Activated protein C (APC) is the key effector protease of the natural anticoagulant protein C (PC) pathway. The first physical preparation of PC was accomplished by Dr. Johan Stenflo in Malmö in 1975 (1). He reported a new vitamin-K dependent protein that was present in a “trailing peak C” seen in elution profiles of prothrombin preparations, and hence was named PC. Dr. Stenflo brought this protein with him from Sweden to the laboratory of Dr. John Suttie in Madison, Wisconsin. There he worked with the postdoctoral fellow Charles Esmon to further characterize this protein as a phospholipid-binding zymogen. Esmon observed that it could be converted into its active serine protease form through limited proteolysis by trypsin, but not by thrombin or factor Xa (2).

In attempts to define the function of this new protein in the coagulation reaction or platelet aggregation, Drs. Kisiel, Ericsson, and Davie in Seattle were the first to note that trypsin-activated PC did not promote thrombin formation or platelet aggregation, but in fact exerted a noticeable anticoagulant effect (3).

This notion of APC as an anticoagulant was confirmed in studies that identified thrombin (employed at much higher concentrations than those used by the Wisconsin group earlier) as a potential activator of PC (4). This discrepancy regarding thrombin as the relevant activator of PC was eventually resolved by demonstrating the existence of a vascular receptor that greatly augmented the in vivo activation of PC by thrombin (5, 6). Later it was identified as thrombomodulin (TM) by Dr. Esmon’s group (7).

After the purification and initial characterization of PC as an anticoagulant protein observations made more than ten years earlier by Drs. Seegers, Marciniak, Murano and Heene could be explained:
- the existence of a prothrombin-derived inhibitor of coagulation that co-purified with preparations of bovine prothrombin (8, 9),
- the conversion by thrombin into a clot-retarding factor of great potency,
- the co-purification with other vitamin K-dependent clotting factors,
- a phospholipid- and Ca²⁺-dependent activity,
- inhibition of coagulation in a species-restricted manner by inhibiting the tenase-complex of factors VIII/IX and the prothrombinase-complex of factors V/X,
● a molecular weight of approx. 80,000,
● slow inhibition in plasma (10).

These observations were entirely consistent with the properties of the newly identified vitamin K-dependent protein termed PC.

While much of the early work on APC was focused on its role in the coagulation reaction, the revived interest in this protease is largely driven by the recognition that APC exerts pleiotropic cytoprotective and anti-inflammatory effects (11–19) on
● vascular endothelial cells,
● neuronal cells, and a variety of
● innate immune cell populations.

This dual effect of the protein C pathway on coagulation and inflammation also forms the basis of the use of APC in the treatment of severe sepsis. The discovery of APC as a potent and life-saving drug in lethal inflammatory disease was triggered by the early observations of Drs. Fletcher (20), Taylor and Hinshaw (21). Running blood through an extracorporeal circuit not only eliminated the need for anticoagulation, but protected dogs from the lethal consequences of endotoxin infusion even if the procedure was terminated before the actual endotoxin administration. Collaborative follow-up studies by Dr. Esmon et al. revealed that low-dose thrombin infusion had the same protective effect (22, 23) and APC was identified as the critical effector.

Eventually this led to the development of recombinant APC as a mortality-reducing treatment for severe sepsis (24, 25).

The majority of APC’s bioactivities, as well as the activation of PC, depend on the interaction of PC/APC with receptors (Fig. 1, Fig. 2) and cofactors that regulate its
● locale of formation,
● site of action,
● substrate selectivity, and
● overall physiologic effect in a given cell type.

This review emphasizes the role of cell surface receptors and substrates that have emerged as the key mediators of the cytoprotective and anti-inflammatory effects of therapeutic APC doses on the outcome of severe inflammatory disease and stroke, but also affect its anticoagulant capacity.

### Protein C

#### Biosynthesis and structure

Human PC is encoded by a single copy gene located on human chromosome 2. The gene spans approximately 14 kb and encompasses nine exons that give rise to an 1800 nucleotide mRNA. No prevalent alternate splice isoforms of PC mRNA, or circulating protein variants derived from alternate splicing have been reported in healthy individuals. The mRNA encodes a 461 amino acid (aa) preprotein containing a 42 aa leader peptide.

