

HUMAN NEUTROPHIL PLASMINOGEN ACTIVATOR IS LOCALIZED IN SPECIFIC GRANULES AND IS TRANSLOCATED TO THE CELL SURFACE BY EXOCYTOSIS

BY JEANNE M. HEIPLE* AND LILIANA OSSOWSKI†

*From the *Department of Physiology, Columbia College of Physicians and Surgeons, New York 10032; and the †Department of Cellular Physiology and Immunology, The Rockefeller University, New York 10021*

Plasminogen activator (PA)¹ is produced and secreted by mouse and human monocytes and neutrophils (1–4). A role for the enzyme in cell migration associated with inflammation has been postulated (5). Recently, a specific PA receptor on the surface of monocytes has been described (6, 7). PA bound to this receptor remains active, providing a mechanism through which a cell can concentrate proteolytic activity on its membrane. This finding suggests PA participation in processes requiring direct, local proteolysis.

It has been shown that PA activity of macrophages and neutrophils can be modulated by agents such as Con A, PMA, or glucocorticoids (1, 3, 5). In these experiments, long-term effects were studied and the enzyme was measured either in whole cell lysates or in the conditioned medium. In a number of other studies (8–12) involving thymocytes, macrophages, basophils, and several tumor cell lines, the subcellular localization of PA has been examined but no uniform conclusion has been reached, nor was a comparison of subcellular PA distribution in resting and activated states in neutrophils or any other cells attempted.

Using a previously published method (13) of neutrophil fractionation, we obtained highly enriched fractions of azurophilic and specific granules and plasma membranes, and we determined PA activity in these fractions. We compared the localization of PA in resting and activated neutrophils.

Our results provide evidence that in resting neutrophils most of PA is associated with the specific granule membranes, and upon activation, PA translocates to the plasma membrane, thus equipping the neutrophil with surface-associated proteolytic activity.

Materials and Methods

Materials. Human blood for isolation of neutrophils was obtained from healthy volunteers. Ficoll, Percoll, Sepharose 4B, and calibration beads were from Pharmacia Fine Chemicals (Piscataway, NJ). Hypaque was from Winthrop Laboratories (New York, NY); Triton X-100 was from Fisher Scientific Co. (Fair Lawn, NJ); ⁵⁷Co-vitamin B12 and Na¹²⁵I

This work was supported by U. S. Public Health Service grant AI-20516 to S. C. Silverstein and CA-40758 to L. Ossowski. Address correspondence to Liliana Ossowski, The Rockefeller University, 1230 York Avenue, New York, NY 10021.

¹ *Abbreviations used in this paper:* PA, plasminogen activator, tPA, tissue-type plasminogen activator, uPA, urokinase-type plasminogen activator, UK, urokinase.

were from New England Nuclear (Boston, MA); and ATP (Na)₂ was from Boehringer Mannheim Biochemicals (Indianapolis, IN). We obtained PMA, *p*-nitrophenyl phosphate, phenolphthalein β -monoglucuronic acid, DFP, and fibrinogen (77% clotting ability) from Sigma Chemical Co. (St. Louis, MO) and chromogenic substrate for urokinase (UK), the L-pyroglutamyl-glycyl-L-arginine-*p*-nitroanilide (S 244 Spectrozyme UK), from American Diagnostica (Greenwich, CT). UK for standards was from Leo Pharmaceuticals (Ballerup, Denmark); plasmin was from Kabi Diagnostica (Stockholm, Sweden); Trasylol (aprotinin) was from FBA Pharmaceuticals (New York, NY). Antibodies to UK- and tissue-type PA (uPA and tPA) were prepared by injecting rabbits with purified antigens, as described (14). All other materials were of reagent grade.

Isolation and Fractionation of Neutrophils. Isolation and fractionation of neutrophils were essentially as described (13). Briefly, after dextran sedimentation of red cells, leukocyte-rich fractions from blood of healthy donors were centrifuged through Ficoll Hypaque as described (13), and the residual red blood cells were lysed by osmotic shock. For disruption, neutrophils were resuspended in relaxation buffer (100 mM KCl, 3 mM NaCl, 1 mM ATP(Na)₂, 3.5 mM MgCl₂, 10 mM Pipes, pH 7.3) at $1-5 \times 10^7$ cells per ml and pressurized with N₂ at 350 psi for 20 min at 4°C in a nitrogen bomb. Nuclei and unbroken cells were removed by centrifugation and the postnuclear supernatants were further fractionated on a discontinuous Percoll gradient (14 ml of Percoll, density 1.120 layered under 14 ml of Percoll, density 1.050 gm/ml, 22 min at 48,000 g, 4°C). The density of the different fractions was estimated from calibration beads centrifuged on a parallel gradient. 1-ml fractions were collected and assayed for protein content (15), β -glucuronidase activity (16), vitamin B12-binding protein content (17), and alkaline phosphatase activity (18). In some experiments (Figs. 1, 3, and 5), PA in the presence of plasminogen, and fibrinolytic activity in the absence of plasminogen were assayed in each individual fraction; in all other experiments the enzyme activities were determined in pools of the fractions under each major peak.

To reexamine the association of PA with the vitamin B12-binding protein marker, those fractions from the original Percoll gradient that contained vitamin B12-binding protein were pooled (total volume, 17.0 ml), diluted with relaxation buffer (final volume, 23 ml), and recentrifuged (48,000 g for 22 min at 4°C). All fractions (0.5 ml each) were reassayed for PA and vitamin B12-binding protein content.

