

Involvement of Amino Acid Residues 423-429 of Human Protein S in Binding to C4b-Binding Protein

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ABSTRACT: Human protein S binds to C4b-binding protein (C4BP) both in plasma and in a system using purified proteins. Amino acid residues 420-434 of the first disulfide loop of the sex hormone binding globulin-like domain of protein S are involved in the interaction of protein S with C4BP. To define the involvement of specific polar amino acids within residues 420-434, we studied in parallel synthetic protein S peptides and recombinant protein S variants containing the same amino acid replacements, K423E, E424K, Q427E and K429E. Synthetic peptide analogs of peptide PSP-420 (residues 420-434) were assayed for binding C4BP and as inhibitors of complex formation. The PSP-420 peptide and the analogous peptide with the substitution E424K, but not the peptides containing the substitutions K423E and K429E, were able to bind C4BP. Recombinant proteins with mutations of K423E, Q427E and K429E showed reduced affinity for C4BP compared to plasma protein S, recombinant wild type protein S, or E424K-protein S. These results suggest that Lys-423, Gln-427 and Lys-429 of protein S are important for normal binding to C4BP. The anti-protein S monoclonal antibody LJ-56, raised against peptide PSP-420, recognizes only free protein S and inhibits complex formation with C4BP. Antibody LJ-56 recognized the E424K and Q427E peptides but not the K423E or K429E peptides. Similarly, the E424K and Q427E protein S mutants were recognized by LJ-56, whereas the K423E and K429E protein S mutants were not recognized. This suggests that both in the peptide PSP-420 and in protein S, Lys-423 and Lys-429 significantly contribute to binding to antibody LJ-56. These results demonstrate that protein S residues 423, 427 and 429, but not residue 424, are involved in binding to both the antibody LJ-56 and to C4BP. When peptides PSP 420 and SL-6 (residues 447-460) with carboxy-terminal amide or carboxylate moieties were compared to their ability to inhibit C4BP-protein S complexation, PSP-420-amide was the most potent. This finding together with the other results described here supports the hypothesis that the residues 420 and 434 in protein S provides a major binding site for C4BP. © 1998 Academic Press

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

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Abbreviations: BSA, bovine serum albumin; C4BP, C4b-binding protein; APC, activated protein C; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; PSP, protein S peptide; TBS, Tris buffered saline; HPLC, high performance liquid chromatography; SAAP, streptavidin conjugated to alkaline phosphatase; p-NPP, p-nitro-phenyl-phosphate; b-C4BP, biotinylated C4b-binding protein

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INTRODUCTION

Activated protein C (APC) is a vitamin K-dependent plasma glycoprotein that inactivates coagulation factors Va and VIIIa (1-4) in a reaction that is enhanced by protein S, another vitamin K-dependent protein (5-8). Patients partially deficient in protein S have an increased risk to develop thrombosis (9-11). Protein S in the immature form is a single-chain molecule and consists of a prepropeptide, a γ -carboxyglutamic acid-rich domain, a thrombin-sensitive domain, four epidermal growth factor-like domains and a sex hormone binding globulin (SHBG)-like domain in the immature form (12-14). In human plasma, protein S occurs in two forms. Approximately 60% forms noncovalent complexes with the C4b-binding protein (C4BP), a regulatory protein of the classical pathway of the complement system. The remaining 40% of plasma protein S is free, and only this form acts as cofactor to APC (9,15,16).

Three sites in the SHBG-like domain have been reported to be involved in the binding of protein S to C4BP (17-21). Walker (17) reported that a synthetic peptide representing the sequence of Gly-605 to Ile-614 of the protein S sequence binds to C4BP. Using recombinant protein S variants we have shown that Leu-608, Asp-609, Asp-611 and Glu-612 were not important for the binding of C4BP (18). Deletion of the whole second disulfide loop, residues (598-625), of the SHBG-like domain (amino acid residues Asp-583 to Ser-635), however, resulted in a reduced binding to C4BP, suggesting that the integrity of the C-terminus in some way affects the binding (19,22). However, deletion of this region also causes a major conformational change of the protein S structure, resulting in a strongly reduced affinity of anti-protein S monoclonal antibody S12 for its epitope (22). Because the S12 epitope is outside the region of residues 583-635, it cannot be excluded that the conformational change induced by the deletion of the whole second disulfide loop, including residues 583-635, causes a loss of binding affinity for C4BP.

Fernández et al. (20) reported that residues 420-434 (peptide PSP-420) of the first disulfide loop, residues (408-434), in the SHBG-like region of protein S are directly involved in binding to C4BP. Linse et al. (23) using a phage display method and synthetic peptides disputed this report and suggested that residues 447-460 constitute a portion of protein S that is important for the interaction with C4BP. To investigate further the involvement of the region of residues 420-434 in the binding to C4BP, we have used synthetic protein S peptides, recombinant protein S mutants and an anti-protein S monoclonal antibody LJ-56 raised against peptide PSP-420. Our studies confirm the involvement of the first disulfide loop in the interaction of protein S with C4BP and suggest that residues Lys-423, Gln-427 and Lys-429 are involved, whereas Glu-424 is not directly involved in binding to C4BP.

