Recycling of the urokinase receptor upon internalization of the uPA:serpin complexes

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The GPI-anchored urokinase plasminogen activator receptor (uPAR) does not internalize free urokinase (uPA) but readily internalizes and degrades uPA:serpin complexes in a process that requires the α_2-macroglobulin receptor/low density lipoprotein receptor-related protein (α2MR-LRP). This process is accompanied by the internalization of uPAR which renders it resistant to phosphatidylinositol-specific phospholipase C (PI-PLC). In this paper we show that during internalization of uPA:serpins at 37°C, analysed by FACScan, immunofluorescence and immunoelectron microscopy, an initial decrease of cell surface uPAR was observed, followed by its reappearance at later times. This effect was not due to redistribution of previously intracellular receptors, nor to the surface expression of newly synthesized uPAR. Recycling was directly demonstrated in cell surface-biotinylated, uPA:PAI-1-exposed cells in which biotinylated uPAR was first internalized and subsequently recycled back to the surface upon incubation at 37°C. In fact, uPAR was resistant to PI-PLC after the 4°C binding of uPA:PAI-1 to biotinylated cells, but upon incubation at 37°C PI-PLC-sensitive biotinylated uPAR reappeared at the cell surface. Binding of uPA:PAI-1 by uPAR, while essential to initiate the whole process, was, however, dispensable at later stages as both internalization and recycling of uPAR could be observed also after dissociation of the bound ligand from the cell surface.
Keywords: internalization/recycling/uPA:serpin complex/ urokinase receptor

Introduction

Binding of urokinase plasminogen activator (uPA) to its specific receptor (uPAR) regulates cell migration and invasiveness (Blasi *et al.*, 1987, 1994; Danø *et al.*, 1994;

Fazioli and Blasi, 1994; Besser *et al.*, 1996). Indeed, invasion and metastasis *in vivo* and basement membranes degradation *in vitro* can be blocked by inhibitors of uPA and antagonists of uPAR (Ossowski and Reich, 1983; Bergman *et al.*, 1986; Hearing *et al.*, 1988; Ossowski, 1988; Ossowski *et al.*, 1991; Quax *et al.*, 1991; Crowley *et al.*, 1993; Min *et al.*, 1996). Moreover, uPA and uPAR are deeply involved in the inflammatory response, regulating cell adhesion and migration and conditioning the recruitment of T-lymphocytes and monocytes through direct signalling mechanisms (Waltz *et al.*, 1994; Bianchi *et al.*, 1996; Gyetko *et al.*, 1996; Resnati *et al.*, 1996; Wei *et al.*, 1996). These properties warrant a detailed analysis of the molecular mechanisms underlying the involvement of uPAR in invasion.

When active uPA or the pro-enzyme pro-uPA are bound to uPAR, they are not internalized and remain on the cell surface (Vassalli *et al.*, 1985; Stoppelli *et al.*, 1985, 1986; Cubellis *et al.*, 1986). When receptor-bound uPA is complexed to the specific plasminogen activator inhibitor type 1 (PAI-1) or to protease nexin-1 (PN-1), the complex is internalized and degraded in the lysosomes (Cubellis *et al.*, 1990; Estreicher *et al.*, 1990; Jensen *et al.*, 1990; Conese *et al.*, 1994). Binding to uPAR is required for internalization and degradation of the uPA:PAI-1 and uPA:PN-1 complexes (Olson *et al.*, 1992; Conese *et al.*, 1994) and internalization and degradation of the uPA: serpin complexes requires a trans-membrane partner which has been identified as a member of the LDL-receptor family: the α_2-macroglobulin receptor/low density lipoprotein receptor-related protein (α2MR-LRP), the epithelial glycoprotein-330 or the VLDL-receptor (Herz *et al.*, 1992; Nykjær *et al.*, 1992; Moestrup *et al.*, 1993; Conese *et al.*, 1994; Argraves *et al.*, 1995; Heegaard *et al.*, 1995). The endocytic α2MR-LRP is expressed by several cultured cells (Moestrup *et al.*, 1992), and binds a variety of ligands, including α2-macroglobulin:proteinase (Moestrup *et al.*, 1990; Moestrup and Gliemann, 1991) and plasminogen activator–serpin complexes (Nykjær *et al.*, 1992; Orth *et al.*, 1992; Conese *et al.*, 1994). The 39 kDa Receptor Associated Protein (RAP), capable of competing for binding with all ligands, the soluble α2MR-LRP and anti-α2MR-LRP antibodies, inhibit uPA:serpin internalization (Nykjær *et al.*, 1992; Conese *et al.*, 1994). Unexpectedly, also uPAR is internalized in the process and also this step is dependent on α2MR-LRP (Conese *et al.*, 1995). We now show that internalized uPAR can be recycled back to the cell surface.

Results

uPA:serpin-internalized uPAR is resistant to release by PI-PLC

PI-PLC releases cell surface uPAR (Ploug *et al.*, 1991) and hence should release the bound ligand quantitatively.

Table I. Comparison of acid-washing and PI-PLC treatment on the release of [^{125}I]uPA, [^{125}I]uPA:PAI-1 or [^{125}I]uPA:rPN-1 from LB6 clone 19 cells and T-lymphocytes

Ligand/treatment	^{125}I-ligand released (%)			
	LB6 clone 19		T-lymphocytes	
	−	+	−	+
uPA/AW	88.6 ± 5.0	88.9 ± 4.5	73 ± 3.0	75 ± 2.0
uPA:PAI-1/AW	82.0 ± 1.0	85.3 ± 2.1	92 ± 4.0	82 ± 8.2
uPA:rPN-1/AW	71.0 ± 3.2	82.0 ± 2.8	ND	ND
uPA/PI-PLC	89.3 ± 4.9	88.8 ± 5.2	99 ± 0.5	95 ± 2.0
uPA:PAI-1/PI-PLC	36.0 ± 4.3	78.0 ± 2.0	94.0 ± 2.3	94.0 ± 3.0
uPA:rPN-1/PI-PLC	27.0 ± 4.1	48.0 ± 1.0	ND	ND

LB6 clone 19 cells (0.2×10^6/well) were incubated with [^{125}I]uPA, [^{125}I]uPA:PAI-1 or [^{125}I]uPA:rPN-1 for 120 min on ice in the presence of control GST (50 μg/ml) (−) or GST–RAP (50 μg/ml) (+), then washed and treated with acid-washing (AW) or PI-PLC (see Materials and methods). T-lymphocytes (1×10^6/ml) were incubated with [^{125}I]uPA or [^{125}I]uPA:PAI-1 for 16 h at 4°C in the presence (+) or absence (−) of 200 nM rRAP. The ligands were used at 6 nM concentration with a specific activity of 21–65.7×10^6 c.p.m./μg. The amount of released ligand is expressed as percentage of the total cell-associated radioactivity before treatments (± standard deviation). Non-specific counts were determined by competition with 200 nM pro-uPA and subtracted. Each value is derived from one experiment in triplicate and was reproduced three times.

We incubated LB6 clone 19 cells or activated human primary T-lymphocytes with radiolabelled ligands at 4°C, and compared the releasing effect of acid-washing (which dissociates the ligand) or PI-PLC treatment (which releases the receptor) (Stoppelli *et al.*, 1986; Ploug *et al.*, 1991) on cell-bound [^{125}I]uPA, [^{125}I]uPA:PAI-1 or [^{125}I]uPA:PN-1. Table I shows that ~90% of the ligand was released with the two methods with both types of cell exposed at 4°C to free [^{125}I]uPA. However, in LB6 clone 19 cells when the ligands were [^{125}I]uPA:PAI-1 or [^{125}I]uPA:rPN-1 complexes, acid-washing released 83% and 71% of the ligands, while PI-PLC released only 36% and 27%, respectively. Treatment with PI-PLC was carried out for 15 min at 37°C, while acid-washing was carried out entirely at 4°C. Since internalization causes PI-PLC resistance of uPAR (Conese *et al.*, 1995), the results in Table I might be explained by a uPAR-dependent internalization of the ligand occurring within the 37°C PI-PLC incubation time (15 min). Indeed, the α2MR-LRP antagonist RAP prevented PI-PLC resistance (Table I). In agreement with this interpretation, activated T-lymphocytes, which express uPAR but not α2MR-LRP (our unpublished results), displayed identical [^{125}I]uPA:PAI-1 release by PI-PLC and by the acid treatment (Table I).

