Removal of domain D2 or D3 of the human urokinase receptor does not affect ligand affinity

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Abstract The main ligand-binding determinant of the human urokinase receptor (uPAR) is located in the amino terminal domain D1, but when isolated this domain presents a 1500 fold lower affinity than the intact three-domain uPAR (D1D2D3) [1]. uPAR mutants missing either domain 2 (D1HD3) or domain 3 (D1D2) were expressed in murine LB6 cells and showed to be properly GPI-anchored to the cell surface. Binding assays with [^{125}I]ATF demonstrated that these mutants possessed a normal (D1D2) or slightly reduced (D1HD3) affinity, indicating that a high ligand-affinity may be achieved by a combination of D1 with domain D2 or D3.

Key words: Urokinase receptor; GPI-anchored protein; uPA

1. Introduction

Binding of urokinase plasminogen activator (uPA) to its specific high-affinity receptor (uPAR) profoundly affects its biochemical and physiological properties (reviewed in [2–4]). Pro-uPA activation, generation of inhibitor-resistant cell surface plasmin, internalization of the uPA/serpins complexes and direct signal transduction influencing cell migration and adhesion, are properties of uPA that are stimulated by or require the binding to uPAR [2–7]. Hence, binding to uPAR mediates the effect of uPA on extracellular proteolysis, cell migration and on the invasive and metastatic phenotype of cancer cells [2–4].

In uPA, the receptor-binding determinants are located in the amino terminus of the molecule, confined to the growth factor domain [8]. This information is not available for uPAR, which is a ca. 55 kDa glycoprotein, product of a 313 residues precursor protein [9,10]. The mature protein is processed at the carboxy terminus (Ser-282/Gly-283) and is substituted by a glycosyl-phosphatidylinositol (GPI) anchor so that the protein remains attached to the plasma membrane in a form that can be released by treatment with a bacterial phospholipase C [11,12]. N-linked glycosylation is responsible for about 40% of the molecular weight [10,13,14]. The position and spacing of cysteines within the amino acids sequence of uPAR has revealed a triple internal repetition, which led to the suggestion of a three-domain structure [15]. Each domain (D1, D2 and D3) is related to a family of single-repeat, GPI-anchored surface proteins that include CD59, the Ly-6 antigens as well as snake neurotoxins [15,16]. The disulfide bond pattern of D1 conforms to the general structure of the Ly-6/neurotoxins protein family and has suggested overall structural homology to CD59 and snake α-bungarotoxin for which a tertiary structural analysis is available [17–19]. The purified amino terminal domain (D1) of uPAR possesses independent ligand-binding activity, although with an affinity 1,500 fold lower than the wild type [1]; the purified carboxy-terminal fragment (D2D3) appears to be devoid of binding activity [1,15]. We have investigated, therefore, the effect of removing domain D2 or D3 on the binding properties of human uPAR. These mutants have been expressed in murine LB6 cells in a GPI-anchored form and found to display an about wild-type affinity for uPA.

2. Materials and methods

2.1. Materials

Recombinant *E. coli* expressed ATF (the amino terminal fragment of uPA: residues 1–143) containing the receptor binding site, was a kind gift of Drs. Anna Brandazza and Marco Soria (DIBIT, Milan). Pro-uPA was obtained from Lepetit SpA, courtesy of Dr. L. Nolli. Phosphatidylinositol-specific phospholipase C (PI-PLC) from *B. cereus* was from Boehringer Mannheim GmbH, Germany. Monoclonal antibodies R2 and R3 [20] were kind gifts from Drs. G. Høyer-Hansen and E. Rønne.

2.2. Cell culture and transfections

Transfected cells were grown as described for LB6 clone 19 cells [9] in the presence of 0.04% G418. Transfections were carried out with the calcium phosphate precipitation technique [21]. Individual clones were isolated and those positive in immunofluorescence for mAb R3 were chosen for further study.