EXPERIMENTAL PROCEDURES

Materials

Restriction endonucleases Bam HI, Xba I, Sal I, Bgl II and Hind III were purchased from Pharmacia Biotechnology (Uppsala, Sweden). T4 DNA Ligase was from Bethesda Research Laboratories (Bethesda, MA, USA). T7 DNA Polymerase was from New England Biolabs (Beverly, MA, USA). All enzymes were used according to the manufacturers' instructions. *Escherichia coli* strains CJ236 and XL-1 blue were from Bio-Rad Corporation (Richmond, CA, USA). APC and protein S/C4BP-depleted plasma were prepared as described (8). C4BP and anti-C4BP monoclonal antibodies 8C11 IgG, directed against the α -chain of C4BP, were prepared as described (24). Rabbit-antihuman protein S IgG conjugated to peroxidase was from Dakopatts (Glostrup, Denmark). Fast Flow Q resin was from Pharmacia (Uppsala, Sweden). Iscovés Dulbeccos Modified Eagles Medium, penicillin G, streptomycin sulfate, glutamine and fetal calf serum were from Gibco (Paisly Park, UK). Trasylol was from Bayer (Leverkusen,

FRG). Vitamin K₁ (Konakion) was from F. Hoffman-La Roche, Ltd. (Basel, Switzerland). Streptavidin conjugated to alkaline phosphatase (SAAP) and immunopure p-nitro-phenyl-phosphate (p-NPP) were from Pierce (Rockford, IL, USA). Biotin-N-hydroxysuccinimide ester (NHS-d-biotin) was from Clontech (Palo Alto, CA, USA). All other reagents were of the highest quality available.

Peptide Synthesis and Characterization

Synthetic peptides (15 amino acids in length) were produced by the simultaneous multiple peptide synthesis method which has been described elsewhere (25). All peptides were synthesized in the carboxy-terminal amide form unless otherwise noted. Two peptides (PSP-420 and SL-6, residues 420-434 and 447-468, respectively) were synthesized in both the carboxy-terminal amide and free carboxyl forms. After synthesis, the peptides were analyzed by reverse-phase high-performance liquid chromatography (HPLC) on a Vydac C-18 column (Alltech Associates, Inc., IL) with a 0.6% acetonitrile linear gradient in 0.1% trifluoroacetic acid. Peptides were purified to homogeneity by preparative HPLC using the best conditions suggested by the analytical chromatography. Amino acid compositions and concentrations of isolated peptides were determined using 24 h hydrolysis in 6 N HCl in evacuated tubes at 110°C and subsequent analysis on a Beckman Model 6300 High Performance Analyzer as described (26). Mass spectroscopic analysis of peptides using the FIB positive ion mass spectra obtained on a VG-ZAB-VSE double focusing mass spectrometer equipped with a cesium ion gun yielded a single peak and an exact expected molecular weight. Solutions of each peptide were prepared as described (27).

Site-Directed Mutagenesis

A 2808 basepair Hind III-Bgl II fragment from the expression vector pMSVPS (18) carrying the protein S cDNA sequence was subcloned into M13mp19 digested with Bam HI and Hind III. The following 19-30 mers were used for mutagenesis: 5'-GCTTCTGGAATAGAGGAAATTATTCAAGAA-3' for the K423E variant, 5'-GGAATAAAGAAAATTATTC-3' for the E424K variant, 5'-GAAATTATTGAAGAAAAG-3' for the Q427E variant, and 5'-ATTCAAGAAGAACAAAATA-3' for the K429E variant. Underlined nucleotides are targets for mutagenesis. Site-directed mutagenesis was performed according to Kunkel (28) and Chang (18).

Cell Culture, DNA Transfection and Expression of Recombinant Protein S Variants

C127 cells (ATCC CRL 1616) were cultured in Iscove's Modified Dulbecco's Medium, supplemented with penicillin G (100 U/ml), streptomycin sulphate (100 µg/ml), glutamine (2 mM), 50 µM β-mercaptoethanol, trasyolol (10 U/ml) and 10% heat-inactivated fetal calf serum, as described (18). Cell cultures were routinely maintained at 37°C in a humidified incubator containing 5% CO₂. One day prior to transfections, cells were trypsinized and seeded with a density of 5 x 10⁵ cells/10 cm dish containing 10 ml complete medium. For transfections, 20 µg of plasmids were used and transfection was performed using a conventional calcium phosphate coprecipitation technique (29). Foci were isolated, assayed for protein S antigen using an ELISA as described (30), and expanded in medium containing 10% fetal calf serum and grown to confluency. Culture medium without fetal calf serum but with vitamin K₁ (5 µg/ml) was added. After an expression period of 48-72 h, culture medium was harvested and stored at -20°C until purification.

