Role of the glycosaminoglycan component of thrombomodulin in its acceleration of the inactivation of single-chain urokinase-type plasminogen activator by thrombin

Gerard A. W. de MUNK*, John F. PARKINSON,†‡ Eleonore GROENEVELD*, Nils U. BANG† and Dingeman C. RIJKEN*§

*IVVO-TNO Gaubius Laboratory, P.O. Box 430, 2300 AK Leiden, The Netherlands, and †Lilly Research Laboratories, Indianapolis, IN, U.S.A.

Thrombomodulin (TM), a membrane proteoglycan on endothelial cells, binds thrombin in a 1:1 complex, accelerates the protein C activation by thrombin, promotes the thrombin inactivation by antithrombin III and inhibits the procoagulant properties of thrombin. The inactivation of single-chain urokinase-type plasminogen activator (scu-PA) by thrombin is accelerated about 70-fold by TM [De Munk, Groeneveld and Rijken (1991) *J. Clin. Invest.* **88**, 1680–1684]. The present study investigates the role of the O-linked glycosaminoglycan moiety of TM in the latter reaction. In the presence of an excess of a fully-glycosylated soluble recombinant human TM mutant (high- M_r rec-TM), 0.11 nM thrombin inactivated 50% of 4.4 nM scu-PA in 45 min at 37 °C. In the presence of a soluble recombinant TM mutant lacking the glycosaminoglycans (low- M_r rec-TM), 1.9 nM thrombin was needed to inactivate 50% scu-PA, as compared with 4.7 nM thrombin in the absence of TM. Using

the scu-PA inactivation assay the dissociation constant for the thrombin-TM interaction was found to be 0.4 nM for high- M_r rec-TM and 14 nM for low- M_r rec-TM. Treatment of high- M_r rec-TM with chondroitinase ABC to digest the glycosaminoglycans decreased the accelerating effect to the level of low- M_r rec-TM. A similar decrease was observed after treatment of solubilized rabbit TM with chondroitinase ABC. As expected, chondroitinase ABC had no influence on the accelerating effect of low- M_r rec-TM. The free glycosaminoglycans obtained by alkaline treatment of TM or chondroitin sulphate A also accelerated the inactivation of scu-PA by thrombin, but about 1000-fold higher concentrations than with TM were needed to obtain the same acceleration. It is concluded that the major glycosaminoglycan of TM plays a pivotal role in the inactivation of scu-PA by the TM–thrombin complex, both in the formation and in the activity of the complex.

INTRODUCTION

The serine proteinase single-chain urokinase-type plasminogen activator (scu-PA), also called pro-urokinase, can, when converted into two-chain urokinase-type plasminogen activator (tcu-PA), activate plasminogen to plasmin. The activator probably has an important role in fibrinolysis and in the maintenance of the haemostatic balance [for reviews, see Lijnen et al. (1987a), Gurewich (1988) and de Munk and Rijken (1990)]. In contrast with plasmin and kallikrein, which cleave scu-PA between Lys-158 and Ile-159, resulting in an active two-chain molecule, thrombin cleaves scu-PA between Arg-156 and Phe-157, resulting in an inactive two-chain molecule (Bernik and Oller, 1977; Ichinose et al., 1986; Gurewich and Pannell, 1987). Thrombin-inactivated scu-PA is very poorly activated by plasmin (Lijnen et al., 1987b).

Thrombomodulin (TM), an endothelial-cell-membrane proteoglycan, binds thrombin with high affinity in a 1:1 complex. The protein stimulates the anticoagulant properties of thrombin by accelerating the activation of protein C and inhibits the procoagulant properties of thrombin [for reviews, see Freyssinet and Cazenave (1988), N. L. Esmon (1987), C. T. Esmon (1989) and Dittman and Majerus (1990)]. Besides these anticoagulant effects, TM may also have an antifibrinolytic effect by accelerating the inactivation of scu-PA by thrombin (De Munk et al., 1991; Molinari et al., 1992).

