

# Cleavage of urokinase receptor regulates its interaction with integrins in thyroid cells

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**Abstract** The urokinase-type plasminogen activator uPA-R can regulate integrin functions by associating with several types of  $\beta$ -subunit. We have recently shown that normal thyroid TAD-2 cells express both a native and a cleaved form of uPA-R which lacks the binding domain for uPA. We found this cleaved form to be present in reduced amounts in papillary and follicular thyroid carcinoma cells and completely absent in cells derived from an anaplastic thyroid carcinoma (ARO). We now report that in normal thyroid cells the intact form of uPA-R strongly associates with  $\beta$ -1 integrins, whereas its cleaved form does not. uPA-R expressed by ARO cells shows a stronger resistance to the cleavage mediated by uPA, plasmin and chymotrypsin than does uPA-R expressed by normal thyroid cells. This resistance to cleavage correlates with the higher level of glycosylation of uPA-R of ARO cells as compared to that of cleavable uPA-R of normal thyroid cells. These results suggest that uPA-R cleavage, which occurs in several cell types, represents a mechanism regulating the interactions of uPA-R with integrins and, possibly, the subsequent integrin-mediated cell adhesion. Moreover we hypothesize that glycosylation regulates uPA-R cleavage and, indirectly, its interaction with integrins.

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**Key words:** Urokinase; Urokinase receptor;  $\beta$ -Integrin; Thyroid

## 1. Introduction

Many events related to tumor growth and metastasis depend on the ability of cells to modulate their adherence to adjacent cells or to the surrounding extracellular matrix (ECM) [1]. In order to promote migration and tissue invasion tumor cells must strictly regulate their adherence to ECM and extracellular proteolysis. Interestingly, the receptor for the urokinase-type plasminogen activator (uPA-R) is able to fulfil both of these functions [2–4]. The role of uPA-R was traditionally seen as the concentration of the proteolytic activity of its ligand (uPA) on the cell surface, thus amplifying plasminogen activation [5]. Recently uPA-R has also been shown to be directly involved in cell adhesion to ECM. In fact, uPA-R can act as a receptor for vitronectin (VTN), a component of ECM associated with malignant tumors [6], and interacts with integrins of the  $\beta$ 1,  $\beta$ 2 and  $\beta$ 3 families [7–9]; uPA-R's interaction with VTN can be increased by binding uPA, independently of its enzymatic activity [6], and, finally, its association with  $\beta$ -integrins can be modulated by ECM components themselves [9].

uPA-R is a highly glycosylated single chain protein. It consists of three different domains: domain 1 ( $D_1$ ), which binds uPA, domain 3 ( $D_3$ ), anchoring uPA-R to the cell surface by a glycosphosphoinositol (GPI) tail, and domain 2 ( $D_2$ ), connecting  $D_1$  to  $D_3$  [3]. The linker region connecting domains 1 and 2 can be cleaved by several proteases such as plasmin, chymotrypsin and uPA itself at physiological concentrations [10,11]. The cleavage occurs in several cell lines, thus expressing on the cell surface the cleaved form of uPA-R ( $D_2+D_3$ ), lacking the uPA binding domain ( $D_1$ ) [12]. Since uPA-R ( $D_2+D_3$ ) cannot bind uPA it has been proposed that the cleavage may be a regulatory step in the cell surface-associated plasminogen activation cascade [11]. Soluble cleaved uPA-R is also unable to bind VTN, even though the binding site for this protein has been assigned to the  $D_2+D_3$  domains [13].

We have recently characterized the plasminogen activation system in thyroid cells [14], showing that normal thyroid cells express both intact uPA-R and its cleaved form. Interestingly, the cleaved form of uPA-R is largely expressed in normal cells, decreases in cells derived from more aggressive thyroid carcinomas and disappears in anaplastic carcinoma cells.

In the present report, we investigated whether uPA-R cleavage can regulate its interactions with integrin adhesion molecules in thyroid cells, and how cell surface uPA-R cleavage, in turn, can be regulated.

