Interference of blood-coagulation vitamin K-dependent proteins in the activation of human protein C

Involvement of the 4-carboxyglutamic acid domain in two distinct interactions with the thrombin–thrombomodulin complex and with phospholipids

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Human protein C is the precursor of a serine proteinase in plasma which contains nine 4-carboxyglutamic acid residues and functions as a potent anticoagulant. It is activated by thrombin in the presence of an essential endothelial-cell-membrane glycoprotein cofactor, thrombomodulin. In a purified human system, vitamin K-dependent proteins such as factor X, prothrombin and prothrombin fragment 1 were able to inhibit protein C activation by the thrombin–thrombomodulin complex, using either detergent-solubilized thrombomodulin or thrombomodulin reconstituted into vesicles consisting of phosphatidylcholine and phosphatidylserine (1:1, w/w). Factors VII and IX and protein S were much less efficient. Prothrombin fragment 1 behaved as a non-competitive inhibitor with apparent K_i values of 4 μM in the absence, and of 2–2.5 μM in the presence, of phospholipids. Heat decarboxylation of fragment 1 abolished its ability to interfere in protein C activation, and high phospholipid concentrations could attenuate its inhibitory effect and were responsible for a gradual loss of the non-competitive character. Fragment 1 also inhibited the activation of 4-carboxyglutamic acid-domainless protein C, a proteolytic derivative of protein C lacking the 4-carboxyglutamic acid residues, without any influence from phospholipids. At high thrombin concentrations, with respect to thrombomodulin, the inhibitory effect of fragment 1 was diminished. Fragment 1, at 3.8 μM , inhibited by 50 % the activation of protein C (0.1 or 0.3 μM) by thrombin. These results suggest that the 4-carboxyglutamic acid domain of vitamin K-dependent proteins can act as a modulator of the protein C anticoagulant pathway through two distinct types of interaction. The functional 4-carboxyglutamic acid domain would be necessary to allow the enhancement of protein C activation in the presence of anionic phospholipids and it could recognize a phospholipid-independent binding site on the thrombin–thrombomodulin complex.

INTRODUCTION

Vitamin K-dependent proteins of plasma share common structural determinants, homologies being particularly striking in the *N*-terminal domain of single-chain molecules or of the light chain of two-chain molecules. In these regions, conserved glutamic acid residues are modified to 4-carboxyglutamic acid residues by a post-translational carboxylation reaction necessitating the presence of vitamin K (Davie, 1987). This modification allows the vitamin K-dependent coagulation factors to interact with acidic tissue phospholipids (Jackson & Nemerson, 1980; Mann, 1984) following a Ca^{2+} -dependent chelation model (Rosing *et al.*, 1987), thus concentrating and localizing coagulation reactions at the site of injury where procoagulant lipids become exposed.

Protein C is the vitamin K-dependent precursor of a serine proteinase, activated protein C, which has potent anticoagulant capacity. Thrombin is the only known physiological activator of protein C in the presence of an

essential endothelial-cell-membrane cofactor, thrombomodulin (Esmon, 1987). Activated protein C exerts its anticoagulant activity by degrading factors Va and VIIIa in the presence of phospholipid and of a vitamin K-dependent cofactor, protein S (Walker, 1984). Thrombomodulin is a membrane glycoprotein which presents structural similarity in its two-thirds *C*-terminal part to the low-density-lipoprotein receptor (Jackman *et al.*, 1986, 1987; Suzuki *et al.*, 1987; Wen *et al.*, 1987). The human thrombomodulin gene is intron-depleted (Jackman *et al.*, 1987) and has been localized to chromosome 20 (Wen *et al.*, 1987). The *N*-terminal one-third of human thrombomodulin contains four hydrophobic segments and does not exhibit significant homology with known proteins; it is the proposed ligand-binding domain. Reconstitution of purified thrombomodulin from human placenta (Freyssinet *et al.*, 1986a) or from rabbit lung (Galvin *et al.*, 1987) into phospholipid vesicles resulted in an increased rate of protein C activation. In these latter studies it was emphasized that the 4-carboxyglutamic acid domain was essential for activation by the

