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Cell-induced potentiation of the plasminogen activation system is abolished by a monoclonal antibody that recognizes the NH_2-terminal domain of the urokinase receptor

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We have raised four monoclonal antibodies recognizing different epitopes within the human cell-surface receptor for urokinase-type plasminogen activator (u-PA). One of these antibodies completely abolishes the potentiation of plasmin generation observed upon incubation of the zymogens pro-u-PA and plasminogen with U937 cells. This antibody, which is also the only one to completely inhibit the binding of DFP-inactivated [^{125}I]-u-PA to U937 cells, is directed against the u-PA binding NH_2-terminal domain of u-PAR, a well-defined fragment formed by limited chymotrypsin digestion of purified u-PAR, demonstrating the functional independence of the u-PA binding domain as well as the critical role of u-PAR in the assembly of the cell-surface plasminogen activation system.

Urokinase receptor; Plasminogen activation; Monoclonal antibody; U937 cell

1. INTRODUCTION

Plasmin, generated from plasminogen by u-PA, is involved in the degradation of extracellular matrix proteins in a variety of processes requiring tissue destruction or cell migration, including cancer invasion. The activity of u-PA is regulated by two fast acting plasminogen activator inhibitors, PAI-1 and PAI-2, and also potentially by a specific cell-surface u-PA receptor (u-PAR) [1–3].

u-PAR was first detected on monocytes and monocyte-like cells by a specific, high-affinity binding of u-PA [4] and its zymogen pro-u-PA [5], and has since been observed on a variety of cultured cells of neoplastic and non-neoplastic origin [6–9]. However, the existence of additional u-PA-binding molecules on the cell surface cannot be excluded [10–12]. We have recently purified and characterized u-PAR from PMA-treated U937 cells [9,13] and cloned a full-length cDNA coding for this receptor [14]. It is an M_r 55–60000 single-chain highly glycosylated protein which is attached to the plasma membrane by a glycosyl-phosphatidylinositol lipid anchor [15]. The mature protein consists of three homologous internal repeats, each of approximately 90 amino acids. An isolated NH_2-terminal chymotryptic fragment, constituting the first of these repeats, binds to the receptor-binding part of u-PA in a chemical cross-linking assay [16].

The virtually inactive pro-u-PA [17,18] can be converted to active u-PA while cell-bound [5], and u-PA thus bound can activate plasminogen [5,19–21]. Concomitant binding of pro-u-PA and plasminogen to cells results in a rapid acceleration of plasmin generation [19].

In order to further study structure–function relationships in the u-PAR molecule and to determine whether the binding of u-PA to u-PAR is responsible for the cellular potentiation of plasminogen activation, we have now developed murine monoclonal antibodies against human u-PAR.

2. EXPERIMENTAL

2.1. *Preparation of monoclonal antibodies*

Purified u-PAR protein [13] was used for immunization of a BALB/c mouse, and cells from the spleen and peripheral lymph nodes were fused with X63-Ag 8.6.5.3. myeloma cells essentially as described [22]. Hybridomas were initially screened by an ELISA, using 2 ng purified u-PAR per well in 96-well plates (Flat bottom high binding capacity, Nunc).

Fusion of the spleen (1000 wells) and lymphnode (90 wells) cells resulted in 24 positive hybridomas. Four of these were selected for cloning on the basis of criteria including the ability of their conditioned media to react with u-PAR in Western blotting and to inhibit binding of [^{125}I]ATF to U937 cells. Antibodies from the resulting clones were purified on a Protein G-Sepharose column (MAb Trap G, Pharmacia). Clones producing antibodies of the indicated subclasses, were

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Abbreviations: u-PA, urokinase-type plasminogen activator; pro-u-PA, zymogenic form of u-PA; u-PAR, u-PA receptor; ATF, NH_2-terminal fragment (1–135) of u-PA; DFP, diisopropyl fluorophosphate; PMA, phorbol 12-myristate 13-acetate; TNP, 2,4,6-trinitrophenol; PBS, phosphate-buffered saline; DSS, disuccinimidyl suberate; BSA, bovine serum albumin; CHAPS, 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

designated R1 (IgG_{2b}, Kappa), R2 (IgG_1, Kappa), R3 (IgG_1, Kappa) and R4 (IgG_1, Kappa).

