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Functions and Physiology of Steroid Binding Proteins

THE GENES FOR SHBG/ABP AND THE SHBG-LIKE REGION OF VITAMIN K-DEPENDENT PROTEIN S HAVE EVOLVED FROM A COMMON ANCESTRAL GENE

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Summary—Sex hormone binding globulin (SHBG) is the most important sex steroid transport protein in human plasma. It is the product of the same single gene as the androgen binding protein (ABP) of testis. Protein S is another protein, which is an important cofactor in the anticoagulation system and, as far as is known today, functionally unrelated to SHBG/ABP. Protein S also has a role in the complement system. A comparison of the human genes for SHBG/ABP and protein S reveals a sequence similarity, which is of a low grade only, between the SHBG/ABP protein and a similar sized COOH-terminal domain of protein S. However, the intron–exon organization exhibits a striking similarity in the two genes, illustrating evolutionary events leading to the appearance of two functionally different proteins from common ancestral genetic elements.

INTRODUCTION

Several small hydrophobic molecules, such as steroid hormones, have been shown to be carried by proteins in the blood. The carrier can be more or less specific for a certain ligand and the capacity can vary from high to low. Albumin is a carrier protein characterized by a high capacity but a rather low affinity for steroid hormones. In 1958 a high affinity/low capacity steroid hormone binding protein was discovered simultaneously by two independent research groups [1, 2]. The protein, sex hormone binding globulin (SHBG), was shown to be the major carrier for the most bioactive androgens (i.e. testosterone and dihydrotestosterone) and estrogens (i.e. estradiol). Since its discovery, there has been a substantial increase in our knowledge about this and subsequently discovered carrier proteins. A major breakthrough came in 1986 when Petra's group in Seattle [3] determined the amino acid sequence of SHBG. This achievement was soon followed by the isolation of the SHBG cDNA [4–6] as well as the characterization of the gene coding for SHBG [7, 8]. At this time it became evident that the androgen binding protein (ABP), produced by the Sertoli cells in the testis, has the same protein backbone as SHBG. Quite unexpectedly, it was also found that SHBG had structural similarities with protein S, a factor in the anticoagulation system. The cloning of the human SHBG/ABP gene and of the human protein S gene, has allowed a comparison of the structure and organization of the two genes. Before presenting the results of our comparison we will give a short overview of the molecular properties of SHBG/ABP and protein S.

CHEMICAL CHARACTERISTICS OF HUMAN SHBG/ABP AND ITS cDNA

The SHBG molecule is produced by the liver, and circulates in plasma as a dimer. Each dimer can bind a single steroid molecule. The monomers are identical, at least with regard to the protein backbone [3, 4, 9]. They may, however, differ somewhat due to post-translational modifications, such as the degree of glycosylation. Even before methods were available for its complete purification, it was found that SHBG is chemically heterogeneous [10]. Since then SHBG has been purified and its heterogeneity studied by several means. SHBG, purified from single individuals, resolves on SDS–PAGE in 80% of the cases into

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two subunit forms with molecular sizes of 52 and 49 kDa, respectively [9, 11]. In the remaining 20% of the cases, an additional form of subunit with a size of approx. 56 kDa is seen [12]. The heterogeneity of the SHBG subunit can most likely be accounted for by differences in glycosylation, since complete enzymatic removal of carbohydrate from SHBG before analysis, yields only a single band on SDS–PAGE [13]. Heterogeneity of SHBG may also be demonstrated by isoelectric focusing. By this method, SHBG is resolved into about ten isoforms [10, 11, 13, 14].

A third form of heterogeneity was discovered when attempts were made to determine the N-terminal amino acid sequence. Edman degradation indicated that a part of the SHBG isolated from plasma was one amino acid residue shorter at the N-terminus [9, 12].

SHBG consists of 373 amino acid residues and the sequence contains two putative glycosylation sites [3]. In addition an *O*-linked oligosaccharide had been experimentally assigned to the threonine residue at position 7 [9, 12]. The structure of SHBG shows several interesting features such as a hydrophobic region with a long sequence with leucine in every second position (residues 267–281) flanked by a repeat (residues 277–292 is a repeat of residues 248–263). This region has been suggested to form a hydrophobic pocket which may be part of the steroid binding site [15].

