

# Phorbol Ester Induces the Biosynthesis of Glycosylated and Nonglycosylated Plasminogen Activator Inhibitor 2 in High Excess over Urokinase-type Plasminogen Activator in Human U-937 Lymphoma Cells

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**Abstract.** The tumor-promoting phorbol ester PMA induces changes in the histiocytic human lymphoma cell line U-937 akin to cellular differentiation (Ralph, P., N. Williams, M. A. S. Moore, and P. B. Litcofsky, 1982, *Cell. Immunol.*, 71:215–223) and concomitantly stimulates the biosynthesis of plasminogen activator inhibitor 2 (PAI 2) and of urokinase-type plasminogen activator (u-PA). PAI 2 is found in a nonglycosylated intracellular and a glycosylated secreted form. The former appears to be identical to PAI 2 previously purified from placental extracts and large-scale U-937 cell cultures. The sixfold increase of PAI 2 antigen measured 24 h after PMA treatment in cell extracts

and conditioned media is accompanied by an equal increase of active PAI 2 mRNA, whereas the 6 to 13-fold increase of u-PA antigen in the same samples is associated with only a 1.5-fold mRNA increase. The increase of PAI 2, but not of u-PA, biosynthesis requires transcription. A 50-fold molar excess of PAI 2 over u-PA is found in both extracts and conditioned media of PMA-treated cells. PAI 2 represents at least 0.3% of total de novo synthesized protein 24 h after induction with PMA. Thus, PAI 2, but not u-PA, is an abundant product of this precursor analogue of the mononuclear phagocyte lineage, and might represent a new marker for monocyte/macrophage differentiation.

**P**LASMINOGEN activators (PAs) are enzymes that convert the inactive zymogen plasminogen into the trypsin-like protease plasmin, which degrades fibrin as well as fibronectin and other extracellular matrix proteins (Danø et al., 1985; Saksela, 1985; Collen, 1980). Two immunologically and genetically distinct PAs are known: urokinase-type PA (u-PA) and tissue-type PA. A substantial body of evidence suggests that PAs play an important role in fibrinolysis (Collen, 1980), inflammation (Vassalli et al., 1976), invasive and metastatic growth (Danø et al., 1985; Markus, 1984; Ossowski and Reich, 1983), and other forms of cell migration (Valinsky et al., 1981). Therefore, much effort has been devoted to the study of the hormonal regulation of PA biosynthesis, using as an assayable endpoint PA activity in conditioned media and/or cell extracts (Danø et al., 1985; Saksela, 1985). Recent results, however, have shown that u-PA is synthesized and secreted as a single chain proenzyme (Wun et al., 1982; Husain et al., 1983) and that many cells synthesize, often under hormonal control, specific PA inhibitors (PAIs) (Seifert and Gelehrter, 1978; Crutchley et al., 1981; Van Mourik et al., 1984; Chapman and Stone, 1985). Hence, the information obtained by PA activity measurement alone is of limited value. Cells of the histiocytic lymphoma cell line U-937 (Sundström and Nils-

son, 1976) differentiate in the presence of PMA and acquire characteristics of monocytes/macrophages (Ralph et al., 1982). Previous work suggested that this differentiation was accompanied by an increase in PAI activity in the conditioned medium (Vassalli et al., 1984; Kruithof et al., 1986). This activity was found to be immunologically related to a PAI found in placental extracts (Vassalli et al., 1984; Kruithof et al., 1986; Kopitar et al., 1985; Åstedt et al., 1985) but different from protease nexin (Vassalli et al., 1984; Kruithof et al., 1986; Scott and Baker, 1983) and a PAI purified from endothelial cells (van Mourik, et al., 1984; Vassalli et al., 1984; Kruithof et al., 1986). Following the nomenclature recommendations of the subcommittee on fibrinolysis of the International Society of Thrombosis and Hemostasis, we refer to the PAI purified first from endothelial cells (Van Mourik et al., 1984) as PAI 1 and to the PAI purified first from placental extracts (Åstedt et al., 1985) as PAI 2. Using the plasminogen-dependent fibrin agar zymography technique (Granelli-Piperno and Reich, 1978) u-PA (but not tissue-type PA) was also found to be produced by U-937 cells (Vassalli et al., 1984). Taking into consideration the recent advances in the biochemistry of the fibrinolytic system, we have studied the coordinate modulation of u-PA and PAI 2 biosynthesis by new, specific, and quantitative methods, determined the relative contribution of transcription, translation, and secretory processes on u-PA and

*I. Abbreviations used in this paper:* PA(s), plasminogen activator(s); PAI(s), PA inhibitor(s); u-PA, urokinase-type PA.

PAI 2 levels in cell extracts and conditioned media, and investigated the distribution of the different molecular forms of both u-PA and PAI 2.

## Materials and Methods

### Materials

Imidazole, Tris, Tween 80, phenol, bovine serum albumin, sodium azide, Triton X-100, boric acid, sodium tetraborate, and lithium chloride were obtained from Fluka AG, Buchs, Switzerland. Bromophenolblue, glycine, ethanol, and trichloroacetic acid were obtained from E. Merck, Darmstadt, Federal Republic of Germany. Pyroglu-gly-arg-p-nitroanilide was from Bachem, Basel, Switzerland. All reagents for polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS PAGE) and nitrocellulose membranes were from Bio-Rad Laboratories, Richmond, CA. Molecular mass standard proteins for SDS PAGE (phosphorylase b, 94 kD; bovine serum albumin, 67 kD; ovalbumin, 43 kD; and carboanhydrase, 30 kD), and Sephadex G-25 were obtained from Pharmacia, Uppsala, Sweden. Sucrose, prestained protein standards employed in immunoblotting experiments (phosphorylase B, 97 kD; bovine serum albumin, 68 kD; ovalbumin, 43 kD; and  $\alpha$ -chymotrypsinogen, 26 kD) and low melting point agarose were from Bethesda Research Laboratories, Inc., Gaithersburg, MD. XAR-5 films for autoradiography and X-omatic intensifying screens were from Kodak, Lausanne, Switzerland. Proteinase K and lithium dodecyl sulfate were from Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany. Oligo dT cellulose type 2 was from Collaborative Research, Inc., Cambridge, MA. NP-40 was from Calbiochem, La Jolla, CA. RPMI 1640 was from Seromed, Biochrom, Federal Republic of Germany. Glutamine was from BioMérieux, Charbonnières-les-Bains, France. FCS was from Gibco, Paisley, United Kingdom. High molecular mass u-PA was from the Green Cross, Osaka, Japan. Plasminogen-rich fibrinogen from bovine origin (containing 3% plasminogen on a weight per weight basis), lot No. 151910, was obtained from Organon Teknika, Oss, The Netherlands. Merthiolate was from Lilly, Indianapolis, IN. Affinity-purified  $^{125}\text{I}$ -protein A and rabbit reticulocyte lysate were from Amersham International, Amersham, United Kingdom. [ $^{35}\text{S}$ ]Methionine and EN $^3$ HANCE were from New England Nuclear, Dreieich, Federal Republic of Germany. Picofluor scintillation fluid was from Packard Instrument Company, Zürich, Switzerland. PMA was from P-L Biochemicals, Inc., Milwaukee, WI (a stock solution of 100  $\mu\text{g}/\text{ml}$  in ethanol was stored at  $-20^\circ\text{C}$ ). Nonfat dry milk (Lorso) was from Stalden, Konolfingen, Switzerland. Donkey IgG anti-rabbit IgG bound to cellulose (Sac-Cell) was from Wellcome, Beckenham, United Kingdom. PBS had the following composition: 8 g/liter NaCl, 0.2 g/liter KCl, 1.44 g/liter  $\text{NaH}_2\text{PO}_4 \cdot 2 \text{H}_2\text{O}$  and 0.2 g/liter  $\text{KH}_2\text{PO}_4$ .

