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Interaction of urokinase-type plasminogenactivator (u-PA) with its cellular receptor (u-PAR) induces phosphorylation on tyrosine of a 38 kDa protein

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We demonstrate by immunoprecipitation that u-PAR is associated with a 38 kDa protein that is phosphorylated on tyrosine after u-PA treatment of cells. As tyrosine phosphorylation is the hallmark of many signal transduction pathways that promote growth and differentiation, these data suggest that u-PA, besides its role as a regulatory protease, might act as a para- or autocrine hormone.

Plasminogen activator; Urokinase; Urokinase-type plasminogen activator receptor; Tyrosine phosphorylation; U-937 cell; Signal transduction

1. INTRODUCTION

Generation of the protease plasmin from its zymogen plasminogen, catalyzed by plasminogen activators (PAs), is frequently associated with biological processes requiring tissue remodelling and cell migration, such as involution of the mammary gland, trophoblast invasion, spermatogenesis, wound healing and malignant growth [1]. The biosynthesis of the components of the plasminogen activating system is under complex hormonal control [2]. Recently a high-affinity receptor for urokinase-type plasminogen activator (u-PAR) was isolated and characterized [3,4]. u-PAR, a 313-residue single polypeptide with five potential N-linked glycosylation sites and a high content of cysteines, is fixed to the plasma membrane via a COOH-terminal glycosyl phosphoinositol (GPI) anchor [5,6] and believed to provide a lever for the regulation of extracellular proteolysis by restricting the activity of u-PA temporally and topologically. As other GPI-linked proteins, such as Ly-6, have been implicated in signal transduction [7] we have explored a similar role of u-PAR. Previous work has shown that u-PA stimulates the differentiation of HL60 cells and mouse cells as well as the migration of bovine endothelial cells and the chemotaxis of human neutrophils [8].

In the present study we demonstrate that u-PA induces a dose-dependent phosphorylation of a 38 kDa protein (p38) on tyrosine in U-937 (histiocytic lymphoma) cells.

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Abbreviations: u-PA, urokinase-type plasminogen activator; u-PAR, u-PA receptor; PMA, phorbol 12-myristate 13-acetate; DFP, diisopropyl fluorophosphate; DSS, disuccinimidyl suberate; PBS, phosphate buffered saline; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; BSA, bovine serum albumin.

2. EXPERIMENTAL

2.1. *Materials*

Chemicals were of the best commercial grade available and purchased from Sigma (St. Louis, MO), Pharmacia (Uppsala, Sweden) or Serva (Heidelberg, Germany). [^{32}P]ATP, [^{35}S]methionine and ^{125}I were obtained from Amersham International. Purified rabbit anti-phosphotyrosine polyclonal antibodies were from Dianova (Hamburg, Germany), PI-specific PLC from Sigma, the protein tyrosine kinase inhibitor herbimycin A (*Streptomyces* spp.) was obtained from Calbiochem Biochemical (San Diego, USA), u-PA from Serono (Freiburg, Germany).

2.2. *Cell culture*

U-937 cells were obtained from the American Type Culture Collection and grown at 37°C in RPMI 1640 medium containing 5% fetal bovine serum. For [^{35}S]methionine labeling of the cellular proteins 2×10^5 U-937 cells/ml were seeded into 9 cm Petri dishes in 8 ml RPMI 1640 containing 10% of the standard methionine concentration, 5% fetal bovine serum and 3 μCi/ml [^{35}S]methionine. Cells were incubated for 3 days and harvested by centrifugation (1,000 × *g*, 10 min, 4°C).

2.3. *Purification of u-PAR*

u-PAR was purified in two steps, consisting of temperature-induced phase separation and affinity chromatography with immobilized u-PA, as previously described [9].

2.4. *Radio-iodination and cross-linking assay*

DFP-inhibited u-PA was iodinated by the Iodo-gen procedure [10]. The iodinated protein was separated from free iodine by gel filtration on a Sephadex G-25 column. The specificity activity of labeled u-PA was approximately 15 μCi/mg protein.

For cross-linking experiments crude cell lysates or purified receptor preparations were incubated with DFP-inactivated radiolabeled u-PA for 1 h at 4°C, followed by incubation with 1 mM DSS for 15 min at room temperature, and with 10 mM CH_3COONH_4 for a further 10 min. Samples were analyzed by SDS-PAGE, followed by radioautography.

2.5. *Preparation of rabbit anti-u-PAR serum*

The protein band corresponding to purified u-PAR was sliced out of the gel after SDS-PAGE and homogenized in 100 μl of elution buffer (50 mM NH_4HCO_3, 0.1% SDS). The first injections (subcutaneous) consisted of 10 μg of protein per animal and were followed by two further treatments (5 μg of protein per animal). The serum was prepared 5 days after the last booster injection.