Soon after the primary structure of SHBG became known, several groups independently cloned the cDNA of human SHBG [4–6]. None of the clones were full-length; they all lacked the nontranslated 5′-end and the translation start site. The longest clone was isolated by Hammond *et al.* [5] and its deduced amino acid sequence contained part of the signal peptide and the complete amino-terminal of the mature protein.

Almost simultaneously with the presentation of the primary structure of human SHBG, Joseph *et al.* [16] presented the cDNA of the ABP of the rat. The deduced amino acid sequence was compared with that of human SHBG and showed a 68% sequence identity. It thus became clear that the two proteins are homologous; the 32% difference in sequence was explained as due to interspecies variation [17]. An ABP is also produced by the Sertoli cell in the human testis [18] and several studies had indicated a high degree of relatedness of the testis protein to SHBG in plasma [19, 20]. The two proteins are not completely identical, however. ABP differs from SHBG in its binding to concavalin A [18, 21]. The differences between SHBG and ABP are most likely due to different posttranslational modifications. The evidence indicating that SHBG and ABP have identical protein backbones, are the demonstration of a correctly sized mRNA in testis hybridizing with liver SHBG cDNA and the presence of a cDNA in a testis library with a sequence identical to that of liver SHBG cDNA [7]. Furthermore, the cDNA clones from the liver as well as the testis library should be products of the same gene since there is evidence that the human haploid genome contains only one copy of the SHBG/ABP gene [7, 8].

During the work with the SHBG/ABP cDNA we discovered another cDNA, different but related to the SHBG/ABP cDNA, which we called SHBG gene related protein (SHBGgrp). It is partly identical with the SHBG/ABP cDNA but it has a unique 5′-end and a major deletion in its 3′-part. The expressed protein corresponding to this alternate cDNA sequence should have a core identical to that of SHBG/ABP but a different N-terminus, so far unknown, and also an entirely different and much shorter COOH-terminal amino acid sequence. The organization of the alternate cDNA is consistent with an alternate splicing of the SHBG/ABP gene and may thus represent a true mRNA [7]. The existence of this form of cDNA has also been demonstrated by Hammond *et al.* [8].

STRUCTURE AND FUNCTION OF HUMAN PROTEIN S

Protein S (S for Seattle) is a plasma glycoprotein circulating in a concentration of approx. 25 mg/l in plasma, half of it in complex with the complement regulatory protein C4b binding protein [22]. Protein S is synthesized by hepatocytes [23], vascular endothelial cells [24] and megakaryocytes [25]. Recently, it was demonstrated that an additional site of synthesis is the Leydig cell of the testis [26].

Protein S was first purified from plasma in 1977 by DiScipio *et al.* [27]. The molecule is a single-chain 70 kDa glycoprotein. The primary structure of human protein S has been deduced from the cDNA [28, 29] and consists of 635 amino acid residues. The precursors of the vitamin K-dependent clotting factors, i.e. factor II, VII, IX, X, and also the anticoagulant

protein, protein C, are all organized in a similar fashion, with domains forming functional entities. They all contain a signal peptide followed by a propeptide, a γ-carboxyglutamic acid (Gla) containing domain, two domains homologous to the epidermal growth factor (EGF), and a large COOH-terminal domain constituting the serine protease. The genes coding for these proteins show a corresponding similarity in their organization. Furthermore, several of the functional units (domains) are encoded by distinct exons. This organization of the genes suggests that they have evolved by exon shuffling [30, 31].

The organization of the protein S molecule agrees fairly well with the general construction plan for the vitamin K-dependent clotting factor precursors (Fig. 1). However, there are three major differences. Protein S contains a unique thrombin sensitive region adjacent to the Gla domain. The domain forms a disulphide loop, in which two peptide bonds can be cleaved by thrombin. Experimental evidence indicates that the domain is important for the conformation of the molecule, the Ca^{2+}-binding properties of the Gla domain, and the ability of the molecule to interact with phospholipids [32].

The thrombin sensitive region is followed by four consecutive EGF-homologous domains, instead of the two normally found in vitamin K-dependent coagulation factor precursors. The first EGF-homologous domain contains β-hydroxyaspartic acid and the following three β-hydroxyasparagine [33]. The latter modified amino acid residue, is unique to these positions in this protein family.

