The structure-function relationship of activated protein C
Lessons from natural and engineered mutations

Karin C. A. A. Wildhagen1; Esther Lutgens1,2; Sarah T. G. B. Loubele1; Hugo ten Cate1; Gerry A. F. Nicolaes1

1Department of Biochemistry, Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, the Netherlands; 2Department of Pathology, Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, the Netherlands; 3Institute for Cardiovascular Prevention (IPEK), Ludwig-Maximilians University Munich, Munich, Germany

Summary
Protein C is the central enzyme of the natural anticoagulant pathway and its activated form APC (activated protein C) is able to proteolyse non-active as well as active coagulation factors V and VIII. Proteolysis renders these cofactors inactive, resulting in an attenuation of thrombin formation and overall down-regulation of coagulation. Presences of the APC cofactor, protein S, thrombomodulin, endothelial protein C receptor and a phospholipid surface are important for the expression of anticoagulant APC activity. Notably, APC also has direct cytoprotective effects on cells: APC is able to protect the endothelial barrier function and expresses anti-inflammatory and anti-apoptotic activities. Exact molecular mechanisms have thus far not been completely described but it has been shown that both the protease activated receptor 1 and EPCR are essential for the cytoprotective activity of APC. Recently it was shown that also other receptors like sphingosine 1 phosphate receptor 1, Cd11b/CD18 and tyrosine kinase with immunoglobulin-like and EGF-like domains are likewise important for APC signalling. Mutagenesis studies are being performed to map the various APC functions and interactions onto its 3D structure and to dissect anticoagulant and cytoprotective properties. The results of these studies have provided a wealth of structure-function information. With this review we describe the state-of-the-art of the intricate structure-function relationships of APC, a protein that harbours several important functions for the maintenance of both humoral and tissue homeostasis.

Keywords
Activated protein C, anticoagulation, cytoprotection, mutagenesis, structure-function

Introduction
Protein C (PC) is a vitamin K-dependent serine protease of 62 kDa, which is present in human plasma at an average concentration of 70 nM (1). PC circulates in plasma as an inactive zymogen and is activated by thrombin bound to the endothelium associated transmembrane receptor thrombomodulin (TM). TM-bound thrombin removes the 158–169 activation peptide of PC via cleavage at R169, thus generating activated PC (APC). The catalytic activity of the thrombin-TM complex is 20-fold higher when PC is bound to thrombin-TM complex and with EPCR are Ca2+-dependent. Once PC is activated, APC has anticoagulant as well as cytoprotective functions, which will be described in detail in the next paragraphs.

Two-dimensional (2D) and three-dimensional (3D) structural protein analyses have shown that PC has a mosaic domain structure containing a N-terminal γ-carboxyglutamic acid (Gla)-domain (residues 1–45), including a helical aromatic segment (residues 38–45), two epidermal growth factor (EGF)-like domains (residues 46–92 and 93–136), a linking peptide (residues 38–45), two epidermal growth factor (EGF)-like domains 2 are likewise important for APC signalling. Mutagenesis studies are being performed to map the various APC functions and interactions onto its 3D structure and to dissect anticoagulant and cytoprotective properties. The results of these studies have provided a wealth of structure-function information. With this review we describe the state-of-the-art of the intricate structure-function relationships of APC, a protein that harbours several important functions for the maintenance of both humoral and tissue homeostasis.

Keywords
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Anticoagulant function of APC

