Urokinase-type plasminogen activator up-regulates the expression of its cellular receptor through a post-transcriptional mechanism

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Abstract We have recently reported that the urokinase-type plasminogen activator (uPA) up-regulates the cell surface expression of its own receptor (uPAR) in several cell types, independently of its enzymatic activity. uPA has no effect on kidney 293 cells which do not express uPAR and then cannot bind uPA. Kidney cells, transfected with the coding region of uPAR cDNA, express very large amounts of uPAR and respond to uPA stimulation by regulating uPAR both at mRNA and protein levels. uPA effect occurs also in the presence of the transcriptional inhibitor dichloro-ribobenzimidazole, whereas it is abolished by the protein synthesis inhibitor cycloheximide. Moreover, uPA-dependent uPAR up-regulation correlates with the increase of a complex between the coding region of uPAR mRNA and an unknown cellular factor. We then propose that uPA regulates uPAR expression at a post-transcriptional level, by promoting the binding of uPAR mRNA to a stabilizing factor.

Key words: Urokinase; Urokinase receptor; Post-transcriptional regulation; mRNA binding protein

1. Introduction

The urokinase-type plasminogen activator (uPA) is a serine protease which promotes extracellular matrix (ECM) degradation by activating plasminogen to plasmin. uPA binds a specific cell surface receptor (uPAR) expressed in a wide variety of cells. uPAR consists of three different domains: the uPA binding domain 1 (D1), the connecting domain 2 (D2) and the cell-anchoring domain 3 (D3) [1,2]. uPAR, besides uPA, binds also an extracellular matrix (ECM) component, vitronectin (VN) [3], and the β-subunit of some integrins [4,5]. uPA activity is regulated by two specific inhibitors: type-1 (PAI-1) and type-2 (PAI-2). PAI-1 competes with uPAR and with specific integrins for the binding to VN [6,7]. These multiple interactions determine the capability of the different components of the uPA-mediated plasminogen activation system to regulate not only cell surface associated proteolysis but also cell adhesion to the ECM, both events being required for cell migration [1,2,8]. In the last few years, in fact, several reports have shown that uPAR, upon binding uPA, is directly involved in chemotaxis [9], cell adhesion and migration [8]. Its role in these processes is not merely due to the physical interaction with different ligands but depends also on its capability to transduce signals inside the cells, even though the signaling mechanisms are still unclear, since uPAR is anchored to the cell surface by a GPI tail [10].

uPAR expression can be regulated by tumor promoters, cytokines, hormones and growth factors, both at transcriptional and post-transcriptional levels [11–14]. The expression of the uPAR gene can be regulated via Sp1, NFkB and AP-1 transcription factors by specific motifs contained within the first 180 bp upstream of the transcriptional start site [15,16]. Recently, a post-transcriptional regulation of uPAR expression has also been described. uPAR mRNA, as well as the mRNA of several cytokines and oncoproteins, contains functional AU-rich elements (AREs) in the 3′-untranslated region (UTR) [17,18]. AREs regulate mRNA degradation by binding stabilizing or destabilizing transacting factors [19–23]; some of these factors have been cloned and characterized. ARE-mediated post-transcriptional regulation has been reported also for other components of the plasminogen activation system, such as uPA [24], PAI-1 [25] and PAI-2 [26]. uPAR mRNA contains also another specific sequence, within the coding region (nucleotides 195–246), which is able to destabilize mRNA upon binding to an unidentified protein [14]. Stability determinants have been identified also within the coding region of few other mRNAs, such as β-tubulin, c-fos, c-myc, MAT-α1 and, very recently, PAI-2 mRNAs [27–31].

uPAR is overexpressed in several tumors, both on the cell surface and in plasma [1,32–35] and plays a fundamental role in tumor cell invasion and metastasis. The mechanism resulting in its high expression in malignant tumor cells has not been elucidated. We have previously shown that uPA up-regulates uPAR expression in different cell types [36]. We now investigate the mechanism underlying uPA-mediated regulation of uPAR expression.

