C4b-Binding Protein Protects Coagulation Factor Va from Inactivation by Activated Protein C†

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ABSTRACT: We investigated the effect of C4BP on APC-mediated inactivation of factor Va (FVa) in the absence and presence of protein S. FVa inactivation was biphasic ($k_{506} = 4.4 \times 10^8$ M^{-1} s^{-1}, $k_{306} = 2.7 \times 10^7$ M^{-1} s^{-1}), and protein S accelerated Arg^{306} cleavage approximately 10-fold. Preincubation of protein S with C4BP resulted in a total abrogation of protein S cofactor activity. C4BP also protected FVa from inactivation by APC in the absence of protein S. Control experiments with CLB-PS13, a monoclonal anti-protein S antibody, indicated that inhibition of FVa inactivation by C4BP was not mediated through contaminating traces of protein S in our reaction systems. Protection of FVa was prevented by a monoclonal antibody directed against the C4BP α-chain. Recombinant rC4BPα comprised of only α-chains also protected FVa, but in the presence of protein S, the level of protection was decreased, since rC4BPα lacks the β-chain responsible for C4BP binding to protein S. A truncated C4BP β-chain (SCR-1+2) inhibited protein S cofactor activity, but had no effect on FVa inactivation by APC in the absence of protein S. In conclusion, C4BP protects FVa from APC-catalyzed cleavage in a protein S-independent way through direct interactions of the α-chaims of C4BP with FVa and/or APC.

Activated factor V (Va)[1] is a cofactor to activated factor X (Xa) in the prothrombin activating (prothrombinase) complex (see refs *1* and *2* for reviews). Factor Va accelerates factor Xa-catalyzed conversion of prothrombin to thrombin by several orders of magnitude. In a similar mechanism, activated factor VIII (VIIIa) is responsible for accelerating factor IXa-catalyzed activation of factor X (*3*). Cofactor activity of factors Va and VIIIa can be inhibited by activated protein C (APC)-mediated cleavage of the heavy chain of these proteins (*4*−*6*). APC cleavage sites in the factor Va heavy chain involve residues Arg^{306}, Arg^{506}, and Arg^{679} (*7*). Cleavage at Arg^{306} and Arg^{506} correlates with loss of cofactor activity of factor Va. Cleavage at Arg^{506} occurs rapidly and results in an intermediate form with a cofactor activity that is approximately 50% of that of noncleaved factor Va, whereas cleavage at Arg^{306} occurs slowly and results in total inactivation of factor Va (*8*).

A naturally occurring mutation in the factor V gene causes a change in amino acid residue 506 from Arg to Gln (*9*−*11*). The presence of this mutation (factor V^{R506Q} or factor V^{Leiden}) is associated with an increased risk for venous thrombosis (*12*, *13*) because the affected factor Va molecule is less sensitive to inactivation by APC, a phenomenon called APC resistance (*14*). In addition, factor V acts as a synergistic cofactor with protein S in the APC-mediated inactivation of factor VIIIa (*15*−*18*). This direct anticoagulant activity of factor V is impaired in the case of factor V^{R506Q} (*18*, *19*).

Factor Va is protected from APC-mediated cleavage at Arg^{506} by factor Xa, and this protection is abolished by protein S (*20*, *21*). Protein S is a cofactor to APC which increases the affinity of APC for negatively charged phospholipids (*22*) and specifically accelerates APC-mediated cleavage at Arg^{306} in factor Va (*23*). Acceleration of cleavage at the Arg^{306} site is accomplished by protein S-mediated topographical and/or conformational changes that localize the active site of APC closer to the membrane surface (*24*). Binding of protein S to C4b-binding protein (C4BP), an important regulator of the classical pathway of complement activation (*25*−*27*), results in a decreased cofactor activity of protein S in APC-catalyzed factor Va inactivation (*26*). C4BP contains several identical α-chains and a single β-chain. Binding of complement component C4b to C4BP is mediated by the α-chains, whereas protein S binds to the β-chain with high affinity, resulting in a 1:1 stoichiometric complex (*28*, *29*).

