Conformational changes in activated protein C caused by binding of the first epidermal growth factor-like module of protein S

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The first epidermal growth factor-like module of human plasma protein S (EGF1, residues 76–116) was chemically synthesized and tested for its ability to inhibit the anticoagulant cofactor activity of protein S for the anticoagulant protease, activated protein C (APC). EGF1 completely inhibited the stimulation of APC activity by protein S in plasma coagulation assays, with 50 % inhibition at approx. 1 μM EGF1, suggesting direct binding of EGF1 to APC. To investigate a direct interaction between EGF1 and APC, fluorescence resonance energy transfer (FRET) experiments were employed. APC labelled in the active site with fluorescein as the donor, and phospholipid vesicles containing octadecylrhodamine as the acceptor, showed that EGF1 association with APC caused an increase in energy transfer consistent with a relocation of the active site of APC from 94 Å (9.4 nm) to 85 Å above the phospholipid surface (assuming $\kappa^2 = 2/3$). An identical increase in energy transfer between the APC active site-bound fluorescein and phospholipid-bound rhodamine was obtained upon association of protein S or protein S–C4b-binding protein complex with APC. The latter suggests the presence of a ternary complex of protein S–C4b-binding protein with APC on the phospholipid surface. To confirm a direct interaction of EGF1 with APC, rhodamine was covalently attached to the α-N-terminus of EGF1, and binding of the labelled EGF1 to APC was directly demonstrated using FRET. The data suggested a separation between the active site of APC and the N-terminus of EGF1 of 76 Å ($\kappa^2 = 2/3$), placing the APC-bound protein S-EGF1 close to, but above, the phospholipid surface and near the two EGF domains of APC. Thus we provide direct evidence for binding of protein S-EGF1 to APC and show that it induces a conformational change in APC.

Key words: anticoagulant, chemical protein synthesis, fluorescence resonance energy transfer.

INTRODUCTION

Human protein S is a physiological anticoagulant plasma protein [1], as demonstrated by a correlation between hereditary protein S deficiency and venous thromboembolism [2]. Protein S is a vitamin K-dependent glycoprotein with a molecular mass of approx. 75 kDa and a plasma concentration of 350 nM [3]. It consists of 635 amino acids and is divided into five regions: the γ-carboxyglutamic acid module (residues 1–37); the aromatic stack module (residues 38–46); the thrombin-sensitive module (residues 47–75); the epidermal growth factor (EGF)-like region (residues 77–242), containing four successive EGF modules; and the sex hormone-binding globulin-like region (residues 243–635). In protein S, three kinds of post-translational modified amino acids have been identified: 11 γ-carboxyglutamic acids in the γ-carboxyglutamic acid module [1], one β-hydroxyaspartic acid in the EGF1 module, and one β-hydroxyasparagine per EGF module 2–4 [4]. The γ-carboxyglutamic acid module is involved in Ca^{2+}-dependent binding to negatively charged phospholipids [1,5]. The roles of β-hydroxyaspartic acid and β-hydroxyasparagine residues are still unclear as it was reported that their hydroxylation was not important for the expression of anticoagulant cofactor activity [6].

Protein S exerts anticoagulant activity by acting as a cofactor to activated protein C (APC) in the proteolytic degradation of the activated cofactors Va and VIIIa of blood coagulation [7,8]. APC cleaves human factor Va by a rapid cleavage at Arg^{506}, a slow phospholipid-dependent cleavage of Arg^{306}, and possibly at Arg^{679} [9,10]. Initially, only a 2-fold effect of protein S on the initial rate of factor Va inactivation by APC was reported [11–13]; however, a 20-fold increase in the rate of phospholipid-dependent cleavage by APC at Arg^{306} in factor Va due to protein S was later demonstrated [14].

Fluorescence resonance energy transfer (FRET) studies have shown that bovine protein S alters the location (height and/or orientation) of the active-site groove of bovine APC relative to the phospholipid surface [15]. Studies of a recombinant APC chimaera containing the prothrombin γ-carboxyglutamic acid module were consistent with the hypothesis that this relocation of the APC active site enhanced APC activity against factor Va [16].

Protein S anticoagulant cofactor activity may be down-regulated by proteolytic cleavage of the thrombin-sensitive module of protein S [17], or by binding of protein S to the complement component C4b-binding protein (C4BP) [18]. The latter involves the β-chain subunit of C4BP [19] and the sex hormone-binding globulin-like region of protein S [20].

The hypothesis that the γ-carboxyglutamic acid/aromatic stack, the thrombin-sensitive module, and EGF1 modules of protein S are functionally important for the APC-cofactor

Abbreviations used: Acm, acetamidomethyl; APC, activated protein C; APTT, activated partial thromboplastin time; C4BP, C4b-binding protein; EGF1, first epidermal growth factor-like module; ESI–MS, electrospray ionization MS; FI-FPR-APC, fluorescein-acetamido-D-phenylalanyl-prolyl-arginyl-APC; FRET, fluorescence resonance energy transfer; OR, octadecylrhodamine; PC, dioleoylphosphatidylcholine; PS, dioleoylphosphatidylserine.

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activity of protein S is supported by a variety of indirect evidence based on studies using monoclonal antibodies against protein S, recombinant chimaeras between human and bovine protein S, recombinant constructs containing various EGF deletions, and site-directed mutagenesis and characterization of a human genetic defect involving a protein S gene lacking the EGF1 [21–25]. Here we demonstrate a structurally important direct interaction between the EGF1 module of protein S and APC by showing that a chemically-synthesized protein S-EGF1 module inhibits the anticoagulant cofactor activity of protein S and relocates the active site of phospholipid-bound APC above the membrane surface. Moreover, we show that a synthetic rhodamine-labelled EGF1 of protein S binds directly to APC.

