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Down-regulation of the clotting cascade by the protein C pathway

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Summary

The protein C pathway provides important biological activities to maintain the fluidity of the circulation, prevent thrombosis, and protect the integrity of the vasculature in response to injury. Activated protein C (APC), in concert with its cofactors and cell receptors, assembles in specific macromolecular complexes to provide efficient proteolysis of multiple substrates that result in anticoagulant and cytoprotective activities. Numerous studies on APC's structure-function relation with its cofactors, cell receptors, and substrates provide valuable insights into the molecular mechanisms and presumed assembly of the macromolecular complexes that are responsible for APC's activities. These insights allow for molecular engineering approaches specifically targeting the interaction of APC with one of its substrates or cofactors. Thus far, these approaches resulted in several anticoagulant-selective and cytoprotective-selective APC mutants, which provide unique insights into the relative contributions of APC's anticoagulant or cytoprotective activities to the beneficial effects of APC in various murine injury and disease models. Because of its multiple physiological and pharmacological activities, the anticoagulant and cytoprotective protein C pathway have important implications for the (patho)physiology of vascular disease and for translational research exploring novel therapeutic strategies to combat complex medical disorders such as thrombosis, inflammation, ischemic stroke and neurodegenerative disease.

Keywords

activated protein C; anticoagulant; factor V; factor VIII; protein S; endothelial protein C receptor; protease activated receptor 1

Introduction

The hemostatic system safeguards the patency of the vasculature. Platelet aggregation, coagulation, and fibrinolysis operate in concert with the endothelium and other vascular cells to arrest bleeding and prevent thrombosis. The coagulation pathway contributes to

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regulation of the hemostatic balance via multiple mechanisms and pathways that ensure a balanced and confined hemostatic response at the site of injury. Despite these feedback mechanisms to fine-tune the hemostatic response, genetic and/or acquired defects often tilt the balance sufficiently to increase the risk for thrombophilia.

In the current model of coagulation,[1,2] clot formation is initiated by the extrinsic pathway with little or no role for the contact system in the initiation of physiological coagulation, although platelet-derived polyphosphates may provide an endogenous activation mechanism for FXII.[3,4] Thrombin formation occurs during two mechanistically different phases. In the first phase, referred to as primary thrombin formation, the extrinsic pathway generates the clot initiated by the tissue factor/FVIIa complex. However, primary thrombin formation is short-lived due to rapid inactivation of the tissue factor/FVIIa initiator complex by tissue factor pathway inhibitor (TFPI). In the second phase, when sufficient thrombin is generated to initiate FXI activation, secondary thrombin formation will continue inside the clot via thrombin-mediated FXI activation and amplification by the intrinsic pathway.[5] This secondary thrombin formation contributes to clot strength and conveys resistance to fibrinolysis via the activation of FXIII and thrombin Activatable finbrinolysis inhibitor (TAFI).[6,7]

Control of coagulation is generally provided by three different mechanisms. First, the γ-carboxyglutamic acid (Gla)-domain, common to most coagulation factors, requires the presence of negatively charged phosphatidylserine for calcium-dependent binding to lipid surfaces.[8] Thus, assembly of the tenase (FIXa, FX, and FVIIIa) and prothrombinase (FXa, FII, and FVa) complex is limited by the presence of negatively charged lipid surfaces (such as on activated platelets).[8,9] Second, serine protease inhibitors (SERPINs) rapidly inhibit activated coagulation factors, thereby blunting coagulation and preventing the escape of active coagulation factors in the circulation.[10] Finally, the protein C pathway actively inhibits coagulation by proteolysis of the tenase and prothrombinase complex cofactors, FVa and FVIIIa, thereby providing a dynamic regulation of coagulation.[11–15]

The focus of this review we will be on the protein C pathway. Because activated protein C (APC) inactivates both FVa and FVIIIa, it has important effects on the down-regulation of both primary and secondary thrombin formation that manifest as potent anti-thrombotic effects in vivo. Furthermore, APC's relatively new activities on cells provide physiological and pharmacological relevant protective effects on the endothelium and the vasculature. Thus APC conveys multiple activities that require assembly of macromolecular complexes with different cofactors, cell receptors and substrates. Structure-function analysis of these APC complexes, discussed in the next sections, provides a unique understanding of how a single enzyme can mediate multiple biologically and therapeutically relevant activities.

The protein C pathway

The protein C pathway provides important contributions to maintain the fluidity of the circulation, prevent thrombosis, and protect the integrity of the vasculature in response to injury. The reactions of the protein C pathway encompass protein C activation on endothelial cells, the anticoagulant protein C pathway on activated platelets, the

cytoprotective protein C pathway on cell membranes, and inactivation of APC by plasma serine protease inhibitors (SERPINs) in the fluid phase (Figure 1). Each of these aspects of the protein C pathway will be discussed in the sections below.