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[1] Abbreviations: Va, activated factor V; Xa, activated factor X; VIIIa, activated factor VIII; APC, activated protein C; C4BP, C4b-binding protein; ACC, *Agkistrodon contortrix contortrix*; rC4BPα, recombinant C4BP containing only α-chains; ELISA, enzyme-linked immunosorbent assay; SCR-1+2, truncated C4BP β-chain; TBS, Tris-buffered saline; BSA, bovine serum albumin.

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In this study, we investigated the effect of C4BP on the APC-mediated inactivation of factor Va in a model system containing purified proteins and phospholipid vesicles in both the absence and presence of protein S. A protein S-independent effect of C4BP on the APC-mediated inactivation of factor Va is described.

EXPERIMENTAL PROCEDURES

Proteins. Human factor V was purified as described previously (*30*) and activated according to the method of Hackeng et al. (*31*). Protein C, the protein C activator purified from *Agkistrodon contortrix contortrix* (ACC), and anti-ACC polyclonal antibody were kindly provided by W. Kisiel (Department of Pathology, University of New Mexico, Albuquerque, NM). Protein C was activated by ACC, after which ACC was removed using anti-ACC polyclonal antibody as described previously (*32*). C4BP was immunopurified from human plasma as described by Hessing et al. (*33*). The DNA encoding the α-chain of C4BP was amplified from a human liver cDNA library by PCR using oligonucleotides C4BPαF (5′CGCGGATCCACATCAGCGAAGCAGCAGGCC3′) and C4BPαR (5′CGCGGATCCCAGCAAGACACCTTTTCCTCC3′). After amplification, the PCR product was cleaved with *Bam*HI (underlined) and cloned in *Bam*HI-cleaved pZem229R. The expression vector pZem229R was kindly provided by E. R. Mulvihill (Zymogenetics Inc., Seattle, WA). The sequence and orientation of the amplified region were confirmed by dideoxy sequencing. This construct (that lacked the C4BP β-chain) was designated rC4BPα. Baby hamster kidney cells were transfected with rC4BPα as described previously (*34*). Expression of rC4BPα was performed in conditioned serum-free medium (UltraCHO, BioWhittaker, Verviers, Belgium), and harvested medium was stored at −20 °C until needed for further use. Purification of rC4BPα was performed using an immobilized monoclonal antibody against the α-chain of C4BP (8C11). The concentrations of C4BP preparations were determined in an ELISA using 8C11 as a capturing antibody and peroxidase-conjugated monoclonal antibody 9H10 directed against the α-chain of C4BP as a detecting antibody. C4BP preparations were subjected to 4 to 15% gradient SDS−PAGE under reducing and nonreducing conditions and visualized by silver staining or transferred to polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) for standard Western blotting procedures using monoclonal antibody 2F12 directed against the α-chain of C4BP followed by a polyclonal peroxidase-conjugated antibody against mouse antibodies (Dako, Glostrup, Denmark). Human prothrombin, protein S, and factor X were purified as described by Hackeng et al. (*31*). Factor X was activated with immobilized Russell's Viper Venom according to the method of Bock et al. (*35*). Monoclonal antibody CLB-PS13 directed against protein S was a generous gift from J. A. van Mourik (Central Laboratory of the Netherlands Red Cross Blood Transfusion Services, Amsterdam, The Netherlands). A truncated β-chain of C4BP fused to a modified tissue plasminogen activator (SCR-1+2) was constructed as described previously (*36*). Purified activated factor V from a patient homozygous for the $Arg^{506} \rightarrow Gln^{506}$ mutation (factor Va^{R506Q}) was obtained as described previously (*37*).

Preparation of Phospholipid Vesicles. Phospholipid vesicles containing 20% phosphatidylserine, 40% phosphatidylcholine, and 40% phosphatidylethanolamine were prepared as described by van Wijnen et al. (*38*).

