A + -Helix of Protein C Inhibitor (PCI) Is a Cell-penetrating Peptide That Mediates Cell Membrane Permeation of PCI*

Received for publication, October 31, 2014, and in revised form, December 5, 2014 Published, JBC Papers in Press, December 8, 2014, DOI 10.1074/jbc.M114.581736

Hanjiang Yang1, Felix Christoph Walmüller1, Bettina Sarg5, Margareta Furtmüller5, and Margarethe Geiger1,2

From the 1Center of Physiology and Pharmacology, Dept. of Vascular Biology and Thrombosis Research, Medical University of Vienna, A-1090 Vienna, Austria and 2Biocenter, Division of Clinical Biochemistry, Innsbruck Medical University, A-6020 Innsbruck, Austria

Background: Extracellular protein C inhibitor (PCI) can cross the cellular plasma membrane.

Results: Testisin (fluid-phase and cell membrane-anchored) cleaves PCI close to its N terminus. N-terminally truncated PCI can no longer be internalized by cells.

Conclusion: Testisin removes helix A +, a cell-penetrating peptide, which mediates cell membrane permeation of PCI.

Significance: Testisin or other proteases could regulate PCI internalization by removing its N terminus.

Protein C inhibitor (PCI) is a serpin with broad protease reactivity. It binds glycosaminoglycans and certain phospholipids that can modulate its inhibitory activity. PCI can penetrate through cellular membranes via binding to phosphatidylethanolamine. The exact mechanism of PCI internalization and the intracellular role of the serpin are not well understood. Here we showed that testisin, a glycosylphosphatidylinositol-anchored serine protease, cleaved human PCI and mouse PCI (mPCI) at their reactive sites as well as at sites close to their N terminus. This cleavage was observed not only with testisin in solution but also with cell membrane-anchored testisin on U937 cells. The cleavage close to the N terminus released peptides rich in basic amino acids. Synthetic peptides corresponding to the released peptides of human PCI (His1–Arg11) and mPCI (Arg1–Ala18) functioned as cell-penetrating peptides. Because intact mPCI but not testisin-cleaved mPCI was internalized by Jurkat T cells, a truncated mPCI mimicking testisin-cleaved mPCI was created. The truncated mPCI lacking 18 amino acids at the N terminus was not taken up by Jurkat T cells. Therefore our model suggests that testisin or other proteases could regulate the internalization of PCI by removing its N terminus. This may represent one of the mechanisms regulating the intracellular functions of PCI.

Protein C inhibitor (PCI; SerpinA5) is a serine protease inhibitor with broad protease reactivity (1). It belongs to clade A of the serpin (serine protease inhibitor) superfamily (1) and was initially identified in human plasma as an inhibitor of the anticoagulant serine protease activated protein C (aPC) (2). Subsequently, PCI was recognized to be an inhibitor of many other serine proteases including blood coagulation factors (3), fibrinolytic enzymes (4, 5), tissue kallikrein (6), acrosin (7), hepatocyte growth factor activator (8), and enteropeptidase (9). Like other serpins, PCI inhibits its target proteases by forming covalent enzyme-serpin complexes (10). PCI is a heparin-binding serpin, and heparin and other glycosaminoglycans can modulate its activity and target enzyme specificity (10–12). Other non-protease ligands like oxidized/negatively charged phospholipids such as phosphatidylinerine, oxidized phosphatidylserine, oxidized phosphatidylethanolamine, and phosphoinositides (13, 14) show heparin-like effects and may enhance the inhibitory activity of PCI (13, 14).

We have shown previously that PCI can be internalized by cells by a phosphatidylethanolamine-dependent mechanism (15). Inside the cell, PCI can translocate to the nucleus. Although we have identified several intracellular proteins interacting with PCI (16, 17), the biological relevance of PCI internalization and nuclear translocation still has to be examined. In addition, glycosaminoglycans and phospholipids present on cell membranes (13, 18) may not only mediate the permeation of PCI through membranes but may also bring PCI into close vicinity of cell membrane-associated serine proteases. To the best of our knowledge, there are only a few publications about the interaction of PCI with membrane-associated serine proteases (9, 19, 20). Among those reports, enteropeptidase is the only membrane-anchored serine protease whose interaction with PCI has been biochemically characterized (9).

The aim of this study was to investigate the interaction of PCI with testisin. Testisin (also referred to as ESP1, TEST5, and PRSS21 (21–23)) is a serine protease that is posttranslationally modified by the addition of a C-terminal glycosylphosphatidylinositol membrane anchor (23, 24). It is highly expressed in male germ cells and sperm (22, 25) and is found in capillary endothelial cells, eosinophils (21), and ovarian cancers (24, 26). Testisin has been described as a putative non-classical type 2 tumor suppressor in testicular cancer because loss of testisin...
expression by germ cell-derived testicular tumors has been observed (22). In contrast, testisin is highly expressed in ovarian tumor cells with little or no expression in normal ovaries and is linked to advanced stage disease in primary human ovarian carcinomas (26). Subsequently Tang et al. (24) demonstrated that enzymatic activity of testisin is critical for transformation of tumor cells and that knockdown of testisin in ovarian cancer cells leads to increased apoptosis and reduced cell growth in soft agar. Testisin-deficient spermatozoa show abnormal function despite subnormal fertility of testisin-deficient mice (27, 28). Those sperm exhibit decreased motility, angulated and curled tails, fragile necks, and increased decapitation (28). Abnormal features may lead to reduced sperm-egg binding and decreased fertility in short term fertility studies (28). Data also show reduced ability of testisin-deficient spermatoozoa to bind to the zona pellucida and fuse with eggs in vitro. This defect could be rescued by treatment of spermatozoa with uterine fluids (27). Interaction of protease inhibitors with testisin may therefore be not only important in tumor invasion and metastasis but also in the development and maturation of spermatozoa.

