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REVIEW ARTICLE

Pathophysiology of the antiphospholipid syndrome: roles of anticardiolipin antibodies in thrombosis and fibrinolysis

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Abstract

Antiphospholipid antibodies (aPL) (anticardiolipin antibodies and lupus anticoagulant) are associated with thrombosis and pregnancy morbidity, the antiphospholipid syndrome (APS). Despite the clear association between aPL and those manifestations, the precise underlying disease mechanisms remain unclear. APL may affect the normal procoagulant and anticoagulant reactions occurring on cell membranes, and also may interact with certain cells, altering the expression and secretion of procoagulant substances. In this article, we review the immunological characteristics of anticardiolipin antibodies and their potential effects on the coagulation and fibrinolytic systems implicated in the development of thrombotic complications in patients with APS.

Key words: anti-β2-glycoprotein I antibodies, antiphospholipid antibodies, coagulation, nicked β2GPI, phospholipid.

INTRODUCTION

Antiphospholipid antibodies (aPL) are a heterogeneous group of circulating immunoglobulins arising in a wide range of infectious and autoimmune diseases. Since the early 1980s, the interest in anticardiolipin antibodies (aCL) has exponentially increased due to their association with thrombosis. The antiphospholipid syndrome (APS) was defined as a clinical disorder characterized by thrombosis and pregnancy morbidity associated to the persistent presence of aCL and/or lupus anticoagulant (LA).[1]

Thrombosis is the major manifestation in patients with aPL, but the spectrum of symptoms and signs associated with aPL has considerably broadened, and other manifestations such as thrombocytopenia, non-thrombotic neurological syndromes, psychiatric manifestations, livedo reticularis, skin ulcers, haemolytic anaemia, pulmonary hypertension, cardiac valve abnormality and atherosclerosis, have also been related to the presence of these antibodies.

Numerous mechanisms have been proposed to explain the thrombotic tendency of patients with aPL, but the pathogenesis seems to be multifactorial[2–9] (Table 1).

In this paper, we review the immunological characteristics of aCL, their potential effects on coagulation and fibrinolysis, and their cell interactions, as possible pathogenic mechanisms implicated in the development of thrombotic complications in patients with APS.

Table 1 Proposed mechanism of antiphospholipid antibody-mediated thrombosis

1	Inhibition of endothelial cell prostacyclin production
2	Procoagulant effects on platelets
3	Impairment of fibrinolysis
4	Interference with the thrombomodulin-protein S-protein C pathway
5	Induction of procoagulant activity on cells (endothelial cells or monocytes)

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ANTICARDIOLIPIN AND ANTIβ2-GLYCOPROTEIN I ANTIBODIES

Anticardiolipin antibodies were detected using immunoassays with solid phase cardiolipin as a putative antigen.[10,11] However, antibodies directed to phospholipid-binding plasma or serum proteins, in particular β2-Glycoprotein I (β2GPI), are present in serum samples and can be components of the sample dilutent and blocking buffer in various types of immunoassay systems.

β2GPI-dependent aCL represents a predictor of future stroke and myocardial infarction in men, and are predictors of arterial thrombosis in patients with APS.[12-14] Also, antiβ2GPI antibodies have been often associated with venous thrombosis.[15] In contrast, aPL associated with infectious diseases binds directly to these negatively charged phospholipids, showing little association with thrombosis.[16-19]

Target antigen for anticardiolipin antibodies

β2GPI is a 50-kDa protein present in normal human plasma at approximately 200 μg/mL. The complete amino acid sequence of human β2GPI, reveals a single polypeptide chain composed of 326 amino acid residues with five oligosaccharide attachment sites.[20] β2GPI is composed of five homologous motifs of approximately 60 amino acids and contains highly conserved cysteines, prolines, and tryptophans. The motif is characterized by a framework of four conserved half-cysteine residues related to the formation of two internal disulphide bridges. These repeating motifs were designated as short consensus repeats (SCR) or as sushi domains. The fifth domain of β2GPI is a modified form that contains 82 amino acid residues and six half cysteines.

In 1999, two individual groups crystallized human β2GPI and characterized its tertiary structure.[21,22] The structure of β2GPI, which is highly glycosylated protein, showed an elongated fish-hook-like arrangement of the globular SCR domains. Both of these crystal analyses consistently indicate that half of domain V deviates strongly from the standard fold, as observed in domain I to IV.

β2GPI binds to solid phase phospholipids through a major phospholipid binding site located in the fifth domain, C^{281}KNKEKKC^{288} close to the hydrophobic loop[23] and the phospholipid binding property is significantly reduced by cleavage of one particular site (K317-T318) on the domain V of β2GPI (nicked β2GPI). This cleavage is generated by factor Xa or by plasmin, with plasmin being more effective.[24]

Anti-β2Glycoprotein I antibodies

The mechanisms by which antiβ2GPI antibodies bind to β2GPI is unclear. It has been proposed that one antibody must bind two β2GPI molecules to obtain considerable avidity, the 'dimerization theory'.[25,26] Another theory is based on the recognition of a cryptic epitope on β2GPI by antiβ2GPI antibodies. This cryptic epitope is only exposed when β2GPI interacts with a lipid membrane composed of negatively charged phospholipids or when adsorbed on a polyoxygenated polystyrene plate treated with γ-irradiation or electrons.[27] Consistent with this story, some reports showed the binding of aPL to β2GPI adsorbed on various commercially available oxidatively modified polystyrene plates, on a nitrocellulose membrane or on an experimentally γ-ray/UV-irradiated polystyrene plate.[28,29] Moreover, an epitope for aPL is exposed on the β2GPI molecule modified with glutaryldialdehyde.[30]

The location of the epitope(s) on β2GPI for antiβ2GPI antibodies from APS patients has been largely discussed. Anti-β2GPI antibodies recognize different epitopes located in all five domains. In 1996, Igarashi *et al.*[31] firstly reported that domain IV or I are candidates for epitopic location on the β2GPI molecule by using a series of deletion mutant proteins of β2GPI. Wang *et al.*[32] showed the presence of antiβ2GPI antibodies directed to domain V. George *et al.*[33] demonstrated that domain IV of β2GPI is one of the major epitopic locations for aCL raised in APS patients. In contrast, Iverson *et al.*[34] reported that antiβ2GPI antibodies, in the major population of APS patients, recognized a particular structure in domain I of β2GPI, and antibody binding was diminished by replacement of a related amino acid locations in the domain. However, it was also shown that some particular mutations made in domain IV also affected antibody binding to β2GPI in antiβ2GPI antibody enzyme-linked immunosorbent assay.[35] Recently, it has been shown that pathogenic aPL binds a cryptic epitope on domain I of β2GPI (G40-R43). This epitope is accessible for aPL only after conformational change which is induced by the binding of β2GPI to a negatively charged surface via a positive-charge patch in domain V of β2GPI.[36,37] Our group revealed that epitopic structures recognized by anti-β2GPI antibodies are cryptic and that electrostatic interaction between domain IV and V are involved in their exposure.[38]

PROPERTIES OF β2-GLYCOPROTEIN I

The *in vitro* properties of β2GPI as a natural procoagulant/anticoagulant regulator have been proposed in a large number of publications, but the specific *in vivo*

role of β2GPI in coagulation remains unknown. Some of the possible mechanisms include the inhibitory effect of β2GPI in the phospholipid-dependent reactions[39-42] (Table 2). But apart from specific haemostatic functions, β2GPI has been suggested to activate lipoprotein lipase, to lower triglyceride levels, to bind to oxidized low-density lipoprotein, to prevent the progression of atherosclerosis, and to bind to non-self particles or apoptotic bodies to allow their clearance.

Despite the regulatory functions of β2GPI in the coagulation cascade *in vitro*, familial deficiency of β2GPI does not represent a risk factor for thrombosis.[43,44] Moreover, plasma levels of β2GPI have been reported to be either normal or elevated in patients with APS.[45,46] Therefore, aCL-associated thrombosis cannot be explained as a result of a secondary β2GPI deficiency, but it is likely that anti-β2GPI antibodies modify the roles of β2GPI.

Shen *et al.*[47] generated β2GPI knockout mice using a homologous recombination approach. When β2GPI heterozygotes on a mixed genetic background were intercrossed, only 8.9% of the resulting 336 offspring possessed both disrupted alleles. Although phenotypes of β2GPI knockout mice were apparently normal, they possessed an impaired *in vitro* ability to generate thrombin relative to wild-type mice, suggesting some roles of β2GPI in thrombin-mediated coagulation.

