

# Introduction of an RRHR motif into chicken urokinase-type plasminogen activator (ch-uPA) confers sensitivity to plasminogen activator inhibitor (PAI)-1 and PAI-2 and allows ch-uPA-mediated extracellular matrix degradation to be controlled by PAI-1

(proteolytic enzymes/protease nexin 1/serine proteinase inhibitor)

JOHN D. SIPLEY, DANIELA S. ALEXANDER, JACQUELINE E. TESTA, AND JAMES P. QUIGLEY\*

Department of Pathology, State University of New York, Stony Brook, NY 11794

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**ABSTRACT** Comparison of the amino acid sequence of the chicken and human urokinase-type plasminogen activators (uPAs) revealed that the putative PAI-binding site found in the variable region 1 (VR1) loop of mammalian PAs is absent in the homologous region of ch-uPA. ch-uPA, unlike mammalian PAs, also appears to be refractory to inhibition by human PAIs and as a naturally occurring PAI-resistant variant, constitutes a unique model system for assessing the functional relevance of the PAI-binding site. Therefore, we molecularly constructed a ch-uPA, ch-uPA<sub>RRHR</sub>, which contains the putative PAI-binding motif RRHR (residues 192–195) in its VR1 loop. As a result of this substitution, the second-order rate constant of inhibition of PAI-1 increased  $\approx 700$ -fold from  $4.50 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$  for wild-type ch-uPA to  $3.02 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$  for ch-uPA<sub>RRHR</sub>, and the ability to form SDS-stable, uPA–PAI-1 complexes increased  $\approx 1000$ -fold. Furthermore, the interaction of ch-uPA<sub>RRHR</sub> with PAI-2 was also substantially enhanced, while the interaction with other members of the serine proteinase inhibitor superfamily, protein nexin 1,  $\alpha_1$ -PI, and C<sub>1</sub>-inhibitor, was unaffected indicating that the RRHR motif is not a general serine proteinase inhibitor binding site. Finally, we show that extracellular matrix degradation by cells expressing ch-uPA<sub>RRHR</sub> is inhibited by PAI-1 in a dose-dependent manner, while matrix breakdown by cells expressing wild-type ch-uPA is unaffected by PAI-1. Thus acquisition of sensitivity to PAI-1 through a structural motif that enhances the specificity of the protease-inhibitor interaction confers to ch-uPA an added level of regulation in the context of the degradative cellular phenotype.

The plasminogen activator (PA)/plasmin system is a proteolytic cascade that leads to effective degradation of extracellular protein components (1, 2) and is initiated by the generation of active urokinase-type PA (uPA). Active uPA converts the inactive zymogen substrate plasminogen to plasmin, an enzyme with broad trypsin-like specificity that can degrade several extracellular matrix (ECM) components (3, 4) and in addition can activate prometalloproteinases (5), latent elastase (6), growth factors (7, 8), and cytokines (9).

Specific regulation of PA catalytic activity is achieved by at least four members of the serine protease inhibitor (serpin) superfamily known as plasminogen activator inhibitors or PAIs (PAI-1, -2, and -3) and protease nexin 1 (PN-1) (10).

Inactivation of uPA and tissue-type PA (tPA) by PAI-1 and PAI-2 results in the formation of stable equimolar PA–PAI complexes (11–15) that are rapidly removed from the circulation (16) and degraded.

PAI-1 binding to the target PAs uPA and tPA is believed to be mediated predominantly by the formation of salt bridges between a series of positively charged residues occupying a surface loop known as variable region 1 (VR1) of the protease, with a complementary series of negatively charged residues in PAI. Deletion of the VR1 residues 296–302 of KHRRSPG (set as <sup>296</sup>KHRRSPG<sup>302</sup>) of tPA produced a mutant that was highly resistant to PAI-1 inhibition (17, 18). Further, mutation of the tPA VR1 sequence <sup>296</sup>KHRR<sup>299</sup> to either Gly-296, Glu-298, and Glu-299, or to <sup>296</sup>AAAA<sup>299</sup>, conferred resistance to PAI inhibition (18, 19). Deletion of the residues <sup>179</sup>RHRGGG<sup>184</sup> in the VR1 region of human uPA (h-uPA) is believed to account for the inability of PAI to inhibit proteolytic activity of this mutant (20). Finally, introduction of <sup>296</sup>KHRR<sup>299</sup> into the VR1 of thrombin, a serine protease inefficiently inhibited by PAI alone, conferred increased sensitivity to PAI inhibition (21), further implicating the basic residue motif in the VR1 region as a PAI binding site.

