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The receptor for the plasminogen activator of urokinase type is up-regulated in transformed rat thyroid cells

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Five rat thyroid cell lines were tested for the expression of the cell surface receptor for urokinase type plasminogen activator (uPA). All tested lines were found to bind uPA, but transformed 1-5G and Ki-Mol cells, which are also high uPA producers, bound at least ten times more uPA, as compared to non-producers, 'normal' TL5 cells. Moreover, it was possible to remove membrane-bound uPA by treating the cells with phosphatidylinositol-specific phopholipase C, suggesting that rat uPAR, like its human counterpart, is linked to the membrane by a glucosyl-phosphatidylinositol anchor. The specificity of the binding was tested by competition with three different synthetic peptides corresponding to amino acids 14–37 of human, rat and mouse uPA. The results indicate also that the receptor binding region of rat uPA is located within the growth factor domain of the molecule and that its expression may be dependent on the transformed state of the cells.

Urokinase; Urokinase receptor; Thyroid cell; Phospholipase C

1. INTRODUCTION

Two distinct plasminogen activators have been characterized in mammals: the urikanse type (uPA) and the tissue type (tPA). Even though the two PAs share the same unique substrate, plasminogen, they are involved in different physio-pathological events [1,2]. It has been suggested that tPA plays a critical role in fibrinolysis, whereas uPA participates in the extracellular proteolysis associated with tissue remodelling and cell migration events, including tumor cell migration and invasion (metastases) [3–6]. Indeed we and others [1,2] have demosntrated that some normal cells that do not secrete uPA, often become uPA producers after transformation [7–9].

It is by now clear that uPA is the key component of a complex system that directs cell-associated plasminogen activation [2]. This system of proteins includes uPA, tPA, several inhibitors (PAIs) and specific cell membrane receptors. The recently characterized cell receptor for uPA (uPAR) is thought to play a central role in this system, by providing a means to productively focus proteolysis at the cell surface [10–12].

The human uPA receptor is a M_r 55–60K protein which binds both single-chain pro-uPA and active two-chain uPA [13] with high affinity (K_d 0.1–1 nM), and is present on the surface of many normal and neoplastic cell types [14]. The receptor-binding region of uPA is located within its amino-terminal growth factor domain [15]. It was recently demonstrated [16] that uPAR is processed at the COOH-terminal region during synthesis and is anchored to the plasma membrane by a glycosyl-phosphatydilinosito moiety.

The regulation of the plasminogen activation system is still poorly understood. Each of the constituent proteins in the system could be independently regulated or, alternatively, some or all of them could be co-regulated, as suggested at least for uPA and uPA-R [17]. In order to approach this problem we are exploring the expression of the plasminogen activation system in a set of rat thyroid cell lines representing different stages of differentiation and/or transformation [18,19]. We previously demonstrated in this system a relationship between transformation and uPA production; we now present data on the expression of the specific receptor uPAR in the same cell lines and its anchorage to the cell membrane.

The study of rat uPAR presents serious difficulties, since purified rat uPA is not available; moreover, iodination of murine uPA, which could bind to rat receptors, is not very efficient and attempts to detect specific binding of radiolabeled murine uPA were unsuccessful [20], whereas human uPA, which is iodinatable, does not crossreact with rodent uPA receptors, due to the

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species specificity of the binding ([20] and our results). In order to circumvent these difficulties, we have used an indirect approach, which consists in using a rat uPA-rich medium for the binding to the receptor, and assaying the uPA activity released after the appropriate treatment of the cells, as described below. In this way we have been able to characterize the rat receptor for uPA.

2. MATERIALS AND METHODS

2.1. *Cell lines*

The cell lines FRTL$_5$ or TL5, FRT, FRA, 1-5G and Kirsten-transformed TL5 (Ki-Mol) are all of rat thyroid origin [7]. TL5 and FRT are 'normal' cells, while FRA, 1-5G and Ki-Mol were derived from in vivo or in vitro transformed cells. Cell culture conditions have been described. The LB6 line is of mouse fibroblast origin [21].