PC is the central enzyme of the natural anticoagulant pathway that helps to control thrombin formation. APC generated by the thrombin-TM complex is able to proteolyse the active coagulation factors V (FVa) and VIII (FVIIIa) (Fig. 1A). Important cofactors in the inactivation of FV and FVIII are respectively protein S and factor V (FV). APC can inactivate FVa by proteolysis of three different peptide bonds at positions R306, R506 and R679 (11–13). The cleavage at R506 is kinetically favoured, protein S-independent and yields a FVa intermediate with decreased factor X (FX) cofactor activity (12). The slower cleavage at R306 is stimulated by protein S and completely inactivates FVa. The exact mechanism through which protein S appears to stimulate cleavage at R306 in FVa remains unclear. It has been proposed that upon binding of protein S to APC the protease domain of APC undergoes a rotational and/or translational movement, thereby decreasing the distance of APC’s active site to the membrane (14, 15). This hypothesis has been challenged, however, by the observation that both R506 and R506 appear to be present at ~80Å from the membrane, whereas only R306 cleavage appears to be dependent upon protein S. Also, mutagenesis studies of FVa in a proposed APC-binding site, for cleavage at R306, showed increased rates of APC-catalysed FVa inactivation, that are apparently independent of the cleavage site:membrane distance (16), nor does protein S stimulate the binding of APC to FVa (17). The cleavage at R679 plays a minor role in the inactivation of FVa (12). APC targets both free FVa and the prothrombinase complex, with the latter inactivation being two orders of magnitude slower (12, 18). APC-catalysed inactivation of FVIIIa, at homologous positions in the FVIII A-domains at R336 and R562 and at a third cleavage site at R740, which can also be cleaved by thrombin (19, 20), is stimulated not only by protein S but also by FV, through an hitherto unknown mechanism (21). It was shown, however, that the presence of the FV B-domain and an R506 cleavage site are required for the cofactor activity of FV in the APC-catalysed inactivation of FVIIIa (22, 23). It should be noted that in contrast to FVa, FVIIIa in solution is subject to rapid decay caused by dissociation of the A2 domain from the rest of the molecule. As a result, the majority of FVIIIa activity (70–80%) is lost spontaneously (24). Remaining FVIIIa activity is then lost by APC-catalysed cleavage at R336. Thus, the greater part of FVIIIa activity is not lost by proteolytic action of APC but rather by spontaneous FVIIIa subunit dissociation. This observation is likewise illustrated by the apparently several fold lower affinity of APC for FVIIIa, as compared to that of FVa (17).

Cytoprotective functions of APC

The widely observed beneficial effects of APC in animal models of sepsis, pulmonary injury, stroke and wound healing (36–44) cannot be explained solely by APC’s anticoagulant effects. APC administration, and not anticoagulants like antithrombin III and TFPI (45, 46), has been successfully used to reduce mortality in severe sepsis patients (Prowess trial [47]). There is, however, controversy as to the general use of APC in the treatment of severe sepsis and septic shock patients since a recent meta-analysis did not confirm the overall beneficial effects of APC on 28-day survival (48). Collectively these and other data have manifested the dual functions of APC in both anticoagulation and cytoprotection. Protease activated receptor 1 (PAR-1) and EPCR were shown to be required for APC-dependent cytoprotective effects. PAR-1, a trans-membrane protein receptor, which is activated upon limited proteolysis, is present on many cell types, including endothelial cells, epithelial cells, myocytes, fibroblasts, astrocytes and neurons (49, 50). Most of these cell types also express EPCR (51–54). APC has cytoprotective effects in different cell types by anti-inflammatory, anti-apoptotic and endothelial barrier stabilising effects. These effects, rather than the anticoagulant activity of APC, likely explain the protective effect of APC administration in animal studies of sepsis, lung injury, myocardial infarction and ischaemic stroke (37–39, 41–44, 55–57). There are still uncertainties with re-
Figure 1: Humoral and cellular effects of APC. A) Anti-coagulation. Upon binding of APC to either FVa or FVIIIa, APC ablates cofactor activity through competition with the respective serine proteases (FXa or FIXa, respectively). Subsequent proteolysis of FVa and FVIIIa renders these cofactors inactive, resulting in irreversible attenuation of thrombin formation and overall downregulation of coagulation. Protein S and FV are important cofactors of APC in this process. B) Anti-inflammation. PAR-1 and EPCR dependent signalling of APC in endothelial cells and leukocytes inhibits NFκB / AP-1 signalling, resulting in an inhibition of inflammatory mediator release and downregulated expression of adhesion molecules. Binding of APC to sEPCR, PR3 and CD11b/CD18 on leukocytes activates PAR-1, resulting in upregulation of SphK-1 and S1P. Subsequently binding of S1P to its receptor S1P1 suppresses the pro-inflammatory activity of leukocytes. These effects of APC result in decreased leukocyte chemotaxis and lower infiltration of leukocytes in the endothelium. C) Anti-apoptosis. Apoptosis can be initiated by binding of TNFα or FasL to death receptors (TNFR1/FasR) on the cell membrane of endothelial cells (extrinsic pathway) or by DNA damage, hypoxia and oxidative stress that increase the permeability of the mitochondrial outer membrane (intrinsic pathway). Activation of both pathways results in activation of caspases. APC inhibits caspase activation, suppresses the expression of pro-apoptotic genes (p53 + Bax) and upregulates the transcription of anti-apoptotic genes (Bcl2). The suppression of Bax and up-regulation of Bcl2 leads to a normalisation of the Bax/Bcl-2 ratio on the mitochondrial membrane and inhibition of apoptosis. D) Endothelial barrier protection. APC protects the endothelial barrier function by induction of SphK-1 and up-regulation of the bioactive lipid S1P. Binding of S1P to S1P1, leads to improved cell survival and barrier protection via activation of the PI 3-kinase/Akt pathway, upregulation of both RAC-1 GTPases and Ang1 and downregulation of Ang2. Ang1 leads via binding to Tie2 to further activation of the PI 3-kinase/Akt pathway and enhancement of endothelial barrier integrity.
gard to the exact roles that EPCR and PAR-1 play in APC-induced cell signalling. Recently, the involvement of other cell-receptors such as Tie2, S1P1, CD11b/CD18, ApoER2 (LRP8) and epidermal growth factor receptor in APC signalling was shown, thereby providing yet additional insights into the molecular mechanism that underlies the cell-protective properties of APC (58–60).

