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The anticoagulant protein C pathway

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Summary

The anticoagulant protein C system regulates the activity of coagulation factors VIIIa and Va, cofactors in the activation of factor X and prothrombin, respectively. Protein C is activated on endothelium by the thrombin-thrombomodulin-EPCR (endothelial protein C receptor) -complex. Activated protein C (APC)-mediated cleavages of factors VIIIa and Va occur on negatively charged phospholipid membranes and involve protein cofactors, protein S and factor V. APC also has anti-inflammatory and anti-apoptotic activities that involve binding of APC to EPCR and cleavage of PAR-1 (protease-activated receptor-1). Genetic defects affecting the protein C system are the most common risk factors of venous thrombosis. The protein C system contains multi-domain proteins, the molecular recognition of which will be reviewed.

Abbreviations: APC, activated protein C; AT, antithrombin; C4BP, CCP, complement control protein domain; C4b binding protein; EPCR, endothelial protein C receptor; EGF, epidermal growth factor; FV, factor V; Gla, γ -carboxy glutamic acid; LamG, laminin G-type domain; PCI, protein C inhibitor; PAR-1, protease activated receptor 1; TAFI, thrombin activatable fibrinolysis inhibitor; TM, thrombomodulin; TSR, thrombin sensitive region;

The protein C anticoagulant pathway regulates blood coagulation

Blood coagulation and platelet-dependent primary hemostasis have evolved as important defense mechanisms against bleeding. The formation of the platelet plug provides the initial occlusion of the vascular lesion. The coagulation system is simultaneously activated by tissue factor (TF), which is exposed to blood. Circulating factor VIIa (FVIIa) binds to TF and the generated FVIIa-TF complex efficiently converts factor IX (FIX) and factor X (FX) to active enzymes FIXa and FXa. FIXa and FXa take part in multi-molecular enzymatic complexes, the tenase and prothrombinase complexes, respectively, which propagate the coagulation process.[1-4] These complexes form on the surface of negatively charged phospholipids, e.g. on activated platelets, and in addition to the enzymes FIXa and FXa, they contain cofactors; activated factor VIII (FVIIIa) in the tenase complex and activated factor V (FVa) in the prothrombinase complex (Fig. 1). FVIIIa and FVa derive from circulating high molecular weight precursor proteins and are converted to their active forms early during the coagulation by thrombin or FXa.[1,4-7] The tenase complex (FIXa-FVIIIa) activates FX, whereas the prothrombinase complex (FXa-FVa) converts prothrombin to thrombin. In the tenase and the prothrombinase complexes, the catalytic efficiencies of the enzymes are several orders of magnitude higher than the intrinsic efficiencies of the enzymes. Very large amounts of thrombin are generated during the coagulation process. Thrombin has multiple procoagulant functions, e.g. it activates FV and FVIII and platelets, it converts fibrinogen to a fibrin clot, and it activates FXIII to an active transglutaminase that cross-links fibrin (Fig. 1).[8,9]

Blood coagulation is controlled by several anticoagulant principles and under normal conditions they prevail over the procoagulant forces. A natural anticoagulant system denoted the protein C pathway exerts its anticoagulant effect by regulating the activity of FVIIIa and FVa, the cofactors in the tenase and prothrombinase complexes, respectively.

The vitamin K-dependent protein C is the key component of the pathway.[4,7,10-13] It circulates as a proenzyme to an anticoagulant serine protease and is activated by thrombin (T) bound to the endothelial membrane protein thrombomodulin (TM). Activated protein C (APC) cleaves and inhibits coagulation cofactors FVIIIa and FVa, which result in downregulation of the activity of the coagulation system. The protein C pathway comprises multiple proteins involved in the different reactions (Fig. 1A). Thus, there are proteins affecting the activation of protein C by the T-TM complex, cofactor proteins that modulate the proteolytic activity of APC and serine protease inhibitors that inactivates APC. The endothelial protein C receptor (EPCR) stimulates the T-TM-mediated activation of protein C on the endothelial cell surface (Fig. 2A and 3A).[12,14] The two cofactors, protein S and the intact form of FV, enhance the anticoagulant activity of APC. Protein S is sufficient for inactivation of FVa (Fig. 2B), whereas regulation of FVIIIa in the tenase complex requires the synergistic contribution of protein S and FV.[7] Protein S is a vitamin K-dependent plasma protein, which in human plasma to 60-70% is bound to the complement regulator C4b-binding protein (C4BP) (Fig. 4). The free form of protein S function as APC cofactor, whereas the complexed form does not.[10,15-17] Protease inhibitors such as the protein C inhibitor (PCI), α 1-antitrypsin, and α 2-macroglobulin inhibit APC in plasma. The inhibition is relatively slow and the half-life of APC in circulation is approximately 20 minutes.[10,11]

Recently, protein C has been shown not only to express anticoagulant activities but also to function as an anti-inflammatory and anti-apoptotic factor.[12,18,19] Moreover, APC has been found to be useful as a therapeutic agent in the treatment of sepsis, the unique combination of anticoagulant, anti-inflammatory and anti-apoptotic properties of activated protein C (APC) presumably being important.[12,18,20-23] Protein C and the other components of the pathway have been intensively investigated and knowledge about

the three-dimensional (3D) structure of the proteins has been gained. The intricate relationships between the structures of the proteins and their functions are in focus in this review.

