Functional characterization of twelve natural *PROS1* mutations associated with anticoagulant protein S deficiency

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ABSTRACT

Background

The molecular mechanisms by which *PROS1* mutations result in protein S deficiency are still unknown for many of the mutations, particularly for those that result in a premature termination codon. The aim of this study was to analyze the functional relevance on mRNA and protein expression of 12 natural *PROS1* mutations associated with protein S deficiency.

Design and Methods

Five mutations were nonsense, three were small frameshift deletions, one was c.258,259AG>GT at the 3' end of exon 3, one was p.M640T and the last two were c.-7C>G and p.L15H, found in double heterozygosis as [c.-7C>G;44T>A]. The apparently neutral variant p.R233K was also analyzed. *PROS1* cDNA was assessed by reverse transcriptase polymerase chain reaction of platelet mRNA. Expression of mutant proteins was determined by site-directed mutagenesis and analyses of transiently transfected *PROS1* mutants in COS-7 cells.

Results

Only cDNA from the normal allele was observed from the five nonsense mutations, the frameshift deletion c.1731delT and from c.258,259AG>GT. Both the normal and the mutated alleles were observed from [c.-7C>G;44T>A], c.187,188delTG and p.M640T. Transient expression analyses of *PROS1* mutants whose mRNA was normally expressed revealed greatly reduced secretion of p.L15H and c.1272delA, mild secretion values of p.M640T and normal secretion levels of c.-7C>G and, as expected, p.R233K.

Conclusions

Whereas the main cause of quantitative protein S deficiency associated with missense mutations is defective synthesis, stability or secretion of the mutated protein, the main mechanism for the deficiency associated with mutations that generate a premature termination codon is not the synthesis of a truncated protein, but the exclusion of the mutated allele, probably by nonsense-mediated mRNA decay.

Key words: mRNA, mutant protein, *PROS1* mutations, protein S deficiency.

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Introduction

Protein S (PS) is a vitamin K-dependent plasma glycoprotein with anticoagulant and antithrombotic properties that plays a crucial role in the regulation of coagulation.[1] PS acts as a co-factor for activated protein C in the inactivation of procoagulant factors Va and VIIIa, which are essential for thrombin generation.[2] PS also has activeted protein C-independent anticoagulant properties by directly inhibiting the tenase and prothrombinase complexes.[3] Besides its confirmed role as an anticoagulant protein, recent findings suggest that PS may also have other important roles in cellular processes and inflammation.[4] Native PS is a multidomain protein that contains a signal peptide, a γ-carboxyglutamic acid-containing domain, a thrombin-sensitive region, four epidermal growth factor-like domains and a C-terminal domain that is homologous to the laminin A globular-like domains.[1] Mature PS circulates in plasma as a single chain, 635-amino acid protein with a molecular weight of approximately 75 kDa. In human plasma, approximately 60% of PS forms a non-covalent equimolar complex with the β-chain of C4BP that is inactive as activated protein C co-factor.[5]

PS deficiency (OMIM: +176880) is a recognized genetic risk factor for thrombosis.[1] It is an autosomal dominant trait with incomplete penetrance and variable clinical and biochemical expression which affects 2% to 8% of patients with thrombosis in Caucasian populations.[6] According to plasma levels of total and free PS antigen and to the functional activity of PS, three types of PS deficiency have been proposed. Type I is a quantitative deficiency characterized by reduced levels of total and free PS antigen, as well as anticoagulant activity; type II is a qualitative deficiency characterized by normal levels of PS antigen but reduced anticoagulant activity; type III PS deficiency, which may be considered as a mild quantitative deficiency,[7] is characterized by normal levels of total PS antigen but reduced levels of free PS antigen and activity. The thrombotic risk associated with heterozygous type I PS deficiency is between 2.5 and 10-fold higher than in the general population.[6,8]

The PS gene (*PROS1*; Gene ID: 5627) spans 101 Kb of genomic DNA in the centromeric region of chromosome3. It is composed of 15 exons that are transcribed in 3.5 Kb of mRNA (NM_000313).[9] There are over 200 *PROS1* defects that segregate with PS deficiency in familial studies[10] but the molecular mechanisms by which these mutations reduce plasma PS levels have been analyzed in fewer than one third of them. Furthermore, while most studies have been focused on the analysis of recombinant *PROS1* missense mutations[11-15] or mRNA analyses from splice-site mutations,[16-25] the effect on mRNA expression of mutations that generate a premature termination codon has been analyzed for only six nonsense or frameshift *PROS1* mutations.[26-31] Analysis of the functional effects of *PROS1* mutations will improve the knowledge of the mechanisms underlying PS function. The purpose of this study was to analyze the functional relevance, both at RNA and protein levels, of 12 natural *PROS1* mutations associated with quantitative PS deficiency and thrombosis in Spanish pedigrees.

