Glycosyl Phosphatidylinositol Anchorage of Tissue Factor Pathway Inhibitor

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Background—The endothelium is a major source of tissue factor pathway inhibitor (TFPI), the endogenous regulator of TF-induced coagulation, and a significant proportion of the expressed TFPI remains associated with the endothelial surface.

Methods and Results—Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment reduced TFPI at the surface of cultured endothelial cells by ~80%, and at least a portion of the TFPI released by PI-PLC contained an intrinsic glycosylphosphatidylinositol (GPI) anchor that is recognized by anti-crossreactive determinant antibodies. Endothelial cells express both of the alternatively spliced forms of TFPI mRNA at a ratio of TFPIβ/TFPIα mRNA of ~0.1 to 0.2. In Chinese hamster ovary (CHO) cells, TFPIα is predominantly secreted, whereas TFPIβ is a GPI-anchored membrane protein. Like TFPIβ, the small proportion of the TFPIα expressed by CHO cells that remains surface associated is also released by PI-PLC treatment, suggesting that it is bound to a separate GPI-anchored protein(s) at the surface of the cells. Conclusions—Both direct (TFPIβ) and indirect (TFPIα) GPI-mediated membrane anchorage is involved in localizing TFPI to the surface of cells. (Circulation. 2003;108:623-627.)

Key Words: coagulation • inhibitors • endothelium-derived factors

Tissue factor pathway inhibitor (TFPI) is the endogenous regulator of TF-induced coagulation. TFPI directly binds and inhibits activated factor X (FXa), and in an FXa-dependent fashion, produces feedback inhibition of the FVIIa/TF catalytic complex. TFPIα, a 41-kDa glycoprotein that contains an acidic N-terminal region followed by 3 tandem, Kunitz-type protease inhibitor domains and a basic C-terminal region, interacts with tissue factor (TF) to form the FVIIa-TFPI-FXa catalytic complex. It is the second Kunitz domain of TFPI that mediates the binding and inhibition of FXa, whereas the first Kunitz domain is responsible for the inhibition of FVIIa bound to TF. Presumably by localizing the molecule to cell surfaces through interactions with anionic phospholipids, glycosaminoglycans, or surface proteins, the C-terminus of TFPIα plays an important role in its inhibitory functions, and C-terminally truncated forms of TFPIα are less potent inhibitors of FXa and FVIIa/TF.1,2,3,4

There is a broad range of TFPI concentrations in the plasma of normal individuals (mean, ~2.0 nmol/L), and platelets carry ~5% of the total blood TFPI. Most of the plasma TFPI circulates as a C-terminally truncated form(s) that is bound to plasma lipoproteins. The major form carried by LDL has a molecular weight of 34 kDa and lacks at least a large portion of the Kunitz-3 domain and the C-terminus of TFPIα. The 41-kDa form of TFPI that circulates with HDL appears to be a similar C-truncated form of TFPI that is disulfide linked to monomeric apolipoprotein-AII. Additional forms of TFPI with less extensive C-terminal truncation and a small amount of full-length TFPIα also circulate in plasma.5,6

The endothelium is presumed to be the major source of TFPI in vivo.6 A significant fraction of the TFPI produced by cultured endothelial cells and present in placental tissue remains membrane associated.7,8-11 In vitro heparin induces the release of a portion of this cell-associated TFPI,7-9,11 and in vivo heparin treatment increases the plasma level of TFPI by 1.5- to 2.5-fold.12 This process could represent the release of TFPI from cell-surface binding sites or the secretion of TFPI from intracellular storage sites.13,14 The bulk of the heparin-resistant, cell-associated TFPI is released by phosphatidylinositol-specific phospholipase C (PI-PLC) treatment in vitro, suggesting that this form of TFPI contains a glycosyl phosphatidylinositol (GPI) membrane anchor or is tightly bound to a surface GPI-containing protein.9,11,15,16 Because cell-bound TFPI is believed to play a critical role in regulating surface FVIIa/TF and FXa activity, the following experiments were undertaken to further define the mechanism(s) responsible for the association of TFPI with cell membranes.7,9,11,17,18
Methods