We have shown previously that targeted disruption of the PCI gene results in infertility of male homozygous mice that is caused by abnormal spermatogenesis (29). This is not surprising because in adult mice PCI is almost exclusively expressed in the reproductive tract. In contrast, human PCI (hPCI) is expressed in many tissues and is present in blood and most body fluids (30). Very high concentrations of hPCI are present in the male and female reproductive tracts (30), and loss of PCI activity in seminal plasma has been shown to be associated with infertility (31). Recently there has been increasing evidence in the literature that loss of PCI expression in cancer cells is associated with increased malignancy and that PCI could act as a tumor suppressor. There are studies showing that PCI expression is decreased or absent in renal, prostate, and ovarian cancers (32–35). In ovarian tumors, the reduction or loss of PCI expression is associated with a more aggressive phenotype of tumors (33, 34). Two single nucleotide polymorphisms identified in the PCI gene are significantly related with risk of papillary thyroid cancer (36). Growth and metastasis of breast cancer cells are suppressed by incubation with exogenous PCI or by overexpression of PCI independently of its serine protease inhibitory activity (37). PCI inhibits growth and migration of breast cancer cells partially by inhibiting cathepsin L activity (38). The mechanism(s) responsible for the protective effects against tumor growth have not been elucidated so far.

Here we investigated the interaction of PCI with testisin and could show that hPCI and mouse PCI (mPCI) act as substrates for testisin. Both hPCI and mPCI were cleaved by testisin at the reactive center loop and at an additional site close to the N terminus. We furthermore showed that the N-terminal peptides of hPCI and mPCI exhibited cell penetrating activity, mediating the internalization of PCI by Jurkat T lymphoma cells.

### MATERIALS AND METHODS

**Cell Culture—** Jurkat T lymphoma cells (Clone E6-1, ATCC number TIB-152) and U937 histiocytic lymphoma cells (ATCC number CRL-1593.2) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Sigma-Aldrich), 20 mM Hepes, 100 μg/ml penicillin, and 100 units/ml streptomycin (full medium) in a humidified atmosphere containing 5% CO₂ at 37 °C. For the internalization experiment, cells were seeded at 1 × 10⁶ cells/ml with 8 ml of full medium into 75-cm² flasks and treated with respective 200 nM proteins for 2 h or 5 μM peptides for 1 h.

**Preparation of Recombinant Proteins—** Expression vectors of recombinant mouse PCI (rmPCI)⁴ and recombinant human PCI (rhPCI) (39) were obtained from the collection of the Department of Vascular Biology and Thrombosis Research, Medical University of Vienna. Expression vectors of ΔR1-18 mPCI (lacking the first 18 amino acids corresponding to helix A+), hPCI-R11G (Arg¹¹ replaced by Gly), hPCI-R354G (Arg⁵⁵⁴ replaced by Gly), mPCI-A18G (Ala¹⁸ replaced by Gly), or mPCI-R352G (Arg⁵⁵² replaced by Gly) were constructed as described previously (39). Briefly, mutated mPCI DNA fragments were prepared by PCR (see primers in Table 1) and cloned into the pET-15b plasmid. Constructs were confirmed by sequencing (Microsynth AG, Balgach, Switzerland). Expression and purification of recombinant proteins were performed as described previously (39).

**Interaction of PCI and PCI Mutants with Testisin—** Activation of testisin and testisin (R&D Systems, Minneapolis, MN) activity assays were performed according to the instructions of the supplier. Inhibition of testisin by PCI was tested on 96-well F16 Black MaxiSorp plates (Nunc, Roskilde, Denmark).

---

⁴ M. Furtmüller, unpublished data.
20 nM testisin was incubated with 200 or 1000 nM (final concentration) rhPCI or rmPCI in the absence or presence of 5 units/ml heparin in 100 μl of testisin buffer (50 mM Tris, 150 mM NaCl, 10 mM CaCl₂, pH 7.5). After a 30-min incubation at 37 °C, 100 μl of 0.2 mM fluorescent substrate I-1295 (Bachem, Bubendorf, Switzerland) dissolved in testisin buffer was added to each well. Plates were read every 15 s at an excitation wavelength of 380 nm and an emission wavelength of 460 nm using a Synergy H4 microplate reader (BioTek, Seattle, WA). The 5-min time point was used to calculate the relative activity. The amidolytic activity of testisin in the absence of PCI was assigned to 1, and for each reaction, the remaining testisin activity was calculated. To detect the possible complex formation between PCI and testisin, different concentrations (7.5–960 nM) of rhPCI or rmPCI, respectively, were incubated with 30 nM testisin for varying times (0–40 min) at 37 °C. The reactions were stopped by adding an equal volume of 2× Laemmli buffer containing 5% 2-mercaptoethanol (40). Samples were heated at 99 °C for 5 min followed by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) and Western blotting. To determine the testisin cleavage sites in human and mouse PCI, WT PCI or PCI mutants (rhPCI, hPCI-R11G, hPCI-R354G, rmPCI, mPCI-A18G, or mPCI-R352G, respectively; 300 nM each) were incubated with 30 nM testisin for 1 h at 37 °C. An equal volume of 2× Laemmli buffer containing 5% 2-mercaptoethanol was added to those samples to stop the reaction followed by SDS-PAGE and Western blotting. 

His Tag Precipitation of Intact rmPCI, Testisin-pretreated rmPCI, or Testisin—rmPCI, testisin-pretreated rmPCI, or testisin (Fig. 1E, labeled as Before Incubation), respectively, was incubated with cobalt beads at 4 °C for 30 min. After the beads were collected by centrifugation (3 min at 500 × g at 4 °C), and the supernatants (Fig. 1E, labeled as Binding Supernatant) were transferred to separate tubes. Cobalt beads were washed gently three times with PBS. PBS containing 250 mM imidazole was applied to elute proteins or peptides (Fig. 1E, labeled as Elution Products) from cobalt beads. 2-μl aliquots of each sample (i.e. rmPCI, testisin-pretreated rmPCI, and testisin) and from each step (i.e. before incubation with cobalt beads, supernatants from the incubation with cobalt beads, and elution with 250 mM imidazole) were directly spotted on polyvinylidene difluoride (PVDF) membrane and subjected to Western blotting using anti-mPCI IgG or anti-penta-His IgG, respectively.

Interaction of PCI with Other Proteases—Urokinase, aPC, thrombin, or enteropeptidase (30 nM each) were incubated with 300 nM rmPCI, respectively, for different periods (10 or 120 min) at 37 °C. The reactions were stopped by adding an equal volume of 2× Laemmli buffer containing 5% 2-mercaptoethanol (40). Samples were heated at 99 °C for 5 min followed by SDS-PAGE and Western blotting.

