Characterization of monoclonal antibodies against human protein C specific for the calcium ion-induced conformation or for the activation peptide region

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Three monoclonal antibodies have been produced that are specific for the activation peptide region in human protein C. These antibodies inhibited the activation of protein C by thrombin and by the thrombin-thrombomodulin complex. A fourth monoclonal antibody specifically recognized the Ca^{2+}-stabilized conformation in protein C. This antibody bound both intact protein C and protein C from which the γ-carboxyglutamic acid-containing region had been removed by limited proteolysis. These results indicate that this antibody recognizes the conformation in protein C stabilized by Ca^{2+} bound to the single binding site that is independent of γ-carboxyglutamic acid.

Protein C *Calcium binding* *Monoclonal antibody* *Activation peptide*

1. INTRODUCTION

Protein C is a vitamin K-dependent glycoprotein that has been purified from bovine and human plasma [1–3]. It is a zymogen of a serine protease [4,5] and is rapidly activated by thrombin in complex with thrombomodulin, an endothelial cell cofactor [6–8]. Activated protein C degrades coagulation factors V_a and $VIII_a$ by limited proteolysis [9–11]. After degradation, factor V_a does not bind factor X_a, nor does it interact with prothrombin, the substrate for factor X_a [12,13]. This anticoagulant function of protein C requires protein S, another vitamin K-dependent protein, as a cofactor [14,15]. In vivo protein C is an important regulator of blood coagulation, as shown by the association of protein C deficiency with early adulthood venous thrombosis [16,17].

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Protein C contains the vitamin K-dependent γ-carboxyglutamic acid (Gla) residues that are required for its Ca^{2+}-binding and biological activity [1,18,19]. In addition to the Gla residues, protein C has one residue of erythro-β-hydroxyaspartic acid, a residue formed by postribosomal hydroxylation of aspartic acid [20]. The function of this modified amino acid is not known. Recently it was demonstrated that protein C, lacking the Gla region, still has one high affinity Ca^{2+}-binding site [19,21]. Ca^{2+}-binding to the Gla independent site induces a conformational change in protein C [21,22]. We have now made a monoclonal antibody againsts an epitope on human protein C which is expressed only in the presence of Ca^{2+}. In the course of this work we also obtained antibodies against the activation peptide region of protein C, suggesting that this is a very immunogenic part of the protein. Here we describe some of the properties of the monoclonal antibody recognizing the Ca^{2+}-induced conformation of protein C and of antibodies directed against the activation peptide region.

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2. MATERIALS AND METHODS

Keyhole limpet hemocyanin (KLH) and 2,2'-azido-di(3-ethylbenzethiazoline)sulfonic acid (ABTS) was obtained from Sigma. Affigel 10 and nitrocellulose papers were from Biorad, DEAE-Sephacel from Pharmacia and maleidobenzoyl-*N*-hydroxysuccinimide ester (MBS) from Pierce. The *p*-nitroanilide substrate S-2238 was obtained from Kabi. The peptide Asp-Pro-Glu-Asp-Gln-Glu-Asp-Gln-Val-Asp-Pro-Arg-Leu-Ile-Asp-Cys was obtained from Peninsula and coupled to KLH as described by Liu et al. [23,24]. Horseradish peroxidase (HRP)-conjugated swine antirabbit immunoglobulin and HRP-conjugated rabbit antimouse immunoglobulin were obtained from Dako. Human protein C was purified and activated as described [10]. Thrombomodulin was a gift from Drs Charles and Naomi Esmon. Bovine thrombin was obtained from prothrombin by activation with the venom from *Oxyuris scutellatus scutellatus* (Sigma) and purified as described by Owen and Jackson [25].

2.1. *Production of monoclonal antibodies*

Mice were immunized with 10 μg of purified human protein C, emulsified in Freund's complete adjuvant. The immunization was repeated after 1 and 3 weeks, the last following emulsification in incomplete Freund's adjuvant. The mouse with the highest antibody titer was selected, and boosted intraperitoneally with 200 μg of human protein C on three consecutive days prior to cell fusion. Cell fusion and production of monoclonal antibodies was performed according to the procedure of Köhler and Milstein with the modifications described previously [26,27]. SP 2/0-Ag 14 or P3-NS1-1 Ag 4 myeloma cells were used for the fusion. Positive clones were subcloned two or three times by limiting dilution, then expanded and injected intraperitoneally into pristine-primed Balb/c mice. After 10–12 days the mice were sacrificed, and the monoclonal antibodies were isolated from the ascites fluid by standard procedures; i.e. ammonium sulfate precipitation followed by ion exchange chromatography on DEAE-Sepharose CL 6B.

