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Phosphorylation of human plasminogen activators and plasminogen

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Abstract Plasminogen (PG), urokinase-type plasminogen activator (u-PA) and tissue-type PA (t-PA) are the main molecules involved in fibrinolysis and in many other physiological and pathological processes. In the present study we report that human t-PA, purified from human melanoma cells, and PG, purified from human plasma, both contain P-Tyr residues, as revealed by immunoblotting analyses with monoclonal anti-P-Tyr antibodies. In addition HPLC amino acid analysis of acid-hydrolyzed t-PA, PG and u-PA, shows that: (i) P-Ser and P-Tyr residues are present in t-PA; (ii) P-Thr and P-Tyr are present in PG; (iii) P-Ser, P-Thr and P-Tyr are present in u-PA. The utilization of monoclonal anti-P-Ser and anti-P-Thr antibodies in immunoblotting experiments has confirmed these data which indicate that phosphorylation is a common feature of PAs and of PG.

Key words: Human tissue-type plasminogen activator; Urokinase-type plasminogen activator; Human plasminogen; Phosphorylation

1. Introduction

Plasminogen (PG), tissue-type plasminogen activator (t-PA) and urokinase-type PA (u-PA) are involved in the plasmin-generating system, one of the physiological proteolytic machineries utilized by tissues and cells to generate localized and directional proteolysis [1,2,3]. Activation of Glu-plasminogen (791 amino acids) to the active enzyme [4] occurs through cleavage of Arg^{560}–Val^{561} peptide bond, near the carboxyl-terminal end of 'kringle' 5 (K5), by PAs. u-PA and t-PA are serine proteases [3,5] secreted as single chain proteins (sct-PA and scu-PA) which are cleaved by plasmin giving rise to two chain t-PA and to two chain u-PA respectively (tct-PA, tcu-PA) [5,6]. PG, t-PA and u-PA structures have in common 'kringle' modules [7], specialized for protein binding, and the carboxyl-terminal region, homologous to the catalytic domain of other serine-proteases, such as prothrombin and trypsin. In addition, PG and t-PA interact with fibrin [8], with fibronectin and its degradation products [9,10] and t-PA is able to promote transformation in vitro [11]. Recently much attention has been given to the phosphorylation of u-PA which might confer specific biological properties to the enzyme [12–17]. This paper reports evidences, obtained by immunoblotting and HPLC amino acid analysis, of the phosphorylation of Ser and Tyr residues in t-PA, of Thr and Tyr residues in PG and of Ser, Thr and Tyr residues in u-PA.

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Abbreviations: t-PA/u-PA, tissue-type/urokinase-type plasminogen activator; PG, plasminogen; SDS-PAGE, sodium dodecyl sulphate-polyacrilamide gel electrophoresis; HPLC, high pressure liquid chromatography; MoAb, monoclonal antibodies; AP, alkaline phosphatase; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate.

2. Materials and methods

2.1. Materials

Human sct-PA (EC 3.4.21) with specific activity of 634,000 IU/mg, from cultured human melanoma cells, and human Glu-PG from pooled human plasma were purchased from Biopool (Umea, Sweden); human tcu-PA (EC 3.4.21.31) with specific activity of 92,719 IU/mg was purchased from Serva (Heidelberg, Germany) and human recombinant t-PA (rt-PA, 0.58 MIU/mg), produced in animal cells, was purchased from Boehringer Ingelheim. The purity of t-PA, u-PA and PG was ascertained by electrophoretic mobility, enzymatic assays and selective inhibition studies.

O-Phospho-DL-serine, *O*-phospho-DL-threonine, *O*-phospho-DL-tyrosine, low molecular weight markers and the monoclonal anti-P-Ser and anti-P-Thr antibodies (MoAb) [18], provided as mouse ascites fluids, were from Sigma (St. Louis, MO, USA).

Goat anti-human t-PA IgGs were from Biopool (Umea, Sweden); rabbit anti-human PG was from Dako (Denmark). The rabbit polyclonal affinity-purified anti-P-Tyr IgG were kindly provided by Dr. Wang (San Diego, La Jolla, CA, USA) [19] and were also purchased from UBI (NY, USA); the anti-P-Tyr MoAb were obtained culturing the 1G2 hybridoma cells [20] obtained from ATCC (USA) with the permission of Dr. Frackelton (Providence, RI, USA); the f-42 monoclonal antibodies against the N-terminal end of human plasma fibronectin were obtained as previously described [21]. Alkaline phosphatase (AP)-conjugated rabbit anti-goat, goat anti-rabbit and anti-mouse IgG, were from Promega (W 153711, USA), as well as the Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrates.

