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Minireview

Transcriptional Regulation of the Urokinase Receptor (u-PAR) – A Central Molecule of Invasion and Metastasis

Tina Fuchs and Heike Allgayer*

Department of Surgery, Grosshadern Clinic,
Ludwig-Maximilians University of Munich,
Marchioninistr. 15, D-81377 Munich, Germany

*Corresponding author

The phenomenon of tumor-associated proteolysis has been acknowledged as a decisive step in the progression of cancer. This short review focuses on the urokinase receptor (u-PAR), a central molecule involved in tumor-associated invasion and metastasis, and summarizes the transcriptional regulation of u-PAR. The urokinase receptor (u-PAR) is a heavily glycosylated cell surface protein and binds the serine protease urokinase specifically and with high affinity. It consists of three similar cysteine-rich repeats and is anchored to the cell membrane *via* a GPI-anchor. The u-PAR gene comprises 7 exons and is located on chromosome 19q13. Transcriptional activation of the u-PAR promoter region can be induced by binding of transcription factors (Sp1, AP-1, AP-2, NF-κB). One current study gives an example for transcriptional downregulation of u-PAR through a PEA3/ets transcriptional silencing element. Knowledge of the molecular regulation of this molecule in tumor cells could be very important for diagnosis and therapy in the near future.

Key words: Cancer / Transcription / Tumor-associated proteolysis / u-PAR.

Introduction

The phenomenon of tumor-associated proteolysis has been acknowledged as a decisive step in cancer progression, invasion and metastasis. One of the proteases which has been implicated in the invasive phenotype of tumor cells is the urokinase-type plasminogen activator (u-PA), a 55 kDa serine protease which, *via* activation of plasminogen to active plasmin, is able to cleave several components of the extracellular matrix including fibrin, fibronectin, proteoglycans and, as the main molecules in basement membranes, laminin and collagen IV (Duffy, 1992). u-PA interacts with factors often summarized as the plasminogen activator system, consisting of the enzyme (u-PA), one substrate (plasminogen), two receptors (u-PAR, plasminogen receptor) and three inhibitors (plasminogen activator inhibitors PAI-1 and PAI-2, protease nexin 1) (Blasi, 1993).

Pro-uPA binds to its specific cell surface receptor u-PAR specifically and with high affinity and becomes cleaved at position 157 by plasmin, which results in the active two-chain form of u-PA. u-PA can catalyze the activation of ubiquitously available plasminogen to plasmin, which in turn can initiate a proteolytic cascade resulting in a highly efficient degradation of extracellular matrix components (Blasi, 1993).

The U-PAR as a Cell Membrane Receptor with Diverse Functions

u-PAR is a 55–60 kDa, heavily glycosylated cell surface receptor with a three-domain structure defined by intrachain disulfide bonds. It consists of three similar cysteine-rich repeats of about 90 amino acids. The amino-terminal domain 1 is responsible for u-PA binding, while the other two domains bind vitronectin (Schvartz *et al.*, 1999). u-PAR is glycosilated at N-residues of glucosamine and sialic acid within the binding site, thereby regulating its affinity (K_d of 0.1–1.0 nM) for u-PA (Behrendt *et al.*,1990). The carboxy-terminal part of u-PAR is anchored to the cell membrane *via* a glycosyl-phosphatidylinositol chain. This GPI-anchor is hypothesized to enable a high intramembrane mobility (Moller, 1993).

The u-PAR plays an important role in many physiological and pathological functions including wound healing, tissue remodeling, and especially tumor cell invasion and metastasis. The ability of the u-PAR to promote these biological effects is a consequence of its diverse functions. First, receptor-bound uPA, as compared to the fluid phase enzyme, activates plasminogen to plasmin much more efficiently, as reflected by a 40-fold decrease in the K_m value of urokinase for its substrate (Ellis *et al.*, 1991). Plasmin can either directly degrade basement membranes and ECM or activate other proteases. ECM degradation is a key step in the process of cancer invasion. Second, u-PAR clears urokinase-inhibitor complexes from the extracellular space *via* an α2-macroglobulin receptor-dependent endocytotic mechanism (Cubellis *et al.*, 1990; Conese *et al.*, 1994). Third, the u-PAR interacts with the extracellular domain of integrins (Wei *et al.*, 1996; Yebra *et al.*, 1996). Integrins mediate adhesion of cells to extracellular matrices as well as intercellular inter-