Design and Methods

PROS1 mutations analyzed

All the mutations analyzed (Table 1) had been identified in the heterozygous state in Spanish patients with quantitative PS deficiency associated with venous thrombotic disease.[7,32] All patients gave their informed consent to participation in this study and all procedures were reviewed and approved by the ethics committee for clinical research of the *Ciutat Sanitària i Universitària de Bellvitge* (CSUB).

Table 1. Mutations analyzed in this study.

Mutation		Amino acid changes			Mean plasma PS[b]			
HUGO nomenclature	Previous nomenclature[a]	HUGO nomenclature	Previous nomenclature[a]	Location	Total mean±SD (n)	Free mean±SD (n)	PSD[c] deficiency	Ref.
[c.-7C>G;44T>A]	[c.143C>G;193T>A][d]	None + p.L15H	None + L-27H	Exon 1	56±4 (2)	21±3 (2)	I	32
c.178G>T	c.324G>T	p.E60X	E19X	Exon 2	52±13 (5)	23±7 (5)	I	32
c.187,188delTG	c.333,334delTG	p.C63QfsX1	Frameshift, 23X	Exon 2	53±8 (8)	28±6 (8)	I	7
c.258,259AG>GT	c.404,405AG>GT	p.V87F	V46F	Exon 3	53±11 (17)	23±12 (17)	I	7
c.698G>A	c.844G>A	p.R233K	R192K	Exon 7	91±16 (6)	77±19 (6)	None[e]	32
c.835C>T	c.981C>T	p.Q279X	Q238X	Exon 8	57±15 (17)	18±10 (17)	I[f]	7
c.1001C>G	c.1147C>G	p.S334X	S293X	Exon 10	39±12 (13)	17±6 (13)	I	7
c.1272delA	c.1418delA	p.K424KfsX22	Frameshift, 405X	Exon 11	57±19 (2)	22±1 (2)	I-III	32
c.1351C>T	c.1497C>T	p.R451X	R410X	Exon 12	59±11 (5)	22±6 (5)	I-III	32
c.1518G>A	c.1664G>A	p.W506X	W465X	Exon 13	60±10 (2)	17±1 (2)	I-III	32
c.1731delT	c.1877delT	p.F577LfsX15	Frameshift, 551X	Exon 14	60±20 (3)	29±6 (3)	I-III	32
c.1919T>C	c.2065T>C	p.M640T	M599T	Exon 15	65±10 (3)	28±3 (4)	I	32

[a]Nomenclature based on the nucleotide and amino acid numbering of Schmidel et al.[9] [b]Mean values, as a percentage of the value in a normal plasma pool, from all heterozygotes for each mutation. [c]Mutation co-segregates with: I, type I PS deficiency; I-III, both type I and type III PS deficiency in the same pedigree. [d]These two mutations were identified in double heterozygosis in the same PROS1 allele. [e]p.R233K mutation was identified in a type III PS deficiency pedigree but it did not co-segregate with the deficient phenotype: only two out of six heterozygotes had type III PS deficiency. [f]p.Q279X was identified in three type I pedigrees and in one pedigree with type I-III PS deficiency.

Here, we have adopted HUGO recommendations for mutation nomenclature (*http://www.hgvs.org/mutnomen/*). Previous nomenclature, based on the mature protein, is also shown in Table 1.

PROS1 mRNA studies

PROS1 mRNA was analyzed from patients who were heterozygotes for both the mutation and at least one transcribed *PROS1* polymorphism (Table 2), in which the allele associated with the mutation was known from previous segregation studies in each pedigree.[7,32] Isolated total RNA from frozen peripheral blood platelets [33] was reverse transcribed by use of random hexamer primers and the First Strand cDNA Synthesis Kit (Amersham Biosciences, Uppsala, Sweden). For each patient, 50 ng of *PROS1* cDNA were amplified by polymerase chain reaction (PCR) with primers overlapping two contiguous exons upstream and downstream of the mutation site (available upon request). Amplified products were analyzed by agarose gel electrophoresis and DNA sequencing in an ABI377 DNA sequencer.