2.6. *Electrophoresis, Western blotting and radioautography*

SDS-PAGE was carried out in slab gels (7.5 or 10%) as described [11]. Samples were reduced immediately before electrophoresis in the presence of 20 mM DTT for 5 min at 95°C or analysed under non-reducing conditions.

Gels were electroblotted onto nitrocellulose sheets which were subsequently blocked with 1% BSA or 30% fetal calf serum. Alkaline phosphatase-conjugated goat anti-rabbit Ig was used as a second antibody and the sheets developed with Nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. Radioautography of ^{125}I, ^{35}S, and ^{32}P-labeled proteins was performed with dried polyacrylamide gels using Konica X-ray film. In some experiments gels containing samples from immune protein tyrosine kinase assays were soaked in 1 N KOH at 55°C for 2 h to hydrolyze phosphate on serine and threonine [12].

2.7. *Immunoprecipitation and immune complex kinase assay*

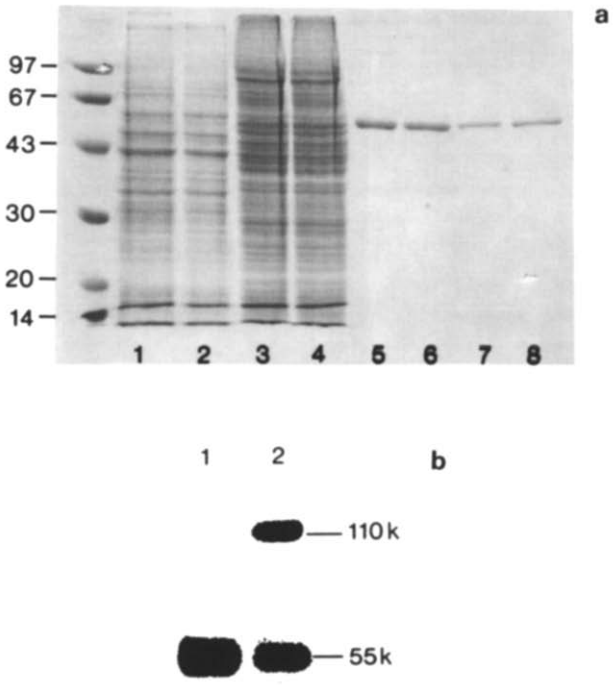
Protein samples were incubated for 2 h at 4°C with 25 μl of protein A Sepharose (Sigma), that had been incubated previously with 10 μl of anti-u-PAR antibody, overnight. The resin was subsequently sedimented by centrifugation and washed twice with 300 μl of 25 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM sodium orthovanadate or 0.5% Nonidet P-40, and twice with buffer without Nonidet P-40. To perform the immune complex kinase assay, the immunoprecipitate was washed further with 25 mM HEPES, pH 7.4, 10 mM $MnCl_2$ containing a protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, and 10^{-6} M each of aprotinin, pepstatin and cystatin). Following the addition of 5 mM *p*-nitrophenylphosphate and 30 nM [^{32}P]ATP the immunoprecipitates were incubated for 10 min at room temperature and subsequently washed for a final time in 20 mM HEPES, pH 7.4. After addition of SDS-sample buffer (with DTT) the immunoprecipitates were boiled and electrophoresed on 10% SDS-PAGE. After the separation the gels were dried and subjected to radioautography.

2.8. *Protein determination*

Protein was quantified with a bicinchoninic acid (BCA) reagent from Pierce, using BSA as a standard.

3. RESULTS AND DISCUSSION

u-PAR purified from U-937 cells was electrophoretically homogeneous (Fig. 1a). Cross-linking with iodinated u-PA revealed a single band corresponding to the complex of u-PA/u-PAR exhibiting a M_r of 110 kDa (Fig. 1b). Antisera raised in rabbits against pure u-PAR recognized the receptor in purified fractions, as well as in crude cell extracts (Fig. 1c). Immunoprecipitation of protein extracts of metabolically ([^{35}S]methionine) labeled native U-937 cells was performed and two main