APC-induced cytoprotection can be attributed to any of or a combination of a number of cellular responses, including an altered inflammatory response, a modified anti-apoptotic response and/or an improved barrier function of the endothelium. The different cytoprotective effects and molecular mechanisms identified so far are described in detail below and are illustrated in Figure 1B–D.

Anti-inflammatory activities

The effects of APC on endothelial cells and leukocytes are summarised in Figure 1B. APC modulates transcription of genes involved in the major inflammatory pathways. In both endothelial cells and leukocytes, APC reduces via PAR-1 and EPCR gene expression levels and functional activity of nuclear transcription factor κB (NFκB) (61). In leukocytes, APC leads via PAR-1, sEPCR, proteinase-3 (PR3) and CD11b/CD18 to increased concentrations of sphingosine kinase-1 (Sphk-1) and sphingosine 1 phosphate (SIP), resulting in suppression of pro-inflammatory activity (55, 59). Both inhibition of NFκB and upregulation of SIP result in inhibition of cytokine signalling, down-regulation of cell surface adhesion molecules and increased expression of anti-inflammatory genes like interleukin 10 (IL-10) (27, 55, 61). Ultimately this results in reduced leukocyte transmigration through the endothelium and less tissue damage in the underlying tissue (55). Recent results from Kerschen et al. have shown that APC targets EPCR-positive CD8+ dendritic cells in mice, which results in a reduction of mortality after induction of endotoxaemia (62).

Anti-apoptotic activity

Apoptosis is an intrinsic program of cell death that occurs in various physiological and pathological situations. It can be induced by both the death receptor (extrinsic) and mitochondrial (intrinsic) pathway. Caspase-8 activation in the extrinsic pathway and release of cytochrome c, apoptosis inducing factor (AIF) and pro-caspases in the intrinsic pathway finally lead to caspase-3 activation and apoptosis (55).

Figure 1C shows a summary of the different anti-apoptotic effects of APC on endothelial cells. APC can reduce apoptosis induced by hypoxia, t-PA and staurosporine in different types of endothelial cells (61, 63, 64). In these experimental conditions, the presence of APC resulted in decreased caspase-3 and –8 activation, reduced DNA degradation and p53 expression, as well as decreased nuclear translocation of AIF, decreased translocation of phosphatidyl serine to the outer cell membrane and normalisation of the Bax/Bcl-2 ratio (27, 55, 65). Via PAR-1 cleavage, APC induces phosphorylation of extracellular signal regulated protein kinases 1/2 and members of the mitogen-activated protein kinase family (66), resulting in restraint of the mitochondrial permeability transition pore, inhibition of cytochrome C release into the cytoplasm and prevention of apoptosis signalling (67).