2. Materials and methods

2.1. Reagents

Mouse monoclonal antibodies R4 were kindly provided by Dr. G. Hoyer-Hansen (Finsen Laboratory, Copenhagen, Denmark) and uPAR cDNA by Dr. MP Stoppelli (IIGB, CNR, Naples, Italy). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were from Bio-Rad (Richmond, CO, USA), polyclonal anti-actin antibody and 5,6-dichloro-1-β-D-ribobenzimidazole (DRB) from Sigma

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(St. Louis, MO, USA). Enhanced chemiluminescence (ECL) detection kit was from Amersham International (Amersham, UK); lipofectamine, Trizol reagent, Superscript II (M-MLV reverse transcriptase (RT)) and geneticin from Life Technologies (Gaithersburg, MD, USA). Polyvinylidene fluoride (PVDF) filters were from Millipore (Windsor, MA, USA). PcDNA3 plasmid was from Invitrogen (San Diego, CA, USA), the PCR kit from Perkin-Elmer (Branchburg, NJ, USA) and T7 and Sp6 polymerases from Promega (Madison, WI, USA).

2.2. Cell culture

293 human kidney cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FCS).

2.3. Inactivation of uPA

uPA was inactivated by 2 h incubation with 10 mM diisopropylfluorophosphate (DFP) [37].

2.4. Western blot

Lung tissues and cells were lysed in 1% Triton X-100/PBS in the presence of protease inhibitors (Sigma); the protein content was measured by a colorimetric assay (Bio-Rad). 30–50 μg of cell lysate proteins and 100 μg of tissue extract proteins were electrophoresed on a 9% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (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 μg/ml. Finally, washed filters were incubated with horseradish peroxidase-conjugated anti-mouse antibody and detected by ECL. Some filters were washed and reprobed with rabbit anti-actin antibodies (1 μg/ml).

2.5. Transfection

The coding region of uPAR cDNA (nt −3–1018) was cloned in the *Eco*RI site of pcDNA3 and the resulting plasmid was named uPAR-pcDNA3. 5×10^6 cells, cultured overnight in 100 mm tissue culture dishes, were transfected with 10 μg of uPAR-pcDNA3 or control vector pcDNA3 and 60 μl of lipofectamine for 5 h at 37°C (5% CO_2). Transfected cells were selected by 1.5 mg/ml geneticin.

2.6. Reverse polymerase chain reaction

Total cellular RNA was isolated by lysing cells in Trizol solution according to the supplier's protocol. RNA was precipitated and quantitated by spectroscopy. 5 μg of total RNA was reversely transcribed with random hexamer primers and 200 U of M-MLV 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. Semi-quantitative polymerase chain reaction (PCR) was performed for 25 cycles at 62°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.

2.7. In vitro transcription

1 μg of linearized plasmid containing the coding region of human uPAR was transcribed 'in vitro' with T7 RNA polymerase according to the supplier's protocol, in the presence of 50 μCi [α-^{32}P]UTP. Precipitation in 1 M NH_4 acetate was performed to remove unincorporated radioactivity. The specific activity of the product was 10^8 cpm/μg. The size of labeled mRNA transcript was confirmed by electrophoresis on a 5% urea gel.

Unlabeled RNA competitors were prepared by 'in vitro' transcription of 10 μg of the linearized plasmid, according to the supplier's protocol, using T7 RNA polymerase for sense uPAR mRNA and SP6 RNA polymerase for antisense uPAR mRNA.

2.8. Gel mobility shift assay

Gel mobility shift assay was performed as previously described [14]. 50 μg of cell lysate proteins or 20 μg of tissue extract proteins was incubated with 4×10^5 cpm of ^{32}P-labeled transcript in a mixture containing 15 mM KCl, 5 mM $MgCl_2$, 0.25 mM EDTA, 0.25 mM dithiothreitol, 12 mM HEPES (pH 7.9), 10% glycerol and 200 ng/μl of *Escherichia coli* tRNA in a total volume of 20 μl at 30°C for 30 min. The mixture was then treated with 50 U of RNase A at 37°C for 30 min and with 5 mg/ml of heparin at room temperature for 10 min to avoid non-specific protein binding. Samples were finally analyzed by 5% native PAGE and autoradiography.

