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Induction of c-*fos* gene expression by urokinase-type plasminogen activator in human ovarian cancer cells

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Abstract

Binding of urokinase-type plasminogen activator (u-PA) to u-PA receptor (u-PAR) induces the rapid and transient expression of c-*fos* in OC-7 ovarian carcinoma cells. The pretreatment of the cells with protein tyrosine kinase (PTK) inhibitors, but not the inactivation of the u-PA active site by DFP (diisopropyl fluorophosphate), abrogates this effect. A soluble u-PAR fragment, expressed in baculovirus-infected Sf9 cells and purified by affinity chromatography, competes for binding of u-PA to u-PAR and inhibits c-*fos* induction. We conclude that activation of u-PAR after interaction with u-PA at the cell surface initiates a transmembrane signal, most likely in conjunction with other still unknown protein(s). This signal generates PTK activity feeding into a signal transduction pathway which activates nuclear transcription factors.

Key words: Immediate early gene expression; Plasminogen activator; Urokinase-type plasminogen activator receptor; Human ovarian cancer cell; Signal transduction

1. Introduction

Invasion and metastasis of solid tumors require the action of tumor-associated proteases which promote the dissolution of the surrounding cellular matrix and basement membranes [1]. u-PA appears to play an important role in these events [2]. In addition to its proteolytic activity, u-PA interacts as a ligand with its specific receptor. Whereas it is generally held that the receptor focuses u-PA activity in space and time [3], recent data indicate that u-PAR, after the binding of u-PA, might interact with other transmembrane proteins in order to transduce a signal [4]. We have shown recently that in histiocytic lymphoma cells (U937) u-PA acts as an effector by activating a protein tyrosine kinase [5]. The distal components of this signalling pathway, which might regulate cell proliferation, are unknown. Likely candidates for such a role could be products of immediate early genes, which are essential for the stimulation of cell proliferation by growth factors [6]. Therefore we investigated the effects of u-PA on c-*fos* gene expression in OC-7 human ovarian cancer cells, and report here that activation of u-PAR by u-PA induces a rapid and transient expression of c-*fos* which is mediated via a tyrosine kinase.

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Abbreviations: u-PA, urokinase-type plasminogen activator; u-PAR, u-PA receptor; PTK, protein tyrosine kinase; PMA, phorbol 12-myristate acetate; DFP, diisopropyl fluorophosphate; PBS, phosphate-buffered saline; SDS, sodium dodecylsulphate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; BSA, bovine serum albumin; FSC, fetal calf serum; SSC, 0.15 M NaCl/0.015 M Na_3-citrate, pH 7.0.

2. Materials and methods

2.1. Materials

Chemicals were of the best commercial grade available and purchased from Sigma (St. Louis, MO), Pharmacia LKB Biotechnology (Uppsala, Sweden), Merck (Darmstadt, Germany) or Serva (Heidelberg, Germany). Radiochemicals were obtained from Amersham International (Little Chalfont, GB), PI-specific PLC was from Sigma, the protein tyrosine kinase inhibitors Herbimycin A (*Streptomyces* spp.) and erbstatin analogue (methyl-2,5-dihydroxycinnamate) were from Calbiochem Biochemicals (San Diego, USA), u-PA was from Serono (Freiburg, Germany), anti-u-PAR monoclonal antibody (product #3936) was purchased from American Diagnostica Inc. (Greenwich, USA), purified rabbit anti-phosphotyrosine polyclonal antibody from Dianova (Hamburg, Germany). The mRNA purification kit was obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden) and the multiple DNA labelling system was from DuPont NEN (Boston, USA). All cell culture media and the FCS were provided by Gibco (Karlstein, Germany).

2.2. Cell culture

OC-7 cells, a cell line isolated from a human cystadenocarcinoma, were provided by Prof. M. Schmitt (Technical University Munich, Germany) and grown in DMEM/Ham's F12 nutrient mix containing 5% FSC.

