STRUCTURE AND FUNCTION OF HUMAN PROTEIN S

STRUKTUUR EN FUNKTIE VAN HUMAAN PROTEINE S

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Trefossa

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CHAPTER 1

GENERAL INTRODUCTION TO HUMAN PROTEIN S, A NATURAL ANTICOAGULANT IN THE CONTROL OF BLOOD COAGULATION

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1 THE NATURAL ANTICOAGULANT SYSTEM

The equilibrium between blood coagulation and fibrinolysis has been defined in 1958 by Astrup as the haemostatic balance. Activation of blood coagulation leads to formation of thrombin and subsequent formation of fibrin, the insoluble protein matrix of a blood clot. Activation of blood fibrinolysis leads to formation of plasmin, the key enzyme that degrades fibrin into soluble degradation products. Disturbance of the haemostatic balance results in a tendency for thrombophilia or haemophilia (reviewed by Furie and Furie, 1988; Davie et al., 1991). One of the regulatory mechanisms in the control of blood coagulation is an anticoagulant system which includes protein C, protein S and thrombomodulin (reviewed by Clouse and Comp, 1986; Esmon, 1987; 1989; 1992; Strickland and Kessler, 1987; Walker, 1984b; 1988; Heeb and Griffin, 1988b; Dahlbäck, 1991) (Fig. 1).

Fig. 1. **The natural anticoagulant system**. EC, endothelial cell; plt, platelet; C4BP, C4b-binding protein; PS, protein S; PS_t, inactivated protein S; T, thrombin; TM, thrombomodulin; PC, protein C; APC, activated protein C; PCI, protein C inhibitor; FVa/FVIIIa, activated factor V/VIII; FVi/FVIIIi, inactivated factor V/VIII.

The physiological importance of the anticoagulant system is that deficiency of either protein C or protein S can lead to thrombotic complications (Griffin et al., 1981; Bertina et al., 1982; Schwarz et al., 1984; Comp et al., 1984; Comp and Esmon 1984).

1.1 HISTORICAL BACKGROUND

In 1960 Seegers' group identified the first inhibitor of blood coagulation which was referred to as autoprothrombin II-A (Mammen et al., 1960). Purification of the bovine protein (Marciniak et al., 1967) showed that it inhibited the factor Xa-mediated activation of prothrombin and it stimulated fibrinolysis (Zolton and Seegers, 1973). In 1976, a vitamin K-dependent protein from bovine plasma was isolated by Stenflo. This protein was called protein C, referring to the third peak that eluted from an ion-exchange chromatography column. Shortly hereafter, Seegers and coworkers (1976) showed that protein C in its activated form was immunologically identical to autoprothrombin II-A.

It was reported that protein C was converted into a serine protease by trypsin or thrombin (Marciniak, 1970; 1972; Kisiel et al., 1976; 1977; Seegers, 1981; Esmon et al., 1976). However, in the presence of calcium thrombin appeared to be a poor activator of purified protein C. Thrombin was therefore probably not of physiological importance for protein C activation unless a cofactor or another activator was involved (Amphlett et al., 1981). In 1981 Esmon and Owen reported that binding of thrombin to an endothelial cell membrane receptor markedly enhanced the activation of protein C. This receptor was referred to as thrombomodulin (Esmon et al., 1982), because it could modulate the properties of thrombin. When thrombin is bound to thrombomodulin it is unable to convert fibrinogen to fibrin and is unable to activate platelets (Esmon et al., 1983). Its coagulant properties are turned into anticoagulant properties (Owen and Esmon, 1981; reviewed by Esmon et al., 1986; Esmon, 1989). The activated form of protein C (APC) inhibits blood coagulation by a unique mechanism: it degradates factor Va, the protein cofactor of the prothrombinase complex, in the presence of calcium (Kisiel et al., 1977; 1979; Walker et al., 1979). Shortly hereafter, it was reported that factor VIIIa, the protein cofactor of the tenase complex, was also degraded by APC

(Vehar and Davie, 1980; Marlar et al., 1982; Fulcher et al., 1984).

In 1980, Walker showed that another vitamin K-dependent protein, protein S, regulates the anticoagulant activity of APC. Protein S was first isolated in 1977 by DiScipio and coworkers and was arbitrarily referred to as protein S, after the city of Seattle, where it was discovered. The cofactor role of protein S for APC was shown to be important for the inactivation of both factor Va (Walker et al., 1981a; Suzuki et al., 1983a; 1984) and factor VIIIa (Gardiner et al., 1984; Walker et al., 1987; Koedam et al., 1988).

The action of activated protein C is regulated by specific inhibitors like protein C inhibitor or plasminogen activator inhibitor-3, α_1-antitrypsin, α_2-macroglobulin and α_2-antiplasmin (Suzuki et al., 1983b; Heeb et al., 1987; Heeb and Griffin, 1988a; 1991; Hoogendoorn et al., 1991). The action of protein S is regulated by complexation with C4b-binding protein, an inhibitor of the complement system (Scharfstein et al., 1978; Fujita et al., 1979).

1.2 BIOSYNTHESIS OF PROTEIN S

Protein S is a glycoprotein (~ 7.8 % of N-linked complex type) with an apparent molecular weight of 75,000 dalton and has been purified from human and bovine plasma (DiScipio et al., 1977; DiScipio and Davie, 1979; Stenflo and Jönsson, 1979). The total concentration of protein S in plasma is approximately 26 μg/ml or 347 nM (Griffin et al., 1992). In plasma, protein S exists in two forms: 40% in the free form and 60% in a reversible complex with C4b-binding protein (Dahlbäck and Stenflo, 1981). Only free protein S acts as a cofactor for APC (Dahlbäck, 1986a). Protein S is synthesized as a single-chain molecule and secreted from human HEP G2 liver cells (Fair and Marlar, 1986); human umbilical vein endothelial cells (Fair et al., 1986); bovine aortic endothelial cells (Stern et al., 1986a); megakaryocytes (Ogura et al., 1987); human colon and breast carcinoma cells (Al-Mondhiry and Wallin, 1989); Leydig cells of human testis (Malm et al., 1991) and osteoblasts (Maillard et al., 1992). Protein S is present in the α-granules of blood platelets and can be released by stimuli like thrombin. This platelet protein S represents 2.5% of total protein S found in blood (Schwarz et al., 1985; 1986).

1.3 ORGANIZATION OF THE PROTEIN S GENES

The existance of an active protein S α and an inactive protein S β gene has been reported (Ploos van Amstel et al., 1987a; 1988). The genes are closely linked together and located on chromosome 3 near the centromere (Ploos van Amstel et al., 1987a; Watkins et al., 1988; Long et al., 1988). They are larger than 80 kb and contain 14 introns and 15 exons (Schmidel et al., 1990; Ploos van Amstel et al., 1990; Edenbrandt et al., 1990). The intron/exon organization of the active protein S α gene reflects the domain structure of protein S and supports the exon-shuffling mechanism as proposed by Gilbert (1978): structural and functional domains of mosaic proteins are encoded by separate exons or sets of exons (Gilbert, 1978; 1985; Blake, 1978; 1979; Patthy 1985; 1987; Rodgers, 1985). The vitamin K-dependent domain, the thrombin-sensitive domain, each of the 4 EGF-like domains of protein S are encoded by a separate exon, while the SHBG-like domain is encoded by 7 exons.

1.4 CLINICAL RELEVANCE OF PROTEIN S

Decreased levels of free protein S can result in thrombotic complications. In 1984 the first patients were reported in which hereditary heterozygous protein S deficiency was associated with (recurrent) venous thrombosis (Schwarz et al., 1984; Comp et al., 1984; 1985; 1986; Comp and Esmon, 1984; Broekmans et al., 1985; Bertina et al., 1985; Kamiya et al., 1986; Engesser at al., 1987). The deficiency is inherited as an autosomal dominant trait (Schwarz et al., 1984; Bertina et al., 1985; Broekmans et al., 1985), which means that heterozygotes are at risk of developing venous thromboembolism. The incidence of heterozygous protein S deficiency in patients with thrombotic disease has been determined to be between 5 and 8% (Broekmans et al., 1985; Gladson et al., 1988). The role of protein S in thrombotic diseases has been reviewed by Kessler and Strickland, 1987; Dolan et al., 1989; Preissner, 1990 and Walker, 1992.

Studying protein S deficiency at the molecular level is complicated by the existence of an active protein S α gene and an inactive protein S β gene (Ploos van Amstel et al., 1987a; 1988). The protein S β gene contain polymorphisms that map near the protein S α gene and segregate with thrombosis. In two families with inherited

protein S deficiency a mutation in the protein S β gene was reported (Ploos van Amstel et al., 1989b). This was based on the observation that the polymorphism did not segregate with the phenotype in one family, whereas it did segregate in the other family. Others, reported that inherited protein S deficiencies were caused by a deletion in exon XIII (Schmidel et al., 1991) and a deletion in the middle of the coding region of the protein S α gene (Ploos van Amstel et al., 1989a).

So far, only two genetic protein S variants have been described. Schwarz and coworkers (1989) reported a protein S variant molecule identified by immunoblotting after gel electrophoresis. The variant had a lower molecular weight. After N-glycanase treatment of the variant and normal protein S, the variant had a molecular weight similar to normal protein S, suggesting the defect to be in one of the glycosylation site. Bertina and coworkers (1990) reported a protein S variant that was not linked to thrombosis and was designated Protein S Heerlen. The variant has a slightly reduced apparent molecular weight, normal APC cofactor activity and normal binding to C4b-binding protein. DNA analysis showed that Ser-460 was replaced by proline in a consensus sequence for N-linked glycosylation of Asn-458. This suggests that the variant is probably not fully glycosylated.

2 STRUCTURAL ASPECTS OF PROTEIN S

The primary structure of bovine and plasma-derived protein S has been determined by amino acid sequence analysis and recombinant DNA techniques (Dahlbäck et al., 1986b; 1986c; Lundwall et al., 1986; Hoskins et al., 1987; Ploos van Amstel et al., 1987b). Protein S is synthesized as a precursor protein of 676 amino acids and consists of the following domains: a prepropeptide, a γ-carboxy glutamic acid-rich (GLA) domain, an aromatic stack domain, a thrombin-sensitive domain, four epidermal growth factor (EGF)-like domains and a sex hormone binding globulin (SHBG)-like domain (Fig. 2). The cDNA encoding for the precursor protein has been expressed in mammalian cells for the production of recombinant protein S and recombinant protein S variants (Malm et al., 1990; Grinnell et al., 1990; Nelson et al., 1991b; Nelson and Long, 1992; Chang et al., 1992a; 1992b; 1992c; 1992d).

Fig. 2. **Primary structure of human protein S**. Amino acid sequence (one-letter code) for human prepro protein S and location of the 14 introns (A-N), indicated by solid arrows. The prepro leader sequence includes residues -41 to -1. Solid bars are disulfide bonds; γ, γ-carboxyglutamic acid (GLA); β, β-hydroxyaspartic acid or β-hydroxyasparagine; *, thrombin-cleavage site in the thrombin-sensitive domain; o, aromatic amino acid residues in the aromatic stack domain; open diamonds indicate potential carbohydrate attachment sites of the Asn-X-Ser/Tyr type. Large numbers denote orders of four epidermal growth factor-like domains, which are followed by the sex hormone binding globulin-like domain.

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2.1 THE SIGNAL PEPTIDE OR PREPEPTIDE

The first exon of the protein S gene encodes for a typical signal peptide or prepeptide: a basic N-terminal region followed by a central hydrophobic and polar C-terminal region (Von Heijne, 1986). These structures are responsible for translocation of proteins across the endoplasmatic reticulum membrane and proper routing of proteins during secretion (Kreil, 1981; Gierasch, 1989; Rapoport, 1991). First, the basic N-terminal region of the elongating polypeptide chain binds to a signal recognition particle composed of six distinct polypeptide chains and a 7 S RNA molecule. Subsequent binding to a receptor or docking protein on the endoplasmatic reticulum results in formation of a ribosome-messenger RNA-signal recognition particle-nascent polypeptide chain complex on the outer membrane of the endoplasmatic reticulum. The signal recognition particle is then released and the nascent polypeptide chain translocates through the lumen of the rough endoplasmatic reticulum. The signal peptide is usually cleaved rapidly from the growing polypeptide chain by a membrane-bound signal peptidase (Blobel et al., 1980; Walter and Blobel, 1982). The cleavage site of the signal peptide of the vitamin K-dependent proteins by the signal peptidase is not known for all vitamin K-dependent proteins. On the basis of homology with known cleavage sites of other proteins, a prediction can be made (Von Heijne, 1986). For factor IX and protein C these sites have been identified at positions -18 and -24, respectively (Bentley et al., 1986; Jorgensen et al., 1987; Foster et al., 1987).

2.2 THE PROPEPTIDE

The propeptide of most vitamin K-dependent proteins and the bone GLA protein contain several conserved residues (Phe at -16, Ala at -10, Leu at -6, Arg at -4 and Arg at -1). This conservation leads to the hypothesis that the propeptide is involved in the γ-carboxylation process (Pan and Price, 1985). Site-specific mutagenesis of the propeptide cDNA of factor IX and protein C showed that the expressed recombinant proteins had incomplete propeptide removal and/or incomplete γ-carboxylation (Jorgensen et al., 1987; Foster et al., 1987). This prosequence was found to bind to the vitamin K-dependent carboxylase associated to the endoplasmatic reticulum membrane (Wu et al., 1991). The propeptide of

prothrombin can adopt an amphipathic α-helix. This structure within the propeptide probably plays a role in the interaction with the vitamin K-dependent carboxylase, an integral membrane protein and the lipid membrane itself (Sandford et al., 1991). Specific glutamic acid residues are converted to γ-carboxy glutamic acid residues. After this modification the protein is released from the membrane and transported to the trans-Golgi, where the propeptide is probably cleaved off by a propeptidase or processing protease. In the case of von Willebrand factor it has been shown that a subtilisin, furin or the paired dibasic amino acid-cleaving enzyme (PACE) is involved in the cleavage of the propeptide (Rehemtulla and Kaufman, 1992). Using site-directed mutants the importance of the dibasic amino acid residues Arg at -1 and Arg/Lys at -2, as well as Arg at -4 for the propeptide cleavage was identified. These three basic residues are conserved in many proteins and in the vitamin K-dependent proteins including protein S. It seems likely that a furin-like enzyme is able to cleave the protein S propeptide. Natural mutants of factor IX or site directed mutants of protein C showed the importance of these three basic amino acid residues at the C-terminal part of the propeptide (Diuguid et al., 1986; Bentley et al., 1986; Foster et al., 1990). Natural and/or artificial mutation in either one of these basic amino acid residues results in an inhibition of the propeptide cleavage. After cleavage post-translational modification like disulfide bond formation and attachment of carbohydrates takes place. The presence of a potential transmembrane type II signal-anchor domain in bovine aspartyl/asparginyl β-hydroxylase suggests that the enzyme is located on the luminal side of the endoplasmatic reticulum (Jia et al., 1992). Finally, the mature protein is secreted into the blood stream.

2.3 THE γ-CARBOXYGLUTAMIC ACID-RICH DOMAIN

The γ-carboxyglutamic acid-rich (GLA) domain (residues 1 to 37) is conserved in the vitamin K-dependent proteins and is located at the N-terminus. A specific monoclonal antibody has been described that recognizes the conserved GLA domain in the vitamin K-dependent proteins (Church et al., 1988). The GLA domain contains 10-12 posttranslationally modified γ-carboxyglutamic acids within the first 42 amino acids. Protein S contains 11 GLA residues, appearing next to each other

or interspaced by basic or hydrophobic residues. In this domain the vitamin K-dependent proteins contain a disulfide loop at Cys-18 to Cys-23, with two GLA residues at positions 20 and 21. This disulfide loop has been reported to be important for calcium binding and for the interaction with the aromatic stack domain (Schwalbe et al., 1989; Soriano-Garcia et al., 1989; 1992). The GLA residues reveal metal binding properties and the protein undergoes conformational changes leading to the expression of membrane binding properties (Nelsestuen, 1978; 1984; Liebman et al., 1987; Prendergast and Mann, 1977; Borowski et al., 1986). The tertiary structure is stabilized by a non-covalent intramolecular bridge between two or three GLA residues with a single calcium ion and regions of the polypeptide backbone (Tai et al., 1984; Furie et al., 1979). However it is still unclear whether some of the GLA residues bind directly to the membrane via calcium bridges or that metal binding to GLA residues changes the structure of adjacent domains, leading to exposure of membrane binding surfaces (Borowski et al., 1986, Mann et al., 1982). Overall, the GLA domain allows the assembly of reversible protein membrane binding via calcium bridges. Vitamin K-dependent γ-carboxylation has been reviewed by Furie and Furie (1990).

2.4 THE AROMATIC STACK DOMAIN

The aromatic stack domain or hydrophobic stack domain (residues 38 to 46) of the vitamin K-dependent proteins is located downstream adjacent to the GLA domain. It is encoded by a single exon and contains the consensus sequence Phe-Trp-X-X-Tyr. The aromatic stack domain in protein S has the sequence: Asp-Tyr-Phe-Tyr-Pro-Lys-Tyr-Leu. The side chains of the conserved aromatic residues are stacked into an aromatic cluster, which stabilizes the protein structure. The aromatic ring structure is surface-exposed, and could serve as a recognition site for a receptor or interact with other aromatic clusters (Park and Tulinsky, 1986). Crystallographic studies of the prothrombin fragment 1 revealed hydrophobic interactions of two molecules forming a tightly linked dimer in the crystal. Due to these highly conserved residues present in the vitamin K-dependent proteins, interaction between the aromatic stacks of pairs of proteins such as APC and protein S has been suggested to be neccesary for correct alignment of the proteins (Harlos et

al., 1987). Furthermore, the aromatic stack interacts in the presence of calcium with the disulfide loop of the GLA domain to form protein structures needed for the interaction of vitamin K-dependent proteins with membranes (Schwalbe et al., 1989; Soriano-Garcia et al., 1989; 1992). For bovine prothrombin fragment 1 it was shown that the residues in the aromatic stack domain are well-ordered in an α-helical structure and that the domain is oriented towards the surface of prothrombin fragment 1 (Park and Tulinsky, 1986). A decapeptide containing six residues of the aromatic stack and four residues of the beginning of the first EGF-like domain of factor IX has been shown to inhibit the binding of factor IX with its endothelial cell receptor, suggesting the aromatic stack domain to be involved in receptor binding (Ryan et al., 1989a; 1989b). Using chimeric proteins of factors IX/X Cheung and coworkers (1991) showed that the involvement of the aromatic stack domain was unlikely to be the major determinant of factor IX for its endothelial cell receptor, but rather the involvement of the GLA domain. Recently Toomey and coworkers (1992) reported that the GLA domain was indeed involved.

2.5 THE THROMBIN-SENSITIVE DOMAIN

The thrombin-sensitive domain (residues 46 to 75) of protein S contains a disulfide loop of 25 amino acids. The domain is encoded by a separate exon and is located between the aromatic stack domain and the first EGF-like domain. A distinct disulfide loop containing 14 amino acid residues has been found in prothrombin between the aromatic stack domain and the first kringle domain, and shown to be important to form a scaffold needed for the proper folding of the GLA domain (Soriano-Garcia et al., 1989). It seems likely that the disulfide loop of protein S is also involved in its interaction with the GLA domain. The thrombin-sensitive domain of protein S is essential for the expression of cofactor activity to APC. Cleavage of peptide bonds in this loop by thrombin converts protein S from a one-chain to a two-chain molecule connected by a disulfide bond. The two-chain molecule has lost its affinity for phospholipids at low calcium concentration ($< 0.2\ \mu M$) and it can not act as a cofactor to APC (Suzuki et al., 1983a; Walker, 1984a; Mitchell et al., 1986). At high calcium concentrations (5-10 mM) the thrombin-cleaved protein S binding affinity to phospholipids is the same as the native molecule suggesting that

the thrombin-sensitive domain can affect the calcium induced conformation of the GLA domain (Schwalbe et al., 1990a; 1990b). When EDTA is present, the peptide bonds in the thrombin-sensitive domain are rapidly cleaved by thrombin. In the presence of 1 mM calcium protein S is almost completely resistant to thrombin cleavage (Dahlbäck, 1983b; Mitchell et al,. 1986). This may suggest that the interaction of the thrombin-sensitive domain and the GLA domain is dependent on binding of calcium to the GLA domain.

2.6 THE EPIDERMAL GROWTH FACTOR-LIKE DOMAINS

Four epidermal growth factor (EGF)-like domains (residues 75 to 242) are present in protein S. These domains are homologous to the cysteine-rich repeat sequence of 53 amino acids found in the precursor of the mouse EGF (Doolittle et al, 1984). Structure-function relationships of EGF modules have been reviewed by Stenflo (1991). EGF is a potent mitogen for various cell lines in vitro and stimulates proliferation and differentation of several skin tissues and epithelia in vivo (Carpenter and Cohen, 1979). The EGF precursor contains 1217 amino acid residues. It is a membrane protein with an extracellular region containing 8 domains homologous to EGF, a hydrophobic stretch and a C-terminal cytoplasmic tail. Trypsin-like enzymes are able to cleave EGF from its precursor (Scott et al., 1983; Gray et al., 1983). The functions of these domains in the vitamin K-dependent proteins are unclear although they may be involved in protein-protein interactions and high affinity interactions of proteins with specific cell surface receptors (Appella et al, 1988).

EGF interaction of APC to protein S has been demonstrated by a monoclonal antibody against the EGF-like domains of APC, which inhibited the APC activity of protein S (Öhlin et al., 1987). Isolated bovine protein C fragments consisting of the GLA and EGF-like domains are able to inhibit the cofactor activity of protein S, suggesting interaction of APC with protein S via the EGF-like domains (Öhlin et al., 1988b). Dahlbäck and coworkers (1990c) demonstrated with a panel of well characterized anti-protein S monoclonal antibodies that the GLA domain, the thrombin-sensitive domain and the first and second EGF-like domains of protein S are essential for the APC cofactor activity.