Inactivation of Factor Va by APC and Determination of Residual Factor Va Activity. Preincubations of C4BP, rC4BPα, anti-C4BP monoclonal antibodies, CLB-PS13, C4BP β-chain SCR-1+2, and protein S were performed for 30 min at 37 °C in Tris-buffered saline (TBS) [50 mM Tris-HCI (pH 7.4) and 150 mM NaCl] containing 0.3% (w/v) bovine serum albumin (BSA) and 3 mM $CaCl_2$. After preincubation, APC was added and the mixture incubated for 3 min at 37 °C. Human factor Va (6 pM) was incubated with APC (20 pM) at 37 °C in TBS containing 0.3% BSA, 5 μM phospholipid vesicles, and 3 mM $CaCl_2$. During inactivation, 15 μL aliquots were taken from the inactivation mixture and residual factor Va activity was immediately determined from the rate of prothrombin activation in 60 μL mixtures containing 1.5 pM factor Va/Vi derived from the inactivation mixtures, 5 nM factor Xa, 500 nM prothrombin, 10 μM phospholipid vesicles, and 3 mM $CaCl_2$, in TBS containing 0.3% BSA. The rate of thrombin formation was proportional to the amount of factor Va.

RESULTS

Expression and Purification of rC4BP. α-C4BP consisting of only α-chains (rC4BPα) was obtained by expression of the α-chain cDNA in baby hamster kidney cells. Expression levels in the medium were determined by an ELISA and were 1−5 μg/mL after being cultured for 3 days. After immunoaffinity purification, rC4BPα appeared on SDS−PAGE as a single band which had the same molecular mass as purified plasma C4BP, a doublet corresponding to the isoform of C4BP containing six or seven α-chains (Figure 1). Due to glycosylation of the subunits that comprise the C4BP molecule, both rC4BPα and purified plasma C4BP appeared under reducing conditions as a diffuse band of approximately 75 kDa corresponding to the α-chain of C4BP (Figure 1). Because of the high level of glycosylation and the low molecular mass of the β-chain, this subunit was not visible on the silver-stained gel of reduced purified plasma C4BP in Figure 1A. Purified plasma C4BP and rC4BPα were also visualized by Western blotting using monoclonal antibody 2F12 directed against the α-chain of C4BP (Figure 1B). On the Western blot, an additional faint band with a molecular mass of approximately 200 kDa was visible in the nonreduced C4BP preparations (Figure 1B). Since rC4BPα did not bind to protein S (data not shown), we conclude that rC4BPα does not contain the protein S-binding β-chain of C4BP.

APC-Mediated Inactivation of Factor Va. To investigate the effect of C4BP and rC4BPα on factor Va inactivation by APC, the residual factor Va activity after inactivation for different periods of time by APC was monitored using a prothrombinase-based assay system (Figure 2). Factor Va activity was stable in the absence of APC during the time period that was investigated (0−12 min). APC inactivated factor Va via a biphasic reaction which, when fitted according to Nicolaes et al. (*8*), yielded second-order rates constants for cleavage at Arg^{506} and Arg^{306} of 4.4×10^8 and