Protection of the endothelial barrier function

In healthy individuals, the intact endothelial layer forms the first natural physical barrier that protects underlying tissues from blood-borne pathogens by controlling passage of molecules and migration of leukocytes into and from the tissues. Excessive or prolonged increases in permeability of the endothelial monolayer barrier, as seen in cases of (chronic) inflammation, may lead to tissue oedema/swelling (68). The effects of APC on endothelial barrier function are summarised in Figure 1D. APC improves the integrity of the endothelial barrier by upregulation of sphingosine kinase-1 (Sphk-1), sphingosine 1 phosphate (S1P), angiopoietin 1 (Ang1), tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (Tie2) and tight junction protein zona occludens (69). APC also stimulates the migration of smooth muscle cells (SMCs) into the endothelium. These SMCs form a sheath around the endothelium that decreases the permeability of the membrane. For APC’s barrier stabilising effects, the presence of the receptors EPCR, PAR-1, sphingosine 1 phosphate receptor 1 (S1P1) and Tie2 is required (69). The improved endothelial barrier limits the infiltration of inflammatory cells through the endothelium into the subendothelial space (55, 61, 70).

Effect of mutations on the function of APC

The dissection of anticoagulant and cytoprotective properties of APC, as has been demonstrated through mutagenesis studies from several groups (17, 70–77), illustrates the different structure-function facets of APC. Several variants have been designed and tested for their functional properties and it appears that specialised surface areas convey different properties to the APC molecule. Meanwhile, numerous natural variants of protein C have been reported that cause functional defects of the protein. In an attempt to provide a 3D properties map of APC, we have collected available structure-function data of APC and have placed these in the perspective of the 3D structure of the protease, in order to better understand the interactions of APC with its many physiological binding partners. Since the naturally occurring type I deficiencies (decreased PC expression levels with normal specific activity) are of less interest from a structure-function point of view, we have restricted ourselves to type II deficiencies (normal PC expression levels with decreased specific activity). It is important to mention that many PC variants are tested for their respective expression lev-
Light chain: Gla-domain mutations

The N-terminal Gla-domain (residues 1–45) confers PC its membrane and EPCR binding capacity and contributes to the binding between APC and protein S. In general, Gla-domain containing proteins bind to phospholipid (PL) vesicles in at least two stages. First, two or three cation binding-sites in the Gla-domain are occupied, resulting in a conformational change of the Gla-domain. Second, a group of four or five divalent cation binding sites with a high specificity for Ca$^{2+}$ become occupied, enabling binding of the protein to acidic PL vesicles. A cluster of hydrophobic side chains that extend away from the core of the protein (F4, L5, and L8, see also Fig. 2) was shown to be part of a PL binding site (78–80). These side chains are flanked by H10, which was suggested to participate in the formation of a pore in the protein that may be important for the formation of a protein-PL ion pair in aqueous medium (81, 82).

In the presence of calcium, seven Ca$^{2+}$-ions occupy a channel through the Gla-domain of APC. These Ca$^{2+}$-ions are important for maintenance of the overall structure of the Gla-domain, for the anticoagulant function of APC and the interaction with EPCR (83) (Fig. 2). A crystal structure of a complex between the Gla-domain of PC and sEPCR has been determined (84). In this structure, PC binds to sEPCR via hydrogen bonds at Gla-residues 7, 25, 29 and via hydrophobic interactions at residues F4, E7 and L8 to EPCR (84). Noteworthy, these interactions are highly specific for the interaction of the hAPC Gla domain and EPCR, as it was shown that hAPC FX- and prothrombin (PT) Gla-domain chimeras do not exhibit any cytoprotection (85).

Both natural (R9H) and engineered (F4Q, L5Q, L8Q, H10P) APC variants show reduced anticoagulant activities, although they have a normal Ca$^{2+}$-dependent conformation of the Gla-domain (78–82, 86). It is likely that these mutants have either suboptimal PL binding or disorientated binding to the PL membrane which results in poor alignment of the active sites towards the complementary cleavage sites of FV/FVa and FVIII/FVIIIa (78, 80). For variants L8Q and R9H, the reduced affinity for EPCR can contribute to the reduced anticoagulant activity (78, 80, 86).