### Cell Culture

U-937 cells, originally developed from the pleural exudate of a diffuse histiocytic lymphoma (Sundstrom and Nilsson, 1976) were grown in suspension culture in RPMI 1640 medium supplemented with 5% FCS, 2 mM glutamate and incubated in a 5%  $\text{CO}_2$  and 95% air atmosphere at  $37^\circ\text{C}$ . Cells were collected by centrifugation, washed three times with PBS, and resuspended at a concentration of  $10^6$  cells/ml in RPMI 1640 containing PMA at the concentrations indicated in the results section. After incubation, media were collected, centrifuged (10 min, 4,000 g and  $4^\circ\text{C}$ ) and stored at  $-70^\circ\text{C}$  until further use. For experiments requiring higher protein concentrations, media were boiled for 5 min in 1% SDS, dialyzed against 0.05% SDS in  $\text{H}_2\text{O}$ , dried, and stored at  $4^\circ\text{C}$  until dissolved in  $\text{H}_2\text{O}$  to a chosen concentration.

### PAI 2 Extraction of Cells after Stimulation with PMA

The adherent cells were washed three times with PBS, scraped off with a rubber policeman, sedimented by centrifugation, suspended in extraction buffer (0.1% NP-40 in PBS), homogenized in a Potter-Elvehjem homogenizer and centrifuged for 15 min at 10,000 g. The supernatants were stored at  $-70^\circ\text{C}$  until further use.

### Activity Assay for u-PA and PAI

u-PA activity was measured using the chromogenic substrate pyroglu-gly-arg-p-nitroanilide. Assays of PAI 2 activity were carried out in 96-well microtiter plates as previously described (Kruithof et al., 1986). 50- $\mu\text{l}$  sam-

ples, suitably diluted with 50 mM imidazole, pH 7.4, 140 mM NaCl, 1 mg/ml albumin, and 0.01% Tween-80 were mixed with 50  $\mu\text{l}$  of a 50 U/ml u-PA solution. After a 10-min incubation at  $37^\circ\text{C}$ , 100  $\mu\text{l}$  of 0.6 mM pyroglu-gly-arg-p-nitroanilide in 0.1 M Tris-HCl, pH 8.8, was added. The increase of absorbance at 405 nm was measured in a micro-ELISA-reader. The results were compared with those of a standard curve of u-PA. The inhibitor activity of the sample was defined as the difference between original activity (50 U/ml) and remaining activity as extrapolated from the standard curve and multiplied by the dilution factor. Activity measurements by the fibrin plate technique were performed as described by Astrup and Müllertz (1952).

### Radioimmunoassay for u-PA and PAI 2 Antigen

The samples to be tested (100  $\mu\text{l}$ , suitably diluted in immunoassay buffer: 50 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 10 mM EDTA, 0.1% albumin) were incubated for 4 h at room temperature in the presence of 100  $\mu\text{l}$  rabbit anti-human u-PA IgG, diluted to a concentration that bound 50% of  $^{125}\text{I}$ -u-PA in the absence of sample. Then 50  $\mu\text{l}$  of  $^{125}\text{I}$ -u-PA (40,000 cpm/ml) was added and the sample incubated overnight at  $4^\circ\text{C}$ . Antigen-antibody complexes were precipitated by 30 min incubation at room temperature with 100  $\mu\text{l}$  Sac-Cell (immobilized donkey IgG anti-rabbit IgG). The cellulose was washed three times with 2 ml of 0.15 M NaCl and bound radioactivity counted in a  $\gamma$ -counter. Results were evaluated using the empirical logit transformation of Rodbard et al. (1974). The radioimmunoassay for PAI 2 was performed essentially like that of u-PA, using rabbit anti-PAI IgG.

### Electrophoretic Procedures

SDS PAGE was performed as described by Laemmli (1970) employing 10% separating gels. Reverse fibrin autography was performed as described by Erickson et al. (1984), using the following modifications. The gel was shaken for 75 min in 1% Triton X-100 and quickly rinsed in  $\text{H}_2\text{O}$  before being placed on a u-PA containing fibrin-agarose underlay, which was prepared as follows. 15 ml of plasminogen-rich fibrinogen (10 mg/ml in 50 mM imidazole, 140 mM NaCl, pH 7.4), 10 ml of 2.5% agarose in  $\text{H}_2\text{O}$  kept at  $54^\circ\text{C}$ , 2 ml of thrombin (1 U/ml in imidazole-NaCl), and 0.54 ml of u-PA (2 U/ml in Imidazole-NaCl, 1% albumin) were mixed and injected immediately between two glass plates separated by 0.75-mm spacers. Proteins were stained by Coomassie Brilliant Blue as described (Laemmli, 1970). Gels were prepared for fluorography using EN $^3$ HANCE, according to the instructions of the manufacturer.

### Concanavalin A (Con A)-Sephacel Chromatography

Conditioned medium of a 3-d culture of phorbol ester-stimulated U-937 cells was concentrated by ultrafiltration through a Pellikon membrane as previously described (Kruithof et al., 1986). The concentrate containing 50 mg of total protein was dialyzed against 0.02 M Tris-HCl, 0.5 M NaCl, pH 7.4, and passed over a 5-ml column of Con A-Sephacel. After washing with the Tris-NaCl buffer, the column was eluted with 0.5 M methyl- $\alpha$ -D-mannopyranoside.

