

Regulation of Urokinase Receptors in Monocytelike U937 Cells by Phorbol Ester Phorbol Myristate Acetate

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Abstract. A specific surface receptor for urokinase plasminogen activator (uPA) recognizes the amino-terminal growth factor-like sequence of uPA, a region independent from and not required for the catalytic activity of this enzyme. The properties of the uPA receptor (uPAR) and the localization and distribution of uPA in tumor cells and tissues suggest that the uPA/uPAR interaction may be important in regulating extracellular proteolysis-dependent processes (e.g., invasion, tissue destruction). Phorbol myristate acetate (PMA), an inducer of U937 cell differentiation to macrophage-like cells, elicits a time- and concentration-

dependent increase in the number of uPAR molecules as shown by binding, cross-linking, and immunoprecipitation studies. The effect of PMA is blocked by cycloheximide. Overall, the data indicate that PMA increases the synthesis of uPA.

PMA treatment also causes a decrease in the affinity of the uPAR for uPA, thus uncovering another way of regulating the interaction between uPA and uPAR. In addition, the PMA treatment causes a modification of migration of the cross-linked receptor in mono- and bidimensional gel electrophoresis.

Two enzymes activate plasminogen into the broad-spectrum protease plasmin: urokinase (uPA)¹ and tissue-type plasminogen activator (12). Available evidence indicates that the activation of plasminogen to plasmin, in addition to regulating intravascular fibrinolysis, also regulates extracellular proteolysis in tissues. Extracellular proteolysis is involved in cellular migration, tissue destruction, and invasiveness (20, 50). In such processes, the attachment and detachment of cells to and from neighbor cells or extracellular matrix must occur via the sequential breakdown and reformation of protein bonds that provide anchorage sites. Urokinase activity has been shown to be required in the degradation of the extracellular matrix, in the invasion of basement membrane by tumor cells, and in many of the transformation parameters of Rous sarcoma virus-transformed chicken embryo fibroblasts in vitro (8, 37, 60). In vivo, inhibitory anti-uPA antibodies block metastasis in two experimental systems: the human HEp3 carcinoma in the chorion allantoic membrane of the chicken embryo (45) and the mouse B16 F10 melanoma injected intravenously into syngeneic animals (29). Immunocytochemical studies have localized uPA within the invasive part of the Lewis lung car-

cinoma in cells that are in proximity to the remnants of the invaded normal tissues (56). In human cells, uPA has been located on the outer surface of several cells (3, 58) and at discrete cell-cell and cell-substratum contact sites (30, 47, 48).

Several normal and malignant cells in culture (4, 13, 26, 32, 57, 58, 63), as well as human tumor specimens (39), have been shown to possess a specific surface receptor for uPA that on some cell types has been identified as a glycoprotein with an *M_r* of 55,000–60,000 (41). The amino acid sequence of uPA responsible for binding to the receptor has been located within the amino-terminal fragment (ATF) of the molecule (58) within residues 12–32 (2). The uPA receptor (uPAR) (10) binds both uPA and pro-uPA and can serve as an activation site for conversion of the single-chain pro-uPA to active two-chain uPA by plasmin (19). This is in keeping with previous observations (57, 63) that showed little, if any, internalization or down-regulation of the uPAR. Actually, receptor-bound uPA dissociates very slowly with a half-life of ~3 h (Cubellis, M. V., and F. Blasi, unpublished observations) and remains active at the surface of the cells (63).

These properties suggest that the uPAR may serve to focalize uPA activity on the cell surface by mediating the activation of pro-uPA and the action of uPA. This, in turn, should allow the cell surface to regulate both intravascular fibrinolysis and the extravascular extracellular matrix and basement membrane degradation (14). The production of uPA by malignant cells and its binding to the uPAR of the same cells

1. *Abbreviations used in this paper:* ATF, amino-terminal fragment; DSS, disuccinimidyl-suberate; PMA, phorbol-12-myristate-13-acetate; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor.

(58) suggest that the latter property might be an integral part of the malignant (invasive) phenotype, facilitating the spread and the invasion of tumors (14).

While a surface proteolytic activity may have important and specific physiological and developmental functions, its activity needs to be carefully controlled; improper regulation might cause a loss of the coordination of different reactions required for cell migration or could provide the cells with the capacity to destroy and/or invade the surrounding tissues.

The expression of the uPA gene is finely regulated by a variety of agents that affect cell growth (7, 11, 20, 21, 38, 59); however, very little is known of the regulation of the uPAR function and synthesis. We have previously observed a specific increase of uPA binding to human U937 cells after differentiation to macrophage-like cells induced by treatment with phorbol-12-myristate-13-acetate (PMA) (41). We now report a detailed analysis of the effect of PMA on uPARs in U937 cells. Our data show that in these cells PMA exerts a complex regulation of the interaction of uPA with uPARs by stimulating the synthesis of uPARs and by decreasing its affinity for uPA.

Materials and Methods

Materials

Human urinary urokinase of high (uPA) and low (33K uPA) M_r and its amino terminal fragment (ATF) were prepared as described (57). PMA (LC Service, Woburn, MA) was dissolved at 1 mg/ml in DMSO and diluted to working concentration with RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated FBS (Gibco Laboratories). Ampholines were purchased from LKB Instruments Inc. (Bromma, Sweden).

Cell Culture

U937 cells (57) were grown in RPMI 1640 medium supplemented with heat-inactivated FCS.

Radio-iodination and Receptor-binding Assay

ATF and uPA iodination with Na^{125}I and binding of iodinated ATF and uPA to U937 cells have been described (57). For each binding assay, 5×10^6 cells were resuspended in 1.0 ml of binding buffer (PBS containing 0.1% BSA), and challenged for 1 h at 23°C with 1.5×10^5 cpm (i.e., ~ 70 fmol) iodinated ligand. Binding saturation curves were obtained by including varying amounts of unlabeled ligand in the binding mixture. Specificity was tested by competition with excess unlabeled ATF, uPA, pro-uPA and low- M_r uPA. When required, cells were acid washed before the binding assay for 3 min in 50 mM glycine-HCl, pH 3.0, 0.1 M NaCl, quickly neutralized, and washed (58).

