX was efficiently activated after the addition of FVIIa to 293 cell monolayers, following a brief incubation with ionomycin to expose the cryptic cell surface TF and/or phosphatidylserine. Inclusion of cycloheximide precluded changes because of de novo protein synthesis. Preincubation of cells with inhibitory anti-TF mAb 9-5B7 blocked the generation of FXa, confirming that FX activation was dependent on TF-FVIIa.

The overall capability of the cells to activate FX, assessed after inhibiting the available TFPI with anti-TFPI IgG, was defined as "total FXa." FXa generation curves for both TFPI cells and TFPI + Cav cells were linear over time for each concentration of FVIIa tested (Fig. 5A). The specific potential of the cells to generate FXa in the presence of TFPI, assessed using non-inhibited assays, was significantly different between the two cell clones (Fig. 5A). Whereas FXa generation increased continuously on TFPI cells, the activation of FX on TFPI + Cav cells stayed at very low levels, and, if it increased slightly, it was with a significant delay and slower rate. The differences of FXa generation were larger than what would be accounted for only by the differences in cell surface TFPI antigen: 45 pg in TFPI cells and 100 pg in TFPI + Cav cells.

The differences between total FXa (open symbols in Fig. 5) and the FXa generated in 15 min in the absence of anti-TFPI IgG (closed squares and circles) represent the amount of FX whose activation was prevented by TFPI, therefore giving the measure of the functional activity of cell surface TFPI (closed triangles). As shown in Fig. 5B, TFPI + Cav cells displayed significantly higher capabilities to prevent FXa generation than TFPI cells for all three concentrations of FVIIa tested ($p < 0.01$). Even after normalization of the values to account for the differences in the cell surface antigen, TFPI on TFPI + Cav
cells was still ~2 times more active in inhibiting FX activation than on TFPI cells. Cave-1 Co-localizes with TF-FVIIa-FXa-TFPI in 293 Cells—Using fluorescence microscopy we studied the spatial relationship between Cave-1, TF, and TFPI in the presence of FVIIa/FX. Cells kept in assay buffer (control) or incubated with FVIIa and FX were fixed with 3% paraformaldehyde in PBS. Cell surface TF was immunostained on non-permeabilized cells with mAb 10H10 followed by donkey anti-mouse IgG-Cy3. EGFP-TFPI was visualized through its intrinsic fluorescence. To detect Cave-1, and thus to observe the relationship between the cell surface TF and TFPI, and the submembrane Cave-1, the cells were permeabilized with 0.1% Triton X-100 and immunostained with rabbit anti-Cav-1 IgG followed by donkey anti-rabbit IgG-Cy3.

In the absence of FVIIa/FX, TFPI cells displayed little to no co-localization between TF and TFPI, and the triple co-localization was scarce (Fig. 6A, a, yellow, and c, white). Quantitatively, 20% of the total TF co-localized with TFPI, and ~15% of the total TFPI co-localized with TF.

After adding FVIIa/FX to TFPI cells the overlap between TFPI and TF increased to 30% of both total TFPI and total TF. TFPI patched with TF over the traces of Cav-1 present (Fig. 6A, d–f) resulting in the odd “rosette” formation (f, arrow). 50–60% of TFPI co-localized with Cav-1, and ~40% of TFPI overlapped both TF and Cav-1 in normal TFPI + Cav cells (Fig. 6B, Control, a–c). After incubation with FVIIa-FX (Fig. 6B, d–f) the patching of TFPI with TF increased to 70–80% co-localization (p < 0.01), and both overlapped Cav-1 (d, yellow, and f, white). Formation of rosettes frequently occurred on the apical surface of TFPI + Cav cells (Fig. 6B, inset in f) where TF, TFPI, and Cav-1 overlapped almost perfectly (white).

Such co-localization was no longer observed if TFPI + Cav cells were incubated with inhibitory anti-TFPI IgG before addition of FVIIa/FX. The activity assays indicated that this treatment blocked the capability of TFPI to inhibit FX activation. Fluorescence microscopy now showed that TF largely failed to co-localize with TFPI and/or Cav-1 (not shown). The percentage of TF overlapping TFPI, Cav-1, or both was brought down to the control levels for all the conditions.