EXPERIMENTAL

Materials

Succinimidyl acetylthioacetate, 5-(iodoacetamido)fluorescein, octadecylrhodamine (OR), and 5(6)-carboxytetramethylrhodamine were purchased from Molecular Probes (Eugene, OR, U.S.A.). Dioleoylphosphatidylcholine (PC) and dioleoylphosphatidylserine (PS) were obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.), and L-3-phosphatidylcholine-1,2-[1-^{14}C]oleoyl was purchased from Amersham (Arlington Heights, IL, U.S.A.). D-Phenylalanyl-L-prolyl-L-arginyl (FPR) chloromethylketone was purchased from Calbiochem (La Jolla, CA, U.S.A.).

Proteins

Human APC was made as described in [13]. Human α-thrombin and protein S were purchased from Enzyme Research Laboratories (South Bend, IN, U.S.A.). C4BP was purified as described in [20]. Active site-directed fluorescein labelling of human APC was performed essentially as described for bovine APC [15]. Thrombin-cleaved protein S was prepared by incubating 10 μM protein S with 10 nM α-thrombin in Hepes-buffered saline [50 mM Hepes (pH 7.4), 150 mM NaCl] containing 1 mM EDTA for 3 h at 37 °C. Subsequently, Mono-Q FPLC was used to remove the α-thrombin, and thrombin-cleaved protein S was eluted with a linear gradient of 150–500 mM NaCl in 50 mM Hepes at pH 7.4. Complete cleavage was confirmed by SDS/12.5 % PAGE under reducing conditions.

Protein S-EGF1 and protein S-EGF1-rhodamine synthesis

The protein S-EGF1 module (residues 76–116) was prepared by stepwise solid-phase peptide synthesis using the *in situ* neutralization/2-(1-*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate-activation procedure for t-butoxycarbonyl chemistry, as described in [26,27]. To overcome refolding difficulties during the oxidation of EGF1 and EGF1-rhodamine, a two-step oxidation procedure was used in which the cysteines involved in the middle disulphide bond (Cys^{85}-Cys^{102}) remained protected with acetamidomethyl (-CH_2-NH-CO-CH_3, Acm) groups after HF-treatment of the peptide resin. In short, EGF1-rhodamine was synthesized as follows: 29 μmol EGF1 peptide resin was treated with trifluoroacetic acid to remove N^{α}-t-butoxycarbonyl from Ile^{76} and was neutralized with 10 % (v/v) diisopropylethylamine in dimethylformamide. 5(6)-Carboxytetramethylrhodamine (44 μmol) was preactivated with 40 μmol diisopropylcarbodiimide in 0.3 ml dichloromethane for 3 min, and added to the peptide resin for 30 min. HF-cleavage of the peptide resin, HPLC purification, two-step oxidative refolding of EGF1-rhodamine, and electrospray ionization MS (ESI–MS) were performed as described in [27]. Selective formation of the first two disulphide bonds (Cys^{80}-Cys^{93} and Cys^{104}-Cys^{113}) was followed by release of the Acm groups and subsequent formation of the third disulphide bond (Cys^{85}-Cys^{102}).

Protein S activity assay

For activated partial thromboplastin time (APTT) assays, 50 μl of protein S-deficient plasma and 50 μl of phospholipid (Platelin LS; Organon Technika Corp., Durham, NC, U.S.A.) were incubated at 37 °C in an ST4 coagulometer (American Bioproducts, Parsippany, NJ, U.S.A.) for 100 s, and then 10 μl of a 3 μM protein S solution was added to yield a final concentration of 150 nM in 200 μl. After an additional 40 s, 40 μl of 1 μg/ml APC in Tris-buffered saline [50 mM Tris (pH 7.4), 100 mM NaCl] containing 5 mM $CaCl_2$/0.1 % BSA (w/w) were added, and clotting was initiated at 180 s by the addition of 50 μl of 25 mM $CaCl_2$ in Tris-buffered saline. The EGF1 module was preincubated with APC (1 μg/ml) for 60 min at room temperature in Tris-buffered saline with 5 mM $CaCl_2$/0.1 % BSA (w/w), before 40 μl was added to the clotting assay. Based on a standard curve of APTT prolongation by APC versus protein S concentration, the inhibition of protein S APC-cofactor activity by synthetic EGF1 peptides was calculated.

FRET measurements

Energy transfer measurements were made using an SLM 8100 photon-counting spectrofluorometer (SLM-Aminco, Rochester, NY, U.S.A.), as described in [15]. The fluorescein dye in fluorescein-acetamido-D-phenylalanyl-prolyl-arginyl-APC (Fl-FPR-APC) was excited at 490 nm (4 nm bandpass) and fluorescein emission was detected at 520 nm (4 nm bandpass) at 25 °C in 5 × 5 mm quartz cuvettes. The preparation of small unilamellar vesicles of PC/PS (80:20, mol/mol) and the determination of the density of OR at the membrane surface (σ, in OR molecules/Å^2) was performed as described in [28]. Coating the cuvettes with PC [29] minimized adsorption of protein to the cuvette walls. In FRET studies, fluorescein was the donor (D) and rhodamine was the acceptor (A) [15]. Four samples were prepared in parallel in Hepes-buffered saline containing 2 mM Ca^{2+} for each energy transfer experiment: cuvettes D (donor-containing) and DA (donor- and acceptor-containing) received Fl-FPR-APC, while cuvettes B (blank) and cuvette A (acceptor-containing) received an equal volume of Hepes-buffered saline (adding FPR-APC in cuvettes A and B did not qualitatively or quantitatively alter the results). The net initial emission intensities were obtained by subtracting the initial intensities of A from DA and of B from D, and were designated $(F_o)_{DA}$ and $(F_o)_D$ respectively. Samples B and D were then titrated with phospholipid vesicles or EGF1 lacking the acceptor (rhodamine), while samples DA and A were titrated with phospholipid vesicles or EGF1 containing the acceptor. Energy transfer was detected by monitoring the acceptor-dependent quenching of the donor emission. Assuming that the absorbance of the donor dye is not altered by the presence of the acceptor dye, the ratio of the donor quantum yields in the D and DA samples is given by:

$$Q_{DA}/Q_D = [(F_{DA}-F_A)/(F_{DA}-F_A)_o]/[(F_D-F_B)(F_D-F_B)_o] \quad (1)$$

where F is the net dilution-corrected emission intensity of a sample at some point in the titration, and the subscript o is used to denote the initial intensity of the sample [28–30].