actions, and modulate transduction of regulatory signals that are central to inflammation, immunity, hemostasis, and tumor progression. u-PAR physically interacts on leukocytes with β2-integrin (Bohuslav *et al.*, 1995), and on fibrosarcoma cells with β1- and β3-integrins (Xue *et al.*, 1997). Current evidence favors a model in which ligand-induced integrin clustering, a central event in integrin activation, promotes caveolin oligomerization leading to release and/or activation of Src-family kinases and initiation of integrin signaling. The presence of u-PAR promotes these events because the extracellular domain of u-PAR binds to β1- and β2-integrins, and the GPI anchor of u-PAR, like that of other GPI-anchored proteins, interacts with cholesterol-rich membrane domains enriched in caveolin and tyrosine kinases (Chapman *et al.*, 1999). Fourth, the u-PAR is chemotactic for human monocytes and mast cells, and this may contribute to inflammatory and tissue remodeling processes, which are also often observed in tumor-infiltrated areas (Resnati *et al.*, 1996; Sillaber *et al.*, 1997).

The Relevance of u-PAR Gene Expression to Invasion and Metastasis

Numerous studies have shown an overexpression of the u-PAR gene in diverse human malignant tumors in contrast to the corresponding normal tissue and/or surrounding stromal cells (Pyke *et al.*, 1991a,b; Jankun *et al.*, 1993; Morita *et al.*, 1998) and suggested u-PAR as a characteristic of the invasive or even the malignant phenotype (Hollas *et al.*, 1991; Bianchi *et al.*, 1994; Wang *et al.*, 1994). Further studies demonstrated u-PAR and the plasminogen activator system to be strong and independent parameters predicting a poor prognosis of cancer patients (Duffy *et al.*, 1990; Jaenicke *et al.*, 1993) suffering from lung (Pedersen *et al.*, 1994), colon (Mulcahy *et al.*, 1994; Ganesh *et al.*, 1994), esophageal and gastric carninoma (Allgayer *et al.*, 1998; Nekarda *et al.*, 1998). Duffy *et al.* (2002) showed that breast cancer patients with high u-PA activity in their primary tumors had a shorter disease-free interval than patients with low u-PA activity.

Experimental evidence implicating u-PAR in tumor invasion and metastasis are as follows: overexpression of a human u-PAR cDNA increased the ability of human osteosarcoma cells to penetrate a barrier of reconstituted basement membrane (Kariko *et al.*, 1993). Ossowski (1988) demonstrated that the invasive potential of tumor cells into a chicken embryo chorioallantoic membrane is correlated with u-PAR-associated proteolytic activity. Another study (Kook *et al.*, 1994) revealed that the expression of an antisense u-PAR cDNA in Hep3 squamous carcinoma cells decreased their invasiveness into a modified chorioallantoic membrane. Furthermore, antisense oligonucleotides inhibiting u-PAR gene expression reduced *in vitro* invasion of transformed human fibroblasts (Quattrone *et al.*, 1995). In glioblastoma, an anti-u-PAR monoclonal antibody effectively blocked matrigel invasion of treated cells (Mohanam *et al.*, 1993). In cultured human lung cancer cell lines, optimum invasiveness was seen only if u-PA, PAI-1 and u-PAR were co-expressed (Liu *et al.*, 1995). High concentrations of PAI-1 also correlate with poor prognosis in patients with breast cancer, including the subgroup with node-negative disease. Possible mechanisms by which PAI-1 contributes to cancer dissemination include modulating cellular adhesion and migration, playing a role in angiogenesis, and stimulating cell proliferation (Duffy *et al.*, 2002). Moreover, Kim *et al.* (1998) have shown elegantly that the expression of the u-PAR gene by tumor cells, besides u-PA and MMP-9, is required for the intravasation of blood vessels. Min *et al.* (1996) reported that u-PAR antagonists prevent tumor growth and angiogenesis, suggesting that u-PAR gene expression is important for tumor and metastasis establishment and outgrowth. Finally, Lakka *et al.* (2001) reported that the transfection of an adenovirus construct (Ad-uPAR) in non-small cell lung cancer cell lines with high levels of u-PAR resulted in a decrease of u-PAR levels by 80–90% , leading to a reduction of invasion and metastasis. Taken together, all of these studies suggest u-PAR as a critical molecule for invasion, intravasation, and metastasis.

