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Urokinase-Type Plasminogen-Activator Receptor Associates to a Cell Surface Molecule in Monocytic Cells

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The monocyte-like THP-1 cells express on their surface the urokinase-type plasminogen activator receptor (uPA-R). This receptor, chemically cross-linked to its possible ligands, migrates, in SDS PAGE, slower than the uPA-R expressed on the epithelial thyroid cell line TAD-2, cross-linked to the same ligands. The different migration corresponds to a difference in molecular weight of 15 kDa. Similar results were obtained with peripheral monocytes and primary cultures of thyroid cells. The molecular weight of the native receptor is about 50 kDa and appears to be identical in these two cell types. Such results suggest that, in monocytic cells, uPA-R associates to a 15 kDa molecule. This molecule is probably linked to the cell surface by a glyco-phospho-inositol anchor since, by phospholipase-C treatment, it is co-eluted with the urokinase-type plasminogen activator receptor from THP-1 cells.

The urokinase-type plasminogen activator (uPA) is a serine protease able to convert plasminogen into plasmin, which can promote the degradation of many components of the extracellular matrix (ECM), directly or activating latent collagenases (1-2). The active form of the enzyme consists of two chains, A and B, linked by a disulfide bridge. The B chain contains the catalytic site of the enzyme and binds specific inhibitors, PAI-1 and PAI-2 (3-4). The A chain, or its N-terminal fragment (ATF), binds the uPA receptor (uPA-R) (5), a heavily glycosylated polypeptide chain, anchored to the cell membrane by a glyco-phospho-inositol (GPI) tail (6).

In the present report we show that uPA-R, bound to its ligands, generates complexes of unexpected high molecular weight in monocytic cells. Such results suggest that, in monocytic cells, uPA-R associates to a 15 kDa molecule. This molecule, very likely, is GPI-linked to the cell surface since, by phospholipase-C treatment (PLC, E.C. 3.1.4.3), it is co-eluted with the urokinase-type plasminogen activator receptor from THP-1 cells.

MATERIALS AND METHODS

Reagents. Human uPA (*M*r=55,000) was obtained from Serono (Denens, Switzerland), recombinant human PAI-2 from Behring (Marburg, Germany). ATF was a kind gift of Dr. F. Blasi. The monoclonal antibody specific for uPA-R was from American Diagnostica Inc. (Greenwich, CT USA). Disuccinimidyl suberate (DSS), Biotin and Iodogen were from Pierce Chemical Co. (Rockford, IL, USA), Na- [^{125}I] and ECL detection kit from Amersham International

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Abbrevations: uPA: urokinase-type plasminogen activator; PAI-2: type-2 plasminogen activator inhibitor; uPA-R: uPA receptor; ATF: N-terminal uPA fragment; GPI: glyco-phospho-inosytol; iPr_2P-F: diisopropylfluorophosphate; DSS: Disuccinimidyl suberate; NaCl/Pi: phosphate buffer saline; DMEM: Dulbecco modified Eagle medium; PtdIns: Phoshatidyl-inositol; PLC: Phospholipase C; PtdIns-PLC: Phoshatidyl-inositol-Phospholipase C.

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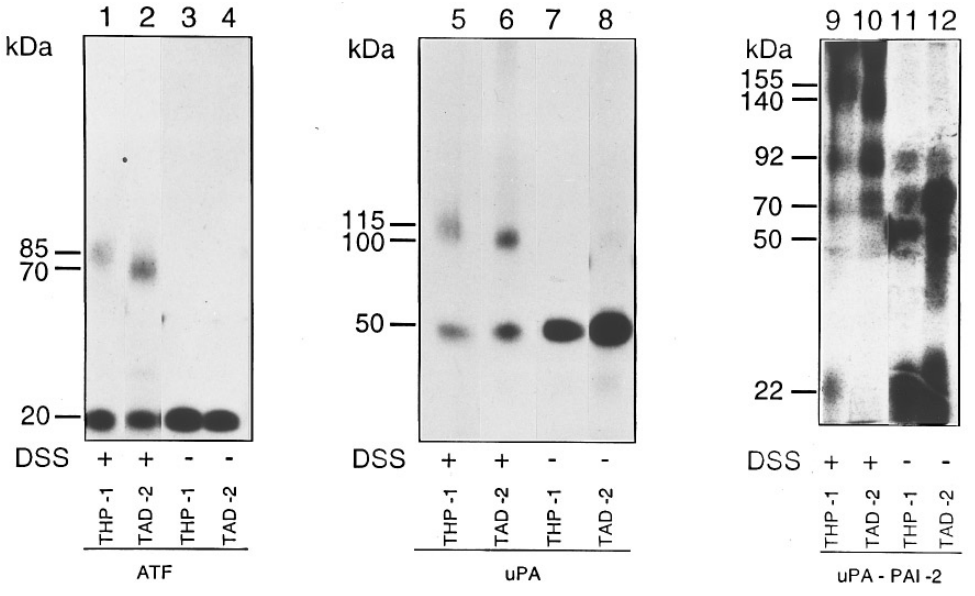