Sf9 insect cells were received from the European Collection of Animal Cell Cultures (ECACC, Porton Down, Wilts, UK) and either propagated in supplemented Grace Insect (TNMFH) medium containing 10% FCS and 0.1% pluronic (Serva, Heidelberg, Germany) or adapted to growth in serum-free medium (Sf 900 II medium) in this laboratory.

2.3. Electrophoresis, Western blotting and autoradiography

SDS-PAGE was carried out in slab gels (7.5, 10 or 12.5%) as described [7]. Samples were reduced immediately before electrophoresis

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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% FCS [5]. Autoradiography of radiolabelled proteins was performed at −70°C with dried polyacrylamide gels using Konica X-ray film.

2.4. Protein determination

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

2.5. Northern blot analysis

Total RNA was isolated from 5×10^7 cells by the guanidinium isothiocyanate method as described in [8]. In some experiments polyadenylated RNA was purified from total RNA on oligo(dT)-cellulose columns using a mRNA purification kit. 20 μg of total RNA or 5 μg of polyadenylated RNA were separated on 1% agarose–18% formaldehyde gels and transferred onto GeneScreen Plus sheets by capillary force. A fragment of the c-*fos* gene (1 kb) was labelled with [^{32}P]dCTP by random priming [9]. The membranes were prehybridised in a solution containing 50% formamide, 1% SDS, 10% dextranesulfate, 1 M NaCl at 42°C for 6 h and then hybridised with a labelled probe (1×10^6 cpm/ml) in the presence of denatured salmon sperm DNA (100 μg/ml) at 42°C overnight. The filters were washed twice with 2 × SSC (each time for 5 min at room temperature), followed by another two washes with 2 × SSC/1% SDS (each time for 30 min at 60°C). Finally the filters were dried at room temperature and exposed to Konica X-ray film at −70°C.

2.6. Construction of a recombinant baculovirus, expressing soluble u-PAR

The complete cDNA of the human u-PAR was obtained from Dr. E.K.O. Kruithof (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland). Using synthetic primers corresponding to the published u-PAR cDNA sequence [10], a 922 bp PCR fragment was amplified corresponding to the amino terminal 285 amino acids of the receptor, lacking the site for attachment of the GPI anchor. The primers were designed so that a 5′-end *Eco*RI site and a 3′-end *Bgl*II site were generated at each end of the fragment. Via these two restriction sites, the truncated u-PAR cDNA was inserted into the polylinker site of the baculovirus transfer vector, pVL1393 (InVitrogen, San Diego, CA, USA) downstream of the polyhedrin promoter.

A recombinant baculovirus was then constructed by co-transfection of Sf9 insect cells with the transfer vector DNA together with linearized viral DNA (Baculogold virus DNA from Pharmingen, Palo Alto, CA, USA). Transfection and isolation of the recombinant virus was performed as described [11]. Soluble u-PAR was produced by infection of Sf9 cells adapted to growth in serum-free medium (Sf 900 II medium; Gibco) with the recombinant virus at a multiplicity of infection of 5. The culture supernatant from infected cells containing the secreted soluble receptor was harvested 60 h post-infection.