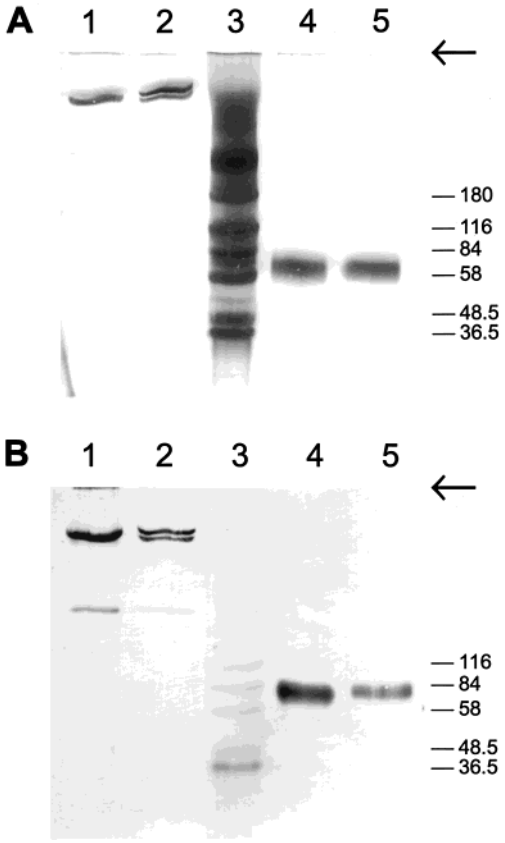

FIGURE 1: SDS−PAGE and Western blotting of rC4BPα and purified plasma C4BP. Purified C4BP preparations were subjected to 4 to 15% gradient SDS−PAGE under nonreducing (lanes 1 and 2) and reducing (lanes 4 and 5) conditions and visualized by silver staining (A) or Western blotting using monoclonal antibody 2F12 directed against the α-chain of C4BP (B): lanes 1 and 4, rC4BPα; lanes 2 and 5, purified plasma C4BP; and lane 3, molecular mass markers (kilodaltons). The arrow indicates the border between the stacking and running gel.

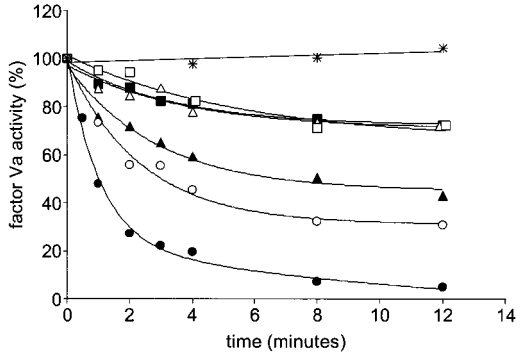

FIGURE 2: APC-mediated inactivation of factor Va. Factor Va (6 pM) was inactivated at 37 °C in a reaction mixture containing 20 pM APC, 5 μM phospholipid vesicles, no protein S (white symbols) or 50 nM protein S (black symbols), and no C4BP (circles), 160 nM C4BP (squares), or 160 nM rC4BPα (triangles). Before addition to the reaction mixture, APC, protein S, C4BP, and/or rC4BPα were preincubated as described in Experimental Procedures. After inactivation for different periods of time, the residual factor Va activity was determined as described in Experimental Procedures. During the time period that was investigated, factor Va activity was stable in the absence of APC (*). Values are the means of at least two separate experiments.

2.7×10^7 M^{-1} s^{-1}, respectively. Factor Va inactivation was inhibited by the addition of 160 nM C4BP, which slowed the initial phase of factor Va inactivation approximately 10-fold. A similar inhibition of factor Va inactivation was observed with 160 nM rC4BPα.

