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Regulation of human protein S gene (PROS1) transcription

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Chapter 5

IL6 induction of Protein S is regulated through Signal Transducer and Activator of Transcription 3 (STAT3)

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Submitted

Summary

The Protein C anticoagulant pathway is an essential mechanism for attenuating thrombin generation by the membrane-bound procoagulant complexes tenase and prothrombinase. In this pathway, Protein S (PS) serves as a cofactor for activated protein C (APC) in the inactivation of coagulation factors VIIIa and Va. The primary site of PS synthesis is the liver. PS circulates in plasma in a free form and in complex with complement component 4b-binding protein (C4BP), thus linking PS to the complement system. C4BP is a known acute phase reactant thereby also suggesting a relation between PS and the acute phase response. Interleukin 6 (IL6), a major contributor to the acute phase response of hepatocytes, has been shown to influence both PS and C4BP gene expression. We show here that IL6 upregulates PS mRNA and protein levels in HepG2 cell culture and that it exerts this effect by induction of PS promoter activity through a direct interaction of Signal Transducer and Activator of Transcription 3 (STAT3) with the PS promoter at a region spanning nucleotides 229 to 207 upstream from the translational start. A possible function for IL6-induced PS expression in cell survival is discussed.

Introduction

The Protein C anticoagulant pathway is indispensable in maintaining the hemostatic balance (1;2). In this pathway, Protein S (PS) functions as a non-enzymatic cofactor for Activated Protein C (APC) in the inactivation of coagulation factors VIIIa and Va (3-6). The clinical importance of PS became apparent by the association of venous thrombo-embolism (VTE) with (partial) PS deficiency (7-9). The major source of circulating plasma PS is the hepatocyte (10). In human plasma, PS circulates in an active free form (40%) and a C4b-binding protein (C4BP)-bound inactive form (60%) (11-13). The acute-phase response (APR) is characterized by a rapid increase of hepatic proteins, which serves to restore the physiological balance and limit the deleterious effects of tissue injury and infection. In the liver, synthesis of several plasma proteins, e.g. α1-antitrypsin, α2-macroglubulin, plasminogen, and fibrinogen, undergoes dramatic changes during the APR (reviewed by Ramadori *et al* (14)). Several of these proteins are also involved in coagulation or fibrinolysis. The changes during the APR are mainly mediated by the action of cytokines, such as IL6, IL1β, and TNFα.

In vitro studies in cultured human hepatoma cell line HepG2, primary Human Umbilical Vein Endothelial Cells (HUVECs), and in human microvascular endothelial cell line HMEC-1, have shown an increased PS production upon IL6 stimulation (15-17). C4BP is an established acute phase reactant with 2-3 fold elevated plasma levels during inflammation (18-21). The C4BP protein contains 6 or 7 identical α-chains and a single β-chain, although 17% of the C4BP molecules lack the β-chain (13). PS is bound to C4BP via the β-chain. Several studies have demonstrated similar or slightly increased total PS levels, but reduced free PS levels in plasma of patients during inflammation (22-24). The observed reduction in free PS levels has been explained as a direct consequence of an increase in the β-chain containing C4BP-form. Other studies, however, provide evidence for stable free PS levels during inflammation, showing only a rise in C4BP α-chain levels, but not in β-chain levels (21;25). The observed discrepancy between the various studies may be explained by a differential regulation of C4BP α- and β-chains in different subpopulations of patients during the APR (26).

The IL6 signalling pathway is well-defined (27-29). In short, IL6 is known to exert its stimulatory effect through IL6-responsive elements (IL6-RE) in the promoters of IL6-responsive genes. Two types of IL6-REs have been identified in genes encoding acute phase proteins. Type I is a binding site for CCAAT/enhancer binding protein (C/EBP) β (NF-IL6,

LAP), whereas the type II IL6-RE is bound by signal transducer and activator of transcription 3 (STAT3) (30). Whilst IL6 upregulates nuclear C/EBPβ levels through both elevated C/EBPβ gene transcription and C/EBPβ phosphorylation (29), increased nuclear STAT3 levels are mainly due to rapid STAT3 phosphorylation (28). C/EBPβ and STAT3 have been shown to affect IL6-induced transcription by binding to a.o. the promoters of the genes encoding C-reactive protein (CRP) (31;32), plasminogen (33), haptoglobin (34), and fibrinogen (35). In this report we investigate the mechanism by which IL6 upregulates PS protein levels.