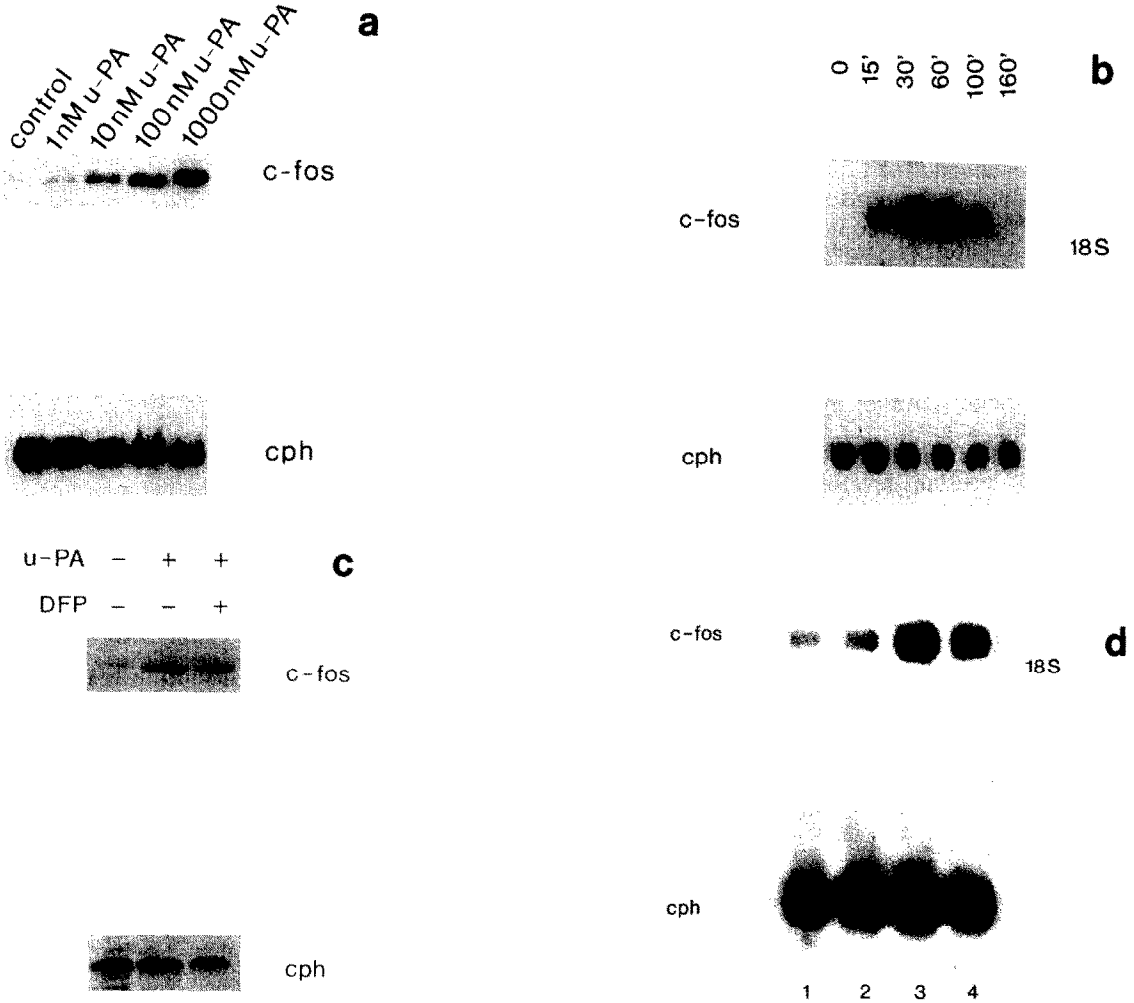

Fig. 1. u-PA-induced c-*fos* gene expression in OC-7 cells. (a) The cells were stimulated with different concentrations of u-PA for 30 min. Total RNA (20 μg) was analysed by Northern blot hybridisation as described in section 2. Human cyclophilin (cph) mRNA levels were assessed as a control. (b) The cells were incubated with 100 nM u-PA for 0–160 min. (c) u-PA was inactivated by the treatment with 20 mM DFP for 2 h at room temperature (right lane). (d) OC-7 cells were pretreated with Herbimycin A (1 μM/2 × 10^5 cells) for 1 h at 37°C before u-PA stimulation (lane 2); lane 1, control; lane 3, stimulation with u-PA (100 nM, 30 min); lane 4, stimulation with PMA (150 nM) for 2 h.

2.7. Purification of recombinant soluble u-PAR

u-PAR was purified from culture supernatants by affinity chromatography on u-PA–Sepharose which was prepared as previously described [5]. The supernatant containing the expressed u-PAR was passed over u-PA–Sepharose in the presence of 1 M NaCl. Bound u-PAR was eluted from the column with 0.2 M acetic acid adjusted to pH 1.8 by the addition of HCl and immediately neutralised with Tris to pH 8.0. Before the assay, u-PAR was dialysed against PBS and concentrated using polyethylene glycol (M_r = 20,000). Amino-terminal amino acid sequence analysis of u-PAR was performed after electrophoretic transfer of the protein from polyacrylamide to Immobilon membranes.