Gasic and coworkers (1992) showed that protein S can act as a potent mitogen for aortic smooth muscle cells (SMC), suggesting a possible contribution of protein S to intimal SMC proliferation after vascular injury. This mitogen activity of protein S was localized on the third and fourth EGF-like domain of protein S, suggesting that these domains might interact with a cell surface receptor. This seems to extend the report of Joseph and Baker (1992), showing that the SHBG-like domain of protein S is homologous to proteins involved in growth and differentiation.

Two postribosomally modified amino acid residues in the EGF-like domains have been identified in protein S (Stenflo et al., 1987). The first domain contains a β-hydroxy aspartic acid residue at Asp-95 and the following three domains each contain a β-hydroxy asparagine residue at positions Asn-136, Asn-178 and Asn-217. The enzyme responsible for hydroxylation has been identified as 2-oxo or 2-keto glutarate-dependent dioxygenase (Stenflo et al., 1989; Derian et al., 1989; Gronke et al., 1989). Cloning of its cDNA suggest that the enzyme is located on the luminal side of the endoplasmatic reticulum (Jia et al., 1992). A consensus sequence for hydroxylation Cys-X-Asp/Asn-X-X-X-X-Tyr/Phe-X-Cys-X-Cys has been noticed by Stenflo and coworkers (1987). The hydroxylation is not complete in the vitamin K-dependent proteins and the function of hydroxylated residues is unknown. Human and bovine protein S contain respectivily 2.2 and 3.0 mol hydroxyaspartic acid residues per mole protein, instead of 4 (Lundwall et al., 1986; Dahlbäck et al., 1986c; Stenlo et al., 1987) All four EGF-like domains seems to be hydroxylated, but each domain contains less than 1 residue per mole indicating incomplete hydroxylation. Site-directed mutagenesis in factor IX of the β-hydroxyaspartic acid at position 64 to neutral or basic residues has been reported to reduce the activity of factor IX (Rees et al., 1988). Derian and coworkers (1989) however, using an inhibitor of β-hydroxylation to produce a recombinant non β-hydroxylated factor IX showed no reduction of the activity of factor IX. In protein S, β-hydroxylation is not required for APC cofactor activity and binding to C4BP (Nelson et al., 1991b). The EGF domains of the vitamin K-dependent proteins have been reported to contain high affinity calcium binding sites in factor IX (Hanford et al., 1990), in protein C (Öhlin et al., 1988a) and in protein S (Sugo et al., 1986; Dahlbäck et al., 1990b).

2.7 THE SEX HORMONE BINDING GLOBULIN-LIKE DOMAIN

Unlike the other vitamin K-dependent proteins, protein S does not have a serine protease domain, indicating that protein S is not an enzyme. Instead of the serine protease domain, protein S has a domain (residues 243-635) homologous to the sex steroid binding proteins: human sex hormone binding globulin (SHBG) and rat androgen binding protein (ABP) (Joseph et al., 1987; Baker et al., 1987; Gershagen et al., 1987). Recently, it was shown that protein S, SHBG and ABP are homologous to the basement membrane proteins, laminin A and merosin and to the integral membrane protein, *Drosophila crumbs* protein (Joseph and Baker, 1992). These proteins are involved in differentiation and development. Homology is also observed in rat agarin and *Drosophila slit* protein.

SHBG is a plasma glycoprotein, which exists as a dimer of two essentailly identical monomers (reviewed by Petra, 1991). The dimer binds only one sex steroid molecule, such as dihydrotestosterone and testosterone with high affinity (Turner et al., 1984). It is a single polypeptide of 373 amino acid residues and probably contains three carbohydrate side chains (Walsh et al., 1986). SHBG can interact with a cell membrane receptor and is regulated by steroid hormone-binding to the protein (Hryb et al., 1990). Human SHBG is produced in the liver. The Sertoli cells of human testis contain a related protein called, androgen binding protein (ABP) (Vigersky et al., 1976). ABP is also present in the rat, but the rat has no SHBG. Cloning studies have shown that human SHBG and human ABP are encoded by the same gene. The genes for SHBG, ABP and the SHBG-like domain of protein S seem to have been evolved from a common ancestral gene (Gershagen et al., 1991). Protein S, SHBG and ABP have common conserved homologies. Two pairs of cysteine residues form two internal disulfide bridges. ABP and SHBG contain a sequence with alternating leucine residues being hypothesized in steroid binding (Walsh et al., 1986). Protein S lacks this hydrophobic sequence and does not have steroid binding properties. Deduced from the cDNA sequence, three N-linked glycosylation sites are present in this domain at Asp-458, Asp-468 and Asp-489, all located in a single exon (Bertina et al., 1990). Several reports have shown that the SHBG-like domain contains binding sites for C4BP (Walker, 1989; Weinstein and Walker, 1990; Nelson and Long, 1992; Chang et al., 1992b; 1992d; Fernández et

al., 1992). They were located at the two internal disulfide loops between residues 401 to 457 and between residues 583 to 635.

3 FUNCTIONAL PROPERTIES OF PROTEIN S

Since protein S is a vitamin K-dependent protein it is anticipated that protein S is able to bind to phospholipid membranes via calcium bridges (DiScipio et al., 1977). Protein S is an anticoagulant protein and serves as a cofactor to APC (Walker, 1980). It has been demonstrated that protein S is able to act as an anticoagulant by directly inhibiting the prothrombinase complex without APC (Mitchell et al., 1988b; Heeb et al., 1992). The action of protein S is regulated by complex formation with C4BP (Dahlbäck and Stenflo, 1981) and by inactivation by thrombin (Suzuki et al., 1983a; Walker, 1984a). In infammatory processes during *Escherichia Coli* infusion in baboons, APC and protein S could protect against disseminated intravascular coagulation and organ damage (Taylor et al., 1987; 1991a).

3.1 *BINDING TO Ca^{2+}-IONS*

The vitamin K-dependent proteins are able to bind calcium via the γ-carboxyglutamic acid-rich (GLA) domain (Stenflo and Suttie, 1977). Formation of GLA residues requires vitamin K and the vitamin K-dependent carboxylase. Once calcium binds to the GLA residues a conformational change occurs and the interaction of the protein with negatively charged phospholipids surfaces takes place (Nelsestuen, 1984). This membrane-binding property is essential for protein S to express APC cofactor activity. Chemical conversion of the GLA residues to γ-methylene glutamic acid residues produces a protein S molecule without APC cofactor activity. It is also unable to bind to phospholipid vesicles and has a decreased quenching of intrinsic fluorescence by calcium (Walker, 1986a).

Cleavage of protein S by thrombin leads to a two-chain molecule with reduced calcium binding capacity at low calcium concentration (Suzuki et al., 1983a; Walker, 1984a). At high calcium concentration (5-10 mM), native and cleaved protein S bind equally well to phospholipids (Schwalbe et al., 1990a; 1990b). This suggests that the thrombin-sensitive domain can be important for the conformation of protein S and for calcium binding of the GLA domain (Walker, 1988).

Recently, Dahlbäck and coworkers (1990b) described a novel type of a high affinity calcium binding site in protein S located in the EGF-like domains. Each domain contains a calcium binding site with a K_d ~ 20 μM, which is low compared to a K_d ~ 0.5 mM of calcium binding to the GLA domain. It is proposed that the calcium binding sites of the EGF-like domains are located at two conserved aspartic acid residues together with the β-hydroxy aspartic acid or β-hydroxy asparagine. This is based on analogy of the 2D NMR spectra of the mouse and human EGF precursor (Makino et al., 1987; Montelione et al., 1987; Cooke et al., 1987).

3.2 INTERACTION WITH BIOMEMBRANES

In the presence of calcium the GLA residues of protein S confer a conformational change leading to the expression of a membrane-binding site. Via this site protein S can bind to membrane surfaces. Walker (1981a) has reported that protein S and APC form a 1:1 molar complex on the surface of phospholipid vesicles. Protein S reduces the apparent dissociation constant between APC and phospholipids about 10-fold. It is also shown that the complex is formed on the platelet surface (Harris and Esmon, 1985; Solymoss et al., 1988) leading to inactivation of membrane-bound factor Va. Protein S bound to platelets can be cleaved rapidly by a platelet-bound calcium-dependent protease (Mitchell and Salem, 1987). Protein S has also been reported to bind to neutrophils (Oates and Salem, 1991); bovine endothelial cells (Stern et al., 1986b); human umbilical vein endothelial cells (Hackeng et al., 1992) and to the surface of platelet-derived microparticles (Tans et al., 1991; Dahlbäck et al., 1991b), where it serves as a receptor to APC. The microparticles are generated when agonists like thrombin induce platelet activation (Sims et al., 1989). The binding of protein S to biomembranes has been reported to be important for expression of the APC cofactor activity of protein S in the inactivation of membrane-bound factor Va (Suzuki et al., 1984; Harris and Esmon, 1985; Stern at al., 1986b; Brett et al., 1988; Tans et al., 1991; Hackeng et al., 1992). This stimulatory effect is approximately 2-fold (Solymoss et al., 1988; Tans et al., 1991; Bakker et al., 1991; Hackeng et al., 1992) which is relatively low compared to the 9-fold stimulating effect for the inactivation of factor VIIIa by APC (Koedam et al., 1988). It seems unlikely that this moderate stimulating effect is caused by the

presence of endogenous protein S since the synthesis and release is too low to interfere significantly with the effect of exogenous protein S (Fair et al., 1986). It has been shown that protein S cleaved by thrombin does not bind to phospholipids at low calcium concentrations (Suzuki et al., 1983a; Walker, 1984a; Mitchell et al., 1986), suggesting the thrombin-sensitive domain can be important for the conformation of the GLA domain. The EGF-like domains are other important domains involved in the interaction of protein S with APC on membranes. It has been suggested that the EGF-like domains of APC and protein S are involved in protein-protein interaction on the phospholipid membrane (Öhlin et al., 1988a; Dahlbäck et al., 1990c). Protein S complexed to C4BP binds equally well to membranes as free protein S (Schwalbe et al., 1990a; 1990b; Hackeng et al., 1992), suggesting that the C4BP-binding site is not located at the N-terminal region of protein S, but in the C-terminal region (Walker, 1989; Nelson and Long, 1992; Chang et al., 1992b).

3.3 COFACTOR ACTIVITY TO ACTIVATED PROTEIN C

Walker (1980) reported that protein S serves as a cofactor to APC in the inactivation of factor Va. When factor Xa is bound to factor Va, APC alone is not able to inactivate factor Va. Protein S however, can reverse this protective effect of factor Xa and thereby modulating the inactivation of factor Va by APC (Solymoss et al., 1988). It is also shown that protein S serves as a cofactor to APC in the inactivation of factor VIIIa (Gardiner et al., 1984; Fulcher et al., 1984; Walker et al., 1987; Koedam et al., 1988). Inactivation of factor VIIIa could be protected by von Willebrand factor (Koedam et al., 1988) and by factor IXa (Bertina et al., 1984). The APC cofactor activity of protein S can be inhibited by antiphospholipid antibodies (Malia et al., 1990), probably by shielding off phospholipid surfaces. On the phospholipid surface protein S serves as an anchor for APC (Harris and Esmon, 1985). It has been suggested that the equimolar complex of APC and protein S form hetero-dimers, probaby existing in the absence of factor Va and VIIIa (Harlos et al., 1987). Nishioka and Suzuki (1990) have shown that APC can interact with the protein S-C4BP complex, suggesting that the binding sites for APC and C4BP are different. Using a panel of anti-protein S monoclonal antibodies Dahlbäck and

coworkers (1990c; Malm et al., 1987) demonstrated that GLA domain, the thrombin-sensitive domain, the first and second EGF-like domains of protein S are required for the APC cofactor activity of protein S.

Marciniak (1970) showed that autoprothrombin IIA or APC activity was species specific, meaning that one species is a poor anticoagulant in the blood of another species. This species specificity is probably not due to substrate specifity but to interaction of APC with protein S. Protein S was observed to act as an APC cofactor in heterologous plasma (Walker, 1981b).

3.4 CLEAVAGE AND INACTIVATION BY THROMBIN

Bovine and human protein S have been reported to be very sensitive to proteolysis by thrombin (Dahlbäck, 1983b; Walker, 1984a; Dahlbäck et al., 1986b; Morita et al., 1986; Mitchell et al., 1986). The single-chain molecule is converted to the two-chain molecule linked together by a disulfide bond between Cys-47 and Cys-72. This thrombin-cleaved protein S has no APC cofactor activity, it has lost its affinity for negatively charged phospholipids at low calcium concentrations and it has a reduced calcium binding. This cleavage has been postulated as a control mechanism for the anticoagulant activity for APC (Suzuki et al., 1983a; Walker, 1984a). It seems unlikely that cleavage of protein S occurs under physiological conditions because calcium protects protein S from inactivation by thrombin. Thrombomodulin was shown to inhibit cleavage by thrombin (Mitchell et al., 1986). Dahlbäck and coworkers (1986b) have reported that in bovine protein S the thrombin cleavage sites are located at Arg-52 and Arg-70, using amino acid sequencing of thrombin-cleaved protein S fragments. For human protein S these have been located at Arg-49, Arg-60 and Arg-70 using site-specific mutagenesis of protein S variants (Chang et al., 1992c). Besides thrombin, protein S has been reported to be sensitive for proteases as plasmin, plasma kallikrein, chymotrypsin and trypsin (Dahlbäck, 1983b; Morita et al., 1986). Platelet-bound calcium-dependent protease and neutrophil elastase have been reported to cleave protein S (Mitchell et al., 1987; Oates and Salem, 1991). Cleavage of protein S has been found in plasma of patients with intravascular coagulation, suggesting activation of the anticoagulant system in these patients (Heeb et al., 1989).

3.5 COMPLEXATION WITH C4b-BINDING PROTEIN

In human plasma, protein S exists in two forms: 60% in an 1:1 complex with C4BP and 40% in a free form (Dahlbäck and Stenflo, 1981; Dahlbäck et al., 1983b; 1984; 1985; Nutkatsuka and Nagasawa, 1987; Hessing, 1991). Only free protein S has APC cofactor activity (Dahlbäck et al., 1986a; Bertina et al., 1985).

C4BP is a component of the classical pathway of the complement system (Scharfstein et al., 1978; Fujita et al., 1979). It is a high molecular weight (530,000-570,000 dalton) multimeric protein with a unique octopus-like structure (Dahlbäck 1983a). The plasma concentration is 155 mg/L or 272 nM (Griffin et al., 1992). It consists of 6 or 7 identical α-chains and 0 or 1 β-chain and exists as a very heterologous population in plasma. The α and β-chains are linked together by disulfide bridges and are in, part very homologous. Suzuki and Nishioka (1988) reported the protein S binding site to be located on the α-chain, while in other reports the β-chain appeared to be the protein S binding site (Hillarp and Dahlbäck, 1988; Hillarp et al., 1989; Hessing et al., 1990). Matsuguchi and coworkers (1989) have reported that the α chain of C4BP is identical to proline-rich protein, a glycoprotein located in chylomicrons with lipid binding properties (Sata et al., 1976). C4BP is an acute phase protein and its concentation in plasma can be increased 4 times by inflammatory processes (Barnum et al., 1990; Stankiewicz et al., 1991). This increase lowers the levels of free protein S in plasma (Comp et al., 1986) The complex of protein S with C4BP is calcium-dependent and non-covalent with a $K_d \sim 10^{-10}$ M (Nelson and Long, 1991a; Dahlbäck et al., 1990a; Griffin et al., 1992). Because of this very low K_d value Dahlbäck and coworkers (1990a) have suggested that a third component might be involved in the equilibrium between protein S and C4BP in plasma. Griffin and coworkers (1992) reported that the majority of the free C4BP in plasma does not contain a β-chain and thus is not able to participate in equilibrium binding to free protein S. Binding sites for C4BP on protein S have been localized in the first (Fernández et al., 1992; Chang et al., 1992d) and in the second disulfide loop of the SHBG-like domain (Walker, 1989; Nelson and Long, 1992; Chang et al., 1992b).

Walker (1986b) has reported a binding protein in bovine plasma, protein S binding protein. It was shown that protein S binding protein could promote the APC

cofactor activity of protein S. The structure, function and existence of this protein in other species remains to be investigated.

3.6 PROTECTION IN INFLAMMATORY PROCESSES

The linkage between coagulation and inflammation is manifest in gram negative septic shock. During septic shock, platelets, leukocytes and monocytes are activated by bacterial endotoxins resulting in the release of cytokines like interleukin-1 and tumor necrosis factor. These cytokines promote the coagulant state of the endothelium by inducing tissue factor expression and to down-regulate thrombomodulin. This can lead to increased coagulation and suppression of the anticoagulant system and ultimately to disseminated intravascular coagulation (reviewed by Esmon et al., 1991). It was shown that APC could prevent the coagulopathic and lethal effects induced by *Escherichia coli* infusion in baboons, suggesting a role of the protein C anticoagulant system in the development of thrombosis during sepsis (Taylor et al., 1987). C4BP, an inhibitor of the complement system is also an acute phase protein which can be elevated during inflammatory disease states including disseminated intravascular coagulation and systemic lupus erythematosus (D'Angelo et al., 1988; Comp et al., 1986; Saeki et al., 1989). This might lead to a decreased level of free protein S and therefore decreased protein S functional activity. Taylor and coworkers (1991a) showed that administration of C4BP during an inflammatory proces in baboons could contribute to fibrin formation, cytokine response and organ damage. Administration of protein S could effectevily prevent these processes, indicating that protein S contributes to the protective effects of APC. An anti-protein S monoclonal antibody which inhibited the APC cofactor activity of protein S was shown to lead to disseminated intravascular coagulation, organ damage and ultimately death when a sublethal dosis of *Escherichia coli* was infused in baboons. This shows the importance of protein S as a potential therapeutic and clinical agent in the treatment of reduced levels of protein S in inflammatory disease states associated with thrombosis (D'Angelo et al., 1988; Comp et al., 1985). It further demonstrates that the anticoagulant system can function as a modulator in inflammatory processes. This modulating effect is different from the effects of another anticoagulant, active site

blocked factor Xa, which could block fibrin formation but failed to prevent shock and organ damage (Taylor et al., 1991b). The anticoagulant system is probably very effective to overcome inflammatory disease states and prevent shock and organ damage.

3.7 ROLE IN THE PROTHROMBINASE COMPLEX

The prothrombinase complex formed both via the intrinsic and extrinsic coagulation pathways consists of calcium, negatively charged phospholipids, the cofactor Va and the enzyme factor Xa (Mann et al., 1982). The complex is inhibited through inactivation of membrane-bound factor Va by APC (Kisiel et al., 1977; 1979; Walker et al., 1979). It was reported that protein S could enhanced the action of APC (Walker, 1981a; Suzuki et al., 1984). Factor Xa is able to protect factor Va from inactivation by APC (Dahlbäck and Stenflo, 1980; Nesheim et al., 1982; Suzuki et al., 1983c) and protein S is able to reverse this protection (Solymoss et al., 1988; Jane et al., 1991). It seems that the GLA-domain of factor X/Xa could protect factor Va from APC (Jane et al., 1991). Mitchell and coworkers (1988b) have described a modified protein S, which was isolated with an anti-protein S monoclonal antibody column. This modified protein S was shown to have intrinsic activity by directly inhibiting the prothrombinase complex without APC. It was further demonstrated that modified protein S competed with prothrombin in the reaction with factor Xa. Inhibition of the APC cofactor activity by prothrombin have also been demonstrated (Mitchell et al., 1988a). Recently, Heeb and coworkers (1992) have shown that protein S can directly inhibit the prothrombinase complex independent of APC. The inhibition was associated by binding of protein S to factor Va in a calcium-dependent, saturable and reversible manner. Binding of protein S to factor Va was partly competed by prothrombin. These results showed the existance of molecular forms of protein S with anticoagulant activity independent of APC.

3.8 ROLE IN FIBRINOLYSIS

Autoprothrombin II-A or APC has been shown to have anticoagulant activity and profibrinolytic activity (Mammen et al., 1960; Zolton and Seegers, 1973). It has been shown that APC is able to neutralize plasminogen activator-1 (PAI-1) and thus

promotes the fibrinolytic activity of APC (Taylor and Lockhart, 1985; Van Hinsbergh et al., 1985; Sakata et al., 1985). It was proposed that protein S could act as an APC cofactor in this process. Protein S was required for the expression of the profibrinolytic effect of APC in blood (De Fouw et al., 1986; D'Angelo et al., 1987). Using purified proteins De Fouw and coworkers (1988) have shown that protein S was not required for the neutralization of PAI-1 by APC. Weinstein and Walker (1991) reported that the profibrinolytic effect of APC correlates with its anticoagulant property and not by its neutralization by PAI-1 using plasma and APC of different species. The profibrinolytic properties through neutralisation of PAI-1 by APC are not protein S-dependent but involves other mechanisms.

4 AIMS AND OUTLINE OF THE PRESENT RESEARCH

Protein S is a natural anticoagulant in the control of blood coagulation. Decreased levels of protein S can lead to uncontrolled coagulation resulting in thrombosis. Purification of protein S often leads to a protein S preparation with proteolytic degraded material. Identification of the proteolytic cleavage sites in protein S can lead to the design of a protein S variant resistant for proteolysis. This will be important to obtain a protein S preparation without degraded material and still retaining full APC cofactor activity. The APC cofactor activity of protein S in blood is regulated by complexation with C4BP. Identification of the C4BP binding sites on protein S can lead to the design of protein S variants which are unable to bind to C4BP. Ultimately protein S variants resistant for proteolysis and without affinity for C4BP can be designed. These protein S variants may be used as potential therapeutic agents in the treatment of protein S deficiencies in trombosis and Gram-negative septic shock.