To confirm that a 15-min treatment at 37°C would be sufficient to internalize the uPA:PAI-1 complex, we performed a detailed time-course experiment of uPA:PAI-1 internalization/degradation and confirmed that such conditions were indeed sufficient to attain almost maximal internalization of the bound uPA:PAI-1, and that this event was suppressed by 200 nM RAP (data not shown). We conclude therefore that the α2MR-LRP-dependent internalization of uPAR occurs very rapidly at 37°C. We also conclude that PI-PLC treatment would not affect internalized uPAR.

Internalization of uPAR is not followed by degradation

LB6 clone 19 cells previously challenged at 4°C with excess uPA:PAI-1, were incubated at 37°C for different times, treated with anti-uPAR antibodies, and subjected to immunofluorescence confocal microscopy. The staining was highly specific, as seen in a series of control experiments (not shown). First, we noticed that the intensity of fluorescence in uPA:PAI-1-treated LB6 clone 19 cells incubated at 37°C for 0–120 min did not decrease over time, as occurred when the uPA antibody was used (not shown; but see Olson *et al.*, 1992). Figure 1 shows selected illustrations of uPAR immunofluorescence taken at different planes of focus. The left series (a, d, g and j) shows a look-through projection of all confocal planes, corresponding to normal epifluorescence. The centre series (b, e, h and k) shows a single confocal plane (0.2 μm) passing through the adhesion plane. The right series (c, f, i and l, higher magnification) is also a single confocal section (0.2 μm) passing through the centre of the cell. On completion of the 4°C incubation (time 0, not shown), and after 10 min at 37°C (Figure 1), staining was concentrated mainly at the cell periphery. After 30 and 60 min at 37°C, most of the previously peripheral uPAR fluorescence was redistributed to a perinuclear position, shown previously by a variety of techniques to be caused by uPAR internalization (Conese *et al.*, 1995). The perinuclear location was specific for cells incubated with uPA:serpins and exposed at 37°C, and was inhibited by the ligand of α2MR-LRP, RAP (not shown; see Conese *et al.*, 1995). When the incubation at 37°C was prolonged to 120 min, uPAR fluorescence did not diminish but was found to be re-concentrated at the cell periphery, at sites reminiscent of contacts with the plastic dish. This is better seen Figure 1, panels c, f, i and l (high magnification). In conclusion, these data showed the striking absence of any time-dependent decrease of uPAR staining over the time frame examined, suggesting that internalization of uPAR may not be followed by its degradation. It is important to point out that after a 1-h incubation at 37°C, these same cells had already degraded ~50% of uPA:PAI-1 (not shown).

Since internalized uPAR is PI-PLC-resistant, treatment of cells with PI-PLC should not eliminate the immunofluorescence signal due to internalized uPAR. We used LB6 clone 19 cells incubated for 90 min at 4°C in the absence of ligands, or in the presence of 10 nM uPA or uPA:PAI-1. In both the absence and presence of ligands, antibodies to uPAR stained the surface of non-permeabilized cells homogeneously (not shown), as previously
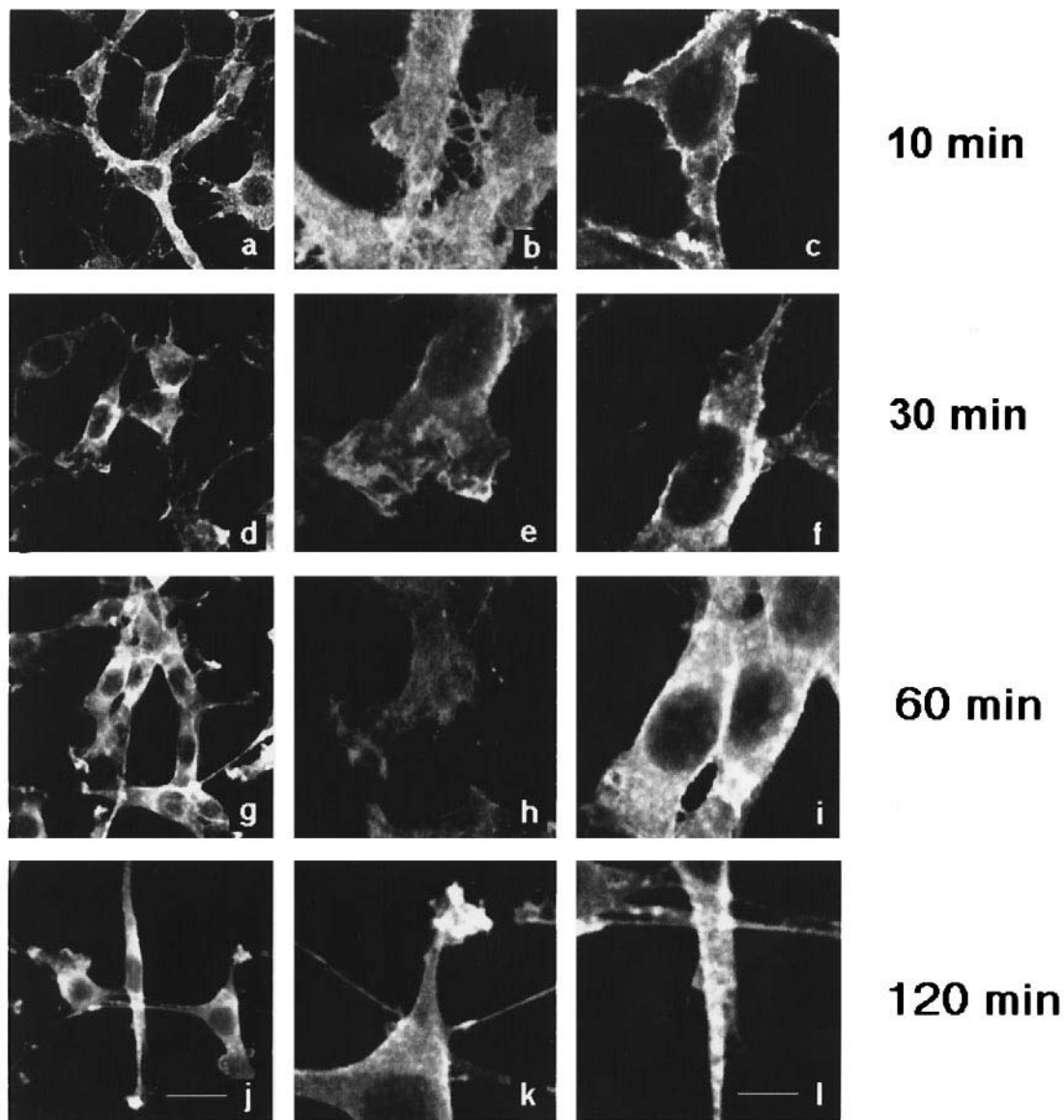
Fig. 1. Confocal immunofluorescence microscopy analysis of uPAR in LB6 clone 19 cells challenged with uPA:PAI-1 and then incubated for 10–120 min at 37°C. Cells were incubated with uPA:PAI-1 for 2 h at 4°C, washed and then incubated at 37°C for 10 min (**a–c**), 30 min (**d–f**), 60 min (**g–i**) or 120 min (j–l). Cells were then washed, permeabilized, treated with 15 μg/ml anti-uPAR R2 monoclonal antibody and developed with fluoresceinated anti-mouse IgG. Bars: 35 μm in the panels a, d, g and j; 12 μm in panels c, f, i and l.

reported by Conese *et al.* (1995). After 90 min at 4°C, the cells were washed, treated with PI-PLC for 15 min at 37°C, and permeabilized. In this case, the signal observed varied depending on the ligand. In the absence of ligands or in the presence of uPA, staining disappeared almost completely (Figure 2, upper and centre panels). When the ligand was uPA:PAI-1, however, uPAR staining disappeared from the cell surface and was replaced by an intracellular punctuate pattern reminiscent of that seen with internalized proteins (Figure 2, lower panel). A similar picture was obtained when the ligand was uPA:rPN-1 (not shown). We conclude that PI-PLC can be employed to study the steps following uPAR internalization, as the enzyme will release the surface-bound, but not the internalized, uPAR.

