THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 24, Issue of June 17, pp. 16696–16700, 1994
Printed in U.S.A.

Mutagenesis of Recombinant Protein C Inhibitor Reactive Site Residues Alters Target Proteinase Specificity*

(Received for publication, March 4, 1994, and in revised form, April 6, 1994)

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Protein C inhibitor (PCI) is a heparin-binding plasma serine proteinase inhibitor (serpin) which is thought to be a physiological regulator of activated protein C. We are using recombinant PCI (rPCI) to study structural determinants of target proteinase specificity. A cDNA encoding full-length PCI has been expressed as a fully active proteinase inhibitor using *Autographa californica* nuclear polyhedrosis virus (baculovirus). rPCI was expressed maximally 4 days after infection and could be expressed either in Sf9 or High-Five™ cells. rPCI bound heparin and was conveniently purified with heparin-Sepharose (eluting >0.5 M NaCl). The rPCI formed sodium dodecyl sulfate-polyacrylamide gel electrophoresis-stable complexes with thrombin and activated protein C (APC). The inhibitory properties of wild-type rPCI and plasma-derived PCI are essentially the same either in the absence or presence of heparin with thrombin, APC, trypsin, and urokinase.

The residues Phe^{353}-Arg^{354}-Ser^{355} (P2-P1-P1′) constitute part of the reactive site loop of PCI with the Arg-Ser peptide bond being cleaved by the proteinase. Using site-directed mutagenesis we studied the contribution of the reactive site FRS for proteinase inhibition in rPCI. Changing the P1 residue Arg^{354} → Met generated a reactive site similar to α_1-proteinase inhibitor which was a much poorer inhibitor of thrombin, APC, trypsin, and urokinase. Changing the P2 residue Phe^{353} → Gly generated a mutant with a reactive site like antithrombin which was better at inhibiting thrombin or urokinase, but was much less active with APC or trypsin. Changing the P1′ residue Ser^{355} → Met generated a reactive site like plasminogen activator inhibitor-1 and this protein inhibits all the proteinases essentially like wild-type rPCI. These results show the importance of PCI's Phe^{353} (P2) and Arg^{354} (P1) in target proteinase specificity, and they further support the concept of reactive site sequences determining serpin function.

Protein C inhibitor (PCI)[1] is a plasma glycoprotein which is a member of the serine proteinase inhibitor (serpin) superfamily of proteins (1–6), whose prototype is α_1-proteinase inhibitor (7, 8). PCI (also known as plasminogen activator inhibitor-3) can be further classified as a heparin-binding serpin (1, 4, 5, 9, 10), along with the proteinase inhibitors antithrombin (historically known as antithrombin III), heparin cofactor II, and protease nexin-I (for a review, see Refs. 5, 6, and 11–13 and references cited therein). Heparin and some other glycosaminoglycans act to increase the rate of proteinase inhibition by these serpins, in some cases as much as several thousand-fold. An important biological function of PCI and other serpins is the regulation of the proteinases involved in hemostasis (14, 15). Thrombin and activated protein C (APC) are believed to be partially regulated by PCI (16–19). Direct evidence for a PCI function from a patient deficiency has not been clinically documented, although *in vivo* animal model clearance studies have clearly shown that PCI inhibits APC (20–22). Other plasma proteins, including α_2-macroglobulin and the serpins α_1-proteinase inhibitor and α_2-antiplasmin, can inhibit APC (23–26), whereas known thrombin inhibitors include α_2-macroglobulin and three other plasma serpins: antithrombin, heparin cofactor II, and α_1-proteinase inhibitor. Thus, there is some redundancy of plasma proteinase inhibitors to react with their various putative target proteinases.

The reactive site Arg^{354}-Ser^{355} peptide bond located near the carboxyl terminus of PCI (termed the P1-P1′ bond using the nomenclature of Schechter and Berger (27)) reacts with the active site of APC and thrombin to form an essentially irreversible bimolecular complex (1, 2). The reactive site P1 Arg residue of PCI is presumably important for proteinase recognition but alone probably does not determine specificity. Additionally, PCI inhibits other proteinases, including kallikrein, factors Xa and XIa, urokinase, tissue plasminogen activator, prostate specific antigen, trypsin, and chymotrypsin (1, 3, 28–33). This broad proteinase inhibition pattern presents a problem in understanding the physiological importance of PCI. Interestingly, antithrombin which has the same Arg-Ser-reactive site sequence (8) inhibits a wide range of proteinases, including thrombin, factor Xa, kallikrein, and trypsin, but does not readily inhibit APC. APC inhibition by PCI-heparin (k_2 of $\sim 1 \times 10^6$ M^{-1} min^{-1} (4)) is 1–2 orders of magnitude lower than other well characterized serpin-proteinase inhibition reactions in the absence or presence of glycosaminoglycans. Some examples of other serpin-proteinase inhibition reactions include: antithrombin-heparin-thrombin (k_2 of $\sim 1 \times 10^9$ M^{-1} min^{-1}), heparin cofactor II-heparin-thrombin (k_2 of $\sim 4 \times 10^8$ M^{-1} min^{-1}), plasminogen activator inhibitor-1 (PAI-1)-tissue plasminogen activator (k_2 of $\sim 3 \times 10^8$ M^{-1} min^{-1}), and α_2-antiplasmin-plasmin (k_2 of $\sim 4 \times 10^7$ M^{-1} min^{-1}) (5, 34, 35). This may be due to evolutionary constraints which allows APC "physiological time" for expression of its anticoagulant action (36).

