[Frontiers in Bioscience 6, d807-819, July 1, 2001]

GENE TARGETING IN HEMOSTASIS: PROTEIN C

Francis J. Castellino

W.M. Keck Center for Transgene Research and the Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556

TABLE OF CONTENTS

1. ABSTRACT

Protein C (PC) is the zymogen form of a serine protease, activated protein C (APC), a naturally occurring anticoagulant. In control of the coagulation of blood, APC functions by attenuating thrombin formation. It serves this role through inactivation, by limited proteolysis, of two important cofactors for overall clot formation, one of which, Factor Va (FVa), stimulates prothrombin activation, and another, Factor VIIIa (FVIIIa), enhances activation of coagulation Factor X (FX). In maintaining the fluidity of blood, APC also indirectly functions in fibrinolysis, in one manner by directly inactivating an inhibitor of plasminogen activation, plasminogen activation inhibitor-1 (PAI-1), and in another manner *via* its role in attenuating thrombin production, with the resulting effect of limiting production of another thrombin-dependent fibrinolytic inhibitor, thrombin activatable fibrinolysis inhibitor (TAFI). PC, and other components of the PC anticoagulant pathway, *e.g.*, protein S (PS), thrombomodulin (Tm), and endothelial cell protein C receptor (EPCR), also can serve as anti-inflammatory mediators, through a number of different thrombin-dependent and thrombin-independent mechanisms. A large number of symptomatic and asymptomatic mutations occur in PC in humans, which express a variety of phenotypes. Generation and characterization of a murine model of a total *PC* gene inactivation has demonstrated that while an untreated total PC deficiency results in neonatal death through DIC-related abnormalities, a valuable resource is now available to study phenotypes of less severe deficiencies of this protein. Such studies will lead to advances in an understanding of the relative role of this protein system in the various pathways in which it has an influence.

2. INTRODUCTION

2.1. Structure of Protein C

Murine PC is translated as a single chain zymogen comprised of 458 amino acids (Figure 1), including a 41-residue signal/propeptide sequence. The mature form of the protein thus contains 417 amino acids. Similarly, the mature form of human PC contains 419 amino acids (Figure 2). Approximately 85-90% of the human protein is found in plasma as a two-chain form consisting of a 155-residue light and 250-residue heavy chains, linked by a single disulfide bond at Cys^{141} and Cys^{277}. In this process, a propeptidase catalyzes cleavage of the protein at residues 156 and 157, resulting in the loss of the Lys-Arg dipeptide. A number of other post-translational modifications are also required to produce the mature zymogen, such as Asn-linked glycosylation at sequence positions 97 (absent in mouse PC), 248, 313(incompletely glycosylated in human PC), and 329, γ-

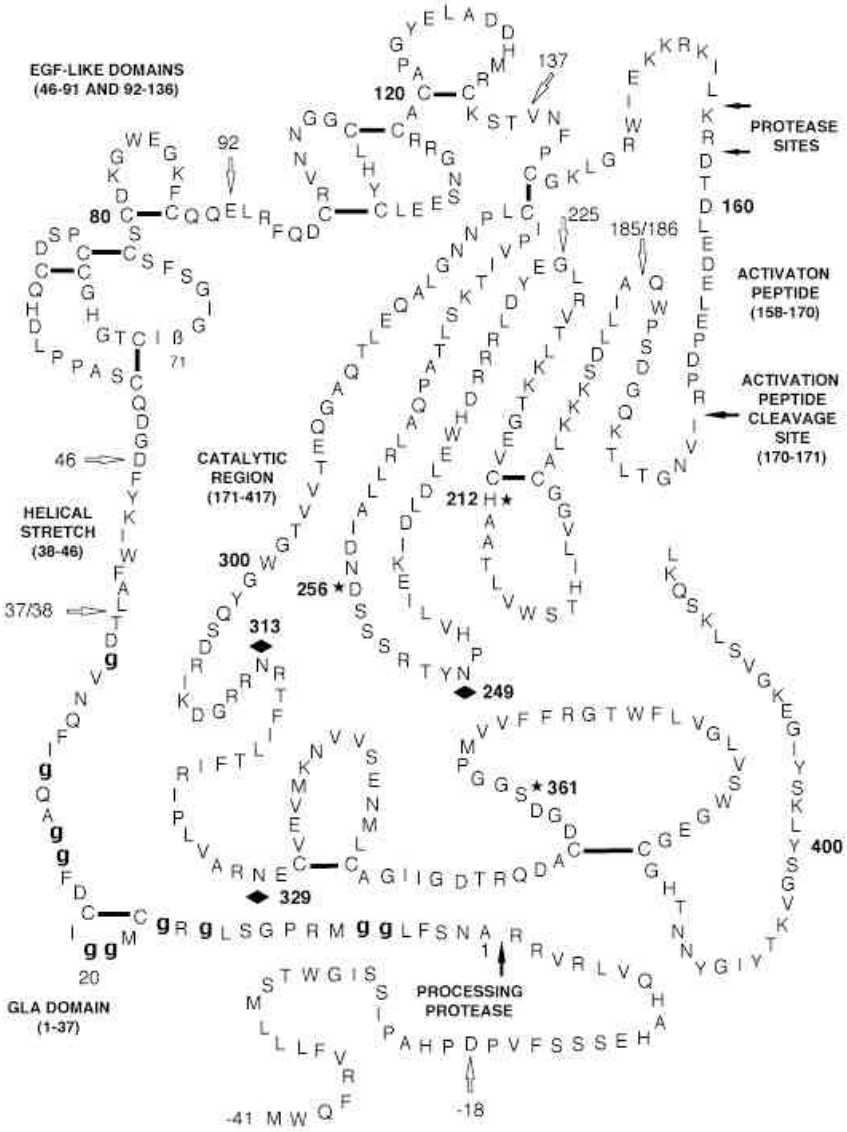

Figure 1. The amino acid sequence of murine PC. A 41 amino acid leader sequence is shown with an intron at position –18. The amino terminal amino acid of the mature protein is Ala, at position +1. The amino terminal γ-carboxyglutamate (Gla) domain (Gla residues are indicated by γ), residues 1-37, is followed consecutively by a short helical stretch (residues 38-46), two epidermal growth factor homology domains (residues 47-92 and 93-137, respectively), and the activation peptide domain (residues 138-185). The $Lys^{156}Arg^{157}$ basic dipeptide that is processed out of the mature protein, which leads to the existence of two-chain PC in plasma, is also present in this domain. The serine protease region begins at residue Ile^{171} and contains the residues of the canonical catalytic triad (His^{212}, Asp^{256}, and Ser^{361}). The positions of introns in the gene, which we have used for convenience in defining the limits of the protein domains, are shown by clear arrows. Sites of proteolytic processing are indicated by filled arrows. Filled diamonds illustrate the sites of N-linked glycosylation consensus sequences.

carboxylation at Glu residues 6, 7, 14, 16, 19, 20, 25, 26, 29, in human PC, and these, along with an additional site, Glu^{35} in mouse PC. Further, ß-hydroxylation occurs at Asp^{71}. With regard to human PC, these processing events occur in an orderly fashion, with γ-carboxylation and core glycosylation occurring in the early endoplasmic reticulum. Endoproteolytic release of the $Lys^{156}Arg^{157}$ dipeptide then takes place late in processing in the Golgi apparatus, as does complex oligosaccharide processing (1).