Internalized uPAR recycles back to the cell surface

U937 monocyte-like cells incubated with uPA:serpin complexes down-regulate their surface uPAR (Olson *et al.*,

1992; Conese *et al.*, 1995). To test for uPAR recycling, U937 cells were exposed to either uPA:PAI-1 or uPA:PN-1 for 90 min at 4°C, washed, further incubated at 37°C, and then subjected to cytofluorimetric analysis with uPAR-specific antibodies. As expected, uPA:PAI-1 and uPA:rPN-1 complexes down-regulate uPAR after 30 min at 37°C (Figure 3, panels A, F and B, G) and this was prevented by the α2MR-LRP antagonist, RAP (Figure 3, panels C and H). When, however, the incubation was continued for 60 or 120 min, the level of uPAR-specific fluorescence reverted to normal and at 120 min the FACScan signal was almost identical to that at time zero (Figure 3, panels D, E and I, J). This result suggests that internalized uPAR might be recycled back to the cell surface.

The events following internalization of uPAR were also analysed by electron microscopy of cryosections prepared for immunocytochemistry, using LB6 clone 19 cells incub-
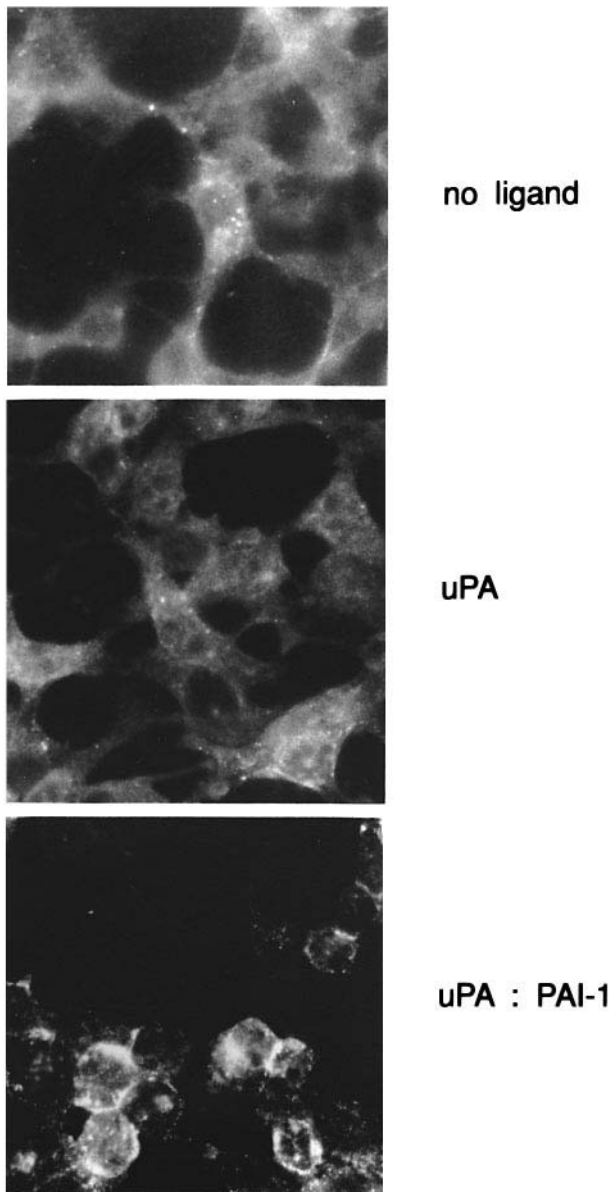
Fig. 2. Immunofluorescence microscopy analysis of uPAR in PI-PLC-treated LB6 clone 19 cells. Incubation of cells with uPA:PAI-1 induces partial resistance to PI-PLC. Cells were incubated with no ligand, 10 nM uPA or 10 nM uPA:PAI-1 complex, for 2 h at 4°C, washed and then incubated 15 min at 37°C with PI-PLC. The cells were then washed, permeabilized and subjected to epifluorescence after treatment with the anti-uPAR monoclonal antibody R2 (see Materials and methods).
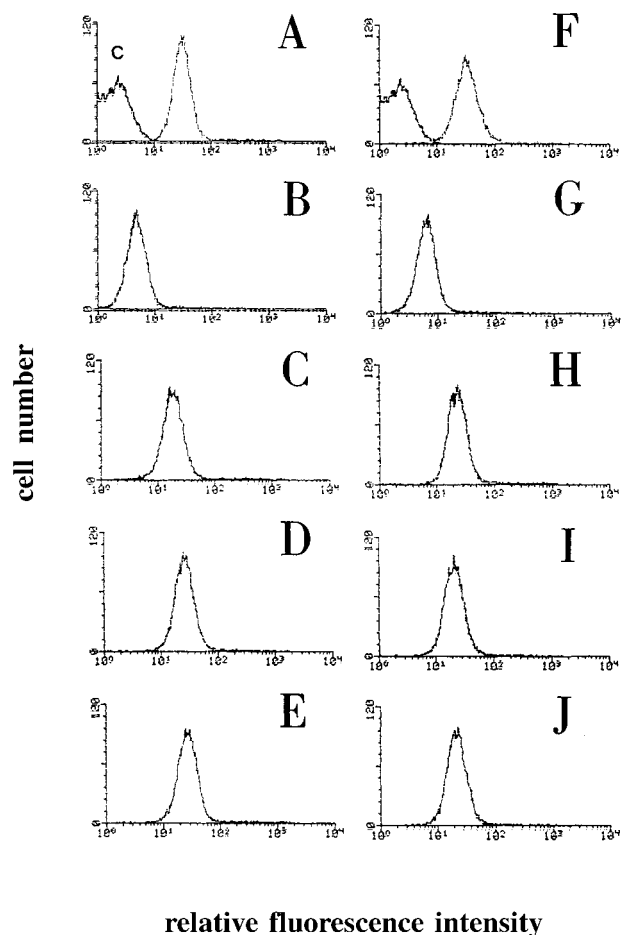

relative fluorescence intensity

Fig. 3. Immunocytofluorimetric analysis of the surface uPAR expression in U937 cells at different times after addition of uPA:PAI-1 or uPA:PN-1. Acid-washed cells were incubated with 10 nM uPA:PAI-1 (A–E) or uPA:PN-1 (F–J) for 90 min at 4°C and then at 37°C for 0 (**A** and **F**), 30 min (**B**, **C**, **G** and **H**), 60 min (**D** and **I**) or 120 min (**E** and **J**). In panels C and H, the cells had received 200 nM RAP in addition to the ligand. The presence of uPAR was analysed by FACS analysis by the use of the anti-uPAR monoclonal antibody R2 (25 µg/ml) (see Materials and methods). Control peak (c) in panel A refers to cells treated with fluorescein-conjugated secondary antibody only.

ated and prepared as described in Materials and methods. After incubation for 2 h at 0°C, with 100 nM uPA:PAI-1, chase for 15 min at 37°C, and treatment with PI-PLC (protocol a, see Materials and methods), most of the labelling (78%) was found over intracellular vacuoles, and 22% on the plasma membrane (Figure 4A). However, after chase–incubation at 37°C for 120 min (protocol b), labelling on the plasma membrane rose to 44% and intracellular vacuolar labelling fell to 56% (Figure 4B). To exclude transport of newly synthesized uPAR to the plasma membrane, all buffers contained 10 µg/ml cycloheximide. Thus, immunoelectron microscopy also

showed that uPAR could at least in part be recycled back to the plasma membrane.