TM contains many N- and O-linked glycans (Bourin et al.,

1986, 1990; Koyama et al., 1991a; Parkinson et al., 1992). One O-linked chondroitin sulphate-like glycosaminoglycan (CSGAG) in TM constitutes a secondary binding site for thrombin (Bourin et al., 1988; Preissner et al., 1990) in addition to the primary binding site on the epidermal-growth-factor domains 5 and 6 of TM (Kurosawa et al., 1988; Hayashi et al., 1990). This CSGAG potentiates inhibition of thrombin's activity towards fibrinogen and other bulky substrates (Bourin et al., 1988; Preissner et al., 1990; Bourin and Lindahl, 1990; Parkinson et al., 1990; Nawa et al., 1990), and is essential for the acceleration of thrombin inhibition by antithrombin III (Preissner et al., 1990; Bourin et al., 1988; Bourin and Lindahl, 1990; Nawa et al., 1990; Koyama et al., 1991b). The CSGAG also significantly enhances protein C activation by the thrombin–TM complex at physiological Ca^{2+} concentrations (Parkinson et al., 1990, 1992). At low Ca^{2+} concentrations, the CSGAG has, however, a less critical function in the activation of protein C (Preissner et al., 1990; Bourin and Lindahl, 1990; Parkinson et al., 1990, 1992).

Various molecular models have recently been designed to illustrate the role of the CSGAG in the interaction between TM, thrombin and different substrates (Bourin and Lindahl, 1990; Preissner et al., 1990; Koyama et al., 1991b). The aim of the present study was to investigate the role of the O-linked chondroitin sulphate-like glycosaminoglycan of TM in the acceleration of the inactivation of scu-PA by thrombin. For that purpose we used two glycoforms of a soluble human recombinant thrombomodulin (rec-TM), a high- M_r rec-TM and a low- M_r

Abbreviations used: TM, thrombomodulin; rec-TM, recombinant thrombomodulin; u-PA, urokinase-type plasminogen activator; scu-PA, single-chain u-PA; tcu-PA, two-chain u-PA; CSGAG, chondroitin sulphate-like glycosaminoglycan; S-2444, Glp-Gly-Arg *p*-nitroanilide.

‡ Present address: Berlex Biosciences, South San Francisco, CA, U.S.A.

§ To whom correspondence should be sent.

rec-TM lacking the O-linked CSGAG (Parkinson et al., 1990, 1992). In addition we modified TM chemically and enzymically.

MATERIALS AND METHODS

Materials

Human scu-PA and low- M_r scu-PA, lacking the N-terminal part of the molecule (the epidermal-growth-factor domain and kringle domain), were isolated from embryonic kidney cell cultures (Badylak et al., 1988) and donated by Dr. J. Henkin (Abbott Laboratories, Abbott Park, IL, U.S.A.). Rabbit lung TM (M_r 74000; Esmon et al., 1982) was purchased from American Diagnostica (Greenwich, CT, U.S.A.). Soluble human high- and low- M_r recombinant thrombomodulins (rec-TM) with M_r 105000 and M_r 75000 respectively were purified from conditioned medium of stable 293 cell transformants, as recently described (Parkinson et al., 1990, 1992). Hirudin was from Pentapharm (Basel, Switzerland). Human plasmin and the chromogenic substrate Glp-Gly-Arg *p*-nitroanilide (S-2444) were from Kabi AB (Stockholm, Sweden). Aprotinin was from Bayer AG (Leverkusen-Bayerwerk, Germany). Human thrombin (T6759), BSA (A7030), chondroitinase ABC (C2905), pepstatin (P4265), leupeptin (L2884), chondroitin sulphate A (C8529) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). A specific activity of 3.3 N.I.H. units/ μ g for thrombin (as indicated by the manufacturer) and M_r 37000 were used for the calculation of concentrations.

Assay of scu-PA

The amount of scu-PA was determined by activating scu-PA (3.7 nM), in 20 mM Tris/HCl (pH 7.5)/100 mM NaCl/3 mM CaCl_2 /0.1% BSA/0.1% Tween-80/hirudin (see below), with plasmin (final concentration 0.1 casein unit/ml or 45 nM) for 20 min at 37 °C. From the activated scu-PA solution, 75 μ l was added to 175 μ l of 0.43 mM S-2444 in 50 mM Tris/HCl/38 mM NaCl/0.01% Tween-80/aprotinin (20 kallikrein-inhibitory units/ml), pH 8.8. The increase in A_{405} was measured during incubation at 37 °C by using a Titertek Multiskan instrument (Eflab Oy, Finland). In this assay scu-PA was thus fully activated before measurement of scu-PA.

Inactivation of scu-PA

The assay for inactivation of scu-PA was performed by incubating 4.4 nM scu-PA with 0–16 nM thrombin in the absence or presence of rabbit TM, TM mutants CSGAG or chondroitin sulphate A in 20 mM Tris/HCl (pH 7.5)/100 mM NaCl/3 mM CaCl_2 /0.1% BSA/0.1% Tween-80 for 45 min at 37 °C. Thrombin was pre-incubated with the components in the incubation buffer for 5 min at 37 °C, immediately before adding scu-PA. The reaction was stopped by adding hirudin (with a final concentration of 0.2 unit/ml or more, depending on the amount of thrombin present in the solution) and scu-PA activity was determined.

