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Separation and Characterization of Nonphosphorylated and Serine-phosphorylated Urokinase

CATALYTIC PROPERTIES AND SENSITIVITY TO PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1*

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Urokinase synthesized by human A431 epidermoid carcinoma cells is phosphorylated on serine (Mastronicola, M. R., Stoppelli, M. P., Migliaccio, A., Auricchio, F., and Blasi, F. (1990) *FEBS Lett.* 266, 109–114). To test the possibility that phosphorylation may have specific effects on urokinase function, the phosphorylated and nonphosphorylated forms of urokinase were separated by Fe^{3+}-Sepharose chromatography. Both forms exhibit indistinguishable K_m and k_{cat} for plasminogen activation. On the other hand, their sensitivity toward the specific plasminogen activator inhibitor type 1 is different as assessed by measuring both the stability of the covalent complex and the residual enzymatic activity. Phosphorylated urokinase was 50% inhibited at a concentration of plasminogen activator inhibitor type 1 4-fold higher than nonphosphorylated urokinase (0.7 *versus* 0.15 nM). Furthermore about 10% of phosphorylated urokinase was resistant to plasminogen activator inhibitor type 1 at a concentration as high as 20 nM. Thus, phosphorylation affects urokinase sensitivity to plasminogen activator inhibitor type 1, therefore resulting in a net, although indirect, increase of urokinase activity. These results suggest the existence of a novel cellular regulatory mechanism of extracellular proteolysis.

Urokinase plasminogen activator (uPA)[1] regulates plasmin-dependent extracellular proteolysis, a process required for cell migration, invasiveness, and fibrinolysis (Reich, 1978; Danø *et al.*, 1985; Blasi *et al.*, 1987).

It has been shown that some cells are equipped with a surface receptor (Blasi, 1988) that allows surface activation of plasminogen to plasmin, via a surface-uPA activity cycle, leading to a strong amplification of the extracellular proteolytic activity (Stephens *et al.*, 1989; Ellis *et al.*, 1989; Cubellis *et al.*, 1990; Blasi *et al.*, 1991). This process is controlled by specific inhibitors of uPA; among these, plasminogen activator inhibitor type 1 (PAI-1), which binds the active two-chain uPA but not the zymogen (Andreasen *et al.*, 1986), is also able to bind uPA when this is receptor-bound, inhibiting its activity and causing its internalization and degradation (Cubellis *et al.*, 1989, 1990; Estreicher *et al.*, 1990; Jensen *et al.*, 1990).

We have reported previously (Mastronicola *et al.*, 1990) that prourokinase (pro-uPA) synthesized by human A431 and HT1080 cells is phosphorylated on serine in at least two sites. In order to assess whether phosphorylation has unique effects on the properties of pro-uPA, we have separated unphosphorylated (Ser-uPA) and phosphorylated (Ser(P)-uPA) uPA synthesized by A431 cells and compared their catalytic activities as well as their sensitivity to PAI-1. We now show that Ser(P)-uPA has a decreased PAI-1 sensitivity, although its enzymatic activity is indistinguishable from that of Ser-uPA.

EXPERIMENTAL PROCEDURES

Materials—Active PAI-1 was purified from HepG2 cells (Wun *et al.*, 1989). The anti-uPA monoclonal antibody 5B4 was described previously (Nolli *et al.*, 1986). Purified recombinant human pro-uPA from *Escherichia coli* was prepared at Farmitalia Carlo Erba, Milan, Italy. Fe^{3+}-Sepharose was obtained from Pharmacia and handled according to instruction from the manufacturer. Plasminogen was purified from human plasma as described previously (Deutsch and Mertz, 1970). Plasminogen-Sepharose was prepared by coupling 1.8 mg of plasminogen to 0.5 g of cyanogen bromide-activated Sepharose (Pharmacia) as described by the manufacturer. S-2390 (H-D-valyl-L-phenylalanyl-L-lysine-*p*-nitroanilide dihydrochloride) and S-2444 (L-pyroglutamyl-glycyl-L-arginine-*p*-nitroanilide hydrochloride) were obtained from Kabi-Vitrum (Sweden). Enlightning was from New England Nuclear. IMUBIND-5-uPA ELISA kit was from American Diagnostica Inc. Geneticin (G418) was from GIBCO.