* This work was supported in part by Research Grants F32-HL08413 (to J. E. P), HL-06530 (to F. C. C.), and 5T32-HL07149 (stipend support to S. T. C.) from the National Institutes of Health and a grant-in-aid from the American Heart Association (to F. C. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PCI, protein C inhibitor; wt rPCI, wild-type recombinant protein C inhibitor; serpin, serine proteinase inhibitor; APC, activated protein C; PAI-1, plasminogen activator inhibitor-1; PAGE, polyacrylamide gel electrophoresis; CAPS, 3-(cyclohexylamino)-propanesulfonic acid.

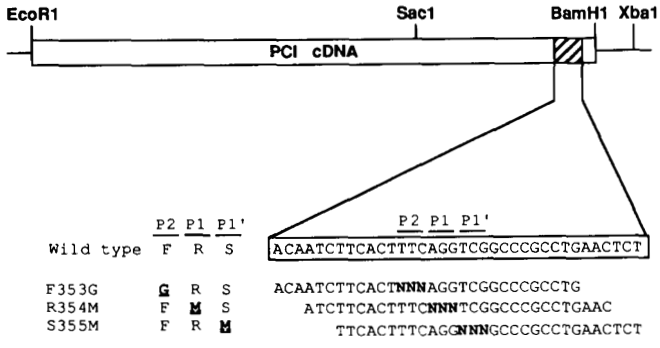

FIG. 1. **Schematic representation of the full-length cDNA clone of PCI and the strategy for mutagenesis of the reactive site region.** Cassette mutagenesis between *Sac*I and *Xba*I was performed to create the reactive site mutants in the P2, P1, and P1′ amino acid position using these designed synthetic oligonucleotides. The nucleotide and predicted amino acid sequence in these sites are shown for wt rPCI and the three PCI mutants F353G, R354M, and S355M. Positions designated by *N* in the oligonucleotides represent equal mixtures of G, A, T, and C used during oligonucelotide synthesis. The cassette was cloned into pVL1392 to obtain full-length PCI, and the protein was expressed as described under "Experimental Procedures."

The work presented here describes the cloning, expression, and characterization of wild-type recombinant protein C inhibitor (wt rPCI) in a Baculovirus expression system. We have used site-directed mutagenesis to examine the importance of the P1, P1′, and P2 reactive site residues in the proteinase reactivity of rPCI with APC, thrombin, trypsin, and urokinase.

EXPERIMENTAL PROCEDURES

Expression of Full-length rPCI—Wild-type human PCI cDNA containing the signal peptide sequence (kindly provided by Dr. Joost C. M. Meijers, Department of Hematology, Utrecht University Hospital, Utrecht, The Netherlands) was subcloned into the baculovirus expression vector pVL1392 (Invitrogen) using the restriction endonuclease sites *Eco*RI 5′ and *Bam*HI 3′ (Fig. 1) (all restrictions enzymes purchased from Promega). Transfer vector (pVL1392) containing wt rPCI and each of the engineered mutations were cotransfected with DNA from *Autographa californica* nuclear polyhedrosis virus (AcNPV) into insect host cells (Sf9, *Spodoptera frugiperda* cells, in complete Grace's medium) using kits from Invitrogen (wt rPCI and F353G rPCI) or PharMingen (R354M, S355M, and S355V rPCIs). Individual plaques were purified and screened for PCI production by immunoblot. Recombinant viral stocks were amplified and used to infect High-Five™ cells (Invitrogen) grown in serum-free medium (ExCell, JRH Biosciences) for protein production.

Expressed proteins were purified 4 days post-infection by incubating the infected medium (50 ml of medium from two T-150 flasks of confluent High-Five™ cells) with 0.5 ml of heparin-Sepharose beads (Pharmacia Biotech Inc.) equilibrated in 20 mM HEPES, 150 mM NaCl, 0.1% polyethylene glycol, 0.02% NaN_3, pH 7.4 (HNPN). The beads were collected by gentle centrifugation, washed twice with HNPN, once with HNPN containing 0.25 M NaCl, and then the rPCI was eluted with HNPN containing 1.0 M NaCl. The protein was then dialyzed against HNPN. Average yield of rPCI was in the range of 170–300 μg of protein.

Reactive Site Region Mutagenesis of rPCI—A fragment of the PCI cDNA was cloned into M13mp19 using *Sac*I and *Xba*I. Uracil-rich single-stranded DNA was prepared and annealed to specific oligonucleotides designed to generate mutations in the reactive site region of PCI (Fig. 1) using the method of Kunkel (37). Individual plaques were purified and sequenced to confirm the introduced nucleotide substitutions. Each generated mutation was cloned into pVL1392 using *Xba*I and *Sac*I to generate full-length PCI containing the single engineered change. The transfer vector was sequenced before transfection to confirm that the desired mutation was present.