2.2. *Antigen binding assay*

Antibody-producing hybridomas were tested by an enzyme-linked immunoadsorbent assay in 96-well Dynatec or Linboro microtiter plates as described previously [27]. The plates were coated with human protein C, or with the synthetic peptide coupled to KLH or with KLH alone. HRP-conjugated rabbit antimouse immunoglobulins were used as a second antibody, with ABTS as a substrate.

Clones specific for the Ca^{2+}-induced conformation of protein C were identified by a solid-phase radioimmunoassay essentially as described by Lewis et al. [28]. 'Removeastrip' microtiter plates (Dynatec) were coated at 4°C overnight with 50 μl affinity-purified rabbit antimouse immunoglobulin (20 μg/ml). After washing with 50 mM Tris-HCl, 0.15 M NaCl, pH 7.4, the plates were incubated for 15 min with 100 μl buffer containing bovine serum albumin (BSA) (10 mg/ml). In the test 40 μl of hybridoma supernatant was added to each well, followed by incubation at ambient temperature for 60 min. Next, using the same buffer, the plates were washed with 2 mM $CaCl_2$ or 2 mM EDTA, and then incubated at 4°C overnight with 5000 cpm (50 μl) per well of ^{125}I-labelled protein C. After extensive washing, radioactivity was measured, and the amount of ^{125}I-protein C bound in Ca^{2+} and EDTA-containing buffer compared.

Rabbit antisera against protein C were tested by ELISA as described above using plates coated with either protein C, KLH-coupled peptide or KLH alone. In this case horseradish peroxidase-conjugated goat antirabbit antibodies were used.

2.3. *Electrophoretic and immunochemical procedures*

SDS-polyacrylamide slab gel electrophoresis was performed in 10–15% gradient gels [29,30]. Gels were either stained with Coomassie brilliant blue or subjected to Western blotting [31], with transfer of the proteins to nitrocellulose paper. Unoccupied protein sites were quenched with 5% teleostean liquid gelatin in 20 mM Tris, pH 7.4, 0.9% NaCl [32]. Subsequent antibody incubations were performed in this buffer and washes were made using the same buffer without gelatin. Transferred proteins were probed with either mouse monoclonal or rabbit antiserum against protein C. When monoclonal antibodies were used, the blots were overlayed with rabbit antiserum against mouse immunoglobulins prior to addition of the labelled an-

tibody. The protein bands were visualized with horseradish peroxidase coupled to an affinity-purified goat antibody against rabbit immunoglobulins and using 3-amino-9-ethylcarbazole (20 mg in 2 ml dimethylformamide in 50 ml 50 M acetate buffer, pH 5.0, containing 20 μl 30% H_2O_2) as a substrate.

2.4. *Inhibition of protein C activation by monoclonal antibodies*

When thrombin-thrombomodulin was used in the activation of purified human protein C, 50 μl (3.7 μg) was mixed with antibody (11 μl, 9.9 μg) in 20 mM Tris-HCl containing 100 mM NaCl, pH 7.4, and supplemented with 1% BSA and 5 mM $CaCl_2$. When thrombin alone was used in the activation the buffer contained 5 mM EDTA instead of $CaCl_2$. After 60 min incubation in 1 ml Eppendorf vials at 37°C, 50 μl of the mixture was transferred to a second vial and then either 10 μl of thrombin (10 U/ml) and 10 μl of buffer, or a mixture of 10 μl of thrombin (1 U/ml) and 10 μl of thrombomodulin (1.78 μg/ml) were added. After incubation for 60 min at 37°C, the thrombin activity was inhibited by the addition of 10 μl antithrombin III (1.43 mg/ml). Next, 50 μl of the reaction mixture was transferred to a 1 ml cuvette containing 850 μl buffer and 100 μl of S-2238 (2 mM), and finally, the protein C amidiolytic activity was measured from the increase in absorbance at 405 nm. Activated or unactivated protein C, mixed with an unrelated monoclonal antibody was used in control experiments.