Two copies of the PS gene are located on chromosome 3. The active PS gene (*PROS1*) shares 96% homology with the inactive pseudogene (*PROS2*) (36-38). Recently, constitutive expression of the *PROS1* promoter was shown to be regulated mainly by Sp1 *in vitro*, and to contain binding sites for various other transcription factors such as the hepatocyte-specific forkhead transcription factor FOXA2, which are possibly involved in tissue-specific or induced expression of the *PROS1* promoter (39). We confirmed earlier findings that PS is upregulated by IL6 in cultured HepG2 cells and complemented these with quantitative PS mRNA data thereby identifying the regulatory mechanism as pre-translational. Subsequently, we identified a region in the PS promoter spanning nucleotides 229 to 207 upstream from the translational start that binds STAT3, one of the nuclear messengers of IL6.

Experimental Procedures

Plasmids --- The generation of *PROS1* promoter reporter constructs is described in Chapter 2. *PROS1* promoter constructs were mutated at a putative STAT3 binding site by use of the QuikChange XL Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA), with forward primer 5'-CCAGCTCCGAAAAGCCGCCTGGTGCTGTCCTTGTTATCAC-3'and reverse primer 5'-GTGATAACAAGGACAGCACCAGGCGGCTTTTCGGAGCTGG-3'. Successful incorporation of the mutations was confirmed by automated sequencing (Beckman).

Cell culture --- The human hepatoblastoma cell line HepG2 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in Minimal essential medium (MEM), 10% Fetal Bovine Serum (FBS), 100 μg/ml penicillin, 100 μg/ml streptomycin, and 1x MEM non-essential amino acids (all purchased from Gibco, Invitrogen). For induction experiments, cells were washed twice with phosphate buffered saline, after

which fresh medium was added that lacked FBS but contained 0.1% human serum albumin and 5 or 100 ng /ml recombinant human IL6 (Strathmann)).

Preparation of nuclear extracts --- Nuclear extracts (NE) were prepared according to the method of Dignam *et al* (40). The final nuclear extract buffer contained 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 100 mM KCl, 0.5 mM DTT, 0.2 mM PMSF and a 1 x concentration of an EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany). Nuclear extracts were aliquoted and frozen at -80°C until further use. Protein concentration of the NE was measured with the BCA assay (Pierce Biotech, Rockford, IL). Nuclear extracts were prepared from HepG2 cells cultured in the absence or presence (30 minutes or 14 hours) of 100ng/ml IL6.

Immunological detection of STAT3 and C/EBPβ --- Nuclear extracts (10 μg protein) were fractionated on a denaturing 7.5% poly-acrylamide gel and transferred onto a nitrocellulose membrane. After blocking for 1 hr in phosphate-buffered saline (PBS) containing 1% nonfat dry milk, 1% bovine serum albumin, and 0.05% Tween 20, the membrane was incubated for 1 hr at room temperature with the first antibody. C/EBPβ and p-STAT3 were detected with the polyclonal antibodies sc-150G (1:1000) and sc-7993 (1:1000), respectively (Santa Cruz Biotech, CA, USA). As secondary antibody, HRP-conjugated rabbit anti-goat IgG was used at a dilution of 1:1000. Enhanced chemoluminescence was used for detection by incubating the membrane for 1 min with freshly mixed 1.25 mM 3-aminophtalhydrazide, 0.2 mM p-coumaric acid, and 0.01% v/v H_2O_2 in 0.1M Tris pH 8.5.

Reporter gene assays --- Cells were transfected at 60-80% confluency. Each transfection was performed in triplicate in 12-wells plates. All assays were conducted with two different DNA preparations of each construct. Transfections were carried out using 3 μl Tfx-20 lipids (Promega) per μg transfected DNA. In each transfection, an equimolar concentration of construct was used, supplemented with pUC13-MCS vector to obtain a fixed amount of transfected DNA. In pUC13-MCS the MCS had been removed by digestion with *Pvu*II and religation. Control vector pRL-SV40 (Promega), expressing the Renilla luciferase, was co-transfected for correction of transfection efficiency in a 1:500 ratio to total transfected DNA. The cell extracts were harvested at 24 hours (HepG2) after transfection. During induction experiments, medium was aspirated and replaced by fresh medium with or without IL6 eight hours after transfection and cell extracts were harvested 48 hrs after IL6 addition. Luciferase activity was measured according to the Dual Luciferase Assay System Protocol (Promega). Cells were lysed in 250 μl Passive Lysis Buffer/well, after which 20 μl was used to measure

luciferase activity. Activity was measured using a Lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany).

