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Regulation of human protein S gene (PROS1) transcription

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Chapter 6

Alternative splicing of Protein S pre-mRNA

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Submitted

Summary

For the first time we report the presence of a Protein S (PS) transcript resulting from alternative splicing of PS pre-mRNA. The splice variant contains an additional 96 base pairs, which maintain the reading frame and originate from an additional exon between exons 1 and 2. The transcript predicts a protein (PSalt) with an additional stretch of 32 amino acids between the pre- and propeptide containing four cysteine residues. The alternatively spliced PS transcript was found in human liver and various cell lines, such as hepatoma cell lines HepG2 and HuH7, megakaryocytic cell line Meg01, and human umbilical vein endothelial cells (HUVEC), and was shown to be relatively abundant. The ratio of wild type transcript versus the alternatively spliced variant was approximately 4 to 1 in human liver. Studies with Protein S expression vectors in COS-1 cells showed that the alternative transcript results in a PS protein, which is retained within these cells. Western blot analysis of cell lysates indicated that this protein S mostly forms high molecular weight aggregates within the cell that can be partly resolved by treatment with a reducing agent. However, in non-transfected HepG2 cells that contain the endogenous PSalt transcript, the high molecular weight species was not detected. The implications of these findings for the regulation of Protein S expression are discussed.

Introduction

Protein S (PS), a vitamin K-dependent plasma glycoprotein, functions as a co-factor for activated protein C (APC) in the proteolytic inactivation of coagulant factors VIIIa and Va by APC (1-4). PS also exhibits direct APC-independent anticoagulant activity by inhibition of the prothrombinase and tenase complexes (5-8). The primary source of circulating plasma PS is the hepatocyte (9), although PS is produced constitutively at low levels by a variety of cell types throughout the body (10-17). In human plasma, PS circulates in a free form (40%) and in complex with C4b-binding protein (C4BP) at a concentration of approximately 0.35 μM (18-20).

Partial PS deficiency was first reported to be associated with venous thrombotic disease in 1984 (21). The disorder inherits as an autosomal dominant trait with incomplete penetrance (22). PS deficiency is classified in three types (23). Type I deficiency corresponds to low levels of both free and total PS, type II PS deficiency is characterized by normal total and free PS levels, but reduced PS activity, and type III is defined by normal total PS levels, but low free PS levels and activity. Many abnormalities in the protein S gene (*PROS1*) have been described underlying hereditary PS deficiency (reviewed in (24;25)). PS deficiency can also be acquired, in which case it may be transient. Several non-genetic and environmental factors such as aging (26) and hormonal status (contraceptive use) influence PS levels (27).

Protein S is synthesized as a single-chain preproprotein of 676 amino acids (aa). Pre- and propeptide are consecutively removed from the aminoterminus of the preproprotein through proteolytic processing, before secretion of the 635 aa long mature protein from the cell. The signal peptide is first cleaved off by a signal peptidase during translocation of the nascent preproprotein to the endoplasmic reticulum. The propeptide is necessary for γ-carboxylation of the N-terminal vitamin K-dependent Gla-domain which is rich in carboxy-glutamic acid residues (28;29). In the trans-Golgi the propeptide is cleaved from the mature protein by a propeptidase or processing protease after completion of this process. The Gla-domain is followed by a thrombin-sensitive region, which is connected to four epidermal growth factor (EGF)-like domains. Whereas the C-terminal domain of other vitamin K-dependent plasma proteins consists of a serine protease domain, the C-terminal domain of PS has two laminin G-type repeats which share significant homology with sex hormone-binding globulin (30).

The PS genetic locus is located near the centromere on chromosome 3. It consists of an active PS gene (*PROS1*) and an inactive pseudogene (*PROS2*), which share 96% homology in their coding sequence. The promoter and the first exon are absent from *PROS2*, rendering it inactive (31-33).