2.2. *Immunoprecipitation of purified u-PAR*

Both ^{125}I-labelled chymotryptic fragments and intact u-PAR [16] were diluted in 0.1 M Tris-HCl, pH 8.1, 0.3 M NaCl, 0.1% BSA, 0.1% CHAPS (reaction buffer) to an activity of 3.5×10^4 cpm in 100 μl and incubated with an equal volume of each of the monoclonal antibodies against u-PAR (10 μg/ml final concentration) or relevant controls for 1 h at 4°C. 50 μl of a 50% suspension of Protein A-Sepharose CL4B (Pharmacia) in reaction buffer was added and the samples incubated for 1 h at 4°C with mixing. The Protein A-Sepharose was recovered by centrifugation and washed in reaction buffer followed by reaction buffer without BSA. The beads were then resuspended in 50 μl SDS-PAGE sample buffer and boiled for 5 min. The samples were analyzed by SDS-PAGE in linear 6–16% gradient slab gels under non-reducing conditions and autoradiography.

2.3. *Miscellaneous procedures*

All other materials and procedures were as described [13,19,21].

3. RESULTS

3.1. *Differential reactivity of four monoclonal antibodies against defined proteolytic fragments and glycosylation variants of u-PAR*

Fig. 1A shows that each of the four antibodies immunoprecipitates the intact u-PAR molecule. Mild chymotrypsin treatment was then used to cleave purified u-PAR at a single site into a u-PA binding fragment of $M_r \approx 16000$ and a fragment of M_r 30–50000 with no u-PA binding activity [16]. The $M_r \approx 16000$ chymotryptic fragment was found to be specifically precipitated by antibody R3, whereas the R1, R2 and R4 antibodies specifically precipitated the M_r 30–50000 fragment (Fig. 1B).

We have previously observed that PMA-treated cells of the subline U937a have, in addition to the M_r 55–60000 u-PAR protein, a glycosylation variant with an apparent M_r of 40–50000 which is not detected in the U937 cell-line [13]. The anti-u-PAR antibodies were found to recognize these two glycosylation variants differently in Western blots (data not shown). R2 and R3 reacted with both glycosylation variants, while R4 reacted primarily with the lower M_r u-PAR variant. There was no detectable binding of the R1 antibody to either u-PAR variant in Western blotting.

The reactivity pattern of the antibodies in these experiments indicates that they recognize different epitopes within u-PAR.