Acetonitrile, triethylamine (TEA), phenylisothiocyanate and amino acid standards (Pierce H) were from Waters Assoc., while Sequanol grade hydrochloric acid was from Pierce (Rockville, IL, USA).

2.2. NaDodSo4-polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium-dodecyl-sulphate (SDS), was performed according to Laemmli [22] using vertical slab gels. The acrylamide concentrations were 3.3% in the stacking gel and 8% in the running one. The samples were diluted in Laemmli's sample buffer in the ratio of 1:1 (v/v).

2.3. Zymographic procedure and immunoblotting of proteins

Zymography of proteins was carried out by a procedure developed by Colombi et al. [23]. The proteins were separated by 8% PAGE in the presence of SDS under non-reducing conditions and electrotransferred on nitrocellulose sheets (Scleicher & Schüll, Germany) according to Laemmli and Towbin, respectively [22,24]. After transfer, the filters were overlaid onto casein plates containing 2 μg/ml of human plasminogen. The lysis bands, developed after 24 h of incubation at 37°C in humidified atmosphere, were photographed using dark-field light. In immunoblotting experiments, after transfer and saturation, the filters were incubated overnight at room temperature with the first antibody which, depending on the experiments, were: goat anti-human t-PA or rabbit anti-human PG (1:500, v/v), polyclonal affinity-purified anti-P-Tyr antibodies (0.3 μg/ml), 1G2 monoclonal anti-P-Tyr antibodies (1:25, v/v), monoclonal anti-P-Ser or anti-P-Thr antibodies (1:10,000, 1:100, v/v, respectively), f-42 monoclonal anti-FN antibodies (5 μg/ml). After washing, the filters were incubated with AP-conjugated rabbit anti-goat IgG or goat anti-rabbit IgG (1:1,000, v/v) or goat anti-mouse IgG (1:7,500, v/v) and the positive bands evidenced with NBT and BCIP substrates [25].

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2.4. Acid hydrolysis of proteins and derivatization of amino acids with PITC

Four sample tubes (6 × 50 mm), each containing 50 or 100 μg of t-PA, PG or u-PA, were placed in a reaction vial and vacuum-dried using the PICO-TAG Work Station [26]. 200 μl of concentrated HCl (37%) and one phenol crystal was added at the bottom of the reaction vial; this reaction vial was purged alternately with nitrogen and after final evacuation to remove O_2, its valve was closed, and the reaction vial itself heated in Work Station oven at 106°C for 7 h, subsequently cooled and vacuum-dried to remove excess HCl. 20 μl of the redry solution (ethanol–water–triethylamine TEA, 2:2:1 v/v) were added to each sample tube, which was then vacuum-dried. 10 μl of the derivatizing solution (ethanol/water/triethylamine/phenylisothiocyanate: 7:1:1:1, v/v) were added to each sample and the tubes were placed in the reaction vial and let stand at room temperature for 10 min; each sample was dried under vacuum and dissolved in 200 μl of sample diluent [27].

2.5. HPLC analyses

The acid-hydrolyzed preparations of amino acids and of the proteins (t-PA or PG or u-PA) after derivation were submitted to HPLC amino acid analysis. Separation of the amino acids was achieved using a Waters PICO-TAG reverse phase column (free amino acid analysis column, 3.9 mm × 300 mm, cod. 10950 Waters, two pumps Mod. 510, Waters, programmed gradient Mod. 680) and a method (developed in Waters' Chicago laboratory) which allows to separate the phospho-amino acids by reducing the concentration of acetonitrile in the initial buffer. The eluents utilized were, eluent A (140 mM NaOAc, pH 6.4; 0.5 ml TEA/l) and eluent B (60% acetonitrile in water).

