Identification of candidate residues for interaction of protein S with C4b binding protein and activated protein C

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Protein S is a plasma factor essential for prevention of thrombosis, partly due to its activity as a cofactor for the plasma anti-coagulant protease-activated protein C. To expand knowledge about structure–function relationships in homologous protein S molecules, studies of protein S from different species have been performed. Protein S anti-coagulant activity in human, monkey, bovine, and porcine plasma has been inactivated by purified human C4b binding protein (C4BP) with dose-dependence, suggesting that each protein S can bind human C4BP and that only the free form of each is anti-coagulant active. Purified porcine protein S has a 10-fold higher K_d for human C4BP than has human protein S. Protein S residues 420–434 provide an essential binding site for the negative regulator C4BP. cDNA

sequences show that protein S residues 420–434 are highly conserved in all four species with the notable exception of Lys-429-Ile in porcine protein S. Differences between porcine and human protein S, e.g. Lys-429-Ile, Lys-43-Ala, Ser-197-Leu, Ser-199-Phe, Glu-463-Gly, Lys-571-Glu, Asn-602-Ile, Gln-607-Pro, may contribute to the decreased affinity of porcine protein S for human C4BP. Moreover, the species specificity of cofactor activities of various species of protein S is determined for human versus bovine-activated protein C, and these results, combined with sequence comparisons, agree with previous evidence that the thrombin-sensitive region and the first epidermal growth factor domain of protein S, i.e. residues 47–116, are responsible for recognition of activated protein C.

INTRODUCTION

Protein S is a vitamin K-dependent plasma protein of 75000 M_r (DiScipio and Davie, 1979) that acts as a non-enzymic cofactor for the anti-coagulant plasma serine protease, activated protein C (APC) (Walker, 1981a, 1984). Protein S is also a direct anti-coagulant that inhibits prothrombinase by binding directly to factors Va and Xa (Heeb et al., 1993). The essential physiologic role of this cofactor in the regulation of thrombosis is demonstrated by reports that 12 to 18 % of young adult patients with recurrent venous thrombotic disease are heterozygous for protein S deficiency (Bertina, 1985; Comp et al., 1986; Gladson et al., 1988), that severe deficiency of protein S can present with purpura fulminans, and that a significant percentage of patients with arterial thrombosis have low levels of free protein S (Thommen et al., 1989). The regulation of protein S activity in human plasma is complex. Approximately 60 % of plasma protein S circulates complexed with C4b binding protein (C4BP) and 40 % of protein S is free (Dahlbäck and Stenflo, 1981). Only the free form of protein S exhibits anti-coagulant cofactor activity (Comp et al., 1986; Dahlbäck, 1986). Thrombin cleavage of bovine protein S at two arginine residues in the so-called thrombin-sensitive loop, i.e. residues 52 and 70, destroys its anti-coagulant cofactor activity. Human protein S has three potentially susceptible arginine residues in this region (residues 49, 60, and 70) (Lundwall et al., 1986). Protein S is present in platelets and endothelial cells, and these cells, as well as platelet microparticles, provide surfaces for the expression of APC and protein S anti-coagulant activity (Schwarz et al., 1985; Fair et al., 1986; Schwalbe et al., 1990). Human genes coding for protein S and for a protein S pseudogene have been sequenced and are designated protein S α and protein S β , respectively (Edenbrandt et al., 1990; Ploos van Amstel et al., 1990; Schmidel et al., 1990).

In the current study, we report the sequences of protein S from rhesus monkey and pig and demonstrate their functional similarity to human protein S in both anti-coagulant cofactor activity and in binding to C4BP, and show that differences in affinity for human C4BP correlate with non-conservative amino acid substitutions in protein S regions implicated in C4BP binding by synthetic peptide studies (Fernández et al., 1993).

EXPERIMENTAL

Materials

All reagent chemicals were of reagent grade or better. When available, molecular biology grade reagents were used. Automated fluorescent terminator sequencing was performed and synthetic oligonucleotides were prepared by Dr. Charles Glass (The Scripps Research Institute). Human C4BP was purified as previously described (Griffin et al., 1992). Human (Gruber et al., 1989) and bovine (Gladson et al., 1989) APC were prepared as described. C4BP was biotinylated by a previously published method (Fernández and Griffin, 1993).