Phorbol Ester-induced Differentiation

U937 cells were resuspended at $0.2\text{--}0.5 \times 10^6/\text{ml}$ and incubated with 150 nM PMA in RPMI 1640 medium (Gibco Laboratories) containing 10% heat-inactivated FBS. Differentiation was routinely followed by estimating the percentage of cells acquiring the property of adhering to the plastic dish and occasionally by the ability to incorporate latex beads by phagocytosis (17). Binding of ^{125}I -ATF or uPA to differentiated cells was carried out as described (57).

Cross-linking of uPAR to Intact Cells

U937 cells (40 ml at $0.5 \times 10^6/\text{ml}$) were induced with 150 nM PMA for 60 h. At this time, nonadherent cells were removed by aspiration; the adherent cells were gently washed and then resuspended by vigorous pipetting. Induced or noninduced cells were first incubated in serum-free RPMI-1640 medium for 2 h at 37°C. One million control or PMA-induced cells, resuspended in 0.2 ml of binding buffer, were incubated in duplicate with

^{125}I -ATF (50,000 cpm, 25 fmol) for 2 h at 4°C with or without unlabeled ATF. The binding step was terminated by washing the cells twice with binding buffer. For cross-linking, 0.2 vol of disuccinimidyl-suberate (DSS) (1.0 mM in DMSO) was added and the cells were incubated 15 min at room temperature. Cross-linking was stopped by the addition of 10 mM ammonium acetate (final concentration) followed by incubation for 10 min at room temperature. The cells were finally washed in binding buffer, centrifuged, and the pellet was resuspended in $50 \mu\text{l}$ of 0.7 M Tris-HCl, pH 6.8, 10% glycerol, 3% SDS, 0.01% bromphenol-blue (35). Each sample was split into two, and β -mercaptoethanol (0.5% final concentration) was added to one set. All samples were boiled for 5 min and analyzed by SDS-PAGE (35) on a 7.5–15% polyacrylamide gel gradient. Gels were dried and exposed to Kodak XAR-5 films.

Immunoprecipitation of the uPA/uPAR Complex

Control or PMA-treated cells (24 h, 150 nM) were incubated overnight with methionine-free medium containing 5% heat-inactivated FCS and [^{35}S]-L-methionine (100 $\mu\text{Ci}/\text{ml}$). Cells were washed in binding buffer, resuspended in the same buffer containing 1.0 nM unlabeled uPA (human urinary urokinase), and incubated for 1 h at room temperature. After this binding step, the cells were washed and cross-linked with DSS, as described above. After washing, the cells were lysed in 20 mM Hepes buffer, pH 7.4, containing 1.0% Triton X-100 and 10% glycerol, and the lysate was centrifuged for 30 min in a microfuge (Eppendorf made by Brinkman Instruments, Inc., Westbury, NY) at 4°C. TCA precipitation was carried out on aliquots of the supernatants. For immunoprecipitation, 2.5×10^7 cpm of the lysate of the cross-linked cells were precipitated with normal rabbit serum and non-specific precipitate was spun down after addition of $50 \mu\text{l}$ of *Staphylococcus aureus* suspension (51). The supernatants were subsequently immunoprecipitated by incubation with anti-uPA IgG (purified by protein A-Sepharose chromatography) overnight at 4°C. *S. aureus* suspension (50 μl) was then added, and the samples were incubated for 30 min at 4°C, and centrifuged. The pellet was washed five times with 40 mM Hepes, pH 7.4, 1.0 M NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA, 0.02% sodium azide, and then washed five more times with 0.1 M Tris-HCl, pH 7.9, 0.5 M LiCl, 0.05% dithiothreitol (DTT). The final pellets were resuspended in loading buffer with β -mercaptoethanol (35), boiled for 5 min, and centrifuged. The supernatants were analyzed by 12.5% SDS-PAGE, followed by fluorography along with ^{14}C -labeled protein molecular mass markers.

Cross-linking Assay of Solubilized Receptor

10^8 PMA-treated or untreated cells were washed, acid treated, and lysed by the addition of 2 ml 0.5% 3-[(3-cholamidopropyl) dimethyl-ammonio]-l-propanesulfonate in 0.1 M Tris-HCl, pH 8.1, 10 mM EDTA, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ Trasylol. After 10 min at 0°C, the lysates were clarified by centrifugation.

2 μl of supernatant was incubated with 42 nM ^{125}I -labeled ATF in a total volume of 6 μl for 1 h at 4°C. 1 μl of 14 mM DSS in DMSO was added, and after 15 min at room temperature, the reaction was stopped by addition of 0.8 μl of 0.1 M ammonium acetate. The samples were left for 1 h, followed by addition of 0.5% SDS, 0.1 M β -mercaptoethanol (final concentrations), boiled for 3 min, and then analyzed by SDS-PAGE (6–16% gradient gel) (35) under reducing conditions and autoradiography.

Bidimensional Gel Electrophoresis

Control and PMA-treated U937 cells were cross-linked to ^{125}I -ATF and extracted (see above). Extracts were analyzed by bidimensional gel electrophoresis (43), using a mixture of 0.8% ampholines, pH 3.5–10, 0.8% ampholines, pH 5–7, and 0.8% ampholines, pH 2.5–5.0. A 7.5% polyacrylamide gel was used in the second dimension. An extract of [^{35}S]methionine-labeled *Escherichia coli* was used as internal marker (46). The molecular mass (in amino acid residues) and isoelectric point of the marker proteins were obtained from the literature (40).

Results

Cell Density and PMA Affect uPA Binding to uPARs on U937 Cells

Treatment with PMA induces U937 cells to differentiate to macrophage-like cells. The cells become initially adherent

to the substratum, express specific surface markers, and acquire complex specialized functions, like phagocytosis (42, 49). PMA-differentiated U937 cells also show an increased binding capacity of uPA to uPARs (57).

To gain information on the mechanisms that regulate the interaction between uPA and uPARs, we have further studied the effect of PMA on U937 cells. ^{125}I -ATF, which has all of the receptor-binding determinants of uPA (57), was used in most of the experiments instead of labeled uPA. Its use allows discrimination from other types of binding sites (including inhibitors) that have been shown to require the catalytic moiety of uPA (1, 5, 24, 31, 64).