Abbreviations used: Tos-Lys- CH_2Cl , tosyl-lysylchloromethane ('TLCK'); PMSF, phenylmethanesulphonyl fluoride; $i\text{Pr}_2\text{P-F}$, di-isopropyl phosphofluoridate; PC, phosphatidylcholine; PS, phosphatidylserine; n-octyl glucoside, 1-*O*-n-octyl β -D-glucopyranoside; GD-protein C, 4-carboxyglutamic acid-domainless protein C; a.t.u. antithrombin unit; APC, activated protein C.

thrombin–thrombomodulin complex, suggesting that interaction of the substrate with membrane is an essential prerequisite of the activation process. This was corroborated by the observation that an IgM lupus anticoagulant, with specificity directed towards anionic phospholipids, was able to neutralize the enhancing effect of phospholipids on purified thrombomodulin activity (Freyssinet *et al.*, 1986b).

Under physiological conditions the mean concentration of circulating protein C is 70 nM (Griffin, 1984), whereas that of prothrombin is 1.5 μ M (Jackson, 1987). This raises the possibility that prothrombin could effectively inhibit the activation of protein C by the thrombin–thrombomodulin complex at the phospholipid membrane interface. In the present study, the ability of human vitamin K-dependent proteins and of prothrombin fragment 1 to interfere in such an activation process was examined in the presence and in the absence of phospholipids. Non-competitive inhibition due to fragment 1 and inhibition of the activation of 4-carboxylglutamic acid-domainless (GD-)protein C suggest the existence of two distinct types of interaction for the functional vitamin K-dependent domain, a direct interaction with anionic phospholipids responsible for an increased rate of protein C activation and a phospholipid-independent interaction with the thrombin–thrombomodulin complex.

MATERIALS AND METHODS

Materials

Human serum albumin (essentially fatty-acid-free), hirudin, Tos-Lys-CH₂Cl, PMSF and iPr₂P-F were products from Sigma, St. Louis, MO, U.S.A. PC and PS from bovine spinal cord were purchased from Lipid Products, Redhill, Surrey, U.K. S-2366 (L-pyroglutamyl-L-prolyl-L-arginine *p*-nitroanilide hydrochloride) was from Kabi Vitrum, Stockholm, Sweden. n-Octyl glucoside was obtained from Boehringer, Mannheim, Germany. α -Chymotrypsin from bovine pancreas (45–70 units/mg of protein) was from Worthington, Freehold, NJ, U.S.A. The kit for protein assay and Chelex resin were products from Bio-Rad, Richmond, CA, U.S.A. Factor II concentrates (PPSB, about 250 prothrombin units/vial) were obtained from the Centre Régional de Transfusion Sanguine, Strasbourg, France, and used to purify human prothrombin by a previously published method (Freyssinet *et al.*, 1982). L- α -[1-¹⁴C]Dipalmitoyl phosphatidylcholine (PC) (110 mCi/mmol) was purchased from New England Nuclear, Boston MA, U.S.A.

Purification of proteins

Human α -thrombin [3000 National Institutes of Health (NIH) units/mg of protein] was obtained by activating purified prothrombin with Taipan-snake venom (Owen & Jackson, 1973; Freyssinet *et al.*, 1986a).

Protein C was purified to homogeneity (as judged by SDS/polyacrylamide-gel electrophoresis) from human plasma cryosupernatant as previously described (Freyssinet *et al.*, 1985).

Human GD-protein C was prepared by limited proteolysis of protein C by α -chymotrypsin as already published (Freyssinet *et al.*, 1986a).

Human blood-coagulation factors II, IX and X were isolated by barium citrate adsorption followed by ion

exchange chromatography and dextran sulphate affinity chromatography (Freyssinet *et al.*, 1982). Human factor VII and protein S were purified by the methods of Broze & Majerus (1981) and Dahlbäck (1983) respectively. Prothrombin fragment 1 was made as described by Owen *et al.* (1974). Decarboxylation of prothrombin fragment 1 was performed as described by Tuhy *et al.* (1979).