We have recently reported that, in contrast to several mammalian uPAs, ch-uPA is resistant to human PAI inhibition and interestingly, lacks any positively charged residues in its corresponding VR1 region (22). Thus ch-uPA would appear to be a natural variant within this family of enzymes in which the putative PAI-binding region is missing from the molecule. In the present report we demonstrate that introduction of the putative PAI-binding site, RRHR, into ch-uPA, results in a gain-of-function alteration rendering the mutant highly susceptible to PAI inhibition. Furthermore, we report that in ECM degradation assays, addition of PAI-1 differentially inhibits matrix degradation mediated by cells expressing the RRHR-containing ch-uPA mutant when compared with cells expressing wild-type (wt) ch-uPA.

## MATERIALS AND METHODS

**Site-Specific Mutagenesis of ch-uPA.** A ch-uPA cDNA subclone was obtained from Jay Degen (Children's Hospital Research Foundation, Cincinnati, OH) and the coding sequence was amplified with synthetic oligonucleotide primers 5'-GCGGAATTCAAAGGAGAAGCTTACCAACATG-3', and 5'-CGCGGATCCTCACTTTGGTTCACGG-3', in a

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Abbreviations: PA, plasminogen activator; uPA, urokinase-type PA; ch-uPA, chicken uPA; h-uPA, human urokinase-type PA; tPA, tissue-type PA; ECM, extracellular matrix; PAI, plasminogen activator inhibitor; rPAI, recombinant PAI; serpin, serine proteinase inhibitor; VR1, variable region 1; PN-1, protease nexin 1; wt, wild type.

\*To whom reprint requests should be addressed.

PCR with Vent<sub>R</sub> DNA Polymerase from New England Biolabs as per the manufacturer's instructions. The PCR product was then digested with *EcoRI* and *BamHI* and inserted into similarly cleaved pBluescript from Stratagene, and transformed into the host strain XL-1 blue (Stratagene). The ch-uPA insert of one clone was sequenced in its entirety and found to match the published sequence (23). A culture of the pBluescript ch-uPA<sub>wt</sub> clone was infected with helper phage VCS-M13 (Stratagene), and single-stranded circular DNA was prepared. A synthetic oligonucleotide 5'-GGATAGCTG-GCATCTTCCGGCGCCACCGTGGTGGCACTGACCAG-TTTCTG-3' was designed to substitute the <sup>192</sup>QNIM<sup>195</sup> sequence of ch-uPA<sub>wt</sub> with RRHR, and insert an additional G. The mutagenic oligonucleotide was 5'-phosphorylated and HPLC purified. Mutagenesis was performed using the Amer-sham Sculptor *in vitro* mutagenesis system. The ch-uPA insert of the ch-uPA<sub>RRHR</sub> mutant was sequenced completely to verify the presence of the desired mutation.

**DNA Sequencing.** DNA sequence was obtained for both strands as described (24).

**Expression and Purification of Recombinant ch-uPA in NS0 and Sf9 Cells.** The mouse myeloma cell line NS0 was the host for the cytomegalovirus promoter-driven expression vector pEE12 obtained from C. Bebbington (Celltech, Slough, U.K.). Untransfected NS0 cells were grown in growth medium [DMEM supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum/1 mM sodium pyruvate/4 mM glutamine/100 units/ml penicillin/0.1 mg/ml streptomycin]. ch-uPA<sub>wt</sub> and ch-uPA<sub>RRHR</sub> inserts were ligated into the *EcoRI* and *BclI* restriction sites of the pEE12 expression vector. NS0 cells were transfected by electroporation then grown in the absence of glutamine as described (25), to select for cells expressing glutamine synthetase encoded by the pEE12 vector. Transfected colonies were screened for ch-uPA<sub>wt</sub> and ch-uPA<sub>RRHR</sub> production. High-level expressors were expanded in culture, transferred to serum-free growth medium, and the conditioned media were collected 24 h later.

*Autographica californica* nuclear polyhedrosis virus polyhedrin promoter-driven expression vector pVL1392 obtained from PharMingen containing ch-uPA cDNA was used to infect *Spodoptera frugiperda* (Sf9) cells. High-level expressors were expanded in culture and transferred to serum-free growth medium, and the conditioned media were collected 24 h later.

The recombinant enzymes from both expression systems were purified by benzamidine-Sepharose affinity and gel filtration chromatography (26). Protein concentration was determined by silver staining of electrophoresed samples compared with known quantities of a protein standard.

**Isolation of Chicken Plasminogen.** Chicken plasminogen was purified from chicken serum by lysine-Sepharose affinity chromatography as described (27). Human glu-plasminogen was purchased from Boehringer Mannheim.

