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Binding of retinoic acid by the inhibitory serpin protein C inhibitor

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The serpin superfamily includes inhibitors of serine proteases and noninhibitory members with other functions (e.g. the hormone precursor angiotensinogen and the hormone carriers corticosteroid-binding globulin and thyroxine-binding globulin). It is not known whether inhibitory serpins have additional, noninhibitory functions. We studied binding of ^{3}H-labeled hydrophobic hormones (estradiol, progesterone, testosterone, cortisol, aldosterone, and all-*trans*-retinoic acid) to the inhibitory serpins antithrombin III, heparin cofactor II, plasminogen activator inhibitor-1, and protein C inhibitor (PCI). All-*trans*-[^{3}H]retinoic acid bound in a specific dose-dependent and time-dependent way to PCI (apparent $K_d = 2.43$ μM, 0.8 binding sites per molecule of PCI). We did not observe binding of other hormones to serpins. Intact and protease-cleaved PCI bound retinoic acid equally well, and retinoic acid did not influence inhibition of tissue kallikrein by PCI. Gel filtration confirmed binding of retinoic acid to PCI in purified systems and suggested that PCI may also function as a retinoic acid-binding protein in seminal plasma. Therefore, our present data, together with the fact that PCI is abundantly expressed in tissues requiring retinoic acid for differentiation processes (e.g. the male reproductive tract, epithelia in various organs), suggest an additional biological role for PCI as a retinoic acid-binding and/or delivering serpin.

Keywords: hormone; protein C inhibitor; retinoic acid; retinoid; serpin.

Serpins (serine protease inhibitors) are a family of closely related glycoproteins that inactivate serine proteases by forming stable, enzymatically inactive 1 : 1 complexes (for a review, see [1,2]). The mechanism of inhibition involves the binding of the so-called reactive-site loop of the serpin by the active site of the protease. Thereafter the serpin undergoes a major conformational change including the insertion of the reactive-site loop into the β sheet A, the cleavage of the reactive-site peptide bond, and the formation of a covalent enzyme–serpin complex [2]. Depending on the enzyme and the serpin involved, the complex can also dissociate and release active enzyme and cleaved inactive serpin [2].

Besides inhibitors of serine proteases, the serpin family also includes members that have no protease inhibitory activity but have other biological functions. The hormone carriers thyroxine-binding globulin [3] and corticosteroid-binding globulin [4–6], the hormone precursor angiotensinogen [7], the tumor suppressor maspin [8], and the neurotrophic pigment epithelium-derived factor [9] belong to the group of noninhibitory serpins.

As far as the inhibitory serpins are concerned, it is generally accepted that they are involved in the regulation of intravascular and extravascular proteolytic activity. There are inhibitory serpins with well-defined biological roles, such as antithrombin III, the most important coagulation inhibitor [10], and C1-inhibitor, an inhibitor of complement activation, of the contact system of kinin formation, and of other clotting and fibrinolytic factors [11]. Deficiencies of these inhibitors have been reported to be associated with distinct clinical symptoms. On the other hand, the biological roles of some members of the serpin family have still not been defined. An example of such a serpin is protein C inhibitor (PCI), which was initially described as an inhibitor of the anticoagulant protease-activated protein C [12]. However, in the meantime it has been shown to inhibit a variety of intravascular and extravascular serine proteases, although the rates of inhibition are rather low compared with other, more specific serpin-type inhibitors [13–18]. We have recently developed a mouse model and generated PCI-deficient mice by targeted disruption of the *PCI* gene [19]. These PCI-knockout mice were normal when born; they grew up and developed normally, and were apparently healthy. However, male homozygous PCI$^{-/-}$ mice were infertile. Heterozygous males and females of all genotypes reproduced normally. Histological analysis of the reproductive organs revealed major changes in the testes of PCI$^{-/-}$ mice with resulting impaired spermatogenesis, suggesting an important role for PCI in male reproduction. However, the precise biological function of PCI is still unclear, especially in humans.

Little is known about whether inhibitory serpins have additional noninhibitory functions, although inhibitory and noninhibitory members of the serpin family share a high degree of homology. In this study we analyzed whether inhibitory serpins could function as hormone carriers. We show that one of the serpins analyzed, PCI, binds [^{3}H]retinoic acid in a specific dose-dependent and time-dependent manner.

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Abbreviations: DCC, dextran-coated charcoal; PAI-1, plasminogen activator inhibitor 1; PCI, protein C inhibitor.