The *PROS1* polymorphisms c.2001A>G (p.P667, dbSNP rs#6123) and c.2551C>A (at the 3'UTR region, rs#9681204) were analyzed by amplification of the cDNA fragment containing exon 15 and restriction analysis with *Bst*XI (c.2001A>G) and *Ava*II (c.2551C>A).

Site-directed mutagenesis and expression of recombinant PS variants

Human PS cDNA subcloned in pcDNA3.1 was used as the template for mutagenesis (primers available upon request), as previously described.[11] Mutant constructs were confirmed by full cDNA sequencing. All *PROS1* cDNA expression vectors were transcribed and translated *in vitro* using the TNT®Coupled Reticulocyte System (Promega, Madison, WI, USA). [^{35}S-Met] synthesized polypeptide chains were analyzed using 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), fixed, dried and revealed by autoradiography. For transient expression of recombinant PS, COS-7 cells were grown in Dulbecc'os modified Eagles's medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco-BRL), 2 mM glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin and maintained at 37 °C in 5% CO_2. Cells were co-transfected by the DEAE-dextran method[11] with 2.0 μg of mock vector, wild type (wt) or mutant PS constructs, 0.5 μg of the β-galactosidase and 0.5 μg of the secreted alkaline phosphatase (SEAP, Clontech, Palo Alto, CA, USA) control vectors. These control vectors were used for normalizing transfection efficiency of recombinant PS in the culture medium and in the cellular extracts, respectively. After 24 hours of transfection, the medium was changed to serum-free medium supplemented with 10 mg/L of vitamin K1, harvested 48 hours later and concentrated 10-fold.[11] Soluble cell extracts were prepared by lysing cells in potassium phosphate buffer 100 mM pH 7.3, 20% (v/v) Triton X-100 and 1 mM dithiothreitol. At least two independent experiments were performed in triplicate, except for the [c.-7C>G;44T>A] double mutant experiment that was performed once.

Western blot analysis

Western blot of 2.5 μg of concentrated media and 3.25 μg of soluble cell extract proteins separated by 7.5% SDS-PAGE under reduced conditions was analyzed by using a rabbit polyclonal antibody against human PS (DAKO, Glostrup, Denmark). Inmunoreactive electrophoretic bands were detected by enhanced chemiluminiscence (ECL Advance™, Amersham) using a VersaDoc® 5000 Imager (BioRad, Hercules, CA) and quantified by Quantity One® software (BioRad). In all assays, 25 ng of commercial purified human plasma PS (Enzyme Research Laboratories, Swansea, UK) were also included. SEAP in the concentrated media was quantitatively assayed with the Great EscAPe SEAP Chemiluminescent Kit (Clontech).

Table 2. Results of mRNA expression studies by reverse-transcriptase polymerase chain reaction.

			Alleles in genomic DNA				Alleles in cDNA			
Mutation	Amino acid chnage	PSD deficiency[a]	PROS1 mutation[b]	SNP[c] c.2001A>G	c.2551C>A	Phase[d]	PROS1 mutation[b]	SNP[c] c.2001A>G	c.2551C>A	Mutated allele exclusion
c.-7C>G;44T>A]	None + p.L15H	I	CT/GA	AG	-	G	CT/GA	AG	-	NO
c.178G>T	p.E60X	I	GT	AG	-	G	G	A	-	YES
c.187,188delTG	p.C63QfsX1	I	TG/delTG	-	CA	-	TG/delTG	-	CA	NO
c.258,259AG>GT	p.V87F	I	AG/GT	AG	-	A	AG	G	-	YES
c.835C>T	p.Q279X	I	CT	-	CA	A	C	-	C	YES
c.1001C>G	p.S334X	I	CG	AG	-	A	C	G	-	YES
c.1351C>T	p.R451X	I-III	CT	AG	-	G	C	A	-	YES
c.1518G>A	p.W506X	I-III	GA	AG	-	A	G	G	-	YES
c.1731delT	p.F577LfsX15	I-III	T/delT	-	CA	C	T	-	A	YES
c.1919T>C	p.M640T	I	CT	AG	-	A	CT	AG	-	NO

[a]Mutation co-segregates with: I, type I PS deficiency;I-III, both type I and type III PS deficiency in the same pedigree. [b]PROS1 mutation alleles in the amplified genomic or cDNA fragments, as determined by DNA sequencing. [c]c.2001A>G or c.2551C>A PROS alleles in the amplified genomic or cDNA fragments, as determined by restriction fragment length polymorphism analysis. [d]Phase (SNP allele associated with mutation) was established from segregation analysis in each pedigree.