A molecular biological approach was followed to the design of recombinant protein S variant molecules with specific deletions or amino acid substitutions. First, a mammalian expression cell system was set up to express a normal recombinant protein S molecule with similar properties as plasma-derived protein S (chapter 2). Chapter 3 describes the use of deletion variants to study the interaction of protein S with C4BP. How will deletion effect the binding to C4BP? Protein S variants with amino acid substitutions were also designed and characterized for C4BP binding

(chapters 2 and 4). Another set of protein S variants was designed to study the effect of mutations of specific amino acid residues in the thrombin-sensitive domain of protein S (chapter 5). Finally, the results described in this thesis are discussed in chapter 6, which includes the presentation of a model between the interaction of protein S with C4BP.

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CHAPTER 2

EXPRESSION AND CHARACTERIZATION OF RECOMBINANT HUMAN PROTEIN S IN HETEROLOGOUS CELLS - STUDIES OF THE INTERACTION OF AMINO ACID RESIDUES LEU-608 TO GLU-612 WITH HUMAN C4b-BINDING PROTEIN

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ABSTRACT

Mouse C127 epithelioid cells were genetically engineered to produce biologically active γ-carboxylated human protein S. A full length human protein S cDNA was cloned into a bovine papilloma virus (BPV) based shuttle vector under the transcriptional control of the Moloney murine sarcoma virus enhancer and the mouse metallothionein promoter. Stable expression was obtained in transfected C127 cells. Expression of γ-carboxylated protein S was dependent on the presence of vitamin K in the culture medium. Protein sequence analysis showed that recombinant and plasma protein S has the same amino terminal sequence. Analysis of specific post-translationally modified amino acids shows that recombinant protein S is fully γ-carboxylated and fully β-hydroxylated. Immunoblotting analysis using polyclonal and monoclonal antibodies shows that recombinant protein S has a slightly higher molecular weight than plasma protein S. After N-Glycanase treatment, identical molecular weights are observed for recombinant and plasma protein S, indicating that the difference is caused by differences in the N-linked carbohydrate side chains. Recombinant protein S also demonstrates normal cofactor activity for activated protein C in a clotting assay. Binding studies with the complement component, C4b-binding protein (C4BP), shows that recombinant protein S binds to C4BP with the same apparent affinity as plasma protein S. Two variant molecules are also tested for their binding to C4BP. The first variant has a replacement of amino acid residue Leu-608 by Val and was designated B variant. The second variant has three alterations, at positions 609, 611 and 612 where the acidic amino acid residues Asp, Asp and Glu were replaced by Asn, Asn and Gln, respectively and this variant was designated C variant. The binding of these variants to C4BP was the same as wild type recombinant protein S. This suggests that amino acid residues Leu-608, Asp-609, Asp-611 and Glu-612 are not essential for binding of the intact full length protein to C4BP.

INTRODUCTION

Human protein S is a vitamin K-dependent plasma glycoprotein (1,2) that functions as a cofactor of activated protein C in the inactivation of the coagulation cofactors

Va and VIIIa (3-5). This anticoagulant property makes protein S an important regulator of blood coagulation. The physiological importance of protein S can be deduced from the observation that patients partially deficient in protein S have an increased risk to develop thrombosis (6-8). The protein S concentration in human plasma is about 25 mg/l of which about 60% is non covalently bound to the complement component C4b-binding protein (C4BP); this C4BP bound protein S is no longer active as cofactor of activated protein C (9,10).

The cDNAs encoding for bovine (11) and human protein S (12-14) have been reported. The human protein S cDNA encodes for a protein S precursor consisting of a 41 amino acid prepropeptide followed by 635 amino acids that form the mature protein S molecule and contain the following domains: an amino-terminal domain containing 11 γ-carboxyglutamyl (gla) residues, a region containing the thrombin-sensitive site, four epidermal growth factor (EGF) like domains (the first EGF-like domain contains a β-hydroxyaspartyl (hya) residue while each of the following three EGF-like domains contains a β-hydroxyasparaginyl (hyn) residue) and a C-terminal loop that shows homology with the sex hormone globulin binding protein (SHGB). This SHGB-like region in protein S is not found in other vitamin K-dependent proteins.

Walker (15) has studied the interaction between human protein S and C4BP using synthetic peptides of the protein S sequence. A peptide based on the amino acid sequence Gly-605 to Ile-614 near the C-terminal part of protein S inhibited the interaction between protein S and C4BP. We have performed site-specific mutagenesis of amino acid residues in this C-terminal part of protein S to study the involvement of these residues in the binding of C4BP to protein S. Two variants were constructed. The first variant has an alteration of amino acid residue Leu-608 to Val-608 and was designated B variant. This variant was based on the observation of Dahlbäck (16) that bovine protein S binds tighter to human C4BP than human protein S. Residue 608 is the only difference in this region between bovine and human protein S. The second variant has three alterations and was designated C variant. The acidic amino acid residues Asp-609, Asp-611 and Glu-612 were altered to Asn-609, Asn-611 and Gln-612, respectively, because these charged residues may be important in the binding to C4BP. We show that these

amino acid residues have no effect on the binding to C4BP, indicating that these amino acid residues are not essential for the binding to C4BP of the intact protein.

MATERIALS AND METHODS

Materials - Restriction endonucleases SmaI, PstI, BamHI, BglI, BglII and EcoRV were purchased from Promega Biotec (Madison, USA) and AccII from Amersham International (Amersham, UK). Calf intestine alkaline phosphatase was from Boehringer Mannheim (Mannheim, FRG). T4 DNA Ligase was from Bethesda Research Laboratories (Bethesda, USA). All enzymes were used according to the manufacturers instructions. *Escherichia coli* strains CJ236 and MV1190 and plasmid pTZ18R were from Bio-Rad Corporation (Richmond, USA). Sequenase Version 2.0 sequencing kit was from USB Corporation (Cleveland, USA). Human protein S and activated protein C were purified from normal pooled plasma as described elsewhere (5). C4b-binding protein was immuno-purified from a barium citrate precipitate of human plasma using anti-C4BP monoclonal affinity chromatography as described (17). Protein S and C4BP depleted plasma was obtained by passing plasma through a Sepharose 4B column to which anti-protein S and anti-C4BP IgG were coupled (5). The depleted plasma contained less than 2% protein S and C4BP as determined by specific ELISAs (18). Thrombin-Sepharose (1 mg/ml gel) was prepared by coupling α-thrombin (Sigma, St. Louis, USA) to CNBr activated Sepharose (Pharmacia, Sweden) according to the manufacturers instructions. Monoclonal anti-protein S antibody S11-Sepharose (1 mg/ml gel) was prepared by coupling S11 to CNBr activated Sepharose. Fast Flow Q resin was obtained from Pharmacia (Sweden). Rabbit anti-human protein S IgG conjugated to peroxidase (1.3 mg/ml) and rabbit anti mouse IgG conjugated to peroxidase (1 mg/ml) were from Dakopatts (Glostrop, Denmark). Immobilon membranes were from Millipore Pty. Ltd. (Bedford, USA). N-Glycanase was from Genzyme Corporation (Boston, USA) and protein S was treated according to manufacturers instructions.

Cloning and Insertion of Protein S cDNA into a Mammalian Expression Vector - A full length cDNA was obtained by fusion of 2 overlapping partial cDNA clones (14). Clone pSUL9 was digested with BglI and BamHI and clone pSUL11 was digested with BglI and PstI. The desired fragments were ligated into the BamHI and PstI-cleaved cloning vector pUC18. The resulting plasmid was designated pSUL and the protein S sequence was verified by the dideoxy chain termination sequencing method (19). An AccII-EcoRV cDNA blunt fragment containing the complete protein S coding region was isolated from pSUL and inserted into the unique SmaI site of the expression vector pSV328A+ (a gift from dr. W. Bovenberg). The resulting plasmid was designated pSVPS and was initially used for transient expression in COS-1 cells and stable expression in wild type CHO K-1 cells and CHO cells deficient in dihydrofolate reductase. pSVPS was cleaved with BglII and BamHI and ligated into the unique BglII site of the plasmid p79-18, which is similar to the

plasmid p8-4 as described by Sarver et al. (20) and designated pMSVPS. Cloning of the BPV-1 genome in this plasmid was as follows. In order to facilitate the growth and production of the full length viral BPV-1 genome in bacteria, pTZX was first constructed from pTZ18R by cleaving pTZ18R with BamHI and inserting the following partially self-complementary oligonucleotide: GAT CTC TCG AGG GAT CCC TCG AGA. In pTZX a BamHI site is now flanked by two XhoI sites, while the reading frame is still intact. The full length viral BPV-1 genome was cleaved at the unique BamHI site and inserted into BamHI cleaved pTZX to yield the plasmid pTZX-BPV. The resulting full length bacterial BPV-1 genome can now be cleaved from pTZX-BPV using either BamHI or XhoI. pMSVPS was cleaved with BamHI and ligated with the BamHI cleaved bacterial BPV-1 genome. The final plasmid was designated pBPVPS. All the cloning steps were performed using standard techniques (21).

Site-Specific Mutagenesis - A 1,300-base pair XbaI-EcoRI fragment from the expression vector pMSVPS carrying the protein S cDNA sequence was subcloned into M13mp19. For the B variant the 19-mer oligonucleotide 5'-CCAGATCCACCTGTACACC-3' and for the C variant the 40-mer oligonucleotide 5'-GTTTAGAAATGGCTTGATTCAGATTCAACTGTACACCATT-3' were used. Underlined nucleotides indicate the positions of the mutations. Site-specific mutagenesis was performed according to Kunkel (22). The M13mp19 clone carrying the protein S cDNA insert was infected into *Escherichia coli* CJ236 (dut^- ung^-) cells and uracil containing single stranded phage DNA was isolated and used as template. After second strand synthesis with T4 DNA Polymerase and ligation with T4 DNA Ligase competent *Escherichia coli* MV1190 (dut^+ ung^+) cells were transformed. Single stranded DNA from individual resultant plaques were isolated and sequenced by the dideoxy chain termination method (19) using the universal M13 forward primer to confirm that the sequence was as expected and that no inadvertent mutations had been introduced. Double stranded phage DNA was isolated and the protein S cDNA insert isolated and recloned into pMSVPS. The BPV-1 genome was isolated after Bam HI digestion as described before and cloned into pMSVPS. The resulting plasmids were finally purified by CsCl gradient centrifugation.

Cell Culture, DNA Transfection and Selection of Stable Transformants - C127 cells (ATCC CRL 1616) were routinely cultured in DMEM supplemented with penicillin G (100 U/ml), streptomycin sulphate (100 μg/ml), glutamine (2 mM) and 10% heat-inactivated fetal calf serum (GIBCO, Paisley, UK). Cultures were maintained at 37°C containing 5% CO_2. The cells were transfected with 15 μg of plasmids by conventional calcium phosphate coprecipitation (23). Briefly, the precipitate was incubated for 20 min at room temperature and added to the cells which were previously subcultured at $5x10^5$ cells/10 cm dish (24 h prior to transfection). After transfection overnight, the cells were rinsed twice with PBS and were allowed to recuperate overnight in complete medium. The cells were then trypsinized, split at an 1:10 ratio and incubated in medium containing only 5%

fetal calf serum. Cells were fed every 3-4 days until foci were visible, usually 12-14 days after starting subculturing. Foci were isolated, grown individually and subcloned using limited dilution (1 cell/well). Expression of γ-carboxylated protein S was initiated by growing the cells in DMEM without serum supplemented with vitamin K_1 (Konakion, Roche, 5 μg/ml) and the clone producing the highest levels of protein S antigen, as determined by a specific ELISA (24), was used for further studies.

Purification and Immunoblotting Analysis - Conditioned media (500 ml) were supplemented with 25 ml 1 M sodium citrate (final concentration 50 mM) and 50 ml 1 M $BaCl_2$ was added slowly (final concentration 100 mM) while stirring on ice. The barium citrate precipitate was collected after 30 min by centrifugation at 5,000 rpm for 10 min, dissolved in 50 ml 100 mM Tris-HCl pH 7.5 containing 100 mM EDTA and dialyzed extensively against 50 mM sodium citrate, reprecipitated by the addition of 5 ml 1 M $BaCl_2$, dissolved in 5 ml 100 mM Tris-HCl pH 7.5 containing 100 mM EDTA and finally dialyzed extensively against 50 mM Tris-HCl pH 7.5 containing 150 mM NaCl. For amino terminal sequence analysis recombinant protein S was further purified by monoclonal antibody S11 coupled to CNBr-activated Sepharose 4B. S11 is probably directed against the thrombin sensitive region of protein S (25). The column (1 ml) was equilibrated with 50 mM Tris-HCl pH 7.5 containing 150 mM NaCl and 5 mM benzamidine, washed with the same buffer and eluted with the same buffer containing 3 M KCNS. The fractions containing recombinant protein S were pooled and dialyzed against starting buffer. The dialysate was applied to an anion exchange Fast Flow Q resin column (2 ml) and further purified according to Grinnell et al. (26). Briefly, recombinant protein S was adsorbed to the column in starting buffer containing 2 mM EDTA, washed with the same buffer without EDTA and eluted with the same buffer containing 5 mM $CaCl_2$. The fractions containing recombinant protein S were pooled, dialyzed against the starting buffer and stored at -20°C.

SDS/PAGE was performed in 10-15% gradient gels according to Laemmli (27) using the Phast system (Pharmacia Biotechnology, Sweden). After electrophoresis, the proteins were stained with silver nitrate (28) or transferred onto Immobilon membranes (29). The membranes were incubated with 5% (w/v) non-fat dry milk (Protifar, Nutricia, The Netherlands) and 0.1% (v/v) Tween 20 (Sigma) in PBS. Protein S antigen was detected using rabbit anti-protein S IgG conjugated to peroxidase (3 μg/ml) or several monoclonal antibodies IgGs (5 μg/ml), followed by rabbit anti mouse IgG conjugated to peroxidase (1 μg/ml).

Inactivation of Protein S by Thrombin - One ml of recombinant or plasma protein S (50 μg/ml) was incubated with 50 μl of thrombin-Sepharose 4B (1 mg/ml) for 1 h at 37°C in 50 mM Tris-HCl pH 7.5 containing 150 mM NaCl. Thrombin-sepharose 4B was removed by centrifugation and inactivated protein S was stored at -20°C until needed.

N-Terminal Sequence, gla and hya/hyn Analysis - N-terminal sequence of plasma and recombinant protein S was analyzed with a 475 A Applied Biosystems (Foster City, USA) pulse liquid sequenator on-line connected with a phenylthiohydantoin (PTH) amino acid analyzer 120 A. Gla was determined by dr. C. Vermeer according to Kuwada and Katayama (30). Hya/hyn was determined by dr. J. Stenflo according to Stenflo et al. (31).

Binding of Protein S and Variants to C4BP - The complex formation between plasma, variants and recombinant protein S and C4BP was measured with a sandwich ELISA using C4BP and a peroxidase conjugated anti-protein S antibody as described before (17). Briefly, 100 μl IgG (10 μg/ml) from monoclonal antibody 8C11, which is directed against the large subunit (α-chain) of C4BP was coated in 50 mM $NaHCO_3$, pH 9.6 overnight at 4°C onto polyvinyl microtiter plates (Costar, Cambrigde, USA). After removal of unbound immunoglobulins, the wells were blocked with 1% (w/v) BSA in 50 mM Tris-HCl pH 7.5 containing 150 mM NaCl and 5 mM $CaCl_2$ for 1 h at room temperature. C4BP was allowed to bind in the same buffer for 2 h at a final concentration of 1 μg/ml. After washing the wells with buffer without BSA to remove unbound proteins, subsequently increasing amounts of protein S were added and incubated for 15 min or 24 h. The wells were washed again with buffer without BSA and bound protein S was detected by anti-protein S IgG conjugated to peroxidase (0.3 μg/ml) and the rate of hydrolysis of nitrophenylphosphate was measured at 492 nm using a Titertek Multiscan (Flow Laboratories Inc., McLean, USA).

Protein S Assays - Protein S antigen was determined using a specific ELISA as described elsewere (24). Briefly, protein S was allowed to bind in the presence of 1% (w/v) BSA and 0.1% (v/v) Tween 20 in PBS (10 mM sodium phosphate pH 7.4, 2.7 mM KCl and 150 mM NaCl) to immunopurified rabbit anti-human protein S IgG (10 μg/ml, 50 mM $NaHCO_3$ pH 9.6) immobilized onto polyvinyl microtiter plates. After overnight binding, unbound proteins were removed and rabbit anti-protein S IgG conjugated to peroxidase (0.3 μg/ml) was added to the wells. Bound conjugate was measured by the rate of hydrolysis of nitrophenylphosphate and the absorbance at 492 nm was measured with a Titertek Multiscan (Flow Laboratories Inc., McLean, USA).
Protein S cofactor activity was determined in an activated partial thromboplastin time system. Briefly, to 50 μl protein S and C4BP depleted plasma was added 50 μl cephalin-kaolin reagent (Boehringer Mannheim), 25 μl activated protein C (5 μg/ml) and 25 μl protein S (0-50 μg/ml) diluted in Michaelis buffer (28.5 mM sodium acetate, 28.5 mM sodium barbital, 116 mM NaCl, pH 7.35) and incubated for 3 min at 37°C. Clotting was initiated by addition of 50 μl of 25 mM $CaCl_2$ and the clotting time measured in an Amelung-Coagulometer KC10.

RESULTS

Expression of Recombinant and Variants of Human Protein S in C127 Cells - The insertion of the full length human protein S cDNA in the expression vector is shown in Fig. 1. In the construct, the human protein S cDNA is under transcriptional control of the mouse metallothionein promoter and the Moloney murine sarcoma virus enhancer elements. In addition, the transcription unit contains the small t intron and early polyadenylation signal from Simian Virus SV 40 (20) and the entire bovine papillomavirus genome. The plasmid was transfected into C127 cells and after 2 weeks foci were isolated, subcloned by limited dilution and assayed for protein S antigen.

Fig.1. **Physical map of plasmid pBPVPS**. The expression vector contains the complete BPV-1 genome ligated to bacterial origin of replication and ampicillin (AMP) resistance sequences of the plasmid pML 2, as described by Sarver et al. (20). The full length human protein S cDNA is flanked 5' by the mouse metallothionein (MMT) promoter and the Molony murine sarcoma virus (MSV) enhancer elements, and 3' by the SV40 polyadenylation recognition and small t antigen splicing signals.

Since protein S is a vitamin K dependent protein, the effect of vitamin K addition was monitored. Fig. 2 shows a maximum in the rate of recombinant protein S antigen production at 5-10 μg/ml of vitamin K_1. Large-scale production of recombinant protein S was obtained in DMEM without serum containing 10 μg/ml of vitamin K_1. Conditioned media were harvested every 48 h and stored at -20°C untill needed. The mean protein S antigen level in the pooled media was 0.5-1 μg/ml for recombinant protein S and the C variant and 0.1-0.2 μg/ml for the B variant.

Fig. 2. **Recombinant protein S antigen levels in transfected C127 cells as a function of vitamin K_1 concentration**. Confluent cells were grown in DMEM without serum containing increasing amounts of vitamin K_1. After 48 h at 37°C conditioned medium was harvested and analyzed for protein S antigen. The experiment was performed in duplicate.

Purification and Immunological Analysis - Recombinant protein S was purified from the culture medium by two successive barium citrate precipitation steps. With this technique partially and fully carboxylated proteins can be isolated. Recombinant

protein S was obtained in a 60% yield with a purity of over 95% as jugded by silver staining after separation with SDS-PAGE (Fig. 3). We have also purified variant and recombinant protein S by applying culture medium, after addition of 5 mM EDTA, directly onto a Fast Flow Q column as described by Grinnell et al. (26). With this one step method 90% of recombinant protein S could be eluted with 5 mM $CaCl_2$. The remaining 5-10% could be eluted with 1 M NaCl, but was not further analyzed. Immunoblotting analysis was performed with rabbit polyclonal and different anti-protein S murine monoclonal antibodies. Fig. 4 (lanes 5-8, polyclonal) shows that recombinant protein S has an apparent molecular weight, which is slighty higher than the molecular weight of plasma protein S. Both plasma and recombinant protein S could be cleaved by thrombin.

Fig. 3. **SDS polyacrylamide gel of recombinant protein S**. Aliquots (100 μl) of conditioned medium (lane 1), barium citrate supernatant (lane 2) and barium citrate precipitate (lane 3) were precipitated with 10% trichloric acetic acid, dissolved in 1 μl 1 M Tris-HCl pH 9.5, separated on a 7.5% gel under non reducing conditions and stained with silver nitrate. Lane 4 represents purified plasma protein S (50 ng).

From the results obtained under reducing conditions (see Fig. 4, lanes 5,7, polyclonal) it can be estimated that at that stage recombinant protein S contains approximately 80% uncleaved form and plasma protein S approximately 50% uncleaved form. Furthermore, recombinant protein S was recognized by different monoclonal antibodies (25). Monoclonal antibody S5, strongly inhibits the APC cofactor activity of protein S and reacts with native protein S, in reduced and non reduced form (lanes 1,3,5,7) and weakly with thrombin cleaved protein S (lanes 2,4,6,8). Monoclonal antibody S9, partially inhibits the cofactor activity, and reacts only with native unreduced protein S (lanes 1,3). Monoclonal antibody S3, which has no effect on the cofactor activity reacts with both native and thrombin cleaved protein S in reduced and non reduced form (lanes 1-8).