Human placenta thrombomodulin was extracted at pH 10.5 and was purified by the method of Jakubowski *et al.* (1986). Extraction at pH 10.5 and a 25% (v/v) methanol precipitation step have been shown to minimize proteolytic degradation of thrombomodulin. At the end of this procedure a supplementary affinity chromatography step on iPr₂P–thrombin–Sephacrose was performed in order to exchange Triton X-100 for n-octyl glucoside [1% (w/v) final concn.]. Thrombomodulin activity was assayed as previously reported (Freyssinet *et al.*, 1986a), except that we used S2366 instead of S2238 (Phe-Pip-Arg *p*-nitroanilide dihydrochloride).

Protein concentrations were determined using the Bio-Rad protein assay, with bovine serum albumin as standard. Protein C and thrombin concentrations were also determined from the measurement of A_{280} ; absorption coefficients ($\epsilon_{1\text{cm}, 280}^{1\%}$) of 14.5 (Kisiel & Davie, 1981) and 18.3 (Fenton *et al.*, 1977) respectively were assumed.

Reconstitution of thrombomodulin into phospholipid vesicles

Unless otherwise stated all the measurements with purified thrombomodulin were performed in 50 mM-Tris (pH 7.5)/0.1 M-NaCl/2 mM-CaCl₂/20 μ M-human serum albumin.

Two procedures of reconstitution were used. In the first one, the dilution method, phospholipid vesicles containing a single phospholipid or a mixture (1:1, w/w) of two phospholipids and tracer amounts of [¹⁴C] phosphatidylcholine were performed as previously described (Freyssinet *et al.*, 1982) in the absence of Ca²⁺, following the method of Huang & Thompson (1974), except that the phospholipid suspension was sonicated three times for 1 min instead of 2 h in order to obtain larger vesicles, the phospholipid concentration being 10 mg/ml. Thrombomodulin in 1% (w/w) n-octyl glucoside was then diluted in phospholipid suspensions in the above buffer (which did not contain albumin) at molar ratios (phospholipid/protein) ranging from 5000 to 100000, assuming an M_r of 780 for the phospholipids. In each case the dilution factor was such that the concentration of n-octyl glucoside was well below its critical micelle concentration, i.e. below 0.73% (Hjelmeland & Chrambach, 1984). This suspension was incubated for at least 30 min at 20 °C, i.e. above the transition temperature of the phospholipids. In the dialysis procedure, organic solvent was carefully evaporated and phospholipids were dissolved in the above buffer containing 2% (w/w) n-octyl glucoside, but no albumin. Thrombomodulin was added to the lipid solution at concentrations such that the molar ratios of phospholipid to protein also ranged from 5000 to 100000. The protein/phospholipid mixtures (0.5 ml samples) were then dialysed against 3 $\times$ 100 vol. of buffer without albumin in dialysis tubing of 12000–14000- M_r cut-off for 36 h at room temperature.

The degree of incorporation of thrombomodulin into phospholipid vesicles was estimated by sucrose density centrifugation as described by Galvin *et al.* (1987), except

that continuous gradients (5–30 %, w/v) were used. The molar ratios of phospholipid to protein were estimated from the concentration of remaining phospholipids obtained by counting the radioactivity of [^{14}C]PC.

Effect of vitamin K-dependent proteins on the activation of protein C by the purified thrombin–thrombomodulin complex

Protein C at various concentrations was activated by the thrombin–thrombomodulin complex, also at different concentrations, thrombomodulin being either pure or reconstituted in phospholipid vesicles, in the presence or in the absence of vitamin K-dependent proteins. The activation was performed in 50 mM-Tris (pH 7.5)/0.1 M-NaCl/2 mM- CaCl_2 /20 μM -human serum albumin in a final volume of 200 μl for 15 min or shorter periods at 37 °C; the reaction was terminated by the addition of 5 a.t.u. of hirudin in 5 μl . The incubation time and/or protein C concentration was adjusted so that less than 20 % of the protein C was activated. Then 795 μl of 0.22 mM-S2366 was added and the rate of hydrolysis was recorded for 3 min at 25 °C at 405 nm. The concentration of APC formed was determined by reference to a standard curve constructed using known amounts of APC.