The nicked form of β2GPI has attracted little attention, since its phospholipid-binding activity was thought to exert the physiological or pathological functions of β2GPI. However, the generation of a nicked form of β2GPI *in vivo* on the surface of activated endothelial cells or platelets may regulate the properties of β2GPI. Plasma levels of nicked β2GPI have been implicated in the pathogenesis of thrombosis. Nicked β2GPI was detected in plasma of patients with disseminated intravascular coagulation and with ischemic stroke.[48,49] The inhibitory effect of nicked β2GPI in extrinsic fibrinolysis has been reported by our group.[49] Lopez-Lira *et al.*[50] demonstrated that the interaction between intact β2-glycoprotein I and glu-plasminogen can participate in plasmin generation both at fibrin and cellular surfaces.

Table 2 Properties of β2-glycoprotein I *in vitro*

Anticoagulant
1 Inhibition of prothrombinase activity (thrombin generation)
2 Inhibition of factor X activation
3 Inhibition of platelets aggregation
Procoagulant
1 Inhibition of protein C activation
2 Inhibition of inactivation of factor Va by activated protein C
3 Inhibition of protein Z anticoagulant pathway.

PATHOGENESIS OF ANTICARDIOLIPIN/ ANTI-β2GPI ANTIBODIES

Experimental models of APS have been developed to investigate the physiologic and pathologic roles of aCL/ antiβ2GPI antibodies in thrombotic complications. Hashimoto *et al.*[51] were the first to report that NZW × BXSB (WB) F1 male mice produce autoantibodies against cardiolipin and that the aCL titre increases with age. Those aCL were β2GPI-dependent and the mice were prone to have myocardial infarction, therefore one of the animal models of APS.

Several studies have shown that aCL derived from both human and murine sources can cause fetal loss in mice when the antibodies were administered either passively or induced actively by immunization with aCL.[52-54] Immunization of animals with human β2GPI induces antibodies that resemble aPL from APS patients, regarding binding characteristics and pathogenic properties.[55-59] In addition, Pierangeli *et al.*[60] tested human affinity-purified aPL from APS patients in a mouse model of microcirculation using the cremaster muscle that allows direct microscopic examination of thrombus formation. They showed that 5/6 aPL enhanced thrombus size and all the six aPL delayed the time of thrombus disappearance, suggesting that the prothrombotic state found in patients with APS may be the result of aPL acting on the endothelium.

Using a photochemically induced model of thrombosis, Jankowski *et al.*[61] revealed that certain aPLs enhance arterial thrombosis by forming β2GPI bivalent complexes with affinity for aPL and that this prothrombotic action is Fc independent.

ROLES OF ANTICARDIOLIPIN ANTIBODIES IN THROMBOSIS

The association between aPL and the occurrence of thrombosis is widely recognized. The effect of aCL in the inhibition of the natural anticoagulant systems, the impairment of fibrinolytic activity and the direct effect of these antibodies on cell functions are proposed mechanisms to explain the thrombotic tendency of patients with aCL.

Interference with the coagulation pathway

Binding of anti-β2GPI antibodies remarkably increases the affinity of β2GPI for negatively charged phospholipids.[62]

Increment of the affinity may modify the physiological function of β2GPI, or inhibit the binding of other phospholipid-binding proteins. Therefore, thrombosis in patients with aCL may be explained, in part, by the interference of aCL with the natural anticoagulant systems, protein C or protein Z.

Protein C system and anticardiolipin antibodies

The protein C system is one of the most important antithrombotic pathways mediated by the vessel wall. Thrombin-thrombomodulin complex activates protein C, leading to the clotting inhibition by proteolytic cleavage of factors Va and VIIIa in the presence of protein S. The impairment of the anticoagulant protein C system can result in the development of thrombosis. The fact that protein C and its cofactor protein S are phospholipid-binding plasma proteins, has made this system one of the most likely to be involved in the pathogenesis of thrombosis in patients with APS.

The interaction between aPL and protein C system has been demonstrated in several studies. APL may inhibit phospholipid-dependent reactions of the protein C pathway in different ways. First, purified aPL have been shown to interfere with the activation of protein C by the thrombin/thrombomodulin complex.[63] Inhibition of thrombin formation by aPL could paradoxically cause a prothrombotic tendency due to insufficient protein C activation.

Second, the proteolytic effect of activated protein C on factors Va/VIIIa can be inhibited by aPL. A reduction of factor Va degradation was found in plasma of patients with LA.[64] Malia *et al.*[65] confirmed the inhibitory effect of IgG purified from patients with aPL on factor Va degradation by activated protein C, and it was also reported that purified IgG/M from aPL positive patients disturb the anticoagulant activity of activated protein C on human endothelial cells.[66] Our group demonstrated that human monoclonal anti-β2GPI antibodies inhibit activated protein C function, confirming the effect of autoimmune aPL on this system.[42] We also showed that aCL may bind to protein C in the presence of both phospholipids and anti-β2GPI antibodies, and that binding activities strongly correlated with anti-β2GPI antibody titres, thus protein C could be a target of aCL leading to protein C dysfunction.[67]

Finally the cofactor effect of protein S in the protein C pathway can be affected. Decreased plasma levels of protein S have been found in patients with APS[4,68] and it was reported that some of the IgG which inhibit factor Va degradation were not directed only against phospholipid-bound protein C but also against phospholipid-bound protein S[5].

Protein Z and anticardiolipin antibodies

Some research works focused on the inhibition of protein Z natural anticoagulant pathway as an additional thrombotic mechanism in APS. However, the physiopathological relation between low plasma protein Z and risk of thrombosis and pregnancy morbidity is far from clear and requires further investigation.

Protein Z is a vitamin K dependent protein that functions as a natural anticoagulant by acting as cofactor for a plasma protease inhibitor for the inhibition of factor Xa.[69] Deficiency in protein Z levels has been found in patients with aPL[70,71] and was associated with arterial thrombosis and fetal loss.[72-75] Forastiero *et al.*[76] showed that aPLs greatly impair the inhibition of factor Xa by protein Z-dependent protease inhibitor in the presence of β2GPI. It is possible that β2GPI–anti-β2GPI antibody complexes compete with protein Z-protease inhibitor-factor Xa for the same phospholipid-binding site, thus interfering with the factor X activation.

Impairment of fibrinolysis by anticardiolipin antibodies

Fibrinolytic reactions involve the formation of plasmin generated from plasminogen and the hydrolytic cleavage of fibrin to fibrin degradation products by plasmin. Plasmin is one of the most potent enzymes, thus the regulation of plasmin generation and activity is highly important to maintain the homeostatic balance *in vivo*. The intrinsic fibrinolysis pathway is mediated by plasminogen pro-activator of blood, pro-urokinase and contact activation components. Factor XIIa triggers this intrinsic fibrinolysis pathway, as well as the intrinsic coagulation. Plasmin formation from plasminogen by tissue plasminogen activator (tPA) is a key event in extrinsic fibrinolysis for the thrombolysis against intravascular blood clots. Endothelial cells, when activated, secrete the inhibitor of plasminogen activator-1 (PAI-1), as well as releasing tPA, to depress fibrinolysis by blocking tPA activity.

Impaired fibrinolysis is a contributing factor for the development of thrombosis, and the effect of aPL in the fibrinolytic system has been investigated, with controversial results probably due to the small size of the cohorts and heterogeneity of subjects. Jurado *et al.*[77] suggested a hypofibrinolytic condition in patients with connective tissue diseases including APS, mainly related to increased PAI-1 levels. In this study, PAI-1 release by endothelial cell stimulation after venous occlusion was greatly enhanced in patients compared with healthy donors, but no difference was found in tPA release. To

avoid the influence of the acute phase nature of PAI-I, Ames *et al.*[4] focused this issue in aPL positive patients without underlying rheumatic diseases. They confirmed the up-regulation of PAI-I levels in female patients with APS and further showed reduced tPA release by endothelial cell stimulation in those subjects, suggesting that tPA/PAI-1 balance was crucial to develop thrombosis in some APS patients.

Several other reports pointed toward a hypofibrinolytic state in APS characterized by elevated PAI-1, suggesting a perturbation of endothelial cells with consequent fibrinolytic impairment, but no direct evidence was reported in the induction of PAI-1 by aPL.[78-80] Antibodies specifically interacting with the catalytic domain of tPA have been found in APS patients, representing a possible cause of hypofibrinolysis.[81]

The effect of lipoprotein (a) [Lp(a)] on the fibrinolytic system has been evaluated. Lp(a) is an apoprotein which shares some sequence homology with plasminogen. Lp(a) inhibits fibrinolytic activity not only by acting as an uncompetitive inhibitor of tPA,[82] but also by increasing PAI-1 expression in endothelial cells.[83] This behaviour confers Lp(a) a prothrombotic potential. Elevated plasma Lp(a) levels in patients with APS have been reported.[84,85] We analysed the relationship between Lp(a) levels and fibrinolytic state showing lower plasma D-dimer but higher PAI-1 levels in patients with elevated Lp(a).[84] These data suggested that the deranged fibrinolysis was related to Lp(a) in patients with APS. Plasma Lp(a) is genetically determined, and it is possible that high Lp(a) levels in patients with APS might account for the impairment of fibrinolysis independently of the presence of aPL.

