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Regulation of Coagulation by a Multivalent Kunitz-Type Inhibitor†

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Blood coagulation proceeds through a series of reactions in which plasma zymogens of serine enzymes are sequentially activated by limited proteolytic cleavage. Mechanistically, the initiation of coagulation has been separated into two pathways, "extrinsic" and "intrinsic", that converge at the activation of factor X with subsequent generation of thrombin proceeding through a single, "common", pathway (MacFarlane, 1964; Davie & Ratnoff, 1964) (Figure 1). In the extrinsic pathway, factor VII_a bound to its cofactor, tissue factor (TF), can activate factor X directly. In the intrinsic pathway, exposure of the contact factors (factor XII, prekallikrein, and high molecular weight kininogen) to a negatively charged surface leads to the activation of factor XI with subsequent activation of factor IX. Activated factor IX, in the presence of its cofactor factor VIII, then cleaves factor X to factor X_a.

Since people individually deficient in one of the contact factors do not bleed, it appears most likely that the extrinsic, or TF-mediated, pathway provides the trigger for physiologic coagulation in vivo. Whereas factor VII and TF may be responsible for the *initiation* of coagulation, however, it is clear that the generation of factor X_a by the factor VII_a/TF catalytic complex alone is not sufficient for ultimate hemostasis since hemophiliacs, who lack either factor IX or factor VIII, suffer a severe hemorrhagic diathesis. The in vivo requirement for additional factor X_a produced through the action of factors VIII and IX_a may be explained by a novel inhibitor that produces feedback inhibition of the VII_a/TF complex. This inhibitor has most recently been called extrinsic pathway inhibitor (EPI) (Rao & Rapaport, 1987) or lipoprotein-associated coagulation inhibitor (LACI) (Broze et al., 1987a). An excellent review of the subject has been published previously (Rapaport, 1989).

Tissue Factor Initiated Coagulation

Early investigators noted that the addition of damaged tissue hastened the clotting of blood. The procoagulant contained in tissue was initially called tissue thromboplastin and more recently TF or factor III (Wright, 1962). Human TF is a MW ~45 000, single-chain, integral membrane protein (Broze et al., 1985; Guha et al., 1986). It is produced constitutively by tissue cells that are normally separated from blood by the vascular endothelium (Maynard et al., 1975, 1977; Weiss et al., 1989, Wilcox et al., 1989). Human factor VII is a single-chain glycoprotein of MW ~50 000 that is present in plasma at trace concentrations (~10 nM) (Broze & Majerus, 1980; Bajaj et al., 1981). The vitamin K dependent γ carboxylation of 10 glutamic acid residues near the N-terminus of factor VII is required for its ability to bind calcium ions and its functional activity (Hagen et al., 1986). Whether zymogen VII possesses intrinsic catalytic activity is controversial (Radcliffe & Nemerson, 1975; Zur et al., 1982; Rao et al., 1986; Rao & Rapaport, 1988; Williams et al., 1989). Factor VII can be activated to a two-chain, disulfide-linked form, factor VII_a, through limited proteolytic cleavage by factor X_a, factor IX_a, factor XII_a (or βXII_a), and thrombin (Broze & Majerus, 1982). Zymogen VII and factor VII_a bind to TF in the presence of calcium ions with equal affinities (Zur et al., 1982; Broze, 1982; Bach et al., 1986), and this TF binding dramatically affects their coagulant activities. First, zymogen VII bound to TF, as opposed to that in solution, is rapidly and preferentially cleaved to factor VII_a by trace concentrations of factor X_a (Nemerson & Repke, 1985; Rao & Rapaport, 1988). Second, the enzymatic activity of factor VII_a is enhanced several thousand fold when it is bound to TF (Silverberg et al., 1977; Bach et al., 1981; Broze et al., 1985). Thus, normal coagulation is presumably initiated when factor VII or factor VII_a in plasma gains access to TF through a laceration in the vessel endothelium at a site of a wound.