**Enzyme and Inhibitor Assays.** Quantitation of uPA activities was performed using the indirect chromogenic substrate assay (28) as described (22). h-uPA, ch-uPA<sub>wt</sub>, or ch-uPA<sub>RRHR</sub> (50 pg) were added to the chromogenic substrate assay at a final concentration of 10 pM, which generates  $\approx 0.3 A_{405}$  linearly over 1 h of incubation. For the uPA inhibition studies enzymes were incubated in PBST [PBS/0.1% (vol/vol) Tween 20] for 45 min at 37°C with varying concentrations of benzamidine, recombinant human PAI-1, or recombinant human PAI-2. For the PAI-1 and PAI-2 inhibition studies, one-half of the PBST reaction mixture was assayed for uPA activity in the chromogenic substrate assay, while the other half was analyzed for uPA-PAI complex formation via casein underlay zymography. Recombinant ch-uPA produced by either the baculoviral expression system or the NS0 expression system behaved equivalently in the PAI-1 inhibition assays.

**Kinetics of Inhibition.** Second-order rate constants were measured under pseudo-first-order conditions. Briefly, en-

zyme and inhibitor were preincubated for periods of time varying from 0 to 90 min at 23°C. Following preincubation the mixtures were diluted in TB (0.1 M Tris, pH 8.1/0.1% Triton X-100), and the residual enzymatic activity was measured in an indirect chromogenic substrate assay. The data were fit to a two exponential model as described (29). The results presented are averaged from three separate experiments and are expressed as mean  $\pm$  SD.

**Casein Underlays.** Free uPA and uPA-PAI complexes were visualized using casein-plasminogen underlays as described (22).

**Preparation of ECM.** Chicken embryo fibroblasts were used to produce [<sup>35</sup>S]methionine-labeled ECMs as described (30).

**Degradation of <sup>35</sup>S-Labeled ECM.** Culture dishes (35 mm) containing radiolabeled ECM were washed twice with serum free growth medium before seeding cells. Transfected or untransfected NS0 cells ( $2 \times 10^6$  cells) were seeded onto the ECM in 2 ml of serum-free growth medium in the absence or presence of uPA inhibitors. Because native, human PAI-1 is relatively unstable at 37°C ( $t_{1/2}$  of 2 h), a recombinant mutant PAI-1 (31) that exhibits increased stability ( $t_{1/2}$  of 145 h) was used in the ECM degradation experiments. The cultures were incubated at 37°C, and at selected time intervals 200  $\mu$ l of the culture media were removed and centrifuged at 10,000 RPM for 2 min, and the supernatants were counted in a scintillation counter. Cell-mediated release of radioactivity into the culture supernatant has been shown previously to be a manifestation of proteolytic degradation of the ECM (30).

**Reagents.** Reagents for enzyme and inhibitor assays and tissue culture were obtained from the previously indicated sources (22). All oligonucleotides were from Operon Technologies (Alameda, CA). Lysine-Sepharose and benzamidine-Sepharose were from Pharmacia. Active, high-molecular-weight h-uPA, and recombinant human PAI-2 were from American Diagnostica (Greenwich, CT). Recombinant human PAI-1 was a kind gift from Robert Gerard (Katholieke Universiteit Leuven, Leuven, Belgium). PN-1 and C<sub>1</sub>-inhibitors were kindly provided by William Van Nostrand, and Sanford Simon (State University of New York, Stony Brook) generously provided the  $\alpha_1$ -PI. Recombinant, stable PAI-1 [mutant 14-1B (31)] was a kind gift from David Ginsburg (University of Michigan, Ann Arbor).

## RESULTS

**Construction of a ch-uPA Containing the RRHR Motif.** The amino acid sequence RRHR, located in the VR1 region on a surface loop of h-uPA, comprises the putative PAI binding site of uPA. This sequence of basic residues is absent in the corresponding region of ch-uPA and has been linked circumstantially with ch-uPA's resistance to inhibition by human PAI (22). This four-residue motif therefore was targeted for substitution into ch-uPA as shown in Fig. 1. An additional glycine was inserted along with RRHR to optimize homology of the mutant ch-uPA<sub>RRHR</sub> with h-uPA. The mutant and wt enzymes

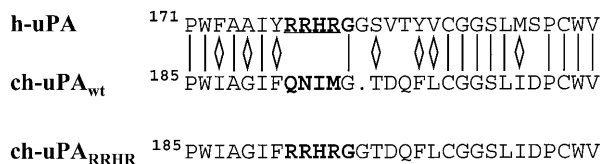


FIG. 1. Alignment of ch-uPA sequence with the putative PAI binding site of h-uPA. ch-uPA was aligned with Pro-171 in h-uPA (32). The positively charged amino acids of the putative PAI binding site in h-uPA (20) are underlined. The ch-uPA amino acid residues targeted for substitution are in boldface type. In the comparison of chicken and human uPA sequences, identical amino acids are indicated by a vertical line and conservative substitutions are indicated by a diamond.

were expressed in NS0 mouse myeloma cells and purified by benzamidine affinity and gel filtration chromatography. The catalytic activities of equivalent amounts of the purified mutant and wt enzymes, are identical as determined by a time- and dose-dependent activation of chicken plasminogen (Fig. 2A). In addition, sensitivity of ch-uPA<sub>wt</sub> and ch-uPA<sub>RRHR</sub> to the reversible, active site PA inhibitor, benzamidine, is nearly identical (Fig. 2B).