Silencing of Cave-1 Expression in EC Decreases the Surface Exposure and Activity of TFPI—The role of Cave-1 in the distribution and function of TFPI in EC was studied by siRNA transfection. EA.hy926 and HUVEC transfected with Cave-1 siRNA duplexes were studied by immunostaining and confocal microscopy. A, distribution of Cave-1 (a, rabbit anti-Cave-1 IgG//goat anti-rabbit IgG-FITC) and cell surface TFPI (b, rabbit anti-TFPI IgG/goat anti-rabbit IgG-FITC) in mock-transfected EA.hy926. Double immunostaining for Cave-1 (a, rabbit anti-Cave-1 IgG/goat anti-rabbit IgG-FITC) and cell surface TFPI (b, rabbit anti-TFPI IgG/goat anti-rabbit IgG-FITC) in mock-transfected EA.hy926 transfected with Cav-1 siRNA. B, double immunostaining for Cave-1 (a, rabbit anti-Cave-1 IgG/goat anti-rabbit IgG-FITC) and cell surface TFPI (b, rabbit anti-TFPI IgG/goat anti-rabbit IgG-FITC) in mock-transfected EA.hy926 transfected with Cav-1 siRNA. C, quantification of fluorescence intensity (arbitrary units) for Cav-1 and cell surface TFPI in HUVEC. D, the positive correlation between fluorescence intensity of Cav-1 and cell surface TFPI in both control (mock-transfected) and Cav-1 siRNA-treated HUVEC was highly significant (r² = 0.86 and 0.95, respectively). Number of cells: n = 201 for mock cells and n = 174 for siRNA cells.
CAVEOLIN-1 MODULATES TFPI FUNCTION

Caveolin-1 Modulates TFPI Function

Depletion of Cav-1 in HUVEC decreases TFPI activity. A and B, tumor necrosis factor-α-stimulated HUVEC promote endogenous TF-dependent activation of FX in the presence of different concentrations of FVIIa. FXa generation over time is shown on mock cells (squares) and Cav-1 siRNA-treated cells (circles) in the presence (open symbols) or absence (closed symbols) of 50 ng/ml inhibitory anti-TFPI IgG, for each of the three concentrations of FVIIa tested (panel A, a–c). As described for 293 cells, the capability of TFPI to prevent FX activation is represented in panel B as a function of the added FVIIa concentration (triangles; a, mock cells; b, Cav-1 siRNA cells). C, TFPI exposed on the cell surface of non-stimulated HUVEC inhibits FX activation by pre-formed TF-FVIIa complexes (exogenous TF) added in the medium. The TFPI specific activity (millinits/pmol) is represented for the three different concentrations of FVIIa and thromboplatin tested. As opposed to Cav-1-depleted HUVEC (circles), TFPI in normal cells (mock cells, squares) preserved almost unchanged its potency against FX activation regardless of the concentration of FVIIa-TF added. All values are mean ± S.D. of triplicate determinations.

FIG. 8. Depletion of Cav-1 in HUVEC decreases TFPI activity. A and B, tumor necrosis factor-α-stimulated HUVEC promote endogenous TF-dependent activation of FX in the presence of different concentrations of FVIIa. FXa generation over time is shown on mock cells (squares) and Cav-1 siRNA-treated cells (circles) in the presence (open symbols) or absence (closed symbols) of 50 ng/ml inhibitory anti-TFPI IgG, for each of the three concentrations of FVIIa tested (panel A, a–c). As described for 293 cells, the capability of TFPI to prevent FX activation is represented in panel B as a function of the added FVIIa concentration (triangles; a, mock cells; b, Cav-1 siRNA cells). C, TFPI exposed on the cell surface of non-stimulated HUVEC inhibits FX activation by pre-formed TF-FVIIa complexes (exogenous TF) added in the medium. The TFPI specific activity (millinits/pmol) is represented for the three different concentrations of FVIIa and thromboplatin tested. As opposed to Cav-1-depleted HUVEC (circles), TFPI in normal cells (mock cells, squares) preserved almost unchanged its potency against FX activation regardless of the concentration of FVIIa-TF added. All values are mean ± S.D. of triplicate determinations.