In 1977, Osterud and Rapaport showed that the VII_a/TF catalytic complex not only activates factor X, the classic extrinsic pathway of coagulation (Nemerson, 1966), but activates factor IX as well. In vitro, the preferred substrate for VII_a/TF is factor X (Zur & Nemerson, 1980; Jesty & Silverberg, 1979), and this remains so in the presence of heparin, which has been shown to increase the rate of factor IX activation

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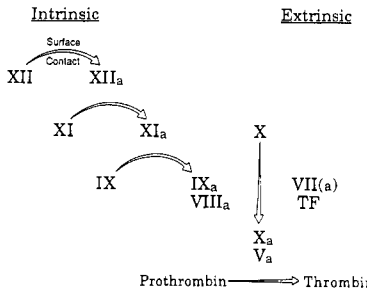

FIGURE 1: Modified cascade scheme of blood coagulation. The intrinsic pathway of coagulation is initiated by exposure of the contact factors—factor XII, prekallikrein, and high molecular weight kininogen—to an appropriate surface with subsequent activation of factor XI by factor XII_a. The extrinsic pathway is initiated by exposure of factor VII or factor VII_a [VII(a) denotes either factor VII or factor VII_a] to TF. Cofactors V and VIII are shown in their activated forms (V_a, $VIII_a$). The requirement for phospholipids and calcium for certain of the reactions is not included.

while having little effect upon the activation of factor X by VII_a/TF (Repke et al., 1981).

When compared to the other activated coagulation factors, factor VII_a is remarkably stable (Radcliffe et al., 1977), remaining in the circulation nearly as long as zymogen VII (Seligsohn et al., 1978). Even antithrombin III in the presence of heparin inhibits factor VII_a at only slow rates (Jesty, 1978; Broze & Majerus, 1980; Kondo & Kisiel, 1987). Thus physiologic regulation of TF-initiated coagulation does not appear to be mediated at the level of the enzyme factor VII_a, and instead LACI, which inhibits the catalytic VII_a/TF complex, may provide the endogenous means for regulating the initiation of coagulation.

LACI History

In 1947, Thomas and Schneider independently showed that the preincubation of tissue thromboplastin with serum prevented the lethal disseminated intravascular coagulation that occurs following thromboplastin infusion into animals. Thomas also noted that this inhibitory effect of serum required the presence of calcium ions, that the inhibitor appeared to bind to the thromboplastin, and that the inhibition could be reversed by calcium ion chelators. The calcium requirement and reversibility of the thromboplastin inhibition were later confirmed by using in vitro coagulation assays (Mann & Hurn, 1949; McClaughry, 1950; Lanchantin & Ware, 1953; Berry, 1957; Hermansky & Vitek, 1960). In a landmark study, Hjort (1957) showed that the serum inhibitor recognized the factor VII–Ca^{2+}–thromboplastin complex, which he called "convertin", rather than factor VII or thromboplastin alone and, using indirect means, suggested that the binding of the inhibitor to convertin was also calcium dependent.

By the 1960s, other investigators had noted that while the clotting of hemophiliac plasma following the addition of large quantities of TF (standard prothrombin time) was indistinguishable from that of normal plasma, the coagulation of hemophiliac plasma was delayed and incomplete following the addition of small amounts of TF (Biggs & MacFarlane, 1951; Biggs & Nossel, 1961). Later it was shown that under these latter conditions the rate of factor X_a generation in hemophiliac plasma, deficient in either factor VIII or factor IX, was much less than in normal plasma (Marlar et al., 1982). Morrison and Jesty (1984) found that only partial activation of factor IX and factor X occurred in normal plasma following the addition of TF and showed that this apparent inhibition of VII_a/TF catalytic activity required the presence of factor X or brief pretreatment of the plasma with factor X_a. The studies

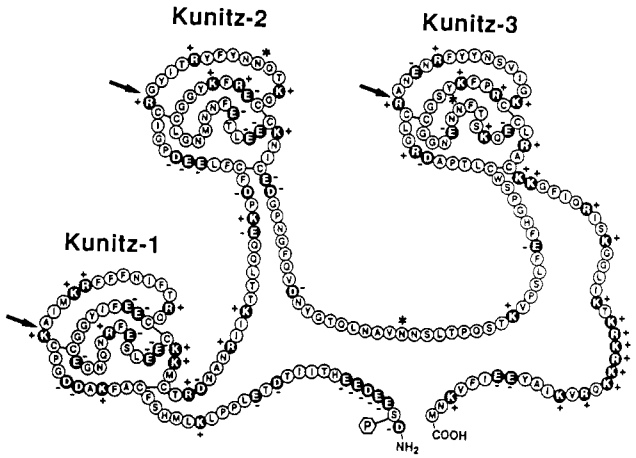

FIGURE 2: Predicted structure of LACI. Arrows indicate the location of the presumed active-site inhibitor clefts (P_1–P_1') for each Kunitz-type domain (Gebhard et al., 1986). The charges of the amino acid side chains are shown (histidine side chains are considered as uncharged). Asterisks indicate the potential sites for N-linked glycosylation, and P represents the site of partial phosphorylation.

of Sanders et al. (1985) documented that both factor X and an inhibitor present in the total lipoprotein fraction of plasma following density centrifugation were needed to produce this VII_a/TF inhibition. Finally, additional reports confirmed these findings and demonstrated that the functional properties of the inhibitor were the same as those previously described for "anticonvertin" by Hjort (Hubbard & Jennings, 1986; Broze & Miletich, 1987a; Rao & Rapaport, 1987).