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EXPERIMENTAL PROCEDURES

Materials

PCI was purified from human urine [15]. Antithrombin III and heparin cofactor II were purified from human plasma

[20]. Plasminogen activator inhibitor-1 (PAI-1) was obtained from Technoclone (Vienna, Austria), and human tissue kallikrein was from Bio-Ass (Münich, Germany). Recombinant human PCI was expressed in *Escherichia coli* and purified (M. Zechmeister-Machhart, J. Malleier, P. Uhrin, M. Hilpert, B. R. Binder & M. Geiger, unpublished results) Mouse protein C inhibitor (mPCI) has similar protease specificity to human PCI but is not a plasma protein.

The tissue kallikrein substrate H-D-valyl-L-leucyl-L-arginine-*p*-nitroaniline dihydrochloride (S-2266) was from Chromogenix (Mölndal, Sweden). [1,2,6,7-^{3}H(n)]Testosterone (92.4 Ci·mmol^{-1}), [2,4,6,7-^{3}H(n)]estradiol (84.1 Ci·mmol^{-1}), [1,2,6,7-^{3}H(n)]progesterone (92 Ci·mmol^{-1}), [1,2,6,7-^{3}H(n)]cortisol (78.4 Ci·mmol^{-1}) [1,2-^{3}H(n)-aldosterone (50 Ci·mmol^{-1}) and [11,12-^{3}H(n)]retinoic acid (51.2 Ci·mmol^{-1}) were from DuPont–NEN. Unlabeled retinoids (all-*trans*-retinoic acid, 9-*cis*-retinoic acid, retinol), pancreatic elastase, activated charcoal, phenylmethanesulfonyl fluoride were from Sigma. Reagents and M_r standards for SDS/PAGE were from Bio-Rad. Dextran T 70 was obtained from Pharmacia. Dextran-coated charcoal (DCC) was prepared as described previously [21]. Charcoal/dextran-treated fetal bovine serum was from HyClone. Human seminal plasma was prepared by centrifugation (3 min, 13 360 ***g***) of liquefied semen samples of healthy volunteers. Seminal plasma from five donors was pooled and stored in aliquots at −70 °C until further use. All other reagents were obtained as indicated below.

Binding of ^{3}H-labeled hormones to purified serpins

Purified serpins (80 μL each, 36 nM to 4.3 μM final concentration) were incubated with ^{3}H-labeled hormones (20 μL each, 11–364 nM final concentration) in a total volume of 110 μL 50 mM Tris/HCl, pH 7.4, containing 50 mM $CaCl_2$ (binding buffer) for 5–60 min at 37 °C. Thereafter tubes were put on ice, and after 15 min 350 μL DCC suspension was added to each sample. After an additional 10 min on ice, tubes were centrifuged, and aliquots (400 μL) of the resulting supernatants were analyzed for ^{3}H radioactivity in a Beckman LS 6500 liquid-scintillation counter using 5 mL Ready Protein® scintillation fluid (Beckman). ^{3}H Radioactivity determined in these supernatants was assumed to be associated with protein and was therefore set equivalent to protein-bound hormone. To account for radioactivity in the supernatants that was not associated with protein, controls without added protein were performed for each concentration of each [^{3}H]hormone.

For analysis of the specificity of [^{3}H]retinoic acid binding to PCI, incubations with [^{3}H]retinoic acid (200 nM) were performed as above but also in the presence of increasing concentrations (up to 10 μM) of unlabeled retinoids (all-*trans*-retinoic acid, 9-*cis*-retinoic acid, retinol). In other experiments, ^{3}H-labeled retinoic acid was incubated with protease-treated PCI in order to investigate whether cleavage or complexation of PCI influences hormone binding. Protease pretreatment of PCI was carried out in the following way. Aliquots of PCI (360 μL, 5.4 μg) were incubated with either tissue kallikrein (2 μL, 0.6 μg) or pancreatic elastase (30 μL, 0.6 μg) for 0, 10, or 60 min at 37 °C. Tissue kallikrein activity was then quenched by the addition of aprotinin (1750 kallikrein inhibitory units·mL^{-1} final concentration), and elastase activity by addition of phenylmethanesulfonyl fluoride (1 mM final concentration). An aliquot of each reaction mixture and each incubation time was analyzed by SDS/PAGE [22] using 1.5-mm slab gels. Protease cleavage was evaluated after silver staining of the gels [23]. Aliquots of the reaction mixtures containing PCI incubated with elastase or tissue kallikrein for 0 or 60 min were used to study [^{3}H]retinoic acid binding as described above.