2.2. *Reagents*

Plasminogen was obtained from Sigma, St. Louis, MO, USA; phosphatidylinositol phospholipase C (PI-PLC), from *B. cereus*, was obtained from Boehringer, Mannheim, Germany. As a source of rat uPA, the supernatant of 1-5G cells, cultured for 4 days in the absence of serum, was employed; 1-5G cells are high uPA producers but do not secrete tPA [7]. In the absence of a purified rat uPA standard, we have measured the enzymatic activity in this medium by radial caseinolysis (see below) by using purified human uPA (Calbiochem, La Jolla, CA, USA) as a standard. The conditioned medium was found to contain an enzymatic activity corresponding to about 1 μg human uPA/ml.

A peptide corresponding to amino acids 14–37 of rat pro-uPA according to the human pro-uPA numbering system was kindly synthesized by Dr. E. Appella using the deduced amino acid sequence of exon 4 of the rat uPA gene (Degen et al., manuscript in preparation) (see Fig. 7). The human and mouse uPA peptides have been previously described [15].

2.3. *Binding assay*

As a source of uPA, we have used the 4 days conditioned medium from 1-5G cells described above. Unless otherwise specified, 1×10^6 cells were plated in 60 mm ∅ dishes (Falcon, Oxnard, CA, USA) and 24 h later they were stripped of surface-bound endogenous uPA by acid treatment [17]. Briefly, cells were washed 3 times with phosphate-buffered saline (PBS), pH 7.4, containing 1 mg/ml of bovine serum albumin (BSA), and incubated for 3 min at 4°C in 50 mM glycine, 100 mM NaCl, pH 3.0; a half volume of 0.5 M HEPES, 100 mM NaCl, pH 7.4, was then added for neutralization. Stripped cells were washed 4 times in PBS-BSA and incubated for 1 h at 37°C in 0.5 ml of 1-5G-conditioned medium, containing 1 mg/ml BSA, in the presence or in the absence of 10^{-4} M uPA-peptides. After 1 h at 37°C, cells were washed 10 times with PBS-BSA and treated again with acid; the eluate was then analyzed by zymography and radial caseinolysis. At each step of the procedure, cells were checked under the microscope to verify the integrity of the cell layer.

Where indicated, cells were lysed in Laemmli [22] buffer (300 μl/dish, 15 min at room temperature) and analyzed as above.

2.4. *PI-PLC treatment*

Acid washed cells were treated with exogenous uPA, as described above, washed 10 times and incubated for 2 h at 37°C with or without 3 U/ml PI-PLC in DMEM (Flow, McLean, VA, USA) containing 2 mg/ml BSA. The supernatants were collected and analyzed by zymography and radial caseinolysis.

2.5. *Zymography*

The method described by Granelli-Piperno and Reich [23], as modified by Vassalli et al. [24], was employed.

2.6. *Radial caseinolysis*

The method of Saksela [25] was used, with minor modifications. Briefly, 5 ml of 1% agar containing 2.0% commercial instant non fat dry milk, as a source of casein, and 0.04 mg/ml plasminogen was layered in 100 mm ∅ Falcon dishes. 3 μl of samples were loaded in wells (3 mm ∅) punched in the agar layer; after 24 h the reaction was stopped and the area of lysis measured.

3. RESULTS

3.1. *Expression of uPA receptors is up-regulated in transformed thyroid cell lines*

In the absence of a radioactive assay, we have detected cellular uPAR indirectly, by assaying cell-bound uPA activity after release with acid or PI-PLC-treatment [13,16]. To establish the validity of the assay, we used murine LB6 cells which do not produce detectable amounts of uPA, do not bind human uPA but bind mouse uPA (our unpublished observations, and below). The mouse and rat uPA sequences are very similar; in particular, in the region between amino acids 11 and 42, which corresponds to the growth factor-like domain, there are only 3 amino acid differences (see Fig. 7). We incubated LB6 cells with the conditioned medium of rat thyroid carcinoma 1-5G cells, that was previously shown, by direct and reverse zymography [7], to contain large amounts of uPA and no tPA nor PAIs. The cell surface-bound uPA was then removed by acid treatment, as described in section 2.