The major site of origin for plasma PC is the liver, with a potential contribution of endothelial cells. Post-translational removal of the leader sequence generates a 419 aa single chain precursor, the majority of which (~80%) is converted into a two-chain form by removal of an internal dipeptide.

- The 35 kD heavy chain contains the protease domain of PC, with a reactive center that includes the canonical, trypsin-like catalytic triade of Ser360, His211, and Asp257.
- The 21 kD light chain contains the N-terminal Gla-domain, and two EGF-hand domains.

Further processing includes β-hydroxylation of an aspartate residue in EGF-domain 1, several sites of N-glycosylation, as well as vitamin K-dependent γ-carboxylation of nine glutamic acid residues within the N-terminal Gla-domain. Circulating PC therefore presents predominantly as a disulfide-linked two-chain form with variable carbohydrate content. No functional differences have yet been described between the single- and two-chain forms.

In healthy individuals, the lower end of the range of PC plasma concentration is about 3 mg/ml, equivalent to approximately 60 nmol/l.

Variances in plasma levels of PC in individuals without diagnosed PC deficiency are in part determined by genetic factors. A recent genome scan identified several novel polymorphisms in the genes for PC itself, the PC receptor (Procr/EPCR), and four additional loci in unrelated genes (26). Remarkably, two of these single nucleotide polymorphisms (SNP) accounted for about 20% of the observed variance.

#### Receptor-mediated activation

The principal physiologic pathway of PC activation resembles an enzyme-substrate interaction in which the enzyme thrombin and the substrate PC are bound to
● membrane surface receptors,
● thrombomodulin (TM) and
● endothelial protein C receptor (EPCR).

These receptor interactions allosterically modulate the conformations of the enzyme as well as the substrate to enable a productive proteolytic reaction that releases a 12 aa activation peptide from the N-terminus of the PC heavy chain. As a result, the activation rate of PC by the thrombin-TM complex is approximately 1000-fold greater than the rate measured for a-thrombin in the absence of TM, and is further augmented ~10–20-fold if the PC zymogen is bound to EPCR.

The structural insights into this mechanism have been exploited to generate conformation-restrained mutants of PC (RR67/82CC and thrombin (R35E) that together yield calcium-independent rates of PC activation similar to that achieved by the thrombin-TM/EPCR complexes (27).

Because of the pronounced effect of the cell surface receptors TM and EPCR on the rate of APC formation, the expression patterns of TM and EPCR largely dictate the spatio-temporal regulation of APC’s biological effects. Interestingly, the Gla-domain of PC mediates binding (29–32) to
● membrane phospholipids and
● membrane-proximal regions of EPCR.

Mutations in the PC Gla-domain can selectively modulate these two interactions, and either abolish EPCR-interaction, or increase by more than an order of magnitude the affinity for phospholipids and thereby increase the apparent anticoagulant potency of APC (33–35).
Dissociation from EPCR releases APC into plasma, where it is inactivated with a relatively long half-life of approximately 20 min through interaction with plasma inhibitors of serine proteases (serpins) (36–41), including

- α1-proteinase inhibitor (α1-PI),
- plasminogen activator inhibitor 1 (PAI-1), and
- PC inhibitor (PCI), and
- some contribution of the non-serpin inhibitor α2-macroglobulin.

PCI-mediated inhibition of APC is accelerated by heparin (36), whereas the formation of the APC-PAI-1 complex is accelerated by the presence of vitronectin (42). PCI also is capable of inhibiting thrombin bound to TM, thereby suppressing APC formation (43). The discovery of PCI in brain lesions of patients with active multiple sclerosis prompted investigations of the potential efficacy of APC in mouse models of the disease (45). Administration of APC indeed ameliorated the multiple sclerosis-like pathologies, although there is little, if any PCI present in the plasma of mice (46). Capture of PAI-1 in a complex with APC enhances overall fibrinolytic activity, and therapeutic administration of high doses of APC is indeed associated with a stimulation of fibrinolysis.

A second mechanism by which APC may augment fibrinolysis is based on the anticoagulant effect of APC on thrombin formation, which would limit activation of the thrombin-activatable fibrinolysis inhibitor (TAFI) by the thrombin-TM complex (44).