**PAI-1 Inhibition of ch-uPA Catalytic Activity and PAI-1-ch-uPA Complex Formation.** The mutant ch-uPA<sub>RRHR</sub> was examined for its sensitivity to the serpin PAI-1 and compared with wt ch-uPA and h-uPA. Equal amounts of catalytically active h-uPA, ch-uPA<sub>wt</sub>, and ch-uPA<sub>RRHR</sub> were incubated with increasing amounts of human recombinant PAI-1 (rPAI-1) and assayed for plasminogen activation in a coupled chromogenic substrate assay, and for SDS-stable complex formation. At a PAI/PA molar ratio of 0.5:1, 50% of the h-uPA activity was inhibited (Fig. 3A). The ch-uPA<sub>wt</sub> was relatively resistant to PAI-1 because a 25-fold molar excess had little effect on catalytic activity. However the ch-uPA<sub>RRHR</sub> mutant required only a 2-fold molar excess of rPAI-1 to yield 50% inhibition.

The interaction of PAI-1 with h-uPA results in the formation of equimolar, SDS-stable complexes, a characteristic of serpin interaction with target proteases (33). However, ch-uPA<sub>wt</sub>

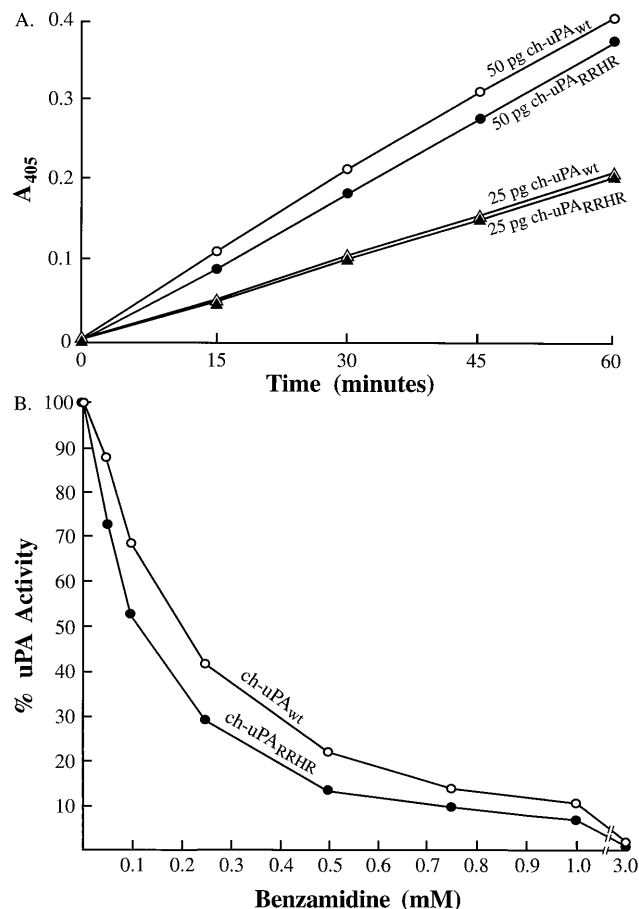


FIG. 2. Activation of chicken plasminogen by ch-uPA<sub>wt</sub> and ch-uPA<sub>RRHR</sub>, and sensitivity of ch-uPA<sub>wt</sub> and ch-uPA<sub>RRHR</sub> to benzamidine. (A) ch-uPA<sub>wt</sub> ( $\Delta$ , 25 pg;  $\circ$ , 50 pg) or ch-uPA<sub>RRHR</sub> ( $\blacktriangle$ , 25 pg;  $\bullet$ , 50 pg) was assayed for plasminogen activation in a coupled chromogenic substrate assay where the cleaved product is monitored at  $A_{405}$  (22). (B) Inhibition of chicken mutant uPA (ch-uPA<sub>RRHR</sub>) and chicken wt uPA (ch-uPA<sub>wt</sub>) by benzamidine. ch-uPA<sub>RRHR</sub> ( $\bullet$ , 50 pg), or ch-uPA<sub>wt</sub> ( $\circ$ , 50 pg) was incubated with varying concentrations of benzamidine and assayed for activity in a chromogenic substrate assay. The activity of ch-uPA in the absence of added benzamidine was assigned a value of 100%.