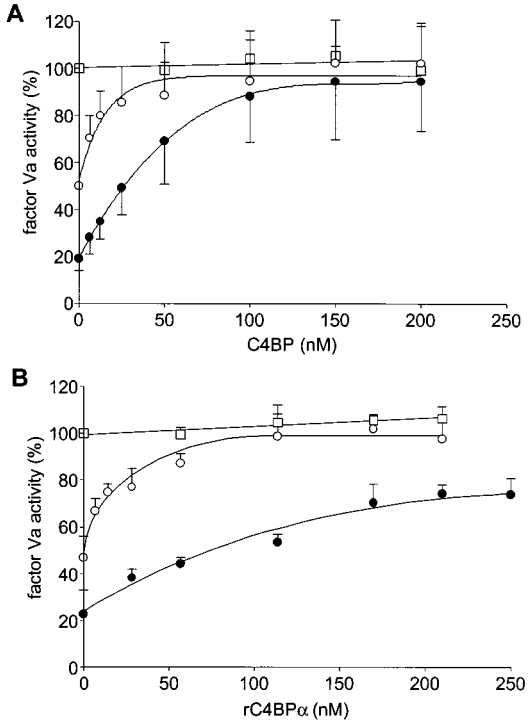

FIGURE 3: Inhibition of APC-mediated factor Va inactivation by C4BP. Factor Va (6 pM) was inactivated at 37 °C for 4 min on 5 μM phospholipid vesicles by 20 pM APC with or without 50 nM protein S and the concentration of C4BP (A) or rC4BPα (B) indicated in the figure. Before addition to the reaction mixture, APC, protein S, C4BP, and/or rC4BPα were preincubated as described in Experimental Procedures. After inactivation, the residual factor Va activity was determined as described in Experimental Procedures: (□) no APC, (○) APC alone, and (●) APC and protein S. Values are the means of at least three separate experiments ± the standard deviation.

Inactivation of factor Va was accelerated by protein S (Figure 2). In the presence of protein S, factor Va was almost completely inactivated by APC after 12 min. Assuming that protein S preferentially stimulated cleavage at Arg^{306} (*23*), we can determine that the level of cleavage of this peptide bond was increased 10-fold by 50 nM protein S. APC-catalyzed factor Va inactivation in the presence of protein S was almost completely inhibited by 160 nM C4BP. In the presence of protein S, rC4BPα only partly protected factor Va from inactivation by APC. This is in agreement with the fact that rC4BPα lacks the protein S-binding β-chain and thus is not able to inhibit the protein S cofactor activity.

Inhibition of APC-Mediated Factor Va Inactivation by C4BP. The concentration dependence of the effect of C4BP and rC4BPα on APC-mediated inactivation of factor Va was investigated by quantifying the residual factor Va activity after a fixed period of inactivation. After inactivation of factor Va by APC for 4 min in the presence of a phospholipid surface, the remaining factor Va activity was 50% in the absence of protein S and 20% in the presence of 50 nM protein S (Figure 3A,B). High concentrations of C4BP (>150 nM) almost completely protected factor Va from inactivation by APC in both the absence and presence of protein S. The C4BP concentrations required for half-maximal protection ($K_{1/2}$) were ∼10 nM in the absence of protein S and ∼40 nM in the presence of protein S (Figure 3A).

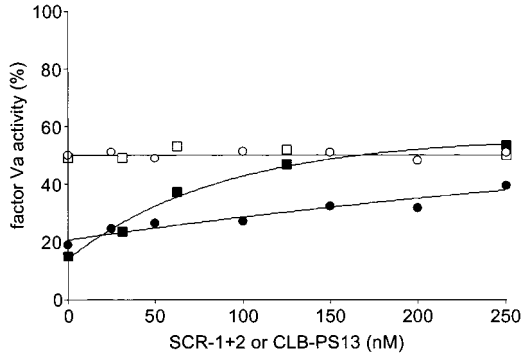

FIGURE 4: Effect of CLB-PS13 or a truncated C4BP β-chain (SCR-1+2) on the protein S cofactor activity in the APC-mediated inactivation of factor Va. Factor Va (6 pM) was inactivated for 4 min at 37 °C in a reaction mixture containing 20 pM APC, 5 μM phospholipid vesicles, no protein S (white symbols) or 50 nM protein S (black symbols), and increasing concentrations of CLB-PS13 (squares) or SCR-1+2 (circles). Before addition to the reaction mixture, APC, protein S, CLB-PS13, and/or SCR-1+2 were preincubated as described in Experimental Procedures. After inactivation, the residual factor Va activity was determined as described in Experimental Procedures. Values are the means of at least two separate experiments.

