Molecular Basis of a Hereditary Type I Protein S Deficiency Caused By a Substitution of Cys for Arg474

By Tomio Yamazaki, Akira Katsumi, Kazuo Kagami, Yoshihiro Okamoto, Isamu Sugiuira, Motohiro Hamaguchi, Tetsuhiro Kojima, Junki Takamatsu, and Hidehiko Saito

The molecular basis for a hereditary type I protein S (PS) deficiency was investigated. DNA sequence analysis in the proband showed a novel missense mutation substituting Cys (TGT) for Arg474 (CGT) that is a highly conserved amino acid residue among the related proteins. This missense mutation cosegregated with the type I PS deficiency in this family. Transient expression studies showed that the secretion of the recombinant Cys-mutant PS was markedly decreased compared with that of the recombinant wild-type PS, reproducing the observed phenotype of type I deficiency. Stable expression and pulse-chase experiments demonstrated an intracellular degradation and an impaired secretion of the recombinant Cys-mutant PS. Furthermore, the substitution of Arg474 by Ala or Glu, but not by Lys, markedly reduced the secretion of the recombinant PS mutants in transient expression studies, suggesting that a positively charged basic amino acid might be needed at residue 474 and might play a key role in the protein structure and conformation of the sex hormone binding globulin-homology domain of the PS molecule. We postulate that the loss of the highly conserved Arg474 might be responsible for the type I PS deficiency inherited in this family.

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duced using an activated partial thromboplastin time (APTT)-based clotting assay. Crossed immunoelectrophoresis was performed according to the method of Camp et al. Plasma PS, purified using barium precipitation, was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli under reducing and nonreducing conditions and then immunoblotted with a polyclonal anti-PS antibody.

DNA-based polymerase chain reaction (PCR) and sequencing of the PS active gene. All the coding exons and intron-exon boundaries of the PS active gene were amplified from the proband's genomic DNA by PCR (DNA-PCR) as described previously, except that exon 15 and the 3' noncoding region of the PS active gene was amplified with primers PSSE15 and PS3NCR (5'-CGCTGCAGTTTATTCACAGTTTGAATTG-3'). The PCR products were gel-purified, subcloned into M13mp18 or pBluescript KSII(+), and sequenced using the dyeodeoxy termination method.

Family studies. We performed family studies using mutagenic DNA-PCR followed by restriction enzyme digestion. Because the C-to-T substitution found in exon 13 of the PS gene (see Results) did not create or destroy any known restriction enzyme site, the mutagenic primer PS13E (5'-GGCATGTAAATGTGACCTTTGAATTG-3') was used to introduce a new EcoRI site (G/AATT) in the amplified DNA fragments of exon 13 from the wild-type allele (italicizing denotes the substituted nucleotides). The expected PCR product (272 bp) would be digested into 22-bp and 250-bp subfragments by EcoRI. In contrast, the C-to-T substitution at nucleotide 1689 destroys this newly created EcoRI site and the PCR product amplified from the mutated allele sequence would remain uncut after EcoRI digestion.

mRNA-based PCR and analysis of PS mRNA. To investigate whether the transcripts from the mutated allele were present in the platelets, we analyzed the proband's PS mRNA using mRNA-based PCR. Total platelet RNA was subjected to a reverse transcription reaction and subsequent PCR amplification (mRNA-PCR), as previously described. Primers PS13E and PSE14A (Table 1) were used in the PCR amplification of the reverse transcribed cDNA. The mRNA-PCR product (253 bp) was subjected to EcoRI digestion as described above. The wild-type mRNA-PCR product would yield 22-bp and 231-bp subfragments, whereas the mutated mRNA-PCR product would not be digested (253 bp).

