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Characterization of the protein Z-dependent protease inhibitor interactive-sites of protein Z

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ABSTRACT

Background: Protein Z (PZ) has been reported to promote the inactivation of factor Xa (FXa) by PZ-dependent protease inhibitor (ZPI) by about three orders of magnitude. Previously, we prepared a chimeric PZ in which its C-terminal pseudo-catalytic domain was grafted on FX light-chain (Gla and EGF-like domains) (PZ/FX-LC). Characterization of PZ/FX-LC revealed that the ZPI interactive-site is primarily located within PZ pseudo-catalytic domain. Nevertheless, the cofactor function and apparent K_d of PZ/FX-LC for interaction with ZPI remained impaired ~6–7-fold, suggesting that PZ contains a ZPI interactive-site outside pseudo-catalytic domain. X-ray structural data indicates that Tyr-240 of ZPI interacts with EGF2-domain of PZ. Structural data further suggests that 3 other ZPI surface loops make salt-bridge interactions with PZ pseudo-catalytic domain. To identify ZPI interactive-sites on PZ, we grafted the N-terminal EGF2 subdomain of PZ onto PZ/FX-LC chimera (PZ-EGF2/FX-LC) and also generated two compensatory charge reversal mutants of PZ pseudo-catalytic domain (Glu-244 and Arg-212) and ZPI surface loops (Lys-239 and Asp-293).
Methods: PZ chimeras were expressed in mammalian cells and ZPI derivatives were expressed in *Escherichia coli*.
Results: The PZ EGF2 subdomain fusion restored the defective cofactor function of PZ/FX-LC. The activities of PZ and ZPI mutants were all impaired if assayed individually, but partially restored if the compensatory charge reversal mutants were used in the assay.
Conclusions: PZ EGF2 subdomain constitutes an interactive-site for ZPI. Data with compensatory charge reversal mutants validates structural data that the identified residues are part of interactive-sites.
General significance: Insight is provided into mechanisms through which specificity of ZPI–PZ–FXa complex formation is determined.

1. Introduction

Protein Z (PZ) is a vitamin K-dependent plasma protein which promotes the inactivation rate of factor Xa (FXa) by the PZ-dependent proteinase inhibitor (ZPI) on negatively charged phospholipids (PC/PS) in the presence of Ca^{2+} by more than three orders of magnitude [1–3]. It has a genetic organization identical to vitamin K-dependent coagulation zymogens [4]. However, PZ has no enzymatic activity, but instead functions as a cofactor to regulate the proteolytic activity of FXa by ZPI on PC/PS vesicles in the presence of Ca^{2+} [1–3]. Similar to other vitamin K-dependent coagulation proteins, PZ has an N-terminal γ-carboxyglutamic acid (Gla) domain that is followed by two epidermal growth factor (EGF)-like domains (light chain homologue) and a C-terminal pseudo-catalytic domain [4]. ZPI is a 72 kDa serpin which binds to the active-site of FXa via its P1-Tyr on the reactive center loop (RCL), thereby trapping it in the form of an inactive and covalently modified serpin–protease complex, a property shared by other inhibitory serpins [1–3,5]. In addition to FXa, ZPI is also a specific inhibitor of factor XIa, in this case however, ZPI does not require PZ and thus effectively inhibits the protease, independent of a cofactor [6]. We recently investigated the mechanism of the cofactor function of PZ by constructing a chimeric PZ derivative in which the pseudo-catalytic domain of the molecule was grafted on the light chain of factor X (PZ/FX-LC). The analysis of the cofactor function and the ZPI-binding properties of PZ/FX-LC chimera indicated that the primary ZPI-interactive site on PZ is located within the C-terminal pseudo-catalytic domain of the cofactor

Abbreviations: PZ, protein Z; ZPI, protein Z-dependent protease inhibitor; RCL, reactive center loop; ZPI-Y387A, a ZPI mutant in which Tyr-387 has been replaced with an Ala; FXa, activated factor X; Gla, γ-carboxyglutamic acid; EGF, epidermal growth factor; PZ/FX-LC, a PZ chimera in which its N-terminal Gla-domain; PZ-EGF2/FX-LC, a PZ chimera in which the first subdomain of EGF2 domain in PZ/FX-LC has been replaced with the corresponding sequence of PZ; PZ/FX-Gla, a PZ chimera in which its N-terminal Gla-domain has been replaced with the Gla-domain of factor X; PZ/FX-EGF2, a PZ chimera in which the first subdomain of EGF2 domain of PZ has been replaced with the corresponding sequence of FX; PEG, polyethylene glycol; BSA, bovine serum albumin