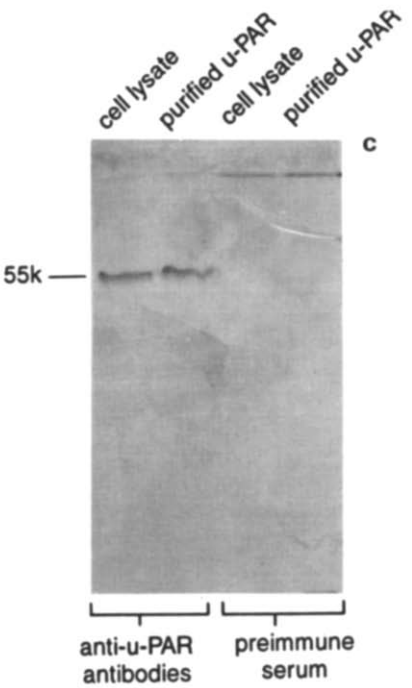


Fig. 1. (a) Purification of u-PAR. The lysis of U-937 cells, temperature-induced phase-separation and affinity chromatography were done as described in section 2. Lanes: 1,2, oily phase fractions; 3,4, water-soluble phase fractions; 5–8, fractions after affinity chromatography. (b) Cross-linking of radio-iodinated u-PA with u-PAR. DFP-treatment of u-PA and chemical cross-linking with DSS were performed as described. Lane 1, cross-linked control with [^{125}I]u-PA; lane 2, cross-linked u-PAR purified by affinity chromatography. (c) Characterization of antibodies raised to u-PAR. 20 μg of cell lysates or 0.5 μg of purified u-PAR were electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose, and probed with immune or preimmune serum (as indicated). Immunoreactive proteins were identified by incubation with alkaline phosphatase-conjugated second antibody.

protein bands were revealed on autoradiograms (Fig. 2a). One of them, according to M_r and immunoreactivity, corresponded to u-PAR, whereas the identity of the other band (M_r 38 kDa: p38) remains unknown. A third minor band co-migrated with the front line and contained material which has not been characterized and is therefore not further considered in the context of this work.

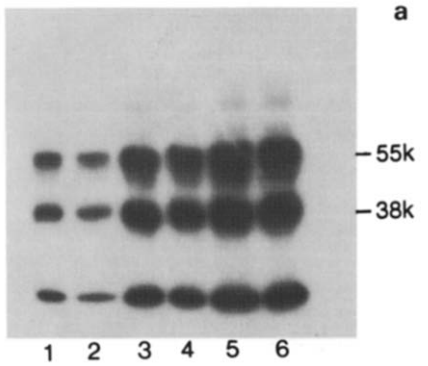
With previous data on the association of several GPI-anchored proteins with protein tyrosine kinase activity in mind [7], we used commercial polyclonal anti-(P)Tyr antibody to test whether it reacted with p38. The results indicate the presence of phosphotyrosine (Fig. 2b).

In vitro phosphorylation of proteins precipitated with anti-u-PAR antibody resulted in labeling of p38. The band was resistant to alkaline hydrolysis, indicating phosphorylation on tyrosine (data not shown). The phosphorylation was dependent on the dose of u-PA in the cell culture medium (Fig. 3a).

In another experiment we removed GPI-linked proteins from the surface of the cells by treatment with PI-specific PLC before immunoprecipitation. This treatment significantly decreased the phosphorylation of p38 (Fig. 3b). Hence, intact GPI-linked u-PAR is necessary for the association with protein and phosphorylation. Preincubation of U-937 cells with the protein kinase inhibitor, herbimycin A, also abolished the u-PA-induced phosphorylation of p38 (Fig. 3b).

Taken together our results demonstrate that u-PAR is (i) associated with p38, a protein that is co-precipitated by polyclonal anti-u-PAR antisera, and (ii) phosphorylated on tyrosine upon the addition of ATP. Some aspects of these findings deserve brief comment.

u-PAR so far has been purified exclusively from cells stimulated with PMA. This treatment increases the number of receptors but concomitantly leads to a lower affinity for u-PA [13,14]. Such an effect could be explained either by the biosynthesis of a modified receptor or the dissociation of a cofactor from the receptor upon addition of PMA. As we wanted to obtain antisera against the constitutive high-affinity receptor we chose to purify it from native U-937 cells and utilize it for the immunization. It seems surprising that p38 did not co-purify with u-PAR on the u-PA-Sepharose. This may be due to the extraction procedure or to the conditions employed during charging and washing of the column. Alternatively p38 could be synthesized at a higher rate than u-PAR, leading to a higher percentage of ^{35}S incorporation, or contain a significantly higher proportion of methionine so that the roughly equal intensity of bands in Fig. 2a could represent similar amounts of radioactivity but not equimolar amounts of protein. If [γ^{32}P]ATP is added to the immunoprecipitates, p38 is phosphorylated on tyrosine. This is demonstrated both by the use of phosphotyrosine-specific monoclonal antibody (Fig. 2b) as well as basic hydrolysis of the modified tyrosine residues (data not shown). The tyrosine phosphorylation is dose dependent (Fig. 3a), abrogated by (i) herbimycin A, an inhibitor of tyrosine kinase, and (ii) treatment of the cells with phospholipase C, which cleaves GPI-linked proteins off the cell surface, establishing an unequivocal link between the occupation of the receptor and tyrosine kinase activity. The phosphorylation may be autocatalytic or due to an enzyme that remains to be identified. Another open question con-