To investigate which Gla-residues are essential for the anti-coagulant activity of APC, different mutants were expressed wherein the nine E residues of the Gla-domain of APC were substituted (83). Kinetic parameters for chromogenic substrate (S-2366) conversion were normal for all variants. Single mutations of Gla-residues at positions 6, 7, 16, 20, 26 or 29 resulted in APC variants with less than 10% of the anticoagulant activity of wt-APC in an APTT based assay. E25D-APC expresses ~20% of wt-APC anti-coagulant activity and Gla-residues 14 and 19 do not contribute to anticoagulant function. Furthermore, E16D-PC and E26K-PC were not activated by the thrombin-TM complex on endothelial cells and showed no binding to sEPCR (86). Collectively, Ca$^{2+}$ interactions are essential for the anticoagulant functions of APC and are influenced by mutations in different regions of the Gla-domain (83, 87, 88).

These aforementioned results are in agreement with the analysis of PC that was isolated from type II PC deficient patients. Both
the E7D (89, 90) and E20A/V34M (Protein C Vermont, [91]) are associated with thrombotic complications, despite the fact that carriers of these mutations have normal PC antigen levels and APC amidolytic activities.

Site directed mutagenesis of R15 shows that this residue is critical for the Ca^{2+}-dependent conformation of the Gla-domain required for PL binding and hence for APC’s anticoagulant activity (92). In agreement with this is the fact that individuals who carry a mutation at R15 (R15W or R15G, with the latter also known as PC Yonago) have an increased thrombotic tendency (93, 94). Mutation of hexapeptide disulfide loop residue C22 to serine, results in a complete loss of anticoagulant activity in an APTT based clotting assay and less than 5% of the activity of wt-APC toward inactivation of FVIIIa (87, 88). Residues R15, C22 and Gla-residues 6, 7, 16, 20, 25, 26 and 29 are thus absolutely required for APC’s anticoagulant function.

The C-terminus of the Gla-domain (residues 25–45) and particularly D35, D36, L38 and A39 were shown to interact with protein S (79). Each of these four motifs was individually replaced by the corresponding PT amino acid residue to investigate their functional importance (95). The amidolytic activities and anticoagulant properties of these mutants were not severely affected in the presence of protein S, except for the L38D mutant which exhibited 20-fold impaired anticoagulant activity as compared to wt-APC in plasma or in protein S dependent assays using purified proteins. Notably, in the absence of protein S, L38D-APC was indistinguishable from wt-APC. L38D-APC binds EPCR with equal binding affinity as wt-APC. All mutants tested in this study (95) inhibited apoptosis to a similar degree and had similar barrier-protective capacities as wt-APC in the absence of protein S.

Light chain: Mutations in EGF domains and linking peptide

The EGF domains of APC have been described to be involved in the interaction of APC with Ca^{2+} and protein S (96–98). In D71E-APC, in which the β-hydroxyaspartic acid was replaced by glutamate, a Ca^{2+}-dependent epitope for the interaction with protein S in the EGF-1 domain was lost, resulting in only 10% of the anticoagulant activity of wt-APC (97).

The so-called “linking peptide” (residues 137–157) precedes the activation peptide, which is excised upon PC activation. A special position here is taken by E149. Mutagenesis of this residue into alanine resulted in a complete loss of anticoagulant activity in an APTT-based clotting assay (75). In an amidolytic assay, probing the conversion of a small chromogenic substrate, and in the absence of protein S, no difference between E149A-APC and wt-APC was observed. In thrombin generation and FVα inactivation studies, E149A-APC required lower concentrations of protein S than wt-APC to achieve half-maximal effects. Taken together, E149A-APC expresses increased anticoagulant activity in the presence of protein S, likely through enhanced affinity for protein S. The improved anticoagulant effect was verified in an in vivo murine thrombosis model (75). Remarkably, the cytoprotective effects of E149A-APC were severely diminished, despite a normal cleavage of PAR-1 and normal binding to EPCR (75). E149A-APC expressed only 6% of the anti-apoptotic activity of wt-APC in a staurosporine-induced apoptosis model in endothelial cells and was unable to down-regulate IL-6 release in lipopolysaccharide (LPS) treated U937 monocytes (75). The molecular mechanism which reconciles the loss of cytoprotective effects, while amidolytic activities and PAR-1 and EPCR-binding are normal, is at present not available.