Purification of Protein S Variants and Immunoblotting Analysis

Protein S variants were purified on an anion-exchange column (Fast Flow Q resin, Pharmacia) as described for wild type recombinant protein S (18) according to the method of Grinnell (31) that yield only fully carboxylated molecules. The purity and integrity of the recombinant proteins was judged by SDS/PAGE on reduced 10% gels and immunoblotted using rabbit anti-protein S polyclonal antibody conjugated to peroxidase (Dako).

Protein S Assays

Protein S antigen was determined by a double polyclonal ELISA assay using rabbit anti-protein S IgG as catching antibody and rabbit anti-protein S IgG conjugated to peroxidase as tagging antibody (30). Dilutions of normal plasma in Tris/0.1% BSA buffer was used as a calibrator. Mutants of protein S were diluted in the same buffer and compared with plasma protein S. Protein S cofactor activity to APC was determined as described (18) in a clotting assay using 50 μ l of protein S-depleted and C4BP-depleted plasma, 25 μ l of APC (5 μ g/ml) in 1% BSA/TBS, 25 μ l protein S sample dilutions, and 50 μ l of cephalin-kaolin reagent (Boehringer Mannheim).

Binding of Recombinant Protein S Variants to C4BP

The complex formation between the recombinant proteins and C4BP was measured with a sensitive ELISA using C4BP and a peroxidase-conjugated anti-protein S antibody as described (18). Briefly, IgG (10 μ g/ml) from monoclonal antibody 8C11, which is directed against the α -chain of C4BP, was coated on microtiter plates (Costar, Cambridge, MA). After blocking the plate, 10 μ g/ml of C4BP was added to the wells, incubated for 1 h and washed extensively. Varying amounts of recombinant protein S (0-100 ng/ml) were added and

incubated overnight at room temperature. Following washing, bound protein S was then detected using anti-protein S IgG conjugated to peroxidase (0.3 μ g/ml in 50 μ l).

Binding of C4BP to Synthetic Protein S Peptides

This binding assay was performed essentially as described previously (20). Briefly, 50 μ l of different synthetic peptides (20 μ M) or native protein S (10 μ g/ml) diluted in 0.02 M sodium carbonate buffer pH 9.0 were coated on microtiter plates for 1 h at 37C. Wells were blocked with 200 μ l of 10% BSA in 0.05 M Tris HCl pH 7.4 containing 0.1 M NaCl (TBS) and then washed three times with 0.2% BSA in TBS, 5 mM CaCl₂ and 0.02% Tween 20 (washing buffer). Serial dilutions containing 50 μ l biotinylated C4BP (b-C4BP) (0-5 μ g/ml in washing buffer) were added to each well and incubated for 2 h at room temperature. Bound biotin was detected with streptavidin alkaline phosphatase and p-NPP substrate at 405 nm.

Inhibition of the Binding of C4BP to Protein S by Synthetic Protein S Peptides

The inhibition of C4BP binding to protein S was studied with two different assays. In the first assay (solid phase inhibition assay), b-C4BP (0.5 μ g/ml) was preincubated with serial dilutions of synthetic peptides (0-500 μ M) for 1 h at room temperature. From this mixture, 50 μ l was added to a microtiter plate that had been coated with 50 μ l of native protein S (10 μ g/ml) in 0.02 M sodium carbonate buffer pH 9.0 for 1 h at 37C and blocked with 200 μ l of 10% BSA in 0.05 M Tris-HCl pH 7.4 containing 0.1 M NaCl (TBS). For the second assay, a fluid phase inhibition assay (20), b-C4BP (0.5 or 1.0 μ g/ml) and native protein S (1 or 2 μ g/ml) were first incubated with serial dilutions of synthetic peptides (0-500 μ M) for 1 h at room temperature. Then from this incubation mixture, 50 or 100 μ l was added to a microtiter plate that had been precoated with an anti-protein S monoclonal IgG antibody (S7) (10

or 20 $\mu\text{g/ml}$) in 0.02 M sodium carbonate buffer pH 9.0 for 1 h at 37C and blocked with 10% BSA in TBS buffer. For both assays, the wells were washed three times with 0.2% BSA in TBS, 5 mM CaCl_2 and 0.02% Tween 20 (washing buffer), and then 50 μl SAAP (1 $\mu\text{g/ml}$) diluted in washing buffer was added and incubated for 30 min at room temperature. Wells were washed six times with washing buffer and 100 μl p-NPP (5 mg/ml) in 0.1 M diethylamine buffer pH 9.0 was added to each well. The change in absorbance at 405 nm was detected kinetically using an EL 312 Microplate reader (Bio-tek Instruments, Inc., Winooski, VT). Each sample was assayed in duplicate and the results averaged.