We describe a protein S splice variant which was found in mRNA isolated from a human liver sample, hepatoma cell lines HepG2 and HuH7, megakaryocytic cell line Meg01, and human umbilical vein endothelial cells (HUVECs). The splice variant contains an additional 96 nucleotides, resulting in a PS protein with 32 aa extra located between the pre- and propeptide. Cloning of this alternative PS transcript in a eukaryotic expression vector and subsequent expression thereof in COS-1 cells resulted in a PS protein (PSalt), which was not secreted, but instead accumulated within the cell, mainly in high molecular weight aggregates.

Experimental procedures

Plasmids --- The PS wild type in expression plasmid pZEM229R (pZEM229R-PSwt) was described previously (34). The alternative PS splice variant was cloned in pZEM229R (pZEM229R-PSalt) as follows. First two overlapping PCR products were generated with one splice variant-specific primer and one wild type-specific primer. In the first PCR, primer PSwt(-14/+5), 5'-TCCGCGCCTTCGAAATGA G-3', which is located in the first exon was used in combination with alternative transcript-specific reverse primer PSalt(+171/+145) 5'-GCATAT AGAATAGAAAAAATTGCATCC-3'. The second PCR was performed with forward primer PSalt (+99/+120) 5'-TTTTATATACAACCGTGCATGC-3' and reverse primer PSwt (+1123/+1103) 5'-CCTCCAGTTGTGATTTTGGA-3'. The overlapping products were combined in a third PCR in which only the outermost primers were used, thereby generating a 1233 bp final product. This PCR product was digested with restriction enzymes BstBI and BlpI (New England Biolabs, Beverly, MA). The 858 bp PSalt BstBI-BlpI fragment was cloned into the pZEM229R-PSwt vector that had previously been linearized with BstBI and BlpI. The resulting pZEM229R-PSalt was sequenced and checked for errors through automated sequencing on the Beckman CEQ2000 system (Beckman Coulter, Fullerton, CA).

Human Liver Sample, Leukocytes and Monocytes --- A human liver sample was obtained from a deceased healthy donor and was a kind gift from E. de Wit at TNO Quality of Life, Leiden

(TNO). Leukocytes and monocytes were isolated from blood from healthy donors as previously described (35).

Cell culture --- The *Cercopithecus aethiops* (African green monkey) kidney cell line, COS-1, and human hepatoma cell line, HepG2, were purchased from the American Type Culture Collection (ATCC, Manassas, VA). COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM), HepG2 cells were grown in Minimal Essential Medium (MEM). Culture media for both cell lines were supplemented with 10% Fetal Bovine Serum (FBS), 100 μg/ml penicillin, and 100 μg/ml streptomycin (all cell culture reagents were purchased from Invitrogen, Carlsbad, CA).

PS measurements --- Total PS antigen levels in culture media were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (36) 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 and human liver 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). PS transcript 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 probes specific for the wild type or alternative PS and a single primer set. The PS (+32/+51) forward primer was 5'-TGCTGGCGTGTCTCCTCCTA-3', the PS (+186/+162) reverse primer was 5'-CAGTTCTTCGATGCATTCTCTTTCA-3', the PSwt probe was TET-5'-CCT GTTGCTTGACAAAAGTTTGCCTCTGA-3'-TAMRA, the PSalt probe was TET-5'-AAGCCGACC AATACAGAAATGCATGCCA-3'-TAMRA. The optimal primer and probe sequences were determined using the ABI Primer Express Program (Applied Biosystems, Foster City, CA). *PROS1* QPCR reactions (Eurogentec, Seraing, Belgium) 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 mRNA standard, the porphobilinogen deaminase gene (PBGD), was carried out in a similar fashion for each RNA sample using 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 sequence detection 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 ($=2^{\Delta Ct}$). PS transcript levels were also determined by densitometric measurement of PCR product using the same PCR conditions and primers as described above. The PCR was allowed to continue for 30, 33 or 36 cycles. The ratio of the alternative versus the wildtype PS transcripts was determined with the Syngene densitometric tools (Syngene, Cambridge, UK).