3. Results

3.1. Tyrosine phosphorylation of purified t-PA and PG

Aliquots of t-PA, from human melanoma cells, or of PG, from human plasma (Fig. 1A,B: from lanes 1–8) were electrophoresed in 8% SDS-PAGE under non-reducing conditions. After electrotransfer on nitrocellulose filters, portions of the filters were amido black-stained (lanes 1), immunoreacted with polyclonal anti-t-PA (Fig. 1A, lane 2) or anti-PG antibodies (Fig. 1B, lane 2), with monoclonal anti-P-Tyr antibodies in the absence (lanes 3) or in the presence of 20 mM P-Tyr (lanes 4) or 20 mM P-Ser, P-Thr (lanes 6 and 7, respectively) and with the secondary AP-conjugated goat anti-mouse antibodies as control (lanes 4). Zymographic analysis of t-PA is reported in lane 8.

In the case of t-PA, the 70 kDa molecule evidenced by amido black and by anti-t-PA (Fig. 1A, lanes 1 and 2, respectively) was also recognized by anti-P-Tyr MoAb (Fig. 1A, lane 3).

Similarly, for PG, the 90 kDa stained protein band reacted with anti-PG antibodies and with anti-P-Tyr MoAb. In both cases, the immunological recognition by anti-P-Tyr antibodies was inhibited by the addition of 20 mM P-Tyr, but not by 20 mM of P-Ser or P-Thr (lanes 5, 6 and 7). In addition to anti-P-Tyr MoAb, also polyclonal anti-P-Tyr antibodies were used in immunoblotting experiments, with similar results, as it has been reported for Tyr-phosphorylation of u-PA [13]. It has also been verified that phospho-amino acids did not interfere with immunological recognition of t-PA, PG and u-PA with other antibodies (not shown).

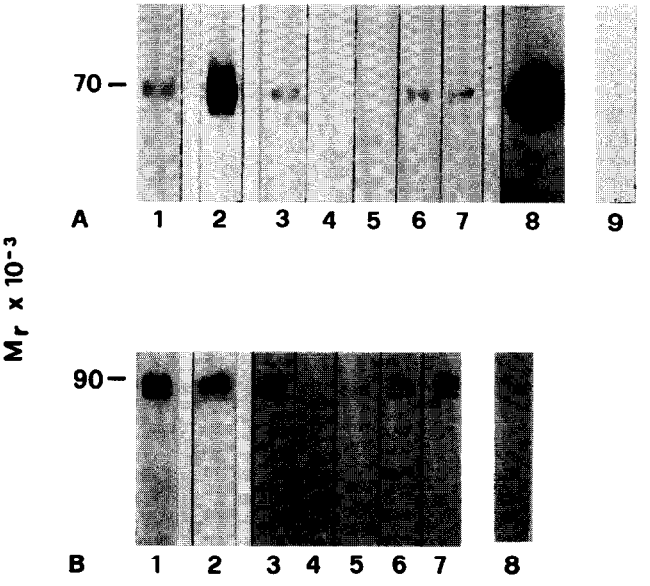

Fig. 1. Tyrosine phosphorylation of purified human t-PA and PG. Aliquots of t-PA, purified from cultured human melanoma cells and of PG, purified from human plasma, were electrophoresed and transferred on nitrocellulose filters (panels A and B, respectively). The single lanes were separated, stained with Amido black (lanes 1), incubated with polyclonal anti-t-PA or anti-PG antibodies (lanes 2, A and B, respectively), with monoclonal anti-P-Tyr antibodies in the absence (lanes 3, A and B) or in the presence of 20 mM P-Tyr (lanes 5, A and B) or 20 mM P-Ser, P-Thr (lanes 6 and 7, respectively, A and B); lanes 4 (A and B) refer to the t-PA and to the PG incubated only with AP-conjugated goat anti-mouse antibodies; lane 8 (panel A) refers to the zymographic analysis of t-PA; lanes 9 and 8 (A and B) refer to t-PA and PG incubated in the presence of f-42 anti-FN MoAb. Aliquots of 0.3 μg of t-PA were loaded in lanes 2 and 8 and of 5 μg in lanes 1, 3, 4, 5, 6, 7 and 9 (panel A). Aliquots of 0.3 and 5 μg of PG were loaded in tracks 2 and 1, 3, 5, 6, 7, 8, respectively (panel B).