To directly test recycling of uPAR to the cell surface, we labelled cell surface uPAR with biotin and then followed its fate after internalization by immunoprecipitation and avidin detection. Surface-biotinylated LB6 clone 19 cells were challenged with uPAR-saturating levels (50 nM) of ATF or uPA:PAI-1 for 120 min at 4°C, washed, incubated at 37°C for 15 min and treated with PI-PLC for 15 min at 37°C. The PI-PLC supernatant and the cell lysate were immunoprecipitated with uPAR antibodies and biotinylated uPAR was detected by avidin-blotting. As shown in Figure 5A, all uPAR was detected in the PI-PLC supernatant when the cells were not exposed to any ligand. Figure 5B shows the results of PI-PLC treatment in ligand-treated cells: when cells were exposed to ATF, uPAR was again found in the PI-PLC supernatant; in contrast, when cells were incubated with uPA:PAI-1, uPAR was found
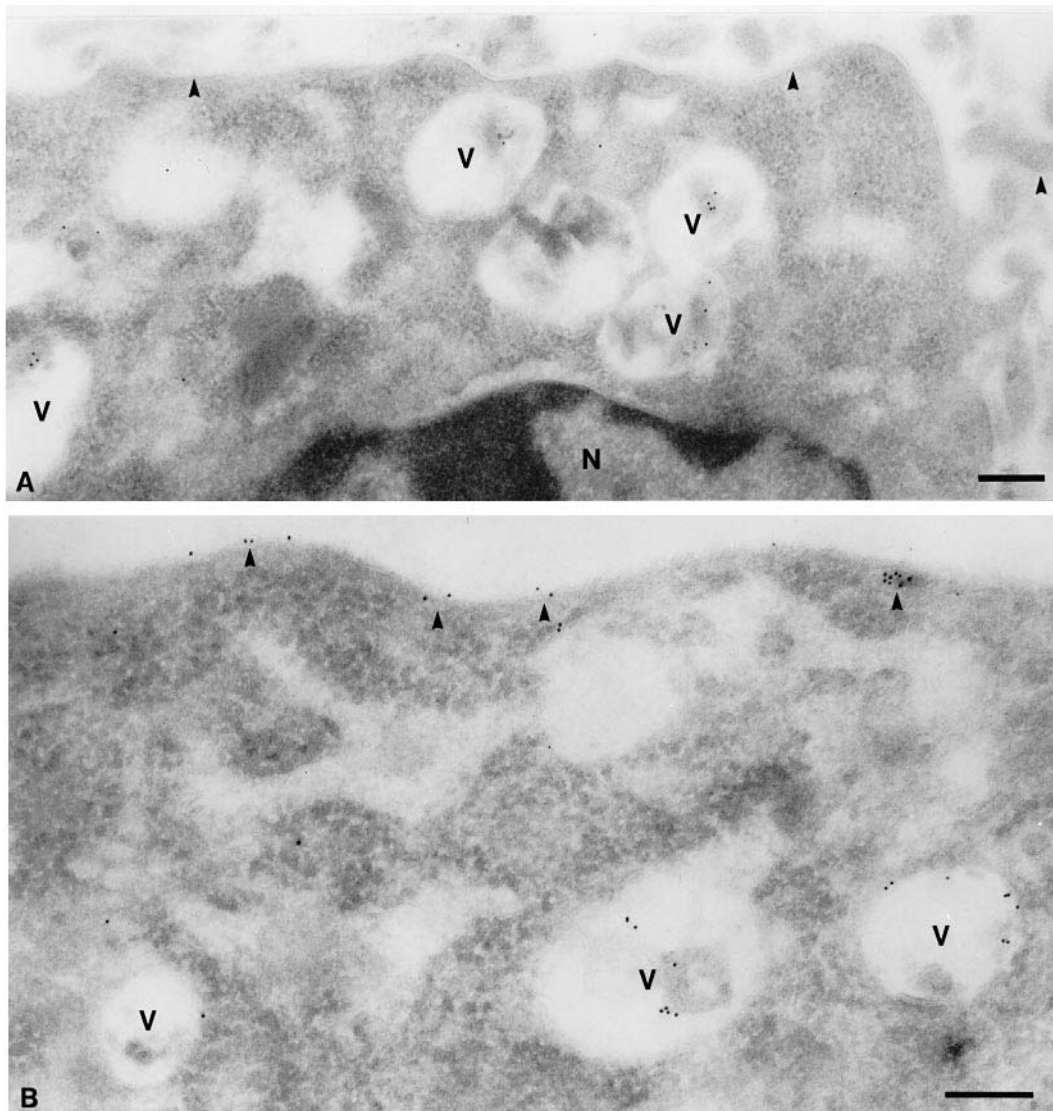
Fig. 4. Immunoelectron microscopic analysis of the fate of internalized uPAR. The cells were incubated as in protocol a (see Materials and methods) (A) with 100 nM uPA:PAI-1 for 2 h at 0°C, chased for 15 min at 37°C and treated with PI-PLC or, according to protocol b (B) after a 120 min chase at 37°C. Electron micrographs from cryosections were prepared for immunocytochemistry as described in Materials and methods. (A) Virtually no labelling is seen on the plasma membrane (arrowheads). Intracellular vacuoles (V) are labelled. No labelling is seen over the nucleus (N). (**B**) Intense labelling is seen on the plasma membrane (arrowheads). Labelling is also seen in vacuoles (V). Bars, 0.25 μm.

mostly in the cell lysate, with only a very small fraction released into the supernatant. To study recycling, the PI-PLC-treated cells were further incubated at 37°C for different time intervals and subjected to a second PI-PLC treatment (now 4°C for 90 min) to prevent re-internalization (these conditions were found efficiently to release surface uPAR; not shown). As shown in Figure 5C, in the case of ATF-challenged cells, PI-PLC supernatants contained little, if any, uPAR. Alternatively, the PI-PLC supernatants of uPA:PAI-1-challenged cells showed minor, though significant, uPAR after only 2 min of incubation at 37°C, and higher levels after 20–30 min of incubation. Concomitantly, uPAR levels were strongly reduced in the cell pellet. The detection of biotinylated uPAR on the cell surface clearly indicates that it can recycle back to that location.

The uPA:PAI-1 ligand, though required to initiate the process, is not required for the internalization/recycling step

The binding of the uPA:serpin complex is required to induce PI-PLC resistance (i.e. internalization) of uPAR (Conese *et al.*, 1995). Since ligand dissociation by acid-washing does not release uPAR (Stoppelli *et al.*, 1986), we can test whether the continuous presence of the ligand is required for uPAR internalization and recycling. Unlabelled uPA or uPA:PAI-1 complex (50 nM) was bound to LB6 clone 19 cells for 120 min at 4°C. The surface-bound ligand was then removed with acid and the washed cells treated with PI-PLC for 15 min at 37°C. The released uPAR was assayed in the PI-PLC supernatant by [^{125}I]ATF cross-linking, SDS–PAGE and autoradiography. The cell pellet was collected, lysed and analysed similarly.
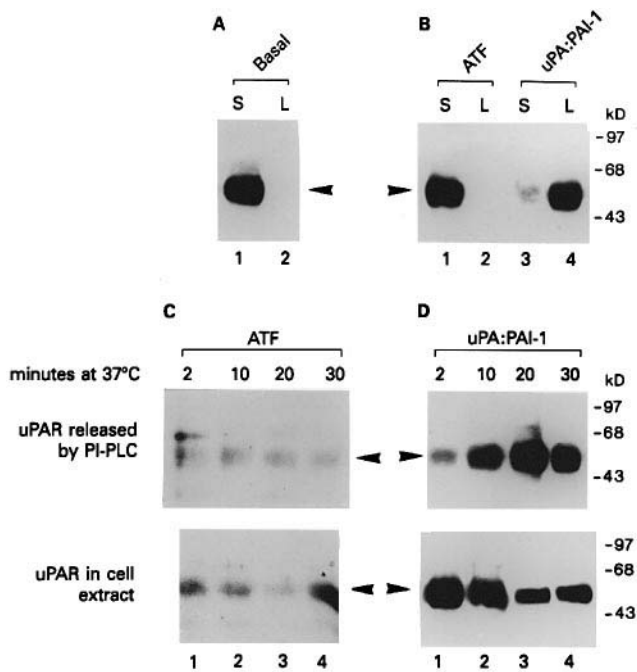
Fig. 5. Recycling of cell surface-biotinylated uPAR in uPA:PAI-1-challenged LB6 clone 19 cells. Cells were surface-biotinylated before treatments and uPAR was detected in the PI-PLC supernatant (S) and cell lysate (L) by immunoprecipitation with anti-uPAR antiserum and avidin detection. (**A**) In untreated cells uPAR was found exclusively in the supernatant after release with PI-PLC. (**B**) In cells incubated with 50 nM ATF or uPA:PAI-1 for 120 min at 4°C, and then at 37°C for 15 min, PI-PLC released uPAR in the supernatant only in the case of ATF. In the case of uPA:PAI-1 treatment, uPAR was PI-PLC-resistant and was found in the cell lysate. (**C**) The cells were treated with ATF, incubated for a further 2–30 min at 37°C and then challenged again with PI-PLC for 15 min at 37°C; they showed neither reappearance of uPAR in the supernatants nor a decrease in the cell lysates. (**D**) In uPA:PAI-1-treated cells, uPAR did reappear on the cell surface as shown by its release by PI-PLC in the supernatant and its decrease in the cell lysate.