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[7]. However, the chimeric cofactor exhibited ~7-fold weaker affinity for ZPI which was also associated with ~6-fold decreased maximal cofactor function in the FXa inhibition assay on the negatively charged phospholipid vesicles in the presence of Ca^{2+} [7]. The molecular basis for the decreased cofactor activity of the PZ/FX-LC chimera was not investigated but the results raised the possibility that there is another interactive-site for ZPI outside the pseudo-catalytic domain of the cofactor.

Recently, the X-ray crystal structure of ZPI in complex with PZ was resolved by two groups [8–10]. Structural data supports our mutagenesis data demonstrating that ZPI makes extensive salt-bridge and hydrophobic interactions with 4 surface loops within the pseudo-catalytic domain of PZ [10]. Interestingly, the structural data further revealed that a hydrophobic residue on ZPI (Tyr-240) is oriented toward the EGF2 domain of PZ, interacting with a hydrophobic cavity in the interface between this domain and the pseudo-catalytic domain of the cofactor [10]. To validate the structural data and identify the site on PZ EGF2 domain that may constitute an interactive-site for ZPI, we grafted the first subdomain (residues forming the first 2 disulfide-stabilized loops) of PZ back onto PZ/FX-LC chimeric cofactor (Fig. 1). Moreover, we substituted the first subdomain of PZ EGF2 domain with the corresponding loops of FXa EGF2 domain. Since an interaction between the Gla-domain of PZ and FXa on PC/PS vesicles has been postulated [2,7], we also prepared a PZ chimera in which the Gla-domain of the cofactor was replaced with the corresponding Gla-domain of FXa (Fig. 1). Characterization of these PZ chimeras in kinetic assays indicates that ZPI interacts with a hydrophobic cavity formed by the first subdomain of PZ EGF2 domain and that the Gla-domain of FXa can functionally substitute for the Gla-domain of PZ on PC/PS vesicles in the presence of Ca^{2+}. Moreover, we mapped the proposed salt-bridge mediated interactive-site of ZPI with the pseudo-catalytic domain of PZ by a compensatory mutagenesis approach, validating the structural data that the interaction of several charged residues of PZ with complementary sites of ZPI contribute to the binding affinity of the cofactor-serpin inhibitory complex formation.

2. Materials and methods

2.1. Construction, mutagenesis and expression of recombinant proteins

Wild-type PZ and a PZ chimera in which the pseudo-catalytic domain of the cofactor was grafted on the light chain of factor X (PZ/FX-LC) were expressed in a mammalian expression/purification vector system as described [7]. A PZ chimera was constructed in which the first EGF2 subdomain of PZ/FX-LC was replaced with the corresponding subdomain of PZ EGF2 domain (Fig. 1). Two other PZ chimeras were prepared. In the first construct, the first EGF2 subdomain of wild-type PZ was replaced with the corresponding FXa EGF2 subdomain. In the second construct, the Gla-domain of PZ was replaced with the corresponding Gla-domain of FXa. The charge reversal mutants of PZ including Glu-244 to Lys (PZ-E244K), Arg-212 to Asp (PZ-R212D) and Arg-298 to Asp (PZ-R298D) were constructed using the same vector system. All mutations were introduced by the PCR mutagenesis approach and the accuracy of all constructs was confirmed by DNA sequencing. The expression vectors, containing a neomycin gene for the selection in mammalian cells with G418, were transfected to human embryonic kidney (HEK-293) cells. Several G418 resistant clones were selected and examined for PZ expression by an ELISA using the HPC4 monoclonal antibody and a polyclonal anti-PZ antibody (Haematologic Technologies Inc., Essex Junction, VT) as described [7]. A high expressing clone for each PZ derivative was identified and 20 L of cell culture supernatant was collected, concentrated and purified by a combination of HPC4 immunoaffinity and Mono Q ion exchange chromatography as described [7]. Concentrations of PZ derivatives were calculated from their absorbance at 280 nm using a molar absorption coefficient of 74,400 M^{-1} cm^{-1} as described [7,11]. The purity of all recombinant cofactors was ensured by SDS-PAGE under non-reducing conditions and the protein preparations were frozen at −80 °C in small aliquots until use.