On the other hand, *in vitro* studies evaluated the direct effect on aPL in the fibrinolytic system. The intrinsic fibrinolytic pathway has been researched and we showed that β2GPI and monoclonal anti-β2GPI antibody suppress intrinsic fibrinolytic activities.[86] This inhibition of fibrinolysis was attributed to a reduced contact activation reaction initiated by factor XIIa.

The *in vitro* effect of β2GPI and monoclonal aCL in the extrinsic fibrinolysis was also investigated. β2GPI, even in the absence of phospholipids, regulated extrinsic fibrinolysis by inhibition of PAI-1 activity but did not directly affect tPA activity. Furthermore, monoclonal aCL inhibit the effect of β2GPI, that is, those monoclonals inhibited the fibrinolytic activity by enhancing PAI-1 activity.[87]

aPL with different specificities have been related to the impairment of the fibrinolytic system in patients with APS. Kolev *et al.*[88] demonstrated that IgG isolated from patients with APS impairs the fibrin dissolution of fibrin with plasmin. It has also been suggested that antibodies that cross-react between prothrombin and plasminogen hinders fibrinolysis and promotes thrombus formation.[89] In conclusion, there is some evidence that modulation of fibrinolysis by aCL may be associated with the prothrombotic tendency found in some patients with these antibodies.

Cellular interaction and anticardiolipin antibodies

β2GPI binds not only to negatively charged substances but also to surface membranes of cell types directly involved in the pathogenic mechanism of the APS, such as activated platelets and endothelial cells.[90-96] In fact, Del Papa *et al.*[96] showed that β2GPI binds to endothelial cells throughout its fifth domain.

Binding of aPL to endothelial cells induces cell activation, triggering a prothrombotic state that could contribute to the hypercoagulability associated with APS. We and others have shown that aPL induces tissue factor activity, antigen or mRNA on endothelial cells as well as on monocytes.[6,7,9] In addition, human monoclonal aCL induced prepro-endothelin-1 mRNA levels, confirming the direct effect of aPL on endothelium regarding endothelin-1 production. These data suggested that endothelin-1 production induced by aPL may play an important role in altering arterial tone and probably contributing to arterial occlusion.[97]

Membranes of activated platelets are an important source of negatively charged phospholipids, which provides a catalytic surface for blood coagulation. Factor Xa and thrombin are generated by the tenase and prothrombinase complexes, respectively, via the catalytic surface of activated platelets and procoagulant microparticles shed by platelet activation. It has been demonstrated that dimeric β2GPI bind to anionic phospholipids exposed on activated platelets and interacts with apolipoprotein E receptor 2, a member of the lipoprotein receptor family present on platelets.[98] Shi *et al.*[91] reported that β2GPI inhibits the generation of factor Xa by activated platelets and that aCL (anti-β2GPI antibodies) interfered with this inhibition.

Great interest has arisen on the signal transduction mechanisms implicated in the induction of procoagulant substances by aPL. The adapter molecule myeloid differentiation protein (MyD88)-dependent signalling pathway and the nuclear factor kappa B (NFκB) have been involved in endothelial cell activation.[99-102] Several groups reported the crucial role of p38 mitogen activated protein kinase (MAPK) in cell activation mediated by

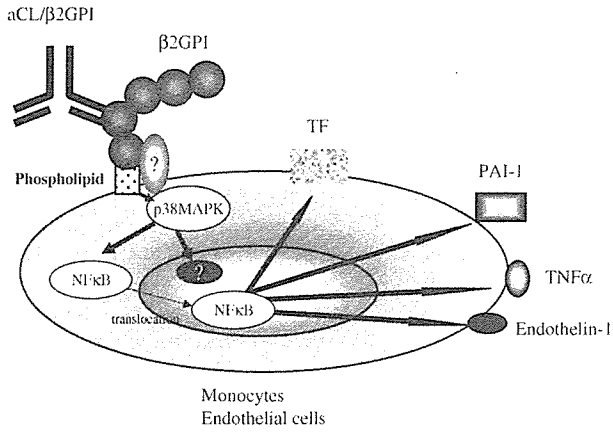

Figure 1 Proposed function of antiphospholipid antibodies on monocytes or endothelial cells. Stimulation of monocytes or endothelial cells by aCL/β2GPI induces p38 MAPK phosphorylation which leads to the nuclear translocation of NFκB and to the transcription of procoagulant substances. aCL/β2GPI, β2 Glycoprotein I dependent anticardiolipin antibodies; p38 MAPK, p38 mitogen activated protein kinase, NFκB: nuclear factor kappa B; TF, tissue factor; PAI-1, plasminogen activator inhibitor-1; TNF-α, tumoral necrosis factor α.

aPL. Our group revealed that monocytes stimulation by monoclonal anti-β2GPI antibodies derived from APS patients induce phosphorylation of p38MAPK, locational shift of NFκB into the nucleus and up-regulation of TF expression.[103] The TF expression occurs only in the presence of β2GPI, suggesting that perturbation of monocyte by anti-β2GPI antibodies is initiated by interaction between the cell and the autoantibody-bound β2GPI (Fig. 1). Almost simultaneously, Vega-Ostertag *et al.* demonstrated increased phosphorylation of p38 MAPK and production of thromboxane B2 in platelets treated with aPL.[104] The same authors also showed the involvement of p38MAPK in the up-regulation of TF in endothelial cells.[105]

Microparticle production is a hallmark of cell activation and endothelial microparticle levels have been found to be increased in patients with aPL. The production of procoagulant microparticles in APS patients may represent a new pathogenic mechanism for the thrombotic complications of this disease.[106,107]

CONCLUSION

The intensive research focused on the mechanisms of thrombosis mediated by aCL has significantly advanced our understanding of the role of these antibodies in clot formation. Classically, APS was considered as a form of coagulopathy because of the interference of aPL in normal coagulation and fibrinolytic reactions. Then, researchers turned upon the function of endothelial cells or other cells which may be modified by aPL.

In recent years, APS pathophysiology has been discussed in 'endotheliology' and in this scenario phospholipid-binding proteins, such as β2GPI, are seen as cofactors that prepare the receptors for the binding of the auto-antibodies to cells. Thus, antibodies against phospholipid binding proteins, irrespective of the functions of these proteins, may alter the properties of bound endothelial cells from antithrombotic to prothrombotic, leading to the production of procoagulant substances and to a prothrombotic state.

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One Novel and One Recurrent Mutation in the PROS1 Gene Cause Type I Protein S Deficiency in Patients With Pulmonary Embolism Associated With Deep Vein Thrombosis

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We investigated the molecular basis of type I protein S (PS) deficiency in two unrelated Japanese families, in which both probands developed pulmonary embolism associated with deep vein thrombosis. Nucleotide sequencing of amplified DNA revealed distinct point mutations in the PROS1 gene of the probands, which were designated protein S Sapporo 1 and protein S Sapporo 2. Additional mutations in the PROS1 gene were excluded by DNA sequencing of all exons and intron/exon boundaries. In the 25-year-old Japanese male patient who carried protein S Sapporo 1, we identified a heterozygous A-to-T change in the invariant ag dinucleotide of the acceptor splice site of intron f of the PROS1 gene. This mutation is a novel splice site mutation that impairs normal mRNA splicing, leading to exon 7 skipping, which was confirmed by platelet mRNA analysis. Translation of this mutant transcript would result in a truncated protein that lacks the entire epidermal growth factor-like domain 3 of the PS molecule. In a 31-year-old Japanese male and his younger brother who each carried protein S Sapporo 2, we detected a previously described heterozygous T-to-C transition at nucleotide position 1147 in exon 10 of the PROS1 gene, which predicts an amino acid substitution of tryptophan by arginine at residue 342 in the laminin G1 domain of the PS molecule. Both mutations would cause misfolding of the PS protein, resulting in the impairment of secretion, which is consistent with the type I PS deficiency phenotype. Am. J. Hematol. 81:787–797, 2006.