Fig. 6. Bound uPA:PAI-1 is not required for the maintenance of uPAR PI-PLC resistance in LB6 clone 19 cells. LB6 clone 19 cells (0.2×10^6 cells/well) were incubated in the absence of ligand (**A**), in the presence of 50 nM uPA or of 50 nM uPA:PAI-1 (**B**) for 2 h at 4°C. The cells were acid-washed in the cold to remove ligands and treated with PI-PLC for 15 min at 37°C. Solubilized uPAR was assayed on the PI-PLC supernatant (S) or on the lysate of the cell pellet (L) by [^{125}I]ATF cross-linking and SDS–PAGE analysis. In (B) the experiment was carried out in the absence (–) or in the presence (+) of 10 μg/ml cycloheximide (see text) to block protein synthesis.

In the absence of ligand, all uPAR was released by PI-PLC (Figure 6A). In uPA:PAI-1-treated cells, the specific [^{125}I]-ATF–uPAR conjugate was found in the cell-associated fraction, but not in the PI-PLC supernatant (Figure 6B). The effect was specific, since in uPA-treated cells, uPAR was found in the PI-PLC supernatant. Thus, although uPA:PAI-1 was required to induce PI-PLC resistance of uPAR at 4°C, its removal did not prevent uPAR internalization.

If the removal of the ligand does not prevent uPAR internalization, it might also not prevent its recycling. To test this point, the two cell lines, U937 and LB6 clone 19, were treated with uPA:serpin complexes at 4°C, then acid-washed and further incubated at 37°C for various times. The cells were then treated with PI-PLC for 15 min at 37°C and released uPAR assayed by [^{125}I]ATF cross-linking, SDS–PAGE and autoradiography. Control, uPA-challenged, LB6 clone 19 cells displayed supernatant uPAR independently of the time of incubation at 37°C (Figure 7A). On the other hand, uPA:PN-1-treated cells showed no uPAR in the PI-PLC supernatant before the 37°C incubation (time zero); upon incubation at 37°C, uPAR reappeared, increasing linearly with time and reaching a maximum at 60 min (Figure 7B). The finding that PI-PLC resistance of uPAR in uPA:PN-1-incubated cells
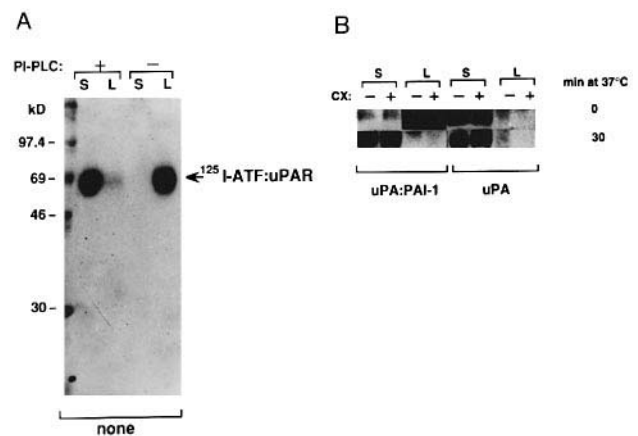
could be reversed upon incubation at 37°C indicates that internalized uPAR can be recycled back to the cell surface, even after dissociation of the uPA:PAI-1 ligand.

Internalization of uPAR is also induced by uPA:serpins in suspension growing monocyte-like U937 cells (Conese *et al.*, 1995). We therefore repeated the above experiment in U937 cells. The uPA-challenged U937 cells (Figure 7C, lane 2), showed a specific [^{125}I]ATF–uPAR adduct in the PI-PLC supernatant independently of the 37°C incubation step. However, [^{125}I]ATF–uPAR adduct was detected in uPA:PAI-1-challenged cells only after incubation at 37°C (Figure 7C, lane 8). Similar results were obtained with cells challenged with uPA:PN-1 complex (Figure 7D). This result again shows that both internalization and recycling of uPAR can occur also in suspension growing cells, after dissociation of the ligand. The experiment also shows that not only uPA:PAI-1, but also uPA:PN-1 complex caused uPAR internalization and recycling.