Materials
Human mammalian gene collection clone 9251 (mgc:9251; image:3902987; gb:BC015514; gi:15930155) containing TFPI cDNA and the human umbilical vein cell (HUVEC)–derived line ECV304 were obtained from American Tissue Culture Collection (ATCC, Manassas, Va.). The HUVEC-derived cell line EA.hy926 was a kind gift from Cora-Jean Edgell (University of North Carolina, Chapel Hill), and HUVECs were purchased from Clonetics BioWhittaker (Walkersville, Md). The Expand high-fidelity polymerase chain reaction (PCR) system and the Lumi-Light Western blotting substrate were from Roche. Dulbecco’s modified Eagle’s medium (DMEM), nonessential amino acids, fetal bovine serum (FBS), and Chinese hamster ovary (CHO)-S cells were from Invitrogen (Carlsbad, Calif). PI-PLC, horseradish peroxidase–labeled goat anti-rabbit immunoglobulin G (IgG) antibodies, Tris, EDTA, and Triton X-114 were from Sigma Chemical. Prestained molecular-weight standards and Affigel-10 were from Bio-Rad. Oligonucleotides were synthesized by the Protein Chemistry Laboratory at Washington University School of Medicine. Anti-cross-reactive determinant (anti-CRD) antiserum was a kind gift from Paul Englund (Johns Hopkins University, Baltimore, Md). The production and characterization of monoclonal anti-TFPI antibodies Mab2H8 and Mab2B12 and of rabbit polyclonal anti-TFPI antisera have been previously described.5

Cell Culture
HUVECs were cultured in essential growth medium-2 (BioWhittaker) supplemented with 2% FBS and studied between passages 3 and 6. ECV304 cells were cultured in medium 199 (BioWhittaker) supplemented with 1 mmol/L pyruvate and 10% FBS, and EA.hy926 cells were cultured in DMEM (BioWhittaker) with 1 mmol/L pyruvate and 10% FBS. CHO-S cells were routinely maintained in DMEM supplemented with 0.1 mmol/L nonessential amino acids and 10% FBS. All cells were cultured at 37°C with 5% CO2, and all media contained penicillin (50 U/mL) and streptomycin (50 μg/mL).

Expression of TFPIα and TFPIβ in CHO-S Cells
Human TFPIα cDNA was amplified from plasmid pBS-SK/TFPI19 by using forward primer 5′-CAC CAT GAT TTA CAC AAT GAA GAA AGT ACA-3′ and reverse primer 5′-TCA CAT ATT TTI AAC AAA ATC TAC TGC ACA-3′. TFPIβ cDNA was amplified from the mgc:9251 clone by using forward primer 5′-CAC CAT GAT TTA CAC AAT GAA GAA AGT ACA-3′ and reverse primer 5′-TTA ACA TAG GCA TTA GAT GCT ATC CCA TCC-3′. The TFPIα and TFPIβ cDNA fragments were cloned into pcDNA3.1D/V5-His-TOPO (Invitrogen) in a manner to prevent expression of the C-terminal V5 and His tags. The direction and sequence of the cDNAs were confirmed by double-strand sequencing. CHO-S cells were transiently transfected with the TFPIα and TFPIβ pcDNA3.1 constructs by using LipofectAMINE 2000 in a 24-well plate, according to the manufacturer’s protocol (Invitrogen). Stable clones expressing TFPIα (CHO-TFPIα) and TFPIβ (CHO-TFPIβ) were isolated in a similar fashion by using G418 selection.