Characteristic of many of the vitamin K-dependent coagulation proteins, PC consists of distinguishable structural elements, such as an amino-terminal Gla domain, followed sequentially by two epidermal growth factor (EGF)-like regions, an activation peptide region, and a serine protease domain (Figures 1 and

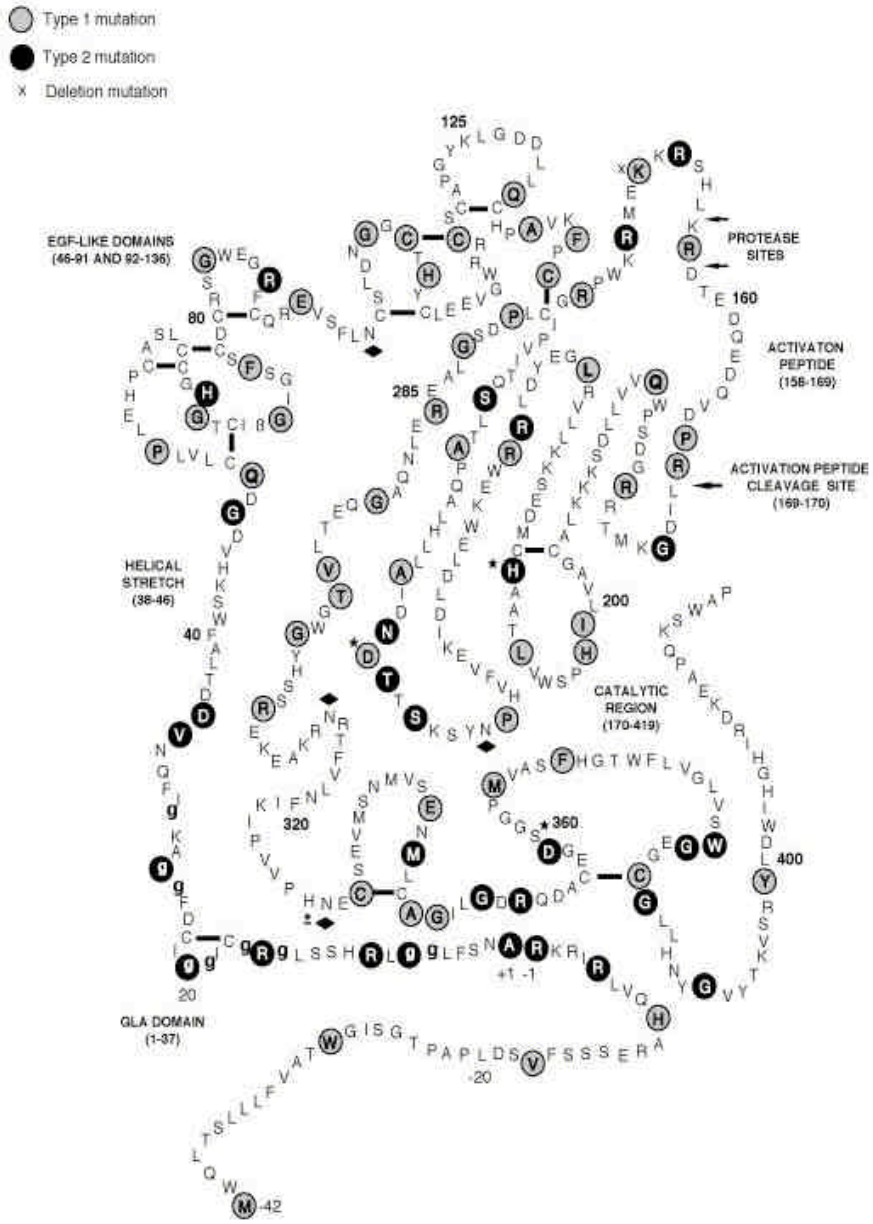

Figure 2. The amino acid sequence of human PC emphasizing the large number of sequence positions in which type 1 (gray circles) and type 2 (black circles) alterations occur in both symptomatic and asymptomatic individuals. All other symbols and designations are as in Figure 1.

2). The ß-hydroxyaspartic acid residue is located within the first EGF module.

2.2. Function of Protein C

PC is a component of the natural anticoagulant system and serves to maintain the fluidity of blood. As a result, it functions in the clot forming and clot dissolving pathways. In order to serve as an anticoagulant, the zymogen, PC, is converted to the serine protease activated Protein C (APC) by thrombin alone, or by the thrombin-thrombomodulin (TM) complex on the endothelial cell surface ((reviewed in (2)). On a molecular basis, this activation step is a consequence of cleavage and release of the amino terminal peptide from the heavy chain of two-chain PC (residues 158-170 in human PC and residues 158-169 in murine PC). The resultant APC, together with Protein S (PS), functions to inactivate by limited proteolysis the coagulation cofactors, Factor (F) V/Va (3) and FVIII/VIIIa (4). In addition, APC serves an indirect profibrinolytic role via its ability to attenuate thrombin formation through its action on FV/Va and FVIII/VIIIa. The resulting diminished thrombin production would thus

lead to lack of production of a thrombin-dependent fibrinolytic inhibitor, *viz.*, a plasma carboxypeptidase B-like enzyme, thrombin-activatable fibrinolytic inhibitor (TAFI). In this manner, PC indirectly stimulates fibrinolysis (5). Further involved in PC and APC function is a transmembrane I-type receptor, EPCR (6). This protein binds both PC (and APC) with high affinity and stimulates its activation by thrombin-TM (7). The highest expression of EPCR is on the endothelium of large vessels, such as the aorta (8), in contrast to TM, which is similarly expressed on small and large vessels. Small amounts of a soluble form of EPCR are present in plasma (9), which inhibits APC activity (10), perhaps providing a mechanism for negative regulation of APC activity in plasma.

In addition to their roles in anticoagulation and fibrinolysis, various components of the PC system have also been implicated in inflammatory diseases, such as the host-defense response to *Escherichia coli*-induced sepsis (11-13), perhaps *via* effects on regulation of thrombin levels, since uncontrolled coagulation results in thrombosis, as well as promotion of cellular proliferation and an inflammatory response. Additionally, certain cytokines, such as tumor necrosis factor (TNF), downregulate TM production on the endothelium, and upregulate tissue factor (TF) production, thereby leading to a thombin-rich environment, further implicating the PC system in the inflammatory response.

2.3. The Protein C Gene

The primary site of PC synthesis is the liver, although significant levels of the mRNA are present kidney (35% of liver) and testis (22% of liver). Both the mRNA and protein were shown to be present in tubular epithelial cells in the renal cortex and in spermatogenic cells in the testis. Lower levels of PC mRNA were present in the epididymis, brain, and lung (14).

The human PC gene, located on chromosome 2p13-14, is comprised of 9 exons and 8 introns, an arrangement that is similar to other vitamin K-dependent coagulation factors, *viz.*, FVII, FIX, FX, FII, PS, and PZ. One difference between PC and these other like genes is the insertion in both the human (15,16) and murine (17) PC genes, as well as the PSα gene (18), of a noncoding exon 1, in which is present the transcriptional start site. This results in a separation of the transcriptional initiation sequence from the translational start codon, which is present on exon 2.

The entire murine PC gene (15.2 kb) has been sequenced, from 414 bp upstream of exon 1 through 80 bp downstream of the translation stop codon (17). This sequence information allowed localization of a number of functional sites. The major transcriptional start site was found 1642 bp upstream of the translation initiation site, with several other minor start sites in that region. This multiplicity of start sites is a usual feature of TATA-less promoters (19), of which PC, FVII (20,21), and FX (22,23) are examples. Other RNA polymerase II promoter regulatory sites seem to be present in the 5' region of the murine PC gene, including two likely CCAAT sites and two probable GC boxes, all within optimal distances from the transcription Cap signal. The GC boxes appear to be needed for transcription initiation of TATAA-less promoters (24). Appropriate transcription termination elements are present in the 3' region of the murine PC gene, including a polyadenylation signal and a downstream sequence that is of assistance in efficient 3' termination of mRNA (17).

Consensus sequences for a number of potential important transcription factor binding sites are present in the 5' promoter region of murine PC (17), upstream of intron 1, including C/EBPß, AP1, AP2, P4, NF1, and GC sites. Intron 1 also contains some high probability consensus sequences for transcription factor binding (17), including potential loci for C/EBPα, C/EBPß, HNF1, HNF3ß, HNF4, HFH1, HFH2, and a nucleotide sequence also found to govern liver specific transcription of the α_2-antiplasmin gene.

In vitro promoter analysis shows that the region between nucleotides -418 and +45 possesses determinants for tissue specificity. The nucleotide stretch between -88 to +45 was sufficient for basal promoter activity of the human gene. Within this minimal TATA-less promoter region, *in vitro* studies show that human PC expression is largely dependent upon liver-specific regions available for binding the transcription factors HNF1 and HNF3, along with the more general factor, NF-1. These factors function synergistically for maximal activation. In addition, an SP1 binding site has also been identified in the promoter region of PC (16,25,26). Natural heterozygous mutations have been identified that disrupt the HNF-1, HNF-3, and NF-1 binding sites, and correspondingly diminish the promoter activity (25). Upstream of this basal promoter area, a strong silencer element exists between nucleotides -162 and -82 and two possible liver-specific enhancer regions, which interact in concert with the proximal promoter elements, were also found between nucleotides -1462 and -162 (16). The murine PC gene has been completely sequenced (17). It contains 15 kb organized in the same manner as the human gene.