Heavy chain: Mutations in the activation peptide and C terminus of the catalytic domain

Since the activation peptide (residues 158–169) is excised during PC activation, mutagenesis in this region is expected to affect PC activation. Three variants have been studied: D167F/D172G-PC, P168V-PC and R169W-PC (96, 99, 100). At saturating Ca^{2+} concentrations, the activation rates of D167F/D172G and P168V mutants and wt-PC by the thrombin-TM complex were comparable, but the mutants required four-fold higher Ca^{2+} concentrations than wt-APC to achieve half-maximal activation rates. When only thrombin was present, Ca^{2+} was not able to influence the activation of the D167F/D172G and P168V mutants, though Ca^{2+} effectively inhibits activation of wt-PC by thrombin (96), R169W-PC cannot be activated by thrombin, but can be efficiently activated by chymotrypsin (100). Residues in close proximity of R169 appear to influence Ca^{2+} affinity and/or the Ca^{2+}-dependent conformation of PC through either direct or indirect mechanisms.

Probing this same region, residues K174, R177 and R178 were mutated to glutamic acid. The activation rates of the mutants by thrombin were 12-fold faster than that observed for wt-PC in the presence of Ca^{2+}, and unchanged in the absence of Ca^{2+}. Addition of TM did not stimulate activation of the PC variants, suggesting that residues 174, 177 and 178 are involved in PC activation by the thrombin-TM complex (101).

Heavy chain: catalytic domain mutations

The well-studied catalytic domain of APC, residues 170–419 (16–254: chymotrypsin numbering is given in parentheses from hereon), contains the catalytic triad, which is responsible for the proteolysis of APC-substrates. Moreover this domain contains substrate binding regions, to which APC substrates must bind prior to their cleavage. Mutations in this domain are therefore likely to directly influence the catalytic efficiency and the binding of substrates to the protease. The 3D structure of the catalytic domain and its various loop regions is shown in Figure 3.
For the anticoagulant activity of APC, especially the cleavage at R506 in FVa is required (73, 102). Protein-protein docking- and mutagenesis studies have revealed that multiple electrostatic contacts between APC and the R506 cleavage site in FVa mediate the efficient contact between FVa and APC (16, 102). The positively charged area in the catalytic domain of APC includes the 191-loop (37-loop), the Ca\(^{2+}\)-binding loop (residues 225–235 (70–80)) and the autolysis loop (residues 302–317 (143–154)) (3, 103). The importance of the electrostatic interactions made by these surface loops was shown by several mutagenesis studies.

Three 191-loop (37-loop) and/or Ca\(^{2+}\)-binding loop mutants were constructed: 2R2A-APC (RR229/230AA (RR74–75AA)), 3K3A-APC (KKK191–193AAA (KKK37–39AAA)) and 5A-APC (combination of both mutants) (73, 74, 102, 104–106). In an APTT-based assay the anticoagulant activities of 2R2A-APC, 3K3A-APC and 5A-APC were 14%, 5% and <3%, respectively, of that of wt-APC, with amidolytic activities being unaffected. These data, together with those from structure-function studies of recombinant R229Q (R74Q) PC, indicate the importance of R229 (R74) for anticoagulant function of APC and are part of a heparin-binding site. In line with this, a K193Q (K39Q) mutation has been described in a type II deficient patient with a history of thrombo-embolic disease (108).

The PC autolysis loop (residues 302–317 (143–154)) is involved in stabilisation of the activation peptide in its Ca\(^{2+}\)-dependent inhibitory conformation, leading to enhanced activation by the thrombin-TM-complex and decreased activation by thrombin (112, 113). The hPC autolysis loop is five residues longer than the autolysis loops of other vitamin K-dependent proteases and four residues longer than the homologous loop in bPC. Replacement of the autolysis loop in hAPC by the shorter bovine form resulted in a four-fold increased catalytic activity. Replacement of the hAPC autolysis loop by that of hFXa resulted in higher Ca\(^{2+}\)-affinity and increased PC activation by thrombin both in the absence (5-fold) and presence (2.5-fold) of TM. Furthermore, from the reduced half-life of these PC variants in plasma it was concluded that the autolysis loop is involved in inhibition by serpins. In both chimeras, substitution of the autolysis loop results in increased inactivation rates of Fv and FVIIIa, while conflicting results were reported for interaction with protein S (112, 113). Studies wherein individual residues of the autolysis loop were mutated (114, 115) showed that APC residues R306 (R147), K308 (K149), K311 (K149c), R312 (R149d) and R314 (R151) are important for FVa cleavage at R506 and residues R306 (R147), K311 (K149c) and R314 (R151) are essential for FVIIIa inactivation by APC.

