Effects of Thrombomodulin and Coagulation Factor V_a-Light Chain on Protein C Activation In Vitro

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Abstract. Protein C activation by thrombin is significantly accelerated by the endothelial cell surface protein thrombomodulin, Factor V_a, or its light chain. In this study we have compared the activation of protein C in the presence of either cofactor and examined the possibility that thrombomodulin and Factor V_a-light chain act together to regulate protein C activation by thrombin. At all concentrations of protein C used, thrombomodulin was 20 times more efficient than Factor V_a-light chain in accelerating protein C activation by thrombin. Protein C treated with chymotrypsin to remove the amino-terminal 41 amino acids that contain the γ-carboxyglutamyl residues was activated by the thrombin-thrombomodulin complex at an identical rate to native protein C, whereas the modified protein C was activated by Factor V_a-light chain and thrombin at only 5% of the rate obtained by using native protein C. Increasing concentrations of Factor V_a-light chain, ≥30 nM, inhibited thrombin-thrombomodulin catalyzed protein C activation with complete inhibition observed at 90 nM Factor V_a-light chain. On the other hand, increasing thrombomodulin concentrations did not inhibit protein C activation by Factor V_a-light chain and thrombin. These reactions in solution mimic, in part, those obtained on endothelial cells where protein C lacking the γ-carboxyglutamyl domain is activated poorly and Factor V_a-light chain at concentrations greater than 50 nM inhibited the activation of native protein C. The results of this study suggest that thrombomodulin and Factor V_a-light chain may act in concert to regulate protein C activation by thrombin.

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Introduction

Two different cofactors have been found to accelerate the rate of protein C activation by thrombin: (*a*) the endothelial cell surface protein thrombomodulin (1, 2), and (*b*) the coagulation protein Factor V_a (3, 4). Thrombomodulin forms an equimolar complex with thrombin, thereby altering the catalytic properties of thrombin (5). In this complex, thrombin fails to clot fibrinogen, activate platelets, or cleave Factor V, whereas its ability to activate protein C is enhanced nearly 1,000-fold. Protein C treated with chymotrypsin to remove the amino-terminal 41 amino acids, which includes all of the γ-carboxyglutamic acid residues (gla-domainless protein C),[1] is activated at the same rate as native protein C by the soluble thrombomodulin-thrombin complex (6). When protein C activation is carried out over endothelial cells, gla-domainless protein C is activated poorly, suggesting that in this setting a component other than thrombomodulin itself is required for rapid protein C activation (6).

Coagulation Factor V_a also accelerates the rate of protein C activation by thrombin; this activity is contained in the light chain of Factor V_a (4). In the current study we have compared the activation of protein C in the presence of thrombomodulin with that in the presence of Factor V_a light chain (FV_a-LC) and have explored the possibility that thrombomodulin and Factor V_a or its light chain act together to regulate protein C activation by thrombin.

Methods

Except where indicated, all chemicals were purchased from Sigma Chemical Co., St. Louis, MO. Human (3) and bovine protein C (7),

1. *Abbreviations used in this paper:* FV_a-LC, Factor V_a light chain; gla-domainless protein C, chymotrypsin-treated protein C that lacks the amino-terminal 41 amino acids, including all of the γ-carboxyglutamyl residues; protein C_a, activated protein C; S2238, D-Phe-pipecolyl-Arg-*p*-nitroanilide.

