Activated Protein C−Protein C Inhibitor Complex Formation: Characterization of a Neoepitope Provides Evidence for Extensive Insertion of the Reactive Center Loop

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ABSTRACT: Protein C inhibitor, a serine proteinase inhibitor (serpin), is the physiologically most important inhibitor of activated protein C. We have made a monoclonal antibody (M36) that binds with equally high affinity to an epitope present in activated protein C−protein C inhibitor complexes and cleaved loop-inserted protein C inhibitor. Insertion of a synthetic N-acetylated tetradecapeptide (corresponding to residues P1−P14 of the reactive center loop) into β-sheet A of the uncleaved inhibitor also exposed the epitope. The antibody had no apparent affinity for native uncleaved inhibitor or for the free peptide. Synthetic P1−P14 analogues, with Arg P13 or Ala P9 substituted to the residues found in mouse protein C inhibitor (Thr and Ile, respectively), were also inserted in β-sheet A. The Arg P13/Thr substitution led to a greatly impaired reactivity with the antibody, whereas the Ala P9/Ile mutation resulted in a modest loss of reactivity with the antibody. These results indicate that complex formation leads to insertion of the reactive center loop in β-sheet A from Arg P14 and presumably beyond Ala P9. Moreover, to the best of our knowledge, this is the first instance where the neoepitope of a complexation-specific monoclonal antibody has been localized to the loop-inserted part of β-sheet A, the part of the serpin where the complexation-induced conformational change is most conspicuous.

Activated protein C (APC)[1] is a vitamin K-dependent serine proteinase that regulates blood coagulation (*1*). Zymogen protein C is activated by thrombin in complex with the integral membrane protein thrombomodulin. APC cleaves factors VIIIa and Va, cofactors of coagulation factors IXa and Xa, respectively, thereby reducing the generation of factor Xa and thrombin. Like other proteinases involved in blood coagulation, APC is inhibited by members of the serine proteinase inhibitor (serpin) family (*2*−*4*). The major serpin inhibitors of APC are protein C inhibitor (PCI) and α_1-proteinase inhibitor, with PCI appearing to be most important physiologically (*5*−*7*). PCI has a rather broad specificity, also inhibiting thrombin, urokinase, prostate-specific antigen, and factor XIa (*8*−*12*). The plasma concentration of APC−PCI complexes is increased in patients with venous thrombosis and disseminated intravascular coagulation (*13, 14*).

Serpins inhibit their target proteinases by a suicide substrate mechanism (*4, 15*). After the enzyme recognizes a potential proteolytic cleavage site in the exposed reactive center loop of the inhibitor, a noncovalent Michaelis-like complex is formed with subsequent formation of a covalent acyl intermediate involving the active site Ser residue of the enzyme and the P1 residue of the inhibitor (Arg 354 in PCI) (*14, 16, 17*). In the following step, residues P1−P14 of the reactive center loop, with the covalently attached enzyme, are inserted into the major β-sheet A of the inhibitor. This presumably leads to a distortion of the active site of the enzyme thereby greatly inhibiting the rate of cleavage of the acyl intermediate and effectively trapping the enzyme (*2*−*4, 18*). In addition to this inhibitory pathway, a minor pathway leads to prompt cleavage of the inhibitor and dissociation of the active enzyme from the cleaved inactive inhibitor (*4, 15*). Crystal-derived structures of enzyme-free cleaved serpins (e.g., α_1-proteinase inhibitor) show complete insertion of the reactive center loop into β-sheet A (*19, 20*). However, the depth to which the loop is inserted in a complex of serpin and acyl-bonded proteinase is still under debate (*15, 21*−*24*). A marked resistance to denaturation is a characteristic feature of cleaved, loop-inserted serpins (often referred to as the relaxed form of the molecule) that sets them apart from the corresponding uncleaved, native (stressed) forms (*18, 25, 40*). Synthetic peptides based on the reactive center loop are able to be inserted into β-sheet A of uncleaved serpins and cause the serpin to adopt the relaxed conformation (*18, 25, 40*).

It has been known for some time that the drastic conformational change that occurs during formation of the enzyme−serpin complex leads to the generation of neoepitopes (*22, 26*−*30*). Typically, these new epitopes are also present on proteolytically cleaved serpins into which the reactive center

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[1] Abbreviations: PCI, protein C inhibitor; APC, activated protein C; RU, response units; hAPC, human activated protein C; hPCI, human protein C inhibitor; mPCI, mouse protein C inhibitor; RCL, reactive center loop; BSA, bovine serum albumin; PSA, prostate-specific antigen; serpin, serine proteinase inhibitor.

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loop is inserted. To date, no antibodies have been identified that recognize neoepitopes covering the inserted strand 4 of β-sheet A, even though it is this region of serpins that undergoes the most striking conformational transition (*4*, *19*, *31*). Such antibodies would be important tools to define the extent of loop insertion in β-sheet A and for simple identification or isolation of the relaxed form of the inhibitor, either bound to the proteinase or free due to cleavage of the acyl intermediate.

Here we describe a mouse monoclonal antibody that recognizes a conformation-dependent neoepitope in APC−PCI complexes and cleaved, loop-inserted PCI but has no affinity for the native/stressed form of the inhibitor. The inserted reactive center loop is part of the epitope recognized by the antibody, but a synthetic peptide corresponding to positions P1−P14 of the loop is not recognized. By swapping mouse and human residues, we localized the epitope to the ArgP13−AlaP9 part of the inserted loop. These results, in conjunction with molecular modeling, indicate that the reactive center loop is inserted to at least the P9 residue.