Animal protein S activity

The ability of animal plasma protein S to act as a cofactor for human or bovine APC was tested using the StaClot Protein S determination kit on the ST4 coagulometer (American Bio-products Company, Parsippany, NJ, U.S.A.) as recommended by the manufacturer. Briefly, 100 μ l of bovine factor Va concentrate was incubated with 100 μ l of protein S-depleted human plasma, 100 μ l of human or bovine APC (50 nM final concn.), and pooled normal human, bovine, porcine, or rhesus monkey plasma diluted in Owren–Koller buffer. After 120 s incubation at

Abbreviations used: C4BP, C4b binding protein; APC, activated protein C; EGF, epidermal growth factor; TBS, Tris-buffered saline; SHBG, sex hormone-binding globulin.

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Figure 1 Partially purified protein S

P, porcine protein S; H, human protein S; M, molecular mass standards. NR, non-reduced; R, reduced.

37 °C, clotting was initiated by addition of 100 μ l of 25 mM CaCl_2 . Prolongation of the clotting time due to protein S in the diluted human or animal plasma was determined. When absolute clotting time in seconds was plotted versus the amount of animal plasma S, standard curves were obtained that were parallel, although displaced (results not shown). It was estimated that pig plasma displayed 0.6 U/ml of protein S activity using bovine APC, while bovine and rhesus monkey plasmas displayed 1.5 U/ml using bovine and human APC. The definition of 1 unit was the amount present in 1 ml of human plasma of protein S cofactor activity towards human APC. These values were in agreement with antigen estimates obtained by densitometry of band immunoblots. Very little cofactor activity for human APC was observed using porcine or bovine plasma as a source of protein S, or for bovine APC using human or rhesus monkey plasma as a source. To study the neutralization of protein S anticoagulant activity by C4BP, the human or animal plasma used as a source of protein S was preincubated for 1 h at 37 °C with varying concentrations of purified human C4BP, then diluted 1:10 prior to the assay of protein S activity. Pooled, citrated rhesus monkey plasma was prepared from blood from eight healthy, normal animals (the kind gift of Dr. F. Bloom, The Scripps Research Institute), whereas pooled bovine and pig plasmas were obtained from Sigma (St. Louis, MO, U.S.A.). Pig plasma was also kindly provided by Dr. J. Brassard (The Scripps Research Institute).

Immunoblotting

Animal or human plasma (1–3 μ l) was run on 8% SDS/PAGE. Protein was transferred to nitrocellulose for 2 h which was then blocked with Tris-buffered saline (TBS)/0.1% casein for 1 h. A 1:500 dilution of rabbit antipeptide antibody, anti-(PSP-1), which was directed against a sequence from the thrombin-sensitive region of protein S (Fernández et al., 1993) was incubated with the blot for 2 h at room temperature. This step was followed by three washes with blocking buffer. ^{125}I -labelled protein S was used for detection (Lämmle et al., 1986).

Purification of porcine protein S

A cocktail of inhibitors (1 mM benzamidine/1 mM di-isopropyl fluorophosphate/50 mg/l soybean trypsin inhibitor) was added to 1.0 l of frozen citrated porcine plasma (Pelfreeze, Rogers, AR, U.S.A.) prior to thawing at 37 °C. The plasma was filtered over Whatman paper and 80 ml of 1 M BaCl_2 were gradually added

while stirring for 1 h at 4 °C, followed by two centrifugations at 7000 *g* for 30 min at 15 °C. The pellet was dissolved in 80 ml of 0.4 M EDTA, pH 7.4, containing the cocktail of inhibitors, dialysed against 50 mM Tris/100 mM NaCl, pH 7.4, and loaded at 1 ml/min onto a Hiload Q-Sepharose 26/10 f.p.l.c. column (Pharmacia-LKB, Piscataway, NJ, U.S.A.). Fractions (4 ml) were eluted with a NaCl gradient from 0.1 to 0.4 M. Protein S antigen was identified by immunoblotting and e.l.i.s.a. as described below. Positive fractions were pooled and loaded onto a human C4BP-Sepharose affinity column (3 mg of C4BP/ml of gel) and eluted with 0.1 M glycine, pH 2.4. The resulting partially purified porcine protein S was > 80% pure (Figure 1) and consisted of the expected doublet at 69 kDa (Dahlbäck, 1983).