The physiologic importance of the protein C system is best illustrated by the manifestation of massive thrombotic complications in infants with protein C deficiency.[16,17] Neonatal purpura fulminans, a rapidly progressing hemorrhagic necrosis of the skin due to microvascular thrombosis, inflammation, and disseminated intravascular coagulation (DIC), is typically observed in severe protein C deficiency, whereas heterozygous protein C deficiency in adults carries a significantly increased risk for venous thrombosis.[18–20] A rare complication referred to as warfarin-induced skin necrosis with clinical symptoms similar to that of purpura fulminans, may present within days after initiation of oral anticoagulant therapy with coumarin derivatives. This is due to a temporary functional protein C deficiency caused by the shorter circulation half-life of protein C (8 hr) compared to the other procoagulant coagulation factors (24–72 hr).[17,18,21] Acquired protein C deficiency is also found in patients with severe infection and sepsis, most likely due to consumption and poor synthesis in the liver, and low protein C levels correlate with poor clinical outcome and death.[22]

Protein C Activation

The protein C zymogen is synthesized in the liver and circulates in plasma at 4 μg/ml, which is equivalent to ~70 nM based on a molecular weight of 62,000 Da. The domain topology of protein C is typical of vitamin K-dependent coagulation factors.[23] The N-terminal protein C light chain contains nine γ-carboxylated Glu residues (Gla-domain) and two epidermal growth factor (EGF)-like domains. The C-terminal heavy chain contains an N-terminal acidic protein C activation peptide that is removed upon activation and the protease domain with a typical His211 (mature protein C numbering), Asp257 and Ser360 active site triad (residues His57, Asp102 and Ser195 in chymotrypsin nomenclature, for a conversion table see[24]).

Protein C is activated by thrombin through limited proteolysis at Arg169. Physiological activation of protein C on the endothelial cell surface requires binding of thrombin to thrombomodulin (TM) and binding of protein C to the endothelial protein C receptor (EPCR) (Figure 1).[14,25–27] The binding surface for TM on protein C shows a partial overlap with the exosite for interactions with FVa, and includes residues in loop 37 (Lys191 and Lys192), loop 60 (Lys217 and Lys218), loop 70–80 (Arg229 and Arg230), and possibly loop 20 (Lys174, Arg177, and Arg178) although the direct interaction of these latter residues with TM remains controversial (loops are referred to by their chymotrypsin numbering[24]).[28–30]

Protein C activation by thrombin in the absence of TM is very inefficient and is inhibited by calcium. Presumably, this limitation ensures that APC generation is initiated only when the clot covers the intact endothelium and thrombin comes in contact with TM.[14] Several residues surrounding the Arg169 activation site in protein C (i.e. P3–P9′ residues relative to Arg169 denoted as P1)[31] are responsible for the inhibitory effect of calcium on the

activation of protein C by free thrombin. Mutation of these residues allows for efficient protein C activation by thrombin in the presence of calcium that is no longer dependent on the presence of TM.[32–34] In vivo proof-of-principal that TM-independent protein C activation by thrombin results in enhanced APC generation was provided by a transgenic mouse (named the "APC^{high}" mouse) expressing human protein C with mutations of the P3 and P3′ residues (Asp167Phe/Asp172Lys).[33,35] Interestingly, increased blood loss after tail amputation in these mice suggest that uncoupling of protein C activation from TM disrupts the regulation of normal thrombus formation.

Inactivation of APC

Inactivation of APC in plasma is driven by serine protease inhibitors (SERPINs) of which protein C inhibitor (PCI), plasminogen activator inhibitor-1 (PAI-1), α_1-antitrypsin and, to a lesser extent, α_2-macroglobulin and α_2-antiplasmin are most relevant for APC (Figure 1).[36] Even though heparin and vitronectin accelerate the reaction of APC with PCI and PAI-1 several orders of magnitude, the reaction of APC with SERPINs is relatively slow, resulting in a ~20 min half-life of APC in the circulation.[36] APC exosites required for interactions of APC with the various inhibitors and heparin largely overlap with those required for interaction with FVa and include residues in loop 37 and the autolysis loop.[36,37] Interestingly, some residues that affect interactions with SERPINs are not shared with FVa and these include Leu194 in loop 37, Lys217 and Lys218 in loop 60, Thr254, and Ser336.[36,38,39]

The anticoagulant protein C pathway

The protein C pathway is best known for its anticoagulant activity that involves proteolytic inactivation of FVa and FVIIIa on negatively charged phospholipid membranes and that is enhanced by cofactors protein S and FV (Figure 1).[40–42] Because APC inactivates both FVa and FVIIIa, it has important effects on the down-regulation of both primary and secondary thrombin formation. Inhibition of primary thrombin formation results in delayed clot formation, whereas inhibition of secondary thrombin formation results in diminished activation of TAFI and subsequently in an enhanced susceptibility of the clot to fibrinolysis. The latter effects of APC on secondary thrombin formation are also referred to as APC's profibrinolytic effects.[43]