Spectral parameters

The quantum yield of Fl-FPR-APC as well as the spectral overlap integrals (J_{DA}) of the Fl-FPR-APC/PCPS-OR and Fl-

FPR-APC/protein S-EGF1-rhodamine donor/acceptor pairs were determined as described [15].

R_o, the distance between a donor and acceptor dye at which energy transfer is 50% efficient, was calculated using eqn. (2):

$$R_o^6 = (8.79 \times 10^{-5}) Q_D J_{DA} \kappa^2 n^{-4} \quad (2)$$

where R_o is in Å, Q_D is the donor dye quantum yield in absence of acceptor dye, J_{DA} is the spectral overlap integral, κ^2 is a geometric factor that depends on relative orientation of donor and acceptor transition dipoles, and n is the refractive index of the medium between the donor and the acceptor dyes.

J_{DA} was calculated using the corrected emission spectrum of the Fl-FPR-APC [$F_D(\lambda)$] and the absorption spectrum of either PCPS-OR or protein S-EGF1-rhodamine ($\epsilon_A(\lambda)$) using eqn. (3):

$$J_{DA} = \int_o^\infty F_D(\lambda)\epsilon_A(\lambda)\lambda^4 d\lambda / \int_o^\infty F_D(\lambda) d\lambda \quad (3)$$

Plane-to-plane FRET: Fl-FPR-APC to PCPS-OR

PCPS-OR vesicles were titrated into samples of Fl-FPR-APC (15 nM) in Hepes-buffered saline containing 2 mM $CaCl_2$ until Q_{DA}/Q_D reached a constant value. Subsequently, protein S, protein S–C4BP, thrombin-cleaved protein S, or synthetic protein S-EGF1 were titrated into the cuvettes. The experiments were performed at two different surface densities of OR. The relative Q_{DA}/Q_D is defined here as (Q_{DA}/Q_D with ligand)/(Q_{DA}/Q_D without ligand), where the ligands are either protein S, or protein S-EGF1 or their derivatives. At the end of the titration, EDTA was added to 5 mM in order to reverse any Ca^{2+}-dependent ligand–Fl-FPR-APC interactions.

The distance of closest approach (L) between the plane formed by the fluorescein donor dyes in membrane-bound Fl-FPR-APC and the plane defined by the OR rhodamine acceptor dyes at the membrane surface was determined using eqn. (4). When the extent of energy transfer between randomly and uniformly distributed donor dyes in one infinite plane and randomly and uniformly distributed acceptor dyes in a parallel infinite plane is small, the first term in the approximate series solution of Dewey and Hammes [31] can be used to solve L:

$$Q_D/Q_{DA} = 1 + (\pi\sigma R_o^2/2)(R_o/L)^4 \quad (4)$$

Point-to-point FRET: Fl-FPR-APC to protein S-EGF1-rhodamine

Protein S-EGF1-rhodamine was titrated into samples of Fl-FPR-APC (50 nM) to detect its association with Fl-FPR-APC. Because high concentrations of protein S-EGF1-rhodamine were used in these experiments, the absorbency of the DA and A cuvettes were measured after each step of the FRET titration after which the fluorescein emission intensities were corrected for the inner filter effect as follows:

$$F_{corr} \approx F_{obs} \text{antilog}[(A_{ex} + A_{em})/2] \quad (5)$$

where A_{ex} and A_{em} are the absorbances of the FRET sample cuvettes at each point during the FRET titration [32].

The distance between donor and acceptor dyes (R) in the Fl-FPR-APC–EGF1-rhodamine complex was determined using eqn. (6) [32]:

$$E = R_o^6/(R_o^6 + R^6) \quad (6)$$

where E is the efficiency of FRET, and is given by eqn. (7):

$$E = 1 - (Q_{DA}/Q_D) \quad (7)$$

RESULTS

Inhibition of protein S cofactor activity by protein S-EGF1

The chemically synthesized EGF1 module was tested for its ability to inhibit the cofactor activity of protein S. After preincubation of synthetic EGF1 with APC in the presence of Ca^{2+}, the APPT clotting time of protein S-depleted plasma to which protein S and APC had been added was shortened in a dose-dependent manner (Figure 1). Thus protein S-EGF1 interfered with protein S stimulation of APC anticoagulant activity. The inhibition of the protein S cofactor activity was half-maximal at 1 μM EGF1 and was $> 95\%$ at protein S-EGF1 50 μM. Using APTT assays with normal plasma or protein S-deficient plasma, protein S-EGF1 did not express cofactor activity, or any anticoagulant or pro-coagulant activity, on its own (results not shown).