Since one of the effects of PMA is a block of U937 cells' proliferation (42), untreated cells ultimately reach a higher cell density. We therefore tested whether cell density could by itself modify the number of uPAR molecules. The number and affinity of uPARs were measured by standard saturation binding experiments and Scatchard analysis. As shown in Table I, the titratable number of uPAR molecules drops from 14,000–18,000 for cells grown at 150,000–300,000 cells/ml to 1,800 for cells grown at 1,500,000 cells/ml. As also shown in Table I, the affinity of the receptors is not affected. A similar effect was observed with U937 cells in which biosynthetic uPA or pro-uPA had been dissociated from the receptors (by acid treatment) before the binding assay was carried out (see Materials and Methods). In this case, the number of ATF-specific uPA binding sites per cell drops from 27,000 (at 180,000 cells/ml) to 9,000 (at 1,500,000 cells/ml). Thus, the decrease in number of uPA-binding sites is not, or at least not only, due to progressive saturation by biosynthetic ligand but rather to a cell density-associated modulation. The nature of this effect will be the object of future investigation. However, to avoid interference between PMA and cell density effects, in the experiments described hereafter, untreated and PMA-treated cells were plated so that they reached the same cell density at the time of the assay.

We have previously reported an increase in the number of uPA binding sites in PMA-treated U937 cells (57), which is accompanied by a decrease in ligand affinity (41). We have now analyzed the time and concentration dependence of these effects. Table II shows a quantitation of the effect of 150 nM PMA at different times of exposure of U937 cells on both the uPAR and on the conversion of the cells from the suspension-growing to the adherent-growing phenotype. By 24 h, essentially all cells are attached and the number of uPARs has

Table I. Effect of Cell Density on the Number and Affinity of uPAR Molecules in U937 Cells

| Cell density | uPARs per cell | | K_d | |
|--------------|-------------------|-----------------|-------------------|-----------------|
| | without acid wash | with acid wash* | without acid wash | with acid wash* |
| | <i>n</i> | <i>n</i> | <i>nM</i> | <i>nM</i> |
| 150,000/ml | 14,000 | ND | 0.2 | ND |
| 180,000/ml | ND | 27,000 | ND | 0.5 |
| 300,000/ml | 18,000 | ND | 0.3 | ND |
| 700,000/ml | 3,500 | ND | 0.1 | ND |
| 750,000/ml | ND | 12,500 | ND | 0.3 |
| 1,500,000/ml | 1,800 | 9,000 | 0.1 | 0.4 |

* U937 cells were treated before the binding assay with 50 mM glycine-HCl, pH 3.0, 0.1 M NaCl (see Materials and Methods) to dissociate any surface-bound uPA.

Table II. Time course of the Effect of PMA on Number and Affinity of uPAR Molecules on U937 Cells

| Duration of PMA treatment* | uPARs per cell [‡] | K_d [‡] | Adherent [§] cells |
|----------------------------|-----------------------------|--------------------|-----------------------------|
| <i>h</i> | <i>n</i> | <i>nM</i> | % |
| 0 | 8,200 | 0.4 | 2 |
| 24 | 270,000 | 4 | 97 |
| 48 | 470,000 | 8 | 99 |
| 72 | 810,000 | 17 | 99 |

* Both control and 150 nM PMA-treated cells had reached a cell density of 500,000/ml at the time of the assay.

[‡] Data obtained by Scatchard plot analysis. Specific binding was calculated by subtracting the counts not displaced by unlabeled ATF.

[§] Counted after gentle washing of the plates and after resuspension of the adherent cells by vigorous pipetting.

increased from 8,000 to 270,000 per cell while the affinity (K_d) is decreased about 10-fold. A further twofold increase in number and decrease in affinity is observed after 48 h and again after 72 h of treatment. In all cases, the binding could be competed for by 50 nM unlabeled ATF (data not shown). Assays carried out after acid dissociation of biosynthetic uPA yielded at each timepoint an approximately twofold higher receptor number with no further change in affinity (not shown).

We next measured the effect of PMA concentration on the amount of bound ^{125}I -ATF to cells at 3 or 24 h of treatment. As shown in Fig. 1 A, PMA treatment induced an increase in ^{125}I -ATF binding both at 3 and 24 h, but only at concentrations above 0.15 nM, with a maximum effect between 15 and 150 nM. These concentrations are within or below the range that promotes U937 cell differentiation (49). As shown in Fig. 1 B, 0.5 nM unlabeled ATF competes with ~50% of the binding in control cells and in cells treated with all PMA concentrations for 3 h. At 24 h of PMA treatment, however, binding of ^{125}I -ATF to cells treated with 1.5 nM or more PMA was no longer competed by 0.5 nM ATF. At 50 nM ATF, 97% or more of the counts are competed at all PMA concentrations tested (data not shown; see, however, Fig. 2 B below).

To test the extent to which the results of Fig. 1 B were due to a change in number and affinity, respectively, of the uPARs, U937 cells were treated with 1.5 or 15 nM PMA for 3 or 24 h and thereafter a saturation binding assay was carried out. As shown in Fig. 2, A, and C, the competition curve for the cells treated for 24 h with PMA is shifted towards higher concentrations in relation to control cells. Scatchard analysis of these data shows (Fig. 2, B and D) that the 24-h treatment with 15 nM PMA changes both the slope and the intercept with the abscissa of the binding isotherm. From this curve, a 40-fold increase in the number of binding sites and a 13-fold decrease of the affinity of the uPAR for ATF (K_d shifted from 0.3 to 4.0 nM) are calculated (data summarized in Table III). A smaller effect is obtained with 1.5 nM PMA for 24 h. A very slight effect is observed after 3 h of treatment at both PMA concentrations. Assays carried out after acid dissociation of biosynthetic uPA yielded qualitatively similar results (not shown).