Affinity of Anti-Protein S Monoclonal Antibody LJ-56 for Synthetic Protein S Peptides and Recombinant Protein S Mutants

Binding of antibody LJ-56 to immobilized peptides was determined as follows: Microtiter wells were coated with 50 μl of different peptides (50 $\mu\text{g/ml}$) in 0.02 M sodium carbonate buffer pH 9.0 for 2 h at room temperature. Wells were blocked with 200 μl of 10% BSA in TBS and subsequently washed with 0.2% BSA and 0.02% Tween 20 in TBS. Wells were incubated with 50 μl of LJ-56 (0-2.9 $\mu\text{g/ml}$) for 1 h at room temperature. Wells were then washed and incubated with 50 μl of biotinylated rabbit anti-mouse IgG (1 $\mu\text{g/ml}$) for 1 h at room temperature. Wells were washed and 50 μl SAAP (1 $\mu\text{g/ml}$) was added to each well and incubated for 30 min at room temperature. Wells were washed and 100 μl substrate p-NPP (5 mg/ml) was added. The change of the absorbance at 405 nm was measured using an ELISA Microplate reader as described earlier. Percentage of LJ-56 binding was calculated by using the observed absorbance value for 2.9 $\mu\text{g/ml}$ LJ-56 as 100%.

Microtiter wells were coated with 100 μl of rabbit anti-protein S IgG (10 $\mu\text{g/ml}$) (Dako) in 0.02 M sodium carbonate buffer pH 9.0 for 18 h at 4C. Wells were blocked with 3% BSA in TBS for at least 30 min at room temperature and

subsequently washed with TBS. Wells were incubated with 100 μl of 100 ng/ml of recombinant protein S variants for 2 h at 37C in blocking buffer. Wells were emptied and washed with TBS, and then 100 μl of anti-protein S monoclonal antibody LJ-56 (0-5 $\mu\text{g/ml}$) was added and incubated for 2 h at 37C. Wells were emptied and subsequently washed with TBS, and 100 μl of rabbit anti-mouse IgG conjugated to peroxidase (1 $\mu\text{g/ml}$) was added and incubated for 1 h at room temperature. The hydrolysis of phenylenediamine was measured at 490 nm using a V_{max} plate reader as described earlier. Percentage of LJ-56 binding was calculated as above.

Table 1. Sequence of Protein S Residues 420-434

	420	425	430
PSP-420	S G I K E I I Q E K Q N K H C		
K423E	- - - E - - - - - - - - - -		
E424K	- - - - K - - - - - - - - - -		
Q427E	- - - - - - - E - - - - - - - -		
K429E	- - - - - - - - - E - - - - - -		

RESULTS

Preparation of Recombinant Mutant Protein S

Recombinant wild type and mutant protein S species were prepared and purified [as described previously (18) and above under Experimental Procedures] in order to assess the importance of residues 423, 424, 427 and 429 (see Table 1) for APC cofactor activity and for binding to C4BP. Each protein S preparation was at least 65% pure based on protein staining patterns of samples analyzed on SDS-PAGE (data not shown) and each protein S preparation appeared to be >80% single chain based on immunoblot analysis. The amount of cleaved protein S found in each preparation was similar, between 10 and 20%. Concentrations of protein S antigen for each protein S preparation were determined using a

protein S ELISA. Parallel dose response curves in the ELISA data were observed for the different protein S forms, allowing quantitation of protein S in each preparation.

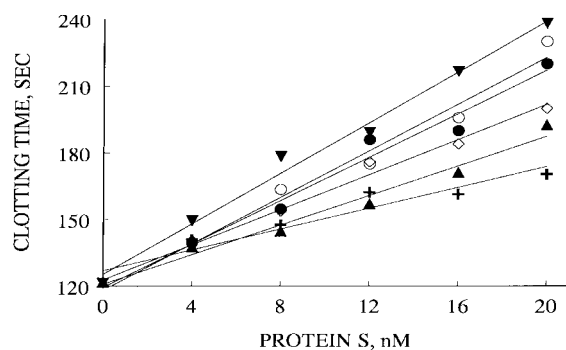


Figure 1. Anticoagulant APC cofactor activity of protein S variants. Increasing amounts of plasma purified protein S (O), recombinant wild type protein S (◇), or protein S variants with E424K (▽), Q427E (+), K429E (▲) or K423E (●) were incubated with APC, and the anticoagulant activity of the mixture was determined in a modified APTT clotting assay using protein S-depleted/C4BP-depleted plasma.

The anticoagulant cofactor activity of each recombinant protein S preparation and of purified plasma-derived protein S was determined. The dose-responses for APC cofactor activity of protein S species with the mutations K423E, E424K, Q427E, and K429E were compared to recombinant wild-type protein S and plasma protein S (Figure 1). Based on the slopes of the dose-response curves (Figure 1), the anticoagulant cofactor activity of each mutant protein S was between 60% and 120% of the protein S controls. The differences in cofactor activity of the mutants cannot solely be explained by the extent of cleavage of the protein S mutants because the amount of cleaved protein S in each preparation was similar. Control experiments with culture medium alone showed no effect on the cofactor activity assay or on the binding of protein S to C4BP.