Transcriptional Activation of u-PAR Gene Expression

The objective to counter u-PAR gene expression in malignant tumors necessarily implies the question as to how it is regulated. The mechanisms of regulation and causes for an upregulation of u-PAR in malignant cells are still the objectives of intensive investigations. Although altered mRNA stability and receptor recycling may be involved, the amounts of u-PAR are controlled mainly at the transcriptional level in malignancies such as colon cancer (Lund *et al.*, 1995; Lengyel *et al.*, 1996; Shetty *et al.*, 1997; Gum *et al.*, 1998). Altered transcription of the gene is the main mediator of u-PAR gene expression brought about by, for example, epidermal growth factor (EGF; Boyd, 1989), basic fibroblast growth factor (FGF; Mignatti *et al.*, 1991), vascular endothelial growth factor (VEGF; Mandriota *et al.*, 1995), transforming growth factor β type 1 (TGF-β1; Lund *et al.*, 1995), phorbol 12-myristate 13-acetate (PMA; Lengyel *et al.*, 1996), IFN-α or IFN-γ, (Wu *et al.*, 2002), protein kinase C (PKC; Ando *et al.*, 1996), protein kinase A (PKA)/c-AMP (Langer *et al.*, 1993; Li *et al.*, 1995), the MAPK- (Lengyel *et al.*, 1997) and the JNK-pathway (Gum *et al.*, 1998).

The u-PAR gene spans seven exons and is located on chromosome 19q13 (Borglum *et al.*, 1992). Transcription of the gene yields a 1.4 kb mRNA or an alternatively spliced variant lacking the membrane attachment peptide sequence (Roldan *et al.*, 1990; Pyke *et al.*, 1993). The human u-PAR promoter sequence was first described by Wang *et al.* (1995) and Soravia *et al.* (1995). Like classic

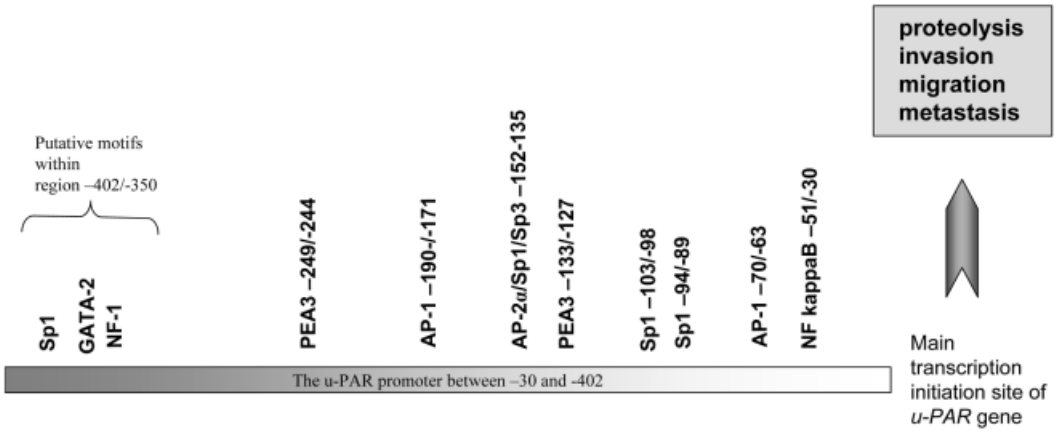

Fig. 1 Overview of Potential Promoter Elements Regulating u-PAR Gene Expression.

'housekeeping genes', it lacks TATA and CAAT boxes and contains a GC-rich proximal sequence with multiple Sp1 consensus elements. Using primer extension analysis, Soravia *et al.* (1995) reported three transcriptional start sites, the most upstream of which – an A following a C – appeared to be the main transcription initiation site and revealed partial similarity to the consensus initiator sequence of the dihydrofolate reductase (DHFR) gene (Means *et al.*, 1990; Soravia *et al.*, 1995). The most proximal 135 base pairs of the human u-PAR promoter showed 68% similarity to the murine u-PAR promoter (Suh *et al.*, 1994).

An overview on the current status on putative or experimentally validated promoter elements regulating the u-PAR gene is given in Figure 1.

First reports of Soravia *et al.* (1995) suggested that the basal expression of the gene is regulated *via* Sp1 motifs proximal and upstream of the transcriptional start site. In colon cancer, both the constitutive and PMA-inducible expression of the gene required a footprinted region located at basepairs -190/-171 of the promoter containing an AP-1 consensus motif bound by Jun-D, c-Jun, c-Fos and Fra-1 (Lengyel *et al.*, 1996). This motif also mediates the induction of u-PAR gene expression *via* the MAPK- and the JNK-pathway (Lengyel *et al.*, 1997; Gum *et al.*, 1998).

