Tissue Factor Encryption

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Abstract—Tissue factor (TF) encryption is the post-translational suppression of TF procoagulant activity (PCA) on the cell surface. There is emerging evidence of encrypted TF in normal blood associated with monocytes and platelets. Expression of this latent TF PCA during the propagation phase of blood coagulation may contribute to hemostasis. One pathway leading to the decryption of TF PCA begins with an increase in cytosolic calcium. A large calcium influx triggers both the exposure of phosphatidylserine and the expression of TF PCA on cell surfaces. The connections between these events are reviewed along with evidence that lipid raft association may also contribute to TF encryption. The last step in the decryption of TF PCA is the proteolytic activation of zymogen factor VII. This event may be a key to understanding the different roles of intravascular and extravascular TF in the process of blood coagulation. (Arterioscler Thromb Vasc Biol. 2006;26:456-461.)

Key Words: coagulation ■ cytosolic calcium ■ factor VII ■ phosphatidylserine ■ tissue factor

The discovery of a thromboplastic activity in tissues inspired the hypothesis that blood coagulation is triggered by contact between intravascular and extravascular factors.1–4 This 19th century model of coagulation is supported by immunohistochemical studies of tissue factor (TF), the protein component of tissue thromboplastin. TF antigen was detected on cells surrounding blood vessels, but it was not visible on either endothelial cells or cells in the bloodstream.5

As important as the intact endothelium is to preventing TF-initiated coagulation, there are compelling reasons to believe this is not the entire story. Foremost is the emerging evidence of TF in normal blood.6–13 Because the levels are generally very low, it is difficult to detect TF antigen on the surface of blood cells.5 However, the membrane-associated TF procoagulant activity (PCA) in blood is easily measured by more sensitive functional assays.6,9

The quantity of TF on blood cells is small relative to that which is present on extravascular cells. This raises an obvious question. Is the level of intravascular TF too low to be of any significance? One study measured an average of \( \approx 30 \) pg/mL for membrane-associated TF in the blood of normal subjects.13 The estimate is based on functional assays of isolated mononuclear cells, platelets, and microparticles. The respective blood levels of the three TF pools were 15.9\( \pm 14.7 \) pg/mL, 10.6\( \pm 5.3 \) pg/mL, and 1.0\( \pm 0.3 \) pg/mL. In a prothrombin time assay, this concentration of pure TF, optimally reconstituted into phospholipids vesicles, clots plasma in \( \approx 60 \) seconds (R. Bach, unpublished, 2005). Thus, it is likely that more than an intact endothelium is required for blood to remain fluid.

TF encryption is the post-translational suppression of TF PCA on the cell surface. It may be the primary mechanism controlling the expression of TF PCA by cells in blood. TF encryption was first observed in cell culture studies. Unperturbed cells express very little TF PCA despite the fact that TF, an integral membrane protein, is on the cell surface where it can bind zymogen factor VII (FVII) as well as activated FVII (FVIIa).14–17 A stimulus is required to express the latent proteolytic activity of the encrypted TF–FVIIa complex.14–17

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There are a number of ways to induce cells to express encrypted TF PCA: freezing and thawing, sonication, proteases, phospholipases, nonionic detergents, apoptosis, complement, and calcium (Ca\(^{2+}\)) ionophores.\textsuperscript{14,17-27} These procedures vary significantly with respect to the level of TF PCA achieved as well as secondary effects on cell structure. In my own work I have a strong preference for Ca\(^{2+}\) ionophores because they rapidly induce the maximum expression of cell surface TF PCA without destroying plasma membrane integrity.