The COOH-terminal part of protein S has no resemblance to a serine protease but, as discussed above, it has a similarity in primary structure with SHBG. This part of protein S is comparable in size to the SHBG molecule. The overall similarity in amino acid sequence between the two proteins is rather low (about 30%), and not evenly distributed along the peptide chain. The four cysteine residues present in SHBG correspond to four of the five cysteine residues present in the COOH-terminal domain of protein S. These four residues in protein S form disulphide loops within this domain. By analogy, it may be suggested that the corresponding cysteines are disulphide bonded in SHBG [4].

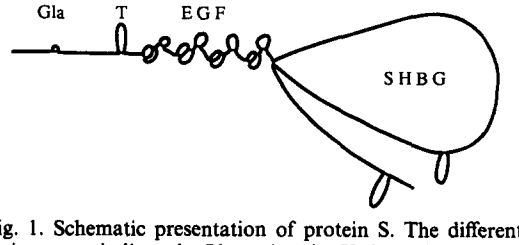

Fig. 1. Schematic presentation of protein S. The different regions are indicated. Gla = vitamin K-dependent γ-carboxy-glutamic acid-rich region; T = thrombin-sensitive region; EGF = the four domains homologous to the EGF precursor protein; SHBG = the C-terminal region similar to sex hormone binding globulin (SHBG).

Protein S acts in two physiological systems in the body. The protein was first realized to be associated with the coagulation–anticoagulation system [34]. It acts as a cofactor to activated protein C (APC) in the proteolytic inactivation of FV_a and $FVIII_a$ [35, 36]. It is believed that protein S and protein C form a 1:1 noncovalent complex on negatively charged phospholipid surfaces, such as activated platelets. Protein S has a higher affinity for phospholipids than APC, and thus protein S may anchor APC to phospholipid membranes and thus enhance the protein C activity.

The second system that involves protein S is the complement system. About 60% of the circulating protein S is noncovalently complexed with C4b-binding protein (C4BP) [22]. C4BP is a plasma protein functioning as a regulator of the classical pathway of the complement system by accelerating the decay of C3-convertase and acting as a cofactor to factor I in the degradation of C4b. The molecule is composed of eight subunits with a total M_w of 570 kDa. In the electron microscope, the C4BP molecule appears as a spider-like structure with seven arms corresponding to seven identical subunits, extending from a central core region [37]. Each arm contains a binding site for a C4BP molecule [38]. Approx. 50% of the C4BP molecules in the circulation are complexed to protein S. The binding site for protein S has recently been localized to the eighth subunit in the C4BP molecule, the β-chain, composed of 235 amino acids [40, 41]. The β-chain contains a region with three so called short consensus repeats (SCR) in its N-terminal.

The SCR is a unit found in several proteins, both complement and noncomplement proteins, the number of repeats ranging from 1 to 30. They are always arranged in tandem, and in some proteins the whole primary structure consists of SCR units. Typically, an SCR is 60 amino acid residues long and has a framework of highly conserved residues in-

cluding four cysteines and one tryptophan as the most commonly occurring residues, and several other partly conserved residues [41, 42]. Protein S is the first vitamin K-dependent protein shown to interact with an SCR-containing protein.

The complex formation between protein S and C4BP is independent of that between C4b and C4BP, and vice versa [43]. When protein S is complexed to C4BP, it is no longer effective as an anticoagulant [44]. Instead, it seems that the protein S–C4BP complex competes with free protein S for binding to APC and forms an inactive protein S–C4BP–APC complex.

COMPARISON OF THE ORGANIZATION OF THE GENES CODING FOR SHBG/ABP AND PROTEIN S

The human SHBG/ABP gene has recently been cloned and characterized by two independent groups [7, 8]. Southern blot analysis has indicated the presence of only one copy of the gene in the human species which also is the case for the ABP gene in the rat [45].

Our group cloned the gene from a human cosmid library. The gene encompasses approx. 3.3 kb and the coding part of the sequence is split into eight exons separated by rather short introns devoid of any conspicuous features except for a sequence, in intron six, similar to the human Alu family of dispersed repeats. All the intron/exon boundaries followed the GT/AG consensus sequence [46]. The first exon codes for a typical signal peptide of 29 residues followed by 8 residues which are identical to the amino-terminal of the published protein sequence [3]. The overall organization of the human SHBG/ABP gene is identical with that of the rat ABP gene [45]. No distinct TATA or CAAT boxes have been identified in the 5′-flanking region of the human or rat gene.