Chondroitinase ABC treatment

Rabbit TM (81 nM, 500 μ l) and low- and high- M_r rec-TM (720 nM, 200 μ l) were incubated overnight at 37 °C with and without chondroitinase ABC in 50 mM Tris/HCl (pH 8.0)/100 mM NaCl/30 μ M sodium acetate, in the presence of 2.5 mM 1,10-phenanthroline, 10 μ g/ml pepstatin, and 20 μ g/ml leupeptin (Parkinson et al., 1990). The chondroitinase ABC concentration

amounted to 250 munits/ml and was enhanced to 500 munits/ml after 6 h.

Alkaline β -elimination

High- M_r rec-TM (1.15 μ M, 15 μ l) was added to 135 μ l of 0.1 M NaCl/0.56 M NaOH/0.1% Tween-80 and incubated for 15 h at 4 °C to release O-linked saccharides from the protein (Bourin et al., 1990). The solution was neutralized with HCl and tested in the inactivation assay of scu-PA. It is assumed that the CSGAG accounts for 10% (w/w) of the high- M_r rec-TM (Bourin et al., 1990).

RESULTS

To compare the accelerating effect of different TM preparations on the thrombin inactivation of scu-PA, various concentrations of thrombin were incubated with 4.4 nM scu-PA for 45 min at 37 °C, in the absence or presence of excess rabbit TM (13.5 nM), human high- M_r rec-TM (13.5 nM) or human low- M_r rec-TM (27 nM). The residual native scu-PA was activated by plasmin and quantified by using S-2444 (Figure 1); 50% of scu-PA was inactivated by 0.10 nM thrombin in complex with rabbit TM, by 0.11 nM thrombin in complex with high- M_r rec-TM, by 1.9 nM thrombin in complex with low- M_r rec-TM and by 4.7 nM thrombin in the absence of TM. All three thrombin-TM complexes, as well as free thrombin, inactivated low- M_r scu-PA (7.3 nM) to the same extent as the intact scu-PA (Table 1). This indicated that the epidermal-growth-factor domain and the kringle domain of scu-PA are not involved in the inactivation reaction.

With increasing concentrations of high- and low- M_r rec-TM and a fixed thrombin concentration the acceleration of the scu-PA inactivation reached a maximum level (Figure 2), at which all the thrombin was assumed to have formed a 1:1 complex with rec-TM. The apparent K_d for this complex was determined as follows (De Munk et al., 1991). The percentages of remaining scu-PA were converted into concentrations of thrombin-rec-TM complex, by reference to the standard curves prepared with various concentrations of thrombin in the presence of an excess of the corresponding rec-TM (Figure 1). The concentrations of free and bound rec-TM were then calculated and double-reciprocal plots (insets to Figures 2a and 2b) were used to

Figure 1 Effect of different types of TM on the inactivation of scu-PA by thrombin

The percentages of scu-PA remaining after incubation for 45 min at 37 °C with various concentrations of thrombin in the absence (●) or in the presence of excess rabbit TM (13.5 nM) (◆), high- M_r rec-TM (13.5 nM) (▲) or low- M_r rec-TM (27 nM) (▼) were quantified with S-2444 after activation with plasmin.

Table 1 Inactivation of low- M_r scu-PA

Data in the Table represent the concentrations of thrombin (nM) inactivating 50% scu-PA in the absence or presence of different types of thrombomodulin (TM). The values for high- M_r scu-PA were taken from Figure 1. For experimental details, see the text.

TM	[Thrombin] (nM)	
	Low- M_r scu-PA	High- M_r scu-PA
Rabbit TM	0.09	0.10
High- M_r rec-TM	0.13	0.11
Low- M_r rec-TM	1.8	1.9
None	5.0	4.7

Figure 2 Effect of various concentrations of the rec-TM glycoforms on the inactivation of scu-PA by thrombin

Thrombin was incubated at a concentration of 0.2 nM with 0–6.7 nM high- M_r rec-TM (a) or at a concentration of 2 nM with 0–54 nM low- M_r rec-TM (b) and 4.4 nM scu-PA for 45 min at 37 °C. Remaining scu-PA was determined. The amounts of free and bound TM were calculated (see the text) and plotted reciprocally to determine the K_d values (insets).

determine the K_d values of the complexes. The apparent K_d for the thrombin–low- M_r rec-TM complex was 14 ± 5 nM and 0.4 ± 0.1 nM ($n = 3$) for the thrombin–high- M_r rec-TM complex.