2.5. *Immobilization of monoclonal antibodies*

The Ca^{2+}-dependent antibody, HPC-4, in 0.1 M morpholinopropane sulfonic acid buffer, pH 7.0, was coupled to Affigel 10 (5.5 mg antibody per ml gel) according to the manufacturer's instructions.

2.6. *Determination of binding constant of the monoclonal antibodies*

The binding constants were determined from measurement of the competition between unlabelled protein C and ^{125}I-labelled protein C for binding to the monoclonal antibodies in a double antibody radioimmunoassay. The data were evaluated with the aid of Scatchard plots [33].

3. RESULTS AND DISCUSSION

The tissue culture supernatants obtained from spleen cells from immunized mice were fused with myeloma cells and were tested with the objective of obtaining monoclonal antibodies against the activation peptide region of protein C and antibodies that specifically recognized the Ca^{2+}-dependent conformation of protein C. After cloning three times, 3 antiactivation peptide antibodies (HPC 1, 5 and 9) and the antibody with the most pronounced Ca^{2+} dependence (HPC 4) were grown as ascitic tumours. The monoclonal antibodies were purified by ammonium sulfate precipitation and DEAE-cellulose chromatography. HPC 1, 5 and 9 from different master wells had different mobilities on agarose gel electrophoresis and were thus unrelated. The properties of the monoclonal antibodies are summarized in table 1. The purified antibodies were tested by solid phase immunoassay with microtiter plates coated with protein C and plates coated with the synthetic peptide. HPC 1 reacted with both the intact protein and the synthetic peptide but not the carrier protein (KLH) (fig.1). In addition, it did not react with activated protein C (not shown). HPC 5 and 9 had the same specificity. On the contrary, HPC 4 did not react with the synthetic peptide. These results indicate that the activation peptide region in protein C is immunogenic, and this is corroborated by the finding that a rabbit antiserum against protein C also reacts with the synthetic peptide (fig.1). The specificity of the monoclonal antibodies was also tested in immunoblotting experiments (fig.2). HPC 1 recognized the zymogen but not activated protein C. Likewise it recognized the heavy chain

Table 1

Properties of monoclonal antibodies against human protein C

Clone	Cell line	IgG samples	Epitope	Binding constant (M^{-1})
HPC 1	SP2-0	IgG1 κ	Act peptide	3×10^8
HPC 4	NS-1	IgG1 κ	Ca^{2+}-dependent	1×10^9
HPC 5	SP2-0	IgG1 κ	Act peptide	1×10^9
HPC 9	SP2-0	IgG2a κ	Act peptide	1×10^9