EXPERIMENTAL PROCEDURES

Proteins. Native PCI was purified as previously described except that affinity chromatography was performed on a Amersham Pharmacia HiTrap affinity column (5 mL) with immobilized antibody (*17*, *32*). The purified PCI was diluted 1:2 with 50 mM Tris-HCl, pH 7.5, and applied to an Affi-Gel 10-monoclonal antibody M36 column (to remove cleaved/complexed PCI; see Results), preequilibrated with 50 mM Tris-HCl, 0.5 M NaCl, pH 7.5. Native PCI was collected, concentrated in an Omegacelle (30 kDa cutoff; Filtron, Northborough, MA) and frozen in aliquots at −70 °C. Reactive site-cleaved, free PCI was prepared from seminal plasma and purified as described for native plasma PCI, except that the M36-Affi-Gel step was omitted (*17*, *32*). The purified cleaved PCI was dialyzed against 50 mM Tris-HCl, 0.1 M NaCl, pH 7.5, concentrated as described above, and frozen at −70 °C. APC was a gift from Chromogenix (Gothenburg, Sweden).

APC−PCI complexes were prepared by incubating APC and PCI at a 1:2 molar ratio for 1 h at 37 °C followed by incubation overnight at room temperature in 30 mM Tris-HCl, 0.4 M NaCl, 0.8 mM EDTA, and 3 mM $CaCl_2$, pH 7.5, containing 4 IU heparin/mL. Uncomplexed APC was removed by immunoaffinity chromatography employing monoclonal antibody M3 (epitope around the active site of APC, unpublished data) after which complexes were isolated by immunoaffinity chromatography employing monoclonal antibody HPC 4 (a Ca^{2+}-dependent antibody that binds to the first EGF-like domain in protein C) (*33*). Both antibodies were coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions. The APC−PCI complex was eluted with 0.1 M sodium acetate buffer, pH 4.0. The pH of the eluate was immediately adjusted to 7.0 with 2 M Tris-HCl buffer, pH 7.5, pooled, and concentrated in a Centricon microconcentrator (30 kDa cutoff; Amicon Ltd, Beverly, MA)

Peptide Synthesis and Protein Sequencing. Overlapping peptides covering residues 83−371 of PCI were synthesized. Tetradecapeptides comprising residues 341−354 (corresponding to residues P1−P14) of human PCI were synthesized either with an unmodified N-terminus or with an acetylated N-terminus. Three N-acetylated tetradecapeptides that encompassed residues P1−P14 of hPCI were synthesized with single amino acid substitutions found in mouse PCI ("mousified" peptides): one with an Arg P13/Thr substitution, one with Ala P9/Ile and one with Thr P6/Ala. The peptides were synthesized with DPfp L-Fmoc amino acids (PerSeptive Biosystems, Framingham, MA) on a Milligen 9050 Plus peptide synthesizer (Perkin-Elmer Corp., Stockholm, Sweden). The peptides were deprotected and cleaved from the resin by treatment with 92−95% anhydrous trifluoroacetic acid containing relevant scavengers. The peptides were then purified by reversed-phase HPLC on a C8 column and eluted with an acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid. Proteins were sequenced using a Perkin-Elmer ABI Procise 494 pulsed liquid-phase sequencer.

Production of Monoclonal Antibodies. Mice were immunized with 10 μg of cleaved PCI emulsified in Freund's complete adjuvant. The immunization was repeated three times with 10 μg of the antigen mixture each time, and the mouse with the highest antibody titer was selected and boostered intraperitoneally with 200 μg of the antigen in Freund's incomplete adjuvant each day for 3 days before the fusion. The splenic cells were fused with SP 2/0-Ag 14 mouse myeloma cells using standard procedures (*32*). The fused cells were harvested in microtiter plates in DMEM medium (ICN, Aurora, OH) with HAT (hypoxanthine, aminopterin, thymidine; Sigma, St. Louis, MO). Anti-APC−PCI complex-specific antibody production was tested in a solid-phase radioimmunoassay. Breakapart microtiter plates (Nunc, Roskilde, Denmark) were coated at 4 °C overnight with 50 μL of affinity isolated rabbit antimouse immunoglobulin (10 μg/mL; Dakopatts AB, Älvsjö, Sweden). The plates were then washed with 50 mM Tris and 150 mM NaCl, pH 7.4 ("Tris buffer"), containing either 2 mM $CaCl_2$ or 2 mM EDTA. The wells were blocked with 100 μL of 10 mg/mL bovine serum albumin (BSA) for 15 min. After the plates were washed with $CaCl_2$ or EDTA-containing Tris buffer, hybridoma supernatant (50 μL) was added and the plates were incubated at room temperature for 1 h then rinsed with the same buffer and incubated for 3 h with ^{125}I-labeled APC−PCI complex, APC or native PCI in Tris buffer containing 1% (w/v) BSA, and either 2 mM $CaCl_2$ or 2 mM EDTA. The plates were washed five times with $CaCl_2$ or EDTA-containing Tris buffer and the bound radioactivity measured in a scintillation counter. Clones of interest were subcloned twice by the limiting dilution method, expanded, and injected intraperitoneally into pristane-primed Balb/c mice. Ascites fluid was harvested after 7−8 days. Monoclonal antibodies were produced on a large scale in a Technomouse apparatus (Integra Biosciences, Wallisellen, Switzerland) and purified on PROSEP-A column using the manufacturer's protocol (Bioprocessing Limited, Durham, United Kingdom).

