Vitamin K-dependent protein S in Leydig cells of human testis

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Protein S is an anticoagulant plasma protein, functioning as a cofactor to activated protein C in the regulation of blood coagulation. In addition, protein S forms a complex with the complement regulatory protein, C4b-binding protein. Protein S is unique among the vitamin K-dependent proteins in being structurally similar to androgen binding proteins. Protein S immunoreactivity was demonstrated in Leydig cells of human testis. In Northern blotting experiments, the presence of protein S mRNA in human testis tissue could be shown. *In situ* hybridization experiments localized protein S mRNA to the Leydig cells, demonstrating transcription of the protein S gene in these cells. Five protein S clones were isolated from a human

testis cDNA library, partially sequenced and characterized by restriction enzyme mapping. Three unique clones contained information for the entire coding sequence and approximately two-thirds of the 5' and 3' non-coding sequences. The results indicate the nucleotide sequences of testis and liver protein S mRNA to be identical. No binding of androgens to protein S could be demonstrated. In conclusion, we demonstrate the presence of protein S immunoreactivity as well as protein S mRNA in the Leydig cells of human testis. These results suggest local synthesis of protein S in Leydig cells of human testis which may be functionally important for local anticoagulation.

INTRODUCTION

Protein S is a vitamin K-dependent plasma protein (concn. 25 mg/l) that functions as a cofactor to activated protein C in the degradation of coagulation factors Va and VIIIa. The physiological importance of protein S as a regulator of blood coagulation is demonstrated by the association of protein S deficiency with thromboembolic disease [1–5]. Most cases of protein S deficiency are due to a hereditary disorder, but acquired deficiencies have also been described [6–10]. The most recent report of acquired protein S deficiency describes a young boy with circulating autoantibodies against protein S who developed a testicular thrombosis [11]. In the circulation, 60–70% of protein S is non-covalently complexed to C4b-binding protein (C4BP), a regulatory protein of the classical complement pathway. Only uncomplexed protein S functions as cofactor to activated protein C [12].

Human protein S cDNA has been cloned and its gene structure elucidated [13–19]. The 75 kDa single-chain molecule is composed of different modules (Figure 1). The N-terminal part encompasses a γ -carboxyglutamic acid (Gla)-rich domain, which binds Ca^{2+} and negatively charged phospholipids. This is followed by a unique thrombin-sensitive region and by four epidermal growth factor (EGF) homologous domains [13,20,21], which contain two different post-translationally modified amino acids, a β -hydroxyaspartic acid (Hya) in the first and β -hydroxyasparagine (Hyn) in each of the following three [21–23]. The EGF-like domains, which contain very-high-affinity Ca^{2+} -binding sites [24], and the thrombin-sensitive region are important for the anticoagulant function of protein S [25]. The C-terminal half of protein S is not similar to the other vitamin K-dependent proteins [13] but displays 30% sequence similarity to two androgen-binding proteins, namely the human plasma protein SHBG (sex hormone binding globulin) and ABP (androgen binding protein) from rat testis [26–28]. It has been suggested that the SHBG-like region of the protein S molecule contains the binding site for C4BP [29].

Protein S has previously been shown to be synthesized by hepatocytes [30], vascular endothelial cells [31,32] and megakaryocytes [33,34]. It has been reported that protein S immunoreactivity was found in human testis (H. P. Schwarz, unpublished work). Using several monoclonal antibodies we can now confirm this observation and, in addition, demonstrate the presence of protein S mRNA in the Leydig cells, i.e. the testosterone-producing cells, of testis.

MATERIALS AND METHODS

Tissue specimens

For immunohistochemistry and *in situ* hybridization experiments, tissue was obtained at operation from ten patients (65–75 years old) undergoing orchiectomy as surgical treatment for carcinoma of the prostate. All specimens were immersion-fixed in Bouin's fixative or in 4% paraformaldehyde solution buffered to pH 7.4 by PBS for 24–36 h within 15 min of removal. The tissues were dehydrated and subsequently embedded in paraffin. Sections were cut at 3 μm thickness and mounted on poly(L-lysine)-coated slides for immunohistochemical studies, or on 3-aminopropyltriethoxysilane-coated slides for *in situ* hybridization [35]. Haematoxylin–eosin-stained sections were examined and found to display normal histological characteristics for testis tissue.