An interesting modifier of APC generation and function is platelet factor 4 (PF4). This chemokine is stored in platelet α-granules and released upon platelet activation (47). Activation of PC by the complex of thrombin and fully glycosylated (chondroitin-sulfate containing) TM is stimulated about 20-fold by PF4 in vitro (48), and physiological levels of PF4 substantially augment APC generation in vivo (49, 50). This stimulating effect depends on the simultaneous interaction of PF4 with the chondroitin-sulfate side chain of TM and the Gla-domain of PC (51). In addition to enhancing APC formation, PF4 may suppress factor V degradation without affecting activation of protease-activated receptor (PAR)-1 by APC and thereby differentially modulates the anticoagulant activity of APC and its ability to activate PAR1-dependent cytoprotective responses (52). Concomitantly, the presence of PF4 shifts the balance between the two known substrates of the thrombin-TM complex, TAFI and PC, towards the activation of PC (53). At sites of ongoing platelet activation, the overall outcome of these effects would therefore result in the localized enhancement of APC formation, maintenance of a high fibrinolytic potential, preserved non-

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**Fig. 1** The structure of human APC as deduced from crystallographic data of Gla-domain less APC, available structure information on related Gla-domain proteins, and in silico modeling as described by Villoutreix et al. (J Comput Aided Mol Des 2001; 15: 13–27): The light chain (dark grey) comprises the Gla-domain and EGF domains 1 and 2. The heavy chain (light grey) forms the protease domain with the catalytic center triad Ser360-Asp257-His211 (red). The last residue of the light chained resolved in crystal 1D structures is E149 (green). Alanine substitution of this residue enhances APC’s anticoagulant activity, but diminishes it’s capacity to signal via the EPCR-PAR1 axis. Mutations of L8 or L38 (purple) in the Gla-domain abolish the interaction with EPCR or protein S, respectively. The RGD motif in the heavy chain (orange) binds β1 and β3 integrins. Alanine substitutions of positively charged lysine and arginine residues constituting a vWF interaction site (magenta) largely eliminate anticoagulant functions, without affecting the cytoprotective functions of APC. The same effect is obtained after constraining the structure of this exosite by introducing an artificial disulfide bond between R222 and D237 (dark blue). APC structure kindly provided by Drs. Laurant Mosnier and Alizar Rezaie.
Anticoagulant activity of APC

The anticoagulant function of APC is based on the limited proteolysis of coagulation factors V and VIII (FV, FVIII), two essential components of the tenase complex of FXa-FVa and the prothrombinase complex of FXa-FVa. Inactivation of FVa requires proteolysis at Arg506 and Arg306. Initial cleavage at Arg506 results in partial inactivation, and facilitates cleavage at the secondary Arg306 site, resulting in a complete loss of FVa procoagulant function. Cleavage of FV at Arg306, and at several other sites (54) is enhanced ~20-fold by the presence of the vitamin K-dependent and Gla-domain-containing cofactor protein S, whereas Arg506 proteolysis is not protein S-dependent.

The physiological relevance of FV cleavage at Arg506 is clearly evident from the thrombotic predisposition of individuals carrying a Arg506Q FV gene polymorphism (FV Leiden) that

- impedes APC cleavage and
- leads to a failure of exogenously added APC to prolong the clotting time in in vitro coagulation assays (APC-resistance).

Although polymorphisms of the Arg306 site have been documented in individuals, it remains unclear to what extent these mutations (i.e., EV Hongkong and EV Cambridge) are associated with thrombosis (55, 56).

FVIII and FV are structurally very similar proteins, and FVIII inactivation by APC occurs at the corresponding positions in FVIII (Arg336 and Arg562). Two features of FVIII inactivation differ from that of FVa:

- Cleavage of either site in FVIII results in a near complete loss of FVIII procoagulant function.
- FVIII proteolysis appears at least in vitro dependent on two co-factors, i.e., the non-enzymatic cofactor protein S and coagulation FV.