Construction of PS cDNA clone. The full length of the coding region of PS cDNA was constructed as follows. The DNA fragment spanning exon 1 to 5 was generated by mRNA-PCR with primers PSE15-2 (5'-GCAAGCTTCTTGGACGACGCGCCACCCTCCTG-3') and PSEA6A (5'-TGAGGGATCTTCTATTTTATGG-3'), in which total platelet RNA obtained from a normal subject was used as the template. This mRNA-PCR product was digested by HindIII and then subcloned into pUC19 (designated pUCHH). The HindIII-Spe I fragment (1,926 bp) of PS cDNA clone M117S (kindly provided by Dr R. Wydro, Integrated Genetics, Framingham, MA) was used in the PCR amplification of the reverse transcribed cDNA. The mRNA-PCR product (253 bp) was subjected to EcoRI digestion and subcloned into pUC19 (designated pUCH). The HindIII-HindIII fragment

Table 1. Oligonucleotide Primers Used in a Mutagenesis of Arg474

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')</th>
<th>Remarks</th>
</tr>
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<tbody>
<tr>
<td>PS12S</td>
<td>CTCCGTCTAGATGGATGTA</td>
<td>Sense primer for outside PCR</td>
</tr>
<tr>
<td>PS14A</td>
<td>AGTGGTGTCACAACCTCCAG</td>
<td>Antisense primer for outside PCR</td>
</tr>
<tr>
<td>PSCysS</td>
<td>CTTGAATATTGTCCATTCAC</td>
<td>Sense primer for Cys</td>
</tr>
<tr>
<td>PSCysA</td>
<td>GTGGAATGACAAATTTCAAG</td>
<td>Antisense primer for Cys</td>
</tr>
<tr>
<td>PSLysS</td>
<td>AATCATTTCCGGGCGG G</td>
<td>Sense primer for Lys</td>
</tr>
<tr>
<td>PSLysA</td>
<td>ACCGGTGAGTGGTTCAATTCAAG</td>
<td>Antisense primer for Lys</td>
</tr>
</tbody>
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of pUCHH (464 bp), which spanned exon 1 to the HindIII site in exon 5, was ligated into the HindIII site of pUCHS, thereby generating the full-length coding region of the PS cDNA (designated pUCPS). The entire coding sequence was confirmed using DNA sequencing.

**Mutagenesis and construction of expression vectors.** To introduce amino acid substitutions of Cys, Ala, Glu, and Lys for Arg474 (PSR474C, PSR474A, PSR474E, and PSR474K, respectively), we used a recombinant PCR technique to alter the CGT codon encoding Arg474 to a TGT codon for Cys, a GCT codon for Ala, a GAA codon for Glu, and an AAA codon for Lys (italicizing denotes the nucleotides substituted). The primers used in the mutagenesis are listed in Table 1. The outside PCR products were digested with XbaI and SalI, subcloned into pUC19 or pBluescript KSII(+) and sequenced to confirm the presence of the desired mutations and the absence of any unexpected mutations. The normal XbaI-SalI fragment of pUCPS (423 bp) was replaced with these recombinant XbaI-SalI fragments to obtain the variant PS cDNAs. The BsrFI-XmaI fragments (2.373 bp) of the wild-type or the variant PS cDNAs, containing a 23-bp noncoding sequence 5' to the initiation codon, a 2.031-bp of the entire coding sequence, a 308-bp segment of the 3' noncoding region, and an 11-bp fragment of the multicloning site of pUC19, were excised and ligated into the XmaI site of the pED vector (kindly provided by Genetic Institute, Cambridge, MA) to construct expression vectors encoding wild-type PS, PSR474C, PSR474A, PSR474E, and PSR474K.

**Transient expression studies.** Wild-type PS and PSR474C were transiently expressed in COS-1 cells. The COS-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 500 ng/mL of vitamin K1, 1 mMol/L glutamine, 100 mg/mL of penicillin, and 100 U/mL of streptomycin. The expression vectors were transfected into COS-1 cells by the liposome-mediated method. The culture media and cell lysates were harvested 48 hours after transfection for PS measurement using an ELISA kit.