E.l.i.s.a. for protein S antigen

Rabbit anti-(PSP-1) antibody (10 μ g/ml) (Fernández et al., 1993) was coated onto Nunc MaxiSorb microtitre plates. The antibody was raised against a synthetic peptide representing human protein S residues 54–67, and it recognized protein S from porcine plasma. Wells were blocked with 200 μ l of 2% BSA in TBS. Protein S-containing fractions were diluted in TBS containing 0.1% BSA, 5 mM CaCl_2 , and 0.02% Tween-20 (dilution buffer). Plates were incubated with 1 μ g/ml monoclonal 163 anti-human protein S that also recognized porcine protein S (the kind gift of Dr. C. Esmon, Oklahoma Medical Research Foundation). Wells were incubated for 1 h at room temperature with 1 μ g/ml biotinylated goat anti-mouse IgG (Pierce). Bound biotin was detected with a 30 min room temperature incubation with 1 μ g/ml streptavidin-conjugated alkaline phosphatase and *p*-nitrophenyl phosphate substrate according to manufacturer's directions (Pierce). Absorbance was monitored in each well with a model EL312 Bio-kinetics e.l.i.s.a. reader and analysed using the Kineticalc software (Biotek, Winooski, VT, U.S.A.).

Determination of apparent affinity constants

Purified human or porcine protein S (10 μ g/ml) was immobilized on microtitre wells (Fernández et al., 1993). The plate was blocked with TBS containing 2% BSA. Wells were then washed and incubated with biotinylated human C4BP (0–50 μ g/ml) in dilution buffer for 2 h at room temperature. Bound biotin was determined as described above. Data were subjected to Scatchard analysis (Scatchard, 1949) using the Enzfitter software (Enzfitter, Cambridge, U.K.).

cDNA cloning

Rhesus monkey and pig liver RNAs were obtained from Clontech (Palo Alto, CA, U.S.A.) and were reverse-transcribed with the cDNA Cycle Kit (Invitrogen, San Diego, CA, U.S.A.) using oligo-dT as primer according to the manufacturer's directions. This single-stranded cDNA product was subjected to two rounds of PCR. In the first round, single-stranded cDNA was incubated with 1 μ M oligo-dT-*Xba* (ProMega, Madison, WI, U.S.A.), 1 μ M *pslh* (gacaagctTGTCCTCCTAGTGCTTCG, nt 159–178) (lower-case bases indicate those which are not derived from the protein S sequence), 5 units of *Taq* DNA Polymerase (ProMega), 200 μ M each dATP, dCTP, dGTP and dTTP, in 50 mM KCl/10 mM Tris, pH 8.8/1.5 mM MgCl_2 /0.1% Triton X-100 under a layer of mineral oil. Thirty cycles were performed for 2 min at 61 °C, 3 min at 72 °C, 1 min at 94 °C, followed by two cycles of 2 min at 61 °C, 3 min at 72 °C. In the second round, 1 μ l of this primary PCR product was reamplified as above, except that the primers were *ps3b* (cagtgaTCAAAGCAACAGGCTTCACA, nt 201–220) and either *ps2bg* (ctgagatcTATAA-

GCAGAGAAAAGATGCC, nt 2171–2151) or pscho2b (cagtgatCACCAAGGACACAGCAAAGG, nt 1739–1720). In other experiments, a 160 bp sequence was amplified as above using the primers pschols (caggaGCTCAATTTACATAGATTAT, nt 1590–1610) and pscho2b. The 3' ends of the cDNAs were obtained through reamplification of the primary PCR product using oligo-dT-*Xba* (ProMega) and pschols. Primers were synthesized according to the reported human protein S α sequence (Lundwall et al., 1986).

Sequencing of porcine and rhesus monkey protein S cDNA

Protein S cDNAs were cloned into the vector pCR1000 (TA Cloning System, Invitrogen) according to the directions of the manufacturer. White colonies were selected and assayed for the presence of protein S cDNA by PCR with pschols and pscho2b as described above. Plasmid DNA was prepared from 3 ml overnight cultures using Qiagen tip-20 columns (Qiagen, Studio City, CA, U.S.A.) and subjected to double-stranded sequencing either manually using Sequenase (U.S. Biochemicals) and 35 S-dATP or *Taq* polymerase and fluorescent terminators on an ABI 380B automated sequencing machine (Foster City, CA, U.S.A.). Some inserts were gel purified, digested with *Sau3AI*, and cloned into pUC19 that had been digested using *BamHI* prior to double-stranded DNA sequencing.