Figure 3 Chondroitinase ABC treatment of rec-TM and rabbit TM

Scu-PA and thrombin were incubated with chondroitinase ABC-treated rabbit TM (◆), high- M_r rec-TM (▲) or low- M_r rec-TM (▼). The broken lines represent curves obtained with the corresponding untreated TM preparations, and were redrawn from Figure 1. Remaining scu-PA was determined.

Figure 4 Inactivation of scu-PA by thrombin accelerated by different CSGAG preparations

Scu-PA was incubated for 45 min at 37 °C with 2 nM thrombin in the presence of various concentrations of intact high- M_r rec-TM (assuming 10% of the M_r is accounted for by CSGAG) (●), CSGAG obtained from high- M_r rec-TM by alkaline β -elimination (▲) and chondroitin sulphate A (◆). Remaining scu-PA was determined.

After treatment with chondroitinase ABC, which destroys the CSGAG moiety, rabbit TM (13.5 nM), high- M_r rec-TM (13.5 nM), and low- M_r rec-TM (27 nM) were incubated with various concentrations of thrombin and 4.4 nM scu-PA for 45 min at 37 °C. Remaining scu-PA was determined. Figure 3 shows that the activities of high- M_r rec-TM and rabbit TM were markedly decreased and fell almost to the activity of low- M_r rec-TM. By contrast, the activity of low- M_r rec-TM was unaffected by chondroitinase ABC (Figure 3). Rabbit TM incubated without chondroitinase ABC as a control showed no significant loss of activity (results not shown).

The O-linked CSGAG was hydrolysed from the peptide chain of high- M_r rec-TM by alkaline β -elimination. The peptide chain was destroyed, as was shown by SDS/PAGE. Various concentrations of high- M_r rec-TM-derived CSGAG (0.2 μ g/ml), chondroitin sulphate A (0–100 μ g/ml), which is closely related to the CSGAG moiety on TM (Bourin et al., 1990; Nawa et al., 1990) or intact high- M_r rec-TM (0–6.7 nM or 0–700 ng/ml, corresponding to about 0–70 ng/ml CSGAG) were incubated with 2 nM thrombin and 4.4 nM scu-PA for 45 min at 37 °C. Remaining scu-PA was measured (Figure 4). Both chondroitin sulphate A and the free CSGAG from TM showed a significant and roughly equal acceleration of the scu-PA inactivation by

Figure 5 Reconstitution of low- M_r rec-TM with CSGAG from high- M_r rec-TM

Scu-PA and thrombin were incubated alone (●), with CSGAG (0.14 μ g/ml or 13.5 nM) obtained by alkaline β -elimination of high- M_r rec-TM (◆) and in the presence of both low- M_r rec-TM (27 nM) and CSGAG (0.14 μ g/ml or 13.5 nM) from high- M_r rec-TM (■). Remaining scu-PA was determined. Broken lines represent inactivation of scu-PA by thrombin in the presence of low- M_r rec-TM (27 nM) (▼) and high- M_r rec-TM (13.5 nM) (▲) (taken from Figure 1).

thrombin. However, high concentrations were needed, about 1000-fold higher than with intact high- M_r rec-TM.

Reconstitution experiments were performed by incubating scu-PA (4.4 nM) and thrombin for 45 min at 37 °C in the presence of both low- M_r rec-TM (27 nM) and CSGAG (0.14 μ g/ml or 13.5 nM), isolated from high- M_r rec-TM. Figure 5 shows that the accelerating effects of the two compounds were additive, but restoration to the level of intact high- M_r rec-TM was not achieved. These results indicate that the polypeptide of rec-TM and the CSGAG must be covalently linked for expression of full activity

DISCUSSION

Recent studies have shown that the anticoagulant protein TM may also have an antifibrinolytic activity by accelerating the inactivation of scu-PA by thrombin (De Munk et al., 1991; Molinari et al., 1992). The present study shows that this property of TM strongly depends on the presence of an O-linked CSGAG, as demonstrated with two glycoforms of human soluble rec-TM as well as with rabbit TM.