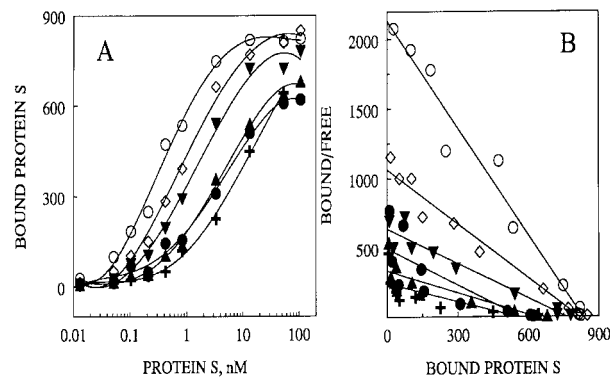


Figure 2. Panel A: Binding of recombinant protein S variants to C4BP. Anti-C4BP monoclonal antibody 8C11 IgG was coated onto microtiter wells to capture C4BP as described in Experimental Procedures. Purified plasma protein S (O), recombinant protein S (◇), or protein S variants with E424K (▽), Q427E (+), K429E (▲) or K423E (●) was added to wells, and bound protein S was detected with anti-protein S IgG as described in Experimental Procedures. Data represent the mean of triplicate values. Panel B: Scatchard plot analysis of binding data from the different mutants of protein S using the same symbols.

Binding of Recombinant Protein S Mutants to C4BP

The binding of the recombinant protein S variants to immobilized C4BP was examined using an anti-C4BP monoclonal antibody IgG (8C11) that is directed against the α -chain of C4BP to capture C4BP. The data in Figure 2 from one of three separate experiments show that the E424K-protein S mutant bound to C4BP with slightly lower affinity than the wild type recombinant protein S molecule. However, the apparent affinity of protein S with mutations of K423E, Q427E or K429E exhibited significantly reduced affinity for C4BP. Apparent K_d values were determined in three separate experiments based on Scatchard plot analyses and the averaged values are given in Table 2. Replacements at residues Lys-423, Gln-427 or Lys-429 caused 5-fold to 10-fold increases in apparent K_d values. The differences in affinity for C4BP of the protein S mutants was not caused by differences in the extent of cleavage of the

mutants because the extent of cleavage was similar for each preparation. Moreover, cleavage does not affect the affinity for C4BP.

Table 2. Apparent Affinity Constant of Protein S Mutants for the Binding to C4BP

	Apparent Kd (nM)
Plasma Protein S	0.5
Wild Type-Protein S	0.7
K423E-Protein S	4.6
E424K-Protein S	1.7
Q427E-Protein S	7.4
K429E-Protein S	3.5

Competition of Synthetic Protein S Peptides and Native Protein S for C4BP

The ability of the synthetic protein S peptides to inhibit native protein S binding to b-C4BP was studied in fluid phase competition assays as described in Experimental Procedures. Figure 3 shows the concentration dependence for the inhibition of the binding of b-C4BP to native protein S by PSP-420 (wild type sequence) (Table 1) and the peptide analogs with replacements E424K, Q427E and K429E. The PSP-420 and E424K peptides inhibited the binding almost completely, with 50% inhibition around 20 μM peptide (20). The negative control peptide with the reverse sequence, residues 434-420, showed no inhibition (Figure 3). Replacement of Gln-427 by Glu had a moderate effect on the binding to C4BP. The K423E and K429E peptides showed only a minor inhibition, suggesting that Lys-423 and Lys-429 are required for normal inhibition of binding.

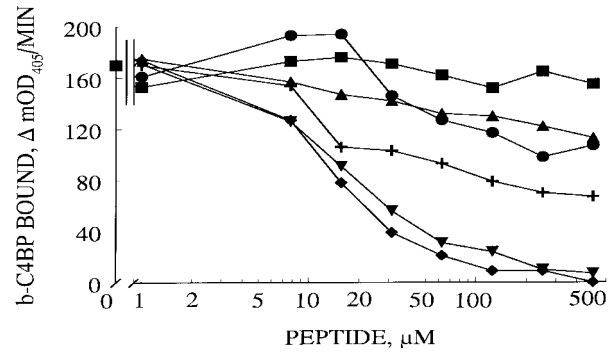


Figure 3. Inhibition of the binding of b-C4BP to protein S by synthetic protein S peptides in fluid phase. PSP-420 peptide (◆), reverse sequence PSP-420 peptide (residues 434-420) (control) (■), E424K peptide (▼), Q427E peptide (+), K423E peptide (●) and K429E peptide (▲) were tested. The fluid phase inhibition assay was performed as described in Experimental Procedures using 0.5 μg/ml b-C4BP and 1.0 μg/ml protein S.