Activation of protein C on endothelium

Protein C is a vitamin K-dependent multi-domain protein composed of a light and a heavy chain, the two chains being disulfide-linked (Fig. 2 and 3). A vitamin K-dependent domain containing γ -carboxy-glutamic acid (Gla) residues and two epidermal growth factor (EGF)-like domains occupy the light chain. The heavy chain contains a short activation peptide and a serine protease domain.[10] Vitamin K-dependent post-translational carboxylation of glutamic acid residues generates the Gla residues. These residues are important for calcium-binding to the Gla domain and for the proper folding of the domain.[24] The correctly folded Gla domain binds negatively charged phospholipid membranes, which is important for the anticoagulant activity of APC. The Gla domain can also bind the endothelial receptor EPCR, an interaction that is important for both the activation of protein C and the generation of anti-inflammatory and anti-apoptotic activities of APC (Fig. 2-4).[12,14] The functions of the EGF domains are not well understood but most likely they are important for interaction with other proteins such as protein S, FVa and FVIIIa. The activation peptide of protein C is released during the activation by the T-TM-EPCR complex, and the serine protease domain converted to its active conformation.

TM is present in all vascular endothelium, the capillary bed having particularly high concentration and a high surface area to blood volume which favors thrombin binding and protein C activation.[12,25,26] TM occupies the functionally important exosite I in thrombin and thereby blocks interactions with other thrombin-binding proteins (Fig. 2 and 3). The procoagulant properties of thrombin are therefore lost upon

binding of thrombin to TM. In addition, the thrombin inhibitors antithrombin (AT) and protein C inhibitor (PCI) efficiently inhibit the TM-bound thrombin.[11,12] The multiple anticoagulant properties of TM, i.e. the conversion of thrombin into an activator of protein C and the accelerated inhibition of thrombin, makes TM a crucial regulator of blood coagulation. TM contains several domains and the molecule is a type I membrane protein (Fig. 2A). The N-terminal type C lectin domain is followed by six EGF domains, a Ser/Thr-rich region, a transmembrane section and a short cytoplasmic tail.[12,25,26] The Ser/Thr-rich region contains a chondroitin sulfate side chain that stimulates the AT- and PCI-mediated inhibition of thrombin that has bound to TM. The EGF domains are important for the activation of protein C. Thrombin binds to EGF5 and EGF6, whereas protein C interacts with EGF4 (Fig. 2 and 3). In this interaction, a positively charged cluster, formed by basic amino acid residues in serine protease domain loops 37, 60, 70, and 148 (minor role), is important.[11,12,25] The activation of protein C is augmented by EPCR which binds the Gla domain of protein C and aligns the substrate protein C with the activating T-TM complex.[12,14] EPCR is a member of the MHC class 1/CD1 family and a type I membrane protein. It contains two α -helices and an eight-stranded β -sheet that create a phospholipid-binding groove. The phospholipid binding is important for the ability of EPCR to bind the Gla domain of protein C (Fig. 3).[27]

Inhibition of coagulation by APC and the role of cofactors

The membrane-bound FV/FVa and FVIIIa are substrates for APC, which cleaves a few peptide bonds in each molecule. FV and FVIII are homologous proteins sharing the domain structure A1-A2-B-A3-C1-C2. The three A-domains are arranged in a triangular fashion and the carbohydrate-rich B domains protrude from the globular A1-A3 region.[6,7,28,29] The B domains of both FV and FVIII are released after cleavage by

thrombin or FXa, the A1-A2-A3-C1-C2 domains forming the active FVa and FVIIIa (Fig. 1-3). The three A-domains are homologous to the three A-domains of ceruloplasmin. The three-dimensional structure of ceruloplasmin is known and forms the basis for theoretical molecular models of the A-domains of FVa and FVIIIa.[30,31] The structure of APC-cleaved bovine FVa lacking the A2 domain is also determined as are structures for the C2 domains in FV and FVIII.[32-34] It is interesting to note that the homology-based models and the experimentally determined 3D structures are in good agreement. For instance, based on the human FVa model we proposed which residues were involved in calcium binding[30] and showed their importance by mutagenesis[35]. The experimentally determined structure of confirmed that the equivalent residues in bovine FVa were indeed coordinating calcium.[34] In both FVIIIa and FVa, the C2 domains contain the major phospholipid-binding sites but a region of the C1 domain of FVa has based on mutagenesis studies also been suggested to be important for binding to phospholipids. In FVIIIa and FVa, the high affinity binding of the respective cofactors, FIXa and FXa, involve the A2 and A3 domains and the details of the interacting sites on participating proteins are beginning to emerge.[28,29,36-38]