PS measurements --- Total PS antigen levels in IL6-stimulated and unstimulated culture media were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (41) with the following modifications. ELISA plates were coated with 10 μg/ml goat anti-human PS IgG (Kordia, Leiden, The Netherlands) overnight at 4°C. A second coating with 2.5% ovalbumin (Sigma-ALDRICH, St. Louis, MO) at 37°C for 1 hour was performed to reduce background absorbance. Immobilized Protein S was detected with horseradish peroxidase-conjugated rabbit anti-human IgG (Dako, Glostrup, Denmark). Absorbance at 450 nm was determined with an Organon Teknika plate reader (Turnhout, Belgium).

RNA-assays --- Total RNA was isolated from cell culture using Trizol reagent (Invitrogen) according to the manufacturers recommendations. Samples were treated with RNAse-free DNAse I (Amersham, Roosendaal, The Netherlands) after which RNA was purified with the RNeasy mini kit (Qiagen, Hilden, Germany). *PROS1* mRNA levels were determined by real-time quantitative PCR analysis (QPCR). First, 1 μg total RNA from each cell line was reverse transcribed using Superscript II reverse transcriptase and random hexamers (Invitrogen). 1/20th of the obtained cDNA was subsequently used in a QPCR reaction with primers and probes specific for *PROS1*. The forward primer was 5'-TGCTGGCGTGTCTCCTCCTA-3', the reverse primer was 5'-CAGTTCTTCGATGCATTCTCTTTCA-3', the probe was TET-5'-CTTCCC GTCTCAGAGGCAAACTTTTTGTC-3'-TAMRA. The primers and probe sequence locations and lengths were determined by using the ABI Primer Express Program (Applied Biosystems, Foster City, CA). *PROS1* QPCR reactions (Eurogentec) were performed in 0.5 ml thin-walled, optical-grade PCR tubes (Applied Biosystems) in a 50 μl final volume, by addition of the following components: 0.25 U AmpliTaq Gold DNA polymerase, 160 nM TaqMan probe, 300 nM of each primer, and 3 mM $MgCl_2$. A QPCR of the internal standard, the porphobilinogen deaminase gene (PBGD), was carried out in a similar fashion for each RNA sample with 4 mM $MgCl_2$. For this QPCR the following primers and probe were used; forward primer 5'-GGCAATGCGGCTGCAA-3', reverse primer 5'-GGGTACCCACGCGATCAC-3', and probe TET-5'-CTCATCTTTGGGCTGTTTTCTTCCGCC-3'-TAMRA. An Applied Biosystems Prism model 7700 instrument monitored the reactions. Thermal cycling conditions consisted of 10 min at 95°C followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. Determinations of cycle threshold (C_T) were performed automatically by the instrument. The results are expressed as fold transcript relative to the internal standard PBGD ($=2^{\Delta Ct}$).

Electrophoretic Mobility Shift Assays (EMSA) --- EMSAs were performed in a 13 µl binding reaction containing 10 µg NE and 195 ng denatured herring sperm DNA. EMSA buffers were purchased from Active Motif (Carlsbad, CA) and used according to the manufacturers recommendations. Double-stranded (ds) oligonucleotides were end-labeled using γ^{32}P-ATP and T4 polynucleotide kinase. The following ds oligonucleotides were used (only the sense strand is given): -229/-207wt 5'-AAAAGCTTCCTGGAAATGTCCTTG-3', -229/-207mt 5'-AAA AGCCGCCTGGTGCTGTCCTTG-3', STAT3cons 5'-GATCCTTCTGGGAATTCCTAGATC-3', C/EBPβ cons 5'-TGCAGATTGCGCAATCTGCA-3'. Reaction mixtures were incubated on ice for 20 min in the presence or absence of an unlabeled competitor. Subsequently the ^{32}P-labeled ds probe was added and the incubation was continued for another 20 min. In antibody supershift experiments, NE was incubated on ice 10 min with the ^{32}P-labeled ds probe after which an anti-STAT3 or anti-C/EBPβ antibody (sc-150x and sc-7993x, Santa Cruz Biotechnology) was added and the incubation was allowed to continue for another 10 min. Samples were loaded on a 5% non-denaturing polyacrylamide gel, which was electrophoresed for 2 hrs at 200V, after which gels were vacuum-dried and exposed to X-ray film.