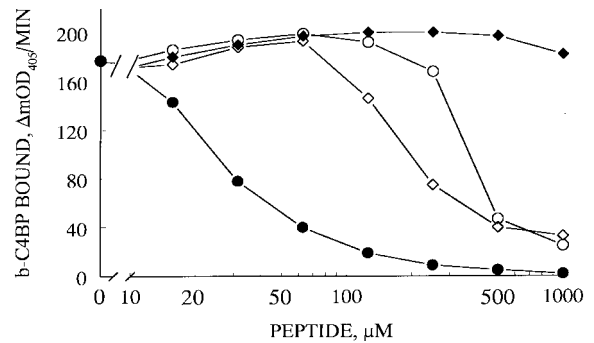


Figure 4. Influence of carboxyl-terminal moiety of peptides on the inhibition of binding of b-C4BP to protein S. Synthetic peptides SGIKEIIQEKQNKHC (PSP-420) and SGIAQFHIDYNN VSSAEGWHVN (SL-6) containing either a carboxy-amide or carboxylate group at the C-terminus were tested using the fluid phase inhibition assay described in Experimental Procedures to measure the inhibition of complex formation between protein S (2.0 μg/ml) and b-C4BP (1.0 μg/ml). PSP-420-carboxy-amide (●), PSP-420-carboxylate (○), SL-6-carboxylate (◇), and SL-6-carboxy-amide (◆).

Influence of the Carboxy-Terminal Moiety of Protein S Peptides on the Inhibition of the Binding of C4BP to Protein S

Apparently conflicting results were reported concerning the ability of synthetic peptides that comprise the same residues of protein S to inhibit

the binding of protein S to C4BP (20) (23). Because these peptides were synthesized in the carboxy-terminal amide or in the carboxyl form, we synthesized two peptides in both the carboxy-terminal amide and carboxyl forms and tested them for their ability to inhibit the binding of C4BP to protein S (Figure 4). PSP-420 in the amide form comprising protein S residues 420 to 434 inhibited the binding of native protein S to C4BP completely with 50% inhibition at 20 μ M peptide as we previously reported (20). The same peptide in the carboxyl form was 20 times less potent compared to PSP-420 in the amide form (Figure 4). The peptide (SL-6) comprising protein S residues 447 to 468 containing a carboxy-terminal was recently reported by Linse et al. (23) to inhibit the binding of protein S to C4BP. This peptide gave a 50% inhibition of the binding of C4BP to protein S at 200 μ M peptide, whereas the same peptide (SL-6) in the carboxy-terminal amide form had no effect at all (Figure 4). These results confirm our published observation and remarkably demonstrated that the use of peptides in either the carboxy-terminal amide or carboxyl form can give very different results.

Binding of C4BP to Synthetic Protein S Peptides

To characterize the importance of specific amino acids for the binding of b-C4BP to synthetic peptides containing residues 420-434, the binding of b-C4BP to various immobilized peptide variants was studied as described under Experimental Procedures. The results show that b-C4BP bound to the E424K peptide, plasma protein S and peptide PSP-420 in the order of decreasing avidity (Figure 5). There was essentially no significant binding to the control peptide with the reverse sequence, i.e., residues 434 to 420, or to the K423E or K429E peptides. Binding to the Q427E peptide was reduced compared to peptide PSP-420 that had the wild type sequence (Figure 5). These data suggest that Lys-423 and Lys-429 in peptides representing residues 420-434 are essential for normal binding to C4BP.

Binding of Anti-Protein S Monoclonal Antibody LJ-56 to Synthetic Protein S Peptides and to Protein S Mutants

The anti-protein S monoclonal antibody, LJ-56, was made against the synthetic peptide PSP-420 as immunogen, and this antibody recognizes only free protein S and inhibits its binding to C4BP (20). To measure the binding of antibody LJ-56 to peptides containing sequences indicated on Table 1, synthetic peptides were coated onto micro titer plates and LJ-56 binding was determined. LJ-56 recognized peptide PSP-420 and the E424K and Q427E peptides with an affinity similar to that for protein S (Figure 6A). In contrast, LJ-56 showed no binding to the K423E or K429E peptides or to the control peptide with the reverse sequence of residues 434 to 420.

LJ-56 was also tested for its ability to bind to recombinant wild type and mutant protein S variants. Figure 5B shows that the E424K and Q427E protein S mutants bound antibody LJ-56 comparably to wild type recombinant and plasma-derived protein S. In contrast, antibody LJ-56 did

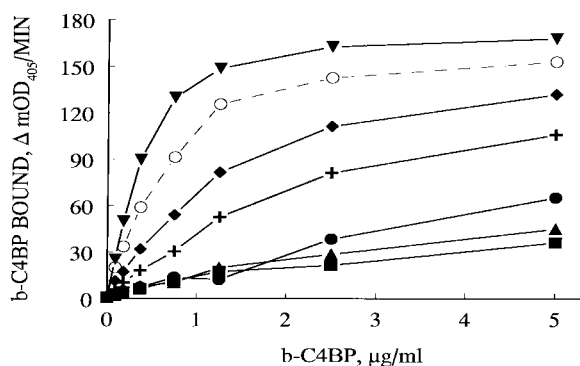


Figure 5. Binding of C4BP to synthetic protein S peptides. Native protein S (10 μ g/ml) (O), peptide PSP-420 (◆), E424K peptide (▼), Q427E peptide (+), K429E peptide (▲), K423E (●) and peptide control containing the reversed sequence of PSP-420 (■) were coated onto micro titer wells, and varying amounts of b-C4BP were added and incubated. Then bound b-C4BP was measured as described in Experimental Procedures. One-hundred percent binding was defined by the maximum binding observed.