APC's anticoagulant activity requires binding of the Gla-domain to negatively charged phospholipid membranes. Although membranes containing phosphatidylethanolamine in addition to phosphatidylserine generally improve APC's lipid-dependent functions, binding of APC to negatively charged phospholipids is relatively poor compared to other vitamin K-dependent coagulation factors.[44] Therefore, anticoagulant activity of APC can be enhanced by strategies aimed at improving APC's affinity for membranes, such as Gla-domain swaps or targeted mutagenesis of the Gla-domain.[45,46]

Inactivation of FVa and FVIIIa

FVa is a non-covalent heterodimeric complex composed of a heavy chain (domains A1-A2) and a light chain (domains A3-C1-C2).[47] Since FVa enhances prothrombinase ~10,000-fold,

inactivation of FVa by APC effectively shuts down thrombin formation.[40,48] Inactivation of FVa involves APC-mediated cleavages at Arg306 and Arg506. The rapid cleavage at Arg506 is kinetically favored over cleavage at Arg306, but results only in partial inactivation of FVa, whereas the slower cleavage at Arg306 results in a complete loss of FVa function.[40,41] The importance of APC-mediated FVa inactivation is clear from the increased risk for thrombosis associated with mutations of the APC cleavage sites in FV (Arg506Gln a.k.a. FV_{Leiden}, Arg306Thr a.k.a. $FV_{Cambridge}$ and Arg306Gly a.k.a. $FV_{Hong\ Kong}$). In fact, FV_{Leiden} is the most common identifiable hereditary risk factor for venous thrombosis among Caucasians.[47,49]

Mutagenesis studies have identified several positively charged exosites on the APC protease domain surface that are required for rapid inactivation of FVa (Figure 2B).[36,37,50,51] This extended FVa exosite on APC is comprised of residues in loop 37 (Lys191, Lys192, and Lys193), loop 60 (Asp214, Glu215 and Arg222), loop 70 (Arg229 and Arg230) and the autolysis loop (Lys306, Lys311, Arg312 and Arg314).[36,37,50,51] Remarkably, these residues primarily contribute to APC cleavage of FVa at Arg506 but have little effect on APC-mediated cleavage at Arg306, suggesting that the FVa Arg306 cleavage site does not rely on APC exosite interactions or that the exosite for Arg306 has not been found yet.[50,51] Instead, protein S enhances APC-mediated cleavage at Arg306 (see below).

In the circulation, tight non-covalent binding (K_D ~0.5 nM) of FVIII to von Willebrand factor (vWF) prevents the incorporation of factor VIII into the tenase complex.[52] FVIIIa dissociates from vWF after activation and enhances FXa formation by the tenase complex approximately 200,000-fold.[53] Despite a domain topology similar to that of FVa, FVIIIa circulates as a heterotrimer and not a heterodimer due to different cleavage patterns that cause their respective activations. As a consequence of being a heterotrimer, FVIIIa is quite unstable with a half-life of only 2 min due to spontaneous dissociation of the A2-domain.[52] Nevertheless, several observations, including stabilization of FVIIIa by FIXa in the tenase complex, support a role for APC in the inactivation of FVIIIa.[54] Homologous to inactivation of FVa, inactivation of FVIIIa by APC occurs upon cleavage at Arg336 and Arg562 but in contrast to FVa, cleavage of FVIIIa at either site results in a complete loss of cofactor activity.[55,56] Both protein S and FV but not FVa enhance inactivation of FVIIIa by APC.[56,57]

Protein S

Protein S is best known for its function as a non-enzymatic cofactor to APC in the inactivation of FVa and FVIIIa. In addition, protein S has APC-independent anticoagulant effects and also has direct (cytoprotective) effects on cells that are independent of its anticoagulant functions but instead require interactions with receptors on cells (the reader is referred to the references provided as a starting point for a more detailed discussion of these protein S activities).[58,59]

The important anticoagulant contributions of protein S are clear from the thrombotic complications and increased risk of venous thromboembolism associated with homozygous and heterozygous protein S deficiency.[60] Functionally, five distinct domains can be

identified that include an N-terminal Gla domain, a thrombin sensitive region (TSR), a repeat of four EGF-like domains, and a sex hormone-binding globulin (SHBG) domain composed of two laminin G-type domains.[23] Protein S predominantly stimulates FVa cleavage at Arg306 by APC but also neutralizes the FXa-mediated protection of FVa against APC cleavage at Arg506.[61,62] Molecular mechanisms for enhanced APC-mediated cleavage of FVa at Arg306 by protein S have been partially elucidated and involve a protein S-induced change in the geometry of APC relative to the membrane. Presumably, protein S binding lowers APC's active site closer to the membrane, placing it in a better position to cleave Arg306.[63] This provides a molecular explanation for how APC can inactivate FVa at Arg306 without extensive exosite interactions between APC and FVa. APC residues that are implicated in interactions with protein S (Figure 2D) include Gla-domain residues 35 to 39 (in particular Leu38), Asp71 that contains a post-translational β-hydroxyaspartic acid modification in EGF1, and potentially the C-terminus of the light-chain.[64–67]