Assays. β -glucuronidase (EC 3.2.1.31) was assayed exactly as described (16) by measuring the liberation of phenolphthalein from 1 mM phenolphthalein monoglucuronic acid; vitamin B12-binding protein was quantitated as described (17) using ⁵⁷Co-cyanocobalamin. In alkaline phosphatase (EC 3.1.3.1) assays, paranitrophenyl phosphate was used as substrate (18). PA activity was measured as previously described (19), using a two step assay in which PA converts plasminogen to plasmin, which in turn degrades ¹²⁵I-fibrin. The assay mixture, which was incubated in wells coated with ¹²⁵I-labeled fibrin, contained an aliquot of the gradient fractions (usually 10 μ l), 300 μ l of 0.1 M Tris-HCl buffer (pH 8.1), and 7 μ g/ml of purified human plasminogen (20). The reaction mixtures were incubated at 37°C, and aliquots were removed at different times for counting. The fibrinolytic activity was measured under identical conditions, but no plasminogen was added to the incubation mixtures. Routinely, all assays were carried out in the presence of 0.01% Triton X-100.

Zymography and Inhibition of PA Activity by Antibody. This procedure, which reveals proteolytic enzyme activity after separation on SDS-PAGE and exposure to an indicator gel, was performed exactly as described (21), except that instead of fibrin, 2% casein was used in the gel overlays. In zymography, the molecular weight and the activity of an enzyme in a given sample can be identified by comparison with a standard sample of known activity. We also examined the type of PA by using antibodies that specifically inhibit the catalytic activity of either tPA or of uPA. 40 μ l aliquots of specific granules and of plasma membranes were mixed with equal volumes of rabbit anti-human uPA or anti-human tPA antibodies (200 μ g/ml); standard preparations of human uPA or conditioned medium from human melanoma-producing tPA, served as controls. The mixtures

were incubated for 40 min at 4°C, and aliquots of the different reaction mixtures were assayed on radioactive fibrin plates for residual PA activity.

Disruption of Granules. To separate the content of specific granules from their membrane components we used the technique described previously (13), in which most of the vitamin B12-binding protein (a soluble component) was liberated into the supernatant upon freezing and thawing. Samples were frozen and thawed three times in succession by transferring them from room temperature to -20°C. The membranes were separated by centrifugation (220,000 g at 4°C). The supernatants and the membranes were assayed for vitamin B12-binding protein content and PA. All the assays for PA were performed in the presence of 0.01% (final concentration) of Triton X-100.

DFP Treatment. Before cavitation, the purified neutrophils were incubated with 3 mM DFP for 15 min on ice, then the cells were pelleted and the DFP-containing supernatants were discarded.

Activation of Neutrophils. After DFP treatment, the neutrophils were resuspended in 20 ml ice-cold, isotonic phosphate buffer (pH 7.4) with 3 mM glucose, and were divided into two equal fractions. 20 ng/ml PMA in DMSO (final concentration 0.007%) was added to one fraction, and the cells were incubated at 37°C for 15 min. Controls, incubated under identical conditions, received only DMSO. The samples were agitated every 5 min, and at the end of incubation they were plunged into ice water and centrifuged (800 rpm) for 10 min at 4°C. Each pellet was resuspended in 10 ml of relaxation buffer and subjected to cavitation and fractionation as described above.

To test the activity of intact neutrophils, aliquots containing 5×10^6 of resting and activated cells (prepared exactly as above, but not treated with DFP before activation) were incubated for 30 min at 37°C in 100 μ l of isotonic phosphate buffer with or without 1 mg/ml of human plasminogen. After incubation, supernatants of cells not exposed to plasminogen were mixed with 1 mg/ml of plasminogen and incubated for 30 min at 37°C. 10- μ l aliquots of each supernatant were assayed for fibrinolytic activity.

Assay of Pro-UK. The assay was carried out essentially as described (14). 100- μ l samples to be tested were mixed with synthetic, chromogenic substrate (S 244), which measures the amidolytic activity of UK. Pro-UK does not have any measurable, amidolytic activity, and only after activation with catalytic amounts of plasmin does it become active (14, 22). We tested the amidolytic activity of samples both in the presence and absence of plasmin, and also in the presence and absence of 10 U/ml of Trasylol. The rest of the experimental procedures were as suggested by the supplier of S 244 substrate, except that all samples were centrifuged at the end of incubation to remove some precipitated material. Standard UK curves of 0.05–5 U were included in all assays.

Results

It has been shown previously (13) that the conditions used for cavitation and fractionation allow the different organelles of the neutrophil to equilibrate at their isopycnic densities, and result in minimal damage to the granule membranes. We have followed this procedure in detail and found that our separation profiles were very similar to those published (13).

Localization of PA and Plasminogen-independent Fibrinolytic Activity in Resting Neutrophils. Fractions obtained from Percoll gradients were analyzed for their content of β -glucuronidase, vitamin B12-binding protein, and alkaline phosphatase. The marker for specific granules (vitamin B12-binding protein) was found in a very sharp, well-defined peak (Fig. 1A), which equilibrated between 1.06 and 1.10 gm/ml of Percoll. As shown previously (13), we found that ~25% of the recovered β -glucuronidase cofractionated with the specific granule fractions (Fig. 1A). As suggested earlier (13), this value is probably an overestimate since the high Percoll concentration in the fractions containing most of the β -glucuronidase activity is inhibitory for the enzyme assay.