PCI Immunodetection—A direct enzyme-linked immunosorbent assay using a rabbit anti-PCI antiserum (1:5000 dilution) and goat anti-rabbit IgG linked with alkaline phosphatase (1:2000 dilution, Sigma) was used to measure rPCI concentrations. Purified human plasma PCI was used as a standard. Samples were incubated overnight in a 96-well microtiter plate at a final volume of 100 μl. The plate was then blocked with 1% milk in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.5). Primary and secondary antibodies diluted in TBST were added for 3 h each at room temperature, with TBST washes between incubations. Bound antibody was quantitated using 1 mg/ml *p*-nitrophenyl phosphate (Sigma) in AP buffer (0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.5) and the absorbance change measured on a V_{max} microplate reader (Molecular Devices). There was an excellent correlation between PCI concentration determined by enzyme-linked immunosorbent assay and that calculated by titration with active site-titrated thrombin. However, for internal consistency and due to the variable activity of some of the rPCI mutants, the enzyme-linked immunosorbent assay was used to determine rPCI concentrations.

Immunoblots were performed by first subjecting samples to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (38) followed by transfer to an Immobilon-P filter (polyvinylidene difluoride, Millipore). The filter was blocked in TBST-milk and incubated overnight with a mouse monoclonal antibody to PCI (1:100 dilution). Goat anti-mouse IgG linked to alkaline phosphatase was used as a secondary antibody, and bands were detected using 0.33 mg/ml nitro blue tetrazolium and 0.12 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in AP buffer.

Protein Sequencing—Approximately 60 pmol of rPCI was subjected to SDS-PAGE taking precautions to minimize protein oxidation that may block the amino terminus (39). The proteins were transferred onto Immobilon-P in buffer containing 10 mM CAPS, 10% methanol, pH 11 at 0.3 amps for 20 min. The blot was stained with Coomassie Brilliant Blue R-250, destained, and washed extensively to remove any residual Tris or glycine. The rPCI band was excised with a sterile scalpel and subjected to automated amino-terminal sequencing using an ABI model 470A sequencer (Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC).

Deglycosylation Reaction—*N*-Linked carbohydrate was cleaved using peptide-*N*-glycosidase F (Oxford GlycoSystems). Plasma PCI and rPCI were boiled for 2 min in a supplied buffer containing 0.5% SDS and 5% β-mercaptoethanol. Upon cooling the solution was made 2% in Triton X-100 and the glycosidase added overnight at room temperature. The sample was then subjected to SDS-PAGE and immunoblotted using a mouse monoclonal antibody to PCI.

Proteinase Inhibition Assays—Plasma PCI was purified as previously described (33) with an additional step of immunoaffinity chromatography on an anti-PCI monoclonal antibody column. Thrombin was prepared as detailed previously (40). Trypsin was purchased from Calbiochem, and a conversion factor of 2972 units/mg was used. Urokinase was purchased from American Diagnostica and a conversion factor of 80,000 units/mg was used. Assays were performed at room temperature in HNPN in the presence of 2 mg/ml bovine serum albumin. For assays in the absence of heparin, 0.1 mg/ml Polybrene (Sigma) was also included in the buffer. Chromogenic substrates for the proteinases were: Lys-carbobenzoxy-Pro-Arg-*p*-nitroanilide (Spectrozyme PCa, American Diagnostica) for APC; carbobenzoxy-L-(g)Glu(α-t-BuO)-Gly-Arg-*p*-nitroanilide (Spectrozyme UK, American Diagnostica) for urokinase; tosyl-Gly-Pro-Arg-*p*-nitroanilide (Boehringer Mannheim) for thrombin and trypsin. Unbleached crude heparin was obtained from Diosynth (Oss, The Netherlands).

Second order inhibition rate constants (k_2, M^{-1} min^{-1}) were measured in triplicate on at least two separate preparations of each serpin. Each rate (reported as mean ± S.D.) was measured from two to eight times under pseudo-first order reaction conditions as described previously (4, 5, 41). Protein concentrations for the various serpin-proteinase inhibition reactions shown in Table I were: 0.5 nM thrombin, 5 nM (r)PCI, antithrombin, and α_1-proteinase inhibitor, 1 μg/ml heparin; 1 nM APC, 10 nM (r)PCI, antithrombin, and α_1-proteinase inhibitor, 10 μg/ml heparin (rPCI's, antithrombin, and α_1-proteinase inhibitor), or 100 μg/ml heparin (plasma PCI); 5 nM urokinase, 100 nM (r)PCI, antithrombin, and α_1-proteinase inhibitor, 10 μg/ml heparin; and 1 nM trypsin, 10 nM (r)PCI, antithrombin, and α_1-proteinase inhibitor, 10 μg/ml heparin. Reaction volumes were 50 μl, and the reaction was started by the addition of 5 μl of proteinase. 50–100 μl of chromogenic substrate containing 1 mg/ml Polybrene was added to block heparin. Following color development, the assay was terminated by adding 50 μl of 50% acetic acid and the absorbance at 405 measured using the V_{max} microplate reader. Rate constants were calculated using the equation $k_2 = (-\ln a)/t[I]$, where a is residual proteinase activity, t is time and [I] is the serpin concentration (4, 5, 41). All inhibition assays were performed by time course analysis to ensure that the kinetic parameters were followed and that at least 50% proteinase inhibition was achieved for calculation of inhibition rates.

