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Tyrosine phosphorylation of human urokinase-type plasminogen activator

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Immunoblotting analysis of purified human urokinase plasminogen activator (u-PA), gives a positive signal when reacted with anti-phosphotyrosine monoclonal antibodies (MoAb anti-P-Tyr); competition with *o*-phospho-DL-tyrosine (P-Tyr) but not *o*-phospho-DL-threonine or serine (P-Treo, P-Ser) completely suppresses this signal. Either the 55 kDa u-PA form and the lower M_r form (33 kDa) derived from the 55 kDa u-PA are Tyr-phosphorylated also the u-PA secreted in the culture media of human fibrosarcoma cells (HT-1080) is phosphorylated in tyrosine as well as u-PA present in tissue extracts of tumors induced in nude mice by HT-1080 cells. These data show that urine purified human u-PA and u-PA produced by human fibrosarcoma cells, in vitro and in vivo, are phosphorylated in tyrosine; furthermore our data show that u-PA is the major Tyr-phosphorylated protein present in these human tumor cells.

Human urokinase-type PA; Tyrosine phosphorylation; HT1080 cell; HT 1080 tumor

1. INTRODUCTION

The urokinase-type plasminogen activator (u-PA, EC 3.4.21.31) is a serine proteinase, which catalyses the conversion of plasminogen to plasmin (EC 3.4.21.7), found in several normal and pathological tissues and in body fluids [1–3]. Many data indicate that u-PA is involved in the directional extracellular proteolysis which may be required in cell migration, tissue destruction, in several normal and pathological processes such as inflammatory reaction, invasive growth of trophoblasts and cancer cells [4,5].

The proenzyme is a single polypeptide chain (scu-PA with an approximate M_r of 55 kDa) containing 411 amino acids organized in four domains: (1) an epidermal growth factor (EGF) domain, spanning amino acids 5–45 with homology to EGF; (2) a 'kringle' domain, from amino acids 46 to 135; (3) a connecting peptide domain from residues 136 to 158; (4) a serine proteinase domain, from amino acids 159 to 411, with associated enzymatic activity [1,3]. The activation of scu-PA to two chain u-PA (tcu-PA, with an M_r of 55 kDa) is obtained by cleavage of the Lys-158–Ile-159 bond: this active tcu-PA consists of two polypeptide chains, linked by a disulphide bridge between cysteine residues 148 and 279 (respectively located in the connecting peptide and in the proteolytic domains). Additional cleavage in the connecting peptide may originate an enzymatically active 33 kDa tcu-PA.

In this paper we present evidence of tyrosine phosphorylation of human u-PA (55 and 33 kDa) purified from urines or produced in vitro and in vivo by human fibrosarcoma HT1080 tumor cells (55 kDa).

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Abbreviations: u-PA, urokinase-type plasminogen activator; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; AP, alkaline phosphatase; NBT, Nitrobluetetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; PBS, phosphate-buffered saline solution.

2. MATERIALS AND METHODS

2.1. *Materials*

Human urokinase (EC 3.4.21.31) with specific activity of 92 719 IU/mg was purchased from Serva (Heidelberg, Germany), human Glu-plasminogen from Biopool (Umea, Sweden), *O*-phospho-DL-serine, *O*-phospho-DL-threonine, *O*-phospho-DL-tyrosine and the low molecular weight markers used as standard in electrophoresis were obtained from Sigma (St. Louis, MO, USA) and Amido black 10B was purchased from Merck (Darmstadt, Germany). The goat anti-human urokinase IgG fraction was purchased from Biopool (Umea, Sweden); the polyclonal affinity-puified anti-P-Tyr antibodies [6–8] were kindly provided by Prof. Comoglio (Torino, Italy), and by Dr. Wang (San Diego, La Jolla, CA, USA); the monoclonal anti-P-Tyr antibodies (MoAb) were obtained culturing the IG_2 hybridoma cells [9–11] purchased from ATCC (USA) with the permission of Dr. Frackelton (Providence, Rhode Island, USA). Alkaline phosphatase (AP)-conjugated rabbit anti-goat and goat anti-mouse IgG, were obtained from Promega (W153711, USA), as well as the Nitrobluetetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrates.