The enhancing effect of FV on protein S-dependent FVIII cleavage has been termed the anticoagulant cofactor function of FV, giving rise to the notion of FV being a bi-functional protein that can exert both pro- and anticoagulant functions (57, 58). A potential explanation for this dichotomy is that initial cleavage of intact pro-cofactor V by APC at Arg506 may generate the anticoagulant co-factor form of FV, whereas ac-
tivation by thrombin forms the procoagulant form of FVa. Consecutive cleavage at Arg306 would abrogate both activities.

One of the few unresolved questions regarding the anticoagulant function of APC is therefore whether the increased thrombosis risk caused by the FV Leiden polymorphism reflects predominantly a loss of the anticoagulant co-factor function of FV (59). There is only limited in vivo evidence for this hypothesis, derived mainly from the fact that “pseudohomozygotic” FV Leiden carriers (carrying one FV Leiden allele and one complete loss of function allele of normal FV) exhibit a similar thrombosis risk than homozygous FV Leiden carriers. A surprising, potentially procoagulant effect of APC has been described that involves the EPCR-dependent proteolysis of tissue factor pathway inhibitor (TFPI) (60). Addition of APC to TNFα-stimulated (to induce tissue factor expression) endothelial-like EAhy926 cells resulted in a dose-dependent increase of tissue factor-dependent FXa generation that was already evident at relatively low APC concentrations (~5 nmol/l). This stimulatory effect of APC was not due to increasing tissue factor expression, but rather was caused by the EPCR-dependent cleavage of TFPI between Kunitz domains 1, which binds to the tissue factor-FVIIa complex, and Kunitz domain 2, which arrests and inhibits the ternary tissue factor-FVIIa-FXa complex. Shedding of TFPI’s Kunitz domain 1 by APC thereby released the TFPI-mediated inhibition of the principal initiation complex of the extrinsic coagulation pathway and resulted in an apparent stimulation of tissue factor activity. The physiological impact of sustaining the activity of the tissue factor-FVIIa-FXa complex on cytokine-stimulated endothelium is unknown, but may involve regulation of the EPCR-dependent modulation of tissue factor signaling.

### Extracellular histones are a novel substrate for APC

Recent work linked the cytoprotective activities of APC to its ability to degrade extracellular histones (61, 62). Exposure of monocyte-like mouse RAW264.7 cells to interferon γ and LPS resulted in the release of a component into the culture medium that rendered it cytotoxic to human endothelial-like EA.hy926 cells. Pre-incubation of the medium with APC prior to adding it to EA.hy926 cultures eliminated this cytotoxic effect. Proteomic analysis identified the APC-sensitive toxic component as histone proteins released from dying cells. Studies in mice and baboons showed that:

- histones accumulated in the plasma of septic animals,
- APC therapy reduced the prevalence of intact histones in blood,
- histone infusion into non-septic animals reproduced some of the lethal pathologies seen in severe inflammation.

The causative role of histone release from damaged tissue in septic animals was ascertained by documenting the protective effect of histone-specific antibodies that blocked the cytotoxic effects of these mediators and improved the survival rate of sepsis. The rate of histone degradation by APC was somewhat enhanced by phospholipid vesicles containing phosphatidyethanolamine. The substrate specificity of APC for individual histones H1–4, the potential role of co-factors in this reaction, and the mechanism by which histones exert their cytotoxic effects on cells remains unknown at present.

The key finding of these studies was that histone release from damaged cells plays a major role in sepsis pathogenesis, especially in progressed disease, when substantial organ damage is imminent.

### EPCR

The search for a specific receptor for APC was prompted by the observation that APC bound in a calcium-dependent manner to endothelial cells with a much higher affinity (~30 nmol/l) than to TM (~1 mmol/l). Although binding required the Gla-domain, APC could not be displaced by homologous vitamin K-dependent proteins, suggesting that binding was not strictly phospholipid-dependent. Expression cloning resulted in the characterization of endothelial protein C receptor (EPCR) (65).

EPCR bears a striking structural similarity to the invariant CD1-type major histocompatibility class 1 molecule, and carries a lipid in the major antigen-presentation groove that is required for efficient binding of APC/PC (66, 67). The receptor binds PC and APC with equivalent affinity (68), and hence is not selective for APC.