APC active site to membrane (plane-to-plane) FRET studies

The EGF1-dependent reduction of the cofactor activity of protein S suggests that EGF1 interacts directly with APC, competing with protein S for a binding site on APC. To determine whether protein S-EGF1 binds to APC, we used a fluorescently labelled derivative of APC (Fl-FPR-APC). Fl-FPR-APC has a fluorescein dye located near the active site via a covalent tripeptidyl linkage. To detect protein S-EGF1 module interactions with APC, we used FRET. The fluorescein moiety in Fl-FPR-APC was used as the FRET donor and rhodamine moieties in PCPS phospholipid vesicles (PCPS-OR) constituted the FRET acceptor. The rhodamine in OR is anchored at the surface of the phospholipid vesicles because the long non-polar hydrocarbon chain of OR is buried in the phospholipid bilayer, while the charge on the rhodamine at neutral pH keeps the dye in the aqueous phase [28–30]. As previously observed [15,16], a decrease in Fl-FPR-APC intensity occurred as a result of energy transfer between the plane of phospholipid-bound donor dyes (Fl-FPR-APC) and the plane of phospholipid-anchored acceptor dyes (PCPS-OR) when Fl-FPR-APC bound to phospholipid vesicles containing OR,

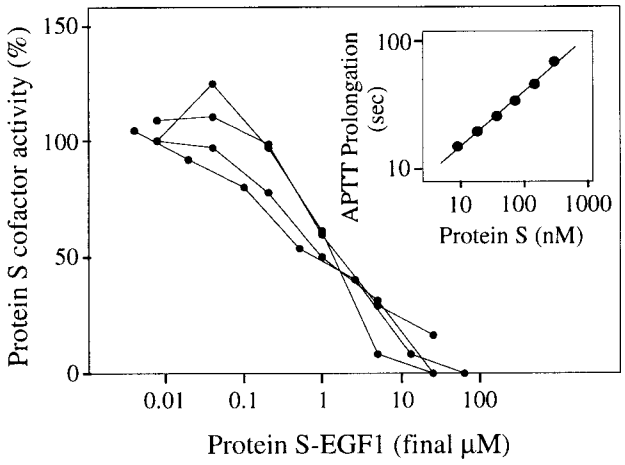

Figure 1 Effect of the synthetic EGF1 of protein S on the cofactor activity of protein S

The cofactor activity of protein S was measured as described in the Experimental section. The data shown are from four independent experiments. The inset contains a protein S calibration curve used for calculation of protein S cofactor activity (%), showing the prolongation of the APTT clotting time of protein S-depleted plasma with APC (81.3 s) by protein S (averages of duplicate measurements are shown).

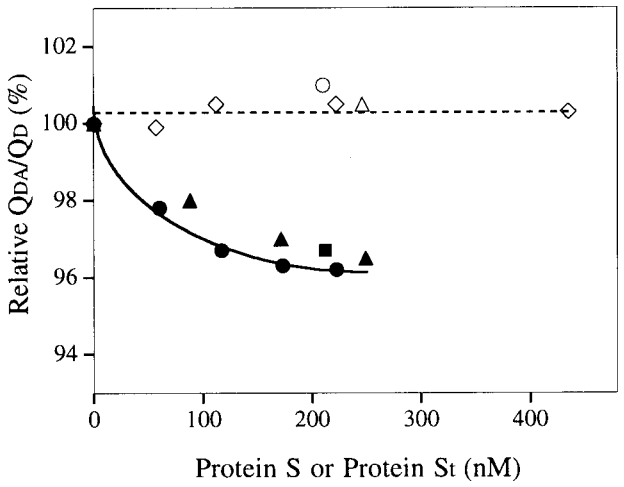

Figure 2 The effect of various forms of protein S on Fl-FPR-APC energy transfer to PCPS-OR vesicles

Samples containing 50 nM Fl-FPR-APC were titrated with PCPS vesicles in the presence or absence of OR until PCPS was in excess and Q_{DA}/Q_D reached a constant value. From this point, designated 100%, the relative Q_{DA}/Q_D [$(Q_{DA}/Q_D$ with protein S)/$(Q_{DA}/Q_D$ without protein S)] was monitored as the preformed Fl-FPR-APC–PCPS-OR complexes were titrated with protein S (●), protein S–C4BP complex (▲), or thrombin-cleaved protein S (◇). At the end of the titration with protein S alone, a twofold excess of C4BP was added (■), and later EDTA was added to 5 mM to release protein S from the membrane (○). At the end of the C4BP–protein S titration, EDTA was added to 5 mM (△). The acceptor density was 2.65×10^{-4} OR/Å^2 for the experiments shown.

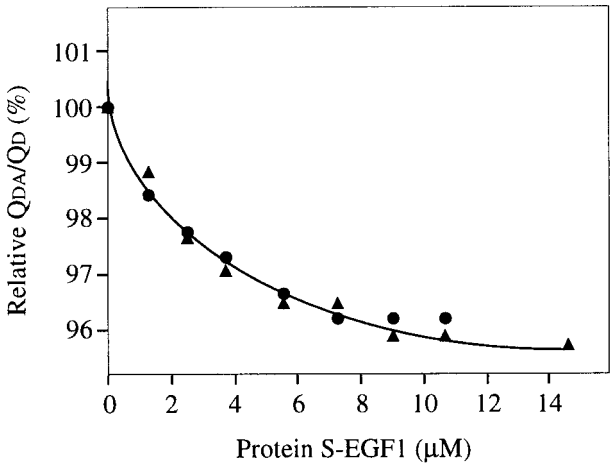

Figure 3 Effect of the synthetic protein S-EGF1 on Fl-FPR-APC energy transfer to PCPS-OR vesicles

