Serine phosphorylation of biosynthetic pro-urokinase from human tumor cells

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Phosphorylation is a potent mechanism regulating the activity of many intracellular enzymes. We have discovered that the product of the human urokinase plasminogen activator gene, pro-uPA, is phosphorylated in serine in at least two human cell lines. Phosphorylation occurs within the cell during biosynthesis, and phosphorylated intracellular pro-uPA is secreted into the medium. Of the secreted pro-uPA molecules, 20–50% are phosphorylated in serine, thus representing a meaningful fraction of the total biosynthetic pro-uPA. Although the sites of phosphorylation have not yet been determined, at least two such sites must exist; in fact plasmin cleavage of phosphorylated single chain pro-uPA yields a two chain uPA in which both chains are phosphorylated. A specific function for pro-uPA phosphorylation has not yet been identified; however, it is tempting to speculate that, as in many other cases, phosphorylation may affect the activity of the enzyme, its response to inhibitors or the conversion of pro-uPA zymogen to active two-chain uPA. This would represent an additional way of regulating extracellular proteolysis, an important pathway involved in both intra- and extravascular phenomena like fibrinolysis, cell migration and invasiveness.

Plasminogen activator; Phosphoserine; Metastasis; Secretion

1. INTRODUCTION

Plasminogen activation (PA) is required intravascularly for digesting fibrin clots, and extravascularly for regulating the interactions between cell surfaces and the protein components of the extracellular matrix and of the basement membrane. The product of the PA reaction, plasmin, can degrade not only fibrin, but also proteins of the extracellular matrix and basement membrane, like proteoglycans, fibronectin, laminin and some collagens; in addition, it is also able to activate latent collagenases [1,2]. Two plasminogen activating enzymes have been described, urokinase (uPA) and tissue plasminogen activator. Specific inhibitors and receptors for both plasminogen activators and plasminogen [3–6] add to the complexity of the plasminogen activation system.

uPa is secreted as a 411 amino acids proenzyme [7–9] (pro-uPA), which can be bound to surface receptors (uPAR) and here be activated by a single proteolytic cleavage [10–12]. In the malignant A431 cell line, all uPAR sites are occupied in an autocrine way by uPA ligand produced by the same cells [13]. Similar results have been reported for a variety of uPA-producing human tumor cells [4]. This property has been suggested to represent a distinctive advantage for migrating or invading cells, providing them with the tools to modify their connections with the surrounding cells and tissues, i.e. through proteolytic modification of the extracellular matrix and basement membrane [1,4,13]. An important support to this view comes from the demonstration of a specific localization of uPA in the focal contacts and in the areas of cell-to-cell contact [14–16]. In addition to high affinity receptors for uPA, cells have a large number of low affinity binding sites for plasminogen [6]. This leads to surface localization of both enzyme and substrate (uPA and plasminogen), and hence to the formation of surface-bound plasmin [16,17]. The surface location of plasmin-directed proteolysis appears to have at least two advantages: receptor-bound single-chain pro-uPA is activated to two-chain uPA at a faster rate than in solution [18] and surface-bound plasmin is resistant to α-2 antiplasmin [6,17]. Receptor-bound activated uPA, on the other hand, is still accessible to inhibition by the specific type-1 inhibitor, at lest in U937 cells [19,34] and inhibition is followed by internalization [34–36].

The regulation of surface uPA, however, is only marginally understood. Synthesis of all components of the plasminogen activating system appears to undergo strict regulation suggesting the existence of multiple levels of control [20]. Other mechanisms affecting the interaction between uPA and its receptor have also been described [21,22].

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Protein phosphorylation is the best-known post-translational mechanism [23] regulating proteins' interactions and functions. In an investigation designed to understand whether phosphorylation occurs in the uPA system, we have discovered that biosynthetic pro-uPA synthesized and secreted from at least two human cell lines is phosphorylated in serine.