Since the plasma concentration of PC is roughly within the range of the Kd for the EPCR-PC binding, the majority of EPCR should always be occupied by PC. As described above, the rate of activation of EPCR-bound PC by the thrombin–TM complex is about 20-fold enhanced over that of unbound, freely circulating PC. On cells co-expressing TM and EPCR, the EPCR-PC complex is therefore exquisitely sensitive to the presence of trace amounts of thrombin captured by TM. Following activation, a substantial fraction of APC remains associated with EPCR, resulting in the existence of an EPCR-bound reservoir of APC that accounted for at least 30% of the total APC generated (69). Elimination of this reservoir by endothelial cell-selective ablation of the EPCR gene abrogated this APC compartment and elicited a prothrombotic and pro-inflammatory state.

Remarkably, circulating plasma APC levels were minimally affected in these animals, suggesting that – at least in mice – the level of freely circulating APC may not be fully informative about its effect on coagulation or inflammation. These findings highlight that the strict spatio-temporal control of pro-coagulant reactions is mirrored in a highly localized generation of balancing anti-coagulant activity. It has been proposed that endocytotic clearance...
of APC through internalization via EPCR might affect the half-life and abundance of APC in the circulation (70), but – given the existence of a substantial, EPCR-bound reservoir of APC on the luminal vascular surface – may not make a large contribution under normal conditions.

In addition, EPCR functions as a receptor for coagulation factors VIIa (68, 71–73) and Xa (74). EPCR-FVIIa interactions may sequester FVIIa from procoagulant phosphatidyserine-rich membrane domains, thereby exerting a FVIIa-dependent anti-coagulant activity (73, 75), and modulate the bioavailability of therapeutically administered recombinant FVIIa (76).

The striking structural similarity with CD1 molecules raises the possibility that EPCR might serve as an as yet undescribed function in the regulation of innate immune cell functions. The major cell populations interacting with CD1-like molecules are natural killer T-cells (NKT) expressing an invariant T cell receptor. Furthermore, the capacity of EPCR to present a lipid in the antigen-presenting groove suggests that it could in theory undergo a similar intracellular lipid exchange as it occurs with cognate CD1 upon encounter of pathogens. In endotoxin-exposed mice, APC therapy was indeed associated with altered abundance and function of a subtype of NKT cells (77). It is unknown whether this observation involves an APC-mediated modulation of a direct interaction between NK cell receptors and EPCR expressed on antigen-presenting dendritic cells, or includes some other EPCR-independent effect of APC on these cells.

EPCR-dependent signaling through PAR

The intracellular signaling processes initiated by the engagement of the protease-activated receptors (PAR)-1, -2, and -4 require the proteolytic removal of a small peptide from the N-terminal, extracellular portion of each receptor. The newly formed N-terminus of the processed receptor functions as a “tethered ligand” that induces conformational changes leading to the activation of heterotrimeric G-proteins. Seminal work by Riewald and colleagues suggested that APC, when bound to EPCR, was capable of activating the prototypic thrombin receptor PAR-1 (78). Remarkably, activation of PAR-1 by thrombin and the EPCR-APC complex elicit qualitatively distinct responses, with APC-initiated signaling eliciting cytoprotective and anti-inflammatory responses in general.

A critical downstream mediator of the signal generated through the APC-EPCR-PAR-1 axis is the increased production of the bioactive lipid, sphingosine-1-phosphate, by sphingosin kinase (sphk-1). The differential effects of APC and thrombin-signaling through PAR-1 correlated with altered cross-talk between PAR-1 and S1P receptors 1 and 3. While the protective effects of APC on endothelial cell permeability were mediated through S1P receptor 1, the permeability-enhancing effects of thrombin required S1P receptor 3. This paradigm has been solidly confirmed in a number of in vitro and in vivo studies (15, 27). In neuronal cells, the protective effects of APC not only depend on EPCR and PAR-1, but required in addition PAR-3 (79–81). It remains to be clarified if APC proteolytically modifies or interacts with PAR-3 directly.