Chemically synthesized protein S-EGF1 titrations of Fl-FPR-APC–PCPS-OR vesicles were performed as described in the Experimental section. Protein S-EGF1 effects on the extent of energy transfer are expressed as a relative Q_{DA}/Q_D [$(Q_{DA}/Q_D$ with peptide)/$(Q_{DA}/Q_D$ without peptide)]. Two acceptor (OR) densities were used: 1.68×10^{-4} OR/Å^2 (●) and 3.38×10^{-4} OR/Å^2 (▲), with absolute Q_{DA}/Q_D values of 0.894 and 0.850 at the start of EGF1 titrations for the lower and higher acceptor densities respectively.

whereas only a very small decrease in Fl-FPR-APC intensity (< 0.5%) was observed during titration with PCPS vesicles without OR (results not shown). The experiments were performed at OR surface densities between 1.68×10^{-4} dyes/Å^2 and 3.38×10^{-4} dyes/Å^2. Under conditions where essentially all of the Fl-FPR-APC was bound to vesicles (40–70 μM PCPS), Q_{DA}/Q_D values decreased from 1.00 to between 0.85 and 0.91, depending on the OR-density (results not shown). At this stage, the Q_{DA}/Q_D value was set on 100% relative Q_{DA}/Q_D. When protein S was then titrated into all 4 cuvettes, additional FRET was observed with a half-maximal effect at approx. 100 nM protein S (Figure 2). This protein S-dependent increase in energy transfer could be fully reversed by the addition of EDTA, but not by the addition of C4BP (Figure 2). Furthermore, when preformed protein S–C4BP complex was titrated into a sample of Fl-FPR-APC–PCPS-OR, the observed increase in FRET was similar to that seen for addition of protein S alone, indicating that C4BP does not inhibit the protein S-APC interaction, and further suggesting that a ternary protein complex of protein S–C4BP–APC is formed on the phospholipid surface (Figure 2). In contrast, when thrombin-cleaved protein S was titrated into a Fl-FPR-APC–PCPS-OR sample, no effect on FRET was observed (Figure 2).

When protein S-EGF1 was added to Fl-FPR-APC–PCPS-OR, a dose-dependent increase in energy transfer (i.e. a reduction in Q_{DA}/Q_D) identical in magnitude to that seen for protein S addition was observed (Figure 3). This EGF1-dependent change in relative Q_{DA}/Q_D was also completely reversible with EDTA (results not shown). The concentration of protein S-EGF1 for its half-maximal change in FRET was approx. 1.5 μM, similar to the concentration for half-maximal inhibition of protein S-cofactor activity by protein S-EGF1 (Figure 1). The 10-fold lower concentration at which native protein S shows a half-maximal increase in energy transfer between Fl-FPR-APC and PCPS-OR (Figure 2), can be likely explained by the high affinity of protein S (γ-carboxyglutamic acid) for negatively charged phospholipids, which provides additional stabilization of the interaction of EGF1 in intact protein S with phospholipid-bound APC.

The protein S-EGF1-dependent alteration in Fl-FPR-APC to PCPS-OR FRET efficiency shows that the EGF1 of protein S binds directly to APC and alters its conformation, as does native protein S.

Protein S-EGF1-rhodamine synthesis

N-terminal rhodamine-labelled protein S-EGF1 module was synthesized to study direct energy transfer between the Fl-FPR-APC and protein S EGF1-rhodamine. After chain assembly and attachment of the rhodamine fluorescent label, ESI–MS of the cleaved peptide yielded a mass of 5074.1 Da, fitting well between the calculated monoisotopic and average mass of 5071.0 Da and 5074.3 Da, respectively, of the reduced EGF1 polypeptide chain with two Acm groups (Cys^{85}, Cys^{102}) and one rhodamine group attached.

After purification using HPLC, the two-step refolding procedure to obtain three disulphides in the orientation 1–3, 2–4 and 5–6 (Cys^{80}-Cys^{93}, Cys^{85}- Cys^{102} and Cys^{104}-Cys^{113}) was performed. After step I, which formed the 1–3 and 5–6 disulphides, the pattern of these two disulphides was confirmed by trypsin cleavage and peptide mapping. Table 1 shows that a homogeneous preparation of EGF1-rhodamine-Acm_2 was obtained with 1–3 and 5–6 disulphide bonds. A small peak at 14.23 min most likely represents uncleaved, but correctly folded material, since any misfolded material would have a shift in retention time [27], and misfolded cleaved EGF1-rhodamine would result in two disulphide-linked peptides with a mass of 5088.3 Da (Figure 4A). It should be noted that only the cleaved EGF1-rhodamine fragments containing the N-terminus absorb at 550 nm, con-

Table 1 Tryptic cleavage of synthetic EGF1–rhodamine-Acm$_2$: pattern of the first two disulphide bonds

The step I refolding product of EGF1–rhodamine-Acm$_2$ (40 μM) was submitted to trypsin cleavage and mass analysis of fragments. Retention times refer to the elution times of the specific fragments from analytical HPLC (C18: 0–60% acetonitrile, 2%/min). Peak areas represent the percentage of total EGF1 fragment-containing peaks. Calculated masses are based on the average isotope composition and were obtained with MacPromass Software. * Rhodamine absorption measured at 550 nm. nd, not detectable.