LACI Structure

LACI was initially isolated from the serum-free conditioned media of HepG2 cells, a human hepatoma cell line (Broze & Miletich, 1987b; Broze et al., 1987a). This LACI has a M_r of 39 000 and was found not only to inhibit the VII_a/TF complex in a factor X_a dependent fashion but also to directly inhibit factor X_a (Broze, 1987). The possible explanation for the multiple inhibitory specificities of LACI was provided with the subsequent isolation and cloning of LACI cDNA (Wun et al., 1988; Girard et al., 1988). The primary structure of LACI predicted by cDNA sequence shows that, after a 24 or 28 amino acid signal peptide, the mature molecule has 276 residues (32 kDa) and contains an acidic amino-terminal region followed by three tandem domains with homology to Kunitz-type protease inhibitors and a basic carboxy-terminal region (Figure 2).

Several posttranslational modifications appear to occur in the LACI molecule. LACI contains N-linked oligosaccharides, but which of the three possible sites of attachment (see Figure 2) are utilized remains to be reported (Novotny et al., 1989a). Experiments using metabolic labeling with $^{35}SO_4$ show that LACI produced by umbilical vein endothelial cells and kidney carcinoma cells (Caki, ATCC HTB46), but not HepG2 cells, contains sulfated N-linked carbohydrate (Novotny et al., unpublished). Thus a sulfated, MW 45 000 coagulation inhibitor recently isolated from the conditioned media of rabbit arterial endothelial cells may represent rabbit LACI (Colburn & Buonassisi, 1988). Whether the sulfation of N-linked carbohydrate in LACI affects its functional properties is not yet known. LACI produced by HepG2 cells and recombinant LACI produced by mouse C127 fibroblasts are partially phosphorylated at serine-2, most likely through the action of casein kinase II (Girard et al., 1989c). Again, what effect this phosphorylation has on the biosynthesis or properties of LACI is unknown.

FIGURE 3: Amino acid sequences of the Kunitz-type domains in LACI and related inhibitors: ITI, human inter-α-trypsin inhibitor, which contains two Kunitz-type domains, and BPTI, bovine basic pancreatic trypsin inhibitor. The arrow indicates the P_1–P_1' active-site cleft. Asterisks overlie the specifically spaced cysteine residues. Conserved amino acid residues are boxed. The boldly underlined amino acids of BPTI are in close contact with trypsin in the trypsin–BPTI complex.

Cells that constitutively express LACI, for example, cultured liver and endothelial cells, contain LACI mRNAs of 4.0 and 1.4 kb (Wun et al., 1988; Girard et al., 1988), which arise by the use of alternative termination and polyadenylation signals during processing (Girard et al., 1989b). Although the 3′ untranslated region of the 4.0-kb message contains 46 AUUU motifs and two UAAUUUAU sequences, which might cause instability of this message (Shaw & Kamen, 1986; Caput et al., 1986), Northern blot analysis of mRNA obtained from actinomycin-treated cells shows that the 4.0- and 1.4-kb messages are both relatively stable (>80% of each message remaining at 7 h).

Characteristics of Kunitz-Type Inhibitors

Kunitz-type serine protease inhibitors are prominently represented in both animal and plant kingdoms, and the most extensively characterized member of this inhibitor family is bovine basic pancreatic trypsin inhibitor, or aprotinin (Trasylol) (Gebhard et al., 1986). With one exception (Sasaki, 1984), Kunitz-type inhibitors contain six specifically spaced cysteine residues (Figure 3). The intramolecular disulfide bridges are presumably responsible for the observed functional stability of Kunitz-type inhibitors despite treatment with a variety of physical and chemical denaturants, including sodium dodecyl sulfate (Gebhard et al., 1986; Broze & Miletich, 1987b). These inhibitors appear to act by the standard mechanism (Laskowski & Kato, 1980) in which the inhibitor feigns to be a good substrate, but after the enzyme binds, subsequent cleavage at the active-site cleft (P_1–P_1') of the inhibitor occurs only very slowly or not at all. The P_1 residue is an important determinant of inhibitory specificity, and alterations of the residue in the P_1 position can profoundly alter the activity of Kunitz-type inhibitors (Wenzel & Tschesche, 1981; see below).