**Stable expression studies.** Wild-type PS and PSR474C were stably expressed in Chinese hamster ovary (CHO) cells. Dihydrofolate reductase-deficient CHO cells (CHO-DUKX; kindly provided by Drs B.C. Furie and B. Furie, New England Medical Center, Boston, MA) were used for the stable expression studies. Cell culture, liposome-mediated transfection, and the establishment of stable transformant clones were performed as described. Pulse-chase experiments of recombinant PS by [35S]-methionine labeling, immunoprecipitation, and electrophoresis were performed as previously described. The levels of radioactivities of the PS bands on the dried gels were measured using a Fujix BAS2000 Bio-Imaging Analyzer (Fuji Photo Film, Tokyo, Japan) as described elsewhere.

**RESULTS**

**Plasma PS assays.** The levels of PS antigen and APC cofactor activity in the family members are shown in Fig 1. The levels of total PS antigen were reduced by approximately 50% in the affected family members, who had markedly reduced levels of free PS antigen and APC cofactor activity, and also showed a decreased peak for free PS in crossed immunoelectrophoresis (data not shown). The PS profiles in these affected individuals were identical with those of a typical case of heterozygous type I PS deficiency. In SDS-PAGE, the plasma PS of the affected individuals showed the same electrophoretic mobility as the normal control and no aberrant band was observed under either reducing or nonreducing conditions (data not shown). The levels of C4BP, protein C, antithrombin, and plasminogen were within the normal range, and no evidence for APC resistance was observed in this family (data not shown).

**Sequencing of the PS gene.** DNA sequencing of the DNA-PCR products of the proband showed only a single nucleotide substitution, a C-to-T exchange, at nucleotide 1689 in exon 13 of the PS gene (data not shown). No other mutation was found in any of the exons, intron-exon junctions, or the 5' and 3' noncoding regions. The C-to-T transition changed the CGT codon to a TGT codon, which would result in the substitution of Arg474 by Cys in the homology domain of PS molecule. The proband was found to be heterozygous for this C-to-T mutation.

**Family studies.** Mutagenic DNA-PCR followed by EcoRI digestion showed an uncleaved, aberrant 272-bp band in the asymptomatic father, son, and nephew of the proband as well as in the clinically affected proband himself and his two siblings (Fig 1). The mother, wife, and daughter of the proband showed only a normal 250-bp band (Fig 1). The mutated allele represented by the 272-bp band, which carried the C-to-T substitution, was found to be inherited through three generations in this family and cosegregated with the phenotype of the type I PS deficiency. All affected individuals were heterozygous for this mutation. The same mutation was not identified in DNA samples obtained from 60 healthy Japanese (120 normal chromosomes).

**Analysis of PS mRNA.** The mRNA-PCR amplification
with primers PS13E and PSE14A followed by EcoRI digestion yielded 253-bp and 231-bp bands in the proband (Fig 2), which represented the transcripts from the mutated and the normal alleles, respectively. The intensities of both bands on the gel were almost equal, indicating the codominant expression of both alleles in the platelets of the proband.

**Transient expression studies.** In transient expression studies with COS-1 cells, the quantities of recombinant wild-type PS in the culture media and cell lysates were 683.5 ± 108.5 and 17.6 ± 4.0 (mean ± SD) ng/10^7 cells/24 h, respectively. The quantities of recombinant PSR474C, PSR474A, and PSR474E in the culture media were markedly decreased compared with those of wild-type PS, whereas recombinant PSR474K was secreted as well as wild-type PS (Fig 3). No significant difference in the amount of recombinant PS in the cell lysates was observed among wild-type PS, PSR474C, PSR474A, PSR474E, and PSR474K (Fig 3).

**Stable expression studies.** The CHO cells producing wild-type PS and PSR474C were pulse-labeled and chased for various periods of time. A quantitative analysis of the pulse-chase data showed that radiolabeled wild-type PS rapidly decreased in the cells with the half-life of 1.1 hours and immediately appeared in the culture media (Fig 4). In contrast, radiolabeled PSR474C slowly disappeared in the cells with the half-life of 3.0 hours and was gradually secreted into the culture media (Fig 4). In addition, the total amount of radioactive wild-type PS was maintained during the 8 hours of chase, whereas that of PSR474C at 8 hours decreased to 56% (Fig 4).