Cell Culture and Labeling Conditions—A431-P1 cells were obtained by transfecting human epidermoid carcinoma cells A431 (Fabricant *et al.*, 1977) with pRSV-uPA DNA, a plasmid containing the human uPA gene driven by the Rous sarcoma virus promoter re-constructed according to our previous description (Nolli *et al.*, 1989). Cotransfection with pRSVneo (Gorman *et al.*, 1982) was used for selection, and clones were selected in G418 (0.8 mg/ml)-containing medium. Clone A431-P1 was found to produce about 10 fold higher levels of pro-uPA than the parental A431 cells (data not shown) and was chosen for further study. Cells were grown in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine

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[1] The abbreviations used are: uPA, urokinase-type plasminogen activator; pro-uPA, prourokinase type plasminogen activator; PAI-1, plasminogen activator inhibitor type 1; Ser(P)-uPA, serine-phosphorylated uPA; Ser-uPA, serine nonphosphorylated uPA; DMEM, Dulbecco's modified minimal essential medium; S-2390, H-D-valyl-L-phenylalanyl-L-lysine-p-nitroanilide dihydrochloride; S-2444 L-pyroglutamyl-glycyl-L-arginine-*p*-nitroanilide hydrochloride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

serum in a 10% CO_2 atmosphere. To metabolically label A431 cells, cells were seeded at a density of 2×10^6/10-cm dish and grown for 24 h in 10 ml of DMEM with 10% fetal bovine serum. After 24 h, the culture medium was removed and replaced with either methionine-free or phosphate-free DMEM containing 5% dialyzed fetal bovine serum. After 8 h, the starvation medium was further replaced with labeling medium (2.5 ml of methionine-free DMEM containing 400 μCi of [^{35}S]methionine or phosphate-free DMEM containing 600 μCi of [^{32}P]orthophosphate) and labeling allowed to proceed for 16–18 h.

Adsorption and Elution of Labeled Conditioned Medium on Fe^{3+}-chelated Sepharose—A previously published protocol was employed (Andersson and Porath, 1986; Michel and Bennet, 1987) with minor modifications. Briefly, 5–100 ml of ^{35}S or ^{32}P conditioned medium of A431-P1 cells was adjusted to pH 3.0 with acetic acid and incubated with 1.0–20.0 ml of Fe^{3+}-chelated Sepharose (1:1, v:v, suspension in 0.1 M acetic acid, pH 3.0, presaturated with 2 mg/ml bovine serum albumin). After a 45-min incubation at room temperature, the sample was centrifuged at 12,000 × *g* for 1 min and the supernatant removed. The pelleted Sepharose was then washed with 0.5 M sodium acetate, pH 3, 0.01% Tween 20, incubated at room temperature for 10 min, and centrifuged. The resulting supernatant is the pH 3 eluted material. This procedure was repeated with the following buffers: 0.5 M sodium acetate buffer at pH 4.0, 5.0, 5.5, 6.0, and 6.5, 1% ammonium acetate buffer, pH 7.0 and 8.0, all containing 0.01% Tween 20. Phosphorylated proteins were then eluted with 200 mM potassium phosphate buffer, pH 8, 0.5 M NaCl, and the gel finally washed with 0.1 M EDTA, 0.05 M Tris-HCl buffer, pH 7.5, 0.5 M NaCl for re-utilization.

Following immunoaffinity purification (see next paragraph), phosphorylated uPA was chromatographed a second time through Fe^{3+}-Sepharose for further purification and eluted with phosphate buffer as described.