Increased ATF Binding Is Due To Increased Synthesis of uPA Receptor

The enhanced uPA binding may be due to an actual increase

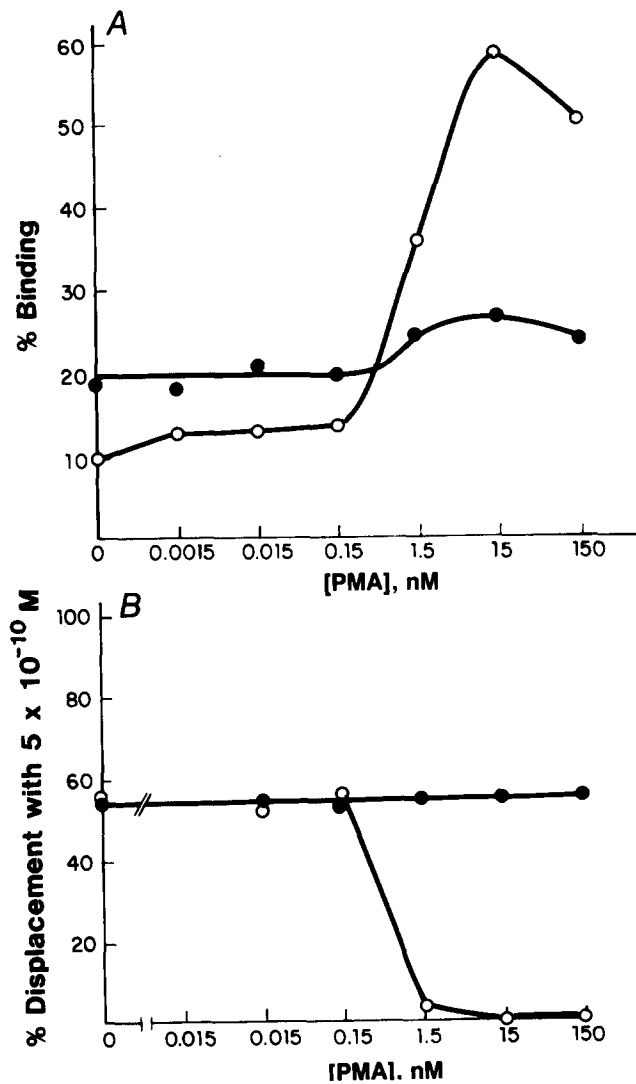


Figure 1. Concentration dependence of PMA effect on the uPA/uPAR interaction after 3 (○) and 24 h (●) of treatment. (A) % ¹²⁵I-ATF specifically bound to 10⁶ U937 cells. (B) % bound ¹²⁵I-ATF that can be displaced by 0.5 nM unlabeled ATF.

in the number of receptor molecules available on the surface. To test this possibility, U937 cells (grown at 10⁶/ml) were treated with 150 nM PMA for 60 h, a time that allows expression of some differentiated functions, like phagocytosis (data not shown). After incubation with the radioactive ATF for 2 h at 4°C, the cells were washed and treated with DSS to cross-link the ligand to the uPAR. Cross-linked products were analyzed by SDS-PAGE after reduction with β-mercaptoethanol. As shown in Fig. 3, ¹²⁵I-ATF (lane 1) can be cross-linked to a cellular component to give a broad band at ~70 kD (lanes 6 and 7). The electrophoretic migration of the ATF-uPAR complex (i.e., its corresponding molecular mass and the diffusiveness of the band) is a specific property of the human uPAR molecule, which is retained with highly purified receptor preparations (41; our unpublished results). Omission of the reduction of the cross-linked products with β-mercaptoethanol does not influence the migration of the complex (not shown). Treatment of U937 cells with PMA in-

creases severalfold the intensity of the ~70-kD band (lanes 4 and 5). When binding to both control (lanes 8 and 9) and PMA-treated cells (lanes 2 and 3) was carried out in the presence of 50 nM unlabeled ATF, the intensity of the cross-linked band is drastically reduced or its presence abolished altogether. In conclusion, this experiment identifies an ~55-kD cellular component that can be cross-linked to ATF and shows that its availability on the surface is increased severalfold after PMA-induced differentiation to macrophages. Similar data have also been obtained using labeled uPA as ligand (not shown). In addition to ATF, unlabeled uPA and pro-uPA, but not the low-*M_r* uPA (which does not bind the uPAR since it is missing the ATF portion [57]), compete for cross-linking to U937 cells (data not shown). In the experiment of Fig. 3, the labeled ¹²⁵I-ATF preparation shows two contaminating bands at ~30,000 and 80,000 D (see lane 1). Experiments carried out with other labeled ATF preparations free from these contaminants give cross-linking results identical to those of Fig. 3 (not shown). These data are in complete agreement with those obtained with purified uPAR (41).

The increase in uPARs was also visualized by immunoprecipitation of metabolically labeled uPAR after cross-linking with uPA using anti-uPA antibodies. Unlabeled uPA was bound to [³⁵S]methionine-labeled U937 cells and then cross-linked with DSS. The cells were lysed and aliquots of the labeled cross-linked extract immunoprecipitated with polyclonal anti-uPA IgG. As shown in Fig. 4, the treatment with PMA causes an increase of a diffuse, specifically immunoprecipitated band that migrates with an *M_r* of ~70,000. uPA (*M_r* ~54,000) is made up of two disulfide-linked chains (28), of which the smaller (A chain, 17 kD) holds the receptor binding site (57). The electrophoretic mobility of the immunoprecipitated band after reduction with β-mercaptoethanol corresponds to the one expected because it is the A chain that is cross-linked to the 55-kD uPAR. The uPA-uPAR complex migrates with a similar *M_r* as the uPA-PAI-2 complex of U937 cells (27). Since PAI-2 is also induced by PMA (27) the ~70-kD band of Fig. 4 could also represent uPA-PAI-2 complex. However, the latter complex (a) has never been observed on the cell surface and (b) would not be formed at the uPA concentrations used in this experiment (15 nM) since the affinity of PAI-2 for uPA is ~2 μM (62). Other fainter bands are immunoprecipitated by the anti-uPA antibodies (Fig. 4), but their intensity appears to be independent of the PMA treatment. These may represent biosynthetic uPA, which is only slightly induced by PMA in U937 cells (our unpublished results), or complexes of different forms of uPA (high- and low-*M_r* uPA) with plasminogen activator inhibitors that are produced by U937 cells (64). More recently, we have used labeled DFP-treated uPA as a ligand in the above experiment. While the ~70 kD band is still increased by PMA, all of the others are no longer present (data not shown).