Several mechanism are currently under investigation how APC may modulate PAR-1 signaling (27), i.e., lipid raft reorganization, control of signal duration, “allosteric” modulation of PAR-association with intracellular signal transducers and/or receptors, and cleavage specificity of APC. Lipid rafts are defined as detergent-resistant domains of the extracellular sheath of the cell membrane with a high content in cholesterol and sphingolipids that serve as spatially defined platforms for the assembly of specific signaling complexes. In vitro studies have shown that EPCR and PAR1 colocalize in caveolin-1-rich rafts (caveolae). Disruption of raft structure by cholesterol depletion or caveolin-deficiency abolishes APC signaling through PAR-1, and binding of APC or PC to EPCR results in a reduced association of EPCR with caveolae; for review see (27). Thus, the ligand-induced partitioning of EPCR out of rafts thus appears to decouple PAR-1 from Gq and G12/13, instead resulting in a predominantly Gi-driven signal. Of note, occupancy of EPCR with PC zymogen also dissociated EPCR from PAR-1-containing rafts, and this was sufficient to convert the response of PAR-1 to thrombin or PAR-1 agonist peptides into an “APC-like” response. Given that the majority of EPCR is constitutively occupied by PC, this implies that under normal conditions the response of cells co-expressing EPCR and PAR1 is always directed to a protective pathway; see article by Shahzad and Issermann (110).

PAR-1 activated by the EPCR-APC complex is retained for a prolonged time on the cell surface and is associated with prolonged elevations of cytoplasmic Calcium-levels, whereas thrombin-activated PAR-1 is rapidly internalized and associated with short-lived Calcium spikes. In line with the above observations, this again suggests that PAR-1 activation via EPCR-APC occurs in a subpopulation of PAR-1 present in a specific subset of rafts that differ in the ability to sustain surface association of PAR-1. A recent study shows that regulation of PAR1 internalization not only affects signal duration, but also alters the coupling to different species of G-proteins (82).

The small population of already thrombin-cleaved PAR-1 that remains associated with the cell surface (and is likely present in a distinct membrane compartment) retains responsiveness to tethered-ligand peptides like SFLLNRN (83). While thrombin exclusively cleaves after the arginine 41 residue to expose the SFLLNRN tethered-ligand domain, the latter contains an additional arginine residue that could at least in theory be a substrate for APC. It is an open question, whether APC – possibly in an EPCR-dependent manner – can productively cleave at this site and activate PAR-1 or even re-activate already thrombin-cleaved PAR-1. In summary, the molecular events governing EPCR-dependent activation of PAR1 by APC are still incompletely understood.

It appears clear that the EPCR-mediated partitioning of PAR-1 into distinct raft-like membrane domains affects the duration of the PAR-1 mediated signal, the association of PAR-1 with specific intracellular signaling platforms, and hence the quality of the PAR-1 response. In particular, the requirement for PAR-3 in at least one paradigm further suggests that additional interactions must exist that govern the PAR response to APC, such as heterodimer
formation between PARs, and crosstalk with other G-protein coupled receptors.

Finally, it is also important to keep in mind that EPCR-dependent signaling through PARs is not unique to APC. Several independent studies have demonstrated that EPCR facilitates productive activation of PAR-1 and PAR-2 by FXa and FVIIa to elicit a variety of intracellular signaling processes (68, 74, 84, 85) and may control the balance between PAR activation by the ternary complex of tissue factor, FVIIa, and FXa, (EPCR-dependent) or the binary TF-FVIIa complex (EPCR-independent) (86).

Mac-1 (αMβ2-integrin) dependent signaling through PAR-1

In murine macrophages derived from in vitro culture of bone marrow cells, APC suppressed LPS-induced IL-6 secretion and the transcription of several inflammatory response genes (STAT3, NFkB, IL12, NOS2A, wnt5a, TRAIL) (87). These anti-inflammatory effects of APC were robustly reproduced by an APC variant that lacked the Gla-domain, which is necessary for APC’s phospholipid-dependent anticoagulant function, as well as for binding to EPCR. Accordingly, the anti-inflammatory effects of normal APC were preserved in cells lacking EPCR, but instead required the αMβ2 integrin Mac1 (CD11b/CD18) and proteolytic activation of the PAR-1 receptor. Inhibition of β1- or β3-integrins had no effect, setting the involved mechanism apart from that described for APC’s effects on endothelial-leukocyte interactions (88).