Key words: protein S; protein S deficiency; DNA sequencing; splice site mutation; missense mutation

INTRODUCTION

Protein S (PS) is a 69-kDa single-chain plasma glycoprotein composed of 635 amino acids. It is primarily synthesized by hepatocytes, but also by endothelial cells, monocytes, megakaryocytes, and osteoblasts [1]. PS functions as a nonenzymatic cofactor for activated protein C (APC) in the specific proteolytic inactivation of procoagulant activated factor V and activated factor VIII on phospholipid membranes [1,2]. In normal individuals, approximately 60% of PS forms a complex with C4b-binding protein (C4bBP), a regulatory component of the complement system [3], and only the remaining 40% is

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able to act as a cofactor for APC. In addition to its APC-dependent anticoagulant activity, PS also expresses APC-independent anticoagulant functions by directly inhibiting prothrombinase and tenase activities [4]. Therefore, PS acts as an anticoagulant protein and its deficiency predisposes the individual to venous thromboembolism.

PS has a multidomain structure, which enables differentiation of the functional domains that correspond to the intron/exon organization of the gene [5]. Starting from the N-terminus, the mature protein contains a vitamin K-dependent γ-carboxyglutamic acid-rich domain or Gla domain mediating $Ca2^{+}$-dependent phospholipid membrane binding (residues 1–46), a region that is sensitive to cleavage by thrombin (TSR) (residues 47–75), 4 successive epidermal growth factor-like (EGF) domains possessing high-affinity calcium-binding sites (residues 76–242), and a large C-terminal sex hormone binding globulin (SHBG)-like region containing 2 laminin G (LG)-type domains (LG1 and LG2) that mediate interactions with C4bBP (residues 243–635) [6].

The functional gene for PS is PROS1 (Mendelian Inheritance in Man No. 176880; URL: http://www.ncbi.nlm.nih.gov/omim/), which is localized close to the centromere on chromosome 3q11.2, spans over 80 kb of genomic DNA, and comprises 15 exons and 14 introns [5]. An inactive pseudogene, PROS2, showing 96.5% identity to exons 2 to 15 of PROS1, is located at 3p11.1 [7].

PS deficiency is inherited as an autosomal dominant trait. The prevalence of PS deficiency is 0.03 to 0.13% of the general Caucasian population [8], while it is estimated to be 1.12% of the general Japanese population [9]. PS deficiency is a recognized risk factor for venous thrombosis and affects 7.3% of nonselected patients with deep vein thrombosis (DVT) [10]. Patients with PS deficiency also have an eightfold increased risk of the development of secondary DVT (associated with surgery, trauma, immobilization, pregnancy, and postpartum period) [11].

Based on measurements of the total (free plus bound-to-C4BP) and free forms of PS, as well as PS activity, PS deficiency has been phenotypically classified into three subtypes. Type I deficiency is characterized by a parallel reduction in total and free PS antigen levels with low PS activity. Type II or qualitative deficiency, a very rare phenotype, is characterized by normal antigen levels and reduced PS activity due to dysfunctional PS in plasma. Type III deficiency is characterized by normal total PS antigen levels, but low free PS antigen levels and low PS activity. According to the Human Gene Mutation Database (HGMD) PROS1 database (URL: http://archive.uwcm.ac.uk/uwcm/mg/search/120721.html) and recent reports [12–15], 189 mutations have been identified thus far in the PROS1 gene, most within families with type I and/or type III PS deficiency. Although this classification has been widely used, a newly proposed classification system suggests that PS deficiency can be categorized as quantitative deficiency (old type I and type III deficiency) and qualitative deficiency (type II deficiency), as reported by the 2000 The International Society on Thrombosis and Hemostasis (ISTH) [16].

Herein, we describe two mutations in the PROS1 gene that caused type I PS deficiency in two unrelated Japanese men from Sapporo with pulmonary embolisms associated with DVT. In the first proband, an A-to-T transversion located at the second nucleotide in the invariant ag dinucleotide of the acceptor splice site of intron f was detected. This mutation, designated as protein S Sapporo 1, is a novel splice site mutation in the PROS1 gene. In the second proband as well as in his younger brother, an inherited T-to-C substitution mutation was identified at nucleotide position 1147 in exon 10 of the PROS1 gene, which is predictive of an amino acid replacement of tryptophan (Trp) by arginine (Arg) at residue 342 in the LG1 domain of PS molecule. This mutation, designated protein S Sapporo 2, is the first reported occurrence of a PROS1 gene mutation in Japan.

MATERIALS AND METHODS

Patient Profiles

Family A. The proband in Family A was a Japanese male, 25 years of age at the time of writing, who was diagnosed with DVT at 15 years old. He was admitted to a local hospital for evaluation of Cushing's disease at the age of 16 years. Diagnostic imaging at that time revealed that he had pulmonary embolism associated with DVT in both lower extremities. Hemostatic studies disclosed type I PS deficiency, while other factors showing predisposition to hereditary thrombophilia were normal. Standard heparin therapy was initiated, followed by warfarin therapy. After a permanent inferior vena cava filter was placed for prophylaxis of the pulmonary embolism, a trans-sphenoidal hypophysectomy was performed without any complications. Following discharge, the patient refused further warfarin treatment out of concern of developing a bleeding tendency. Hence, low-dose aspirin therapy was started instead of warfarin and he has not experienced additional thrombotic events to this point. His family history is positive for thrombotic episodes, as his father died from myocardial infarction

at 45 years old and his mother presented with DVT at delivery, whereas his elder brother has had no thrombotic episodes. A PROS1 gene analysis of all the family members was not consented to; therefore, the proband was the only member available for genetic investigation.

Family B. The proband in family B, a 31-year-old Japanese male, was hospitalized due to a sudden onset of pulmonary embolism associated with DVT in the right lower extremity. Hemostatic studies prior to initiation of heparin therapy identified that he had type I PS deficiency, while other factors showing predisposition to hereditary thrombophilia were normal. He was treated with standard intravenous heparin therapy in conjunction with placement of a temporary inferior vena cava filter to prevent pulmonary embolism. After the temporary inferior vena cava filter was removed, warfarin therapy was administered to maintain PT-INR at 2.5–3.0. Following discharge from the hospital, he remains under warfarin therapy and no thrombotic episodes have been seen to this point. The parents of the proband are divorced and no clinical information for his father is available. Neither the mother or younger brother of the proband has experienced an episode of thrombosis.

METHODS

Collection and Processing of Blood Samples

After receiving informed consent, venous blood samples were drawn from the patients and family members, as well as control individuals. Blood specimens from the proband of family B were taken when warfarin therapy was being given. All samples were collected in a 1/10 volume of 3.2% sodium citrate and kept frozen at −80°C until assayed. Genomic DNA was prepared from peripheral blood leukocytes using a QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

Assays for Plasma PS

The concentrations of total and free PS antigens were measured using monoclonal antibody-based enzyme-linked immunosorbent assay kits (Asserachrom total and free protein S, respectively; Diagnostica Stago, Asnières, France). PS activity was determined with a Staclot Protein S kit (Diagnostica Stago).

PCR Amplification of PROS1 Gene

All exons and intron/exon boundaries of the PROS1 gene were amplified by polymerase chain reaction (PCR) using an Expand High Fidelity PCR system (Roche Diagnostics Systems, Mannheim, Germany). Amplification was carried out in 50-μl reaction mixtures comprising 100 ng of genomic DNA, 200 μM of dNTPs, 20 pmol of each primer, 1.5 mM of $MgCl_2$, 1× Expand PCR buffer I, and 1.3 U of Expand High Fidelity PCR System enzyme mix (*Taq* DNA-*Pwo* DNA polymerase). The primers used for PCR for exons 1, 2, 9, and 12 were previously reported by Tatewaki et al. [17], those for exons 3, 5/6 (for co-amplified exon 5, intron E, and exon 6), 7, 8, and 14 were previously reported by Hirose et al. [18], and those for exons 4, 10, 11, 13, and 15 were previously reported by Simmonds et al. [19]. Each of these primers has gene specificity to anneal only to the PROS1 gene and do not amplify sequences in the PROS2 gene, except for exon 14. To remove the PCR products of exon 14 derived from PROS2, the amplified fragments were digested with *Bcl* I (Promega, Madison, WI) and separated by agarose gel electrophoresis according to the method of Iwaki et al. [20]. Undigested fragments relevant to PROS1 exon 14 were purified and then used for subsequent sequencing. The thermal profile consisted of an initial denaturing step at 94°C for 2 min, followed by 10 cycles for 15 s at 94°C, 30 s at 64°C, and 45 s at 72°C, followed by 20 cycles with the extension step increased by 5 s each time and a final elongation step at 72°C for 7 min.