2. MATERIALS AND METHODS

2.1. *Materials*

Bovine serum albumin, aprotinin, benzamidine, phenyl-methyl-sulfonyl-fluoride (PMSF), sodium ortho-vanadate and plasmin were purchased from Sigma. Affigel used for antibody coupling and electrophoresis reagents were from BioRad. [^{35}S]Methionine, [^{32}P]-orthophosphate and ^{14}C molecular weight protein markers were obtained from Amersham. Enlightning was from New England Nuclear.

Human high molecular weight uPA (120 000 U/mg) and the monoclonal antibody 5B4, specific for the A chain of human uPA, have been described [12,24]. They were kind gifts of Dr M.L. Nolli and F. Parenti (Lepetit SpA).

Cell culture medium (Dulbecco's modified Eagle's medium, DMEM), fetal bovine serum, dialysed serum, methionine-free and phosphate-free DMEM, glutamine, were from Gibco.

2.2. *Cell culture and labeling*

A431 human epidermoid carcinoma cells [25] were obtained from Ira Pastan. HT1080, human fibrosarcoma cells [15], were obtained from Keld Danø. Both cell lines were grown in DMEM supplemented with 10% fetal bovine serum (FBS) in a 10% CO_2 atmosphere.

Cells were seeded at a density of 1.5×10^6 in a 10 cm plastic dish and grown for 24 h in 10 ml DMEM with 10% FBS. After 24 h, the medium was aspirated and substituted with either methionine-free or phosphate-free DMEM containing 5% dialysed FBS. After 6 h starvation, this medium was further substituted either with 2 ml methionine-free DMEM containing 400 μCi of [^{35}S]methionine, or with 2 ml of phosphate-free, serum-free DMEM, containing 600 μCi [^{32}P]orthophosphate. Unless stated otherwise, cells were labeled for 18 h.

2.3. *Acid wash and cell lysates*

Labeled cells were acid-washed as previously described [13] and washed with phosphate buffered saline. The cell pellet was lysed by vortexing them for 30 s in lysis buffer (20 mM Hepes, pH 7.5, 1% Triton X-100, 10% glycerol) (0.5 ml lysis buffer/10 cm plate), centrifuging the extract at 10 000 rpm at 4°C for 30 min. Both acid washes and cell lysates were stored frozen until use.

2.4. *Immunoprecipitation*

Media and acid washes were supplemented with one third of their volume of 0.2 M potassium phosphate buffer, pH 7.0, containing 2.0 M NaCl and 0.4% Triton X-100. The volumes used for each immunoprecipitation were 0.3 ml for the ^{35}S-labeled or 1.0 ml for the ^{32}P-labeled conditioned medium and 0.5–2.0 ml for the cell lysate.

Immunoprecipitation was carried out with agarose-bound monoclonal antibody 5B4 as previously described using as control glycine-blocked agarose [13]. The immunoprecipitates were supplemented with 20 μg/ml bovine serum albumin and precipitated with 20% trichloroacetic acid, the pellet washed with ether and acetone, suspended in two-fold concentrated Laemmli buffer with β-mercaptoethanol, boiled for 5 min and analysed by SDS-polyacrylamide (12.5%) gel electrophoresis [26]. Similarly treated ^{14}C-molecular weight markers were run alongside. ^{32}P-gels were directly dried under vacuum while ^{35}S-gels were fixed in 25% methanol-10% acetic acid, embedded in Enlightning (NEN) and dried. The dried gels were exposed to Kodak X-OMAT X-ray films.

2.5. *Plasmin activation of pro-uPA*

The pellet resulting from the TCA precipitation of the eluted immunoprecipitates was resuspended in 30 μl of 0.1 M Hepes buffer, pH 7.4, in the presence of adequate concentrations of previously titrated plasmin and incubated for 30 min at 37°C. At the end of the incubation, 30 μl of two-fold concentrated Laemmli buffer [26] were added, and the sample was boiled and analysed by 12.5% SDS-PAGE (see above).

