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C4b-Binding Protein (C4BP) β-Chain Short Consensus Repeat-2 Specifically Contributes to the Interaction of C4BP with Protein S

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Abstract: C4b-binding protein (C4BP) regulates the complement system and the anticoagulant activity of protein S. Protein S can bind to C4BP, resulting in a decreased cofactor activity of protein S for anticoagulant activated protein C. C4BP contains several identical α-chains and a single β-chain. Each chain contains Short Consensus Repeats (SCRs). By making chimeras of β-chain SCRs fused to tissue-type plasminogen activator (tPA chimeras), we found that β-chain SCR-2 contributed to the interaction of β-chain SCR-1 with protein S (van de Poel RHL, Meijers JCM, Bouma BN. *J Biol Chem* 274:15144-15150, 1999). Chimeras containing C4BP α-chains with SCR-1, SCR-1+2 or SCR-1+2+3 replaced by their β-chain counterpart had affinities for protein S similar to C4BP (Härdig Y, Dahlbäck B. *J Biol Chem* 271:20861-20867, 1996). This was not in agreement with the finding that β-chain SCR-2 contributed to the interaction and could be explained by the possibility that α-chain SCR-2 in the α-chain chimeras contributed comparable with β-chain SCR-2 in the tPA chimeras. To investigate this we constructed a tPA chimera containing β-chain SCR-1 and α-chain SCR-2 (β1α2). Binding studies showed that β1α2 had a lower affinity compared with SCR-1+2, indicating that α-chain SCR-2 did not contribute to the interaction. The difference with the α-chain chimeras may be explained by the fact that the α-chain chimeras were linked by their C-terminal cysteines, resulting in multiple binding sites in a single molecule. Thereby, the effect of a lower affinity of each α-chain chimera may have been masked. The studies performed here help to clarify the apparent inconsistencies in two previous reports about the contribution of the SCR-2 domain in C4BP to protein S binding. In conclusion, β-chain SCR-2 specifically contributes to the interaction of SCR-1 with protein S.

Keywords: protein S, C4b-binding protein, protein C, blood coagulation, complement

INTRODUCTION

C4b-binding protein (C4BP) regulates the classical pathway of complement activation (1-5). It accelerates C2a decay from the C3-convertase (C4b2a) complex (4,6) and promotes degradation of C4b by factor I (2-4,7). C4BP also binds to anticoagulant vitamin K-dependent protein S in a high affinity 1:1 stoichiometric complex (8-11). By binding of protein S to C4BP, the cofactor function of protein S for anticoagulant activated protein C (APC) in the inactivation of coagulation factors Va and VIIIa is decreased (12,13). Binding of protein S to C4BP does not interfere with the inhibition of complement activation. C4BP contains six or seven identical α-chains (M_r 70,000) and 80-85% of the C4BP molecules contain an additional single β-chain (M_r 45,000) that binds to protein S (8,14,15). Both the α- and β-chains contain cysteine residues in their C-terminal part that form the interchain disulfide bridges in the so-called core region of C4BP (16). Under normal conditions approximately 60% of total protein S is in complex with C4BP and 40% is free (17). C4BP levels can increase up to four-fold during an acute phase response, and due to a mechanism of differential regulation of α- and β-chain expression by which mainly the expression of α-chains increases, free protein S levels remain stable (17). The α- and β-chains are composed of short consensus repeats (SCRs), also called complement control protein (CCP) modules or

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Sushi domains (16,18). SCRs have been shown to have complement C3b/C4b binding properties (19) in complement regulatory proteins such as factor H and Decay Accelerating Factor. However, SCRs have also been found in noncomplement regulatory proteins such as β2-glycoprotein I and the β-subunit of coagulation factor XIII in which the function of SCRs is unknown (for reviews see references 19 and 20). The α-chain of C4BP contains eight SCRs, the β-chain contains three SCRs. It is known from previous studies that the protein S binding site is localized within the NH_2-terminal SCR unit (SCR-1) of the β-chain (21-25). Chimeras containing C4BP α-chains with SCR-1, SCR-1+2 or SCR-1+2+3 replaced by their β-chain counterpart had affinities for protein S comparable with plasma C4BP (24). In our previous study using chimeras of β-chain SCRs fused to the NH_2-terminus of a modified tissue-type plasminogen activator (tPA chimeras, reference 26), we found that β-chain SCR-2 contributed to the interaction of SCR-1 with protein S (25). The experiments using the α-chain chimeras showed a different effect compared with the tPA chimeras. To investigate whether α-chain SCR-2 in the α-chain chimeras contributed in a similar way as β-chain SCR-2 in the tPA chimeras to the interaction of protein S with β-chain SCR-1, a tPA chimera was constructed containing β-chain SCR-1 and α-chain SCR-2 (designated β1α2). This report helps to clarify the apparent inconsistencies in two previous reports about the contribution of SCR-2 in the C4BP to protein S binding. It was found that the contribution to the affinity for protein S was specific for β-chain SCR-2, since this could not be substituted by SCR-2 of the α-chain of C4BP.