3.2. HPLC based phospho-amino acid analysis of t-PA and PG

Fig. 2 (panels A and B) reports the relevant portions of the HPLC chromatograms of the amino acid standards before and after hydrolysis, respectively. As reported in Table 1 the recovery of phospho-amino acids after acid hydrolysis was: 37.1% for P-Ser, 89.1% for P-Thr and 13.8% for P-Tyr. Fig. 3 reports the analysis of the acid hydrolysis of 10 μg of each protein; panel A, B and C refer to t-PA (143 pmol), PG (111 pmol) and u-PA (182 pmol), respectively. The analysis revealed the presence of P-Tyr residues in t-PA, PG and u-PA, of P-Ser residues in both PAs and of P-Thr residues in PG and u-PA. The

Table 1
Comparison of the amino acid standards analyzed in HPLC

		Retention time (min)	Peak area	Loaded (pmol)	Recovered	
					pmol	%
Amino acids	P-Ser	3.24	252,212	500	500	100
	P-Thr	4.37	370,422	500	500	100
	P-Tyr	5.82	398,651	500	500	100
Acid hydrolyzed amino acids	P-Ser	3.39	187,295	1000	371	37.1
	P-Thr	4.35	660,124	1000	891	89.1
	P-Tyr	5.83	110,705	1000	138	13.8

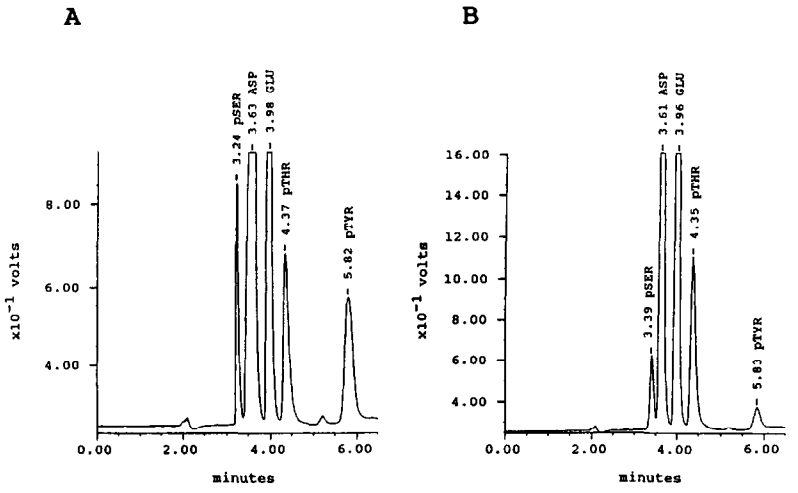

Fig. 2. HPLC analysis of amino acid standards. Panel A refers to the not-hydrolyzed and panel B to the acid-hydrolyzed amino acid standards. Panel A: 500 and 1,250 pmol of phosphorylated and not phosphorylated amino acids were respectively loaded for HPLC analysis. Panel B: twice the amounts reported in panel A. Values given are peak retention times in minutes.

phospho-amino acids concentration corresponding to the peaks detected by this analysis has been directly evaluated by the computerized system associated to the HPLC apparatus taking into account the recovery of phospho-amino acids after hydrolysis (see Table 1); the pmol obtained were the following: 145, 77 and 105 for P-Tyr in t-PA, PG and u-PA, respectively; 141 and 50 for P-Ser in t-PA and in u-PA; 128 and 95 for P-Thr in PG and in u-PA (Table 2).

The concentration of protein hydrolyzate was evaluated from the average percentage yield of the different amino acids obtained from each protein and compared to the respective number of amino acid residues present in each molecule. As reported in Table 2, the amount of acid hydrolyzed proteins were: 76% for t-PA (109/143 pmol), 73% for PG (81/111 pmol) and 84% for u-PA (153/182 pmol).

Considering the ratio between P-amino acid pmol and hydrolyzed protein pmol, the results obtained suggest that only a limited number (1 or 2) of the Ser, Thr and Tyr out of those present in the proteins are susceptible to phosphorylation. The results obtained are in line with those obtained by Mastronicola et al. [12] in the case of P-Ser of u-PA where two phosphorylation sites have been demonstrated.

3.3. *Immunological recognition of P-Ser and P-Thr residues in t-PA, PG and u-PA*

The P-Ser and the P-Thr residues detected by HPLC analysis were also immunologically recognized by monoclonal anti-P-Ser and anti-P-Thr antibodies (Fig. 4). As expected, t-PA and u-PA reacted with anti-P-Ser MoAb (lanes 1 and 5, respectively); PG and u-PA reacted with anti-P-Thr MoAb (lanes 3 and 6, respectively) and incubation with the secondary antibody did not give any signal for t-PA, PG and u-PA (lanes 2, 4 and 7, respectively).