In one experiment, 150 μg of *E. coli*-made recombinant pro-uPA was added to excess of Fe^{3+}-chelated Sepharose as described above. In this case, the amount of pro-uPA in each fraction was quantitated by protein determination (Bio-Rad).

Affinity Purification of Pro-uPA—^{32}P- and ^{35}S-labeled pro-uPAs were purified by immunoaffinity chromatography of the pooled supernatants from Fe^{3+}-Sepharose chromatography according to a previously described procedure (Nolli *et al.*, 1986; Stoppelli *et al.*, 1986). This single-step purification method yields electrophoretically pure radioactive uPA and pro-uPA, although the product contains minor amounts of nonradioactive impurities like bovine serum albumin. Before the immunoaffinity purification of pro-uPA, each supernatant was neutralized when required and incubated with the 5B4 monoclonal antibody bound to agarose in the presence of 50 mM potassium phosphate, 0.5 M NaCl, 0.01% Tween 20 buffer as described (Stoppelli *et al.*, 1986). The immunoprecipitates were then analyzed by 12.5% polyacrylamide gel electrophoresis under reducing conditions followed by autoradiography (Laemmli, 1970). The ^{35}S-containing polyacrylamide gels were subjected to fluorography with Enlightning before drying and exposure.

Activation of Pro-uPA and Determination of the uPA Enzymatic Activity—For all experiments, a solid-state activation procedure was employed. Immunoaffinity purified labeled pro-uPA (about 2 mg of protein, equivalent to 200,000 cpm) was incubated for 2 h at room temperature in the presence of 75 ml of plasminogen-Sepharose (corresponding to about 100 mg of plasminogen) in 10 mM Hepes buffer, pH 7.5, and the reaction products were then analyzed by SDS-PAGE. The use of plasminogen rather than plasmin allows a more reproducible and gentle activation of pro-uPA, probably started by traces of contaminating uPA or plasmin.

The uPA content of each preparation was determined by both measuring its enzymatic activity and its immunoreactivity using a preparation of recombinant activated pro-uPA as a standard.

The enzymatic activity of uPA was measured with 0.3 mM of the plasmin substrate S-2390, in the presence of 1.0 μM human plasminogen, using a 50 mM Tris-HCl buffer, pH 7.5. Alternatively the direct substrate S-2444 (0.6 mM) was employed in the presence of 25 μg/ml aprotinin in 50 mM Tris-HCl, pH 8.8, 38 mM NaCl, 0.01% Tween 20. Both reactions were performed at 25 °C and quantitated by measuring the absorbance at 400 nm.

Amounts of Fe^{3+}-Sepharose-purified Ser-uPA and Ser(P)-uPA exhibiting the same enzymatic activity were also quantitated by a commercial enzyme immunoassay (see "Materials").

Formation of uPA·PAI-1 Complexes—Plasmin-activated ^{35}S-labeled u-PA was incubated with different concentrations of PAI-1 to produce a sodium dodecyl sulfate-stable complex under standard conditions (Andreasen *et al.*, 1986). The relative concentrations of uPA and PAI-1 are specified in the figure legends. The samples were incubated at room temperature for 1 h and fractionated on a SDS-polyacrylamide (12.5%) gel under reducing conditions to assess the extent of SDS-stable complex formation. The results were quantitated by scanning the autoradiographic film with a Beckman DU-25 spectrophotometer.

To assess residual uPA enzymatic activity in the presence of different concentrations of PAI-1, after the formation of the uPA·PAI-1 complex, the reaction mixture was diluted to 500 μl and the uPA activity measured under the conditions described in the previous paragraph.