2.6. *Phosphoamino acid separation*

In order to determine the nature of the phosphorylated amino acid, the medium of 5 ^{32}P-labeled A431 cell dishes was pooled, immunoprecipitated [13] and analysed by SDS-PAGE as described above. The 47 kDa pro-uPA band was cut out and boiled in 1 ml of 1% SDS for 15 min, homogenized and centrifuged at 4500 rpm for 15 min. The supernatant was collected, supplemented with 5 vols of cold acetone, incubated at −15°C for 30 min, centrifuged again and the pellet dried. The protein pellet was washed with a 1:1 ether-ethanol mixture and suspended in 1 ml of 6 N HCl. The samples were hydrolysed at 110°C for 90 min, diluted with water and lyophilized overnight. The dried samples were taken up in 20 μl water containing 2 mg/ml each of phosphoserine, phosphothreonine and phosphotyrosine, and subjected to monodimensional thin layer electrophoresis at 1000 V at 4°C on Macherey-Nagel 100 μm thin layer plates, in acetic acid/pyridine water (50:5:945), pH 2.5, for 1 h. After the run, the cellulose plates were dried, stained with ninhydrin and exposed to a Kodak X-OMAT X-ray film to detect ^{32}P-amino acids.

2.7. *Pulse-chase experiments*

For these experiments, PMA-treated (50 ng/ml) A431 cells were used, since they produce about 10 fold more pro-uPA (27). Cells were starved and labeled with ^{32}P orthophosphate or [^{35}S]methionine. The cells were washed twice with phosphate-free, methionine-free DMEM and subjected to two sets of acid washes (see above). Two of each group of dishes were supplemented with lysis buffer as described above and the resulting lysates were frozen. Serum-free DMEM (2 ml) containing unlabeled phosphate were added to each of the remaining plates which were further incubated at 37°C. The cells were then acid-washed again and the cell pellets lysed (see above). Both cell lysate and incubation supernatants were immunoprecipitated and analysed by SDS-PAGE (see above).

3. RESULTS

3.1. *Secreted pro-uPA is phosphorylated in serine*

Pro-uPA secreted from A431 cells can be biosynthetically labeled when the cells are incubated in phosphate-free medium and [^{32}P]orthophosphate (Fig. 1). This is not peculiar of A431 cells and can be observed also with fibrosarcoma HT1080 cells. In both cell lines, metabolically labeled with either ^{32}P or ^{35}S the same 47 kDa band is specifically immunoprecipitated with anti-u-PA monoclonal antibody. Its migration corresponds to the M_r of pro-uPA secreted from ^{35}S methionine-labeled A431 cells [13]. Thus pro-uPA secreted from at least two tumor human cell lines is phosphorylated.

We next analysed whether pro-uPA is phosphorylated at a single or at multiple sites. Media from ^{32}P-labeled A431 cells were immunoprecipitated with anti-uPA antibodies, the precipitate treated with plasmin which, under controlled conditions, cleaves pro-uPA in two fragments (A and B chains) of about 30 and 17 kDa [13], and analysed by SDS-PAGE under reducing con-