Mutations in 214-loop (60-loop)

hAPC is, in contrast to its human counterpart, nearly completely resistant to inactivation by AAT. Homology modelling and site-
directed mutagenesis studies of the serine protease domains of bAPC and hAPC identified the 214-loop (residues 214–220) (60-loop (residues 60–65)) as being probably involved in interaction with AAT, heparin and PCI (116). Three PC mutants, E215S/S216R (E60aS/S61R), S336E (S173E) and K217N/K218D (K62N-K63D) were generated (104, 116, 117). Mutant APC amidolytic activities were unaffected, except for S336E (S173E) which had a reduced Km, which suggests that a glutamate residue at position 336 (173) improves the fit of the substrate in the catalytic cleft (104, 116, 117). K217N/K218D (K62N-K63D)-APC had slightly lower anticoagulant activity compared to wt-APC and showed undetectable binding to heparin (104, 117). Both E215S/S216R (E60aS/S61R)-APC and S336E (S173E)-APC are resistant to AAT (116). E215S/S216R (E60aS/S61R)-APC was 2–3 times more efficient inhibited by PCI than wt-APC in the absence as well as the presence of heparin (116). Inhibition of K217N/K218D (K62N-K63D)-APC by PCI was 3–5 times more efficient in the absence of heparin and two times less efficient in the presence of heparin. These results indicate that residues E215 (E60a), S216 (S61) and S336 (S173) influence the interaction of APC with heparin and/or AAT and residues K217 (K62) and K218 (K63) are important for heparin stimulation of APC inhibition by PCI (104, 116, 117).

Active site mutants

A much studied APC variant (17, 71, 73, 118) is S360A (S195A)-APC, wherein the active site serine S360 (S195) is mutated to alanine. Consequently, this APC is unable to proteolyse substrates (71). Remarkably, in plasma-based assays, S360A (S195A)-APC still has 15–25% of the anticoagulant activity of wt-APC. S360A (S195A)-APC had a dose-dependent effect on the activity of the purified prothrombinase complex (17, 119), in particular at low prothrombinase concentrations (119). APC had a dose-dependent effect on the activity of the purified prothrombinase complex (117, 119), in particular at low prothrombinase concentrations (119). APC inhibition of FVa by S360A (S195A)-APC is independent of protein C and/or AAT and residues K217 (K62) and K218 (K63) are important for heparin stimulation of APC inhibition by PCI (104, 116, 117).

Other catalytic domain mutants

An interesting engineered APC variant is C222-C237 (C67-C82)-APC in which a disulfide bond was introduced between two anti-parallel β-structures consisting of residues that form the Ca²⁺-binding loop of APC (70, 120). Binding of Ca²⁺ to this loop results in overall stabilisation of the catalytic domain. As a result, PC changes to a thrombin-(hyper) activatable conformation in which recognition by the thrombin-TM complex and the subsequent activation of PC are enhanced (70, 120). Due to the engineered disulfide bond, C222-C237 (C67-C82)-PC is trapped in the thrombin-(hyper) activatable conformation, even in the absence of Ca²⁺, resulting in a 80-fold increased activation rate in the absence of Ca²⁺ and TM. The amidolytic activity of the mutant and its inactivation by PCI were unaffected (120). C222-C237 (C67-C82)-APC has strongly reduced anticoagulant activity in both purified and plasma-based clotting assays (70). Two-fold higher concentrations of C222-C237 (C67-C82)-APC are required to reach cytoprotection that is comparable to wt-PC, likely due to lower EPCR affinity. These results show that occupation of the Ca²⁺-binding loop is not necessary for the expression of cellular activities. It is not known why the introduction of a disulfide bond abolishes the anticoagulant activity of APC, but a changed conformation of the APC catalytic domain may likely impair the interaction of C222-C237 (C67-C82)-APC with FVa and FVIIIa (70).