MATERIALS AND METHODS

Proteins

C4BP was immunopurified from human plasma as described (27). Protein C was purified and activated as described previously (28). Protein S was purified from prothrombin concentrates as described (29). SCR-tPA chimeras containing β-chain SCRs were expressed and immunopurified as described in our previous report (25).

Cloning Procedure

The first NH_2-terminal SCR from the β-chain (β1) and the second NH_2-terminal SCR from the α-chain (α2) of C4BP were amplified by PCR amplification using the DNA construct containing the β/α-chain chimera designated β1α as described (24) as a template. The oligonucleotides used for construction of the β1α2 chimera were SCR1βF2 (5' TTT **AGA TCT** GAG CAC TGT CCA GAG CTT CCT CCA GTG 3') and SCR2αR (5' T TTC TCG AGT TTC ACA TTG TGG GAG AGG AT 3'). After amplification, the PCR product was cleaved with *Bgl*II (boldface) and *Xho*I (underlined) and cloned in *Bgl*II/*Xho*I-cleaved expression vector Zpl7 containing a modified tissue-type plasminogen activator (tPA) (30). The sequence of the amplified region was confirmed by dideoxy sequencing.

Cell Line and Culture Conditions

Transfection of baby hamster kidney cells was performed as described previously (31). Expression of all constructs 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 Recombinant Proteins

Purification of chimeric SCR-tPA constructs was performed as described previously (26) using a monoclonal antibody against tPA. Concentrations of chimeric SCR-tPA constructs were determined using an ELISA system that determines tPA concentration (Imulyse tPA, Kordia, Leiden, The Netherlands). Purified

constructs were applied to 10% SDS-PAGE under nonreducing conditions, and stained by Coomassie brilliant blue.

Ligand-Binding Assays

The binding of protein S to immobilized SCR-tPA chimeras, binding of SCR-tPA chimeras to immobilized protein S, stoichiometry of the interaction between protein S and β1α2 and the competition of SCR-tPA chimeras with immobilized C4BP for protein S binding was investigated using the same experiments as described in our previous report (25). These assays were all performed in the presence of 5 mM $CaCl_2$. Purified SCR-tPA chimeras and protein S were immobilized using polyclonal antibodies directed against tPA and protein S, respectively. The binding of protein S to immobilized tPA chimeras was performed with a minor modification: purified SCR-tPA constructs at a coating concentration of 10 nM were used instead of culture supernatant.

Clotting Assay

The effect of SCR-tPA chimeras on protein S cofactor activity was performed using an Activated Partial Thromboplastin Time (APTT)-based assay as described previously (25). Prior to the clotting assay, protein S and the tPA chimeras were preincubated for 30 min in the presence of 3 mM $CaCl_2$.

RESULTS

Expression and Purification of Chimeras

In order to investigate if the contribution of SCR-2 to the interaction of protein S with SCR-1 is specific for SCR-2 from the β-chain, a chimera was constructed containing SCR-1 from the β-chain and SCR-2 from the α-chain of C4BP fused to the NH_2-terminus of a modified tPA. Baby