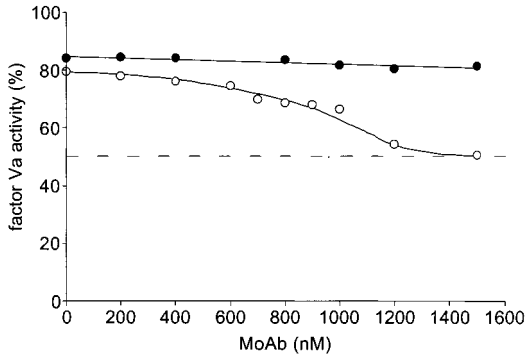

FIGURE 5: Effect of monoclonal antibodies against C4BP on C4BP-mediated protection of factor Va in the absence of protein S. C4BP (100 nM) was preincubated for 30 min at 37 °C with increasing concentrations of monoclonal antibody 9F6 (○) or 9HI0 (●) directed against the α-chain of C4BP. C4BP preincubated with antibody was subsequently added to APC (20 pM) and the mixture incubated for an additional 3 min at 37 °C. C4BP/monoclonal antibody/APC preincubation mixtures were added to factor Va (6 pM) in the presence of 5 μM phospholipid vesicles, and factor Va was inactivated for 4 min at 37 °C. After inactivation, the residual factor Va activity was determined as described in Experimental Procedures. Values are the means of two separate experiments. The dashed line indicates the factor Va activity in the presence of APC without C4BP.

To investigate whether the protective effect of C4BP was mediated by the α-chains, the same experiment was performed with rC4BPα (Figure 3B). rC4BPα was as effective as C4BP in protecting factor Va from inactivation by APC in the absence of protein S (Figure 3B). However, in the presence of protein S, rC4BPα was much less effective in protecting factor Va ($K_{1/2}$ = 160 nM). The remainder of the inactivation was most likely caused by the protein S cofactor activity to APC that could not be inhibited by rC4BPα.

The possibility that the C4BP-mediated effect was due to a contamination of the used purified components (e.g., APC and factor Va) with trace amounts of protein S was excluded by performing experiments with CLB-PS13, a monoclonal antibody directed against protein S. CLB-PS13 inhibited protein S activity, and at a concentration of 250 nM, the level of factor Va inactivation was restored to that obtained in the absence of protein S (Figure 4). Thus, CLB-PS13 inhibited the protein S cofactor activity but had no effect on the inactivation of factor Va by APC in the absence of protein S. Addition of 250 nM CLB-PS13 in the absence of protein S did not have an effect on the C4BP-mediated protection of factor Va (data not shown).

Effect of a Truncated C4BP β-Chain (SCR-1+2) on the Protein S Cofactor Activity in the APC-Mediated Inactivation of Factor Va. A truncated C4BP β-chain (SCR-1+2) consisting of the first two amino-terminal short consensus repeats (SCRs) from the β-chain fused to a modified tissue plasminogen activator (*36*) inhibited the protein S cofactor activity but had no effect on APC-mediated inactivation of factor Va in the absence of protein S (Figure 4). This shows that the α-chain of C4BP was responsible for the protection of factor Va from inactivation by APC in both the absence and presence of protein S, whereas the truncated chain only inhibited inactivation of factor Va in the presence of protein S.

Effect of Antibodies Directed against C4BP on C4BP-Mediated Protection of Factor Va. The C4BP-mediated protection of factor Va from APC-mediated inactivation was prevented by preincubation of C4BP with monoclonal antibody 9F6 directed against the α-chain of C4BP (Figure 5, ○). After preincubation of 100 nM C4BP with 0−1500 nM 9F6, the factor Va activity decreased from 80% in the absence of 9F6 (maximum protection by 100 nM C4BP in this experiment) to 50% in the presence of 1500 nM 9F6 (no protection).

Strikingly, 50% inhibition of the C4BP-mediated protection was observed at an approximately 7−8-fold molar excess of 9F6 over C4BP, corresponding to the number of α-chains present in C4BP. Another monoclonal antibody directed against the α-chain of C4BP (9H10) did not affect the C4BP-mediated protection of factor Va (Figure 5, ●).

Inactivation of Factor Va^{R506Q}. To investigate whether C4BP protects a specific site from APC-mediated cleavage, the same experiments whose results are shown in Figure 2 were performed with activated factor V purified from plasma of a patient homozygous for the $Arg^{506} \rightarrow Gln^{506}$ mutation (factor Va^{R506Q}). Since factor Va^{R506Q} is resistant to APC, 25-fold higher concentrations of APC were needed to inactivate factor Va^{R506Q} within a time frame similar to that of normal factor Va. As shown in Figure 6, 500 pM APC resulted in an inactivation of factor Va^{R506Q} that was almost complete after 12 min. Addition of 200 nM C4BP inhibited APC-mediated inactivation of factor Va^{R506Q} more than 4-fold, indicating that C4BP also prevented cleavage at Arg^{306}.