Analysis of α-1-Antitrypsin (A1AT) Interaction with Testisin—To analyze whether testisin also interacted with other serpins, 30 nM testisin was incubated with 300 nM serum-derived human A1AT (Sigma-Aldrich) for different time periods (0–40 min) at 37 °C. The reactions were stopped by adding an equal volume of 2× Laemmli buffer containing 5% 2-mercaptoethanol (40). Samples were heated at 99 °C for 5 min followed by SDS-PAGE and Western blotting.

Inhibition of aPC by PCI—Inhibition of aPC amidolytic activity by PCI was tested on 96-well microtiter plates. 0.2 nM aPC was incubated with 10 or 50 nM (final concentration) rhPCI, rmPCI, hPCI-R11G, hPCI-R354G, mPCI-A18G, mPCI-R352G, or ΔR1-A18 mPCI, respectively, in the absence or presence of 5 units/ml heparin in 100 μl of aPC buffer (25 mM Hepes, 137 mM NaCl, 3.5 mM KCl, 3 mM CaCl₂, 1% BSA, pH 7.4). After a 30-min incubation at 37 °C, 100 μl of 0.4 mM chromogenic substrate S-2366 (Chromogenix, Milano, Italy) dissolved in aPC buffer was added to each well. The absorbance at 405 nm was determined in a Synergy H4 microplate reader. The 30-min time point was used to calculate the relative activity. The amidolytic activity of aPC in the absence of PCI was assigned to 1, and for each reaction, the remaining aPC activity was calculated.

Interaction of PCI with Cellular Testisin—U937 cells (1 × 10⁷ cells/ml) were incubated in RPMI 1640 medium without or with 2 units/ml phosphoinositide phospholipase C (PI-PLC; Calbiochem) at 37 °C for 30 min. Media and cells were separated by centrifugation (5 min at 500 × g at 4 °C). For Western blotting experiments, the cells were washed and resuspended in Celllytic cell lysis buffer (Sigma-Aldrich), and the media were concentrated (20–25 times) with Millipore Amicon Ultra-4 centrifugal filter units with Ultracel-10 membrane. Samples were boiled with an equal amount of 2× Laemmli buffer containing 2-mercaptoethanol followed by SDS-PAGE and Western blotting using anti-testisin IgG. For rmPCI cleavage experiments, media were collected, and cells were resuspended in RPMI 1640 medium at a final concentration of 1 × 10⁷ cells/ml. 20 nM rmPCI was incubated with those media or cell suspensions at 37 °C for 0–4 h. After incubation, samples were mixed with 2× Laemmli buffer containing 2-mercaptoethanol and heated at 99 °C for 5 min followed by SDS-PAGE and Western blotting using anti-mPCI IgG.

SDS-PAGE and Western Blotting—SDS-PAGE was performed according to the method of Laemmli (40) using 10% acrylamide gels. Following electrophoresis, the proteins were transferred onto PVDF membranes. The membranes were blocked with blotting buffer (5% skim milk powder and 0.05% Tween 20 in PBS, pH 7.4) for 1 h at room temperature. Rabbit anti-mPCI IgG (diluted 1:1000; homemade), rabbit anti-hPCI IgG (diluted 1:1000; homemade), monoclonal mouse anti-penta-His IgG (diluted 1:500; Abgent, San Diego, CA), mouse monoclonal anti-A1AT(diluted 1:2000; Fisher Scientific GmbH), rabbit anti-actin IgG (diluted 1:2000; Sigma-Aldrich), monoclonal mouse anti-lamin B2 IgG (diluted 1:2000; Abcam, Cambridge, UK), and monoclonal mouse anti-Hsp 90 IgG (diluted 1:2000; Qiagen, Hilden, Germany), rabbit anti-testisin IgG (diluted 1:500; Abgent, San Diego, CA), mouse monoclonal anti-A1AT(diluted 1:2000; Fisher Scientific GmbH), rabbit anti-actin IgG (diluted 1:2000; Sigma-Aldrich), monoclonal mouse anti-lamin B2 IgG (diluted 1:2000; Abcam, Cambridge, UK), and monoclonal mouse anti-Hsp 90 IgG (diluted 1:2000; Exbio, Prague, Czech) were used, respectively, in blotting buffer. The respective secondary antibodies were horseradish peroxidase-conjugated donkey anti-rabbit IgG (diluted 1:5000; GE Healthcare Handels GmbH) and horseradish peroxidase-conjugated sheep anti-mouse IgG (diluted 1:5000; GE Healthcare Handels GmbH). The signal was detected using SuperSignal West Femto or SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific,

A+ -Helix of PCI Mediates Its Internalization by Cells

JANUARY 30, 2015 • VOLUME 290 • NUMBER 5 ASBMB • JOURNAL OF BIOLOGICAL CHEMISTRY 3083
A+–Helix of PCI Mediates Its Internalization by Cells

Rockford, IL) with a FluorChem HD2 imaging system (ProteinSimple, Santa Clara, CA).

Cell Lysis and Subcellular Fractionation—Jurkat T cells were incubated in full medium with 200 nm rmPCI, testisin-cleaved rmPCI, or AR1-A18 mPCI, respectively, at 37 °C for 2 h. 1 × 10^7 cells were harvested and washed three times with PBS. To prepare whole cell lysates, cell pellets were resuspended and incubated in Celllytic M cell lysis buffer at room temperature for 10 min and applied to electrophoresis. Subcellular fractions were prepared as described previously (41) with some modifications. In brief, cell pellets were resuspended in 125 μl of buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.34 mM sucrose, 10% glycerol, 1 mM DTT, 0.1% Triton X-100) containing 10 μl of protease inhibitor mixture P8340 (Sigma-Aldrich) and incubated for 8 min. Intact nuclei were collected by low speed centrifugation (5 min at 1300 × g at 4 °C), and the supernatant (cytosol) was transferred to another tube. The nuclei were washed once with buffer A plus 1/10 volume P8340 and then lysed in 125 μl of hypotonic buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 1/10 volume P8340) for 30 min. Insoluble chromatin and the soluble fraction (soluble nucleus) were separated by centrifugation (5 min at 1700 × g at 4 °C). Insoluble chromatin, which was washed once with buffer B, was suspended in 125 μl of micrococcal nuclease buffer (10 mM Tris, pH 9.5, 10 mM KCl, 1 mM CaCl₂) containing 5 units of micrococcal nuclease (Sigma-Aldrich) and incubated at 37 °C for 15 min. 12.5 μl of 10 mM EGTA (1 mM final concentration) was added to the mixture to stop the reaction. Nuclease-digested chromatin (chromatin-bound proteins) and nuclease-resistant (nuclear envelope) components were separated by centrifugation (5 min at 1700 × g at 4 °C). The nuclease-resistant, insoluble fraction (nuclear envelope) was solubilized in 125 μl of 1× Laemmli buffer. If not mentioned, all fractionation procedures were performed on ice.