Immunohistochemical techniques

Specimens were subjected to immunohistochemical analysis using the peroxidase/antiperoxidase (PAP) method with both polyclonal and monoclonal antibodies [36]. The polyclonal rabbit anti-(human protein S) (described in [9]) and monoclonal mouse anti-(human C4BP) (M104) (described in [37]) antisera were used at dilutions of 1:800 and 1:100 respectively. The monoclonal mouse anti-(human protein S) antibodies, abbreviated as HPS 21, HPS 34, HPS 41 and HPS 54 [25], could be diluted to 2 $\mu\text{g}/\text{ml}$, but the highest signal/noise ratio was obtained with

Abbreviations used: C4BP, C4b-binding protein; Gla, γ -carboxyglutamic acid; EGF, epidermal growth factor; SHBG, sex hormone binding globulin; ABP, androgen binding protein; PAP, peroxidase/antiperoxidase.

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Figure 1 Schematic model of protein S demonstrating the localization of the epitopes for monoclonal antibodies HPS 21, HPS 41 and HPS 54

The different regions of the protein S molecule are indicated. Gla, γ -carboxyglutamic acid-rich region; Thr, thrombin-sensitive region; EGF, the four domains homologous to the EGF protein precursor; SHBG, C-terminal half of the molecule structurally similar to SHBG. a and b denote the location of probes used in *in situ* hybridization.

sections stained with an antibody concentration of 50 $\mu\text{g}/\text{ml}$. The antibodies were diluted in 50 mM Tris/HCl, 0.15 M NaCl, pH 7.5. For HPS 21, 2 mM Ca^{2+} was added. HPS 21 recognized a Ca^{2+} -dependent epitope in the Gla-rich region, HPS 41 and HPS 34 reacted with an epitope in the third or fourth EGF-like domain, whereas HPS 54 recognized a Ca^{2+} -dependent epitope in the first or second EGF-like domain (Figure 1) [25].

To verify the specificity of the immunochemical staining the following control experiments were performed: (i) elimination of primary antibody from the reaction, (ii) substitution of the primary antibody with non-immune normal rabbit IgG, normal mouse IgG or buffer solution, and (iii) pretreatment of the primary antibody at a concentration of 50 $\mu\text{g}/\text{ml}$ (performed only with HPS 34) for 18 h with a 5-fold molar excess of purified protein S prior to incubation of the tissue section. Structures that were stained by the PAP procedure with the protein S antibodies, but were not stained in the three control experiments, were considered to be stained specifically by present standards [38].

Extraction of human testis and protein quantification

One decapsulated human testis (17.3 g) was frozen in liquid nitrogen within 1 h of orchiectomy. The frozen tissue was crushed and added to extraction buffer, and immediately homogenized in a Polytron homogenizer (Kinematica G.m.b.H.). The buffer was 50 mM Tris/HCl, 0.15 M NaCl, 10 mM benzamidine, 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethanesulphonyl fluoride, 5 mM ϵ -aminocaproic acid, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ antipain, 50 $\mu\text{g}/\text{ml}$ soy bean trypsin inhibitor and 1% Tween 20; 12 ml was used for one testis. The homogenate was centrifuged (4 °C) in a Sorvall SS-34 rotor at 36000 g for 30 min and the supernatant collected. The concentrations of protein S, protein C and C4BP in the extract and in the patient plasma were determined as earlier described [9].

Northern blotting, cDNA cloning and nucleotide sequencing

RNA from testis and liver was prepared as previously described [39]. Total RNA (20 μg) was electrophoresed in 1.1% agarose gels, run in the presence of formaldehyde, and transferred to Hybond N filters (Amersham). The membranes were probed with the *Eco*RI fragment of clone M117S, which encoded the mature protein and 45 bp of the 5' untranslated region [13]. The

probe was labelled using the multiprime DNA labelling system (Amersham) to a specific radioactivity of more than 10^8 c.p.m./ μg . Hybridizations were done at 42 °C in $5\times$ SSC, 15 mM sodium citrate, pH 7.0, 50% formamide, 10% dextran sulphate, 100 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA and 0.5 g/l dried milk powder. The filters were washed at 67 °C in $0.5\times$ SSC, 0.5% SDS for approx. 60 min and subjected to autoradiography.