Triton X-114 Fractionation of Membrane-Associated Protein
Forty-eight hours after transfection, CHO-S cells cultured in CD CHO serum-free medium supplemented with hyoxanthine and thymidine (Invitrogen) were detached with cell dissociation buffer (Invitrogen) and washed with phosphate-buffered saline (PBS). Cells (5×106/mL) were lysed with 1% Triton X-114 in 0.1 mol/L Tris–HCl, pH 7.5, and 10 mmol/L EDTA with repeated mixing on ice for 15 minutes. Cell debris was removed by centrifugation at 14,000g × 2 minutes at 4°C. Phase separation was induced by incubation of the cleared detergent lysate for 5 minutes at 37°C, followed by brief centrifugation at 14,000g at room temperature to separate the detergent and aqueous phases. The detergent pellet was extracted with acetone, and the membrane proteins were resuspended in reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The detergent extract derived from 2.5×106 cells was loaded in each lane for separation by 10% SDS-PAGE and Western blotting with rabbit polyclonal anti-TFPI antibodies with chemiluminescent detection.

Quantitative RT-PCR-Based Gene Expression Analysis
Real-time reverse transcription (RT)-PCR was used to determine the relative amount of TFPIα and TFPIβ mRNA in HUVE, ECV304, and EA.hy926 cells. Primers for human TFPIα and TFPIβ were designed by using Primer Express software (Applied Biosystems). For TFPIα, the forward and reverse primers were 5′-TAT GGA ACC CAG CTC AAT GCT-3′ and 5′-ACC CCA TGA GGC ACC GTG AA-3′, respectively. For TFPIβ, the forward and reverse primers were 5′-CCA AGG TTC CAA GCC TTG TTG-3′ and 5′-ATG AAT GCA GAA GCC GTC TGG-3′, respectively. Total RNA was isolated from the cells with the RNaseasy mini-kit and QIAshredder (Qiagen) and treated with reagents from the RNase-free DNase set (Qiagen). For each sample, 2 μg total RNA was reverse-transcribed by using the SuperScript first-strand synthesis system (Invitrogen) with oligo-dT primers. TFPIα and TFPIβ real-time PCR was performed in separate tubes in triplicate with 1 μL of the 20-μL RT reaction products by using the SYBR green dye kit in an ABI Prism 5700 sequence detector (Applied Biosystems). RT-PCR and subsequent calculations with the average threshold cycle method were performed, following the manufacturer’s protocols (Applied Biosystems).

Determination of Cellular TFPI Expression
CHO-TFPIα, CHO-TFPIβ, HUVE, ECV304, and EA.hy926 endothelial cells were allowed to grow to 90% confluence in their appropriate media. Complete medium was then withdrawn, and the cells were incubated overnight in the appropriate serum-free medium. The cells were washed 3 times in PBS, harvested in unincellular suspension by treatment with cell dissociation buffer (Invitrogen), washed again 3 times in PBS, and resuspended at 1×106 cell/mL in appropriate serum-free medium. Cell suspensions (0.5 mL) were then treated at 37°C in different ways: (1) incubated in serum-free medium for 1 hour; (2) incubated with heparin (5 U/mL) for the last 20 minutes of a 1-hour incubation; (3) incubated with PI-PLC (1 U/mL) for 1 hour; and (4) incubated with heparin (5 U/mL) for 20 minutes after the cells had been incubated 1 hour with PI-PLC (1 U/mL) and washed twice. At the end of the incubations, cells were centrifuged by centifugation (250g × 5 minutes) for flow cytometric analysis. In the case of HUVECs, supernatants were centrifuged a second time to remove cell debris, concentrated 2.5-fold (Centricron YM10, Millipore), and stored at −20°C for subsequent TFPI immunosassay.

Flow Cytometry
Cells (5×106) were resuspended in 200 μL of ice-cold PBS containing 0.08% (wt/vol) sodium azide, 25 mmol/L EDTA, and 3% (wt/vol) bovine serum albumin and incubated with mouse monoclonal anti-TFPI Kunitz-1 (Mab288, 5 μg/mL) for 30 minutes. After being washed 3 times with the same buffer, the cells were incubated for an additional 30 minutes on ice in the dark with phycoerythrin-conjugated F(ab), sheep anti-mouse IgG (Sigma). The labeled cells were washed twice and then analyzed by fluorescence-activated cell sorting flow cytometry (FACScan, Becton Dickinson). Regions appropriate for each of the tested cell types were defined by using forward light scatter and side-angle light intensity dot plots. All measurements were performed with the same instrument settings, and at least 5×105 events were analyzed by using CellQuest software (Becton Dickinson). Results were expressed as mean fluorescence intensity (MFI), and the difference between the surface TFPI of cells that had been treated in various ways was assessed by using Kolmogorov-Smirnov statistical analysis (CellQuest, Becton Dickinson). Cells incubated with isotype control IgG, with the primary antibody alone, with the secondary antibody alone, and with neither were used as negative controls.
Immunoaffinity Isolation of TFPI Released From ECV304 by PI-PLC