Analysis of the Internalization of Fluorescein Isothiocyanate (FITC)-labeled Peptides by Jurkat T Cells—Human A+–helix peptide (HRHHPREMKKK; corresponding to the cleaved N-terminal peptide of hPCI), mouse A+–helix peptide (RRHSHSKKAKKESVGA; corresponding to the cleaved N-terminal peptide of mPCI), and TAT peptide (GRKKRRQRRRPQ) (42) were synthesized and labeled with FITC (GenScript, Piscataway, NJ). Jurkat T cells were incubated with 5 μM human A+–helix-FITC, mouse A+–helix-FITC, TAT–FITC, dextran–FITC, or free FITC, respectively, at 37 °C for 1 h, and cells were washed and fixed with 4% paraformaldehyde for 20 min at room temperature. Fluorescence-activated cell sorting (FACS) analysis was performed with a FACSCalibur flow cytometer (BD Biosciences). Further data analysis was done with Flowing Software (43). For confocal microscopy, the fixed cells were suspended with Vectashield mounting medium containing 1.5 ng/ml DAPI (Vector Laboratories, Burlingame, CA) and transferred onto slides. Confocal images were taken by a Zeiss LSM-510 Meta confocal microscope system (Zeiss, Jena, Germany). Zeiss LSM Image Browser software was applied for further analysis of images.

Edman Degradation—Peptide sequencing was performed on an Applied Biosystems Inc. Model 492 Procise protein sequenator. The proteins were blotted onto PVDF membranes, and typically samples were run for 5–10 cycles as required for an unambiguous identification.

RESULTS

PCI Is a Testisin Substrate—We analyzed the interaction between PCI and testisin by SDS-PAGE followed by Western blotting using anti-PCI IgGs. Incubation of PCI with testisin resulted in a time-dependent decrease in the molecular weight of PCI. No complex formation of PCI with testisin was observed (Fig. 1A). To confirm the hypothesis that there is no complex formation between PCI and testisin, we also used antibodies against testisin, but none of the available antibodies was suitable for mouse testisin. Additionally we evaluated the possible inhibition of testisin by PCI using a fluorogenic substrate. PCI was not able to inhibit testisin either in the absence or in the presence of heparin (not shown). To investigate whether testisin interacts with other serpins, we incubated testisin with different concentrations of A1AT for different time periods at 37 °C. Neither cleavage of A1AT nor testisin-A1AT complex formation was detected by Western blotting (Fig. 1B).

Testisin Cleavage of PCI Leads to N-terminal Peptide Release—As shown in Fig. 1A, testisin-cleaved PCI is about 4–5 kDa smaller than intact PCI, suggesting that the potential cleavage site(s) are located close to the C terminus and/or N terminus of PCI. To determine the testisin cleavage site within the PCI molecule, we performed Western blotting using anti-penta-His IgG, which recognizes the N-terminal His tag of recombinant PCI, and aPC inhibition assay to determine whether testisin-cleaved PCI contains a functional reactive site. Anti-penta-His IgG recognized intact PCI but not testisin-cleaved PCI (Fig. 1C). Testisin-cleaved PCI did not inhibit aPC either in the absence or in the presence of heparin (not shown). These data suggest that testisin cleaves PCI at two sites: at the N terminus and at the reactive site close to the C terminus. To confirm a cleavage of PCI by testisin close to its N terminus, Edman degradation was performed. New N-terminal sequences of hPCI and mPCI were identified, demonstrating that testisin cleaved hPCI between Arg^1^ and Val^12^ and mPCI between Ala^18^ and Val^19^, respectively (Fig. 1D). To confirm and validate these results, we performed mutagenesis of both the N-terminal and the C-terminal cleavage sites in human and mouse PCI and studied cleavage of these mutants by testisin. As can be seen from Fig. 1C, testisin-treated human and mouse PCIs in which either the N-terminal or the C-terminal cleavage sites were mutated exhibited a higher molecular weight as compared with the cleaved wild type forms, suggesting that the introduced mutations prevented cleavage at the respective sites. Furthermore testisin-treated N-terminally mutated human (hPCI-R11G) and mouse (mPCI-A18G) PCIs were still recognized by anti-penta-His IgG, which was not the case with wild type and C-terminally mutated human and mouse PCI (Fig. 1C, lower panels). The peptides that are generated by testisin cleavage of PCI correspond essentially to the A+–helix of hPCI and mPCI, respectively. His tag precipitation revealed that with intact PCI both PCI antigen and the His tag were present in the fraction bound to and eluted from the cobalt beads, whereas with testisin-treated PCI, only the His tag was detected in this fraction, and PCI antigen was found in the
“binding supernatant” (=unbound fraction). These data suggest that the A+ -helix peptide can be separated from the mPCI core domain after testisin cleavage (Fig. 1E). To study whether the N terminus of PCI could be released by other proteases, we also incubated PCI with urokinase, aPC, thrombin, or enteropeptidase for 10 or 120 min, respectively. As shown in (Fig. 1F),
incubation of mPCI with urokinase, aPC, or enteropeptidase, respectively, also resulted in the appearance of a cleaved PCI form. However, the bands seen after 2-h incubation had a slightly higher molecular weight as compared with the band seen with testisin treatment. Furthermore they were still recognized by anti-penta-His IgG. These data suggest that urokinase, aPC, and enteropeptidase do not cleave the N terminus of mPCI, although we cannot completely rule out the presence of an N-terminally cleaved mPCI form in the aPC-PCI complex because of the lower sensitivity of the anti-penta-His antibody as compared with the anti-mPCI IgG. Here thrombin was used as a control because there is a thrombin cleavage site at the N terminus of the recombinant protein from pET-15b vector.

