

Urokinase-type plasminogen activator up-regulates the expression of its cellular receptor

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Abstract The expression of the receptor for the urokinase-type plasminogen activator (uPAR) can be regulated by several hormones, cytokines, tumor promoters, etc. Recently, it has been reported that uPAR is capable of transducing signals, even though it is lacking a transmembrane domain and a cytoplasmic tail. We now report that uPAR cell surface expression can be positively regulated by its ligand, uPA, in thyroid cells. The effect of uPA is independent of its proteolytic activity, since inactivated uPA or its aminoterminal fragment have the same effects of the active enzyme. The increase of uPAR on the cell surface correlates with an increase of specific uPAR mRNA. Finally, uPA up-regulates uPAR expression also in other cell lines of different type and origin, thus suggesting that the regulatory role of uPA on uPAR expression is not restricted to thyroid cells, but it occurs in different tissues, both normal and tumoral. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Urokinase; Urokinase receptor; Plasminogen activation

1. Introduction

The urokinase-type plasminogen activator (uPA) and its cellular receptor (uPAR) belong to one of the most efficient proteolytic systems active in the extracellular environment. Active uPA, by converting plasminogen into plasmin, promotes a proteolytic cascade which can lead to the degradation of most extracellular matrix (ECM) proteins [1,2]. The presence of uPAR highly potentiates cell surface proteolytic activity mediated by uPA [3].

uPAR plays a prominent role also in cell adhesion [4]. In fact, the receptor binds vitronectin (VTN), a specific component of the ECM [5], and interacts with integrins of the $\beta 1$, $\beta 2$, $\beta 3$ and $\beta 5$ families [6–8], often modifying their substrate specificity [6].

uPA is a two-chain serine protease; the uPAR-binding region of uPA resides in the aminoterminal region of the A chain, whereas the catalytic domain is contained in the B chain [1,2]. Therefore, uPA retains its proteolytic activity even after binding its receptor. In recent years, it has been clearly shown that uPA, upon binding uPAR and independently of its proteolytic activity, can activate cell-signalling pathways involved in cell adhesion, cell migration and chemotaxis [4].

uPAR is a heavily glycosylated single-chain protein with an molecular weight (MW) of 45–60 kDa. It is anchored to the cell surface by a GPI tail [9] and therefore lacks a transmembrane domain and a cytosolic tail, but it is capable of transmitting extracellular signals inside the cell, probably by association with other cell membrane molecules, such as integrins [6], caveolin [10] or unidentified adapters [11].

uPAR expression can be regulated by tumor promoters, cytokines, hormones and growth factors, both at transcriptional and post-transcriptional levels [12]. We have previously shown that uPA, upon binding uPAR, increases thyroid cell surface expression of αv and $\beta 3$ integrins, which form receptors for VTN [13]. In this report we show that uPA can regulate also the cell surface expression of its receptor and investigate the mechanism underlying this regulatory effect.

2. Materials and methods

2.1. Reagents

Mouse monoclonal antibodies R4 were kindly provided by Dr. G. Hoyer-Hansen (Finsen Laboratory, Copenhagen, Denmark). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were from Bio-Rad (Richmond, CO, USA), FITC-labelled anti-mouse IgG from Jackson (West Grove, PA, USA) and polyclonal anti-actin antibody from Sigma (St. Louis, MO, USA). Enhanced chemoluminescence (ECL) detection kit was from Amersham International (Amersham, England); Trizol reagent and Superscript II (RNase H reverse transcriptase) from Life Technologies (Gaithersburg, MD, USA). Polyvinylidene fluoride (PVDF) filters were from Millipore (Windsor, MA, USA) and the PCR kit from Perkin-Elmer (Branchburg, NJ, USA).

2.2. Cell culture

TAD-2 cell line was derived from human fetal thyroid cells [14], ARO cells from anaplastic thyroid carcinoma [14], WRO and NPA from follicular and papillary thyroid carcinoma, respectively [15]. These cell lines were cultured in RPMI 1640 medium (Flow, McLean, VA, USA) supplemented with 10% fetal calf serum (Gibco). Melanoma A2058 cells, 293 human kidney cells and a primary culture of human fibroblasts were grown in DMEM supplemented with 10% fetal calf serum.

2.3. Cell stripping

Acid treatment of cells was performed as previously described [16], in order to strip surface-bound endogenous uPA.