The surface exposure of TFPI correlated positively and highly significantly with the expression of Cav-1, for both EA.hy926 (not shown) and HUVEC, as indicated by the correlation analysis of the fluorescence intensity performed on 201 cells (mock) and 174 cells (Cav-1 siRNA) following double immunostaining for TFPI and Cav-1 (Fig. 7D). The fluorescence intensity for TFPI similar with mock cells. The remainder displayed fluorescence intensity of 30% (±7) of the mock cells levels.

The surface exposure of TFPI correlated positively and highly significantly with the expression of Cav-1, for both EA.hy926 (not shown) and HUVEC, as indicated by the correlation analysis of the fluorescence intensity performed on 201 cells (mock) and 174 cells (Cav-1 siRNA) following double immunostaining for TFPI and Cav-1 (Fig. 7D). The fluorescence intensity for TFPI similar with mock cells. The remainder displayed fluorescence intensity of 30% (±7) of the mock cells levels.
Caveolin-1 Modulates TFPI Function

Our strategy involved first the expression of tagged full-length TFPI and Cav-1 in HEK293, a cell line that is naturally deficient of TFPI and Cav-1. We found that the properties and functional activity of EGFP-TFPI matched those of the endogenous TFPI in EA.hy926 cells. We thus confirmed that EGFP-TFPI is a valid fluorescent indicator of native TFPI. Next we confirmed by immunofluorescence and freeze-fracture EM that the expression of Cav-1 in HEK293 induced the appearance of caveolae proper, as it was described for other cell types (21, 33).

Analysis of HEK293 expressing TFPI, Cav-1, or both proteins, by live cell and (immuno)fluorescence microscopy, and by functional assays, revealed novel aspects of TFPI distribution and function. In the absence of Cav-1, TFPI had predominant intracellular localization and was largely secreted. Without affecting the overall levels of TFPI, Cav-1 expression instrumented a shift in the distribution of TFPI, with the inhibitor becoming predominantly associated with the plasma membrane surface and less secreted into the medium.

FRAP results showed that cell surface TFPI was significantly less laterally mobile in the presence of Cav-1. Under standard culture conditions Cav-1 becomes highly immobile once it reaches the plasma membrane, and shows very limited lateral diffusion and exchange with the intracellular pool (34). The fluorescence recovery profiles of EGFP-TFPI in TFPI + Cav cells (present paper, Fig. 3) and caveolae-associated GFP-tagged Cav-1 (35) are strikingly similar. We suggest that Cav-1/caveolae retain and stabilize TFPI on the membrane surface, hence the increased surface exposure and decreased lateral mobility of EGFP-TFPI in Cav-1 expressing cells.

The functional impact of TFPI localization in caveolae was analyzed through the inhibition of TF-FVIIa activity, as reflected by inhibition of FXa generation. Activation of FX by a pre-formed mixture of 10 nM FVIIa, which is the equivalent of the plasma level of FVII (7), and thromboplastin (crude TF) was almost completely inhibited by the TFPI on TFPI + Cav cells, but not on TFPI cells. As observed on the 293 cell clones that express different levels of TFPI and Cav-1, the inhibitory activity of TFPI was dependent on the level of antigen only when Cav-1 was also present. In the absence of Cav-1, varia-

\[ \text{Fig. 9. Triple immunolabeling for TFPI, TF, and Cav-1 in EC shows the formation of the TF-FVIIa-FXa-TFPI complex.} \]

\[ \text{A, HUVEC; B, EA.hy926.} \]

\[ \text{Non-permeabilized cells, either mock-transfected (a–d) or Cav-1 siRNA-transfected (e–h) were incubated with mAb TPPIK-9 and goat anti-human TF IgG, followed by anti-mouse IgG-FITC (green) and donkey anti-goat IgG-Cy5 (blue). The cells were then permeabilized and labeled with rabbit anti-Cav-1 IgG/anti-rabbit IgG-Cy3 (red). Triple co-localization appears white in both panels (merge, d and h, yellow arrows). Bars, 20 μm.} \]
tions of cell surface TFPI antigen had no noticeable impact upon the functional capabilities of the inhibitor. Accordingly, equal or even larger amounts of TFPI were always far less active against exogenous TF in TFPI cells.