In order to assess a possible effect of vitamin K-dependent proteins on APC amidolytic activity, activation of protein C was performed as described above but in the absence of vitamin K-dependent proteins. Vitamin K-dependent proteins were only added after activation was stopped by hirudin, and a further incubation of 15 min at 37 °C was allowed before APC activity was measured.

Endothelial-cell-bound thrombomodulin

Endothelial cells from human saphenous vein were cultured in the presence of human serum in 35 mm-diameter culture wells as described by Klein-Soyer & Cazenave (1986). They were used at confluent density and endothelial-cell-bound thrombomodulin was assayed after the culture medium had been removed. The cells were washed at 37 °C with 3 $\times$ 1 ml of a solution containing 5 mM-Hepes, 11.9 mM- NaHCO_3 , 0.42 mM- NaH_2PO_4 , 137 mM-NaCl, 5.3 mM-KCl, 5.5 mM-glucose, 2 mM- CaCl_2 , 1 mM- MgCl_2 and 50 μM -human serum albumin, adjusted to pH 7.3 and to an osmolarity of 0.295 osm. The activation of 0.1 μM - or 0.3 μM -protein C was carried out in the above solution for 45 min at 37 °C in the cell-culture incubator in the presence of 3 nM-thrombin and in the presence or in the absence of fragment 1, the final volume being 1 ml. At the end of the reaction, 900 μl of the supernatant was collected and 5 a.t.u. of hirudin was added to block residual thrombin. Chromogenic substrate S2366 was added in order to obtain a final concentration of 0.1 mM; the rate of hydrolysis was then measured for 5 min at 37 °C at 405 nm. The concentration of APC formed was determined as described above.

Data analysis

When necessary, data were plotted by using linear-regression analysis.

RESULTS

The influence of several vitamin K-dependent proteins or fragment on the activation of protein C by thrombin

was investigated in three different systems, with purified thrombomodulin, with thrombomodulin reconstituted in phospholipid vesicles and in the presence of human endothelial cells in culture.

Reconstitution of thrombomodulin into phospholipid vesicles

In order to optimize the degree of reconstitution of thrombomodulin into phospholipid vesicles, two methods were compared at the same protein-to-phospholipid molar ratios as described in the Materials and methods section. In both cases the degree of reconstitution was greater than 85 % in 1:1 phosphatidylcholine/phosphatidylserine vesicles. Upon sucrose density centrifugation in the absence of phospholipid, thrombomodulin was recovered in the bottom fractions [25–30 % (w/v) sucrose]. In the absence of protein, phospholipids remained in the top fractions (5–10 % sucrose). When both components were mixed, thrombomodulin was found in the 5–20 % sucrose part of the gradient after reconstitution by the dilution method and in the 5–15 % sucrose part after reconstitution by the dialysis method. The displacement of thrombomodulin, due to the presence of phospholipids, demonstrates a direct interaction between these two components. The rather broad distribution of reconstituted thrombomodulin was probably due to the heterogeneous density of the liposomes, which could contain various proportions of protein. This reflects the heterogeneous character of the reconstitution, more pronounced in the case of the dilution procedure.

In both cases, thrombomodulin activity was almost fully recovered only when the liposomes containing thrombomodulin were disrupted by n-octyl glucoside [1 % (w/v) final concn.] before assay, suggesting that thrombomodulin molecules were distributed on both sides of the membrane. We were able to estimate that 40–55 % of thrombomodulin molecules could be localized on the inner side of the vesicles. Phospholipid-to-protein molar ratios ranging from 12000:1 to 50000:1 gave the best conditions of reconstitution for both procedures. For all the following reconstitution experiments, the dilution procedure was preferred, owing to its simplicity and rapidity.