In FVa, three APC-cleavage sites have been identified at positions Arg306, Arg506 and Arg679 (Fig. 2 and 3).[6,7] The cleavages at the first two sites demonstrate interesting differences with regard to kinetics, requirement of APC cofactors, and remaining activity after cleavage. The cleavage at Arg506 is less dependent on the presence of protein S and the phospholipid composition than the Arg306 cleavage. Moreover, it is kinetically favored over the Arg306 cleavage, is inhibited by FXa bound to FVa, and only results in partial loss of FXa cofactor activity.[7] In contrast, the Arg306 cleavage is fully dependent on negatively charged phospholipid, not inhibited by FXa bound to FVa, strongly stimulated by protein S and results in severe loss of FVa activity. Total loss of FVa activity

is caused by the dissociation of the A2 fragments after cleavage at Arg306.[39] The differences in the cleavages at Arg306 and Arg506 are due to different involvement of an exosite in the SP domain of APC in the two cleavages (Fig. 2B).[11,40] A positively charged cluster in the SP domain of protein C formed by basic residues in loops 60, 37, 70 and 148 (Fig. 2B and 3B) presumably interacts with a negatively charged region in FVa located adjacent to the Arg506 site. Elimination of this positive cluster by mutagenesis reduces the rate of cleavage of the Arg506 site but has no effect on the Arg306 site. The same cluster in APC binds negatively charged heparin, which at high concentrations specifically inhibits the APC-mediated cleavage at Arg506.

The concentration of the tenase complex is much lower than that of the prothrombinase complex as the FVIII concentration is approximately 100-fold lower than that of FV.[4] This may be the reason why in the APC-mediated regulation of the tenase activity, not only protein S but also the non-activated form of FV serve as cofactor to APC.[7] It has been shown that the FV molecule is cleaved by APC at Arg306 and Arg506 during the FVIIIa inactivation, at least the cleavage at Arg506 being important for the ability of FV to serve as APC cofactor in the reaction.[7] Moreover, the last section of the B domain of FV is important for the APC cofactor activity of FV, as is an intact junction between the B domain and the A3 domain. Upon full activation of FV by thrombin, when the B-A3 junction is disrupted by the thrombin-mediated cleavage at Arg1545, the anticoagulant APC cofactor activity of FV is lost. Thus, FV is a Janus-faced protein, having the ability to express both pro- and anticoagulant functions depending on proteolysis by either pro- or anticoagulant enzymes such as thrombin/FXa or APC, respectively.[7]

Multiple functions of vitamin K-dependent protein S

Protein S contains several domains: an N-terminal vitamin K-dependent Gla domain, a thrombin-sensitive region (TSR), four EGF-like domains and two laminin G-type (LamG) domains (Figs. 2, 4, and 5).[10,15,16] The Gla domain has high affinity for negative phospholipids and protein S and APC form a membrane-bound complex, the Gla-domain, TSR, EGF1, and EGF2 being important for the interaction.[10,15,16,41] Protein S has been suggested to decrease the distance between the active site of APC and the phospholipid membrane. This may be of importance for proper localization of APC in relation to the cleavage sites in FVa and FVIIIa. The LamG-domains are also important for the synergistic function of protein S and FV during the APC-mediated degradation of FVIIIa.[10,15,16,40]

The binding of protein S to C4BP is of high affinity in particular when calcium is present.[10,15-17] Both LamG-domains of protein S are involved in the binding and several areas in both LamG domains have been suggested to contribute to the binding (Fig. 5). C4BP has an octopus-like structure comprising seven α -chains and a single β -chain (Fig. 4).[17] The chains are composed of CCP (complement control protein) domains arranged in tandem, eight in the α -chain and three in the β -chain. Each α -chain binds a C4b molecule (activated complement protein C4) and converts it into a substrate for factor I, a complement regulatory enzyme in blood. Thus, C4BP is an important regulator of the classical complement pathway. The β -chain CCP1 contains the binding site for protein S, a hydrophobic patch (I16, V18, V31 and I33) being particularly important (Fig. 5).