Immunoassays. The following monoclonal antibodies were used: M11-5 (*32*), M52, and M36 against PCI and HPC 4 against protein C (*33*). APC−PCI complexes were measured by a microtiter-based time-resolved immunofluorimetric assay (DELFIA immunoassay) with antibody HPC 4 as the detector (*34*). The samples were incubated with biotinylated monoclonal catcher antibody M36 (2 μg/mL, 50 μL) in a nonadsorptive Sero-Wel microtiter plate (Bibby Sterilin Ltd.,

Staffs, United Kingdom). Samples were then transferred to a streptavidin-coated microtiter plate (Wallac, Upplands Väsby, Sweden) and incubated for 1 h. For detection, Eu^{3+}-labeled HPC 4 (0.2 μg/mL, 25 μL) was used. After a 1 h incubation, the wells were washed with Wash buffer (Wallac) and Enhancement solution (Wallac) was added (200 μL/well). Fluorescence was measured 5 min later with a DELFIA plate fluorometer (Wallac). To measure total PCI, native/stressed and/or cleaved/relaxed, the assay was modified to use biotinylated M52 as the catcher and Eu^{3+}-labeled M11-5 as the detector. Exposure of the cleavage-specific neoepitope in PCI was studied in an assay with biotinylated M36 as catcher and Eu^{3+}-labeled M11-5 as detector.

Affinity Chromatography. Native PCI, reactive site-cleaved PCI, or APC−PCI complex (30 μg) was chromatographed on an Affi-Gel 10 column (BioRad, Hercules, CA) containing immobilized monoclonal antibody M36. The column was preequilibrated with 50 mM Tris-HCl, 0.5 M NaCl, pH 7.5, and a flow rate of 0.1 mL/min was used. Bound proteins were eluted with 0.1 M glycine−HCl, 0.5 M NaCl, pH 2.7, and the pH of the eluate was immediately adjusted to 7.0. The concentrations of native, cleaved, and complexed PCI in the eluate were measured with the DELFIA assay as described.

Generation of Reactive Center Loop Peptide−PCI Complexes. To induce the relaxed conformational state, PCI was incubated with a synthetic N-acetylated tetradecapeptide comprising residues P1−P14 of the reactive center loop (*35*). Native PCI (14 μg) in 50 mM Tris-HCl, 0.5 M NaCl, pH 7.5, was diluted 1:2 with 1 M Tris-HCl and incubated with the N-acetylated tetradecapeptide with the human reactive center loop sequence or with one of the "mousified" peptides (100-fold molar excess in 2.1 μL of formic acid) for 24 h at 37 °C. As a control, the tetradecapeptide without an N-acetylated N-terminus was used. Aliquots were removed at intervals during the incubation and immediately frozen at −70 °C. The PCI activity in each aliquot was determined as the inhibition of the amidolytic activity of APC toward the substrate S-2366 (Chromogenix, Gothenburg, Sweden). The assay was performed in microtiter plate wells (E.I.A/R.I.A Plate, Corning Inc., Corning, NY) to which had been added 100 ng of APC in 200 μL of 20 mM Tris-HCl, 0.1 M NaCl, 0.1% (w/v) BSA, pH 7.5, and heparin (4 IU/mL). PCI (100 ng, 1 μL) was added, and the samples incubated for 1 h at 37 °C after which S-2366 (2.5 mM, 50 μL) was added and the enzymatic activity measured by monitoring the absorbance at 405 nm in a UVmax kinetic microplate reader (Molecular Devices, Gothenburg, Sweden). APC−PCI complex formation and exposure of the neoepitope in the modified inhibitor were tested in the DELFIA assay as described above.

Fluorescence Spectroscopy. Tryptophan fluorescence measurements of PCI and reactive center loop peptide−PCI complexes were performed with a Perkin-Elmer LS 50-B spectrofluorometer. Aliquots of PCI or the complex (100 μL, 27 μg PCI) were diluted with 900 μL of 50 mM Tris-HCl, 0.1 M NaCl, pH 7.4, containing various concentrations of guanidine−HCl (0−6.0 M). Excitation was at 295 nm, and emission spectra were recorded from 310 to 450 nm. Formation of the denatured form of the inhibitor was followed by measuring the increase in emission wavelength maximum as a function of the guanidine−HCl concentration. The data are presented as $(\lambda_{native} - \lambda)/(\lambda_{native} - \lambda_{denatured})$, where λ is the emission wavelength maximum of native PCI (λ_{native}), unfolded PCI ($\lambda_{denatured}$), and the individual sample (λ).

BIAcore Biosensor Assay. The interaction of monoclonal antibody M36 with native PCI, reactive site-cleaved PCI, and the APC−PCI complex was studied by surface plasmon resonance in a BIAcore 2000 apparatus (Biacore, Uppsala, Sweden) using biotinylated M36 that had been captured on a streptavidin-coated SA sensor chip SA (Biacore AB). The amount of immobilized antibody was 1500−2000 response units (RU). The association of native PCI, reactive site-cleaved PCI, and APC−PCI complex with M36 was studied at several concentrations in a flow buffer comprising 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% (w/v) BSA. Protein concentrations were determined by amino acid analysis after acid hydrolysis. Aliquots of the protein stock solutions were diluted in flow buffer, and 90 μL was injected at 30 μL/min and the association phase followed for 5 min. The dissociation phase was monitored at 30 μL/min for 10 min. The system was regenerated by two 5 μL pulses of 100 mM glycine, 500 mM NaCl, pH 2.75, at a flow rate of 5 μL/min followed by a 5 μL pulse of 40 mM octylglucoside. Data were evaluated using the program Biaevaluation 3.0 (Biacore AB). Association and dissociation phases were evaluated simultaneously using eq 1 for the association phase and eq 2 for the dissociation phase:

$$R(t) = R_0 + (R_{max}ck_{on}/(ck_{on} + k_{off}))(1 - \exp(-(ck_{on} + k_{off})t)) \quad (1)$$

$$R(t) = C\exp(-k_{off}t) \quad (2)$$

where C is the amplitude of the dissociation process, k_{on} and k_{off} are the association and dissociation rate constants, R_0 is the response signal at $t = 0$, R_{max} is the capacity of the immobilized antibody, and c is the protein concentration.