**Calcium Influx and Phospholipid Asymmetry**

The first step in the Ca\(^{2+}\) ionophore-induced decryption of TF PCA is a release of Ca\(^{2+}\) from internal stores. This is followed by a second and larger influx of extracellular Ca\(^{2+}\) across the plasma membrane. TF remains encrypted on HL-60 cells until the concentration of cytosolic Ca\(^{2+}\) exceeds the basal level by >4-fold.\textsuperscript{28} Once this threshold is crossed, the decryption process is triggered and TF PCA is fully expressed in \(\approx\)30 seconds.\textsuperscript{21}

In the plasma membrane of quiescent cells phosphatidylserine (PS) is sequestered on the inner leaflet of the bilayer.\textsuperscript{29} PS asymmetry is maintained with an expenditure of energy in the form of ATP hydrolysis. At least 2 lipid transporters are required to create and sustain this steady state. Flippase is the ATP-dependent aminophospholipid translocase catalyzing the inward transport of PS. Floppase is the less specific ATP-dependent translocase catalyzing the outward flow of phospholipids.\textsuperscript{30}

An increase in cytosolic Ca\(^{2+}\) of the same magnitude that decrypts TF PCA also disrupts PS asymmetry.\textsuperscript{26} The Ca\(^{2+}\) influx inhibits flippase activity. At the same time the activity of scramblase, the Ca\(^{2+}\)-dependent transporter catalyzing the bidirectional movement of lipids, is enhanced.\textsuperscript{29,30} The net result is the rapid appearance of PS on the cell surface. The process is reversible as long as the basal cytosolic Ca\(^{2+}\) level is re-established before flippase is destroyed by calpain.\textsuperscript{14,31}

The exposure of HL-60 cells to a Ca\(^{2+}\) ionophore induces the expression of TF PCA and the appearance of PS on the cell surface.\textsuperscript{14,21,23,28} The coincidence of TF decryption and PS exposure does not prove these events are coupled. However, it has been known for decades that PS accelerates coagulation reactions on membrane surfaces.\textsuperscript{29,32-35} Furthermore, the binding of annexin V to PS on the surface of a Ca\(^{2+}\)-ionophore-stimulated cell inhibits the expression of decrypted TF PCA.\textsuperscript{23} Therefore, the loss of PS asymmetry appears to be connected to the process of TF decryption.

In addition to Ca\(^{2+}\) ionophore stimulation there are biologically relevant cell-activating events that can disrupt PS asymmetry. The prototype is platelet activation by collagen and thrombin. These stimuli induce Ca\(^{2+}\) influx and PS exposure,\textsuperscript{29,36} as well as the decryption of platelet-associated TF PCA.\textsuperscript{12,13} PS also appears on the surface of cells undergoing apoptosis,\textsuperscript{37} and TF PCA is decrypted when cells become apoptotic.\textsuperscript{26}

Platelet activation by thrombin and collagen occurs in seconds, whereas apoptosis is a slow process requiring hours. Furthermore, in cells from individuals with Scott syndrome, a rare bleeding disorder, the rapid exposure of PS on activated platelets is defective, but the slow appearance of PS on apoptotic lymphocytes is normal.\textsuperscript{38,39} Thus, the initial steps of these cell-activating processes are distinct. However, the common results of PS exposure and membrane blebbing suggest some degree of overlap in later stages.

The role of abnormal PS exposure in human diseases is the subject of a recent review.\textsuperscript{20,30} There is evidence of increased cell surface PS in antiphospholipid syndrome, sickle cell anemia, thalassemia, bacterial and viral infections, malaria, uremia, diabetes, preeclampsia, and cancer. In each case, thrombotic complications are commonly observed that may be caused at least in part by the loss of PS asymmetry.

**PS Effects on TF-FVIIa Structure and Function**

The kinetics of the TF-FVIIa catalyzed conversion of zymogen factor X (FX) to activated FX (FXa) provides some insight into the mechanism of the PS effect on TF PCA. Stimulating cells with a Ca\(^{2+}\) ionophore increases the V\(_{\text{max}}\) and decreases the k\(_{\text{m}}\) for this reaction. Similar changes in the kinetic parameters are produced by the addition of PS to TF-containing phospholipid vesicles.\textsuperscript{14} A discussion of how the interaction between FX and PS can affect the kinetics of FX hydrolysis by TF-FVIIa is beyond the scope of this review.\textsuperscript{32-35} Instead, I focus on the evidence for associations between PS and TF-FVIIa that may increase the V\(_{\text{max}}\) of the reaction.