Molecular interaction between APC, protein S, and FVa

Since limited structural information is available, the perceived assembly of APC with protein S and the interactions with FVa remain highly speculative.[24,68–70] An APC-FVa model for cleavage at Arg506 (Figure 2A), based on the interaction of the extended positively charged exosite of APC (Figure 2B) with a negatively charged region on FVa that includes Asp513, Asp577, and Asp578 in the A2-domain and Asp659, Asp660, Glu661, Glu662, and Asp663 that follows the A2-domain (Figure 2C), projects the APC Gla-domain rather far away from FVa.[51,70] In complex with protein S and FVa, the APC Gla-EGF1 domains are anticipated to be orientated in closer proximity to FVa, with a flexible conformation of the APC protease domain that bends down to Arg506 (or Arg306). This hypothesis is consistent with biochemical data that indicates binding of APC to the FVa light chain.[71,72] Thus, protein S is likely to have important implications for the spatial orientation APC in the ternary APC-protein S-FVa complex.

The APC-cofactor function of protein S involves a complex set of interactions of protein S with APC and factor Va.[73] The minimal structure of protein S to support APC cofactor activity requires the Gla-domain, the TSR and EGF1 (~30% compared with native protein S) although EGF2 and part of the SHBG domain provide additional interactions with APC and FVa and are required for full protein S cofactor activity.[74–76] Direct APC-binding to protein S seems contained to protein S EGF1 with important contributions of Asp78, Gln79, Asp95, and Thr103 (Figure 2E).[75,77] The protein S Gla-domain, TSR and EGF-2 are unlikely to contribute to direct interactions with APC but rather play a supporting structural role. Important residues in these supporting domains identified thus far include Glu36 in the Gla-domain and Arg49 and Gln52 in the TSR.[69,74–78]

The SHBG domain of protein S is projected to extend above the protease domain of APC since protein S contains two additional EGF-like domains compared to APC. The contributions of the SHBG-domain to protein S cofactor activity seem contained to the C-terminus of the SHBG laminin G2-domain and are likely derived from mediating interactions with FVa rather than APC.[79] Especially residues Lys630, Lys631, and Lys633 in the G2-domain seem to provide important contributions for binding of protein S to FVa,

which possibly facilitates directing the APC protease domain to the FVa cleavage sites consistent with protein S decreasing the distance of the APC active site to the membrane.[63,80]

The cytoprotective protein C pathway

In addition to its anticoagulant activity, APC can convey direct effects on cells, collectively referred to as APC cytoprotective activities that require EPCR and PAR1.[11,81–84] Dependent on the cell type and injury, these cellular activities of APC include anti-apoptotic and anti-inflammatory activities, alteration of gene expression profiles and protection of endothelial barrier function. Other receptors may also contribute to APC-initiated signaling such as sphingosine-1-phosphate receptor 1 (S1P1), apolipoprotein E receptor 2 (ApoER2), CD11b/CD18 ($\alpha_M\beta_2$; Mac-1; CR3), PAR-3, and Tie2, whereas APC's ability to inactivate extracellular histones is presumably independent from APC's cell signaling effects.[85,86]

The currently prevailing paradigm for APC's direct cytoprotective actions on endothelial cells is that when PAR1 and EPCR are colocalized in caveolin-1 enriched lipid rafts or caveolae, APC binding to EPCR permits non-canonical PAR1 activation at Arg46 to initiate cytoprotective signaling.[11,87–89] It is important to realize that there are several fundamental distinctions between PAR1 activation by APC and thrombin on endothelial cells. Foremost, the functional outcome is different. Thrombin activation of PAR1 results in proinflammatory and endothelial barrier disruptive effects, whereas PAR1 activation by APC results in cytoprotective actions that include endothelial barrier stabilization. The reasons for this functional contrast become evident when taking the fundamental differences in PAR1 activation and signaling between these two proteases in consideration. PAR1 activation by thrombin occurs after cleavage of the canonical Arg41 site after which the tethered-ligand starting at Ser42 promotes G-protein dependent signaling that includes activation of barrier disruptive Ras homolog gene family member A (RhoA). In contrast, activation of PAR1 by APC occurs through cleavage of the non-canonical Arg46 site after which the tethered-ligand starting at Asn47 mediates β-arrestin 2-dependent barrier protective Ras-related C3 botulinum toxin substrate 1 (Rac1) activation (Figure 3).[89,90] Synthetic peptides representing the sequences of the different tethered ligands exposed after cleavage of PAR1 at Arg41 (thrombin receptor activating peptide (TRAP)) or Arg46 (TR47) also recapitulate the remarkable differences in PAR1 signaling. TRAP induces typical phosphorylation of ERK1/2 but TR47 does not. Instead, TR47 but not TRAP induces phosphorylation of Akt in endothelial cells that is linked to cytoprotective functions.[89] The fact that different ligands induce different signaling pathways via the same receptor of which one employs G protein-dependent signaling (TRAP) and the other initiates β-arrestin 2-dependent signaling (TR47) is highly indicative that PAR1 can induce biased signaling (Figure 3).[91]

Presumably the thrombin generated TRAP-like tethered-ligand induces a subset of PAR1 conformations that preferentially employs G protein-dependent signaling, whereas the APC generated TR47-like tethered-ligand induces a different spectrum of PAR1 conformations that recruit β-arrestin 2-dependent signaling. Thus, non-canonical activation of PAR1 by APC at Arg46 and canonical activation of PAR1 by thrombin at Arg41 can understandably

mediate the often opposite effects of thrombin and APC because each protease generates different tethered-ligand agonists that utilize different signaling pathways with different functional consequences.