To measure optimum heparin concentrations for (r)PCI inhibition of thrombin and APC, the same concentrations of serpin and proteinase as

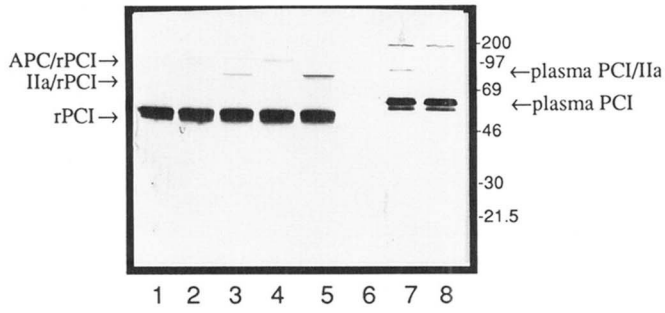

FIG. 2. **SDS-resistant complex formation of proteinases with rPCI.** Thrombin (IIa) and APC were incubated with wt rPCI (supernatant of infected High-Five™ cells concentrated in a Centriprep-10 tube) or plasma PCI (250 nM) in culture medium with 1 μg/ml heparin for 15 min at 37 °C. The samples were boiled in SDS-PAGE sample buffer and loaded onto a 10% acrylamide gel under nonreducing conditions and then visualized by immunoblot with a monoclonal antibody against PCI as described under "Experimental Procedures." *Lane 1*, rPCI alone; *lane 2*, rPCI and APC (10 nM); *lane 3*, rPCI and thrombin (10 nM); *lane 4*, rPCI and APC (20 nM); *lane 5*, rPCI and thrombin (20 nM); *lane 6*, control β-galactosidase-infected medium with thrombin (20 nM); *lane 7*, plasma PCI and thrombin (10 nM); *lane 8*, plasma PCI alone. Migration positions for molecular size markers: myosin (molecular mass = 200 kDa), phosphorylase *b* (molecular mass = 97 kDa), bovine serum albumin (molecular mass = 69 kDa), ovalbumin (molecular mass = 46 kDa), carbonic anhydrase (molecular mass = 30 kDa), and trypsin inhibitor (molecular mass = 21.5 kDa).

above were used with a final reaction volume of 100 μl. Final heparin concentrations varied from 0.01 to 1000 μg/ml. Following termination of the reaction, the microtiter plate was subjected to centrifugation at 3000 rpm for 10 min to precipitate heparin-Polybrene complexes which form at very high heparin concentrations (4, 5, 41). 100 μl was transferred from each well to a fresh microplate and the absorbance at 405 nm was measured. The inhibition rate (k_2, M^{-1} min^{-1}) was calculated for PCI at each heparin concentration, and the data presented are mean values of duplicate assays performed three times with three separate preparations of each serpin.

RESULTS

Expression and Characterization of rPCI—The expression vector pVL1392-PCI was constructed as described under "Experimental Procedures." Cotransfection of Sf9 cells with pVL1392-PCI and baculovirus DNA resulted in the production of recombinant viruses which were screened by plaque assay. Sf9 (and High-Five™) cells were then infected with the purified recombinant virus and supernatants were harvested. Time courses showed that harvesting the medium four days post-infection resulted in the highest yield of immunoreactive rPCI (data not shown). Wild-type rPCI was initially characterized from the harvested medium of the High-Five™ cells, grown in serum-free medium, and the protein further characterized following partial purification with heparin-Sepharose.

Thrombin and APC were incubated with both cell-free medium containing wt rPCI and plasma PCI. Both proteinases formed bimolecular complexes with wt rPCI which were stable to SDS-PAGE (Fig. 2). The apparent M_r values of the rPCI-proteinase complexes formed were lower than that of plasma PCI-proteinase complexes. A control with conditioned medium from High-Five™ cells infected with β-galactosidase showed no proteinase complex formation. Similar complexes were seen with the three rPCI mutants (data not shown).

Purified rPCI had a lower apparent molecular weight than plasma PCI as determined by immunoblot (Fig. 3). A majority of the discrepancy was shown to be due to incomplete *N*-glycosylation of the rPCI. Plasma and purified rPCI were digested with peptide-*N*-glycosidase F, resulting in both proteins migrating with similar apparent molecular weights (Fig. 3). With the exception of incomplete *N*-glycosylation, rPCI was apparently processed normally, as amino-terminal sequencing for 17

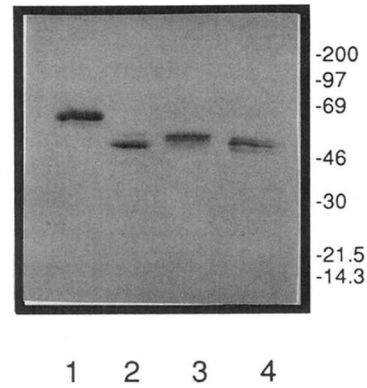

FIG. 3. **Immunoblot of plasma rPCI and PCI before and after treatment with peptide-*N*-glycosidase F.** Plasma PCI and wt rPCI were treated with peptide-*N*-glycosidase F as described under "Experimental Procedures." *Lane 1*, plasma PCI; *lane 2*, rPCI; *lane 3*, plasma PCI treated with peptide-*N*-glycosidase F; *lane 4*, rPCI treatment with peptide-*N*-glycosidase F. Migration positions for molecular size markers are included.

cycles gave the correct amino-terminal sequence of human PCI (HRHHPREMKKRVEDLHV). Wild-type rPCI (and the three rPCI mutants) also retained heparin binding ability, eluting from heparin-Sepharose at >0.5 M NaCl.