Relative amounts of PS were normalized for the corresponding SEAP values from culture media and actin/β-galatosidase values from cell lysates.

PS enzyme-linked immunosorbent assay

Wild-type and mutated PS in culture media were quantified by ELISA using the Asserachrom® total and free protein S kits (Diagnostica Stago, France) with some modifications. Briefly, 25 μg of total protein in phosphate-buffered saline were used in both assays. Moreover, a standard curve of 150, 100, 50, 12.5 and 6.25 ng of purified human plasma PS (Enzyme Research Laboratories) in phosphate-buffered saline was prepared (*Online Supplementary Figure S1*). All secreted PS values were normalized for transfection efficiency as previously described and quantified through the standard curve. At least two independent experiments were performed in triplicate.

Results

mRNA analysis of PROS1 mutations

Electrophoretic analysis of amplified *PROS1* cDNA from the patients revealed normal-sized fragments in all cases, thus excluding the presence of stable mRNA with exon skipping or other splicing alterations that could significantly alter cDNA size. As indicated in Table 2, direct sequencing of amplified cDNA fragments revealed the absence of the mutated allele in cDNA sequences from heterozygotes for c.178G>T, c.258,259AG>GT, c.835C>T, c.1001C>G, c.1351C>T, c.1518G>A, and c.1731delT mutations. Furthermore, exclusion of the mutated allele was confirmed by restriction fragment length polymorphism analysis of the transcribed polymorphisms c.2001A>G and c.2551C>A in heterozygotes for at least one of these polymorphisms and the disease-associated mutation. This analysis showed that only the allele associated with the wild type sequence, but not the one associated with the mutation, was present in the amplified cDNA fragment (Table 2). Mutated and normal alleles were both observed in cDNA sequences from [c.-7C>G;44T>A], c.187, 188delTG and c.1919T>C heterozygotes.

Transient expression analysis of PS mutants

Transient expression analysis of *PROS1* mutants in COS-7 cells was performed for [c.-7C>G;44T>A] and p.M640T mutations that did not result in allelic exclusion (Table 2), and for c.1272delA (not available for mRNA studies) and p.R233K mutations. The effect of the single mutants c.-7C>G and c.44T>A (p.L15H) was also analyzed. Although mRNA from c.187,188delTG was present, we did not consider it necessary to analyze this PS mutant that was expected to result in a truncated protein of only 64 residues. As shown in Figure 1, *in vitro* transcription-translation of all PS mutants, except c.1272delA, revealed [^{35}S-Met]-labeled proteins of 68 kDa, which is the molecular weight of native PS without post-translational modifications. cDNA containing the c.1272delA mutation was translated into a 49 kDa protein, which is the molecular weight of the truncated protein (p.K424KfsX22) that is expected to result from this frameshift mutation.

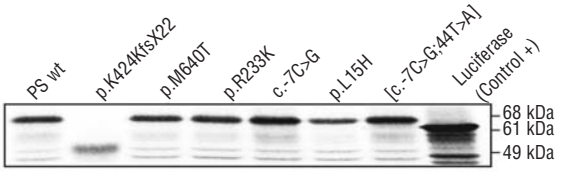


Figure 1. ***In vitro*** **transcription-translation of wild-type and mutated PS. Autoradiography of 7.5% SDS-PAGE of [^{35}S]methionine-labeled wild-type (wt) and mutated PS, translated in a reticulocyte system. According to the molecular weight marker run in the gel, on the right we indicate the molecular weights corresponding to native PS (68 kDa), to the luciferase control (61 kDa) and to the p.K424KfsX22 mutant (49 kDa), without post-translational modifications.**