Fig. 4. **Immunoblotting analysis of protein S**. Aliquots (50 ng) of plasma (lanes 1,2,5,6) or recombinant (lanes 3,4,7,8) protein S were treated with thrombin-Sepharose (lanes 2,4,6,8), separated on 10-15% gradient gels under non-reducing (lanes 1-4) and reducing (lanes 5-8) conditions, electrotransferred, and visualized with anti-protein S IgG conjugated to peroxidase (3 μg/ml) or monoclonal antibodies S3, S5, and S9 (5 μg/ml), followed by rabbit anti-mouse IgG conjugated to peroxidase (0.1 μg/ml).

Binding of Protein S and Variants to C4BP - The binding of plasma, variant and recombinant protein S to C4BP was examined using a monoclonal antibody against C4BP (8C11) which does not interfere with the binding site of C4BP for protein S (17). This antibody was used as capture antibody for C4BP. Then protein S was allowed to bind during various incubation times and the bound protein S was measured using rabbit anti-protein S IgG conjugated to peroxidase. The dose response curves for two different binding times are shown in Fig. 5 for plasma and recombinant protein S and in Fig. 6 for the two variants. From these data, the apparent dissociation constant for plasma, variant and recombinant protein S at 50% binding, can be estimated to be approximately $5x10^{-10}$ M.

Fig. 5. **Binding of protein S to C4BP**. Monoclonal antibody 8C11 was coated onto microtiter wells to catch C4BP (1 μg/ml). Increasing amounts of plasma (circles) or recombinant (triangles) protein S were allowed to bind to C4BP for 15 min (closed symbols) or 24 h (open symbols). Bound protein S was detected with rabbit anti-protein S IgG conjugated to peroxidase (3 μg/ml). The experiment was performed in duplicate.

Fig. 6. **Binding of variant protein S to C4BP**. Monoclonal antibody 8C11 was coated onto microtiter wells to catch C4BP (1 μg/ml). Increasing amounts of recombinant (triangles), B variant (squares) or C variant (diamonds) were allowed to bind to C4BP for 30 min (closed symbols) or 18 h (open symbols). Bound protein S was detected with rabbit anti-protein S conjugated to peroxidase (3 μg/ml). The experiment was performed in duplicate.

Analysis of N-Terminal Sequence, gla and hya/hyn Content - The N-terminal sequence of purified recombinant and plasma protein S was analyzed with a pulse liquid phase sequenator. Both preparations gave the same amino terminal sequence of ala-asn-ser-leu-leu--- as reported earlier by DiScipio and Davie (2) and predicted from the cDNA sequence (12-14).

Content of γ-carboxyglutamyl acid residues gave a gla/glu ratio of 0.22 indicating a carboxylation level of 11 residues per recombinant molecule.

Content of β-hydroxylated aspartyl/asparaginyl acid residues was 2.5 and 2.8 mol hya/mol protein for plasma and recombinant protein S, respectively.

Protein S Functional Assay - The cofactor activity of recombinant protein S to activated protein C was tested using an assay based upon the ability of protein S to enhance the anticoagulant activity of activated protein C. In this assay the prolongation of the clotting time by activated protein C is dependent on the protein S concentration in the plasma (Fig. 7). In the absence of activated protein C, protein S has no influence on the clotting time (not shown). Recombinant protein S in this assay contains approximately 80% of the native uncleaved form compared to plasma protein S, which contains approximately 50% native uncleaved form (Fig. 4, lanes 5,7, polyclonal). The experiment shows that on a molar basis, recombinant protein S has a 2-3 fold higher cofactor activity than plasma protein S.

Fig. 7. **Protein S functional assay**. The protein S dependent prolongation of the clotting was measured in an activated partial thromboplastin time system using protein S and C4BP depleted plasma, activated protein C, kaolin and cephalin. Increasing amounts of plasma (circles) or recombinant (triangles) protein S and thrombin treated protein S (open symbols) were added. Clotting was initiated by the addition of $CaCl_2$ and the clotting time was measured. The experiment was performed in duplicate.

DISCUSSION

To study the structure function relationships of human protein S, a full length human protein S cDNA was brought to expression in C127 mammalian cells. An expression level of approximately 0.5-1 μg/ml of recombinant protein S was obtained and recombinant protein S was purified by two successive barium citrate adsorption steps. For N-terminal amino acid and hya determination protein S was further purified by monoclonal anti-protein S antibody (S11) affinity and Fast Flow Q anion exchange chromatography. Purified recombinant protein S was characterized and compared to purified human plasma protein S. Analysis on non reduced SDS-polyacrylamide gels indicated a slightly higher molecular weight for recombinant protein S and variants (not shown) compared to plasma protein S.

Treatment of recombinant and plasma protein S with N-Glycanase, which is known to remove N-linked carbohydrate chains, resulted in similar molecular weights (not shown). This suggests that the difference in molecular weight can be explained by a difference in carbohydrate side chain composition. This is probably caused by a different composition of the carbohydrate chains in the C127 cells compared to human liver cells. The carbohydrate composition seems to be cell specific because no differences were observed when kidney COS-1 and Chinese Hamster Ovary K-1 cells were used to express recombinant protein S (unpublished observations). Grinnell et al. (26) recently reported isoforms of recombinant human protein S with different biologic properties. Their data show that recombinant protein S produced in hamster AV12 cells has the same molecular weigth as plasma protein S. However, recombinant protein S from human kidney 293 cells has a lower molecular weigth, caused by different glycosylation. These results suggest differences in the glycosylation machinery between human kidney 293 cells and human liver cells.

Recombinant protein S was further characterized by immunoblotting with monoclonal antibodies against protein S. Several conformation specific monoclonal antibodies were tested and all reacted with recombinant protein S. This suggests that the monoclonal antibodies have the same specificity for recombinant and plasma protein S and that the epitopes recognized by the monoclonal antibodies are present on recombinant protein S.

N-terminal sequence analysis of plasma and recombinant protein S gave identical amino acid sequences of ala-asn-ser-leu-leu--- as reported by DiScipio and Davie (2). This suggests correct proteolytic processing of the prepropeptide by the C127 cells.

Analysis of the hya content gave 2.5 and 2.8 residues of hya/hyn per molecule for plasma and recombinant protein S, respectively. This indicates recombinant protein S to be equally hydroxylated as plasma protein S.

Analysis of the gla content of recombinant protein S revealed a gla/glu ratio of 0.22 which corresponds to 11 residues of γ-carboxyglutamic acid per molecule, meaning full carboxylation of recombinant protein S (12-14). Human protein C, another vitamin-K dependent protein, expressed in C127 cells, has also been reported to be fully carboxylated (33). The vitamin-K dependent carboxylase activity in C127 cells seems to direct the complete carboxylation of specific glutamic acid residues of the protein S molecule to full extent.

Human protein S acts as a cofactor to activated protein C in the inactivation of factors Va and VIIIa (3-5). As shown in Fig. 7 recombinant protein S, on a molar basis, has a higher APC cofactor activity as purified plasma protein S. This observation might be explained by a difference in proteolytic degradation, which can result in inactivation of the cofactor activity. The plasma protein S used in our study contains approximately 50% proteolytic degraded protein S, whereas the recombinant protein S contains approximately 20% degraded material. This could explain a 2-3 fold higher cofactor activity for recombinant protein S. When plasma or recombinant protein S were degraded by thrombin and tested in the clotting assay, the cofactor activity was completely lost, in agreement with previous reports (9,10). Malm et al. (34) reported that the recombinant protein S had the same biological activity as plasma protein S, suggesting the same extent of degradation for their recombinant protein S and for the plasma protein S preparation used. Grinnell et al. (26) reported two isoforms of recombinant protein S, one with a cofactor activity 2.5 fold higher then plasma protein S. This recombinant protein S preparation also contained proteolytically degraded material.

Finally, we examined the binding of variant and recombinant protein S to C4BP. For this purpose we used a binding assay using a monoclonal antibody against C4BP,

not interfering with the interaction of C4BP with protein S as mentioned before. Our analysis demonstrates that both variant and recombinant protein S molecules bind to C4BP in a way similar to purified plasma protein S (Figs. 5 and 6). The apparent K_d was estimated to be about $5x10^{-10}$ M in the presence of 5 mM $CaCl_2$, which is in good agreement with recent reports (35,36). The binding of plasma, variant and recombinant protein S to C4BP is identical both at short and long incubation times (Figs. 5 and 6). The rate at which the C4BP-protein S complex is formed is similar for all four proteins (not shown). This suggests that recombinant and variant protein S bind to C4BP with the same affinity as plasma protein S.

The protein S peptide Gly-605 to Ile-614 reported by Walker (15) enhanced the anticoagulant activity of APC in human plasma and showed binding to C4BP in a purified system with a K_d of 5 μM. The enhancing effect on the anticoagulant activity was lost when Asp-609 was replaced by gly in the peptide, suggesting that Asp-609 of the peptide may be important for the interaction with C4BP. Our results, however, indicate that amino acid residues Leu-608, Asp-609, Asp-611 and Glu-612 of human protein S are not essential for the binding to C4BP of the intact protein. The anticoagulant activity of short synthetic peptides corresponding to this region is probably not due to mimicry of the C4BP binding region of the intact protein. The binding of the peptide is about 1,000 fold weaker than the binding of intact protein S to C4BP, which is in the nanomolar range as reported by several groups (35,36). This suggests that a larger region of the protein S molecule is needed for optimal binding to C4BP. It is also possible that more domains contribute to the binding site as recently described by Fernández and Griffin (37) using synthetic peptides of protein S which bind to C4BP. The expression system we described here will be useful for further studies of the binding site(s) for C4BP on protein S.

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CHAPTER 3

INTERACTION BETWEEN HUMAN PROTEIN S AND HUMAN C4b-BINDING PROTEIN USING DELETION VARIANTS OF RECOMBINANT HUMAN PROTEIN S

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ABSTRACT

Human protein S interacts noncovalently with human C4b-binding protein (C4BP). We have studied this interaction using deletion variants of recombinant human protein S. Two deletion variants were constructed by restriction enzyme digestion and in vitro site-specific mutagenesis of the human protein S cDNA. The variants were stably expressed in C127 cells. Recombinant proteins were purified using Fast Flow Q anion-exchange chromatography. The activated protein C (APC) cofactor activity, C4BP binding properties and reactivity to different monoclonal antibodies against human protein S were examined. One variant (E variant), which has a deletion of the third epidermal growth factor (EGF)-like domain (deletion of exon VII, corresponding to amino acid residues Asp-160 to Asp-202) expresses normal APC cofactor activity in a plasma system. This activity could be inhibited by the addition of purified C4BP. The second variant (L variant), which has a deletion of the C-terminal loop of the sex hormone binding globulin (SHBG)-like domain (deletion of exon XV, corresponding to amino acid residues Asp-583 to Ser-635) also expresses normal APC cofactor activity in plasma. This activity could only be partially inhibited by the addition of purified C4BP. Binding of the recombinant proteins to C4BP was studied in a system using purified proteins. The E variant binds to C4BP with an affinity similar as recombinant wild type protein S (apparent $K_d \sim 10^{-10}$ M). The L variant shows a reduced affinity for binding to C4BP (apparent $K_d \sim 10^{-7}$ M). Reactivity of the deletion variants with three different Ca^{2+}-independent anti-protein S monoclonal antibodies was also tested. These monoclonal antibodies do not interfere with the APC cofactor activity and C4BP binding of protein S. Two monoclonal antibodies (S5 and S17) react with a similar affinity with the E and L variant as with recombinant wild type protein S. One monoclonal antibody (S12), which is directed to an epitope which is sensitive to the replacement of Ser-460 by proline reacts with the E variant and recombinant wild type protein S but failed to react with the L variant under non-denaturing conditions. However, S12 did recognize the L variant after denaturing SDS/PAGE indicating that the epitope of S12 is still present. This suggests that deletion of the C-terminal loop induces a conformational change which effects the epitope for S12. Therefore although our results suggest that the C-terminal loop of the SHBG-like

domain of human protein S is involved in the interaction with C4BP, we cannot exclude the possibility that deletion of the C-terminal loop induces a conformational change that results in a loss of binding affinity for C4BP elsewhere in the protein S molecule.

INTRODUCTION

Human protein S (M_r 75.000) is a vitamin K-dependent plasma glycoprotein that functions as a cofactor of activated protein C (APC) in the inactivation of coagulation factors Va and VIIIa (1-5). This anticoagulant property makes protein S an important regulator of blood coagulation. The physiological importance of protein S can be deduced from the observation that patients partially deficient in protein S have an increased risk to develop thrombosis (6-8). The cDNAs encoding for bovine (9) and human protein S (10-12) have been reported. The recent resolution of the intron/exon organization of the human protein S gene indicates the presence of 14 introns and 15 exons. The human protein S gene encodes for a leader sequence followed by protein modules homologous to a γ-carboxyglutamic acid domain, a thrombin-sensitive domain, four EGF-like domains and a SHBG-like domain (13-15). In human plasma, protein S is complexed noncovalently with C4b-binding protein (C4BP). Approximately 40% of plasma protein S is free and only this form functions as a cofactor to APC (6,16,17).

C4BP (M_r 530.000-570.000) is a component of the classical pathway of the complement system (18). It accelerates the proteolysis of C4b by factor I (19) and inhibits C3 convertase (20). C4BP exists in human plasma as a heterogeneous population of molecules. Six or seven identical α-chains (21-23) and zero or one β-chain (24,25) are covalently linked by disulfide bridges near the C-terminal part of each chain. The β-chain appears to contain the protein S binding site (24-26).

Using synthetic protein S peptides the C4BP binding site on protein S has been localized in different parts of the molecule. First Walker (27) demonstrated that a synthetic peptide based on the sequence of Gly-605 to Ile-614 in the C-terminal region bound to C4BP in a system using purified proteins. Recently, Fernández and Griffin (28) reported that synthetic protein S peptides based on the amino acid sequence in the center of the SHBG-like domain inhibited the binding of C4BP to

protein S, suggesting that this region of the protein S molecule is involved in the binding of C4BP. Using site-specific mutagenesis we found that changing the amino acid residues Leu-608, Asp-609, Asp-611 and Glu-612 to Val-608, Asn-609, Asn-611 and Gln-612 had no effect on the binding of protein S to C4BP, suggesting that these amino acids are not essential for the interaction of protein S with C4BP (29). However, Nelson and Long (30) described several mutants of protein S in the C-terminal part with a decreased affinity for C4BP. Two of these mutants however lacked APC cofactor activity, whereas other mutants contained free sulfhydryl groups.

We have constructed and characterized deletion variants of recombinant protein S. One variant, designated E variant lacks the third EGF-like domain and the other variant, designated L variant lacks the C-terminal part of the SHBG-like domain. These deletion variants have normal APC cofactor activity in a clotting assay. The E variant binds to C4BP, while the L variant shows a greatly reduced binding. However, in contrast to wild type recombinant protein S and the E variant, the L variant is not recognized by an anti-protein S monoclonal antibody that does not recognize a genetic variant of protein S, Protein S Heerlen, which has a proline in stead of a serine at position 460. Together these results suggest that a part of the C-terminal loop (between amino acid residues Asp-583 to Ser-635) of protein S is either directly involved in the interaction with C4BP or that deletion of the C-terminal loop results in a loss of the binding affinity elsewhere in the protein S molecule. The possibility that two binding sites are needed for optimal binding of C4BP to protein S can not be excluded.

EXPERIMENTAL PROCEDURES

Materials - Restriction endonucleases Bam HI, Xba I and Eco RI were purchased from Promega Biotec (Madison, WI, USA). Restriction endonuclease Bsm I was from New England Biolabs (Beverly, MA, USA). Calf intestine alkaline phosphatase was from Boehringer Mannheim (Mannheim, FRG). T4 DNA Polymerase was from Bio-Rad Corporation (Richmond, CA, USA). T4 DNA Ligase was from Bethesda Research Laboratory (Bethesda, MD, USA). All enzymes were used according to the manufacturers instructions. *Escherichia coli* strains CJ236 and MV1190 were from Bio-Rad Corporation (Richmond, CA, USA). Sequenase Version 2.0 sequencing kit was from USB Corporation (Cleveland, Ohio, USA). APC and rabbit anti-protein S IgG and protein S

and C4BP depleted plasma were prepared as described (5). C4BP and anti-C4BP monoclonal antibody 8C11, directed against the α-chain of C4BP was prepared as described (31). Rabbit anti-human protein S IgG conjugated to HRP was from Dakopatts (Glostrup, Denmark). Fast Flow Q resin was from Pharmacia (Uppsala, Sweden). Iscoves Modified Dulbeccos Medium, pennicilin G, streptomycin sulphate, glutamine and fetal calf serum were from GIBCO (Paisley, UK). Vitamin K_1 (Konakion) was from F. Hoffman-La Roche Ltd. (Basel, Switserland).

Preparation of Anti-Protein S Monoclonal Antibodies - Murine monoclonal antibodies against human protein S were prepared according to standard procedures as will be described elsewhere (Bertina, R.M., Van Wijngaarden, A. and Nieuwenhuizen, W., in preparation). The monoclonal antibodies S5, S12 and S17 bind equally efficient to native protein S (purified from human plasma), thrombin-inactivated protein S and Gla-domainless protein S. They recognize three different epitopes on protein S as concluded from competition experiments and they do not effect the APC cofactor activity of protein S or its affinity for C4BP. Antibody-rich ascites fluid was obtained after peritoneal injection of cloned hybridoma cells in Balb-C mice. IgGs were purified from ascites fluid using protein A Sepharose chromatography. IgG from monoclonal antibody S5 was conjugated to HRP using N-succinimidyl 3-(2-piridyl-thio) propionate (SPDP), as previously described (32).

Construction of Protein S Variants - Standard recombinant DNA techniques were performed as described by Sambrook and colleagues (33). Construction of the E variant was achieved by Bsm I digestion of the expression plasmid pMSVPS (29). Desired fragments were isolated from agarose gels and ligated. The resulting plasmid which has now a deletion of the third EGF like domain, while the reading frame is still intact, was verified by restriction enzyme digestion and dideoxy sequencing (34). Construction of the L variant was achieved by "loop-out" site-specific mutagenesis. A 1300-base pair XbaI-EcoRI fragment from the expression vector pMSVPS (29) carrying a part the protein S cDNA sequence was subcloned into M13mp19. The 40-mer fusion oligonucleotide 5'-AGCAGAGAAAAGATGCCTTA TGGAAGGCCACCCAGGTATG-3' was used for mutagenesis. Site-specific mutagenesis was performed according to Kunkel (35). The M13mp19 clone carrying the protein S cDNA insert was infected into *Escherichia coli* mutant CJ236 (dut^- ung^-) cells and uracil containing single stranded phage DNA was isolated and used as template. After second strand synthesis with T4 DNA Polymerase and ligation with T4 DNA Ligase competent *Escherichia coli* MV1190 (dut^+ ung^+) cells were transformed. Single stranded DNA from individual resultant plaques was isolated and sequenced by the dideoxy chain termination method (34) using the universal M13 forward primer to confirm the sequence was as expected and that no inadvertent mutations had been introduced. Double stranded phage DNA was isolated and the protein S cDNA insert isolated and recloned into pMSVPS. The BPV-1 genome was isolated after Bam HI digestion from pTZX-BPV as described (29) and cloned into pMSVPS. The resulting

plasmids were finally purified by CsCl gradient centrifugation.

Cell Culture and DNA Transfection - C127 cells (ATCC CRL 1616) were cultured in Iscoves Modified Dulbeccos Medium, supplemented with penicillin G (100 U/ml), streptomycin sulphate (100 μg/ml), glutamine (2 mM) and 10% heat-inactivated fetal calf serum. Cultures were maintained at 37°C in an incubator containing 5% CO_2. For transfections 20 μg of plasmids were used and cells were transfected by conventional calcium phosphate coprecipitation technique (36). Briefly, the precipitate was incubated for 20 min at room temperature and added to the cells which were subcultured at $5x10^5$ cells/10 cm dish 24 hours prior to transfections. After transfections the cells were rinsed twice with 10 mM sodium phosphate pH 7.4 containing 2.7 mM KCl and 150 mM NaCl (PBS) and allowed to recuperate overnight in complete medium. The cells were then trypsinized, split at a 1:10 ratio and incubated in medium containing only 5% fetal calf serum. Cells were fed every 3-4 days until foci were visible, which was usually 12-14 days after starting of subculturing. Foci were isolated and assayed for protein S antigen. Cells were than expanded in 1400 cm^2 rollerbottles (Falcon, Lincoln Park, NJ, USA) and grown in medium containing 10% fetal calf serum to confluency. Culture medium (100 ml) without serum, but with vitamin K_1 (Konakion, 5 μg/ml) was added and harvested every 48-72 hours and stored at -20°C until purification.

Purification of Recombinant Protein S and Variants - Recombinant proteins were purified on an anion-exchange column (Fast Flow Q resin, Pharmacia) as described for wild type recombinant protein S (29,37). Briefly, to 500 ml of conditioned culture medium EDTA and benzamidine were added to 5 mM. The medium was filtered to remove cellular debris and passed through a 5 ml column which was equilibrated with 50 mM Tris HCl pH 7.5 containing 150 mM NaCl and 5 mM benzamidine. The column had a flow rate of 25 ml/hour and after extensively washing the column to remove unbound proteins with starting buffer, recombinant proteins were eluted with starting buffer containing 5 mM $CaCl_2$. The fractions containing protein S as assessed by an ELISA were pooled, dialyzed against 50 mM Tris HCl pH 7.5 containing 150 mM NaCl and stored at -20°C until needed. The purity and integrity of the recombinant proteins was judged by SDS/PAGE on reduced 10-15% gradient gels according to Laemmli (38) and silver staining (39) using the Phast System (Pharmacia Biotechnology, Uppsala, Sweden) or immunoblotted using anti-protein S monoclonal antibody S12 conjugated to HRP.