2.4. Human PC Deficiencies

2.4.1. Single PC Deficiencies

A large number of PC deficiencies that lead to type I (a parallel reduction in PC antigen and PC activity) and type II (reduction in PC activity with up to normal levels of PC antigen) abnormalities have been described, some as compound deficiencies. The most recent updated database of mutations is available (27) and many of the locations of those observed are summarized in Figure 2. Mutations have been found in virtually all domain regions of the human PC molecule and many of these are illustrated in Figure 2. Among some notable mutations are those in the propeptide region, *viz.*, Arg^{-1}, Arg^{-5}, which affect propeptide processing of the protein (28-30); Gla^{7}, Gla^{20}, and Gla^{26} of the γ-carboxyglutamate domain, which influence binding of Ca^{2+} and phospholipid to the protein (and subsequent conformation and activation) (31-34), as well as an Arg^{15} mutation in this same domain, which results in an inability of PC to adopt a Ca^{2+}-dependent

conformation (33). A $His^{66}Asn$ mutation in EGF1 results in an additional potential N-linked glycosylation site and an Arg^{157} mutation at the start of the activation peptide causes a loss of ability for proper processing to the two-chain PC zymogen. An Arg^{169} mutation affects activation peptide cleavage. Mutations in two of the residues of the serine protease catalytic triad, *viz.*, His^{211} and Asp^{255} substantially attenuate protease activity of APC. A mutation at Arg^{229} results in impaired activation of PC by the thrombin/Tm complex. A $Ser^{252}Asn$ mutation creates a potential new glycosylation site. An Asp^{359} mutation disrupts the interaction of this residue with the heavy chain amino terminal Leu^{170} and, as a consequence, impairs the serine protease activity of APC (35).

The prevalence of an asymptomatic PC deficiency is as high as 1/500 in the overall population (36), with approximately 10-fold higher prevalence in patients with thrombotic episodes. A heterozygous deficiency of PC carries a risk of venous thromboembolism and one of these defects is present in as many as 5% of patients presenting with venous thrombosis. However, whether PC deficiency is a significant risk factor for arterial thrombosis is less certain. Homozygous PC deficiencies are extremely rare (1/200,000-1/400,000) and cause fatal coagulopathies, including DIC, as well as *purpura fulminans* and skin necrosis.

A type of PC deficiency results from a genetic defect in one of the physiological substrates of APC, FV. Here, heterozygotes or homozygotes for a point mutation in the FV gene result in an abnormal protein FV-Leiden containing an $Arg^{506}Gln$ mutation. This is a functional prothrombotic cofactor form of FV, but one that is not efficiently inactivated by APC since this mutation occurs at one of the cleavage sites on FV for APC. Thus, this mutation results in a thrombotic phenotype (37). This mutation has been found to be one of the highest genetic risk factors for thrombosis, and reaches approximately 20% of all symptomatic PC deficiencies (38). Heterozygotes for the FV-Leiden mutation are 5-10-fold at increased risk for thrombosis. Homozygotes have a 50- 100-fold increased risk of venous thrombosis (39).

2.4.2. Compound PC Deficiencies

A PC deficiency is now well recognized as one of the hereditary thrombophilias, and this condition has been known to occur in patients in coexistence with other acquired and/or congenital deficiencies of coagulation factors. Of course, compound deficiencies of PC with other vitamin K-dependent coagulation factors are observed in generalized cases of vitamin K deficiencies. For example, in one reported case defective *gamma*-carboxylation machinery inside the hepatocyte led to a multiple deficiency of prothrombin, as well as FVII, FIX, FX, and PC, in a 15 month old female. This individual presented with numerous hemorrhagic events, with multiple bruises and hematomas (40). Similarly, two families with a hereditary defect in the vitamin K-2,3-epoxide reductase complex produced offspring with perinatal intracerebral haemorrhage resulting from bnormal levels of all vitamin K-dependent coagulation factors (41).

There exist more specific examples of patients with selective multiple deficiencies involving PC. A case report describing presentation of ischemic necrosis in a toe revealed a patient with congenital afibrinogenemia, combined with a heterozygous Type 1-PC deficiency (42). In another case, an acquired compound deficiency of these two proteins was reported resulting from orthotopic liver transplantation. Here, transmission of a heterozygous PC deficiency, combined with dysfibrinogenemia, resulted from transplantation with an affected donor liver, leading to severe thromboses in the recipient (43). Additionally, an inherited combined dysfibrinogenemia and PC deficiency was found in a 48-year old male who suffered from severe atherosclerosis of the aorta and lower limb arteries (44).

Cases of a combined heterozygous deficiency of two anticoagulants, PC (52% activity) and antithrombin-III (38% activity), led to arterial occlusion, a less frequent complication in the case of either single deficiency. The more common presentation, venous thrombosis also occurred, and subsequent amputation of a limb resulted (45). In another case, thromboses of venous cerebral sinuses were found (46). A combined PC deficiency with another anticoagulant, heparin cofactor II (HCII), has also been described (47).

An inherited combined PC/PS deficiency has also been reported (48-50), presenting, in various cases, with recurrent venous thrombosis and ischemic cerebral stroke. The affected family showed three afflicted members over three generations. All were characterized by low PC antigen and activity and normal PS antigen, with a dysfunctional protein (49). Another case of this same combined deficiency state revealed a fatal case of mesenteric arterial thrombosis in a 46 year-old woman (51). This latter patient possessed a type 2 PC deficiency and a type 1 PS defect.

A combined PC/FV-Leiden deficiency, characterized by spontaneous venous thromboembolism, has been reported by several groups of investigators (38,52-54). It was found that the prevalence of the FV-Leiden syndrome occurred in approximately 19% of symptomatic PC-deficient probands. These double-deficient patients presented with more than twice the thrombotic episodes than those with either single deficiency (38).

Recurrent restenosis after coronary angioplasty occurred in a patient with PC deficiency, combined with high FVIIa levels (55). Further, congenital PC deficiency with elevated levels of FVII in a 29-year old male led to myocardial infarction, suggesting that low PC levels enhanced the procoagulant activity of FVII, a situation that may play a role in the development of arterial thrombosis (56). On the other hand, a combined FVII (50% activity and antigen) and PC (50% activity and antigen) deficiency was discovered in a 21 year-old female with peripheral pulmonary artery stenosis, also accompanied by dyspnea, progressive pulmonary hypertension, and hemoptysis (57).

Homozygous type 1 FIX deficiency (1% antigen and activity), combined with a type 1 PC deficiency (27%

activity and antigen), was found in a 1-year old male (58). While the hemophilic trait was evident because of the low FIX levels, plasma assays showed that the coagulation system was activated likely due to stimulation of the extrinsic coagulation system by low PC levels. For this same reason, thrombogenicity was further enhanced upon treatment of the patient with FIX concentrates for the hemophiliac condition.

A type 1 deficiency of PC, with combined hyperhomocysteinemia, was found in a 16-year-old female. She developed a sinus sagittalis thrombosis. In addition, her father, with this same combined defect, experienced a transient ischemic attack (59). Both sisters possessed only the PC deficiency, and did not display clinical symptoms. While it is known that both of these factors present independent risks for thromboembolism, these results suggest that together they act to significantly enhance thrombogenesis.

Regarding combined deficiencies with components of the fibrinolytic system, a family with a congenital homozygous PC deficiency, combined with heterozygous dysplasminogenemia, was found in a 21-year old male presenting with recurrent thrombosis (60). This patient avoided the thrombotic problems frequently noted with PC defects during the neonatal period, likely due to the presence of a low PC activity.

Triple combined deficiencies of PC have also been reported. A case of a heterozygous type 1 PC deficiency (ca., 45% activity) has been studied, combined with a type 2 deficiency of ATIII (ca., 50% activity) and a type 1 deficiency of HCII (ca., 47% activity). While this patient was asymptomatic (61), he ultimately presented with spontaneous pulmonary embolism (62). In another case, venous thrombosis occurred in a PC-deficient patient, combined with type III von Willebrand's disease and a simultaneous ATIII deficiency (63). In a related situation, a deficiency of PC (10-28%), along with the presence of the thrombotic FV-Leiden and prothrombin G20210A mutations, led to mesenteric venous thrombosis in a 64-year old male (53). This latter mutation, in the 3'-untranslated region of the prothrombin gene, allows higher levels of prothrombin to occur, thus further enhancing the likelihood of venous thrombosis, and possibly arterial thrombosis. (64).