Molecular Modeling. Models for uncleaved hPCI were performed as described previously (*36*). Homology models of cleaved human PCI (hPCI) and X-ray structures of human APC (hAPC) were obtained from the Protein Data Bank (*37*−*39*). A test model for cleaved mouse PCI was developed using comparative model building with the cleaved hPCI structure as initial template. This process was initiated to investigate regions in mouse and hPCI that differ and should thus be involved in stimulating antibody production. The test model was built using a standard procedure with the program Homology (Biosym-MSI, San Diego, CA).

Other Methods. Monoclonal antibodies were labeled with Eu^{3+}−DTTA chelate using an Eu-labeling kit (Wallac) according to the manufacturer's instructions. Monoclonal antibodies were biotinylated with NHS-LC-Biotin (Pierce, Rockford, IL) following the instructions of the manufacturer. APC, PCI, and APC−PCI were labeled with ^{125}I (Amersham Pharmacia AB, Stockholm, Sweden) using the chloramine T method described by Amersham. SDS-PAGE and western blotting were performed using standard methods.

RESULTS

Monoclonal Antibodies Specific for Relaxed PCI. To produce monoclonal antibodies specific for the reactive site-

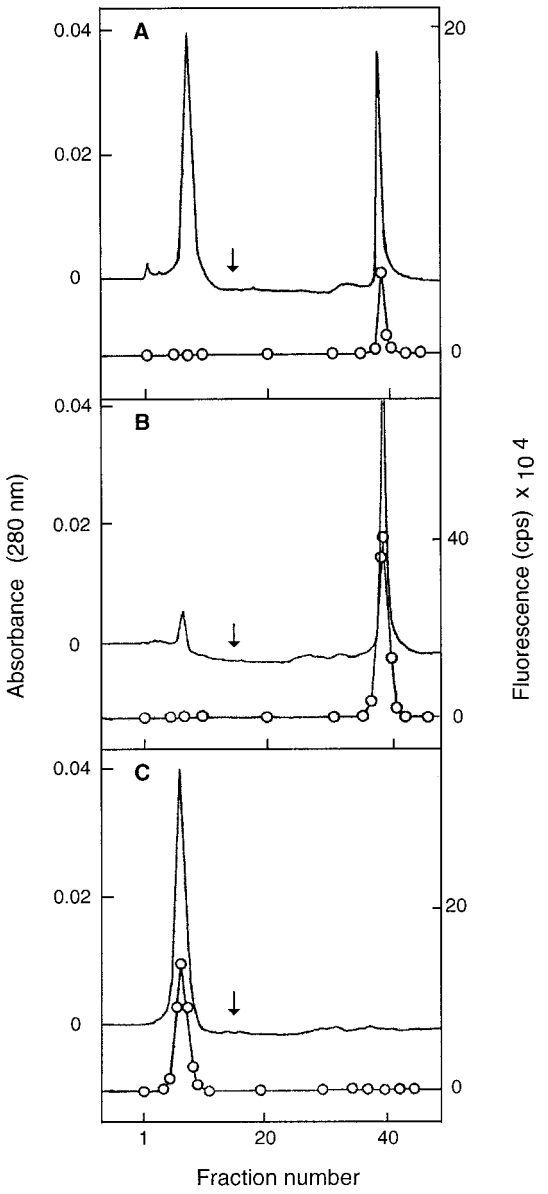

FIGURE 1: Affinity chromatography on a column of immobilized antibody M36: (A) APC−PCI complex; (B) reactive site-cleaved PCI; (C) native PCI. Bound material was eluted with 0.1 M glycine−HCl, 0.5 M NaCl, pH 2.7, applied as indicated by arrows. The effluent was analyzed with a DELFIA immunoassay with antibody M52 as catcher and Eu^{3+}-labeled HPC 4 (against APC (A)) or M11-5 (against PCI (B and C)) as detector. Absorbance at 280 nm (−). Concentrations of PCI or APC−PCI complex or reactive site-cleaved PCI as determined with a DELFIA assay (○).

cleaved form of PCI, mice were immunized with cleaved, relaxed PCI (*17*). Clones were screened with ^{125}I-labeled uncomplexed, stressed PCI, with ^{125}I-labeled cleaved/relaxed PCI, and with PCI in complex with ^{125}I-labeled APC. Clones that bound complexed and cleaved PCI but not the uncomplexed form of the inhibitor were selected. One antibody (M36) with this specificity was characterized in detail. The epitope of the antibody appeared to be conformation-dependent as it reacted very weakly with both cleaved and complexed PCI (both reduced and unreduced samples) in western blot experiments (not shown). Immunoaffinity chromatography employing immobilized antibody M36 showed that both cleaved and complexed inhibitor bound tightly to the column, whereas the native/stressed inhibitor