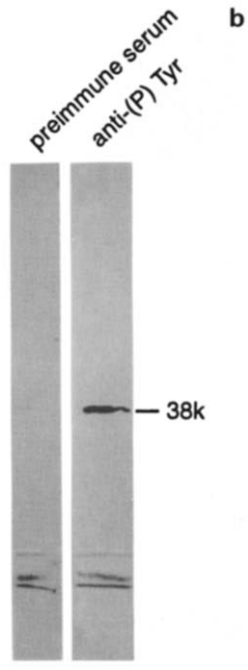


Fig. 2. (a) Immunoprecipitation of in vivo-labeled U-937 cells. Cells were biosynthetically labeled with [^{35}S]methionine and immunoprecipitation using anti-u-PAR was done as described. All samples were resolved by SDS-PAGE, after which the gels were dried and analysed by autoradiography. Different amounts of cell lysates were used for immunoprecipitation: lanes 1,2, 25 μg protein; lanes 3,4, 50 μg protein; lanes 5,6, 100 μg protein. (b) Western blotting of co-immunoprecipitated proteins with anti-(P)Tyr antibodies. The lysate of 2×10^6 U-937 cells preincubated with 100 nM u-PA for 1 h at 37°C was used for immunoprecipitation with anti-u-PAR antibodies. Commercial polyclonal anti-(P)Tyr antibody and alkaline phosphatase-conjugated second antibody were used for Western blot analysis.

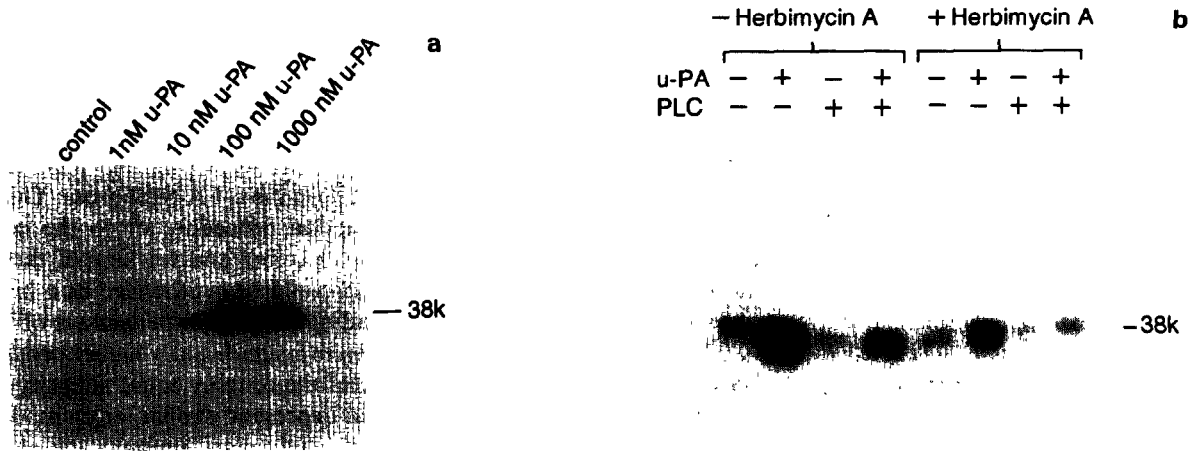


Fig. 3. (a) Dose-dependency of p38 protein phosphorylation on the concentration of u-PA. U-937 cells (2×10^6) were incubated at 37°C for 1 h in the presence of different concentrations of u-PA. After that the immunoprecipitation and phosphorylation were done as described. (b) Effect of treatment of U-937 cells with PI-PLC ($2\ \mu l/2 \times 10^5$ cells) and herbimycin A ($1\ \mu M/2 \times 10^5$ cells) on co-precipitation and phosphorylation of p38. The cells were treated with the indicated amount of drugs for 1 h at 37°C. Immunoprecipitation and in vitro kinase assay were performed as described.

cerns the biological function of p38 tyrosine phosphorylation. As the u-PA receptor has been implicated in signal transduction [5,15], tyrosine phosphorylation may be an early event in the activation of a u-PAR-mediated signal transduction pathway. Alternatively it may play a role in the mechanism of receptor internalization. Current experiments are directed towards the elucidation of this question.

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