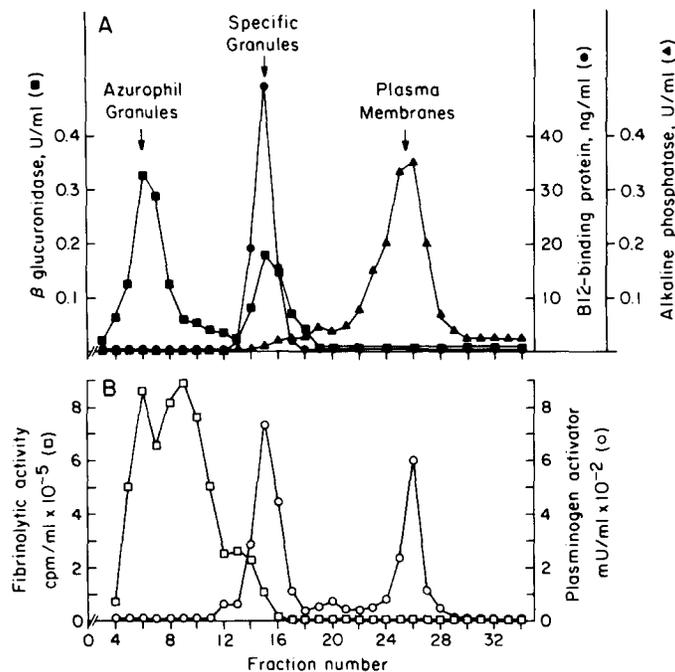


FIGURE 1. Distribution of enzyme markers (A) and PA and fibrinolytic activity (B) in fractions from a discontinuous Percoll gradient. (A) Postnuclear supernatant (see Materials and Methods) was layered on a discontinuous Percoll gradient, and after centrifugation 1-ml fractions were collected by aspiration from the bottom of the gradient. Aliquots of each sample were assayed for β -glucuronidase, vitamin B12-binding protein, and alkaline phosphatase (see Materials and Methods). (B) Undiluted aliquots (10 μ l) were assayed for PA and fibrinolytic activity. The results are the average of two determinations which did not vary by >5%. The results shown are from cells that were incubated for 15 min at 37°C before cavitation. The plasma membrane-associated PA activity was 40% of the total.

PA activity was determined in every fraction across the gradient. This activity was concentrated in two areas that corresponded to the specific granule and the plasma membrane peaks (Fig. 1B). Routinely, only a minor fraction of PA activity (0–21% in five individual experiments) was associated with the plasma membrane; the rest (79–100% of total PA activity) was in the specific granules (Table I). No PA activity was detected in the azurophilic granules (Fig. 1B). From the comparison of results illustrated in Figs. 1, A and B, it was apparent that the distribution of PA and vitamin B12-binding protein was very similar, suggesting that the two proteins reside in the same subcellular compartment. Plasminogen-independent fibrinolytic activity was predominantly associated with the azurophilic granules (Fig. 1B); a small part of the activity cofractionated with a portion of the specific granule peak (Fig. 1B).

Due to the possibility of the presence of other granule compartments in the specific granule fractions (23), we reexamined the association of PA with the specific granules by pooling the fractions containing vitamin B12-binding protein from the original Percoll gradient, and then diluting and recentrifuging the pooled fractions. As shown in Fig. 2, under experimental conditions designed to improve the separation of specific granule fractions, PA and vitamin B12-binding

TABLE I
Distribution of PA in Subcellular Fractions

Exp.	Plasminogen activator (mU UK) in:		
	Specific granules	Plasma membranes (% total)	
1	1,890	279	12.9
2	2,160	0	0
3	549	150	21.4
4	1,500	12	0.8
5	1,800	66	3.5

10- μ l aliquots of pooled fractions of specific granules and plasma membranes isolated from cavitates in five individual experiments were assayed for PA activity. The numbers represent total PA activity in the specific granules or in the plasma membranes; the calculation is based on duplicate assays that differed by <10%.

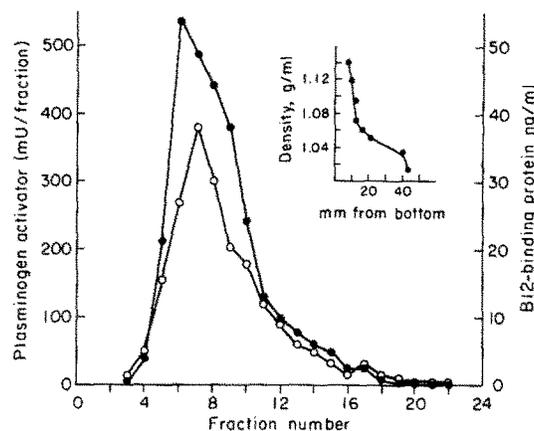


FIGURE 2. Distribution of vitamin B12-binding protein and PA activity in fractions from the attenuated Percoll gradient. Fractions containing vitamin B12-binding protein were pooled, diluted, and recentrifuged (see Materials and Methods). The density profile of the attenuated gradient is shown in the inset. Fractions (0.5 ml), were collected by aspiration from the bottom of the gradient. Aliquots were assayed for vitamin B12-binding protein (○) and PA activity (●) as in Fig. 1.

protein were still superimposable, reconfirming the initial observation that PA is localized in the specific granules.