A human testis cDNA library in $\lambda\text{gt} 11$ (Clontech) was screened using standard procedures [40]. Probing was done with the same probe and labelling with the same system as described above. Hybridizations were done at 68 °C in $6\times$ SSC, 100 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA, $5\times$ Denhardt's solution, 0.1% SDS and 0.05% PP_i. The filters were washed at 68 °C in $0.2\times$ SSC, 0.05% PP_i and 1.0% SDS for approx. 60 min and subjected to autoradiography. Hybridizing plaques were plaque-purified and the recombinant phages prepared by the liquid culture procedure [40]. The inserts of the cDNA clones were subcloned in both M13mp18 and pUC 18. M13mp18 templates for sequencing were prepared by a standard procedure [40] and the nucleotide sequence was determined using a modified dideoxy chain termination method [41]. The sequences were analysed using the Genetics Computer Group Software [42]. pUC 18 plasmids containing the protein S cDNA inserts were purified using Qiagen (Diagen) and cleaved with different restriction enzymes under conditions recommended by the manufacturers.

Oligonucleotide probes

Oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer using standard β -cyanoethylphosphoramidite chemistry. Experiments using digoxigenin-labelled probes were done with two anti-sense oligonucleotides: 5'-AGGATAAGCATTAGTTGACTGACGTGCAGC-3', corresponding to amino acids 48–58 in the thrombin-sensitive region (labelled a in Figure 1), and 5'-CCAAGTGTACACCATTAATATTCACCTCCATGC-3' corresponding to amino acids 597–608 in the C-terminal part of the protein S molecule (labelled b in Figure 1). The probes were labelled using a 3'-end labelling kit for a tailing reaction with digoxigenin-11-dUTP and dTTP supplied by Boehringer-Mannheim G.m.b.H. (Mannheim, Germany). Labelling reactions were performed according to the manufacturer's recommendations: 100 pmol of each oligonucleotide was labelled at 37 °C for 15 min and precipitated with ethanol. In addition, a 30-mer oligo(dT) probe was synthesized and used as positive control for *in situ* hybridization. A 30-mer sense oligonucleotide corresponding to the thrombin-sensitive region served as negative control (5'-GCTGCACGTCACTCAACTAATGCTTATCCT-3').

In situ hybridization

In situ hybridization was performed using probes conjugated with digoxigenin. Tissue sections were dewaxed in xylene and hydrated in successively decreasing concentrations of ethanol. Pretreatment of the tissue sections was carried out by a procedure based on earlier described methods [43]. The sections were subsequently exposed to various solutions in the following order: PBS for 2×5 min, 0.2 M HCl for 20 min, PBS for 2×5 min, protease type VIII in PBS (100 $\mu\text{g}/\text{ml}$) for 15 min at 37 °C, 4% paraformaldehyde in PBS for 5 min, PBS for 3×5 min, and 0.25% acetanhydride in 0.1 M triethanolamine containing 0.15 M NaCl for 10 min followed by $2\times$ SSC for 5 min. After denaturation in $2\times$ SSC at 95 °C for 8 min, prehybridization was carried out in hybridization buffer ($4\times$ SSC containing 10% dextran sulphate, $1\times$ Denhardt's solution, 400 $\mu\text{g}/\text{ml}$ freshly

Figure 2 Immunohistochemical visualization of protein S in Leydig cells of human testis

Clusters of Leydig cells in the interstitium can be seen (marked with arrowheads). The non-stained germ cell epithelium is marked with arrows. Low-power (a) and medium-power (b–d) photomicrographs of human testis, showing the occurrence of protein S immunoreactive cells. The mouse anti-(human protein S) monoclonal antibodies HPS 21 (a, $\times 40$; b, $\times 600$), HPS 41 (c, $\times 500$) and HPS 54 (d, $\times 375$) were used for the PAP procedure.

denatured salmon sperm DNA, 250 $\mu\text{g}/\text{ml}$ tRNA and 30% deionized formamide) for 2 h at 42 $^{\circ}\text{C}$.