human (8) and bovine (9) Factor V, human (10) and bovine thrombin (11), rabbit thrombomodulin (2), and human antithrombin III (12) were isolated as previously reported. The proteins used were all homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gla-domainless protein C was prepared by limited proteolysis of bovine protein C with chymotrypsin (6). Human (4) and bovine (9) FV_a-LC were isolated from thrombin-activated Factor V by ion-exchange chromatography, as indicated. Protein C activation by thrombin (1–10 nM) in the presence of cofactors was carried out at 37°C for 15 min in 20 mM Tris pH 7.4 containing 0.15 M NaCl, 2.5 mM $CaCl_2$, and 5 mg/ml bovine serum albumin. At the end of the activation period, the thrombin was neutralized by adjusting the reactions to contain hirudin 40 U/ml and antithrombin III 350 μg/ml. Activated protein C (protein C_a) was then determined by measuring the rate of hydrolysis of D-Phe-pipecolyl-Arg-p-nitroanilide (S2238, Kabi Diagnostica, Stockholm, Sweden) at 25°C, in a Beckman DU8 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) at 405 nM. Reaction mixtures contained 0.2 mM S2238, 20 mM Tris buffer, pH 7.4, 0.1 M NaCl, and 1 mg/ml bovine serum albumin. The concentration of protein C_a was determined by reference to a standard curve constructed by using known amounts of protein C_a. The protein C_a preparations used to construct the standard curves were obtained by fully activating human protein C (moles product per mole C_a per second $[K_{cat}] = 10\ s^{-1}$), bovine protein C ($K_{cat} = 30\ s^{-1}$), and gla-domainless bovine protein C with thrombin-thrombomodulin complex. In some instances activated protein C was assayed by measuring hydrolysis of benzoyl arginine ethyl ester as previously reported (4).

Results

Direct comparison of the kinetic properties and requirements for protein C activation using thrombomodulin and FV_a-LC

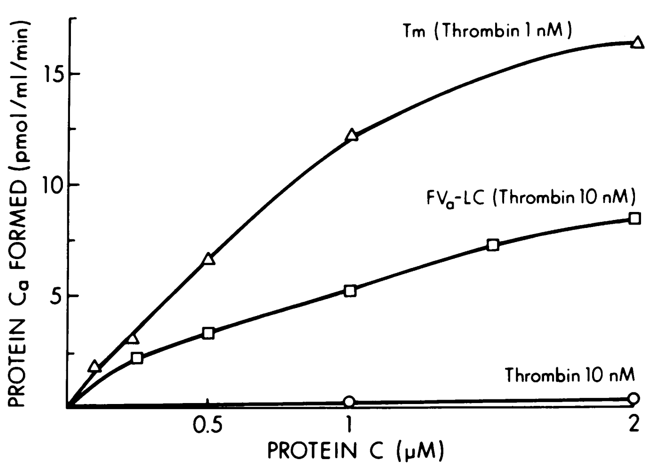


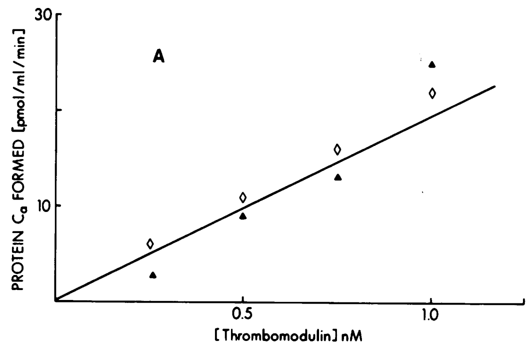
Figure 1. Comparison of thrombomodulin and FV_a-LC as cofactors for human protein C activation by thrombin. Reaction mixtures contained varying concentrations of protein C in the presence of 10 nM thrombin alone (○), 10 nM thrombin and 75 nM FV_a-LC (□), or 1 nM thrombin and 2 nM thrombomodulin (Tm) (△). Incubations were performed at 37°C for 15 min in a 20 nM Tris buffer, pH 7.4, containing 0.1 M NaCl, 2.5 mM $CaCl_2$, and 5 mg/ml bovine serum albumin. Protein C activation was terminated using a final concentration of 40 U/ml hirudin and 350 μg/ml antithrombin III, and the amount of protein C_a assayed using S2338 as described in Methods.