DNA Sequence Analysis

PCR products were separated by electrophoresis on 2% agarose gels and purified using a QIAquick Gel Extraction Kit (Qiagen). Purified templates were sequenced using a Big Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Foster City, CA) with the same primers as used for PCR, according to the manufacturer's protocol, and the results were analyzed with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). All amplified segments were sequenced in both directions. Exon sequence numbering of the PROS1 gene was done according to the method of Schmidel et al. [5], as adopted by Biguzzi et al. [14] [PROS1 cDNA NCBI GenBank accession number NM_000313.1 (+1 corresponds to the A of the ATG translation initiation codon)]. Amino acid residues of PS were numbered according to the method presented in the 2000 ISTH protein S database [16].

PCR-Restriction Fragment Length Polymorphism (RFLP) Analysis

To detect an A-to-T transversion located on the second invariant ag nucleotide of the acceptor splice

site of intron f in the PROS1 gene of the family A proband and normal control individuals, we used a mutagenic PCR strategy for PCR-RFLP analysis (*Bgl* II PCR-RFLP analysis). PCR analyses were carried out using a sense primer (5′-CTAGAAAGG-AACATGGCAAACATCAA-3′) and a partially mismatched antisense primer (5′-GCTTCAAAGAG-CATTCATCCAGATC-3′: underlining denotes the substituted nucleotide), which introduced a *Bgl* II (New England BioLabs, Beverly, MA) recognition site into only the wild-type PCR products. Subsequently, the PCR products were digested with *Bgl* II and analyzed by 2% agarose gel electrophoresis with ethidium bromide. To detect an 1147 T-to-C mutation in the proband of family B, as well as his family members and normal control individuals, *Acc* I PCR-RFLP analyses were performed. PCR products spanning exon 10 from the DNA samples were digested with the restriction enzyme *Acc* I (New England BioLabs) and analyzed by 2% agarose gel electrophoresis with ethidium bromide.

Computer-Assisted Splice Site Prediction

To predict the result of an A-to-T transversion in the acceptor splice site of intron f in the PROS1 gene of the family A proband after PROS1 mRNA splicing, computer-assisted analysis of splice site prediction was performed using the Splice Site Prediction program provided by Neural Network (URL: http://www.fruitfly.org/seq_tools/splice.html).

PROS1 Gene Expression Analysis and PROS1 cDNA Sequence Analysis

To investigate the possibility that the A-to-T transversion identified 2 bp upstream of exon 7 in the PROS1 gene of the family A proband caused aberrant splicing of PROS1 mRNA, platelet cDNA analysis was performed. Total platelet RNA containing PROS1 mRNA was isolated from citrated blood obtained from the family A proband and normal control individuals. Total platelet RNA (2 μg) was extracted with TRIZOL Reagent (Invitrogen, Carlsbad, CA), then reverse transcribed to cDNA using SuperScript II (Invitrogen) with Oligo dT primers in a total volume of 20 μl. An aliquot of 5 μl of the cDNA reaction mixture was amplified with the primers PROS1-6F (sense primer: 5′-ACA-CCTGGAAGTTACCACTG-3′, complementary to the sequence in exon 6) and PROS1-8R (antisense primer: 5′-CCATCACAATAGCAAGTGTAACC-3′, complementary to the sequence in exon 8), which resulted in a 275-bp fragment encompassing exon 7 in the case of correct splicing. Purified products were applied for cycle sequencing in both directions using the primers PROS1-6F and PROS1-8R.

TABLE I. Total and Free Protein S Antigens and Activity Levels in Members of Two Unrelated Families with Type I Protein S Deficiency

Family member	Total PS antigen	Free PS antigen	PS activity
Family A			
Proband[a,b]	51	19	13
Mother	40	21	19
Brother	54	33	18
Family B			
Proband[a,b,c]	31	27	9
Mother[a]	102	80	72
Brother[a,b]	57	37	20
References	66–110	62–95	62–106

Note: Total and free protein S (PS) antigens and PS activity are expressed as a percentage of normal.
[a]Family member available for PROS1 gene analysis.
[b]Family member with PROS1 gene mutation.
[c]On warfarin at time of blood sampling.

RESULTS

Protein S Sapporo 1

Analysis of plasma PS phenotype in family A. Total and free PS antigens and PS activity levels of the family A proband, as well as his mother and elder brother were examined (Table I). Plasma concentrations of both total and free PS antigens were decreased (to 51 and 19%, respectively, of normal) in the proband, while plasma PS activity was reduced to 13% of normal, indicating that he had type I PS deficiency. The same pattern was found in the mother and elder brother, suggesting that they had also type I PS deficiency.

Identification of an A-to-T mutation at the second invariant nucleotide of the acceptor splice site of intron f of the PROS1 gene. PCR-amplified samples of genomic DNA from the family A proband and a normal control individual were sequenced. In the proband, a heterozygous A-to-T transversion was found at the second base in the invariant ag dinucleotide of the natural acceptor splice site of intron f (Fig. 1), which we termed protein S Sapporo 1. There were no other mutations in any of the exons or intron/exon boundaries of the PROS1 gene.

***Bgl* II PCR-RFLP analysis.** A mutagenic PCR assay followed by restriction enzyme *Bgl* II digestion revealed two bands of 230 and 25 bp from the normal allele and an undigested 255-bp band from the mutant allele (Fig. 2A). The family A proband demonstrated 2 bands of 255 and 230 bp, indicating that he carried this mutation in a heterozygous state

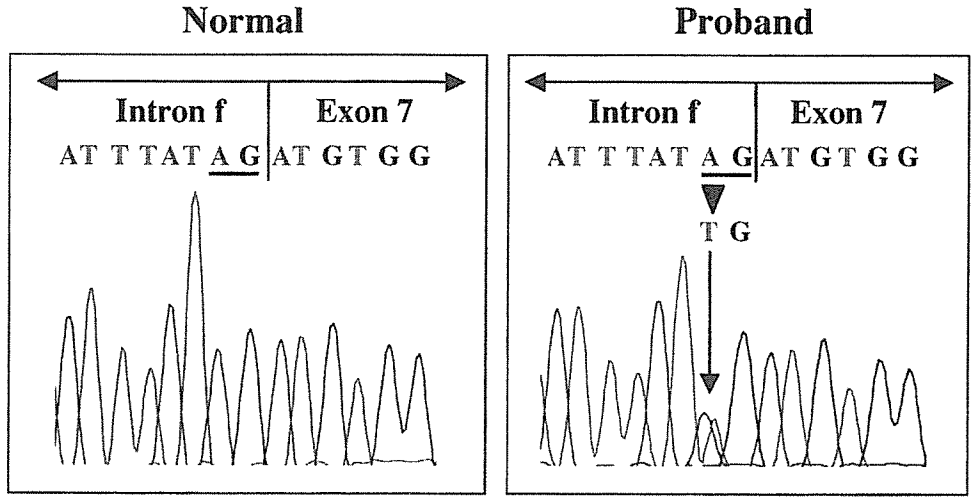

Fig. 1. DNA sequence analysis of the region around the junctions of intron f and exon 7 of the PROS1 gene from the proband of family A. PCR products encompassing the acceptor splice site of intron f of the PROS1 gene from a normal control individual and the proband were directly sequenced. In the proband, a heterozygous A-to-T transversion was found at position −2 of the invariant ag dinucleotide of the splicing acceptor site of intron f and is indicated by arrows. The consensus ag dinucleotide in the acceptor splice site is shown by an underline. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(Fig. 2B). DNA samples obtained from 50 healthy Japanese individuals (100 chromosomes) showed only a 230-bp band, demonstrating that the A-to-T mutation at the second invariant nucleotide of the acceptor splice site of intron f was not a polymorphism (Fig. 2B and data not shown).

Computer-based splice site prediction. To predict the possible effect of the mutation on PROS1 mRNA splicing, a computer-assisted splice site prediction analysis of the region surrounding the intron f acceptor splice site was performed using the Neural Network Splice Site Prediction program. The mutation was found to elicit the disappearance of an acceptor splice site (data not shown).

Platelet PROS1 mRNA expression analysis and PROS1 cDNA sequence analysis for the family a proband. To confirm the occurrence of aberrant mRNA splicing in the mutated allele of the proband, PROS1 mRNA expression analysis was performed using platelet mRNA. Reverse transcriptase (RT)-PCR products encompassing exon 7 were obtained with the primers PROS1-6F and PROS1-8R and then analyzed using 2% agarose gel electrophoresis. In the proband, an aberrant 149-bp band was detected in addition to the expected 275-bp band that was found in normal individuals (Fig. 3A). Direct sequencing of the normal sized RT-PCR product showed a normal sequence with correct splicing from exon 6 to exon 7, while that of the aberrant sized band revealed a skipping of exon 7 (Fig. 3B). This skipping in the mutant allele induced an in-frame deletion of 126-bp segments responsible for a deletion of 42 amino acids corresponding to the EGF3 domain of mature PS (Fig. 3C).