3. Results

3.1. uPA increases uPAR expression in uPAR-transfected cells

We have previously shown that inactive uPA up-regulates cell surface expression of its receptor in several cell types except in kidney 293 cells which do not express uPAR and then cannot bind uPA [36]. uPAR expression can be regulated transcriptionally, by specific motifs contained in the promoter region of the uPAR gene [15,16], or post-transcriptionally, by determinants present within the 3′ untranslated region and in the coding region of uPAR mRNA [14,17,18]. We then stably transfected only the coding region of uPAR cDNA in 293 cells in order to investigate its role in the uPA-mediated regulation of uPAR expression at protein and mRNA levels.

293 cells, transfected with uPAR cDNA (uPAR-293, Fig. 1,

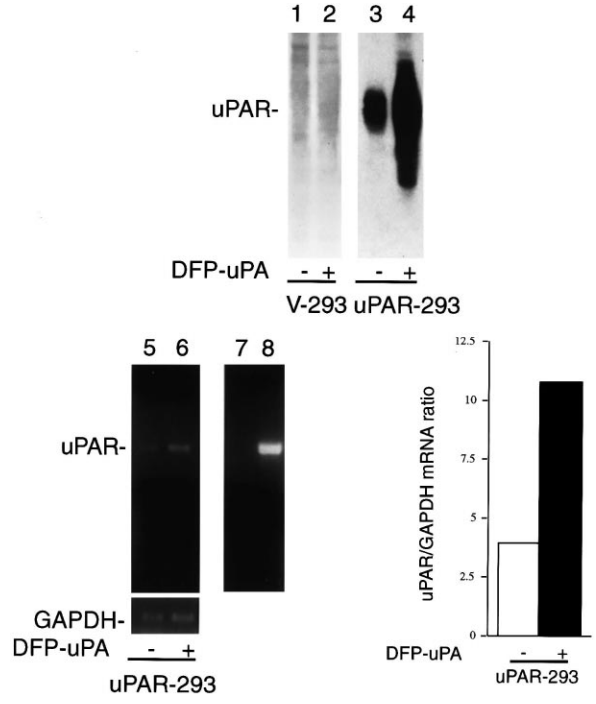

Fig. 1. uPA regulates uPAR expression in uPAR-transfected cells. 293 cells were stably transfected with pcDNA3 vector (V-293, lanes 1, 2) or with the coding region of uPAR cloned in pcDNA3 (uPAR-293, lanes 3, 4, 5, 6). Upper panel: transfected cells were treated with (lanes 2, 4) or without (lanes 1, 3) 5 nM DFP-uPA for 16 h at 37°C and lysed in 1% Triton X-100. 5 μg of total proteins of V-293 and uPAR-293 cell lysates was analyzed by 9% SDS–PAGE and Western blot with 1 μg/ml of an anti-uPAR monoclonal antibody. Lower panel: uPAR-293 cells were treated with (lane 6) or without (lane 5) 5 nM DFP-uPA for 16 h at 37°C and lysed in Trizol. Total RNA was prepared, reverse-transcribed and amplified by 25 cycles PCR using primers for uPAR or for GAPDH, as a control for DNA loading. Buffer (lane 7) and 10 ng of uPAR-cDNA (lane 8) were amplified as a negative and a positive control, respectively. PCR products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide, followed by photography under ultraviolet illumination. The levels of uPAR mRNA in lanes 5, 6 were estimated by densitometric scanning and normalized against GAPDH loading controls.