In kinetic terms, Kunitz-type inhibitors typically produce slow, tight-binding, competitive, and reversible inhibition of the form:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^* \qquad (1)$$

where E is enzyme, I is inhibitor, EI is the initial collision complex with a K_i(initial) = k_2/k_1, and EI* is the final complex that develops slowly from EI and is of higher affinity (Morrison, 1982; Antonini et al., 1983; Broze et al., 1987b). The K_i(final) of the final EI* complex equals $K_i[k_4/(k_3 + k_4)]$. "Slow" implies the inhibition is not immediate, and "tight-binding" refers to the fact that these inhibitors produce significant inhibition at concentrations near that of the enzyme being inhibited. This latter property can make the determination of kinetic constants technically difficult because, as the inhibitor concentration approaches that of the enzyme, the total inhibitor concentration can no longer be used as a measure of free inhibitor concentration. Specific techniques, however, are available for appropriate kinetic analysis (Cha, 1975, 1980; Williams, 1979; Bieth, 1980).

Table I: Effect of Heparin on the Inhibition of Factor X_a and Trypsin by LACI

enzyme	heparin (1.0 unit/mL)	K_i(initial) (nM)	k_3 (min^{-1})	k_4 (min^{-1})	K_i(final) (nM)
factor X_a	–	26	1.4	0.035	0.630
	+	3	1.0	0.005	0.015
trypsin	–	18	2.2	0.080	0.630
	+	22	5.0	0.090	0.390

LACI Inhibition of Factor X_a

LACI produces direct inhibition of factor X_a by binding at or near its serine active site (Broze, 1987; Broze et al., 1988). This factor X_a–LACI interaction has 1:1 stoichiometry, does not require the presence of calcium ions, and can be reversed by treatment with sodium dodecyl sulfate or high concentrations of the serine protease inhibitor benzamidine (Broze et al., 1987a, 1988). LACI is also a potent inhibitor of trypsin but does not significantly affect the activities of leukocyte elastase, urokinase, activated protein C, tissue plasminogen activator, thrombin, or kallikrein and inhibits plasmin and chymotrypsin only modestly (Broze et al., 1987b).

The derived kinetic constants for LACI inhibition of human factor X_a and bovine trypsin are shown in Table I. Heparin enhances the inhibition of factor X_a by LACI 40-fold by decreasing the K_i(initial) of the initial collision complex (EI) and lowering the k_4 of the final EI* complex (see eq 1) (Crecelius et al., submitted for publication). This effect of heparin upon the factor X_a–LACI interaction is not due to a conformational change in LACI with subsequent enhanced binding to all target enzymes, since the inhibition of trypsin by LACI is not affected by heparin. The second Kunitz domain of LACI has recently been identified as the structure in LACI responsible for factor X_a inhibition (Girard et al., 1989a), which is consistent with previous studies reporting that the modification of arginine residues (the P_1 residue in the second Kunitz domain) in LACI abrogates its inhibition of factor X_a (Warn-Cramer et al., 1988).

The importance of LACI as an inhibitor of factor X_a in vivo is not known. Its plasma concentration is low (~2.5 nM) and 10-fold below the K_i of its initial encounter complex with factor X_a. Plasma LACI, however, may represent only a small fraction of the total endogenous LACI that is readily available to interact with factor X_a (see below), and the association of LACI with the heparin-like glycosaminoglycans on the surface of endothelial cells may markedly enhance its inhibitory potency against factor X_a (Table I). Since factor X_a–LACI binding is reversible, one would expect the eventual transfer