Retention time (min)	Peak area 214 nm (%)	Peak area 550 nm* (%)	Mass (observed)	Mass (calculated)	Identity (residue)
11.12	34	nd	2205.0	2205.5	98–116 (C^{104}-C^{113}; C^{102}-Acm)
14.23	9	6	5070.1	5070.3	Rh-76–116 (2 S-S, $C^{85,\ 102}$-Acm)
14.67	44	69	2882.5	2882.8	Rh-76–97 (C^{80}-C^{93}; C^{85}-Acm)
15.15	13	25	2581.3	2582.5	Rh-76–94 (C^{80}-C^{93}; C^{85}-Acm)

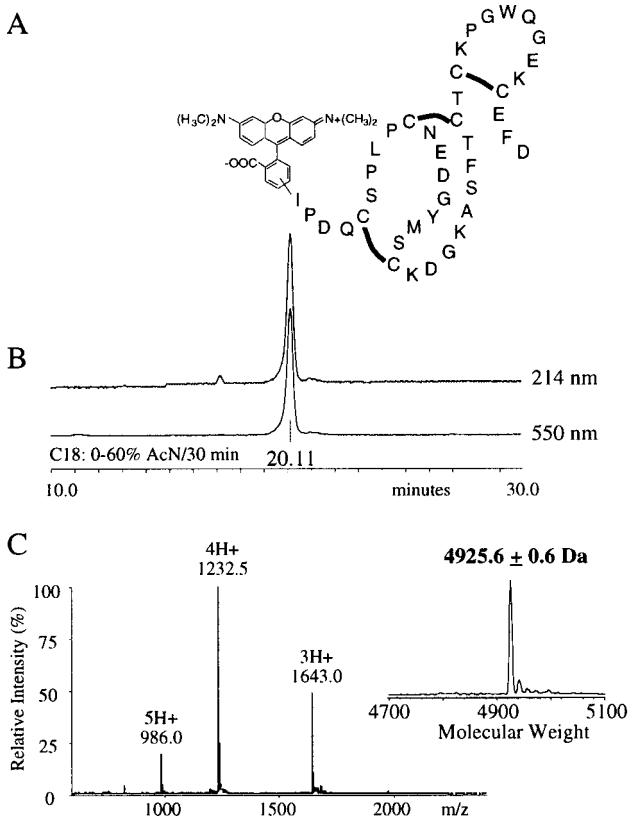

Figure 4 Chemical synthesis of protein S EGF1-rhodamine

(**A**) The sequence and rhodamine-labelling position at Ile^{76} of protein S-EGF1 (76–116). (**B**) Analytical HPLC (C18 reversed phase; 0–60% acetonitrile, 2%/min) of purified refolded EGF1-rhodamine monitored at 214 nm and 550 nm. (**C**) ESI–MS raw data and mass reconstruction for the purified refolded EGF1-rhodamine [peak 20.11 in (**B**)]. The *m/z* at 986.0, 1232.5 and 1643.0 yielded a peptide mass of 4926 Da, the expected mass for the disulphide-bonded EGF1-peptide with one rhodamine dye attached.

sistent with rhodamine labelling at the N-terminus only. Subsequently, EGF1-rhodamine-Acm$_2$ was incubated with iodine in the presence of 15% acetic acid which removed the two Acm groups, allowing formation of the third disulphide bond. EGF1-rhodamine was HPLC-purified to homogeneity (Figure 4B), and the mass of the final product was 4926 Da (ESI–MS) (Figure 4C), a value between the calculated monoisotopic and average mass of 4922.8 Da and 4926.2 Da, respectively, for the disulphide bonded EGF1 module with one rhodamine label attached.

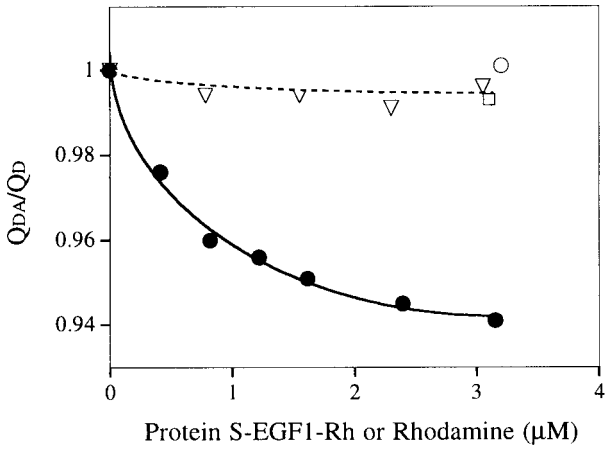

Figure 5 Energy transfer from Fl-FPR-APC to protein S-EGF1-rhodamine

Samples containing 50 nM Fl-FPR-APC were titrated with EGF1-rhodamine (●) or rhodamine (open triangles), and fluorescein-to-rhodamine energy transfer was measured as described in the Experimental section. At the end of the titration, EDTA was added to reverse any Ca^{2+}-dependent changes (○, □).

Fl-FPR-APC to protein S-EGF1-rhodamine (point-to-point) FRET studies

In addition to the described plane-to-plane FRET studies between the plane of donor fluorescein dyes and the plane of acceptor rhodamine dyes, we performed point-to-point FRET experiments in the absence of phospholipids, in which EGF1-rhodamine was added to the DA and A cuvettes, whereas EGF1 was added to the D and B cuvettes (see the Experimental section). As a result of energy transfer between the Fl-FPR-APC fluorescein and the protein S-EGF1-rhodamine, Q_{DA}/Q_D values decreased in a dose-dependent fashion on addition of protein S-EGF1-rhodamine, with half-maximal FRET occurring at approx. 1 μM of protein S-EGF1-rhodamine (Figure 5). To determine if non-specific binding of the rhodamine dye caused energy transfer to Fl-FPR-APC, a titration with free rhodamine was performed. No energy transfer between Fl-FPR-APC and free rhodamine was observed at any comparable rhodamine concentration (Figure 5), thereby indicating that energy transfer between Fl-FPR-APC and protein S-EGF1-rhodamine required the direct binding of the EGF1 moiety of protein S-EGF1-rhodamine to APC. The energy transfer between donor and acceptor dye due to binding of EGF1 to APC was fully reversible by the addition of EDTA (Figure 5), thereby confirming that protein S-EGF1 association with APC is Ca^{2+}-dependent.

Table 2 Donor-acceptor separation

Values of 2/3 and 1.4 were assumed for κ^2 and the refractive index (η) respectively. *n*, number of experiments.