2.4. Western blot

Cells were lysed in 1% Triton X-100 in PBS and the protein content was measured by a colorimetric assay (Bio-Rad, Richmond, CO, USA). 50 μ g of total protein was electrophoresed on a 9% SDS-PAGE and transferred onto a PVDF membrane. The membrane was blocked with 5% non-fat dry milk, and probed with R4 anti-uPAR antibody, at a concentration of 1 μ g/ml. Finally, washed filters were incubated with horseradish peroxidase-conjugated anti-mouse antibody and detected by ECL. Some filters were washed and re-probed with rabbit anti-actin antibodies (1 μ g/ml).

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2.5. Immunofluorescence

Cells were washed and harvested by PBS-EDTA 10 mM. After three washes in $\text{Ca}^{2+}\text{Mg}^{2+}$ -containing PBS, 5×10^5 cells were incubated with 1 $\mu\text{g}/\text{ml}$ of R4 anti-uPAR monoclonal antibody for 1 h at 4°C . Purified mouse immunoglobulins were used as a negative control. Cells were then washed in PBS-0.1% BSA and incubated with an FITC-labelled rabbit anti-mouse IgG for 30 min at 4°C . Finally, cells were washed and analyzed by flow cytometry using a FACScan (Becton Dickinson, Mountain View, CA, USA).

2.6. Reverse polymerase chain reaction

Total cellular RNA was isolated by lysing cells in Trizol solution. RNA was precipitated and quantitated by spectroscopy. 5 μg of total RNA was reversely transcribed with random hexamer primers and 200 U of RNase H reverse transcriptase. 1 μl of reversely transcribed DNA was then amplified using uPAR specific 5' sense (CTG CGG TGC ATG CAG TGT AAG) and 3' antisense (GGT CCA GAG GAG AGT GCC TCC) 21-mer cDNA primers or GAPDH specific 5' sense (TTC ACC ACC ATG GAG AAG GCT) and 3' antisense (ACA GCC TTG GCA GCA CCA GT) 20-mer cDNA primers as a control. PCR was performed for 25 cycles at 63°C in a thermocycler, and the reaction products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide, followed by photography under ultraviolet illumination.

3. Results

3.1. uPA increases uPAR antigen in thyroid cells

TAD-2 cells express large amounts of uPAR and can specifically bind uPA [17]. We previously reported that these cells express also a truncated form of the receptor, which lacks the uPA-binding domain and shows an MW of 35 kDa [17].

We investigated if uPA is able to affect uPAR expression in these cells.

TAD-2 cells were stripped of endogenous uPA by acid treatment, incubated with or without 50 nM uPA for 2 h and 24 h in the absence of serum and finally lysed. The 2 h incubation was preceded by a 22 h serum starvation in order to equalize the basal expression of uPAR in both treatments.

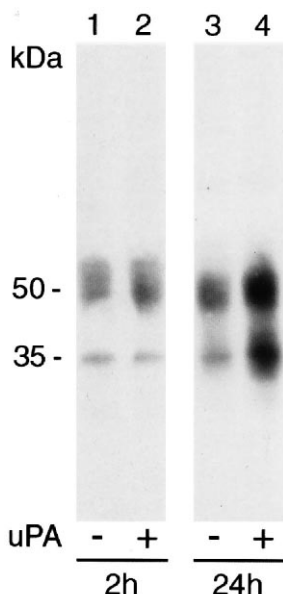


Fig. 1. uPA increases uPAR expression in thyroid cells. TAD-2 cells were stripped of endogenous uPA by acid treatment, incubated with (lanes 2, 4) or without (lanes 1, 3) 50 nM uPA for 2 h (lanes 1, 2) or 24 h (lanes 3, 4), lysed and analyzed by Western blot with R4 anti-uPAR monoclonal antibody.