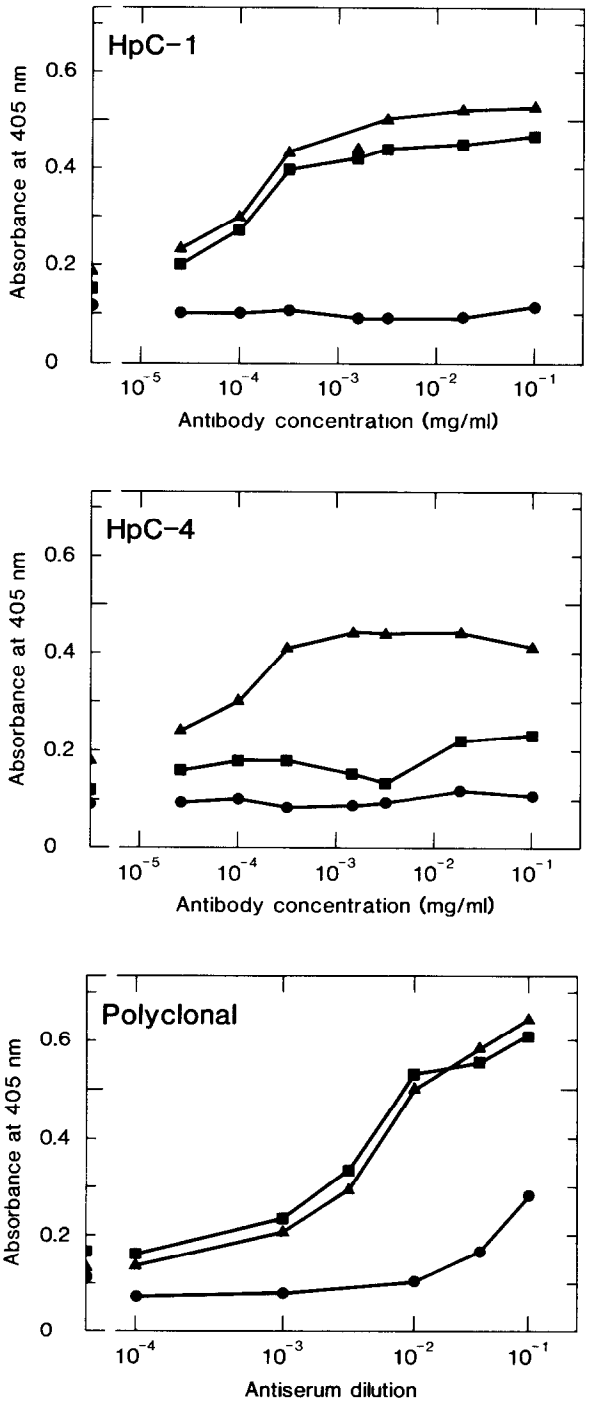

Fig.1. Antigenic specificity of monoclonal antibodies as evaluated by ELISA assay. The microliter plates were coated with protein C (▲—▲), synthetic peptide (see section 2) coupled to KLH (■—■) or with KLH only (●—●).

of the unactivated protein but not of the activated protein. The antibodies HPC 5 and HPC 9 had the same specificity.

The antibodies specific for the Ca^{2+}-induced conformation in protein C were tested by solid phase radioimmunoassay. Measurements were made of the binding of ^{125}I-labelled protein C to microtiter plates previously coated with affinity-purified rabbit antimouse immunoglobulins. The binding of the radiolabelled tracer in Ca^{2+} and EDTA containing buffer was compared (fig.3). The binding of protein C to the antibodies directed against the activation peptide region was not influenced by the presence of Ca^{2+}. In contrast, HPC 4 bound protein C with high affinity in the presence of Ca^{2+}, but only weakly in the presence of EDTA. In immunoblotting experiments, HPC 4 reacted with both the zymogen and the active enzyme prior to reduction of disulfide bridges but did not recognize the electrophoretically separated light and heavy chains (fig.2).

The ability of the monoclonal antibodies to inhibit the activation of protein C was investigated. Thrombin was used for the activation both alone in EDTA-containing buffer and in complex with thrombomodulin in Ca^{2+}-containing buffer. As seen in fig.4, activated protein C had a slightly higher amidolytic activity in the presence of Ca^{2+} than in EDTA-containing buffer [34]. The three monoclonal antibodies against the activation pep-

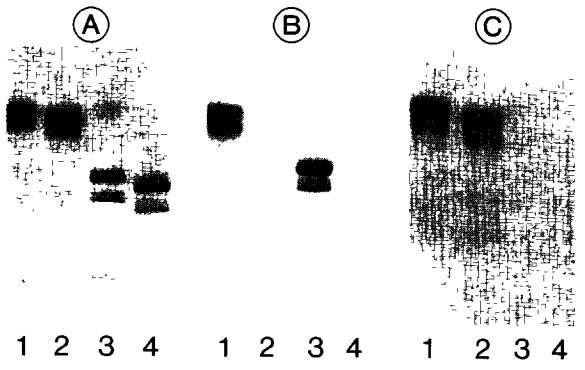

Fig.2. Immunoblotting with rabbit antiserum against protein C (A), monoclonal antibody HPC 1 (B) and monoclonal antibody HPC 4 (C). Lanes 1 and 2, unreduced samples; lanes 3 and 4, samples reduced with β-mercaptoethanol. Lanes 1 and 3, protein C; lanes 2 and 4, activated protein C.