not bind to protein S that contained the mutations of K423E or K429E (Figure 6B). These data demonstrate that Lys-423 and Lys-429 but not Glu-424 or Gln-427 are essential for the binding of monoclonal antibody LJ-56 to its epitope in both synthetic peptides and in the region of protein S comprising residues 420-434.

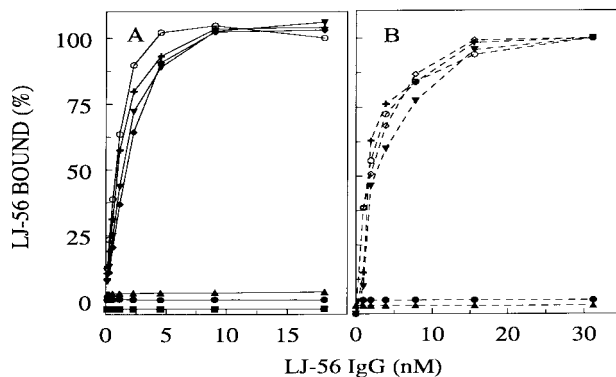


Figure 6. Binding of anti-protein S monoclonal antibody LJ-56 to synthetic protein S peptides (A) and recombinant protein S variants (B). Panel A: Varying amounts of antibody LJ-56 were added to the immobilized peptides PSP-420 (◆), reversed sequence PSP-420 (residues 434-420) (■), E424K (▼), Q427E (+), K423E (●) and K429E (▲) or to protein S (O). Bound antibody LJ-56 was detected with biotinylated rabbit anti-mouse IgG as described in Experimental Procedures. Panel B: Increasing amounts of antibody LJ-56 were added to protein S variants [same symbols for designation of specific amino acid replacements as for peptide variants in (A)], purified plasma protein S (O) or recombinant wild type protein S (◇), and then binding of antibody LJ-56 to the various protein S species was determined.

DISCUSSION

Human protein S forms a reversible complex with human C4BP which downregulates the APC cofactor activity of protein S because only free protein S is active as a cofactor to APC. Several reports suggested that the disulfide loop at the carboxy-terminal end of the SHBG-like domain (amino acid residues 583-635) of protein S is involved in its binding to C4BP (17-19,22). An alternative binding site in protein S involving residues 408-434 was subsequently reported (20), and it was suggested that this region of protein S interacts with residues 31-45 of the β -chain of

C4BP (32). The amino acid sequence between residues 420 to 434 was found to be highly conserved in human, rhesus monkey and bovine protein S, each of which interacts with human C4BP in a similar manner (33). Recently another region of protein S comprising residues 447-460 was reported to be involved in the interaction with C4BP (23). Studies of the binding of recombinant chimeric molecules containing domains of protein S and factor IX indicated that only the SHBG-like domain of protein S (residues 243-635) that comprises all reported regions to be involved in the interaction with C4BP is required for normal binding of protein S to C4BP (34).

To study the involvement of specific amino acids in the region of residues 420-434 of protein S in its interaction with C4BP in more detail, we used synthetic protein S peptide analogs and, in parallel, recombinant protein S variants containing the same amino acid substitutions (Table 1). The binding to C4BP of the recombinant protein S mutants and of the various peptide analogs was examined. The E424K protein S mutant binds to C4BP with an affinity similar to wild type recombinant protein S, suggesting that replacement of Glu-424 by Lys has no marked effect on the binding and that Glu-424 is not required for binding to C4BP. The K423E, Q427E and K429E mutants each showed 5-fold to 10-fold reduced affinity for C4BP, indicating that Lys-423, Gln-427 and Lys-429 are important for normal binding to C4BP. The observed increase in apparent K_d , in terms of the change in free energy for the association of protein S with C4BP, is consistent with the loss of buried ion pairs involving the side chains of Lys-423 or Lys-429 or of a high energy hydrogen bond(s) involving the side chain carboxamide of Gln-427. Essentially the same conclusions were reached based on studies of the ability of the various synthetic peptides to compete with protein S for binding to C4BP (Figure 3). Both peptide PSP-420 and the E424K peptide are able to prevent binding almost completely (Figure 3). The Q427E peptide competes less well, suggesting that Gln-427 is perhaps weakly

involved in the binding of protein S to C4BP or necessary for optimal peptide conformation. The K423E and K429E peptides do not compete, suggesting that Lys-423 and Lys-429 are involved in the binding. Interestingly, a nonconservative amino acid substitution Lys-429-Ile was reported in porcine protein S that has reduced affinity for human C4BP (33). Based on results reported here, this substitution is predicted to cause the reduced affinity of porcine protein S for human C4BP.

