Thrombosis Research 125 (2010) e33–e39

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Membrane binding and anticoagulant properties of protein S natural variants

Marcello Baroni [a,*], Giulia Pavani [b], Diego Marescotti [a], Tahar Kaabache [c], Delphine Borgel [d], Sophie Gandrille [e], Giovanna Marchetti [a], Cristina Legnani [f], Armando D'Angelo [b], Mirko Pinotti [a], Francesco Bernardi [a]

[a] Department of Biochemistry and Molecular Biology, ICSI, University of Ferrara, Ferrara, Italy
[b] Coagulation Service and Thrombosis Research Unit, IRCCS H S. Raffaele, Milano, Italy
[c] INSERM Unité de Recherche 765, Paris, France
[d] Univ Paris-Sud, IFR 141, Faculté de Pharmacie, Orsay, F-91405, Paris, France
[e] AP-HP Hôpital Européen Georges Pompidou, Service d'Hématologie Biologique, Paris; Université Paris Descartes, UFR des Sciences Pharmaceutiques et Biologiques, Paris, France
[f] Dept. Angiology and Blood Coagulation, University Hospital S. Orsola-Malpighi, Bologna, Italy

ARTICLE INFO

Article history:
Received 8 April 2009
Received in revised form 17 September 2009
Accepted 21 September 2009
Available online 29 October 2009

Keywords:
Thrombophilia
protein S deficiency
protein S domains
protein S activity
protein S-membrane binding
oxidized phospholipids

ABSTRACT

Introduction: Protein S (PS) is a vitamin K-dependent plasma glycoprotein with a key role in the control of coagulation pathway on phospholipid membranes. We compared anticoagulant and membrane binding properties of PS altered by natural mutations (N217S, DelI203D204) affecting the epidermal growth factor like-domain 4 (EGF4) and causing PS deficiency.

Materials and methods: Binding of recombinant, immunopurified PS (rPS) to several conformation-specific antibodies, to C4BP and to phospholipid liposomes was investigated by ELISA. PS binding to cells was analysed by flow cytometry. PS inhibitory activities were studied in plasma and purified systems.

Results and conclusions: Conformational changes produced by mutations were revealed by mapping with calcium-dependent antibodies. The immunopurified recombinant mutants (rPS) showed at 200-800nM concentration reduced inhibition of coagulation (rPS217S, 10.2-17.3%; rPSDelI203D204, 5.8-8.9% of rPSwt) in FXa 1-stage clotting assay with APC. In thrombin generation assays the inhibition of ETP was reduced to 51.6% (rPS217S) and 24.1% (rPSDelI203D204) of rPSwt. A slightly shortened lag time (minutes) was also observed (rPS217S, 2.58; rPSDelI203D204, 2.33; rPSwt, 3.17; PS deficient plasma, 2.17).

In flow cytometry analysis both mutants efficiently bound apoptotic cells in adhesion or in suspension. The affinity for phosphatidylserine-rich vesicles (apparent Kd: rPSwt 27.7 ± 1.6 nM, rPS217S 146.0 ± 16.1 nM and rPSDelI203D204 234.1 ± 28.1 nM) was substantially increased by membrane oxidation (10.9 ± 0.6, 38.2 ± 3.5 and 81.4 ± 6.0 nM), which resulted in a virtually normal binding capacity of mutants at physiological PS concentration.

These properties help to define the molecular bases of PS deficiency, and provide further elements for PS-mediated bridging of coagulation and inflammation.

© 2009 Elsevier Ltd. All rights reserved.

Introduction

Protein S (PS) [1–3] is a modular glycoprotein exerting anticoagulant activity as non-enzymatic cofactor of activated protein C (APC) in the proteolytic inactivation of blood coagulation factors Va (FVa) and VIIIa (FVIIIa). PS also expresses anticoagulant activity in the absence of APC acting as a cofactor of full-length TFPI [4], in the inhibition of TF_FVIIa-catalyzed FX activation [5]. Binding of negatively charged phospholipids through the N-terminal γ-carboxyglutamic acid-rich (GLA) domain of PS and APC [6,7] or the C-terminal tail (TFPI) is crucial for the activity of these proteins. The PS molecule also includes a thrombin-sensitive region (TSR), four epidermal growth-factor (EGF) like-domains with high affinity Ca^{2+} binding sites, and a C-terminal portion homologous to steroid hormone-binding globulin (SHBG domain).