Protein S Assays - Quantification of recombinant protein S and variant antigen were determined by ELISA as described elsewere using rabbit anti-protein S IgG as catching antibody and rabbit anti-protein S IgG conjugated to HRP as tagging antibody (40).
Protein S cofactor activity was determined in a clotting assay. Briefly, to 50 μl protein S and C4BP depleted plasma was added 50 μl cephalin-kaolin reagent (Boehringer Mannheim, Mannheim,

FRG), 5 μl activated protein C (5 μg/ml) and 45 μl protein S or variants (0-10 μg/ml) diluted in Michaelis buffer (28.5 mM sodium acetate, 28.5 mM sodium barbital, 116 mM NaCl, pH 7.35) and incubated for 3 min at 37°C. Clotting was initiated by addition of 50 μl of 25 mM $CaCl_2$ (prewarmed at 37°C) and the clotting time determined in an Amelung-Coagulometer KC10. The complex formation of recombinant protein S and variants with C4BP was studied in this system as follows. A fixed amount of C4BP (final concentration 5.9 μg) was incubated with increasing amounts of recombinant proteins (final concentration 0-0.5 μg) in 45 μl 50 mM Tris-HCl pH 7.5 containing 150 mM NaCl for 2 h at room temperature prior to addition to the protein S and C4BP depleted plasma.

Binding of Recombinant Protein S and Variants to C4BP - The complex formation between the recombinant proteins and C4BP was measured with a sensitive ELISA using C4BP and a HRP conjugated anti-protein S antibody as previously described (29). Briefly, 100 μl anti-C4BP monoclonal antibody 8C11 IgG (10 μg/ml), which is directed against the α-chain of C4BP was coated in 50 mM $NaHCO_3$, pH 9.6 overnight at 4°C onto polyvinyl microtiter plates (Costar, Cambridge, MA, USA). After washing the wells to remove unbound IgGs, the wells were blocked with 150 μl 1% (w/v) BSA in 50 mM Tris-HCl pH 7.5, 150 mM NaCl (TBS) containing 5 mM $CaCl_2$ for 1 h at room temperature and 100 μl C4BP was allowed to bind for 2 h at room temperature with a final concentration of 1 μg/ml. Binding of protein S and variants was examined in two different types of experiments. In the first experiment a fixed amount of recombinant protein S variant (60 ng) in TBS containing $CaCl_2$ was added and incubated for several time periods. In the second experiment increasing amounts of recombinant protein S variant were added and incubated for 18 h at room temperature. After washing the wells to remove unbound proteins, bound protein S variants were incubated with 100 μl anti-protein S IgG conjugated to HRP (0.3 μg/ml) for 2 h at room temperature. After washing the wells to remove unbound proteins, 100 μl of 0.1 M sodium phosphate containing 0.04% 1,2-phenylenediamine dihydrochloride and 0.015% hydrogen peroxide was added. The hydrolysis of p-nitrophenylphosphate was stopped by the addition of 50 μl 2 N sulfuric acid and the absorbance was measured at 490 nm using a V_{max} plate reader (Molecular Devices Corporation, Menlo Park, CA, USA).

Binding of Recombinant Protein S and Variants to Anti-Protein S Monoclonal Antibodies - The affinity of recombinant protein S and variants for different anti-protein S monoclonal antibodies was estimated by incubating 100 μl of increasing dilutions of recombinant protein S and variants in wells coated with the relevant monoclonal antibody (10 μg/ml in 0.1 M sodium carbonate, pH 9.0). After 16 hours incubation at room temperature, wells were emptied, washed 4 times and incubated with 100 μl of a 1/5000 dilution of polyclonal rabbit anti-protein S antibody conjungated to HRP (Dakopatts) for 60 min at room temperature. After washing the wells, HRP activity was measured

kinetically at 450 nm using p-nitrophenylphosphate as substrate. Samples were diluted in a buffer containing 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 3 mM EDTA and 0.2% Tween 20. Phosphate-buffered saline containing 0.2% Tween 20 was used as washing buffer.

RESULTS

Construction, Expresssion and Purification of Protein S Variants - Table 1 summarizes the deletion variants of protein S used in this study. The variants were expressed in C127 cells and foci producing protein S antigen as determined by ELISA were isolated. Cells were grown in rollerbottles to confluency in culture medium without serum, but containing vitamin K_1. The culture medium was harvested every 48-72 hrs. The mean antigen level in the pooled media was approximately 0.1-0.2 μg/ml for both variants as determined by the ELISA. Because the two deletions did not result in a loss of epitopes for the polyclonal anti-protein S antibodies (see later) this ELISA will detect wild type and deletion variants with equal efficiency. The variant proteins were purified from the culture medium using a Fast Flow Q anion-exchange chromatography column. The purified proteins were separated by SDS-PAGE under reducing conditions using 10-15% gradient gels. Silver staining of the gels shows that the deletion variants have the predicted lower molecular weights (Fig. 1). This heterogeneity in molecular weights is caused by limited proteolysis of protein S.

Table 1. *Deletion variants of protein S*

Variant	Alterations	Number of amino acids
rec	**non**	**635**
E	**del D160-D202**	**593**
L	**del D583-S635**	**582**

Deletion variants were constructed by restriction enzyme digestion and by in vitro site-specific mutagenesis of the protein S cDNA as described in the text. Numbering of the amino acid residues is according to refs. 9-12. Rec, recombinant protein S; E, variant lacking the third EGF-like domain; L, variant lacking the C-terminal loop of the SHBG-like domain.

Fig. 1. **SDS/PAGE analysis of deletion variants of protein S.** Aliquots (10-50 ng) of plasma protein S (lanes 1 and 4), recombinant protein S (lane 5), E variant (lane 2) and L variant (lane 3) were separated on a 10-15% SDS gel under reducing conditions and stained with silver nitrate. Molecular weights markers are shown in kilodaltons on the right.

Protein S Cofactor Activity to APC - The cofactor activity to APC was measured in an activated partial thromboplastin time system using protein S and C4BP depleted plasma and increasing amounts of the recombinant protein S variants. In this assay, the prolongation of the clotting time by APC is dependent on the protein S concentration in the plasma. In the absence of APC, protein S has no influence on the clotting time. C4BP itself has no influence on the prolongation of the clotting time induced by APC (not shown). Fig. 2 demonstrates that the deletion variants have normal cofactor activity to APC compared to wild type recombinant protein S. Addition of a two to eight-fold molar excess of purified C4BP inhibits the cofactor activity of wild type recombinant protein S and the E variant almost completely. The L variant, however, was only inhibited for approximately 50%.

Fig. 2. **APC cofactor activity of protein S variants.** The protein S dependent prolongation of the clotting time was measured in an activated partial thromboplastin time system using protein S and C4BP depleted plasma, APC, kaolin and cephalin. Increasing amounts of recombinant protein S *(circles)*, E variant *(diamonds)* or L variant *(squares)* in the presence *(open symbols)* or absence *(closed symbols)* of C4BP (two to eight-fold molar excess) were added. Clotting was initiated by the addition of $CaCl_2$ and the clotting time was measured. All data are the mean of duplicate values.

Binding of Protein S and Variants to C4BP - The binding of recombinant protein S and variants to C4BP was examined using a monoclonal antibody against C4BP (8C11) which is directed against the α-chain of C4BP. This antibody was used as capture antibody for C4BP as described before (29). The binding was performed in 50 mM Tris-HCl pH 7.5, containing 150 mM NaCl and 5 mM $CaCl_2$. Fig. 3 shows the time course of binding of recombinant protein S and variants to C4BP. The rate at which recombinant protein S and the E variant bind to C4BP is similar, while the L variant binds only very weakly to C4BP. The dose-response curves in Fig. 4 show that the E variant (panel A) binds to C4BP with the same affinity as the wild type recombinant protein S. The apparent dissociation constant (K_d) can be estimated to be $\sim 10^{-10}$ M. The L variant (panel B) shows a greatly reduced affinity with an apparent $K_d \sim 10^{-7}$ M.

Fig. 3. **Time course of the binding of recombinant protein S and variants to C4BP.** Anti-C4BP monoclonal antibody 8C11 IgG was coated onto microtiter wells to catch C4BP (1 μg/ml). A fixed amount (60 ng) of recombinant protein S *(circles)*, E variant *(diamonds)* or L variant *(squares)* was added and incubated for the indicated time periods. Bound proteins were detected with rabbit anti-protein S IgG conjugated to HRP (0.3 μg/ml). Each datapoint is the mean of duplicate values.

Fig. 4. **Binding of deletion variants of protein S to C4BP.** Anti-C4BP monoclonal antibody 8C11 IgG was coated onto microtiter wells to catch C4BP (10 μg/ml). Recombinant protein S *(circles)*, E variant *(diamonds)* or L variant *(squares)* was added in increasing amounts (panel A: 0.1 - 100 ng/ml and panel B: 0.1 - 10,000 ng/ml) and incubated for 18 h at room temperature. Bound proteins were detected with rabbit anti-protein S IgG conjugated to HRP (0.3 μg/ml). All data are the mean of duplicate values.

Binding of Recombinant Protein S and Variants to Anti-Protein S Monoclonal Antibodies - The binding of recombinant protein S and variants to three different Ca^{2+}-independent anti-protein S monoclonal antibodies was examined to study the possibility that the deletions would effect the conformation of the protein S molecule. Fig. 5 shows the binding curves for recombinant protein S and the two deletion variants. Monoclonal antibodies S5 and S17 bind to the variants equally well as recombinant protein S. However, monoclonal antibody S12 binds only to recombinant wild type protein S and the E variant and not to the L variant. This observation would suggests that the epitope of S12 is located in the deleted C-terminal region of protein S. S12 could however, recognize the L variant after denaturing SDS-PAGE (not shown) indicating that the epitope is not located in the C-terminal region of the protein S molecule.

Fig. 5. **Binding of deletion variants of protein S to anti-protein S monoclonal antibodies.** Anti-protein S monoclonal antibodies S5, S12 and S17 IgGs were coated onto microtiter plates. Recombinant protein S *(circles)*, E variant *(diamonds)*, L variant *(squares)* or protein S as present in normal pooled plasma *(triangles)* was added in increasing amounts. The protein S concentration is expressed in ng/ml as determined by the polyclonal protein S ELISA. Bound proteins were detected with rabbit anti-protein S IgG conjugated to HRP. All data are the mean of triplicate values.

Further, the results of monoclonal antibodies S5 and S17 indicate that at saturating protein concentrations, wild type protein S and the variants are recognized equally efficient by the polyclonal anti-protein S conjugate. This suggests that these two deletions will not result in the loss of epitopes for the polyclonal anti-protein S antibodies.

DISCUSSION

To study the interaction between protein S and C4BP we have constructed two deletion variants of recombinant human protein S. One variant, lacking the third EGF-like domain was designated E variant and the other variant, lacking the C-terminal loop of the SHBG like domain was designated L variant. The deletion variants were stably expressed in C127 cells and the recombinant proteins were purified from the culture medium using a Fast Flow Q column with a yield of about 80%. On reduced SDS gels the isolated deletion variants show the predicted lower molecular weights compared to recombinant protein S (Fig. 1). Recombinant protein S has a higher molecular weight than plasma protein S due to different glycosylation. We have previously shown, that this has no effect on the APC cofactor activity and C4BP binding ability (29).

The deletion variants have normal APC cofactor activity in a clotting assay (Fig. 2), indicating that the deletion variants are functionally active and that essential parts of the protein have been properly folded. This also indicates that the third EGF-like domain and the C-terminal part of the SHBG-like domain of protein S are not involved in the expression of the APC cofactor activity of protein S.

The ability of the deletion variants to bind to C4BP was studied in a system using the purified proteins (Figs. 3 and 4). Recombinant protein S and the E variant bind to C4BP with the same affinity (apparent $K_d \sim 10^{-10}$ M), whereas the binding affinity of the L variant was greatly reduced (apparent $K_d \sim 10^{-7}$ M). This is approximately 1000-fold lower than previously reported values (29,41-43). Performing the binding assay in the reversed order (wells coated with rabbit anti-protein S IgG, recombinant proteins, C4BP, anti-C4BP monoclonal antibody 8C11 IgG and rabbit anti-mouse IgG conjugated to HRP) gave essentially the same results (not shown).

These results suggest that the Asp-583 to Ser-635 deletion either removes an important region in protein S involved in the binding of C4BP or severely effects the affinity of another binding site (localized outside the deleted area).

The interaction of C4BP with the protein S variants was also evaluated in human plasma by measuring the APC cofactor activity of protein S as a function of the C4BP concentration added. As mentioned before both the deletion variants have normal APC cofactor activity. Addition of an excess of purified C4BP resulted in a loss of APC cofactor activity for recombinant protein S and the E variant, due to formation of inactive protein S-C4BP complexes. The APC cofactor activity of the L variant was only partially inhibited by the addition of C4BP, indicating that some complex formation could take place for which an apparent dissociation constant of $\sim 10^{-7}$ M could be estimated.

Walker (27) reported that the protein S peptide Gly-605 to Ile-614 binds to C4BP about 1000-fold weaker than intact protein S. The peptide also enhanced the APC cofactor activity in plasma, suggesting competition of the peptide with plasma protein S for binding to C4BP. The flanking protein S peptides Met-599 to Glu-607, Leu-608 to Lys-616 and Leu-616 to Ser-624 were also shown to enhance the APC cofactor activity but two-fold weaker than the Gly-605 to Ile-614 peptide. We have previously shown that within this C-terminal loop the amino acid residues Leu-608, Asp-609, Asp-611 and Glu-612 are not essential for the binding to C4BP (29). Therefore, a larger region than the aforementioned Gly-605 to Ile-614 peptide may be needed for optimal binding of protein S to C4BP. Deletion of the entire C-terminal end, from Asp-583 to Ser-635, resulted in a strongly reduced binding to C4BP, suggesting that an essential element is present in this region. That this element is located entirely within the protein S sequence Gly-605 to Ile-614 as Walker proposed seems unlikely. Recently, Nelson and Long (30) described several mutants in the C-terminal region of protein S. This region contains a disulfide bridge between amino acid residues Cys-598 and Cys-625. Two protein S mutants (deletion of Cys-598, which results in an unpaired Cys-625 and deletion of Gln-607 to Ser-635, which results in a unpaired Cys-598) were shown to have a reduced affinity for binding to C4BP. Deletion of a greater part of the C-terminal loop between amino acid residues Tyr-577 to Ser-635, resulted in a reduced

affinity, but this deletion lead to a protein without APC cofactor activity. Our L variant, which has a deletion of Asp-583 to Ser-635 showed reduced binding to C4BP but still had full APC cofactor activity. Together these results suggest that the C-terminal region is important for the binding of C4BP to protein S.

To study the possibility that the deletions would effect the conformation of the protein S molecule we tested the affinity of wild type protein S and the two deletion variants for three different Ca^{2+}-independent anti-protein S monoclonal antibodies. These monoclonal antibodies do not interfere with the APC cofactor activity and C4BP binding of purified protein S. Fig. 5 showed that two of these monoclonal antibodies (S5 and S17) bind to the E and L variant and to recombinant protein S with very similar affinities, whereas monoclonal antibody S12 does not bind to the L variant. However, S12 recognized the L variant upon immunoblotting after denaturing SDS-PAGE indicating that the epitope is not localized in the C-terminal region (Asp-583 to Ser-635). We have shown previously (44) that the epitope of monoclonal antibody S12 is sensitive for the replacement of amino acid residue Ser-460 by proline in human protein S, because S12 did not recognize a protein S variant (even after denaturation) in which Ser-460 is replaced by a proline residue. This variant, Protein S Heerlen has normal APC cofactor activity and also the distribution of the protein S molecule over C4BP in plasma seems normal. The failure of S12 to bind to Protein S Heerlen and to the non-denatured L variant suggests that deletion of the C-terminal loop induces such a conformational change in the region close to Ser-460 that the epitope for S12 is lost. These data also imply that the conformational change induced by deletion of the C-terminal loop might also effect the binding of C4BP if this would be located elsewhere in the protein S molecule. In this concept, the peptide used by Walker (27) might interfere with the interaction of the C-terminal loop with other parts of the molecule resulting in a loss of the binding capacity for C4BP elsewhere in the protein S molecule. Therefore, it is of interest that recently a C4BP binding site was reported in the center of the SHBG domain, relatively close to amino acid residue Ser-460 (28). Monoclonal antibody S12 itself does not interfere with the binding of C4BP to protein S, suggesting that the S12 epitope and the Ser-460 region are not involved in the binding to C4BP.

In conclusion, our results suggest that a part of the C-terminal region (between amino acid residues Asp-583 to Ser-635) of protein S is either directly involved in the interaction with C4BP or that deletion of this region results in a loss of binding affinity elsewhere in the protein S molecule. The possibility that more binding sites for C4BP are present in the protein S molecule still cannot be excluded.

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CHAPTER 4

THE INVOLVEMENT OF AMINO ACID RESIDUE LYS-429 OF HUMAN PROTEIN S IN THE BINDING TO HUMAN C4b-BINDING PROTEIN AS REVEALED BY SYNTHETIC PEPTIDES AND SITE DIRECTED MUTAGENESIS

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ABSTRACT

Human protein S binds to C4b-binding protein (C4BP) both in plasma and in a system using purified proteins. Recently, it was shown that the second disulfide loop of the C-terminal part of the sex hormone binding globulin (SHBG)-like domain (amino acid residues 583-635) of human protein S is an essential element in the binding of protein S to C4BP (Walker, 1989; Chang et al., 1991 and Nelson and Long, 1992). In addition, the amino acid residues 420-434 (PSP-420 peptide) of the first disulfide loop of the SHBG-like domain were shown to be involved in the interaction of protein S with C4BP (Fernández and Griffin, 1991). To study the involvement of the first disulfide loop in the interaction with C4BP in more detail we have used synthetic protein S peptides and recombinant protein S variants to investigate the role of some amino acid residues essential for this interaction. Three synthetic mutant peptides of the PSP-420 peptide (14 amino acids in length) were used. The same amino acid substitutions that were changed in the peptide were introduced in protein S by site directed mutagenesis. These substitutions were: Glu-424 to Lys, Gln-427 to Glu and Lys-429 to Glu. The synthetic peptides and recombinant variants were designated respectively E424K, Q427E and K429E peptide or variant protein. The E424K, Q427E and PSP-420 peptides were able to bind to C4BP, whereas the K429E peptide did not bind, indicating that Lys-429 is important for the binding to C4BP. In competition assays, the E424K, Q427E and PSP-420 peptides, but not the K429E peptide inhibited the binding of biotinylated C4BP to protein S. In concordance with this the K429E recombinant variant protein was not able to bind to C4BP whereas the other two variant proteins did bind. These results suggest that Lys-429 of human protein S is important for the binding of protein S to C4BP. An anti-protein S monoclonal antibody LJ-56, which was raised against the PSP-420 peptide, recognizes only free protein S and inhibits complex formation with C4BP, recognized the E424K and Q427E peptides but not the K429E peptide. Similarly with this the E424K and Q427E variant proteins were recognized by LJ-56, whereas the K429E variant protein was not recognized This indicates both in the peptide and in the protein Lys-429 is involved in the binding to LJ-56. These results confirm that the first disulfide loop of the SHBG-like domain of protein S is involved in the binding to C4BP and that within this loop Lys-429 is

important for the binding to C4BP and recognition by LJ-56. LJ-56 recognizes its epitope also in a protein S variant (L variant), where the second disulfide loop has been deleted. Since the epitope of LJ-56 closely coincides with the binding site for C4BP, it seems probably that in the L variant the binding site for C4BP is exposed. Because the L variant was shown to have a strongly reduced affinity for C4BP it may be concluded that both the first and the second disulfide loop of the SHBG-like domain of protein S are involved in the interaction with C4BP.

INTRODUCTION

Activated Protein C (APC) is a vitamin K-dependent glycoprotein that inactivates coagulation factors Va and VIIIa (1-4). This inactivation is enhanced by protein S, another vitamin K-dependent protein (5-8). The latter is believed to be an important step in the control of blood coagulation because patients partially deficient of protein S have an increased risk to develop thrombosis (9-11). From its primary structure it is deduced that protein S 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 (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% is free and only this form acts as cofactor to APC (15,16).

Two sites in the SHBG-like domain have been reported to be involved in the binding of protein S to C4BP (17-21). First Walker (17) showed that a synthetic peptide representing 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 of the SHBG-like domain (amino acid residues Asp-583 to Ser-635) however, resulted in a strongly reduced binding to C4BP, suggesting that the second disulfide loop in some way is involved in the binding as we and others have shown (19,20). 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. Because

the S12 epitope is outside the 583-635 region, it can therefore not be excluded that the conformational change induced by the deletion of the whole second disulfide loop also results in a loss of binding affinity for C4BP.

Recently, it was shown using synthetic protein S peptides that residues 420-434 (PSP-420) of the first disulfide loop in the SHBG-like region are also involved in the binding to C4BP (21). It was shown that PSP-420 bound stronger to C4BP than the peptide described by Walker (17). To further investigate the involvement of this region in the binding to C4BP we have used synthetic protein S peptides, recombinant proteins and an anti-protein S monoclonal antibody LJ-56 raised against PSP-420. Our studies confirm the involvement of the first disulfide loop in the interaction of protein S with C4BP and indicate that in this region especially Lys-429 is involved whereas Glu-424 and Gln-427 had no effect of binding to C4BP. Moreover, our studies show that both the first and the second disulfide loop of the SHBG-like domain of protein S may be both involved in the interaction with 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 and C4BP depleted plasma was prepared as described (8). C4BP and anti-C4BP monoclonal antibodies 8C11 IgG, directed against the α-chain of C4BP were prepared as described (22). Rabbit-anti human protein S IgG conjugated to peroxidase was from Dakopatts (Glostrup, Denmark). The L variant was isolated as described (20). Fast Flow Q resin was from Pharmacia (Uppsala, Sweden). Iscoves Dulbeccos Modified Eagles Medium, pennicilin G, streptomycin sulfate, glutamine and fetal calf serum were from Gibco (Paisly Park, UK). Trasylol was from Bayer (Leverkusen, FRG). Vitamin K_1 (Konakion) was from F. Hoffman-La Roche Ltd. (Basel, Switserland). Streptavidin conjugated to alkaline phosphatase (SAAP) and immunopure-p-nitro-phenyl-phosphate (p-NPP) was 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 (14 amino acids in length, Fig.1) were produced by the simultaneous multiple peptide synthesis method which has been described

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

Fig. 1. **Amino acid sequences of protein S peptides**. The first line is the normal human protein S sequence. Dots are identical amino acids. Underlined amino acid residues are targets for substitutions. Numbering of the amino acid residues is according to refs. 12-14.

elsewhere (23). All peptides were synthesized in the carboxy terminal amide form. 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 by subjection to 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 (24). 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 (25).