RESULTS AND DISCUSSION

Preliminary experiments suggested that phosphorylated pro-uPA isolated from A431 cells, when activated to two-chain uPA, is hampered in its interaction with PAI-1 (data not shown). To better investigate this phenomenon, we have attempted to separate phosphorylated from nonphosphory-

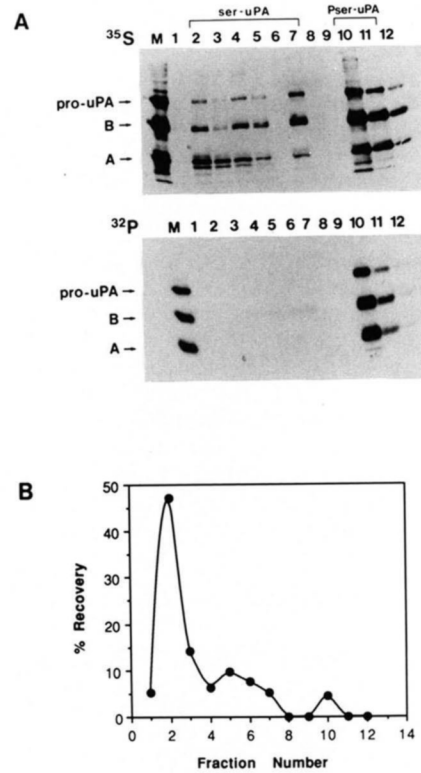

FIG. 1. **Adsorption and elution of pro-uPA on Fe^{3+}-chelated Sepharose.** *Panel A*, 5 ml of conditioned medium from ^{35}S- or ^{32}P-labeled human A431-P1 cells was adjusted to pH 3.0 with acetic acid, incubated with Fe^{3+}-chelated Sepharose, and washed as described under "Experimental Procedures." The resulting fractions were immunoaffinity-purified with 5B4 anti-uPA antibody and analyzed by 12.5% polyacrylamide SDS-gel electrophoresis under reducing conditions followed by autoradiography. *Lane M* shows the unfractionated uPA purified from 1 ml of A431-P1-labeled conditioned medium. *Lane 1* shows the nonretained material. Lanes 2–7 show the eluates in acetate buffers at pH 3.0 (*lane 2*), pH 4.0 (*lane 3*), pH 5.0 (*lane 4*), pH 5.5 (*lane 5*), pH 6.0 (*lane 6*), and 6.5 (*lane 7*). *Lane 8* shows the supernatant of the wash with 1% ammonium acetate, pH 7.0. *Lane 9* shows the supernatant of the 1% ammonium acetate wash, pH 8.0. *Lanes 10* and *11*, two sequential elution steps with 0.2 M potassium phosphate, 0.5 M NaCl, pH 8.0. *Lane 12* is the supernatant of the final wash with 0.05 M Tris, 0.1 M EDTA, 0.5 M NaCl, pH 7.5. The bands labeled *A* and *B* correspond to the low and high molecular weight subunits of two-chain uPA, respectively. *Panel B*, elution of purified activated recombinant pro-uPA made in *E. coli* on Fe^{3+}-chelated Sepharose. Conditions and buffers as for *panel A*. The content of pro-uPA was estimated by the $OD_{280\,nm}$ and is expressed in percent of the total pro-uPA applied to the column.

TABLE I

Kinetic parameters of phosphorylated and nonphosphorylated uPA

Plasminogen-Sepharose-activated, unfractionated, and ser- and Pser-uPA were quantitated and assayed in the presence of different concentrations of plasminogen as described under "Experimental Procedures." K_m and V_{max} values were estimated from double-reciprocal plots. The values reported represent the average of three determinations with the standard deviation.