(a) Distance, plane-to-plane, APC-active site to membrane surface (L)*

Donor (fluorescein)	Acceptor (rhodamine)	Absorption maximum (nm)	J_{DA} (10^{15} $M^{-1}\cdot cm^{-1}\cdot nm^4$)	R_0 (Å)	*n*	Distance (Å)
Fl-FPR-APC	PCPS-OR	564	3.62	50.3	5	94 ± 5
Fl-FPR-APC + protein S	PCPS-OR	564	3.62	50.3	2	85
Fl-FPR-APC + EGF1	PCPS-OR	564	3.62	50.3	2	84

(b) Distance, point-to-point, APC-active site to protein S-EGF1 (R)†

Donor (fluorescein)	Acceptor (rhodamine)	Absorption maximum (nm)	J_{DA} (10^{15} $M^{-1}\cdot cm^{-1}\cdot nm^4$)	R_0 (Å)	*n*	Distance (Å)
Fl-FPR-APC	EGF1-Rh	554	3.86	50.9	3	76 ± 3

* Experiments were performed with Fl-FPR-APC bound to PCPS vesicles, with OR densities (σ) between 1.68 and 3.38 $\times$ 10^{-4}/Å^2.
† No PCPS vesicles were present in these experiments.

Spectral parameters and donor-acceptor separations

From the amount of energy transfer between fluorescence donor and fluorescence acceptor dyes, the separation between the dyes can be calculated according to the Experimental section. R_o, the distance between donor and acceptor dyes at which FRET efficiency is 50%, was calculated as described previously [15], assuming that the refractive index of the medium between the donor and acceptor dyes (η) is 1.4 and the orientations of the transition dipoles of the dyes were random during the lifetime of the excited state of the donor dye ($\kappa^2 = 2/3$). Q_D, the quantum yield of human Fl-FPR-APC in the absence of acceptor, was found experimentally to be 0.30. For the Fl-FPR-APC/PCPS-OR donor/acceptor pair, the spectral overlap integral J_{DA} was found to be 3.62×10^{15} $M^{-1}\cdot cm^{-1}\cdot nm^4$, yielding an R_o of 50.3 Å. For the Fl-FPR-APC/EGF1-rhodamine donor/acceptor pair, a spectral overlap determination (results not shown) resulted in a calculated J_{DA} of 3.86×10^{15} $M^{-1}\cdot cm^{-1}\cdot nm^4$, yielding an R_o of 50.9 Å. In Table 2 the spectral parameters of both donor/acceptor pairs are summarized as well as the results of the FRET experiments.

FRET measurements at different OR densities revealed that the distance of closest approach of the active site fluorescein of human APC to the phospholipid surface was 94 Å, identical to that described for bovine and human APC [15,16]. This distance was calculated assuming that $\kappa^2 = 2/3$, and the uncertainty in this determination due to orientation effects is approx. 10%, for reasons detailed earlier [15,16].

The addition of human protein S to the Fl-FPR-APC–PCPS-OR sample increased the extent of FRET between the fluorescein dye in the active site of APC and the rhodamine dyes at the membrane surface. This increase in energy transfer results from a cofactor-dependent movement of the active site, which is either translational or/and rotational. If solely translational (i.e. $\kappa^2 = 2/3$), the magnitude of energy transfer yields a distance of closest approach of the active site of APC to the membrane surface of 85 Å, essentially equivalent to that previously reported for the protein S–Fl-FPR-APC–PCPS-OR complex [15,16]. Thus the topography of the membrane-bound APC and the magnitude of the protein S-dependent conformational change that relocates the APC active site are the same for the human and bovine proteins. Strikingly, the binding of the chemically synthesized protein S-EGF1 module, as well as protein S–C4BP complex, to membrane-bound APC elicits a similar change in the active site topography of APC to that caused by native protein S, reducing the distance of closest approach of the active site of APC to the phospholipid surface to 84 Å, based on calculations assuming $\kappa^2 = 2/3$.

When the chemically synthesized rhodamine-labelled protein S-EGF1 module bound to Fl-FPR-APC, FRET was observed, thereby demonstrating directly the binding of protein S-EGF1-rhodamine to APC. The low efficiency of FRET shows that the fluorescein in the active site of APC is located far from the rhodamine attached to the bound protein S-EGF1 domain. The donor-acceptor separation is approx. 76 Å, assuming $\kappa^2 = 2/3$. The locations of this rhodamine and the protein S-EGF1 module on the APC molecule are unknown, and hence the exact location of the protein S-EGF1-rhodamine dye relative to the membrane surface is unknown. However, because APC, like homologous vitamin K-dependent clotting factors, is approx. 90 Å in length [33], this 76 Å distance suggests that the protein S-EGF1-rhodamine module binds to APC near the surface of the phospholipid membrane.

DISCUSSION

The importance of protein S as an antithrombotic factor, expressing cofactor activity in the inactivation of coagulation cofactor Va by APC, is clinically well established, although the structure–function relationships of this essential anticoagulant protein are not thoroughly understood. The EGF1 module of protein S was synthesized, purified and folded to yield the native disulphide linkages. Amino acid analysis and ESI–MS showed the correct amino acid composition and molecular mass, and the correct disulphide pairing was confirmed, suggesting that synthetic protein S-EGF1 reflects its structure in protein S [27].

Hence, we used the chemically synthesized protein S-EGF1 module to investigate the interactions of human protein S with APC. Stimulation of anticoagulant APC activity by protein S was completely inhibited by preincubation of APC with purified synthetic protein S-EGF1, strongly suggesting that EGF1 directly participates in the binding of protein S to APC.