Negatively charged phosphatidylserine is under normal conditions not exposed on the surface of cells but located in the inner leaflet of the cell membrane. Certain situations result in exposure of phosphatidylserine on the cell surface, e.g. apoptosis or platelet activation. Protein S can bind to the negatively charged phospholipids that are exposed on the surface of apoptotic cells and on platelet microparticles. [42,43] The Gla

domain mediates the binding, which is dependent on calcium. Protein S bound to the apoptotic cell surface stimulates phagocytosis of the apoptotic cells through mechanisms yet to be defined.[44] The complex protein S-C4BP also binds to the apoptotic cells but this is in contrast found to counteract the phagocytic process.[45] Protein S and the protein S-C4BP complex may have other important functions on the apoptotic cell surface, e.g. controlling the coagulation and complement pathways.

Protein S is homologous to the anti-apoptotic protein Gas6 (product of the growth arrest specific gene number 6), the two proteins having similar domain organization. Gas6 is vitamin K-dependent and as the name implies, the Gas6 synthesis is induced by growth arrest, e.g. by serum starvation of cultured cells.[46-48] Gas 6 has several biological effects on cells, e.g. it inhibits apoptosis, stimulates mitogenesis and growth. Endothelium, fibroblasts and smooth muscle cells express Gas6, but in contrast to protein S, the liver does not express it. Gas6 binds and stimulates tyrosine kinase receptors Axl, Sky, and c-Mer.[46-48] The Gla-domain of Gas6 binds apoptotic cells and stimulates phagocytosis. Protein S has also been reported to function as a ligand for the Sky receptor. However, the physiological significance of this has been questioned because only protein S from certain species can stimulate the Sky receptor.[49]

The protein C system and venous thrombosis

Individuals with complete inherited protein C deficiency suffer from severe micro-vascular thrombotic disease (purpura fulminans) already in the neonatal period.[10,50] This clearly illustrates that the protein C system is vitally important to keep the blood in a fluid state and the circulatory system open. The prevalence of protein C deficient alleles is around 1/600 in the population and thus complete deficiency occurs in approximately 1/200.000-1/300.000 newborn and heterozygous deficiencies in 1/300.[4]

Heterozygous protein C deficiency is associated with approximately 5-fold increased risk of venous thrombosis. Similar risk of thrombosis affects individuals with heterozygous protein S deficiency. The most common gene defect associated with venous thrombosis is the FV Leiden mutation (APC resistance), which is present in 20-40% of thrombosis patients.[4] The FV Leiden mutation (G1691A) replaces Arg506 with a Gln. The protein C system is compromised in two ways by the FV Leiden mutation. 1/ the degradation of mutant FVa by APC is impaired because one of three APC cleavage sites is missing in FVa Leiden. 2/ the degradation of FVIIIa is poor because FV Leiden cannot be cleaved at Arg506. Therefore, FV Leiden is a poor cofactor to APC in the degradation of FVIIIa.[7]

FV Leiden mutation, which is predominantly found in whites, is approximately 30,000 years old. The prevalence of FV Leiden in the general population varies geographically. In America, where the population is of mixed ethnic background the prevalence is approximately 5% in the north and somewhat lower in the south. In Europe there is a north to south gradient with highest prevalence (10-15%) in the north and lowest in the south (approximately 2%). Individuals with heterozygous FV Leiden have approximately 5-fold increased risk of venous thrombosis, whereas homozygotes have around 50-fold increased risk. [4,51] In contrast, FV Leiden is not a risk factor for arterial thrombosis. The high prevalence in certain populations suggests that the FV Leiden allele have provided a survival advantage during evolution. In fact, even today women with FV Leiden have reduced bleeding tendency after delivery. In the history of mankind this must have been a major survival benefit.

Anti-inflammatory and anti-apoptotic effects of protein C

Components of the protein C pathway have biological effects other than those strictly referred to as being anticoagulant.[16,18,25,52] However, the molecular

mechanisms of these activities and their physiological importance are not yet fully understood. It has been found that the lectin domain of TM has anti-inflammatory properties, down-regulating NFκB and the MAP kinase pathways, and also decreases leukocyte adhesion and extravasation.[52] TAFI (thrombin activatable fibrinolysis inhibitor), a carboxypeptidase B that removes C-terminal lysine residues from fibrin is activated by the T-TM complex.[25] TAFI inhibits fibrinolysis and inactivates the anaphylatoxins C3a and C5a. Both protein C and APC directly inhibit the adhesion of neutrophils to the endothelial cell surface and the trans-migration of neutrophils. In addition, APC has direct anti-inflammatory and anti-apoptotic properties that seem to depend on the presence of both EPCR and protease activated receptor 1 (PAR-1) in the membrane (Fig. 4).[18,53] PAR-1, which is primarily cleaved by thrombin, is a seven-transmembrane domain, G-protein-coupled receptor. After cleavage, the novel N-terminus of PAR-1 activates itself by an intra-cellular signaling event. Recently, it was shown that thrombin is several orders of magnitude (10^4) more potent than APC in cleaving PAR-1, which raises doubts as to the physiological significance of the APC-mediated PAR-1 cleavage *in vivo*.[54]