### Immunoblotting

Immunoblot analysis was performed according to Towbin et al. (1979) including the modifications of Johnson et al. (1984). After SDS PAGE the proteins were electrophoretically transferred to a nitrocellulose sheet using a Transblot cell (Bio-Rad Laboratories) containing 17 mM Tris, 67 mM glycine, pH 8.3, 0.07% SDS, and 17% methanol. Experiments using  $^{125}\text{I}$ -labeled antigen established that transfer was more than 95% under these conditions. The nitrocellulose sheet was shaken successively in the following solutions: 1  $\times$  10 min in 50 mM Imidazole, 140 mM NaCl, pH 7.4; 2  $\times$  15 min in BLOTTO (0.02 M Tris, 0.5 M NaCl, and 50 g/liter nonfat dry milk, adjusted to pH 7.5; Johnson et al., 1984); then incubated overnight at  $4^\circ\text{C}$  in 50 ml BLOTTO containing 1.2  $\mu\text{g}/\text{ml}$  of immunoaffinity-purified anti-PAI IgG (Kruithof et al., 1986) or 0.4  $\mu\text{g}/\text{ml}$  of anti-u-PA IgG (Wun et al., 1982) followed by 2  $\times$  15 min in BLOTTO; 4 h at room temperature with  $^{125}\text{I}$ -protein A (10  $\mu\text{Ci}$  of a 30-mCi/mg preparation) in BLOTTO; and 2 h with BLOTTO. The nitrocellulose sheet was rinsed with water, dried, and exposed to Kodak XAR film. Thereafter the radioactive bands were cut out of the nitrocellulose and counted in a  $\gamma$ -counter (Howe and Hershey, 1981; Vaessen et al., 1981). Alternatively, after incubation with the anti-PAI IgG, the nitrocellulose was washed twice with BLOTTO and incubated for 3 h with goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad Laboratories) used at a dilution of 1:200, washed twice with BLOTTO,

and peroxidase activity stained with the reagent provided by Bio-Rad Laboratories.

### **[<sup>35</sup>S]Methionine Biosynthetic Labeling and Immunoprecipitation**

Cells were collected by centrifugation (10 min, 400 g), washed three times with PBS, resuspended in methionine-free RPMI 1640, and starved for methionine for 2 h. Cells were then incubated for 4 h at 37°C with [<sup>35</sup>S]methionine (50 μCi/ml) and PMA (10 ng/ml). Subsequently, 10% vol/vol of regular RPMI 1640 was added. After 24 h more of incubation, the media were harvested and the cells extracted as described above. Total protein in cellular extracts and conditioned media was precipitated by adjustment to 10% trichloroacetic acid and incubated on ice overnight. The precipitates were recovered by centrifugation, washed three times with 10% trichloroacetic acid, and counted for total incorporation of radioactivity. Immunoprecipitation was performed essentially as described (Mihara and Blobel, 1980) except that Sac-Cell was used instead of protein A-Sepharose for the recovery of antigen-antibody complexes.

### **RNA Extraction**

RNA was prepared by a combination of SDS/proteinase K digestion and phenol/chloroform extraction. Cells were incubated for 15 min at 37°C in extraction buffer (25 mM Tris-HCl, pH 7.35, 0.1 M NaCl, 7.5 mM EDTA, 20 μg/ml polyvinylsulfate, 2.4% SDS, and 250 μg/ml proteinase K; Lizardi and Engelberg, 1979). 1 vol of cell lysate, collected in a 50-ml tube (Falcon Labware, Oxnard, CA), was mixed with 1 vol of phenol/chloroform/ether (50:50:1), homogenized using a polytron homogenizer for 15 s at the highest speed setting, vigorously shaken for 30 min, and centrifuged at 4,000 g for 10 min. The aqueous phase was collected, adjusted to 0.2 M NaCl, mixed with an equal volume of isopropanol cooled to -70°C, and centrifuged at 10,000 g for 10 min. The pellet was then dissolved in H<sub>2</sub>O, reprecipitated with ethanol, and redissolved in 0.07 M lithium dodecyl sulfate in water. The mixture was adjusted to 2.8 M lithium chloride, incubated at 4°C for 4 h, and centrifuged at 11,000 rpm for 10 min. After dissolving of the pellet in H<sub>2</sub>O, the solution was ethanol precipitated and stored at -70°C. Poly(A)<sup>+</sup> RNA was selected by hybridization to oligo dT cellulose essentially as described by Aviv and Leder (1972), and following the instructions of the manufacturer (Collaborative Research, Inc.).

### **In Vitro Translation**

In vitro biosynthesis of protein was performed in a rabbit reticulocyte cell-free system, as described by Pelham and Jackson (1976), using a reagent kit from New England Nuclear. 1 μg of poly(A)<sup>+</sup> RNA in 25 μl total reaction volume was translated for 90 min. Total radioactivity incorporated into protein was subsequently determined by precipitation in 10% trichloroacetic acid. The proportion of specific PAI 2 translated was determined by immunoprecipitation as described above and by Maniatis et al. (1975).

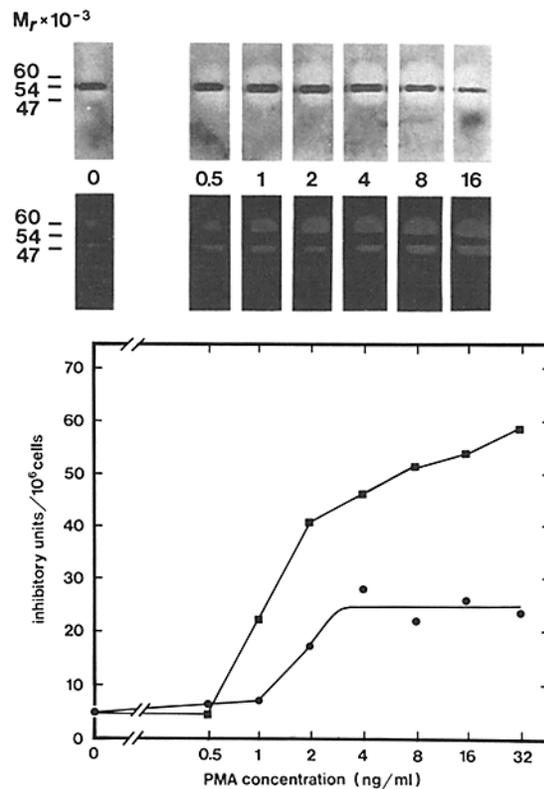
### **Northern Blot Hybridization**

pUK 0321, a recombinant pUC 9 derivative (Vieira and Messing, 1982) containing u-PA cDNA, was isolated from a cDNA library constructed from mRNA from HT 1080 fibrosarcoma cells (Hession, K., H. Meade, W.-D. Schleuning, unpublished results) by screening with a mixed oligonucleotide probe, synthesized according to the published protein sequence of u-PA (Günzler et al., 1982). Electrophoresis was performed using 1% agarose gels containing 20% formaldehyde and RNA transferred to nitrocellulose filter paper. Hybridization with radioactive pUK 0321 (labeled by nick translation; Maniatis et al., 1975) and washing of the filters was performed as described (Thomas, 1980).