Two glutamic acid residues at position 330 (167) and 333 (170), located in helix 325–333 (helix 162–170) are not conserved in homologous regions of other vitamin-K-dependent proteins. Replacement of these residues by alanine yielded variants that were indistinguishable from wt-APC except for their procoagulant properties (118). E330A (E167A)-APC and E333A (E170A)-APC show normal interactions with EPCR, but display dramatically impaired cleavage of PAR-1. Thus, both E330 (E167) and E333 (E170) are required for the interaction of APC with PAR-1 and mutation of these residues results in a loss of cytoprotective activity (118).

To investigate the role of N-linked glycosylation on APC functions, four recombinant PC variants in which the asparagines at 97, 121, 248 (93), 313 (150) and 329 (166) were mutated to glutamine, were generated (5, 121). Elimination of the latter three glycosylation sites increased activation by the thrombin-TM complex and improved anticoagulant activity in both a modified APTT assay and in a thrombin generation assay (5, 121). Notably, the N329Q (N166Q) mutation influenced the cytoprotective function of APC. In an endothelial barrier permeability assay, N329Q (N166Q)-APC was up to six-fold more efficient than wt-APC and in staurosporine-induced endothelial cell apoptosis, N329Q (N166Q)-APC completely inhibited apoptosis at a 30-fold lower concentration than wt APC (121). Possibly the absence of a N-linked glycan chain at N329 enhances the access of residues E330 and E333 to PAR-1 and EPCR-dependent PAR-1 activation by APC is up-regulated, resulting in increased cytoprotection (121).

E357 (E192) is involved in binding of macromolecular substrates. Engineered E357Q (E192Q)-APC showed two- to three-fold improved Fva inactivation, but slightly reduced anticoagulant activity in plasma compared to wt-APC, likely due to an observed
280-fold faster inhibition by AAT (122). In contrast to wt-APC, this variant is effectively inactivated by pancreatic trypsin inhibitor, TFPI and AAT in both the presence and absence of heparin. These findings hint at an evolved function of E357 (E192) in order to prevent fast inhibition by serpins, at the cost of a reduced FVa inactivation (122).

Conclusion

APC is a protease with both anticoagulant and cytoprotective functions. Several areas on the surface of (A)PC are involved in the many interactions between (A)PC and its ligands (Fig. 4). Mutagenesis studies proved the importance of a number of residues in the Gla-domain for the anticoagulant function of PC. The correct Ca2+-dependent conformation of this domain is essential for its binding to PL membranes and EPCR. Several studies have been performed to study the mechanism of PL binding for Gla-domains in general and for PC in particular. Detailed structure-function data have provided a rational basis for protein engineering of PC, which has resulted in variants of PC with improved membrane binding, which may prove effective in the pharmacological use of which has resulted in variants of PC with improved membrane binding, which may prove effective in the pharmacological use of PC (76, 77, 86). Residues in 191-loop (37-loop) the Ca2+-binding loop and the autolysis loop of the protease domain are important for electrostatic interactions between APC and FVas, the main physiological APC substrate in coagulation. Residues interacting with protein S are situated in the helical aromatic segment (residue 38), EGF domain (residue 71), linking peptide (residue 149) and autolysis loop. For the PC activation by thrombin and the thrombin-TM-complex, residues in close proximity to residue 169 are important, as well as residues in the Gla-domain (residue 16 and 26), 191-loop (37-loop), 214-loop (60-loop), the Ca2+-binding loop and the autolysis loop. The interaction of PC with heparin, PCI and AAT is mediated by residues in 191-loop (37-loop), 214-loop (60-loop) and residue 336 (173). The autolysis loop and residues 357 (192) and 360 (195) are important for the interaction of PC with serpins.

Mutagenesis studies have indicated that the more recently discovered cytoprotective functions of APC can be linked to residues 149, 329 (166), 330 (167), 333 (170) and 360 (195), which are essential for cytoprotection. The effects of a number of other APC variants, which have been characterised for their anticoagulant properties, have not been investigated so far in the context of their cytoprotective properties and this research is currently ongoing. Considering the interest from both the academia and the pharmaceutical industry in PC, it is desirable and likely that a completed structure-function description of the cytoprotective properties of APC will become available in the next years. This will aid in the engineering of a yet safer variant of APC devoid of increased bleeding risks, for use in cell-protection in a number of relevant clinical conditions of which severe sepsis appears the most prominent.

Conflict of interest

None declared.

References