Two plausible explanations for the PS effect on V\(_{\text{max}}\) can be excluded. First, FVIIa binds to both encrypted TF and decrypted TF.\textsuperscript{17} Therefore, in the presence of excess FVIIa the formation of TF-FVIIa complexes per se is not the limiting step. Second, the crystal structure of the complex between the soluble extracellular domain of TF (sTF) and FVIIa yields an estimate of \(\approx 80 \text{ Å}\) for the distance from the membrane surface to the catalytic site.\textsuperscript{40} Therefore, a PS-induced conformational change in the active site of FVIIa is unlikely.

Another way for PS to increase V\(_{\text{max}}\) starts with the assumption that encrypted TF-FVIIa is completely inactive with respect to protein substrate hydrolysis. In this hypothesis the decryption of TF PCA is a conversion from inactive TF-FVIIa to proteolytic TF-FVIIa. In other words the PS effect on V\(_{\text{max}}\) is an increase the number of functional catalytic sites. There is evidence supporting this 2-state model of TF encryption.\textsuperscript{14,17,21}

TF is a member of the cytokine receptor superfamily.\textsuperscript{41} This family of integral membrane proteins includes human growth hormone (GH) receptor (hGHR) and erythropoietin receptor (EPOr). The soluble extracellular domains of both receptors (shGHR, sEPOr) crystallize as homodimers in the presence of ligand.\textsuperscript{42,43} The same basic structural motifs are shared by all members of the cytokine receptor superfamily. Therefore, self-association “is likely to be a relatively common feature of the family as a whole.”\textsuperscript{42} This property provides a plausible mechanism for the PS-induced transition from inactive to active TF-FVIIa.

The self-association of cell-surface TF has been demonstrated by chemical cross-linking.\textsuperscript{21,44} This cross-linking is reduced if the cytoplasmic domain is deleted, and it is
eliminated entirely if a chimeric TF is created by attaching the TF extracellular domain to a nonhomologous transmembrane domain.44 Similarly, cross-linking of TF on cells is prevented by Ca$^{2+}$ ionophore stimulation.21 Thus, both the transmembrane and cytoplasmic domains are necessary for optimal TF self-association on an unperturbed cell, and a Ca$^{2+}$ influx diminishes this interaction.

One way for TF quaternary structure to regulate TF function is suggested by the crystal structures of sTF-FVIIa, shGH$_2$, and sEPO$_2$. The sTF-FVIIa complex contains an extended surface of intermolecular contacts. The area of this interface is estimated to be 1810 $\AA^2$.40 Most of the remaining unoccupied surface on the TF extracellular domain is the binding site for protein substrates, FX and factor IX (FIX).45–47 The regions of shGH$_2$ and sEPO$_2$ corresponding to the FX/FIX docking site on TF are the homodimer interfaces.

If the quaternary structure of TF$_2$ resembles that of shGH$_2$ and sEPO$_2$, then the FVIIa binding site on TF is exposed, but the protein substrate binding site is buried. Thus, the proteolytic activity of the TF-FVIIa complex may be inhibited. The properties of encrypted TF-FVIIa are consistent with this model. As noted, FVIIa does in fact bind to encrypted TF. Also, the functional assay data suggest that the FX/FIX binding site on encrypted TF-FVIIa is not fully functional because either the complex of tissue factor pathway inhibitor (tFPI) and FXa (tFPFI-FXa) does not bind at all or it has a much lower affinity for encrypted TF-FVIIa.21

A direct interaction between PS and TF may induce changes in TF structure. Lys$^{165}$ and lys$^{166}$ are 2 essential amino acids residing in the TF extracellular domain.48,49 Chemical modification of these residues inhibits TF PCA but does not interfere with FVII/FVIIa binding.48 In the crystal structure of sTF, these lysines are in a flexible solvent-exposed loop on the side of TF opposite the FVII/FVIIa binding site.48 The loop is near the C-terminal end of the extracellular domain. This places lys$^{165}$ and lys$^{166}$ adjacent to the cell surface when TF is anchored in the membrane. The conversion of either lysine to alanine significantly reduces the in vitro effect of PS on TF PCA.48 This evidence suggests the possibility of electrostatic interactions between PS polar head groups and lys$^{165}$/lys$^{166}$. Such an association could change TF quaternary structure by altering either the conformation of the homodimer interface or the orientation of the extracellular domain relative to the membrane surface. Thus, PS exposure and binding to TF may transform encrypted TF-FVIIa into a protease.