Characterization of PA. To characterize the type of PA produced by neutrophils, we chromatographed aliquots taken from pooled fractions of each of the three major cytoplasmic organelles on SDS-PAGE, removed the SDS, and exposed the gel to an indicator overlay that contained casein and plasminogen (21). Lysis zones in the overlay corresponded to the areas where PA was present in the gel. No lysis zones formed in lanes that contained aliquots of the azurophilic granules (Fig. 3, lane 2). As explained below, serine proteases, such as elastase and chymotrypsin, were inhibited by the pretreatment of neutrophils with DFP before cavitation. Aliquots of specific granules and plasma membranes produced lysis zones (Fig. 3, lanes 3 and 4). The specific granules had more activity than plasma membranes. This activity comigrated with that of a standard human urinary UK (lane 5) and not with tPA (lane 1), suggesting that the PA present

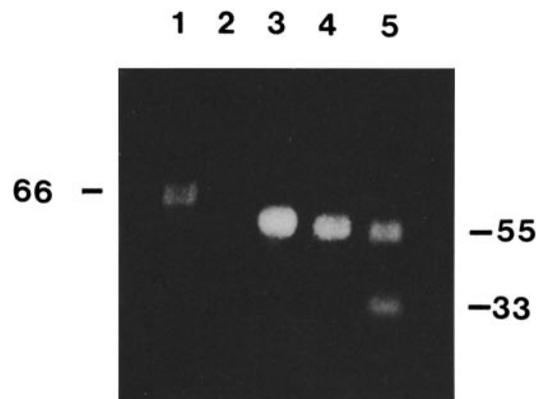


FIGURE 3. Identification of PA-type in the different cellular fractions. 10 ml aliquots of peak fraction of azurophilic granules, specific granules, plasma membranes, and 10 μ l of \sim 1 U/ml of uPA (human urinary UK) and tPA (human melanoma-conditioned medium) were electrophoresed on SDS-PAGE. The gel was washed for 1 h in Triton X-100 and incubated for 11 h on top of an indicator gel that contained casein and plasminogen. Light bands represent lysis zones in the indicator gel. Lane 1, tPA; lane 2, azurophilic granules; lane 3, specific granules; lane 4, plasma membranes; lane 5, uPA. Aliquots of subcellular fractions were derived from neutrophils exposed to DFP, a treatment that greatly reduces the content of serine proteases in the azurophilic granules (lane 2). Molecular mass markers are shown.

in both organelles was of the UK type. We reconfirmed this result by showing that the catalytic activity of the enzyme obtained from specific granules and from plasma membranes of neutrophils can be completely inhibited by antibodies to human UK (Table II).

The Effect of DFP Pretreatment on Plasminogen-independent Fibrinolysis and on the Recovery of PA Activity. Our standard fractionation procedure included a step in which isolated, intact neutrophils were exposed to a brief treatment (15 min at 4°C) with 3 mM DFP. Without DFP pretreatment the fractionation pattern of markers other than PA was not altered. However, none or only a trace of PA activity, associated exclusively with the plasma membrane fractions, could be recovered from these cells (Fig. 4). In the cells not pretreated with DFP the total plasminogen-independent fibrinolysis, associated mainly with the azurophilic, and to a much lesser degree with the specific granule fractions, was increased by about sevenfold (Fig. 4). The difference in fibrinolytic activity between the DFP pretreated and untreated cells showed that, in addition to PA, neutrophils contain a major serine protease activity, and that the different subcellular compartments in which these proteases reside are accessible to DFP.

We resolved the paradox of PA remaining active in cells pretreated with DFP by showing that PA was stored in these cells in a pro-enzyme, single-chain form (Table III). Pro-UK does not have detectable amidolytic activity when assayed with a specific, chromogenic substrate (14, 22, 24). It can be converted to an active form through catalytic activity of plasmin (14). As can be seen in Table III, aliquots of specific granules and plasma membranes were devoid of activity unless incubated with catalytic amounts of plasmin. Trasylol (which inhibits plasmin activity and therefore the conversion of pro-UK to active enzyme) completely inhibited the cleavage of the chromogenic substrate; plasmin alone

TABLE II
The Effect of Anti-uPA and Anti-tPA Antibodies on PA in Specific Granules and Plasma Membranes

Source of PA	IgG	PA (mU/ml)	Inhibition (%)
Specific granules	None	229.0	—
	Anti-uPA	0.0	100.0
	Anti-tPA	228.0	0.4
Plasma membranes	None	248.0	—
	Anti-uPA	0.4	99.8
	Anti-tPA	251.0	0.0
uPA	None	385.0	—
	Anti-uPA	7.4	98.1
	Anti-tPA	367.0	4.7
tPA	None	301.0	—
	Anti-uPA	298.0	1.0
	Anti-tPA	2.8	99.1

40- μ l aliquots of neutrophil-specific granules from resting cells, plasma membranes from activated cells, human uPA and human tPA were mixed with equal volumes of anti-uPA or anti-tPA rabbit IgG (200 μ g/ml) diluted in phosphate buffer with 1 mg/ml BSA. The mixtures were incubated for 40 min at 4°C. Duplicate aliquots (10 μ l) were assayed for PA activity in radioactive fibrin plates. The variability between duplicates was always <5%.

did not cleave the substrate. Results similar to those presented in Table III were obtained with aliquots of plasma membranes from resting neutrophils (results not shown). The specific granule PA of activated neutrophils was below the level of detection in this assay.