## 2. Materials and methods

### 2.1. Reagents

Mouse monoclonal antibodies R<sub>4</sub>, recognizing an epitope on uPA-R domains 2+3, S1, directed to reduced and alkylated uPA-R and the anti-uPA c15 [11] were kindly provided by Dr. G. Hoyer-Hansen (Finsen Laboratory, Copenhagen, Denmark). Rabbit polyclonal antibodies against  $\beta$ 1 and  $\beta$ 3 integrin chains were provided by Dr. G. Tarone (University of Turin, Italy) and uPA-R cDNA by Dr. M.P. Stoppelli (IIGB, CNR, Naples, Italy). Enhanced chemiluminescence (ECL) detection kit was from Amersham International (Amersham, UK) and Lipofectamine from Gibco BRL (Life Technologies, MD, USA). Polyvinylidene fluoride (PVDF) filters were from Millipore (Windsor, MA, USA) and protein A-Sepharose from Pharmacia (Uppsala, Sweden). Collagen, laminin, vitronectin and fibronectin were from Collaborative Research (Bedford, MA, USA).

### 2.2. Cell culture

TAD-2 and ARO cell lines were derived from human fetal thyroid cells [15] and from anaplastic thyroid carcinoma [16] respectively. Both cell lines were cultured in RPMI 1640 medium (Flow, McLean, VA, USA) supplemented with 10% fetal calf serum (Gibco).

### 2.3. Immunoprecipitation

Immunoprecipitation was performed as previously described [7]. Cells ( $2.5 \times 10^6$ /sample) were washed twice with microtubule stabilization buffer (0.1 M PIPES, pH 6.9, 2 M glycerol, 1 mM EGTA, 1 mM magnesium acetate), and then extracted in 0.2% Triton X-100 supplemented with protease inhibitors. The insoluble residue, enriched in

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cytoskeleton-associated proteins, was solubilized in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% deoxycholate, 0.1% SDS, 1% Triton X-100 and protease inhibitors), and preincubated with non-immune serum and protein A-Sepharose, for 2 h at 4°C. After centrifugation, supernatants were incubated with 3 µl of antisera to β1 and β3 integrins or with 3 µl of non-immune serum, for 2 h at 4°C. After 30 min incubation with protein A-Sepharose, immunoprecipitates were washed, and analyzed by 9% SDS-PAGE and Western blot, using R<sub>4</sub> monoclonal anti-uPA-R antibody, at a concentration of 1 µg/ml.

#### 2.4. Western blot

Cells were lysed in 1% Triton X-100 in PBS and protein content was measured by a colorimetric assay (Bio-Rad, Richmond, CO, USA). 50 µg of total proteins or immunoprecipitated samples (see above) were 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 R<sub>4</sub> anti-uPA-R antibody, at a concentration of 1 µg/ml. Finally, washed filters were incubated with horseradish peroxidase-conjugated anti-mouse antibody (Bio-Rad) and detected by ECL.

#### 2.5. Transfection

uPA-R cDNA was cloned in the *EcoRI* site of pcDNA3 (Invitrogen, San Diego, CA, USA) and the resulting plasmid was named uPA-R-pcDNA3.  $5 \times 10^6$  cells, cultured overnight in 100 mm tissue culture dishes, were transiently transfected with 10 µg of uPA-R-pcDNA3 or control vector pcDNA3 and 60 µl of Lipofectamine for 5 h at 37°C (5% CO<sub>2</sub>). Cells were analyzed at 72 h after transfection.