FIG. 1. Cross-linking of ATF, uPA, and uPA-PAI-2 complexes to THP-1 and TAD cells. THP-1 cells (lanes 1,3,5,7,9,11) and TAD-2 cells (lanes 2,4,6,8,10,12) were incubated with [^{125}I]ATF (lanes 1–4), iPr$_2$P-F-treated [^{125}I] uPA (lanes 5–8), or [^{125}I] uPA–PAI-2 preformed complexes (lanes 9–12). Cells were then treated with DSS (lanes 1,2,5,6,9,10) or medium alone (lanes 3,4,7,8,11,12), lysed and analyzed by 10% SDS-PAGE and autoradiography.

(Amersham, England), Phosphatidylinositol Phospholipase C (PtdIns-PLC) from Boehringer (Mannheim, Germany). Type IV collagenase was from SIGMA (St. Louis, MO USA), PVDF from Millipore (Windsor, MA USA) and Protein A-Sepharose from Pharmacia (Uppsala, Sweden).

Cell culture. Human monocytic leukemia THP-1 (7) and fetal thyroid TAD-2 (8) cell lines were cultured in RPMI 1640 medium (Flow, McLean, VA, USA). Monocytes were grown in Dulbecco Modified Eagle Medium (DMEM) (Biowhittaker Verviers, Belgium) and primary cultures of thyroid in Ham's F12 medium (GIBCO, Gaithesburg, MD, USA). The different media were supplemented with 10% fetal calf serum (GIBCO).

Thyroid primary cultures were obtained as previously described (9) from the unaffected contralateral lobes of thyroid papillary carcinomas after total thyroidectomy. Monocytes were prepared from peripheral blood. Blood mononuclear cells, obtained by Ficoll-Hypaque gradient, were plated in culture dishes and incubated overnight in a 5% CO_2 atmosphere at 37°C. Adherent monocytes were washed and used for binding experiments.

Cell surface biotinilation. Cells were washed with phosphate buffered saline (NaCl/Pi: 0.14 M NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2) and incubated with NHS-LC-biotin 0.5 mg/ml in NaCl/Pi for 30° at room temperature with constant gentle agitation. The supernatant was then removed and the reaction stopped by the addition of glycine, 50 mg/ml in NaCl/Pi, for 10′ at room temperature. Finally, cells were washed with NaCl/Pi and lysed in 1% Triton X-100.

Iodination and inactivation of uPA. uPA was radiolabeled by iodogen and Na-[^{125}I] and inactivated by diisopropylfluorophosphate (iPr$_2$P-F) as described (10).

Preparation of uPA–PAI complexes. uPA-PAI complexes were formed by incubating 10^{-9}M labeled uPA with a 20 fold molar excess of PAI at room temperature for 1h.

Binding assay. Cells, stripped of surface-bound endogenous uPA by acid treatment (11), were incubated for 2 h at 4°C with [^{125}I]ATF, iPr$_2$P-F treated [^{125}I]uPA (10^{-9} M) or [^{125}I]uPA-PAI-2 preformed complexes. Cells were then washed, subjected to different treatments (see below) and lysed with 1% Triton X-100.

Cross-linking. Cross-linking was performed after binding as previously described (11).

Immunoprecipitation. Iodinated or biotinilated samples were incubated 2 h at 4°C with 10 μg/ml of an anti-uPA-R polyclonal antibody or with 10 μl/ml of normal mouse serum. Immunocomplexes were then precipitated by protein A-Sepharose as previously described (11), eluted by boiling for 10 min in Laemmli buffer (12) and loaded on a 10% SDS-polyacrylamide gel. The [^{125}I] labeled proteins were detected by autoradiography of the dried gel. The biotin labeled proteins were transferred onto a PVDF membrane. The membrane was blocked for 1 h at room temperature with 5% non fat dry milk, 1% ovalbumin, 5% fetal calf serum and 7.5% glycine. The filter was then washed in washing solution (NaCl/Pi containing 0.1% Tween 20) and incubated for 1 h at room temperature with horseradish