Effect of vitamin K-dependent proteins on the activation of protein C in a purified system

Several vitamin K-dependent proteins or derivatives were first assayed for their ability to interfere in the activation of protein C by the thrombin–thrombomodulin complex in the presence and in the absence of phospholipids. Table 1 reveals that only prothrombin fragment 1 and, to a lesser extent, prothrombin and factor X, were able to inhibit protein C activation in the absence of phospholipids. When thrombomodulin was reconstituted into phospholipid vesicles, these three proteins produced similar inhibitory effects, whereas factors IX and VII and protein S were much less potent. None of these vitamin K-dependent proteins, including fragment 1, had any influence on the amidolytic activity of activated protein C at their highest concentration tested, i.e. 20 μM , either in the absence or in the presence of phospholipids at a molar ratio to thrombomodulin of 12500.

In order to study in more detail the mechanism of this inhibition, the substrate-dependence of protein C activa-

Table 1. Concentration of vitamin K-dependent proteins required to achieve 50% inhibition of protein C (0.6 μM) activation by the thrombin–thrombomodulin complex (5 nM) in the presence or absence of phospholipids (phospholipid:thrombomodulin 12500 mol/mol)

Other experimental details were as described in the Materials and methods section. Abbreviation: n.i., 50% inhibition not achieved at 15 μM .

Protein	Concentration of vitamin K-dependent proteins required for 50% inhibition of protein C activation (μM)	
	Activating complex ...	Thrombin–thrombomodulin (5 nM) + phospholipids
Fragment 1	4.2	2.7
Prothrombin	7.8	2.9
Factor X	7.5	3.0
Factor IX	n.i.	14.0
Factor VII	n.i.	n.i.
Protein S	n.i.	10.1

tion by the 10 nM-thrombin–thrombomodulin complex was examined. These experiments were performed in the presence and absence of 5 μM -fragment 1 and in the absence and presence of phospholipids at a molar ratio to thrombomodulin of 12500 in this last case. The double-reciprocal plots of the data (Fig. 1) suggested that fragment 1 behaved as a non-competitive inhibitor either in the presence or in the absence of phospholipids with respective apparent K_i values of 2.5 μM and 4.5 μM . The apparent K_m was of the order of 2 μM (Freyssinet *et al.*, 1986a).

To confirm this observation, the fragment 1-dependence of the inhibition of protein C activation was also examined in the absence and presence of phospholipids (Figs. 2a and 2b). Again, non-competitive inhibition was suggested by the Dixon plot of these data with an apparent K_i of approx. 4 μM without lipids. With reconstituted thrombomodulin, at a phospholipid-to-protein molar ratio of 6000, the non-competitive character was less pronounced, probably owing to direct interactions of fragment 1 with phospholipids. However, an apparent K_i of 2.0–2.5 μM could still be estimated.

Heat decarboxylation of fragment 1 abolished its ability to inhibit protein C activation. This observation favours the involvement of the 4-carboxyglutamic acid domain in the above non-competitive inhibition. In support of this hypothesis, phospholipids at molar ratios to thrombomodulin greater than 50000 could attenuate the inhibitory effect of fragment 1, probably because of increased direct fragment 1–membrane interactions due to excess phospholipids. Furthermore, we observed that, at a phospholipid-to-thrombomodulin molar ratio of 100000, the inhibition due to fragment 1 was no longer non-competitive, at least at protein C concentrations lower than 3 μM (Fig. 3).

Since fragment 1 could inhibit the activation of protein C in a non-competitive manner, we thought it interesting to examine its effect on the activation of GD-protein C

Fig. 1. Inhibition of human protein C activation by prothrombin fragment 1 in the presence and absence of phospholipid: double-reciprocal plot of the data

Protein C, at various concentrations, was activated by the thrombin–thrombomodulin complex (10 nM), either in the absence or in the presence of 5 μM -fragment 1 and either without phospholipid or after reconstitution of thrombomodulin into PC/PS (1:1, w/w) vesicles at a lipid:protein molar ratio of 12500 as described in the Materials and methods section. The buffer was 50 mM-Tris, pH 7.5, containing 0.1 M-NaCl, 20 μM -human serum albumin, 5 mM- CaCl_2 and 0.02% NaN_3 . The assay of generated APC and other experimental details were as described in the Materials and methods section. The K_m for protein C was $\sim 2 \mu\text{M}$. ■, No phospholipid, no fragment 1; □, no phospholipid, 5 μM -fragment 1; ▲, phospholipid present, no fragment 1; △, phospholipid present, 5 μM -fragment 1.