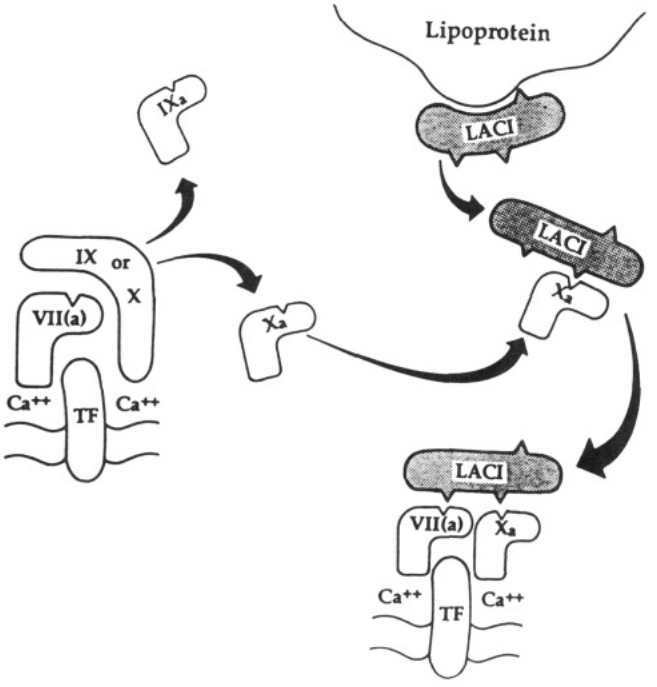

FIGURE 4: Proposed mechanism for the inhibition of the VII_a/TF complex by LACI. VII(a) denotes either factor VII or factor VII_a. TF apoprotein is shown spanning a phospholipid membrane. The indentations represent the active sites for factor VII_a and factor X_a, and the protrusions represent the three Kunitz-type domains of LACI. In the factor X_a–LACI complex, the active site of factor X_a is bound to the second Kunitz domain of LACI. In the final quaternary X_a-LACI–VII_a/TF complex, factor X_a is bound at its active site to Kunitz-2 and factor VII_a is bound at its active site to Kunitz-1. Not shown is the alternative pathway for formation of the final quaternary complex in which LACI binds to a preformed factor X_a–VII_a/TF complex. The mechanism of the association of LACI with lipoproteins is not known. (Reprinted with permission from Girard et al. (1989a). Copyright 1989 Macmillan Magazine, Ltd.)

of factor X_a from this complex to its major plasma inhibitors, antithrombin III, α_1 proteinase inhibitor, and α_2 macroglobulin, which form essentially irreversible bonds with factor X_a (Pratt & Pizzo, 1986; Colman et al., 1987). Indeed, it is remarkable that the factor X_a dependent inhibition of VII_a/TF by LACI is quite stable (Broze & Miletich, 1987a). This suggests that the affinity of factor X_a binding in the putative X_a–LACI–VII_a/TF inhibitory complex (see below) may be considerably greater than the affinity of factor X_a binding to LACI alone or, alternatively, that factor X_a is protected from protease inhibitors other than LACI in the milieu of VII_a/TF. In this regard, the presence of VII_a/TF but not TF alone reportedly protects factor X_a from inactivation by antithrombin III (Jesty, 1986).

LACI Inhibition of the Factor VII_a/Tissue Factor Complex

The proposed mechanism for the factor X_a dependent inhibition of VIIα/TF involves the formation of a quaternary X_a–LACI–VII_a/TF complex (see Figure 4) (Broze, 1987; Broze et al., 1988). This inhibitory complex could result from the initial binding of factor X_a to LACI with subsequent binding of the factor X_a–LACI complex to VII_a/TF, or alternatively, LACI could bind to a preformed factor X_a–VII_a/TF complex. This hypothesis explains the need for the persistent presence of factor X_a for VII_a/TF inhibition (Rao & Rapaport, 1987) and also explains the requirement for active factor X_a (Broze & Miletich, 1987a), since active-site-inactivated factor X_a will not bind to LACI. Further support for this mechanism of inhibition is provided by the

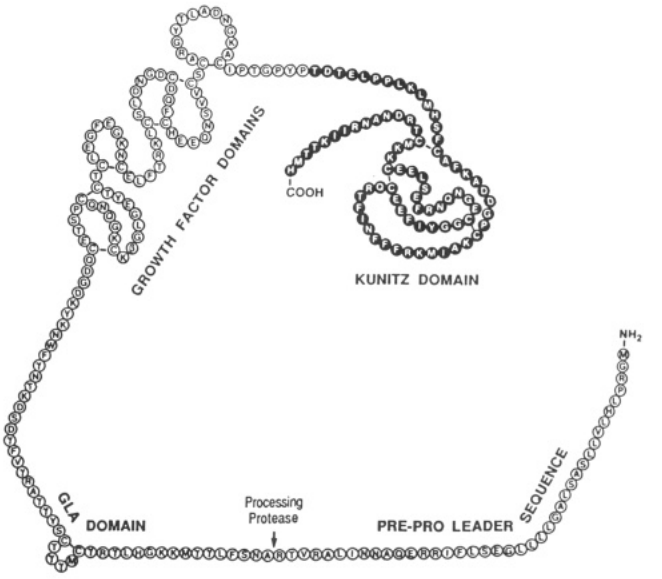

FIGURE 5: Structure of the $X_{LC}LACI_{K1}$ hybrid protein. The layout of the factor X light-chain structure is taken from Leytus et al. (1986). The LACI-derived portion of the molecule is shaded. The arrow indicates the presumed cleavage site of the signal peptidase.

observation that factor X_a lacking its amino-terminal γ-carboxyglutamic acid containing domain (GD-X_a), which is required for calcium binding (Morita & Jackson, 1986), binds to and is inhibited by LACI but the subsequent GD-X_a–LACI complex does not inhibit VII_a/TF (Broze et al., 1988; Warn-Cramer et al., 1988). This result is also consistent with the suggestion of Hjort (1957) that the binding of the inhibitor (the factor X_a–LACI complex) to VII_a/TF is calcium dependent.