RESULTS

Neutralization of animal protein S by human C4BP

Preliminary experiments indicated that human APC was able to use the protein S in human or rhesus monkey plasma and that bovine APC could use the protein S in porcine or bovine plasma as cofactor in anti-coagulant activity assays, while the opposite combinations were ineffective (results not shown). These data verify that all four species have protein S APC-cofactor activity and confirm previous reports that the protein S–APC interaction is species-specific (Walker, 1981b).

Since complex formation between human protein S and human C4BP inhibits protein S anti-coagulant cofactor activity, the

Figure 2 Effect of purified human C4BP on the protein S (PS) anti-coagulant activity of human (◆), rhesus monkey (□), bovine (△), and porcine (○) plasmas

Varying concentrations of purified human C4BP were incubated with pooled normal human or rhesus monkey plasma for 60 min at 37 °C. Protein S anti-coagulant cofactor activity was determined as described in the Methods section, and values were calculated as the percentage activity relative to a standard curve based on normal human plasma dilutions. Linear extrapolations of initial slopes are shown by broken lines.

Figure 3 Immunoblotting analysis of protein S (PS) in plasma from denaturing gels

Plasma (1 μ l) was run on SDS/polyacrylamide gels and blotted with anti-(PSP-1). H, human plasma; M, rhesus monkey plasma; P, porcine plasma; B, bovine plasma.

Figure 4 Binding of human C4BP to immobilized protein S

Indicated concentrations of biotinylated human C4BP were added to microtitre plate wells coated with (a) porcine or (b) human protein S (PS). Bound biotin was detected as described. Insets: Scatchard analysis of binding data. Apparent dissociation constants were calculated from the slopes using the Enzfitter software.

ability of purified human C4BP to neutralize protein S cofactor activities in the different plasmas was studied. Figure 2 shows the effect of preincubation of human or rhesus monkey plasma used as a source of protein S with increasing concentrations of purified human C4BP prior to assaying protein S activity in the APC cofactor assay. The results in Figure 2 demonstrate that human C4BP potentially neutralized monkey, porcine, bovine, and human protein S activity. Note that consistent with previous reports in human (Dahlbäck, 1986) and in rabbit plasmas (He and Dahlbäck, 1993), it was not possible to block all human protein S activity by addition of human C4BP. When the curves

Figure 5 Predicted human (hum), bovine, porcine, rabbit, and rhesus monkey protein S (PS) amino acid sequences

Alignments of protein sequences inferred from the cDNA sequences were performed using the University of Wisconsin Genetics Computer Group software package (Devereux et al., 1984). Blanks indicate identity between sequences; dashes indicate undetermined sequence. Deletions are indicated by a '*'.

in Figure 2 were linearly extrapolated to minimum attainable protein S activity (i.e. to zero for bovine, rhesus monkey and porcine plasmas, and to approx. 19% for human plasma), the intercepts on the x-axis were at approx. 80 nM C4BP for human plasma, 95 nM C4BP for rhesus monkey plasma, 140 nM for bovine plasma, and 330 nM for porcine plasma. Neutralization of porcine protein S by human C4BP was therefore apparently considerably less efficient than for other species.

K_d of human C4BP for porcine protein S

The higher concentration of human C4BP needed to neutralize most of the protein S activity in porcine plasma might result from lower binding affinity for human C4BP or else from markedly higher levels of total or free protein S in porcine plasma. To estimate the similarity of total antigen levels of protein S in the four plasma types, immunoblotting analysis was performed (Figure 3). During SDS/PAGE, complexes of protein S were denatured so that the various circulating forms of protein S migrated to the same position (Dahlbäck and Stenflo, 1981) and thus the intensity of the fastest migrating band in Figure 3 yielded an estimate of the total protein S antigen. Since labelled protein S was used for detection (Lämmle et al., 1986), the other bands represented affinity labelling of plasma proteins that