Site Directed Mutagenesis - A 2808 basepair Hind III-Bgl II fragment from the expression vector pMSVPS (20) carrying the protein S cDNA sequence was subcloned into M13mp19 digested with Bam HI and Hind III. The following 19-mers were used for mutagenesis: 5'-GGAATAAAGAAAATTATTC-3' for the E424K variant, 5'-GAAATTATTGAAGAAAAAG-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 (26). The M13mp19 clone carrying the protein S insert was infected into CJ236 (dut^-ung^-) cells and uracil-containing single stranded phage DNA was isolated and used as template. After second strand synthesis with T7 DNA Polymerase and ligation with T4 DNA Ligase competent XL-1 blue (dut^+ung^+) cells were transformed. Single stranded DNA from individual resultant plaques was isolated and sequenced by the dideoxy chain termination method (27) to confirm that the sequence was as expected and that no inadvertent mutations had been introduced. Double-stranded phage DNA was isolated from bacterial cells and a part of the protein S insert containing

the mutated sequences was isolated after Sal I and Xba I digestion. The protein S insert was cloned into pMSVPS, digested with Xba I and Sal I. The BPV-1 genome was isolated after Bam HI digestion of pTZX-BPV as described before (18) and cloned into pMSVPS. The resulting plasmids were finally purified by precipitation with polyethylene glycol (28).

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, trasylol (10 U/ml) and 10% heat-inactivated fetal calf serum as described before (18). Cell cultures were routinely maintained at 37°C in a humidified incubator containing 5% CO_2. One day prior to transfections, cells were trypsinized and seeded with a density of $5x10^5$ 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). Briefly, the DNA-calcium phosphate precipitate was formed during a 20 min incubation at room temperature and added to the cells. Transfections were carried out overnight in the incubator. After transfections the cells were washed twice with 10 mM sodium phosphate pH 7.4 containing 2.7 mM KCl and 150 mM NaCl (PBS) and allowed to recuperate overnight in complete medium. Cells were washed, trypsinized and seeded at a 1:5 ratio and incubated in medium containing only 5% fetal calf serum. Cells were fed every 3-4 days in medium containing 5% fetal calf serum until foci were visible, usually 12-14 days after subcultering. 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 K_1 (Konakion, 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). The purity and integrity of the recombinant proteins was jugded by SDS/PAGE on reduced 12.5% gels and immunoblotted using rabbit anti-protein S polyclonal antibody conjugated to peroxidase (Dakopatts).

Protein S Assays - Protein S antigen was determined by a double polyclonal ELISA using rabbit anti-protein S IgG as catching antibody and rabbit anti-protein S IgG conjugated to peroxidase as tagging antibody (30).
Protein S cofactor activity to APC was determined in a clotting assay using protein S and C4BP depleted plasma, cephalin-kaolin reagent (Boehringer Mannheim), APC and increasing amounts of protein S variants (18).

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 μg/ml) from monoclonal antibody 8C11, which is directed against the α-chain of C4BP was coated in 50 mM $NaHCO_3$ pH 9.6 overnight at 4°C onto polyvinyl microtiter plates (Costar, Cambrigde, MA). After washing of unbound IgGs, the wells were blocked with 1% (w/v) BSA in 50 mM Tris-HCl pH 7.5 containing 150 mM NaCl and 5 mM $CaCl_2$ for 1 h at room temperature. C4BP was allowed to bind for 2 h with a final concentration of 1 μg/ml. Increasing amounts of recombinant proteins (0-150 ng/ml) were added and incubated for 18 h at room temperature. Bound proteins were allowed to bind to anti-protein S IgG conjugated to peroxidase (0.3 μg/ml) for 2 h at room temperature. After washing the wells to remove unbound proteins, 100 μl of 0.1 M sodium phosphate / 0.2 M sodium citrate pH 5.0 containing 0.04% 1,2-phenylenediamine dihydrochloride and 0.015% hydrogen peroxide was added. The hydrolysis of phenylenediamine was stopped by adding 50 μl 2 N sulfuric acid. The absorbance was measured at 490 nm using a V_{max} plate reader (Molecular Devices Corporation, Menlo Park, CA, USA).

Binding of C4BP to Synthetic Protein S Peptides - This assay has been described before (31). Briefly, 50 μl of different synthetic peptides (20 μM) or native protein S (10 μg/ml) diluted in 0.02 M sodium carbonate buffer pH 9.0 were coated on microtiter plates for 1 h at 37°C. Wells were blocked with 200 μl of 10% BSA in 0.05 M Tris HCl pH 7.4 containing 0.1 M NaCl (TBS). Wells were washed 3 times with 0.2% BSA in TBS, 5 mM $CaCl_2$ and 0.02% Tween 20 (washing buffer). Serial dilutions containing 50 μl biotinylated C4BP (bC4BP) (0-5 μg/ml in washing buffer) were added to each well and incubated for 2 h at room temperature. Wells were washed 3 times with washing buffer, then 50 μl/well of streptavidin conjugated to alkaline phosphatase (SAAP) (1 μg/ml) diluted in washing buffer was added and incubated 30 min at room temperature. Wells were washed 6 times, then 100 μl of p-nitrophenylphosphate (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 using EL 312 Microplate (Bio-tek Instruments, Inc., Winooski, VT, USA). Results of the assay are given as delta A 405 nm/min. Each sample was assayed in duplicate and the results averaged.

Inhibition of the Binding of C4BP to Protein S by Synthetic Protein S Peptides - The inhibition was studied with two different assays. In the first assay a mixture of bC4BP and synthetic peptides was added to protein S which was immobilized onto microtiter wells (solid phase inhibition assay). In the second assay a mixture of bC4BP, protein S and synthetic peptides were added to an anti-protein S monoclonal antibody (S7, which does not interfere with the binding of protein S to C4BP, J.A. Fernández and J.H. Griffin, unpublished results) which was immobilized onto microtiter plates (fluid phase inhibition assay). For the solid phase inhibition assay, bC4BP (0.5 μg/ml) had been

incubated with serial dilutions of synthetic peptides (0-500 μM) for 1 h at room temperature. From this mixture, 50 μl was added to a microtiter plate coated with 50 μl of native protein S (10 μg/ml) in 0.02 M sodium carbonate buffer pH 9.0 for 1 h at 37°C and blocked with 200 μl of 10% BSA in 0.05 M Tris-HCl pH 7.4 containing 0.1 M NaCl (TBS). For the fluid phase inhibition assay, bC4BP (0.5 μg/ml) and native protein S (1 μg/ml) had been incubated with serial dilutions of synthetic peptides (0-500 μM) for 1h at room temperature. From this incubation mixture, 50 μl was added to a microtiterplate precoated with IgG of an anti-protein S monoclonal antibody (S7) (10 μg/ml) in 0.02 M sodium carbonate buffer pH 9.0 for 1 h at 37°C and blocked with 10% BSA in TBS buffer. For both assays the wells were washed 3 times with 0.2% BSA in TBS, 5 mM $CaCl_2$ and 0.02% Tween 20 (washing buffer) and 50 μl SAAP (1 μg/ml) diluted in washing buffer was added and incubated for 30 min at room temperature. Wells were washed 6 times with washing buffer and 100 μl p-nitrophenylphosphate (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, USA). Results of the assay were are given as delta A 405 nm/min. Each sample was assayed in duplicate and the results averaged.

Affinity of Anti-Protein S Monoclonal Antibody LJ-56 for Synthetic Protein S Peptides - Microtiter wells were coated with 50 μl of different peptides (20 μM, 50 μg/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 (292 μg/ml) for 1 h at room temperature at dilutions 1/100 to 1/12,800. Wells were washed and incubated with 50 μl of biotinylated rabbit anti-mouse IgG (1 μg/ml) for 1 h at room temperature. Wells were washed and 50 μl SAAP (1 μg/ml) was 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 EL Microplate reader as described earlier.

Affinity of Anti-Protein S Monoclonal Antibody LJ-56 for Recombinant Protein S Variants - Microtiter wells were coated with 100 μl of rabbit anti-protein S IgG (10 μg/ml) in 0.02 M sodium carbonate buffer pH 9.0 for 18 h at 4°C. Wells were emptied and blocked with 2% BSA in TBS for at least 30 min at room temperature and subsequently washed with TBS. Wells were incubated with 100 μl of a fixed amount of recombinant protein S variants (50 ng for E424K variant and 100 ng for wild type, Q427E, K429E and L variant) for 18 h at room temperature. Wells were emptied, subsequently washed with TBS and 100 μl of serial dilutions of anti-protein S monoclonal antibody LJ-56 (0-10 μg/ml) was added and incubated for 2 h at room temperature. Wells were emptied, subsequently washed with TBS and 100 μl of rabbit anti-mouse IgG conjugated to peroxidase (1:1000) was added and incubated for 1 h at room temperature. The hydrolysis of nitrophenylphosphate was measured at 490 nm using a V_{max} plate reader as described earlier.

RESULTS

Binding of Recombinant Protein S Variants to C4BP - The binding of the recombinant protein S variants to immobilized C4BP was examined using an anti-C4BP monoclonal antibody IgG (8C11), which is directed against the α-chain of C4BP. This antibody was used to capture C4BP. The binding was performed in 50 mM Tris-HCl pH 7.5 containing 150 mM NaCl and 5 mM $CaCl_2$. Fig. 2 shows that the E424K and Q427E variants bind to C4BP with the same apparent affinity as the wild type recombinant molecule. The K429E variant, at concentrations up to 50 ng/ml, failed to bind to C4BP.

Fig. 2. **Binding of recombinant protein S variants to C4BP.** Anti-C4BP monoclonal antibody 8C11 IgG was coated onto microtiter wells to catch C4BP (1 μg/ml). Recombinant protein S *(circles)*, E424K variant *(diamonds)*, Q427E variant *(squares)* or K429E variant *(triangles)* were added with increasing amounts in the presence of calcium. Binding to C4BP was for 18 h at room temperature. Bound proteins were detected with anti-protein S IgG conjugated to peroxidase (0.3 μg/ml). The data of the experiment are the mean of duplicated values.

Binding of C4BP to Synthetic Protein S Peptides - The binding of biotinylated C4BP to immobilized synthetic protein S peptides was examined in microtiter wells. Fig. 3 shows the binding curves of the peptides. PSP-420 binds weaker then the E424K peptide and stronger than the Q427E peptide. The K429E peptide failed to bind to C4BP.

Fig. 3. **Binding of C4BP to synthetic protein S peptides.** Native protein S (10 μg/ml) *(open squares)*, PSP-420 peptide *(circles)*, E424K peptide *(diamonds)*, Q427E peptide *(squares)* and K429E peptide *(triangles)* were coated onto microtiter wells (20 μM). Increasing amounts (0-5 μg/ml) of biotinylated C4BP were added and bound biotinylated C4BP were measured with SAAP/pNPP. The data of the experiment are the mean of duplicated values.

Competition of Synthetic Protein S Peptides and Native Protein S for C4BP - The ability of the synthetic protein S peptides to compete with native protein S for binding to C4BP was studied in solid and fluid phase competition assays. Fig. 4 shows dose-reponse studies for the inhibition of the binding of C4BP to native protein S by the normal peptide PSP-420 and the mutant peptides E424K, Q427E and K429E in solid and in fluid phase. Panel A shows the results of the solid phase assay. The PSP-420 and E424K peptides inhibit the binding almost completely, while the Q427E peptide inhibits less. This shows that replacement of Glu-424 to Lys had no effect and Gln-427 to Glu had minor effect on the binding to C4BP. The K429E peptide does not show inhibition, indicating that Lys-429 is involved in the binding. Panel B shows the results of the fluid phase assay. The PSP-420 and E424K peptide inhibit the binding almost completely. The Q427E and K429E peptides do not inhibit the binding, suggesting Gln-427 and Lys-429 to be involved in the binding.

Fig. 4. **Inhibition of the binding of C4BP to protein S by synthetic protein S peptides in solid and fluid phase.** The assay was performed as described in "Experimental Procedures". Increasing amounts of PSP-420 peptide *(circles)*, E424K peptide *(diamonds)*, Q427E peptide *(squares)* and K429E peptide *(triangles)* were tested. Panel A: solid phase; panel B: fluid phase. All data are mean values of the percentage of inhibition of bC4BP to immobilized protein S from 3 different experiments performed in duplicate.

Affinity of Anti-Protein S Monoclonal Antibody LJ-56 for Synthetic Protein S Peptides and Recombinant Protein S Variants - Recently, we have developed an anti-protein S monoclonal antibody LJ-56, which is raised against the PSP-420 peptide and recognizes only free protein S and inhibits the binding to C4BP (31). Protein S peptides or protein S variants (via a polyclonal anti-protein S antibody) were coated onto microtiter wells and increasing amounts of LJ-56 were added.

Fig. 5 shows that LJ-56 recognizes PSP-420 peptide, the E424K and Q427E peptides with equal affinity. The K429E peptide was not recognized by LJ-56.

Fig. 6 (panel A) shows that the E424K and Q427E variant proteins were able to bind to LJ-56 comparable as wild type recombinant protein S, while the K429E variant protein did not bind. Panel B shows that the L-variant (which lacks a part of the C-terminal end) binds to LJ-56 with an affinity similar as wild type recombinant protein S.

Fig. 5. **Affinity of anti-protein S monoclonal antibody LJ-56 for synthetic protein S peptides.** In microtiter wells increasing amounts of LJ-56 were added to the different immobilized peptides PSP-420 *(circles)*, E424K *(diamonds)*, Q427E *(squares)* and K429E *(triangles)*. Bound LJ-56 was detected with biotinylated rabbit anti-mouse IgG as described in "Experimental Procedures".

Fig. 6. **Affinity of anti-protein S monoclonal antibody LJ-56 for recombinant protein S variants.** - In panel A, a fixed amount of wild type recombinant protein S *(circles)*, E424K variant protein *(diamonds)*, Q427E variant protein *(squares)* and K429E variant protein *(triangles)* and in panel B, a fixed amount of wild type recombinant protein S *(closed circles)* and L variant *(open circles)* were added to immobilized rabbit anti-protein S IgG coated onto microtiter wells. Increasing amounts of LJ-56 was added and detected with rabbit anti-mouse IgG conjugated to peroxidase as described in "Experimental Procedures".

DISCUSSION

Human protein S forms with human C4BP a reversible complex, which down-regulates the APC cofactor activity of protein S, because only free protein S is active as a cofactor to APC. Several reports have shown that the second disulfide loop of the C-terminal end of the SHBG-like domain (amino acid residues 583-635) of protein S is involved in its binding to C4BP (17-20). Later studies raised the possibility that this region was not directly involved in the binding of C4BP but that this region was an essential element in maintaining the correct protein structure for a binding site located elsewhere in the protein S molecule (20). Studies with an anti-protein S monoclonal antibody (S12) suggested that deletion of this region causes a conformational change of the protein S structure resulting in the loss of affinity for its epitope, which is probably located in the Ser-460 region. The possibility that such a conformational change also results in a loss of affinity for binding to C4BP elsewhere in protein S therefore can not be excluded. The presence of such an alternative binding site was reported recently (21). It is localized in the first disulfide loop of the SHBG-like domain. To study the interaction of protein S and C4BP in the first disulfide loop of the SHBG-like domain (amino acid residues 420-434) of protein S in more detail, we have used synthetic protein S peptide analogs and recombinant protein S variants (Fig. 1). The protein S peptides were purified by preparative HPLC (24) and the recombinant protein S variants were purified by anion-exchange chromatography (18).

The recombinant protein S variants have comparable molecular weights as wild type recombinant protein S after SDS/PAGE and immunoblotting analysis (not shown). Furthermore, in a modified activated partial thromboplastin time (APTT) system the recombinant protein S variants posses cofactor activity to APC (not shown). This indicates that replacement of Glu-424 by Lys, Gln-427 by Glu and Lys-429 by Glu does not influence the APC cofactor activity of protein S.

The binding of the recombinant protein S variants and synthetic protein S peptides to C4BP was examined. Fig. 2 shows the binding of the recombinant protein S variants to C4BP in the presence of calcium. The E424K and Q427E variants bind to C4BP with an affinity similar as the wild type recombinant protein, suggesting that replacement of Glu-424 by Lys or Gln-427 by Glu has no effect on the binding

and are therefore not required for binding to C4BP. The K429E variant does not bind to C4BP, suggesting Lys-429 is important in the binding to C4BP. Essentially the same results were obtained with the synthetic protein S peptides (Fig. 3).

The ability of the synthetic peptides to compete with protein S for the binding to C4BP was tested in solid and fluid phase assays. Panel A (solid phase) in Fig. 4 shows that the E424K, Q427E and PSP-420 peptides competed with protein S for the binding to C4BP. The competition of the E424K peptide was more pronounced than the PSP-420 peptide. Both peptides were able to prevent binding almost completely. The Q427E peptide competes less, suggesting that Gln-427 is probably weakly involved in the binding of protein S to C4BP. The K429E peptide does not compete, suggesting that Lys-429 is involved in the binding. Panel B (fluid phase) in Fig. 4 shows that the PSP-420 and E424K peptides compete with native protein S for C4BP, comparable as the results in the solid phase assay. The K429E and Q427E peptides do not compete with native protein S for C4BP suggesting Lys-429 and Gln-427 not to be involved in the binding to C4BP. The effect of the Q427E peptide in solid phase is different from the effect observed in the fluid phase experiment. The results of the fluid phase studies show no effect of the Q427E peptide suggesting the Q427E peptide is not able to compete with native protein S for C4BP in fluid phase. The Q427E peptide probably has a weaker affinity than native protein S for C4BP. In the solid phase the Q427E peptide does show binding to C4BP as it competes with native protein S, but in the fluid phase native protein S is able to displace the Q427E peptide from C4BP.

Anti-protein S monoclonal antibody LJ-56 was developed against the PSP-420 peptide and its further characterization showed that LJ-56 only recognizes free protein S and is able to inhibit the binding of protein S to C4BP (31). Fig. 5 shows that LJ-56 recognizes the PSP-420, E424K and Q427E peptides with equal affinity. The K429E peptide is not recognized by LJ-56, suggesting that Lys-429 is important for the recognition by LJ-56. Reactivity of LJ-56 to the recombinant protein S variant proteins gave essentially the same results (Fig. 6). LJ-56 recognizes the E424K and Q427E variant proteins in a way similar as the wild type protein, whereas the K429E variant was not recognized. The substitution of Lys-429 by Glu probably induces a conformational change in the peptide and in the variant

protein, resulting in a loss of recognition by LJ-56. These data suggest that Lys-429 of protein S is essential for the binding to C4BP and for the recognition of LJ-56.
LJ-56 was able to recognize a recombinant protein S variant (L variant) that lacks the second disulfide loop of the SHBG-like domain of protein S. This suggests that the affinity of LJ-56 for its epitope is not influenced by deletion of the second disulfide loop in the C-terminal region of protein S. Since the epitope of LJ-56 closely coincides with the binding site for C4BP this suggests that in the L variant the binding site for C4BP is exposed. Previous study with another monoclonal antibody (S12) had suggested that deletion of the C-terminal end caused a major conformational change in the protein structure resulting in a loss of the epitope for S12. It could therefore not be excluded that this conformational change also effected the binding affinity for C4BP elsewhere in the protein S molecule. We have shown that the L variant has a greatly reduced affinity for C4BP (20). The fact that LJ-56 still recognizes the L variant suggests however, that the binding site for C4BP could still be exposed. This suggests that two binding sites, one in the first and one in the second disulfide loop of the SHBG-like domain of protein S might form the binding site for C4BP. Whether these two binding sites acts independently or cooperatively is not known at present. Due to the low amounts of variant proteins, it is not possible at this stage to determine which disulfide loop of the SHBG-like domain of protein S is the major binding site for C4BP.
Our results show that combinations of mutant peptides and recombinant protein S variants are powerfull tools to study the contribution of individual amino acid residues. The results of the mutant peptides are in good agreement with the results of the recombinant variant proteins, suggesting the mutant peptides probably to be in the same conformation as in their corresponding sequence in the recombinant protein variants. An advantages of use of peptides is that peptides are much faster to test than constructing and expressing recombinant protein variants to obtain similar results. It is obvious that for studying specific functional properties the recombinant variant molecule is preferred.
Currently, other mutations are made in this region to determine other relevant amino acid residues responsible for the binding of protein S to C4BP.

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CHAPTER 5

CONSTRUCTION AND CHARACTERIZATION OF THROMBIN-RESISTANT VARIANTS OF RECOMBINANT HUMAN PROTEIN S

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ABSTRACT

Protein S is a vitamin K-dependent plasma protein that functions as a cofactor to activated protein C (APC) in the inactivation of coagulation factors Va and VIIIa. This anticoagulant property requires the presence of Ca^{2+} ions and negatively charged phospholipids. Protein S, purified from bovine and human plasma, migrates as a doublet on sodium dodecyl sulphate/polyacrylamide gel electrophoresis under reduced conditions. This heterogeneity in molecular weight has been explained by limited proteolysis of protein S. The single chain molecule is converted to an inactive two chain molecule with the two chains linked together by a disulfide bridge between Cys-47 and Cys-72. This modification therefore may represent an important regulatory step in blood coagulation.