Fig. 2. Fragment 1-dependence of the inhibition of protein C activation in the absence (a) and in the presence (b) of phospholipid

Conditions of activation were as described in the legend of Fig. 1 and in the Materials and methods section. (a) Dixon plot of the data obtained for the activation of protein C by the thrombin–thrombomodulin complex (10 nM) in the absence of phospholipids. △, 0.6 μM -protein C; ▲, 1.8 μM -protein C. Data were plotted using linear regression analysis; the K_i was 4 μM . (b) Dixon plot of the data obtained under the same conditions but after reconstitution of thrombomodulin into PC/PS (1:1, w/w) vesicles at a lipid:protein molar ratio of 6000 as described in the Materials and methods section. □, 0.6 μM -protein C; ■, 1.8 μM -protein C. Data were plotted using linear regression analysis; a K_i value of 2–2.5 μM could be estimated.

Fig. 3. Inhibition of protein C activation by fragment 1 in the presence of phospholipids at high concentration

Protein C, at two concentrations (□, 1 μM ; Δ , 3 μM) was activated by the thrombin–thrombomodulin complex (10 nM) after reconstitution of thrombomodulin into PC/PS (1:1, w/w) vesicles at a lipid:protein molar ratio of 100 000 as described in the Materials and methods section.

Table 2. Effect of fragment 1 on the activation of GD-protein C and of protein C by the thrombin–thrombomodulin complex

The phospholipid:thrombomodulin molar ratio was 12 500 when phospholipids were present; thrombin and thrombomodulin concentrations were 14.2 nM and 10 nM respectively. Other experimental details were as described in the Materials and methods section.

Phospholipids	Fragment 1 (9 μM)	<i>v</i> (pmol/min per ml)	
		Protein C (0.9 μM)	GD-protein C (0.8 μM)
—	—	26.4	13.3
—	+	6.2	5.4
+	—	165.3	14.3
+	+	26.2	4.2

Table 3. Effect of thrombin concentration on the inhibition of protein C activation by fragment 1

The thrombomodulin concentration was 10 nM, the phospholipid:thrombomodulin molar ratio was 12 500 when lipids were present, and the protein C concentration was 0.6 μM . Other experimental details were as described in the Materials and methods section.

Phospholipids	[Thrombin] (nM)	<i>v</i> (pmol/min per ml)		
		Control (buffer)	Fragment 1 (20 μM)	Inhibition (%)
—	0.25	2.5	0.1	96
—	5	16.3	2.3	86
—	100	30.1	9.5	68
+	0.25	65.5	2.1	97
+	5	201.3	26.9	87
+	100	212.2	71.4	66

Fig. 4. Inhibition of protein C activation by prothrombin fragment 1 in the presence of endothelial-cell-bound thrombomodulin

Protein C, at two concentrations (□, 0.1 μM ; Δ , 0.3 μM) was activated by thrombin (3 nM) at the surface of cultured human endothelial cells from saphenous veins in the presence of prothrombin fragment 1 at various concentrations. The conditions of protein C activation and of measurement of generated activated protein C are detailed in the Materials and methods section.

(Table 2). As shown on this table, the inhibition of the activation of GD-protein C by fragment 1 was clear, although somewhat less important than that of protein C. In the presence of phospholipids, which had no enhancing effect on the activation of GD-protein C (Freyssinet *et al.*, 1986a), a similar extent of inhibition due to fragment 1 was observed. Therefore these last results confirm the non-competitive character of the inhibition of protein C activation by the thrombin–thrombomodulin complex. When thrombin was varied over a wide range of concentrations with respect to thrombomodulin, the inhibitory effect of fragment 1 was much more pronounced at low thrombin-to-thrombomodulin molar ratios than at high ratios (Table 3). This observation favours a decreased capacity of thrombomodulin to interact with thrombin in the presence of fragment 1.