Another way for PS to increase $V_{max}$ is suggested by the interactions between TF-FVIIa and the membrane. TF is tethered to the surface by the membrane-spanning domain. When PS is exposed, FVIIa is linked to the cell surface via the N-terminal $\gamma$-carboxyglutamic acid domain. These points of contact restrict the orientation of the TF-FVIIa complex relative to the membrane surface. Any additional connections between TF-FVIIa and the membrane, such as the direct association of TF and PS, will restrict the orientation further and fix the distance from the membrane surface to the catalytic site on TF-FVIIa. The precise alignment of this active site with the scissile peptide bonds in membrane-bound FX/FIX is required for the maximum rate of hydrolysis.40 Therefore, the PS effect on $V_{max}$ may be caused in part by an increase in the rate of catalysis.

The evidence just described suggests that PS exposure may enhance TF-FVIIa function by directly altering TF-FVIIa structure. A PS-induced change in TF quaternary could increase the number of active catalytic sites. Also, PS exposure may increase the rate of catalysis by optimizing the orientation of the TF-FVIIa catalytic site. Because the 2 mechanisms are not mutually exclusive, both may contribute to the PS-induced increase in $V_{max}$.

**Evidence Against the Model**

It is important to note that the evidence connecting TF quaternary structure and TF PCA is circumstantial. The same can be said of the case for the link between PS exposure and TF decryption. Thus, final judgment on these hypotheses awaits more conclusive evidence. An example of the continuing controversy is the conclusion of report of Donaté et al$^{52}$ that TF dimerization does not inhibit TF PCA. Several sTF proteins, ie, recombinant derivatives of the TF extracellular domain (residues 1 to 219) were used in this study. To promote dimerization, a leucine zipper motif (LZ) was attached to the carboxyterminus of sTF. A soluble chimeric construct composed of sTF$^{1–220}$, a short polypeptide linker region, and LZ (TF$_2$-LZ) was characterized with respect to self-association and FVIIa binding. As expected, TF$_1$-LZ was primarily a dimer at the concentrations tested. FVIIa binding to TF$_1$-LZ was not inhibited by dimerization. One molecule of FVIIa bound to each subunit of the dimer. In accord with previous work, another sTF construct, sTF$_{1–218}$, remained monomeric in the aqueous solution.53

The amidolytic and FXase activities of TF$_1$-LZ-FVIIa, (TF$_1$-LZ-FVIIa)$_2$, and sTF$_{1–218}$-FVIIa were measured in aqueous solution. The amidolytic activities were identical. However, the FXase activities of TF$_1$-LZ-FVIIa and (TF$_1$-LZ-FVIIa)$_2$ were 5-fold greater than the FXase activity of sTF$_{1–218}$-FVIIa. This evidence is the basis for the conclusion that sTF self-association does not impair sTF PCA.

Experiments supporting the hypothesis that TF self-association inhibits TF PCA have used full-length TF on cell surfaces. The evidence against this hypothesis comes from functional assays performed in aqueous buffer in the absence of phospholipids. There are fundamental differences between protein–protein interactions in aqueous solution and on membrane surfaces that may account for the apparent discrepancy. The main difference is the limit on movement imposed by attachment to a surface. Membrane-anchored TF as well as membrane-bound FVIIa and FX are not free to rotate about the axis perpendicular to the membrane surface. Restricting movement to the plane of the membrane may be an important factor in TF self-association, FVIIa binding to TF, and catalytic complex assembly.