Localization of PA Within Specific Granules. Freezing and thawing granules, conditions which release the majority of the vitamin B12-binding protein from the granule matrix, failed to release PA into the supernatant fraction. In three out of three experiments, 100, 78, and 73% of PA activity remained associated with the granule membranes.

Distribution of PA in Activated Neutrophils. It has been previously established (25) that activation of neutrophils with 8×10^{-8} M PMA can induce selective exocytosis of specific granules. Since PA was found to be associated with this compartment, we were interested in examining whether activation could alter the pattern of its distribution.

Isolated neutrophils were pretreated with DFP, one aliquot was treated with PMA, the other served as control. Both were incubated at 37°C for 15 min and subsequently subjected to standard fractionation procedures. We found that PMA treatment caused a dramatic reduction (from >70 down to <14%) in PA activity of specific granules. This reduction was similar to that found in vitamin B12-binding protein when granules from resting neutrophils were compared with activated cell granules, (Fig. 5).

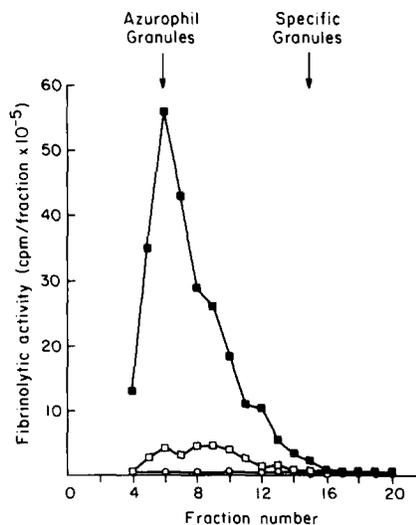


FIGURE 4. PA and fibrinolytic activity in azurophilic granules obtained from neutrophils not pretreated with DFP. Neutrophils were isolated exactly as described in Materials and Methods except that they were not pretreated with DFP before cavitation. The fractionation on discontinuous Percoll gradient and assays were done exactly as in Fig. 1, A and B. (○) PA, (■) fibrinolytic activity. For comparison, fibrinolytic activity present in fractions from DFP-pretreated cells (shown in Fig. 1B) is also represented (□).

TABLE III
Amidolytic Activity of PA from Specific Granules and Plasma Membranes

Neutrophil fraction	Reaction mixtures*			
	Sample alone	Sample with plasmin	Sample with plasmin and Trasylol	Plasmin alone
Specific granules (Resting)	<0.05	2.6	<0.05	<0.05
Plasma membranes (Activated)	<0.05	2.2	<0.05	<0.05

100- μ l aliquots of peak fractions (specific granules or plasma membranes) were mixed with Spectrozyme S-244, some samples were mixed with 1 μ g of plasmin in the presence or in the absence of 10 U of Trasylol, the volume was brought up to 1 ml with phosphate buffer (pH 7.4), and the samples were incubated for 2 h at 37°C. Optical density was measured at 405 nm. Unit values were calculated from a standard, UK curve of 0.05–50 U.

* Units per fraction amidolytic activity.

In this experiment, plasma membranes of control neutrophils contained 27% of the total PA. This value (and the value shown in Fig. 1B) are somewhat greater than the values in Table I. In these experiments the resting neutrophils were incubated for 15 min at 37°C, conditions which may have caused partial activation. While most of the vitamin B12-binding protein was found in the supernatant of the activated cells (not shown and reference 13), >85% of the

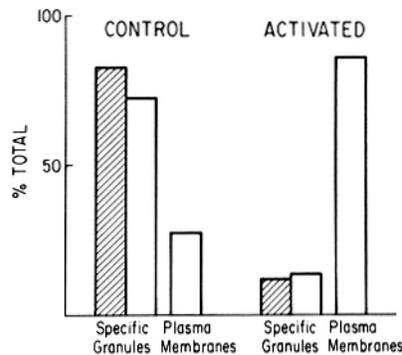


FIGURE 5. Translocation of PA from specific granules to plasma membranes. Isolated neutrophils were divided into two parts; one was exposed to 20 ng/ml of PMA for 15 min at 37°C, the other served as a control. After cavitation and separation on a Percoll gradient, each fraction was assayed for specific markers and PA activity. The results show activities in pooled peak fractions of specific granules and plasma membranes, and are expressed as the percent of total activity. For comparison, vitamin B12-binding protein content in control and activated neutrophils was assayed in parallel. (▨) B12-binding protein, (□), PA.

total PA activity was found in the plasma membrane fractions. This would be expected of a protein which appears to be associated with the specific granule membrane, and indicates that during activation PA translocates from the specific granule to the plasma membrane. PA from activated neutrophils (both in specific granules and in plasma membranes) was of the uPA type, as judged by zymography (not shown) and by inhibition by specific antibody (Table II).