Hybridizations were done at 37 $^{\circ}\text{C}$ overnight with probes in hybridization buffer (1–5 fmol/ μl). Following a rinse in $4 \times \text{SSC}$ for 2×10 min at room temperature, the stringency was increased by washing in $0.2 \times \text{SSC}$ for 4×15 min at 5 $^{\circ}\text{C}$ below T_m [44]. This step was followed by incubation in $1 \times \text{SSC}$ for 10 min, at room temperature, to achieve stabilization. Sections were then rinsed in 100 mM Tris/HCl, pH 7.5, containing 0.15 M NaCl, for 10 min and preblocked by incubation in 2% normal sheep serum for 30 min at room temperature. Excess serum was decanted and, after incubation with polyclonal sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Boehringer), diluted 1:500 in 100 mM Tris/HCl, pH 7.5, 150 mM NaCl containing 0.03% Triton X-100 and 1% normal

sheep serum (3 h in a sealed humidified chamber at room temperature) and washed in the same buffer without Fab fragments. After equilibration in 100 mM Tris/HCl, pH 9.5, 100 mM NaCl, for 10 min, the same buffer containing 50 mM MgCl_2 was added for 2×5 min and the development reaction initiated by the addition of the substrate (170 $\mu\text{g}/\text{ml}$ 5-bromo-4-chloro-3-indolyl phosphate, 340 $\mu\text{g}/\text{ml}$ Nitroblue Tetrazolium, 1 mM levamisole; final concentrations given) [45]. After incubation at room temperature for between 20 min and 36 h, the reaction was stopped by adding 10 mM EDTA in 10 mM Tris/HCl, pH 7.5, containing 0.15 M NaCl for 30 min.

Coverslips were mounted using Mount-quick Aqueous (Daido Sangyo). An oligo(dT) probe served as a positive hybridization control. As negative controls we used hybridization without probe and with sense probe, and RNAase pretreatment of the

tissue sections (0.1 mg/ml in $2 \times$ SSC, 10 mM MgCl_2 for 60 min at 37°C).

Binding studies

The binding studies were performed essentially according to Fernlund and Gershagen [46]. Protein S (final concentration 0.5 mM) was incubated with [^3H]testosterone ([1,2,6,7- ^3H]testosterone; Amersham) (20000 c.p.m.), [^3H]dihydrotestosterone ([1,2- ^3H]dihydrotestosterone; Amersham) (20000 c.p.m.), [^3H]androstendione ([1,2,6,7- ^3H]androst-4-ene-3,17-dione; Amersham) (20000 c.p.m.) or [^3H]pregnenolone ([7- ^3H]pregnenolone; NEN) (20000 c.p.m.) in 100 mM Hepes buffer, pH 7.4, containing 0.15 M NaCl, 0.05% NaN_3 , 0.1% gelatin and either 1 mM CaCl_2 or 1 mM EDTA for 1 h at room temperature. The incubation volume was 200 μl . The bound and free fractions of the steroids were separated by adding 200 μl of ice-cold dextran-coated charcoal. After incubation for 20 min at 0°C , the mixtures were centrifuged and the radioactivity in 200 μl of the supernatant (bound fraction) was measured. As a positive control we used purified human SHBG (final concentration 0.5 nM) (gift from Dr. P. Fernlund) or human serum albumin (final concentration 0.5 mM) instead of protein S.

RESULTS

Protein S immunoreactivity in Leydig cells

Cells displaying protein S immunoreactivity were found in sections of testis tissue (Figure 2). The immunostaining was confined to the cytoplasm of the Leydig cells, and all Leydig cells were positive. No staining was seen of the seminiferous tubules, the spermatozoa, the Sertoli cells, the blood vessels or the connective tissue. The protein S-positive Leydig cells were specifically immunoreactive with both the polyclonal rabbit antiserum and the monoclonal mouse antibodies, as judged by comparison using pairs of consecutive thin sections (not shown). All four monoclonal antibodies, HPS 21 (against the Ca^{2+} -dependent epitope in the Gla-rich domain), HPS 34, HPS 41 (react with EGF-like domain three or four respectively) and HPS 54 (recognizes the first or second EGF-like domain), gave the same staining pattern. No immunostaining was observed in any of the three control experiments (see the Materials and methods section), whether using the polyclonal or the monoclonal antibodies. Also, experiments with monoclonal antibodies against C4BP were negative, i.e. no staining of Leydig cells or other structures in testis tissue could be observed.