When assessing the inhibitory potency of TFPI against endogenous TF, we used concentrations of FVIIa meant to span both sides of 10 nM (the equivalent of the plasma level of FVII). The higher concentration of FVIIa (50 nM) was intended to mimic locally increased levels of FVIIa. These may occur in normal circumstances, because of FVIIa interactions with phospholipids and/or proteoglycans (7), or in pathological conditions where a hypercoagulable state arises. Regardless of the concentration of FVIIa added, equimolar amounts of TFPI prevented twice as much FXa generation in the presence of Cav-1. The increase in TFPI activity was independent of the level of TF, which was also not affected by Cav-1 expression. TFPI + Cav cells were able to keep FX activation at very low levels for both 10 and 50 nM added FVIIa, preventing, in both cases, ~85% of total FX activation. The fact that TFPI cells were capable, in similar conditions, to inhibit only ~20% of FX activation suggests that the presence of Cav-1 was essential for the preservation of the TFPI inhibitory potential, especially when high levels of FVIIa, and consequently FXa, were present. Altogether, these findings suggest that, when expressed in naturally deficient cells such as HEK293, Cav-1 actively enhanced the anticoagulant activity of TFPI, probably through a combination of increased TFPI antigen retention on the cell surface and microenvironment modifications brought about by the formation of caveolae.

Next we verified that Cav-1 played a similar role in EC. To the best of our knowledge, this is the first direct proof that caveolae (or Cav-1) regulate the activity of TFPI against TF-FVIIa in EC. Using two well characterized duplex RNA oligonucleotides (30, 31), we achieved both Cav-1 depletion and considerable reduction of cell surface TFPI antigen and activity in both HUVEC and EA.hy926. This indicates that Cav-1 on the cytosolic face of the membrane might control the exposure of TFPI on the plasmalemma surface.

Cav-1 depletion decreased the capability of TFPI to downregulate the activity of endogenous TF-FVIIa, an effect that was more visible for high FVIIa concentrations. Similar with 293 cells, equimolar levels of TFPI were ~3 times less potent in preventing TF-FVIIa-dependent FX activation in the absence of Cav-1. As a result of combined lower cell surface TFPI antigen and diminished functionality, the procoagulant activity of Cav-1-depressed EC could increase by as much as ~5 times.

TF-bearing circulating microparticles derived from leukocytes and/or other blood cells probably represent a very significant source of procoagulant species in several diseased states. We sought to find out whether cell surface TFPI on EC could inhibit the procoagulant activity of preformed TF-FVIIa complexes, used as an in vitro equivalent of microparticle-associated TF-FVIIa. Accordingly, we assayed in HUVEC the inhibitory activity of TFPI against several mixtures of FVIIa (including the 10 nM equivalent of plasma FVII) and crude TF. Depletion of Cav-1 decreased by ~6-fold the potency of cell surface TFPI to block FX activation. Furthermore, high levels of FVIIa-TF and, consequently large amounts of FXa, could overwhelm the inhibitory capability of TFPI in the absence of Cav-1.

The nature of the interaction(s) that keeps TFPI in caveolae is under investigation in our group. TFPI associates through a direct or indirect glycosylphosphatidylinositol anchor with lipid rafts (10, 17), and both cholesterol and sphingolipids influence the association of TFPI with lipid rafts in EC.2 Because caveolae are highly enriched in cholesterol and sphingolipids, it is conceivable that these lipids mediate the caveolar distribution and function of TFPI. Whether such a mechanism is instrumental only in cells that express caveolins, as suggested by the lack of effect of cholesterol depletion on the inhibition of TF-FVIIa by TFPI in HEK293 (36), remains to be determined.

As indicated by the fluorescence microscopy, a high percentage of the cell surface TF and TFPI becomes co-localized with the submembrane Cav-1 during and/or after the formation of the quaternary complex. Blocking TFPI-FXa and TFPI-FVIIa interactions with anti-TFPI IgG prevents the redistribution of TF and the overlap of the complex with Cav-1. We suggest that the complex either forms within caveolae or reaches them afterward. Regardless of the mechanism, the association of TFPI with caveolae plays the determinant role in targeting the TF-FVIIa-FXa-TFPI complex to caveolae.