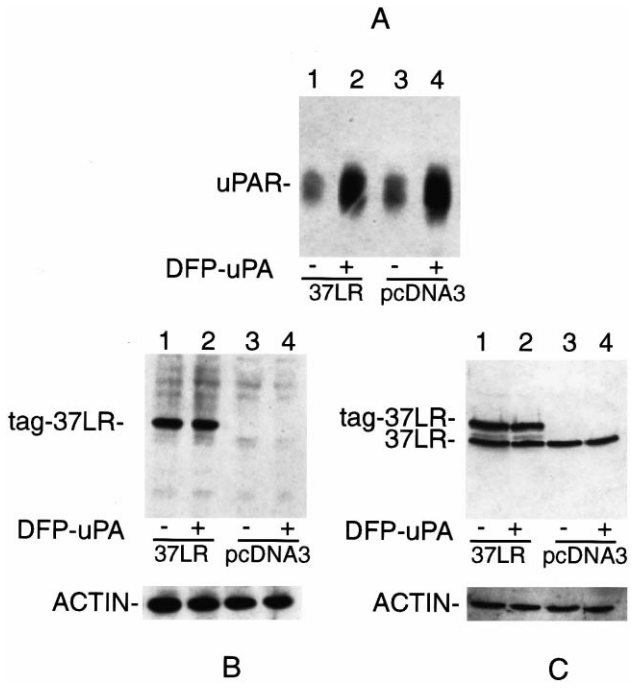

Fig. 2. uPA does not up-regulate uPAR expression in uPAR-293 cells through a transcriptional mechanism. uPAR-293 cells were transiently transfected with the cDNA of 37LR cloned into pcDNA3 (lanes 1, 2) or with the vector alone (lanes 3, 4). Cells were treated with (lanes 2, 4) or without (lanes 1, 3) 5 nM DFP-uPA for 16 h at 37°C and lysed. 1 μg (A) or 30 μg (B, C) of total proteins was analyzed by 9% SDS–PAGE and Western blot with anti-uPAR (A), anti-tag (B) and anti-37LR (C) antibodies. Filters B and C were hybridized with anti-actin polyclonal antibodies as a control for protein loading; actin was undetectable in filter A where 1 μg of total proteins was loaded.

upper panel, lanes 3, 4) or with the pcDNA3 vector alone (V-293, Fig. 1, upper panel, lanes 1, 2) as a control, were treated with (lanes 2, 4) or without (lanes 1, 3) 5 nM DFP inactivated uPA for 16 h at 37°C. uPA was inactivated in these and in all the following experiments in order to observe only the effects mediated by its interaction with uPAR, thus excluding those due to its enzymatic activity. Cells were then lysed and analyzed by 9% SDS–PAGE and Western blot using an anti-uPAR monoclonal antibody (upper panel, lanes 1–4). uPAR-transfected 293 cells synthesized a large amount of receptor, which increased after uPA treatment (lanes 3, 4). As expected, control cells V-293 did not express uPAR even after uPA treatment (lanes 1, 2). In this experiment a very small amount of total proteins (5 μg) was analyzed, since uPAR-293 cells express huge amounts of uPAR, therefore the same filters could not be hybridized with actin, as a control for protein loading; however, 30 μg of the same samples was analyzed by Western blot with anti-actin antibodies, showing the same total protein concentration (not shown).

We then investigated if the observed increase in uPAR expression correlated with the increase in uPAR mRNA by semi-quantitative RT-PCR and densitometric scanning (Fig. 1, lower panel). uPAR-293 cells were treated with (lane 6) or without (lane 5) 5 nM DFP-uPA for 16 h at 37°C and lysed. Total RNA was prepared, reverse-transcribed and amplified by 25 cycles PCR in the presence of primers for uPAR or for GAPDH as a loading control (lanes 5, 6). Buffer (lane 7) or uPAR cDNA (lane 8) were amplified as negative and positive controls, respectively. The analysis of PCR products by agarose gel showed the presence of uPAR mRNA in uPAR-transfected cells (lane 5) and its three-fold increase after treatment with DFP-uPA (lane 6), thus demonstrating that the uPA-mediated increase of uPAR expression observed by Western blot analysis was due to the increase of the specific uPAR mRNA.

These results indicate that the expression of uPAR cDNA lacking 3′ regulatory untranslated regions and under the control of a viral promoter (CMV) can still be regulated by uPA.

3.2. *uPA regulates uPAR expression at a post-transcriptional level in uPAR-transfected cells*