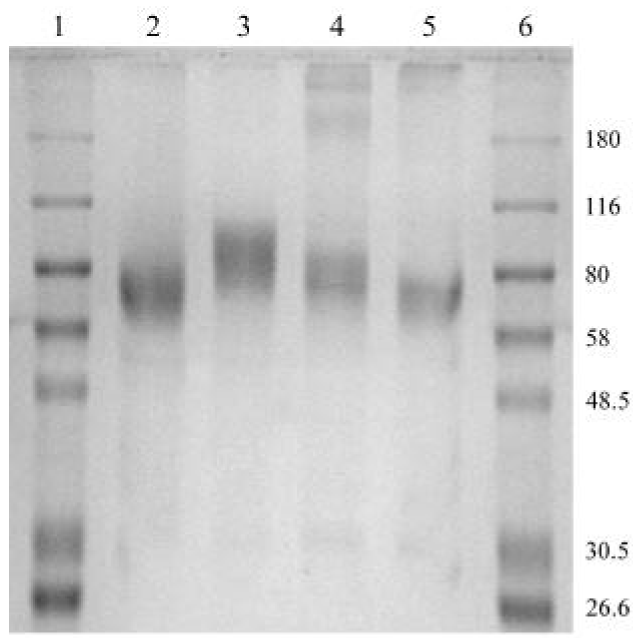

Figure 1. SDS-PAGE of SCR-tPA chimeras. Purified tPA chimeras (1.5 μg) were applied to 10% SDS-PAGE gel under nonreducing conditions. Proteins were visualized by Coomassie brilliant blue staining. *Lane 1* and *6*, molecular weight markers (kDa); *lane 2,* SCR-1-tPA; *lane 3*, SCR-1+2-tPA; *lane 4*, SCR-1+3-tPA; *lane 5*, SCR-β1α2-tPA.

hamster kidney cells were transfected with the expression vector containing the SCR-tPA chimeras. Expression levels in the medium were detected using a tPA ELISA system and were 1-5 μg/ml after 3 days of culture. After purification of the constructs with an immobilized monoclonal antibody against tPA, the constructs were applied to 10% SDS-PAGE under nonreducing conditions and stained by Coomassie brilliant blue (Figure 1). As seen in our previous report (25), the SCR-tPA constructs appeared as diffuse bands which is probably caused by heterogeneous glycosylation of the SCRs and the tPA module. The construct β1α2 migrated as a band with an estimated molecular weight of 70,000-75,000, which was slightly higher then SCR-1. In principle, one would expect the construct β1α2 to have a mobility approximately the same as SCR-1+2 and SCR-1+3. This was not the case however, and is most likely due to glycosylation that is known to considerably affect the mobility shift of proteins.

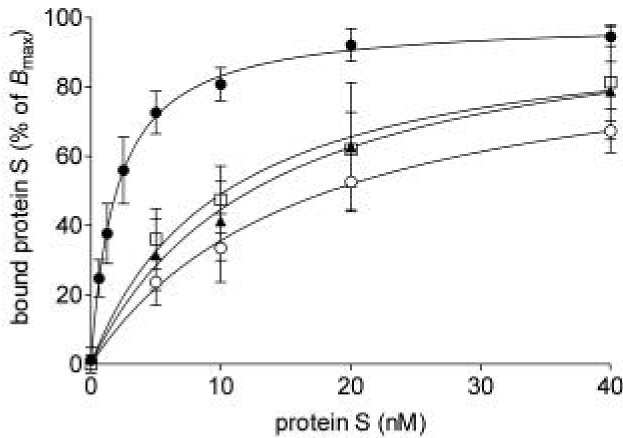

Figure 2. Binding of protein S to immobilized chimeras. SCR-tPA constructs were immobilized to microtiter wells using polyclonal antibodies against tPA. After 2 hours of incubation with increasing concentrations of human protein S, bound protein S was detected using peroxidase-conjugated polyclonal antibodies against protein S. After correction for background absorbance, bound protein S was expressed as a percentage of B_{max}. Values are displayed as means (±; SD) of three separate experiments. ○, SCR-1; ●, SCR-1+2; ▲, SCR-1+3; □, β1α2.

Binding of Protein S to Immobilized Chimeras

The interaction of the SCR-tPA constructs with protein S was investigated using a direct binding assay in which protein S was allowed to bind to immobilized SCR-tPA chimeras (Figure 2). The results were expressed as a percentage of maximum binding (B_{max}) for each construct. Apparent dissociation constants of the binding of protein S to tPA chimeras were 16.9 nM for SCR-1, 2.0 nM for SCR-1+2 and 13.5 nM for SCR-1+3, respectively. These values were similar to our previous findings with these constructs (25). The affinity for the binding between protein S and C4BP is in the range between 2 and 5 nM (10,24). The apparent dissociation constant for the binding of protein S to β1α2 was 10.2 nM. These results showed that protein S had an affinity for β1α2 comparable with SCR-1 and SCR-1+3.