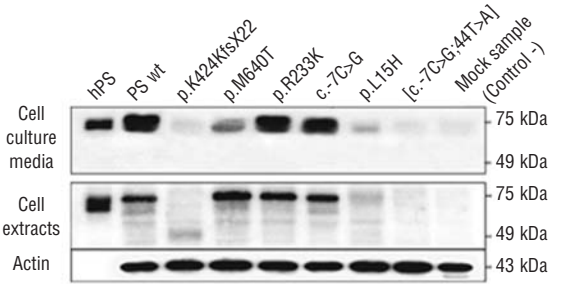


Figure 2. Western blot analysis of transiently expressed wild-type and mutated PS in COS-7 cells. Western blot of purified human plasma PS (hPS), wild-type (wt) and mutated PS secreted from transfected COS-7 cells (cell culture media) or retained in the cells (cellular extracts).

As illustrated in Figure 2, western blot analyses of PS mutants in COS-7 cell extracts indicated that the electrophoretic mobility of p.M640T, p.R233K and c.-7C>G PS was similar to that of wild-type PS. No band or, in some experiments a faint one, was observed from [c.-7C>G;44T>A] and p.L15H (c.44T>A) mutants. As in the *in vitro* transcription–translation, a 49 kDa band was observed from the p.K424KfsX22 mutant. In culture media, bands with the same electrophoretic mobility as that of wild-type PS were observed from the p.M640T, p.R233K and c.-7C>G mutants. A faint band, which could be due to background COS-7 PS, was observed from p.K424KfsX22, [c.-7C>G;44T>A] and p.L15H mutants, thus indicating the absence of significant amounts of secreted proteins.

Mean normalized results of the semi-quantitative densitometric analysis of the recombinant PS electrophoretic bands in the cell culture media and in the cell extracts showed that, in soluble cell extracts, PS from p.L15H and [c.-7C>G;44T>A] mutants was absent or undetectable (below 2% of wild-type PS). Values from p.K424KfsX22 truncated protein were 33±14% of wild-type PS. In contrast, the value from the p.M640T mutation was signifi-

cantly higher than the wild-type PS (155±11%). p.R233K and c.-7C>G PS relative values were very similar to wild-type PS (105±10% and 105±11%, respectively). In culture media, PS p.K424KfsX22 was undetectable and p.L15H and [c.-7C>G;44T>A] mutants showed low relative expression values of 21±11% and 21±5% respectively, while the value for p.M640T was half that of the wild-type (42±6%). Finally, the relative PS values obtained for p.R233K and c.-7C>G were similar to wild-type PS (109±4% and105±14%).

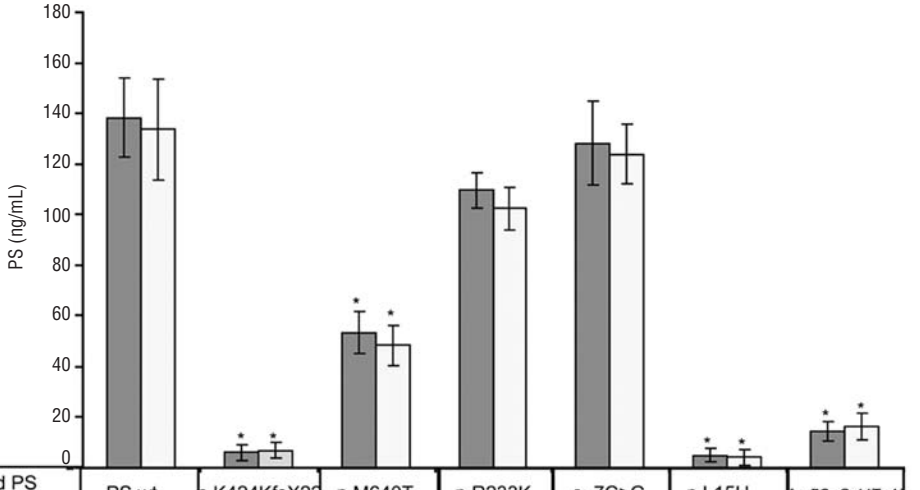
Quantification of mutant PS secretion by ELISA

In order to confirm the western blot results, PS in the culture media was quantified by ELISA. As shown in Figure 3, the results obtained (which were similar for total and free PS ELISA) agreed well with those calculated from the western blots for secreted PS. The lowest secretion values (around 10% of wild-type PS) were obtained with the p.K424KfsX22 and p.L15H mutants, and with the double mutant [c.-7C>G;44T>A]. Between 40% and 50% of wild-type PS was obtained with the p.M640T mutant. Protein concentrations similar to wild-type PS were obtained with the c.-7C>G and p.R233K mutants.