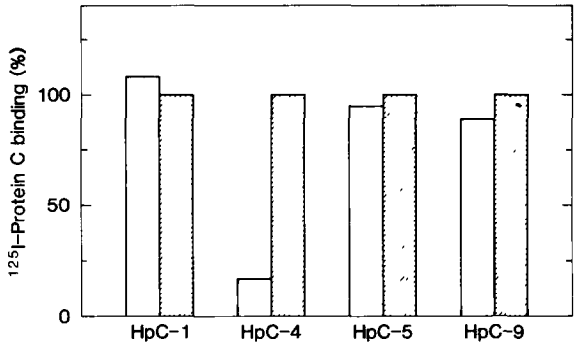

Fig.3. Binding of ^{125}I-protein C to monoclonal antibodies from ascites fluid as determined by solid phase radioimmunoassay. The assay was performed in Ca^{2+}-containing buffer (filled bars) or in EDTA-containing buffer (open bars). The binding in the Ca^{2+}-containing buffer was arbitrarily assigned a value of 100% in each experiment. Blank figures did not exceed 15% of the monoclonal binding in the Ca^{2+}-containing buffer.

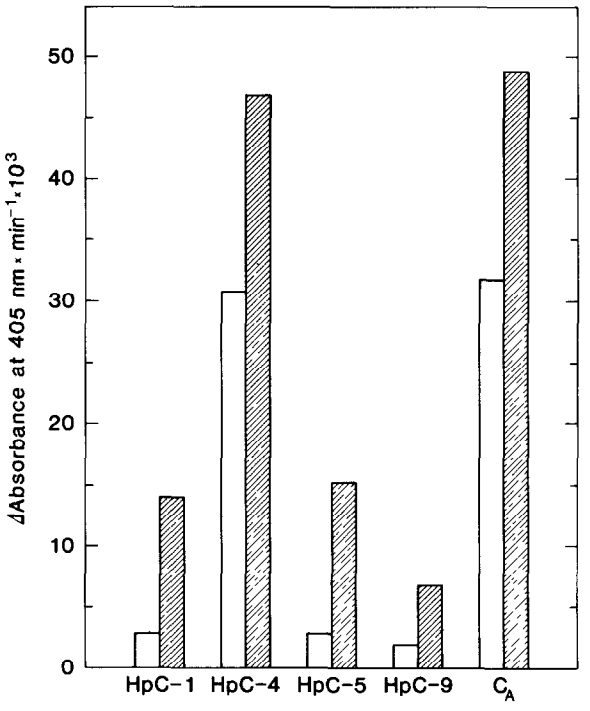

Fig.4. Effect of monoclonal antibodies on activation of protein C. Protein C was incubated with antibodies and activated with either thrombin alone in EDTA-containing buffer (open bars) or with the thrombin-thrombomodulin complex in Ca^{2+}-containing buffer (filled bars). Activated protein C was measured with S-2238 as described in section 2. C_A, activated protein C alone.

tide region inhibited the activation of protein C both by thrombin and by thrombin-thrombomodulin. On the other hand, HPC 4 did not inhibit the activation of protein C by the thrombin-thrombomodulin complex.

Protein C from which the Gla-region had been removed by limited proteolysis with chymotrypsin still bound one Ca^{2+} with high affinity. Binding of this single Ca^{2+} induced an easily monitored conformational change in the protein [21,22]. Gla-domainless protein C was prepared as described by Esmon et al. [19] characterized by SDS-polyacrylamide gel electrophoresis and its binding to HPC 4 studied. Protein C, activated protein C and Gla-domainless protein C all bound to a column with immobilized HPC 4 in the presence of Ca^{2+} and could then be eluted with EDTA-containing buffer (fig.5). HPC 4 thus recognized the conformational change induced in protein C by the Gla-independent Ca^{2+} binding site.

We have previously noted that the activation peptide region in bovine protein C is very immunogenic [27]. The synthetic peptide corresponding to residues 1 to 15 in the heavy chain of human protein C that we have used was synthesized with a proline residue in position 2 [2]. More recent reports have found a threonine residual in this position in the heavy chain of human protein C [35,36]. There may thus be additional clones producing antibodies directed against the activation peptide in protein C that were not detected with our screening procedure. The activation peptide has 7 charged amino acids and should thus be very immunogenic as predicted by the method of Hopps and Wood [37]. Recently Suzuki et al. characterized 13 monoclonal antibodies against human protein C [38]. In this case however none was directed against the activation peptide region. The reason for this is unknow, but it may be due to different immunization procedures.