Relative contributions of APC's anticoagulant and cytoprotective activities

Because of its multiple biologic activities, APC and the protein C pathway components have important roles in complex and challenging medical disorders and provide potential opportunities for pharmacologic treatment strategies in thrombosis, inflammation, and ischemic stroke.[15,92,93] Although APC conveys beneficial effects in numerous different in vivo disease models, not all APC activities are necessarily beneficial. Based on the notion that the substrates and cofactors for APC's anticoagulant activity (phospholipids, protein S and FVa) differ from APC's cytoprotective effects (EPCR and PAR1), engineered APC mutants with cytoprotective-selective activities or anticoagulant-selective activities allowed the interrogation of the differential requirements for APC beneficial activities in vivo.[66,94,95] Targeted disruption of the interaction of APC with protein S (Leu38Asp-APC) or with FVa results in cytoprotective-selective APC mutants such as, Arg222Cys/Asp237Cys (stabilizing the 70–80 loop, Arg229Ala/Arg230Ala-APC, Lys191Ala/Lys192Ala/Lys193Ala (a.k.a. 3K3A-APC) or a combination of the latter two (a.k.a. 5A-APC) (Figure 4).[11,64,94–97] Targeted disruption of APC binding to EPCR while leaving phospholipid binding relatively intact (Leu8Gln-APC) or disruption of a region on APC that is required for cleavage of PAR1 (Glu330Ala and Glu333Ala) yields anticoagulant-selective APC mutants.[98,99] Glu149Ala-APC, another anticoagulant-selective mutant provides a challenging test of our current understanding of the cytoprotective protein C pathway as its lack of cytoprotective activities remains enigmatic.[66] Cytoprotective-selective but not anticoagulant-selective APC mutants provide beneficial effects in models of inflammation, sepsis, and ischemic stroke, whereas anticoagulant-selective but not cytoprotective-selective APC mutants prevent thrombosis, generally consistent with the concept that APC's cytoprotective activities protect cells and APC's anticoagulant activities prevent occlusive thrombosis.[66,93,94] For instance, cytoprotective-selective 3K3A-APC or 5A-APC reduce mortality in bacteremia and LPS-induced endotoxemia but anticoagulant-selective Glu149Ala-APC fails to reduce mortality in these settings, indicating that the anticoagulant activity of APC that contributes to bleeding is dispensable for mortality reduction in sepsis.[66,94] Comparable results are obtained for APC protection in ischemic stroke and neurodegenerative disease.[93] In contrast, cytoprotective-selective 5A-APC fails to delay time to first occlusion in an acute carotid artery thrombosis model, whereas anticoagulant-selective Glu149Ala-APC effectively delays time to first occlusion as anticipated.[66] Interestingly, and to contrast expectations, anticoagulant-selective Glu149Ala-APC but not cytoprotective-selective 5A-APC mitigates toxicity induced by lethal total body irradiation.[100] Thus, depending on insult or disease model some activities of APC mediate beneficial effects, whereas other APC activities are dispensable or even harmful.

Concluding remarks

A concerted effort by many has resulted in several important discoveries for the protein C pathway in the last decade. These important advances include novel insights into the

structure-function relation of APC with its multiple cofactors, receptors and substrates. The discovery of a novel cytoprotective protein C pathway that is independent of APC anticoagulant activities and conveys activities directly on cells through interactions with cellular receptors such as EPCR and PAR1 exemplifies another major advance in the last decade. The subsequent search for molecular mechanisms to explain these remarkable effects of APC on cells has provided some initial clues on the fundamental differences and contrasting functional effects between APC and thrombin-mediated initiation of PAR1-dependent cell signaling. Nevertheless, many unanswered questions still remain. The notion that PAR1 can initiate biased signaling with different and often opposite outcomes provides an intriguing challenge for ongoing and future basic and translational research on the protein C pathway and PAR1 structure-function. Biased signaling by PAR1 is especially relevant and helpful for interpretation of recent outcomes of large phase III clinical trials that evaluated the anti-thrombotic effects of PAR1 antagonists as these PAR1 antagonists were associated with increased bleeding, especially intracranial bleeding.[101] In this regard second generation PAR1 compounds that antagonize PAR1-dependent G protein-mediated signaling but not β-arrestin 2-dependent signaling may provide therapeutic relevant entities, especially since APC, although an anticoagulant, prevented bleeding in the brain associated with profibrinolytic therapy.[102]