Transient transfection --- For PS production purposes, COS-1 cells were cultured in 75 cm^2 flasks. At 70% confluency cells were transfected with 7.2 pmol pZEM229R-PSwt or pZEM229R-PSalt and 100 µl transfection reagent Tfx-20 (Promega). Mock transfections contained 7.2 pmol pcDNA3 (Invitrogen) and 76 µl Tfx-20. Eight hours after transfection, cells were washed with phosphate buffered saline (PBS), after which cells were overlayed with 10 ml Opti-MEM Glutamax production medium (Invitrogen) containing 10 mg/l vitamin K (Konakion, Roche), 100 µg/ml penicillin, and 100 µg/ml streptomycin. Conditioned medium and cell lysates were harvested after a 10 hour incubation period. Three ml lysate buffer consisting of 2 mM $CaCl_2$, 50 mM Tris/HCl (pH 7.6), 0.5% Nonidet P40, 20% glycerol, 0.5 mM phenyl methyl sulfonyl fluoride (PMSF) and 4 x complete EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany), was used per 75 cm^2 flask. For immunofluorescence purposes, cells were grown to 70% confluency in 6 well plates, in which 18 mm glass dishes had been placed. Transfection conditions were identical as described above, but scaled down for transfections in 6 well plates. Eight hours after transfection cells were washed and medium was replaced with Opti-MEM. Cells were allowed to grow for another 5, 10, 15 or 20 hours. After the designated incubation time conditioned medium was aspirated and cells were washed twice with PBS.

In situ *immunofluorescent detection of PS* --- Cells were fixed *in situ* with 3% formaldehyde during a 30 min incubation period at 4°C. To allow penetration of antibodies into the cell, cells were permeabilized with 0.1% Triton in PBS. The glass dishes, which were overgrown

with cells, were then incubated at room temperature for 30 min with 50 µl 10 µg/ml primary antibody (goat-anti-human PS IgG (Kordia, Leiden, The Netherlands)). 50 µl 0.4 µg/ml FITC-conjugated secondary antibody (donkey-anti-goat IgG FITC (Santa Cruz)) was added after an additional three PBS washes, and reactions were incubated for 30 min in the dark at room temperature. The glass dish was put on a microscopic slide and coverslips were mounted over the sections with 2.5% 1.4-diazabicyclo-(2,2,2)-octane (DABCO).

Western Blot analysis --- Polyacrylamide gel electrophoresis was performed on 7.5% SDS/PAGE under reducing and non-reducing conditions according to Laemmli (37). Protein transfer to PVDF membranes (Millipore Corporation, Bedford, MA) was carried out overnight through a wet blot system (Bio-Rad, Hercules, CA). Goat-anti-human PS IgG (Kordia) was used to detect PS protein. Horseradish peroxidase (HRP)-conjugated rabbit-anti-human PS IgG (Dako, Glostrup, Denmark) in combination with a chemiluminescent peroxidase blotting substrate (SuperSignal West, Pierce, Rockford, IL) was used to visualize the immobilized antibodies.

Results

A novel transcript is produced from the PROS1 gene --- For the purpose of measuring PS protein and transcript levels in cell culture medium and RNA isolates, we used a sensitive PS ELISA and real-time quantitative PCR (QPCR), respectively. During optimisation of the real-time PCR assay on human liver cDNA with primers located in *PROS1* exon 1 and 2, the presence of a second PCR product was noticed; the expected 154 bp wild type PS (PSwt) product and an additional product of approximately 250 bp. Purification and sequence analysis of the unexpected product revealed that it was PS-specific and that the additional 96 base pairs originated from intron A (Figure 1A). The sequence was compared with the human expressed sequence tag (EST) database at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). From a total of 32 PS-specific ESTs covering this region, three entries contained (part of) the inserted sequence. The novel exon is located approximately 1.3 kb upstream from exon 2 and is flanked by consensus splice sites (Figure 1B).