Thrombin and APC Inhibition by rPCI in the Absence and Presence of Heparin—Thrombin and APC inhibition time courses under pseudo-first order conditions were then performed comparing wt rPCI to plasma PCI. In the absence of heparin, thrombin, and APC, inhibition by plasma PCI and partially purified wt rPCI were essentially indistinguishable (k_2, M^{-1} min^{-1}): 1.42 ± 0.67 × 10^6 and 1.25 ± 0.71 × 10^6 for thrombin with plasma PCI and wt rPCI, respectively; and 1.85 ± 0.29 × 10^5 and 1.38 + 0.36 × 10^5 for APC with plasma PCI and wt rPCI, respectively. Wild-type rPCI displayed a typical serpin heparin-dependent curve in the inhibition of both thrombin and APC (Fig. 4). The maximum inhibition activity (k_2) was similar between wt rPCI and plasma PCI. However, wt rPCI required less heparin to achieve maximum inhibitory activity. In the inhibition of thrombin, wt rPCI had maximal activity at 0.5 μg/ml heparin compared with 1 μg/ml for plasma PCI. With APC this difference was slightly greater, with maximum wt rPCI inhibition at 10 μg/ml heparin compared with 100 μg/ml for plasma PCI.

Inhibition of Thrombin, APC, Urokinase, and Trypsin by rPCI Mutants—Wild-type rPCI and the three reactive site mutants were tested for their ability to inhibit four different serine proteinases (Table I). Plasma PCI, antithrombin and α_1-proteinase inhibitor were used as control serpins. Wild-type rPCI and plasma PCI showed no difference in their abilities to inhibit thrombin, APC, trypsin, or urokinase in the presence of optimal heparin. Under the assay conditions used, wt rPCI and plasma PCI are better inhibitors of thrombin than of APC and of trypsin than of urokinase (Table I).

R354M rPCI which changes the P1 residue Arg → Met generated a reactive site similar to α_1-proteinase inhibitor. The activity of R354M rPCI was dramatically reduced for each proteinase compared with wt rPCI, but it was quite similar and was not significantly different from α_1-proteinase inhibitor with the four proteinases assayed (Table I). Although the k_2 value was half that of wt rPCI, R354M rPCI was best at inhibiting trypsin of the four proteinases tested and is a better inhibitor than wt rPCI against chymotrypsin (data not shown).

Changing the P2 residue from Phe → Gly, F353G rPCI, generated a mutant with a reactive site like antithrombin which, relative to wt rPCI, was a better inhibitor of thrombin and urokinase, but was less active with APC or trypsin (Table I).

The ratio of proteinase inhibition between wt rPCI and F353G rPCI shifted from 25 to 396 for thrombin/APC and from 10 to 1.5 for trypsin/urokinase, respectively. F353G rPCI was still a better inhibitor of APC and urokinase than was antithrombin, but antithrombin was a much better inhibitor of both thrombin and trypsin.

S355M rPCI which changes the P1′ residue from Ser → Met generated a reactive site similar to PAI-1. S355M rPCI inhibits thrombin, APC, trypsin, and urokinase with essentially the same k_2 values as wt rPCI (Table I). A second rPCI P1′ Ser mutant (S355V rPCI) also had the same k_2 (M^{-1} min^{-1}) values as wt rPCI with thrombin and APC, 1.18 ± 0.32 × 10^7 and 6.79 ± 2.16 × 10^5, respectively, which suggests that the P1′ position may not play a crucial role in the activity of PCI.

DISCUSSION

Protein C inhibitor has been a difficult protein to study owing to its scarcity in plasma, instability during purification from plasma, and a lack of naturally occurring functional mutations.

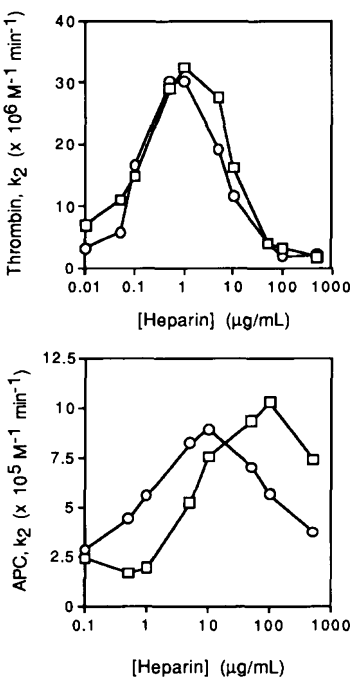

FIG. 4. **Heparin-dependent inhibition of thrombin and APC by rPCI and plasma PCI.** Thrombin (*top panel*) and APC (*bottom panel*) were incubated in the presence of a 10-fold molar excess of wt rPCI (○) and plasma PCI (□) with increasing concentrations of heparin as detailed under "Experimental Procedures." The inhibition rate constant (k_2) was calculated as M^{-1} min^{-1} for PCI at each heparin concentration.

These studies were undertaken to achieve expression of a recombinant form of PCI in order to begin to understand its putative target proteinase specificity with particular emphasis on its ability to inhibit both APC and thrombin. Several serpins which are closely related to PCI have been expressed, including antithrombin (42–46), heparin cofactor II (47–49), α_1-proteinase inhibitor (50), α_1-anti-chymotrypsin (51), and PAI-1 (34, 52, 53). Using a baculovirus system, full-length rPCI can be expressed in Sf9 or High-Five™ cells, and the recombinant protein is fully active. With the exception of incomplete *N*-glycosylation, rPCI was apparently processed normally by the insect cells, and it had the same amino-terminal sequence as plasma PCI. rPCI formed bimolecular complexes with proteinases in a manner that was accelerated by heparin. Inhibition of four proteinases (thrombin, APC, urokinase, and trypsin) showed no differences between wt rPCI and plasma PCI. However, the amount of heparin required to maximally accelerate proteinase inhibition by rPCI was slightly less than that of plasma PCI. Although we cannot explain this apparent difference at the present time, it may be due to a reduced glycosylation that might result in slightly better binding of rPCI to heparin.