Reversion to PI-PLC sensitivity might also be explained by cell surface redistribution of either an intracellular pool or of newly synthesized receptors. The latter possibility seems unlikely because identical results were obtained in two cell lines, where different regulatory mechanisms govern uPAR synthesis. In human U937 cells, $\sim 5 \times 10^4$ uPAR are expressed by the endogenous gene, while in murine LB6 clone 19 cells, $\sim 5 \times 10^5$ human receptors are expressed by the transfected SV40-driven cDNA (Stoppelli *et al.*, 1985; Roldan *et al.*, 1990). We nevertheless tested the effect of the protein synthesis inhibitor cycloheximide, in the temperature-dependent reversion to PI-PLC sensitivity of LB6 clone 19 cells. The data showed that treatment of cells with cycloheximide (10 μg/ml, blocking protein synthesis by 90% as shown by ^{35}S metabolic labelling of cells and TCA precipitation of lysates) added 30 min before incubation with uPA:PAI-1, had no influence on the result (Figure 6B). Thus, recovery of PI-PLC
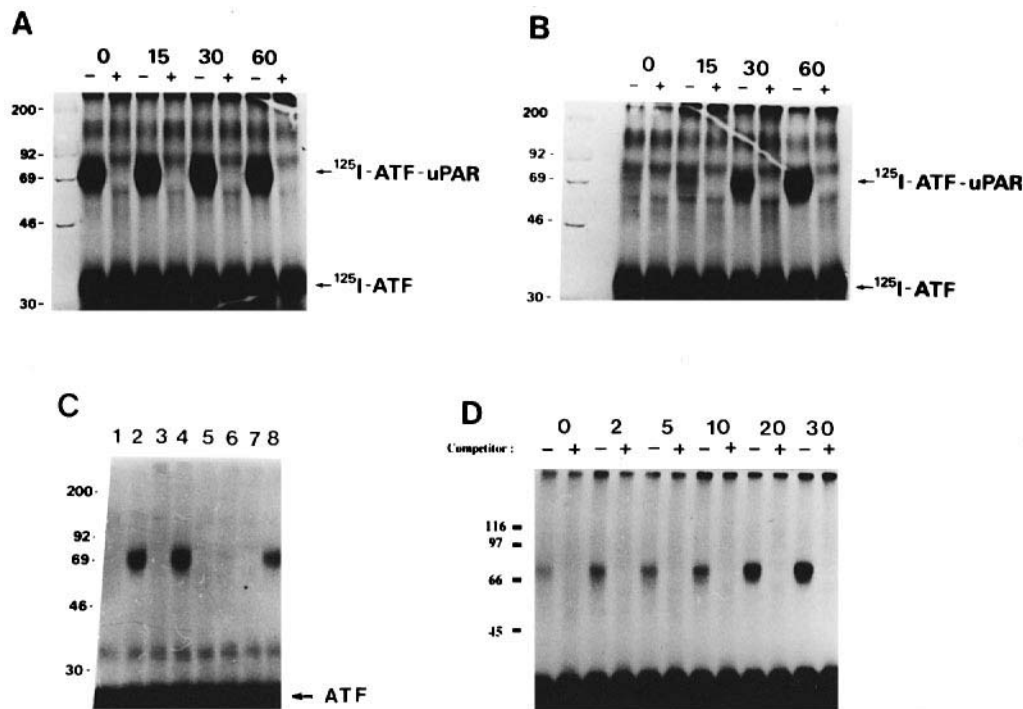
Fig. 7. Incubation at 37°C reverses the PI-PLC resistance of uPAR in U937 and LB6 clone 19 cells challenged with uPA:PAI-1 or uPA:PN-1 complexes. (**A**) Acid-washed LB6 clone 19 cells were incubated on ice with 10 nM uPA for 90 min. Cells were warmed to 37°C for the indicated times, transferred back to ice, acid-washed and treated with PI-PLC (5 U/ml) for 15 min at 37°C. Solubilized uPAR was assayed by cross-linking to [^{125}I]ATF, SDS–PAGE and autoradiography. Specificity was indicated by competition of [^{125}I]ATF cross-linking with 100 nM unlabelled pro-uPA (lanes marked +). (**B**) Conditions as in (A), except that the cells had been incubated with 10 nM uPA:PN-1. (**C**) Acid-washed U937 cells were incubated on ice with 10 nM uPA (lanes 1–4) or 10 nM uPA:PAI-1 (lanes 5–8) for 60 min. Half of the cells were left on ice (lanes 1 and 2, and 5 and 6) and the other half warmed to 37°C for 120 min (lanes 3 and 4, and 7 and 8). All samples were acid-washed, treated with PI-PLC (3 U/ml) for 15 min at 37°C, and supernatants cross-linked to [^{125}I]ATF and analysed by SDS–PAGE and autoradiography. In lanes 1, 3, 5 and 7, excess unlabelled pro-uPA was added for specificity control. (**D**) Acid-washed U937 cells were incubated with 10 nM uPA:PN-1 on ice for 90 min. Cells were warmed to 37°C for the indicated times, transferred back to ice, acid-washed, treated with PI-PLC (5 U/ml) for 15 min at 37°C and the supernatants assayed for the presence of solubilized uPAR as above. Specificity was indicated by competition of [^{125}I]ATF binding to uPAR with 100 nM unlabelled pro-uPA (lanes marked +).

sensitivity did not depend on cell surface exposure of newly synthesized molecules.

That reversion to PI-PLC sensitivity could not be due to surface re-distribution of a previously intracellular uPAR, was also shown by the absence of such a pool. Data in Figure 6A and B show that the lysates of LB6 clone 19 cells either not exposed to ligand or treated with free uPA did not contain any detectable uPAR after treatment with PI-PLC, in agreement with the immuno-fluorescence results of Figure 2. Indeed, uPAR was found in the lysate before and in the supernatant after PI-PLC treatment. Finally, we have previously also shown efficient cross-linking to [^{125}I]ATF with intracellular uPAR (Møller *et al.*, 1992, 1993); therefore the failure to detect an intracellular uPAR could not depend on a limitation of the assay.

Discussion

Recycling
The ability to internalize and degrade uPA:serpin complexes is a unique property of uPAR in view of the absence of a trans-membrane and cytosolic domain. This can only occur because the uPAR-bound uPA:PAI-1 can interact with proteins of the LDL-receptor family such as α2MR-

LRP and VLDL-R (Herz *et al.*, 1992; Nykjær *et al.*, 1992, 1994a; Heegaard *et al.*, 1995). In the process, uPAR itself is internalized through an α2MR-LRP-requiring mechanism (Conese *et al.*, 1995). When we analysed the internalization and degradation of uPA:PAI-1 through uPAR immunofluorescence, we were surprised to find that the signal of internalized uPAR neither decreased nor diminished with time (Figure 1), as would occur for the signal of the ligand (Olson *et al.*, 1992). In fact, uPAR was not degraded but was recycled back to the cell surface, as shown by the following experiments. Immunocyto-fluorimetry with uPA:serpin-incubated U937 cells showed that, at 37°C, the uPAR signal after an initial decrease (due to the internalization) could be recovered at later times (Figure 3). Similar results were obtained by immuno-electron microscopy in EDTA-detached LB6 clone 19 cells (Figure 4). Finally, we surface-labelled uPAR with biotin in LB6 clone 19 cells, incubated the cells at 37°C with uPA:PAI-1 to force uPAR internalization, and treated cells with PI-PLC to eliminate non-internalized uPAR. Upon further incubation at 37°C, biotinylated uPAR re-appeared at the cell surface (Figure 5). Finally, the uPA:PAI-1 and uPA:PN-1-induced resistance of uPAR to release by PI-PLC was reversed in time-dependent manner on incubation at 37°C (Figures 6 and 7). All these data

show that uPAR can recycle. The uPA:PAI-1 ligand, however, does not recycle (our unpublished data).

Steps involved in uPA:PAI-1 clearance

From these and previous results, we can distinguish four steps in the internalization of uPA:PAI-1: (i) binding of uPA:PAI-1; (ii) induction of PI-PLC resistance of uPAR; (iii) endocytosis; and (iv) recycling. While binding of uPA:PAI-1 is essential to initiate the process, the other two steps do not require its continuous presence.

Binding of uPA:PAI-1 to cell surface. In cells that contain both uPAR and α2MR-LRP, uPA:PAI-1 at low physiological concentrations will preferentially bind to uPAR because of its higher affinity (Cubellis *et al.*, 1990; Nykjær *et al.*, 1994a). Indeed, ATF and not RAP, competes for binding of uPA:PAI-1 (Nykjær *et al.*, 1992; Olson *et al.*, 1992; Conese *et al.*, 1994).

PI-PLC resistance of uPAR. Indirect data point to the formation of a quaternary complex between uPA:PAI-1, uPAR and α2MR-LRP. Not only uPAR, but also α2MR-LRP antagonists prevent both degradation of uPA:PAI-1/uPA:PN-1 (Nykjær *et al.*, 1992; Conese *et al.*, 1994) and uPAR internalization. Since the two moieties of uPA:PAI-1 can contact both uPAR and α2MR-LRP, respectively, a quaternary complex might be formed in which the uPA:PAI-1 complex bridges uPAR and α2MR-LRP. This step might occur also at 4°C. However, no direct demonstration of the formation of the quaternary complex has so far been made. Interestingly, uPAR becomes PI-PLC-resistant already in the 4°C step, as shown by experiments such as those described in Figures 6 and 7 (not shown) in which the whole experiment was carried out at 4°C, including PI-PLC release (90 min). The mechanism of PI-PLC resistance is not clear, but uPAR may either undergo a conformational change or be shielded from PI-PLC by, for example, α2MR-LRP.

Internalization of the components of the quaternary complex. When the temperature is raised to 37°C, the interaction of uPAR-bound uPA:PAI-1 with α2MR-LRP triggers the internalization of the ligand and its degradation, as well as the internalization and recycling of uPAR. However, at this point the presence of the ligand is no longer required for uPAR internalization (Figures 6 and 7). Thus, an initial step in which binding of the uPA:serpin complex is essential to trigger the subsequent interaction with α2MR-LRP is followed by the ligand-independent induction of PI-PLC resistance of uPAR and then by the actual internalization.