This AP-1 consensus motif is also required for induction of u-PAR gene expression brought about by the K-ras oncogene (Allgayer *et al.*, 1999). A substantial reduction of endogenous u-PAR protein and u-PAR-mediated proteolysis was observed in HCT116 clones in which activated K-*ras* had been deleted. In gelshift and CAT-reporter analysis we detected a decrease of the binding of c-Jun, JunD, c-Fos and Fra-1 in the K-ras-knockout clones, and this was paralleled by a severe reduction of promoter activity when the AP-1-consensus motif within promoter region -190/-171 was deleted. These results suggest that activated K-*ras* regulates u-PAR and u-PAR-mediated proteolysis in colon cancer, at least in part *via* the -190/-171 region of the promoter bound by AP-1-transcription factors.

Ras uses a variety of functionally diverse effectors that transduce signals through multiple pathways. The binding of effector proteins involves at least interactions with the effector loop (amino acids 32 – 40) of Ras. The use of multiple effector pathways by Ras has been demonstrated through the use of constitutively activated mutants of Ras with point mutations in the effector domain. Muller *et al.* (2000) showed that constitutively active V12 H-Ras and Rho-A lead to an increased transcription from the u-PAR promoter. Thereby the use of Ras-effector-loop mutants indicated that signaling *via* multiple Ras effectors is necessary for the maximum activation of u-PAR transcription. Okan *et al.* (2001) further showed that a constitutively activated RalA mutant (RalA 72L) stimulates u-PAR transcription. RalA is one of two highly similar (85% identity) GTPases of the Ras subfamily. The stimulation of u-PAR transcription required the presence of the ATF2-like AP-1 site at 70 bp and the c-Jun binding motif at -184 bp upstream of the major transcriptional start site. Activation of transcription is inhibited by a dominant-negative mutant of c-Src, indicating that c-Src is a downstream effector of RalA. These data showed activation of u-PAR trancription by RalA through an AP-1-dependent mechanism.

Another footprinted region (-148/-124) of the u-PAR-promoter containing putative binding sites for (mismatched) Sp1, AP-2 and PEA3 binding motifs (Lengyel *et al.*, 1996) was shown to be bound by an AP-2α-like protein closely related to, but not identical with, authentic AP-2α, Sp1 and Sp3 transcription factors. The region required for binding were identified as -152/-135 (Allgayer *et al.*, 1999a). Binding of the AP-2α-like protein was found to be important for a constitutively high u-PAR promoter activity in a highly invasive colon cancer cell line and for PMA-stimulated u-PAR expression in a cell line with low constitutive u-PAR expression. Interestingly, a dominant-negative AP-2 expression construct not only reduced u-PAR promoter activity and u-PAR gene expression but also substantially inhibited u-PAR-mediated proteolysis. These results suggest that an inhibition at the transcriptional level can be used to suppress u-PAR-me-

diated proteolysis, thereby potentially inhibiting invasion and metastasis.

The binding of Sp1 transcription factor to region -152/-135 of the u-PAR promoter was shown to be important in part for PMA-induced u-PAR promoter activity, but, more interestingly, for the induction of u-PAR gene expression by the *c-src* oncogene in colon cancer (Allgayer *et al.*, 1999b). In SW480 colon cancer clones stably expressing a constitutively active Src (Y-*c-src*527F), increased u-PAR protein and laminin degradation paralleling elevated Src activity was evident as compared to parental cells. Nuclear run-on experiments indicated that the increased u-PAR protein was due largely to transcriptional activation. While transient transfection of SW480 cells with Y-*c-src*527F induced a u-PAR-CAT-reporter, mutations preventing Sp1-binding to promoter region -152/-135 abolished this induction. Mobility shift assays revealed increased Sp1 binding to region -152/-135 with nuclear extracts of Src-transfected SW480 cells. Finally, the amounts of endogenous u-PAR in resected colon cancers significantly correlated with Src-activity. These data suggest that u-PAR gene expression and u-PAR-mediated proteolysis are regulated by Src, which requires the promoter region (-152/-135) bound by Sp1, thus demonstrating for the first time that transcription factor Sp1 is a downstream effector of Src.