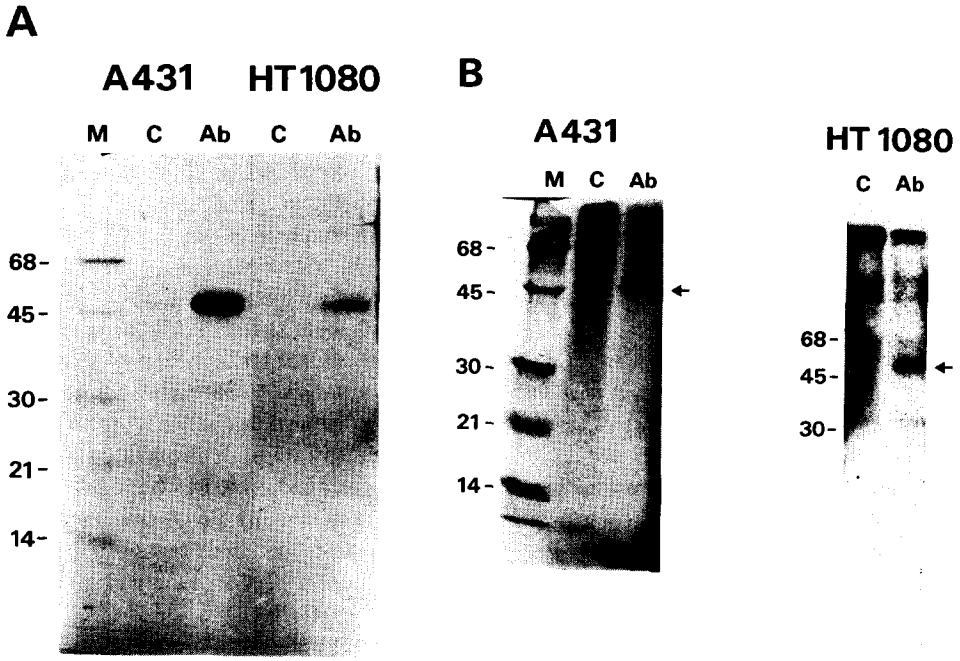

Fig. 1. The pro-uPA secreted by A431 cells is phosphorylated. Immunoprecipitation of ^{35}S- (part A) and ^{32}P-labeled (part B) A431 medium using 5B4 monoclonal antibody coupled to affigel (lane Ab). In lanes C medium was precipitated with glycine-blocked agarose. Lane M: molecular weight markers.

ditions. As shown in Fig. 2A, two bands of the expected size are labeled, indicating that phosphorylated residues are present on both the amino- and the carboxy-terminal moieties of pro-uPA.

In order to identify the type of phosphorylated amino acid in pro-uPA, ^{32}P-labeled A431 medium was immunoprecipitated, run in an SDS-PAGE, the band cut out, eluted and subjected to acid hydrolysis and