Cell Surface-associated PA in Resting and Activated Neutrophils. To assess the cell surface-associated PA activity of intact cells, resting and activated neutrophils were incubated for 30 min at 37°C in the presence or absence of 1 mg/ml of plasminogen. The activation of neutrophils was carried out exactly as described, except that in this experiment, the cells were not pretreated with DFP. Aliquots of supernatants containing plasminogen were assayed directly for fibrinolytic activity. Supernatants of cells incubated in absence of plasminogen were first mixed with 1 mg/ml of plasminogen, incubated for 30 min at 37°C, and only then assayed for fibrinolytic activity. No detectable activity was found in the samples obtained from cells incubated in the absence of plasminogen, regardless of the state of activation of the cells. This indicates that no PA was released into the supernatants during the 30 min incubation period. In contrast, the supernatants from cells incubated in the presence of plasminogen had measurable levels of fibrinolytic activity. More importantly, the activated neutrophils were 12-fold more efficient than the resting cells in converting plasminogen to plasmin (Table IV), indicating the presence of an active PA on the surface of these cells.

The level of cell surface-associated PA of resting and activated neutrophils was also compared using a monoclonal anti-uPA IgG and ¹²⁵I-labeled F(ab)₂ fragments of anti-mouse IgG (P. Detmers, unpublished results). It was determined that activated neutrophils bind approximately five to seven times more anti-uPA IgG, confirming, by an independent approach, the presence of an increased level of PA on the surface of activated neutrophils.

TABLE IV
Generation of Plasmin by Resting and PMA-activated Neutrophils

Neutrophils	Incubation of cells	Fibrinolytic activity*
Resting	Without plasminogen [‡]	0.0
	With plasminogen	6.0
Activated	Without plasminogen [‡]	0.0
	With plasminogen	70.0

Aliquots ($5 \times 10^6/100 \mu\text{l}$ of phosphate buffer) of resting and activated neutrophils were incubated for 30 min at 37°C with or without 1 mg/ml of human plasminogen.

* cpm/ml $\times 10^{-4}$

[‡] Before assaying for fibrinolytic activity, a fraction of each supernatant taken from cells incubated in buffer alone was mixed with and incubated with plasminogen, using conditions identical to those described above. The lack of activity in these samples indicates that no PA was released into the supernatant of either resting or activated cells during the 30 min of incubation.

Discussion

By using the previously described procedure of neutrophil fractionation (13), we have identified the fractions that contain the azurophilic granules, the specific granules, and the plasma membranes and have assayed each fraction for PA activity; most of the activity was found in the specific granules; only a small amount was present in the plasma membrane (Table I). PA activity was detected only in neutrophils that were pretreated with DFP before fractionation (Fig. 1 and Table I). Since PA is a serine protease, and therefore should be inhibited by DFP, this finding may seem paradoxical. However, our results (Table III) showed that PA was present in these cells in the form of an amidolytically inactive proenzyme that could not bind DFP (14, 22, 24) and consequently was protected from inactivation by this compound.

Neutrophils are known to contain a number of different proteases, some of them serine proteases (26–29). The plasminogen-independent activity of DFP-treated cells was sevenfold less than that of untreated control cells (Fig. 4). In individual Percoll gradient fractions, the level of fibrinolytic activity was reduced by 3–11-fold. These results indicate that a large proportion of the fibrinolytic activity is contributed by serine proteases such as elastase and chymotrypsin, which are sensitive to DFP. It is likely that, when not inhibited, these activities are responsible for the degradation of neutrophil PA during fractionation.

As stated in Results, without DFP pretreatment a small proportion of PA (at most 1% of that present in DFP-treated cells) was sometimes recovered; this activity was always associated with the plasma membranes. In a number of published studies of cells other than neutrophils, PA was found in the plasma membrane or plasma membrane-like structures (8, 11, 12), but in view of our findings, which clearly show that other cell proteases may have a crucial effect on the quantity and the distribution of PA, these results should be reexamined.

Two points concerning the quantitation of PA in neutrophils should be

discussed. We found that the total PA activity of the cavitate (before it was applied to a Percoll gradient) was only 40–60% of the sum of activities recovered from the individual fractions (not shown). This may be due to the presence of inhibitors of PA which upon fractionation were distributed into separate (most likely soluble) compartments. Such inhibitors have been shown to be present in a number of cells, including macrophages (30). The presence of inhibitors makes the calculation of enzyme recovery in different steps of fractionation somewhat uncertain. Secondly, were active PA (in addition to pro-PA) present in the cells, it would have been inactivated by the DFP pre-treatment, and we would have no way of assessing what proportion of the total enzyme it might represent. This does not seem to be the case, since in a number of recent reports using different cells, it has been shown that PA is produced as an amidolytically inactive pro-enzyme (14, 31–34), suggesting that this may be a general pathway of PA synthesis; neutrophils should not be different with respect to this property. In addition, as shown in Table IV, the level of surface-associated PA activity of resting neutrophils is very low, even in cells that have not been pretreated with DFP. This finding indicates that the subcellular distribution of PA found in DFP-pretreated cells reflects the genuine localization of this enzyme and does not result from a selective inhibition by DFP of the surface, plasma membrane-bound PA.

As shown in Results, before activation PA appears to be associated with the membrane of the specific granules. Although the precise nature of this association has not been identified, we found that treatment of the specific granules with Triton X-100 enhances PA activity by three- to fivefold (data not shown). This suggests that PA is localized in the inner face of the specific granule membrane, and that during fusion of the specific granules with plasma membrane, which is a result of activation, it may become expressed on the extracellular face of the plasma membrane. Our results support this possibility. We found that upon activation, PA activity of the specific granules is reduced from 70 to 14% of total (Fig. 5). This reduction is very similar to that of vitamin B12-binding protein (Fig. 5), a component of the specific granule matrix. However, in contrast to vitamin B12-binding protein which can be quantitatively recovered from the supernatant of the activated neutrophils, most of the PA (~85% of total) in activated neutrophils is found associated with the plasma membrane. This distribution is very similar to that described for cytochrome-*b*, another component associated with the membrane of neutrophil-specific granules (13).