bound to protein S, such as C4BP (Dahlbäck and Stenflo, 1981) and factor V (Heeb et al., 1993). Immunoblotting of SDS gels of serial dilutions of plasma followed by autoradiography and densitometry yielded the estimate that porcine plasma contains 72% of the total human protein S antigen concentration (results not shown). Although these results were only semi-quantitative due to the possibility of differential recognition of animal protein S by anti-human protein S antibodies, the data were consistent with the assumption that the concentrations of protein S differed by, at most, a few-fold. This suggested that the difference in the concentration of human C4BP needed to neutralize the protein S cofactor activity in these two plasmas did not result from a markedly higher amount of protein S circulating in porcine plasma, but was caused by a higher K_d of human C4BP for porcine protein S than for human protein S.

The hypothesized higher K_d of purified porcine protein S compared with human protein S for human C4BP was confirmed directly using Scatchard analysis of solid-phase binding assays employing purified proteins. In the presence of calcium, human protein S bound to human C4BP-coated wells with an apparent K_d of 0.5 ± 0.05 nM, while partially purified porcine protein S bound to these wells with an apparent K_d of 9.2 ± 1.1 nM (Figure 4), confirming that the lesser ability of human C4BP to neutralize porcine protein S resulted from lower affinity interaction. The

difference in K_d did not arise from the acid treatment of the affinity-purified porcine protein S, since glycine-treated human protein S displayed a difference of only 10% in the K_d compared with buffer-treated human protein S (results not shown).

Sequences of rhesus monkey and porcine protein S

To examine possible amino acid sequences important for the species specificity of protein S cofactor activity for APC and for the differences in affinity for human C4BP, the cDNAs for rhesus monkey and porcine protein S were cloned from liver RNAs and subjected to double-stranded DNA sequencing. The sequences have been deposited in the GenBank database under accession numbers L31380 and L31379, respectively. The rhesus monkey and porcine protein S cDNA sequences were aligned with the sequences for human protein S α (Lundwall et al., 1986) and bovine protein S (Dahlbäck et al., 1986) (results not shown). Fifty-six nucleotide differences between monkey and human protein S were observed in the coding region, corresponding to 3.0% difference. One of these, the C to T transition at nucleotide 308, was present in only 5/13 clones sequenced in that region and therefore may represent a polymorphism. The above data suggest that in rhesus monkey liver, as in human liver, there is only one expressed protein S gene. A single porcine cDNA sequence was observed that differed from human protein S α at 223 positions in the coding region, corresponding to 10.3% difference.

The inferred amino acid sequences for the rhesus monkey, porcine, bovine, rabbit, and human protein S α are aligned in Figure 5. The rhesus monkey cDNA coded for a polypeptide with a typical prepropeptide sequence and a processed protein S molecule of 635 residues that aligns perfectly with the human protein S sequence of 635 residues without insertions or deletions. The porcine sequence coded for a similar protein with a single amino acid deletion relative to human protein S in the same position as the deletion in bovine protein S (Dahlbäck et al., 1986) (Figure 5). Many of the sequence differences are common to human and rhesus monkey protein S as compared with the bovine and porcine protein S. These are indicated schematically as asterisks in Figure 6. The distribution of such common changes is non-uniform. Only one such change occurs in the γ -carboxyglutamic acid-containing domain, Asp-38-Glu. There are none in the aromatic stack region. Four such dual changes are found in the short thrombin-sensitive loop, namely Arg-49-Gly, Gln-52-Arg, Thr-53-Ala, and Gln-61-Leu. The first epidermal growth factor (EGF)-like domain has six: Ser-81-Asn, Tyr-90-Phe, Ser-92-Thr, Lys-97-Gln, Thr-103-Ile, and Pro-106-Ser. There are no common changes in the second EGF-like domain and only one in the third EGF-like domain, Leu-195-Pro. The fourth EGF-like domain has two such common changes, Thr-223-Ser and Ser-245-Pro. The sex hormone binding globulin (SHBG)-like domain has 39 changes that differentiate the porcine/bovine sequences from the human/rhesus monkey sequences. These include the substitution at residue 422, which is Ile in human and monkey protein S, but Val in porcine, rabbit, and bovine. This residue lies within the peptide 420–434 that we have implicated as part of the C4BP binding site in protein S (Fernández et al., 1993). The above groups of amino acid changes are significant in view of the species specificity displayed by protein S in its cofactor activity towards APC. A recent report (He and Dahlbäck, 1993) has indicated that bovine APC is able to utilize rabbit protein S as a cofactor under appropriate conditions. The sequence of rabbit protein S (He and Dahlbäck, 1993) is included in Figure 5 for reference. Of the above-listed residue differences, rabbit protein S shares Arg-49-Gly, Gln-52-