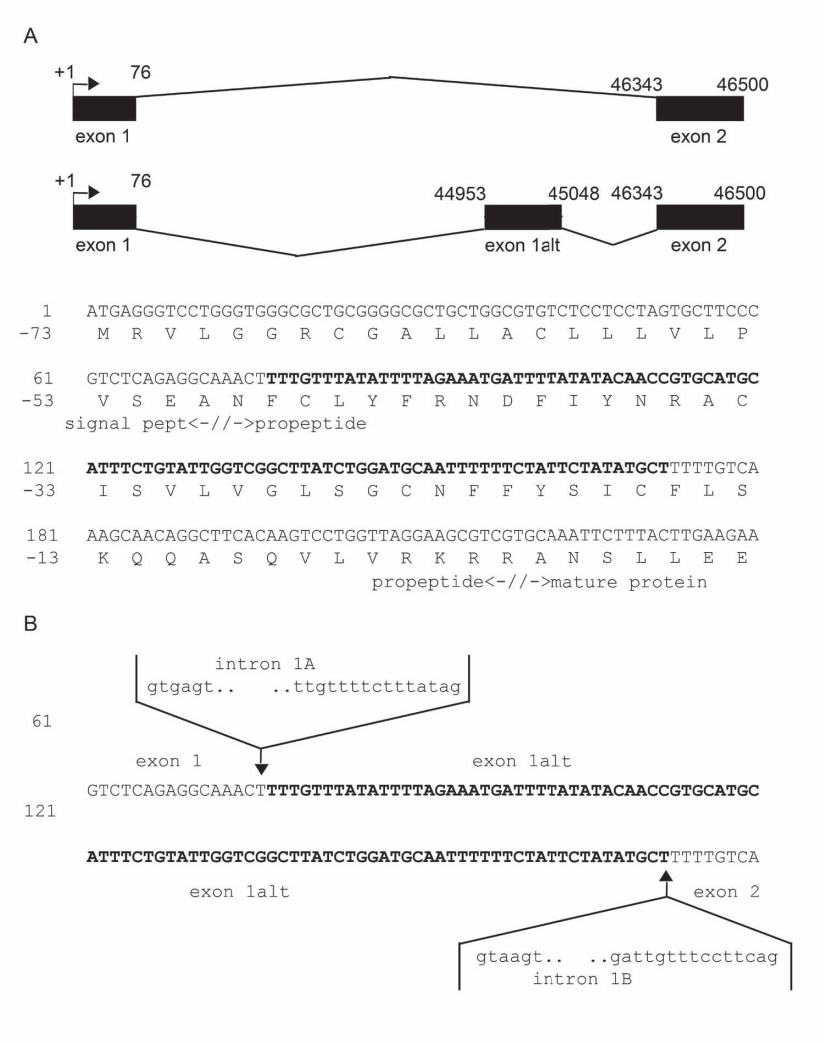

Figure 1 Location and sequence of the alternative exon in *PROS1*. (**A**) Schematic representation of the splice pattern of the standard and alternative transcript in the region of exons 1 and 2. The exons and introns are not shown to scale, the sequence coordinates are based on GenBank accession number AC117474. The sequence shows the relevant part of the alternative transcript. The nucleotide sequence of the alternative exon is shown in bold and is underlined. The putative junctions between signal peptide and propeptide and between propeptide and the mature protein have been indicated below the amino acid sequence. See the main text for a discussion on propeptide processing. Nucleotide numbering starts at the A of the startcodon, amino acid numbering starts at the first amino acid of the mature standard protein. (**B**) Partial cDNA sequence of the PSalt transcript showing the position of the introns and their splice consensus sequences.