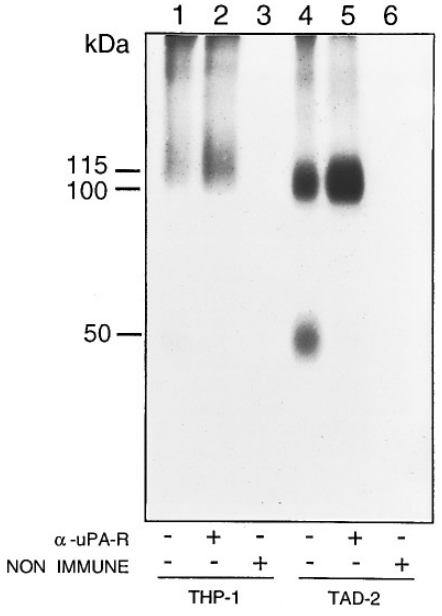

FIG. 2. Immunoprecipitation of uPA-R cross-linked to [^{125}I] uPA in THP-1 and TAD-2 cells. THP-1 cells (lanes 1–3) and TAD-2 cells (lanes 4–6) were incubated with inactivated [^{125}I] uPA, treated with DSS, lysed (lanes 1,4), and immunoprecipitated with 10 μg/ml of an anti-uPA-R polyclonal antibody (lanes 2,5) or with 10 μl/ml of nonimmune serum (lanes 3,6). The immunocomplexes were analyzed by 10% SDS-PAGE and autoradiography.

peroxidase-conjugated streptavidin diluted 1:1500. Finally, the filter was washed three times with washing solution for 10′ at room temperature. Samples were stained using the ECL detection kit.

PtdIns-PLC treatment. Cells were treated with 1 U/ml of Phoshatidyl-inositol-Phospholipase C (PtdIns-PLC) as described (13).

RESULTS AND DISCUSSION

Cross-linking of uPA-R bound to its ligands. THP-1 cells were treated with or without the cross-linking agent DSS after binding iodinated ATF, uPA or preformed uPA-PAI-2 complexes. SDS-PAGE analysis, under non reducing condition, showed that ATF, 20 kDa m.w., (Fig.1, lane 3), uPA, 50 kDa m.w., (Fig.1, lane 7) and uPA-PAI-2 complex, 92 kDa m.w., (Fig.1, lane 11) generated cross-linked products of 85 kDa (Fig. 1, lane 1), 115 kDa (Fig.1, lane 5) and 155 kDa respectively (Fig.1, lane 9). Since the molecular weight of uPA-R is about 50 kDa, all the observed cross-linked bands, obtained by uPA-R bound to ATF, uPA or uPA-PAI-2 complex, were 15 kDa higher than expected. Such a result could have several possible explanations. It could be due to an artefact of the cross-linking tecnique, to a different glycosilation of uPA-R in this particular cell line, or to cross-linking of an unknown molecule to uPA-R. In order to verify the first hypotesis, the cross-linked bands obtained in THP-1 cells were compared with the corresponding cross-linked molecules obtained in a different cell type, the epithelial thyroid TAD-2 cells. Iodinated ATF, uPA and uPA-PAI-2 complexes (Fig.1, lanes 4,8,12), cross-linked to TAD-2 cells (Fig.1, lanes 2,6,10), all gave bands with the expected molecular weight, 15 kDa lower than the corresponding bands in THP-1 cells, thus excluding an artefact due to the cross-linking technique.

Similar results were obtained on adherent monocytes and thyroid primary cultures, thus indicating that the cross-linking results were not due to an altered behaviour in culture of the two cell lines (data not shown).

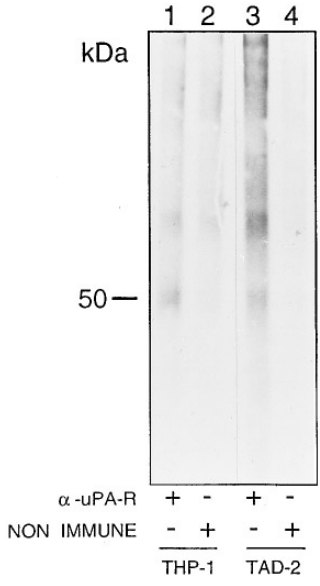

FIG. 3. Immunoprecipitation of biotinilated uPA-R in THP-1 and TAD-2 cells. Lysates of surface-biotinilated THP-1 cells (lanes 1,2) and TAD-2 cells (lanes 3,4) were immunoprecipitated with 10 μg/ml of an anti-uPA-R polyclonal antibody (lanes 1,3) or with 10 μl/ml of a nonimmune serum (lanes 2,4). The immunocomplexes, electrophoresed on a 10% SDS-PAGE and transferred onto a PVDF membrane, were stained using the ECL detection kit.

Immunoprecipitation of uPA-R cross-linked to [^{125}I] uPA in THP-1 and TAD-2 cells. Cross-linking of ATF, uPA and uPA-PAI-2 complexes to uPA-R of THP-1 and TAD-2 cells was verified by immunoprecipitation. The m.w. differences of cross-linked molecules between the two cell lines were independent from the type of ligand, therefore only lysates of THP-1 (Fig.2, lanes 1-3) and TAD-2 cells (Fig.2, lanes 4-6) cross-linked to iodinated uPA were immunoprecipitated with a polyclonal antibody against uPA-R. Analysis by SDS-PAGE showed that the antibody precipitated the cross-linked products of both cell lines (Fig.2, lanes 2 and 5 respectively).