Alterations of the P1 reactive site residue of many serpins strongly influence inhibition activity. α_1-Proteinase inhibitor Pittsburgh, a natural mutation of Met^{358} → Arg, has changed its target proteinase specificity from elastase to thrombin (54). Mutation of Leu^{444} → Arg in heparin cofactor II resulted in enhanced thrombin but diminished chymotrypsin inhibitory activities (49). Mutagenesis of the P1 residue in PAI-1 showed that a basic residue (Lys or Arg) was required for significant inhibition of urokinase (34, 53). Our data confirm the importance of the P1 Arg of PCI in that mutation of Arg^{354} → Met displayed a significant loss of inhibition activity toward proteinases. The inhibition profile of R354M rPCI is very similar to the α_1-proteinase inhibitor, which further indicates that a role of the P1 residue is to resemble a proteinase substrate. Likewise, α_1-proteinase inhibitor Pittsburgh (P1 Met^{358} → Arg) is a better APC inhibitor than naturally occurring α_1-proteinase inhibitor (55, 56). Thus, it can be generally concluded that the P1 Arg in PCI is important for APC (and other "trypsin-like" proteinases) inhibition.

Using peptide *p*-nitroanilide substrates and chloromethyl ketone-containing peptide inhibitors, the specificity of APC for the P2 position was found to be large hydrophobic residues like Phe (and Leu), whereas a P2 Gly failed to react (57). Interestingly, this is the exact difference in the *P2*-P1-P1′ sequence between PCI (FRS) and antithrombin (GRS). Our study demonstrated that changing the P2 Phe → Gly converted PCI into a better thrombin inhibitor which is only 16-fold less reactive than the antithrombin-thrombin reaction in the presence of

TABLE I
Proteinase inhibition rates of rPCI mutants

Serpin[b]	Proteinase (k_2 × M^{-1} min^{-1})[a]					
	Thrombin (IIa)	APC	IIa/APC	Trypsin (T)	Urokinase (UK)	T/UK
pPCI (FRS)	3.25 ± 0.31 × 10^7	1.03 ± 0.14 × 10^6	32	1.02 ± 0.18 × 10^6	7.83 ± 2.1 × 10^4	13
wt rPCI (FRS)	2.43 ± 0.19 × 10^7[c]	9.90 ± 3.6 × 10^5[c]	25	1.04 ± 0.05 × 10^6[c]	1.03 ± 0.43 × 10^5[c]	10
F353G rPCI (GRS)	5.30 ± 0.60 × 10^7[d]	1.34 ± 0.78 × 10^5[d]	396	3.17 ± 2.2 × 10^5[d]	2.06 ± 0.98 × 10^5[d]	1.5
R354M rPCI (FMS)	5.70 ± 1.6 × 10^4[d]	2.21 ± 1.0 × 10^4[d]	2.6	5.39 ± 2.4 × 10^5[d]	5.68 ± 3.1 × 10^3[d]	95
S355M rPCI (FRM)	2.70 ± 0.01 × 10^7[e]	8.17 ± 1.7 × 10^5[e]	33	1.05 ± 0.08 × 10^6[e]	3.32 ± 0.37 × 10^4[e]	32
AT (GRS)	8.55 ± 0.14 × 10^8	<1.5 × 10^1		1.09 ± 0.08 × 10^7	8.59 ± 0.53 × 10^3	1270
α_1-PI (PMS)	5.46 ± 6.1 × 10^4[f]	4.84 ± 6.5 × 10^4[f]	1.1	2.52 ± 0.44 × 10^6[f]	8.96 ± 0.52 × 10^3[f]	280

[a] All proteinase-serpin inhibition assays contained heparin as detailed under "Experimental Procedures."
[b] Reactive site sequence (P2-P1-P1′) shown in parentheses: pPCI, plasma PCI; wt rPCI, wild-type rPCI; F353G rPCI, rPCI with Phe^{353} → Gly; R354M rPCI, rPCI with Arg^{354} → Met; and S355M rPCI, rPCI with Ser^{355} → Met; AT, antithrombin; α_1-PI, α_1-proteinase inhibitor.
[c] Not significantly different from plasma-derived PCI.
[d] Significantly ($p < 0.05$) different from wt rPCI.
[e] Not significantly different from wt rPCI.
[f] Not significantly different from R354M rPCI.

optimal heparin. Furthermore, there was more than a 7-fold reduction in APC inhibition by F353G rPCI compared with PCI. F353G rPCI was also a better inhibitor of urokinase, but it was a worse inhibitor of trypsin. A mutant α_1-anti-chymotrpysin with a P2-P1-P1′ reactive site sequence of LRS (the wild-type sequence is LLS) was recently shown to inhibit APC comparable with PCI, further suggesting that a hydrophobic P2 residue in concert with a P1 Arg is an effective recognition sequence for APC (56). The major conclusion in our study suggests that a P2 Gly like that found in F353G rPCI and in antithrombin is not well tolerated for effective APC inhibition, but it is excellent for thrombin inhibition.