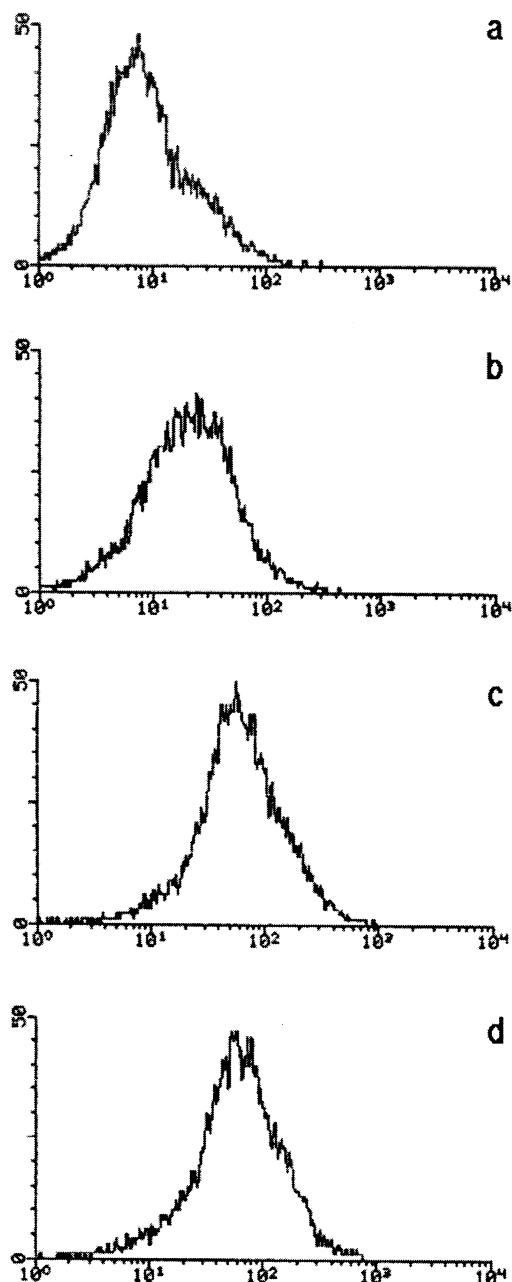


Fig. 2. uPA increases uPAR cell surface expression. Acid-treated TAD-2 cells were incubated for 24 h with 5 nM (c) or 50 nM (d) uPA or without uPA (b). Cells were then harvested by EDTA, incubated with R4 anti-uPAR monoclonal antibody (b, c, d) or with non-immune serum as a control (a). Cells were finally incubated with FITC-labelled rabbit anti-mouse IgG and analyzed by flow cytometry. Mean fluorescence intensity values = a: 14.09; b: 29.72; c: 85.54; d: 84.39.

Western blot analysis of total lysates with R4 anti-uPAR monoclonal antibody showed that 2 h of uPA treatment (Fig. 1, lane 2) slightly increased uPAR expression (50 and 35 kDa MW) as compared to the untreated control (Fig. 1, lane 1), whereas 24 h of uPA treatment (Fig. 1, lane 4) strongly increased uPAR expression as compared to the control (Fig. 1, lane 3). In lane 4, the ratio between the 50 and 35 kDa forms is different compared to the other lanes probably because the longer incubation with uPA can generate the

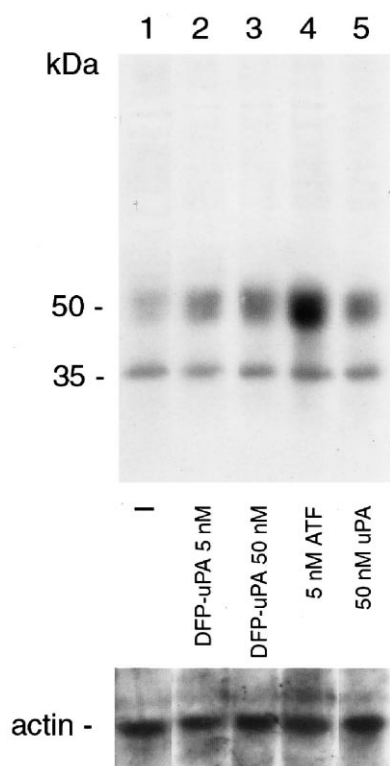


Fig. 3. uPA-mediated uPAR increase is independent of uPA proteolytic activity. TAD-2 cells were stripped of endogenous uPA by acid treatment and incubated for 24 h with medium (lane 1), 5 or 50 nM DFP-uPA (lanes 2, 3 respectively), 5 nM ATF (lane 4), 50 nM uPA (lane 5). Cells were then lysed and analyzed by Western blot with R4 anti-uPAR monoclonal antibody. Bottom panel: same filter hybridized with anti-actin polyclonal antibodies as a control for protein loading.

truncated form of uPAR by proteolytic cleavage. This experiment was performed three times in duplicate with similar results.

We then investigated whether the observed increase of uPAR in total cell lysates reflected a higher cell surface expression of the receptor. TAD-2 cells treated with 5 or 50 nM uPA for 24 h were harvested by 10 mM PBS-EDTA, incubated with R4 anti-uPAR monoclonal antibody and analyzed by flow cytometry. Cell surface expression of uPAR was strongly up-modulated (about 3-fold) after 24 h of uPA treatment (Fig. 2c,d), as compared to the untreated control (Fig. 2b). No differences were observed between 5 and 50 nM uPA treatments (Fig. 2c,d). These data showed that uPA increases uPAR expression on the cell surface.

It should be noted that TAD-2 cells produce moderate amounts of PAI-1 [17] which can lead to the internalization of the uPA-uPAR complex. Therefore it is possible that the observed increase in the uPAR level induced by uPA was underestimated.