#### 2.6. Deglycosylation assay

Cells ( $5 \times 10^6$ ) were lysed in 0.5 ml of 1% Triton X-100 supplemented with protease inhibitors. Lysates were then boiled for 3 min in the presence of 0.5% SDS and 1.6 mM dithiothreitol. Aliquots of the denatured samples (20 µl) were adjusted to include 200 mM sodium phosphate, pH 8.6, 1.5% Triton X-100, 10 mM EDTA, up to a volume of 60 µl. The samples were then incubated with or without 2 units of *N*-glycosidase F at 37°C for 20 h. Finally they were treated with 25 mM iodoacetamide for 1 h at 20°C and analyzed by Western blot using 10 µg/ml of the monoclonal antibody (S1) which reacts with reduced and alkylated uPA-R.

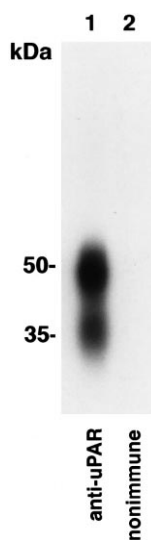


Fig. 1. uPA-R in normal thyroid cells. Biotinylated cell surface antigens of TAD-2 cells were immunoprecipitated with 10 µg/ml of R<sub>4</sub> anti-uPA-R monoclonal antibody (lane 1) or 10 µg/ml of non-immune Ig (lane 2). Immunoprecipitated samples were electrophoresed on 9% SDS-PAGE and transferred onto PVDF membrane. Biotinylated antigens were detected by streptavidin and ECL.

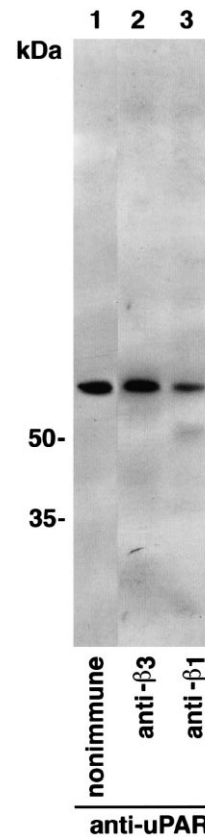


Fig. 2. Cleaved uPA-R and integrins. TAD-2 cell lysates were immunoprecipitated with polyclonal antisera against β3 (lane 2) and β1 (lane 3) chains or with non-immune serum (lane 1). Immunoprecipitated samples were electrophoresed on 9% SDS-PAGE and analyzed by Western-blot with 1 µg/ml of R<sub>4</sub> anti-uPA-R antibody.

### 3. Results

#### 3.1. Cleaved uPA-R and integrins

Normal thyroid TAD-2 cells express equivalent amounts of both native and cleaved forms of uPA-R (Fig. 1, lane 1) and detectable amounts of β1 and β3 integrins [17]. We investigated whether uPA-R cleavage in this cell line can interfere with its association to these integrins.

RIPA lysates of TAD-2 cells, enriched in cytoskeleton-associated proteins [7], were immunoprecipitated with polyclonal antisera against β1 and β3 chains and analyzed by Western blot, using R<sub>4</sub> monoclonal antibody, which recognizes both forms of uPA-R. ECL staining showed the presence of intact uPA-R (MW 50 kDa) in the samples immunoprecipitated with anti-β1 (Fig. 2, lane 3), whereas the receptor was undetectable in the sample immunoprecipitated with anti-β3 antibodies (Fig. 2, lane 2) and in the control sample immunoprecipitated with non-immune serum (Fig. 2, lane 1). Cleaved uPA-R (MW 35 kDa) was absent in all immunoprecipitated samples (Fig. 2, lanes 1–3). However, the signal of intact uPA-R was extremely weak, just above the threshold of detection. It is therefore possible that slightly lower amounts of cleaved uPA-R, coprecipitated with β1 integrins, could have escaped observation. In order to exclude this possibility, uPA-R was overexpressed in TAD-2 cells by transfection with uPA-R cDNA. Transfected cells expressed very high amounts of both forms of uPA-R (Fig. 3, lane 1), to such an extent that

the amount of endogenous uPA-R became negligible (Fig. 3, lane 2). Lysates of transfected TAD-2 cells were immunoprecipitated with an anti- $\beta 1$  antiserum and analyzed by Western blot with R<sub>4</sub> anti-uPA-R antibody. Large amounts of intact uPA-R co-precipitated with  $\beta 1$  integrins, giving rise to a strong and readily detectable signal, whereas cleaved uPA-R was present in a much smaller amount (Fig. 3, lane 3).