In experiments performed to determine binding constants for the interaction of retinoic acid with PCI, [^{3}H]retinoic acid was diluted with unlabeled retinoic acid to yield a specific activity of 2.5 Ci·mmol^{-1}. Different concentrations of this [^{3}H]retinoic acid (62 nM to 2.5 μM final concentration) were incubated with PCI (160 nM final concentration) in a total volume of 90 μL binding buffer at 37 °C. After 60 min, tubes were put on ice, and after 15 min 350 μL DCC suspension was added to each sample. After an additional 10 min on ice, tubes were centrifuged and aliquots (400 μL) of the resulting supernatants were analyzed for ^{3}H radioactivity. Data obtained were plotted in a Scatchard plot as [bound retinoic acid] vs. [bound retinoic acid]/[free retinoic acid], and from this plot the apparent dissociation constant (K_d) and the number of binding sites (n) per molecule of PCI were determined from the slope ($-1/K_d$) and intercept on the *x*-axis (n[PCI]).

Assay for the amidolytic activity of tissue kallikrein

Inhibition of the amidolytic acivity of tissue kallikrein by PCI was tested on 96-well microtiter plates. Tissue kallikrein (1.5 nM final concentration) was incubated without or with PCI (8.8–35 nM final concentration) in the absence or presence of heparin (30 μg·mL^{-1} final concentration) and in the absence or presence of all-*trans*-retinoic acid (500 nM final concentration) at 37 °C in 50 μL NaCl/P_i, pH 7.4, containing 0.2 g·L^{-1} KCl, 0.1 g·L^{-1} $MgCl_2$, and 0.13 g·L^{-1} $CaCl_2$. After 60 min, 50 μL S-2266 (0.4 mM final concentration; dissolved in the same buffer) was added to each well and after further incubation for 3–5 h at 37 °C, the D_{405} was determined in an ELISA reader (Anthos reader 2001).

Gel filtration of PCI-bound [^{3}H]retinoic acid on Sephadex G-200

[^{3}H]Retinoic acid (5 μL, 20 μM) was incubated with either purified urinary PCI (50 μL, 10 μg·mL^{-1}) or seminal plasma (50 μL) in duplicate tubes. After incubation for 60 min at 37 °C in the dark, one tube was additionally exposed to intense UV light (365 nm) for 15 min in order to cross-link bound retinoic acid to protein [24]. Unbound [^{3}H]retinoic acid was removed by DCC treatment performed as described above. Thereafter samples were subjected to gel filtration on a Sephadex G-200 column (1.5 × 40 cm) equilibrated in 0.01 M phosphate buffer containing 0.14 M NaCl, pH 7.4 (NaCl/P_i). Fractions of volume 1.5 mL were collected and analyzed for protein (A_{280}), ^{3}H radioactivity, and PCI antigen (ELISA). In control experiments, purified recombinant human PCI (1 mL, 34 μg·mL^{-1}) was used instead of urinary PCI and incubated for 60 min at 37 °C with either [^{3}H]retinoic acid (10 μL, 20 μM) or buffer alone. Thereafter samples were subjected to DCC treatment

to remove free [^{3}H]retinoic acid, and the unbound fractions were analyzed by gel filtration on Sephadex G-200 as above.

Analytical methods

Concentrations of heparin cofactor II and PCI were estimated from A_{280} using absorption coefficients (ε_{280}, 1%) of 11.7 for heparin cofactor II [25], and 14.1 for PCI [13]. The concentration of PAI-1 was determined by ELISA (Technoclone) as were the concentrations of PCI [26] in the fractions eluted from the Sephadex G-200 column. The concentration of antithrombin III was determined by the method of Lowry *et al*. [27]. Molar concentrations of serpins were calculated using M_r values of 59 000 for antithrombin III [28], 66 000 for heparin cofactor II [25,28], 50 000 for PAI-1 [28], and 57 000 for PCI [13,15,28].

RESULTS

In screening experiments performed to identify possible interactions between inhibitory serpins and hydrophobic hormones, we analyzed binding of ^{3}H-labeled steroids (aldosterone, cortisol, estradiol, progesterone, and testosterone) and all-*trans*-[^{3}H]retinoic acid to four different purified inhibitory serpins (antithrombin III, heparin cofactor II, PAI-1 and PCI). We used a binding assay system in which free ^{3}H-labeled hormones are removed by adsorption to DCC [21]. After centrifugation, protein bound ^{3}H-labeled hormones remain in the supernatant and their concentration can be determined in a liquid-scintillation counter. Results obtained for comparable serpin concentrations are summarized in Table 1. The amount of serpin-bound hormone is given for each hormone concentration and each serpin relative to the radioactivity present in the buffer control (serpin concentration = 0), which was given a value of 1. It can be seen that, in most hormone/serpin combinations, this amount was close to one, indicating that there was no association of radioactivity with protein. Only when [^{3}H]retinoic acid was incubated with PCI was appreciable association of radioactivity with protein seen. The amount of protein-bound radioactivity was dependent on the PCI concentration (not shown), and was at least twice as high as the buffer control even at the lowest serpin concentration used (48 nM). At the highest PCI concentration used (191 nM) about fivefold more radioactivity was present in the DCC supernatant compared to the buffer control.