The zymographic analysis of the eluted material (Fig. 1) showed that the acid treatment was able to remove most receptor-bound uPA (lane 2), while only a small amount of the enzyme remained bound to the cells (lane 4). No uPA was observed in the absence of exogenous rat uPA, thus confirming that LB6 cells do not produce the enzyme (lanes 1 and 3).

To assay for the presence of uPAR in rat cells, semi-confluent FRT, 1-5G, FRA and Ki-Mol cells were first stripped of endogenous uPA by acid treatment; TL5 cells were subjected to the same treatment, even though they do not produce uPA. Cells were then incubated

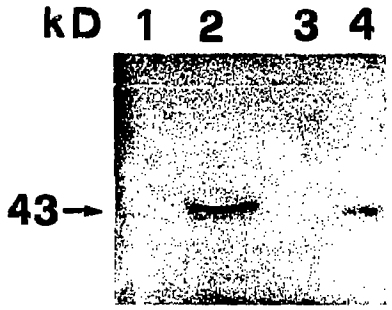

Fig. 1. Binding of uPA to LB6 cells. Zymographic analysis of acid eluates (lanes 1 and 2) and cell extracts (lanes 3 and 4) of LB6 cells pre-incubated with (lanes 2 and 4) or without (lanes 1 and 3) the uPA-rich, 4-days-conditioned medium of 1-5G cells. In this figure and in the following ones, the molecular weight was estimated from the migration of standards.

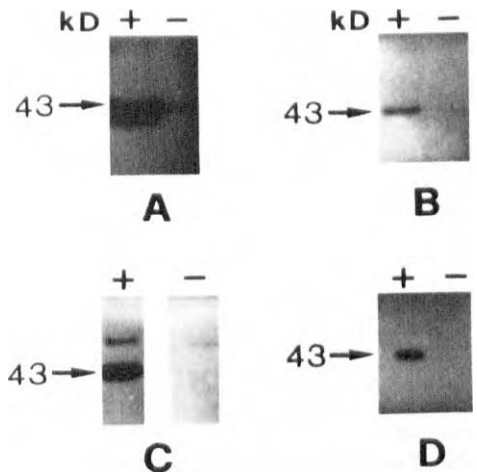

Fig. 2. Binding of uPA to semi-confluent rat thyroid cells stripped of endogenous uPA. Zymographic analysis of the eluates from stripped cells, incubated with the uPA-rich 1-5G medium and eluted with an acidic (+) or a neutral (–) buffer. Cells: A, 1-5G; B, TL5; C, FRT; D, FRA.

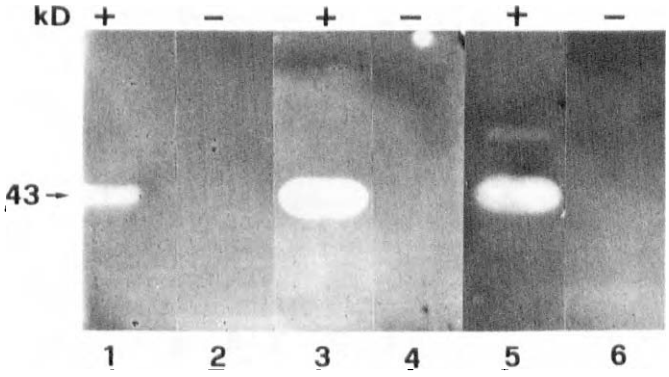

Fig. 3. Binding of uPA to 1×10^6 rat thyroid cells. Zymographic analysis of acid (+) or neutral (–) eluates from TL5 (lanes 1 and 2), 1-5G (lanes 3 and 4) and Ki-Mol cells (lanes 5 and 6), treated as described in Fig. 2. The eluates of 1-5G and Ki-Mol cells were diluted 1:10 before the assay.

with exogenous uPA, washed and stripped again, as described; the eluted material was analyzed by zymography. Fig. 2 shows that acid treatment released a 43K band from all the tested lines, showing that they are able to bind uPA. An additional band at 67K was observed in FRT cells (panel C). In contrast to the 43K band, this band was not inhibited by amiloride, a specific inhibitor of uPA but not of tPA [26], and may therefore represent endogenous tPA bound to cells or to the extracellular matrix.