One of the most highly conserved amino acid residues in the serpin reactive site sequences is the P1′ Ser (7, 8). In some instances, as in antithrombin, it has been found that the P1′ Ser is crucial for inhibitory action possibly due to hydrophobic and size constraints of the reactive site loop (44). In contrast it was found that all amino acids at the P1′ position, with the exception of Pro, were well tolerated in PAI-1 (where the wild-type P1′ is Met) (34). The P1′ PCI mutants prepared here changed $Ser^{355} \rightarrow$ Met or Val and they showed no substantial changes in inhibition activity. Comparing both amino acid residue volumes and hydrophobic indexes (given in parentheses) of these amino acid residues (Ser (89 Å^3, 0.3), Met (163 Å^3, −1.3), and Val (140 Å^3, −1.5)) show that a variety of possibilities are tolerated at the P1′ position. Our data support a concept that the P1′ site of PCI is flexible regarding size constraint and hydrophobic character, and it may be similar to PAI-1.

In summary, we have expressed recombinant PCI and have provided some observations about target proteinase inhibition due to PCI's reactive site sequence at the P2, P1, and P1′ positions. Interestingly, PCI is the only serpin that possesses an Arg residue at the P3′ position, similar to fibrinogen, which may mediate ionic interactions with Glu^{39} and Glu^{192} of thrombin (58–60), and Glu^{192} of APC (36). Future PCI mutagenesis studies will examine the importance of both P3 and P3′ residues in an attempt to make a PCI molecule that has enhanced APC but diminished thrombin inhibitory properties. A better understanding of PCI interactions with its various putative plasma target proteinases, especially APC and thrombin, will be useful as new therapeutic agents are developed and tested directly dealing with the anticoagulation and anti-inflammation properties of the protein C system.

Acknowledgments—We thank Professors Elmer M. Price and Susan T. Lord for invaluable advice throughout this investigation, Rebecca A. Shirk, Professor Dougald M. Monroe and Laura L. Neese for helpful suggestions, Dr. Joost C. M. Meijers for providing the PCI cDNA, and Judy Phelps and Professor Vann Bennett (Howard Hughes Medical Institute, Duke University) for performing the rPCI sequence analyses.