How is uPAR internalized in a α2MR-LRP-dependent way if the ligand is no longer necessary at the time of the internalization? This might either occur through direct contact between uPAR and α2MR-LRP, independently of the uPA:PAI-1 bridge, or through the intervention of other, hitherto unknown and uncharacterized molecules. A direct interaction between uPAR and α2MR-LRP has no experimental basis, but would not be surprising given the large size of α2MR-LRP and the heterogeneity of its many ligands (Krieger and Herz, 1994). The interaction of uPAR with another protein is also plausible, since uPAR itself has been reported to interact not only with uPA, but also with other molecules like vitronectin, integrins, caveolin

and an unidentified adaptor molecule (Wei *et al.*, 1994, 1996; Bohuslav *et al.*, 1995; Resnati *et al.*, 1996).

Recycling. While we have shown here the recycling of uPAR, we have no information on the mechanism involved. Also, while α2MR-LRP is known to be a recycling receptor (Krieger and Herz, 1994), no information is available on whether it recycles in the case of the uPA:PAI-1 internalization/degradation process. If so, the recycling routes of the two receptors still need to be defined.

Several unsolved problems persist therefore in uPA:PAI-1 internalization. For example, the mechanism of dissociation of uPA:PAI-1 from uPAR and α2MR-LRP, the endocytotic and recycling routes (preliminary data suggest that uPAR and α2MR-LRP can be observed within the same endocytic vesicles; E.I.Christensen, unpublished observations), the molecular basis for the sorting of uPA:PAI-1 to lysosomes versus uPAR and α2MR-LRP to recycling, are issues that need to be addressed in the future.

Functional relevance

The ability of uPAR to recycle provides the plasminogen activation system with a novel property which is highly relevant to its central function in the regulation of cell migration. The aggressive, invasive phenotype of cancer cells is strongly tied to uPAR expression (Hearing *et al.*, 1988; Ossowski, 1988; Crowley *et al.*, 1993; Grøndahl-Hansen *et al.*, 1995; Min *et al.*, 1996) and the uPA/uPAR system appears to be essential in cell recruitment in inflammatory response (Bianchi *et al.*, 1996; Gyetko *et al.*, 1996; Resnati *et al.*, 1996). Several of the individual functions of uPAR have been shown to be involved in the invasive behaviour of cancer: pro-uPA activation, chemotaxis, cell adhesion, cell recruitment, etc. Moreover, PAI-1 itself inhibits cell adhesion and migration, through its ability to bind vitronectin and prevention of its interaction with integrins (Deng *et al.*, 1996; Stefansson and Lawrence, 1996; L.Kjøller, L.Ossowski and P.Andreasen, personal communication). On the other hand, vitronectin itself is a ligand for uPAR and this provides an additional adhesion mechanism for cells (Wei *et al.*, 1994). Thus, uPA/uPAR influence cell migration via both proteolytic, i.e. plasminogen activation (Cubellis *et al.*, 1986; Stoppelli *et al.*, 1986; Ellis *et al.*, 1989; Stephens *et al.*, 1989), and non-proteolytic mechanisms, i.e. by direct signalling (adhesion and chemotaxis) (Busso *et al.*, 1994; Resnati *et al.*, 1996; Wei *et al.*, 1996). PAI-1, on the other hand, inhibits both proteolytic activity of uPA and the integrin-mediated cell adhesion and migration (Deng *et al.*, 1996; Stefansson and Lawrence, 1996; L.Kjøller, L.Ossowski and P.Andreasen, personal communication). Thus, the plasminogen activating system is strongly involved in the attachment/detachment machinery of cells, a process obviously essential for cell migration. Indeed, uPA$^{-/-}$ homologous recombinant mice are deeply impaired in inflammatory cell recruitment (Gyetko *et al.*, 1996), and the uPAR-binding domain of uPA blocks VEGF and bFGF-induced angiogenesis (Min *et al.*, 1996). In this context, the ability of uPAR to induce internalization and degradation of PAI-1 and to recycle back to the cell surface appears to be a very important function in the regulation of the attachment/detachment machinery. The

uPA/uPAR/PAI-1 system therefore appears to be able to inform cells on when, how and where to move. It provides a 'clear' signal through plasminogen activation and uPAR occupancy, a 'stop' signal via PAI-1, and a 'walk' signal through uPA:PAI-1 lysosomal degradation and the concomitant surface reappearance of naked uPAR.

Materials and methods

Materials

Two-chain uPA was obtained from Lepetit SpA (Milan, Italy), courtesy of Dr M.L.Nolli. ATF, the amino-terminal fragment of uPA (residues 1–143) containing the receptor binding site (Stoppelli *et al.*, 1985) was obtained from Abbott Laboratories (Chicago, IL), courtesy of Dr Jack Henkin. Recombinant pro-uPA was obtained from Dr Paolo Sarmientos (Farmitalia, Italy). Anti-uPAR monoclonal antibodies R2, R3 and R4 (Rønne *et al.*, 1991) were purified on a Protein G–Sepharose column using a commercial kit (mAbTrap™ G, Pharmacia LKB, Sweden) from hybridoma cell culture supernatant received from Drs E.Rønne and G.Høyer-Hansen (Copenhagen, Denmark). Recombinant active PAI-1 (Sherman *et al.*, 1992) was a kind gift from Drs David Ginsburg (Ann Arbor, MI) and Tor Ny (Umeå, Sweden); recombinant PN-1 (rPN-1) was a generous gift of Dr Randy Scott (Incyte Co., CA). Preparation of the 39 kDa α2MR-LRP ligand RAP has been described previously (Nykjær *et al.*, 1992). GST and GST-RAP were kindly provided by Dr J.Herz (Herz *et al.*, 1991). Phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus cereus* was from Boehringer Mannheim (Germany). Benzamidine Sepharose 6B was from Pharmacia (Uppsala, Sweden); dimethyl-diphenylpolysiloxane and cycloheximide were from Sigma Chemical Co. (St Louis, MO). The cross-linker disuccinimidyl-suberate and sulfo-NHS-biotin were obtained from Pierce Chem Co. (Rockford, IL).

Cell-lines and cell cultures

Growth conditions for the human monocyte-like U937 and for the murine LB6 clone 19 cells, a mouse cell line expressing the human uPA-receptor, have been previously described (Picone *et al.*, 1989; Roldan *et al.*, 1990).

Mononuclear cells were obtained from the buffy coats of healthy donors by Ficoll gradient sedimentation (Pharmacia, Uppsala, Sweden). Monocytes were separated by adhesion on plastic dishes (60 min at 37°C, repeated once). T-lymphocytes were then isolated by panning on anti-CD16 antibodies to remove natural killer cells and passage through nylon wool fibres to remove B-lymphocytes. T-lymphocytes were stimulated with 50 µg/ml PMA and 10 U/ml IL-2 for 48–72 h to induce the synthesis of uPAR as previously described (Nykjær *et al.*, 1994b).

Iodinations

Iodination of ATF or uPA with Iodogen (Pierce Chemical Co.) has been described (Behrendt *et al.*, 1990). Specific activities of the proteins ranged between 2.5×10^5 and 4.0×10^6 c.p.m./pmol. Iodinated uPA retaining enzymatic activity was purified by affinity chromatography on benzamidine–Sepharose 6B (Holmberg *et al.*, 1976).

Formation of uPA:serpin complexes

Labelled uPA:PAI-1 complexes were formed combining benzamidine–Sepharose-purified ^{125}I-labelled or unlabelled uPA and a 20-fold molar excess of PAI-1 at room temperature for 1 h. The inhibitor:uPA ratio was experimentally determined to convert >90% of the uPA to the complex form as determined by SDS–PAGE analysis (not shown). Similarly, uPA:rPN-1 was formed at a 1:50 ratio as previously described (Conese *et al.*, 1994). Purified, preformed uPA:PAI-1 complex for immunoelectron microscopy experiments was a kind gift of Dr Peter A.Andreasen and was prepared as described before (Nykjær *et al.*, 1994a).