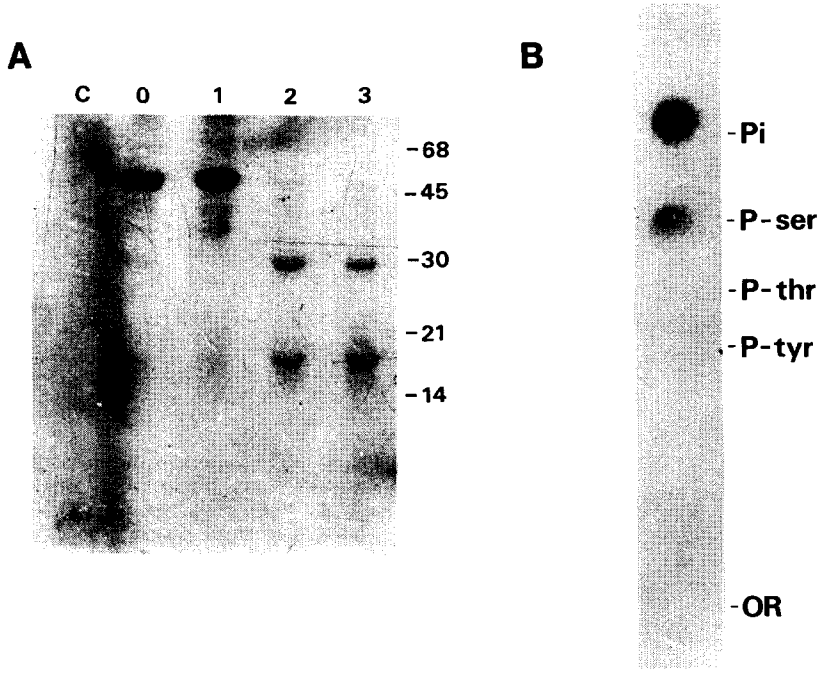

Fig. 2. (A) Limited digestion of the immuno-precipitated ^{32}P-pro-uPA with plasmin. Duplicate immunoprecipitates from the labeled medium are treated (lanes 2, 3) or not (lanes 0, 1) with plasmin (see section 2). C, control immunoprecipitate in which labeled medium was incubated with glycine-blocked agarose. (B) Determination of the phosphorylated amino acid in ^{32}P-pro-uPA. The immunoprecipitated 32P medium was subjected to acid hydrolysis (see section 2) and analysed by high voltage electrophoresis on thin layer plates, in the presence of unlabeled phosphoamino acid markers.

phosphoamino acid analysis. As shown in Fig. 2B, a radioactive spot migrates with the phosphoserine marker. The experiment has been repeated several times on different pro-u-PA preparations with essentially the same result. Thus pro-u-PA is phosphorylated in serine. Amino acid analysis of biosynthetic pro-uPA also showed that phosphoserine was the only phosphoamino acid. Considering two phosphorylation sites per molecule, this analysis allowed us to estimate that between 20 and 50% of the pro-uPA molecules are phosphorylated (data not shown).

3.2. *Phosphorylation of pro-uPA occurs intracellularly*

To exclude that phosphorylation of pro-uPA occurs within or in contact with the culture medium, we added exogenous unlabeled two-chain uPA to the medium of A431 cells. The use of two-chain uPA allows us to distinguish it from the endogenous single-chain pro-uPA by SDS-PAGE under reducing conditions. In no case labeling of the exogenous two-chain uPA was observed (Fig. 3). A fainter band of about 30 kDa, probably the B chain of uPA, is present in the immunoprecipitates independent of the addition of exogenous uPA. It probably represents degradation of biosynthetic pro-uPA, an event often observed in conditioned media.

We then investigated whether phosphorylated pro-uPA could be detected within the cells. Since A431 cells have receptor-bound pro-uPA which can be dissociated by an acid wash [13], we acid-washed ^{32}P-labeled A431 cells and immunoprecipitated the lysate of the acid-washed cells. In both ^{35}S- and ^{32}P-labeled cells a 47 kDa pro-uPA band was observed, indicating that the intracellular pro-uPA is also phosphorylated (data not shown, but see Fig. 4, lane 3). These data concord with the idea that phosphorylation of pro-uPA occurs within the cells, prior to secretion.

The amount of pro-uPA produced in culture is in general very low even in A431 cells which are among the best producers and this poses a problem in the immunoprecipitation of cell lysates requiring large numbers of cells and of radioactive phosphate. However, pro-uPA synthesis in A431 cells can be increased several-fold by treatment with the tumor promoter phorbol-12-myristate-13-acetate (PMA) [27]. PMA treated A431 cells secrete more pro-uPA than controls cells, but the PMA treatment does not modify the fraction of phosphorylated pro-uPA, nor the ratio of phosphorylation between the A and B chains (data not shown). Using PMA treatment of A431 cells all the results described before could be reproduced and the experimental protocol simplified by the higher production of pro-uPA.

To prove that phosphorylation occurs intracellularly, pulse-chase experiments have been carried out. PMA-treated A431 cells have been starved for phosphate overnight and labeled for 7 h with ^{32}P, the cells were acid-washed to dissociate any bound pro-uPA and incubated for 16 h in fresh medium containing unlabeled phosphate. As shown in Fig. 4 (left panel), at the end of

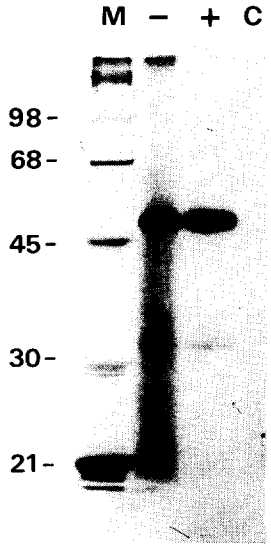

Fig. 3. Exogenous two-chain uPA is not phosphorylated in A431 cell medium. Immunoprecipitation of the ^{32}P-labeled medium of A431 cells to which no (lane –) or 7.5 μg of unlabeled two-chain uPA (lane +) had been added during the labeling period. C, control immunoprecipitate (see section 2); M, molecular weight markers.

