

*Advances in Basic, Laboratory and Clinical Aspects of Thromboembolic Diseases\****FACTOR V AND PROTEIN S AS COFACTORS TO ACTIVATED PROTEIN C**

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**ABSTRACT**

**Background and Objective.** Activated protein C (APC)-resistance, the most common risk factor for venous thrombosis described so far, is due to a single point mutation in the factor V gene. As a result, inactivation of factor-activated factor V by APC is impaired, which leads to a hypercoagulable state and a lifelong increased risk of thrombosis. The importance of protein S as an anticoagulant protein is illustrated by the association between protein S deficiency and venous thrombosis. The objective of this article is to examine the most recent advances on the role of factor V and protein S as cofactors to activated protein C.

**Evidence and Information Sources.** The material examined in the present review includes several personal papers in this field, and articles and abstracts published in journals covered by the Science Citation Index®.

**State of art.** Factors V and VIII are homologous, high molecular weight glycoproteins with similar functional properties. Factors Va and VIIIa bind to negatively charged phospholipid and function as high affinity receptors/cofactors for factors Xa and IXa, respectively. Factors Va and VIIIa account for at least a 10<sup>3</sup> increase in the rate of activation of prothrombin and factor Xa, respec-

tively. The potent anticoagulant activity of APC is mediated by the degradation of factors VIIIa and Va, resulting in inhibition of both Xase and prothrombinase activities. APC specifically degrades the membrane-bound activated forms of factors V and VIII, whereas the unactivated factors V and VIII are poor substrates for APC. Mature human protein S is a single chain glycoprotein composed of multiple domains, including a thrombin-sensitive region. Protein S acts as a cofactor to activated protein C. This function of protein S is lost upon thrombin cleavage, suggesting that the thrombin-sensitive region interacts with APC on the phospholipid surface.

**Perspectives.** Recent data suggest that factor V and protein S work in synergy as phospholipid-bound cofactors to APC in the degradation of factor VIIIa and that factor VIIIa is preferred over factor Va as APC-substrate. Thus complicated multi-molecular complexes, which are the result of protein-protein as well as protein-phospholipid interactions, appear to form the basis for efficient cleavage and inhibition of factors VIIIa and Va.

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Blood coagulation is activated in response to vascular injury.<sup>1,2</sup> Binding of factor VII to tissue factor initiates a series of proteolytic reactions which lead to the formation of thrombin. At sites of vascular injury thrombin converts soluble fibrinogen to a fibrin network, activates platelets and in a positive feedback reaction stimulates coagulation by activation of factor V and factor VIII. Factor Va and factor VIIIa bind to negatively charged phospholipids exposed on activated platelets and serve as receptor sites for the proteolytic enzymes, factor IXa and factor Xa, respectively.<sup>3-5</sup> Explosive amplification of the reactions generates high local concentrations of thrombin, which results in coagulation of blood. *In vivo*, several anticoagulant mechanisms control blood coagulation,

ensuring that clot propagation does not lead to occlusion of the vasculature.<sup>6,9</sup> One of these natural anticoagulant systems is the protein C system, which is initiated by thrombin after its binding to the endothelial cell surface protein thrombomodulin (TM).<sup>6,7,10</sup> Binding of thrombin to TM is associated with a loss of most of the procoagulant abilities of thrombin. Instead, thrombin becomes a potent activator of protein C, a vitamin K-dependent zymogen to a serine protease which circulates in plasma. Activated protein C (APC) cleaves and inactivates the membrane-bound activated forms of coagulation factors V and VIII (Va and VIIIa), whereas the circulating, non activated forms of factors V and VIII are poor substrates for APC. The anticoagulant activity of APC is potentiated by two

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plasma proteins, protein S and factor V. Protein S is a vitamin K-dependent plasma protein that is unrelated to the serine protease family.<sup>7,10</sup> In human plasma, approximately 60% of protein S circulates bound to C4b-binding protein (C4BP), an inhibitor of the classical complement pathway.<sup>7,11</sup> Only free protein S expresses APC cofactor activity. The ability of factor V to function as a cofactor in the protein C system was discovered recently.<sup>12,13</sup>