## **Results**

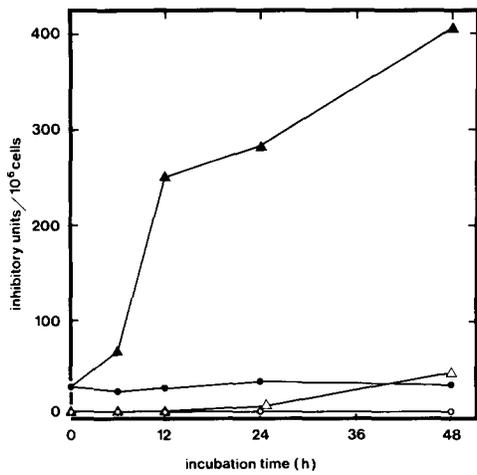
### **PMA-induced Biosynthesis of PAI 2 and u-PA in U-937 Cells Is Time- and Dose-Dependent**

The treatment of U-937 cells with increasing concentrations of PMA resulted in a concentration-dependent accumulation of PAI 2 in the culture medium. If PAI 2 activity was measured 24 h after stimulation, maximal induction was ob-



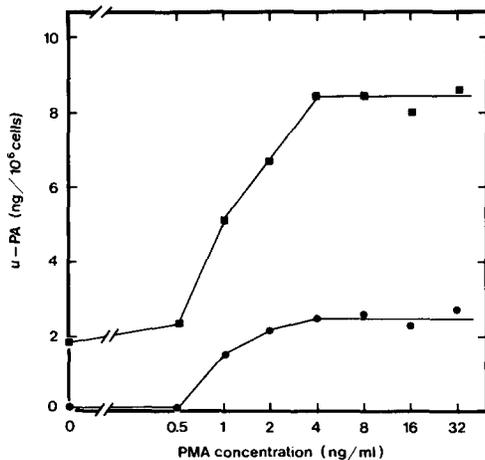
**Figure 1.** Biosynthesis of PAI 2 and u-PA activity by U-937 cells; response to increasing doses of PMA. (Top) Demonstration of u-PA and PAI 2 activity in conditioned media by reverse fibrin autography (Erickson et al., 1984). 100 μl conditioned media, mixed with 20 μl 6× sample buffer were charged per track; underlays were incubated for 11 h at 37°C. u-PA activity is visualized as a clear band at *M<sub>r</sub>* 54,000, PAI 2 activity as two opaque zones at *M<sub>r</sub>* 47,000 and 60,000. (Middle) 17-h incubation at 37°C. (Bottom) PAI 2 activity measured in the conditioned media 24 (solid circle) and 48 h (solid square) after PMA treatment.

served at a concentration of 4 ng/ml (Fig. 1, bottom). If PAI 2 activity was measured after 48 h, the dose-response curve did not reach a plateau. Separate experiments employing trypan blue exclusion as a criteria for cell viability, however, suggested that this was due to a release of intracellular PAI 2 from a proportionately increasing number of dead cells. The effect of PMA was also visualized by reverse fibrin autography (Fig. 1, top). Under the chosen conditions, which are described in detail in Materials and Methods, both u-PA and PAI could be visualized by this technique. After 11 h of incubation, activation of plasminogen by exogenous u-PA added to the indicator gel was not sufficient to produce lysis. Where u-PA was present in the separation gel, however, a lysis zone was generated at a position corresponding to 54 kD (Fig. 1, top). After 17 h of incubation, fibrin in the indicator gel was completely lysed, except in positions where PAI activity had been present. The position of the two opaque bands that increased after phorbol ester treatment corresponded to 50 and 60 kD (Fig. 1, middle). In separate experiments with purified PAI 2, we established that PAI 2 can be visualized by this technique, although its activity is partially destroyed by SDS. The increase of PAI 2 activity was found to be time-dependent both in cellular extracts and conditioned media (Fig. 2). Conspicuously, the large majority of activity (85%)

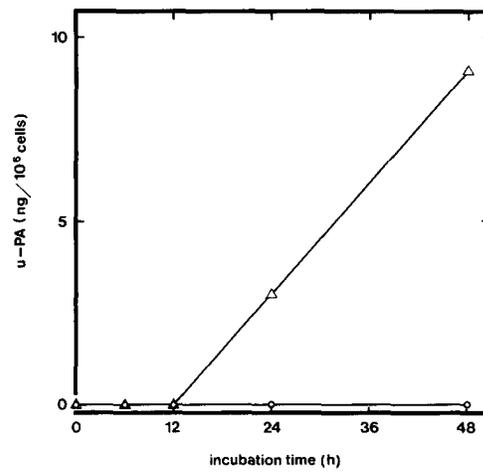


**Figure 2.** Accumulation of PAI 2 activity in extracts (solid triangle) and conditioned media (open triangle) of U-937 cells after treatment with PMA (10 ng/ml) and in extracts (solid circle) and conditioned media (open circle) of untreated controls.

was present in cellular extracts. U-PA activity, present in the conditioned media, was evidenced by the sharp lysis zone corresponding to 54 kD (Fig. 1). By comparing the width of the lysis zone with lysis zones generated by standard amounts of u-PA, this activity could be estimated to represent  $\sim 1$  ng u-PA/ml. It could therefore not be determined by the colorimetric assay, which reaches its sensitivity limit at  $\sim 10$   $\mu$ g u-PA/ml (our unpublished results). Using the more sensitive fibrin plate technique (Astrup and Müllertz, 1952) with which concentrations as low as 0.2 ng/ml can be determined, there was, nevertheless, no u-PA activity detectable. This was most probably due to the presence of high excess of PAI 2 and not to the fact that u-PA was present in its pro-form, since pro-u-PA is also detectable in this assay system (our unpublished results), apparently because it is converted into u-PA by the presence of trace amounts of plasmin. We therefore quantified u-PA antigen by a radioimmunoassay. Maximal release of u-PA into the medium occurred at the same PMA concentration as that of PAI 2 (Fig. 3). U-PA levels only started to rise 12 h after stimulation by PMA (Fig. 4).



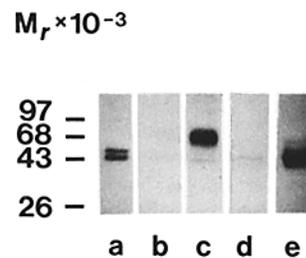
**Figure 3.** u-PA antigen in media conditioned by U-937 cells in response to increasing doses of PMA as determined by a radioimmunoassay; 24 (solid circle) and 48 h (solid square) after treatment.



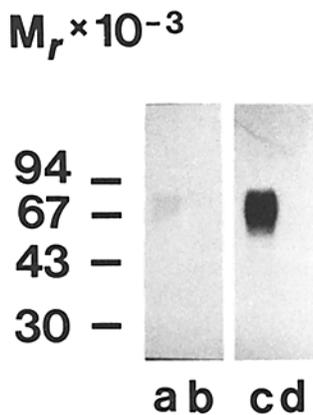
**Figure 4.** Time-dependent accumulation of u-PA antigen (as determined by radioimmunoassay) in media conditioned by U-937 cells treated with 10 ng/ml of PMA (open triangle). Media of untreated control cells (open circle).