Results

IL6 induces PROS1 mRNA and PS protein levels in HepG2 --- PS circulates in normal plasma at a concentration of about 0.33 µM (13). Hepatocytes are the largest contributors to the systemic levels of PS (10) therefore, hepatoma cell line HepG2 was used as a model system. PS mRNA and protein levels under basal culture conditions were compared with those after induction with 5 or 100 ng IL6 per ml culture medium (Figure 1). A dose-dependent increase in PS mRNA and protein levels in HepG2 by IL6 became detectable after 16 hrs. Total fibrinogen antigen levels were measured as a positive control. Fibrinogen is an established acute phase reactant (42-44), and its levels increased from 3.5 nM to 8.6 or 16.6 nM after induction with 5 or 100 ng/ml IL6 for 24 hours, respectively.

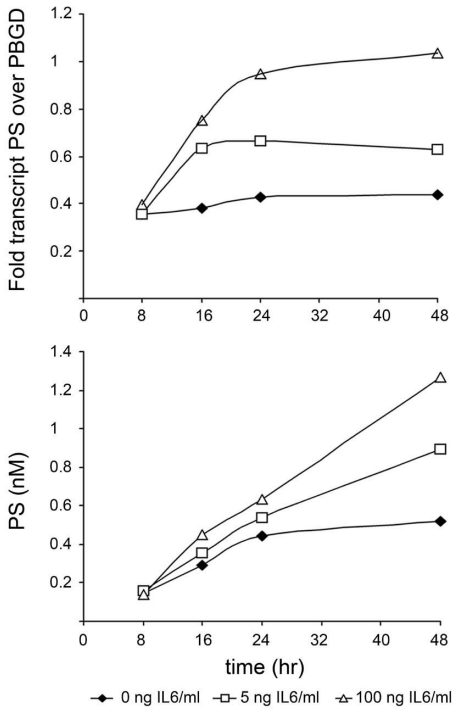

Figure 1 Induction of PS mRNA and protein expression by IL6. In the top panel, the expression of *PROS1*-derived mRNA in HepG2 cells is shown relative to that of the housekeeping gene PBGD, encoding porphobilinogen deaminase. The incubation time (x-axis) is plotted against the *PROS1:PBGD* transcript ratio (y-axis). The ratio of *PROS1* over *PBGD* transcripts was determined by real-time quantitative PCR (qPCR). In the lower panel, the production of PS over time is shown. The PS concentration in the culture medium was determined by ELISA. All values represent the average of at least 3 pooled independent experiments.

Phosphorylation of STAT3 is induced and maintained by IL6 in HepG2 cells --- The signal transduction pathway of IL6 signal has two main cellular targets: the transcription factors STAT3 and C/EBPβ that mediate most of the IL6-induced alterations in gene expression. Strongly increased nuclear STAT3 levels stimulate transcription of target genes and are due to rapid STAT3 phosphorylation by janus kinases (JAKS) that are stimulated by binding of IL6 to its cognate receptor (28). As a consequence, induction of gene transcription by binding of phosphorylated STAT3, which leads to dimerization, occurs rapidly after IL6 signalling. On

the other hand, increased C/EBPβ activity is achieved by increased transcription of the structural gene as well as by phosphorylation of the protein (29), hence increased *trans*-activation of gene promoters by C/EBPβ generally occurs later in time and lasts longer (45). Therefore we tested nuclear extracts from HepG2 cells, which had been stimulated with IL6, for the presence of both C/EBPβ and phosphorylated STAT3 (Figure 2). Whereas C/EBPβ levels remained similar, phosphorylated STAT3 levels were increased at both time points tested, 30 minutes and 14 hours. The presence of strong STAT3 phosphorylation after 14 hrs incubation with IL6 is in concordance with the sustained *PROS1* mRNA induction.

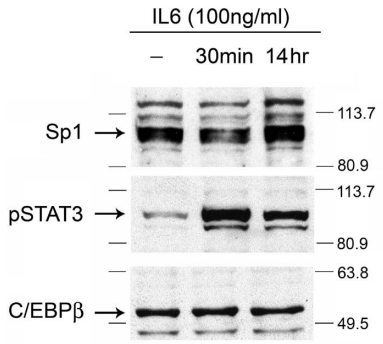

Figure 2 Effect of IL6 treatment on several transcription factors. The effect of IL6 treatment on the transcription factors Sp1 (as an unchanged loading control), STAT3 and C/EBPβ is shown using Western blot analysis. Antibodies specific for Sp1, C/EBPβ and the phosphorylated form of STAT3 were used.