The protein C system and the treatment of severe sepsis

Blood coagulation is activated during sepsis by, which is TF expressed on the endothelium and monocytes/macrophages in response to cytokines (e.g. TNF, IL-1 and IL-6).[18,55] Protein C is consumed during development of severe septic shock and the drop in the plasma level of protein C may contribute to the development of micro-vascular thrombosis and DIC (disseminated intravascular coagulation). In addition, the expression levels of TM and EPCR on endothelium decrease during sepsis. APC can counteract the deleterious effects associated with sepsis. Thus, in a study of severe sepsis (PROWESS)

recombinant APC (drotrecogin alfa–Xigris) gave a 19.4% reduction in the relative risk of death and an absolute reduction of 6.1%.[21] Thus, the protein C system plays a role in the defense against sepsis, the anticoagulant as well as anti-inflammatory and anti-apoptotic properties of APC presumably being crucial.

Concluding remarks

The elucidation of the molecular mechanisms of the intricate protein C system has not only provided insights into a fascinating molecular world but also knowledge of great medical relevance. Insights have been gained in the structure-function relationships of macromolecular complexes important for the activation of protein C, the regulation of tenase and prothrombinase complexes, and the cell-surface interactions with EPCR/PAR-1. Many unanswered questions remain and some are particularly challenging, e.g. the molecular mechanism of the synergistic APC cofactor activity of FV and protein S that regulates FVIIIa in the tenase complex and elucidation of the cell-surface and intra-cellular events associated with the anti-inflammatory and anti-apoptotic functions of the protein C system.

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Legends to figures

Figure 1

Schematic representation of blood coagulation and the protein C anticoagulant system.

Section A demonstrates a scheme of blood coagulation reactions together with the balancing anticoagulant reactions of the protein C pathway. The coagulation scheme is incomplete as the interaction between FVIIa and tissue factor (TF) and the FVIIa-TF mediated activations of FIX and FX are not shown. In section B, a schematic model of the prothrombinase complex is shown to illustrate the principle of multi-molecular enzyme complexes forming on the surface of negatively charged phospholipid membranes. The enzyme FXa and the cofactor FVa assemble on the surface of negatively charged phospholipid to form the prothrombinase complex, which efficiently converts prothrombin to thrombin and fragment 1.2.

Figure 2

Schematic model of certain reactions of the protein C anticoagulant system. A, Activation of protein C by thrombin bound to thrombomodulin (TM). EPCR (endothelial protein C receptor) binds the Gla-domain of protein C and helps orient protein C to the T-TM complex. **B,** Degradation of FVa by APC in the presence of protein S, which serves as an APC cofactor. APC cleaves FV at three sites, Arg306, Arg506 and Arg679, which results in dissociation of degradation fragments from the FVa molecule and loss of FVa activity.

Figure 3

3D overview of the protein C activation and the FVa degradation. A, The figure illustrates the complex (determined by X-ray crystallography) between thrombin (T) (solid surface magenta) and EGFs 4-6 of thrombomodulin (TM) (ribbon in green, blue and white

respectively) and a theoretical model of protein C (solid surface). The Gla domain of protein C is bound to EPCR (ribbon, magenta). In EPCR, a phospholipid (green) is bound in a groove. Some key loops of protein C denoted 37, 60, and 70 are coloured. The white dashed lines indicate that the protein contains other domains. The red line in TM running toward thrombin represents the chondroitin sulfate side chain. AP, activation peptide approaching the active site of thrombin (arrow); SP, serine protease domain. **B**, A theoretical model of human FVa and the X-ray structure of APC-cleaved bovine FVa are shown. Two APC cleavage sites, R306 and R506, in FVa are indicated. APC is docked at position R506 and slightly moved apart to facilitate presentation of the figure. The loops in APC that are important for the interaction (loops 37 and 148) are blue. The A2 domain dissociates after cleavage at Arg306 and FVi remains. For references and discussion please see the text.