3. RESULTS AND DISCUSSION

3.1. Targeted Deletion of the *PC* Gene in Mice

3.1.1. Nature of the Targeting Vector

The targeting vector, illustrated in Figure 3A, was designed to replace all exons (2-9) of the murine PC gene with the neomycin (*neo)* gene, using homologous recombination at the PC chromosomal locus. The cytidine deaminase gene *(cda)* cassette was employed for negative selection of random integrations. Southern blots (Figure 3B) of *EcoR*I-digested DNA extracted from genomic DNA of wild-type *(WT* or $PC^{+/+}$*)*, heterozygous *(*$PC^{+/-}$*)*, and null *(*$PC^{-/-}$*)* mice, used with the 5' external probe (Figure 3A), and blots of *BamH*I-digested genomic DNA of these same mice, used with the 3' external probe (Figure 3A), indicated that proper targeting occurred. Northern blot analysis of total RNA extracted from these mice (Figure 3C) showed the absence of *PC* mRNA in the $PC^{-/-}$ animals, thus further demonstrating that there is a total deficiency of PC in these mice. In association with this, PC activity was undetectable in plasma of 17.5 dpc and neonatal $PC^{-/-}$ mice.

3.1.2. Embryonic Development of $PC^{-/-}$ Mice (65)

The number of $PC^{-/-}$ offspring from breedings of $PC^{+/-}$ parents were underrepresented in the postnatal day 1 pool. Out of a total of 207 mice, only 8% (expected, 25%) were found to possess the $PC^{-/-}$ genotype. Careful monitoring of the births subsequently proved this to be due to consumption by the mother of $PC^{-/-}$ pups that were born dead. Examination of the genotypes of 84 cesarean section-derived pups from similar breedings at gestation day 17.5 showed that 26% (expected, 25%) of the resulting embryos possessed the $PC^{-/-}$ genotype. This indicated that the embryos died between gestational age (dpc) 17.5 and birth. These $PC^{-/-}$ embryos usually lived only a few hours. It has become clear in many subsequent analyses that normal embryogenesis and development occurs in $PC^{-/-}$ mice and neonatal death occurs from a coagulopathy associated with the trauma of birth.

3.1.3. Characterization of $PC^{-/-}$ Mice

$PC^{-/-}$ neonates grossly presented with bruising within the brain, and blood was present in the subdural cavity. Signs of DIC were present upon inspection of the abdominal cavity, with severely anemic livers.

Microscopically, $PC^{-/-}$ embryos showed normal blood vessel development during development in a variety of organs, but some evidence of thrombotic and necrotic changes were found, especially in the brains and livers. Microscopic analysis of dead $PC^{-/-}$ 1 day neonates showed widespread coagulopathy, manifested by severe bleeding (Figure 4A) and fibrin deposition (Figure 4B) in the brain, as compared to wild-type embryos at the same age (Figures 4C and 4D). Interstitial fibrin deposits were also found in livers of these $PC^{-/-}$ neonates, as well as necrosis of hepatic tissue (Figures 4E and 4F), characteristics absent in age-matched wild-type embryos (Figure 4G and 4H). In addition, microscopic examination revealed some fibrin deposition in kidney, heart, and lung (not shown).

In order to determine the timepoint of fibrin deposition during embryonic development, embryos at various stages of development were examined. At 13.5 dpc, a small amount of fibrin was found throughout the telencephalic region of the brain (Figures 5A and 5B) in $PC^{-/-}$ embryos, but not in age matched wild-type embryos (Figures 5C and 5D). A very small level of interstitial fibrin was seen in the livers of $PC^{-/-}$ embryos of this age (not shown). No signs of degradation of the brains or livers of these animals were noted.

At 17.5 dpc, more extensive bleeding (Figure 6A) and fibrin deposition (Figure 6B) was evident in the brains of $PC^{-/-}$ mice. No evidence for such characteristics were found in age-matched wild-type embryos (Figures 6

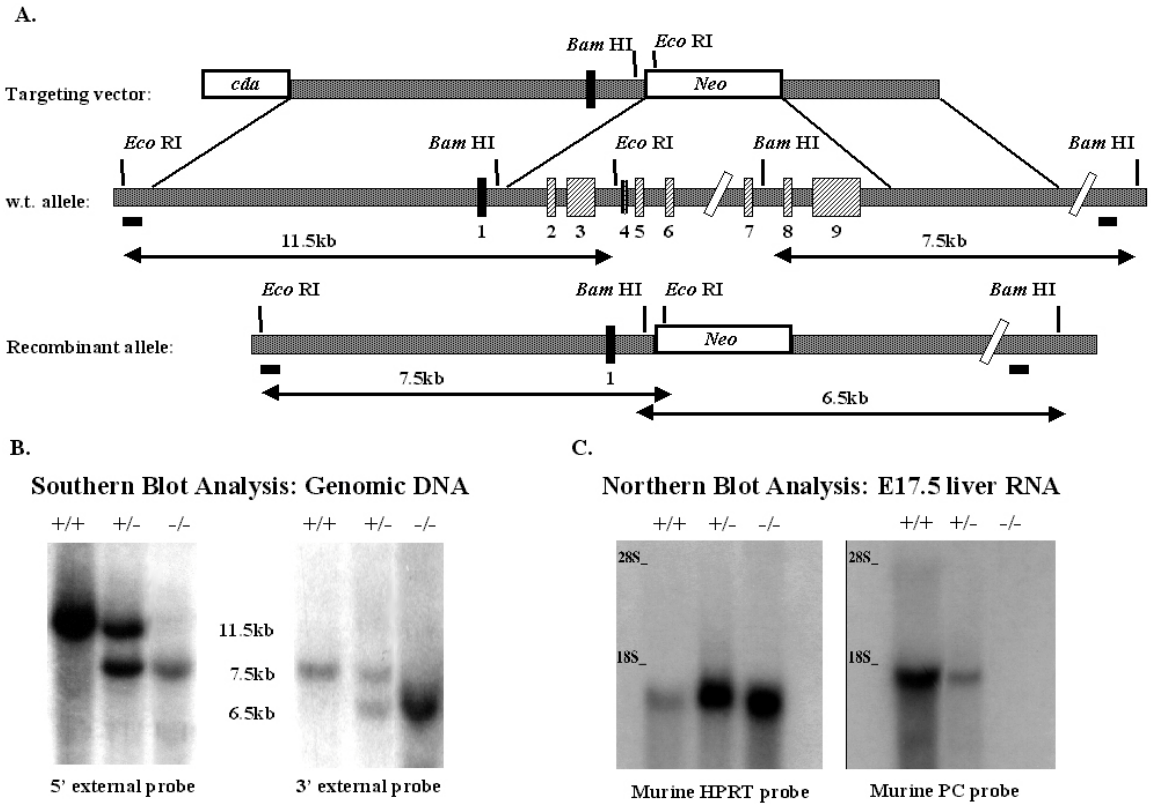

Figure 3. Targeted gene replacement at the murine *PC* locus. A. Targeting vector for *PC* gene disruption. The top line illustrates the targeting vector. Homologous flanking regions are shaded gray. The neo^R selection cassette cloned between the flanking regions was designed to replace the entire coding region of *PC* upon homologous recombination. An *Eco*RI restriction site was introduced from the polylinker of the neo^R cassette. A cda negative selection cassette, cloned upstream of the 5' flanking region, was used to select against random integrants. The second line indicates the wild-type murine *PC* allele. The first untranslated exon is presented as a darkened block while the other exons are presented as lighter shaded boxes. The 5' and 3' extended probes are indicated by the darkened boxes below the gene. The recombinant allele is indicated in the third line. The differential restriction fragments that result from the diagnostic digests are indicated. The slanted bars indicate a location where a break was placed in a large noncoding region. B. Southern blot analysis of DNA extracted from $PC^{+/+}$, $PC^{+/-}$, and $PC^{-/-}$ genomic DNA. *Eco*RI-digested DNA was used with the 5' external probe and *BamH*I digested DNA was used with the 3' external probe. C. Northern blot analysis of total RNA extracted from $PC^{+/+}$, $PC^{+/-}$, and $PC^{-/-}$ E17.5 livers. The murine *HPRT* cDNA and murine *PC* cDNA probes were used ((taken from (65)).

C and 6D). Interstitial fibrin, as well as signs of necrosis were observed in the livers of the PC null animals (Figures 6E and 6F), but not in wild-type age-matched controls (Figures 6G and 6H). Leukocyte infiltration was also found in the brains of $PC^{-/-}$ animals (Figure 6A).

Since PC is a major factor in regulating thrombin levels, uncontrolled fibrin formation likely occurs in $PC^{-/-}$ mice, leading to consumptive coagulopathy, and a bleeding phenotype ultimately results. The fact that fibrinogen depletion has occurred, at least at 17.5 dpc, in these latter mice, was shown by the fact that plasma samples from 17.5 dpc $PC^{-/-}$ embryos were not able to clot upon thrombin addition. This suggests that all fibrinogen was consumed by the high levels of thrombin present in the plasmas of these animals.