PCI Is Cleaved by Testisin on U937 Cells—The experiments above have shown that PCI is cleaved by testisin in solution in purified systems. Because testisin is a glycosylphosphatidylinositol-anchored protein, we also analyzed whether this cleavage can occur on the cell membrane. We incubated U937 cells, which express testisin (Fig. 2A), with rmPCI. As shown in Fig. 2B, rmPCI incubated with U937 cells is cleaved (left panel) and cannot be detected with anti-penta-His IgG (right panel), suggesting that the N terminus of rmPCI was cleaved by incubation with U937 cells. We used PI-PLC to release testisin from the membrane. As can be seen from Fig. 2C, testisin can be detected in the conditioned medium of PI-PLC-treated U937 cells. PI-PLC treatment largely reduced the cleavage of rmPCI by U937 cells (Fig. 2D): PI-PLC-pretreated U937 cells or media from untreated cells did not affect the molecular weight of mPCI upon incubation for 1 and 4 h as compared with control. Incubation of mPCI with media from PI-PLC-pretreated U937 cells led to partial degradation of PCI after 1 and 4 h, indicating that cells preincubated with PI-PLC lost membrane-anchored testisin. After 4 h the PCI cleaving activity on the cell surface seemed to be partially recovered.

The A+ -helix of PCI Is Necessary for the Internalization of the Serpin by Jurkat T Cells—Previous studies have shown that the absence of the A+-helix does not influence the inhibitory activity or the heparin binding of PCI (44, 45). The released A+-helix peptides are rich in basic amino acids, one of the characteristics for cell-penetrating peptides (42). In a previous study, we have shown that PCI is internalized by cells in a phosphatidylethanolamine-dependent manner and can translocate to the nucleus (15). Therefore we hypothesized that the A+-helix peptides might play a role in cell membrane translocation of PCI. To verify this, we first analyzed the A+-helix peptides of hPCI and mPCI using an on line cell-penetrating peptide analysis tool (46). The A+-helix peptides of hPCI and mPCI scored 0.71 and 1.44, respectively, which indicate a high probability of cell-penetrating peptide function. We therefore investigated whether testisin-cleaved PCI or PCI preincubated with testisin-expressing U937 cells could still be internalized by cells. In all internalization experiments, we used mPCI and human cells (Jurkat T cells) to be able to differentiate between internalized PCI and endogenous PCI. As a positive control, we used intact rmPCI, which was taken up by Jurkat T cells and translocated to the nucleus (Fig. 3). In contrast, testisin-cleaved rmPCI could not be detected in the whole cell lysate (Fig. 3A) or in any subcellular fraction (Fig. 3B). Also rmPCI preincubated with U937 cells was not detected inside Jurkat T cells (Fig. 3C).

From these experiments, we cannot exclude that cleavage of mPCI at other than the Ala18-Val19 site (e.g. at the reactive site) and/or another reaction of testisin interferes with PCI internalization. We therefore expressed and purified truncated mPCI
A+ -Helix of PCI Mediates Its Internalization by Cells

(JANUARY 30, 2015 • VOLUME 290 • NUMBER 5 • JOURNAL OF BIOLOGICAL CHEMISTRY 3087)

FIGURE 3. Internalization of intact mPCI, testisin-cleaved mPCI, and mPCI preincubated with U937 cells by Jurkat T cells. Jurkat T cells were incubated with PBS (lanes 1), 200 nM (final concentration) mPCI (lanes 2), testisin-pretreated mPCI (A and B, lanes 3), or mPCI preincubated with U937 cells (C, lanes 3) for 2 h. Cell lysates and subcellular fractions were prepared as described under “Materials and Methods.” The whole cell lysates (A) and subcellular/nuclear fractions (B and C) were analyzed by SDS-PAGE and Western blotting. IgGs against mPCI, β-actin, lamin B2, or Hsp 90, respectively, as indicated in the figure were applied.

(A) Whole cell lysate
mPCI
β-Actin

Jurkat cells incubated with

1 PBS
2 200 nM rmPCI
3 200 nM testisin-cleaved rmPCI (A, B), or 200 nM rmPCI preincubated with U937 cells (C)

(B) Conditioned media Cytosol Soluble Chromatin bound Envelope
mPCI
Lamin B2
Hsp 70

(C) Conditioned media Cytosol Soluble Chromatin bound Envelope
mPCI
Lamin B2
Hsp 90

(ΔR1-A18 mPCI; lacking the N-terminal A+ -helix) to mimic testisin-cleaved mPCI (Fig. 4A). The recombinant protein contained a His tag at the N terminus. As shown in Fig. 4B, the mutant was recognized by anti-mPCI IgG as well as by anti-penta-His IgG. It was functionally active toward aPC, and its activity was stimulated by heparin (Fig. 4C). Subcellular fractions were prepared from Jurkat T cells incubated with rmPCI or ΔR1-A18 mPCI and applied to Western blotting using anti-mPCI IgG (Fig. 4D). rmPCI was detected in the nuclear envelope fraction of Jurkat T cells (Fig. 4D). ΔR1-A18 mPCI was not detected in any of the subcellular fractions (Fig. 4D), thereby verifying that the A+ -helix peptide of mPCI is essential for its internalization by Jurkat T cells.

The A+ -helix of PCI Is a Cell-penetrating Peptide—Although PCI lacking the A+ -helix cannot be internalized by cells, we could not conclude that the A+ -helix peptide of PCI has cell-penetrating properties. To verify this, we studied the internalization of synthesized FITC-labeled human and mouse A+ -helix peptides by cells. FITC-labeled HIV TAT peptide, a well known cell-penetrating peptide (42); free FITC; and dextran-FITC were used as controls. Jurkat T cells treated with FITC-labeled human and mouse A+ -helix peptides or FITC-labeled TAT peptides were analyzed by FACS and confocal microscopy and compared with untreated cells. As a negative control, cells treated with an equal molar amount of free FITC or dextran-FITC, respectively, were used (Fig. 5). FACS analysis revealed strong fluorescence signals in cells treated with human or mouse A+ -helix-FITC, and an even stronger signal in cells incubated with TAT-FITC was observed. Cells treated with free FITC or dextran-FITC showed a small shift compared with untreated cells (Fig. 5A). Confocal microscopy analysis of Jurkat T cells treated with human or mouse A+ -helix peptides, respectively, revealed that the A+ -helix peptides exhibited cytosolic and nuclear localization (Fig. 5B).