Figure 6 Locations of differences in protein S molecules

The inferred protein S structures are shown schematically with disulphide bonds assigned as in the human sequence. Amino acid residues shared by human and rhesus monkey which are different from residues common to both porcine and bovine protein S (see Figure 5) are indicated by asterisks. Glu, γ -carboxyglutamic acid domain; TSR, thrombin-sensitive region; EGF, EGF-like domain; SHBG, SHBG-like domain.

Arg, Thr-53-Ala, Ser-81-Asn, Lys-97-Gln, Thr-103-Ile, Thr-223-Ser, and Ser-245-Pro.

DISCUSSION

In this study, functional differences between protein S molecules from different species are used to identify specific residues that may be important for these two protein S functions. Rhesus monkey and porcine protein S in plasma are able to serve as cofactors for human and bovine activated protein C respectively. The restriction of human or rhesus monkey protein S cofactor activity to human APC and the corresponding restriction of porcine and bovine protein S cofactor activity to bovine APC suggest that residues common to these pairs of protein S sequences are likely to be involved in interactions with APC. Recent evidence that rabbit protein S can act as a cofactor for bovine APC and the report of the sequence for rabbit protein S (He and Dahlbäck, 1993) can be used to narrow further the list of most likely candidate residues.

The relationship of protein S structure to its binding to APC and C4BP can be analysed by comparison of the sequences and functional properties of rhesus monkey and porcine protein S to those of human and bovine protein S. A peptide from the SHBG domain of protein S representing residues 420–434 provides an essential site for interaction between human protein S and human C4BP (Fernández et al., 1993). The current study shows that the sequence of this region is highly conserved in human, rhesus monkey, and bovine protein S (Figure 5), each of which interact with human C4BP in a similar manner (Figure 2) as does rabbit protein S which binds to human C4BP (He and Dahlbäck, 1993). Since the overall protein S sequences are so similar, the observed difference in K_d (Figure 4) is assumed to result from interspecies sequence differences in regions that

Table 1 Conservation of protein S C4BP binding peptides

Protein S (PS) sequences were obtained from Dahlbäck et al. (1986), He and Dahlbäck (1993) and Lundwall et al. (1986) or cloned as described. Gas6 sequences were obtained from Manfioletti et al. (1993). Alignments were performed using PILEUP from the University of Wisconsin Genetics Computer Group package (Devereux et al., 1984).

	420	434	605	614
Bovine PS	SG VKEIIQEKqN khC		GVQvDLD EAI	
Porcine PS	SG VKEIIQEIqN khC		GVpLDLD EAI	
Human PS	SG IKEIIQEKqN khC		GVQLDLD EAI	
Monkey PS	SG IKEIIQEKqN khC		GVeLDLD EAI	
Rabbit PS	SG VKEIIqqkqk khC		GVQLDLD EAI	
Human Gas6	tt IqEtVkvNtr mqC		rrlLDLD EAa	
Mouse Gas6	Sa IqEtVkaNtk mqC		GkiLDLD tAs	
Consensus	SG I-E---EN-N --C		GVQLDLD EAI	

interact with C4BP. Inspection of the sequences of the four species of protein S in the region corresponding to human residues 420–434 (Figure 5, Table 1) indicates that a non-conservative amino acid substitution, Lys-429-Ile, occurs in porcine protein S relative to the other molecules. This substitution could indeed result in reduced affinity for human C4BP.