Binding of Chimeras to Immobilized Protein S

A binding assay was performed in which SCR-tPA constructs were allowed to bind to immobilized protein S. The results of the binding experiments of SCR-tPA chimeras to immobilized protein S are presented in Figure 3. Binding of each SCR-tPA construct was expressed as a percentage of B_{max} to protein S. The apparent dissociation constants for the binding of tPA chimeras to protein S were 23.1 nM for SCR-1, 1.5 nM for SCR-1+2 and 14.0 nM for SCR-1+3, respectively. These apparent dissociation constants were somewhat lower as previously reported (25), but showed the same effect of β-chain SCR-2 in the interaction of protein S with SCR-1. The reason for the discrepancy of the apparent dissociation constants for SCR-1, SCR-1+2 and SCR-1+3 compared with our previous study (25) is unknown but it did not affect the conclusion that β-chain SCR-2 contributed to the interaction of protein S with β-chain SCR-1. The apparent dissociation constant for the binding of β1α2 to protein S was 14.0 nM. These results showed that β1α2 had an affinity for protein S comparable with SCR-1 and SCR-1+3.

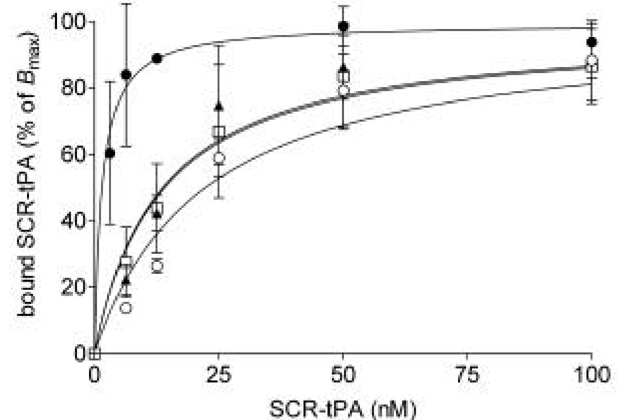

Figure 3. Binding of chimeras to immobilized protein S. Human protein S was immobilized to microtiter wells using polyclonal antibodies. After 2 hours of incubation with increasing concentrations of SCR-tPA chimeras, bound chimeras were detected using polyclonal antibodies against tPA. After correction for background absorbance, bound constructs were expressed as percentages of B_{max}. Values are displayed as means (± SD) of three separate experiments. ○, SCR-1; ●, SCR-1+2; ▲, SCR-1+3; □, β1α2.

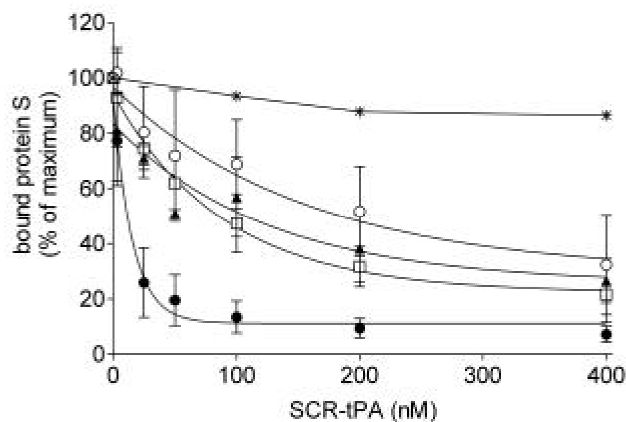

Figure 4. Competition of chimeras with immobilized C4BP for protein S binding. C4BP was immobilized to microtiter wells using monoclonal antibodies against the C4BP α-chain. After 1 hour of preincubation of human protein S with SCR-tPA chimeras, protein S was allowed to bind to immobilized C4BP for 2 hours. Bound protein S was detected using peroxidase-conjugated polyclonal antibodies against protein S. After correction for background absorbance, bound protein S was expressed as a percentage of maximum binding in the absence of SCR-tPA chimeras. Values are displayed as means (± SD) of three separate experiments. ○, SCR-1; ●, SCR-1+2; ▲, SCR-1+3; □, β1α2; ✳, tPA.

Stoichiometry of the Interaction between Protein S and ***b****1****a****2*

The stoichiometry of the interaction between protein S and β1α2 was analyzed using a fluid phase binding assay as described (25). A value of 1.3 was found for the ratio of maximum binding of β1α2 and the protein S concentration used in this fluid phase binding assay (mean of two separate determinations), which is in agreement with the existence of a single protein S binding site on β1α2. This was also found for SCR-1, SCR-1+2 and SCR-1+3 (25).