Similar to the paradigm established for APC-signaling via the EPCR-PAR-1 axis in endothelial cells, proteolytic activation of PAR-1 by APC correlated with a co-localization of PAR-1 and Mac-1 in cholesterol-rich membrane domains and produced an increase in intracellular production of sphingosin-1-phosphate (SIP). The anti-inflammatory effects elicited by APC in wild-type mouse monocytes, which were strictly dependent on expression of Mac1, could be reproduced by administration of synthetic SIP-receptor agonists in both wild type- and integrin-deficient cells, providing convincing evidence that PAR1-initiated SIP-production is a down-stream effector mechanism triggered by the Mac-1-APC interaction.

The inhibitory effect on TRAIL and wnt5a transcription, although somewhat modest, suggests that these earlier described, EPCR-independent effects of APC, may also be attributed to a Mac-1-dependent mechanism (89, 90). Whether the RGD-sequence in human APC, and the conserved RGQ motif in mice are required and sufficient for a productive integrin-APC interaction was not tested in these studies, but appears a likely candidate mechanism. A clinically important aspect of these observations is that they could potentially explain the failure of therapeutic approaches targeting CD11b/CD18 function in inflammatory diseases like asthma, inflammatory bowel disease, rheumatoid arthritis, and multiple sclerosis (91), because they would likely also suppress beneficial effects of endogenous APC, mediated through Mac-1.

A different, indirect interaction between APC/PC and Mac1 is mediated by binding of soluble forms of EPCR to proteinase 3 (PR3) on the surface of activated neutrophils (92). Under conditions of inflammation, the soluble form of EPCR is mostly generated by metalloprotease-dependent shedding of membrane-associated EPCR (93,94), with a potential contribution of alternate mRNA splicing (95). PR3 is an abundant neutrophil granule protein that is released upon activation, and then associates with Mac-1 on the neutrophil surface. Soluble EPCR-APC and EPCR-PC complexes both bind to Mac-1 associated PR3, indicating that this particular sequestration of APC/PC on Mac-1 likely occurs through a different mechanism as opposed to the direct binding described on monocytes (87). The involvement of these interactions in the modulation of in vivo neutrophil migration/recruitment by APC is unclear.

Signaling through apolipoprotein E receptor 2

A 1985 report (96) described the binding of purified APC to washed bovine platelets. Specific binding to unstimulated platelets only occurred in the presence of protein S. Binding to thrombin-stimulated platelets did not require addition of protein S, but could be blocked with an antibody raised against protein S. Of note, binding could not be competed for with an excess of PC zymogen, and APC did not elicit a platelet shape change or serotonin release typically
ApoER2 and GP1bα have been implicated as a receptor pair for the complex between pathogenic anti-antibodies and their cognate antigen, β2-glycoprotein 1 (β2GPI) that occur in patients with antiphospholipid syndrome. In a subset of patients, the occurrence of auto-antibodies seems associated with non-Leiden APC resistance and it is possible that this prothrombotic effect might be mechanistically related to the interactions of PC/APC with these receptors on platelets (98–102). Finally, apoER2 and GP1bα both have been reported to mediate the association of coagulation factor XI with platelets (103), which would position this intrinsic factor to facilitate the activation of the intrinsic pathway of coagulation by platelet-released polyphosphates (104). It is formally possibly that APC/PC may competitively inhibit this interaction.

The receptor-ligand interaction between apoER2 and APC is not limited to platelets, but may also play a role in the function of innate immune cells. Experiments performed on human U937 monocytic cancer cells showed that APC ligation of apoER2 triggered the typical intracellular signaling cascade associated with the Reelin pathway operating in neuronal cells (105). Activation of the reelin pathway did not require an interaction of APC with EPCR or PAR-1. In contrast, static adhesion of U937 cells to immobilized APC was inhibited by EPCR-blocking antibodies, consistent with a cooperative effect of apoER2 and EPCR in this process. In addition to its function in static adhesion, EPCR also is known to be important for the ability of APC to inhibit the LPS-induced pro-coagulant tissue factor activity of U937 cells (106). This anticoagulant and EPCR-dependent activity of APC also required the function of a RAP1 sensitive co-receptor, presumably again apoER2 (105). The in vivo role of APC/PC binding to apoER2 is unknown.