Proof for a direct interaction between EGF1 and APC was obtained using fluorescence spectroscopy to monitor energy transfer between fluorescein in the active site of APC and rhodamine at the vesicle surface. Specifically, addition of synthetic protein S-EGF1 increased the energy transfer between membrane bound fluorescein-labelled APC and rhodamine on the bilayer, showing that the EGF1, through a direct interaction with APC, changed the orientation of the APC-active site above the phospholipid surface.

The interaction between EGF1 and APC was confirmed with N-terminal rhodamine-labelled protein S-EGF1. Direct energy transfer between fluorescein in the active site of APC and rhodamine attached to protein S-EGF1 was observed as a result of a physical interaction of protein S-EGF1 with APC.

When protein S was added to fluorescein-labelled APC bound to rhodamine-labelled phospholipid vesicles, an increase in energy transfer between fluorescein and rhodamine was observed, as was seen for the bovine APC/protein S system [15], and recently, for the human APC/protein S system [16].

Interestingly, the addition of C4BP did not reverse the increase of energy transfer by protein S. Moreover, the addition of protein S–C4BP complex induced the same increase in energy transfer as did protein S alone. This strongly suggests a phospholipid-bound ternary protein complex of APC with protein S and C4BP. The existence and importance of this ternary complex was previously suggested based on anticoagulant functional studies, in which the protein S–C4BP complex functioned as an inhibitor for the APC-cofactor activity of protein S [34].

Thrombin-cleaved protein S lost the ability to increase the energy transfer between fluorescein-labelled APC and rhodamine at the phospholipid surface. This failure to relocate the APC active site may be due to failure to bind to APC, and may help explain its well known inability to stimulate APC activity.

The distances of closest approach between the APC active site and the phospholipid surface were calculated under the various FRET conditions, and were found to be identical to those in other studies [15,16]. The distance between the APC active site and the phospholipid surface of 94 Å was unaffected by thrombin-cleaved protein S, but was decreased to 85 Å due to addition of protein S, protein S–C4BP complex, and synthetic protein S-EGF1 (assuming $\kappa^2 = 2/3$). A schematic overview of the observed data is shown in Figure 6.

It has been established that the relocation of the APC active site contributes to the anticoagulant cofactor activity of protein S [15,16]. Yet the C4BP–protein S complex and protein S-EFG1 elicit the same increase in APC-to-membrane energy transfer (Table 2), without a stimulation of APC-activity (this study and [18–20]). Since both translational and rotational movements can alter FRET efficiency, one possible explanation for this apparent discrepancy is that the conformational changes in membrane-bound APC caused by protein S-EGF1 and the protein S–C4BP complex differ from that caused by protein S, even though the observed changes in FRET efficiency are very similar. Other interactions may also influence the observed stimulation of APC activity by protein S, and hence may contribute to the lack of cofactor activity of protein S-EGF1 alone and protein S–C4BP, such as an absence of interactions between APC and its substrate factor Va. Indeed, protein S can bind to factor Va through its C-terminal sex hormone-binding globulin-like region [35,36], and this interaction is inhibited by association of C4BP to protein S [35]. Thus C4BP might block potential protein S–factor Va interactions in the APC–protein S–C4BP complex. In the case of the synthetic EGF1, the lack of cofactor activity could be due, in part, to the absence of a protein S–factor Va binding site(s) in the APC–EGF1 complex.

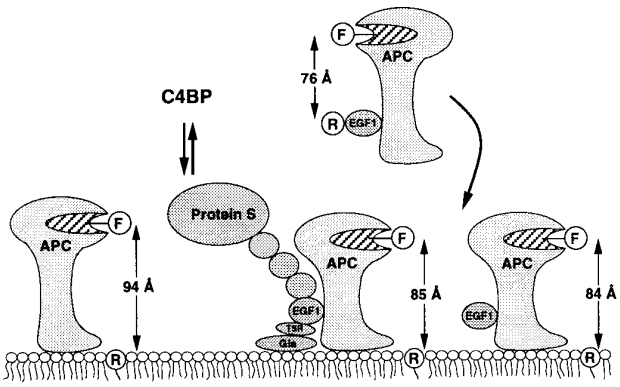

Figure 6 Protein S-EGF1 binding to activated protein C

The active site of APC is located 94 Å ($\kappa^2 = 2/3$) above the phospholipid surface ([15,16] and this study). Addition of protein S, protein S–C4BP complex, or protein S-EGF1 module, resulted in a relocation of the APC active site to approx. 85 Å above the phospholipid surface, if κ^2 is assumed to be 2/3, in all cases. The distance between the fluorescein in the APC-active site and the rhodamine at the N-terminal of the EGF1 module was calculated to be 76 Å ($\kappa^2 = 2/3$), placing the EGF1 module of protein S bound to APC, close to the phospholipid surface. Abbreviations: F, fluorescein; Gla, γ-carboxyglutamic-acid-rich module; R, rhodamine, TSR, thrombin-sensitive module.

The conformational changes that cause an increase in APC-to-membrane energy transfer may not be precisely equivalent in the APC complexes with protein S, protein S-EGF1 and protein S–C4BP, but it is clear from the observed EGF1-dependent increase in energy transfer that the synthetic EGF1 module of protein S interacts directly with APC. Thus while a variety of studies provided indirect evidence that the EGF1 module of protein S binds to APC [21–25], the present paper provides the first direct biophysical evidence that the EGF1 of protein S binds to APC and is capable of causing conformational changes which can relocate the active site of APC above the phospholipid surface. Furthermore, these results demonstrate the feasibility and versatility of using chemically synthesized protein modules that can be labelled with fluorescent probes at specific residues (e.g. rhodamine at residue 76 of protein S-EGF1) to characterize structure–function relationships of coagulation proteins.

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