These results clearly show that the intact form of uPA-R (50 kDa) strongly associates with  $\beta 1$  integrins, whereas its cleaved form interacts poorly with them.

### 3.2. Resistance of uPA-R to cleavage

Since cleavage of uPA-R could regulate uPA-R-integrin interactions, we investigated whether the cleavage itself is regulated by the cell.

We have previously shown that cleaved uPA-R is absent in a cell line derived from an anaplastic thyroid carcinoma (ARO cells), expressing only the intact form of uPA-R with a MW slightly higher (55 kDa) than that of uPA-R of TAD-2 cells (50 kDa) [14]. We therefore investigated whether the absence of cleaved uPA-R was the result of a resistance of the intact receptor to proteolytic cleavage.

Cl5, an antibody able to inactivate endogenous uPA, strongly decreases the formation of cleaved uPA-R on the cell surface of monocyte-like cell lines [11]. Therefore TAD-2 and ARO cells were grown in the presence of cl5, in order to obtain TAD-2 cells expressing most uPA-R in the intact form, as well as ARO cells (Fig. 4, lanes 1–2, 7–8). Cl5-treated cells were incubated with uPA, plasmin and chymotrypsin, enzymes able to cleave the linker region of domains 1 and 2 of uPA-R [10]. Cells were then lysed and analyzed by Western blot with R<sub>4</sub> anti-uPA-R antibody. uPA and plasmin partially cleaved uPA-R of TAD-2 cells, causing a decrease in the amount of the intact form and a proportional increase of the amount of the cleaved form (Fig. 4, lanes 3 and 4); higher doses of plasmin and chymotrypsin almost completely cleaved

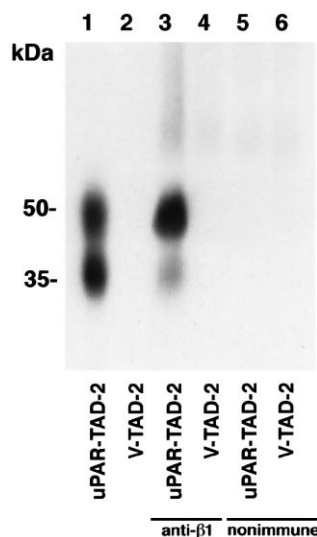


Fig. 3. Cleaved uPA-R and  $\beta 1$  integrins in transfected TAD-2 cells. TAD-2 cells, transfected with uPAR-pcDNA3 (lanes 1,3,5) or pcDNA3 vector (lanes 2,4,6), were lysed (lanes 1,2) and immunoprecipitated with antiserum against  $\beta 1$  integrin chain (lanes 3,4) or with non-immune serum (lanes 5,6). Immunoprecipitated samples were electrophoresed on 9% SDS-PAGE and analyzed by Western blot with 1  $\mu$ g/ml of R<sub>4</sub> anti-uPA-R antibody.