3.1.4. Mouse Models of Compound PC Deficiencies

The availability of mice with separate deficiencies of a variety of genes allows development, through mating strategies, of animals with compound gene deficiencies. We have utilized this approach to generate animals with targeted compound hemostasis-related gene defects in order to examine the effects of multigenetic factors on hemostasis and also to attempt to evaluate whether rescue of unfavorable phenotypes of single gene deficiencies can occur with other gene alterations.

3.1.4.1. Mice with a PC/FXI Double Deficiency (66)

In order to determine whether the fibrin deposition previously observed in $PC^{-/-}$ murine embryos and neonates was mediated through the FXI pathway, mice doubly deficient in PC and FXI ($PC^{-/-}/FXI^{-/-}$) were generated. Several of these mice survived the early lethality observed in the $PC^{-/-}$ neonates, but this appeared to be random. The oldest animal lived for three months and was sacrificed upon presentation with severe inflammation, edema, and gangrene on its extremities such as its nose, tail

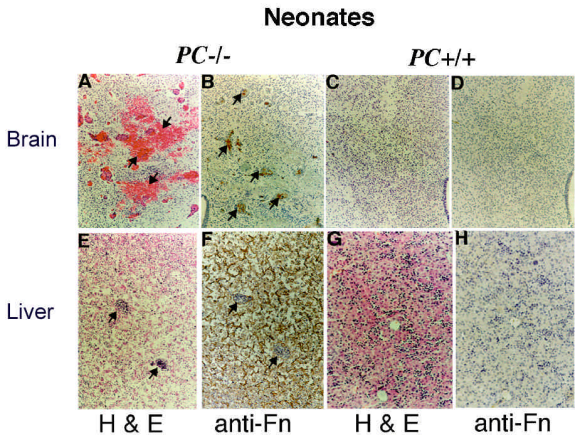

Figure 4. Analysis of tissue sections from neonatal PC-deficient mice. Brain (A-D) and liver (E-H) sections of $PC^{-/-}$ (A, B, E, F) and wild-type ($PC^{+/+}$; C, D, G, H) tissues stained with hematoxylin & eosin (H & E) (A, E, C, G) and anti-fibrin(ogen) (anti-Fn) (B, D, F, H) show extensive bleeding (A, arrows) surrounding the clotted vessels indicated by the arrows in panel B in $PC^{-/-}$ mice. The wild-type neonatal control sections (C, D) do not demonstrate these abnormalities in the corresponding region of the brain near the lateral ventricle. The $PC^{-/-}$ neonatal liver sections (E, F) indicate severe necrosis with anemia and increased levels of fibrin(ogen) deposition. These abnormalities are not observed in the corresponding neonatal wild-type ($PC^{+/+}$) stained sections (G, H). Taken from (65)).

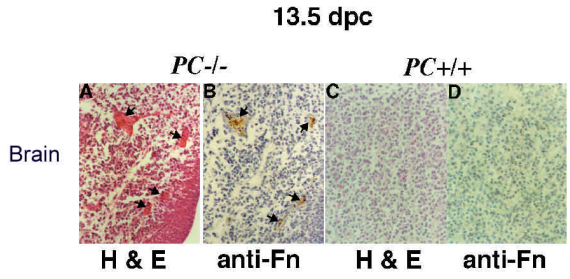

Figure 5. Analysis of brain sections from 13.5 dpc PC-deficient mice. Sections of $PC^{-/-}$ (A, B) and wild-type ($PC^{+/+}$; C, D) tissue stained with H & E (A) and anti-Fn (B) show clotted vessels in the telencephalic region. Clotting did not occur in the corresponding sections of the wild-type control (C, D). Taken from (65).

(Figure 7A) and paws (Figure 7B). Most PC-/-/FXI-/- animals were sedentary and significantly growth-retarded (Figure 7C). At necropsy, the surviving animals showed a wide range of pathologies, among which were the presence of fluid in the thoracic cavity, enlarged lymph nodes (Figure 7D), enlarged hearts, ventricular infarction, atrial clotting and fibrosis, and thrombosis near the *truncus pulmonalis*. Hemmorhagic lesions in the lungs were also apparent, as well as atherosclerotic changes in the large vessels (Figure 7E).

Upon sacrifice or natural death, all of these $PC^{-/-}/FXI^{-/-}$ mice demonstrated massive systemic fibrin deposition with concomitant hemorrhage and fibrosis, as confirmed through histological analyses. The hearts of $PC^{-/-}/FXI^{-/-}$ mice often contained multiple small and large intravascular, epicardial, and lymphatic thrombus formation, as observed by positive anti-fibrin (Figure 8A, B) immunostaining. Activated platelets were present within the thrombi (Figure 8C). Inflammatory cell infiltrates (neutrophils, macrophages, mast cells) were seen surrounding affected vessels and within areas of degeneration and repair. Lungs (Figure 8D) demonstrated varying degrees of septal thickening, consolidation, alveolar hemorrhage, and fibroblast infiltration into the alveolar spaces, as compared to $PC^{+/+}/FXI^{-/-}$ controls (Figure 8E). Collagen deposition and focal mineralization were observed around these fibrotic lesions. In addition, acute and chronic inflammation (neutrophils, leukocytes, macrophages, giant cells) (Figure 8F), were evident. Livers consistently demonstrated intravascular thrombosis (Figure 8G, H) with multifocal areas of hyperplasia, mineralization, fibrosis, and hemorrhage.

This combined deficiency state provided the first clear indication that the course of a severe thrombotic disorder could be manipulated by blocking the FXI-dependent intrinsic blood coagulation pathway and provided the first opportunity to study a total PC deficiency in an adult animal.

3.1.4.2. Mice with a PC/FVII Double Deficiency (67)

$PC^{+/-}$ and $FVII^{+/-}$ matings were established to evaluate whether the additional absence of FVII had an influence on the clot deposition found in $PC^{-/-}$ embryos. In addition, examination of the resulting doubly homozygous mice would allow evaluation of the ability of the FXI-dependent intrinsic coagulation system, alone, to deposit intravascular clots under conditions wherein FVa and FVIIIa, generated solely through the intrinsic system were not inhibited. Embryos of the combined $FVII^{-/-}/PC^{-/-}$ genotype were present at their expected Mendelian frequency at all times examined (12.5 dpc, 14.5 dpc, and 17.5 dpc), showing that the double deficiency of these coagulation factors did not lead to embryonic lethality. However, the $FVII^{-/-}/PC^{-/-}$ mice displayed a phenotype that was different from each of the single total deficiency animals.

At 12.5dpc, $FVII^{-/-}/PC^{-/-}$ embryos, while appearing developmentally normal, demonstrated an intra- and extravascular coagulopathy, present in liver (Figure 9A) and brain (Figure 9B), as well as the neural canal, caudal artery, pulmonary cavity, dorsal aorta, and left hindlimb bud (not shown). These coagulopathies progressed in 14.5 dpc embryos (Figure 9C-F), with fibrin deposition and bleeding observed in the snout, lateral ventricle, and spinal column. Hemmorhage and edema occurred by 17.5 dpc, along with collagen deposition and the presence of CD45-positive leukocytes (not shown). Histological analyses of atrial sections confirmed the presence of blood in the atria, fibrin deposition, and internal collagen invasion (not shown) around these areas of fibrin resulting in mortality immediately following birth. Figure 9G&H shows the hemmorhage and fibrin deposition, respectively, in hindbrain sections of these 17.5 dpc embryos. $FVII^{+/-}/PC^{-/-}$ embryos demonstrated a less

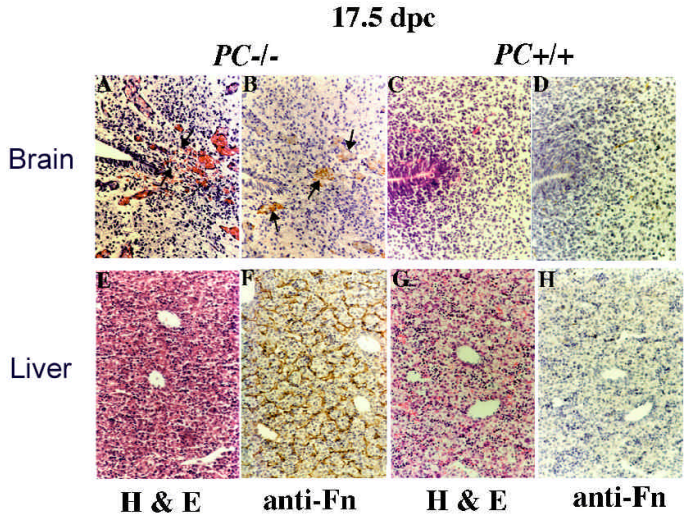

Figure 6. Analysis of tissue sections from 17.5 dpc PC-deficient mice. Brain (A-D) and liver (E-H) sections of $PC^{-/-}$ (A, B, E, F) and wild-type ($PC^{+/+}$; C, D, G, H) tissues stained with H & E (A, E, C, G) and anti-Fn (B, D, F, H). In the $PC^{-/-}$ brain, a minor bleeding event is seen near the lateral ventricle (A, arrow). The surrounding clotted vessels are indicated by the arrows in B. The wild-type control stains (C, D) do not show clotted vessels or bleeding. In the $PC^{-/-}$ liver, interstitial fibrin(ogen) deposition is evident (F). The wild-type liver (H) demonstrates no fibrin(ogen) deposition in this region. Taken from (65).