2.2. *Preparation of HT-1080 conditioned media*

Human fibrosarcoma cells (HT-1080, ATCC, CCL 121, Rockville, MD, USA) producing u-PA [12], were grown in a 24-well plate (Costar) up to confluency in growth medium (minimum essential

medium, MEM, with Earle's salts containing 10% fetal calf serum, purchased from Gibco); the monolayer was washed 3 times with phosphate-buffered saline (PBS) and after 24 h incubation at 37°C under serum-free conditions, the conditioned medium was harvested, centrifuged at 2000 rpm × for 15 min and frozen in aliquots.

2.3. *HT-1080 tumor induction in nude mice and preparation of tumor tissue extracts*

Nude mice were subcutaneously (s.c.) injected with HT-1080 cells and starting from the appearance of a detectable tumor mass, animals were sacrificed at different times: tumors and organs were collected and kept frozen at −80°C. For the preparation of tumor tissue extracts, the specimens were thawed, washed in PBS, cleaned from fat and necrotic tissues, weighed and, after addition of 0.1% (Vol/Vol) Triton X-100 in 0.1 M Tris-HCl, pH 8.0, were sonicated in ice; the homogenates were centrifuged at 12 000 × *g* for 5 min and the supernatants frozen in aliquots.

2.4. *Zymographic procedure and immunoblotting of proteins*

Zymography of proteins was carried out with a procedure developed by Colombi et al. [13]. The proteins were separated by 8% polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) under non-reducing conditions and electrotransferred on nitrocellulose sheets (Scheicher and Schüll, Germany) according to Laemmli and Towbin, respectively [14,15]. 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 a 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-u PA IgG (1:500), polyclonal affinity-purified anti-P-Tyr antibodies (0.3 μg/ml) and 1G2 monoclonal anti-P-Tyr antibodies (1:25). After washing, the filters were incubated, with AP-conjugated rabbit anti-goat IgG (1:1000) or goat anti-mouse IgG (1:7500) and the positive bands evidenced with NBT and BCIP substrates [16].

3. RESULTS

3.1. *Tyrosine phosphorylation of purified u-PA*

Aliquots of 3 and 1 μg of u-PA (Fig. 1, lanes 1 and 2), loaded in triplicate (A, B and C), and one of 0.02 μg (D) were electrophoresed in 8% SDS-PAGE under non-reducing conditions. After electrotransfer on nitrocellulose filters, portions of the filter were Amido black-stained (panel A), immunoreacted with polyclonal anti-uPA IgG (panel B) or with monoclonal anti-P-Tyr antibodies (panel C). Zymographic analysis of u-PA is reported in panel D.

The 55 kDa stained protein band, also reacts with anti-u-PA antibodies and with anti-P-Tyr MoAb. Partially degraded, enzymatically active, 33 kDa u-PA also gives a positive response with anti-u-PA and anti-P-Tyr.

As reported in Fig. 2, the positive 55 kDa band evidenced with anti-P-Tyr MoAb (lane 1) is suppressed when 20 mM P-Tyr is added at the first step of im-

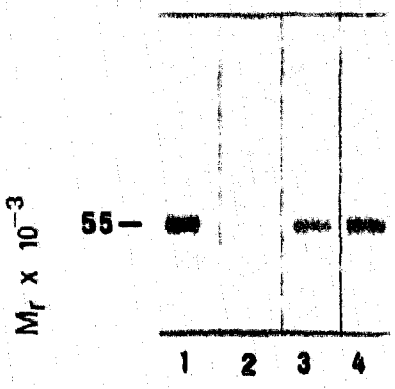

Fig. 2. Specificity of immunoblotting with anti-P-Tyr MoAb. 1 μg/tracks of purified u-PA were electrophoresed and transferred to nitrocellulose filters. The single lanes were separated and incubated with monoclonal anti-P-Tyr antibodies in the absence (lane 1) or in the presence of 20 mM P-Tyr (lane 2) or 20 mM P-Treo, P-Ser (lanes 3 and 4 respectively). The positive bands were evidenced as reported in section 2.