### Molecular Heterogeneity of Extracellular and Intracellular PAI 2 and u-PA Antigen: an Assessment by Quantitative Immunoblot Analysis and Biosynthetic Labeling

Reverse fibrin autography (Fig. 1) revealed a molecular heterogeneity of PAI 2. To study the different forms of PAI 2 in more detail and to quantify each form separately, we established a quantitative immunoblot assay, using  $^{125}$ I-labeled protein A as indicator protein, as previously described (Vaessen et al., 1981; Lämmle et al., 1986). Extracellular PAI 2 was almost exclusively (but not entirely) found as a 60-kD species and was increased approximately sixfold after 24 h of PMA stimulation (Fig. 5, lanes b and c). Intracellular PAI 2 consisted of a closely spaced doublet of 47 kD and increased approximately sevenfold under the same conditions (Fig. 5, lanes d and e). The intracellular form was predominant (85% of the total antigen) both before and after PMA stimulation. The increase in PAI 2 activity and antigen was matched by a proportional increase in biosynthetically labeled PAI 2 in the conditioned medium 24 h after PMA treatment (Fig. 6). Thus, PMA did not stimulate the processing and subsequent secretion of the 60-kD form of PAI 2 from an existing storage pool, but stimulated its de novo synthesis. The proportion of PAI 2 antigen in relation to total newly



**Figure 5.** Quantitative immunoblotting assay of PAI 2 antigen in conditioned media (lanes b and c) or cell extracts (lanes d and e) of U-937 cells 24 h after treatment with 10 ng/ml of PMA (lanes c and e) or of untreated controls (lanes b and d). Purified PAI 2 (lane a). By comparison with a standard curve constructed in a parallel experiment the following amounts (b-d expressed per  $10^6$  cells) were obtained: lane a, 150 ng; lane b, 46 ng; lane c, 275 ng; lane d, 295 ng; lane e, 1960 ng.

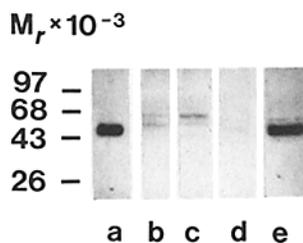


**Figure 6.** Biosynthetically  $^{35}\text{S}$ -labeled PAI 2 from conditioned media of U-937 cells 24 h after PMA treatment (lanes *c* and *d*) or from nontreated controls (lanes *a* and *b*). 100  $\mu\text{l}$  of conditioned medium were subjected to immune precipitation using affinity-purified anti PAI 2 IgG (lanes *a* and *c*) or nonimmune IgG (lanes *b* and *d*). Immune precipitates were analyzed by SDS PAGE, followed by treatment with  $\text{EN}^3\text{HANCE}$ , drying, and exposure for 3 wk to Kodak XAR film at  $-70^\circ\text{C}$ .

synthesized protein as measured by trichloroacetic acid-precipitable, versus -immunoprecipitable radioactivity increased 10-fold from 0.03 to 0.3% in cellular extracts and about sevenfold from 3 to 20% in conditioned media (data not shown). SDS PAGE of immunoprecipitates obtained from cellular extracts revealed, besides bands migrating like PAI 2, considerable amounts of contaminating protein even after extensive washing of the immunoprecipitates. This indicates that U-937 cells contain protein(s) that bind to IgG (probably the Fc receptor and/or other unknown proteins). This is also the most likely reason for our failure to establish a radioimmunoassay for intracellular PAI 2 antigen. In contrast to the band migrating like PAI 2, however, the bands representing contaminating proteins did not appear to increase after PMA treatment (data not shown). Intracellular u-PA antigen displayed a molecular mass indistinguishable from that of commercial urokinase (Fig. 7, lanes *a*, *d*, and *e*), and increased approximately 10-fold after 24 h of PMA treatment. Extracellular u-PA antigen was found predominantly as a heterogeneous form with molecular masses ranging from 60 to 70 kD (Fig. 7, lanes *b* and *c*) and increased approximately sixfold. Zymography indicated that this form was inactive (Fig. 1). In contrast, the active u-PA revealed in Fig. 1 was hardly visible after immunoblotting, apparently because its concentration was close to the lower limit of detection of this test (below 1 ng/ml).

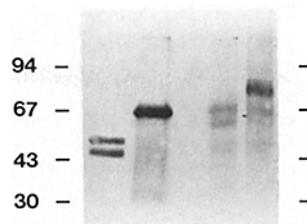
#### The 60-kD Form of PAI Is Glycosylated

To investigate the possibility that the molecular heterogeneity of PAI 2 was caused by differential glycosylation, a mix-



**Figure 7.** Quantitative immunoblot assay of u-PA antigen in conditioned media (lanes *b* and *c*) or cell extracts (lanes *d* and *e*) of U-937 cells 24 h after treatment with 10 ng/ml PMA (lanes *c* and *e*) or of untreated controls (lanes *b* and *d*). Purified high molecular mass u-PA (lane *a*). By comparison with

a standard curve constructed in a parallel experiment, the following amounts (lanes *b-d* expressed per  $10^6$  cells) were obtained: (lane *a*), 40 ng; lane *b*, 1 ng; lane *c*, 2.8 ng; lane *d*, 6 ng; lane *e*, 39 ng.



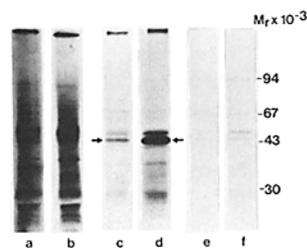
methyl- $\alpha$ -D-mannopyranoside. The fraction not retarded by the column (left) and the eluted fraction (right) were subjected to SDS PAGE in a 10% gel, either before or after a 15-min incubation with a five-fold excess of low relative molecular mass u-PA, electrotransferred to nitrocellulose and revealed by immunoperoxidase staining using immunopurified rabbit anti-PAI antibodies and goat anti-rabbit IgG antibodies conjugated to horseradish peroxidase.

**Figure 8.** Separation of the 60- and 47-kD forms of PAI 2 by affinity chromatography on Con A-Sepharose. Crude, concentrated U-937-conditioned medium was passed over a column of Con A-Sepharose as described in Materials and Methods. After washing, the column was eluted with 0.5 M

ure of the two forms was passed over a column of Con A-Sepharose. Only the 60-kD form was retained by the column, whereas the 47-kD doublet passed unretarded (Fig. 8). Thus, the 60-kD form contains carbohydrates that bind to Con A-Sepharose, whereas the 47-kD form does not (Fig. 8). The functional activity of the two forms of PAI 2 was similar, since both were able to form complexes with low relative molecular mass u-PA (Fig. 8). Furthermore, the kinetics of inhibition of u-PA by the two forms of PAI 2 was identical (unpublished observations). The relative molecular masses of the complexes of both glycosylated and nonglycosylated PAI 2 with low molecular mass u-PA are less than the sum of their relative molecular masses. This implies cleavage of the inhibitor during complex formation as discussed by Kruithof et al. (1986).