We used these results to analyze the interaction of PCI and retinoic acid in more detail. Binding of ^{3}H radioactivity was competed for in a concentration-dependent manner by

Table 1. Binding of ^{3}H-labeled hormones to inhibitory serpins. Different concentrations of ^{3}H-labeled hormones were incubated without or with different serpins for 1 h at 37 °C. Unbound [^{3}H]hormones were removed by adsorption to DCC, and radioactivity of an aliquot of the supernatant was determined for each hormone concentration, for each serpin, and for the buffer controls (serpin concentration = 0). Values for [^{3}H]hormone bound are given relative to the buffer control, which was given a value of 1. ATIII, Antithrombin III; HCII, heparin cofactor II; ND, not determined.

		[^{3}H]Hormone bound			
Hormone	Hormone concn (nM)	ATIII (212 nM)	HCII (198 nM)	PAI-1 (145 nM)	PCI (191 nM)
Aldosterone	364	1.0	1.09	0.99	1.6
	182	1.04	1.05	1.13	1.68
	91	1.0	1.16	1.05	1.63
	45	1.02	1.1	1.08	1.74
Cortisol	236	1.27	1.23	1.04	1.46
	118	1.0	1.25	1.02	1.45
	59	1.01	1.2	0.96	1.45
	29	1.1	1.31	0.99	1.47
Estradiol	182	1.04	1.08	1.15	2.28
	91	1.06	1.04	1.2	2.65
	45	1.0	ND	1.25	2.14
	23	0.83	1.04	1.09	1.54
Progesterone	182	0.9	1.08	0.97	1.68
	91	0.84	1.34	1.6	1.53
	45	0.81	1.02	1.42	1.46
	23	0.92	1.36	1.11	1.83
Testosterone	182	1.06	1.28	1.49	2.07
	91	0.99	1.35	1.67	2.18
	45	0.96	1.31	1.49	1.98
	23	0.98	1.23	1.45	1.64
Retinoic acid	364	1.14	2.07	1.88	5.08
	182	1.07	2.33	1.9	4.33
	91	1.09	1.75	0.9	4.66
	45	0.94	1.5	1.19	5.18

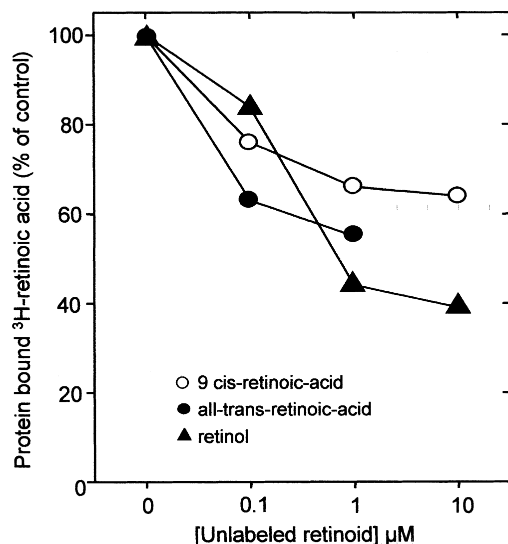

Fig. 1. Binding of [^{3}H]retinoic acid to PCI: competition by unlabeled retinoids. PCI (191 nM final concentration) was incubated with [^{3}H]retinoic acid (200 nM) in the absence and presence of different concentrations of unlabeled all-*trans*-retinoic acid, 9-*cis*-retinoic acid or all-*trans*-retinol for 1 h at 37 °C. Thereafter protein-bound radioactivity was determined as described in Experimental procedures.