β -glucuronidase, a marker for azurophilic granules, was undetectable in supernatants from activated cells and was not noticeably reduced in the region of the Percoll gradient containing azurophilic granules.

Low concentrations of PMA cause a selective exocytosis of the specific granules (25) and result in PA accumulation in the neutrophil supernatants several hours after treatment (1). We have shown that the appearance of PA on the surface of the cells after activation is an early event that temporally precedes the release of the enzyme into the supernatant. This was documented in fractionated cells that were preexposed to DFP, a treatment that does not affect subsequent activation (35), as well as in intact, untreated neutrophils (Table IV). These results support

the theory suggesting that membrane-associated PA may have an important role in processes requiring locally high levels of proteolytic activity (4, 6, 7, 36).

The question of how the pro-uPA is initially converted to an active enzyme is still under debate. Recent findings have shown that pro-uPA, which is inactive in the amidolytic assay and unable to bind DFP, can activate plasminogen (22, 24, 37). Moreover, there are some indications (38) that membrane-bound PA may be less sensitive to macromolecular inhibitors of proteases. Should these findings be confirmed, they would mean that translocation of PA from the specific granule to the plasma membrane would be all that is required to endow the neutrophil surface with an important proteolytic activity.

Summary

The subcellular localization of plasminogen activator (PA) in human neutrophils was studied. The cells were disrupted by nitrogen cavitation and fractionated on Percoll density gradients into three major components containing the plasma membranes, the specific granules, and the azurophilic granules. The biochemical markers we used to identify these organelles were alkaline phosphatase, vitamin B12-binding protein, and β -glucuronidase, respectively. Using the radioactive fibrin plate method, PA activity and plasminogen-independent fibrinolytic activity were measured.

In resting neutrophils, PA was associated mainly with the membranes of the specific granules. In five individual experiments the activity of this fraction varied from 79 to 100% of the total; the remaining activity was found to be associated with the plasma membrane, and no activity was present in the azurophilic granules. In neutrophils that were activated by exposure to PMA (20 ng/ml for 15 min at 37°C), the total recoverable PA activity remained unchanged; however, the main peak of activity (85% of total) shifted from the specific granules to the plasma membranes. The magnitude of the reduction of the enzyme in the specific granules paralleled that of vitamin B12-binding protein. PMA-activated, intact neutrophils had ~12-fold more surface-bound PA activity than resting cells.

Recovery of PA activity from neutrophils was critically dependent on pretreatment of the intact cells with DFP before cavitation; 100-fold more PA activity was detected in DFP-pretreated cells. At the same time, this pretreatment reduced the plasminogen-independent fibrinolytic activity by approximately sevenfold. We determined that PA present in the neutrophils is of the urokinase (UK) type and that the enzyme is produced and stored as a pro-UK, a form insensitive to DFP inhibition. The reduction in the level of proteases (measured as fibrinolytic activity) and the resistance of pro-UK to DFP are most likely the two major reasons for the greatly improved recovery of PA from the DFP-pretreated cells.

These findings show that in resting neutrophils PA is stored in the specific granules, and that during activation, it translocates to the outer surface of the plasma membranes, thus equipping the cell with an ecto-proteolytic potential.

We would like to thank S. Nicholson, H. Russo, and G. Bertuzzi for excellent technical assistance; Dr. J. Gabay for assistance and helpful discussions; Dr. S. C. Silverstein for

support and encouragement; and Dr. Z. A. Cohn for helpful discussions and critical reading of the manuscript.

Received for publication 12 May 1986.