does not appear to form stable complexes with human PAI-1 (22). To test for complex formation of ch-uPA<sub>RRHR</sub> with PAI-1, increasing amounts of rPAI-1 were incubated with both wt and mutant ch-uPA and the mixtures were analyzed by casein underlay zymography (Fig. 3B and C). In the absence of rPAI-1, the lytic zone due to ch-uPA activity appears at  $\approx 40$  kDa (Fig. 3B and C, lane 1). With increasing concentrations of rPAI-1, the ch-uPA<sub>RRHR</sub> activity at 40 kDa is diminished and a corresponding activity at 80–85 kDa, the electrophoretic position of the uPA–PAI-1 complex, progressively increases (Fig. 3B, lanes 2–6). Even though uPA and PAI-1 form inactive complexes, uPA activity is seen in the underlays at 80–85 kDa because prolonged incubation of the uPA–PAI complex eventually leads to hydrolysis of the uPA acyl-intermediate of PAI, yielding active enzyme and an inactive PAI (34, 35). When 1 fmol of ch-uPA<sub>RRHR</sub> is incubated with 10 fmol of rPAI-1, all of the ch-uPA<sub>RRHR</sub> activity shifts up to the uPA–PAI-1 complex (Fig. 3B, lane 7). In contrast, ch-uPA<sub>wt</sub> under identical incubation conditions did not exhibit significant formation of uPA–PAI-1 complexes, even when 1000 fmol rPAI-1 was added (Fig. 3C, lane 7). Only when 5000 fmol rPAI-1 were added to ch-uPA<sub>wt</sub> were complexes formed (Fig. 3C, lane 8). Thus, the addition of the RRHR motif confers to uPA an  $\approx 1000$ -fold enhanced ability to form SDS-stable complexes with PAI-1. The extent of uPA–PAI-1 complex formation detected on casein underlays was confirmed by Western blot analysis, with anti-ch-uPA polyclonal antiserum. A lack of activity at 40–45 kDa on casein underlay zymography corresponded with a lack of immunoreactivity at 40–45 kDa on Western blots, indicating that all of the ch-uPA<sub>wt</sub> or ch-uPA<sub>RRHR</sub> had shifted into complex formation and that little or no catalytically inactive (non-PAI-1-reactive) recombinant ch-uPA was present in the preparations (data not shown).

The increase in susceptibility to PAI inhibition and SDS-stable complex formation of ch-uPA<sub>RRHR</sub> versus ch-uPA<sub>wt</sub> was analyzed further by measuring the second-order rate constant of PAI-1 inhibition. Table 1 shows that ch-uPA<sub>RRHR</sub> was inhibited by PAI-1 at a 671-fold higher association rate ( $k_1$ ) than ch-uPA<sub>wt</sub>. The introduction of the RRHR motif into ch-uPA thus confers a substantially increased rate of inhibition by PAI-1,  $3.02 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ , which is in the range of reported values for the rapid rate of PAI-1 inhibition of h-uPA (Table 1).

#### Reactivity of ch-uPA<sub>RRHR</sub> and ch-uPA<sub>wt</sub> with Other Serpins.

It was of interest to determine if the substitution of the RRHR motif into the VR1 region of ch-uPA also rendered the avian enzyme more sensitive to other serpins. PAI-2, a serpin that like PAI-1 reacts specifically with mammalian uPAs, was incubated with ch-uPA<sub>RRHR</sub> and ch-uPA<sub>wt</sub>. Formation of SDS-stable complexes between the uPAs and human rPAI-2 was monitored as described for uPA–PAI-1 complexes. At a 15- to 25-fold molar excess of rPAI-2 over ch-uPA<sub>RRHR</sub>, a 40–60% diminishment in the 40-kDa uPA activity was observed with a corresponding appearance of a 75- to 80-kDa complex. In contrast, ch-uPA<sub>wt</sub> was completely resistant to rPAI-2 up to a 250-fold molar excess (data not shown).

The mutant and wt enzymes were also incubated with PN-1, another serpin that inhibits uPA. As shown in Fig. 4, complex formation occurs to the same extent with both enzymes. Likewise, complex formation between ch-uPA<sub>wt</sub> and the serpins  $\alpha_1$ -PI, or C<sub>1</sub>-inhibitor required large excesses of the serpins but nevertheless were unaffected by introduction of the RRHR motif (data not shown). Thus the differential sensitivities observed when ch-uPA<sub>wt</sub> and ch-uPA<sub>RRHR</sub> are incubated with PAIs are not manifested when the enzymes are incubated with other serpins indicating that the RRHR motif is not a general serpin binding site.