Protein S Sapporo 2

Analysis of plasma PS phenotype in family B. Total and free PS antigens and PS activity levels in plasma from the family B proband on warfarin, as well as from his mother and younger brother, were examined (Table I). The plasma concentrations of both total and free PS antigens were decreased (to 34 and 9% of normal, respectively) in the proband, while plasma PS activity was also reduced to 5% of normal, which indicated that the proband likely carried type I PS deficiency. A similar pattern was found in his younger brother, who had not received warfarin, suggesting that he had also type I PS deficiency. The mother had normal levels of total and free PS antigens, along with normal PS activity, indicating that she was a normal individual.

Identification of a T-to-C mutation at nucleotide position 1147 in PROS1 gene. PCR-amplified samples of genomic DNA from a normal control individual, as well as from the proband and his mother and younger brother, were sequenced. A single point mutation, a T-to-C transition at nucleotide number 1147, was identified in exon 10 of the PROS1 gene of the proband, which we termed protein S Sapporo 2. The proband and his younger brother were heterozygous for this mutation, which altered the TGG codon coding Trp342 to CGG coding Arg (Fig. 4 and data not shown). No other mutations were

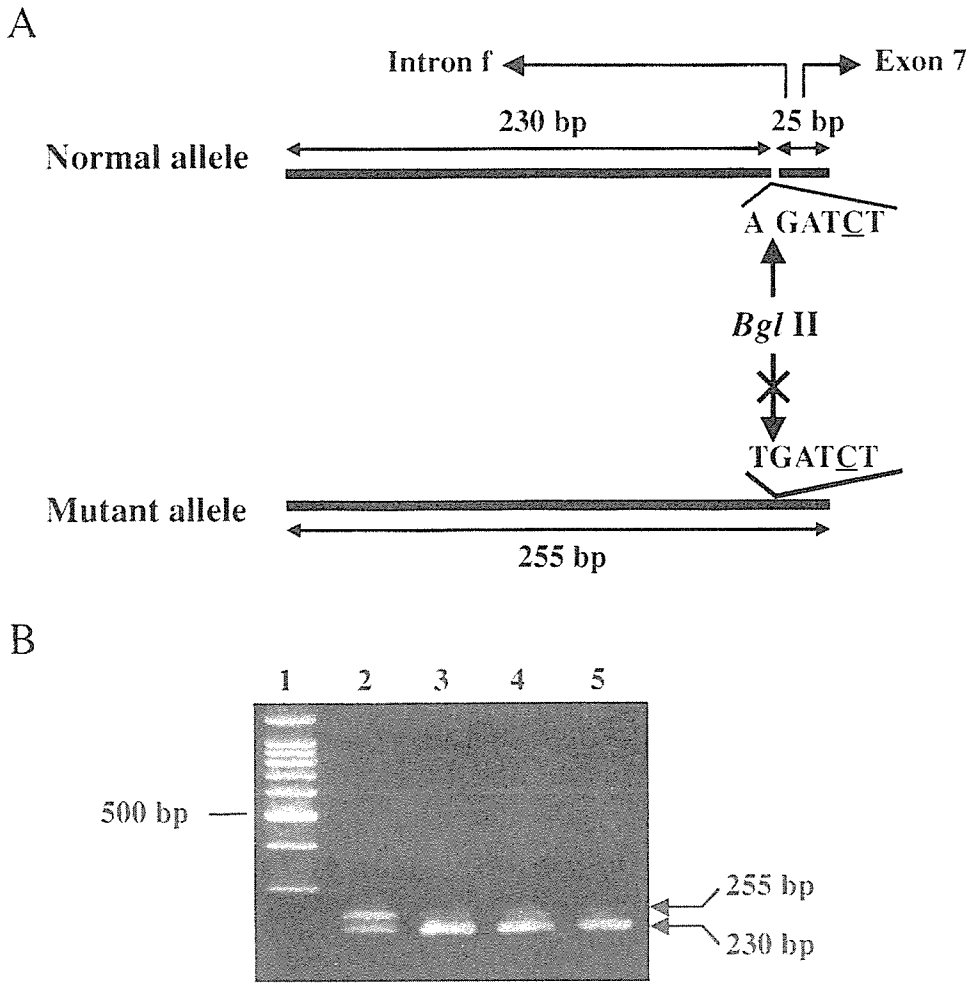

Fig. 2. *Bgl* II PCR-RFLP analysis of the proband in family A. (A) Schematic digestion pattern of PCR products encompassing the acceptor splice site of intron f of the PROS1 gene with restriction endonuclease *Bgl* II. Mismatch PCR analysis was performed using the mutated oligonucleotide designed to introduce a *Bgl* II restriction site in the normal allele, but not in the mutant allele. The amplified DNA fragment was 255 bp. The PCR fragment derived from the normal allele was cut into two fragments of 230 and 25 bp. The mutagenic PCR procedure generated a new *Bgl* II restriction site only in the wild-type PCR fragment by changing the sequence from AGATGT to AGATCT (substituted nucleotide shown by the underline). The A-to-T mutation at the –2 position of the acceptor splice site in intron f destroyed the newly created *Bgl* II recognition site and the PCR product amplified from the mutated allele remained uncut after enzyme digestion. (B) Electrophoretic patterns following *Bgl* II digestion. The proband showed two bands of 255 and 230 bp, indicating that he was heterozygous for the observed mutation. Lane 1, 100-bp ladder DNA marker; lane 2, proband; lanes 3 to 5, normal individuals.

Fig. 3. Exon 7 skipping of PROS1 mRNA from the mutant allele of the proband of family A. (A) Analysis of PROS1 mRNA expression in the proband. RT-PCR products encompassing exon 7 with the primers PROS1-6F and PROS1-8R were analyzed using 2% agarose gel electrophoresis. The RT-PCR products of normal subjects showed only a single band of the expected size (275 bp). In the proband, in addition to the expected band, an aberrant smaller band (149 bp) was also observed. Lane 1, 100-bp ladder DNA marker; lanes 2 and 3, normal individuals; lane 4, proband. (B) Nucleotide sequences of the normal sized and aberrant RT-PCR products detected in the proband. The normal sized RT-PCR product was found to carry only the normal sequence, while sequencing of the aberrant RT-PCR product revealed a skipping of exon 7. (C) Schematic diagram indicating exon 7 skipping induced by an A-to-T substitution of the second base of the invariant ag dinucleotide of the acceptor splice site of intron f in the proband. The skipping of exon 7 in the mutated allele resulted in an in-frame deletion of 126-bp segments, i.e., the deletion of 42 amino acids that construct the EGF3 domain of the PS molecule. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