3. Results and discussion

3.1. u-PA-induced c-fos gene expression

Northern blot analysis demonstrated that u-PA-induced c-*fos* expression was dose-dependent (Fig. 1a) and that the increase in mRNA started at physiological concentrations of the ligand.

Of additional interest was the time-course of the induction, in comparison to other effectors [12]. As shown in Fig. 1b, the mRNA level reached a maximum after 30 min treatment and declined to undetectable levels after 120 min. This type of kinetics resembles the one previously described in the context of mitogen-induced c-*fos* expression [13], but not the type observed after induction with muscarinic receptor agonists and platelet-activating factor where transcription stated within 1 min and detectable signals were observed as early as 5 min after ligand addition [14].

In order to exclude a potential contribution of proteolytic activity to the mechanism of induction, u-PA was inactivated with DFP (Fig. 1c). This material induced the same level of expression, corroborating previous findings that demonstrated the involvement of proteolytically inactive u-PA in signalling events [4,5]. Stimulation of tyrosine kinases is a common denominator of various signalling pathways (including signalling via GPI-anchored proteins, [15]) ultimately leading to the activation of nuclear transcription factors [16]. Pretreatment of cells for 20 min with Herbimycin A, an inhibitor of protein tyrosine kinase activity, completely inhibited u-PA-induced signal transduction (Fig. 1d). The same results were obtained with the erbstatin analogue (data not shown). PMA-treated cells served as a positive control.

3.2. Expression and purification of recombinant truncated u-PAR

Recombinant truncated u-PAR, purified by affinity chromatography on u-PA–Sepharose, was subjected to SDS-PAGE and Western blot analysis. Under non-reducing conditions one prominent band (M_r ~ 35 kDa) was stained by Coomassie blue R-250 (Fig. 2a) and revealed by Western blot analysis using polyclonal and monoclonal anti-u-PAR antibody (data not shown). The amino acid sequence of purified Sf9-u-PAR was determined by