DISCUSSION

We have studied the effect of C4BP on the APC-mediated inactivation of factor Va and showed that purified plasma C4BP inhibited inactivation of factor Va by APC in both the absence and presence of protein S (Figures 2 and 3A). The fact that C4BP-mediated protection of factor Va from inactivation by APC was also observed at a 10-fold higher phospholipid concentration and that C4BP did not affect the cofactor activity of factor Va in prothrombin activation (data not shown) excludes the possibility that our findings were

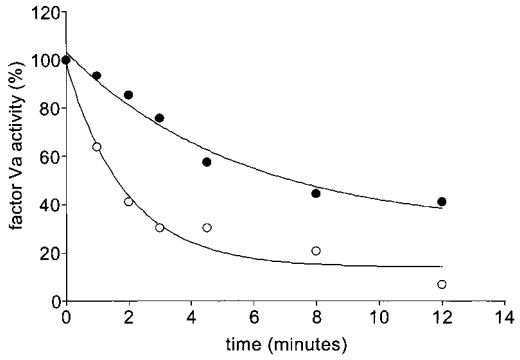

FIGURE 6: Effect of C4BP on the time course of factor Va^{R506Q} inactivation by APC. Factor Va^{R506Q} (6 pM) was inactivated at 37 °C in a reaction mixture containing 500 pM APC, 5 μM phospholipid vesicles, and no C4BP (○) or 200 nM C4BP (●). Before addition to the reaction mixture, APC was incubated with C4BP for 3 min at 37 °C. After inactivation for different periods of time, the residual factor Va activity was determined as described in Experimental Procedures. Values are the means of at least two separate experiments.

caused by direct competition of proteins for binding to a limited phospholipid surface. It is also unlikely that the C4BP-mediated effect was due to scavenging of trace amounts of protein S present as contamination in the reaction systems, since CLB-PS13, an antibody that inhibits the protein S cofactor activity (Figure 3), affected neither inactivation of factor Va by APC in the absence of protein S nor C4BP-mediated protection of factor Va. However, C4BP-mediated protection against inactivation by APC in the absence of protein S was specifically inhibited by a monoclonal antibody against the α-chain of C4BP (9F6, Figure 5). Taken together, these results exclude the possibility that the effect of C4BP was due to an artifact or was mediated through contaminating traces of protein S present in the reaction systems and indicate that the C4BP-mediated protection of factor Va from inactivation by APC was caused by specific interactions of C4BP with APC and/or factor Va.

A recombinant C4BP that lacked the β-chain (rC4BPα) also inhibited APC-catalyzed factor Va inactivation (Figures 2 and 3B). In the presence of protein S, rC4BPα was, however, less effective than C4BP in protecting factor Va. Since rC4BPα lacks the protein S-binding β-chain, this was most likely caused by the fact that rC4BPα does not inhibit the protein S cofactor activity, thus leading to a decreased level of factor Va protection compared with that of purified plasma C4BP.

Although the inhibition of APC-mediated inactivation of factor Va at high concentrations of purified plasma C4BP (Figure 3A) or rC4BPα (Figure 3B) in the absence of protein S was almost complete, the inactivation continued slowly upon prolonged incubation (Figure 2). The remainder of the inactivation may be due to cleavage at Arg^{306}, which is known to proceed at a slow rate (*8*). This may also explain why rC4BPα, which lacks the protein S-binding β-chain, did not protect factor Va in the presence of protein S to the same extent as purified plasma C4BP which protected factor Va against APC with the same effectiveness in the absence and presence of protein S. Since protein S increases the level of inactivation of factor Va by specifically accelerating cleavage at Arg^{306} (*8*), the decreased level of protection by rC4BPα compared with that by purified plasma C4BP may be caused by accelerated cleavage at Arg^{306}.