2.3. Mutants and plasmids construction

In the following description, amino acid residues and nucleotide positions are taken from the previously published uPAR cDNA sequence [9]. Construct D1HD3 was made from the uPAR *Eco*RV mutant in an Okayama-Berg vector [22]. The sequence coding for domain D2 (amino acids 93–191) was removed by PCR. As upstream primer, the sequence corresponding to nucleotides 32–60 of the human uPAR cDNA was used. For the downstream primer, a sequence was used overlapping the 3′ end of domain D1 (nucleotides 375–388) and the 5′ end of domain D3 (nucleotides 685–711) thus looping out domain D2 (nucleotides 389–710). In addition, we have inserted between residues 92 and 192, i.e. between D1 and D3, the hinge region

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Abbreviations: uPA, urokinase-type plasminogen activator; pro-uPA, pro-urokinase; uPAR, urokinase-type plasminogen activator receptor; GPI, glycosyl-phosphatidylinositol; ATF, amino-terminal fragment of uPA; PI-PLC, phosphatidylinositol-specific phospholipase C; DSS, *N,N′*-disuccinimidyl suberate; FITC, fluorescein isothiocyanate; D-MEM, Dulbecco's Modified Eagle's Medium; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline

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Gly-Gly-Gly-Ser, by inserting the sequence ACTACCACCACC in the downstream primer. Both primers contained appropriate restriction sites (*Nru*I and *Pvu*II). The plasmid coding for the uPAR *Eco*RV mutant [22] was double digested with *Nru*I and *Pvu*II and replaced with the PCR product cut with the same enzymes. As a result, this construct contains a Gly-Gly-Gly-Ser hinge inserted between residues 92 and 192, i.e. between D1 and D3.

Construct D1D2, missing the 3rd domain, was constructed from the same starting plasmid as D1HD3. The sequence coding for domain D3 (amino acids 192-274) was removed by PCR using an upstream primer corresponding to position 32–60 and a downstream primer (nucleotides 670–684+935–956) looping out domain D3 and fusing domain D2 to the GPI-anchor attachment and signal sequence. The plasmid coding for the uPAR *Eco*RV mutant was double digested with *Nru*I and *Eco*RV; the PCR product was also double digested with *Nru*I and *Eco*RV. The cleaved plasmid and PCR product were ligated by standard methods.

All mutations were verified by DNA sequencing of the PCR-cloned constructs and by restriction mapping of the junctions of the final expression clones.

2.4. Immunofluorescence

Cells were placed and grown on glass slides in 24 well plates. The medium was removed, the cells were fixed with 3% paraformaldehyde, washed and stained with anti-uPAR antibody R3 and then with rhodamine-conjugated swine anti-mouse IgG (Dakopatts). Co-staining with fluorescein-conjugated phalloidin was used to visualize actin. In the experiments in which the effect of PI-PLC was tested, the cells were incubated with 5 IU/ml of PI-PLC in D-MEM for 15 min at 37°C, then washed with PBS containing 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.1% BSA, fixed and processed as above.

2.5. Binding assay

Iodination of ATF with Iodogen (Pierce Chemical Co.) has been described before [10]; the iodinated ATF had a specific radioactivity of 75,000 to 150,000 cpm/ng, depending on the preparation. Cells were plated on gelatin coated 96 well plates (80,000 cells/well) one day before the experiment. On the day of experiment, the cells were incubated for 30–45 min at 37°C in binding buffer (50 mM HEPES, pH 7, 0.1% BSA in D-MEM), then transferred on ice; the binding buffer was replaced with increasing concentrations of [^{125}I]ATF in the presence or absence of 100 fold excess unlabeled pro-uPA or recombinant ATF and the cells were incubated on ice for 3 h. The cells were washed 3 times with cold PBS containing 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.1% BSA. Then, 1% SDS was added and the radioactivity in the SDS-lysate was measured.

2.6. Metabolic labelling, immunoprecipitation and SDS-PAGE

Cells were metabolically labeled as previously described [23], with a 15 min labeling with [^{35}S]-*Trans*Label and a chase-time of 3 h. After lysis and preclearing of cell lysates, immunoprecipitation was performed using a mixture of mAbs R2 and R3 at 4°C [23]. Immunocomplexes were recovered with GammaBind G-Sepharose (Pharmacia), resuspended in SDS-PAGE sample buffer, separated on 12.5% SDS-PAGE and autoradiographed.