An IL6 responsive element is present in the PROS1 promoter --- Subconfluent HepG2 cells were transfected with promoter-reporter gene plasmids containing a *PROS1* 5' region of varying length, after which the cells were either induced with IL6, or grown further under basal conditions (Figure 3a). A *PROS1* promoter region of 261 bp remained IL6-responsive. However, transfections with the shortest promoter-reporter gene construct, PS197, did not result in increased luciferase levels upon IL6 induction. These results indicate that an IL6-responsive element within the *PROS1* promoter is located between 261 and 197 bp upstream from the translational startcodon. The stimulatory effect of IL6 on PS261 was not augmented with increasing promoter size.

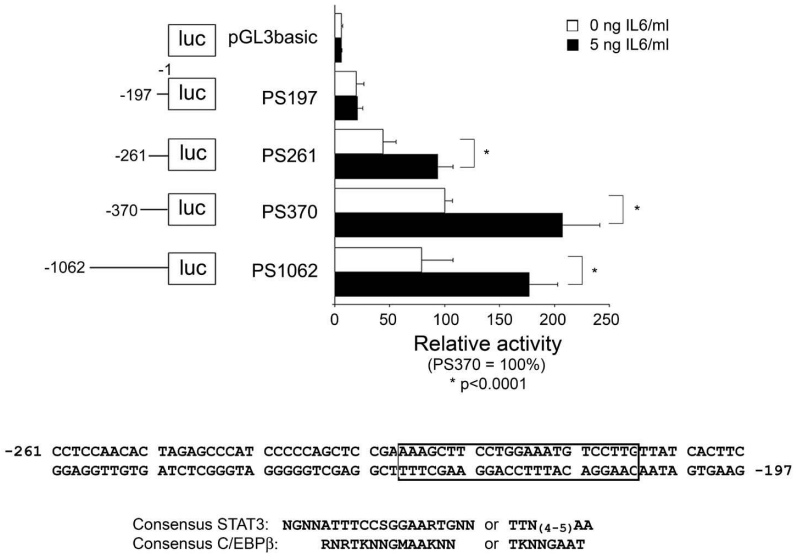

Figure 3 Localization of the IL6-responsive element in the *PROS1* promoter. (**A**) Histogram showing *PROS1* promoter activity, as assessed using luciferase constructs, in the absence or presence of a physiological concentration of IL6 (5 ng/ml). A schematic representation of each construct is shown on the left of the histogram. Statistical significance was determined with the Student's T-test. (**B**) Sequence of the *PROS1* promoter between -261 and -197 containing the IL6 responsive element. The ds oligonucleotide used in the EMSA assays is indicated.

The IL6 response is mediated by binding of STAT3 to the IL6-RE in the PROS1 promoter --- Computational analysis using various databases on the internet such as MatInspector professional (http://www.genomatix.com), Transfac (http://www.gene-regulation.com), and tfsitescan (http://www.ifti.org) showed that both a STAT3 and a C/EBPβ binding consensus are located in the -261 to -197 bp region in the *PROS1* promoter (Figure 3b). Increased binding of a protein complex to ds oligonucleotide probe -229/-207wt, was observed in extracts from IL6-treated HepG2 cells (Figure 4, left panel). The majority of this protein-DNA complex was shifted with a STAT3 antibody, leaving a weak signal at the original location. The mutated unlabeled ds oligonucleotide, -229/-207mt, was unable to compete with the PS wildtype probe for protein binding, whereas unlabeled ds oligonucleotides -229/-207wt and STAT3cons containing the wild type and STAT3 consensus sequence were efficient competitors (Figure 4). The C/EBPβ consensus oligo did not influence protein binding to the -229/-207 oligo probe. However, a slight shift from the original complex in nuclear extracts from untreated HepG2 cells was observed with a C/EBPβ antibody, suggesting that the

residual band present in the STAT3 supershift might contain comigrating C/EBPβ-probe complexes (Figure 4, right panel).