were initiated to determine how these two cofactors augment or regulate protein C activation. In the absence of either cofactor, thrombin activates protein C slowly. Using 1 nM human thrombin, protein C activation was accelerated from 0.01 mol/min per mol to 16 mol protein C_a/min per mol of thrombin by 2 nM thrombomodulin (Fig. 1). At a saturating concentration of human FV_a-LC (90 nM), protein C_a formation was 20 times less rapid (0.75 mol protein C_a/min per mol of thrombin). At all protein C concentrations used, thrombomodulin caused a 20-fold greater acceleration of protein C activation than human FV_a-LC. The activation of bovine protein C and bovine gla-domainless protein C by thrombin in the presence of either cofactor was also compared. When thrombomodulin was varied from 0.25 to 1 nM in the presence of 10 nM bovine thrombin, the activation of intact and gla-domainless protein C was indistinguishable as shown in Fig. 2 *A*.[2] When human FV_a-LC was used as a cofactor instead of thrombomodulin, gla-domainless protein C was activated by human thrombin at only 5% the rate of intact protein C (Fig. 2 *B*). A mixture of intact and gla-domainless protein C was activated by thrombin and human FV_a-LC at the same rate as intact protein C, thus excluding the presence of an inhibitor to the FV_a-LC in the gla-domainless protein C preparation.

That thrombomodulin is 20 times more efficient than FV_a-LC allowed us to study the effects of adding human FV_a-LC on the thrombin-thrombomodulin-catalyzed activation of protein C. For these experiments we used conditions where FV_a-LC contributed minimally to protein C activation (using 1 nM thrombin). FV_a-LC in concentrations up to 25 nM had no effect on protein C activation by 2 nM thrombomodulin and 1 nM thrombin. However, at FV_a-LC concentrations ≥30 nM there was significant reduction in the activity of the thrombin-thrombomodulin complex, and at 90 nM FV_a-LC, complete inhibition was observed. Fig. 3 shows the inhibition of thrombin-thrombomodulin complex by 30 and 90 nM FV_a-LC at different protein C concentrations. Increasing protein C concentration from 0.25 to 3 μM had no effect on the inhibitory activity of FV_a-LC, suggesting that FV_a-LC does not inhibit by binding protein C in place of the thrombin-thrombomodulin complex. Although FV_a-LC did not stimulate activation of gla-domainless protein C, it did inhibit the ability of thrombomodulin to activate this molecule.

Inhibition of protein C activation could not be completely reversed by increasing thrombomodulin concentrations (2–128 nM), although at high thrombomodulin concentrations some acceleration of the rate of protein C activation was observed (Table I). When the concentration of both thrombin and thrombomodulin were increased in the presence of inhibitory concentrations of FV_a-LC, there was a linear increase in the rate of protein C activation. We were unable to completely overcome

2. Rabbit thrombomodulin is approximately twofold more active with bovine thrombin as compared with human thrombin using either native or gla-domainless protein C.



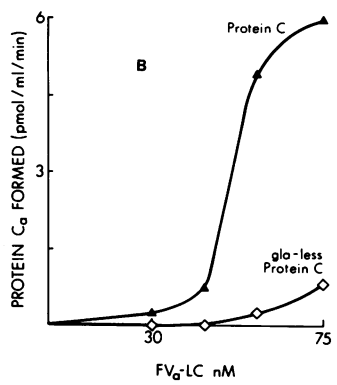


Figure 2. The activation of native and gla-domainless bovine protein C by thrombin in the presence of thrombomodulin or FV_a-LC. (*A*) The activation of intact and gla-domainless protein C by thrombin-thrombomodulin complex. Assay mixtures contained increasing concentrations of thrombomodulin in the presence of 10 nM bovine thrombin and 1 μM intact (▲), or gla-domainless protein C (◇). The reaction was terminated after 15 min, using hirudin and antithrombin III, and the amount of protein C_a measured as described in Methods. (*B*) The activation of native and gla-domainless bovine protein C by thrombin and FV_a-LC. Assay mixtures were as in *A*, except that increasing concentrations of FV_a-LC were used as the thrombin cofactor instead of thrombomodulin. Other details are as in *A*.

the inhibition using up to 400 nM thrombin-thrombomodulin complex. We also studied whether thrombomodulin could inhibit FV_a-LC-dependent protein C activation. We used con-