How does Cav-1, a protein located on the inner surface of the membrane, influence localization and function of outer surface proteins such as TFPI and TF? In normal conditions, lipid-associated proteins, TFPI included, reside very briefly within transient rafts, which are extremely small in size and very dynamic (37). Clustering of the small rafts and their associated proteins leads to the formation of larger, less mobile rafts that can be further stabilized by Cav-1. When cells are exposed to FVIIa/FX, TFPI forms complexes with TF-FVIIa/FXa regardless of the presence of Cav-1. In Cav-1-deficient cells, which likely contain less cholesterol in the membrane (35), the strength of the interaction between the complex and rafts may be too low, or the residence time too short, to "lock" the quaternary complex in the condensed cholesterol-sphingolipid domains. If the complex dissociates, a dynamic equilibrium between the formation and the dissociation of the complex would be expected, with a significant fraction of TF-FVIIa being left free to generate FXa at any given moment. This would explain the continuous generation of FXa in Cav-1-deficient cells. The presence of Cav-1, which poses a barrier into the lateral mobility of TFPI, would produce a longer lasting inhibitory effect once the complex is stabilized in the cholesterol-sphingolipid-rich environment of caveolae/rafts.

Our results suggest that caveolae could concentrate the interacting molecules in particular regions of the cell surface and facilitate the formation of the quaternary complex. The process may have high biological relevance because it identifies Cav-1 and/or caveolae as a key factor in the regulation of TFPI-dependent inhibition of TF-driven coagulation pathway in EC. As such, our findings convey a novel function to caveolae, namely active regulation of hemostasis.

Acknowledgments—We thank Dr. D. Qu and Dr. C. Esmon in our institute for the pSVNeo-Cav1/HPC4ep rDNA and mAb HPC4, Dr. J. Morrissey for anti-TF antibodies, Dr. T. Hamuro for mAb TF-PK-9, Dr. J. Crawley (Imperial College, London, United Kingdom) for initial work on EGFP-TFPI chimeras, and Dr. C.-J. Edgell for the hybrid cell line EA.hy926. We are grateful to Dr. C. Esmon for continued support and suggestions. We thank Drs. C. Esmon, R. McEvers, and J. Morrissey for critical reading of the manuscript.

REFERENCES

1. Drakes, T. A., Cheng, J., Chang, A., and Taylor, F. B., Jr. (1993) Am. J. Pathol. 142, 1458–1470
2. Contrino, J., Hair, G. A., Schmeizl, M. A., Rickles, F. R., and Kreutzer, D. L. (1994) Am. J. Pathol. 145, 1315–1322
3. Crawley, J., Lupu, F., Westmuckett, A. D., Severs, N. J., Kakkar, V. V., and Lupu, C. (2000) Arterioscler. Thromb. Vasc. Biol. 20, 1362–1373
4. Westmuckett, A. D., Lupu, C., Roquefeuil, S., Krausz, T., Kakkar, V. V., and Lupu, F. (2000) Arterioscler. Thromb. Vasc. Biol. 20, 2474–2482
5. Roque, M., Reis, E. D., Fuster, V., Padurean, A., Fallon, J. T., Taubman, M. B., Chessbro, J. H., and Badimon, J. J. (2000) J. Am. Coll. Cardiol. 36, 142, 25–31

2 C. Lupu, X. Hu, and F. Lupu, unpublished results.
2303–2310

6. Ruf, W., and Mueller, B. M. (1996) Curr. Opin. Hematol. 3, 379–384

7. Rottingen, J. A., Enden, T., Camerer, E., Iversen, J. G., and Prydz, H. (1995) J. Biol. Chem. 270, 4650–4660

8. Broze, G. J., Jr., Warren, L. A., Novotny, W. F., Higuchi, D. A., Girard, J. J., and Miletich, J. P. (1988) Blood 71, 335–343

9. Nordfang, O., Bjorn, S. E., Valentim, S., Nielsen, L. S., Wildgoose, P., Beck, T. C., and Hedner, U. (1991) Biochemistry 30, 10371–10376