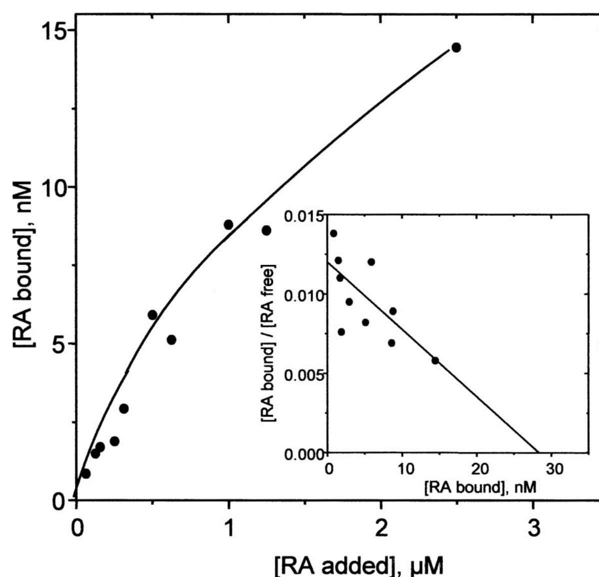

Fig. 2. Dose-dependent binding of [^{3}H]retinoic acid to PCI. PCI (35 nM final concentration) or binding buffer alone was incubated with different concentrations of [^{3}H]retinoic acid (as shown on the abscissa) for 60 min at 37 °C. Total protein-bound radioactivity was determined after DCC adsorption of unbound hormone as described in Experimental procedures. The respective buffer controls were subtracted. Inset: data shown in the figure were replotted in a Scatchard plot and the apparent K_d and the number of binding sites were calculated from the slope ($-1/K_d$) and x-intercept (n[PCI]) of this plot, respectively. RA, retinoic acid.

excess unlabeled all-*trans*-retinoic acid, 9-*cis*-retinoic acid, and retinol (Fig. 1), indicating that binding of [^{3}H]retinoic acid to PCI is specific and that, in addition to all-*trans*-retinoic acid, PCI binds other retinoids. Binding of [^{3}H]retinoic acid to purified PCI was furthermore time-dependent (not shown), and dose-dependent and saturable (Fig. 2). Scatchard analysis of these binding data revealed an apparent K_d of 2.43 µM and ≈ 0.8 binding sites per molecule of PCI.

We also studied binding of [^{3}H]retinoic acid to protease-treated PCI. Incubation of PCI with tissue kallikrein (Fig. 3A) or pancreatic elastase (Fig. 3B) resulted in a time-dependent decrease in intensity of the 57 000-Da band (intact PCI) and in the appearance of lower-molecular-mass bands representing cleaved PCI (insets). Under the assay conditions used no serpin–protease complexes were seen in the silver-stained gels (not shown). When binding of [^{3}H]retinoic acid to intact and protease-treated (60 min) PCI was studied, similar results were obtained (Fig. 3A,B), suggesting that the binding site for retinoic acid is not affected by either protease. As binding of retinoic acid to the serpin could also influence serpin activity, the effect of retinoic acid on the inhibitory activity of PCI was analyzed using tissue kallikrein as a target protease and a low-molecular-mass substrate to quantify tissue kallikrein activity. As can be seen from Fig. 4, tissue kallikrein inhibition by PCI was very similar in the absence and presence of retinoic acid. Also the inhibitory effect of heparin on the tissue kallikrein inhibition by PCI [15] did not seem to be influenced by retinoic acid.

To confirm binding of retinoic acid to PCI and to estimate the M_r of [^{3}H]retinoic acid after 1 h incubation with purified urinary PCI, gel filtration on Sephadex G-200 was performed. The elution profile revealed only one peak of ^{3}H radioactivity (Fig. 5A). This peak was eluted at a position corresponding to an M_r of > 200 000. When urinary PCI was incubated for 1 h with [^{3}H]retinoic acid and thereafter exposed to UV light for direct cross-linking of bound [^{3}H]retinoic acid, the ^{3}H-radioactivity peak at this position was approximately threefold higher (Fig. 5B). In addition, a second small peak corresponding approximately to the M_r of

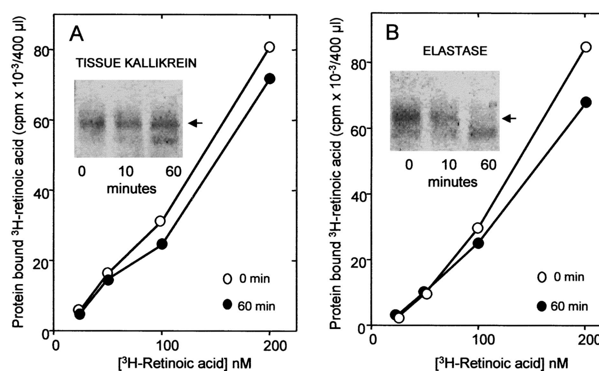

Fig. 3. Effect of protease treatment on binding of [^{3}H]retinoic acid to PCI. PCI was incubated with tissue kallikrein or pancreatic elastase for 0, 10 or 60 min as described in Experimental procedures. Cleavage of PCI by these proteases was confirmed by analyzing aliquots of each reaction mixture in silver-stained SDS/polyacrylamide gels (insets). The arrows indicate the migration distance of intact PCI. Separate aliquots from the 0 min or 60 min incubations with tissue kallikrein (A) or elastase (B) were used for [^{3}H]retinoic acid-binding studies performed as described in Experimental procedures.