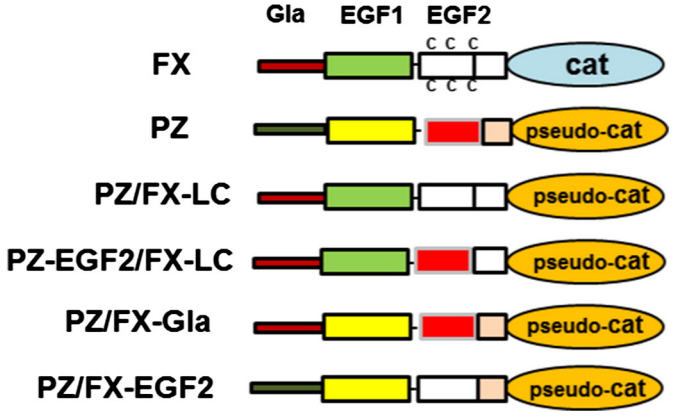

Fig. 1. Cartoons of factor X and PZ chimeras used in the study. Cat represents factor X catalytic domain; pseudo-Cat represents PZ pseudo-catalytic domain; LC represents factor X light chain.

Wild-type ZPI was prepared in *E. coli* using the SUMO fusion expression/purification system and characterized as described [12]. A ZPI mutant in which an Ala substituted the native P1-Tyr-387 of the serpin (ZPI-Y387A) was constructed by standard PCR mutagenesis methods and expressed using the same vector system. The compensatory charge reversal mutants of ZPI including Lys-239 to Glu (ZPI-K239E) and Asp-293 to Arg (D293R) were constructed and expressed in the same vector system. Concentrations of ZPI derivatives were calculated from their absorbance at 280 nm using a molar absorption coefficient of 31,525 M^{-1} cm^{-1} as described [3].

Human plasma FXa was purchased from Haematologic Technologies Inc. (Essex Junction, VT). Phospholipid vesicles containing 80% phosphatidylcholine and 20% phosphatidylserine (PC/PS) were prepared as described [13]. The chromogenic substrate Spectrozyme FXa (SpFXa) was purchased from American Diagnostica (Greenwich, CT).

2.2. Inhibition assay

A discontinuous assay method was used to measure the second-order association rate constants (k_2) for the ZPI inhibition of FXa under pseudo-first-order conditions as a function of increasing concentrations of the PZ chimeras as described [7,12]. Briefly, FXa (0.75 nM) was incubated with ZPI (5 nM) and different concentrations of each PZ derivative (0.3–20 nM) on PC/PS vesicles (25 μM) in 0.1 M NaCl, 0.02 M Tris–HCl (pH 7.5), 0.1% polyethylene glycol 8000 (PEG 8000), 0.1 mg/mL bovine serum albumin (BSA) and 5 mM Ca^{2+} (TBS/Ca^{2+}) for 1.5–8 min in 50 μL volumes in 96-well polystyrene plates at room temperature. The inhibition reactions were stopped by the addition of 50 μL SpFXa (0.2 mM final) in TBS containing 50 mM EDTA and the remaining enzyme activity was measured with a V_{max} Kinetics Microplate Reader (Molecular Devices, Menlo Park, CA) at 405 nm. The cofactor concentration dependence of the ZPI inhibition of FXa indicated that the PZ concentrations used are saturating under these conditions. The observed pseudo-first-order (k_{obs}) rate constants were calculated from a first-order rate equation and the second-order rate constants (k_2) were calculated from the slope of plots of k_{obs} values versus PZ–ZPI complex concentrations as described [7,12]. The apparent dissociation constants ($K_{d(app)}$) of the PZ derivatives for interaction with ZPI were estimated from the hyperbolic dependence of k_{obs} values on PZ concentrations in the presence of a fixed concentration of ZPI (5 nM) on PC/PS vesicles in TBS/Ca^{2+}.

Similar methods were employed to evaluate the PC/PS dependence of the cofactor effect of each PZ derivative (20 nM) in catalyzing the ZPI (5 nM) inhibition of FXa (0.75 nM) in the presence of 5 mM Ca^{2+}.