The increase in uPARs observed in these experiments might result from a PMA-induced translocation of uPARs to the surface or from an increase in their synthesis. Two experiments were carried out to discriminate among these possibilities. In the first, U937 cells (grown at 0.5 × 10⁶/ml) were treated with 150 nM PMA for 24 h in the presence or absence of cycloheximide (10 or 50 μg/ml), and the extent of ¹²⁵I-ATF binding and biological effect of PMA (i.e., the

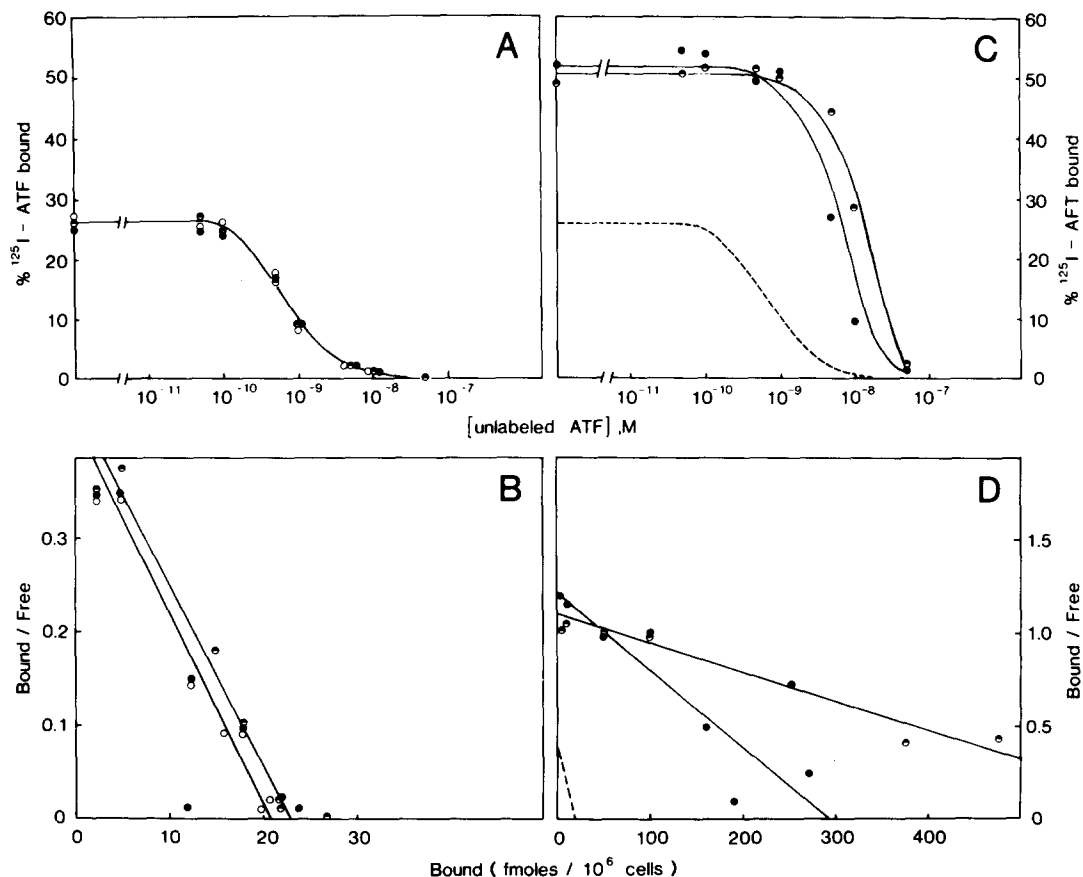


Figure 2. Effect of PMA treatment on the interaction of ^{125}I -ATF with uPAR. (○) Control cells; (●) cells treated with 1.5 nM PMA; (●) cells treated with 15 nM PMA. (A) Binding competition plot with control cells or with cells treated 3 h with PMA. (B) Scatchard plot of the data of A. (C) Binding competition plot with cells treated for 24 h with PMA. (D) Scatchard plot of the data of C. Dashed lines reproduce the data of control cells.

percentage of adherent cells) was estimated. As shown in the histogram of Fig. 5, cycloheximide completely prevents the PMA-induced adhesion of U937 cells as well as the increase in ^{125}I -ATF binding capacity. This suggests that increased uPA binding is the result of increased receptor synthesis rather than of translocation of cytoplasmic receptors to the outer surface.

To further investigate this point, we carried out cross-linking experiments with whole cell extracts of control and PMA-treated cells. This procedure has been previously de-

veloped and used to assay for uPAR during its purification (41). We cross-linked protein extracts to ^{125}I -ATF and analyzed the complex by gel electrophoresis (Fig. 6). In this experiment, the band obtained with PMA-treated extract (lane 1) is approximately twofold stronger than that obtained with control extract. However, the extract from the PMA-treated cells was diluted before cross-linking to contain an eightfold lower protein concentration than the extract from control cells. The quantitation and the specificity of the assay was controlled by using different extract concentrations and by displacement with appropriate uPA analogs (not shown). Thus, whole cell extracts of PMA-treated U937 cells contain at least 10-fold more uPAR specific activity than do control cells. The slightly different electrophoretic mobility of the PMA-treated ATF-uPAR complex (compare lanes 1 and 2) does not affect this conclusion and is dealt with in the next paragraph.

On the whole, the experiments show that PMA treatment of U937 cells results in an increase in number of total uPA binding sites, that is blocked by cycloheximide treatment, and in an increase of metabolically labeled uPARs as well as of uPARs detected by cross-linking radioactive ATF. We therefore conclude that PMA increases the synthesis of uPARs. In further support of this conclusion, PMA treatment of U937 was instrumental in uPAR purification (41; our unpublished observations).