10. Lupu, C., Goodwin, C. A., Westmuckett, A. D., Emeis, J. J., Scully, M. F., Kakkar, V. V., and Lupu, F. (1997) Arterioscler. Thromb. Vasc. Biol. 17, 2964–2974

11. Ott, I., Miyagi, Y., Miyazaki, K., Heeb, M. J., Mueller, B. M., Rao, L. V., and Ruf, W. (2000) Arterioscler. Thromb. Vasc. Biol. 20, 874–882

12. Lupu, C., Poulsen, E., Roquefeuil, S., Westmuckett, A. D., Kakkar, V. V., and Lupu, F. (1999) Arterioscler. Thromb. Vasc. Biol. 19, 2251–2262

13. Bajaj, M. S., Kuppuswamy, M. N., Saito, H., Spitzer, S. G., and Bajaj, S. P. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8869–8873

14. Lupu, C., Kruithof, E. K., Kakkar, V. V., and Lupu, F. (1999) Thromb. Haemostasis 82, 1652–1658

15. Hansen, J. B., Naalsund, T., Sandset, P. M., and Svensson, B. (2000) Thromb. Res. 100, 413–417

16. Sevinsky, J. R., Rao, L. V., and Ruf, W. (1996) J. Cell Biol. 133, 293–304

17. Mast, A. E., Acharya, N., Malecha, M. J., Hall, C. L., and Dietzen, D. J. (2002) Arterioscler. Thromb. Vasc. Biol. 22, 2099–2104

18. Ott, I., Andrasssy, M., Ziegelhansberger, D., Geith, S., Schomig, A., and Neumann, F. J. (2001) Blood 97, 3721–3726

19. Rottingen, J. A., Enden, T., Camerer, E., Iversen, J. G., and Prydz, H. (1995) J. Biol. Chem. 270, 4650–4660

20. Fra, A. M., Williamson, E., Simons, K., and Parton, R. G. (1994) J. Biol. Chem. 269, 30745–30748

21. Fra, A. M., Williamson, E., Simons, K., and Parton, R. G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8655–8659

22. Krajewska, W. M., and Maslowska, I. (2004) Cell Mol. Biol. Lett. 9, 195–220

23. Westmuckett, A. D., Kakkar, V. V., Hamuro, T., Lupu, F., and Lupu, C. (2001) Thromb. Haemostasis 86, 1547–1554

24. Stearns, D. J., Kurosawa, S., Sims, P. J., Esmen, N. L., and Esmen, C. T. (1988) J. Biol. Chem. 263, 826–832

25. Feng, X., Gaeta, M. L., Madge, L. A., Yang, J. H., Bradley, J. R., and Pober, J. S. (2001) J. Biol. Chem. 276, 8341–8349

26. Xu, J., Qu, D., Esmen, N. L., and Esmen, C. T. (2000) J. Biol. Chem. 275, 6938–6944

27. Caplice, N. M., Mueske, C. S., Kleppe, L. S., Peterson, T. E., Broze, G. J., Jr., and Simari, R. D. (1996) Circ. Res. 83, 1264–1270

28. Simionescu, N., Lupu, F., and Simionescu, M. (1983) J. Cell Biol. 97, 1592–1600

29. Pelkmans, L., Kartenbeck, J., and Helenius, A. (2001) Nat. Cell Biol. 3, 473–483

30. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) Nature 411, 494–498

31. Nichols, B. J. (2002) Nat. Cell Biol. 4, 374–378

32. Zhang, J., Piro, O., Lu, L., and Broze, G. J., Jr. (2003) Circulation 108, 623–627

33. Vogel, U., Sandvig, K., and van Deurs, B. (1998) J. Cell Sci. 111, 825–832

34. Lipincott-Schwartz, J., Snapp, E., and Kenworthy, A. (2001) Nat. Rev. Mol. Cell. Biol. 2, 444–456

35. van Deurs, B., Roepstorff, K., Hommelgaard, A. M., and Sandvig, K. (2003) Trends Cell Biol. 13, 92–100

36. Dietzen, D. J., Jack, G. G., Page, K. L., Tetzloff, T. A., Hall, C. L., and Mast, A. E. (2003) Thromb. Haemostasis 89, 65–73

37. Jacobson, B., and Dietrich, C. (1999) Trends Cell Biol. 9, 87–91