Scu-PA was 50% inactivated in 45 min with 4.7 nM free thrombin and only 0.10 or 0.11 nM thrombin respectively in the presence of excess rabbit TM or human high- M_r rec-TM, indicating that the two TM preparations had equal accelerating effects. In the presence of an excess human low- M_r rec-TM, lacking the O-linked CSGAG (Parkinson et al., 1990, 1992; Koyama et al., 1991a), 1.9 nM thrombin was needed to inactivate 50% scu-PA, thus showing only a slight accelerating effect.

The conclusion that only the CSGAG is responsible for the difference in activity of high- M_r and low- M_r rec-TM was supported by the experiments in which treatment of high- M_r rec-TM with chondroitinase ABC led to the level of activity of the low- M_r form. The free CSGAG component, obtained from high- M_r rec-TM by alkaline β -elimination, as well as chondroitin sulphate A also accelerated the inactivation of scu-PA by thrombin, in agreement with a recent paper of Molinari et al. (1992). However, on a carbohydrate weight basis, 1000-fold more free CSGAG was required than intact high- M_r rec-TM to obtain the same effect. Reconstitution experiments showed that the addition of CSGAG to low- M_r rec-TM did not achieve the

accelerating activity of high- M_r rec-TM. This indicates that the CSGAG should be linked to the TM polypeptide chain for the expression of the full activity of TM.

The accelerating effects of both high- M_r and low- M_r rec-TM were dose-dependent and showed an apparent K_d for their complexes with human thrombin of 0.4 and 14 nM respectively, somewhat lower than the K_d values for bovine thrombin of 3 and 16 nM respectively (Parkinson et al., 1990). It can be concluded that the CSGAG on TM plays a pivotal role in the acceleration of the inactivation of scu-PA by thrombin, both by providing a secondary binding site for thrombin (Bourin et al., 1988; Preissner et al., 1990), leading to a higher affinity for thrombin, and by increasing the activity of the TM-thrombin complex towards scu-PA.

The action of TM in the scu-PA inactivation reaction is probably based on induction of a conformational change in thrombin upon binding to the epidermal-growth-factor modules 5 and 6 of TM (Musci et al., 1988). Additional conformational change in thrombin caused by binding to the secondary binding site on the CSGAG moiety (Bourin et al., 1988; Preissner et al., 1990) might occur. An additional explanation for the pivotal role of the CSGAG could be that the CSGAG provides a binding site for high- and low- M_r scu-PA, resulting in a cyclic ternary complex between TM, thrombin and scu-PA. The idea of direct binding between TM and scu-PA is supported by the finding that active-site-blocked or thrombin-derived tscu-PA can inhibit the TM acceleration of the inactivation of scu-PA (Molinari et al., 1992). On the other hand, in the inactivation of thrombin by antithrombin III, which is accelerated by TM, the CSGAG moiety is also essential (Preissner et al., 1990; Bourin and Lindahl, 1990; Koyama et al., 1991a), but binding of antithrombin III to TM could not be detected (Preissner et al., 1990).

If TM binds scu-PA directly, the model used for the estimation of the dissociation constants of the TM-thrombin complex would be inappropriate. However, as the K_d values obtained with rabbit TM (De Munk et al., 1991) and with high- M_r rec-TM (Figure 2a) were very close to 0.5 nM, a value obtained in the literature by independent methods (Owen and Esmon, 1981; Haley et al., 1989), the putative binding between TM and scu-PA is probably weak with regard to the strong binding between TM and thrombin and does not significantly affect the estimation of the K_d of the latter interaction.

In addition to the elucidation of some structure-function relationships in TM, this study may have interesting physiological implications. As discussed previously (Koyama et al., 1991a; Parkinson et al., 1992), glycosylation of TM *in vivo* may vary in a species-, tissue- or organ-specific manner. By modulating the CSGAG on TM, cells could regulate their scu-PA-inactivating potential and thereby u-PA activity-dependent processes, such as fibrinolysis as well as cell migration, tissue destruction and remodelling (De Munk et al., 1991). Finally, potential anti-fibrinolytic effects of TM molecules should be considered when they are tested for anti-thrombolytic therapy (Nawa et al., 1992).

We thank Dr. J. Henkin and Dr. A. A. Sasahara, Abbott Laboratories, Abbott Park, IL, U.S.A., for generously providing high- and low- M_r scu-PA. This study was supported by the Netherlands Heart Foundation (grant 87.041 and Established Investigatorship of D. C. Rijken).