### Factors Va and VIIIa, substrates for activated protein C

Factors V and VIII are homologous, high molecular weight glycoproteins with similar functional properties.<sup>3,4</sup> The plasma concentration of factor V (10 mg/L) is 50-100 times higher than that of factor VIII (0.1-0.2 mg/L). In their circulating precursor states, factors V and VIII express no or very little procoagulant activity. Both factor V and factor VIII are synthesized as single chain precursor proteins with molecular weights around 300,000. They contain two types of internal repeats, three A-modules and two C-modules. In factor V and factor VIII, two A-modules occupy the amino terminal regions, whereas a third A-module and two C-modules constitute the carboxy-terminal parts of the molecules. The central portions of factors V and VIII, the B-modules, are very rich in carbohydrate and contain both N- and O-linked sugars. Unlike the rest of the molecules, the B-modules of factor V and factor VIII manifest no significant amino acid sequence similarity. During activation of factor V and factor VIII by thrombin, at least three peptide bonds are cleaved in each molecule.<sup>3,4</sup> In the case of factor V, the amino- and carboxy-terminal fragments form a calcium-dependent, non covalent complex constituting factor Va (composition A1-A2/A3-C1-C2) (Figure 1). During activation of factor VIII a peptide bond between A1 and A2 is cleaved in addition to cleavages between the A2- and B-domains and B- and A3-domains. Factor VIIIa is thus a heterotrimer (composition A1/A2/A3-C1-C2), whose subunits are held together by non-covalent  $Me^{2+}$ -dependent bonds.

Factors Va and VIIIa bind to negatively charged phospholipid and function as high affinity receptors/cofactors for factors Xa and IXa, respectively. Prothrombin and factor X are more rapidly activated ( $10^5$  higher rates) by the prothrombinase (factor Xa, factor Va, phospholipid and  $Ca^{2+}$ ) and Xase (factor IXa, factor VIIIa, phospholipid and  $Ca^{2+}$ ) complexes, respectively, than by the corresponding enzymes alone. This is the result of a lowering of the  $K_m$  for the substrates (prothrombin and factor X) by the phospholipid and an increase in the  $V_{max}$  by the respective receptor (factors Va and VIIIa).<sup>5</sup> Factors Va and VIIIa account for at least a  $10^3$  increase in the rate of activation of prothrombin

and factor Xa, respectively. The potent anticoagulant activity of APC is mediated by the degradation of factors VIIIa and Va, resulting in inhibition of both Xase and prothrombinase activities. APC specifically degrades the membrane-bound activated forms of factors V and VIII, whereas unactivated factors V and VIII are poor substrates for APC. Several peptide bonds in the heavy chains of factors Va and VIIIa are cleaved by APC, which leads to loss of respective cofactor activity.<sup>3,4</sup> In factor Va, the loss of cofactor activity is associated with peptide bond cleavages at Arg 306, Arg 506, and Arg 679 (Figure 1). Recently, it was demonstrated that cleavage at Arg 306 is characterized by a low  $K_m$  (20 nM) and a  $k_{cat}$  of  $0.96 s^{-1}$ , whereas the 506 cleavage has a higher  $K_m$  (196 nM) and a  $k_{cat}$  of  $0.37 s^{-1}$ .<sup>14</sup> This means that the 506 cleavage is preferred over the 306 cleavage, but full inactivation of normal factor Va requires cleavage at both 506 and 306. In APC-resistance, which is the most common risk factor for venous thrombosis described so far, a single point mutation leads to the replacement of Arg 506 with a Gln.<sup>10,16,17</sup> As a result, the inactivation of factor Va by APC is impaired, which leads to a hypercoagulable state and a lifelong increased risk of thrombosis.