	K_m	V_{max}	k_{cat}
	μM	$\mu mol\ min^{-1}\ mg^{-1}$	s^{-1}
Unfractionated	0.91 ± 0.08	15.28 ± 1.73	12.73 ± 1.5
Ser-uPA	1.00 ± 0.07	14.83 ± 1.58	12.36 ± 1.4
Pser-uPA	0.92 ± 0.11	16.86 ± 0.38	14.05 ± 0.2

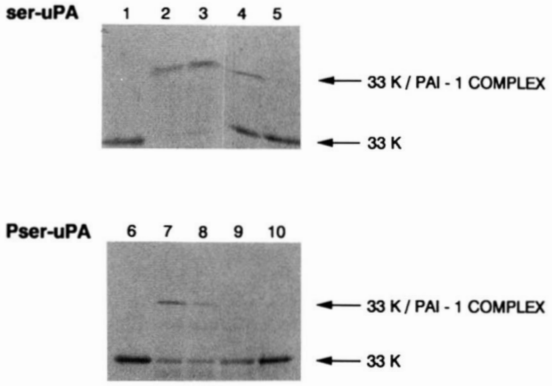

FIG. 2. **Formation of an SDS-stable complex between Ser(P)- and Ser-uPA and PAI-1 at different PAI-1:uPA ratios.** ^{35}S-Labeled Ser(P)- and Ser-uPA were purified, activated, and employed in each reaction with PAI-1 at a concentration of 2 nM. The samples were then fractionated by SDS-PAGE under reducing conditions as described under "Experimental Procedures." The PAI-1:uPA molar ratio was 0 (*lanes 1* and *6*), 20 (*lanes 2* and 7), 4 (*lanes 3* and *8*), 2 (*lanes 4* and *9*), and 0.4 (*lanes 5* and *10*)

TABLE II

Quantitation of the SDS-stable uPA·PAI-1 complex formed with Ser- and Pser-uPA

These data report the extent of 33-KDa uPA B chain complexed with PAI-1 (complex) in Ser- and Pser-uPA and expressed as percent of the radioactivity recovered in the 33-kDa band in the absence of PAI-1. The numbers were obtained by scanning the autoradiography shown in Fig. 2. The percent recovered refers to the total amount of radioactivity estimated to be on the gel following the reaction with PAI-1. The concentration of PAI-1 is expressed in molar ratio to uPA (PAI-1:uPA).

PAI-1:uPA	uPA	% in 33 kDa	% in complex	% recovered
0	Ser-uPA	100	0	100
0.4	Ser-uPA	100	5	105
2	Ser-uPA	75	22.5	105
4	Ser-uPA	18	53	82.6
20	Ser-uPA	3	52	58
0	Pser-uPA	100	0	100
0.4	Pser-uPA	93.3	0	99.2
2	Pser-uPA	65.6	13	99.1
4	Pser-uPA	47	8.2	92.6
20	Pser-uPA	35	35	87

lated uPA with Fe^{3+}-Sepharose, a matrix already used to isolate phosphorylated peptides and proteins (Andersson and Porath, 1986; Michel and Bennet, 1987). To this goal we have used the uPA-overexpressing cell line, A431-P1, obtained by stably transfecting the A431 cell line with a recombinant human uPA gene driven by a constitutive viral promoter. Immunoprecipitation of the ^{35}S- and ^{32}P- conditioned medium from A431-P1 cells with anti-uPA antibody yielded reproducibly ~10-fold more uPA than the parental cells, showing that in these cells phosphorylation is not limiting in pro-uPA synthesis (data not shown). To test the reliability of the Fe^{3+}-Sepharose chromatography, the ^{32}P- or the ^{35}S-labeled conditioned media from A431-P1 were adsorbed to the matrix and batch-eluted at different pH values as described under "Experimental Procedures." The 12.5% SDS-polyacrylamide gel analysis under reducing conditions of the immunoprecipitated samples shows that ^{35}S- and ^{32}P-labeled purified pro-uPA preparations (in both cases containing about 50% of two-chain uPA as shown by the presence of the 33- and 17-kDa bands) have a different elution pattern (Fig. 1). The ^{35}S-labeled pro-uPA/uPA preparation binds to the matrix and can be partially eluted with buffers in the pH range 3–7 and partially (about 60% of the applied radioactivity) by potassium phosphate at pH 8.0 (*lanes 10–11* in *panel A*, Fig. 1). On the other hand, the ^{32}P-labeled pro-uPA/uPA was almost exclusively eluted by the potassium phosphate buffer at pH 8.0 (*lanes 10* and *11*). This result shows that the majority of the phosphorylated pro-uPA/uPA specifically interacts with the matrix and can only be eluted by competition with potassium phosphate. In addition, it also shows that nonphosphorylated pro-uPA (*i.e.* not labeled with ^{32}P) was eluted in fractions 1–7 (Fig. 1). In agreement with these results, in a separate experiment we have observed that in an unlabeled pro-uPA/uPA preparation purified from the same cells, and subjected to the same treatment, phosphoserine residues were present only in the potassium phosphate eluate (data not shown). Finally, to assess the extent of nonphosphorylated pro-uPA contaminating the Fe^{3+}-Sepharose retained fraction, we chromatographed *E. coli*-made recombinant human pro-uPA on Fe^{3+}-Sepharose. We found that only 4% of this material was retained by the $Fe3^{+}$-Sepharose (Fig.1, *panel B*). Overall these results both indicate that Fe^{3+}Sepharose chromatography is a reliable technique for separating Ser-uPA from Ser(P)-uPA (Fig. 1, *panel A*) and that in A431-P1 conditioned media, a large proportion of uPA is in a phosphorylated form.