The deleted region in SHBGgrp corresponds completely to exon 7 and the joining of exon 6 with exon 8 introduces a frameshift. The abberant 5′-end in SHBGgrp could be assigned to a region approx. 1.5 kb upstream from the first exon in SHBG/ABP. The finding of this exon, in combination with the lack of common promoter elements in the 5′-flanking region of the SHBG gene, introduced a new dimension regarding the regulation of the expression of this gene. This matter is discussed in our original publication on the SHBG gene [7]. A hypothetical model of the organization of the gene, with its alternative transcription and translation is given in Fig. 2.

The human protein S gene has recently been cloned and its organization determined [47–49]. Edenbrandt *et al.* [47] used a human protein S cDNA fragment to screen three genomic libraries. The positive clones were purified and subjected to restriction enzyme mapping. The map suggested the existence of two copies of the gene which is in agreement of the results of Ploos van Amstel *et al.* [50] who named them PS-α and PS-β, respectively. They also demonstrated that both genes were localized on chromosome 3. Although extremely similar to the PS-α gene, the PS-β gene has a number of critical changes causing frameshifts and stop codons and is, therefore, a nonfunctional pseudogene.

The isolated clones represented the thrombin sensitive domain, the four EGF-homologous

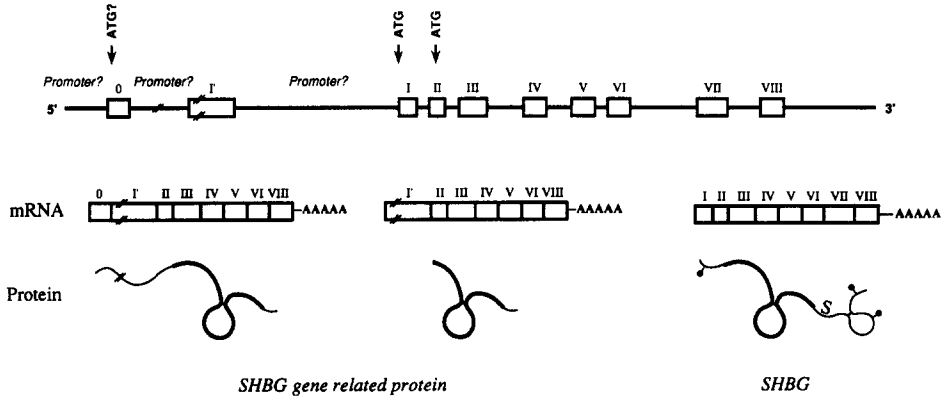

Fig. 2. The organization of the human SHBG gene, its alternative spliced transcripts and their translated proteins. The exons are numbered by roman numerals. The alternative 5′-exon is denoted I′ and the putative exon containing a translation start codon, is denoted 0. In SHBG the proposed steroid binding site is indicated by an S and the locations of the carbohydrate groups are marked with filled circles. From Ref. [7] with permission from Oxford University Press.

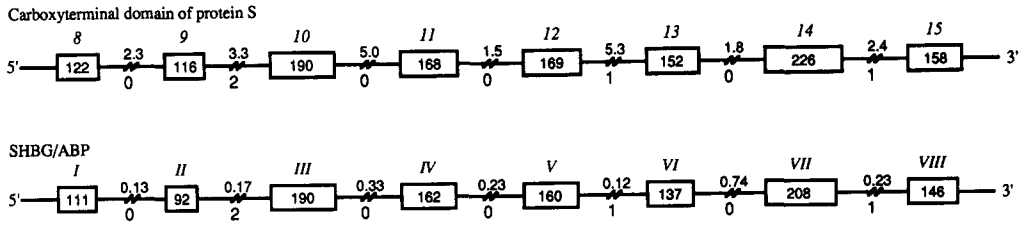

Fig. 3. Schematic illustration of the gene-organization of the carboxyterminal region of protein S α and SHBG. Exons are indicated by boxes inside of which the length of the respective exon is given. The length given for the last exon in the two genes, respectively, corresponds to translated nucleotides. The splicing type of intron is indicated by numbers 0 to 2 below each intron where type 0 is intron located between codons, type 1 is intron located between the first and second codon nucleotide, and type 2 is intron located between the second and third codon nucleotide.