The concentrations of protein S, protein C and C4BP were determined in an extract of testis. One testis was extracted in 12 ml of buffer and the concentrations of protein S, protein C and C4BP in the extract were found to be 11, 2.8 and 3.5% of the normal plasma levels respectively. The concentrations in the plasma from the patient of protein S, protein C and C4BP were 104, 78 and 140% respectively of control levels.

Identification of transcript in testis

A Northern blot of RNA prepared from human testis and liver was probed with a human liver cDNA for protein S. Figure 3 shows that protein S is transcribed in the testis. The mRNA is approximately 3.5 kb, which is indistinguishable from the size of the liver transcript. The band corresponding to protein S mRNA in liver was clearly seen after overnight exposure, whereas the band in the testis lane was relatively weak, even after a 1 week exposure.

Protein S mRNA in Leydig cells

In situ hybridization experiments using probes against human protein S showed cytoplasmic staining of the Leydig cells (Figure 4). The pattern was identical to that seen in the immunohistochemical studies described above. No staining of the testicular tissue surrounding the Leydig cells was seen. The two probes that were used, one located in the thrombin-sensitive region of the protein S molecule and one in the SHBG-like region, gave identical results. The staining of the Leydig cells was specific; control experiments without probe or with sense probe, or RNAase pretreatment of the tissue sections, gave negative results.

Figure 3 Northern blot analysis of protein S mRNA in testis and liver

Approximately 20 μg of total RNA from human testis (lane 2) and human liver (lane 3) was probed by the *Eco*RI fragment of the cDNA M117 S from human liver. λ DNA digested with *Hind*III was used as reference; the approximate sizes in bp of the different bands are indicated. Lane 2 was exposed overnight, lane 3 for 1 week. The arrow indicates the application slot.

Figure 4 *In situ* hybridization of protein S mRNA in human testis

Notice the cytoplasmic staining of clusters of Leydig cells. Oligonucleotide a (see Figure 1) was used ($\times 300$).

Figure 5 Schematic model of protein S cDNA clones from human testis

Protein S cDNA from human liver [13,19], with the sequence corresponding to the mature protein (bold line) indicated, is shown in the lower part of the figure. The hatched part denotes the leader sequence (L). The lengths of the liver cDNA and testis clones correspond to their sizes. The arrows show the regions that were sequenced. Verified restriction enzyme sites in the testis cDNA clones are indicated. The cDNA corresponding to the human protein S cDNA M117 S, that was used as a probe in the Northern blot and in the screening of the testis library, is also indicated.

Characterization of protein S cDNA clones

Approximately 4×10^5 plaques of the testis cDNA library were screened with the protein S cDNA probe from liver. Five positive clones, designated PST1–PST5 (PST1 = cDNA clone number 1 for protein S from a human testis library), were isolated and characterized by partial nucleotide sequencing and restriction enzyme fragment mapping (Figure 5). Clone PST1 was identical to PST5, whereas PST3 was identical to PST4. The nucleotide sequencing and restriction enzyme mapping suggested the transcript from testis to be identical to that from liver. PST1 and PST5 contain a 1755 bp fragment corresponding to Ala¹⁸ of the leader peptide to Leu⁵⁶⁶ of the mature protein. PST2 contains a 2144 bp fragment encoding the mature protein starting at Val¹⁶¹ and a 0.7 kb portion of the 3' untranslated region. PST3 contains a 1098 bp fragment beginning approximately 70 bp before the initiation methionine and ending at amino acid 300. Together, the cDNA clones encode the whole translated protein S molecule.