The question of whether C4BP specifically protects Arg^{506} against APC-mediated cleavage was addressed using factor Va^{R506Q}. As shown in Figure 6, C4BP also inhibits APC-mediated inactivation of factor Va^{R506Q}. Thus, C4BP does not protect a specific site from APC-mediated cleavage, but inhibits APC activity independent of cleavage site. Since C4BP affects neither the amidolytic activity of APC nor factor VIIIa inactivation by APC,[2] it is likely that the mechanism by which C4BP inhibits APC-mediated factor Va inactivation involves interactions of C4BP with secondary binding sites on APC or factor Va, required for formation of the APC−factor Va complex.

Interestingly, a truncated form of the β-chain of C4BP (SCR-1+2) described previously (*36*) inhibited the protein S cofactor activity, whereas in the absence of protein S, SCR-1+2 did not affect factor Va inactivation (Figure 3). This confirms that protection of factor Va by C4BP is indeed mediated by the α-chains of C4BP, and that the β-chain only inhibits the cofactor activity of protein S. The fact that SCR-1+2 only contains the first two amino-terminal short consensus repeat (SCR) modules (fused to a modified tissue plasminogen activator; *36*) may explain why it was not a very potent inhibitor of the protein S cofactor activity. Although binding of protein S to APC is not prevented by C4BP (*27*), steric hindrance may be involved in the inhibition of protein S cofactor activity in APC-mediated factor Va inactivation. Reduced steric hindrance due to the smaller size of SCR-1+2 compared with C4BP may explain the weak inhibition of protein S cofactor activity by SCR-1+2.

The inhibitory effect of C4BP on the APC-mediated inactivation of factor Va was not found in a previous study by Dahlbäck (*26*), who used a similar system to measure the effect of C4BP on the inactivation of factor Va. This discrepancy may be explained by the concentrations of APC and C4BP that were used. Dahlbäck used APC concentrations that were 10−50 times higher than those in our study. Since the C4BP concentrations used were similar in the two studies, the APC concentrations used by Dahlbäck may have abrogated the C4BP-mediated inhibition of factor Va inactivation. Interestingly, the level of C4BP-mediated protection in our system was decreased at increasing APC concentrations (not shown). The experiments described here were performed at low concentrations of APC and factor Va to mimic a physiological situation.

The finding that C4BP protected factor Va from APC-mediated inactivation may play a role in the pathogenesis of thrombosis. C4BP is an acute phase reactant (*39*, *40*), and C4BP concentrations can increase up to 4-fold in an acute phase reaction. Increased C4BP levels could result in an inhibition of factor Va inactivation, and may therefore explain the thrombotic events that can occur during an acute phase response (*40*−*42*). It should be noted that activation of the complement system during inflammatory reaction results in the generation of C4b. C4b binds to the α-chain of C4BP (*43*), and this may influence the protective effect of C4BP on the inactivation of factor Va.

The protection of factor Va from APC-mediated inactivation in our purified system was observed at physiological concentrations of C4BP, implying that factor Va inactivation

[2] Robbert H. L. van de Poel, Joost C. M. Meijers, and Bonno N. Bouma, manuscript submitted for publication.

would hardly occur in plasma. However, in an activated partial thromboplastin time-based clotting assay using C4BP/protein S double deficient plasma as described previously (*36*), addition of 200 nM C4BP did not affect the prolonging of the clotting time induced by 30 nM APC (not shown). Our experiments in the system containing purified proteins were carried out at an APC concentration of only 20 pM, and increasing the concentration of APC resulted in a decrease in the protective effect of C4BP on factor Va. The fact that we did not observe a protective effect of C4BP in the plasma milieu could therefore be due to the high concentrations of APC that were needed to induce a prolonging of the clotting time. In addition, prothrombin may have masked the protective effect of C4BP on factor Va in plasma, because prothrombin itself was recently shown to protect factor Va against inactivation by APC (*44*).

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