There is no reason to assume a priori that the quaternary structures of dimeric TF$_1$-LZ in solution and dimeric TF on a cell surface are the same. The relative positions of the shared domains may be different. The evidence that TF$_1$-219 is a monomer in aqueous solution$^{53}$ suggests that the energy of
association for TF-LZ dimerization comes predominately from the LZ–LZ interaction. The experimental results of Doñate et al suggest there are no stable contacts between the sTF(1–20) domains of the chimeric TF-LZ dimer. FVIIa binding is unimpaired and FX is hydrolyzed, albeit very slowly. If the FX docking sites on the sTF subunits of (TF1–LZ-FVIIa), were tightly associated or even held in close proximity, then steric hindrance would prevent FX binding and hydrolysis. Thus, in the soluble chimeric dimer the sTF(1–20) domains appear to be unconnected and free to rotate independently about their LZ tails.

There is evidence, in addition to the cross-linking data, of changes in TF structure and function when encrypted TF is converted to decrypted TF. The rate of chemical modification of the essential lysine residues in the substrate binding site, lys165 and lys166, is significantly increased after decryption. This result is consistent with a change in the chemical environment of lys165 and lys166. Furthermore, the binding of TFPI-FXa to encrypted TF-FVIIa is impaired relative to its association with decrypted TF-FVIIa. This may be the result of either a reduction in affinity or blocked access to the site. The details of this restriction on TFPI-FXa binding are undefined. It could be imposed by TF self-association, binding of another regulatory protein to the site, or conformational events. At present the only relevant evidence is the correlation between TF dimerization and TF encryption.

Waxman et al demonstrated significant quantitative differences between sTF-FVIIa in solution and TF-FVIIa on a membrane surface. The form of the enzyme used in these experiments was active site-inhibited FVIIa (FVIIai). The cofactor was either sTF1–219 in water or full-length TF (TF1–263) reconstituted into phospholipid bilayers. In each case the stoichiometry of the complex was 1:1. Pressure dissociation experiments yielded a Kd for the sTF1–219–FVIIai complex of 0.59 nM in aqueous solution. By the same technique the Kd for the TF1–263–FVIIai complex on phosphatidylcholine vesicles was 7.3 pM. The molar volume change of association was not known. Subsequent work has demonstrated that palmitoylation of cytoplasmic cystines can target integral membrane proteins to lipid rafts.

Two studies have compared catalytic complex assembly in solution and on membrane surfaces. Ruf et al estimated the enhancement of sTF1–219–FVIIa proteolytic activity by acidic vesicles to be more than 100-fold. Waxman et al detected no FX hydrolysis by sTF1–219–FVIIa in the absence of acidic phospholipid vesicles. However, when optimum levels of PS-containing vesicles were added to the reaction, the FXase activity of sTF1–219–FVIIa was only 4% of the activity expressed by TF1–263–FVIIai anchored on the surface of PS-containing vesicles. Both studies concluded that the relevant substrate at physiological concentrations is FX bound to a phospholipid surface.

These differences in functionally significant protein–protein interactions underscore the difficulties in comparing FXase reactions in solution with FXase reactions on a membrane. The physiologically relevant catalytic complex is formed when membrane-associated TF-FVIIa binds membrane-associated FX. Thus, membrane-anchored TF may be required in experiments testing the hypothetical connection between TF self-association and TF encryption on the cell surface.

**Lipid Rafts**

PS asymmetry may not be all that is required for TF encryption in vivo. Another possible contribution is suggested by the evidence of an association between TF and lipid rafts. The sequestering of TF in specific membrane microdomains was first proposed in 1975. When cultured cells are exposed to a protease, cell viability is maintained and most of the TF is shed in microparticles comprising only a small fraction of the plasma membrane. It is clear from this result that the distribution of TF on the cell surface is not random. Later it was shown that cys245 on the cytoplasmic tail of TF is acylated. At that time of the discovery the functional significance this posttranslational modification was not known. Subsequent work has demonstrated that palmitoylation of cytoplasmic cystines can target integral membrane proteins to lipid rafts.

Localization of an integral membrane protein in a lipid raft can promote self-association by mass action. Interactions with other components of this membrane microdomain may also contribute to the energy of oligomerization. It was recently demonstrated that disrupting lipid rafts increases in the basal expression of TF PCA. This result is consistent with the model wherein binding to lipid rafts promotes TF encryption via enhanced self-association.