Perhaps the most striking advancement in the last decade that impacts our current view of APC's multiple activities and the regulation thereof relates to the structure-function analysis of the protein C pathway. The notion that APC's anticoagulant activity requires the APC Gla-domain to bind phospholipids and the APC protease domain to interact with FVa and FVIIIa aided by protein S versus APC's cytoprotective activities that require the APC Gla-domain to bind to EPCR and the APC protease domain to interact with PAR1, led to new interrogations of the extended exosite on the protease domain of APC that is required for interactions with FVa and FVIIIa. Observations that the FVa exosite on APC is not required for APC interactions with PAR1 but instead that APC interaction with PAR1 requires a negatively charged region on the other side of the protease domain provided a way to separate APC anticoagulant activities from its cytoprotective activities. Pharmacological applications of these activity-selective APC mutants have allowed for unique insights into the relative contributions and requirements of anticoagulant versus cytoprotective activities of APC for its beneficial effects in numerous in vivo injury and disease models. In addition, activity-selective APC mutants allow for the exploration of new avenues in translation, preclinical and clinical research. For instance, the cytoprotective-selective APC 3K3A mutant has recently entered phase I clinical testing for applications in ischemic stroke.[103]

In summary, APC has multiple activities that require assembly of APC in macromolecular complexes supported by interactions of APC with cofactors and by exosite interactions on the protease domain of APC with its different substrates. These exosites interactions are overlapping or partially overlapping for some substrates, whereas for other substrates they are unique and non-overlapping. Although the novel advances of the last decade provide unique insights into how a single enzyme can mediate multiple biologically and therapeutically relevant activities, information on spatial orientation of the various ternary APC-cofactor-substrate complexes is limited and much remains unknown. Overall, the protein C pathway provides plentiful opportunities for basic research on the structure-

function and molecular mechanisms of its multiple activities, as well as exciting avenues for translational research with potential therapeutic applications in complex diseases, such as the treatment of thrombosis, ischemic stroke, inflammatory disease, atherosclerosis, and vascular disease.

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Abbreviations

APC	activated protein C
ApoER2	apolipoprotein E receptor 2
EPCR	endothelial protein C receptor
FVa	activated factor V
FVIIa	activated factor VIIa
FVIIIa	activated factor VIII
PAR	protease activated receptor
PS	protein S
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homolog gene family member A
S1P1	S1P receptor 1
SHBG	sex hormone-binding globulin domain
TAFI	thrombin Activatable fibrinolysis inhibitor
TRAP	thrombin receptor activating peptide
TSR	thrombin sensitive region

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Learning Goals

1. The protein C pathway provides multiple important functions to maintain a regulated balance between hemostasis and host defense systems.
2. APC's anticoagulant activities prevent thrombosis whereas APC's cytoprotective activities protect cells.
3. APC's different activities require assembly of different macromolecular complexes with different cofactors that can be targeted by mutagenesis to obtain activity-selective APC mutants.
4. Anticoagulant-selective and cytoprotective-selective APC mutants allow insights into the relative contributions of these APC activities to beneficial effects in various murine injury and disease models.