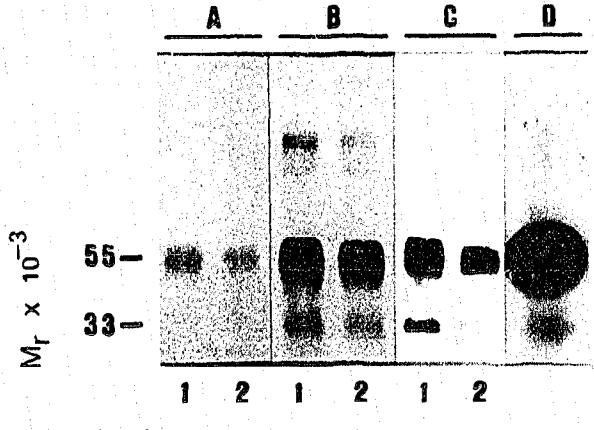

Fig. 1. Tyrosine phosphorylation of purified human u-PA. Commercially available research grade u-PA, purified from human urines, was tested at 3 and 2 μg/track (lanes 1 and 2 respectively); after electrotransfer to nitrocellulose filters these were stained with Amido black (panel A) and treated with polyclonal anti-u-PA anibodies (panel B) or monoclonal anti-P-Tyr antibodies (panel C); one aliquot (0.02 μg/track) was also tested in zymography (panel D).

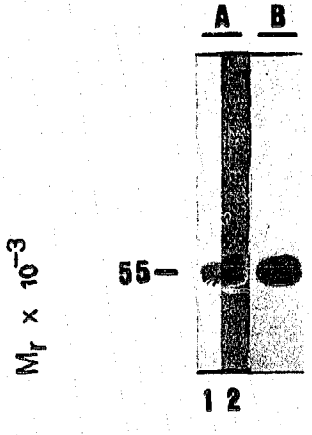

Fig. 3. Phosphorylated tyrosine residues in human u-PA secreted in vitro by human fibrosarcoma cells. The conditioned media of HT1080 cells, collected and prepared as described in section 2, was analyzed (30 μl/track) in immunoblotting against monoclonal anti-P-Tyr or polyclonal anti-u-PA antibodies (lanes A 1 and 2, respectively) and in zymography (lane B).

munoreaction with the MoAb. Addition of P-Treo or P-Ser does not suppress the positive band.

These results show that u-PA is phosphorylated in Tyr residues (Fig. 1, panel C, lane 1) and, in particular, in the B chain containing the proteolytic domain, since no Tyr is present in the connecting peptide domain which is present in the 33 kDa u-PA [1].

3.2. *Tyrosine phosphorylation of u-PA produced by HT-1080 cells in vitro and in vivo*

HT1080 human fibrosarcoma cells producing u-PA were grown in vitro and in vivo and the u-PA produced was characterized by the presence of P-Tyr residues.

30 μl aliquots of conditioned, serum-free medium from HT1080 cells were loaded on two tracks (Fig. 3, A and B) and submitted to electrophoresis and transfer to nitrocellulose filters.

Lane B was overlayed on agar-casein plasminogen plates for the enzymatic detection of u-PA activity while lane A was divided into two portions: one (lane A, 1) immunoreacted with anti-P-Tyr MoAb and one (lane A, 2) with anti-u-PA IgG.

The results obtained indicate that u-PA secreted by HT1080 cells is Tyr-phosphorylated and it represents the main P-Tyr protein secreted in vitro by these cells.

The analysis was also extended to tumors induced in nude mice by HT1080 cells. Nude mice, s.c. injected with HT-1080 cells, were sacrificed at different times starting from the appearance of a detectable tumor mass. The tumor was excised and the tumor extract analysed. Fig. 4, as an example, reports the results obtained by the analysis of one tumor which, after 30 days from the injection of HT1080 cells, was 8.8 mm in diameter. 3 μl aliquots of the tumor extracts (10.5 μg of total protein) was analysed by immunoblotting and by zymography. Panel A of Fig. 4 reports the PA activity (lane 2) and the immunoreaction using anti-u-PA IgG (lane 1). Panel B reports the immunoreaction using anti-P-Tyr rabbit polyclonal IgG. Panel C reports the immunoreaction with anti-P-Tyr monoclonal antibody in the absence (lane 2) or in the presence of 20 mM P-Tyr (lane 3) or P-Treo (lane 4) or P-Ser (lane 5); lane 1 reports the immunoreaction in the presence of the second antibody (AP-conjugated anti-mouse IgG) in the absence of the first antibody. Also in this case it is evident that only a 55 kDa band superimposing with u-PA and with PA activity shows P-Tyr residues. The high MW bands, evidenced with the monoclonal are the mouse IgG present in the tumor extract recognized by AP-conjugated anti-mouse IgG.