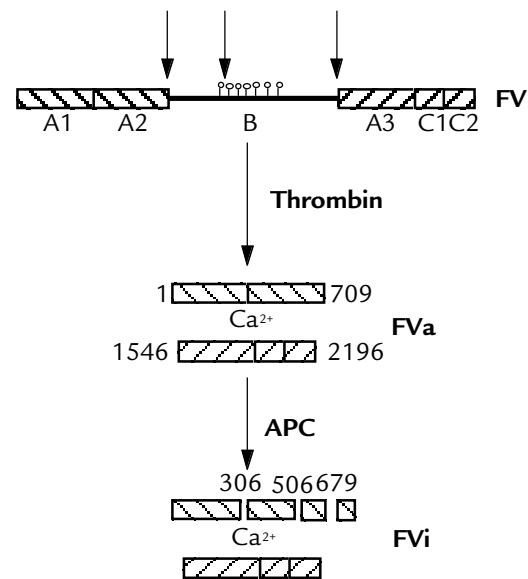


Figure 1. Schematic models of factor V. Factor V contains three A-modules, one B-module and two C-modules. Thrombin cleaves three peptide bonds, as indicated by the arrows. In factor Va, the A1-A2 containing heavy chain and the A3-C1-C2 composed light chain form a calcium-dependent complex. Factor Va is a substrate for APC, which cleaves the peptide bond at Arg 306, Arg 506 and Arg 679. The cleavage at 506 is preferred and this is also the site that is mutated in APC-resistance, a mutation which predicts replacement of the Arg with a Gln.<sup>16</sup>

### **Protein S, a cofactor to activated protein C**

Mature human protein S is a single chain glycoprotein (635 amino acids in length) composed of multiple domains (Figure 2).<sup>7,10</sup> It has a Gla-domain, a thrombin-sensitive region, four EGF-like domains, and a carboxy-terminal region which is unrelated to the serine proteases, but homologous to sex hormone binding globulin (SHBG). The Gla-module binds multiple  $\text{Ca}^{2+}$ -ions, and the  $\text{Ca}^{2+}$ -stabilized structure has a high affinity for negatively charged phospholipid membranes. The thrombin-sensitive region contains two cysteines which form a disulphide bridge. Two peptide bonds in this region are sensitive to proteolysis by thrombin. After cleavage by thrombin, the Gla-module remains attached to the rest of protein S via the disulphide bond. In thrombin-cleaved protein S, the Gla-module cannot undergo the calcium-mediated conformational change required for biological activity, indicating that the thrombin-sensitive region is intimately involved in the folding of the Gla-module. The APC cofactor function of protein S is lost upon thrombin cleavage. This suggests that the thrombin-sensitive region interacts with APC on the phospholipid surface, a hypothesis which has also derived support from experiments using monoclonal antibodies.<sup>18</sup>

The importance of protein S as an anticoagulant protein is illustrated by the association between protein S deficiency and venous thrombosis.<sup>7,10</sup> Protein S functions as a cofactor to APC in the degradation of both factor Va and factor VIIIa, but its mechanism of action is not yet completely understood.<sup>19-23</sup> Protein S has the highest affinity for negatively charged phospholipids among the vitamin K-dependent proteins,<sup>24</sup> and it has been shown to increase the affinity of APC for this type of membrane.<sup>21,25,26</sup> Although it has not been possible to demonstrate an interaction between protein S and APC in fluid phase, they form a complex with 1:1 stoichiometry on the lipid surface. *In vivo*, the protein S-APC interaction may occur on the surface of platelets, platelet microparticles and on endothelial cells.<sup>27-31</sup> The APC-cofactor activity of protein S is weak in purified systems, e.g. it only enhances the APC-mediated degradation of factor Va by a factor of two.<sup>19</sup> Factor Xa binding to factor Va leads to protection of factor Va from APC-mediated proteolysis, and protein S has been reported to abrogate this protective effect by making the substrate factor Va available for proteolysis by APC, although there is some controversy in the literature on this topic.<sup>20,32</sup> Factor IXa exerts a similar protective effect on factor VIIIa and protein S counteracts this protection as well.<sup>33</sup> Thus, the function of protein S may be to make the substrates Va and VIIIa available for APC-mediated proteolysis. In a recent investigation by Rosing *et al.*,<sup>32</sup> it was demonstrated that protein S specifically accelerates the slow APC-

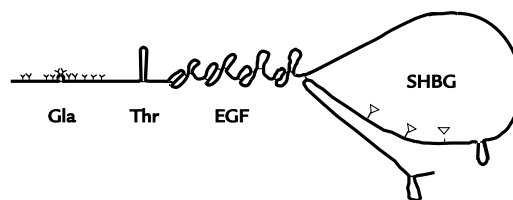


Figure 2. Schematic representation of human protein S. Protein S is composed of different modules. It contains a Gla-module, the thrombin-sensitive region, four EGF-modules and a region which is homologous to sexual hormone binding globulin (SHBG-like).