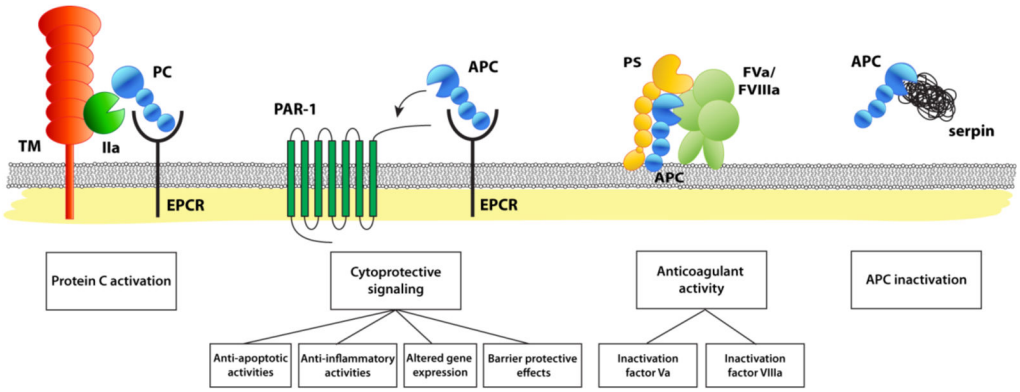

Figure 1. Reaction of the protein C pathway

Schematic representation of the protein C pathway reactions, from left to right: protein C activation, the cytoprotective protein C pathway, the anticoagulant protein C pathway and the inactivation of APC by serine protease inhibitors (SERPINs). *Protein C activation*: physiologic activation of protein C (PC) by the thrombin (IIa)-thrombomodulin (TM) complex occurs on the surface of endothelial cell membranes when protein C is bound to the endothelial receptor (EPCR). Since protein C and APC have a similar affinity for EPCR, after activation APC can remain bound to EPCR to initiate cytoprotective signaling. *The cytoprotective protein C pathway*: APC's direct effects of on endothelial cells require the cellular receptors EPCR and PAR1. These cellular activities of APC include anti-apoptotic and anti-inflammatory activities, alteration of gene expression profiles, and protection of endothelial barrier functions and are collectively referred to as APC's cytoprotective activities. *The anticoagulant protein C pathway:* APC anticoagulant activities involve proteolytic cleavages of FVa and FVIIIa. Different protein cofactors, such as protein S (PS), FV, and various lipid cofactors (e.g. phosphatidylserine, phosphatidylethanolamine cardiolipin, glucosylceramide, etc.), enhance the inactivation of FVa and FVIIIa by APC. *APC inactivation*: Inactivation of APC in plasma by serine protease inhibitors (SERPINs) is slow, which contributes to a remarkably long circulation half-life of APC (~ 20 min). Most important inhibitors of APC in plasma are protein C inhibitor (PCI), plasminogen activator inhibitor-1 (PAI-1), and α_1-antitrypsin and, to a lesser extent, α_2-macroglobulin and α_2-antiplasmin.

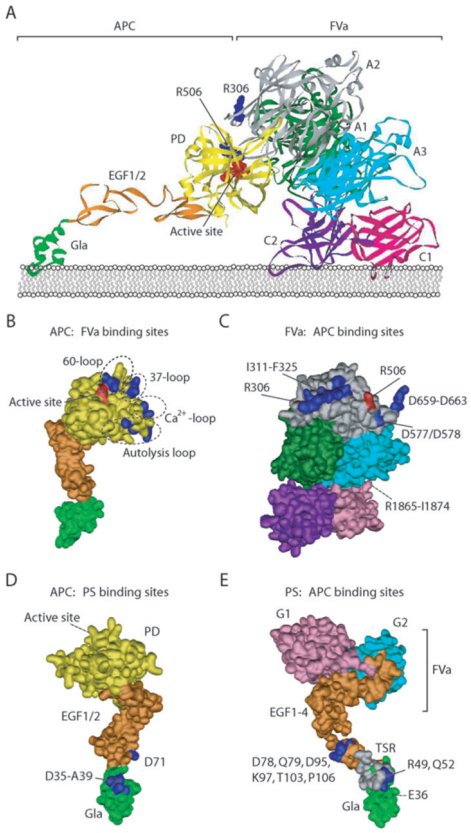

Figure 2. Schematic structural model of the anticoagulant protein C pathway

(**A**) Proposed model for the APC-mediated inactivation of FVa at Arg506 based on the interaction of an extended basic exosite on the protease domain (PD) of APC with negatively charged residues on the surface of FVa (adapted from Pellequer et al.[70]). In this model, the protease domain of APC (yellow) interacts with the A1 (green) and A2 (gray) domains of FVa. The interactions between APC and FVa position Arg506 (blue) of FVa in the active site pocket (red) of APC. The Gla-domain of APC (green) and the C1 (pink) and C2 (purple) domains of FVa interact with the phospholipid layer. Although biochemical data strongly support additional interactions between the APC Gla-domain and the FVa light chain (A3-C1-C2), especially residues within the A3 (light blue) sequence Arg1865-Ile1874, the extended projection of these domains in this model illustrate the need for a more flexible orientation of the APC protease domain to accommodate this (see text for additional details). (**B**). Schematic overview of the extended exosite (blue) on the surface of the APC protease domain, comprised of residues in loop 37, loop 60, the calcium-binding loop (loop70–80) and the autolysis loop that are required for interactions with FVa to accommodate cleavage at Arg506. (**C**) Schematic overview of the residues on the FVa surface (blue) that are involved in interactions of FVa with APC. Important residues are located on the A2 domain surrounding the Arg306 and Arg506 cleavage sites (red) and between the A1 and A3 domain at the back of the protein (not visible). (**D**) A schematic overview of APC residues implicated in the interaction of APC with protein S. Important residues (blue) in the Gla-domain (Asp35-Ala39) and the EGF1-domain (Asp71) are highlighted. (**E**) Schematic overview of the protein S residues (blue) implicated in the interaction of protein S with

APC. Important residues (blue) in the Gla-domain (Glu36), TSR (Arg49 and Gln52), and EGF1-domain (Asp78, Gln79, Asp95, Lys97, Thr103 and Pro106) are highlighted.