2.7. Cross linking of ATF to whole cells

Cells were plated for 24 h on gelatin coated 6 well plates (500,000 cells/well) as above. Cross-linking was performed as described before [13]. Briefly, on the day of the experiment the cells were incubated for 30 min at 37°C in binding buffer and transferred on ice. Binding buffer was replaced with either 10 or 0.1 nM [^{125}I]ATF in binding buffer and the cells were incubated on ice for 3 h. The cells were washed twice with cold PBS containing 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.1% BSA and incubated 15 min at room temperature with 2 mM *N,N'*-disuccinimidyl suberate (DSS) (Pierce Chemical Co.) in PBS; the reaction was stopped with 10 mM ammonium acetate. Then the cells were scraped from the wells and centrifuged. Re-

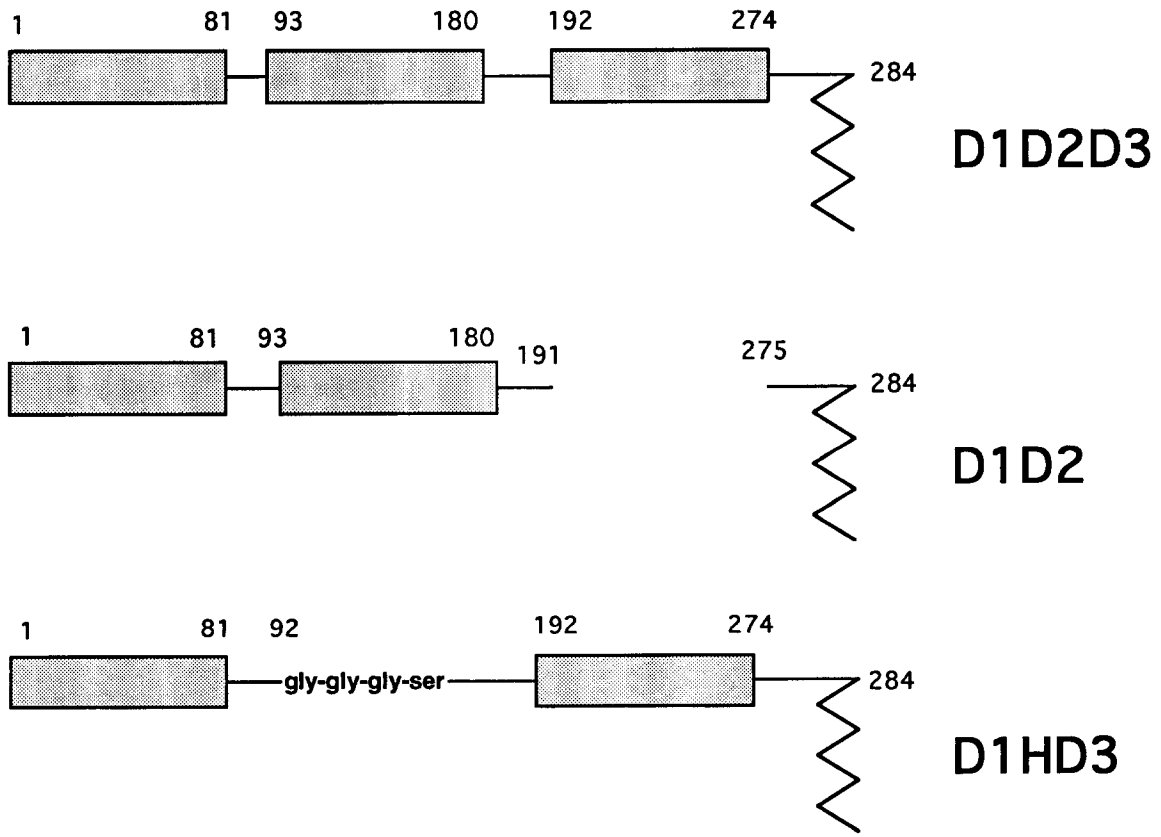

Fig. 1. Schematic representation of the three-domains wild type human uPAR and of the two deletion mutants. The numbers indicate amino acids residues in mature uPAR. The wavy line to the right shows the GPI-anchor. The rectangles represent the remaining domains, the lines the linker regions. Terminology on the right is based on which of the three domains is retained: D1D2D3 is the symbol for wild type uPAR. Mutant D1HD3 contains a four amino acids, Gly-Gly-Gly-Ser hinge (indicated).

ducing SDS-PAGE sample buffer was added to the pellet, samples were boiled, run in 12.5% SDS-PAGE and the gel autoradiographed.