ECV304 cells that had reached confluence in a T75 culture flask were washed twice with PBS and then treated with PI-PLC (1 U/mL) in 4 mL serum-free medium at room temperature. After 1 hour, the medium was collected by filtration (0.22 µm) to remove debris and passed through a 200-µL column of Mab2H8-Affigel (2 mg/mL). After the column was washed with 0.1 mol/L NaCl and 0.05 mol/L Tris-HCl, pH 7.5, the bound TFPI was eluted with 8 mol/L urea and 0.02 mol/L Tris-HCl, pH 7.5. Twenty microliters of reduced samples was evaluated by 12% SDS-PAGE and Western blotting with chemiluminescent detection with a rabbit polyclonal anti-TFPI and anti-CRD antisera.

TFPI Immunoassay

Wells of a microtiter plate (Nunc, Fisher Scientific) were incubated overnight at room temperature with 150 µL mouse monoclonal anti-TFPI Kunitz-2 (Mab2B12, 20 µg/mL) and then washed (200 µL) with PBS containing 0.05% Tween 20 (PBST). Duplicate samples (100 µL) were applied to the wells and incubated for 2 hours. After the wells were washed with PBST (200 µL ×4), a solution (150 µL) containing biotin-conjugated (EZ-link-Sulfo-NHS-LC-Biotin, Pierce) Mab2H8 (4 µg/mL) was incubated in the wells for 1.5 hours. The wells were washed with PBST (200 µL ×4), and 150 µL of a 1:100 dilution of streptavidin–horseradish peroxidase complex (Pierce) in PBST was applied. After 1 hour, the wells were washed with PBST (200 µL ×4), and 200 µL of TMB liquid substrate (Sigma) was added. After 5 minutes, 0.5 mol/L H2SO4 (100 µL) was added to stop the reaction, and the absorbance at 450 nm was measured with a Vmax microtiter plate reader and Spectramax Pro software (Molecular Devices). Purified, recombinant TFPI expressed in Escherichia coli was used as the standard and produced a linear response between concentrations of 0 and 40 ng/mL.

Results and Discussion

Endothelial Cell–Associated TFPI

The association of TFPI with endothelial cells was investigated in initial experiments (Figure 1). Heparin (5 U/mL) treatment of HUVECs increased the TFPI concentration in the medium modestly but did not reduce cell surface TFPI significantly. Thus, the TFPI released by heparin comes from intracellular stores or is removed from untreated cells during flow cytometric analysis. Previous studies have shown that this heparin-releasable TFPI is TFPI\(\text{H251}\). PI-PLC (1 U/mL) treatment released substantial TFPI into the medium and reduced cell surface TFPI by \(\approx 80\%\) (\(P<0.001\)). Subsequent exposure of the cells to heparin (5 U/mL) after PI-PLC treatment released a greater amount of TFPI than did heparin treatment alone and further reduced cell surface TFPI (\(P<0.001\)). This suggests that a portion of the TFPI released by PI-PLC treatment remains associated with the cell surface in a manner that is sensitive to heparin. Studies with ECV304 and EA.hy926 cells produced the same results (not shown), and our work with cultured endothelial cells largely mirrors that of Mast et al using placental tissue.