Effect of prothrombin fragment 1 on the activation of protein C in the presence of cultured human vascular endothelial cells

Fragment 1 was also able to interfere in the activation of protein C by thrombin in the presence of endothelial-cell-bound thrombomodulin (Fig. 4). Although the nature of this inhibition was more difficult to determine, owing to lack of precision at low fragment 1 concentrations, 50 % inhibition was achieved at 3.8 μM -fragment 1 at either 0.1 μM - or 0.3 μM -protein C. These results agree with those obtained by using the purified systems described above.

DISCUSSION

It seems now well established that, *in vivo*, the activation of protein C by thrombin is under cellular control, requiring an endothelial-cell-membrane cofactor, thrombomodulin (Esmon, 1987). In a previous study we observed that reconstitution of thrombomodulin into

phospholipid vesicles resulted in an increased rate of protein C activation. This enhancing effect was only significant when anionic phospholipids were present in the vesicles and when protein C had its 4-carboxyglutamic acid domain (Freyssinet *et al.*, 1986a). In the present study *in vitro*, involving purified components, it was demonstrated that human prothrombin, factor X and, particularly, prothrombin fragment 1, can interfere in the activation of human protein C. Inhibition was observed not only in the presence of thrombomodulin reconstituted in phospholipid vesicles, but also with thrombomodulin on its own and with endothelial-cell-bound thrombomodulin.

Fragment 1 behaved as a non-competitive inhibitor of protein C activation by the thrombin–thrombomodulin complex either in the presence or in the absence of phospholipids with K_i values of the order of 2.5 and 4 μM respectively. Since heat decarboxylation of fragment 1 abolished its inhibitory effect and phospholipids in excess diminished this effect, it seems reasonable to hypothesize that the 4-carboxyglutamic acid domain is involved in these interactions. However, the non-competitive character of the inhibition of the activation of protein C noticed in the presence or absence of phospholipids and the inhibition of the activation of GD-protein C suggest that the 4-carboxyglutamic acid domain of fragment 1 interacts primarily with a region of the thrombin–thrombomodulin complex different from the protein C-binding site. This is corroborated by the observed decreased capacity of thrombomodulin to interact with thrombin in the presence of fragment 1.

In the presence of phospholipids, a lower K_i and a less pronounced non-competitive character testify to a probably more complex situation where fragment 1 could inhibit protein C activation in two different ways: by direct interaction with the thrombin–thrombomodulin complex and by competition with protein C for binding to phospholipid. The situation was even more complex in the presence of endothelial-cell-bound thrombomodulin, where the capacity of cultured endothelium to participate in the initiation of procoagulant reactions (Nawroth & Stern, 1986) precluded any investigation of the possible interference of vitamin K-dependent procoagulant proteins in the activation of protein C by thrombin. However, fragment 1 also behaved as an inhibitor in this activation process, although the mechanism could not be further investigated owing to lack of precision.

These results suggest that, in addition to its ability to interact with anionic phospholipids (Mann, 1984), following a chelation model (Rosing *et al.*, 1987), the 4-carboxyglutamic acid domain of plasma vitamin K-dependent proteins can also interact with proteins such as the thrombin–thrombomodulin complex. Another possibility would be that some lipids could remain tightly bound to thrombomodulin during the purification process. But in this case one would expect a competitive inhibition between protein C and fragment 1 for binding to phospholipids. In another study it was noticed that treatment of purified thrombomodulin with phospholipase C had no influence on its cofactor activity (Freyssinet *et al.*, 1986b). Self-association of prothrombin fragment 1 (Jackson *et al.*, 1987) and the formation of heterodimers between the vitamin K-dependent proteins (Harlos *et al.*, 1987) have been recently demonstrated. However, the K_i values of the present study are at least 25 times smaller than the K_d for fragment 1 self-

association, and the formation of protein C-fragment 1 heterodimers would have resulted in an uncompetitive inhibition rather than in a non-competitive process.