domains, the SHBG-homologous domain as well as the 3′ untranslated sequence. Despite several rescreenings no clones representing regions 5′ to the thrombin-sensitive domain were found. According to the restriction maps, many of the clones were partially overlapping and together they represented approx. 50 kb of the protein S α-gene and 25 kb of the β-gene. These clones were used for a further analysis of the organization of exons and introns. Technical details of the characterization of the human protein S genes have been thoroughly described by Edenbrandt *et al.* [47]. The whole protein S α-gene including the 5′-end has recently been characterized by two independent groups [48, 49]. The gene consists of 15 exons, including 8 which code for the SHBG homologous COOH-terminal domain. The domains of protein S that can be functionally or structurally identified are encoded by separate exons. The 5′ untranslated region and the propeptide are encoded by a single exon as is the propeptide/Gla domain. The thrombin-sensitive domain and the four EGF-homologous domains are each encoded by a separate exon. Exons are separated by introns which can be of three types depending on the position of the intron in the codon triplet. Type 0 introns are located between codons, type 1 introns after the first nucleotide and type 2 introns after the second nucleotide in the codon. Except for the intron between the thrombin-sensitive domain and the first EGF-homologous domain, which is of type 0, all introns separating exons in this region of the gene, are of type 1. The part of the protein S α-gene that we characterized contains 12 of the exons. All of the intron/exon junctions follow the GT/AG rule [46]. The sizes of the introns were determined by restriction mapping, and was found to range between 0.1 to >10 kb.

The thrombin-sensitive region as well as the four EGF-homologous domains were found to be coded by separate exons. This correlation between domains and exons agrees well with the organization of the other vitamin K-dependent coagulation factors [30, 31]. Furthermore, all of these exons were separated by introns of type 1. This homogeneity in type of intron suggests that this part of the protein S α-gene has been assembled by exon shuffling [51]. A prerequisite for such shuffling, is that all introns are of the same type to avoid frameshifts when exons are inserted or deleted. Type 1 introns are also present in the genes of the other proteins which contain domains of this type and are involved in blood coagulation and fibrinolysis.

The SHBG-homologous domain of protein S α is coded for by seven exons in the protein S α-gene. The sizes of the corresponding exons in this region of protein S and SHBG are almost identical (Fig. 3). The exons are separated by introns of all three types and with identical types in the corresponding positions in the two genes. These facts strongly suggest that this domain in protein S α and SHBG have evolved from a common ancestral gene. The ancestor of SHBG and the SHBG-homologous protein S domain has presumably been linked to the 5′-part of the protein S α-gene as one block by a shuffling that occurred prior to the duplication of the ancestral protein S gene [47–49].

The duplication of the protein S gene is probably a recent event. Phylogenetic studies on apes has demonstrated the presence of two protein S genes only in the species closely related to man (i.e. chimpanzee and gorilla) whereas orangutan has a single copy of the gene [48].

As mentioned earlier the overall similarity between SHBG and the SHBG-homologous domain in protein S is about 30%. When the similarity is analysed by a diagonal plot, in which the positions of the introns are marked, it becomes apparent that the similarity is not evenly distributed. The amino acid sequences

encoded by exons 9, 11 and 14 in protein S and the—corresponding—exons II, IV and VII in SHBG are much less similar than those encoded by the other related exons.

Finally, an additional notable similarity between the SHBG/ABP and protein S gene, is the lack of conventional promoter elements in their respective 5′-flanking regions. For both genes a hypothetical nontranslated exon, located upstream from the first coding exon, have been suggested [7, 48, 49]. The transcription of the two genes could thus both be regulated from a region upstream from this nontranslated exon.

The genes for protein S and SHBG/ABP illustrate nature's ability to create a diversified set of proteins. Although, both genes probably originated from a common ancestral gene, they have under the influence of genetic recombination in concert with mutations, eventually been transformed into two genes coding for proteins with different primary structures and acting in entirely different physiological systems.

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