3.2. uPA-mediated uPAR increase is independent of uPA proteolytic activity

The effect of uPA on uPAR expression could be due to the proteolytic activity of the enzyme or to its interaction with the receptor. uPAR expression was then investigated after incubation of TAD-2 cells with uPA inactivated by DFP treat-

ment or with the aminoterminal fragment of uPA (ATF), corresponding to the uPAR-binding region of uPA.

TAD-2 cells were stripped of endogenous uPA by acid treatment, incubated for 24 h with medium (Fig. 3, lane 1), 5 or 50 nM DFP-inactivated uPA (Fig. 3, lanes 2, 3 respectively), 5 nM ATF (Fig. 3, lane 4), 50 nM uPA, as a positive control (Fig. 3, lane 5) and finally lysed. Western blot analysis of total lysates with R4 anti-uPAR monoclonal antibody showed that both DFP-inactivated uPA (Fig. 3, lanes 2, 3) and ATF (Fig. 3, lane 4) are capable of increasing uPAR expression as compared to the untreated control (Fig. 3, lane 1). Same results were obtained in two different experiments, performed in duplicate.

These results clearly show that the increase of uPAR expression depends on the interaction of uPA with its receptor, since it occurs even in the absence of any uPA proteolytic activity.

3.3. uPA increases uPAR mRNA in thyroid cells

We then investigated by RT-PCR if the observed increase of uPAR expression was related to an increase of uPAR mRNA (Fig. 4). TAD-2 cells were incubated with (Fig. 4, lane 2) or without (Fig. 4, lane 1) 5 nM DFP-uPA for 24 h. Total RNA was prepared, reverse transcribed and amplified by 25 cycles PCR in the presence of primers for uPAR or for GAPDH as a control (Fig. 4, lanes 1, 2). Buffer (Fig. 4, lane 3) or uPAR cDNA (Fig. 4, lane 4) were amplified as negative and positive control, respectively. The analysis of PCR products by agarose gel showed the increase in uPAR mRNA in cells treated with DFP-uPA (Fig. 4, lane 2) as compared to untreated cells (Fig. 4, lane 1) in which basal uPAR mRNA was below the threshold of detection.

3.4. uPAR increase mediated by uPA occurs in different cell types

uPA effect was also studied in tumor and normal cell lines of different origin in order to investigate if it was restricted only to TAD-2 cells. TAD-2 thyroid cells (Fig. 5, lanes 1, 2), WRO follicular thyroid carcinoma cells (Fig. 5, lanes 3, 4),

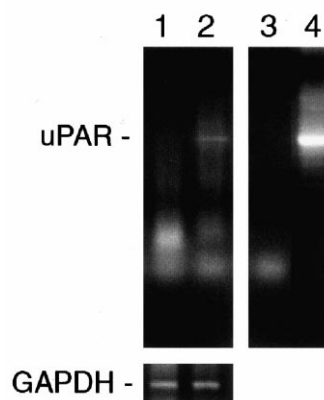


Fig. 4. uPA increases uPAR mRNA in thyroid cells. TAD-2 cells were incubated with (lane 1) or without (lane 2) 5 nM DFP-uPA for 24 h. PCR was performed with 1 μ l of reverse transcribed cDNA (lanes 1, 2) or without DNA as negative control (lane 3) in the presence of primers for uPAR or for GAPDH. 10 ng of uPAR cDNA was amplified as a positive control (lane 4). PCR products were analyzed by 1% agarose gel containing ethidium bromide, followed by photography under ultraviolet illumination.