Discussion

Quantitative PS deficiency is usually associated with different types of *PROS1* mutations that impair *PROS1* gene expression by several mechanisms related to DNA transcription, mRNA processing, protein synthesis, stability and secretion pathways. Nevertheless, the molecular mechanism by which a given *PROS1* mutation results in quantitative PS deficiency is largely unknown. In this context, this study was aimed at analyzing the effect on mRNA and protein expression of 12 natural *PROS1* mutations associated with inherited quantitative PS deficiency in Spanish pedigrees. An apparently neutral variant (p.R233K) was also analyzed. Eight of these mutations are nonsense or frameshift deletions that create a premature termination codon. We, therefore, first determined their expression at the mRNA level in order to analyze whether they were subjected to nonsense-mediated mRNA decay, a mechanism that degrades spliced mRNA that prematurely terminates translation and, consequently, prevents the synthesis of potentially toxic proteins.[34] The remaining mutations were missense or regulatory mutations. Their expression, as well as that of the nonsense mutations not subjected to nonsense-mediated mRNA decay or whose RNA could not be obtained, was analyzed at the protein level.

Analysis of cDNA sequences revealed the absence of detectable amounts of the mutated allele from heterozygotes for the five nonsense mutations, the frameshift deletion c.1731delT (p.F577LfsX15) and the splice site and missense mutation c.258,259AG>GT (p.V87F), at the 3' end of exon 3. In the case of p.R451X and p.W506X, this was in agreement with previous reports.[26,28] Interestingly, all the nonsense mutations that showed allelic exclusion, but p.Q279X, are localized more than 50 nucleotides upstream of an exon-exon junction. By contrast, c.187,188delTG, which results in a premature termination codon at position 23, at 40 nucleotides from the junction between exons 2 and 3, is expressed at the mRNA level, as are the double



ng/mL Secreted PS (% respect to PS-WT)	PS wt	p.K424KfsX22	p.M640T	p.R233K	c.-7C>G	p.L15H	[c.-7C>G;44T>A]
Free PS ELISA	138.33±15.49 (100%)	6.12±3.15 (4.42%)	53.41±8.06 (38.61%)	109.66±6.92 (79.27%)	128.22±16.70 (92.69%)	4.99±2.67 (3.61%)	14.39±3.80 (10.40%)
Total PS ELISA	133.56±19.88 (100%)	6.97±3.26 (5.22%)	48.37±8.14 (36.22%)	102.32±8.32 (76.61%)	23.80±11.71 (92.69%)	4.17±3.14 (3.12%)	16.35±5.09 (12.24%)

Figure 3. PS values of secreted recombinant PS, as measured by ELISA. Mean and standard error values, in ng/mL after normalization for transfection efficiency, of secreted wild-type (wt) and mutated PS to the COS-7 cell culture media. Dark bars represent values obtained with the free PS ELISA and light bars represent values obtained with the total PS ELISA assay. * Statistically significant differences ($p<0.001$) between PS levels in mutated and wild-type PS, assessed by the unpaired Student's t-test.

mutant [c.-7C>G;44T>A] and p.M640T. This agrees with the 50 bp nonsense-mediated mRNA decay rule, which states that premature termination codons that are followed by an intron more than 50-55 nucleotides downstream generally elicit nonsense-mediated mRNA decay.[35] The only mutation that does not follow the 50 bp nonsense-mediated mRNA decay rule is c.835C>T (p.Q279X), at only 12 bp upstream of the exons 8 and 9 junction. Though the mechanism by which this mutation elicits nonsense-mediated mRNA decay is unknown, *in silico* analysis with the ESE-finder (*http://exon.cshl.org/ESE*)[36] and the RESCUE-ESE (*http://genes.mit.edu/burgelab/rescue-ese*)[37] programs revealed that c.835C>T abolishes a strong exon splicing enhancer site for SF2/ASF and reduces the score for SC35 and SRp40 splicing factors. This could result in defective splicing of intron 8, exon skipping, frameshift and introduction of another premature termination codon. Unfortunately, we could not test this hypothesis because of the lack of availability of new fresh blood samples needed to perform mRNA studies in a medium that inhibits nonsense-mediated mRNA decay. Similarly, the absence of mutated mRNA from c.258,259AG>GT indicates that what causes PS deficiency is not the p.V87F substitution that would result from this mutation if normally spliced, but an alteration of the normal splicing of intron 3. This agrees with the observation that this mutation reduces the consensus value of the wild type donor splice site of intron 3 to a value lower than the one calculated for the c.259G>C (p.V87L) mutation,[7] which also results in reduced mRNA expression.[18]