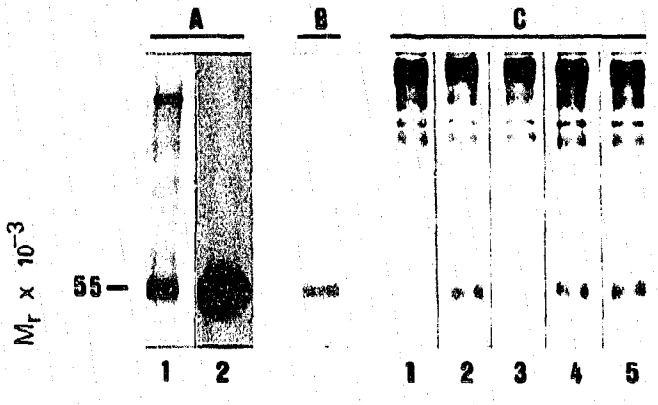

Fig. 4. Phosphorylated tyrosine residues in human u-PA present in tissue extracts of tumors induced in nude mice by HT-1080 cells. The tumor tissue extract (10.5 μg/track), obtained as described in section 2, was reacted in immunoblotting with anti-u-PA and with anti-P-Tyr antibodies (panels A and B, respectively, lanes 1) and was analyzed also in zymography (panel A, lane 2); the same specimen was probed with anti-P-Tyr MoAb (panel C, lane 2) and with AP-conjugated goat anti-mouse antibodies as control of the positive bands recognized by the second antibody (panel C, lane 1); moreover the specificity of the monoclonal anti-P-Tyr antibody was tested probing the blots in the presence of 20 mM P-Tyr, P-Treo, P-Ser (lanes 3, 4 and 5, respectively).

All these data, indicate that the u-PA present in tissue extracts of HT1080 tumors, induced in nude mice, contains P-Tyr residues and that u-PA is the main tyrosine-phosphorylated protein detectable.

4. DISCUSSION

Protein phosphorylation is one of the major general mechanisms by which intracellular events in mammalian tissues are controlled by external physiological stimuli; different cellular functions are controlled by a network of regulatory pathways mediated by phosphorylation and dephosphorylation. All protein kinases discovered prior to 1980 were known to phosphorylate proteins on serine or threonine residues. In 1980 Hunter and Sefton [17] showed that the protein product of the src oncogene was a tyrosine kinase and in the subsequent years the products of many other oncogenes and the receptors of several polypeptide growth factors were found to possess such activity [18]. The development of specific anti P-Tyr polyclonal and monoclonal antibodies [6,7,9,19] has helped to overcome the difficulty in distinguishing Tyr-phosphorylation from P-Ser or P-Treo residues becoming a powerful tool to identify protein tyrosine kinases with autocatalytic activity and cellular protein substrates. It is known that the dynamic equilibrium between the phosphorylation and the dephosphorylation [20] of proteins and/or enzymes may turn on or off signals in the cell and that hormone regulated enzymes can be post-translationally modified by phosphorylation [21]. Modulation of u-PA activity is part of the cellular response to growth stimuli and increased production of u-PA is associated with many spontaneous tumors and also with tumor virus-transformed cells [4,22,23]. The recent preliminary report, by Mastronicola et al. [24], of serine phosphorylation of u-PA suggests that this enzyme might modulate its activity via phosphorylation. In this paper we report that

purified human u-PA and u-PA produced in vitro by HT1080 cells and secreted into the conditioned medium, as well as the one present in tissue extracts of tumors induced in nude mice by HT1080 cells, shows P-Tyr residues recognized by anti P-Tyr antibodies inhibited by P-Tyr but not by P-Ser or P-Treo. The results obtained also show that, in vitro or in vivo, the only phosphorylated tyrosine protein detectable with our assay system is u-PA. Since it is known that the u-PA secreted under serum-free conditions by HT1080 cells consists for 90% of scu-PA [25], our results indicate that pro-u-PA is Tyr-phosphorylated. As already shown [12], the u-PA activity evidenced in the culture media of HT1080 cells and detectable in zymography is due to the activation of the proenzymes in the assay plate. The positive 33 kDa band evidenced with purified u-PA also shows that tcu-PA is Tyr-phosphorylated too and in particular in the proteolytic domain located in the B chain [1] of the molecule; we cannot exclude Tyr-phosphorylation of the A chain of the 55 kDa u-PA. Work is in progress to verify the possible involvement of phosphorylation in the modulation of the enzymatic activity.

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