In control experiments, the anti-fibronectin monoclonal antibody f-42 (see Figs. 1 and 4), as well as control mouse IgG (not shown), did not evidence any positive signal when tested against t-PA and PG (Fig. 1, panels A and B, lanes 9 and 8) and against u-PA (Fig. 4, lane 8).

4. Discussion

Recent studies, performed in our [13] and other laboratories [12,14–17], have shown that human urokinase-type PA can be phosphorylated in Tyr and/or Ser residues. In this paper, we have shown by two different approaches, HPLC and immunoblotting, that both PAs contain P-Ser residues, that u-PA and PG contain P-Thr residues and that, in addition to u-PA, also t-PA and PG contain P-Tyr residues.

The computer search of phosphorylation consensus sequences [28], present in these molecules, evidenced several consensus sequences for phosphorylation by Ser/Thr kinases (cyclic nucleotide-dependent protein-kinase, casein-kinase II and protein kinase C) in the domains of PAs and of PG except for the finger domain of t-PA; in particular, 13 sequences have been evidenced in u-PA, 17 in t-PA and 22 in PG. Considering the Tyr-phosphorylation consensus sequences [(R,K)x(2,3)

Table 2
Summary of the data obtained by HPLC phospho-amino acid analysis of t-PA, PG and u-PA.

Loaded protein	Phospho-amino acids (pmol)		Hydrolyzed protein		P-amino acids (pmol)
			%	pmol	Hydrolyzed protein (pmol)
t-PA 10 μg/143 pmol	P-Ser	141	76	109	1.30
	P-Tyr	145			1.33
PG 10 μg/111 pmol	P-Thr	128	73	81	1.58
	P-Tyr	77			0.95
u-PA 10 μg/182 pmol	P-Ser	50	84	153	0.32
	P-Thr	95			0.62
	P-Tyr	105			0.68

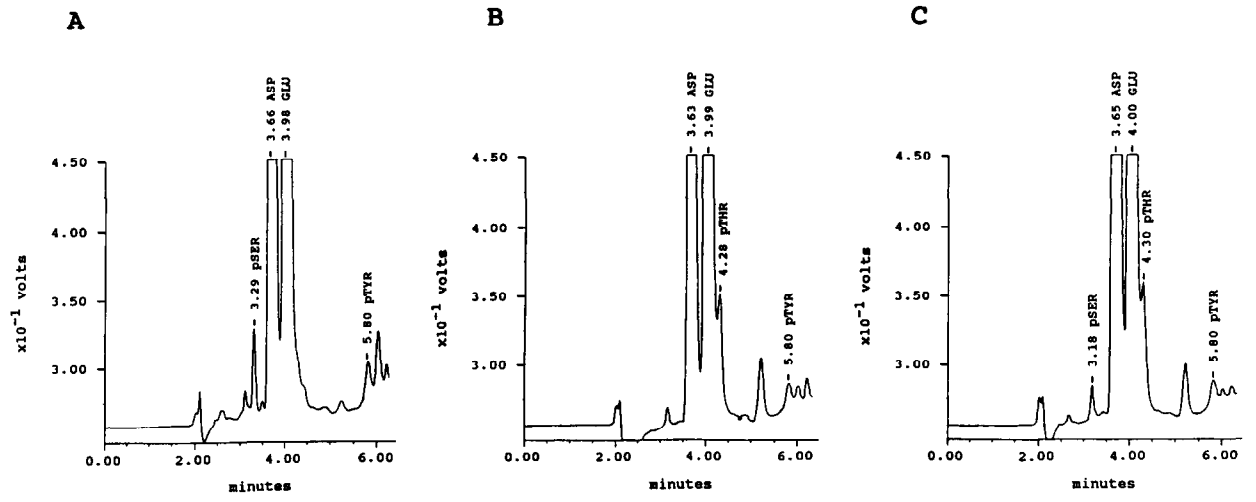

Fig. 3. HPLC-based phospho-amino acid analysis of acid-hydrolyzed t-PA, PG and u-PA (panel A, B and C, respectively). Values given are peak retention times in minutes: P-Ser, 3.29 and P-Tyr, 5.80 for t-PA (panel A); P-Thr, 4.28 and P-Tyr, 5.80 for PG (panel B); P-Ser, 3.18, P-Thr, 4.30 and P-Tyr, 5.80 for u-PA (panel C). The pmoles values corresponding to the peak areas have been directly evaluated by the computerized system of the HPLC apparatus taking into account the recovery of each phospho-amino acid of the acid hydrolyzed standard preparation (see the text, Tables 1 and 2).