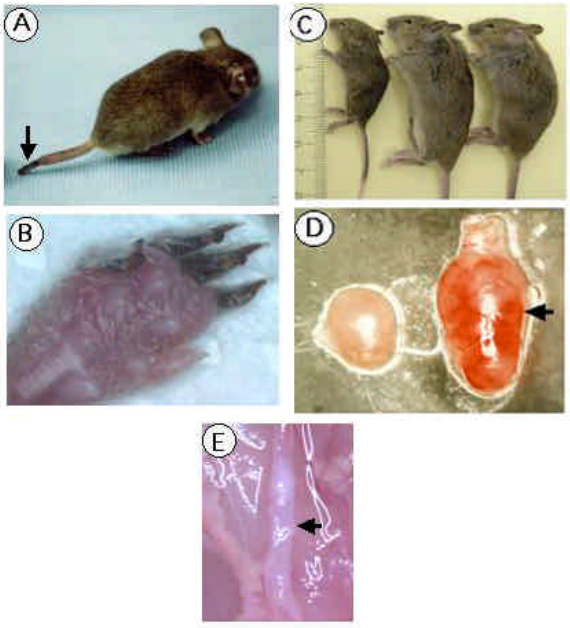

Figure 7. Gross appearance of $PC^{-/-}/FXI^{-/-}$ animals. The oldest surviving $PC^{-/-}/FXI^{-/-}$ mouse (94 days), presented with (A) gangrene on the tail tip (arrow) and toes (B), and severe inflammation and edema on the paws (B). (C) The majority of the $PC^{-/-}/FXI^{-/-}$ animals (left) were significantly growth retarded compared to their $PC^{+/-}/FXI^{-/-}$ (middle) and $PC^{+/+}/FXI^{-/-}$ (right) littermate. (D) A common attribute observed in these animals was enlargement and apparent hemorrhage of lymph nodes (arrow). (E) Atherosclerotic areas were seen in some of the larger vessels such as the abdominal and thoracic aorta (arrow). Taken from (66).

severe phenotype compared to the $FVII^{-/-}/PC^{-/-}$ embryos, suggesting a gene dosage effect.

The augmented coagulopathy resulting in $PC^{-/-}$ animals that arises from a second *FVII* deficiency is likely due to increased FXa and thrombin generation from at least two effects, *viz.*, (1) the loss of the ability of TFPI to inhibit FXa, since this inhibition is dependent on the presence of FVIIa, and (2) the loss of negative control of production of FVa and thrombin by the absence of APC. Further, the discovery of fibrin deposition in embryos in the absence of fetal FVII shows that coagulation occurs to a substantial degree with the FXI-dependent pathway.

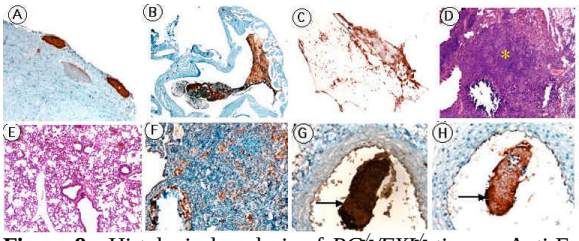

Figure 8. Histological analysis of $PC^{-/-}/FXI^{-/-}$ tissue. Anti-Fn immunostaining indicated thrombus deposition (brown areas) in various regions of the heart, such as (A) the epicardium (100X) and (B) atria (40X). (C) Anti-P-selectin immunostaining showed activated platelets within the thrombus (400X). (D) Lung tissue demonstrated focal areas of consolidation and mineralization (*) (H&E, 100X) as compared to a (E) $PC^{+/+}/FXI^{-/-}$ control (100X). (F) Macrophage infiltration (brown), visualized by anti-Mac3 immunostaining (200X), was also observed. Taken from (66). (E, F) Livers showed intravascular thrombi (arrows) that were visualized by anti-fibrin(ogen) (G, arrow) and anti-vonWillebrand factor (H, arrow) immunostaining (400X).

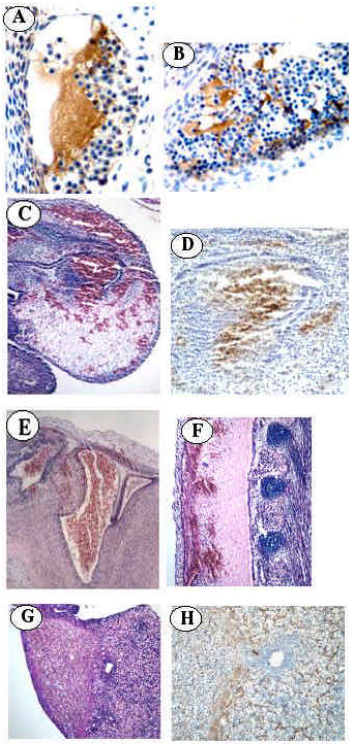

Figure 9. Histological and histochemical analysis of $FVII^{-/-}/PC^{-/-}$ mouse embryos. Anti-Fn immunostaining of 12.5 dpc $FVII^{-/-}/PC^{-/-}$ embryo sections revealed fibrin deposition (brown areas) in brain vessels (A) and liver interstitia (B), characteristics not observed at these early embryonic stages in $PC^{-/-}$ embryos. At 14.5 dpc, hemorrhage (red, C) and fibrin deposition (brown, D) is seen in the snout, plus bleeding in the lateral ventricle (E) and behind the spinal column (F). H&E staining of hindbrain sections of 17.5 dpc $FVII^{-/-}/PC^{-/-}$ embryo sections indicate areas of hemmorhage (G). Anti-fibrin(ogen) immunostaining of a parallel section demonstrates fibrin deposition (brown, H). This figure was adapted from (67).

4. PERSPECTIVE

The roles of components of the PC anticoagulation pathway in regulating clot formation are well known, but this system has been more recently revealed to possess unanticipated functions in control of fibrinolysis and in mediating the inflammatory response. Since the PC system principally governs the levels of thrombin, and thrombin, besides its coagulant properties, is being continually revealed to be a major factor in cell signaling responses, it is expected that the PC system will be shown to have a much more expanded role in many other pathways. This is in addition to the probable cell signaling roles of PC and APC that result from its binding to its own cell receptor, EPCR. Mice with a total deficiency of PC do not generally survive the neonatal period, as a result of the expected coagulopathies, but will allow the study of a variety of phenotypes with single and combined less severe deficiencies of this protein. Thus, the next several years should be an important period in the study of many thrombin-dependent, fibrin-dependent, and inflammatory-dependent maladies, among many other types of pathophysiological events, which are directly or indirectly influenced by levels of PC and APC.

5. ACKNOWLEDGEMENTS

These studies were supported by grant HL-19982 from the National Institutes of Health, the W.M. Keck Foundation, and the Kleiderer-Pezold family endowed professorship.