REFERENCES

1. Suzuki, K., Nishioka, J., and Hashimoto, S. (1983) *J. Biol. Chem.* **258,** 163–168
2. Suzuki, K., Deyashiki, Y., Nishioka, J., Kurachi, K., Akira, M., Yamamoto, S., and Hashimoto, S. (1987) *J. Biol. Chem.* **262,** 611–616
3. Suzuki, K., Deyashiki, Y., Nishioka, J., and Toma, K. (1989) *Thromb. Haemostasis* **61,** 337–342
4. Pratt, C. W., and Church, F. C. (1992) *J. Biol. Chem.* **267,** 8789–94
5. Pratt, C. W., Whinna, H. C., and Church, F. C. (1992) *J. Biol. Chem.* **267,** 8795–8801
6. Pratt, C. W., and Church, F. C. (1993) *Blood Coagul. & Fibrinolysis* **4,** 479–490
7. Carrell, R. W., and Travis, J. (1985) *Trends Biochem. Sci.* **10,** 20–24
8. Huber, R., and Carrell, R. W. (1989) *Biochemistry* **28,** 8951–8966
9. Geiger, M., Priglinger, U., Griffin, J. H., and Binder, B. R. (1991) *J. Biol. Chem.* **266,** 11851–11857
10. Kuhn, L. A., Griffin, J. H., Fisher, C. L., Greengard, J. S., Bouma, B. N., Espana, F., and Tainer, J. A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 8506–8510
11. Olson, S. T., and Björk, I. (1992) in *Thrombin: Structure and Function* (Berliner, L. J., ed) pp. 159–217 Plenum Press, New York
12. Tollefsen, D. M. (1992) *Adv. Exp. Med. Biol.* **313,** 167–176
13. Cunningham, D. D., Wagner, S. L., and Farrell, D. H. (1992) *Adv. Exp. Med. Biol.* **313,** 297–306
14. Roberts, H. R., and Lozier, J. N. (1992) *Hosp. Pract.* **VOLUME,** 97–111
15. Furie, B., and Furie, B. C. (1992) *N. Engl. J. Med.* **326,** 800–806
16. Esmon, C. T. (1987) *Science* **235,** 1348–1352
17. Esmon, C. T., Taylor, F. B., and Snow, T. R. (1991) *Thromb. Haemostasis* **66,** 160–165
18. Esmon, C. T. (1992) *Arterioscler. Thromb.* **12,** 135–145
19. Walker, F. J., and Fay, P. J. (1992) *FASEB J.* **6,** 2561–2567
20. Hoogendoorn, H., Nesheim, M. E., and Giles, A. R. (1990) *Blood* **75,** 2164–2171
21. Espana, F., Gruber, A., Heeb, M. J., Hanson, S. R., Harker, L. A., and Griffin, J. H. (1991) *Blood* **77,** 1754–1760
22. Scully, M. F., Toh, C. H., Hoogendoorn, H., Manuel, R. P., Nesheim, M. E., Solymoss, S., and Giles, A. R. (1993) *Thromb. Haemostasis* **69,** 448–53
23. Heeb, M. J., Schwarz, P., White, T., Lammle, B., Berrettini, M., and Griffin, J. H. (1988) *Thromb. Res.* **52,** 33–43
24. Heeb, M. J., and Griffin, J. H. (1988) *J. Biol. Chem.* **263,** 11613–11616
25. Heeb, M. J., Gruber, A., and Griffin, J. H. (1991) *J. Biol. Chem.* **266,** 17606–17612
26. Hoogendoorn, H., Toh, C. H., Nesheim, M. E., and Giles, A. R. (1991) *Blood* **78,** 2283–2290
27. Schechter, I., and Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **27,** 157–162
28. Geiger, M., Huber, K., Wojta, J., Stingl, L., Espana, F., Griffin, J. H., and Binder, B. R. (1989) *Blood* **74,** 722–8
29. Espana, F., Gilabert, J., Estelles, A., Romeu, A., Aznar, J., and Cabo, A. (1991) *Thromb. Res.* **64,** 309–20
30. Laurell, M., Christensson, A., Abrahamsson, P.-A., Stenflo, J., and Lilja, H. (1992) *J. Clin. Invest.* **89,** 1094–101
31. Ecke, S., Geiger, M., Resch, I., Jerabek, I., Stingl, L., Maier, M., and Binder, B. R. (1992) *J. Biol. Chem.* **267,** 7048–7052
32. Meijers, J. C. M., Kanters, D. H. A. J., Vlooswijk, R. A. A., van Erp, H. E., Hessing, M., and Bouma, B. N. (1988) *Biochemistry* **27,** 4231–4237
33. Pratt, C. W., Macik, B. G., and Church, F. C. (1989) *Thromb. Res.* **53,** 595–602
34. Sherman, P. M., Lawrence, D. A., Yang, A. Y., Vandenberg, E. T., Paielli, D., Olson, S. T., Shore, J. D., and Ginsburg, D. (1992) *J. Biol. Chem.* **267,** 7588–7595
35. Shieh, B.-H., and Travis, J. (1987) *J. Biol. Chem.* **262,** 6055–6059
36. Rezaie, A. R., and Esmon, C. T. (1993) *J. Biol. Chem.* **268,** 19943–19948
37. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol.* **154,** 367–382
38. Laemmli, U. K. (1970) *Nature* **227,** 680–685
39. Hunkapiller, M. W., Lujan, E., Ostrander, F., and Hood, L. E. (1983) *Methods. Enzymol.* **91,** 227–236
40. Church, F. C., and Whinna, H. C. (1986) *Anal. Biochem.* **157,** 77–83
41. Whinna, H. C., Choi, H. U., Rosenberg, L. C., and Church, F. C. (1993) *J. Biol. Chem.* **268,** 3920–3924
42. Stephens, A. W., Siddiqui, A., and Hirs, C. H. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 3886–3890
43. Wasley, L. C., Atha, D. H., Bauer, K. A., and Kaufman, R. J. (1987) *J. Biol. Chem.* **262,** 14766–14772
44. Stephens, A. W., Siddiqui, A., and Hirs, C. H. (1988) *J. Biol. Chem.* **263,** 15849–52
45. Gillespie, L. S., Hillesland, K. K., and Knauer, D. J. (1991) *J. Biol. Chem.* **266,** 3995–4001
46. Gettins, P. G. W., Fan, B., Crews, B. C., Turko, I. V., Olson, S. T., and Streusand, V. J. (1993) *Biochemistry* **32,** 8385–8389
47. Ragg, H., and Preibisch, G. (1988) *J. Biol. Chem.* **263,** 12129–12134
48. Blinder, M. A., Marasa, J. C., Reynolds, C. H., Deaven, L. L., and Tollefsen, D. M. (1988) *Biochemistry* **27,** 752–759
49. Derechin, V. M., Blinder, M. A., and Tollefsen, D. M. (1990) *J. Biol. Chem.* **265,** 5623–5628
50. Hopkins, P. C., Carrell, R. W., and Stone, S. R. (1993) *Biochemistry* **32,** 7650–7
51. Rubin, J., Wang, Z. M., Nickbarg, E. B., McLarney, S., Naidos, N., Schoenberger, O. L., Johnson, J. L., and Cooperman, B. S. (1990) *J. Biol. Chem.* **265,** 1199–1207
52. Lawrence, D. A., Strandberg, L., Ericson, J., and Ny, T. (1990) *J. Biol. Chem.* **265,** 20293–20301
53. York, J. D., Li, P., and Gardell, S. J. (1991) *J. Biol. Chem.* **266,** 8495–8500
54. Owen, M. C., Brennan, S. O., Lewis, J. H., and Carrell, R. W. (1983) *N. Engl. J. Med.* **309,** 694–698
55. Heeb, M. J., Bischoff, R., Courtney, M., and Griffin, J. H. (1990) *J. Biol. Chem.* **265,** 2365–2369
56. Hermans, J. M., and Stone, S. R. (1993) *Biochem. J.* **295,** 239–45
57. Stone, S. R., and Hofsteenge, J. (1985) *Biochem. J.* **230,** 497–502
58. Le Bonniec, B. F., MacGillivray, R. T. A., and Esmon, C. T. (1991) *J. Biol. Chem.* **266,** 13796–13803
59. Le Bonniec, B. F., and Esmon, C. T. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 7371–7375
60. Bode, W., Turk, D., and Karshikov, A. (1992) *Protein Sci.* **1,** 426–471