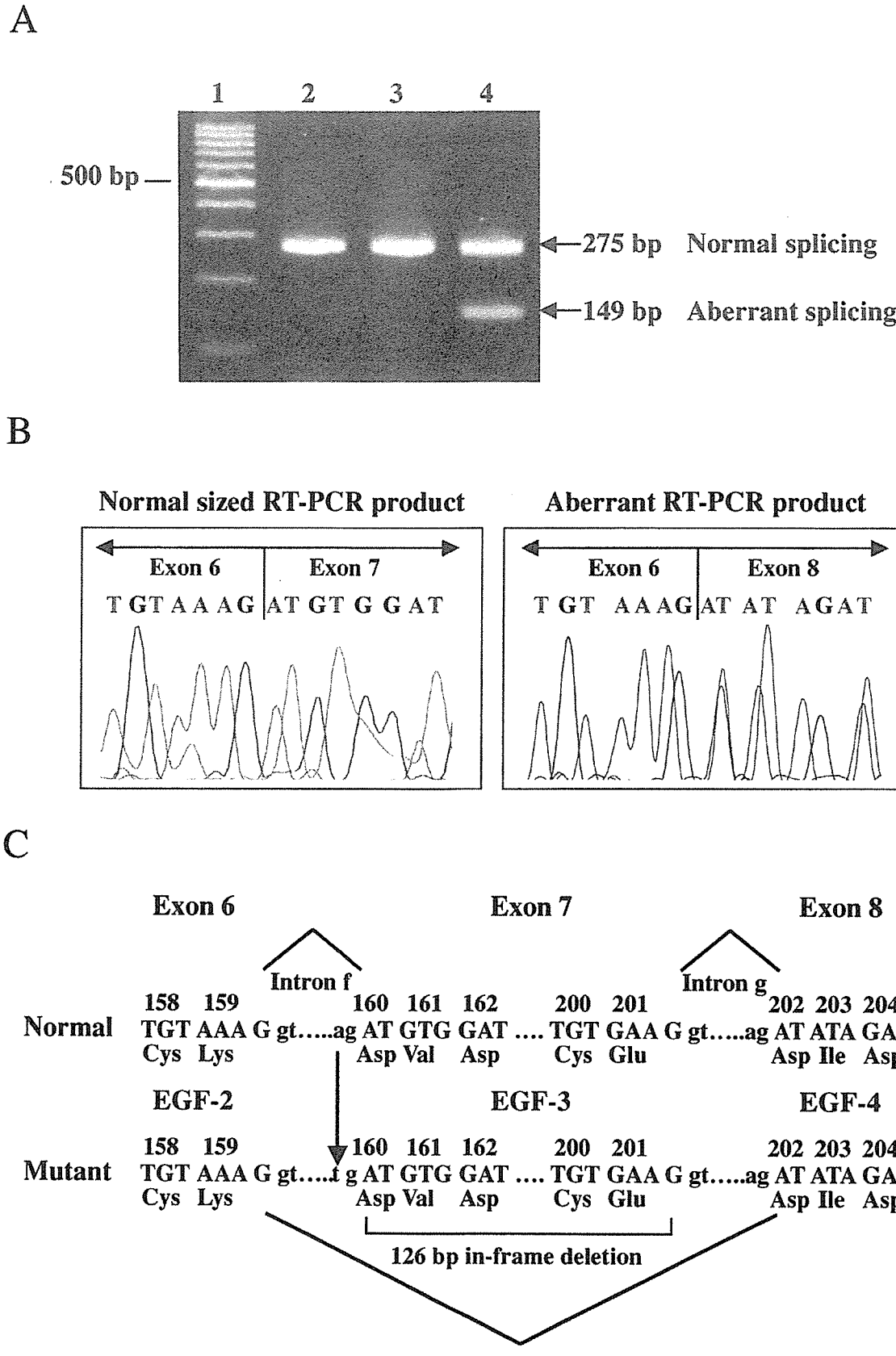
A
1
2
3
4
500 bp
275 bp Normal splicing
149 bp Aberrant splicing
B
Normal sized RT-PCR product
Aberrant RT-PCR product
Exon 6
Exon 7
T GT A A AG AT GT G G AT
Exon 6
Exon 8
T GT AAAG AT AT AGAT
C
Exon 6
Exon 7
Exon 8
Intron f
Intron g
158 159 160 161 162 200 201 202 203 204
Normal
TGT AAA G gt.....ag AT GTG GAT .... TGT GAA G gt.....ag AT ATA GAT
Cys Lys Asp Val Asp Cys Glu Asp Ile Asp
EGF-2
EGF-3
EGF-4
158 159 160 161 162 200 201 202 203 204
Mutant
TGT AAA G gt.....t g AT GTG GAT .... TGT GAA G gt.....ag AT ATA GAT
Cys Lys Asp Val Asp Cys Glu Asp Ile Asp
126 bp in-frame deletion

Figure 3.

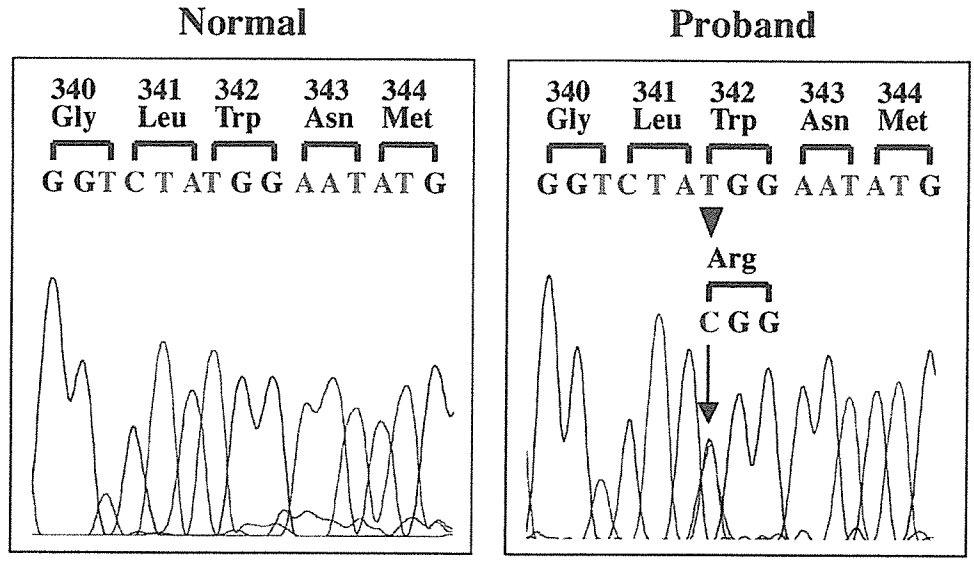

Fig. 4. DNA sequence analysis of exon 10 of the PROS1 gene in the proband of family B. PCR products spanning exon 10 of the PROS1 gene from a normal control individual and the proband were directly sequenced. In the proband, a heterozygous T-to-C transition at nucleotide position 1147, changing Trp to Arg at residue 342 of the mature PS protein, was observed. The position of the substitution is indicated by arrows. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

detected in the other exons or intron/exon boundaries of the PROS1 gene.

***Acc* I PCR-RFLP analysis.** *Acc* I digestion of the amplified products spanning exon 10 revealed an undigested 279-bp band from the normal allele, and cleaved 229- and 50-bp bands from the mutant allele (Fig. 5A). The proband and his younger brother demonstrated two bands of 279 and 229 bp, showing that they were heterozygous for the observed mutation (Fig. 5B). We also screened 100 alleles from 50 normal Japanese individuals for this mutation using *Acc* I cleavage of the amplified exon 10. None showed the 229-bp fragment, demonstrating that the T-to-C mutation at nucleotide position 1147 was not a polymorphism (Fig. 5B and data not shown).

DISCUSSION

The genetic basis of two type I PS deficiency patients from two unrelated families residing in Sapporo was analyzed by DNA sequencing and PCR-RFLP. In both patients, a normal allele and a mutant allele with a single base substitution were identified, confirming heterozygosity of the PS deficiency in affected individuals.

In the proband of family A, DNA sequencing showed a heterozygous single base mutation that affected the acceptor splice site in the intron f/exon 7 boundary, where the consensus ag sequence was replaced by tg (Fig. 1) and designated it as protein S Sapporo 1. Protein S Sapporo 1 is a novel mutation of the PROS1 gene that is not described in the 2000 ISTH database of PS mutations [16], the HGMD PROS1 database, or recent reports [12–15]. The proband, as well as his mother and elder brother, were phenotypically diagnosed with type I deficiency (Table I). Therefore, the mutation in the proband appeared to be inherited from his mother and by the elder brother, although genetic analysis of the family members was not performed. It is unlikely that this mutation is associated with a polymorphism, since it was not identified in the PROS1 gene in samples from 50 healthy individuals examined by *Bgl* II PCR-RFLP analysis (Fig. 2B and data not shown).

Ten mutations in the PROS1 gene, which affect the invariant dinucleotide of the intron donor or acceptor splice sites, have been described to date [12–14,16]. The majority result in a cryptic splice site activation and/or exon skipping, although a failure of PROS1 mRNA expression from the affected allele (allelic exclusion) has been reported in three cases [21–23]. Since the mutant acceptor splice site of intron f was not detected by the Neural Network Splice Site Prediction program, we speculated that the identified mutation would cause aberrant mRNA splicing. To investigate the effect on mRNA splicing by the mutant allele in the proband, we performed PROS1 mRNA analysis using platelet mRNA. RT-PCR products encompassing exon 7 demonstrated two bands of the expected and smaller sizes, suggesting that aberrant PROS1 mRNA splic-

