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Characterization of recombinant human protein C inhibitor expressed in *Escherichia coli*

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Abstract

The serine protease inhibitor (serpin) protein C inhibitor (PCI; also named plasminogen activator inhibitor-3) regulates serine proteases in hemostasis, fibrinolysis, and reproduction. The biochemical activity of PCI is not fully defined partly due to the lack of a convenient expression system for active rPCI. Using pET-15b plasmid, Ni^{2+}-chelate and heparin-Sepharose affinity chromatography steps, we describe here the expression, purification and characterization of wild-type recombinant (wt-rPCI) and two inactive mutants, R354A (P1 residue) and T341R (P14 residue), expressed in *Escherichia coli*. Wild-type rPCI, but not the two mutants, formed a stable bimolecular complex with thrombin, activated protein C and urokinase. In the absence of heparin, wt-rPCI-thrombin, -activated protein C, and -urokinase inhibition rates were 56.7, 3.4, and 2.3×10^4 M^{-1} min^{-1}, respectively, and the inhibition rates were accelerated 25-, 71-, and 265-fold in the presence of 10 μg/mL heparin for each respective inhibition reaction. The stoichiometry of inhibition (SI) for wt-rPCI-thrombin was 2.0, which is comparable to plasma-derived PCI. The present report describes for the first time the expression and characterization of recombinant PCI in a bacterial expression system and demonstrates the feasibility of using this system to obtain adequate amounts of biologically active rPCI for future structure–function studies.

Keywords: Serpin; Recombinant protein C inhibitor; Thrombin; Heparin; Purification

1. Introduction

Protein C inhibitor (PCI; systematic name *SERPINA5*; also named plasminogen activator inhibitor-3) is a 57 kD glycoprotein that inhibits numerous serine proteases including activated protein C, thrombin (free and bound to thrombomodulin), urokinase, acrosin, and plasma kallikrein [1–10]. The physiological function of PCI in blood coagulation and fibrinolysis has been widely studied but its physiological relevance is not fully understood in part due to its apparent dual anti- and pro-coagulant activities [11–15]. Interestingly, PCI is also critically involved in reproduction since male homozygous PCI knock-out mice are infertile and show impaired spermatogenesis [16]. In humans, seminal plasma is a major source of PCI where it acts as the inhibitor of acrosin [17], human kallikrein 2 [18] and the plasminogen activators, urokinase and tissue plasminogen activator [5]. PCI would locally prevent non-appropriate proteolytic activities that could interfere with normal reproduction mechanism. Recently, PCI has been studied in cancer biology using PCI-transfected MDA-MB-

Abbreviations: APC, activated protein C; IPTG, isopropyl-beta-D-thiogalactopyranoside; LB medium, L-broth medium; Ni-NTA, nickel-nitrilotriacetic acid; rPCI, recombinant protein C Inhibitor; P1-rPCI, R355A rPCI; P14-rPCI, T341R rPCI

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435 breast cancer cells and PCI increased both adhesion and motility to vitronectin and fibronectin [19]. Protein C inhibitor has also been identified in malignant prostate, which suggests a potential role for PCI in cancer progression [20].

Protein C inhibitor belongs to the *ser*ine *p*rotease *in*hibitor superfamily (serpin), which contains more than 400 members [21–23]. Serpins share the same overall structure, composed of 3 β sheets and 9 α helices [21,22]. Their inhibitory activity follows an original scheme where the target protease interacts with the reactive site loop of the serpin and cleaves it at the reactive site P1–P1′ (according to the Schechter and Berger nomenclature [24]). This interaction leads either to the formation of a covalent complex between the protease and the serpin where both proteins become inactive (the inhibitory pathway) or to the generation of an inactive irreversible clipped form of the serpin while the protease recovers its activity (the substrate pathway). PCI interaction with proteases is characterized predominantly by the formation of a covalent complex, although some clipped forms of PCI have been identified in biological fluids as well as in in vitro experiments [25]. Besides its ability to regulate protease activities, PCI also binds heparin [26–28] and retinoic acid [29]. Many PCI–protease inhibition reactions are increased by heparin [26–28]. The binding of retinoic acid to PCI has no effect on its inhibitory activity but this interaction may have a role in hormone regulation [29]. The three-dimensional structure of clipped PCI was recently described, and Huntington et al. proposed independent binding sites for heparin and retinoic acid [30].