When the same number of cells were compared TL5 cells were found to bind less than one-tenth of uPA, as judged by the intensity of the zymographic band, in comparison to 1-5G and Ki-Mol cells (Fig. 3). In the absence of added uPA, the zymographic assay was negative (not shown), thus demonstrating that the amount of endogenous uPA produced during the assay period was negligible.

3.2. *Release of uPA-uPAR complexes by treatment with PI-PLC*

To study the anchorage of uPAR to the cell surface, the effect of PI-PLC treatment was investigated in 1-5G and TL5 cells. Semi-confluent cultures were treated with acid, incubated with exogenous uPA, washed and treated with PI-PLC, as described. The culture meidum was then analyzed by zymography (Fig. 4). PI-PLC was able to remove a large amount of receptor-bound uPA from 1-5G cells; the small band observed in cells incubated without PI-PLC was most probably due to the production of endogenous uPA during the incubation period and/or to shedding of membrane bound uPA. Lysates of PI-PLC-treated cells show, correspondingly, much less uPA as compared to untreated cells, thus indicating that most receptor-bound uPA had been removed by the treatment (not shown). Similar results were obtained with TL5 cells (Fig. 5); moreover, in the absence of exogenous uPA, the culture medium of TL5 cells, did not contain any uPA activity after PI-PLC-treatment, showing that PI-PLC itself is not contaminated by a caseinolytic activity.

3.3. *Specificity of the binding of uPA to rat thyroid uPAR receptors*

The specificity of the binding was confirmed by incubating acid-stripped 1-5G cells with exogenous uPA, in the presence or in ht eabsence of different amounts of rat uPA peptide 14–37, and quantitating the acid-elutable material by radial caseinolysis. The eluted material was previously tested by direct and reverse zymography, with or without amiloride, to exclude the presence of tPA and PAIs. The results (Fig. 6) show that increasing amounts of rat peptide gradually displace rat uPA, until a maximum of about 65–70% at a concentration of 10^{-4} M. We could not use higher amounts of peptide, due to its limited availability.

In order to check the species specificity of the rat receptor, the binding was performed also in the presence

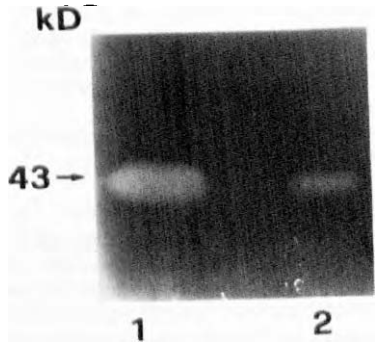

Fig. 4. PI-PLC treatment removes uPA bound to 1-5G cells. Zymographic analysis of 1:10 diluted culture medium of 1-5G cells incubated with uPA and treated with PI-PLC (lane 1) or without (lane 2), for 2 h at 37°C.

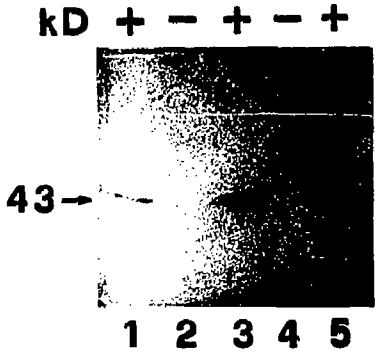

Fig. 5. PI-PLC treatment removes uPA bound to TL5 cells. Zymographic analysis of the incubation medium of TL5 cells, incubated with (+) or without (−) uPA and treated with PI-PLC (lanes 3, 4 and 5) or without (lanes 1 and 2). Cells analyzed in lane 5 were incubated with uPA in the presence of 10^{-4} M rat uPA peptide 14–37.