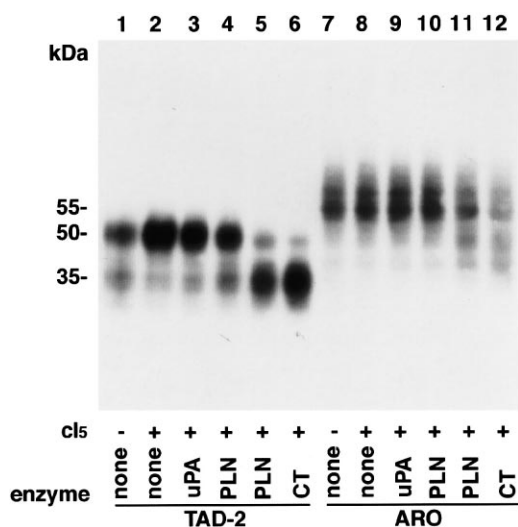


Fig. 4. Resistance of uPA-R to cleavage. ARO and TAD-2 cells, grown in the presence (lanes 2–6 and 8–12) or in absence (lanes 1,7) of cl5 antibody, were incubated with medium (lanes 1–2,7–8) or treated with  $10^{-8}$  M uPA (lanes 3,9),  $10^{-7}$  M and  $10^{-6}$  M plasmin (lanes 4,10 and 5,11, respectively), 1 mg/ml chymotrypsin (lanes 6,12) for 2 h at 37°C. Cells were then lysed, electrophoresed on 9% SDS-PAGE and analyzed by Western blot with 1  $\mu$ g/ml of R<sub>4</sub> antibody.

uPA-R, which appeared only in the cleaved form (Fig. 4, lanes 5 and 6).

In ARO cells, which produce only traces of uPA [14], no modifications of the higher molecular weight uPA-R were observed after treatment with cl5, as expected (Fig. 4, lanes 7 and 8). uPA and low doses of plasmin did not cleave uPA-R (Fig. 4, lanes 9 and 10), and higher doses of plasmin and chymotrypsin were only partially effective in cleaving the receptor (Fig. 4, lanes 11 and 12), whereas high doses of plasmin and chymotrypsin seemed to induce a broader degradation of uPA-R, since the decrease of the intact form did not correspond to a proportional increase of a single cleaved form, as observed in TAD-2 cells (Fig. 4, lanes 5–6, 11–12).

All together, these results indicate that uPA-R expressed by ARO cells is particularly resistant to uPA-, plasmin- and chymotrypsin-dependent cleavage as compared to uPA-R of normal thyroid cells, which can be cleaved very efficiently by all these proteolytic enzymes.

### 3.3. uPA-R glycosylation and cleavage resistance

The difference in molecular weight between cleavage-resistant uPA-R, expressed by ARO cells, and cleavable uPA-R of TAD-2 cells could be due to a different level of glycosylation. Therefore TAD-2 and ARO cell lysates were incubated with or without endoglycosidase F (PGNase F) and analyzed by Western blot with a monoclonal antibody, S1, recognizing both forms of reduced and alkylated uPA-R. Intact uPA-Rs in untreated TAD-2 and ARO samples migrated differently in a 9% SDS-PAGE, showing a molecular weight difference of about 5 kDa, as expected (Fig. 5, lanes 1 and 2). After deglycosylation with PGNase F, intact uPA-R of the two cell lines showed the same molecular weight (30 kDa) (Fig. 5, lanes 3 and 4), which suggests a higher glycosylation of uPA-R of ARO cells than of uPA-R expressed by TAD-2 cells. The molecular weight of the cleaved form of uPA-R expressed

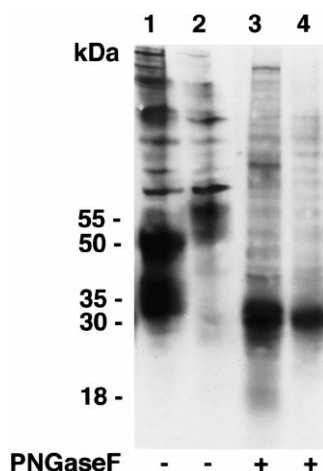


Fig. 5. uPA-R glycosylation and cleavage resistance. TAD-2 (lanes 1,3) and ARO cell (lanes 2,4) lysates were incubated with (lanes 3,4) or without (lanes 1,2) 2 U of PGNase F for 20 h at 37°C and then analyzed by Western blot with 10 µg/ml of monoclonal antibody S1.

by TAD-2 cells after deglycosylation was 18 kDa (Fig. 5, lane 3).

The higher glycosylation of ARO uPA-R could protect the receptor from the effect of proteolytic enzymes, thus explaining its resistance to cleavage.