By expressing altered forms of recombinant LACI in which the P_1 residue at the active-site cleft in each Kunitz domain was individually changed, Girard et al. (1989a) showed that the Kunitz-2 domain (Figure 2) is responsible for factor X_a binding and inhibition and that both the Kunitz-1 and Kunitz-2 domains were required for factor X_a dependent inhibition of VII_a/TF. The inability of the Kunitz-2 mutant to inhibit VII_a/TF is further confirmation that the binding of factor X_a to LACI is a prerequisite for VII_a/TF inhibition. The fact that the Kunitz-1 mutant failed to inhibit VII_a/TF, yet bound and inactivated factor X_a normally, is consistent with the notion that this Kunitz domain binds to and inhibits factor VII_a in the final quaternary X_a–LACI–VII_a/TF complex (Figure 4). The LACI with an altered P_1 residue in its third Kunitz domain inhibited factor X_a and VII_a/TF in a manner equivalent to native LACI. Unless a portion of Kunitz-3 unrelated to its active-site cleft is required for these properties, this suggests that yet another binding/inhibitory function of LACI may remain to be discovered.

Factor X–LACI Hybrid Protein Inhibits the Factor VII_a/Tissue Factor Complex

Both the amino-terminal, γ-carboxyglutamic acid containing domain of factor X_a and the Kunitz-1 domain of LACI are required for the inhibition of VII_a/TF by the factor X_a–LACI complex (see above). To determine whether the linkage of limited portions of factor X_a and LACI is sufficient for VII_a/TF inhibition, a recombinant cDNA encoding a fusion protein containing the preproleader sequence, γ-carboxyglutamic acid domain, and "growth factor" domain of factor X (Leytus et al., 1986) followed by the Kunitz-1 domain of LACI has been constructed (Figure 5). The hybrid protein

expressed in C127 mouse fibroblasts and designated $X_{LC}LACI_{K1}$ is a potent direct inhibitor of VII_a/TF in the absence of factor X_a (Girard et al., submitted for publication). In a modified prothrombin time assay, 50% inhibition of apparent TF activity occurs at concentrations of 35 ng/mL $X_{LC}LACI_{K1}$ and 2.5 μg/mL LACI. Moreover, the inhibitory effect of LACI in this assay is due at least in part to its direct inhibition of factor X_a, since the same concentration of LACI (but not $X_{LC}LACI_{K1}$) prolongs the coagulation time of plasma induced to clot by contact activation (activated partial thromboplastin time; aPTT). $X_{LC}LACI_{K1}$ produced by cells grown in the presence of warfarin, which prevents the vitamin K dependent γ-carboxylation of glutamic acid residues in the factor X light chain, lacks inhibitory activity. Thus the formation of the factor X_a–LACI complex through the binding of the Kunitz-2 domain in LACI with the active-site serine in factor X_a may serve to juxtapose the factor X_a light chain and LACI Kunitz-1 domains, thereby producing a complex with much greater affinity for VII_a/TF than LACI alone. Whether $X_{LC}LACI_{K1}$ and/or the factor X_a–LACI complex compete directly with factor IX and factor X for the substrate binding site(s) on VII_a/TF or instead interact with VII_a/TF through an alternative site (or sites) remains to be established.

Lipoprotein Association of Plasma LACI

In 1981, Carson noted that plasma lipoproteins inhibited the catalytic activity of VII_a/TF. Shortly thereafter, Dahl et al. (1982) demonstrated that anticonvertin activity migrated as two high molecular weight peaks following gel filtration of plasma, which in retrospect may have represented low-density lipoproteins (LDL) and high-density lipoproteins (HDL). The lipoprotein association of LACI was subsequently established (Sanders et al., 1985; Broze & Miletich, 1987a) and its relative distribution in LDL > HDL ≫ very low density lipoproteins (VLDL) was determined (Hubbard & Jennings, 1987).

When analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, LACI purified >500 000-fold from plasma consists of multiple forms with predominant proteins of M_r 34 000 and 40 000 and less abundant bands of higher apparent molecular weight (Novotny et al., 1989a). With reduction, the multiple bands are consolidated into a tightly spaced doublet at M_r 36 000 and a faint diffuse band at M_r 7000. Western blot analysis showed that the M_r 40 000 and higher molecular weight forms contain apolipoprotein AII (apo-AII) as well as LACI, whereas the M_r 34 000 band represents apparently uncomplexed LACI, as originally suggested by Warn-Cramer et al. (1987). The major form of LACI in LDL is M_r 34 000 (uncomplexed), that in HDL is M_r 40 000 (LACI/apo-AII complexes), and VLDL contains both M_r 34 000 and 40 000 forms. The possible participation of other proteins or peptides in the higher molecular weight LACI/apo-AII complexes has not been excluded.

The mechanism responsible for the formation of the mixed disulfide LACI/apo-AII complexes is not known, but interestingly similar complexes between apolipoprotein E (apo-E) and apo-AII have been described (Weisgraber & Mahley, 1978) and both the LACI/apo-AII and apo-E/apo-AII complexes reside in the larger sized HDL particles, i.e., HDL_1 (Weisgraber & Mahley, 1986; Novotny et al., 1989a). Apo-AII normally exists as a disulfide-linked homodimer of MW 14 000. The apo-AII monomer contains a single cysteine near its amino terminus, which presumably is involved in its linkage to LACI. Since the LACI/apo-AII complexes in plasma possess factor X_a and VII_a/TF inhibitory activity (Novotny et al., 1989a), and since Kunitz-3 of LACI may not be required for these functions (Girard et al., 1989a), it appears most likely that cysteines in this third Kunitz domain are involved in the disulfide bridge formation with apo-AII.

The means by which LACI associates with lipoproteins is not clear. Apo-AII could direct the lipoprotein incorporation of LACI/apo-AII complexes, but M_r 34 000 LACI lacking apo-AII is also found in lipoproteins, LDL in particular. Possible mixed disulfide binding of the M_r 34 000 LACI with a small, lipophilic peptide, however, has not been excluded. The predicted primary structure of the LACI molecule itself does not contain obvious regions of hydrophobicity or amphipathic α helix (Rall et al., 1982) to explain its lipid association. LACI purified from HepG2 cells and rLACI produced by mouse C127 cells are M_r 39 000, rather than the M_r 34 000 of plasma LACI uncomplexed with apo-AII, and this disparity appears to be related to the extent of N-linked glycosylation (Novotny et al., 1989a). Whereas the LACI purified from plasma reincorporates into lipoproteins following incubation with plasma, LACI isolated from these cultured cells does not associate with plasma lipoproteins (Novotny et al., unpublished). Thus some form of additional or alternative posttranslational processing, which occurs in vivo but not in HepG2 or C127 cells in vitro, may be required for the lipoprotein-binding property of LACI.

LACI in Blood

LACI is synthesized by cultured cells derived from a variety of tissues including liver (Broze & Miletich, 1987a; Wun et al., 1988), endothelium (Bajaj et al., 1987), the monocyte-like cell line U937 (Rana et al., 1988), lung, bladder, and kidney (Crecelius and Broze, unpublished). Which cell type is responsible in vivo for the maintenance of plasma LACI levels is not known, but the endothelium is an attractive candidate given its mass and location. Unlike levels of tissue plasminogen activator and von Willebrand factor, which are also synthesized in endothelial cells, LACI levels are not increased by infusion of DDAVP and venous occlusion (Sandset et al., 1988; Warr et al., 1989; Novotny et al., 1989b).

Platelets have been shown to release LACI following stimulation with thrombin or a calcium ionophore (A23187) (Novotny et al., 1988). Assuming a normal platelet count (3×10^8/mL), only ~8% of the LACI in blood is sequestered in platelets. At the site of a wound, however, the contribution of LACI released from platelets could be substantial due to platelet aggregation at the developing thrombus. Indeed, the concentration of LACI in blood escaping from a superficial laceration (template bleeding time) reaches a level severalfold that of venous blood obtained simultaneously by venipuncture (Novotny et al., 1988). This increase is presumably related to the release of platelet LACI, although the contribution of other cells, including perhaps endothelial cells, cannot be excluded.

Sandset et al. (1988) reported that heparin administration in vivo increases the concentration of LACI in plasma, and Thomas et al. (1980) may have been describing this same phenomenon when they noted that the antithrombin III independent anti-factor X_a activity of plasma was increased following the infusion of a heparin analogue. This effect of heparin is dramatic, with antigenic LACI levels increasing an average of 3.75-fold 10 min after 2500 units of intravenous heparin and as much as 10-fold following the infusion of 7500 units of heparin (Novotny et al., 1989b). Since the ex vivo addition of heparin to blood or plasma does not change its LACI level, the in vivo effect of heparin appears to be related to the release of LACI from intra- or extracellular stores. Heparin-induced release of LACI from cultured HepG2 cells can be demonstrated in vitro (Crecelius et al., unpublished).