**ECM Degradation by Cells Expressing Mutant or wt ch-uPA.** The participation of the RRHR motif in controlling a cellular phenotype was assessed by monitoring the effect of

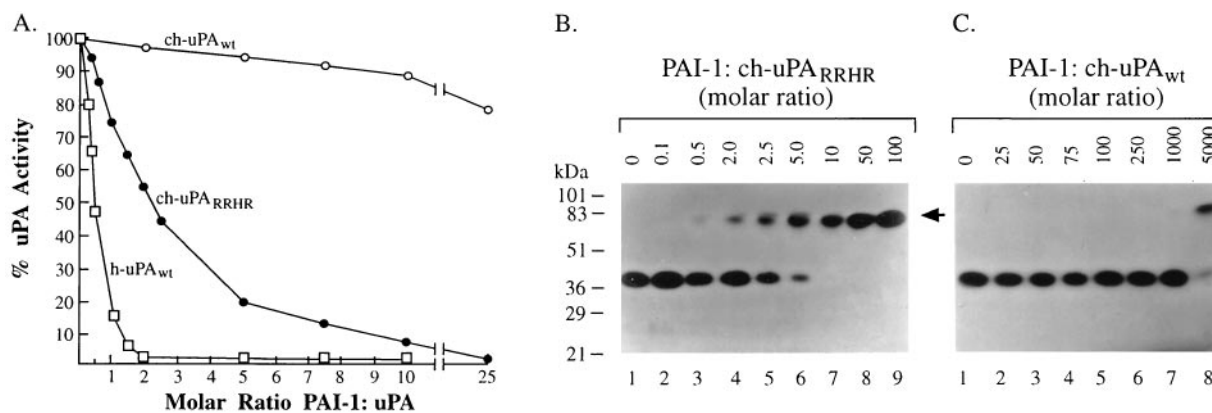


FIG. 3. The effect of PAI-1 on h-uPA, ch-uPA<sub>wt</sub>, and ch-uPA<sub>RRHR</sub> catalytic activity and electrophoretic mobility. ch-uPA<sub>RRHR</sub> (A and B, 50 pg), or ch-uPA<sub>wt</sub> (A and C, 50 pg) was incubated either without (B and C, lane 1), or with human rPAI-1 in the various molar amounts indicated and analyzed for residual uPA activity (A) and electrophoretic mobility by casein-plasminogen underlay (B and C). The underlay was incubated at 37°C for 3 days. The positions of prestained molecular weight markers are given in kDa on the left. The arrowhead indicates the position of the uPA-PAI-1 complexes.

PAI-1 on ECM degradation mediated by cells producing mutant or wt ch-uPA. Extensive matrix degradation was observed over a 48-h time course when transfected NS0 cells expressing recombinant wt ch-uPA (NS0-ch-uPA<sub>wt</sub>) or mutant ch-uPA (NS0-ch-uPA<sub>RRHR</sub>) were cultured on radiolabeled matrices (Fig. 5). Matrix breakdown by untransfected NS0 cells is 30–35% of that observed with NS0-ch-uPA<sub>wt</sub> or NS0-ch-uPA<sub>RRHR</sub>, indicating that the catalytic activity of ch-uPA is responsible for much of the degradation. In addition, NS0 cells producing an active site mutant of ch-uPA, which is catalytically inactive, degrade the matrix at a rate similar to that of untransfected NS0 cells (data not shown). These results are consistent with previous work which has shown that uPA is a major catalytic mediator of degradation of these matrices (30).

When NS0-ch-uPA<sub>RRHR</sub> cells are added to the labeled matrices in the presence of nanomolar levels (2–10 nM) of a recombinant stable PAI-1 (31), the extensive and progressive degradation of the ECM is inhibited in a dose-dependent manner to the levels seen with untransfected NS0 cells (Fig. 5A). In contrast, stable PAI-1 had no effect on the ability of NS0-ch-uPA<sub>wt</sub> cells to degrade ECM (Fig. 5B). Matrix degradation by these cells was extensive and progressive in either the absence or presence of up to 10 nM stable PAI-1, but was inhibited by the reversible uPA inhibitor, benzamidine. Thus, the differential sensitivity to PAI-1 observed *in vitro* with purified mutant and wt molecules can be recapitulated in a physiological, cellular setting, indicating that the presence of the RRHR motif on a uPA molecule can allow for PAI-1-mediated control of a cellular phenotype, namely ECM degradation.

## DISCUSSION

Several studies have implicated a sequence of basic residues in the VR1 surface loop of both tPA (17–19) and uPA (20) as the major, if not only, PAI-1 binding site on these two serine proteases. ch-uPA would appear to be an appropriate test

for demonstrating a requirement of the basic residue motif for PAI-1 reactivity and also for examining its possible physiological significance. ch-uPA is a potent activator of plasminogen (38), possesses the identical domain structure of all mammalian uPAs, (23, 39), and has been directly linked to the invasive phenotype (30, 38), but surprisingly its VR1 region, although positionally homologous to h-uPA, is completely devoid of basic residues (Fig. 1). Furthermore, ch-uPA appears to be refractory to mammalian PAI-1 and PAI-2 (22). Introduction into ch-uPA of the precise RRHR motif that is found in human, bovine, porcine, and ovine uPAs renders the resulting ch-uPA  $\approx 700$ -fold more rapidly inhibitable by PAI-1 (Table 1) and  $\approx 1000$ -fold more readily able to form SDS-stable uPA-PAI-1 complexes (Fig. 3B).