3. Results and discussion

Fig. 1 shows a schematic representation of the three-domains wild type human uPAR and of the two deletion mutants constructed. Notice that D1HD3 contains a four amino acids (Gly-Gly-Gly-Ser) linker-hinge region between D1 and D3. This hinge region was introduced after having failed to express a hinge-less D1D3 mutant possibly for improper folding (data not shown). The sequence of the hinge region was chosen as this sequence appears to confer adequate mobility in other recombinant proteins made of two domains [24]. All constructs retain the N-terminal domain (D1) that can be specifically recognized by the R3 monoclonal antibody [20],

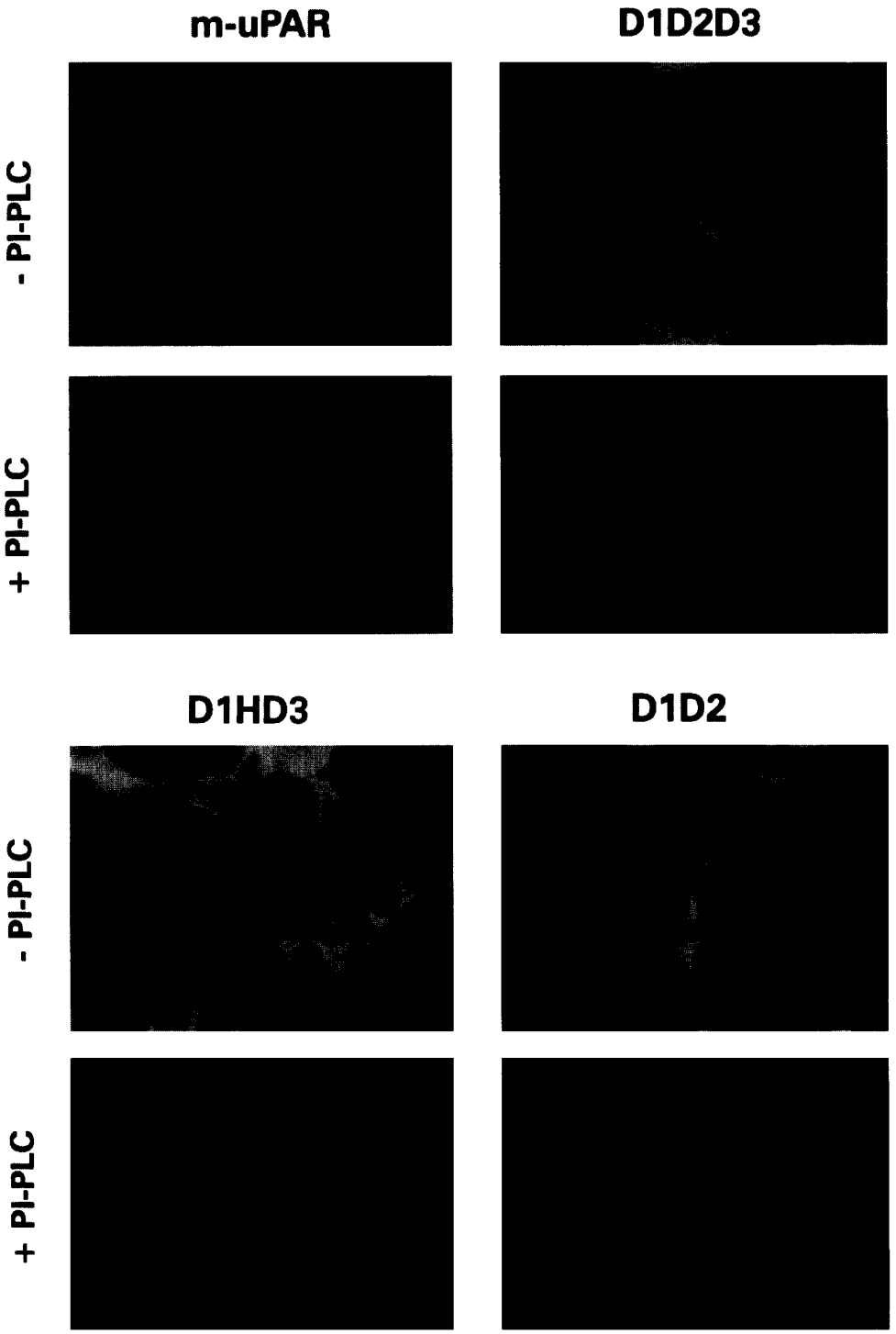

Fig. 2. Immunofluorescence analysis of the expression and PI-PLC sensitivity of transfected human uPAR and deletion mutants D1D2 and D1HD3. Parental LB6 cells (m-uPAR) and LB6 cells expressing wild-type uPAR (D1D2D3), deletion mutant D1D2 or D1HD3 were either untreated (−PI-PLC) or treated with 5 IU PI-PLC (+PI-PLC), incubated with mAb R3 (20 μg/ml) and then stained with a rhodamine-conjugated anti-mouse IgG. FITC-phalloidin staining (not shown) was used to demonstrate the presence of cells in the uPAR-negative samples.

as well as the C-terminal sequence containing the GPI anchor-attachment and signal sequence [12]. Therefore all uPAR mutants can be recognized by antibody R3 in immunofluorescence and should be sensitive to PI-PLC; sensitivity to PI-PLC should eliminate the positive immunofluorescence reaction, as a proof of cell-surface GPI-anchoring.

Immunofluorescence with R3 antibody on non-permeabilized cells, treated or not with PI-PLC, was used to test for the expression of uPAR mutants on the surface of individual clones of LB6 cells. As shown in Fig. 2, the untransfected LB6 cells expressing only murine uPAR (m-uPAR) did not yield a fluorescent signal, while all the others did. Treatment of each clone with PI-PLC resulted in the disappearance or clear reduction of the R3-positive staining (Fig. 2). Therefore, we conclude that the mutants were expressed on the cell surface of LB6 cells and that they were PI-PLC sensitive and hence GPI-anchored to the membrane.