(D,E)x(2,3)Y, (K)x(2)(D)x(3)Y], only one is present in the proteolytic domain of u-PA, (amino acids 211–218), t-PA (amino acids 396–403) and of PG (amino acids 504–511) and other two are present in the first and in the fifth kringle of PG (amino acids 84–91 and 298–304, respectively). In the case of u-PA, these data are consistent with those, previously reported [13], which showed the presence of Tyr-phosphorylation in the proteolytic domain of the 33 kDa mini-two chain u-PA.

From the quantitative analysis on the level of phosphorylation obtained by HPLC, it can be concluded that only a limited number (1 or 2) of the Ser and Thr residues, present in the molecules analyzed, should be susceptible to phosphorylation in agreement with previous data on Ser-phosphorylation of u-PA molecules secreted by tumor cells [12].

The data reported open the questions not only on the biological significance of phosphorylation of these molecules, but also on the kinases responsible for the phosphorylation of these secreted proteins.

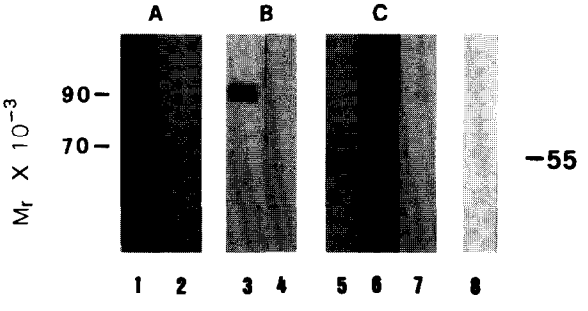

Fig. 4. Immunoblotting analysis of PG and PAs for the evaluation of P-Ser and P-Thr residues. Aliquots of human t-PA, PG and u-PA were analysed by immunoblotting with monoclonal anti-P-Ser and anti-P-Thr antibodies. Panel A, lane 1, refers to t-PA (5 μg) reacted with anti-P-Ser antibodies, panel B, lane 3 refers to PG (5 μg) reacted with anti-P-Thr antibodies; panel C, lanes 5 and 6, refer to u-PA (2 μg) reacted with anti-P-Ser and anti-P-Thr antibodies, respectively; lanes 2, 4 and 7 refer to t-PA (5 μg), PG (5 μg) and u-PA (2 μg) reacted as control only with the secondary antibodies; lane 8 refers to u-PA (2 μg) reacted with the f-42 anti-FN MoAb.

Concerning the role of phosphorylation, it has been previously shown that phosphorylation of u-PA decreases its affinity with PA inhibitors (PAI type 1 and 2), thus indirectly increasing the urokinase enzymatic activity (12, 14–16) which might favour the invasive properties of tumor cells.

In this line, Tyr and Ser phosphorylation of u-PA has been shown to be associated respectively with the activation of pp60src and of protein kinase C in metastatic [14] and in tumor cells [12,16]. In addition a shift in the isoelectric point of u-PA (from 9.2 to 7.6), likely associated with phosphorylation, has been reported as a function of tumor growth in the plasma of Balb/C mice injected with metastatic murine cells [23].

Concerning phosphorylation of secreted proteins (i.e. osteopontin, IGFBP-1, fibrinogen, [29]), this might occurr via intracellular or extracellular kinases, as it has been shown in the case of the β amyloid precursor protein, whose phosphorylation may be due to the luminal kinases present in the central vacuolar pathway [30] or to ecto-protein kinases present in the extracellular compartment [31,32]. No data, however, are available on the kinases involved in t-PA, PG and u-PA phosphorylation and on its role on the multiple biological functions exerted by these proteins.

In particular we are interested in verifying the possible role of phosphorylation in relationship with their PG-independent enzymatic activities [10,33,34] and mitogenic effect exerted on human fibroblasts [35], also considering the interaction with PA receptors and inhibitors.

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