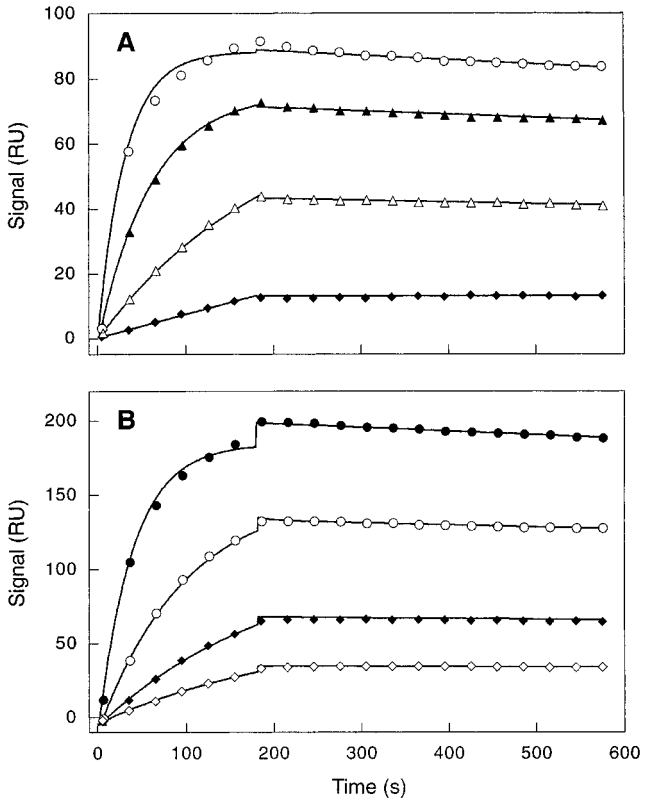

FIGURE 2: Adsorption and desorption kinetics for binding of reactive site-cleaved PCI (A) and the APC−PCI complex (B) to antibody M36 as monitored by surface plasmon resonance. Experiments were performed using 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% (w/v) BSA as running buffer at a flow rate of 30 μL/min. The final concentrations were 4.6 (○), 2.3 (▲), 1.2 (△), and 0.6 (◆) nM for reactive site-cleaved PCI and 17.6 (●), 8.8 (○), 4.4 (◆), and 2.2 (◇) nM for the APC−PCI complex. The protein was injected at $t = 0$, and binding to the antibody is apparent during the association phase (180 s). The protein-containing buffer was then replaced by running buffer, and dissociation of the protein from the antibody was followed. The solid curves were calculated using eqs 1 and 2 (see Experimental Procedures).

did not bind to the immobilized antibody (Figure 1). Binding of cleaved/complexed PCI to antibody M36 was not Ca^{2+}-dependent. Even when the native inhibitor was chromatographed on resin that was heavily substituted with antibody M36 so as to bind ~36 nmoles of cleaved PCI/mL, there was no retention of the native/stressed form of the inhibitor. The affinity of antibody M36 for cleaved and complexed PCI was measured by surface plasmon resonance in a BiaCore (Figure 2). M36 bound to both the APC−PCI complex and cleaved PCI with high affinities, K_d = 8.5-($\pm$2.7) $\times$ 10^{-11} M and K_d = 2.9($\pm$0.8) $\times$ 10^{-11} M, respectively. The affinity of the uncomplexed, stressed PCI for M36 was too low to measure (not shown).

Induction of the Complexed/Relaxed State of PCI. Insertion of a synthetic tetradecapeptide with an acetylated N-terminus corresponding to the reactive center loop (residues P1−P14) induces the relaxed state in native/stressed serpins, for example, α_1-proteinase inhibitor and antithrombin (*18, 25, 30, 35*). Native PCI incubated with a 100-fold molar excess of nonacetylated peptide retained full inhibitory activity toward APC, whereas incubation with the N-acetylated tetradecapeptide resulted in peptide incorporation and a complete loss of inhibitory activity. Consistent with this, PCI complexed with the N-acetylated peptide did not form

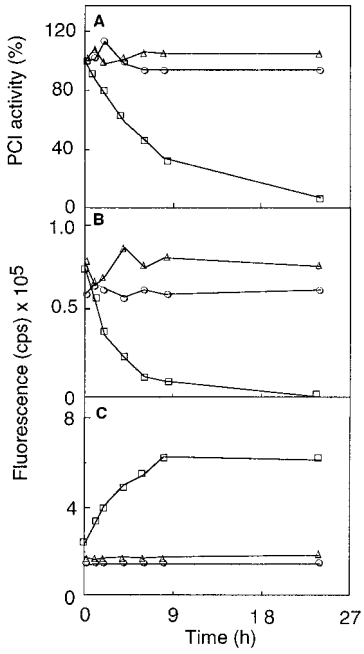

FIGURE 3: Conversion of native/stressed PCI to the relaxed, inactive form by incubation with an N-acetylated reactive center loop peptide. (A) Inhibition of amidolytic activity of APC by native/stressed PCI. Incubation of native PCI with N-acetylated peptide destroyed the inhibitory activity of PCI, whereas incubation with peptide that had not been acetylated had no effect. (B) APC−PCI complex formation studied in a sandwich immunoassay (M36 biotinylated and HPC 4 Eu^{3+}-labeled). Complexes were formed on incubation of APC with PCI and with PCI that had been incubated with the nonacetylated reactive center loop peptide, whereas incubation with the N-acetylated peptide destroyed the activity of PCI. (C) Induction of neoepitope(s) in PCI by incubation with the N-acetylated peptide as measured in a sandwich immunoassay (biotinylated M36 and Eu^{3+}-labeled M11−5). Incubation with the non-acetylated peptide did not induce the epitope. (○) PCI, (△) PCI + RCL peptide, and (□) PCI + N-acetylated RCL peptide.