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REFERENCES

[1] Stenflo, J. (1976) J. Biol. Chem. 251, 355–363.
[2] Kisiel, W. (1979) J. Clin. Invest. 64, 761–769.
[3] Kisiel, W. and Davie, E.W. (1981) Methods Enzymol. 80, 320–332.
[4] Esmon, C.T., Stenflo, J., Suttie, J.W. and Jackson, C.M. (1976) J. Biol. Chem. 251, 3052–3056.
[5] Kisiel, W., Ericsson, L.H. and Davie, E.W. (1976) Biochemistry 15, 4893–4900.
[6] Esmon, C.T. and Owen, W.G. (1981) Proc. Natl. Acad. Sci. USA 78, 2249–2252.
[7] Esmon, C.T. and Esmon, N.L. (1984) Semin. Thromb. Hemostas. (Mammen, E.F. and Esmon, C.T. eds) vol.10, pp.122–130.
[8] Salem, H.H., Maruyama, I., Ishii, H. and Majerus, P.W. (1984) J. Biol. Chem. 259, 12246–12251.
[9] Walker, F.J., Sexton, P.W. and Esmon, C.T. (1979) Biochim. Biophys. Acta 571, 333–342.
[10] Suzuki, K., Stenflo, J., Dahlbäck, B. and Teodorsson, B. (1983) J. Biol. Chem. 258, 1914–1920.
[11] Vehar, G.A. and Davie, E.W. (1980) Biochemistry 19, 401–410.
[12] Comp, P.C. and Esmon, C.T. (1979) Blood 54, 1272–1281.
[13] Dahlbäck, B. and Stenflo, J. (1980) Eur. J. Biochem. 107, 331–335.
[14] Walker, F.J. (1980) J. Biol. Chem. 255, 5521–5524.