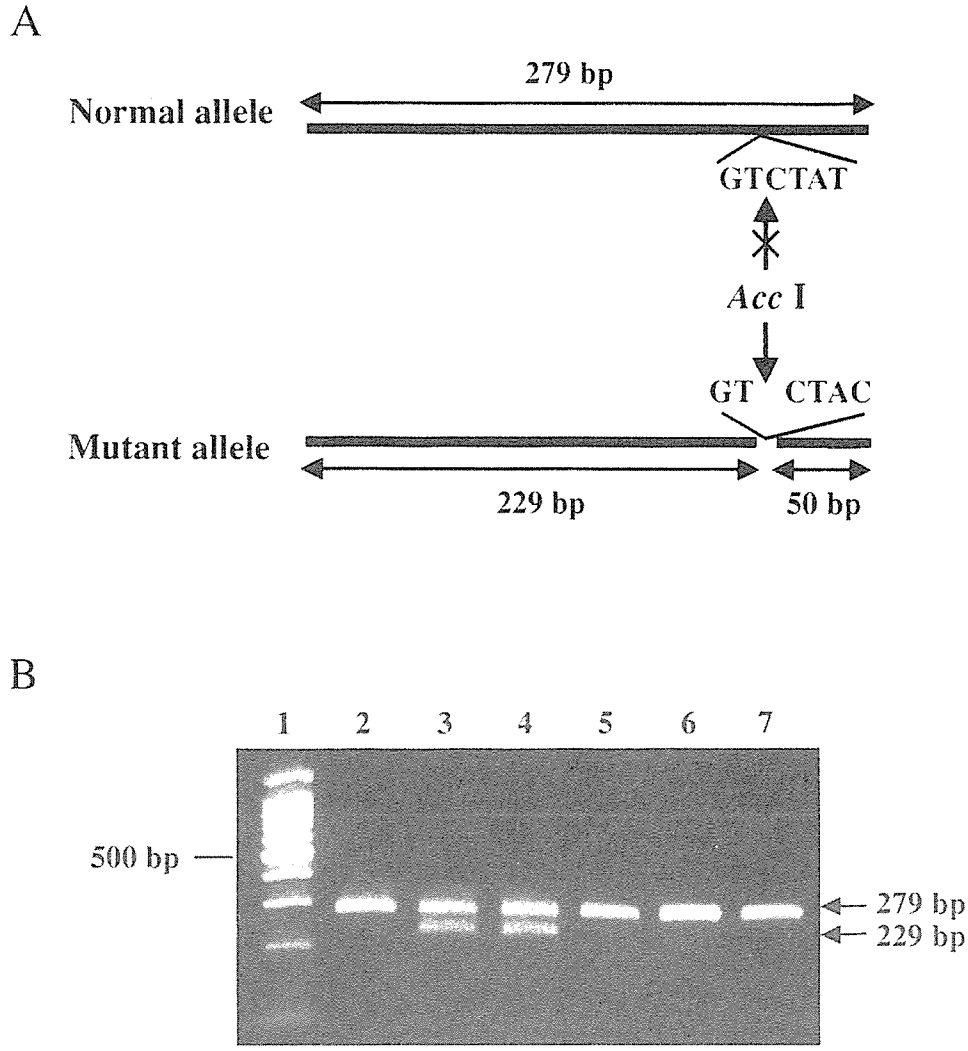

Fig. 5. *Acc* I PCR-RFLP analysis of the proband of family B and his family members. (A) Schematic digestion pattern of a PCR fragment spanning exon 10 and adjacent introns with the restriction endonuclease *Acc* I. The amplified DNA fragment was 279 bp. The PCR product derived from the normal allele remained intact after digestion, because there was no recognition site for *Acc* I. The mutant fragment was cleaved into two fragments of 229 and 50 bp. The T-to-C transition at nucleotide position 1147 created an *Acc* I restriction site by changing the sequence from GTCTAT to GTCTAC. (B) Electrophoretic patterns following enzyme digestion. The proband and his younger brother each showed two bands of 279 and 229 bp, indicating that they were heterozygous for the observed mutation. Lane 1, 100-bp ladder DNA marker; lane 2, mother; lane 3, proband; lane 4, brother; lanes 5 to 7, normal individuals.

ing had occurred and allelic exclusion had not in the mutant allele (Fig. 3A). Direct sequencing of the aberrant RT-PCR product from the proband showed that the mutant impaired normal mRNA splicing, leading to the deletion of the whole exon 7, with exon 6 splicing directly on exon 8 (Figs. 3B and C). The skipping of the 126 bp of exon 7 caused an in-frame deletion (Fig. 3C), resulting in a truncated PS with the entire EGF3 domain eliminated. Translation of this transcript would lead to a truncated protein lacking 42 internal amino acid residues, including six cysteine residues involved in the formation of three disulfide bridges and a consensus sequence associated with high-affinity Ca^{2+} binding, both of which play important roles in proper folding during assembly of the ternary structure of the EGF3 domain [24]. In addition, a pairwise linkage of EGF domains, such as EGF3 to EGF4 of PS, has been shown to play a critical role in achievement of a native conformation [25,26]. Furthermore, Drackenberg et al. very recently reported that the EGF3 domain of PS in a bent conformation could induce a domain-domain interaction between EGF2 and EGF4 [27]. Accordingly, loss of the EGF3 domain would alter the ternary structure of the PS molecule. Thus, if the above-mentioned transcribed PS variants are formed, they are likely retained and show proteolytic degradation in the endoplasmic

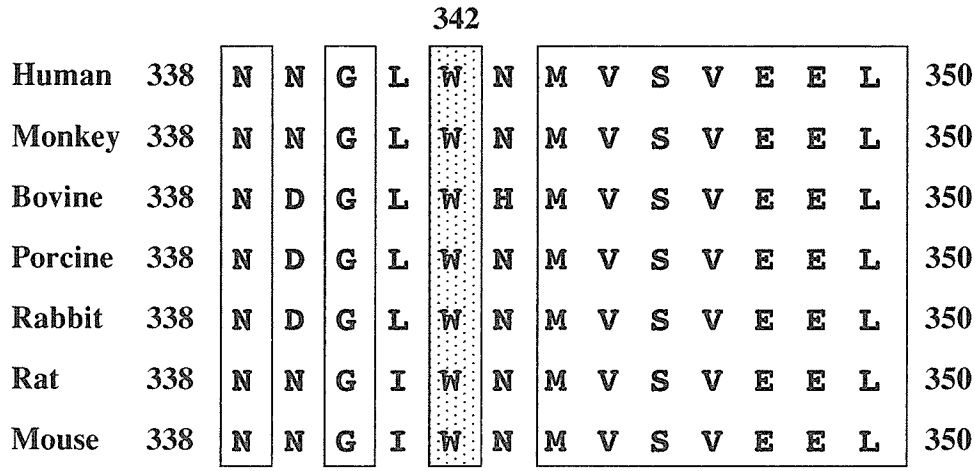

Fig. 6. Partial alignments of the amino acid sequences in the LG1 domain associated with human, monkey, bovine, porcine, rabbit, rat, and mouse PS. Identical amino acids are denoted by open boxes between human PS and PS in other mammalian species. Trp (W) at codon 342 remained conserved in all the species and is indicated by shaded boxes. The NCBI accession numbers for PS amino acid sequences aligned in this study, except for porcine PS, are as follow: human, P07225; monkey, Q28520; bovine, P07224; rabbit, P98118; rat, P53813; mouse, Q08761. The porcine PS amino acid sequence was obtained from the report by Greengard et al. (Biochem J 1995;305:397–403).

reticulum, as reported for other structurally abnormal proteins [28,29].

In the family B proband and his younger brother, who were phenotypically diagnosed with type I PS deficiency (Table I), a heterozygous T-to-C missense mutation in exon 10 at nucleotide position 1147 was identified, which predicted a Trp342-to-Arg substitution by DNA sequencing (Fig. 4 and data not shown) and *Acc* I PCR-RFLP analysis (Fig. 5). We designated this mutation protein S Sapporo 2. We screened 50 unrelated normal Japanese controls (100 alleles) using *Acc* I PCR-RFLP analysis and found that none were positive for the mutation, suggesting that it is not a common Japanese polymorphism. An identical mutation was previously described in a European family associated with type I and/or type III PS deficiency [30]. Since the mother of the present proband was not a carrier of the mutation, it is likely that the proband and his brother had inherited it from their father.

Trp342 is in the LG1 domain of the SHBG-like region of PS and close to the interface with the LG2 domain. By aligning the amino acid sequences of residue 342 and the flanking regions of human PS with those of other mammalian PS, we observed that Trp342 is strictly conserved in mammalian PS and located at the beginning of a β-strand in the LG1 domain (Fig. 6). Trp is a hydrophobic amino acid with an aromatic side chain. The sequential residues L_{341}WNMVSV_{347} are hydrophobic/aromatic stretches of residues and have been predicted to be buried in the tertiary structure [31]. Thus, Trp342 might play a key role in the structure and conformation of the LG1 domain. Trp342 replacement by positively charged Arg would markedly alter the conformation by interfering with correct protein folding and/or contributing to destabilizing effects [30], which lead to a degradation of the mutant protein and impairment of intracellular transport, resulting in a type I PS deficiency phenotype [28,29].

In summary, we identified one novel mutation in the acceptor splice site of intron f in the PROS1 gene and one recurrent missense mutation in exon 10 of the gene. Both mutations would cause misfolding of the PS protein, resulting in the impediment of secretion.

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