REFERENCES

- Badylak, S. F., Voytik, S., Klabunde, R. E., Henkin, J. and Leski, M. (1988) *Thromb. Res.* **52**, 295–312.
- Bernik, M. B. and Oller, E. P. (1977) *Thromb. Haemostasis* **38**, 136.
- Bourin, M.-C. and Lindahl, U. (1990) *Biochem. J.* **270**, 419–425.
- Bourin, M.-C., Boffa, M.-C., Björk, I. and Lindahl, U. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5924–5928.

- Bourin, M.-C., Öhlin, A.-K., Lane, D. A., Stenflo, J. and Lindahl, U. (1988) *J. Biol. Chem.* **263**, 8044–8052
- Bourin, M.-C., Lundgren-Åkerlund, E. and Lindahl, U. (1990) *J. Biol. Chem.* **265**, 15424–15431
- de Munk, G. A. W. and Rijken, D. C. (1990) *Fibrinolysis* **4**, 1–9
- de Munk, G. A. W., Groeneveld, E. and Rijken, D. C. (1991) *J. Clin. Invest.* **88**, 1680–1684
- Dittman, W. and Majerus, P. W. (1990) *Blood* **75**, 329–336
- Esmon, C. T. (1989) *J. Biol. Chem.* **264**, 4743–4746
- Esmon, N. L. (1987) *Semin. Thromb. Haemostasis* **13**, 454–463
- Esmon, N. L., Owen, W. G. and Esmon, C. T. (1982) *J. Biol. Chem.* **257**, 859–864
- Freyssinet, J.-M. and Cazenave, J.-P. (1988) in *Protein C and Related Proteins. Biochemical and Clinical Aspects* (Bertina, R. M., ed.), pp. 91–105, Churchill Livingstone, Edinburgh, London, Melbourne and New York
- Gurewich, V. (1988) *Semin. Thromb. Haemostasis* **14**, 110–115
- Gurewich, V. and Pannell, R. (1987) *Blood* **69**, 769–772
- Haley, P. E., Doyle, M. F. and Mann, K. G. (1989) *J. Biol. Chem.* **264**, 16303–16310
- Hayashi, T., Zushi, M., Yamamoto, S. and Suzuki, K. (1990) *J. Biol. Chem.* **265**, 20156–20159
- Ichinose, A., Fujikawa, K. and Suyama, T. (1986) *J. Biol. Chem.* **261**, 3486–3489
- Koyama, T., Parkinson, J. F., Aoki, N., Bang, N. U., Müller-Berghaus, G. and Preissner, K. T. (1991a) *Br. J. Haematol.* **78**, 515–522
- Koyama, T., Parkinson, J. F., Sié, P., Bang, N. U., Müller-Berghaus, G. and Preissner, K. T. (1991b) *Eur. J. Biochem.* **198**, 563–570
- Kurosawa, S., Stearns, D. J., Jackson, K. W. and Esmon, C. T. (1988) *J. Biol. Chem.* **263**, 5993–5996
- Lijnen, H. R., Stump, D. C. and Collen, D. C. (1987a) *Semin. Thromb. Haemostasis* **13**, 152–159
- Lijnen, H. R., Van Hoef, B. and Collen, D. (1987b) *Eur. J. Biochem.* **169**, 359–364
- Molinari, A., Giorgetti, C., Lansén, J., Vaghi, F., Orsini, G., Faioni, E. M. and Mannucci, P. M. (1992) *Thromb. Haemostasis* **67**, 226–232
- Musci, G., Berliner, L. J. and Esmon, C. T. (1988) *Biochemistry* **27**, 769–773
- Nawa, K., Sakano, K.-I., Fujiwara, H., Sato, Y., Sugiyama, N., Teruuchi, T., Iwamoto, M. and Marumoto, Y. (1990) *Biochem. Biophys. Res. Commun.* **171**, 729–737
- Nawa, K., Itani, T., Ono, M., Sakano, K.-I., Marumoto, Y. and Iwamoto, M. (1992) *Thromb. Haemostasis* **67**, 366–370
- Owen, W. G. and Esmon, C. T. (1981) *J. Biol. Chem.* **256**, 5532–5535
- Parkinson, J. F., Grinnell, B. W., Moore, R. E., Hoskins, J., Vlahos, J. C. and Bang, N. U. (1990) *J. Biol. Chem.* **265**, 12602–12610
- Parkinson, J. F., Vlahos, J. C., Yan, S. C. B. and Bang, N. U. (1992) *Biochem. J.* **283**, 151–157
- Preissner, K. T., Koyama, T., Müller, D., Tschopp, J. and Müller-Berghaus, G. (1990) *J. Biol. Chem.* **265**, 4915–4922