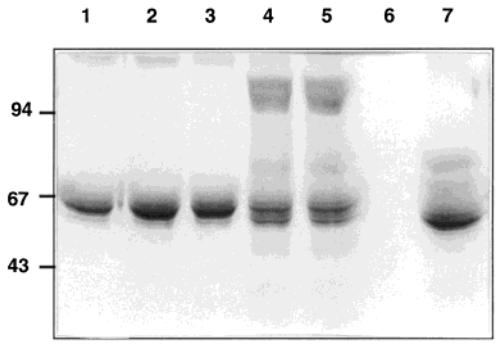

FIGURE 4: SDS-PAGE demonstrating complex formation between APC and either native PCI or native PCI that had been incubated with the non-acetylated reactive center loop peptide or PCI that had been incubated with the N-acetylated peptide: lane 1, native PCI; lane 2, native PCI incubated with reactive center loop peptide; lane 3, native PCI incubated with the N-acetylated reactive center loop peptide; lane 4, APC and native PCI; lane 5, APC and native PCI incubated with the non-acetylated reactive center loop peptide; lane 6, empty lane; lane 7, APC and native PCI incubated with the N-acetylated reactive center peptide. Apparent molecular mass (kDa) is shown on the left.

complexes with APC as demonstrated by both an antibody-based sandwich assay (Figure 3) and a gel shift mobility assay (Figure 4). Insertion of the peptide also induced the M36 epitope (Figure 3).

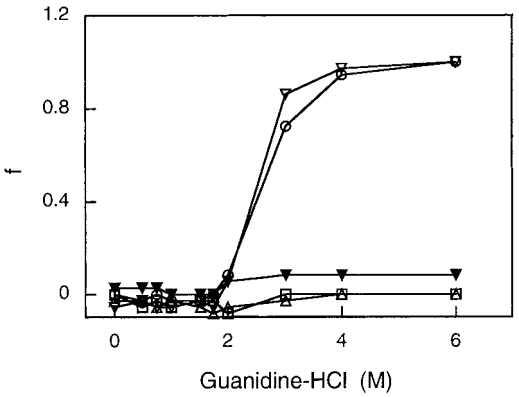

FIGURE 5: Fraction of PCI denatured as a function of the concentration of guanidine−HCl as monitored by fluorescence emission. Native PCI (○), native PCI incubated with the non-acetylated human reactive center loop peptide (▽), native PCI in complex with the N-acetylated peptide (□), native PCI complexed with the N-acetylated ArgP13Thr peptide (△), and reactive site-cleaved PCI (▼). Amount of unfolded PCI was calculated as $f = (\lambda_{native} - \lambda)/(\lambda_{native} - \lambda_{denatured})$, where $\lambda_{native} = 336$ nm and $\lambda_{denatured} = 354$ nm are the emission maximum wavelengths of native and denatured PCI, respectively, and λ is the maximum wavelength of the emitted light in the individual sample.

The relaxed form of other serpins is more resistant to denaturation than the native/stressed form (*18*, *24*, *34*). To test whether this is also the case for PCI, the native/stressed form and the cleaved/relaxed form (induced either by complex formation with the synthetic N-acetylated reactive center loop peptide or by complex formation with APC and subsequent cleavage) were incubated with various concentrations of guanidine−HCl and the fluorescence emission spectra recorded. The emisson maximum of native/stressed PCI was at 336 nm, whereas after denaturation with guanidine, the quantum yield decreased and the wavelength maximum increased to 354 nm. The denaturation curve for native/stressed PCI showed a half-maximum between 2.0 and 2.5 M guanidine−HCl (Figure 5). An identical result was obtained with PCI that had been incubated with nonacetylated tetradecapeptide. In contrast, cleaved PCI and PCI complexed with the N-acetylated tetradecapeptide were entirely resistant to denaturation with guanidine−HCl over the concentration range studied (up to 6.0 M).

Identification of the Epitope for Antibody M36. Antibody M36 recognizes a conformation-dependent, presumably nonlinear epitope as judged by its very weak reaction with the denatured inhibitor on western blots (not shown). The antibody also bound a proteolytic fragment that was obtained by digestion of the cleaved, loop-inserted PCI with the lysine-specific endoproteinase (results not shown). This fragment, which was isolated by HPLC, had Ser83 at the N-terminus (amino acids are numbered from the N-terminus of mature PCI). SDS-PAGE and the amino acid composition of the fragment suggested that it continued to position 354 (i.e., to the Arg P1 residue). Overlapping synthetic peptides (including the reactive center loop peptide, residues P1−P14) covering this part of the molecule did not compete with the cleaved inhibitor for binding to M36, even at a 10^4-fold molar excess over the labeled detector that consisted of cleaved PCI consistent with the results obtained by western blotting. We therefore speculated that the conformation-dependent epitope is located on pleated sheet A, where the complexation-induced conformational transition is most conspicous,