Figure 4

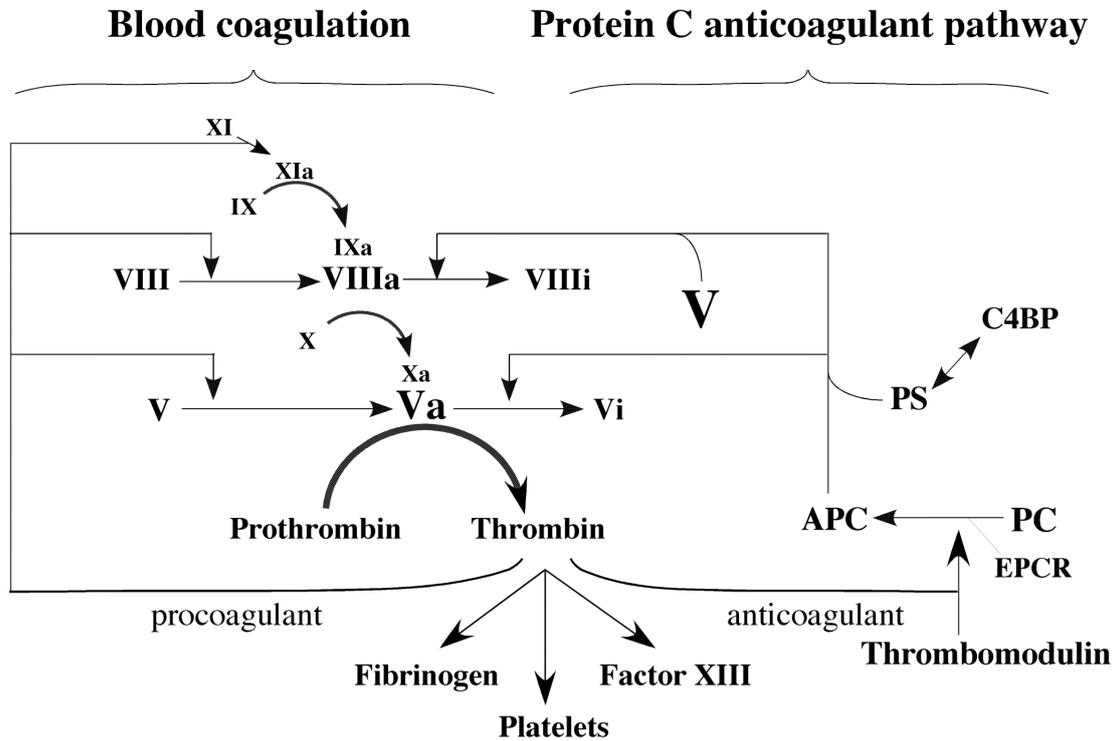
Schematic models of APC-mediated activation of PAR-1 and protein S binding to apoptotic cells. **A**, The Gla domain of APC binds EPCR and the active site of APC cleaves PAR-1 (protease activated receptor 1). The PAR-1 polypeptide spans the phospholipid membrane seven times. The cytoplasmic part of PAR-1 binds to G-proteins. The cleavage of PAR-1 creates a novel N-terminus, which activates PAR-1. This results in the activation of the intracellular G-proteins and generation of anti-inflammatory and anti-apoptotic responses (see text for references). **B**, Negatively charged phosphatidylserine is exposed on the surface of apoptotic cells. Free protein S and the protein S-C4BP complex bind to the cell surface via the Gla-domain of protein S. This provides the potential for regulation of both coagulation and the complement systems on the surface of apoptotic cells. In addition, the bound protein S stimulates phagocytosis of the apoptotic cells.[44] The octopus-like shape of C4BP is illustrated, the arrows indicating the α - and β -chains.

Figure 5

Structures of the β -CCP1 of C4BP, protein S and APC. A model of CCP1 (magenta) of the C4BP β -chain has been created and a region rich in solvent exposed hydrophobic residues (in yellow) has been demonstrated to play a crucial part in the interaction with protein S.[56] Protein S is composed of several domains and theoretical models for several regions have been reported, e.g. the region comprising the Gla (yellow), the TSR (thrombin sensitive region) (blue) and EGF1 have been predicted. The structures of the other EGF domains are unknown. The C-terminal region of protein S is composed of two LamG domains (green and white). The model was originally predicted using the laminin X-ray structure and recently refined using the X-ray structure of Gas6 (unpublished data). The areas of the LamG domains that have been proposed as important for the interaction with C4BP are shown in red on the protein S model.[40] Protein S is a cofactor for APC and a model of full length APC based on a combination of the X-ray structure of the Gla-domain less APC molecule is shown in order to help visualize the overall dimensions of the system.