The PS GLA domain, a "unique sensor" for activated cell surfaces [8], provides a Ca^{2+} dependent binding site for anionic phospholipid surfaces, such as those abundantly exposed when platelets and endothelial cells are activated or damaged during coagulation and inflammation. Phospholipids containing polyunsaturated fatty acids represent one of the major targets for oxidation, and the atherosclerotic vessels are known to accumulate a large quantity of oxidized phospholipids [9]. Phospholipid membrane oxidation enhances both the APC anticoagulant function and the PS cofactor activity in the FVa inactivation [10], thus modulating the hemostatic balance.

* Correspondence author. Department of Biochemistry and Molecular Biology, University of Ferrara, via Fossato di Mortara 74, 44100, Ferrara, Italy. Tel.: +39 0532 974424; fax: +39 0532 974484.
E-mail address: brnmcl@unife.it (M. Baroni).

0049-3848/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.thromres.2009.09.015

Although the central role of membrane surface in the coagulation cascade is well known [2], and the anticoagulant functions of several coagulation proteins increase after lipid oxidation [11], as during inflammatory state, the interaction of PS with phospholipids in oxidative conditions requires investigation.

In human plasma, 60% of PS forms a non-covalent complex with the regulatory complement protein C4b-binding protein β(+) isoform (C4BP) [12–14], providing additional links between coagulation and inflammation processes.

Two mutations, both located in the EGF4 domain, the Ile203-Asp204 deletion [15] and the Asn217Ser [16,17] substitution affecting the β-hydroxylation site, have been described by us and others in PS deficient families with recurrent thrombosis [18]. Recently [19], the N217S was found to cause abolition of Ca^{2+} binding to EGF4 and alterations in structure and function of non-contiguous EGF1.

In the present study, both EGF4 natural variants were employed as tools to investigate the effects of domain alteration on PS conformation, interaction and multiple inhibitory functions.

Methods

Reagents

APC and PS were purchased from Hematologic Technologies (HTI, Vermont, USA).

PS-deficient plasma and factor X activator from Russell viper venom (RVV-X) were from Diagnostica Stago (Asnières, France).

Synthetic 1,2-diacyl-snglycero-3-phosphocholine OLEOYL18:1 (phosphatidylcholine), 1,2-diacylsn-glycero-3-[phospho-L-Serine] DIOLEOYL 18:1 (phosphatidylserine), and 1,2-diacyl-sn-glycero-3-phosphoethanolamine DIOLEOYL 18:1 (Δ9-cis) (phosphatidylethanolamine) were from Avanti Polar Lipids (Coger, Paris, France). Pore-size polycarbonate membranes were from Poretics Corporation (Livermore, CA).

Specific fluorogenic substrates for thrombin were from ICN Biomedicals (Irvine, CA, USA).

Polyclonal peroxidase-conjugated anti-human PS antibody (p-AbHRP), polyclonal rabbit anti-human PS antibody and polyclonal FITC-conjugated anti-rabbit antibody were purchased by Dako, (Glostrup, Denmark).

Sepharose 12 column and Mono Q column were obtained by Pharmacia Biotech (Uppsala, Sweden).

Microtiter plate were from Nunc, Immuno plate Maxisorp, from Polylabo (Strasbourg, France).

Expression of recombinant PS variants and purification

The expression vector for the rPSDell203D204 variant was already available for the study [15]. The N217S substitution was introduced into the full-length human PS cDNA cloned into pZEM229r (pPSwt) through the Quick Change Site-Directed mutagenesis kit (Stratagene, La Jolla, USA) with the following primers: 5'GCTAGCTTTGTGTCAGTTACCCTG-GAGGTTAC3' and 5'GTCTTGTGAAGATGAATGCTCTGAGAAC 3'.

Baby Hamster Kidney (BHK21) cells, cultured in the presence of 5 μg/ml vitamin K1 (Sigma-Aldrich, Milan, Italy), were stably transfected as previously described [15]. High-level rPS producer clones were selected by ELISA. SV40-immortalized fibroblasts and EBV-transformed lymphoblastoid cell line (LCL) were cultured in DMEM and in RPMI 1640 supplemented as reported [20].

rPS variants were purified from conditioned medium by ion-exchange and immunoaffinity chromatography as previously described [21]. A NaCl gradient (0.15-1 M) was used to elute the rPS molecules from the Mono Q column. The protein concentration was evaluated in triplicate by monitoring the absorbance at 280 nm using an ε (1%, 1 cm) value of 9.5 (rPSwt, 8.00 μM; rPS217S, 6.22 μM; rPSDell203D204, 5.11 μM). The relative amount of recombinant proteins was also confirmed by ELISA using 4.0 μg/mL of sheep and rabbit polyclonal anti-human PS antibodies (from HTI and Dako, respectively).