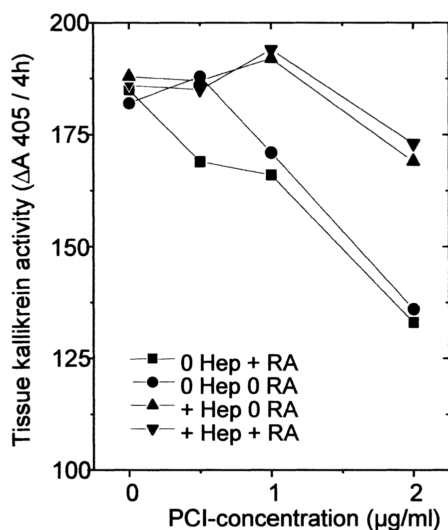

Fig. 4. Effect of retinoic acid on the inhibition of tissue kallikrein by PCI. Tissue kallikrein (1.5 nM final concentration) was incubated with different concentrations of PCI in the absence and presence of heparin (30 $\mu g \cdot mL^{-1}$ final concentration) and in the absence and presence of retinoic acid (500 nM final concentration) for 1 h at 37 °C. Thereafter remaining enzymatic activity was determined from the cleavage of S-2266 as described in Experimental procedures. Hep, heparin. RA, retinoic acid.

PCI was observed (Fig. 5B). Similar data were also obtained when [^{3}H]retinoic acid was incubated in seminal plasma and then analyzed by gel filtration (Fig. 5C,D). Without exposure to UV light, there were two small peaks of ^{3}H radioactivity, one with an M_r of > 200 000 and one of ≈ 65 000 (Fig. 5C). The latter was eluted at the same position as the main PCI antigen peak. Low concentrations of PCI antigen were also measurable at the position of the first radioactive peak. On cross-linking by UV light exposure, the radioactivity in the first peak increased ≈ 10-fold (Fig. 5D). In the experiments using purified urinary PCI (Fig. 5A,B), we were not able to detect PCI antigen in any of the fractions by our ELISA system [26]. To determine the elution position of purified PCI, we therefore used recombinant human PCI (rhPCI) expressed in *E. coli*, which is available in larger quantities (Fig. 5E,F). rhPCI was incubated without (Fig. 5E) or with [^{3}H]retinoic acid (Fig. 5F). Thereafter samples were treated with DCC, and then subjected to gel filtration on Sephadex G-200. As determined by ELISA, two peaks of PCI antigen were eluted in both experiments, the first peak corresponding to an M_r of > 200,000, the second to an M_r of ≈ 65 000. On SDS/PAGE, purified rhPCI used in these experiments migrated as a single band with a, M_r of 46 000 (inset Fig. 5E). Preincubation of PCI with [^{3}H]retinoic acid (Fig. 5F) did not change the elution profile of rhPCI on Sephadex G-200. The [^{3}H]retinoic acid radioactivity (● in Fig. 5F) was predominantly associated with the M_r > 200 000 form of rhPCI. Results obtained with rhPCI are therefore consistent with those obtained with urinary PCI, where the majority of [^{3}H]retinoic acid was also eluted at a position corresponding to an M_r of > 200 000. Our data suggest that retinoic acid predominantly associates with a high-M_r form of PCI, which possibly represents a polymer. Elution profiles obtained on incubation of [^{3}H]retinoic acid in seminal plasma furthermore support the hypothesis that PCI may function as a retinoic acid-binding protein not only in purified systems but also in biological fluids.

DISCUSSION

The serpin family includes members that are inhibitors of serine proteases and are involved in the regulation of the activities of intravascular and extravascular protease systems [1,2]. Other members of the serpin family have no apparent protease inhibitor activity, but have acquired other noninhibitory functions [4–9]. As it is not known whether inhibitory serpins have additional noninhibitory functions, we analyzed binding of hydrophobic hormones to inhibitory members of the serpin family. In initial screening experiments, when ^{3}H-labeled hormones were incubated with purified serpins, dose-dependent association of [^{3}H]retinoic acid with PCI was seen, whereas in all other [^{3}H]hormone–serpin combinations analyzed (six different hormones and four different serpins) protein associated radioactivity was much lower (Table 1). We therefore concluded that PCI may function as a binding protein for retinoic acid, although from these initial experiments we cannot definitely exclude other possible hormone–serpin interactions. Studying the interaction of PCI with retinoic acid in more detail, we showed that binding of all-*trans*-[^{3}H]retinoic acid to PCI is specific, as it can be competed for in a dose-dependent manner by unlabeled all-*trans*-retinoic acid. In addition, binding of all-*trans*-[^{3}H]retinoic acid could also be competed for by unlabeled 9-*cis*-retinoic acid and retinol, suggesting that PCI not only interacts with retinoic acid but also with other retinoids in a similar fashion.