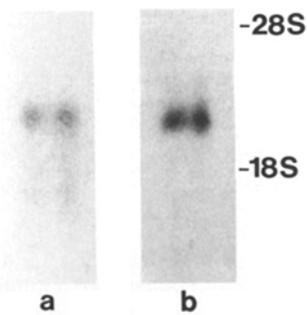
#### The Increase of PAI 2 Requires Gene Transcription and Is Accompanied by an Equal Increase of mRNA

When cells were cultured for 24 h in the presence of PMA and either  $\alpha$ -amanitine (10  $\mu\text{g/ml}$ ) or cycloheximide (1  $\mu\text{g/ml}$ ), PAI 2 activity in both cellular extracts and conditioned media was decreased 80 and 85%, respectively, with respect to controls that had been treated with PMA alone.



**Figure 9.** Protein biosynthesis in a rabbit reticulocyte-derived cell-free system directed by mRNA from U-937 cells that were maintained for 24 h in serum-free RPMI (lanes *a*, *c*, and *e*) or serum-free RPMI supplemented with 10 ng/ml PMA (lanes *b*, *d*, and *f*). After 90-min reaction time, total

protein of 1  $\mu\text{l}$  of the sample was precipitated with trichloroacetic acid (lanes *a* and *b*) and 24  $\mu\text{l}$  of the sample were subjected to immunoprecipitation, using anti-PAI 2 IgG (lanes *c* and *d*) or nonimmune IgG (lanes *e* and *f*). Precipitates were solubilized by boiling in  $1\times$  sample buffer, containing 10 mM dithiothreitol, electrophoresed through SDS polyacrylamide gels (Lämmli, 1970), treated with  $\text{EN}^3\text{HANCE}$ , dried, and exposed for 5 d to Kodak XAR-film. The PAI 2 band is marked with an arrow. The band above represents an artifact, which frequently contaminates [ $^{35}\text{S}$ ]-methionine.



**Figure 10.** Northern blot analysis of mRNA from U-937 cells maintained for 24 h in serum-free RPMI (lane *a*) or serum-free RPMI supplemented with 10 ng/ml PMA (lane *b*). 10 ng poly(A)<sup>+</sup> RNA were loaded per slot, electrophoresed through a 1% agarose gel containing 20% formaldehyde, transferred to nitrocellulose (Thomas, 1980), and hybridized to a u-PA cDNA probe

that had been <sup>32</sup>P-labeled by nick translation to a specific activity of  $0.5 \times 10^8$  cpm/ $\mu$ g. Filters were washed (Thomas, 1980) and exposed to Kodak XAR film at  $-70^\circ\text{C}$ .

The increase of u-PA antigen was unaffected by  $\alpha$ -amanitin but reduced 60% by cycloheximide. The relative amount of PAI 2 mRNA in induced and noninduced cells was compared by *in vitro* translation experiments. mRNA from both cell types was used to direct protein biosynthesis in a rabbit reticulocyte-derived cell-free system. PAI 2 antigen was immunoprecipitated from the mixtures and analyzed by SDS PAGE. The relevant bands were cut out, counted, and compared with total incorporated counts (measured by trichloroacetic acid precipitation). Upon PMA stimulation, the quantity of PAI 2 antigen synthesized in the *in vitro* translation system increased sixfold. The proportion of PAI 2-specific mRNA increased from 0.04 to 0.25% (Fig. 9). The levels of u-PA mRNA were too low for characterization by *in vitro* translation and immunoprecipitation. We therefore resorted to Northern blot analysis. The specificity of this assay was previously established using mRNA from HT 1080 fibrosarcoma cells and Bowes melanoma cells. Whereas mRNA from the former line, a known producer of u-PA, gave a strong band at about 22 S, the mRNA of the latter line, which does not produce u-PA antigen, gave a negative result (Schleuning, W. D., and R. Medcalf, unpublished observations). The increase of u-PA-specific mRNA in induced versus noninduced U 937 cells was only 1.5 times, as compared with a 6- to 13-fold increase in intra- and extracellular u-PA antigen (Fig. 10).

## Discussion

The association of PA biosynthesis with a variety of biological processes that involve cell migration and tissue remodeling has been extensively documented (Danø et al., 1985; Saksela, 1985; Vassalli et al., 1976; Markus, 1984; Ossowski and Reich, 1983). The classical example of a migratory cell is the macrophage. Its PA was first described by Unkeless et al. (1974) and subsequently studied by Hamilton et al. (1976), Vassalli et al. (1976), and Vassalli and Reich (1977). In these early reports, however, the molecular species of PA were not identified, and proenzyme activation and specific inhibition not investigated. U-937 cells were chosen to reexamine this work, because they are akin to precursors of the mononuclear phagocyte lineage and differentiate in the presence of PMA, acquiring monocyte/macrophage enzymatic and serological markers (Ralph et al., 1982). Macrophages and U-937 cells synthesize pro-u-PA and a PAI 2 immunologi-

cally related to a PAI in placental extracts (Åstedt et al., 1984; Kopitar et al., 1985; Vassalli et al., 1984; Kruithof et al., 1986). Our results provide a quantitative assessment of the modulation of this synthesis during differentiation and demonstrate the need of immunological assays, since u-PA activity was undetectable in unfractionated conditioned media or cellular extracts.

PAI 2 activity and PAI 2 antigen measurements correlated reasonably well. Assuming a specific activity of  $1 \times 10^5$  inhibitory units (IU)/mg (based on a specific activity of u-PA of  $1 \times 10^5$  U/mg, equimolar complex formation, and a relative molecular mass of PAI 2 close to u-PA) the measured activities corresponded approximately to the quantity of antigen present (Figs. 1 and 5). Hence, most PAI 2 is fully active in both cellular extracts and conditioned media and is not present in a latent form or complexed to PA, like PAI 1 purified from endothelial cells (Hekman and Loskutoff, 1985; Sprengers et al., 1984).