DISCUSSION

In the present study, we investigated the interaction of PCI with testisin. We could show that mPCI and hPCI are cleaved by testisin but do not form SDS-stable complexes with the protease. Consistently we were not able to detect inhibition of testisin by PCI. Testisin-cleaved PCI had no inhibitory activity toward aPC, suggesting cleavage of PCI at its reactive site. In addition, testisin also cleaved PCI close to its N terminus. As judged from Edman degradation of the remaining core protein, this cleavage occurred between Ala18 and Val19 in mPCI and between Arg11 and Val12 in hPCI, resulting in the release of a peptide essentially corresponding to the A+ -helix of PCI. The fact that testisin cannot remove the N terminus of mPCI-A18G and hPCI-R11G further confirms those new cleavage sites (Fig. 1D). We could show cleavage of PCI not only for testisin in solution but also for glycosylphosphatidylinositol-anchored testisin on U937 cells, suggesting that it may also occur in vivo. Other proteases that cleave PCI at the reactive center loop site do not trim the N terminus of PCI (Fig. 1D). This suggests that if the A+ -helix has a certain function this function would be regulated by testisin. The A+ -helix is rich in basic amino acids and has been suggested to play a role in heparin binding (47). However, later studies using rhPCI mutants have shown that the H-helix but not the A+ -helix functions as the heparin binding site of PCI (44). The A+ -helix is not necessary for the inhibitory
activity of PCI because hPCI lacking the A+ helix sequence has inhibitory activity similar to that of wild type hPCI (45). Also mPCI lacking A+ helix has inhibitory activity similar to that of intact PCI and can be stimulated by heparin (Fig. 4C).

We have shown before that PCI can penetrate through cell membranes in a process requiring the membrane phospholipid phosphatidylethanolamine (15). PCI contains a functional nuclear localization signal,5 and once inside the cell, PCI translocates to the nucleus (15). At present, it is still too early to speculate about intracellular functions of PCI; however, we

5 B. Sokolikova, J. M. Malieier, F. C. Wahlmüller, J. Breuss, D. Rieger, K. Einfinger, M. Furtmüller, and M. Geiger, manuscript in preparation.

FIGURE 4. Purification and characterization of truncated mPCI lacking the N terminus and its internalization by Jurkat T cells. A, Coomassie Blue R-250-stained SDS-polyacrylamide gels of purified WT mPCI (45.8 kDa) and ΔR1-A18 mPCI (43.8 kDa). B, WT mPCI and ΔR1-A18 mPCI were analyzed by SDS-PAGE and Western blotting with rabbit anti-mPCI IgG (left panel) and anti-penta-His IgG (right panel). C, inhibitory activity of WT mPCI and ΔR1-A18 mPCI. aPC (1 nM) was incubated for 30 min at 37 °C without or with different concentrations (10 and 50 nM) of WT mPCI or ΔR1-A18 mPCI in the absence (upper panel) or presence of heparin (5 units/ml; lower panel), respectively. The activity of aPC was determined from the cleavage of S-2366. Data shown in the graph are means from three independent experiments, and error bars represent S.E. D, Jurkat T cells were incubated with PBS (lanes 1), 200 nM mPCI (lanes 2), or 200 nM ΔR1-A18 mPCI (lanes 3), respectively, for 2 h at 37 °C. Cell lysates and subcellular fractions were prepared and analyzed by SDS-PAGE and Western blotting. IgGs against mPCI, lamin B2, or Hsp 90, respectively, as indicated in the figure were applied.
FIGURE 5. **Internalization of A+–helix-FITC by Jurkat T cells.** Jurkat T cells were incubated for 1 h with 5 μM human A+–helix-FITC peptide, mouse A+–helix-FITC peptide, TAT-FITC peptide, dextran-FITC, or free FITC, respectively. After washing and fixation by paraformaldehyde, cellular fluorescence was analyzed by FACS (A) or confocal microscopy (B). Cell nuclei were stained with DAPI (blue). The scale bar represents 5 μm (40×; numerical aperture, 1.3; oil objective).
A+−Helix of PCI Mediates Its Internalization by Cells

have identified several intracellular/nuclear proteins that interact with PCI (16, 17).

In the human system, PCI is present in many tissues and in most body fluids. Assuming that PCI has in fact intracellular functions, its permeation through the phospholipid bilayer has to be regulated by a mechanism(s) allowing cell membrane penetration of PCI into certain cells while excluding it from others. Peptides rich in basic amino acids such as the A+−helix of PCI have been shown to function as cell-penetrating peptides because of their ability to cross phospholipid membranes. We therefore hypothesized that the A+−helix might be involved in PCI internalization and that testisin, a protease that removes the A+−helix of PCI, could prevent PCI internalization. Because we have identified intracellular interaction partners of PCI in Jurkat T cells (16, 17), we used these cells to study internalization of PCI and of different truncated PCI forms. We can show that PCI pretreated with testisin or preincubated with testisin-expressing U937 cells is no longer internalized by Jurkat T cells. Also a truncated mPCI mutant lacking the A+−helix is not internalized by Jurkat T cells. To further support the hypothesis that the A+−helix of PCI is responsible for PCI internalization, we used FITC-labeled synthetic peptides with the amino acid sequences of the A+−helix of hPCI and mPCI, respectively, and studied their internalization into Jurkat T cells. We could show by flow cytometry as well as by confocal laser-scanning microscopy that these peptides are taken up by Jurkat T cells.

Taken together our results indicate that the A+−helix of PCI is a cell-penetrating peptide that mediates the internalization of PCI by cells. Our data furthermore suggest that proteases such as testisin could regulate the process of PCI internalization through removal of the A+−helix of PCI. This mechanism could be relevant in the male reproductive tract because PCI by cell-penetrating protein C inhibitor. J. Biol. Chem. 269, 74, 231–242

1. Inoue, M., Kanbe, N., Kurosawa, M., and Kidokoro, H. (2011) Cloning and tissue distribution of a novel serine protease esp-1 from human eosinophils. Biochem. Biophys. Res. Commun. 392, 307–312

2. Hooper, J. D., Nicol, D. L., Dickinson, J. T., Eyle, H. J., Scarmar, A. L., Normyle, J. E., Stuttgen, M. A., Douglas, M. L., Loveland, K. A., Sutherland, G. R., and Antalis, T. M. (1999) Testisin, a new human serine proteinase expressed by preemiotic testicular germ cells and lost in testicular germ cell tumors. Cancer Res. 59, 3199–3205