Figure 1

A



B

Prothrombinase complex

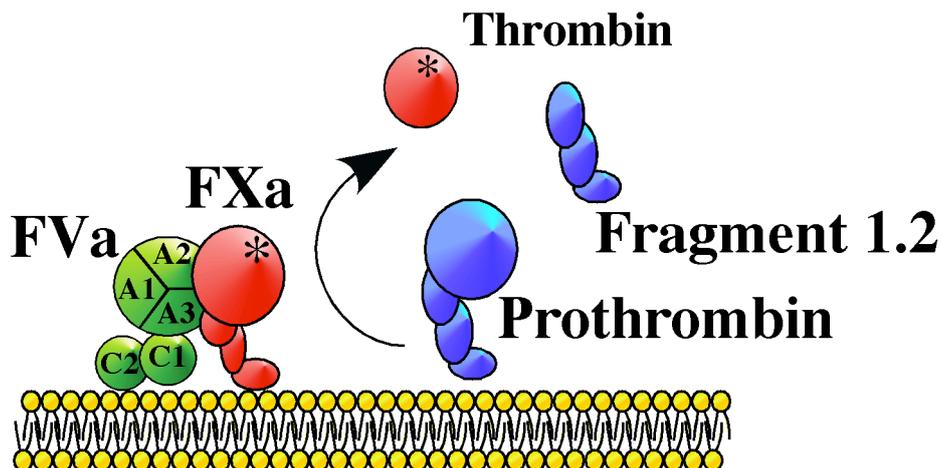
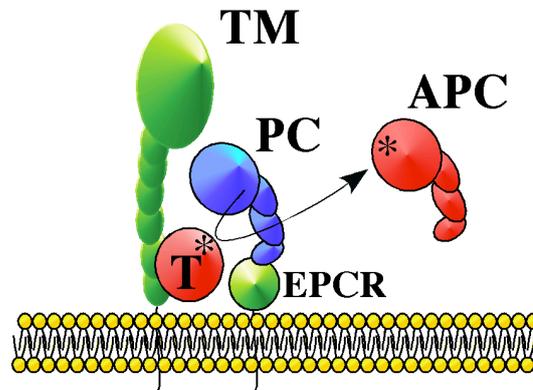