We next estimated the molecular weight of the uPAR mutants by immunoprecipitation with anti-uPAR antibodies and SDS-PAGE analysis of ^{35}S-labeled extracts. Fig. 3A shows the SDS-PAGE analysis obtained after immunoprecipitation of the whole cell lysates. The lane labeled m-uPAR represents the negative control LB6 cells pattern. In comparison, LB6 clone 19 (wild type uPAR, indicated as D1D2D3) gave the expected 45–55 kDa broad band characteristic of human uPAR [9–13,23]. Both mutants D1HD3 and D1D2 gave instead of a single band, two strong bands of about 30 and 35 kDa (Fig. 3A, arrows). The 30 kDa band might represent incompletely glycosylated mutant uPAR, as observed intracellularly in other cells [10]. To check this point, D1HD3 and D1D2 expressing cells were metabolically labeled with [^{35}S] and treated with PI-PLC; both the lysed cell pellet and the PI-PLC supernatants were subjected to immunoprecipitation with anti-uPAR antibodies. Fig. 3B shows that the 30 kDa band was observed exclusively in the cell lysates of both mutants, while the higher molecular weight band was observed in the PI-PLC supernatant. Thus D1HD3 and D1D2 express both a PI-PLC sensitive, cell surface uPAR, and a PI-PLC resistant uPAR of lower molecular weight which might represent intracellular, incompletely glycosylated molecules. Therefore, despite the high level of expression, only part of the two mutant receptors appears to be properly exposed. This behaviour parallels that of PMA-treated human U937 cells in which a portion of the overexpressed uPA receptors is not properly glycosylated and does not reach the cell-surface [10].

We next analysed the ligand-binding properties of the uPAR mutants. We performed cross-linking analysis with DSS [13] on living cells after binding to 0.1 or 10 nM [^{125}I]ATF and analysed the formation of ATF-uPAR adducts by SDS-PAGE of the cell extracts and autoradiography. The formation of adducts of the expected size was observed with wild type uPAR-expressing cells as well as with the mutants D1HD3 and D1D2 at both ligand concentrations (Fig. 4A,B). The migration of the mutant adducts was faster than wild-type in agreement with the lower molecular weight of the mutant receptors. No specific adduct was observed in the case of the negative control LB6 cells. It must be noticed that at 0.1 nM ATF (Fig. 4B), visualization of a cross-linked adduct required a longer exposure time of the gel to the X-ray film in the mutants than in the wild type.