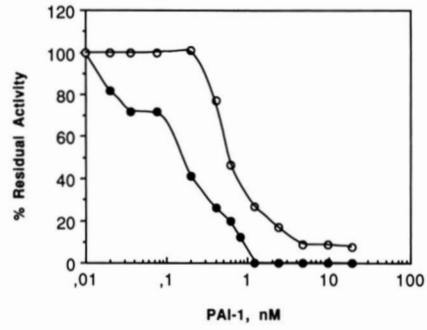

FIG. 3. **Inhibition of Ser(P)-uPA and Ser-uPA plasminogen activation by PAI-1.** Ser(P)-uPA and Ser-uPA at a concentration of 0.4 nM were incubated with increasing concentrations of PAI-1 (0.01–20 nM) for 1 h at 25 °C and the residual enzymatic activity assayed at 1.0 μM plasminogen, as described under "Experimental Procedures." Residual activity is expressed as percent of the activity measured in the absence of PAI-1. The results represent the average of triplicate samples with less than 10% deviation.

In order to compare Ser(P)-uPA and Ser-uPA with respect to the enzymatic activity and PAI-1 sensitivity, [^{35}S]methionine-labeled conditioned medium from A431-P1 cells was fractionated by the above procedure and fractions containing Ser-uPA and Ser(P)-uPA collected and immunoaffinity-purified with anti-uPA-agarose (see "Experimental Procedures"). The resulting samples were analyzed by SDS-PAGE after complete conversion of pro-uPA to two-chain uPA with

plasminogen-Sepharose. The uPA content of each sample was assessed by quantitating the enzymatic activity in the presence of plasminogen and S-2390 (see "Experimental Procedures"). In a separate experiment, two Ser-uPA and Ser(P)-uPA preparations exhibiting the same enzymatic activity were compared using a commercial ELISA kit (see "Experimental Procedures"). In these samples the ratio of the concentration values obtained by enzymatic activity to the concentration values measured by the ELISA assay ranged from 0.95 to 1.29 for unfractionated, from 0.75 to 0.9 for Ser-uPA, and 0.9 to 0.78 for Ser(P)-uPA (not shown).

The enzymatic activity and the PAI-1 sensitivity of the thus quantitated Ser-uPA and Ser(P)-uPA were then analyzed.

Double-reciprocal plots at different plasminogen concentrations gave almost identical estimates of K_m, V_{max}, and k_{cat} for the unfractionated enzyme, Ser(P)-uPA and Ser-uPA (K_m, 0.91–1.0 μM; V_{max}, 14.83–16.86 μmol min^{-1} mg^{-1}; k_{cat}, 12.36–14.05 s^{-1}; see Table I). Using S-2444 as a substrate for direct uPA activity, again no difference in V_{max} nor K_m was observed (not shown). We conclude, therefore, that phosphorylation does not in itself affect the activity of uPA.