Calculation of binding constants revealed an apparent K_d of 2.43 µM and 0.8 retinoic acid-binding sites per molecule of PCI, suggesting a 1 : 1 stoichiometry. The calculated K_d is one to two orders of magnitude higher than values previously calculated for interactions of serum retinol-binding protein and intracellular binding proteins with retinoids [29], but it is in the same order of magnitude as the K_d for the interaction of retinol with interphotoreceptor retinol-binding protein [30].

For the hormone-binding serpin, corticosteroid-binding globulin, it has been shown that cleavage by leukocyte elastase within the so-called stressed loop, a region harboring the reactive site in inhibitory serpins [2], reduced its affinity for cortisol ≈ 10-fold [5]. It has been suggested that this may represent a mechanism for local delivery of the hormone at sites of inflammation [5]. On the other hand, proteolytic cleavage did not influence the affinity of thyroxine-binding globulin, another hormone-binding serpin, for thyroid hormone [5]. These results suggest different locations of the hormone-binding sites within these two serpins [6]. In our studies, proteolytic cleavage of PCI had no effect on binding of [^{3}H]retinoic acid (Fig. 3). In addition, retinoic acid did not seem to influence the inhibitory activity of PCI (Fig. 4). Both findings support the hypothesis that the reactive-site loop of PCI is not involved in binding of retinoic acid. However, at present, we do not have kinetic data on either the affinities of intact vs. cleaved PCI for retinoic acid or the effect of retinoic acid on the inhibitory activity of PCI. Therefore, we cannot completely exclude a contribution of the reactive-site loop in retinoic acid binding by PCI.