uPA could regulate uPAR expression in uPAR-293 cells at a transcriptional level, by activating the CMV promoter or at a post-transcriptional level, by promoting uPAR mRNA stabilization. To test the first hypothesis, uPAR-293 were transiently transfected with the cDNA of an unrelated protein, the 37 kDa precursor of the laminin receptor (37LR), provided with a T7-tag tail and cloned in pcDNA3, under the control of the CMV promoter, as in the case of uPAR cDNA. Double transfected cells were treated with 5 nM DFP-uPA, then lysed and analyzed by 9% SDS–PAGE and Western blot with anti-uPAR, anti-T7-tag and anti-37LR antibodies (Fig. 2). Western blot with anti-uPAR antibodies (panel A) showed that uPA up-regulates uPAR expression also in uPAR-293 cells transfected with 37LR-cDNA or with the vector alone, as a control (Fig. 2A, lanes 2, 4). Western blot with anti-T7-tag and anti-37LR antibodies (Fig. 2B and C, respectively) showed that, in the same cells, uPA did not increase the expression of tagged 37LR (B and C, lanes 2). Endogenous 37LR, as expected, was not modulated by uPA (Fig. 2C, lanes 2, 4). These experiments indicate that uPA does not induce activation of the CMV promoter which controls both uPAR and 37LR cDNA transcription in double transfected 293 cells.

We then examined the effect of uPA on uPAR mRNA stability, to test the hypothesis of a post-transcriptional mechanism of regulation (Fig. 3). Firstly, we analyzed the decay curve of uPAR mRNA in uPAR-293 cells by adding the transcription inhibitor DRB and harvesting RNA at various time points (upper panel). Semi-quantitative RT-PCR analysis of RNA and densitometric scanning showed 40% and 80% of uPAR mRNA degradation after 8 and 16 h of 0.1 mM DRB treatment, respectively (Fig. 3, upper panel, lanes 4, 5).

We then compared uPAR mRNA decay in DFP-uPA-treated or untreated uPAR-293 cells (Fig. 3, lower panel). Cells were stimulated with (lower panel, lanes 2, 4) or without (lower panel, lanes 1, 3) 5 nM DFP-uPA for 16 h at 37°C, in order to allow the induction of the initial transcripts, and then arrested transcriptionally with DRB for 16 h. Semi-quantitative RT-PCR analysis of total RNA and densitometric scanning showed that in the absence of uPA, uPAR mRNA was decreased to the same extent as in the upper panel, whereas uPA-induced up-regulation of uPAR mRNA was maintained also in the absence of transcription (Fig. 3, lower panel, lanes 3, 4), thus confirming a post-transcriptional regulation. Therefore, the observed uPA-dependent increase of uPAR mRNA was due to the increased stability of the mRNA and not to neosynthesis.