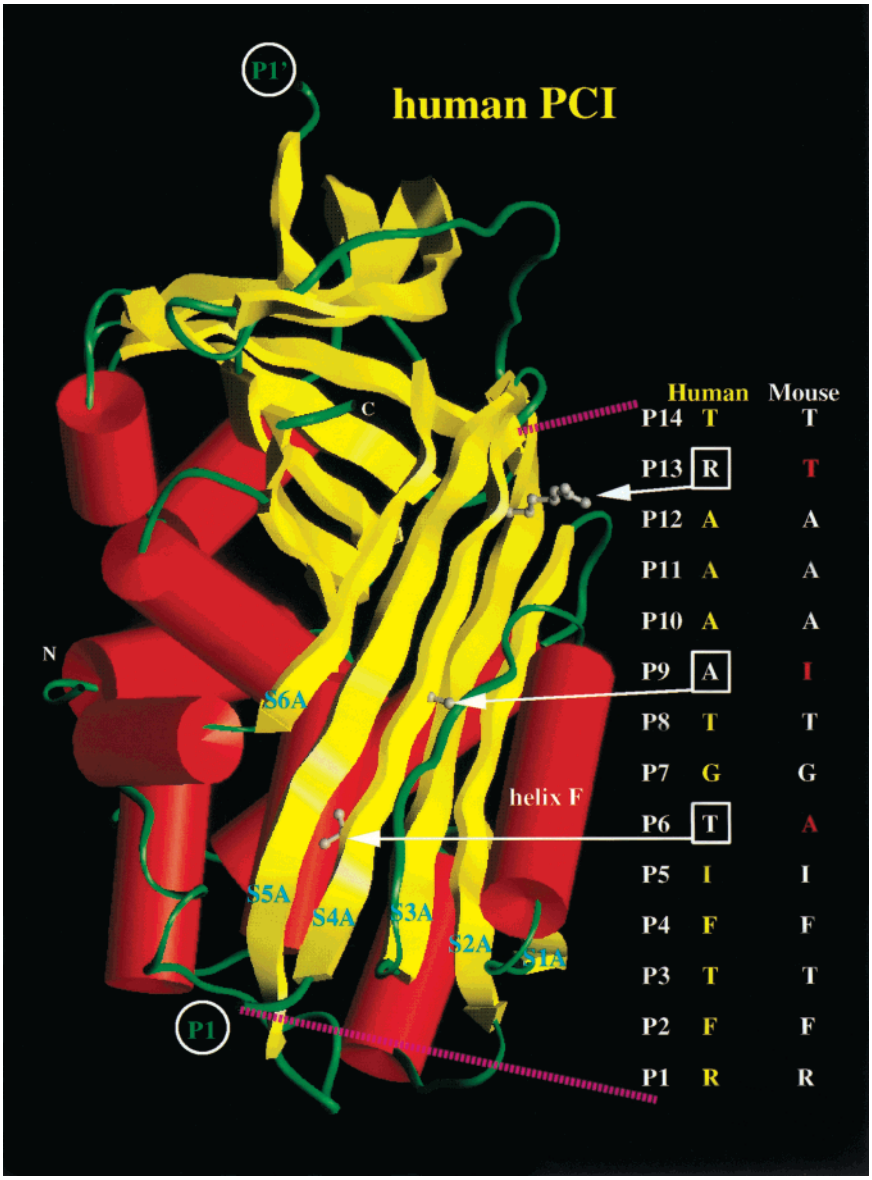

FIGURE 6: Molecular model of human PCI. The model shows the reactive center loop inserted as strand S4 in β-sheet A of the inhibitor. The sequences of the human and mouse reactive center loops are shown.

and particularly that the epitope involves the reactive center loop peptide.

The sequence identity between human and mouse PCI is 65%, and thus, a model for mouse PCI (mPCI) based on the theoretical model of cleaved human PCI (hPCI) could easily be derived. Similarly, a preliminary model of uncleaved mPCI could be predicted based upon the X-ray structures of related uncleaved serpin molecules. In the inserted reactive center loop peptide of mouse PCI there are three substitutions (hArg P13/mThr, hAla P9/mIle, hThr P6/mAla)(Figure 6). Interestingly, the parts of other strands in β-sheet A predicted to surround Arg P13 (residue 342) are highly conserved whether one considers fully exposed or buried residues. Only two additional solvent-exposed substitutions were identified by such modeling, hPCILys 189/mPCIGlu and hPCIVal 333/mPCIMet. This suggested that the mouse immune system might generate antibodies directed against Arg P13 (see below), Ala P9 (position 346), or Thr P6 (position 349). Arg P13 is fully exposed in the model. Ala P9 is not directly exposed to the solvent. However, the long side chain of Ile seems to slightly dislocate a few side chains in the C-terminal region of helix F of mouse PCI (Figure 6), suggesting a slight difference in conformation between the human and mouse proteins in this short loop segment. The P6 residue is indicated to be inaccessible to solvent. The Thr to Ala substitution in this position is easily tolerated in the 3D model of mouse PCI, and it is very unlikely that antibodies develop against this amino acid. Thus, the key difference between the mouse and human molecules in this region appears to be the hArg to mThr mutation in the P13 position.

The three "mousified" N-acetylated peptides, based on the sequence of mPCI (ArgP13Thr, AlaP9Ile, ThrP6Ala), were incorporated by the stressed/native form of PCI. This was concluded from the loss of inhibitory activity (not shown), the induction of the neoepitope (AlaP9Ile, ThrP6Ala), and for the ArgP13Thr peptide, the increased stability to denaturation by guanidine−HCl (Figures 5 and 7). The epitope of the M36 antibody was probed in a sandwich assay. PCI in complex with either the N-acetylated wild-type human tetradecapeptide or each of the three mutated peptides was incubated first with the biotinylated M36 and then with a labeled antibody (M11-5) directed against a separate epitope

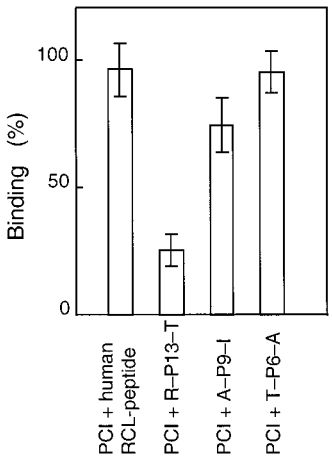

FIGURE 7: Binding of monoclonal antibody M36 to the binary complexes of PCI with synthetic N-acetylated reactive center loop (RCL) peptide or corresponding peptides with substitutions based on the mouse PCI sequence (see Figure 6). The binding of M36 to reactive center loop-cleaved relaxed PCI is denoted 100%. The bars represent the average of four determinations (±2 SD).

on PCI (expressed by both the stressed and relaxed forms of the inhibitor). For the complex formed with the ArgP13Thr peptide, binding was reduced by 80% compared to the wild-type reactive center loop peptide despite the fact that the peptide had induced the relaxed conformation in the inhibitor. Binding to the complex formed with peptide AlaP9Ile was reduced by ~20%. Binding was not influenced by the Thr P6/Ala substitution (Figure 7). These results strongly suggest that the epitope is located in the reactive site loop peptide around ArgP13 and presumably extends to the AlaP9. As the antibody is conformation-dependent, one or both adjacent strands in β-sheet A (or surrounding loop segments) are presumably also involved in the monoclonal antibody−PCI interaction.