The protein S sequence comprising residues 605 to 614 has also been implicated in the binding of protein S to C4BP (Walker, 1989). Inspection of the four protein S sequences in this region (Figure 5, Table 1) indicates a high degree of conservation in this area. Site-directed mutations affecting residues 608, 609, 611, and 612 do not affect the ability of human protein S to interact with C4BP (Chang et al., 1992; Nelson and Long, 1992). At minimum, these data suggest that residues 608, 609, 611, and 612 are not essential for C4BP binding. Table 1 shows a comparison of the 420–434 and 605–614 peptides of protein S from all five species to the equivalent regions of human and mouse gas6, a growth arrest specific protein made by fibroblasts that is > 40% identical to protein S overall (Manfioletti et al., 1993). The region at 605–614 is extremely well conserved between protein S and gas6, while homology at 420–434 is much weaker (Table 1). Since it is unlikely that gas6 also binds to C4BP, this provides further evidence against a functional role for these residues in C4BP binding.

Overall, the three-dimensional structures of the five protein S molecules from various species must be extremely similar, given the degree of sequence identity (> 80%), the identical number and position of cysteine residues, and the difference in polypeptide length of only one residue. Since human C4BP has ~20-fold lower affinity for porcine than for human, bovine, or rhesus monkey protein S (Figures 2 and 4), it is interesting to note the residues that are identical in the last three molecules but different in porcine protein S. Inspection of the sequence data in Figure 5 shows that there are 23 such residues. Nineteen of these also differ from rabbit protein S. Since previous studies (Walker, 1989; Fernández et al., 1993) have suggested that synthetic peptides with sequences representing 32–46, 187–200, 353–368, 420–434, and 605–614 inhibit interaction of human protein S with human C4BP to varying degrees, the following amino acid differences between human and porcine protein S are particularly noted: Lys-43-Ala, Ser-197-Leu, Lys-429-Ile, and Gln-607-Pro. It is therefore speculated that one or more of these noted differences, but especially Lys-429-Ile and perhaps Gln-607-Pro, may be responsible for much of the decreased affinity exhibited by porcine protein S for human C4BP. Other residue differences

such as Gly-370-Glu could conceivably also play a role, but the observation that synthetic peptides representing other regions such as that comprising residues 359–372 fail to inhibit binding of the two proteins (Fernández et al., 1993) does not support this possibility.

Human and rhesus monkey protein S are active cofactors for human APC but not for bovine APC, while the converse is true for bovine and porcine protein S. Rabbit APC is more active with bovine than with human APC (He and Dahlbäck, 1993). Inspection of the sequence data in Figure 5 for residues which are common to human and monkey protein S and whose identity is different from residues that are common to porcine and bovine protein S detects 47 such residues. The integrity of the thrombin-sensitive loop, residues 47–72, is essential for protein S anti-coagulant cofactor activity (Dahlbäck et al., 1990), and interactions between APC and protein S may involve the EGF-like domains (Stenflo, 1991). It is unlikely that the SHBG domain is involved in protein S-APC interactions since deletion of the C terminal 30 residues (Nelson and Long, 1992) or the entire SHBG domain (Chang et al., 1993) has no effect on protein S cofactor activity. The shared amino acid changes for human/rhesus monkey versus bovine/porcine protein S in these regions involve: Arg-49-Gly, Gln-52-Arg, and Gln-61-Leu in the thrombin-sensitive loop; Ser-81-Asn, Ser-92-Thr, Lys-97-Gln, Thr-103-Ile, and Pro-106-Ser in the first EGF-like domain; none in the second EGF-like domain; only Leu-195-Pro in the third; and Thr-223-Ser in the fourth EGF-like domain, with Ser-245-Pro nearby. Among these, the rabbit sequence has the same amino acid as the human and monkey protein S only at residue 106; at most of the others it has the same residue as the porcine and bovine protein S. The superior ability of rabbit protein S to act as a cofactor for human APC compared with bovine APC (He and Dahlbäck, 1993) suggests that one or more of the protein S residues, 61, 92, or 106, may play a pivotal role in its interaction with APC. Interestingly, only three such identified amino acid differences in the region of the second, third, and fourth EGF-like domains are found, Leu-195-Pro, Thr-223-Ser, and Ser-245-Pro, at which the rabbit sequence had Pro, Ser, and Pro, respectively, which suggests that these domains are not likely to be responsible for the observed species specificity of protein S for APC. Taken together, the above data suggest that Arg-49, Gln-52, Gln-61, Ser-81, Ser-92, Lys-97, Thr-103, and Pro-106 are likely candidates for residues in human protein S that may interact with APC.

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