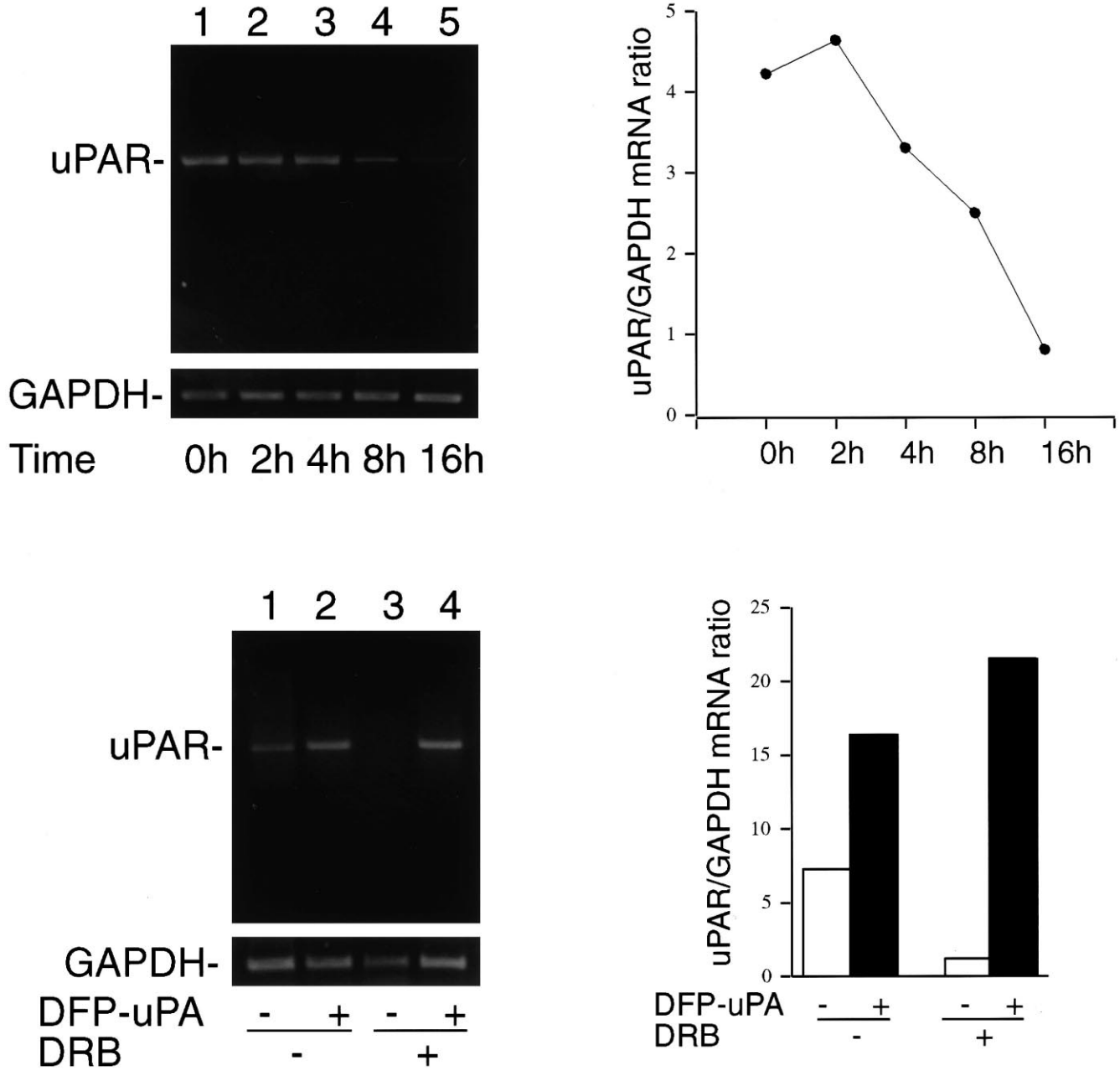

Fig. 3. uPA promotes the stability of uPAR mRNA. Upper panel: uPAR-293 cells were treated with 0.1 mM DRB in the absence of serum, and lysed after 0, 2, 4, 8 and 16 h. Lower panel: uPAR-293 cells were incubated with (lanes 2, 4) or without (lanes 1, 3) 5 nM DFP-uPA for 16 h at 37°C and then with 0.1 mM DRB (lanes 3, 4) or buffer (lanes 1, 2) for further 16 h and lysed. Total RNA was reverse-transcribed and amplified by 25 cycles PCR using primers for uPAR or for GAPDH, as a control for DNA loading. PCR products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide, followed by photography under ultraviolet illumination. The levels of uPAR mRNA were estimated by densitometric scanning and normalized against GAPDH loading controls.

3.3. Post-transcriptional mechanism of uPA-mediated regulation of uPAR expression in uPAR-transfected cells

To investigate the regulatory mechanism involved in uPAR mRNA stability, we examined whether the uPAR mRNA binds to transacting factor(s). We used a gel mobility shift assay to detect transacting factor(s), conferring lability or stability on uPAR mRNA in uPAR-293 cells. Total lysates of cells treated with or without 5nM DFP-uPA for 16 h were incubated with ^{32}P-labeled uPAR mRNA and treated with RNase A. The analysis on a 5% non-denaturing polyacrylamide gel (Fig. 4, upper panel) showed a single RNase resistant RNA–protein complex in unstimulated cells (lane 1); this complex was up-regulated by uPA treatment (lane 2). Binding of labeled uPAR mRNA was competed by a 100-fold excess of cold sense uPAR mRNA (lanes 3, 4), whereas it was not affected by cold antisense uPAR mRNA (lanes 5, 6). Labeled uPAR mRNA was not protected from RNase action in the absence of cell lysate (lane 7). Densitometric scanning showed a three-fold increase of the complex in uPA-treated cells as compared to untreated cells (Fig. 4, lower panel). These results indicate that the coding region of uPAR mRNA is able to bind specifically a cellular factor present in uPAR-293 cells

and that uPA up-regulates the formation of the resulting mRNA–protein complex. Therefore, the increased level of the complex could be related to the increased stability of uPAR mRNA induced by uPA.