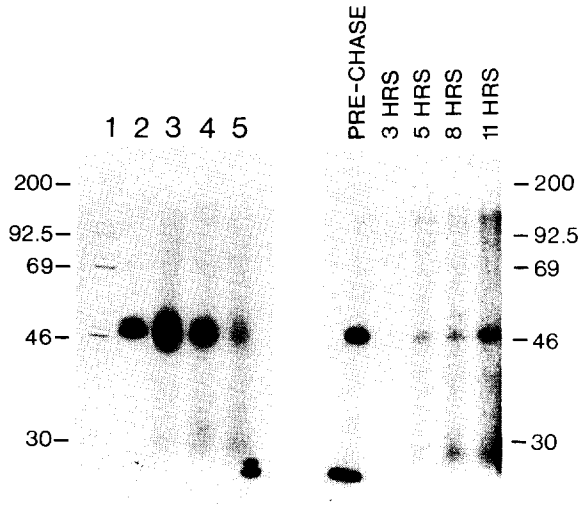

Fig. 4. Pulse-chase experiment of pro-uPA phosphorylation and secretion in PMA-treated A431 cells. (Left) PMA-treated A431 cells were pulsed for 7 h with ^{32}P orthophosphate, acid washed and chased with normal DMEM for 16 h. Lane 1, molecular weight markers; 2, immunoprecipitation from the 7 h labeling medium; 3, immunoprecipitation of the 7 h labeled acid washed cells lysate; 4, immunoprecipitation from the medium after 16 h of chase; 5, immunoprecipitation of the acid-washed cell lysate after 16 h chase (see section 2). (Right) PMA-treated A431 cells were labeled for 18 h with ^{32}P orthophosphate, the labeling medium substituted with normal DMEM and the cells chased for 3, 5, 8 and 11 h. At each time-point the medium was immunoprecipitated (see section 2). The lane 'pre-chase' shows immunoprecipitation of the medium at the end of the 18 h labeling period.

the labeling period both medium and acid-washed cells contain phosphorylated pro-uPA (lanes 2, 3). After 16 h chase, phosphorylated pro-uPA appears in medium and decreases in the cell lysate (lanes 4, 5). Thus the phosphorylated pro-uPA moves from the cells into the medium.

A time course of the secretion was carried out: PMA-treated A431 cells pre-labeled with ^{32}P for 18 h were acid washed and incubated into fresh serum-free and phosphate-free medium, and the medium analysed for phosphorylated pro-uPA at different time points. As shown in Fig. 4 (right panel) no pro-uPA is visible after 3 h, but the 47 kDa band appears after 5 h and increases its intensity thereafter. The time course of secretion is not appreciably slower than that of ^{35}S-labeled PMA-treated A431 cells.

In conclusion, intracellular phosphorylated pro-uPA moves from cells to medium and thus represents phosphorylation of pro-uPA during its biosynthesis and before secretion; however, we cannot exclude that part of it does not derive from the phosphorylation of internalized extracellular pro-uPA.

4. DISCUSSION

In this paper we have shown that the product of the human uPA gene, pro-uPA, is phosphorylated in serine in human tumor cell lines. Serine phosphorylation occurs intracellularly, and the phosphate group is conserved in the secreted form of pro-uPA. The actual phosphorylated serine residues have not yet been identified: however, our preliminary data show that at least two phosphorylation sites exist, as both the A and B plasmin-generated chains are phosphorylated. Between 20 and 50% of the secreted pro-uPA molecules are phosphorylated representing a considerable fraction of the pro-uPA output of human A431 cells. The identity of the pro-uPA phosphorylating enzyme is at present not known. Sequences resembling substrates for different protein kinases are present in pro-uPA. The chemical identification of the phosphorylated residues is now being pursued.