Release of surface-bound ligand

Surface-bound ligands were detached from the plasma membrane either by dissociation with acid-washing (Stoppelli *et al.*, 1986) or by incubating cells with 5 U/ml PI-PLC (Ploug *et al.*, 1991) at 37°C for 15 min.

PI-PLC sensitivity of uPAR

Approximately 2×10^6 U937 cells (previously incubated under conditions indicated in the individual experiments) were resuspended in 0.1 ml binding buffer (RPMI-1640 containing 50 mM HEPES, pH 7.4 and

1 mg/ml BSA) and treated with 5 U/ml PI-PLC at 37°C for 15 min or at 4°C for 90 min. On completion of incubation, the supernatants were recovered by centrifugation and assayed for solubilized uPAR by cross-linking to [^{125}I]ATF and SDS–PAGE analysis (see below). In the case of LB6 clone 19 cells, 0.2×10^6 cells were used, but methods and conditions were otherwise identical. In some experiments, after PI-PLC treatment and collection of the supernatants, the pellets were lysed in lysis buffer (0.1 M Tris–HCl, pH 8.1, 10 mM EDTA, 1% Triton X-114 and 1% aprotinin) and cross-linked to [^{125}I]ATF as described below.

Cross-linking to [^{125}I]ATF

Cell lysates or supernatants were cleared at 15 000 *g* for 10 min at 4°C and tested for uPAR by cross-linking to [^{125}I]ATF (50 000 c.p.m., 1500 c.p.m./fmol), using 1 mM DSS (disuccinimidyl suberate), as previously described (Picone *et al.*, 1989). The samples were analysed by SDS–PAGE (12% acrylamide) under non-reducing conditions (Laemmli, 1970). The specificity of the ATF:uPAR conjugate was shown in all cases by competition with 100 nM unlabelled two-chain uPA or pro-uPA.

Immunodetection of surface-biotinylated uPAR

Biotinylation of cell surface proteins was carried out on 70–80% confluent LB6 clone 19 cells. After three washes with phosphate-buffered saline (PBS), cells were incubated with 0.5 mg/ml sulfo-NHS-biotin for 30 min at room temperature. The labelling solution was removed and the procedure repeated once. The cells were then washed three times with PBS, treated with ligands and PI-PLC as described in the Results section and lysed. The lysate and the PI-PLC supernatant were supplemented with antibodies (monoclonals R2, R3 and R4 1 µg/ml each), incubated for 1 h at 4°C with gentle rocking. Protein G–Sepharose (50 µl of a 10% suspension) was added, the mixture further incubated 1 h at 4°C and centrifuged. The precipitate was washed, supplemented with SDS-sample buffer, heated, centrifuged and the supernatant subjected to SDS–PAGE in 12.5% acrylamide. After transfer onto nitrocellulose filter (Schleicher & Schuell, Dassel, Germany), the blot was blocked in PBS–3% BSA for 1 h at room temperature. The membrane was then washed six times with TBS–0.5% Tween 20 (TTBS) and incubated in PBS containing 0.5 mM CaCl₂, 0.5 mM MgCl₂, 1 M glucose, 10% (v/v) glycerol, 0.3% BSA and [^{125}I]streptavidin (0.6 µCi/ml; Amersham) for 30 min at room temperature. After six washes in TTBS, the membrane was exposed to X-ray film at –80°C.

Light microscopic procedures

LB6 Clone 19 cells were studied either by conventional epifluorescence or confocal microscopy: in both cases, the specimens were processed as described (Conese *et al.*, 1995). Briefly, 0.2×10^6 cells were plated onto 24-well Costar plates containing 1.4 cm² round glass coverslips. Cells were fixed in 3% formaldehyde (from paraformaldehyde) in PBS pH 7.6–2% sucrose, for 10 min at 4°C to avoid permeabilization. For permeabilization, coverslips were soaked, after fixation, for 3–5 min in HEPES–Triton X-100 buffer (20 mM HEPES, pH 7.4, 3 mM MgCl₂ and 0.5% Triton X-100). Primary monoclonal anti-uPAR antibody R2 was used at 15 µg/ml in TBS–0.2% bovine serum albumin; a fluorescein-tagged secondary antibody (Protos Immunoresearch, San Francisco, CA) was used at a 1:200 dilution in TBS–0.2% bovine serum albumin.

Conventional epifluorescence was carried out on a Zeiss Axiophot microscope and photomicrographs were recorded on a Kodak T-MAX 400 film exposed at 1000 ISO (Eastman Kodak, Rochester, NY). For confocal microscopy, a Sarastro 2000 confocal laser scanning system (Molecular Dynamics, Sunnyvale, CA) attached to a Zeiss Axioskop fluorescence microscope was employed. The objectives used were a plan-apochromatic 63×1.4 and a plan-apochromatic 100×1.3 (Carl Zeiss). Optical sections of 0.29 µm thickness were saved as 512×512 pixel images and then processed on a McIntosh computer. Final images were printed on photographic quality paper with a Kodak DL-7700 sublimation ink printer.

Cytofluorimetric analysis

Acid-washed-U937 cells (1×10^6) were incubated with uPA:PAI-1 or uPA:PN-1 complexes for 2 h at 4°C and then transferred to 37°C for different times. Cells were then washed twice with PBS, and incubated in 0.1 ml PBS and 25 µg/ml of anti-uPAR monoclonal antibody R2. After 30 min of incubation at 4°C, cells were washed twice with PBS and resuspended in PBS containing a 1:50 dilution of fluorescein-conjugated antibody against mouse IgG (Dakopatts, Copenhagen, Denmark). After 30 min at 4°C, cells were washed twice and analysed by flow cytofluorimetry with a FACScan apparatus (Becton-Dickinson, San Jose, CA). To express the data, the cell number was plotted against

the log of the mean fluorescence intensity, with ~5000 cells being measured at each determination. The negative control was obtained incubating the cells in the absence of the primary antibody.

Electron microscopy
LB6 clone 19 cells (5×10^6) were pretreated for 30 min with 10 µg/ml cycloheximide and incubated (protocol a) for 2 h with 100 nM uPA:PAI-1 at 0°C, washed, treated with 5 U/ml PI-PLC at 37°C for 15 min and then fixed. To demonstrate possible recycling (protocol b), cells were washed after the incubation step with PI-PLC, further incubated for 120 min in the presence of cycloheximide at 37°C, and then fixed following detachment from coverslips with EDTA. The cells were fixed with 0.1% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 1 h and subsequently post-fixed for up to 18 h in 2% formaldehyde in the same buffer. The cells were embedded in 15% gelatin, then infiltrated with 2.3 M sucrose containing 2% formaldehyde for 30 min and frozen in liquid nitrogen. Ultrathin cryosections, 70–90 nm, were obtained with a RCS Reichert Ultracut S cryoultramicrotome at approximately –100°C and collected on 300 Mesh Ni grids. The sections were incubated overnight at 4°C with polyclonal affinity-purified rabbit anti-uPAR IgG (5 µg/ml) and subsequently with 10 nm goat anti-rabbit-gold (BioCell, Cardiff, UK) at 4°C for 2 h. The sections were finally contrasted with methyl cellulose containing 0.3% uranyl acetate (Tokuyasu, 1978; Griffiths *et al.*, 1984) and studied in a Philips EM208 or Philips CM100 electron microscope.

Immunogold distribution over the cells was determined as follows: ~25 electron micrographs were taken at random but including as much cytoplasm and cell surface as possible from each of the two groups (a and b) at a primary magnification of ×11 500 and then enlarged 3-fold. Gold particles were counted over the plasma membrane, over cytoplasmic vacuoles, and over the nucleus. The cytoplasmic areas analysed in the two groups as determined by point-counting were essentially identical: (a) 1210 µm^2 and (b) 1215 µm^2. The total number of gold particles counted was 5298. The background labelling which was determined by the number of particles present over the nucleus was 0.16/µm^2 in protocol a and 0.10/µm^2 in protocol b.

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