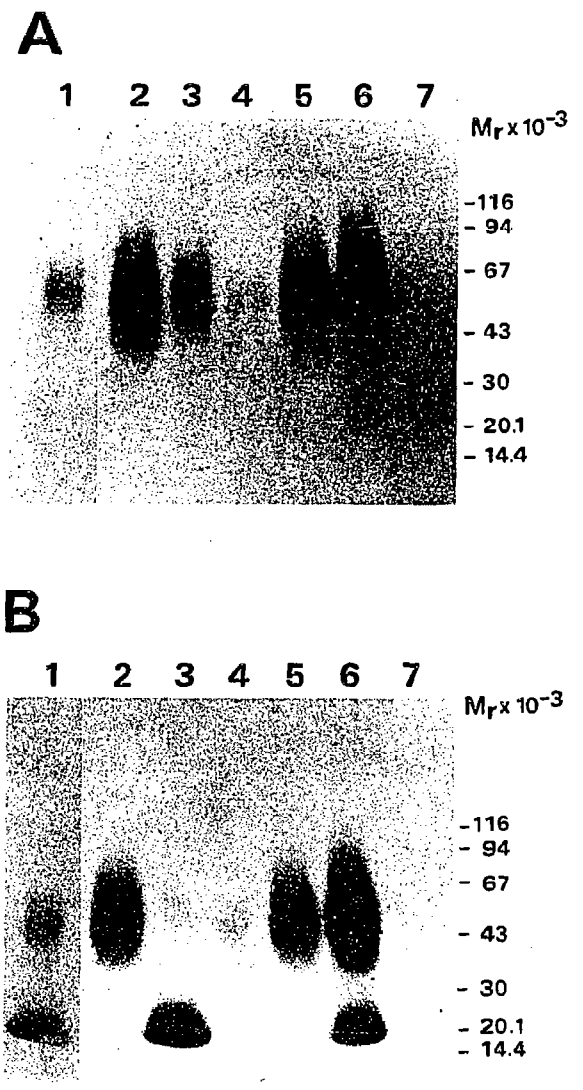

Fig. 1. Immunoprecipitation of [^{125}I]u-PAR and chymotryptic fragments by monoclonal antibodies. Purified u-PAR (30 μg/ml) from U937 cells was treated with chymotrypsin (40 ng/ml) for 7 h at 37°C. Both intact u-PAR (1–2 μg) and the chymotryptic fragments of u-PAR (1–2 μg) were ^{125}I-labelled and immunoprecipitated, as described in section 2. Panel A. Immunoprecipitation of intact [^{125}I]u-PAR. Lane 1 is [^{125}I]u-PAR alone, and lanes 2–7 represent the immunoprecipitated material using R2, R3, R1, R4, a pool of the 4 monoclonal antibodies, and anti-TNP monoclonal antibody, respectively. Panel B. Immunoprecipitation of [^{125}I]chymotryptic fragments of u-PAR. Lane 1 is [^{125}I]u-PAR fragments alone, whereas lanes 2–7 represent the immunoprecipitated material using the same antibodies as above. The molecular weights of standard proteins are indicated.

3.2. *Inhibition of the cell-associated enhancement of pro-u-PA-dependent plasminogen activation*

Pro-u-PA dependent plasminogen activation has been shown to be greatly enhanced in the presence of U937 cells [19]. Fig. 2 demonstrates that preincubation of the cells with antibody R3 completely abolished this enhancement while the antibodies R1 and R2 were without any effect. Concentrations of these two antibodies up to 50 μg/ml were also without effect on the generation of plasmin in this system. Antibody R4 gave a partial inhibition of the enhancement of plasmin generation (Fig. 2D), although this inhibitory effect was never more than approximately 50% at higher antibody concentrations (up to 50 μg/ml, data not shown). However, if cells which had been pre-incubated with R4 as before were further incubated with pro-u-PA for 15 min prior to the addition of plasminogen, the inhibitory effect of R4 on the enhancement of plasmin generation