Competition of Chimeras with Immobilized C4BP for Protein S Binding

SCR-tPA constructs were allowed to compete with immobilized plasma purified C4BP to bind to protein S. Purified human protein S (0.5 nM) was preincubated with increasing concentrations of SCR-tPA constructs and then allowed to bind to immobilized purified human C4BP. Bound protein S was expressed as a percentage of maximum binding in the absence of SCR-tPA chimeras in Figure 4. Preincubation of protein S with SCR-1, SCR-1+2, SCR-1+3 or β1α2 resulted in an inhibition of protein S binding, and a 50% inhibition of protein S binding was observed at 185 nM SCR-1, 12 nM SCR-1+2, 107 nM SCR-1+3 and 85 nM β1α2, respectively. Thus, higher concentrations of β1α2 were necessary to yield a 50% inhibition of protein S binding to immobilized C4BP compared with SCR-1+2. The concentrations of SCR-1, SCR-1+2 and SCR-1+3 at which a 50% inhibition of binding of protein S occurred were comparable with the concentrations found in our previous report (25). As a control, tPA alone did not inhibit the binding of protein S to C4BP.

Protein S Cofactor Activity in Plasma

The effect of the SCRs on the cofactor activity of protein S was tested by preincubation of 20 nM protein S (final concentration) with increasing concentrations of SCR-tPA constructs. Subsequently, C4BP- and protein S depleted plasma was added and the clotting time was determined in the presence of 30 nM APC. The residual cofactor activity of protein S after preincubation with SCR-tPA chimeras is shown in Figure 5. SCR-1+2 yielded a 50% inhibition of protein S cofactor activity at a concentration of approximately 20 nM. A 50% inhibition of protein S cofactor activity by β1α2 was achieved at higher concentrations, approximately 138 nM. As a comparison, the inhibition of protein S cofactor activity by SCR-1 and SCR-1+3 is also displayed in Figure 5. Concentrations higher than 175 nM SCR-1 and SCR-1+3 were necessary to yield a 50% inhibition of protein S cofactor activity. The values for SCR-1, SCR-1+2 and SCR-1+3 that were necessary to yield a 50% inhibition of protein S cofactor activity were in agreement with our previous report (25). Like SCR-1 and

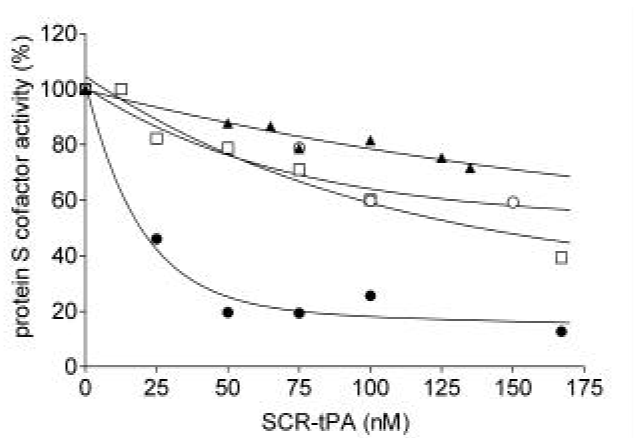

Figure 5. Protein S cofactor activity in plasma. Human protein S (final concentration 20 nM) was preincubated for 30 min with SCR-tPA chimeras and added to a mixture of C4BP- and protein S-depleted plasma and 30 nM APC. After addition of 25 mM $CaCl_2$, the clotting time was determined. Protein S cofactor activity was expressed as percentage of maximum activity in the absence of SCR-tPA constructs. Values are displayed as means of at least two experiments. ○, SCR-1; ●, SCR-1+2; ▲, SCR-1+3; □, β1α2.

SCR-1+3, β1α2 was a less potent inhibitor of the protein S cofactor activity compared with SCR-1+2.