Quality control of protein preparations

rPS preparations were analysed for purity and for the presence of thrombin-cleaved PS by 4-12% gradient SDS/PAGE under reducing conditions and silver staining.

γ-Carboxylation was evaluated through ion-exchange chromatography of alkaline hydrolyzates in accordance with the method of Price [22]. The Gla content of recombinant PS molecules, estimated with standard (rPSwt = 8.0 ± 0.3; rPS217S = 7.8 ± 0.1; rPSDell203D204 = 9.3 ± 0.2) or ratio method (rPSwt = 7.4 ± 0.3; rPS217S = 7.2 ± 0.1; rPSDell203D204 = 8.6 ± 0.2), was lower than the theoretical one (n = 11), as observed in other *in vitro* expressed vit K-dependent proteins [23,24].

Polymerization analysis of rPS preparations was carried out by gel filtration chromatography on a Sepharose 12 column, at a flow rate of 0.25 mL/min, in 50 mM Tris, 150 mM NaCl, 2 mM $CaCl_2$ (pH 7.4) and by migration on PAGE (4-20% PAA gradient) under native condition.

FXa 1-stage clotting assay

Assay was performed in accordance to Saller et al [25]. Briefly, 50 μM phosphatidylcholine/phosphatidylserine/phosphatidylethanolamine (PC/PS/PE, 60/20/20) vesicles, purified human APC (final concentration 7.3 nM), 0.1 μg/mL RVV-X and increasing concentrations of rPS (200-800 nM) were incubated in PS depleted plasma for 2 min at 37 °C. All reagents were diluted in TBS containing 1 mg/mL gelatin and 5 mg/mL bovine serum albumin (BSA). Coagulation was triggered by addition of 25 mM $CaCl_2$ and clotting time measured. For each experimental PS concentration the results were expressed as ratio [25]: clotting time with APC + rPS/clotting time with APC only. The relative activity of 200-400-800 nM PS mutants was calculated as apparent concentration on a calibration curve made with rPSwt and expressed as percentage of rPSwt.

Inhibition of thrombin generation in plasma

PS deficient plasma (Hyphen BioMed, <1% of PS) was centrifuged at 20000 g at 4 °C for 50 min [26] and reconstituted with 350 nM rPS variants or PS purified from plasma (HTI, Vermont, USA), with or without polyclonal rabbit anti-human PS [4]. Calibrated automated thrombin activity measurement was conducted in accordance to Hemker et al [27] in an microtiter plate fluorometer (Fluoroskan Ascent, ThermoLabsystems, Helinski, Finland) using the Thrombinoscope software (Synapse BV, Maastricht, The Netherlands). Coagulation was triggered by re-calcification in the presence of 3.5 pM recombinant TF and 10 μM phospholipids. Thrombin generation was evaluated overtime by using a specific fluorogenic substrate (Z-Gly-Gly-Arg-AMC). Thrombin generation was evaluated as reported [28]. The inhibition was evaluated through the lag time and the area under the curves of thrombin generated (endogenous thrombin potential, ETP).

Conformational mapping in monoclonal antibody-binding assays

The conformation of the rPS variants was investigated by testing monoclonal antibodies specific for $CaCl_2$-dependent conformation of GLA (mAb5 and HPS21), TSR (mAb3 and HPS67) and EGF1 (HPS54) domains. We also used an EDTA-dependent antibody (HPS8) able to recognize an epitope in the TSR and/or GLA regions, as inferred by better recognition of the uncleaved PS respect the TSR-cleaved PS (D'Angelo, unpublished results). Microtiter plates, coated in Na_2CO_3/$NaHCO_3$ (pH 9.0) with each antibody, were incubated with increasing concentrations of rPS in presence of 2 mM EDTA (HPS8) or 5 mM

Download English Version:

https://daneshyari.com/en/article/3029165

Download Persian Version:

https://daneshyari.com/article/3029165

Daneshyari.com