The transcription factor NF-κB has also been implicated in the regulation of the u-PAR. In a recent study, Wang *et al.* (2000) implicated NF-κB bound to a non-consensus NF-κB motif (-51/-30) in the constitutive expression of the u-PAR gene in HCT116 colon cancer cells. Co-transfection with a dominant-negative IκB-kinase-2 expression vector reduced u-PAR promoter activity up to 75%, demonstrating that this region is required for promoter activity.

Transcriptional Downregulation of u-PAR

Although many reports showed a transcriptional activation of u-PAR gene expression by diverse promoter motifs, there is not much known about potential silencer elements. One example for transcriptional suppression has been given by Hapke *et al.* (2001), indicating a PEA3-element at -248 bp as a mediator of integrin-induced suppression: the adhesion receptor β3-integrin downregulated u-PAR in CHO cells, an overexpression of β3-integrin leading to a reduction of u-PAR mRNA and u-PAR promoter activity. Downregulation of u-PAR expression can be increased by clustering of αvβ3-integrin, which was achieved after ligation with immobilized vitronectin or the antibody LM609. After transient transfection, a CAT reporter driven by 398 bp of 5'-flanking sequence of the u-PAR promoter was significantly reduced by a β3-integrin expression construct, whereas transfection of αv-integrin alone did not show any effect on the u-PAR promoter. This indicated that the u-PAR promoter contains a transcription factor binding site that might account for a β3-integrin-mediated repression of u-PAR gene transcription in CHO cells. Using 5'-deletion fragments of the u-PAR promoter and the β3-integrin expression vector, a sequence between -398 and -197 bp of the u-PAR promoter was identified to be essential for β3-integrin-mediated u-PAR gene suppression. Deletion of the *PEA3/ets* motif at position -248 prevented the ability of β3-integrin to downregulate the u-PAR promoter. Furthermore, an expression vector encoding PEA3 inhibited the activity of the wild type but not the activity of the mutated form. In β3-integrin-overexpressing cells, nuclear factors showed enhanced binding activity for the *PEA3/ets* site and PEA3 was identified by an anti-PEA3 antibody which inhibited DNA-protein complex formation. These experiments suggest a *PEA3/ets* motif at -248 bp as a transcriptional repressor of the u-PAR gene (Hapke *et al.*, 2001).

Recently, a new tumor suppressor gene (*pdcd4*) inhibiting neoplastic transformation of epithelial cells has been identified, however, its role in invasion and mechanisms of regulation has not been investigated yet (Cmarik *et al.*, 1999; Hsu *et al.*, 2000; Yang *et al.*, 2001). Preliminary studies of our group implicate that Pdcd4 might downregulate u-PAR gene expression at least in part by inhibiting u-PAR gene transcription, and a region containing putative binding sites for Sp-1, GATA-2 and NF-1 (-402/-350 bp), but also the PEA3/ets motif at -248 bp might be mediators of this suppression (Leupold *et al.*, 2002)

Conclusion

In summary, the u-PAR is a central molecule of invasion and metastasis with clinical-prognostic value that has been shown to be regulated especially at the transcriptional level by diverse *cis*-elements and *trans*-acting factors. These mediate multiple means of induction or suppression by different regulators involving, for example, signaling cascades, oncogenes, or even potentially tumor suppressor genes. When speculating about developing future therapeutic strategies based on this knowledge, it is interesting to hypothesize that, besides already existing u-PAR-inhibitory strategies at the protein level (antibodies, small molecular compounds; Wilhelm *et al.*, 1994; Renatus *et al.*, 1998; Aguirre-Ghiso *et al.*, 1999) or efforts to inhibit at the signal transduction level (*e.g.* MAPK-, Src- and K-Ras-inhibition) even a direct targeting of transcriptional mechanisms could be considered.

To support this notion, in a first clinical study comparing transcription factor binding to u-PAR promoter region -152/-135 in 145 patients with resected colorectal or gastric cancers in primary tumors and corresponding normal mucosae, we suggested a tumor-specific transactivation of u-PAR gene expression by this promoter region in about 60% of cases (Schewe *et al.*, 2001). Therefore, it might be possible in the future to identify subgroups of patients where a targeting of certain promoter regions with a preference for tumor cells could be speculated. It

should be attractive to further develop targeting methods such as triplex binding oligonucleotides which might be appropriate to be applied as targeting tools at the transcriptional level.

Ultimately, there is to be expected an exciting development in next few years to extend the knowledge on u-PAR and its regulators as molecular markers to improve the prediction of an individual patient's clinical outcome, define approaches in the treatment of cancer invasion and metastasis, and define appropiate target subpopulations of patients.

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