For bovine protein S two cleavage sites for thrombin have been identified at Arg-52 and Arg-70. Human protein S contains at Arg-49, Arg-60 and Arg-70 three potential cleavage sites in this region. Whether cleavage occurs at all three sites is not known. To study the role of these arginine residues in human protein S, we have replaced them by leucine or isoleucine. All seven possible variants were constructed: three variants with single mutations, three variants with double mutations and one variant with a triple mutation. On reduced SDS polyacrylamide gels the single and double variants migrate as a doublet just like the wild type protein S. The triple variant migrates as a single band at a molecular weight corresponding to the upper band of the doublet. The upper band of the single and double variants but not of the triple variant could be converted into the lower band by thrombin treatment. All variants showed cofactor activity to APC in a clotting assay. After thrombin treatment, this cofactor activity was abolished for the single and double variants, while the triple variant retained its cofactor activity completely, suggesting resistance to thrombin. This shows that cleavage can take place at all three arginine sites and that cleavage at each of these sites results in the loss of APC cofactor activity. Finally, all variants bind to C4b-binding protein with an affinity similar as the wild type recombinant molecule. The variants described here will be important for further studies on the thrombin-sensitive domain of human protein S. The thrombin resistant variant (triple variant) could be very useful for standardization of the APC cofactor activity of protein S.

INTRODUCTION

Human protein S (M_r 75.000) is a vitamin K-dependent plasma glycoprotein (1,2). In human plasma, protein S is complexed noncovalently to C4b-binding protein (C4BP), a component of the classical pathway of the complement system (3). Approximately 40% of plasma protein S is free and only this form can function as a cofactor to activated protein C (APC), thereby promoting the degradation of the procoagulant factors Va and VIIIa (4-7). This anticoagulant property makes protein S an important regulator of blood coagulation. The physiological importance of protein S can be illustrated from the observation that patients partially deficient in protein S have an increased risk to develop thrombosis (8-10).

From the cDNAs encoding for bovine (11) and human protein S (12-14) it is inferred that protein S is synthesized as a single chain molecule. Protein S consists of a leader sequence followed by protein modules homologous to a γ-carboxyglutamic acid domain, a thrombin sensitive domain, four epidermal growth factor-like domains and a sex hormone binding globulin-like domain.

In human protein S the thrombin-sensitive domain contains three potential cleavage sites for thrombin, namely after Arg-49, Arg-60 and Arg-70. Which of these is actually cleaved and what the effect of cleavage for the APC cofactor activity is, is not known. Bovine and human protein S have been reported to be very sensitive to proteolysis by thrombin (15-18). In bovine protein S thrombin cleaves after Arg-52 and Arg-70, thus producing a two-chain molecule linked together by a disulfide bond between Cys-47 to Cys-72. Thrombin-cleaved protein S has lost its APC cofactor activity. Therefore it has been proposed that this cleavage may represent a physiological mechanism for the control of the protein S cofactor acitivity to APC; however physiological concentrations of calcium might protect protein S against cleavage.

Because most of the purified protein S preparations contain a variable amount of two-chain protein S it is very difficult to use these preparations for the quantitative evaluation of functional aspects of purified protein S. Therefore we decided to study the possibility to produce a protein S variant which is resistant to thrombin cleavage, but still retains full APC cofactor activity and binding affinity for C4BP. Seven variants were constructed, where the arginines were replaced by leucine or

isoleucine. We show that cleavage at each arginine residue results in a loss of the APC cofactor activity of protein S.

EXPERIMENTAL PROCEDURES

Materials - Restriction endonucleases Bam HI and Hind III were purchased from Pharmacia Biotechnology (Uppsala, Sweden). T7 DNA Polymerase was from New England Biolabs (Beverly, MA, USA). T4 DNA Ligase was from Bethesda Research Laboratory (Bethesda, MD, USA). All enzymes were used according to the manufacturers instructions. *Escherichia coli* strains CJ236 and MV1190 were from Bio-Rad Corporation (Richmond, CA, USA). Sequenase Version 2.0 sequencing kit was from USB Corporation (Cleveland, OH, USA). APC, rabbit anti-protein S IgG and protein S and C4BP depleted plasma were prepared as described (7). Thrombin-Sepharose was prepared by coupling 1 mg of thrombin (Sigma, St Louis, MO, USA) per ml of CNBr-activated Sepharose (Pharmacia) according to manufacturers instructions. C4BP and anti-C4BP monoclonal antibody 8C11 IgG, directed against the α-chain of C4BP was prepared as described (28). Rabbit anti human protein S IgG conjugated to peroxidase was from Dakopatts (Glostrup, Denmark). Fast Flow Q resin was from Pharmacia (Uppsala, Sweden). Iscove's Modified Dulbecco's Medium, pennicilin G, streptomycin sulphate, glutamine and fetal calf serum were from GIBCO (Paisley, UK). Vitamin K_1 (Konakion) was from F. Hoffman-La Roche Ltd. (Basel, Switserland). Trasylol was from Bayer (Leverkusen, FRG).

Construction of Protein S Variants - Standard recombinant DNA techniques were performed as described by Sambrook et al (29). Construction of the variants was achieved by site-specific mutagenesis. A 430-base pair BamHI-HindIII fragment from the expression vector pMSVPS (26) carrying a part of the protein S cDNA sequence was subcloned into M13mp19. The following 19-mer oligonucleotides were used for mutagenesis: 5'-TGA AAA GAG AGA AGA CAA A-3' for Arg-49 to Leu, 5'-GTG GAC TGA AGT GCA GCA C-3' for Arg-60 to Leu and 5'-ACA CAG CTT ATT AGG TCA G-3' for Arg-70 to Ile. For the double and triple variants combinations of two or three oligonucleotides were used. Site-specific mutagenesis was performed according to Kunkel (30) and the mutations were verified by DNA sequencing according to Sanger et al. (31).

Cell Culture, DNA Transfection and Purification 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), 10% heat-inactivated fetal calf serum and trasylol (10 KIE/ml). Cultures were maintained at 37°C containing 5% CO_2. For transfections 20 μg of plasmids were used and cells were transfected by a conventional calcium phosphate coprecipitation technique (32) as described (26). After transfection and isolation of cell lines secreting protein S variants, recombinant proteins were purified from the cell culture medium

on an anion-exchange column (Fast Flow Q resin, Pharmacia) as described for wild type recombinant protein S (25,26).

Treatment of Recombinant Protein S Variants with Thrombin - One ml of purified protein S variant (4-40 μg/ml) was incubated with 1/20 vol (50 μl) of thrombin-Sepharose for 1 h at 37°C in 50 mM Tris-HCl pH 7.5 containing 150 mM NaCl. Thrombin-Sepharose was removed by centrifugation. Supernatants, containing the thrombin-treated protein S were stored at -20°C until used.

Protein S Assays - Protein S antigen was determined using a specific ELISA as described elsewere (33) using rabbit anti-protein S IgG as catching antibody, and rabbit anti-protein S IgG conjugated to peroxidase (Dakopatts), as tagging antibody. Protein S cofactor activity was determined in a clotting assay as described (26).

Binding of Recombinant Protein S and Variants to C4BP - The complex formation between the recombinant proteins and C4BP was measured with a sensitive ELISA using anti-C4BP monoclonal antibody 8C11 IgG, purified C4BP and a peroxidase conjugated anti-protein S antibody as described (26).

RESULTS AND DISCUSSION

Protein S circulates in blood as a single chain molecule. Upon isolation, the single chain form can be converted into a two-chain form by limited proteolysis, which results in a loss of its APC cofactor activity. For bovine protein S cleavage takes place after Arg-52 and Arg-70 in the so-called thrombin-sensitive domain. In this domain human protein S contains three arginine residues suggesting three potential cleavage sites for thrombin. It is not known which cleavages are involved in the regulation of the APC cofactor activity of human protein S. To study this we have constructed the following seven variants: three single variants (R49L, R60L and R70I), three double variants (R49L/R60L, R60L/R70I and R49L/R70I) and one triple variant (R49L/R60L/R70I)(Fig. 1).

The variants were stably expressed in C127 cells, a mammalian cell expression system which was previously used to express recombinant protein S with full APC cofactor activity and C4BP binding capacity (26). The level of expression varied between 0.1 to 1 μg/ml. Recombinant protein S and the variants were purified using a Fast Flow Q column and a final yield of about 80-90 % was obtained.

	47		50						55						60						65						70		72
Bovine	-	-	G	-	-	R	A	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-
Human	C	L	R	S	F	Q	T	G	L	F	T	A	A	R	Q	S	T	N	A	Y	P	D	L	R	S	C			
R49L	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
R60L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-			
R70I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	-			
R49L/R60L	-	-	L	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-			
R60L/R70I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	-	-	I	-			
R49L/R70I	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	-			
R49L/R60L /R70I	-	-	L	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	-	-	I	-			

Fig. 1. **Amino acid sequence of bovine, human and variants of protein S.** The second line is the human protein S sequence. Dots are identical amino acid residues. Underlined amino acid residues are targets for site-specific mutagenesis. The cysteine residues at positions 47 and 72 are covalently linked by a disulfide bond. Numbering of the amino acids is according to refs. 11-14.

On reduced SDS gels, purified recombinant protein S and the single and double variants all migrate as a doublet. In the case of the triple variant only one band, at the heigth of the upper band of the doublet, is observed (Fig. 2, lanes 13,14). The upper band of the doublet for recombinant protein S and the single and double variants all could be converted into the lower band by thrombin treatment. The band of the triple variant however, was not converted into a lower band, indicating that this triple variant is resistant to thrombin. The observation that the single chain form (upper band) of all three double variants could be converted into two-chain forms (lower band) indicates that all three potential cleavage sites can be hydrolyzed by thrombin (Fig. 2, lanes 7-12).

Fig. 2. **Western blot analysis of variants of protein S: effect of thrombin.** Aliquots (10-50 ng) of purified protein S variants (lanes 1 to 14) and recombinant protein S (lanes 15 and 16), were separated on a 10-15% SDS polyacrylamide gel under reducing conditions. Uneven lanes are untreated proteins and even lanes are thrombin-treated proteins (see Experimental Procedures). A, R49L; B, R60L; C, R70I; D, R49L/R60L; E, R60L/R70I, F, R49I/R70I; G, R49L/R60L/R70I; H, recombinant wild type protein S. After blotting, the proteins were detected with rabbit anti-protein S IgG conjugated with peroxidase (1.5 μg/ml).

The ability of the variants to serve as a cofactor for APC was tested in a clotting assay. As shown in Table 1, recombinant protein S and all variants show APC cofactor activity. When the proteins were treated with thrombin, this APC cofactor activity was lost except in the case of the triple variant, which retained its APC cofactor activity. This demonstrates that cleavage at each of the three arginine residues results in a loss of APC cofactor activity. From these experiments it cannot be concluded whether all three cleavage sites are equally sensitive for the action of thrombin.

Table 1. *APC cofactor activity of protein S variants.*

Variant	Final concentration (μg/ml)	Clotting time (sec)	Clotting time (sec) after thrombin treatment
R49L	**0.60**	**80**	**72**
R60L	**2.38**	**90**	**75**
R70I	**0.60**	**79**	**75**
R49L/R60L	**1.12**	**88**	**80**
R60L/R70I	**0.60**	**79**	**72**
R49L/R70I	**1.12**	**86**	**80**
R49L/R69L/R70I	**1.19**	**84**	**85**
Recombinant	**2.38**	**91**	**70**

Variants were constructed by in vitro site-specific mutagenesis as described in the text. APC cofactor activity of the variants (final concentration: 0.60-2.38 μg/ml) was determined in a clotting assay as described in "Experimental Procedures". The clotting time in the absence of protein S is 72 ± 2 seconds. Numbering of the amino acid residues is according to refs. 12-14. R = arginine (arg), L = leucine (leu), I = isoleucine (ile).

In Fig. 3 a dose-response curve of the APC cofactor activity of recombinant protein S and the triple variant is shown. At each concentration tested, the triple variant shows a higher APC cofactor activity (as defined by prolongation of the clotting time) than recombinant protein S. This is most likely due to the absence of cleaved protein S in the triple variant preparation in contrast to cleaved protein S present in the preparation of recombinant wild type protein S (Fig. 2, lanes 15, 16). Upon treatment with thrombin the dose-response curve of the triple variant remains unchanged, whereas the APC cofactor activity of recombinant protein S is significantly decreased.

Fig. 3. **Protein S functional activity.** The protein S dependent prolongation of the clotting time was measured in an activated partial thromboplastin time system using protein S and C4BP depleted plasma, APC, kaolin and cephalin. Increasing amounts of recombinant protein S *(circles)* or the triple variant *(triangles)* treated without *(open symbols)* or with *(closed symbols)* thrombin were added. Clotting was initiated by the addition of $CaCl_2$ and the clotting time was measured. The results are the means of duplicate values.

The single and double variants also have APC cofactor activity (Table 1). Fig. 2 demonstrates that varying amounts of the single and two-chain forms of protein S are present in the preparations. Treatment of these preparations with thrombin followed by analysis on reduced SDS gels indicated that the upper migrating band present in each preparation was converted into the lower band.

The thrombin cleavage sites in bovine protein S were identified using amino acid

terminal sequences of the isolated bovine protein S fragments obtained after thrombin treatment (16). Three bovine protein S fragments were identified: Ala-1 to Arg-52, Ala-53 to Arg-70 and Ser-71 to Ser-635, indicating that the cleavage sites were located after Arg-52 and Arg-70. From our data it can be concluded that human protein S can be cleaved by thrombin after Arg-49, Arg-60 and Arg-70. Human and bovine protein S share 82% homology between Cys-47 and Cys-72 in the thrombin-sensitive domain and both proteins contain three arginine residues in this region. Therefore it remains possible that bovine protein S can also be cleaved after Arg-60. Although the corresponding fragments were not identified, an intermediate band was observed on SDS gels, between Ala-1 to Arg-52 and Ala-1 to Arg-70, that could represent the Ala-1 to Arg-60 fragment (16).

Finally, we examined the binding of the variants to C4BP. Fig. 4 shows that in the presence of calcium all seven variants bind to C4BP with the same affinity as wild

Fig. 4. **Binding of variants of protein S to C4BP.** Anti-C4BP monoclonal antibody 8C11 IgG was coated onto microtiter wells to catch C4BP (1μg/ml). Increasing amounts (0-100 ng/ml) of recombinant protein S *(open circles)* or variants *(R49L, closed diamonds; R60L, closed squares; R70I, closed triangles; R49L/R60L, closed circles; R60L/R70I, open diamonds; R49L/R70I, open squares; R49L/R60L/R70I, open triangles)* were added. Binding to C4BP was for 18 h at room temperature. Bound protein S variants were detected with anti-protein S IgG conjugated to peroxidase (0.3 μg/ml). The results are the mean of duplicate values in two different experiments.

type recombinant protein S. This suggests that the alteration of the arginine residues does not influence the binding affinity of protein S for binding to C4BP, and that the binding site(s) on the protein S molecule for C4BP are still available.

In conclusion, we have succeeded to construct a protein S variant (the triple variant) which is completely resistant to inactivation by thrombin which still retains full APC cofactor activity and which has an unaltered affinity for binding to C4BP. This variant will be very useful for the quantitative studies of the APC cofactor activity of protein S without interference of thrombin. In addition, the triple variant can be used for standardization of the APC cofactor activity of protein S.

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CHAPTER 6

GENERAL DISCUSSION ON THE STRUCTURE/FUNCTION RELATIONSHIPS OF HUMAN PROTEIN S

INTRODUCTION

Patients deficient in protein S have an increased risk to develop thrombosis (Comp and Esmon, 1984; Comp et al., 1984; Schwarz et al., 1984; Bertina et al., 1985; Broekmans et al., 1985). Protein S acts as a cofactor to activated protein C (APC) (Walker, 1980). The action of protein S is regulated by thrombin and C4b-binding protein (C4BP).

Thrombin cleaves protein S from a single chain to a two chain form (Dahlbäck, 1983; Suzuki et al., 1983; Walker, 1984; Morita et al., 1986; Mitchell et al., 1986). This thrombin-cleaved protein S has no APC cofactor activity (Suzuki et al., 1983; Walker, 1984; Mitchell et al., 1986).

C4BP forms a complex with protein S (Dahlbäck and Stenflo, 1981). Free protein S has APC cofactor activity and protein S in complex with C4BP has no cofactor activity to APC (Bertina et al., 1985; Dahlbäck, 1986a). Localization of the thrombin cleavage site(s) and the C4BP binding site(s) on protein S would lead to the design of recombinant protein S variant molecules resistant to thrombin and unable to bind to C4BP. These second generation protein S molecules can be usefull as potent therapeutic agents.

INTERACTION OF PROTEIN S WITH C4b-BINDING PROTEIN

To study the interaction of protein S with C4BP synthetic protein S peptides and recombinant protein S variant molecules were used. Additionally, specific anti-protein S monoclonal antibodies were used to study the conformation of the designed protein S variant molecules.

Two sites involved in the interaction of protein S with C4BP were reported (Walker, 1989; Fernández et al., 1992). The first binding site was located at the second disulfide loop (residues 605-614) of the sex hormone binding globulin (SHBG)-like domain of protein S and the second binding site at the first disulfide loop (residues 420-434). Using synthetic protein S peptides Walker (1989) showed that the first binding site for C4BP was located between Gly-605 and Ile-614. Site-specific mutagenesis of amino acid residues in this region was performed to study their involvement in the binding to C4BP (Chapter 2). Two variant protein S molecules were constructed. The first variant (B variant) had a replacement of residue Leu-608

by Val and the second variant (C variant) had 3 replacements. The acidic amino acid residues Asp-609, Asp-611 and Glu-612 were replaced by Asn, Asn and Gln, respectively. Binding of these variants to C4BP showed that the variants bind in a similar way as wild type recombinant protein S. This suggests that these residues are not essential for the binding to C4BP and that a larger region of protein S is needed for optimal binding to C4BP.

In Chapter 3 two deletion variants of protein S were constructed. The first variant (E variant) lacks the third epidermal growth factor (EGF)-like domain (Asp-160 to Asp-202) and the second variant (L variant) lacks the C-terminal part of the sex hormone binding globulin (SHBG)-like domain (Asp-583 to Ser-635). The E variant binds to C4BP similar as the wild type recombinant protein S ($K_d \sim 10^{-10}$ M), while the L variant shows a greatly reduced binding ($K_d \sim 10^{-7}$ M). This suggests that deletion of the C-terminal part either removes or severely effects an important binding site in protein S for interaction with C4BP. Nelson and Long (1992) have reported several mutants made in the C-terminal end of protein S. It was shown that the mutants had reduced affinities for C4BP. Some mutants had no APC cofactor activity, possibly due to severe deletions in the protein S molecule.

Three different Ca^{2+}-independent anti-protein S monoclonal antibodies were examined in Chapter 3 to study the possibility that deletions would effect the conformation of protein S. Two antibodies, S5 and S17, bind to the purified deletion variants and recombinant protein S with very similar affinities, whereas one antibody, S12, does not bind to the L variant. However, after denaturing SDS-PAGE and immunoblotting S12 could recognize the L variant, indicating the epitope is not localized in the C-terminal part. It was previously reported (Bertina et al., 1990), that the epitope of S12 is sensitive for the replacement of amino acid residue Ser-460 by Pro, because S12 did not recognize a protein S variant (even after denaturation) in which Ser-460 was replaced by Pro. This suggests that deletion of the C-terminal part induces such a conformational change in the region close to Ser-460 that the epitope of S12 is lost. This could also mean that the binding site for C4BP is not localized in the C-terminal region of protein S, but that deletion of the C-terminal region influences another binding site for C4BP elsewhere in the protein S molecule.

Recently, Fernández and coworkers (1992) reported a binding site located at the first disulfide loop (residues 420-434) of the SHBG-like domain, which is relatively close to Ser-460. Location of this second binding site was based on the use of a synthetic protein S peptide (residues 420-434). This peptide was shown to bind to C4BP which a higher affinity than the peptide described by Walker (1989). In Chapter 4 the role of specific amino acid residues in this region was studied. Both synthetic mutant protein S peptides were synthesized and site-directed recombinant protein S variant molecules were constructed. The role of three amino acids was studied. Glu-424, Gln-427 and Lys-429 were replaced by Lys, Glu and Glu, respectively. The protein S mutant peptides and recombinant proteins were designated E424K, Q427E and K429E peptide or variant protein. Replacement of Glu-424 by Lys in the peptide and in the protein did not interfere with binding to C4BP, suggesting that Glu-424 is not important for the binding of protein S to C4BP. Replacement of Gln-427 to Glu in the protein showed that the variant could still bind to C4BP similar as wild type recombinant protein S. For the peptide however, a reduced binding to C4BP was found. In a competition assay native protein S could prevent binding to C4BP. This indicates that replacement of Gln-427 by Glu is probably not involved in the binding to C4BP. Replacement of Lys-429 to Glu in the peptide and in the protein competively prevented binding to C4BP, suggesting that Lys-429 is important in the binding of protein S to C4BP.
An anti-protein S monoclonal antibody LJ-56, which was raised against the protein S peptide (residues 420-434) recognizes only free protein S and is able to inhibit the binding of protein S to C4BP. The recognition of LJ-56 by the protein S peptides and variants was studied (Chapter 4). LJ-56 could recognize the E424K and Q427E peptide and variant protein, whereas the K429E peptide or protein was not recognized. Replacement of Lys-429 by Glu probably induces a conformational change in the peptide and in the protein, resulting in a loss of recognition by LJ-56. These data suggest that Lys-429 of protein S is essential both for the binding to C4BP and for the recognition of LJ-56. Further studies showed that LJ-56 could recognize the L variant (which lacks a part of the C-terminal end), indicating that the epitope of LJ-56 and thus also the binding site for C4BP is exposed on the L variant despite the fact that the L variant showed a reduced affinity for C4BP.