PS splice variant PSalt is present in various cell lines and human liver --- cDNA from human liver and human hepatoma cell lines HuH7 and HepG2, megakaryocytic cell line Meg01, cervical carcinoma cell line HeLa, Human Umbilical Vein Endothelial Cells (HUVECs), and freshly isolated human leukocytes and monocytes was assayed for the presence of PSalt-encoding mRNAs (Figure 2). PS transcripts were found in all cell lines and human liver. Unexpectedly, low levels of the PSwt product were also found in reactions containing leukocytic and monocytic cDNA. However, this product did not appear until 33 or more PCR cycles had been performed. Therefore, we regard the potential of these cell types to produce PS mRNA as negligible. Quantitative analysis of transcript ratios through real-time QPCR and densitometric measurement of PCR products revealed that PSalt-specific transcripts represented approximately 20% of the total PS transcripts in the human liver sample (Table 1). Lowest PSalt transcript levels were found in cDNA isolated from HUVEC cells (0.3-5.7%). Overall, the alternative transcript represented a significant fraction of the total PS-specific mRNA pool in most cell types expressing PS.

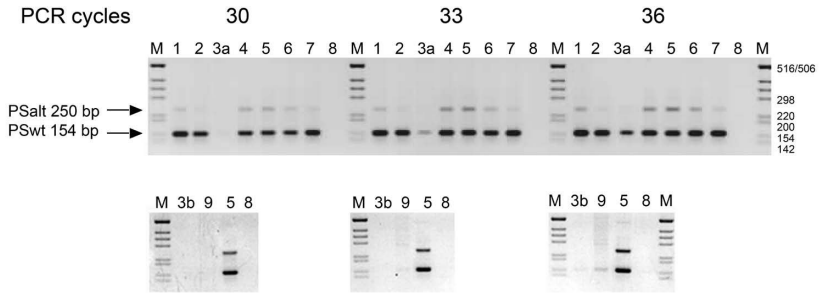

Figure 2 RT-PCR analysis of the standard and alternative transcripts in various cell types. First strand cDNA synthesis was primed using random hexamers and part of the product was amplified for a varying number of cycles using Protein S-specific primers located in exons 1 and 2. The products were separated on agarose gel. The lower panels represent a separate experiment. 1; HuH7. 2; HUVEC. 3a; PBMC leukocytes donor 1. 4; HeLa. 5; human liver. 6; Meg01. 7; HepG2. 8; blank. 3b; PBMC leukocytes donor 2. 9; 95% pure monocytes donor 3.

Recombinant PSalt protein expression --- The additional sequence in the PS splice variant was determined to be in frame with the rest of the PSwt sequence and encoded an additional 32 amino acids. This stretch of amino acids is located between the pre- and propeptide, just downstream of the predicted signal peptide cleavage site between the Ala and Asn-residues at

		% PSwt or PSalt transcript per cell type					
Technique	product	**HuH7**	**HUVEC**	**HeLa**	**Liver**	**Meg01**	**HepG2**
QPCR	PSwt	90.8	99.7	73.0	72.9	81.0	93.8
	PSalt	9.2	0.3	27.0	27.1	19.0	6.2
Densitometry	PSwt	91.3	94.3	86.2	84.8	89.8	93.9
	PSalt	8.7	5.7	13.8	15.2	10.2	6.1

Table 1 PS transcript composition per cell type. Transcript ratios were determined with two different methods; quantitative real-time PCR (QPCR) and densitometric analysis of PCR endproduct. In densitometric analysis a correction for product size was applied.

positions -50 and -49, respectively, and contains four cysteine residues (Figure 1A). This raised several questions regarding the intracellular processing of PSalt. A full-length PSalt-encoding cDNA was therefore cloned into a eukaryotic expression vector and protein expression of PSalt and Pswt was studied in COS-1 cells. COS-1 cells only produce very low levels of endogenous PS and readily produce high levels of protein from transfected expression vectors. PS levels were measured in conditioned media and lysates from COS-1 cells, which had been transfected with either pZEM229R-PSwt or pZEM229R-PSalt (Table 2). *In situ* immunofluorescent detection of PS confirmed the presence of PS protein in cells that had been transfected with either construct (Figure 3). After a 5 hour transfection with PS constructs, PSalt appeared to accumulate to a higher level within cells than PSwt. However, after a 10 hour transfection period the observed difference in fluorescence intensity between cells transfected with PSwt and PSalt had disappeared.