Table III. Effect of PMA Treatment on Number and Affinity of uPAR Molecules in U937 Cells*: Role of Time of Treatment and PMA Concentration

| Treatment | uPARs per cell | K_d |
|------------------|----------------|-----------|
| | <i>n</i> | <i>nM</i> |
| Control‡ | 12,500 | 0.3 |
| PMA 1.5 nM, 3 h | 14,300 | 0.3 |
| PMA 15 nM, 3 h | 14,900 | 0.3 |
| PMA 1.5 nM, 24 h | 165,000 | 1.2 |
| PMA 15 nM, 24 h | 475,000 | 4.0 |

* Data calculated from the experiment of Fig. 2. Specific binding was calculated after subtracting the counts not competed by 50 nM ATF unlabeled ATF. ‡ Both treated and PMA-treated U937 cells had reached a cell density of $\sim 500,000/\text{ml}$ at the time of the assay.

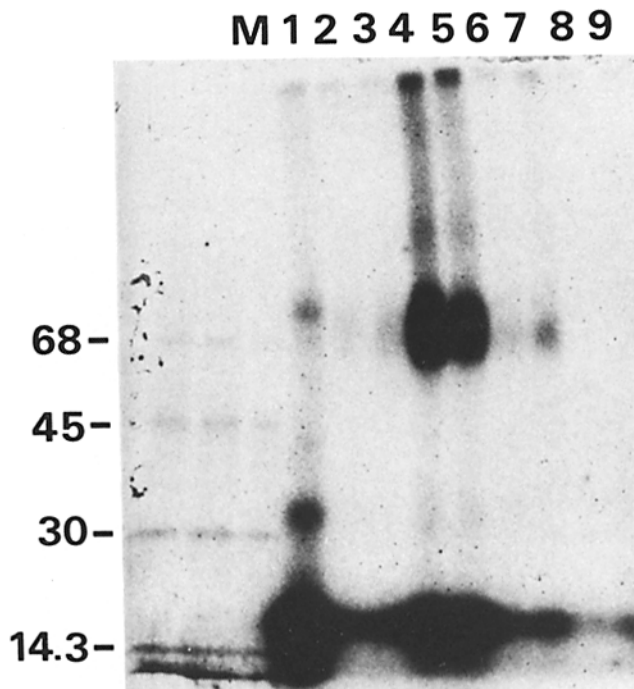


Figure 3. SDS-PAGE (7.5–15% gradient) of the products of cross-linking of ^{125}I -ATF with U937 cells. Effect of PMA. ^{125}I -ATF was bound to control and PMA-treated (150 nM, 60 h) U937 cells, and cross-linking was performed with DSS (see Materials and Methods). The samples were denatured and reduced in the presence of β -mercaptoethanol. (Lane 1) ^{125}I -ATF preparation used for binding; (lanes 2 and 3) binding to PMA-treated cells in the presence of 50 nM unlabeled ATF; (lanes 4 and 5) binding to PMA-treated cells; (lanes 6 and 7) binding to untreated U937 cells; and (lanes 8 and 9) binding to untreated cells in the presence of 50 nM unlabeled ATF. The M_r (in kD) of markers in lane M are indicated to the left.

PMA Treatment Increases the Heterogeneity of the uPAR

PMA treatment of U937 cells, in addition to increasing the intensity of the cross-linked uPAR band (Fig. 3), also causes a slight retardation of its migration. While this is not particularly evident in monodimensional gels (Figs. 3 and 6), bidimensional analysis shows the heterogeneity of the complex in a more striking way. Control and PMA-treated (150 nM, 24 h) U937 cells were cross-linked to ^{125}I ATF, lysed, and analyzed by bidimensional gel electrophoresis. The results are shown in Fig. 7. The cross-linked uPA–uPAR complex of control cells (A) migrates as a double spot of similar molecular mass and different isoelectric point (C, 1 and 2). The complex from PMA-treated cells (B) separates into three components, one of which coincides with the control (C, 2) while two others (C, 3 and 4) have lower mobility and a more acidic isoelectric point. The reasons for uPAR heterogeneity are not clear. Posttranslational modifications might account for it but have not yet been investigated.

Discussion

In this paper, we have analyzed the effect of PMA on the uPARs of U937 cells.

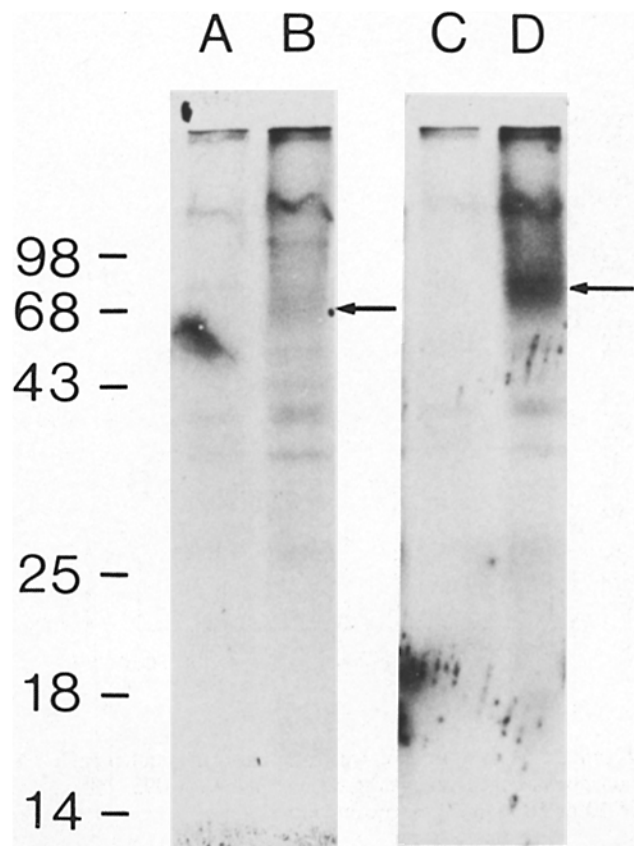


Figure 4. Immunoprecipitation by anti-uPA IgG of the cross-linked uPA/uPAR complex of U937 cells. Effect of PMA. Control or PMA-treated [^{35}S]methionine-labeled U937 cells were incubated with uPA (15 nM), cross-linked with DSS, and lysed. Aliquots of the labeled extracts were adsorbed with normal rabbit serum and immunoprecipitated with anti-uPA IgG (lanes B and D) or with pre-immune IgG (lanes A and C) and subjected to electrophoresis on a 12.5% SDS-polyacrylamide gel (see Materials and Methods). (Lanes A and B) Untreated cells; (lanes C and D) cells treated with 150 nM PMA for 24 h.