Previous studies with S12 had suggested that deletion of the C-terminal region caused a major conformational change in protein S leading to loss of affinity for the epitope of S12 under non-denaturing conditions. It can therefore not be excluded that this conformational change also effected the binding site for C4BP. Our studies suggest the existence of two binding sites for C4BP, one in the C-terminal region and one in the middle of the SHBG-like domain. These binding sites might act cooperatively. This would result in a decreased affinity for C4BP if one of the two binding sites is mutated. It is also possible that there is only one binding site and that mutation of the other site results in such a conformational change of the protein that the affinity for C4BP is lost.

MODEL OF THE INTERACTION BETWEEN HUMAN PROTEIN S AND C4BP

In Fig. 1 a schematic model of the interaction of human protein S with C4BP based on the current knowledge and the results described in this thesis is proposed. In this model the following facts and hypotheses are included:

- Binding of C4BP to protein S involves the β-chain of C4BP (Hillarp and Dahlbäck, 1988).
- The C-terminal end (residues 583-635) of protein S is involved in the interaction with C4BP (Walker, 1989; Nelson and Long, 1992; Chapter 3) and in this region Leu-608, Asp-609, Asp-611 and Glu-612 are not important (Chapter 2).
- The first disulfide loop of the SHBG-like domain of protein S (residues 420-424) is involved in the interaction with C4BP (Fernández et al., 1992) and in this loop Lys-429 is important (Chapter 4).
- Anti-protein S monoclonal antibody S12 is sensitive for the replacement of Ser-460 by Pro (Bertina et al., 1990) and its epitope is lost when the C-terminal end is deleted (Chapter 3).
- Anti-protein S monoclonal antibody LJ-56 raised against the protein S peptide residues 420-434 recognizes only free protein S and inhibits complex formation of protein S with C4BP (Fernández et al., 1992) and is sensitive for the replacement of Lys-429 by Glu (Chapter 4).

Fig. 1. **Schematic representation of a model for the interaction of protein S with C4BP**. Amino acid residues are abbreviated using the one-letter code. S12 and LJ-56 are anti-protein S monoclonal antibodies. The epitope of S12 is sensitive for the replacement of Ser-460 by Pro and the epitope of LJ-56 is sensitive for the replacement of Lys-429 by Glu. The protein S peptide (420-434) reported by Fernández and coworkers (1992) is shown as a straight line and the protein S peptide (605-614) reported by Walker (1989) is shown as a dashed line. β, the β-chain of C4BP, which is located between the first and second disulfide loop of the SHBG-like domain of protein S.

In Fig. 2 two possible primary structures of the L variant of protein S are presented. Deletion of the C-terminal end (residues 583-635) of protein S results in a strongly reduced interaction with C4BP (Nelson and Long, 1992; Chapter 3), but does not influence the binding of LJ-56 with protein S (Chapter 4). Deletion however, induced a conformational change in protein S resulting in the loss of epitope recognition by S12 (Chapter 3). This loss leads to either a direct change (model A) or to shielding off (model B) of the region close to Ser-460.

Fig. 2. **Schematic representations of the L-variant of human protein S**. Amino acid residues are abbreviated using the one-letter code. Deletion of the C-terminal end (residues 583-635) of protein S induces a conformational change in protein S leading to either a direct conformational change (model A) or to shielding off (model B) of the region close to Ser-460. In both models the epitope of anti-protein S monoclonal antibody LJ-56 is not influenced by the deletion, but is sensitive for the replacement of Lys-429 by Pro. A reduced binding of protein S with C4BP occurs when the C-terminal end of protein S is deleted or when Lys-429 is replaced by Glu in native protein S.

INACTIVATION OF PROTEIN S BY THROMBIN

The thrombin cleavage sites of bovine protein S have been reported to be located at Arg-52 and Arg-70 in the thrombin-sensitive domain of protein S (Dahlbäck et al., 1986b). Comparison of the cDNA sequence of the bovine molecule with the human molecule shows that human protein S contains three potential arginyl peptide bonds in the thrombin-sensitive domain (Dahlbäck et al., 1986c; Lundwall et al., 1986; Hoskins et al., 1987; Ploos van Amstel et al., 1987). These are located after Arg-49, Arg-60 and Arg-70. To study the role of these arginine residues in the regulation of the protein S activity, they were altered to Leu or Ile (Chapter 5). Seven possible variants were constructed: three variants with single mutations, three variants with double mutations and one variant with a triple mutation. Isolation of the variants showed that all variants, except for the triple variant, have a doublet. The triple variant showed only the upper band of the doublet. After thrombin treatment the upper band of the doublet was converted into the lower band for the single and the double variants. The upper band of the triple variant could not be converted into the lower band, indicating that the triple variant is resistant to thrombin. These results further suggest that all three arginyl peptide bonds can be cleaved by thrombin. Which peptide bond is cleaved first or randomly is not known.

In a clotting assay all variants showed APC cofactor activity, which was lost after thrombin treatment. The triple variant however retains its cofactor activity, after thrombin treatment. These data show that the triple variant is resistant to thrombin and may be an important tool to study the role of protein S in the anticoagulant system (no interference by inactivation of proteolytic enzymes). Furthermore it might be important to use the triple variant for the standardization of the APC cofactor activity, since an international standard for protein S is not available at the moment. In addition, the design of a triple variant resistant to thrombin with full APC cofactor activity and without C4BP binding ability would be an ideal potent therapeutic agent.

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SUMMARY

Protein S is a vitamin K-dependent plasma protein that acts as a cofactor to activated protein C (APC). APC, another vitamin K-dependent plasma protein is responsible for the inactivation of coagulation factors Va and VIIIa. The physiological importance of protein S and protein C has been deduced from observations in patients deficient of either one of these proteins, who are at high risk to develop thrombosis. A part of protein S (60%) circulates in blood in complex with C4b-binding protein (C4BP). Only free protein S has APC cofactor activity. Protein S can be inactivated by thrombin, which hydrolyzes peptide bonds in a disulfide loop in the N-terminus of protein S. This thesis describes investigations regarding the localization of sites on the protein S molecule which are essential for C4BP binding of protein S and identification of the peptide bonds in protein S that are cleaved by thrombin and are involved in the inactivation of protein S.

Chapter 1 gives an overview of the structural aspects and the functional properties of protein S. The primairy structure of protein S as deduced from the cDNA sequence has been constructed, mostly based on homology in other proteins. Certain functions can be addressed to different domains. The domains and functions of protein S are discussed.

In *chapter 2* the expression of the cDNA encoding for protein S in mammalian cells is described. The resulting recombinant protein S has been isolated from the culture medium and characterized. Recombinant protein S has APC cofactor activity and could bind to C4BP in a way similar as plasma protein S. Monoclonal antibodies against plasma protein S also recognize recombinant protein S. N-terminal amino acid analysis showed that recombinant protein S has the same amino acid sequence as plasma protein S. Furthermore, recombinant protein S is γ-carboxylated and β-hydroxylated in equal amounts as plasma protein S. Recombinant protein S was different in N-linked glycosylation but this has no effect on the functions of the protein. Deduced from the literature the involvement of 4 amino acid residues in the C-terminal end was studied for binding to C4BP. The residues Leu-608, Asp-609, Asp-611 and Glu-612 were altered to Val, Asn, Asn and Gln, respectively. It is shown that alterations of these amino acids have no effect on

the binding to C4BP.

In *chapter 3* two deletion variants of protein S were constructed and studied. The first variant (E variant) has a deletion of the third epidermal growth factor-like domain and the second variant (L variant) has a deletion of the C-terminal end of protein S. Both deletion variants have APC cofactor activity. Binding to C4BP showed that the E variant binds to C4BP and that the L variant showed a greatly reduced binding, suggesting that the C-terminal part is involved in the binding to C4BP. The variants were also tested for their reactivity for different anti-protein S monoclonal antibodies. Two of these antibodies (S5 and S17) reacted with the E and L variant, but one antibody (S12) failed to react with the L variant. The epitope of S12 has been described to be sensitive for the replacement of Ser-460 by Pro. The L variant could be recognized by S12 after denaturation, suggesting that the epitope is not located in the C-terminal end of protein S. These results showed that the C-terminal end is either directly involved in the binding to C4BP or that it induces a conformational change in the protein S molecule and thereby affecting another binding site elsewhere in the molecule for C4BP. It can not be excluded however, that more than one binding site is present on the protein S molecule that act cooperatively.

Chapter 4 describes the results regarding studies of amino acid residues in the first disulfide loop of the SHBG-like domain of protein S for binding to C4BP. Two different methods were used: synthetic protein S peptides and recombinant protein S variants. In this disulfide loop three amino acid residues were studied. Glu-424, Gln-427 and Lys-429 were altered to Lys, Gln and Glu, respectivily. It is shown that Glu-424 and Gln-427 in the peptide and in the protein are not important for the binding to C4BP. Lys-429 however is important for the binding. The peptides and the proteins were also tested for their reactivity to LJ-56, a monoclonal antibody directed against the protein S peptide PSP-420 (residues 420-434). LJ-56 was reported to recognize only free protein S and could inhibit the binding of protein S to C4BP. LJ-56 could recognize the peptides and proteins where Glu-424 and Gln-427 were altered. Alteration of Lys-429 resulted in a decreased affinity for LJ-56, suggesting Lys-429 is important for the recognition of LJ-56. Furthermore, LJ-56 reacted with the L variant which lacks the C-terminal region. Since the epitope of

LJ-56 closely coincides with the binding site for C4BP, this suggests that in the L variant the binding site for C4BP is exposed. Because the L variant was shown to have reduced binding for C4BP this suggests that both the first and the second disulfide loop of the SHBG-like domain of protein S are involved in the interaction with C4BP.

Chapter 5 describes the inactivation of protein S by thrombin. In the thrombin-sensitive domain of protein S three potential cleavage sites are located. By alterations of these sites one by one or in combinations the role of these cleavage sites could be studied. Seven possible variants were constructed: three variants with single mutations, three variants with double mutations and one variant with a triple mutation. All variants (except the triple variant) showed a doublet after reduced SDS-PAGE, suggesting the presence of inactived material. All variants have APC cofactor activity, which was lost after thrombin treatment, except for the triple variant which retained its APC cofactor activity. Form these results it can be concluded that all three cleavage sites are important for the inactivation by thrombin. Since most protein S preparations contain degraded inactived material it is now possible to isolate a protein S molecule without inactived material. This can be important for further studies on the structure and function of protein S.

Chapter 6 deals with the results describe in the thesis, including the presentation of a model of the interaction between protein S and C4BP.

SAMENVATTING

Proteïne S is een vitamine K-afhankelijk plasma eiwit dat funktioneert als cofactor voor geaktiveerd proteïne C. Aktief proteïne C (APC) is ook een vitamine K-afhankelijk plasma eiwit dat verantwoordelijk is voor de inaktivering van de stollingsfactoren Va en VIIIa. Het fysiologisch belang van proteïne S and proteïne C blijkt uit het feit dat afwezigheid van één van deze eiwitten een trombose neiging tot gevolg kan hebben. Een deel van proteïne S (60%) komt in plasma voor in komplex met C4b-bindingseiwit (C4BP). Alleen vrij proteïne S heeft cofactor aktiviteit voor APC. Proteïne S kan geïnaktiveerd worden door trombine, dat een aantal peptidebindingen in een lusje in het N-terminale deel van proteïne S kan hydrolyseren. De resultaten van dit proefschrift beschrijven het onderzoek naar de lokalizatie van bindingsplaatsen op het proteïne S molekuul essentieël voor de binding aan C4BP en identificatie van peptide bindingen in het proteïne S molekuul betrokken bij de inaktivatie door trombine.

Hoofdstuk 1 geeft een overzicht van de strukturele aspecten en funktionele eigenschappen van proteïne S weer. Afgeleid van het cDNA is de primaire struktuur van proteïne S gekonstrueerd op grond van homologieën met andere eiwitten. Aan de verschillende domeinen kunnen bepaalde funkties worden toegeschreven. De verschillende domeinen en funkties van proteïne S worden uitvoerig besproken.

In *hoofdstuk 2* werd met behulp van een expressiesysteem het cDNA koderend voor proteïne S tot expressie gebracht in zoogdiercellen. Dit recombinant proteïne S werd uit het kweekmedium geïsoleerd en gekarakteriseerd. Het recombinant proteïne S bleek APC cofactor aktiviteit te bezitten en kon een komplex vormen met C4BP op dezelfde wijze als het plasma proteïne S. Monoklonale antilichamen opgewekt tegen plasma proteïne S bleken ook te worden herkent door recombinant proteïne S. N-terminale aminozuur volgorde liet zien dat recombinant proteïne S dezelfde volgorde had als plasma proteïne S. Verdere analyse liet zien dat het recombinant proteïne S gecarboxyleerd en gehydroxyleerd was in gelijke hoeveelheden als plasma proteïne S. Het recombinant proteïne S bleek anders geglycosyleerd te zijn, maar dit had geen invloed op de funkties van het molekuul.

Op grond van literatuur gegevens werden in het C-terminale deel van proteïne S 4 aminozuren bestudeerd met betrekking tot binding aan C4BP. De aminozuren leucine-608, asparaginezuur-609, asparaginezuur-611 en glutaminezuur-612 werden met behulp van mutagenese veranderd in valine, asparagine, asparagine en glutamine, respektivelijk. Het bleek dat deze aminozuren geen invloed hadden op de binding aan C4BP.

In *hoofdstuk 3* werden twee deletie varianten van proteïn S gekonstrueerd en bestudeerd. De eerste variant (E variant) had een deletie in het derde groei factor-achtig domein en de tweede variant (L variant) had een deletie van het C-terminale deel van proteïne S. Beide varianten hadden APC cofactor aktiviteit. Binding aan C4BP liet zien dat de E variant kon binden aan C4BP, maar dat de L variant een sterk verminderde binding liet zien. Hieruit kan gekonkludeerd worden dat het C-terminale deel belangrijk is voor de binding aan C4BP. De varianten werden ook bekeken op reaktiviteit met drie anti-proteïne S monoklonale antilichamen. Twee monoklonale antilichamen (S5 en S17) reageerden met de varianten terwijl een monoklonaal antilichaam (S12) niet meer reageerde met de L variant. Van het epitoop van S12 is gerapporteerd dat het gevoelig is voor de substitutie van serine-460 door proline. De resultaten laten zien dat het C-terminale deel direkt betrokken is bij de binding aan C4BP of dat deletie, een konformatie teweeg brengt, die andere bindingsmogelijkheden in het proteïne S molekuul kan beïnvloeden. De mogelijkheid van meerdere bindingsplaatsen kan niet worden uitgesloten.

In *hoofdstuk 4* worden de resultaten beschreven omtrent het effect van aminozuren in de eerste disulfide lus van het SHBG-achtig domein van proteïne S. Daartoe werden twee methoden gebruikt: synthetische proteïne S peptiden en recombinant proteïne S varianten met aminozuur veranderingen. In de eerste disulfide lus werden drie aminozuren bestudeerd. Glutaminezuur-424, glutamine-427 en lysine-429 werden veranderd in lysine, glutaminezuur en glutaminezuur, respectivelijk. Het bleek dat glutaminezuur-424 en glutamine-427 in zowel het peptide als in het eiwit, geen invloed hadden op de binding aan C4BP. Lysine-429 daaren tegen bleek wel belangrijk te zijn. De peptiden en recombinant eiwitten werden ook bestudeerd ten aanzien van hun reaktiviteit voor het anti-proteïne S monoklonaal antilichaam LJ-56.

LJ-56 is opgewekt tegen het proteïne S peptide 420-434, dat binding aan C4BP vertoonde en alleen vrij proteïne S herkent. Het bleek dat LJ-56 de peptiden en het eiwit waarin glutaminezuur-424 en glutamine-427 waren veranderd net zo goed herkende als het onveranderde eiwit. Na verandering van lysine-429 werd het eiwit echter niet herkent door LJ-56, hetgeen duidt op de belangrijkheid van dit aminozuur voor de herkenning van LJ-56. Verdere studies met LJ-56 gaven aan dat LJ-56 met de L variant (die een deel van de C-terminus mist) kon reageren. Aangezien het epitoop van LJ-56 samenvalt met de C4BP bindingsplaats, betekent dit dat in de L variant de bindingplaats voor C4BP geëxposeerd is. Omdat de L variant een verminderde C4BP binding heeft, suggereert dit dat zowel de eerste als de tweede disulfide loop van het SHBG-achtig domein van proteïne S betrokken is bij de interactie met C4BP.

In *hoofdstuk 5* werd bestudeerd hoe trombine het proteïne S molekuul kon inaktiveren. In het trombine-gevoelige domein (residuen 47 tot 72) zijn er drie potentiële splitsingsplaatsen voor trombine. Door deze plaatsen één voor één te veranderen of in kombinatie kan de rol van deze residuen worden bestudeerd. Alle zeven mogelijke varianten werden gekonstrueerd: drie varianten met enkele mutaties, drie varianten met dubbele mutaties en een variant met drie mutaties. Alle varianten (behalve de variant met drie mutaties) bleken na SDS gel elektroforese onder gereduceerde omstandigheden, een dubbele band te vertonen, hetgeen duidt op afbraak. De varianten bleken APC cofactor aktiviteit te bezitten en na trombine behandeling was de aktiviteit verdwenen, behalve voor de triple variant die zijn aktiviteit behield. Uit deze resultaten blijkt dat alle drie de peptidebindingen belangrijk zijn voor de cofactor aktiviteit van proteïne S. Dit kan belangrijk zijn voor verder onderzoek van proteïne S. Er kan nu een proteïne S molekuul geïsoleerd worden dat niet voor een deel is afgebroken door de inwerking van trombine.

In *hoofdstuk 6* worden de resultaten van dit proefschrift in een breed verband met elkaar besproken. Er wordt bovendien een model gepresenteerd met betrekking tot de interaktie tussen proteïne S en C4BP.

ABBREVIATIONS

Abbreviations and nomenclature as recommended by the IUPAC-IUB Commision on Biochemical Nomenclature (Eur J Biochem 1984; 138:9-37) are used in most cases. Amino acid residues are abbreviated using either the one or three-letter code. Special abbreviations:

APC	activated protein C
bC4BP	biotinylated C4b-binding protein
BPV	bovine papilloma virus
BSA	bovine serum albumin
C	carboxyl
C4BP	C4b-binding protein
cDNA	complementary deoxyribonucleic acid
DMEM	Dulbeccos modified Eagles medium
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
GLA	γ-carboxyglutamic acid
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
Hya	β-hydroxy aspartic acid
Hyn	β-hydroxy asparagine
IgG	immunoglobulin G
M_r	relative molecular weight
N	amino
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PSP	protein S peptide
SAAP	streptavidin conjugated to alkaline phosphatase
SDS	sodium dodecyl sulphate
SHBG	sex hormone binding globulin
TBS	tris buffered saline
Tris	tris (hydroxy-methyl) aminoethane

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CURRICULUM VITAE

The author of this thesis was born on the 5th September 1958 in New Nickerie, Surinam. After succesfully attending the primary school (1964-1970) in New Nickerie and the secondary school (1970-1974) in New Nickerie and Paramaribo he moved to The Netherlands. After graduation from the high school (1974-1977) in The Hague, he studied biochemistry at the polytechnic school (1977-1981) in Delft. During 1981-1987 he worked as a research assistant at the Department of Fibrinolysis of the Gaubius Institute TNO in Leiden on the structure/function relationships of tissue-type plasminogen activator and studies on plasminogen activator inhibitor type 1. In fulfilment of the requirements for the doctoral degree of the University of Utrecht, he worked at the Department of Haematology of the University Hospital Utrecht (1987-1992) on the structure and function of human protein S. From January 1993 he will be working as a research associate at the Department of Pathology of the University of New Mexico, School of Medicine in Albuquerque, NM, U.S.A.

STRUCTURE AND FUNCTION OF HUMAN PROTEIN S

Glenn T.G. Chang

Human protein S is a vitamin-K dependent plasma glycoprotein with anticoagulant properties. It acts as a cofactor to activated protein C (APC) in the degradation of the procoagulation cofactors Va and VIIIa. Patients deficient in either protein C or protein S are considered to be at high risk to get thrombotic complications.

In human blood, protein S is for 60% in complex with C4b-binding protein (C4BP), a component of the complement system. The protein S-C4BP complex has no APC cofactor activity. To study the interaction between protein S and C4BP a number of protein S variants was constructed using molecular biological techniques. After characterization, two sites on protein S were found to be involved in the interaction with C4BP. These sites were localized between amino acid residues Asp-583 to Ser-635 at the carboxyl-terminal end and at amino acid residue Lys-429 in the middle of the sex hormone binding globulin-like domain. The protein S variants with reduced binding to C4BP possess APC cofactor activity and can be usefull in the treatment of patients deficient in protein S.

Upon purification from plasma, protein S is very sensitive for inactivation by proteases like thrombin. This leads to a protein S preparation without APC cofactor activity. Three thrombin-cleavage sites on human protein S were identified at Arg-49, Arg-60 and Arg-70. Furthermore, a protein S variant resistant to thrombin was obtained. This protein S variant has full APC cofactor activity and can be usefull to study the role of protein S in the anticoagulant system without the interference of proteolytically inactive protein S. In addition, a thrombin-resistant protein S variant molecule with full APC cofactor activity and without binding ability to C4BP would be an ideal potent therapeutic agent.

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