In order to obtain quantitative information, binding isotherm studies with whole cells at 4°C were carried out with

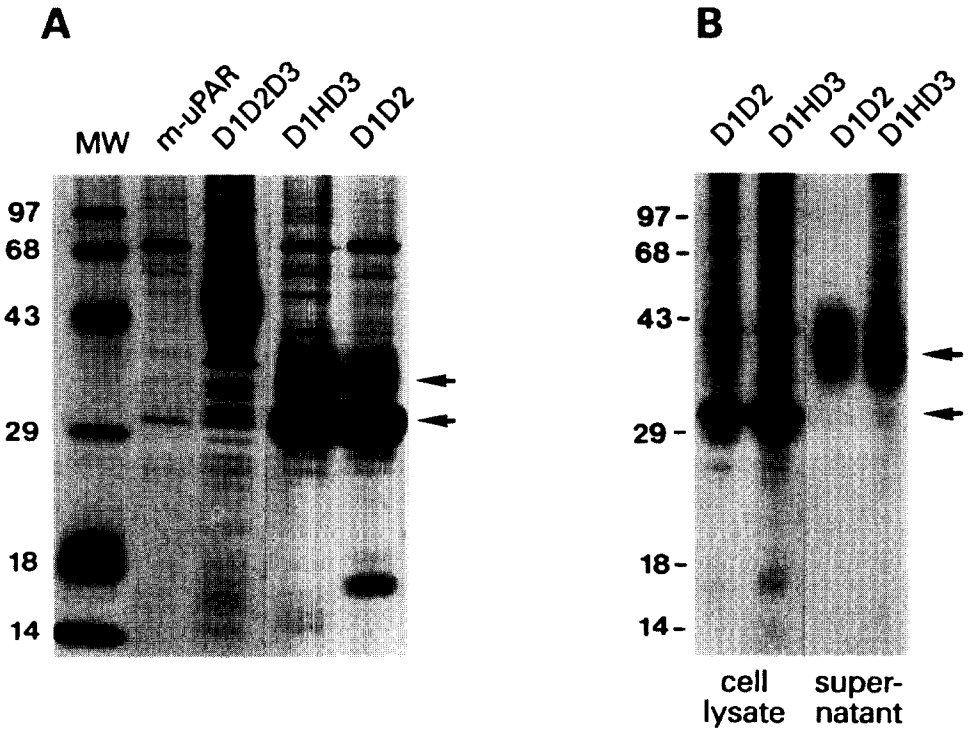

Fig. 3. Immunoprecipitation analysis of uPAR mutants expressed in LB6 cells. (A) Parental LB6 cells (m-uPAR) or LB6 cells expressing either wild-type uPAR (D1D2D3), deletion mutant D1D2 or D1HD3 were pulse-labeled with [^{35}S]-TransLabel for 15 min and chased for 3 h. Cells were lysed, immunoprecipitated with anti-uPAR antibodies R2 and R3 and analysed by SDS-PAGE. (B) ^{35}S-labeled cells were pretreated with PI-PLC; both the lysed cell pellet and the PI-PLC supernatants were immunoprecipitated with anti-uPAR antibodies R2 and R3 and analysed by SDS-PAGE. Molecular weight markers are shown in kilodaltons on the left of each panel.

Table 1
Analysis of the [^{125}I]ATF-binding properties of LB6 cells expressing wild-type (D1D2D3), and mutant uPAR (D1HD3 and D1D2)[a]

Construct	n[b]	K_d (nM) mean (±S.D.)	n uPAR[c] mean±S.D.
D1D2D3 (wild type)	6	2.62 (±1.1)	5.1 (±1.5)×10^5
D1HD3	2	7.9	1.6×10^5
D1D2	3	1.5 (±1.23)	0.92 (±0.1)×10^5

[a][^{125}I]ATF binding was assessed by Scatchard analysis over a range of concentrations from 0.001 to 10 nM in triplicate wells. Specifity of binding was controlled in a parallel competition experiment by using a 100-fold molar excess of unlabeled ATF.
[b]Number of experiments performed.
[c]Number of uPA receptors per cell.