3.4. Cycloheximide abrogates uPA-mediated up-regulation of uPAR expression in uPAR-transfected cells

uPA stimulation of uPAR-293 cells was also performed in the presence of cycloheximide, an inhibitor of protein synthesis, in order to investigate if uPA promotes the synthesis of factors involved in the increased stability of uPAR mRNA (Fig. 5). uPAR-293 cells were treated with (lanes 2, 4) or without (lanes 1, 3) 5 nM DFP-uPA for 6 h in the presence (lanes 3, 4) or in the absence (lanes 1, 2) of 10 μg/ml cycloheximide and lysed. Semi-quantitative RT-PCR analysis of total mRNA showed that 6 h of DFP-uPA treatment were sufficient to increase uPAR mRNA in uPAR-293 cells (lanes 1, 2). However, uPA, in the presence of cycloheximide, was not able to increase uPAR mRNA (lanes 3, 4), thus indicating that uPA effect requires protein synthesis.

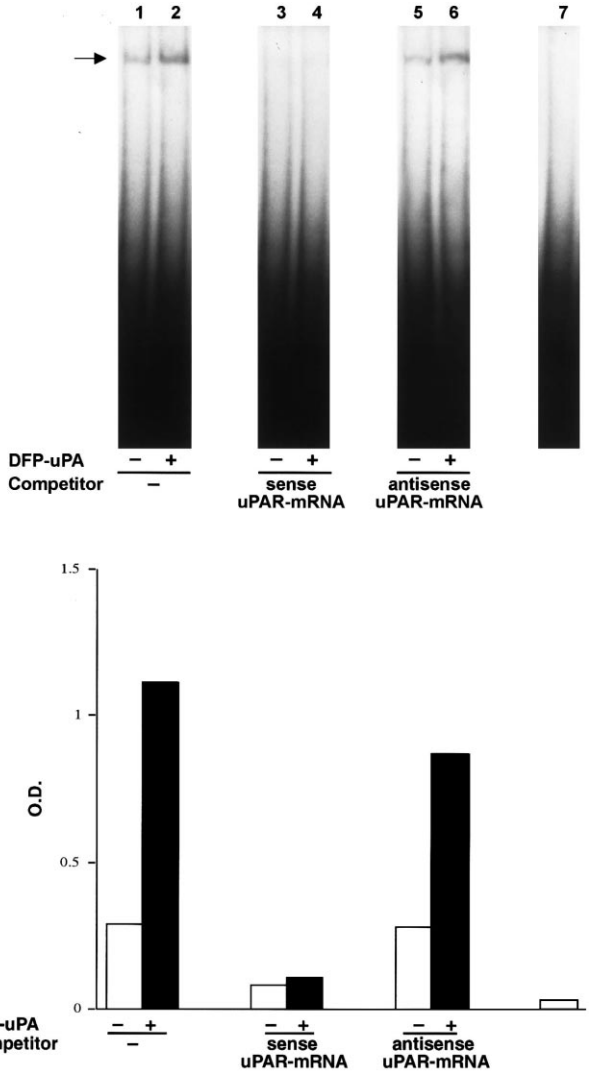

Fig. 4. uPA up-regulates a uPAR mRNA–protein complex, identified by gel mobility shift assay, in uPAR-293 cells. Cells were treated with (lanes 2, 4, 6) or without (lanes 1, 3, 5) 5 nM DFP-uPA for 16 h and lysed. 50 μg of total proteins was incubated with ^{32}P-labeled uPAR mRNA in the absence of a competitor (lanes 1, 2), in the presence of a 100-fold excess of cold sense uPAR mRNA (lanes 3, 4) or cold antisense uPAR mRNA (lanes 5, 6). The mixtures and the probe alone, as a control (lane 7), were then treated with RNase A and analyzed by 5% non-denaturing polyacrylamide gel and autoradiography. The arrow indicates the RNA–protein complex. Lower panel: The levels of uPAR mRNA–protein complex were estimated by densitometric scanning.