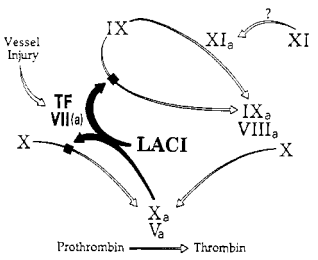

FIGURE 6: Scheme of blood coagulation including LACI-mediated feedback inhibition of VII_a/TF. VII(a) denotes factor VII or factor VII_a. The cofactors V and VIII are shown in their activated forms (V_a, $VIII_a$). The requirement for phospholipids and calcium for certain of the reactions is not included. See text for discussion.

There is a broad range of plasma LACI concentrations in normal individuals (60–180 ng/mL, mean 113 ng/mL) (Sandset et al., 1989; Warr et al., 1989; Novotny et al., 1989b), and modest alterations in the plasma concentration of LACI have been described in certain clinical conditions [reviewed by Broze et al. (1990)]. The physiologic relevance of these differences between the plasma LACI levels in health and disease is difficult to interpret. The severalfold increase in LACI concentrations following heparin treatment suggests that LACI in plasma represents only a fraction of the total LACI that can be readily mobilized and raises the possibility that other physiological or pathological mechanisms may similarly affect the concentration of LACI in plasma or at local sites. If LACI plays a critical role in regulating coagulation, the fact that individuals with abetalipoproteinemia have very low plasma LACI levels (<20%; Novotny et al., unpublished) and yet do not have an increased risk of thrombosis suggests that plasma may not be the most important reservoir of LACI.

Predicted Effect of LACI upon Coagulation

The properties of LACI suggest that it would function through a novel feedback mechanism to inhibit the VII_a/TF catalytic complex (Broze et al., 1988). A formulation of coagulation including the proposed effect of LACI is shown in Figure 6. Coagulation is initiated when damage to blood vessels at the site of a wound allows the exposure of blood to the TF produced constitutively by cells beneath the endothelium. The factor VII or factor VII_a present in plasma then binds to this TF, and the VII_a/TF complex activates some factor X to X_a and some factor IX to IX_a. With the generation of factor X_a, however, the inhibitory effect of LACI becomes manifest and prevents further production of factor X_a and factor IX_a by VII_a/TF. Then additional factor X_a can only be produced through the alternative pathway involving factor IX_a and factor VIII, where the factor IX_a results from the initial action of VII_a/TF and its supplemental production by factor XI_a.

Thus LACI-induced feedback inhibition of the VII_a/TF complex can explain the clinical need for both extrinsic and intrinsic (factors VIII, IX, XI) coagulation pathways and is consistent with the in vitro results of Marlar et al. (1982), showing deficient factor X_a production in hemophiliac plasma induced to clot by small quantities of TF. In addition, data to the present are consistent with the notion that, in normal hemostasis at least, VII_a/TF is responsible for an initial burst of factor X_a generation, which provides sufficient thrombin to induce the local aggregation of platelets and the activation of the critical coagulation cofactors factor V and factor VIII. That ultimate and persistent hemostasis, however, requires the continued production of additional factor X_a through the action of factor IX_a and factor VIII is consistent with the bleeding, frequently delayed in onset, seen in hemophiliacs (Roberts & Jones, 1990). The fact that patients with factor XI deficiency suffer a variable but usually mild bleeding diathesis (Roberts & Jones, 1990) suggests that under certain conditions the initial quantity of factor IX_a produced by VII_a/TF is insufficient and additional factor IX_a generated by factor XI_a is needed for normal hemostasis. The mechanism by which this factor XI_a is produced in vivo, however, has not been determined.

We stress that the predicted in vivo role of LACI is simply that—a prediction based on its known in vitro properties. Documentation of its physiologic importance remains to be provided and is an area of active research. Further, although significant progress has been made over the past few years in the characterization of LACI, many questions remain unanswered. For example, what is the mechanism for LACI's association with lipoproteins in plasma? What function, if any, does the third Kunitz-type protease inhibitor domain in LACI serve? What is the source and mechanism of the release of LACI in vivo by heparin? Clearly, our knowledge concerning this inhibitor is in its infancy, and future research will hopefully answer the questions posed above and, most importantly, establish the physiologic role of LACI.

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