Figure 2

A Activation of protein C



B FVa degradation

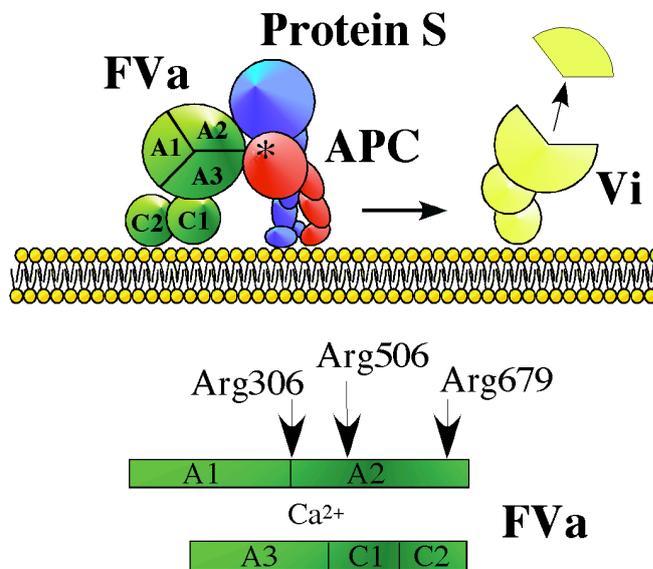


Figure 3

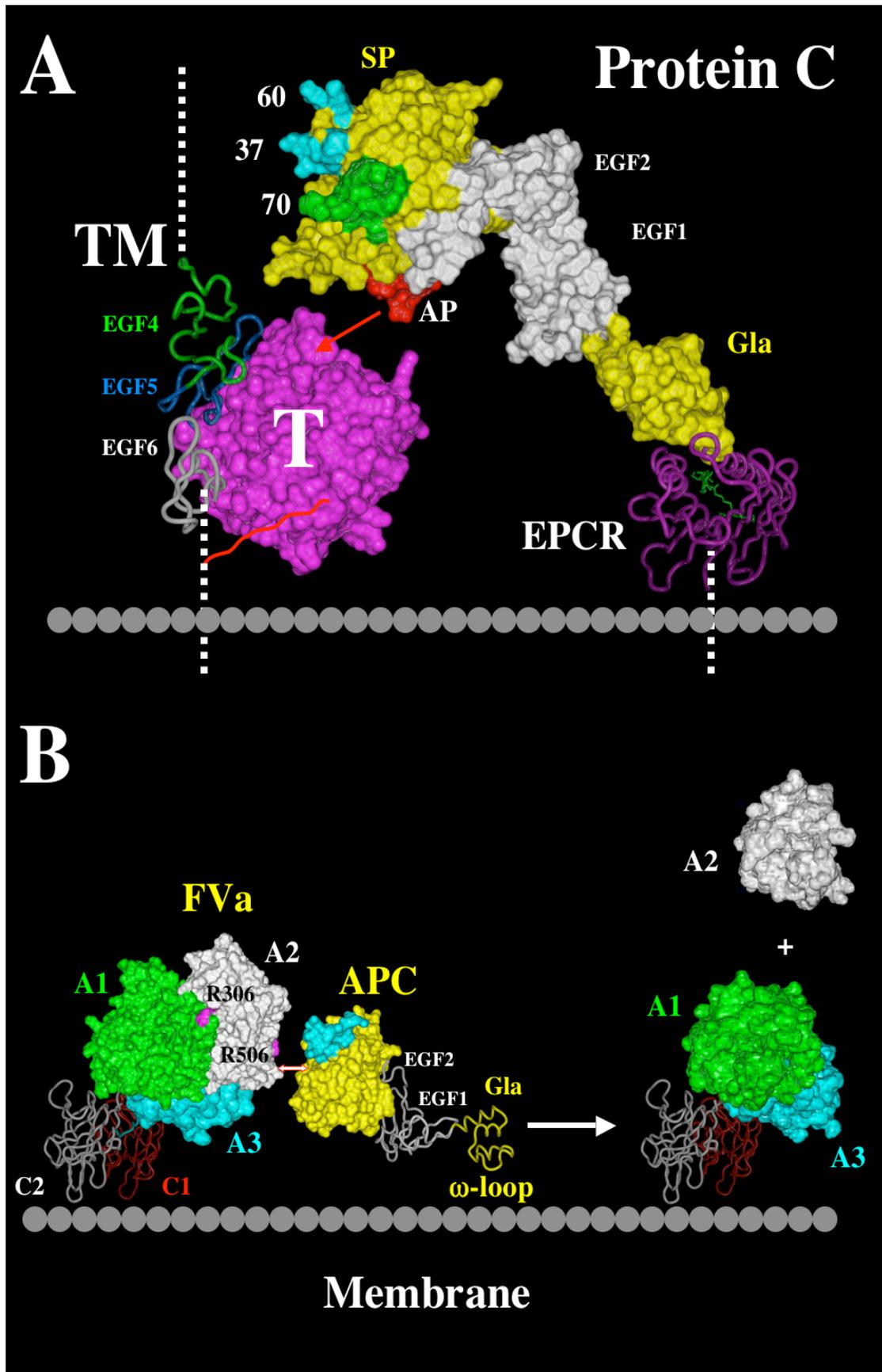
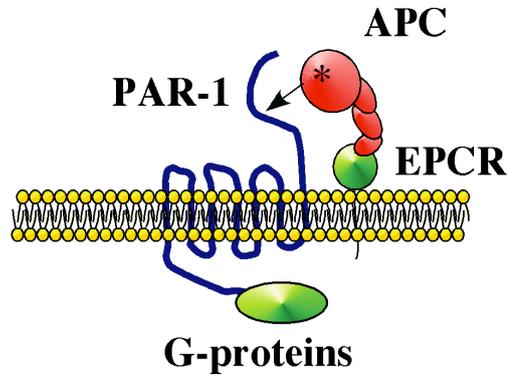


Figure 4

A Activation of PAR-1 by APC



B Protein S on apoptotic cells

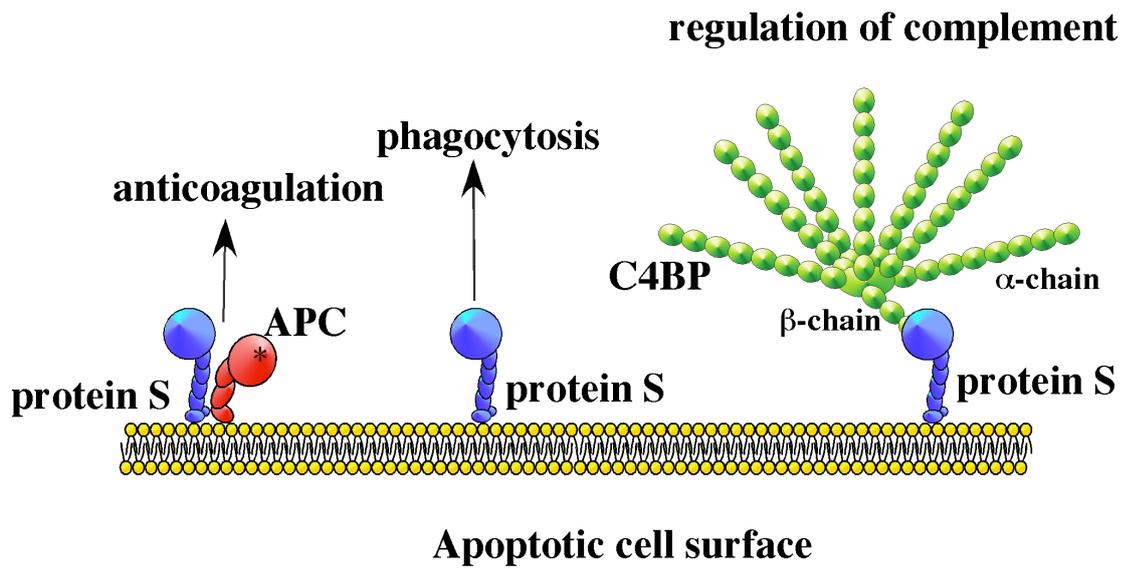


Figure 5