mediated cleavage at Arg306, whereas the Arg506 cleavage was unaffected by protein S. Moreover, it was shown that factor Xa protects Arg506 from inactivation by APC, whereas the Arg306 cleavage was unaffected by factor Xa. As a consequence, the factor Va incorporated in the prothrombinase complex is protected from APC-mediated inactivation. The same authors also documented that in the presence of both factor Xa and protein S, there was almost no difference in the rate of APC-catalyzed inactivation of normal factor Va or of factor Va with the Arg506 to Gln mutation. This may explain why APC-resistance is only a weak risk factor for venous thrombosis.<sup>17</sup>

The anticoagulant activity of APC is species specific.<sup>7,34</sup> Thus bovine APC does not prolong the clotting time of human plasma to any major extent. Walker demonstrated that the presence of bovine protein S restored the ability of bovine APC to inhibit coagulation of human plasma, suggesting that the protein S-APC interaction may be involved in species specification of the anticoagulant activity of APC.<sup>22</sup> Later studies have shown that the species-specificity is restricted in the sense that bovine APC does not work with human protein S, whereas human APC functions with protein S of both human and bovine origin.<sup>35</sup> Recently, we found that sequences in the thrombin-sensitive region and in the first EGF-like domain of protein S determine the species-specificity of protein S.<sup>36</sup>

### **Factor V and protein S as synergistic cofactors in the degradation of factor VIIIa**

We have recently found that factor V and protein S function in synergy to support APC-mediated degradation of factor VIIIa in a purified system.<sup>12</sup> In the presence of both factor V and protein S, APC inhibited factor VIIIa activity efficiently, whereas APC alone or together with factor V was without effect. The combination of APC and protein S was less potent than when factor V was also included in the reaction. It was possible to inhibit the APC-

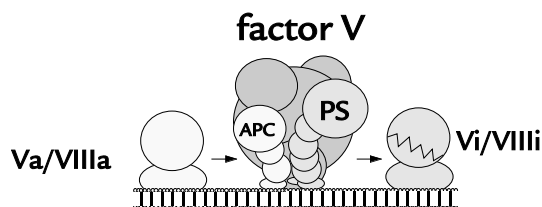


Figure 3. Speculative molecular model of APC-mediated degradation of factor Va and factor VIIIa. A complex formed between protein S and factor V on the membrane surface may constitute a binding site for APC. APC efficiently inhibits blood coagulation through degradation of factors VIIIa and Va.

cofactor activity of the factor V-protein S mixture by certain monoclonal antibodies against protein S or factor V. Factor Va did not express anticoagulant cofactor activity, and excess factor Va over factor V did not inhibit the APC-cofactor function of the factor V-protein S mixture in degradation of factor VIIIa. These results suggested that factor V and protein S work in synergy as phospholipid-bound cofactors to APC in the degradation of factor VIIIa and that factor VIIIa is preferred over factor Va as APC-substrate.

APC binds to negatively charged phospholipid with a  $K_d$  of approximately  $7 \times 10^{-8}$  M.<sup>21,37,38</sup> Protein S enhances the affinity of the APC binding by approximately tenfold. Factor Va has been reported to have a similar effect and to increase the affinity of APC for the membrane by approximately 10 times.<sup>37</sup> It is noteworthy that intact factor V is also claimed to increase the affinity of APC for the membrane. This may be important for expression of the recently identified anticoagulant activity of intact factor V.<sup>12,13</sup> Even though APC interacts with factor V, it is much more efficient in cleaving activated cofactor factor Va than the precursor form factor V.<sup>3,4,39</sup> The basis for the specificity of this reaction is presumably related to the very different molecular presentation of factor Va and factor V on the membrane. Since APC cleaves the heavy chain of factor Va, it must be oriented close to the active site of APC. In intact factor V (containing the large B-domain), the APC-sensitive bonds are probably not accessible to the active site of APC.<sup>40</sup> Both protein S and factor V are known to associate with platelets and endothelial cells, and they probably interact on the membrane surface.<sup>3,28,29,31,41</sup> Presumably, APC in turn binds to both protein S and factor V on the membrane. APC and protein S may interact with both factor V and factor Va on the membrane because both molecules have binding sites for factor Va which may be different from the ones interacting with intact factor V.<sup>37,42-46</sup> Compli-

cated multi-molecular complexes, that are the result of protein-protein as well as protein-phospholipid interactions, appear to form the basis for efficient cleavage and inhibition of factors VIIIa and Va (Figure 3).

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