6. REFERENCES

1. McClure, D. B., J. D. Walls & B. W. Grinnell: Post-translational processing events in the secretion pathway of human protein C, a complex vitamin K-dependent antithrombotic factor. *J Biol Chem* 267, 19710-19717 (1992)
2. F. J. Castellino: Human protein C and activated protein C. Components of the human anticoagulation system. *Trends Cardiovasc Med* 5, 55-62 (1995)
3. Kisiel, W. & E. W. Davie: Protein C. *Meth Enzymol* 80, 320-332 (1981)
4. Vehar, G. A. & E. W. Davie: Preparation and properties of bovine factor VIII (antihemophilic factor). *Biochemistry* 19, 401-410 (1980)
5. Bajzar, L., M. Kalafatis, P. Simioni & P. B. Tracy: An antifibrinolytic mechanism describing the prothrombotic effect associated with factor V-Leiden. *J Biol Chem* 271, 22949-22952 (1996)
6. Fukudome, K. & C. T. Esmon: Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor. *J Biol Chem* 269, 26486-26491 (1994)
7. Stearn-Skurosawa, D. J., S. Kurosawa, J. S. Mollica, G. L. Ferrell & C. T. Esmon: The endothelial cell protein C receptor augments protein C activation by the thrombin-thrombomodulin complex. *Proc Natl Acad Sci USA* 93, 10212-10216 (1996)
8. Fukudome, K., X. F. Ye, N. Tsuneyoshi, O. Tokunaga, K. Sugawara, H. Mizokami & M. Kimoto: Activation

mechanism of anticoagulant protein C in large blood vessels involving the endothelial cell protein C receptor. *J Exp Med* 187, 1029-1035 (1998)
9. Xu, J., D. F. Qu, N. L. Esmon & C. T. Esmon: Metalloproteolytic release of endothelial cell protein C receptor. *J Biol Chem* 275, 6038-6044 (2000)
10. Liaw, P. C. Y., P. F. Neuenschwander, M. D. Smirnov & C. T. Esmon: Mechanisms by which soluble endothelial cell protein C receptor modulates protein C and activated protein C function. *J Biol Chem* 275, 5447-5452 (2000)
11. Taylor, F. B., A. Chang, C. T. Esmon, A. D'Angelo, S. Vigano-D'Angelo & K. E. Blick: Protein C prevents the coagulopathic and lethal effects of Escherichia coli infusion in the baboon. *J Clin Invest* 79, 918-925 (1987)
12. Taylor, F., A. Chang, G. Ferrell, T. Mather, R. Catlett, K. Blick & C. T. Esmon: C4b-binding protein exacerbates the host response to *Escherichia coli*. *Blood* 78, 357-363 (1991)
13. Taylor, F. B., D. J. Stearns-Kurosawa, S. Kurosawa, G. Ferrell, A. C. K. Chang, Z. Laszik, S. Kosanke, G. Peer & C. T. Esmon: The endothelial cell protein C receptor aids in host defense against Escherichia coli sepsis. *Blood* 95, 1680-1686 (2000)
14. Yamamoto, K. & D. J. Loskutoff: Extrahepatic expression and regulation of protein C in the mouse. *Am J Pathol* 153, 547-555 (1998)
15. Plutzky, J., J. A. Hoskins, G. L. Long & G. R. Crabtree: Evolution and organization of the human protein C gene. *Proc Natl Acad Sci USA* 83, 546-550 (1986)
16. Miao, C. H., W. T. Ho, D. L. Greenberg & E. W. Davie: Transcriptional regulation of the gene coding for human protein C. *J Biol Chem* 271, 9587-9594 (1996)
17. Jalbert, L. R., E. D. Rosen, A. Lissens, P. Carmeliet, D. Collen & F. J. Castellino: Nucleotide structure and characterization of the murine gene encoding anticoagulant protein C. *Thromb Haemost* 79, 310-316 (1998)
18. Ploos van Amstel, H. K., P. H. Reitsma, C. P. E. van der Logt & R. M. Bertina: Intron-exon organization of the active human protein S gene PSa and its pseudogene PSß: Duplication and silencing during primate evolution. *Biochemistry* 29, 7853-7861 (1990)
19. Geng, Y. & L. F. Johnson: Lack of an initiator element is responsible for multiple transcriptional initiation sites of the TATA-less mouse thymidylate synthase promoter. *Mol Cell Biol* 13, 4894-4903 (1993)
20. Idusogie, E., E. D. Rosen, P. Carmeliet, D. Collen & F. J. Castellino: Nucleotide structure and characterization of the murine blood coagulation factor VII gene. *Thromb Haemost* 76, 957-964 (1996)
21. Stauffer, D. R., B. N. Chukwumezie, J. A. Wilberding, E. D. Rosen & F. J. Castellino: Characterization of transcriptional regulatory elements in the promoter region of the murine blood coagulation factor VII gene. *J Biol Chem* 273, 2277-2287 (1998)
22. Cooper, A., Z. Liang, F. J. Castellino & E. D. Rosen: Cloning and characterization of the murine coagulation factor X gene. *Thromb Haemost* 83, 732-735 (2000)
23. Wilberding, J. A. & F. J. Castellino: Characterization of the murine coagulation factor X promoter. *Thromb Haemost* 84, 1031-1038 (2000)
24. Blake, M. C., R. C. Jambou, A. G. Swick, J. W. Kahn & J. C. Azizkhan: Transcriptional initiation is controlled by upstream GC-box interactions in a TATAA-less promoter. *Mol Cell Biol* 10, 6632-6641 (1990)
25. Tsay, W., Y. M. Lee, S. C. Lee, M. C. Shen & P. J. Chen: Characterization of human protein C gene promoter: Insights from natural human mutants. *DNA Cell Biol* 15, 907-919 (1996)
26. Tsay, W., Y. M. Lee, S. C. Lee, M. C. Shen & P. J. Chen: Synergistic transactivation of HNF-1 alpha, HNF-3, and NF-I contributes to the activation of the liver-specific protein C gene. *DNA Cell Biol* 16, 569-577 (1997)
27. Reitsma, P. H., F. Bernardi, R. G. Doig, S. Gandrille, J. S. Greengard, H. Ireland, M. Krawczak, B. Lind, G. L. Long, S. R. Poort, H. Sato, H., I. Witt, N. Sala, N. & D. N. Cooper: Protein C deficiency: A database of mutations, 1995 update. On behalf of the Subcommittee on Plasma Coagulation Inhibitors of the Scientific and Standardization Committee of the ISTH. *Thromb Haemostas* 73, 876-889 (1995)
28. Huber, P., T. Schmitz, J. Griffin, M. Jacobs, C. Walsh, B. Furie & B. C. Furie: Identification of amino acids in the g-carboxylation recognition site of the propeptide of prothrombin. *J Biol Chem* 265, 12467-12473 (1990)
29. Girolami, A., P. Simioni, B. Girolami, A. Marchiori, D. S. Millar, P. Bignell, V. V. Kakkar & D. N. Cooper: A novel dysfunctional protein C (protein C Padua-2) associated with a thrombotic tendency. Substitution of cys for arg-1 results in a strongly reduced affinity for binding of Ca++. *Br J Haematol* 85, 521-527 (1993)
30. Gaussem, P., S. Gandrille, J. Duchemin, J. Emmerich, M. Alhencgelas, M. F. Aillaud & M. Aiach: Influence of six mutations of the protein C gene on the gla domain conformation and calcium affinity. *Thromb Haemostas* 71, 748-754 (1994)
31. Zhang, L. & F. J. Castellino: A g-carboxyglutamic acid variant (g^6D, g^7D) of human activated protein C displays greatly reduced activity as an anticoagulant. *Biochemistry* 29, 10828-10834 (1990)
32. Zhang, L., A. Jhingan & F. J. Castellino: Role of individual gamma-carboxyglutamic acid residues of activated human protein C in defining its *in vitro* anticoagulant activity. *Blood* 80, 942-952 (1992)
33. Zhang, L. & F. J. Castellino: Influence of specific g-carboxyglutamic acid residues on the integrity of the calcium-dependent conformation of human protein C. *J Biol Chem* 267, 26078-26084 (1992)
34. Zhang, L. & F. J. Castellino: The contributions of individual *gamma*-carboxyglutamic acid residues in the calcium-dependent binding of recombinant human protein C to acidic phospholipid vesicles. *J Biol Chem* 268, 12040-12045 (1993)
35. Greengard, J. S., C. L. Fisher, B. Villoutreix & J. H. Griffin: Structural basis for type-I and type-II deficiencies of antithrombotic plasma protein C. Patterns revealed by 3-dimensional molecular modelling of mutations of the protease domain. *Proteins-Struct Funct Genet* 18, 367-380 (1994)
36. Tait, R. C., I. D. Walker, P. H. Reitsma, S. Islam, F. McCall, S. R. Poort, J. A. Conkie & R. M. Bertina: Prevalence of protein C deficiency in the healthy population. *Thromb Haemostas* 73, 87-93 (1995)
37. Bertina, R. M., B. P. C. Koeleman, T. Koster, F. R. Rosendaal, R. J. Dirven, H. Deronde, P. A. Vandervelden