Biotinilation of uPA-R in THP-1 and TAD-2 cells. uPA-R glycosilation variants, with slightly different m.w., have been described (14). Therefore it was investigated whether the altered m.w. of cross-linked uPA-R-uPA in THP-1 reflected a different molecular weight of uPA-R in THP-1 cells as compared to TAD-2 cells. Cell surface antigens of both cell types were biotinilated and immunoprecipitated by a polyclonal antibody against uPA-R. The immunoprecipitated samples, electrophoresed on a 10% SDS-PAGE and transferred onto a PVDF membrane, were detected by ECL. The m.w. of uPA-R (50 kDa) was identical in both cell lines (Fig.3, lanes 1,3). Since cross-linking of identical receptors to the same ligands should give products of the same m.w., it was reasonable to hypothesize that, in THP-1 cells, DSS cross-links an unknown 15 kDa surface molecule to uPA-R occupied by ATF, uPA or uPA-PAI-2 complexes.

PtdIns-PLC experiments. uPA-R is anchored to the cell membrane by a glyco-phosphoinositol (GPI) tail (6). In order to assess whether the 15 kDa uPA-R associated molecule had a similar cell anchoring, we investigated its sensitivity to PtdIns-PLC treatment. [^{125}I]uPA treated TAD-2 and THP-1 cells were incubated with DSS (Fig.4, lanes 3,6); aliquotes were then eluted with PtdIns-PLC (Fig.4, lanes 2,5). Other cell samples, viceversa, after binding uPA, were first eluted with PtdIns-PLC, then DSS was added to the PtdIns-PLC eluates, enriched in uPA-R-uPA complexes (Fig.4, lanes 1,4). Analysis by SDS-PAGE revealed that

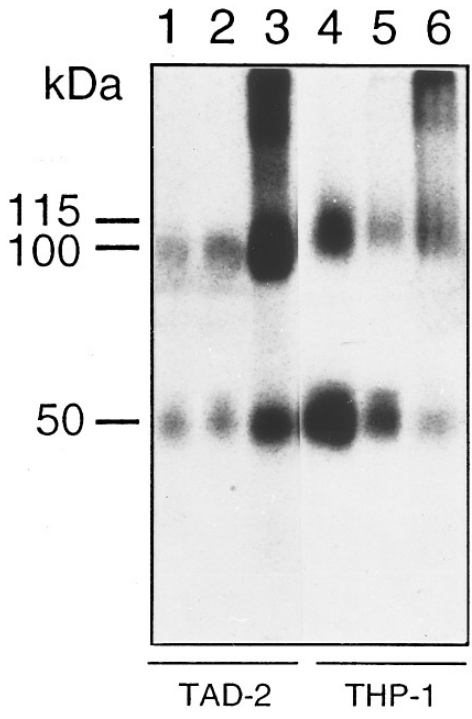

FIG. 4. Release by PtdIns-PLC of cross-linked uPA–PAI-2 complexes from THP-1 cells. TAD-2 cells (lanes 1–3) and THP-1 cells (lanes 4–6) were incubated with inactivated uPA, treated with DSS (lanes 2,3,5,6), and then incubated with (lanes 2,5) or without (lanes 3,6) PtdIns-PLC. Aliquots of uPA-treated cells were incubated directly with PtdIns-PLC, and DSS was added to the PtdIns-PLC eluates (lanes 1,4). Finally, PtdIns-PLC eluates (lanes 1,2,4,5) and PtdIns-PLC untreated cells (lanes 3,6) were analyzed by 10% SDS-PAGE and autoradiography.

uPA-R cross-linked to uPA in solution (Fig.4, lane 4), or on the cell surface (Fig.4, lane 6) and uPA-R cross-linked to uPA on the cell surface and eluted by PtdIns-PLC (Fig.4, lane 5) gave rise to identical bands of 115 kDa in THP-1 cells. All these bands were higher than the corresponding ones in TAD-2 cells (Fig.4, lanes 1,2,3). These results strongly suggest that the molecule, which associates to uPA-R in THP-1 cells, is released from cell-surface by PtdIns-PLC and therefore is a GPI-linked protein as uPA-R.

CONCLUSION

In this report we show that uPA-R associates to a 15 kDa GPI-anchored cell surface protein in cells of monocytic origin and not in epithelial thyroid cells. It has been previously reported that cross-linking of GPI-anchored proteins could induce tyrosine-phosphorylation (15) and recently, many reports suggest that uPA-R occupancy could activate signal transduction pathways in monocyte-like cells (16-18). We therefore hypotesize that the first step in the signal transduction pathway mediated by uPA-R is the association of uPA-R to a 15 kDa GPI-linked protein.

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