#### 4. Discussion

uPA-R is expressed in a wide variety of normal and tumor cells. Its overexpression inhibits the normal ligand binding function of  $\beta 1$  integrins, thus decreasing cell adhesion to some components of the ECM, such as fibronectin (FNT) and collagen (CGN), and increasing cell adhesion to VTN, a component of ECM associated with malignant tumors [7]. These effects are induced by the physical interaction of uPA-R with active  $\beta 1$  integrins and caveolin [7]. Moreover, uPA-R can be 'activated' by uPA, which promotes cell surface proteolytic activity [5] and uPA-R binding to VTN [6]. All these events must be tightly regulated, since they can increase the invasive potential of cells [1]. We have therefore investigated whether uPA-R cleavage could represent a regulatory step of these uPA-R activities.

We have previously shown that a cleaved form of uPA-R, lacking the uPA binding domain, is strongly expressed on the surface of normal thyroid cells and in benign thyroid tumors, whereas it is reduced or absent in more aggressive tumor thyroid cells, such as follicular and anaplastic thyroid carcinoma-derived cells [14].

We now report the association of intact uPA-R with  $\beta 1$  integrins in thyroid cells as well, and show that uPA-R cleavage abolishes most of this association. The observation that very low amounts of cleaved uPA-R still interact with  $\beta 1$  integrins could indicate that cleaved uPA-R retains its ability to bind integrins, but with a much lower affinity than intact uPA-R. Therefore uPA-R cleavage represents the physiological mechanism regulating the physical interaction between uPA-R and  $\beta 1$  integrins. Since uPA-R interaction with  $\beta 1$  integrins plays an important role in integrin-mediated adhesion and signaling [7,18], we suggest that both these processes

are indirectly regulated by uPA-R cleavage. Our hypothesis is also supported by the recent observation that soluble intact uPA-R functionally interacts with  $\beta 2$  integrins in the binding to I-CAM molecule on endothelial cells, whereas the soluble cleaved form does not [19].

Cleaved uPA-R disappears in more aggressive carcinoma cells, such as follicular and anaplastic carcinomas [14]. We now report that anaplastic carcinoma ARO cells express a cleavage-resistant form of uPA-R, which is more glycosylated than the cleavage-sensitive uPA-R expressed by normal thyroid TAD-2 cells. We propose that glycosylation could regulate uPA-R cleavage by masking uPA-R sites sensitive to proteolytic enzymes such as uPA and plasmin. Indeed, glycosylation variants of uPA-R have been widely described in the past [20], although they have only been related to a lower affinity of uPA-R for its traditional ligand uPA [21].

uPA-R cleavage could thus represent a sort of negative regulation of the uPA-R-mediated invasive potential of the cell. In normal cells, uPA cleaves uPA-R, thus reducing cell surface proteolytic activity and cell adhesion to VTN, and, at the same time, allowing integrin-mediated cell binding to physiological components of ECM (FNT and CGN). In fact, intact uPA-R is required for binding to active uPA, VTN and  $\beta 1$  integrins. In addition, cleaved uPA-R is not simply an inactive degradation product of uPA-R, but a chemoattractant for macrophages, T-lymphocytes, etc. [22,23], and therefore could be actively involved in immunological processes. Aggressive tumor cells could escape this regulatory mechanism by more heavily glycosylating uPA-R, thus becoming advantaged in the process of migration and tissue invasion. In fact, uPA is able to bind cleavage-resistant uPA-R, thus increasing both cell surface proteolysis and uPA-R-mediated, high affinity cell adhesion to VTN. Moreover, intact uPA-R is able to decrease integrin-mediated cell adhesion to FNT and CGN, by interacting with  $\beta 1$  integrins.

The characterization of uPA-R cleavage as a regulatory mechanism of uPA-R 'activities', and its regulation by glycosylation, could represent an important step in the elucidation of the role of uPA-R in tumor invasion and metastasis.

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