Binding studies

Protein S was incubated with a constant amount of [³H]testosterone, [³H]dihydrotestosterone, [³H]androstendione or [³H]pregnenolone in the presence of either Ca²⁺ or EDTA, and the amount of steroid bound to protein S was determined. SHBG and human serum albumin were used as positive controls and yielded binding in agreement with previous results [47]. There was no specific binding of the steroids to protein S, whether the experiments were performed in the presence of Ca²⁺ or EDTA (results not shown).

DISCUSSION

Extrahepatic synthesis of protein S has been described previously. Human megakaryocytes [33], human endothelial cells [31,32] and a human erythroleukaemia cell line [34] have been shown to synthesize and secrete protein S. A precursor of protein S has been reported to be produced by breast and colon cancer cells [48]. Extrahepatic synthesis of other vitamin K-dependent proteins has also been described, e.g. factor VII by human monocyte/macrophage cells [49,50]. However, protein S is the first vitamin K-dependent protein shown to be synthesized in the Leydig cells of testis. Immunoreactivity was seen using a polyclonal rabbit anti-(protein S) antiserum as well as several

monoclonal antibodies directed against different parts of the molecule. The concentration of protein S in a testis extract, when related to its plasma concentration, was 3–4-fold higher than corresponding levels of protein C and C4BP. This suggests that a considerable amount of protein S is synthesized in the testis and that the protein is predominantly in its free form, a hypothesis further strengthened by the lack of C4BP immunoreactivity in testis tissue.

The partial nucleotide sequence of the protein S cDNA from testis was identical to that of protein S cDNA from liver, and the restriction map gave the expected fragments. Together with the identical sizes of the mRNAs from testis and liver, this suggests that protein S in testis is identical to protein S from liver. The Leydig cells appear to be the site for the synthesis. Using two different oligonucleotide probes corresponding to different regions of the molecule, protein S mRNA was specifically identified in these cells.

Our immunohistochemical and *in situ* hybridization studies have been performed on tissue from older men. The Leydig cells constitute only a few per cent of the total testis cell population in this age group. The Northern blot of testis protein S mRNA had to be exposed to autoradiography for 1 week, whereas liver protein S mRNA was equally 'black' after an overnight exposure. Moreover, the intensity of staining of Leydig cells and hepatocytes in our immunohistochemical studies was similar. These data, although semi-quantitative in nature, would tend to indicate that the level of protein S expression per cell is of the same order of magnitude in the hepatocyte as in Leydig cells.

In males, Leydig cells are the major source of testosterone. Once secreted into the blood, most of the testosterone is bound to SHBG, a plasma protein produced in the liver [51] and possibly also in the testis [52]. SHBG and rat ABP, produced in the Sertoli cells [53], display sequence similarity with the C-terminal half of protein S [27, 52]. The SHBG and rat ABP genes and the corresponding part of the protein S gene show similar intron–exon organization [17–19,52,54]. However, the region of protein S corresponding to the proposed steroid binding site in rat ABP has a low degree of similarity with ABP and SHBG, which may explain the apparent lack of steroid binding to protein S [55,56]. Binding of steroids other than testosterone, dihydrotestosterone, androstendione and pregnenolone, however, cannot be excluded.

The physiological significance of protein S synthesized in the Leydig cell is not yet known. A possible function may be that

protein S is of importance for local anticoagulation in the testis. Production in Leydig cells may provide high local concentrations of protein S in the blood vessels in the testis and in the venules draining the organ. That this may be of functional significance was recently suggested by a report describing an 11-year-old boy who developed a massive testicular thrombosis and was found to have circulating antibodies against protein S [11]. Although the testes are susceptible to frequent trauma, testicular thrombosis is very rarely experienced. Another possible function for protein S in the testes is suggested by the homology between the C-terminal half of protein S and the ABPs. It is tempting to speculate on possible functions related to the androgen hormones, but at present no experimental evidence supports the idea of an interaction between any steroid and protein S. A third possible function of protein S in the testis is suggested by a recent study showing protein S to stimulate DNA synthesis in cultured smooth muscle cells [57]. It remains to be elucidated whether the synthesis of protein S in the Leydig cells has a growth regulating role in spermatogenesis.

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