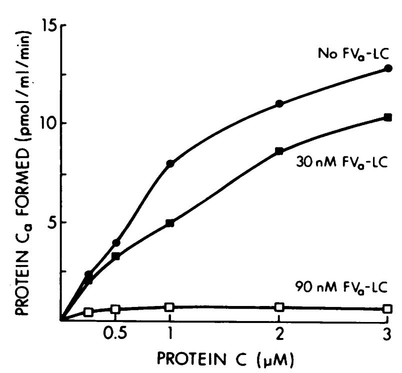


Figure 3. The inhibition of thrombin-thrombomodulin catalyzed protein C activation by FV_a-LC. Reaction mixtures contained 1 μM human protein C, 1 nM thrombin, and 2 nM thrombomodulin in the presence of no FV_a-LC (●), 30 nM FV_a-LC (■) or 90 nM FV_a-LC (□). Activation was carried out at 37°C for 15 min. Protein C_a was measured as described in Methods.

Table I. The Effect of Thrombomodulin on FV_a-LC-Stimulated Protein C Activation by 5 nM Thrombin

FV_a-LC	Thrombomodulin	Protein C_a formed
nM	*nM*	*pmol/min/ml*
120	None	2.7
120	1	2.7
120	4	2.9
120	16	3.2
120	32	3.5
120	64	4.7
120	128	6.7
None	128	55.0

Reaction mixtures contained the items indicated in 0.02 ml, 20 mM Tris–HCl, pH 7.4, 0.1 M NaCl and 2.5 mM $CaCl_2$. Protein C formation was measured using S2238 as described in Methods.

ditions where FV_a-LC itself was active but where the thrombin-thrombomodulin activity was inhibited by FV_a-LC. In concentrations varying from 2 nM to 1 μM, thrombomodulin did not inhibit the FV_a-LC-dependent activation of protein C by 5–20 nM thrombin. For example, at 5 nM thrombin protein C_a was formed at 2.7 pmol/ml per min in the presence of 120 nM FV_a-LC as shown in Table I. As thrombomodulin was increased from 1 to 128 nM the rate of protein C_a formation was not inhibited; in fact, it slowly increased to 6.7 pmol/ml per min. This rate is much less than that observed with thrombomodulin alone (55 pmol/ml per min). When intact human Factor V_a was used in these experiments instead of isolated FV_a-LC, no inhibition or stimulation of thrombin-thrombomodulin activity was noted.

In contrast to the aforementioned results obtained using human FV_a-LC, neither bovine FV_a nor its isolated light chain accelerated the rate of protein C activation by thrombin, furthermore bovine FV_a-LC did not inhibit the ability of thrombomodulin to activate protein C.

Discussion

Our results indicate that purified rabbit thrombomodulin is much more efficient than human FV_a-LC in activating protein C. These differences are even larger when the influence of the endothelial cell membrane on thrombomodulin activity is considered, as thrombomodulin activity is increased 20- to 50-fold when thrombomodulin is embedded in the endothelial cell membrane (6). Despite these large differences in potency, the relative in vivo importance of these two cofactors cannot be assessed since the concentrations of thrombin, thrombomodulin, and Factor V_a or its light chain at particular sites are unknown. Furthermore, our studies used rabbit thrombomodulin with human FV_a-LC and human or bovine protein C. Different results may be obtained when it is possible to do these experiments with all human proteins. We believe that an all human system will be qualitatively similar to the results shown here since thrombomodulin activity is present on human umbilical vein endothelium and Factor V_a and FV_a-LC produce similar effects on cells as those reported here (13). Studies in patients with congenital Factor V deficiency would be difficult to interpret since these patients lack not only the potential protein C activating capacity of Factor V_a but are also deficient in thrombin generation. We speculate that in the microvasculature, where endothelial cell density is great (14), thrombomodulin must be a major determinant of protein C activation.