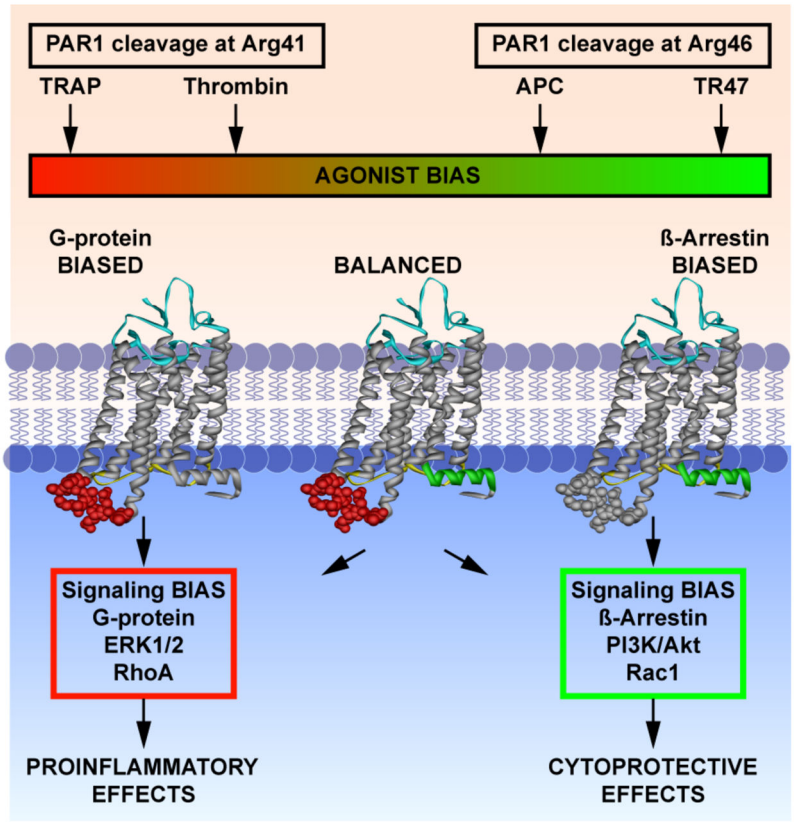

Figure 3. Induction of biased signaling by canonical and non-canonical activation of PAR1

Schematic representation of the fundamental and functional differences between APC and thrombin-mediated PAR1 activation. APC cleavage of PAR1 at Arg46 and the tethered-ligand sequence generated by this cleavage starting at Asn47 (TR47) induces a subset of PAR1 conformations that prefer signaling mediated by β-arrestin-2 involving activation of the PI3K-Akt pathway and that result in activation of barrier protective Rac1. In contrast, thrombin cleavage of PAR1 at Arg46 and the tethered-ligand sequence generated by this cleavage starting at Ser42 (TRAP) induces a subset of PAR1 conformations that prefer G protein-mediated signaling involving activation of the ERK1/2 and that result in activation of barrier disruptive RhoA. Thus, depending on the activating ligand, PAR1 can recruit different signaling pathways that result in different functional outcomes, which has been labeled “biased agonism.” The agonist bias is thus directly related to the cleavage sites of the tethered-ligand and the new N-terminal sequence as represented by the TRAP peptide that exists after cleavage at Arg41 or the TR47 peptide that exists after cleavage at Arg46. This figure was originally published in *Blood*.[89] L.O. Mosnier, R.K. Sinha, L. Burnier, E.A. Bouwens, J.H. Griffin. Biased agonism of protease-activated receptor 1 by activated protein C caused by noncanonical cleavage at Arg46. Blood 2012;120:5237-46. © the American Society of Hematology.

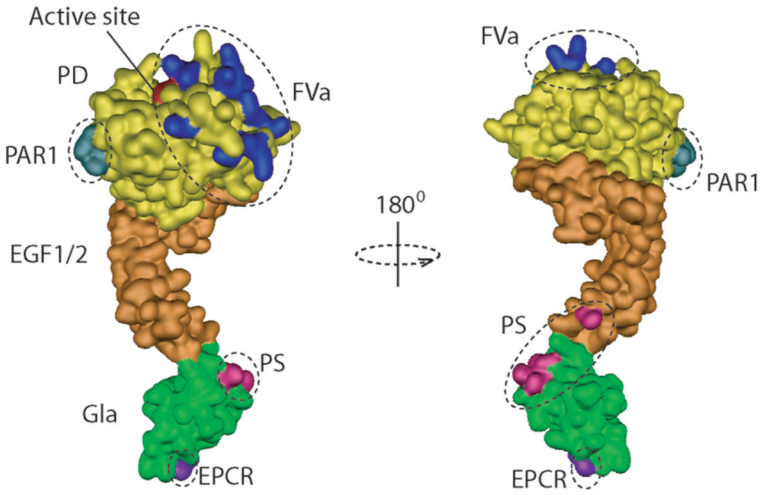

Figure 4. Schematic representation of the different structural requirements for APC's anticoagulant and cytoprotective activities

Anticoagulant activity of APC requires binding of the Gla-domain (green) to phospholipid surfaces, interaction with protein S (PS) mediated by residues on the Gla-domain and EGF1-domain (pink), and interactions of exosite residues (blue) on the APC protease domain (PD) with FVa. In contrast, cytoprotective activity of APC requires binding of the Gla-domain to EPCR (indicated by Leu8 (purple)), and interactions of a region on the opposite side of the FVa exosite on protease domain of APC that involves residues Glu330 and Glu333.