To determine whether the TFPI released by PI-PLC contains a GPI anchor or is bound by a separate GPI-linked protein, ECV304 cells were washed and treated with PI-PLC (1 U/mL). The released TFPI was immunoaffinity-isolated and evaluated by Western blotting with the use of polyclonal anti-TFPI and anti-crossreactive-determinant (anti-CRD) antibodies (Figure 2A). The PI-PLC–cleaved TFPI migrated...
with an apparent molecular weight of 45 kDa and was recognized by both anti-TFPI and anti-CRD antibodies. The major epitope involved in anti-CRD recognition is the inositol 1,2-cyclic monophosphate that is generated on PLC cleavage of a GPI anchor. Recognition of a protein by anti-CRD antiserum after PI-PLC cleavage is virtually unequivocal evidence for the presence of a GPI anchor. Thus, at least a portion of the TFPI produced by endothelial cells contains an intrinsic GPI anchor.

**TFPIβ Is an GPI-Anchored Protein**

A search of the National Center for Biotechnology GenBank showed that the cDNA sequences for mouse (gb:AF016313) and human (gb:AF021834) TFPIβ had been deposited by Chang et al and that the TFPI gene (Hs2 5422, gi:2204416) contains an exon encoding the alternative C-terminal amino acid sequence of TFPIβ. This exon precedes that encoding the Kunitz-3 domain of TFPIα. The algorithm of A.L. Veuthey from the Swiss Institute of Bioinformatics (http://us.expasy.org/tools; select DGPI) predicts that the C-terminal amino acid sequence of TFPIβ (but not of TFPIα) following residue 181 of mature TFPIα contains the appropriate signal to direct attachment of a GPI anchor: VTKEGTN V terminal sequence that directs appropriate cleavage and attachment of a GPI anchor.

**GPI-Linked Endothelial TFPI**

HUVE, ECV304, and EA.hy926 cells contain both TFPIα and TFPIβ mRNA by quantitative RT-PCR at a ratio of TFPIβ:TFPIα mRNA of 0.08, 0.17, and 0.20, respectively. The extent to which TFPIα and TFPIβ contribute to the PI-PLC–releasable TFPI on these endothelial cells, however, is unclear. Both TFPIα, presumably bound to a separate GPI-linked protein(s), and TFPIβ are released by PI-PLC treatment (Figure 4). The proteins cannot be distinguished by size, because the TFPIα and TFPIβ expressed by CHO cells and the TFPI released by PI-PLC from HUVE, ECV304, and EA.hy926 endothelial cells all migrate with the same apparent molecular weight (~45 kDa) on SDS-PAGE gels (Figure 2 and not shown). Only a small amount of the TFPIα produced by CHO-TFPIα cells remains at the cell surface, but the putative GPI-linked protein(s) with which TFPIα associates might be expressed at a much higher concentration on endothelial cells than on CHO cells. Previous publications have reported the recognition of endothelial surface and placental TFPI by anti-peptide antibodies against the Kunitz-3 domain and the C-terminus of TFPIα, respectively. Thus, at least some of the endothelial surface TFPI appears to be TFPIα. Our studies, however, suggest that TFPIβ might also contribute significantly to the surface TFPI of endothelial cells (Figure 2A).

In endothelial cells, post–PI-PLC treatment with heparin further decreased cell surface TFPI (Figure 1). This phenomenon was seen in CHO-TFPIα cells, in which only a small quantity of TFPI remained cell associated, but not in CHO-TFPIβ cells (Figure 4). At first glance, this result would suggest that the Kunitz-3 domain and/or C-terminus present in TFPIα, but not TFPIβ, are required for the heparin–inhibitable interaction of TFPI with the cell surface after PI-PLC treatment. A firm conclusion, however, cannot be drawn because of the dramatically different surface levels of TFPIα and TFPIβ and the possibility that the CHO-TFPIβ cells might have expressed additional surface TFPI during the course of the experiment.

Deletion of the TFPI Kunitz-1 domain in mice, which affects the function of both TFPIα and TFPIβ, leads to a consumptive coagulopathy and intrauterine lethality. The
relative physiological importance of TFPIα and TFPIβ, however, is not known. Ongoing studies characterizing mice individually lacking TFPIα or TFPIβ should address this issue.

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References
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