It is not known where encrypted TF and decrypted TF reside in the plasma membrane. From the evidence presented, I would expect encrypted TF to be sequestered in lipid rafts and decrypted TF to be released after cell stimulation into the liquid disordered phase of the membrane (Figure). If palmitoylation of cys245 enhances the association of TF with lipid rafts, then this post-translational modification may be important for TF encryption.

The association with lipid rafts may also affect a TF function that is independent of TF PCA. The role of TF in coagulation-dependent and coagulation-independent cell signaling is the subject of a recent review. When the TF cytoplasmic domain is deleted, protease activated receptor 2 (PAR-2)-dependent angiogenesis is enhanced in mice. Also, ocular tissue neovascularure from diabetic patients shows PAR-2 colocalization with phosphorylated TF. These results are the basis for the hypothesis that the dephosphorylated cytoplasmic domain of TF is a negative regulator of PAR-2 signaling. So far no direct connection between TF encryption and TF-mediated coagulation-independent signaling has been established. However, the evidence that palmitoylation of cys245 is a negative regulator of TF phosphorylation suggest the possibility that encrypted TF is an inhibitor of PAR-2 signaling.

One of the pleiotropic effects of statins is a decrease in thrombotic events. This may be caused, in part, by a reduction in TF gene expression. Another possible connection between cholesterol and TF PCA is suggested by the evidence that reducing cholesterol in the plasma membrane by methyl-β-cyclodextrin extraction impairs a cell’s ability to expose PS. This treatment also diminishes the decryption of TF PCA on HL-60 cells by Ca2+ ionophore stimulation.
Decryption of TF PCA on the cell surface via Ca\(^{2+}\) influx. In this model of TF-initiated coagulation the expression of TF PCA is initiated by an agonist-induced increase in cytosolic Ca\(^{2+}\). Both capacitative and noncapacitative Ca\(^{2+}\) influxes are required to achieve the necessary level of cytosolic Ca\(^{2+}\). The Ca\(^{2+}\) increase inhibits flipflopase activity and activates scramblase activity. The net result is the loss of PS asymmetry. In addition, Ca\(^{2+}\) influx triggers the release of TF from lipid rafts into the liquid disordered phase. Whereas the total number of TF molecules on the cell surface remains unchanged, the local density decreases. Thus, a change in TF quaternary structure may be driven by increasing PS and decreasing TF surface densities.

Bach, unpublished, 2005). Thus, statins may inhibit TF-initiated coagulation by suppressing both TF gene expression and the decryption of TF PCA.

Autoactivation of FVII

TF PCA is expressed by decrypted TF-FVIIa. Therefore, proteolytic activation of FVII must occur at some point in the decryption process. One way to convert FVII to FVIIa is via an autocatalytic reaction on the cell surface.\(^6\) A trace of FVIIa is already present in normal plasma.\(^8\) Therefore, the appearance of decrypted TF in blood will result in the formation of a few decrypted TF-FVIIa complexes. Because both the enzyme (TF-FVIIa) and the substrate (TF-FVII) are attached to the membrane, the rate of autoactivation is a function of their surface densities and mobilities. High substrate density and mobility in the vicinity of the enzyme will favor a burst of TF-FVII→TF-FVIIa conversion. Therefore, the autocatalytic activation of FVII may be an efficient way to convert TF-FVII to TF-FVIIa on extravascular cells where TF surface density is relatively high. However, the low TF surface density on blood cells will result in a much slower rate of autoactivation.\(^9\)

Failure to promote FVII autoactivation could inhibit the ability of intravascular TF to initiate coagulation. An alternative pathway for FVII cleavage emerges when FXa is generated by the expression of extravascular TF PCA. FXa can activate FVII either before or after it is bound to TF.\(^7\) Thus, the participation of intravascular TF in hemostasis may be confined to the propagation phase of coagulation. This property of intravascular TF may account for at least some of the efficacy of FVIIa as a hemostatic agent.\(^7\) Furthermore, the suppressed proteolytic activity of encrypted intravascular TF-FVIIa may explain why infusions of FVIIa do not trigger thrombosis.

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