3. Honda, A., Yamagata, K., Sugiyama, S., Watanabe, K., and Baba, T. (2002) A mouse serine protease TESP5 is selectively included into lipid rafts of sperm membrane presumably as a glycosylphosphatidylinositol-anchored protein. J. Biol. Chem. 277, 16976–16984

4. Tang, T., Kmet, M., Corral, L., Vartanian, S., Tobler, A., and Pappkoff, J. (2005) Testisin, a glycosyl-phosphatidylinositol-linked serine protease, promotes malignant transformation in vitro and in vivo. Cancer Res. 65, 868–878

REFERENCES

1. Meijs, J. C., and Herwald, H. (2011) Protein C inhibitor. Semin. Thromb. Hemost. 37, 349–354

2. Marlatt, R. A., and Griffin, J. H. (1980) Deficiency of protein C inhibitor in combined factor V/VIII deficiency disease. J. Clin. Invest. 66, 1186–1189

3. Meijs, J. C., Kanters, D. H., Vlooswijk, R. A., van Erp, H. E., Hessing, M., and Bouna, B. N. (1988) Inactivation of human plasma kallikrein and factor XIa by protein C inhibitor. Biochemistry 27, 4231–4237

4. España, F., Berrettini, M., and Griffin, J. H. (1989) Purification and characterization of plasma protein C inhibitor. Thromb. Res. 55, 369–384

5. Geiger, M., Huber, K., Wojta, J., Stingl, L., Expana, F., Griffin, J. H., and Binder, B. R. (1989) Complex formation between urokinase and plasma protein C inhibitor in vitro and in vivo. Blood 74, 722–728

6. Ecke, S., Geiger, M., Resch, I., Jerabek, I., Stingl, L., Maier, M., and Binder, B. R. (1992) Inhibition of tissue kallikrein by protein C inhibitor. Evidence for identity of protein C inhibitor with the kallikrein binding protein. J. Biol. Chem. 267, 7048–7052

7. Zheng, X., Geiger, M., Ecke, S., Bielek, E., Donner, P., Eberspächer, U., Schleuning, W. D., and Binder, B. R. (1994) Inhibition of acrosin by protein C inhibitor and localization of protein C inhibitor to spermatozoa. Am. J. Physiol. Cell Physiol. 267, C466–C472

8. Hayashi, T., Nishioka, J., Nakagawa, N., Kamada, H., Gabazza, E. C., Kobayashi, T., Hattori, A., and Suzuki, K. (2007) Protein C inhibitor directly and potently inhibits activated hepatocyte growth factor activator. J. Thromb. Haemost. 5, 1477–1485

9. Prohaska, T. A., Wahlmüller, F. C., Furthmüller, M., and Geiger, M. (2012) Interaction of protein C inhibitor with the type II transmembrane serine protease enteropeptidase. PLoS One 7, e39262

10. Suzuki, K., Nishioka, J., Kusumoto, H., and Hashimoto, S. (1984) Mechanism of inactivation of activated protein C by protein C inhibitor. J. Biochem. 95, 187–195

11. Geiger, M., Priglinger, U., Griffin, J. H., and Binder, B. R. (1991) Urinary protein C inhibitor. Glycosaminoglycans synthesized by the epithelial kidney cell line TCL-598 enhance its interaction with urokinase. J. Biol. Chem. 266, 11851–11857

12. Ecke, S., Geiger, M., and Binder, B. R. (1992) Glycosaminoglycans regulate the enzyme specificity of protein C inhibitor. Ann. N.Y. Acad. Sci. 667, 84–86

13. Malleier, J. M., Oskolkova, O., Bochkov, V., Jerabek, I., Sokolikova, L., Perkmann, T., Breuss, J., Binder, B. R., and Geiger, M. (2007) Regulation of protein C inhibitor (PCI) activity by specific oxidized and negatively charged phospholipids. Blood 109, 4769–4776

14. Wahlmüller, F. C., Sokolikova, B., Rieger, D., and Geiger, M. (2014) New lipid interaction partners stimulate the inhibition of activated protein C by cell-penetrating protein C inhibitor. Thromb. Haemost. 111, 41–52

15. Baumgartner, P., Geiger, M., Zieseniss, S., Malleier, J., Huntington, J. A., Hochrainer, K., Bielek, E., Stoeckelhuber, M., Lauber, K., Scherfeld, D., Schville, P., Wäldele, K., Beyer, K., and Engelmann, B. (2007) Phosphatidylethanolamine critically supports internalization of cell-penetrating protein C inhibitor. J. Cell Biol. 179, 793–804

16. Furthmüller, M., Schmid, A. J., Sarg, B., Lindner, H., and Geiger, M. (2011) Intracellular interaction partners of protein C inhibitor (PCI). J. Thromb. Haemost. 9, Suppl. s2, Abstr. P-TH-166

17. Sokolikova, B., Malleier, J. M., Binder, B. R., and Geiger, M. (2011) Protein C inhibitor and IFCl interaction in prostate cells. J. Thromb. Haemost. 9, Suppl. s2, Abstr. P-TH-164

18. Priglinger, U., Geiger, M., Bielek, E., Vanyak, E., and Binder, B. R. (1994) Binding of urinary protein C inhibitor to cultured human epithelial kidney tumor cells (TCL-598). The role of glycosaminoglycans present on the luminal cell surface. J. Biol. Chem. 269, 14705–14710

19. Hobson, J. P., Netzel-Arnett, S., Szabo, R., Réhault, S. M., Church, F. C., Normyle, J. F., Stuttgen, M. A., Douglas, M. L., Loveland, K. A., Sutherland, G. R., and Antalis, T. M., and Bugge, T. H. (2004) Mouse DESC1 is located within a cluster of seven DESC1-like genes and encodes a type II transmembrane serine protease that forms serpin inhibitory complexes. J. Biol. Chem. 279, 46981–46994

20. Szabo, R., Netzel-Arnett, S., Hobson, J. P., Antalis, T. M., and Bugge, T. H. (2005) Matriptase-3 is a novel phylogenetically preserved membrane-anchored serine protease with broad serpin reactivity. Biochem. J. 390, 231–242
A+Helix of PCI Mediates Its Internalization by Cells