Galvin *et al.* (1987) and Kurosawa *et al.* (1987) have respectively found that reconstitution of rabbit lung thrombomodulin into neutral phospholipid vesicles and that proteolytic fragmentation of the thrombomodulin molecule by elastase expose a site capable of recognizing the 4-carboxyglutamic acid domain of protein C. They observed that the major fragment (M_r 54000) released upon elastase treatment retained cofactor activity, but had lost its capacity to interact with phospholipid vesicles. They noticed that fragment 1 or prothrombin interfered in protein C activation by thrombin only when thrombomodulin was reconstituted into vesicles containing a mixture of anionic and neutral phospholipids. These authors also found that, above 1 μM -protein C, i.e. 10 times the K_m they measured in the presence of PC/PS vesicles (8:2, w/w), significant deviation from single Michaelis–Menten kinetics occurred. Here we report different conclusions obtained with human placenta thrombomodulin. The K_m remained at 2 μM , but we could only explore a protein C concentration range not greater than 5 times the apparent K_m . We also detected a binding site for the 4-carboxyglutamic acid domain on the thrombin–thrombomodulin complex, presumably located on the thrombomodulin molecule itself, since excess thrombin could attenuate the inhibitory effect of fragment 1. The non-competitive character of this inhibition observed with purified thrombomodulin or with thrombomodulin reconstituted into vesicles containing either neutral phospholipids (results not shown) or a 1:1 mixture of anionic and neutral phospholipids and the inhibition of the activation of GD-protein C suggest that this region is different from the protein C activation centre. Our results also suggest that this 4-carboxyglutamic acid-binding region would always be accessible. Therefore it could be argued that human thrombomodulin could be modified by proteolysis in a manner resembling that of rabbit thrombomodulin by elastase. However in this case, and contrary to what we have observed, human thrombomodulin would have lost its capacity to interact with phospholipids. Of course, despite this observation, other almost undetectable proteolytic alterations cannot be totally ruled out. Species specificity might also explain these discrepancies. Bovine and rabbit lung thrombomodulins showed similar behaviour upon reconstitution into phospholipid vesicles (Galvin *et al.*, 1987). Furthermore the rabbit molecule (Esmon *et al.*, 1982) and to a lesser extent the bovine one (Suzuki *et al.*, 1986) have an unusually high proline content. From cDNA studies, it has been shown that the greatest structural difference between human and bovine thrombomodulin occurs in the Ser+Thr-rich region which also contains a high density of proline residues. This domain is 20% shorter in the human component (Jackman *et al.*, 1987).

Other authors have shown that the 4-carboxyglutamic acid domain of factor X has anticoagulant and anti-thrombotic properties, probably due to its ability to interfere in factor VIIa–tissue-factor-catalysed activation of factors IX and X, half-maximal inhibition occurring at concentrations ranging from 0.2 to 0.8 μM . In addition they observed that this domain could block factor X activation on the endothelial surface and prevent thrombin formation in response to factors IXa and Xa in a

Wessler venous stasis model (Nawroth *et al.*, 1986). Another group of investigators noticed that prothrombin fragment 1 could inhibit the tissue-factor pathway when this membrane molecule was reconstituted into vesicles containing a mixture of acidic and neutral phospholipids (Forman *et al.*, 1984). Hence our results, together with the above observations, suggest that the 4-carboxyglutamic acid domain of vitamin K-dependent proteins can act as a modulator not only of the coagulant system but also of the protein C anticoagulant pathway. The differences in the ability of the various vitamin K-dependent proteins to interfere in protein C activation might just arise from steric hindrance due to other domains of these molecules. Therefore it could be deduced that the 4-carboxyglutamic acid domain of protein C is involved in two distinct types of interaction. This functional entity would be necessary to allow the enhancement of protein C activation in the presence of anionic phospholipids and it could recognize a phospholipid-independent binding site on the thrombin-thrombomodulin complex.

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