**Does an APC-selective receptor exist?**

Two early studies (107, 108) investigating the effects of APC on human monocytic cells provided seminal insights into the potential role of APC as a modifier of the innate immune response to severe inflammation and infection. APC was found to inhibit the release of TNFα following stimulation of monocytes with LPS, interferon γ, or phorbol ester, prevent the down-modulation of the β2-integrin Mac-1 and of the CD14 receptor for LPS. The inflammation-triggered production of reactive oxygen intermediates and expression of inflammatory cell surface receptors (ICAM-1, CD59, MHC class II/HLA-DR, II2-receptor) were not affected by APC. These data were the first to indicate that APC is neither a generalized suppressor of inflammatory monocyte responses, nor a selective inhibitor of LPS-triggered responses, but rather appears to suppress specific cytokine responses (i.e. TNFα and IL-6), while maintaining functions critical for an effective host response to bacterial infection.

A second interesting observation was that these activities required an intact proteolytic activity since neither the zymogen PC, nor inactive APC, in which the active site was blocked with disopropyl-fluorophosphate (DFP), elicited these responses. A follow-up study showed that an intact proteolytic center was in fact necessary for the physical binding of APC to a monocyte receptor that was likely distinct from EPCR. The bound APC could not be displaced by PC zymogen, DFP-APC, or PPACK-APC in which the active center is blocked by an even smaller molecule, and was not affected by other APC interacting molecules, such as FVa, TM, or protein S. This was remarkable, as it suggested that this as yet still unidentified receptor could distinguish not only between APC and the PC zymogen, but could in addition discern APC with an intact catalytic center from catalytically inactive or blocked APC. The subsequent identification of EPCR, PAR-1, β1/β3 integrins, Mac-1, and apoER2 as receptors for APC raises the question whether one of these candidates fits the profile of the APC-selective receptor described in the studies of Grey and Hancock (107, 108), or whether there is still room for discovery.

EPCR does not discriminate between APC and PC zymogen, and thus clearly is not an APC-selective receptor. APC-inte-
grin interactions are an interesting candidate for a specific receptor, since only APC, but not PC bound these integrins (88). This is likely because removal of the activation peptide in the PC heavy chain may be necessary to make the integrin-binding RGD-like motif of APC accessible for productive interaction. However, it appears to differ from the putative APC-specific receptor described on monocytes (108) because a proteolytically inactive form such as S360A-APC still could engage neutrophil β1- and β3-integrins, whereas this variant did not bind to human monocytes. ApoER2 was responsible for the adhesion of U937 cells and platelets to immobilized APC or PC, and the static adhesion of platelets could also be inhibited by an excess of soluble PC (97) indicating that binding per se was not APC-selective. Whether PC can similarly compete with APC for productive interaction with apoER2 in U937 cells (105) has not been established.

The remaining candidate is the β2-integrin Mac-1. Physical binding of APC or Gla-domain deleted APC to macrophages was completely absent in cells lacking Mac-1 (87), providing indirect evidence that Mac-1 might be identical to the APC-specific receptor described earlier in human monocytes (108). However, given that RGD-mediated APC’s interactions with β- and β3-integrins did not require intact proteolytic activity, it remains to be resolved through direct binding and competition studies whether Mac-1 is indeed identical to the APC-specific receptor detected in the early studies.

Conclusions

The discoveries of receptors for APC that transmit the anti-inflammatory and cytotoxic effects of this protease have opened new avenues of investigation into the physiological role of APC and the therapeutic use of recombinant APC. While the anticoagulant function of the protein C pathway is fairly well understood, the physiologic role of the various receptor interactions is still far from clear. A large number of recombinant mutants of APC have been generated that may be used to dissect the relative importance of the different mechanisms involved in APC interactions with FV, protein S, EPCR, and integrins (109). Similar mutational approaches will likely be forthcoming to generate variants that are selectively defective in their ability to degrade histones, activate PARs, or interact with apoER2.

Such APC variants constitute a growing arsenal for identifying the critical interactions, downstream pathways, and cellular targets on which the beneficial effects of therapeutic APC in various diseases are based.

Conflict of interest

The author declares that he has no conflict of interest.

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