(a) We have observed a time- and concentration-dependent increase in ATF binding of U937 cells upon addition of PMA. At 150 nM PMA and 24 h of treatment, a 34-fold increase of uPAR molecules can be measured (Table II). This increase has been measured by direct binding (Figs. 1 and 2), cross-linking (Fig. 3), and immunoprecipitation (Fig. 4).

(b) Three types of evidence suggest that the increase in uPA-binding sites is the result of increased synthesis of uPAR. First, an increase in metabolically labeled uPAR is noticed after PMA treatment (Fig. 4). Second, the PMA-induced increase in the number of ATF binding sites is blocked by the protein synthesis inhibitor cycloheximide (Fig. 5). Third, the uPA-binding protein is increased not only on the membrane (Fig. 3) but also in whole cell extracts (Fig. 6).

(c) After PMA treatment, a decrease in affinity of uPAR for ATF is also observed (Table II). This is not due to induction by PMA of a binding protein with different specificity, as demonstrated by the competition with unlabeled ATF, uPA, and pro-uPA, but not with low- M_r uPA. The biochemical nature of the affinity change has not yet been understood. Several models could be envisaged on the basis of present

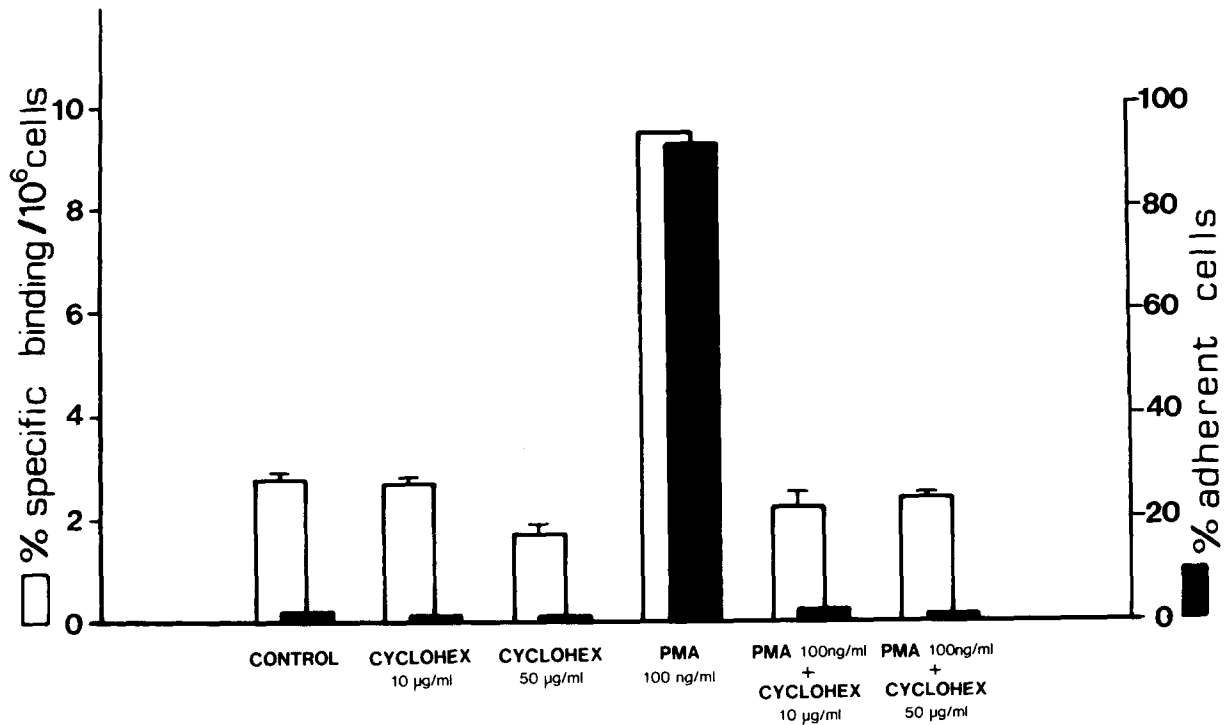


Figure 5. Effect of protein synthesis inhibitor cycloheximide on the PMA-induced increase in ¹²⁵I-ATF binding (open bars) and on the conversion from suspension-growing to adherent U937 cells (solid bars). PMA treatment was carried out for 24 h in the presence or absence of 10 or 50 µg/ml cycloheximide.

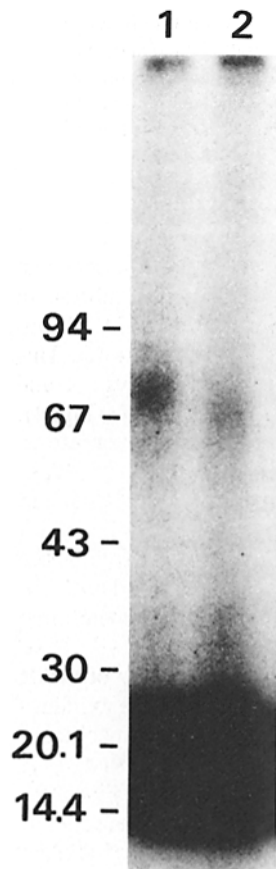


Figure 6. Cell-free binding of ¹²⁵I-ATF to total cell extracts of PMA-treated (lane 1) and control (lane 2) U937 cells. Undiluted (control) or eightfold diluted (PMA-treated) lysates of acid-treated cells were incubated with ¹²⁵I-ATF, cross-linked with DSS, and then analyzed by SDS-PAGE (6-16%) under reducing conditions.

knowledge and of the available data. A posttranslational modification, the synthesis of a regulatory subunit and oligomerization of receptor, is a mechanism capable of modifying the affinity of receptors for their ligands.