The potential regulatory importance of phosphorylation is not known. Phosphorylation may modify the general conformation of the enzyme by adding acid charges and hence change the mutual positions of particular amino acid residues. In the case of pro-uPA, one can imagine several possibilities: (i) Phosphorylation may affect the charge-relay system of the active site; the substrate (plasminogen) binding region, which again would affect enzymatic activity; the inhibitors binding region, which might affect binding of two-chain uPA to PAI-1 or PAI-2. We have no data at the moment on this point. The presence of one phosphorylation site in the B chain (Fig. 2) is consistent with such possibilities. (ii) Phosphorylation of residues at or near the activation region might lead to a modifcation of the rate of single-chain to two-chain conversion. At least three cleavage points have been observed in pro-uPA synthesized by in vitro cultured cells. One or more such breaks can occur on the same pro-uPA molecule. The first type of cleavage (Lys-158) generates the active two-chain uPA [28,29]. The second type, at Lys-135, liberates at low M_r uPA or pro-uPA (the carboxy terminus) and an amino terminal fragment (residues 1–135). The former has all the enzymatic activity, the latter can bind the receptor with high affinity [11]. The third type (thrombin-catalysed) produces a non-activatable low M_r pro-uPA [30]. The presence of phosphorylated residues also on the A chain is consistent with phosphorylation regulating pro-uPA processing. Our data show that phosphorylated pro-uPA can be cleaved by plasmin, but more precise concentration dependence studies will have to be carried out. (iii) Phosphorylation of residues in the growth factor domain might affect the ability of pro-uPA to bind the receptor. The presence of a phosphorylated residue on the A chain is consistent with this possibility, as the receptor-binding domain is located on the A chain of uPA [11].

An accurate analysis of all these possibilities will require separation of phosphorylated from nonphosphorylated pro-uPA molecules. We are currently investigating this possibility.

If the receptor has a direct role in pro-uPA phosphorylation, one would assume that pro-uPA should associate with the receptor within the cell; however, the only interaction between pro-uPA and receptor to be described is an autocrine one following secretion of pro-uPA from A431 cells [13]. In addition, the little available information on the structure of the uPA receptor does not support the possibility that the receptor itself has protein kinase activity. The uPA receptor is a heavily glycosylated protein of 313 amino acid residues with little if any intracytoplasmic domain with no sequence homology to known protein kinases [31]. Thus in conclusion, binding to the uPA receptor may not be involved directly in pro-uPA phosphorylation.

Protein phosphorylation is a general regulatory mechanism for the activity of enzymes [32]. Hormones and growth factors utilize intracellular and/or membrane receptors to transduce regulatory signals that profoundly affect cell metabolism and cell fate. Phosphorylation and dephosphorylation of specific amino acid residues in target proteins, catalyzed by specific protein kinases and protein phosphatases, has a major role in these pathways [32,33]. The activity of these enzymes is often regulated by metabolic signals, like Ca^{2+}, cyclic AMP, diacylglycerol. Therefore, a further alternative regulation of extracellular proteolysis might be achieved by regulating the state of phosphorylation and possibly the activity of the plasmin-forming enzyme. Plasmin, and hence plasminogen activators, are important elements in a variety of physiological, pathological and therapeutical

conditions like fibrinolysis, cellular migration and invasiveness [1,2]. If phosphorylation represents a way of regulating uPA activity, it will not only provide a novel mechanism of regulation of extracellular proteolysis, but will also open new paths in devising novel types of drugs to control as different disease states as thrombosis and cancer metastasis.

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