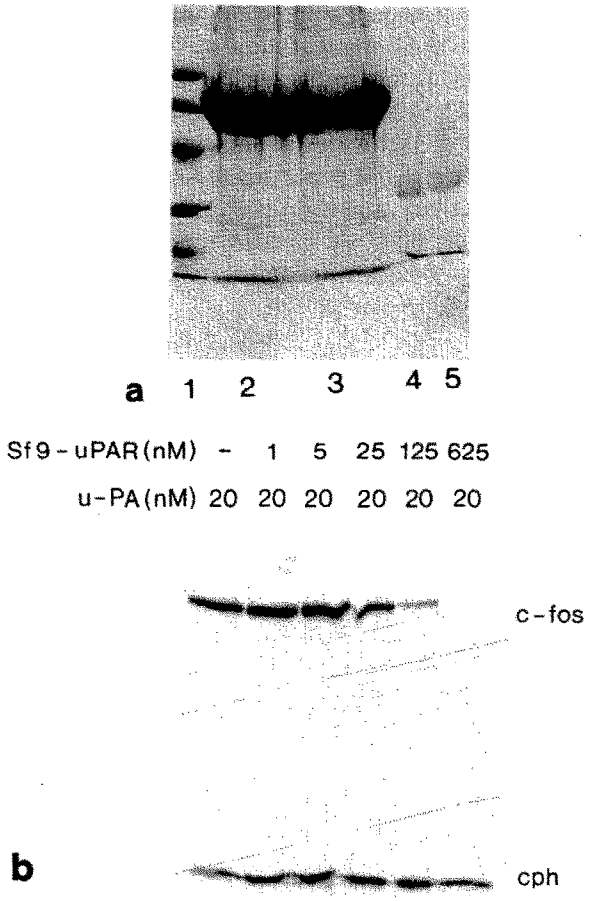

Fig. 2. (a) Purification of recombinant Sf9-u-PAR by affinity chromatography. Details of the purification are described in the text. The samples were stained by Coomassie blue R-250. Lane 1, fraction after affinity chromatography; lane 2, molecular weight markers (20, 30, 43, 67 and 97 kDa). (b) u-PA (20 nM) was preincubated with increasing concentrations of recombinant u-PAR and then incubated with the cells as described in the text. RNA extraction and Northern blot analysis were carried out as described in section 2.

automated Edmann degradation and shown to correspond to a real one. The reduced M_r of the fragment indicated incomplete glycosylation during heterologous expression. These data established uniquivocally that the purified material consisted of authentic, truncated u-PAR.

3.3. Competition for binding by soluble u-PAR

Recombinant u-PAR was used in order to obtain some further evidence for c-*fos* expression by u-PA induction. For this purpose a competition assay was performed. u-PA was preincubated with increasing concentrations of recombinant Sf9-u-PAR and then incubated with OC-7 cells for 30 min. The induction of c-*fos* expression in all probes was evaluated by Northern blot analysis. The data presented in Fig. 2b, clearly show that recombinant u-PAR competed with binding of u-PA to u-PAR and interfered with u-PA-induced c-*fos* expression in a dose-dependent fashion. A 30-fold molar excess of recombi-

nant Sf9-u-PAR almost completely prevented the induction. Our results allow the following conclusions. (i) u-PA transduces signals to the nuclear transcriptional apparatus via u-PAR. This effect most likely requires the interaction of u-PAR with still undefined additional protein(s). (ii) The signal is generated independently of u-PA proteolytic activity, and (iii) involves the activation of a tyrosine kinase. What we described represents probably a novel pathway of growth control that might be accessible to therapeutic intervention.

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References

[1] Alitalo, K. and Vaheri, A. (1982) Adv. Cancer Res. 37, 111–158.
[2] Danø, K., Anderson, P.A., Grøndahl-Hansen, J., Kristensen, P., Nielsen, L.S. and Skriver, L. (1985) Adv. Cancer Res. 44, 139–266.
[3] Kristensen, P., Eriksen, J., Blasi, F. and Danø, K. (1991) J. Cell Biol. 115, 1763–1771.
[4] Odekon, L.E., Sato, Y. and Rifkin, D.B. (1992) J. Cell Physiol. 150, 258–263.
[5] Dumler, I., Petri, T. and Schleuning, W.-D. (1993) FEBS Lett. 322, 37–40.
[6] Distel, R.J. and Spiegelman, B.M. (1990) Adv. Cancer Res. 55, 37–55.
[7] Laemmli, U.K. (1970) Nature 227, 680–685.
[8] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1990) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
[9] Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
[10] Roldan, A.L., Cubellis, M.V., Masucci, M.T., Behrendt, N., Lund, L.R., Danø, K., Appella, E. and Blasi, F. (1990) EMBO J. 9, 467–474.
[11] King, L.A. and Possee, R.D. (1992) The Baculovirus Expression System: A Laboratory Guide, Chapman & Hall, London.
[12] Molinar-Rode, R., Smeyne, R.J., Curran, T. and Morgan, J.I. (1993) Mol. Cell. Biol. 13, 3213–3220.
[13] Herschman, H.R. (1991) Annu. Rev. Biochem. 60, 281–319.
[14] Ransone, L.J. and Verma, J.M. (1990) Annu. Rev. Cell Biol. 6, 539–557.
[15] Stefanova, I., Horejsi, V., Ansotegui, I.J., Knapp, W. and Stockinger, H. (1991) Science 254, 1016–1019.
[16] Tripathi, Y.B., Lim, R.W., Fernandez-Gallardo, S., Kandala, J.C., Guntaka, R.V. and Schukla, S.D. (1992) Biochem. J. 286, 527–533.