Incubation time (hr)	PS in medium (nM)			PS in lysate (nM)		
	-/-	PSwt	PSalt	-/-	PSwt	PSalt
10	0.028	1.037	0.030	0.076	0.222	0.423
20	0.045	1.612	0.055	0.089	0.264	0.573
30	0.048	1.865	0.066	0.103	0.232	0.460

Table 2 PS levels in COS-1 conditioned medium and cell lysate. Cells were grown in 75 cm^2 flasks. At 70% confluency cells were transfected with 7200 fmol pcDNA3 (-/-), pZEM229R-PSwt (PSwt), or pZEM229R-PSalt (PSalt), and then cultured in 10 ml Opti-MEM production medium for the designated incubation time.

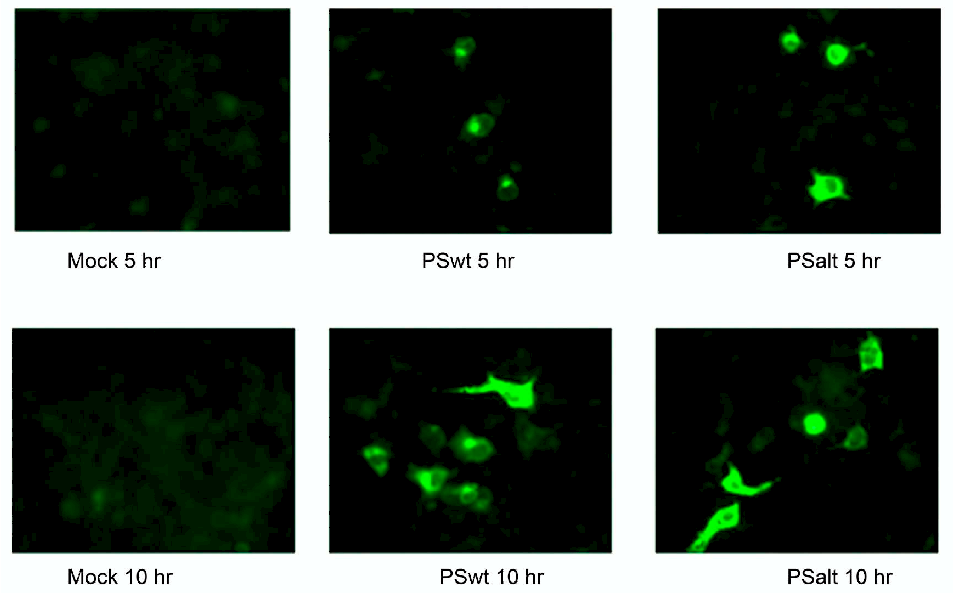

Figure 3 Immunofluorescent labeling of PS antigen in transfected COS-1 cells *in situ*. COS-1 cells were transfected with expression constructs expressing either the standard Protein S, PSwt, or the alternative Protein S, PSalt. Cells were grown on glass coverslips, fixed, permeabilized and stained with a PS-specific antibody 5 and 10 hrs after transfection.

PS-immunoreactivity was not present in medium from COS-1 cells that had been transfected with pZEM229R-PSalt, whereas it was present in medium from COS-1 cells transfected with pZEM229R-PSwt (Figure 4a). PS antigen was detected in lysates from COS-1 cells transfected with either the wild type or alternative construct. The PS protein in lysates from pZEM229R-PSalt transfections had a much higher propensity to form high molecular weight complexes. Some of the PSalt in these complexes could be reduced to the approximate size of the monomeric protein by addition of DTT, presumably by reduction of intermolecular disulfide bonds present in the cysteine-rich extra segment in the alternative protein (Figure 4b). The apparent molecular weight of the liberated PSalt appeared to be slightly greater than that of PS in lysates of cells transfected with PSwt, as is visible in the immunoblot analysis in Figure 4b. The larger molecular weight of PSalt, in combination with its propensity to form aggregates, might indicate that the cysteine-rich domain is still present at the aminoterminus of the protein, although another difference in post-translational