25. Hooper, J. D., Bowen, N., Marshall, H., Cullen, L. M., Sood, R., Daniels, R., Stuttgen, M. A., Normyle, J. F., Higgs, D. R., Kastner, D. L., Ogbourne, S. M., Pera, M. F., Jazwinska, E. C., and Antalis, T. M. (2000) Localization, expression and genominc structure of the gene encoding the human serine protease testisin. Biochim. Biophys. Acta 1492, 63–71

26. Shigemasa, K., Underwood, L. J., Beard, J., Tanimoto, H., Ohama, K., Parnsey, T. H., and O’Brien, T. J. (2000) Overexpression of testisin, a serine protease expressed by testicular germ cells, in epithelial ovarian tumor cells. J. Soc. Gynecol. Investig. 7, 358–362

27. Yamashita, M., Honda, A., Ogura, A., Kashiwabara, S., Fukami, K., and Baba, T. (2008) Reduced fertility of mouse epididymal sperm lacking Prss21/Tesp5 is rescued by sperm exposure to uterine microenvironment. Genes Cells 13, 1001–1013

28. Netzler-Arnett, S., Bugge, T. H., Hess, R. A., Carnes, K., Stringer, B. W., Scarmar, A. L., Hooper, J. D., Tonks, I. D., Kay, G. F., and Antalis, T. M. (2009) The glycosylphosphatidylinositol-anchored serine protease PRSS21 (testisin) imparts marine epididymal sperm cell maturation and fertilizing ability. Biol. Reprod. 81, 921–932

29. Uhrin, P., Dewerchin, M., Hilpert, M., Chrenek, P., Schöfer, C., Zechmeister-Machhart, M., Krönke, G., Vales, A., Carmeliet, P., Binder, B. R., and Geiger, M. (2000) Disruption of the protein C inhibitor gene results in impaired spermatogenesis and male infertility. J. Clin. Investig. 106, 1531–1539

30. Geiger, M. (2007) Protein C inhibitor, a serpin with functions in- and outside vascular biology. Thromb. Haemost. 97, 343–347

31. He, S., Lin, Y. L., and Liu, Y. X. (1999) Functionally inactive protein C inhibitor in seminal plasma may be associated with infertility. Mol. Hum. Reprod. 5, 513–519

32. Cao, Y., Becker, C., Lundwall, A., Christensson, A., Gadaleanu, V., Lilja, H., and Bjartell, A. (2003) Expression of protein C inhibitor (PCI) in benign and malignant prostatic tissues. Prostate 57, 196–204

33. Bijsmans, I. T., Smits, K. M., de Graeff, P., Wisman, G. B., van der Zee, A. G., Slangen, B. F., de Bruïne, A. P., van Engeland, M., Sieben, N. L., and Van de Vijver, K. K. (2011) Loss of SerpinA5 protein expression is associated with borderline tumors. Int. J. Cancer 128, 2491–2499

34. Madani, F., Lindberg, S., Langel, U., Futaki, S., and Gräslund, A. (2011) Reduced fertility of mouse epididymal sperm lacking Prss21/Tesp5 is rescued by sperm exposure to uterine microenvironment. Genes Cells 13, 1001–1013

35. Shigemasa, K., Underwood, L. J., Beard, J., Tanimoto, H., Ohama, K., Parnsey, T. H., and O’Brien, T. J. (2000) Overexpression of testisin, a serine protease expressed by testicular germ cells, in epithelial ovarian tumor cells. J. Soc. Gynecol. Investig. 7, 358–362

36. Brenner, A. V., Neta, G., Sturgis, E. M., Pfeiffer, R. M., Hutchinson, A., Yeager, M., Xu, L., Zhou, C., Wheeler, W., Tucker, M. A., Chanock, S. J., and Sigurdsson, A. J. (2013) Common single nucleotide polymorphisms in genes related to immune function and risk of papillary thyroid cancer. PLoS One 8, e57243

37. Asanuma, K., Yoshikawa, T., Hayashi, T., Akita, N., Nakagawa, N., Hamada, Y., Nishioka, J., Kamada, H., Gabazza, E. C., Ido, M., Uchida, A., and Suzuki, K. (2007) Protein C inhibitor inhibits breast cancer cell growth, metastasis and angiogenesis independently of its protease inhibitory activity. Int. J. Cancer 121, 955–965

38. Fortenberry, Y. M., Brandal, S., Bialas, R. C., and Church, F. C. (2010) Protein C inhibitor regulates both cathepsin L activity and cell-mediated tumor cell migration. Biochim. Biophys. Acta 1800, 580–590

39. Réhault, S. M., Zechmeister-Machhart, M., Fortenberry, Y. M., Malleier, J., Binz, N. M., Cooper, S. T., Geiger, M., and Church, F. C. (2005) Characterization of recombinant human protein C inhibitor expressed in Escherichia coli. Biochim. Biophys. Acta 1748, 57–65

40. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 268, 680–685

41. Méndez, J., and Stillman, B. (2000) Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis. Mol. Cell. Biol. 20, 8602–8612

42. Madani, F., Lindberg, S., Langel, U., Futaki, S., and Gräslund, A. (2011) Mechanisms of cellular uptake of cell-penetrating peptides. J. Biophysics. 2011, 414729

43. Sahraneshin Samani, F., Moore, J. K., Khosravani, P., and Ebrahimi, M. (2014) Features of free software packages in flow cytometry: a comparison between four non-commercial software sources. Cytotechnology 66, 555–559

44. Elisen, M. G., Maseland, M. H., Bouma, B. N., and Meijers, J. C. (1996) Role of the A+helix in heparin binding to protein C inhibitor. Thromb. Haemost. 75, 760–766

45. Li, W., Adams, T. E., Kjellberg, M., Stenflo, J., and Huntington, J. A. (2007) Structure of native protein C inhibitor provides insight into its multiple functions. J. Biol. Chem. 282, 13759–13768

46. Gautam, A., Chaudhary, K., Kumar, R., Sharma, A., Kapoor, P., Tyagi, A., Open Source Drug Discovery Consortium, and Raghava, G. P. (2013) In silico approaches for designing highly effective cell penetrating peptides. J. Transl. Med. 11, 74

47. Kuhn, L. A., Griffin, J. H., Fisher, C. L., Greengard, J. S., Bouma, B. N., España, F., and Tainer, J. A. (1990) Elucidating the structural chemistry of glycosaminoglycan recognition by protein C inhibitor. Proc. Natl. Acad. Sci. U.S.A. 87, 8506–8510