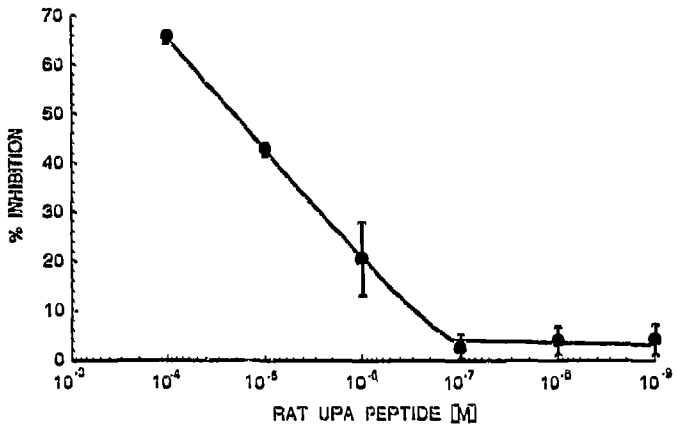

Fig. 6. Inhibition of binding of rat uPA to 1-5G cells, in the presence of increasing amounts of rat uPA peptide 14–37. Average values ± S.D., from triplicate samples.

of mouse and human peptides, at the highest concentration used with the rat peptide (10^{-4} M). The mouse peptide was slightly less effective than the rat one, while the human peptide showed, as expected, little inhibitory capacity (Table I).

4. DISCUSSION

The plasminogen activator of the urokinase type (uPA) plays an important role in cell migration and tissue remodelling, e.g. in the reabsorption of the mammary gland after lactation [1]; we therefore found it interesting to investigate the uPA system in a set of rat thyroid cells representing different stages of differentiation and/or transformation. The thyroid gland originates in fact in the pharingeal floor and begins at about the fifth week of fetal life to migrate caudally until it reaches its adult location in the low anterior neck [32]. Moreover, the architecture of the gland may vary considerably under the effect of physiologic and pathologic stimuli.

In this study, we have demonstrated the presence of uPA receptors in the same set of cells, by using an indirect assay. We found that uPAR is present in all the thyroid lines we have tested and that the amount of bound uPA varied from line to line. Whereas transformed cells, 1-5G and Ki-Mol, express very high levels of uPAR, fully differentiated TL5 cells have an at least 10 times lower amount. This observation parallels our previous data on the production of uPA by the different lines; this is particularly interesting since the Ki-Mol cells are derivative of TL5 cells: thus, as in the case of uPA [7], also the synthesis of uPAR appears to be increased by Ki-ras in rat thyroid cells.

On the other hand, the expression of uPAR could be related not only to a transformed phenotype, but also to an undifferentiated phenotype: TL5 in fact are the only cells, among those we tested, that in culture maintain the characteristic parameters of thyroid differentiation. Further investigation is, however, required to verify the possible role of uPAR in thyroid differentiation and/or transformation.

We have also studied the anchorage of the protein to the cell membrane. The human uPAR is attached to the membrane via a glycosyl-phosphatidylinositol molecule

Table I

Radial caseinolytic assay of uPA released by acid treatment of 1-5G cells[a]

Treatment of cells	Area of lysis (mm^2)[b]	% of inhibition
uPA + neutral buffer wash	0	–
uPA peptide[c] + neutral buffer wash	0	–
uPA + glycine buffer, pH 3.0 wash	55.0 ± 1[d]	–
uPA + human uPA peptide + glycine buffer, pH 3.0 wash	42.0 ± 1	23.7 ± 1.8
uPA + mouse uPA peptide + glycine buffer, pH 3.0 wash	30.0 ± 1	45.5 ± 1.8
uPA + rat uPA peptide + glycine buffer, pH 3.0 wash	22.0 ± 4.2	60.0 ± 5.4

[a] Radial caseinolytic assay [25] of 4 days-conditioned medium of 1-5G cells incubated with uPA for 1 h in the presence or in the absence of 10^{-4} M uPA peptides from different species (see Fig. 7) and treated thereafter with neutral buffer or glycine buffer, pH 3.0.
[b] Average values, with standard deviations, from three experiments in duplicate.
[c] Any of the three uPA peptides.
[d] This value was assumed as 100%.