Intracellular and extracellular PAI 2 exhibit the same specific activity (Kruithof, E. K. O., unpublished data) but differ in their molecular masses. Experiments involving fixation of the protein to Con A-Sepharose demonstrated that secreted 60-kD PAI 2 is a glycoprotein, whereas the two intracellular forms (which do not bind to Con A-Sepharose) are either glycosylated differently, or not at all. Current concepts of protein compartmentalization imply that secretory proteins are synthesized on bound ribosomes and core-glycosylated co-translationally during insertion into the rough endoplasmic reticulum (reviewed by Dunphy and Rothman, 1985). The N-linked precursor oligosaccharide chain contains glucose, mannose, and *N*-acetyl glucosamine. In the Golgi stack the high mannose precursor chains are partially degraded and remodeled by several glycosyltransferases. All forms along this pathway bind Con A. Should this scheme be verified for all secretory proteins, it would appear as if the intracellular PAI 2 is not targeted to the secretory pathway. Then the high intracellular concentration of PAI 2 suggests an intracellular function, which is at present unknown. It was shown in two important recent contributions that translocation and modification of a yeast secretory protein may happen posttranslationally in an ATP-dependent process (Waters and Blobel, 1986; Hansen et al., 1986). Such a mechanism has so far not been found in animal cells but likewise has not been rigorously excluded. It can therefore not be ruled out that PAI 2 is translated, stored in an unmodified form, and channeled into the secretory pathway in response to still undefined signals.

Intracellular u-PA antigen migrated on SDS PAGE similar to urinary high molecular mass u-PA. Surprisingly, extracellular u-PA antigen was found predominantly in an enzymatically inactive form of three closely spaced bands of 67–70 kD. We considered the possibility that these bands presented an artifact due to unspecific binding of the antibody employed. This, however, appears unlikely for the following reasons: (a) immunoaffinity-purified antibodies were used throughout the experiment; (b) the bands were absent in controls where nonimmune IgG was employed; and (c) a surplus of urokinase antigen competed for binding (data not shown). There is so far no clue to the origin, the function, or the biological significance of these forms. Judging from the molecular mass, it might represent a complex of low molecular mass u-PA with an inhibitor, which, however, does not react with

anti-PAI 2 antisera. Alternatively, it might represent a partially degraded form of a complex of u-PA with its cellular binding site (Vassalli et al., 1985; Stoppelli et al., 1985; Stoppelli et al., 1986). This cellular binding site (also called the u-PA receptor) constitutes an important new element of the plasminogen-activating enzymatic system and an attractive subject for future work. We note that Stoppelli et al. (1986), in their study on the u-PA receptor, have observed u-PA antigen of similar relative molecular mass as that described here in conditioned media of A-431 cells, and have likewise failed to present a fully satisfactory interpretation. We are presently making efforts to determine the definite identity of this abnormally migrating u-PA antigen.

Calculations of the second order rate constant of complex formation (Kruithof et al., 1986) and the concentrations of enzyme and inhibitor in cellular extracts (Figs. 2, 6, and 8) indicate that almost all u-PA should form complexes with PAI 2 within minutes if in an active form. As this is not the case, pro-u-PA appears to be unable to react with PAI 2. Stimulation of u-PA and PAI 2 biosynthesis after PMA treatment was time- and dose-dependent. u-PA antigen increased to the same extent in the presence and absence of  $\alpha$ -amanitine. A 1.5-fold increase of u-PA-specific mRNA was observed by Northern blot analysis. To account for the total increase of antigen, however, one would have to also assume an increased translation rate, or perhaps a decreased degradation of u-PA antigen in untreated cells. Alternatively, a higher increase of mRNA could have occurred transiently and thus would not have been observed after 24 h. This is unlikely, however, because  $\alpha$ -amanitine did not block the increase in u-PA antigen. No experiments were performed to discriminate between these possibilities. 24 h after PMA treatment, PAI 2 antigen and activity increased sixfold in conditioned media and about sevenfold in cellular extracts. About 85% of PAI 2 was found in cellular extracts. The 80% inhibition of this increase by  $\alpha$ -amanitine indicates a contribution of de novo transcription. Representing at least 0.3% of total protein synthesized 24 h after PMA induction, PAI 2 has to be considered a major product of this monocytic cell. PAI 2 is present in a 50-fold molar excess over u-PA. It will be interesting to see whether authentic macrophages or other PA-producing cells also produce such large surplus of inhibitor, and how modulation of PAI 2 biosynthesis contributes to the extent of pericellular proteolysis in cell migration and tissue remodeling.

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## References

Åstedt, B., I. Lecander, T. Brodin, A. Lundblad, and K. Löw. 1985. Purification of a specific placental plasminogen activator inhibitor by monoclonal antibody and its complex formation with plasminogen activator. *Thromb. Haemostasis*. 53:122-125.

Astrup, T., and S. Müllertz. 1952. The fibrin plate method for estimating fibrinolytic activity. *Arch. Biochem. Biophys.* 40:346-351.

Aviv, H., and P. Leder. 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA*. 69:1408-1412.

Chapman, H. A., and O. L. Stone. 1985. Characterization of a macrophage-derived plasminogen activator inhibitor. *Biochem. J.* 230:109-116.

Collen, D. 1980. On the regulation and control of fibrinolysis. *Thromb. Haemostasis*. 43:77-89.

Crutchley, D. J., L. B. Conanan, and J. R. Maynard. 1981. Human fibroblasts produce inhibitor directed against plasminogen activator when treated with glucocorticoids. *Ann. N.Y. Acad. Sci.* 370:609-616.

Danø, K., P. A. Andreasen, J. Grøndahl-Hansen, P. Kristensen, L. S. Nielsen, and L. Skriver. 1985. Plasminogen activators, tissue degradation and cancer. *Adv. Cancer Res.* 44:139-266.

Dunphy, W. G., and J. E. Rothman. 1985. Compartmental organization of the Golgi stack. *Cell*. 42:13-21.

Erickson, L. A., D. A. Lawrence, and D. J. Loskutoff. 1984. Reverse fibrin autography: a method to detect and partially characterize protease inhibitors after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal. Biochem.* 137:454-463.

Granelli-Piperno, A., and E. Reich. 1978. A study of proteases and protease-inhibitor complexes in biological fluids. *J. Exp. Med.* 148:223-234.

Günzler, W. A., G. J. Steffens, F. Ötting, S. A. Kim, E. Frankus, and L. Flohè. 1982. The primary structure of high molecular mass urokinase from human urine. The complete sequence of the A chain. *Hoppe-Seyler's Z. Physiol. Chem.* 363:1155-1165.

Hamilton, J., J.-D. Vassalli, and E. Reich. 1976. Macrophage plasminogen activator: induction by asbestos is blocked by anti-inflammatory steroids. *J. Exp. Med.* 144:1689-1694.

Hansen, W., P. D. Garcia, and P. Walter. 1986. In vitro protein translocation across the yeast endoplasmic reticulum: ATP dependent posttranslational translocation of the prepro- $\alpha$ -factor. *Cell*. 45:397-406.

Hekman, C. M., and D. J. Loskutoff. 1985. Endothelial cells produce a latent inhibitor of plasminogen activator that can be activated by denaturants. *J. Biol. Chem.* 260:11581-11587.