Since it was confirmed that [c.-7C>G;44T>A] and p.M640T are not affected by nonsense-mediated mRNA decay, their functional consequences, as well as those of c.1272delA and p.R233K, were analyzed by means of transient transfection experiments in COS-7 cells. According to the results obtained, we can cluster these mutations into three groups: c.-7C>G and p.R233K with normal secretion values, p.M640T which showed mildly reduced secretion values, and p.L15H and p.K424KfsX22 which showed undetectable secretion of the mutated protein.

In the first group, the amounts of c.-7C>G and p.R233K recombinant PS were similar to those of the wild-type PS, thus indicating similar levels of synthesis and secretion. This indicates that these mutant proteins are neutral variants of *PROS1*.[10] In the case of p.R233K this was not surprising, given the conservative nature of the amino-acid replacement.[1] The p.M640T mutation resulted in a 60% reduction of protein secretion compared to that obtained with wild-type PS but, by contrast, there was 40% more mutant PS than wild-type PS in the cell extract. This suggests that the mutant protein is normally synthesized but accumulates in the cell, probably because of a secretion and degradation defect caused by the synthesis of a structurally abnormal protein, as already postulated for the substitution of the highly conserved hydrophobic Met 640 residue of the second laminin A globular domain β-strand by a polar one.[1]

The lowest amounts of PS were obtained with mutations p.L15H, the double mutant [c.-7C>G;44T>A] and c.1272delA (p.K424KfsX22); the protein was absent or present at very low levels in both the cell extract and the cell media. These results indicate that these mutations cause quantitative PS deficiency by reduced synthesis or increased clearance of the mutant proteins. Furthermore, the fact that c.-7C>G showed normal PS secretion, indicates that PS deficiency in the [c.-7C>G;44T>A] double heterozygotes is caused by the c.44T>A (p.L15H) mutation. As recently reviewed,[1] the substitution of a polar residue for a hydrophobic one in the signal peptide compromises its properties as a signal sequence for correct membrane translocation and protein secretion. With regard to the c.1272delA mutant, we detected some truncated p.K424KfsX22 mutant protein in the cell extract. Since no mRNA expression studies could be performed, we cannot exclude that this mutation results in nonsense-mediated mRNA decay but if it does not, it would also result in quantitative PS deficiency, probably because the truncated protein is degraded intracellularly.

We also observed a good correlation between recombinant PS secretion and plasma PS levels in the heterozygotes for the mutations analyzed. Thus, while p.R233K was found in patients with normal PS levels, heterozygotes for p.M640T showed mean free (28±3.4%) and, particularly, total (65±9.5%) PS values higher than the mean values (21±9.5 for free PS and 53±13.7 for total PS) in 74 heterozygotes for mutations that result in a more severe secretion defect, such as p.L15H or c.1272delA, or that are subjected to nonsense-mediated mRNA decay. Furthermore, the fact that heterozygotes for four of these *more severe* mutations show either type I or type III PS deficiency phenotypes suggests that, at least in these pedigrees, the PS deficiency phenotype is unrelated to the *PROS1* mutation genotype.

In summary, the present results indicate that whereas the main mechanism for quantitative PS deficiency associated with missense mutations is impaired synthesis, stability or secretion of the mutated protein, mutations that result in a premature termination codon result in a quantitative PS deficiency mainly because of exclusion of the mutated allele, probably by nonsense-mediated mRNA decay.

Authorship and Disclosures

Conception and design of the study: BH, PGF, MPR and NS; acquisition and/or analysis of data: BH, XM, MCM, GN, PD, PGF, MPR and NS; drafting the article: BH, MPR and NS; critical revision of the article: all authors; all authors approved the final, submitted version of the manuscript. The authors reported no potential conflicts of interest.

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