The chicken mutant uPA, ch-uPA<sub>RRHR</sub>, was similarly more sensitive to another PA-specific serpin, PAI-2, but exhibited little or no difference from wt ch-uPA in its reactivity to the serpins PN-1,  $\alpha_1$ -PI, and C<sub>1</sub>-inhibitor. The very low efficiency of ch-uPA inhibition and complex formation with PN-1 and the absence of any differential effect of PN-1 on ch-uPA<sub>RRHR</sub> and ch-uPA<sub>wt</sub> (Fig. 4) indicate that the RRHR motif may mediate

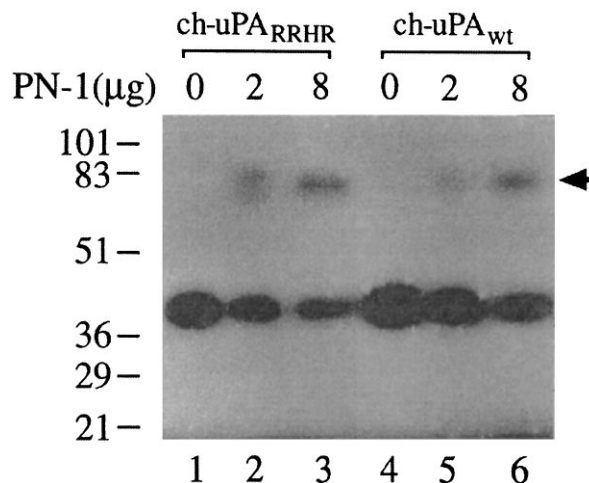


FIG. 4. The effect of PN-1 on the electrophoretic mobility of ch-uPA<sub>RRHR</sub>, and ch-uPA<sub>wt</sub>. Fifty picograms of ch-uPA<sub>RRHR</sub> or ch-uPA<sub>wt</sub> was incubated for 45 min at 37°C without (lanes 1 and 4, respectively) or with 2 or 8  $\mu$ g PN-1 (lanes 2 and 5 and lanes 3 and 6, respectively). After incubation, the samples were analyzed by zymography as in Fig. 3. The positions of prestained molecular weight markers are given in kDa on the left. The arrowhead indicates the position of uPA-PN-1 complexes.

Table 1. Second-order rate constant of PAI-1 inhibition of uPA

Enzyme	$k_1, M^{-1}\cdot s^{-1}$
ch-uPA <sub>wt</sub>	$4.50 \pm 1.81 \times 10^4$ (n = 3)
ch-uPA <sub>RRHR</sub>	$3.02 \pm 1.76 \times 10^7$ (n = 3)
h-uPA*	$1.8 \times 10^7$ to $1.6 \times 10^8$

\*h-uPA rate constant values were taken from references 36 and 37.