Having a convenient recombinant PCI expression system allowing high-level expression of PCI and the production of PCI mutants would provide a new tool for further structure/function studies. A recombinant PCI was obtained using baculovirus and insect cells [31–34]. There have been other rPCI expression systems described including baby hamster kidney cells [35] and human embryonic kidney cells [36]. However, insect cell expression and other eukaryotic systems can be somewhat challenging and time-consuming, while a bacterial expression system provides an easy alternative for rapid protein production and at a lower cost. This work reports the expression of wild-type recombinant PCI (wt-rPCI) using an *Escherichia coli* system. By site-directed mutagenesis, we also prepared two inactive rPCI mutants, R354A-rPCI (P1-rPCI) and T341R-rPCI (P14-rPCI). We assessed the activity of wt-rPCI and the non-inhibitory mutants with thrombin, activated protein C, and urokinase in the absence and the presence of heparin.

2. Materials and methods

2.1. Materials

Human α-thrombin and urokinase were respectively purchased from Haematologic Technologies Inc. (Essex Jct.,VT, USA) and American Diagnostica (Greenwich, CT, USA). Human wild-type recombinant activated protein C (APC) was a gift from Dr. Brian W. Grinnell (Lilly Research Laboratories, Indianapolis, IN, USA). Human plasma PCI was from Affinity Biological Inc. (Ontario, Canada). Tos-Gly-Pro-Arg-pNA and Pefachrome PCa were obtained from Pentapharm (Basel, Switzerland) and S-2444 substrate was from Chromogenix (Milano, Italy). Heparin was from Diosynth Inc. (Oss, the Netherlands). Imidazole and polybrene were from Sigma-Aldrich Co. (St Louis, MO, USA); lysozyme from Worthington Biochemical Co. (Freehold, NJ, USA). PolyHis (0.2 μg/μl, Novagen, Madison, WI, USA) and human PCI (0.1 μg/μl) monoclonal antibodies were diluted in non-fat milk. Anti-mouse peroxidase conjugate antibody (1/15,000 in non-fat milk) was from Sigma-Aldrich Co. (St Louis, MO, USA).

2.2. Cloning and mutagenesis of PCI

The cDNA for human PCI was cloned between the *Nde*I and *Bam*HI restriction sites in the pET-15b plasmid (Novagen). This vector includes a 6xHis tag followed by a sequence for further thrombin cleavage at the 5′ terminal of the polylinker. Point mutations were introduced according to the QuickChange Site-directed Mutagenesis Kit procedure (Stratagene), using the following primers to generate P1 and P14 mutants respectively: 5′-GCG GCA GCC ACG GGG ACA ATC ATC TTC ACT TTC GCG TCG GCC CGC CTG-3′ (forward R354A), 5′-CAG GCG GGC CGA CGC GAA AGT GAA GAT TGT CCC CGT GGC TGC CGC-3′ (reverse R354A) to 5′-GCT GTG GTG GAG GTG GAC GAG TCG GGA CGG AGA GCA GCG GCA GCC-3′ (forward T341R), 5′-GGC TGC CGC TGC TCT GCG TCC CGA CTC GTC CAC CTC CAC CAC AGC-3′ (reverse T341R). Supercompetent XL1-Blue cells were transformed by the heat shock method, according to the manufacturer's instructions, the positive clones were selected with ampicillin, and the plasmids were then purified and sequenced by the University of North Carolina Sequencing Core Facility to verify the incorporation of the mutations.

2.3. Expression and purification of recombinant PCI

Recombinant PCI in pET-15b was expressed in *E. coli* BL21(DE3)plyS grown at 30 °C in 2 L of LB with 50 μg/ml carbenicillin and 34 μg/ml choramphenicol. At an OD_{600} of 0.6–0.8, IPTG (isopropyl-beta-D-thiogalactopyranoside) was added to a final concentration of 1 mM. Five hours after the addition of IPTG, cells were collected by centrifugation at 4000×*g* for 20 min and frozen at −80 °C. Recombinant PCI was purified under native conditions according to the Qiagen procedure for purification of His-tagged proteins. Cells were thawed for 1 h on ice, brought to a final volume of 40 ml with lysis buffer containing 40 mg lysozyme and incubated on ice for an hour. Cells were