Several distinct features of the two protein C activation systems are clearly resolved by this study. First, the activation of protein C by the thrombin-thrombomodulin complex and the FV_a-LC in the presence of thrombin involves recognition of different structural features on the protein C molecule. Specifically, whereas FV_a-LC requires the presence of the gla-domain, the thrombin-thrombomodulin complex does not. The γ-carboxyl glutamyl residues in coagulation factors are considered to be calcium binding sites. The calcium-dependent activation of protein C by the thrombin-thrombomodulin complex is mediated via calcium binding to a gla-domain-independent site (15). Our current results indicate that the region of protein C containing the γ-carboxyglutamyl residues is involved in substrate recognition by the light chain and thrombin, but this involvement does not depend on calcium ions. Indeed, calcium binding to the gla-residues may inhibit protein C activation in the presence of light chain. High calcium ion concentrations ($\geq$7.5 mM) do inhibit the reaction (unpublished results).

These obvious differences between the two activation systems raise the question of whether the two activators might function synergistically. Previous studies had suggested possible synergistic interaction between thrombomodulin and an undefined component of the endothelial cell membrane (6). Kinetic studies indicated that this component functioned by interacting through the gla-domain of protein C to decrease the Michaelis constant for protein C activation. Our finding that effective FV_a-LC-dependent activation of protein C is dependent on the gla-domain, is compatible with the hypothesis that the light chain provides this substrate binding site on the cell surface. Attempts to test this model in solution resulted in FV_a-LC inhibition of thrombin-thrombomodulin catalyzed protein C activation. Although these results are not compatible with a simple model in which thrombomodulin binds to FV_a-LC and the light chain provides a part of the substrate binding site, they do indicate an interaction between thrombomodulin and light chain. The interaction appears to be high affinity since inhibition occurs at nanomolar concentrations. One possible explanation for the observed inhibition is that in the absence of the membrane surface, the geometry of the complex is altered relative to that formed on the membrane surface. Support for this concept comes from the observation that low levels of Factor V_a light chain over the endothelial cell surface enhance protein C activation approximately twofold, however, as in solution, high concentrations are inhibitory (13). Since the levels of Factors V and V_a or the light chain on the cell surface before Factor V_a addition is indeterminate, the twofold stimulation of protein C activation probably represents a minimum estimate. It is clear, however, that high light chain concentrations inhibit protein C activation. These findings are most easily interpreted in terms of multiple binding sites for FV_a-LC at or near the protein C activation complex. Although the physiological significance of these interactions is not immediately apparent, it is possible to postulate a control mechanism. Low levels of FV_a-LC or intact Factor V_a stimulate thrombomodulin activity on endothelial cells and confer the ability to discriminate substrates, thereby promoting protein C_a generation. Thus, the protein C_a formed may then hydrolyze the Factor V_a heavy chain (16) leaving the FV_a-LC behind. As the FV_a-LC accumulates, inhibition of thrombomodulin activity could then result, preventing continued protein C activation once sufficient Factor V_a has been inactivated. That the thrombin-thrombomodulin complex is subject to inhibition in vivo is suggested by experiments in dogs where it was shown that infusion of thrombin stimulated conversion of $\sim$10% of protein to C to C_a (17). When the thrombin infusion was stopped, plasma levels of protein C_a fell at the same rate as found when protein C_a itself was infused, implying that the thrombin-thrombomodulin complex was rapidly inhibited. The contribution of antithrombin III, FV_a-LC, or other factors in the inactivation of the thrombin-thrombomodulin complex in vivo is unknown. The studies provided here suggest the possibility of Factor V_a involvement in the regulation of thrombomodulin activity. Further investigations of the physiological role of Factor V_a in protein C activation will have to be addressed in animal model experiments. Although the concentrations of proteins used in our studies are probably higher than those achieved in vivo, it must be remembered that protein C activation in vivo occurs on cell surface receptors where proteins are concentrated. The finding that human but not bovine Factor V_a or the isolated light chain accelerates protein C activation by thrombin could explain the well-documented observation that Factor V_a activity is rapidly lost during in vitro clotting of human but not bovine blood (18).

Acknowledgments

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