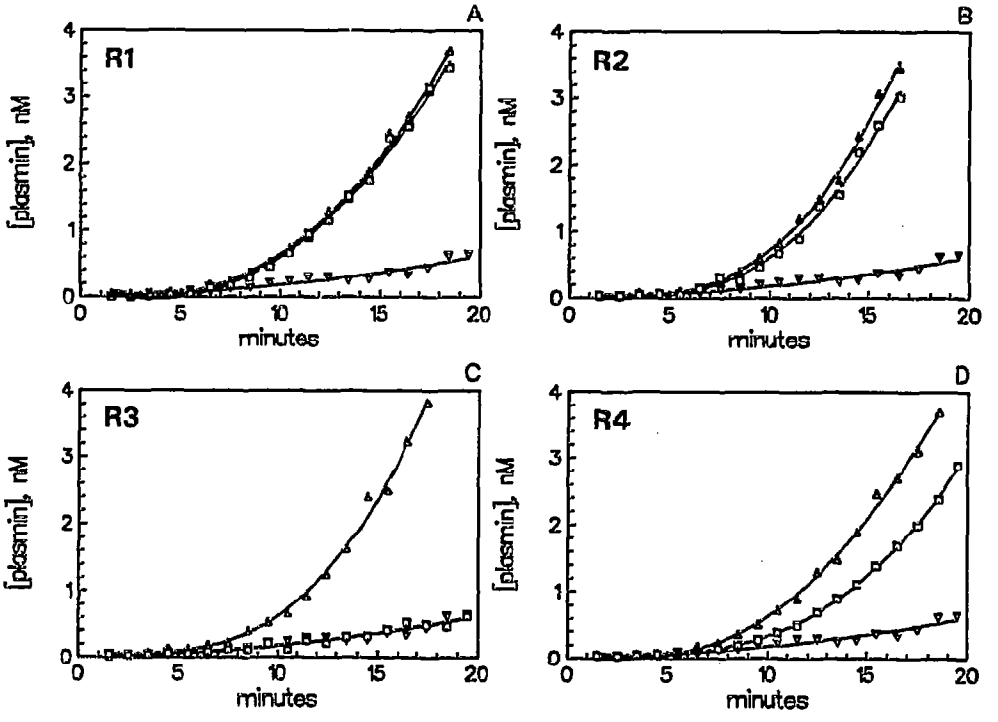

Fig. 2. The binding of pro-u-PA to cellular u-PAR is essential for enhanced plasmin generation. Acid-washed U937a cells were pre-incubated for 30 min in the presence or absence of 2 μg/ml of each of the monoclonal antibodies R1 (panel A), R2 (panel B), R3 (panel C), and R4 (panel D), prior to incubation with pro-u-PA (1.2 nM), plasminogen 2 (0.14 μM) and the fluorogenic plasmin substrate H-D-Val-Leu-Lys-7-amido-4-methylcoumarin (0.2 mM). Plasmin generation was determined from measurements of the rate of change in fluorescence, which were compared to calibration curves constructed using active-site titrated plasmin [21]. Plasmin generation is shown in the absence of cells (▽), and in the presence of U937a cells pre-incubated with the various antibodies (□) and without antibodies (△). Control experiments showed that plasminogen activation in the absence of cells was unaffected by any of the antibodies.

was abolished. Under the same conditions, the effect of antibody R3 as unchanged.

3.3. *Inhibition of u-PA binding to u-PAR on intact cells*

As shown in Fig. 3, the antibody R3 could completely abolish the binding of [^{125}I]DFP-u-PA and subsequent formation of cross-linked complex with u-PAR on the surface of U937 cells. In contrast, no effect was observed with the antibodies R2 and R4 (Fig. 3, lanes 5 and 7), and only a slight inhibition of binding with R1 (Fig. 3, lane 4).

4. DISCUSSION

A previous study has shown that the cellular binding of pro-u-PA potentiates the plasminogen activation system, as concomitant binding of pro-u-PA and plasminogen to the cell surface resulted in a rapid acceleration of plasmin generation [19]. The dependence of this effect on the cellular binding of pro-u-PA was demonstrated by the inhibition of the acceleration by ATF, the NH_2-terminal fragment of u-PA. It was proposed that u-PAR was responsible for the potentiation on the basis of the following correlations: that ATF binds to the cell surface with similar characteristics to intact u-PA, ATF can be cross-linked to purified u-PAR, and that ATF cross-links exclusively to u-PAR on the cell surface. Nevertheless, the dependence of the effect on molecules other than u-PAR could not be excluded, as the correlation assumes that the cross-linking procedure used is able to detect all interactions between ATF and u-PA binding molecules. This is not necessarily the case, however, as in the murine system, for example, u-PA is not cross-linked to any cell-surface proteins despite its specific cellular binding [8] (Solberg, H. and Høyer-Hansen, G., unpublished). The observation here that the antibody R3 completely abolishes the cell-induced potentiation of plasmin generation now presents directe evidence that this functional effect is absolutely dependent upon u-PAR.