To assess PAI-1 sensitivity, the ability of activated Ser(P)-uPA and Ser-uPA to form an SDS-stable complex at different PAI-1:uPA ratios was examined. Equal amounts of uPA were incubated with increasing amounts of PAI-1, and complex formation was analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 2, an uPA·PAI-1 complex is observed with both Ser-uPA and Ser(P)-uPA; however, in the latter case, this occurs at higher PAI-1:uPA ratios. Since the formation of a uPA·PAI-1 complex is associated with the disappearance of free uPA, we have measured the ^{35}S radioactivity contained in the uPA·PAI-1 complex and expressed as percent of the radioactivity recovered in the 33-kDa band in the absence of PAI-1. As shown in Table II, the amount of Ser-uPA·PAI-1 complex is higher than the amount of Ser(P)-uPA·PAI-1 complex at all PAI-1:uPA ratios.

The data also show a relevant loss of radioactivity at the maximum PAI-1 concentration used (40 nM).

As reported in Table II, the extent of radioactivity loss is higher in the Ser-uPA (58% recovery at a 20:1 ratio) than in the Ser(P)-uPA (87% recovery at the 20:1 ratio)-containing samples. Although the nature of the loss has not been investigated, we can speculate that it may be due to some degradation of labeled uPA. However, the selective disappearance of the uPA·PAI-1 complex band in the Ser(P)-uPA-containing samples shows that phosphorylation reduces the stability of the uPA·PAI-1 complex.

A decreased reactivity of Ser(P)-uPA toward PAI-1 was also confirmed by measuring uPA residual enzymatic activity at different PAI-1 concentrations. As shown in Fig. 3, the amount of residual plasminogen-activating activity was reproducibly higher for Ser(P)-uPA than for Ser-uPA (IC_{50}, 0.7 and 0.15 nM, respectively). Also, the amount of PAI-1 required for complete abolition of uPA activity of Ser(P)-uPA was at least 20-fold higher (20 nM) than for Ser-uPA (1 nM). As expected, unfractionated uPA gave intermediate results showing that it is a combination of Ser-uPA and Ser(P)-uPA (not shown). When the residual uPA activity was tested with the substrate S-2444, an about 3-fold difference in IC_{50} for Ser(P)- and Ser-uPA was detected (10 nM *versus* 28 nM, respectively).

In conclusion, phosphorylated uPA appears to be impaired in its interaction with PAI-1, whereas its activity is not significantly modified.

Based on the elution profile of ^{35}S-uPA (Fig. 1), the enzyme synthesized by A431 carcinoma cells appears to be a (about 60:40) mixture of phosphorylated and nonphosphorylated molecules. In addition, Ser(P)-uPA may be heterogeneous, containing a small fraction (10%) of highly PAI-1-resistant material. Since serine phosphorylation of uPA occurs at at least two sites (Mastronicola *et al.*, 1990), it is possible that different phosphorylation sites affect PAI-1 resistance in a different way. This remains to be determined.

The effect described in this paper suggests that phosphorylation may indirectly regulate the activity of cellular uPA, introducing a novel regulatory mechanism in extracellular proteolysis. In view of the observed direct role of PAI-1 in inhibition, internalization, and degradation of receptor-bound u-PA (Cubellis *et al.*, 1989, 1990; Jensen *et al.*, 1990), phosphorylation of u-PA may therefore result in longer lasting surface proteolytic activity.

Resistance of the fully active phosphorylated uPA to PAI-1 suggests that in addition to the active site at least another site is involved in the formation of the uPA·PAI-1 complex. This finding might be exploited in the production of novel, *i.e.* PAI-1-resistant, thrombolytic derivatives of pro-uPA based on the above described mechanism with higher *in vivo* activity and possibly longer half-life.

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