processing could also cause the observed migratory shift. Unfortunately we do not know whether the PS identified in the cell lysates represents fully processed PS or an intermediate form of PS, which still contains the propeptide. If the cysteine-rich domain is still present in the intracellular PSalt, the propeptide which is C-terminal from the PSalt-specific sequence, should also still be present in this protein. Interestingly, the high molecular weight complexes were not detected in non-transfected HepG2 cells, that also express the PSalt-specific mRNA (Figure 4a).

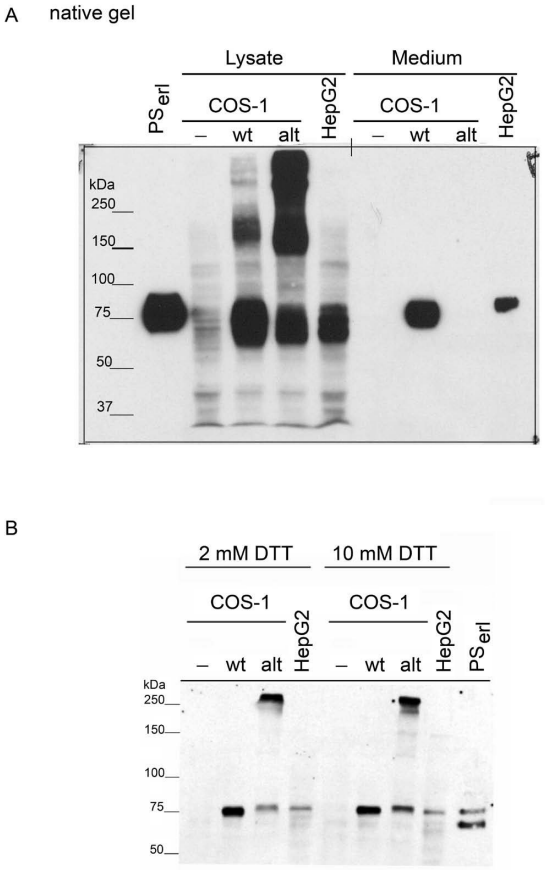

Figure 4 Western blot analysis of wild type and alternative Protein S. (**A**) shows a blot with denatured, but non-reduced proteins. Note the intense staining of the high molecular wight portion of the blot in the lane with PSalt and the absence of any staining in the lane with the growth medium of Psalt-transfected cells. PSerl represents purified Protein S from Enzyme Research Laboratories. HepG2 indicates lysate and medium from human HepG2 cells that show endogenous expression of Protein S and PSalt-encoding mRNAs. (**B**) shows a similar blot with reduced proteins. Note the somewhat higher molecular weight of the free PSalt compared to PSwt.

Discussion

We detected an alternatively spliced mRNA encoding a variant of the anti-coagulant Protein S in human liver and various cell lines. The Protein S splice variant, PSalt, contains an additional in-frame exon between the first two exons which encode the PS signal peptide (exon 1) and the propeptide and the Gla-domain (exon 2). Our results demonstrate that the additional 32 aa have a major impact on the maturation of the PSalt protein. The PSalt mRNA comprises a significant fraction of the total PS mRNA in most cell types that were tested and, most significantly, in human liver. Human liver mRNA had the highest level of PSalt transcript, whereas mRNA isolated from HUVEC cells had the lowest PSalt content. The difference between PSalt transcript levels in hepatocytic cell lines, HuH7 and HepG2, and those found in human liver may occur through various processes. Firstly, differences in growth conditions between cell culture and the *in vivo* liver may contribute to differences in levels of mRNA splice variants. Secondly, whereas mRNA from HuH7 and HepG2 culture contains only transcripts from a single origin, a human liver sample contains not only RNA from hepatocytes, but also from other cell types. Cell type-specific PS pre-mRNA splicing may be influenced by differences in composition of the spliceosome and splicing regulators, thereby resulting in different levels of mRNA splice variants (38;39).