& P. H. Reitsma: Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 369, 64-67 (1994)
38. Koeleman, B. P., P. H. Reitsma, C. F. Allaart & R. M. Bertina: Activated protein C resistance as an additional risk factor for thrombosis in protein C-deficient families. *Blood* 84, 1031-1035 (1994)
39. B. Dahlback: New molecular insights into the genetics of thrombophilia. Resistance to activated protein C caused by Arg506 to Gln mutation in factor V as a pathogenic risk factor for venous thrombosis. *Thromb Haemost* 74, 139-148 (1995)
40. Vicente, V., R. Maia, I. Alberca, G. P. Tamagnini & A. Lopez-Borrasca: Congenital deficiency of vitamin K-dependent coagulation factors and protein C. *Thromb Haemost* 51, 343-346 (1984)
41. Oldenburg, J., B. von Brederlow, A. Fregin, S. Rost, W. Wolz, W. Eberl, S. Eber, E. Lenz, R. Schwaab, H. H. Brackmann, Effenberger, W., U.Harbrecht, U., L. J. Schurgers, C. Vermeer & C. R. Muller: Congenital deficiency of vitamin K dependent coagulation factors in two families presents as a genetic defect of the vitamin K-epoxide-reductase-complex. *Thromb Haemost* 84, 937-941 (2000)
42. Hanano, M., H. Takahashi, M. Itoh & A. Shibata: Coexistence of congenital afibrinogenemia and protein C deficiency in a patient. *Am J Hematol* 41, 57-60 (1992)
43. Cransac, M., J. Carles, P. H. Bernard, P. Malavialle, G. Freyburger, S. Winnock & J. Saric: Heterozygous protein C deficiency and dysfibrinogenemia acquired by liver transplantation. *Transpl Int* 8, 307-311 (1995)
44. Gandrille, S., P. Priollet, L. Capron, M. Roncato, J. N. Fiessinger & M. Aiach: Association of inherited dysfibrinogenaemia and protein C deficiency in two unrelated families. *Br J Haematol* 68, 329-337 (1988)
45. Sansores-Garcia, L. & A. Majluf-Cruz: Arterial and venous thrombosis associated to combined deficiency of protein C and antithrombin III. *Am J Hematol* 57, 182-183 (1998)
46. Gouault-Heilmann, M., P. Quetin, M. Dreyfus, S. Gandrille, J. Emmerich, C. Leroy-Matheron & M. Guesnu: Massive thrombosis of venous cerebral sinuses in a 2-year-old boy with a combined inherited deficiency of antithrombin III and protein C. *Thromb Haemost* 72, 782-783 (1994)
47. Bernardi, F., C. Legnani, F. Micheletti, B. Lunghi, P. Ferraresi, G. Palareti, R. Biagi & G. Marchetti: A heparin cofactor II mutation (HCII Rimini) combined with factor V Leiden or type I protein C deficiency in two unrelated thrombophilic subjects. *Thromb Haemost* 76, 505-509 (1996)
48. Ozbek, N., N. K. Tokel & S. M. Kayiran: Inherited combined deficiency of proteins C and S. *Eur J Haematol* 63, 138-139 (1999)
49. Hashizume, T., N. Tsushima, H. Matsuo, A. Ito, K. Oozono & T. Sakata: Inherited heterozygous protein C deficiency and dysfunctional protein S with recurrent venous thrombotic diseases: a study of three generations of a Japanese family. *Intern Med* 31, 1197-1200 (1992)
50. Koller, H., G. Stoll, M. Sitzer, M. Burk, B. Schottler & H. J. Freund: Deficiency of both protein C and protein S in a family with ischemic strokes in young adults. *Neurology* 44, 1238-1240 (1994)
51. Atkins, K. A. & J. L. Zehnder: Mesenteric artery thrombosis: A case report of combined protein S and protein C deficiency. *Am J Hematol* 58, 246-247 (1998)
52. Gandrille, S., J. S. Greengard, M. Alhencgelas, I. Juhanvague, J. F. Abgrall, B. Jude, J. H. Griffin & M. Aiach: Incidence of activated protein C resistance caused by the Arg 506 Gln mutation in factor V in 113 unrelated symptomatic protein C-deficient patients. *Blood* 86, 219-224 (1995)
53. Hertzberg, M. S., T. Underwood & E. J. Favaloro: Mesenteric vein thrombosis secondary to combined protein C deficiency and double heterozygosity for factor V Leiden and prothrombin G20210A. *Amer J Hematol* 62, 199-200 (1999)
54. Nowak-Gottl, U., R. Junker, W. Kreuz, A. von Eckardstein, A. Kosch, N. Nohe, R. Schobess & S. Ehrenforth: Risk of recurrent venous thrombosis in children with combined prothrombotic risk factors. *Blood* 97, 858-862 (2001)
55. Sakata, T., Y. Goto, K. Kario, Y. Katayama, T. Matsuyama, H. Kato & T. Miyata: Recurrent restenosis after percutaneous transluminal coronary angioplasty in a patient with congenital protein C deficiency and high activated factor VII level. *Thromb Res* 81, 687-691 (1996)
56. Kario, K., T. Matsuo, S. Tai, S. Sakamoto, T. Yamada, T. Miki & M. Matsuo: Congenital protein C deficiency and myocardial infarction:concomitant factor VII hyperactivity may play a role in the onset of arterial thrombosis. *Thromb Res* 67, 95-103 (1992)
57. Takeuchi, Y., Y. Saito, H. Ikenouchi, T. Sugimoto, T. Sugoh, Y. Sakata, M. Matsuda & O. Takamiya: Combined factor VII and protein C deficiency found in a patient with peripheral pulmonary artery stenosis accompanied by progressive pulmonary hypertension and hemoptysis. *Thromb Res* 51, 117-126 (1988)
58. Negrier, C., M. Berruyer, A. Durin, N. Philippe & M. Dechavanne: Increased thrombin generation in a child with a combined factor IX and protein C deficiency. *Blood* 81, 690-695 (1993)
59. Franken, D. G., A. Vreugdenhil, G. H. Boers, A. Verrips, H. J. Blom & I. R. Novakova: Familial cerebrovascular accidents due to concomitant hyperhomocysteinemia and protein C deficiency type 1. *Stroke* 24, 1599-1600 (1993)
60. Manabe, S. & M. Matsuda: Homozygous protein C deficiency combined with heterozygous dysplasminogenemia found in a 21-year-old thrombophilic male. *Thromb Res* 39, 333-341 (1985)
61. Jobin, F., L. Vu & M. Lessard: Two cases of inherited triple deficiency in a large kindred with thrombotic diathesis and deficiencies of antithrombin III, heparin cofactor II, protein C and protein S. *Thromb Haemost* 66, 295-299 (1991)
62. Jobin, F., L. Vu & J. M. Biogonesse: Follow-up: a young man with three deficiencies of antithrombotic proteins, asymptomatic until now, spontaneously develops pulmonary embolism. *Thromb Haemost* 67, 730 (1992)
63. Bowen, D., H. Dasani, B. Yung & A. Bloom: Deep venous thrombosis and pulmonary embolism in a patient with type III von Willebrand's disease, protein C and antithrombin III deficiency. *Br J Haematol* 81, 446-447 (1992)

64. Arruda, V. R., J. M. Annichino-Bizzacchi, M. S. Goncalves & F. F. Costa: Prevalence of the prothrombin gene variant (nt20210A) in venous thrombosis and arterial disease. *Thromb Haemost* 78, 1430-1433 (1997)
65. Jalbert, L. R., E. D. Rosen, L. Moons, J. C. Y. Chan, P. Carmeliet, D. Collen & F. J. Castellino: Inactivation of the gene for anticoagulant protein C causes lethal perinatal consumptive coagulopathy in mice. *J Clin Invest* 102, 1481-1488 (1998)
66. Chan, J. C. Y., J. G. Ganopolsky, I. Cornelissen, M. A. Suckow, M. J. Sandoval-Cooper, E. C. Brown, F. Noria, D. Gailani, E. D. Rosen, V. A. Ploplis & F. J. Castellino: The characterization of mice with a targeted combined deficiency of protein C and factor XI. *Am J Pathol* 158, 469-479 (2001)
67. Chan, J. C. Y., I. Cornelissen, D. Collen, V. A. Ploplis & F. J. Castellino: Combined factor VII/protein C deficiency results in intrauterine coagulopathy in mice. *J Clin Invest* 105, 897-903

Key words: protein C deficiency, anticoagulation, gene targeting, inflammation, embryonic development, double gene deficiencies, Review

Send correspondence to: Dr. F. J. Castellino, Department of Chemistry & Biochemistry, 229 Nieuwland Science Hall, University of Notre Dame, Notre Dame, Indiana 46556. Tel: 219-631-6456. Fax:219-631-8017; E-mail: castellino.1@nd.edu