LB6 transfectants of wild type (D1D2D3) and mutant uPAR. Specific binding was defined as the amount of [^{125}I]human ATF ligand that could be displaced in the presence of 100 fold molar excess unlabeled human pro-uPA or recombinant ATF. In all cases, specific binding amounted to at least 80% of total binding. The negative control LB6 cells displayed no specific binding at ATF concentrations below 3 nM and very low levels (10–20% of total) at concentrations of 10 nM or more. Mutants D1D2 and D1HD3, however, displayed specific binding for ATF (again at least 80% of total binding). The binding isotherm data were subjected to Scatchard plot analysis to calculate the affinity for ATF and the number of receptors per cell. Representative data are reported in Table 1. Wild-type uPAR-expressing cells displayed 513,000 receptors/cell, with a calculated K_d of about 2 nM, in agreement with previously published data [14]. Mutant D1D2 displayed a reduced binding capacity (91,000 receptors/cell), with an essentially identical binding affinity (1.5 nM), while mutant D1HD3 had a somewhat lower binding affinity, 7.9 nM, and 161,000 receptors/cell.

These results show that mutant receptors displaying an about wild-type affinity can be generated even when domain D2 or D3 are deleted. Interestingly, our data show that domain D2 and D3 are interchangeable in this respect, since a combination of D1 with both D2 or D3 yields a high affinity binding site. The difference in K_d measured between D1D2 and D1HD3 (2 vs. 7 nM) may be due to folding problems in the latter, and in any case is only minor. However, the presence of all three domains is not required for attaining a wild-type binding affinity.

The major ligand-binding domain of uPAR is located in domain D1 [15]. However, the isolated D1 domain has an over thousand fold lower affinity than the three-domains receptor [1]. In conclusion, the behaviour of mutants D1D2 and D1HD3 suggests that the achievement of high ligand affinity requires at least two of the three domains. Since the isolated D2+D3 domain does not show any binding activity [1], the presence of domain D1 is probably required to reach high affinity. However, D2 and D3 can inter-changeably cooperate with D1 to achieve high affinity.

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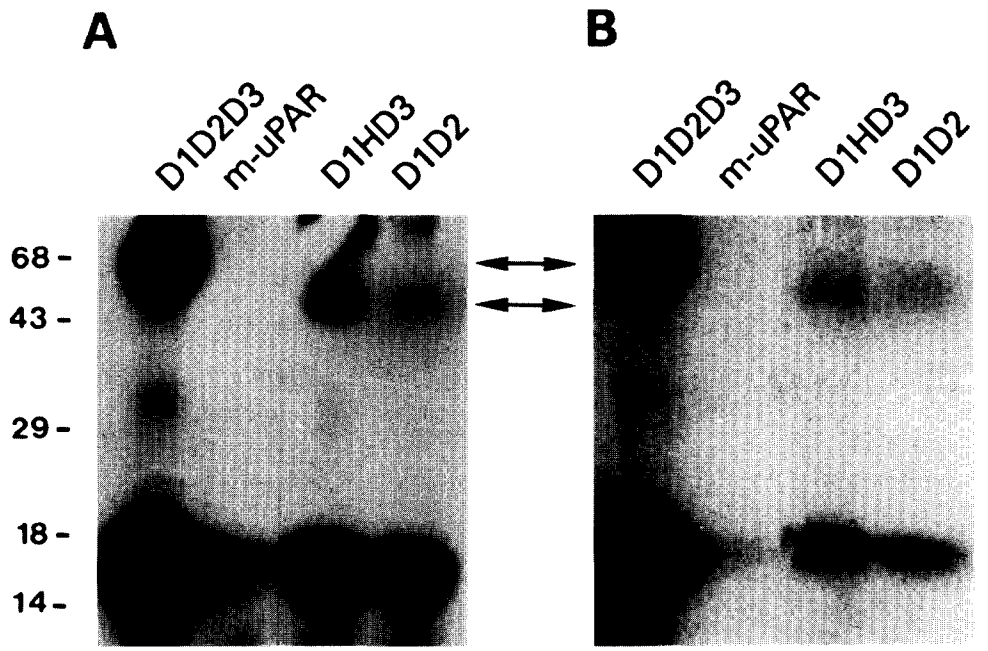

Fig. 4. [^{125}I]ATF cross-linking analysis on living cells. Parental LB6 cells (m-uPAR), LB6 cells expressing either wild type uPAR (D1D2D3), deletion mutant D1D2 or D1HD3 were incubated with 10 nM (A) or 0.1 nM (B) [^{125}I]ATF. Crosslinking was performed with DSS; cells were then lysed and extracts were resolved by SDS-PAGE and autoradiography. (A) 10 nM [^{125}I]ATF and overnight exposure of the film. (B) 0.1 nM [^{125}I]ATF, 10 days exposure.

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