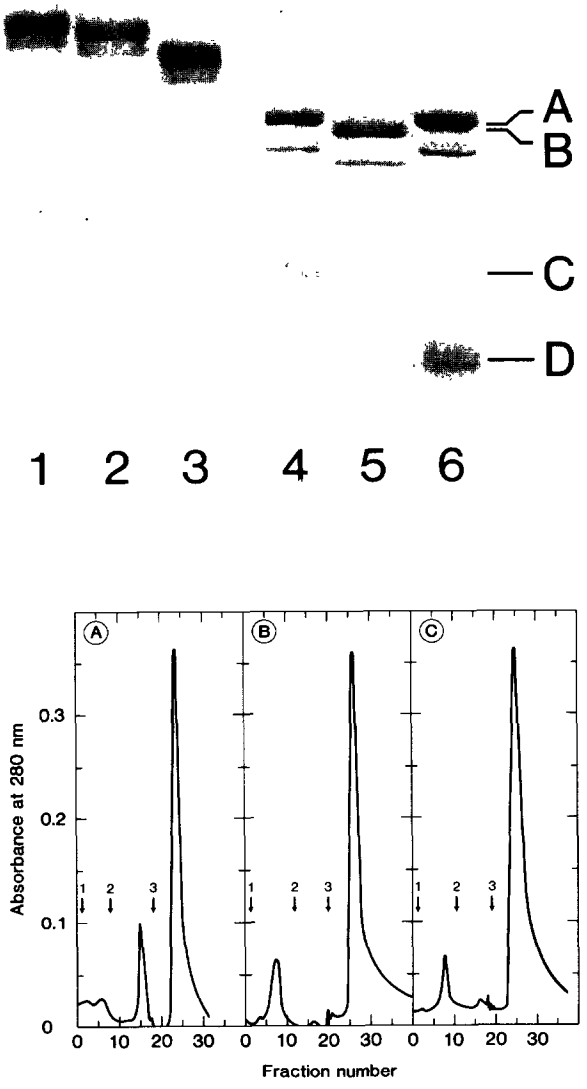

Fig.5. Binding of protein C, activated protein C and Gla-domainless protein C to immobilized HPC 4. Top, SDS-polyacrylamide gel electrophoresis of samples prior to chromatography. 1 and 4, protein C; 2 and 5, activated protein C; 3 and 6, Gla-domainless protein C. 1–3, unreduced samples and 4–6, samples reduced with β-mercaptoethanol. A, heavy chain of protein C; B, heavy chain of activated protein C; C, light chain of protein C and activated protein C; D, light chain of Gla-domainless protein C. Below, chromatography of protein C (A), activated protein C (B) and Gla-domainless protein C (C) on a column (0.4 × 10 cm) of HPC 4 immobilized on Affigel 10. The column was equilibrated with 50 mM Tris-HCl, pH 7.4, containing 5 mM $CaCl_2$ and 0.1 M NaCl. Samples were applied at arrow 1 and the column was washed with buffer containing 2 M NaCl at arrow 2. Elution was started at arrow 3 with the same buffer but containing 5 mM EDTA instead of $CaCl_2$.

[15] Walker, F.J. (1984) Semin. Thromb. Hemostas. (Mammen, E.F. and Esmon, C.T. eds) vol.10, pp.131–138.
[16] Griffin, J.H., Evatt, B., Zimmerman, T.S., Kleiss, A.J. and Wideman, C. (1981) J. Clin. Invest. 68, 1370–1373.
[17] Griffin, J.H. (1984) Semin. Thromb. Hemostas. (Mammen, E.F. and Esmon, C.T. eds) vol.10, pp.162–166.
[18] Stenflo, J. (1984) Semin. Thromb. Hemostas. (Mammen, E.F. and Esmon, C.T. eds) vol.10, pp.109–121.
[19] Esmon, N.L., DeBault, L.E. and Esmon, C.T. (1983) J. Biol. Chem. 258, 5548–5553.
[20] Drakenberg, T., Fernlund, P., Roepstorff, P. and Stenflo, J. (1983) Proc. Natl. Acad. Sci. USA 80, 1802–1806.
[21] Johnson, A.E., Esmon, N.L., Lane, T.M. and Esmon, C.T. (1983) J. Biol. Chem. 258, 5554–5560.
[22] Sugo, T., Björk, I., Holmgren, A. and Stenflo, J. (1984) J. Biol. Chem. 259, 5705–5710.
[23] Liu, F.T., Zinnecker, M., Hamaska, T. and Katz, D. (1979) Biochemistry 18, 690–697.
[24] Green, N., Alexander, H., Olson, A., Alexander, S., Shinnick, T.M., Sutcliffe, J.G. and Lerner, R.A. (1982) Cell 28, 477–487.
[25] Owen, W.G. and Jackson, C.M. (1973) Thromb. Res. 3, 705–714.
[26] Borrebaeck, C.A.K. and Eylar, M.E. (1981) J. Biol. Chem. 256, 4723–4725.
[27] Sugo, T., Persson, U. and Stenflo, J. (1985) J. Biol. Chem., in press.
[28] Lewis, R.M., Furie, B.C. and Furie, B. (1983) Biochemistry 22, 948–954.
[29] Blobel, G. and Dobberstein, B. (1975) J. Cell Biol. 67, 835–851.
[30] Maizel, J.V. (1971) Methods Virol. 179–246.
[31] Burnett, W.N. (1981) Anal. Biochem. 112, 195–203.
[32] Sarvis, C.A. (1984) Electrophoresis 5, 54–55.
[33] Scatchard, G. (1949) Annu. NY Acad. Sci. 51, 660–672.
[34] Steiner, S.A., Amphlett, G.W. and Castellino, F.J. (1980) Biochem. Biophys. Res. Commun. 94, 340–347.
[35] Miletich, J.P., Leykam, J.F. and Broze, G.J. (1983) Blood 62, suppl.1, 306a.
[36] Foster, D. and Davie, E.W. (1984) Proc. Natl. Acad. Sci. USA 81, 4766–4770.
[37] Hopp, T.P. and Woods, K.R. (1981) Proc. Natl. Acad. Sci. USA 78, 3824–3828.
[38] Suzuki, K., Matsuda, Y., Kusumoto, H., Nishioka, J., Terada, M., Yamashita, T. and Hashimoto, S. (1985) J. Biochem. 97, 127–138.