Howe, J. G., and J. W. B. Hershey. 1981. A sensitive immunoblotting method for measuring protein synthesis initiation factor levels in lysates of *Escherichia coli*. *J. Biol. Chem.* 256:12836-12839.

Husain, S. S., V. Gurewich, and B. Lipinski. 1983. Purification and partial characterization of a single chain high molecular weight form of urokinase from human urine. *Arch. Biochem. Biophys.* 220:31-38.

Johnson, D. A., J. W. Gautsch, J. R. Sportsman, and H. Elder. 1984. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. *Gene Anal. Tech.* 1:3-8.

Kopitar, M., B. Rozman, J. Babnik, V. Turk, D. E. Mullins, and T. C. Wun. 1985. Human leukocyte urokinase inhibitor—purification, characterization and comparative studies against different plasminogen activators. *Thromb. Haemostasis*. 54:750-755.

Kruithof, E. K. O., J.-D. Vassalli, W.-D. Schleuning, R. J. Mattaliano, and F. Bachmann. 1986. Purification and characterization of a plasminogen activator inhibitor from the histiocytic lymphoma cell line U-937. *J. Biol. Chem.* 261:11207-11213.

Lämmle, B., M. Berrettini, H. P. Schwarz, M. J. Heeb, and J. H. Griffin. 1986. Quantitative immunoblotting assay of blood coagulation factor XII. *Thromb. Res.* 41:747-759.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.

Lizardi, P. M., and A. Engelberg. 1979. Rapid isolation of RNA using proteinase K and sodium perchlorate. *Anal. Biochem.* 98:116-122.

Maniatis, T., A. Jeffrey, and D. G. Kleid. 1975. Nucleotide sequence of the rightward operator of phage. *Proc. Natl. Acad. Sci. USA*. 72:1184-1188.

Markus, G. 1984. The role of haemostasis and fibrinolysis in the metastatic spread of cancer. *Semin. Thromb. Haemostasis*. 10:61-70.

Mihara, K., and G. Blobel. 1980. The four cytoplasmically made subunits of yeast mitochondrial cytochrome c oxidase are synthesized individually and not as a polypeptide. *Proc. Natl. Acad. Sci. USA*. 77:4160-4164.

Ossowski, L., and E. Reich. 1983. Antibodies to plasminogen activator inhibit tumor metastasis. *Cell*. 35:611-619.

Pelham, H. R. B., and R. J. Jackson. 1976. An efficient mRNA dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* 67:247-251.

Ralph, P., N. Williams, M. A. S. Moore, and P. B. Litcofsky. 1982. Induction of antibody dependent and non-specific tumor killing in human monocytic leukemia cells by non-lymphocyte factors and phorbol ester. *Cell. Immunol.* 71:215-223.

Rodbard, D. 1974. Statistical quality control and routine data processing for radioimmunoassay and immunoradiometric assay. *Clin. Chem.* 20:1255-1270.

Saksela, O. 1985. Plasminogen activator and regulation of pericellular proteolysis. *Biochim. Biophys. Acta*. 823:35-65.

Scott, R. W., and J. B. Baker. 1983. Purification of human protease nexin. *J. Biol. Chem.* 258:10439-10444.

Seifert, S. C., and T. D. Gelehrter. 1978. Mechanism of dexamethasone inhibition of plasminogen activator in rat hepatoma cells. *Proc. Natl. Acad. Sci. USA*. 75:6130-6133.

Sprengers, E. D., J. H. Verheijen, V. W. M. Van Hinsbergh, and J. J. Emeis. 1984. Evidence for the presence of two different fibrinolytic inhibitors in human endothelial cell conditioned medium. *Biochim. Biophys. Acta*. 801:163-170.

Stoppelli, M. P., A. Corti, A. Soffientini, G. Cassani, F. Blasi, and R. K. Assoian. 1985. Differentiation enhanced binding of the amino terminal fragment of human urokinase plasminogen activator to a specific receptor on U-937 monocytes. *Proc. Natl. Acad. Sci. USA*. 82:4939-4943.

Stoppelli, M. P., C. Tacchetti, M. V. Cubellis, A. Corti, V. J. Hearing, G. Cassani, E. Appella, and F. Blasi. 1986. Autocrine saturation of pro-urokinase

receptors on human A431 cells. *Cell*. 45:675-684.

Sundström, C., and K. Nilsson. 1976. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int. J. Cancer*. 17:565-577.

Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA*. 77:5201-5205.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA*. 76:4350-4354.

Unkeless, J. C., S. Gordon, and E. Reich. 1974. Secretion of plasminogen activator by stimulated macrophages. *J. Exp. Med.* 139:834-850.

Vaessen, R. T. M. J., J. Kreike, and G. S. P. Groot. 1981. Protein transfer to nitrocellulose filters: a simple method for quantitation of single proteins in complex mixtures. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 124:193-196.

Valinsky, J. E., E. Reich, and N. M. Le Douarin. 1981. Plasminogen activator in the bursa of Fabricius: correlations with morphogenetic remodeling and cell migrations. *Cell*. 25:471-476.

Van Mourik, J. A., D. A. Lawrence, D. J. Loskutoff. 1984. Purification of an inhibitor of plasminogen activator (antiactivator) synthesized by endothelial cells. *J. Biol. Chem.* 259:14914-14921.

Vassalli, J.-D., and E. Reich. 1977. Macrophage plasminogen activator: induction by products of activated lymphoid cells. *J. Exp. Med.* 145:429-437.

Vassalli, J.-D., D. Baccino, and D. Belin. 1985. A cellular binding site for the *M*<sub>1</sub> 55'000 form of the human plasminogen activator urokinase. *J. Cell Biol.* 100:86-92.

Vassalli, J.-D., J. M. Dayer, A. Wohlwend, and D. Belin. 1984. Concomitant secretion of prourokinase and of a plasminogen activator-specific inhibitor by cultured human monocytes-macrophages. *J. Exp. Med.* 159:1653-1658.

Vassalli, J.-D., J. Hamilton, and E. Reich. 1976. Macrophage plasminogen activator: modulation of enzyme production by antiinflammatory steroids, mitotic inhibitors, and cyclic nucleotides. *Cell*. 8:271-281.

Vieira, J., and J. Messing. 1982. The pUC plasmids, an m13mp7 derived system for insertion, mutagenesis and sequencing with synthetic universal primers. *Gene*. 19:259-268.

Waters, M. G., and G. Blobel. 1986. Secretory protein translocation in a yeast cell free system can occur posttranslationally and requires ATP hydrolysis. *J. Cell. Biol.* 102:1543-1550.

Wun, T.-C., L. Ossowski, and E. Reich. 1982. A proenzyme form of human urokinase. *J. Biol. Chem.* 257:7262-7268.