DISCUSSION

Protein S binds to the β-chain of C4BP (15,27,32), and the binding site has been localized to residues within the first NH_2-terminal SCR module of the β-chain (21,22,24,33). Two studies were previously performed in order to investigate the contribution of other C4BP SCR modules to the interaction of C4BP with protein S (24,25). Using tPA chimeras containing β-chain SCRs, we found that SCR-2 contributed to the interaction of protein S with SCR-1 (25). The interaction of protein S with the β-chain SCRs has also been studied using C4BP α-chain chimeras in which the NH_2-terminal SCR modules 1, 1+2 or 1+2+3 were replaced by their β-chain counterpart (24). Using the α-chain chimeras, it was found that the binding affinities of all chimeras were comparable with plasma C4BP and hence there was no contributional role ascribed to β-chain SCR-2. Since we concluded from the study using the tPA chimeras that β-chain SCR-2 contributed to the interaction (25), the findings in these two separate studies were not in agreement. This difference could be explained by the possibility that in the α-chain chimeras, α-chain SCR-2 contributed to the interaction of β-chain SCR-1 with protein S as well. Possibly, α-chain SCR-2 could exert a similar function like β-chain SCR-2 implying that the contributional role for β-chain SCR-2 as described (25) was not specific for β-chain SCR-2. To answer this question, we constructed a tPA chimera containing β-chain SCR-1 (β1) and α-chain SCR-2 (α2). With this construct (designated β1α2) we performed the same experiments as described in our previous report (25). The binding of protein S to immobilized SCRs and the binding of SCRs to immobilized protein S were investigated, and competition experiments were performed in which the SCRs were allowed to compete with immobilized C4BP for protein S binding. Finally, the effect of the tPA chimeras on the cofactor activity of protein S for APC was investigated.

The binding of protein S to immobilized SCRs (Figure 2) occurred in a 1:1 stoichiometry with an affinity for β1α2 that was comparable with SCR-1 and SCR-1+3 while protein S bound to immobilized SCR-1+2 with a stronger affinity that was comparable with C4BP. The apparent dissociation constants for SCR-1, SCR-1+3 and SCR-1+2 were similar to the values described previously (25). The affinity of the binding of β1α2 to immobilized protein S (Figure 3) was also comparable with SCR-1 and SCR-1+3 while SCR-1+2 bound to immobilized protein S with a stronger affinity that was comparable with C4BP. The apparent dissociation constants found for SCR-1, SCR-1+3 and SCR-1+2 were lower as previously reported (25), but showed the same contributional effect of β-chain SCR-2 in the interaction of protein S with SCR-1. The reason for the discrepancy of the apparent dissociation constants for SCR-1, SCR-1+2 and SCR-1+3 compared with our previous study (25) is

unknown. To confirm the results of the direct binding assays, competition experiments were performed in which the SCRs were allowed to compete with immobilized C4BP for protein S binding (Figure 4). The binding of protein S to immobilized C4BP was inhibited by β1α2 at a concentration comparable with SCR-1 and SCR-1+3, while SCR-1+2 was a more potent inhibitor. This was in agreement with our previous study (25) and was also shown in the APTT-based assay that measures protein S cofactor activity in which the effect of the SCRs on the cofactor activity of protein S was investigated (Figure 5). In agreement with our previous study (25), SCR-1+2 was a strong inhibitor of protein S cofactor activity while SCR-1 and SCR-1+3 were less potent inhibitors, comparable with β1α2.

The results of the direct binding assays, the competition experiments and the studies on the effect of the SCRs on the cofactor activity of protein S clearly showed that β1α2 had an affinity for protein S that was comparable with SCR-1 and SCR-1+3, while SCR-1+2 had a stronger affinity. Apparently, SCR-2 derived from the α-chain in the construct β1α2 did not contribute to the interaction of β-chain SCR-1 with protein S. Thus, the additional effect of β-chain SCR-2 shown in our previous report using the tPA chimeras (25) and also found in this report appeared to be specific for the β-chain since α-chain SCR-2 was not able to contribute to the interaction of β1α2 with protein S. The difference found with the α-chain chimeras may be explained by the fact that the α-chain chimeras were polymeric molecules containing 7-8 subunits. The N-terminus of each subunit contained the SCR(s) derived from the β-chain, and two cysteines were present in the C-terminus that linked the subunits together in a single molecule. By combining multiple (7-8) binding sites for protein S in a single molecule, the effect of a lower binding affinity of each individual α-chain chimera that had SCR-1 replaced by the β-chain counterpart may have been masked (24).

We conclude that the second NH_2-terminal SCR unit of the C4BP β-chain contributes specifically to the interaction between protein S and the first NH_2-terminal SCR unit of the β-chain. This function has been found in our previous study (25) and in this study we showed that this function is specific for β-chain SCR-2.

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