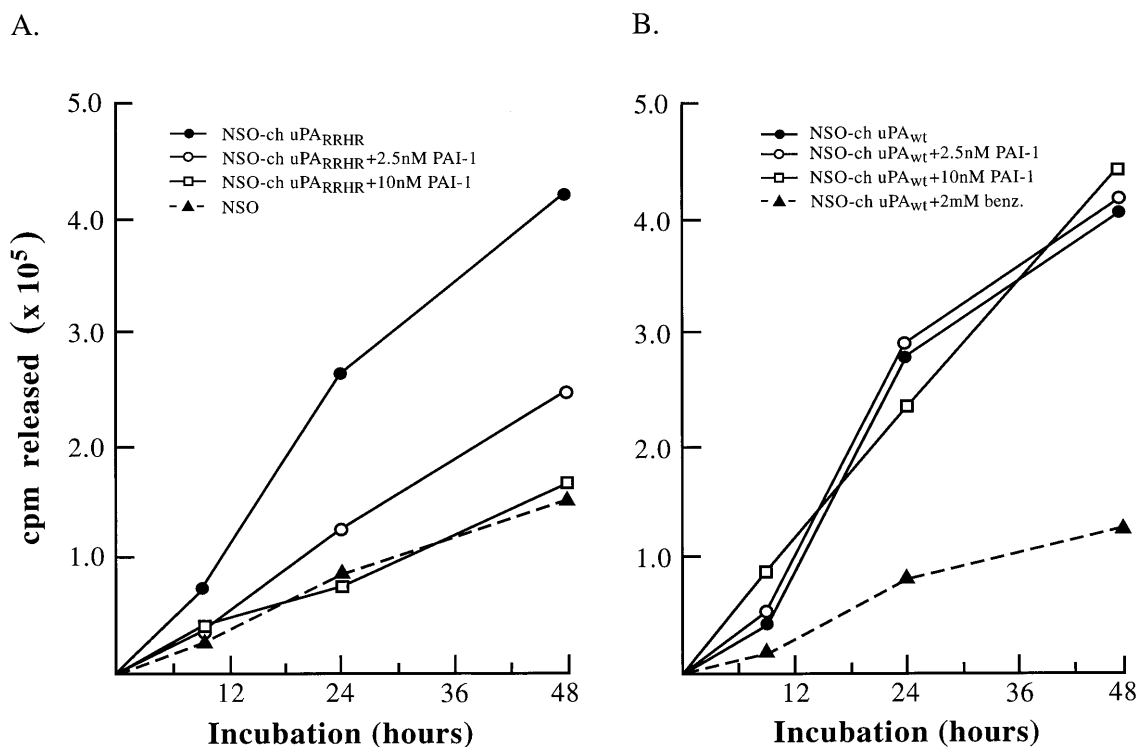


FIG. 5. Effect of stable PAI-1 on ECM degradation by transfected NS0 cells expressing ch-uPA<sub>RRHR</sub> and ch-uPA<sub>wt</sub>. Transfected or untransfected NS0 cells were seeded ( $2 \times 10^6$  cells/2 ml) onto <sup>35</sup>S-labeled ECM ( $9 \times 10^5$  cpm/35-mm dish) in the absence or presence of recombinant, stable PAI-1 (31), or benzamidine (benz.). The cultures were incubated at 37°C in serum-free growth medium and at the indicated times, 200  $\mu$ l of the medium was removed and centrifuged to remove cell debris, and the supernatant was counted. The total radioactivity released into the culture supernatant was calculated for each time point after subtracting the cpm released from the control dishes incubated in the absence of cells. The radioactivity released from the ECM in the absence of cells totaled 5–10% that in the presence of untransfected NS0 cells ( $0.04$ – $0.20 \times 10^5$  cpm/35-mm dish). (A) ECM degradation mediated by untransfected NS0 cells or NS0 cells transfected with and expressing the ch-uPA mutant (NS0-ch-uPA<sub>RRHR</sub>). Recombinant, stable PAI-1 was added to the cultures at the indicated concentrations. (B) Degradation of radiolabeled ECM by NS0 cells transfected with and expressing wt ch-uPA (NS0-ch-uPA<sub>wt</sub>). Recombinant stable PAI-1 or benzamidine was added to the cultures as indicated.

a specific electrostatic interaction only with PAI-1 and possibly PAI-2. It may be that the relative electrostatic and hydrophobic composition of the VR1 region of specific serine proteases is one of the evolutionary modes of selecting out those serpins that will function physiologically as highly specific and highly efficient inhibitors of a given serine protease.

Possibly the more physiologically significant gain of function that results from the mutational introduction of the RRHR motif into the VR1 region of ch-uPA is the ability of PAI-1 to modulate ECM degradation mediated by cells expressing and secreting catalytically active ch-uPA<sub>RRHR</sub>. It has been shown previously that when highly transformed chicken cells (RSV-CEF) are cultured on connective tissue matrix, they produce elevated levels of active, two-chain ch-uPA (30, 40). The uPA was shown to mediate almost complete degradation of the matrix either indirectly when plasminogen was present and plasmin was generated, or when plasminogen was not available, the active uPA directly cleaved specific ECM components such as fibronectin (38, 41). Even in the presence of serum, a rich source of serpins, the transformed RSVCEF cultures continued to degrade the matrix via a uPA-dependent mechanism (30). When the RRHR motif is introduced into ch-uPA, matrix degradation by cells expressing this mutant can now be controlled by the presence of nanomolar levels of PAI-1 in the system (Fig. 5). The reduction in ECM breakdown brought about by 10 nM PAI-1, compared with the lack of a PAI-1 effect on ch-uPA<sub>wt</sub>-expressing cells, indicates that the RRHR motif under these defined conditions can control cellular phenotype.

The absence of a PAI binding site in native ch-uPA raises the question of how the plasminogen activating and degradative

potential of ch-uPA is regulated in the different *in vivo* tissue remodeling and pathological situations where active uPA might be generated. Avian PAI homologues have not yet been described, and thus the precise negative regulatory mechanisms for ch-uPA are unknown. Perhaps ch-uPA, despite its many structural and catalytic similarities with uPAs from other species, is inhibited through a unique mechanism. It may be that specific cofactors are utilized that enhance the binding of avian serpins to uPA, substituting for the missing RRHR motif. It is interesting that thrombin, which also does not contain a prototypic basic residue motif in its VR1 region and does not react efficiently with PAI-1, is enhanced 100- to 200-fold in its reactivity to PAI-1 by the cofactors vitronectin and heparin (42, 43). It is also possible that avian serpins exist that interact with avian uPA via a different surface loop and through a different array of amino acid residues, but still culminating in the reactive center of the serpin inserting into the active site of the enzyme yielding complete inhibition. The exact mechanism of how ch-uPA is regulated awaits the isolation and identification of specific avian uPA inhibitors.

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