(d) PMA causes an increase in the heterogeneity of the uPAR leading to a decrease in electrophoretic mobility of uPAR and increase of the acid charges (Fig. 7). Whether this is due to synthesis of regulatory subunits or to posttranslational modification remains to be seen.

uPAR and Differentiation

PMA induces the differentiation of monocyte-like U937 cells into macrophage-like cells. This entails a block of cell division, a major phenotypic change with expression of specific macrophage functions, like phagocytosis, and synthesis of surface markers (42, 49). The observed increase of uPAR synthesis and decrease in affinity may be part of this differentiation program. In fact, we have previously shown that nonadherent and adherent PMA-treated U937 cells show different levels of induction of uPAR even though they have been exposed to the same dose of PMA (57).

Two Populations of uPARs

Induction in U937 cells of uPAR synthesis and decrease of affinity, two effects that should have opposite regulatory consequences, arouse further interest. The PMA-induced decrease in affinity is a novel regulatory mechanism capable of modifying the ratio between soluble and surface-bound uPA; i.e., regulating the location of uPA. This effect might cause

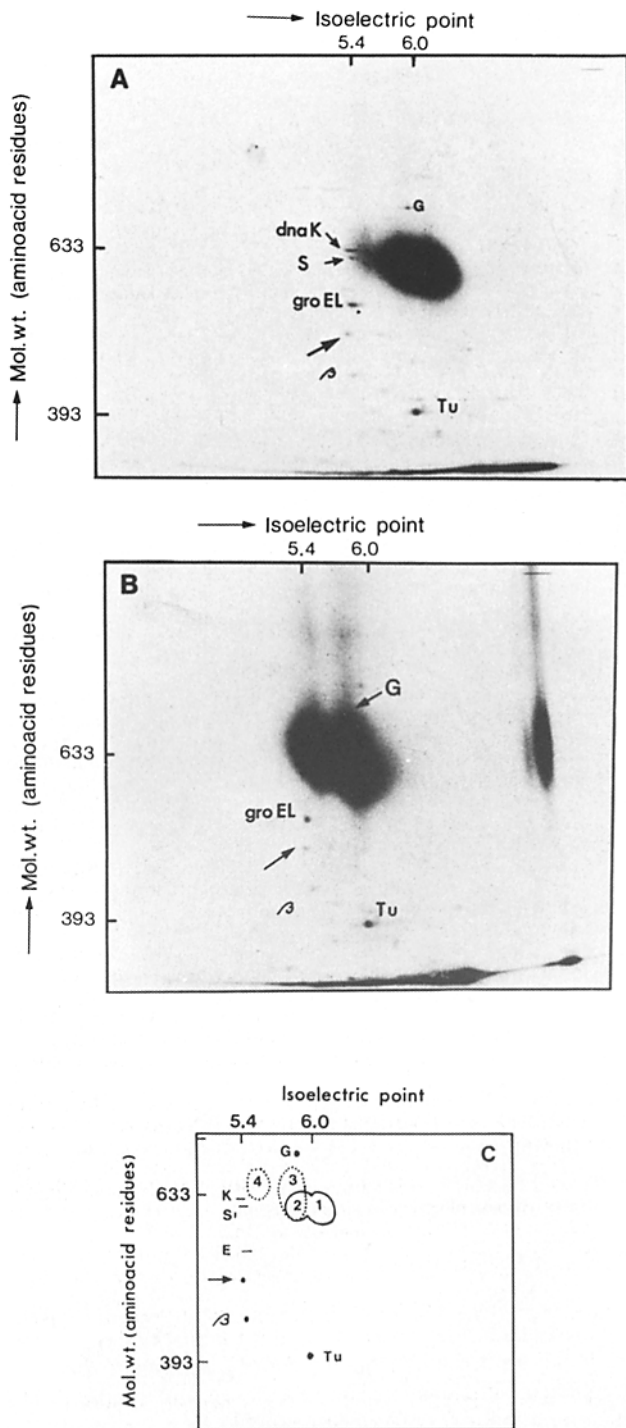


Figure 7. Bidimensional gel electrophoresis of ^{125}I -ATF cross-linked control (A) and PMA-treated (100 nM, 72 h) (B) U937 cells. The internal markers are indicated. The arrow represents an unidentified marker protein. C shows a reconstructed composite picture of A and B.

an inversion of the medium/cell ratio of uPA activity as observed in PMA-treated transformed mouse fibroblasts (7). No information is available at this time on the physiological significance of the apparently contradictory combination of the above effects of PMA on uPARs. It is possible that the effects on synthesis and affinity of uPARs normally take place

either in different cells or in the same cells, but in response to different stimuli.

Although nothing is yet known of the physiological signal for the affinity-regulating mechanism, it seems to us that it may be connected with the level and the localization of the uPA activity on the cell surface. On the other hand, PMA is a fast regulator of the activity of several types of receptors. Binding of epidermal growth factor (36, 55), insulin (61), and somatomedin (44) is affected by PMA treatment. Down-regulation and increased internalization (6, 16, 22, 34, 55) have been reported. PMA activates protein kinase C (15), which in turn phosphorylates the epidermal growth factor receptor at a specific threonine residue, causing a decrease of the affinity for epidermal growth factor (18, 23, 25, 33, 34). The effect of PMA on uPAR affinity suggests that the uPAR also is directly or indirectly affected by protein kinase C.

The presence of two populations of receptor molecules appears to be rather common in mammalian cells (9, 52-54). The modulation of the uPAR molecules may represent an important feature in the regulation of extracellular proteolysis and thus of the degradation of extracellular matrix and basement membrane components, processes that are at the core of cell migration and invasiveness. Since uPARs are present in several blood and endothelial cells, their regulation might also significantly affect intravascular fibrinolytic activity in physiological, pathological, and pharmacological conditions (10).

The authors are grateful to Angelo Corti and Giovanni Cassani for the generous gift of ATF and anti-uPA antibodies.

E. L. Kajtaniak was supported by a fellowship of Lepetit SpA. R. Picone and M. R. Mastronicola were supported by fellowships of the Associazione Italiana Ricerche sul Cancro (AIRC). This work was supported by grants of the Consiglio Nazionale delle Ricerche (Italy), PF Oncologia, and PF Ingegneria Genetica e Basi Molecolari delle Malattie Ereditarie, and by the Danish Cancer Society, the Danish Medical and Natural Sciences Research Councils, the Danish Biotechnology program, and the Carlsberg Foundation.

Received for publication 25 July 1988, and in revised form 28 September 1988.

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