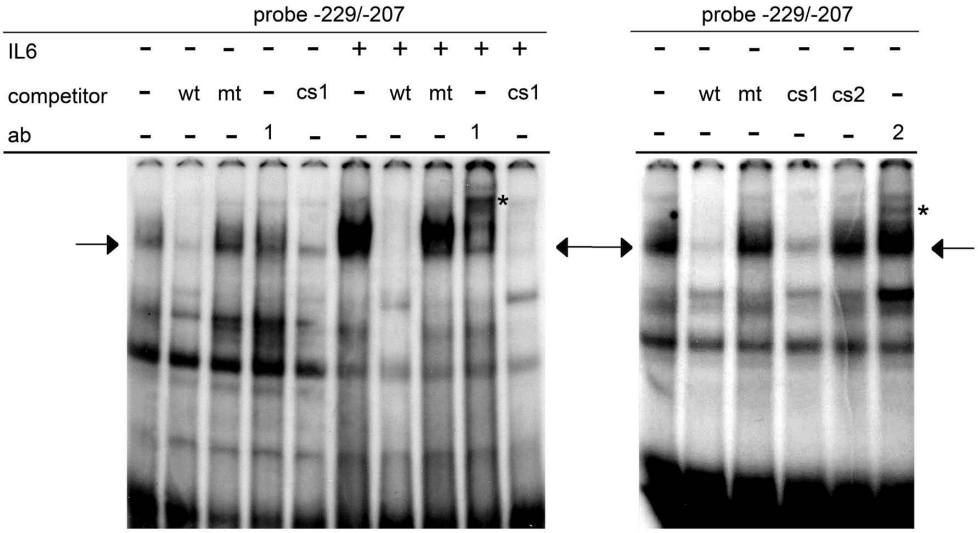

Figure 4 EMSA of the IL6-responsive element in the *PROS1* promoter. EMSA (Electrophoretic Mobility Shift Assay) using a ds oligonucleotide representing the IL6-responsive element and using nuclear extracts from HepG2 cells treated with or without 100 ng/ml IL6. The location of the IL6-specific band is indicated by an arrow. Non-labeled ds competitor oligonucleotides were used at 100 fold molar excess. wt = wild type, mt = mutant, cs1 is a STAT3 consensus oligonucleotide, cs2 is a C/EBPβ consensus oligonucleotide (see the experimental section for the sequences of the oligonucleotides). Antibody 1 was a p-STAT3-specific antibody and antibody 2 was a C/EBPβ-specific antibody.

The expression of mutated promoter-reporter gene constructs in HepG2 cell culture was analysed to investigate the contribution of STAT3 and possibly C/EBPβ to transcriptional upregulation of PS levels during IL6 induction. Although basal promoter activity remained unaltered upon mutation of the -229/-207 region, the mutations resulted in abolishment of the IL6 response in all promoter-reporter gene constructs (Figure 5). This underlines our preliminary conclusion from the data obtained with the wild type *PROS1*-reporter gene constructs that the site at -229/-207 is the only IL6-RE in the proximal *PROS1* promoter.

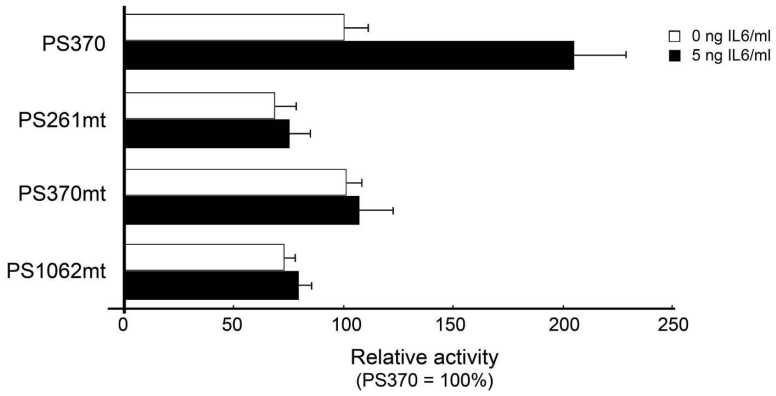

Figure 5 Activity of the *PROS1* promoter after mutagenesis of the IL6-responsive element. Histogram showing the luciferase activity of the wild type –370 construct and of three constructs with a mutated IL6-responsive element in the presence of a physiological concentration IL6 (5 ng/ml).