DISCUSSION

Our results demonstrate that PCI, the prime inhibitor of APC, shares the properties characteristic of serpins such as α_1-proteinase inhibitor, antithrombin, C-1 esterase inhibitor, and plasminogen activator inhibitors types 1 and 2 (*4, 18, 35*). The native/stressed form of PCI is more easily denatured by guanidine than the cleaved/relaxed form of the inhibitor. Moreover, the cleaved/relaxed form does not form complexes with or inhibit proteinases. We also demonstrated that the relaxed form of PCI can be induced without proteolytic cleavage by insertion into the major β-sheet A of a synthetic peptide corresponding to residues P1−P14 of the reactive center loop. The specificity of this reaction is illustrated by the fact that only the N-acetylated tetradecapeptide is inserted into the pleated sheet (*18, 25, 35*). Like the cleaved inhibitor, the peptide-inserted form of the inhibitor does not form complexes with its cognate proteinase.

Opening up of β-sheet A, which allows insertion of the cleaved reactive site loop, is the most conspicuous conformational transition that ensues after reactive center loop cleavage (*2, 18*). Loop insertion also leads to more overt exposure to solvent of the upper part of the β-sheet than of the lower part of the sheet that is protected by α-helix F and its connecting loops. The structural transitions associated with loop insertion expose epitopes that can stimulate antibody production. The same neoepitopes are presented on both the complexed and cleaved forms of an inhibitor, and most of them are conformation dependent (*26, 40*). Such neoepitopes have been identified in antithrombin, plasminogen activator inhibitor 2, and C1-esterase inhibitor, but their positions have not been established (*26, 27, 29, 30, 35*). Monoclonal antibodies against neoepitopes are potentially important tools for characterizing the conformational transition that is accompanied by proteinase−serpin complex formation and for the identification and isolation of cleaved or complexed inhibitor (*22, 30*).

Antibody M36 was identified by a screening procedure designed to identify monoclonal antibodies that specifically recognize neoepitopes in PCI that are present in the relaxed form of the inhibitor. Such antibodies should bind PCI whether in complex with APC or already dissociated from the proteinase and should have no affinity for the native/stressed form of PCI. We identified candidate antibodies and characterized M36 in detail. The epitope of M36 was demonstrated to be conformation-dependent as it did not bind synthetic peptides per se that spanned the parts of interest of the PCI sequence, nor did it stain PCI, neither native nor cleaved, in western blots. The neoepitope was exposed upon insertion of the synthetic reactive center loop in stressed PCI, which enabled us to determine its position on the surface of the molecule. A sequence comparison between mouse and human PCI established that three residues in the inserted part of the reactive center loop (P6, P9, and P13) differed between the two species. Synthetic, N-acetylated tetradecapeptides, comprising residues P1−P14 with single amino acid substitutions based on the mouse PCI sequence, were inserted into human PCI. All three peptides induced the loop-inserted conformation in PCI that is incompatible with inhibition of complex formation with APC. Yet, the antibody M36 did not react with the Arg P13/Thr mutation. Considering the specificity of the loop-insertion process and the fact that the ArgP13Thr peptide induces the same functional state of PCI as the Arg P13 peptide, the conformation of the two loop-inserted forms of the inhibitor is presumably identical. Moreover, molecular modeling of cleaved human PCI demonstrated that the side chain of Arg P13 is solvent exposed. We infer that the neoepitope is localized around Arg P13 and that it probably extends to adjacent strands in β-sheet A. Finally, a comparison of the cleaved mouse and human PCI molecules suggested that ArgP13 is the major candidate residue in this region of human PCI for forming a binding surface with mouse antibodies.

Together, the experimental and modeling data provide additional insight into the possible orientation of APC within the covalent APC−PCI complex. The overall size of APC, PCI, and an antibody has to be considered. The antibody M36 interacts with the area of PCI around Arg P13 while still sensitive to the region of Ala P9. It binds cleaved PCI and the stable APC−PCI complex with nearly identical affinities. Thus, it seems clear that the proteinase in the stable complex cannot be immobilized on top of helix F or around the N-terminal part of strand 2A or the C-terminal part of stand 5A as there would not be enough room to accommodate both APC and M36. Attempts to dock APC to cleaved PCI (with a fully inserted reactive center loop) were fraught with difficulty. The active site Ser of the proteinase (Ser 195) could not be close enough to the P1 residue to form a

covalent bond without serious distortion of both the active site of the enzyme and the serpin structure. However, insertion of the reactive center loop to residues P4−P6 would obviate this problem. This degree of loop insertion would also be consistent with the antibody data as there would be no steric clash between enzyme and antibody. It also seems to be consistent with the fluorescence energy transfer data that indicate extensive insertion of the reactive center loop with acyl-bonded enzyme (*15*, *23*). Our data are thus consistent with a model where APC, as a result of reactive center loop insertion to residues P6−P4, is moved almost to the other side of PCI (as compared to the Michaelis-like complex). The proteinase would thus be positioned adjacent to the N-terminal part of strand 5A.

NOTE ADDED IN PROOF

After this manuscript was submitted, the first crystallographic structure of a serpin−proteinase complex was reported (Huntington, J. A., Read, R. J., and Carrell, R. W. (2000) *Nature 407*; 923−926).

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