In SDS-PAGE under nonreducing conditions, radiolabeled PSR474C from the cells and culture media migrated as a monomer (Fig 5). The molecular weight of PSR474C was apparently higher than that of wild-type PS under nonreducing conditions; however, there was no difference in their molecular weights when the samples were reduced (Fig 5).

**DISCUSSION**

We have identified a single base exchange, a C-to-T transition, in exon 13 of the PS gene of a patient with a hereditary type I PS deficiency. This mutation results in the substitution of Arg474 (CGT) by Cys (TGT). No other nucleotide alteration was identified in the rest of the coding region, splice junctions, and 5' and 3' noncoding regions of the PS gene of the proband. A gross gene abnormality was ruled out by Southern blot analysis, as previously reported. Family studies by mutagenic DNA-PCR and EcoRI digestion showed the cosegregation of this missense mutation with the phenotype of the type I PS deficiency in family members (Fig 1). It seems very likely that the C-to-T substitution in exon 13 is the causative mutation of the type I PS deficiency inherited in this family. All affected members in this family were found to be heterozygous for this missense mutation. Family studies proved that the proband's mother did not have the C-to-T mutation and that no one was homozygously affected in this family, even though the proband's parents were first cousins (Fig 1).

The mRNA-based analysis showed that transcripts from the mutated allele carrying the C-to-T mutation in exon 13 apparently were present in the platelets of the proband (Fig 2). Both transcripts from the normal allele and the mutated allele were almost codominantly expressed, indicating that this missense mutation does not affect the accumulation of the mutated mRNA and that the type I PS deficiency is not due to a defect in the steps before mRNA expression.

To verify that the substitution of Arg474 by Cys (PSR474C) does indeed give rise to the observed phenotype, expression vectors bearing wild-type PS and PSR474C
cDNA were transfected into COS-1 cells or CHO cells. Such mammalian expression systems allow one to assess the structural as well as functional consequences of the mutation on the protein’s biosynthesis. In transient expression studies in COS-1 cells, the amount of PSR474C secreted into the culture media was markedly decreased compared with that of wild-type PS (Fig 3), reproducing the phenotype of the type I deficiency. The identical result was obtained when CHO cells were used in transient expression studies (data not shown). The stable expression and pulse-chase experiments in CHO cells showed that the half-life of PSR474C in the cells (3.0 hours) was longer than that of wild-type PS (1.1 hours; Fig 4), showing impaired intracellular transport or processing of PSR474C in the secretion pathway. Furthermore, the decrease in the total amount of radioactive PSR474C indicated its intracellular degradation. These results clearly showed that the substitution of Arg474 by Cys causes the type I PS deficiency.

Under nonreducing conditions, PSR474C synthesized by CHO cells had an apparently higher molecular weight than did wild-type PS; however, there was no difference in their molecular weights when the samples were reduced (Fig 5), suggesting a formation of disulfide bonds between an unpaired Cys of PSR474C and a small molecular weight protein or an improper folding of PSR474C in which an additional Cys at residue 474 might be involved. The plasma of the proband and affected family members did not contain such an abnormal size PS by immunoblotting (data not shown), indicating that this PS mutant with a higher molecular weight was not present in detectable levels in their plasma. Although it is not clear, of course, whether this aberrant molecule is actually synthesized in vivo, the observation that there was no evidence for this variant PS in the plasma suggested its early degradation in the circulation or its impaired secretion from PS-producing cells in vivo. Further investigations are needed to solve this interesting problem.

By aligning the amino acid sequences of residue 474 and the flanking regions of the human PS with those of other...
Fig 6. Multiple alignment of human PS, bovine PS, mouse PS, rabbit PS, porcine PS, monkey PS, human SHBG, rat androgen binding protein (ABP), human Gas6, mouse Gas6, human laminin, and human merosin. Identities between mammalian PS and the other related proteins are denoted by open boxes. Arg474 of mammalian PS and its corresponding residue in the other related proteins are indicated by the shaded box.

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Molecular basis of a hereditary type I protein S deficiency caused by a substitution of Cys for Arg474

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