Discussion

Plasma proteins that are upregulated during the APR can be roughly divided into three major groups: proteins with an increased level of about 50% (e.g. ceruloplasmin and complement factor-3), those with an increase of two-three fold (haptoglobin, fibrinogen, C4BP), and a group of proteins which responds with a rapid increase of up to 5-fold to 1000-fold (CRP and serum amyloid A (SAA)) (18;46-49). We found a 2-fold increase in PS levels in medium after 48 hours incubation of HepG2 cell culture with 5 ng IL6 per ml medium (Figure 1), which is in agreement with a previous report (15). Both *PROS1* mRNA and PS protein levels were elevated up to 48 hours of IL6 stimulation. Protein S thus seems to belong to the second category of acute phase proteins.

That not only PS protein levels but also *PROS1* mRNA levels were upregulated by IL6 indicated that the mechanism by which IL6 upregulates PS levels was at the transcriptional level. Since IL6 induction is known to be mediated primarily by STAT3 and C/EBPβ, we tested the levels of these two transcription factors in IL6-induced nuclear extracts and found that total C/EBPβ levels remained unchanged throughout time, whereas phosphorylated STAT3 levels were increased, also after prolonged IL6 stimulation (Figure 2). This result is in

concordance with the sustained higher PS levels in IL6-treated versus untreated HepG2 cell culture.

In this study we identified an IL6-RE in the *PROS1* promoter at a region approximately 220 bp upstream from the *PROS1* translational startcodon. This region contains both the hallmarks of the STAT3 and the C/EBPβ binding sites (Figure 3). We identified STAT3 as the main transcription factor binding to the IL6-RE (Figure 4). C/EBPβ may also bind to this region, but its contribution appears to be relatively minor. Mutation of the STAT3 binding site in the *PROS1* promoter completely abolished the IL6 response in transfection experiments with promoter-reporter gene constructs (Figure 5). This indicates that there are no additional functional STAT3 or C/EBPβ binding sites in the region investigated through which the IL6 effect is mediated. The putative overlap between the STAT3 and the C/EBPβ binding sites around position –220 makes it difficult to make definitive statements about the role of the latter protein in the induction of *PROS1* transcription in HepG2 cells. Overlapping binding sites might constitute an efficient way to create an IL6-responsive element that is active both at early and late time points of IL6 stimulation.

Although *PROS1* is clearly IL6-responsive in HepG2 cell culture, patient studies indicate that total systemic PS levels are only slightly increased or remain at a similar level during inflammation. Moreover, in a subpopulation of patients free PS levels are reduced during inflammation due to increased C4BP β-chain levels (22). Also in patients with sepsis, free PS levels (measured as PS activity) were decreased (50). In both circumstances, inflammation or sepsis, IL6 is a major player and its induction of PS transcription does apparently not outweigh the above-mentioned *in vivo* effects. It could be argued that the effect on PS anti-coagulant activity would even be worse in the absence of the mild induction of *PROS1* transcription by IL6. Nevertheless, the possibility that the main function of *PROS1* transcriptional regulation by IL6 may lie in another aspect of the inflammatory response should also be considered.

IL6 has been implicated in a variety of cellular processes with a diverse array of regulatory roles. Dependent on the target cell, IL6 may induce various and sometimes contrasting responses. For instance, IL6 has a pro-inflammatory function and it can induce cytotoxic

effects, but it is also a cell survival factor. Several studies have demonstrated neuroprotective effects of IL6, which are mediated by the stimulation of growth factors and STAT3 (51-55). Recently, Liu *et al* reported a neuroprotective effect of intravenously injected PS on cortical neurons during ischemic injury in mice (56). The authors speculated that PS may be working through a similar mechanism as its structural homologue, growth arrest-specific gene 6 (GAS6), which induces cell survival through a signalling pathway mediated by the Tyro3/Axl membrane receptor family. Stimulation of local PS synthesis by IL6 may therefore explain part of the cell survival properties reported for IL6. In addition, increasing evidence suggests a function of PS in macrophage phagocytosis of apoptotic cells both directly (57;58) and indirectly by directing complement to the surface of apoptotic cells (59;60). Exactly what this new role for PS entails has not yet been fully clarified. Based on the cited literature, it seems plausible that the upregulation by IL6 of local and not systemic PS levels may contribute to processes such as the cell survival of cells in the inflamed region. Obviously, this hypothesis needs to be tested in future research.

Acknowledgements

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