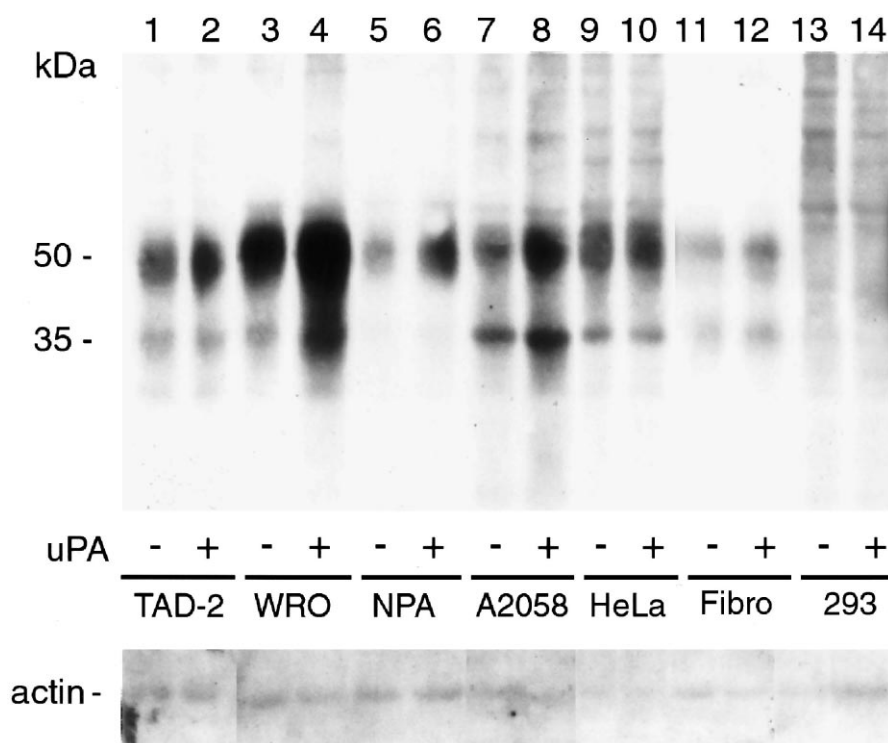


Fig. 5. uPAR increase mediated by uPA occurs in different cell types. TAD-2 thyroid cells (lanes 1, 2), WRO follicular thyroid carcinoma cells (lanes 3, 4), NPA papillary thyroid carcinoma cells (lanes 5, 6), A2058 melanoma cells (lanes 7, 8), HeLa epidermoid carcinoma cells (lanes 9, 10), primary cultures of fibroblasts (lanes 11, 12) and 293 kidney cells (lanes 13, 14) were treated with (lanes 2, 4, 6, 8, 10, 12, 14) or without (lanes 1, 3, 5, 7, 9, 11, 13) 5 nM DFP-uPA for 24 h, lysed and analyzed by Western blot with R4 anti-uPAR monoclonal antibody. Lower panel: same filter hybridized with anti-actin polyclonal antibodies as a control for protein loading.

NPA papillary thyroid carcinoma cells (Fig. 5, lanes 5, 6), A2058 melanoma cells (Fig. 5, lanes 7, 8), HeLa epidermoid carcinoma cells (Fig. 5, lanes 9, 10) and primary cultures of fibroblasts (Fig. 5, lanes 11, 12) were treated with (Fig. 5, lanes 2, 4, 6, 8, 10, 12) or without (Fig. 5, lanes 1, 3, 5, 7, 9, 11) 5 nM DFP-uPA for 24 h, lysed and analyzed by Western blot with anti-uPAR antibodies. uPA treatment increased uPAR expression in all the different cell lines, independently on their normal or tumor origin.

uPAR-negative 293 kidney cells [6] cannot bind the enzyme and, as expected, were not sensitive to uPA effect (Fig. 5, lanes 13, 14).

4. Discussion

uPAR and its ligand uPA have been implicated in several pathological and physiological processes which require cell migration, in particular tumor cell invasion and metastasis. In fact, uPAR and uPA play a central role in cell-associated proteolysis [3] and in cell adhesion [4], which are required for cell migration. Enhanced levels of uPAR have been described in several types of tumor, both on the cell surface and in plasma [3,18–20]. It is to be thus expected that the expression of uPAR is strictly controlled. In this report we investigated if uPAR could be regulated by its own ligand. We showed that indeed the interaction of uPA with uPAR up-regulates the expression of the receptor. The active enzyme is also able to cleave its receptor, thus removing the uPA-binding domain [21]; this proteolytic activity could explain previous results showing a reduction in the specific binding of DFP-uPA in cells pre-treated with active uPA [22,23]. Therefore, uPA can

regulate both uPAR expression and uPAR functions [24] and its final effect depends on the balance between these two regulatory activities.

It has been reported that uPAR expression can be efficiently regulated: the high lability of uPAR mRNA allows a rapid down-regulation of protein synthesis and provides an efficient mechanism for transient expression [25]. uPA, which 'in vivo' could be provided by tumor-infiltrating stromal cells [3], counteracts this regulation, thus promoting the overexpression of uPAR observed, for instance, in several types of tumor.

Enhanced adherence to VTN has been associated to increased invasiveness of tumor cells [26]. uPAR can act as VTN receptor [5]; its affinity for VTN is enhanced after binding uPA [27]. uPAR and integrin receptors for VTN can be co-regulated [28] and we have previously reported that uPA, in thyroid cells, increases the expression of integrin receptors for VTN [13]. It is then possible that uPA, upon binding to uPAR, modulates the expression of integrin and non-integrin receptors for VTN, thus playing a prominent role in regulating cell adhesion to VTN.

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