Anti-protein S monoclonal antibody LJ-56 was developed against the PSP-420 peptide, and it selectively recognizes free protein S and is able to inhibit the binding of protein S to C4BP (20). LJ-56 recognizes protein S, peptide PSP-420, and the E424K peptide with similar affinity. The K423E and K429E peptides are not recognized by LJ-56. Reactivity of LJ-56 with recombinant protein S species gave essentially similar results as no binding of LJ-56 to K423E-protein S or K429E-protein S was observed. This suggests that Lys-423 and Lys-429 are essential for epitope recognition by LJ-56 and for normal binding to C4BP.

Three distinct regions in protein S are now reported to be involved in the binding to C4BP (35): the carboxy-terminal region (residues 605-614 (17,19,22)), the region between residues 447-460 (23) and the region between residues 413-433 (20). The carboxy-terminal region might however be indirectly involved in the binding of C4BP to protein S because deletion of amino acid residues Asp-583 to Ser 635 caused a major conformational change in the protein S structure resulting in a strongly reduced affinity of anti-protein S monoclonal antibody S12 for its epitope (22). Because the S12 epitope is outside the region of residues 583-635, it cannot be excluded that the conformational change induced by the deletion of the whole carboxy-terminal disulfide loop including residues 583-635 causes a loss of binding affinity for C4BP. In conflict with the report of Walker (1989), in two separate studies synthetic peptides comprising residues 595-628 (23) or 605-614 (20) had no effect on the binding

affinity of C4BP to protein S, further suggesting that the carboxy-terminal region of protein S is not directly involved in the binding to C4BP.

The obvious primary determinants of a peptide's ability to inhibit protein-protein interactions are the sequence of amino acids and their configuration at the α -carbon. However, significant "end effects" can sometimes arise involving the chemical natures of the amino and carboxy termini. For example, we found that the anticoagulant potency of a synthetic peptide containing residues 493-506 of factor V was reduced by more than an order of magnitude when the carboxy terminal amide group (CONH_2) was replaced by a carboxylate group (CO_2H) (36). As demonstrated here, the nature of the carboxy terminal moiety markedly influences the ability of protein S peptides containing residues 420-434 and 447-468 to inhibit binding of protein S to C4BP (Figure 4). Because Linse et al. (1997) reported that the later peptide was a good inhibitor while the former peptide, in apparent conflict with our report (20), was a poor inhibitor, we decided to synthesize these peptides in both the carboxy-terminal amide and carboxylate forms and to compare their inhibitory effects on the binding of C4BP to protein S in the same experiment. Peptide PSP-420 in the amide form was confirmed to have a potent inhibitory effect on the binding of C4BP to protein S (20), whereas the same peptide in the C-terminal carboxylate form was 20 times less potent (Figure 4). Peptide SL-6 (residues 447-468) with a C-terminal carboxylate was used in the study of Linse et al. (23). This peptide had only a modest effect on the binding of C4BP to protein S and was 10 times less potent compared to PSP 420 in the carboxy-terminal amide form. Peptide, SL-6 in the carboxy-terminal amide form had no inhibitory effect at all (Figure 4). Clearly there are remarkable end effects for these two peptides. Hence, our results conflict with those of Linse et al. (1997) and indicate that the 420-434 region of protein S is the predominant region directly involved in the binding of protein S to C4BP. The minor effect of peptide SL-6 with a C-terminal

carboxylate on the binding of C4BP if significant might be an indirect one and be related to the fact that the 447-460 region is close to the 420-434 region. In contrast to peptides with a carboxy-terminal carboxyl group, peptides with a carboxy-terminal amide resemble native protein structures in which each amino acid is connected to the adjacent amino acid by peptide bonds that involve amino groups (37). Moreover, a carboxyl group introduces an extra negative charge that might affect the conformation of the peptide or the binding of the peptide to a protein. Based on this, it is always highly advisable to use peptides with a carboxy-terminal amide group to avoid end effect artefacts.

Our results show that parallel studies using peptide analogues and recombinant protein S variants containing the same amino acid replacements provide powerful tools to study the contribution of individual amino acid residues to protein-protein interactions. The results obtained using peptide analogs are in good agreement with the results obtained using site-directed mutagenesis, suggesting the various peptides can achieve the same conformation as the corresponding sequence in the recombinant wild type or mutant proteins. An advantage of using peptides is that peptides are much faster to test than purified mutant proteins; however, the use of valid screening methods using conditioned media containing site-directed mutant proteins could offset some of these advantages, depending on the protein under study. Moreover, it appears that for studying certain specific functional properties of large multifunctional proteins such as protein S, the recombinant mutant molecule is preferred.

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