Previously, we identified a transcription start site (TSS) in HUVECs, which is not employed for transcriptional initiation in other cell types (40). It is tempting to speculate that the TSS location in the *PROS1* promoter is somehow involved in the lower level of alternative splicing in these cells, as it has been shown that usage of different TSSs may lead to different splicing patterns downstream (41-44). However, alternative splicing of pre-mRNA is mostly due to the usage of multiple alternate promoters, leading to the incorporation of different first exons in the mature mRNAs. In the case of the *PROS1* transcripts in HUVECs, it is just the length of the 5' untranslated region (5'UTR) of the first exon that is different in the mRNAs.

Recombinant human PSalt was expressed by COS-1 cells, in which it was retained in large aggregates, whereas recombinant human PSwt was readily secreted into the medium. We believe that the accumulation of recombinant PSalt protein within the cell is a consequence of the altered sequence and folding of the region normally encoding the prepropeptide of

Protein S. In addition, some specific properties of the COS-1 cells may be responsible for the observed results, as similar high molecular weight complexes were not detected in HepG2 cells that apart from normal PS also should express PSalt from endogenous mRNAs. The presence of an extra sequence in this region of Protein S is not predicted to affect the cleavage site of the signal peptide. However, the cleavage site of the propeptide may be less accessible to the peptidase responsible for its cleavage, because of an altered three-dimensional structure of this region. This is expected to be mainly due to intra- or intermolecular disulfide bond formation by the four cysteine residues contained within the alternative peptide sequence. In addition, the γ-carboxylase complex may not be able to bind efficiently to the propeptide, because of steric hindrance by the cysteine-rich stretch located directly upstream. Fluorescent in situ antibody staining showed that at longer transfection times (10 hours) the observed difference in fluorescence intensity between cells that had been transfected with PSwt or PSalt had disappeared. Possibly, PSalt was actively degraded within the cell.

The high molecular weight complexes, which were found in immunoblot assays of lysates from COS-1 cells transfected with pZEM229R-PSalt, could be partly dissociated by addition of DTT. This may be explained if the cysteines within the alternative protein sequence form intermolecular disulfide bridges. We could not exclude the possibility that the observed complexes contained proteins other than PSalt, leaving open the possibility that PSalt interacts with other proteins within the cell. Cultured HepG2 cells contain both normally and alternatively spliced PS mRNA and secrete fully processed PS in medium. In contrast to what was observed in the lysates of COS-1 cells transfected with PSalt the lysates of HepG2 cells did not contain high molecular weight PS antigen. Whether this is related to differences between COS-1 cells and HepG2 cells or to the simultaneous presence of wt presence will need further investigation.

Alternative exons have been found in the same position in mRNAs from the Factor VII and Protein Z genes, which encode two other liver-expressed Vitamin K-dependent coagulation factors (45;46). The length and sequence of these exons is completely different, suggesting that it is mainly their location that matters. The significance of these findings has not yet been studied. GAS6 is a platelet protein with a domain structure highly similar to PS. No similar alternative transcripts have been reported for the GAS6 mRNA. However, the first

intron of *PROS1* is 46 kb long, whereas that of the GAS6 gene is only 91 bp in length, leaving no room for alternative splicing within the latter intron.

The presence of an alternative transcript resulting in a non-secreted form of PS suggests that alternative splicing might be used to regulate PS plasma levels *in vivo* and thus may contribute to the intraindividual variation in PS levels. However, it is as yet unknown whether this alternative splicing event is modulated *in vivo*. This awaits further investigations.

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