The partial inhibition of the potentiation of plasmin generation observed with R4 is apparently contradictory to its lack of effect on [^{125}I]DFP-u-PA binding. This is most likely a consequence of the dynamic nature of the assay system used to detect cellular plasminogen activation, in which we observe not only the enzymatic reactions but also the time-dependent cellular binding of pro-u-PA and plasminogen. The effect of R4 could be explained by an altered rate of association of pro-u-PA with u-PAR, possibly caused by steric hindrance, which would not be detected in the binding experiments performed under equilibrium conditions. The observa-

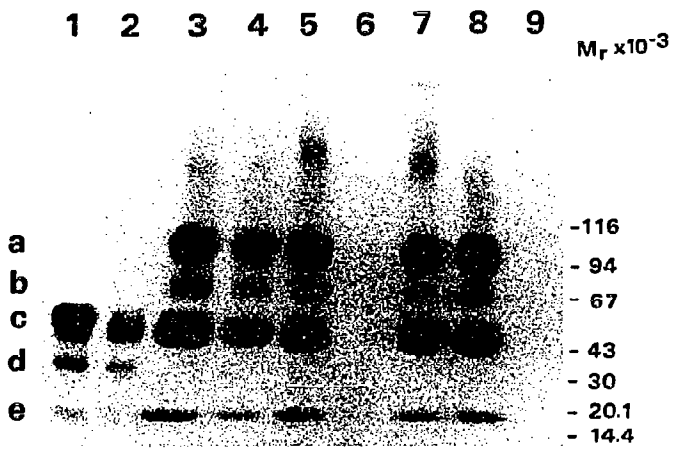

Fig. 3. Inhibition of binding and chemical cross-linking of [^{125}I]DFP-u-PA to u-PAR on intact cells by monoclonal antibodies. 5×10^6 U937a cells were pre-incubated with buffer alone (lane 3), 25 μg/ml of each of the monoclonal antibodies R1 (lane 4), R2 (lane 5), R3 (lane 6), R4 (lane 7) for 1 h at 4°C, followed by the addition of [^{125}I]DFP-u-PA (final concentration 7 nM) and incubation for another hour at the same temperature. The cells were then washed 3 times and subjected to cross-linking with DSS, followed by 3 washes, Triton X-114 lysis, SDS-PAGE and autoradiography. Also shown are [^{125}I]DFP-u-PA without incubation (lane 1), [^{125}I]DFP-u-PA cross-linked alone (lane 2), pre-incubation with an excess of cold u-PA (700 nM, lane 9), and with a monoclonal antibody of irrelevant specificity (anti-TNP 25 μg/ml, lane 8). The u-PA preparation used in this experiment contains minor amounts of low molecular weight u-PA (M_r 33000) and ATF; due to the u-PAR binding capacity of ATF, but not of low molecular weight u-PA, the following bands are seen after cross-linking; (a) cross-linked complex between [^{125}I]DFP-u-PA and u-PAR; (b) cross-linked complex between [^{125}I]ATF and u-PAR; (c) high molecular weight [^{125}I]DFP-u-PA; (e) [^{125}I]ATF. Also indicated are (d) low molecular weight [^{125}I]DFP-u-PA, as well as the molecular weights of protein standards.

tion that R4 was without effect on plasmin generation when pro-u-PA was pre-incubated with the cells supports this view.

Recent cross-linking studies have shown that a 16 kDa chymotryptic fragment derived from purified u-PAR can bind to u-PA independently of the rest of the protein. This fragment includes the NH_2-terminus of the protein, probably extending to residue 87, and constitutes the first of three internal repeats in the receptor molecule [16]. The complete inhibition of [^{125}I]DFP-u-PA binding to whole cells by the antibody R3 demonstrates that this NH_2-terminal domain is indeed accessible for ligand binding in the membrane-associated receptor. The lack of effect of the three other monoclonal antibodies, all recognizing different epitopes within the rest of the u-PAR molecule, supports the view that the two domains constituting this part of the molecule do not contribute directly to the ligand-binding process. Therefore these observations, together with those of the effect of R3 on the potentiation of plasmin generation, provide independent evidence that the NH_2-terminal domain of u-PAR constitutes the functionally distinct ligand binding domain of the molecule.

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