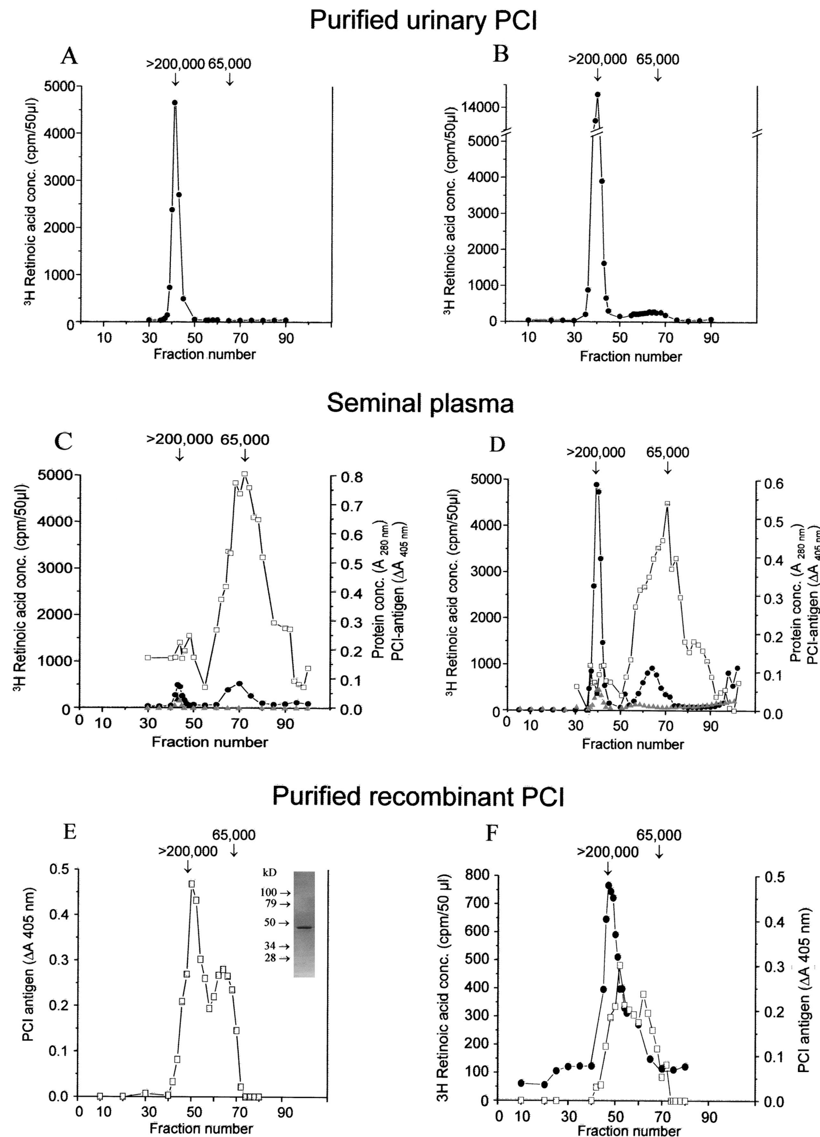

Fig. 5. Gel filtration of PCI-bound retinoic acid on Sephadex G-200. (A,B) [^{3}H]Retinoic acid was incubated for 1 h with purified urinary PCI. To remove unbound [^{3}H]retinoic acid, one sample (A) was immediately treated with DCC, and one sample (B) after cross-linking of [^{3}H]retinoic acid to PCI by UV light exposure. Thereafter samples were subjected to Sephadex G-200 chromatography. Fractions were collected and analyzed for ^{3}H radioactivity (●), protein (not detectable), and PCI antigen (not detectable). (C,D) [^{3}H]Retinoic acid was incubated in seminal plasma. After 1 h, one sample (C) was immediately treated with DCC, and one sample after UV light exposure (D). After gel filtration on Sephadex G-200, collected fractions were analyzed for protein (A_{280}; ▲), PCI antigen (□), and ^{3}H radioactivity (●). (E, F) Purified recombinant PCI was incubated for 1 h without (E) or with (F) [^{3}H]retinoic acid. After DCC treatment, samples were applied to the Sephadex G-200 column. Fractions were collected and analyzed for PCI antigen (□) and ^{3}H radioactivity (●). Experimental details are described in Experimental procedures. In all panels the elution positions of sizing standards are indicated. Note: different specific activity of [^{3}H]retinoic acid in (F) compared with (A–D).

We have confirmed binding of retinoic acid to PCI by gel filtration on Sephadex G-200 using purified urinary and recombinant PCI. These experiments furthermore revealed that part of the PCI antigen is present in a high-M_r form (> 200 000). From the results obtained with recombinant PCI, we can exclude the possibility that this PCI form represents a complex with a protease, because, on SDS/PAGE, only one band, corresponding to an M_r of 46 000, was detected. The size of this band is consistent with the size calculated for nonglycosylated recombinant PCI from its amino-acid composition. It is therefore likely that the high-M_r form of PCI is a noncovalent polymer. Polymerization of serpins is not unusual and has been described previously [31–34]. Our data also suggest that retinoic acid preferentially binds to the high-M_r form of PCI. However, more and quantitative data are needed to definitely determine the identity of the high-M_r PCI form as well as its affinity for retinoids as compared with the active PCI monomer (M_r = 57 000).

Taken together our data strongly suggest that PCI may be a novel retinoic acid-binding protein. Our hypothesis is also supported by the fact that PCI is abundantly expressed in organs and tissues requiring retinoic acid for maturation and differentiation processes [26,35–37]. So far, the highest concentrations of PCI have been found in seminal plasma [28,35,36], and PCI synthesis has been shown throughout the male reproductive tract [35,36]. *In situ* hybridization experiments have furthermore revealed that *PCI* mRNA is present in the spermatogonia layer of the seminiferous tubules in testes (unpublished observations), suggesting a role for PCI in spermatogenesis. In fact, male homozygous PCI$^{-/-}$ mice recently generated in our laboratory are infertile because of abnormal spermatogenesis [19]. Whether or not the observed increased proteolytic activity is sufficient cause for these disturbances is unclear at present. Abnormal delivery of retinoids in the complete absence of PCI may be an alternative explanation. We have furthermore identified PCI in megakaryocytes and platelets [26] as well as in other bone marrow cells (M. J. Prendes, E. Bielek, M. Bechmeister-Machhart, B. R. Binder, M. Geiger, unpublished data). Also the epidermis contains PCI antigen, and keratinocytes in culture synthesize PCI [37]. Therefore

the tissue localization of PCI would be consistent with a possible role for it in the local storage and/or delivery of retinoic acid in retinoic acid-dependent tissues. So far, only limited information is available on extracellular retinoic acid-binding proteins (for a review, see [38]). We therefore believe that our findings may prompt research in new directions not only in the field of serpins but also in the field of signal transduction by retinoids.

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