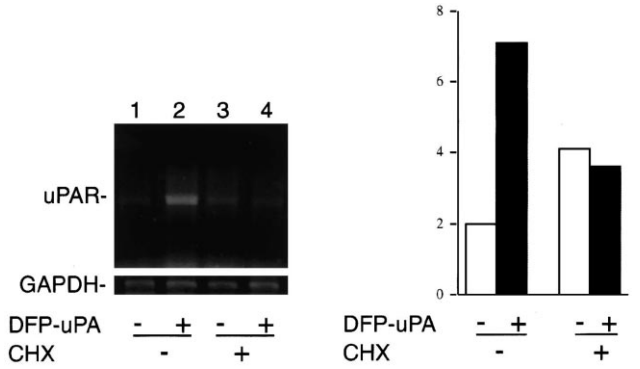

Fig. 5. Cycloheximide abrogates uPA-mediated up-regulation of uPAR expression in uPAR-transfected cells. uPAR-293 cells were treated with (lanes 2, 4) or without (lanes 1, 3) 5 nM DFP-uPA for 6 h in the presence (lanes 3, 4) or in the absence (lanes 1, 2) of 10 μg/ml cycloheximide. Total RNA was reverse-transcribed and amplified by 25 cycles PCR using primers for uPAR or for GAPDH, as a control for DNA loading. PCR products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide, followed by photography under ultraviolet illumination. The levels of uPAR mRNA were estimated by densitometric scanning and normalized against GAPDH loading controls.

4. Discussion

uPAR and its ligand uPA have been implicated in several pathological and physiological processes which require cell migration, such as tumor cell invasion and metastasis. Enhanced expression of uPAR has been described in several types of tumor, both on the cell surface and in plasma [1,32–35]. Therefore, it should be expected that the expression of uPAR is strictly controlled. In fact, uPAR expression can be regulated by various agents, which appear to act at both transcriptional and post-transcriptional levels. We have previously shown that uPA, upon binding uPAR, is able to increase the expression of its receptor on the cell surface of different cell types [36]. The same observation has been reported in a breast carcinoma cell line [38]; in this report, a correlation was also found between the levels of uPA and uPAR and the activity of the transcriptional factor Sp1 in breast carcinoma tissues, thus suggesting that uPA regulates uPAR expression at a transcriptional level, by increasing the activity of the transcriptional factor Sp1. We then investigated if uPA can also act at a post-transcriptional level, like other agents (PMA, TGFβ, etc.), showing that uPA can modulate uPAR expression in cells transfected only with the coding region of the receptor under the control of a viral promoter, which is not activated by the enzyme. The arrest of transcription did not affect the uPA capability of regulating uPAR expression, thus proving that uPA can act not only at a transcriptional level but also at a post-transcriptional level, by promoting uPAR mRNA stability. The increase in uPAR expression induced by uPA requires protein synthesis and cor-

relates with the increase in a uPAR mRNA–protein complex. All together these observations suggest that uPA is able to regulate uPAR expression at a post-transcriptional level by promoting the binding of the coding region of uPAR mRNA to a stabilizing factor. We do not know, at the moment, if uPA induces neosynthesis of this factor or activates a mechanism which renders an already present factor free to bind uPAR mRNA. Whichever is the mechanism, uPA, upon binding to uPAR, is able to activate cell signaling even though the receptor lacks a transmembrane and intracytoplasmic domain [10].

The presence in the mRNA coding region of sequences regulating mRNA stability has been described only in few other cases [27–31]. It has been reported that c-myc contains a destabilizing sequence in the message that can be inactivated by a 75 kDa stabilizing protein [29]; the same mechanism could also occur in uPA-dependent uPAR mRNA stabilization.

A regulatory determinant has been described also in the coding region of uPAR mRNA (nt 195–246); this determinant is able to bind an as yet uncharacterized cytoplasmic protein which appears to destabilize uPAR mRNA in human mesothelioma cells [14]. This finding is not in contrast with our observation. Indeed, it is possible that the same determinant binds both stabilizing and destabilizing regulatory proteins, such as the regulatory determinants (AREs) present in the untranslated region (UTR) of several mRNAs [19–23]. The identification of the binding site for the stabilizing factor will elucidate this point.

Investigations on the occurrence 'in vivo' of the regulatory mechanism observed in uPAR-transfected 293 cells will elucidate its importance in the uPA-uPAR-dependent invasiveness of metastatic cells.

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