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RuPA        1099TGT GGC TGT CAG AAC GGA GGA GTA TGT GTG TCC TAC AAG TAC TTC TCC AGC ATT CGA AGA TGC AGC TGC CCA AAG AAA TTC AAA GGG GAG CAC TGT
              11Cys Gly Cys Gln Asn Gly Gly Val Cys Val Ser Tyr Lys Tyr Phe Ser Ser Ile Arg Arg Cys Ser Cys Pro Lys Lys Phe Lys Gly Glu His Cys

RuPA GF Peptide           14Gln Asn Gly Gly Val Cys Val Ser Tyr Lys Tyr Phe Ser Ser Ile Arg Arg Cys Ser Ala Pro Lys Lys Phe
14Gln-37Phe[33Ala]

MuPA            --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- Arg --- --- --- --- --- --- --- Arg --- --- Gln --- --- --- ---
PuPA            --- --- --- Leu --- --- --- Lys --- --- --- --- --- --- --- --- Asn --- Gln --- --- --- --- --- --- --- --- Gln --- --- --- ---
BuPA            --- --- --- Leu --- --- --- Thr --- Met --- Asn --- --- --- --- --- --- His Trp --- Asn --- --- --- --- --- Gly --- Gln --- ---
HuPA            --- Asp --- Leu --- --- --- Thr --- --- --- Asn --- --- --- --- Asn --- His Trp --- Asn --- --- --- --- --- Gly --- Gln --- ---
CuPA            --- Gln --- Leu --- --- --- Thr --- Ile Thr --- Arg Phe --- --- Gln --- Lys --- --- Leu --- --- Glu Gly Tyr Gly --- Leu --- ---
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Fig. 7. Sequence of the rat uPA receptor-binding domain and cross-species sequence identity. (Top line) Partial nucleotide sequence of exon 4 of the rat uPA (RuPA) gene; the first nucleotide shown is number 1099 when numbered from the proposed site of transcription initiation (Degen et al., manuscript in preparation). (Second line) The deduced amino acid sequence of the rat uPA growth factor domain numbered according to the human pro-uPA numbering system. (Third line) The rat uPA synthetic peptide ^{14}Gln-^{37}Phe[^{33}Ala]; note that ^{33}Cys was replaced with Ala to restrict the type of intramolecular disulfide linkages. The murine (MuPA) [27,28], porcine (PuPA) [29], baboon (BuPA) [30], human (HuPA) [31], and chicken (CuPA) [9] uPA growth factor-like domains are listed below; residues identical to the rat sequence are indicated with dashes.

[16]; therefore, by treating the cell with PI-PLC it is possible to release a soluble receptor, as well as any ligand bound to it. Also the rat uPAR appears to be attached to the cell surface through a glycolipid linkage since treatment of TL5 and 1-5G cells with PI-PLC releases previously bound uPA (Figs. 4 and 5). The result is much clearer in the case of TL5 which do not produce endogenous uPA; uPA removed by PI-PLC-treatment can therefore only be derived from exogenous uPA bound to the receptor. In 1-5G cells, endogenous uPA produced during the 2 h incubation with PLC may interfere with the assay of the bound exogenous uPA; however, the difference in the amount of uPA released in the presence or in the absence of PLC-treatment was very clear.

The specificity of the binding was confirmed by incubating cells with uPA in the presence of an excess of the synthetic rat uPA peptide 14–37, which contains the receptor binding domain. The peptide was able to strongly inhibit uPA binding, even though the inhibition was not complete and was obtained only at relatively high concentrations of the peptide. This was probably due to the low affinity of the peptide, because of conformational differences with the native ligand [15]. The ability of the peptide to compete for binding also demonstrates the conservation of the mechanism of interaction between uPA and its receptor. In addition, we showed that the corresponding mouse peptide, which contains two amino acid substitutions (Fig. 7), could also compete for rat uPA-binding, while the human peptide, which contains seven amino acid substitutions (Fig. 7), was much less effective. Human uPA is indeed a poor inhibitor also of the binding of mouse uPA to the mouse receptor [20].

In conclusion, our data show that uPAR is present on the membrane of rat thyroid cell lines and that transformation causes a marked increase in its surface expression. The rat receptor has the same properties as that of human cells, both in normal and transformed cells. It binds the same region (the growth factor domain) of uPA, is species specific and is attached via a glycolipid anchor.

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