References

1. Granelli-Piperno, A., J.-D. Vassalli, and E. Reich. 1977. Secretion of plasminogen activator by human polymorphonuclear leukocytes. Modulation by glucocorticoids and other effectors. *J. Exp. Med.* 146:1693.
2. Unkeless, J., S. Gordon, and E. Reich. 1975. Secretion of plasminogen activator by stimulated macrophages. *J. Exp. Med.* 139:834.
3. Vassalli, J.-D., J. Hamilton, and E. Reich. 1977. Macrophage plasminogen activator: induction by Concanavalin A and phorbol myristate acetate. *Cell.* 11:695.
4. Chapman, H. A., Z. Vavrin, and J. B. Hibbs. 1982. Macrophage fibrinolytic activity: identification of 2 pathways of plasmin formation by intact cells and of a plasminogen activator inhibitor. *Cell.* 28:653.
5. Vassalli, J.-D., J. Hamilton, and E. Reich. 1976. Macrophage plasminogen activator: modulation of enzyme production by anti-inflammatory steroids, mitotic inhibitors and cyclic nucleotides. *Cell.* 8:271.
6. Vassalli, J.-D., D. Baccino, and D. Belin. 1985. A cellular binding site for the M_r 55,000 form of the human plasminogen activator, urokinase. *J. Cell Biol.* 100:86.
7. Stopelli, M. P., A. Corti, A. Soffientini, G. Cassani, F. Blasi, and R. K. Assoian. 1985. Differentiation-enhanced binding of the amino-terminal fragments of human urokinase plasminogen activator to a specific receptor on U937 monocytes. *Proc. Natl. Acad. Sci. USA.* 82:4939.
8. Fulton, R. J., and D. A. Hart. 1981. Characterization of plasma membrane-associated plasminogen activator on thymocytes. *Biochim. Biophys. Acta.* 642:345.
9. Lemaire, G., J.-C. Drapier, and J.-F. Petit. 1983. Importance, localization and functional properties of the cell associated form of plasminogen activator in mouse peritoneal macrophages. *Biochim. Biophys. Acta.* 755:332.
10. Dvorak, H. F., N. S. Orenstein, A. M. Dvorak, M. E. Hammon, R. O. Roblin, J. Feder, C. F. Schott, J. Goodwin, and E. Morgan. 1977. Isolation of the cytoplasmic granules of guinea pig basophilic leukocytes: identification of esterase and protease activities. *J. Immunol.* 119:38.
11. Quigley, J. P. 1976. Association of a protease (plasminogen activator) with a specific membrane fraction isolated from transformed cells. *J. Cell Biol.* 71:472.
12. Laug, W. E., B. Dewald, J. Schnyder, and M. Baggiolini. 1983. Subcellular distribution of plasminogen activator in cultured human fibrosarcoma cells. *Cancer Res.* 43:22.
13. Borregaard, N., J. M. Heiple, E. R. Simons, and R. A. Clark. 1983. Subcellular localization of the *b*-cytochrome component of the human neutrophil microbicidal oxidase: translocation during activation. *J. Cell Biol.* 97:52.
14. Wun, T.-C., L. Ossowski, and E. Reich. 1982. A proenzyme form of human urokinase. *J. Biol. Chem.* 257:7262.
15. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* 193:265.
16. Brittinger, G., R. Hirschorn, S. D. Douglas, and G. Weissman. 1968. Studies on lysosomes. XI. Characterization of a hydrolase-rich fraction from human lymphocytes. *J. Cell Biol.* 37:394.
17. Gottlieb, C., K.-S. Lau, L. R. Wasserman, and V. Herbert. 1965. Rapid charcoal

- assay for intrinsic factor (IF), gastric juice unsaturated B12 binding capacity, antibody to IF, and serum unsaturated B12 binding capacity. *Blood*. 25:875.
18. DeChatelet, L. R., and M. R. Cooper. 1970. A modified procedure for the determination of leukocyte alkaline phosphatase. *Biochem. Med.* 4:61.
 19. Strickland, S., and W. H. Beers. 1976. Studies on the role of plasminogen activator in ovulation. In vitro response of granulosa cells to gonadotropins, cyclic nucleotides, and prostaglandins. *J. Biol. Chem.* 251:5694.
 20. Deutsch, D. G., and E. T. Mertz. 1970. Plasminogen purification from human plasma by affinity chromatography. *Science (Wash. DC)*. 170:1095.
 21. Granelli-Piperno, A., and E. Reich. 1978. A study of proteases and protease-inhibitor complexes in biological fluids. *J. Exp. Med.* 148:223.
 22. Gurevich, V., R. Pannell, S. Louie, P. Kelley, R. L. Suddith, and R. Greenlee. 1984. Effective and fibrin-specific clot lysis by a zymogen precursor form (pro-urokinase). A study in vitro and in two animal species. *J. Clin. Invest.* 73:1731.
 23. Dewald, B., V. Bretz, and M. Baggiolini. 1982. Release of gelatinase from a novel secretory compartment of human neutrophils. *J. Clin. Invest.* 70:518.
 24. Lijnen, H. R., C. Zamarron, M. Blaber, M. E. Winkler, and D. Collen. 1986. Activation of plasminogen by pro-urokinase. II. Kinetics. *J. Biol. Chem.* 261:1266.
 25. Estensen, R. D., J. G. White, and B. Holmes. 1974. Specific degranulation of human polymorphonuclear leukocytes. *Nature (Lond.)*. 248:347.
 26. Ohlsson, K., and I. Ohlsson. 1976. The neutral protease of human granulocytes: isolation and partial characterization of granulocyte elastase. *Eur. J. Biochem.* 42:519.
 27. Rindler-Ludwig, R., and H. Braunsteiner. 1975. Cationic proteins from human neutrophil granulocytes: evidence for their chymotrypsin-like properties. *Biochim. Biophys. Acta.* 379:606.
 28. Ohlsson, K., and I. Ohlsson. 1973. The neutral proteases of human granulocytes: isolation and partial characterization of two granulocyte collagenases. *Eur. J. Biochem.* 36:473.
 29. Pontremoli, S., E. Melloni, M. Michetti, O. Sacco, B. Sparatore, F. Salamino, G. Damiani, and B. L. Horecker. 1986. Cytolytic effects of neutrophils: role for a membrane-bound neutral proteinase. *Proc. Natl. Acad. Sci. USA.* 83:1685.
 30. Vassalli, J.-D., J.-M. Dayer, A. Wohlwend, and D. Belin. 1984. Concomitant secretion of prourokinase and of plasminogen activator-specific inhibitor by cultured human monocytes-macrophages. *J. Exp. Med.* 159:1653.
 31. Nielsen, L. S., J. G. Hansen, L. Skriver, E. L. Wilson, K. Kaltoft, J. Zeuthen, and K. Dano. 1982. Purification of zymogen to plasminogen activator from human glioblastoma cells by affinity chromatography with monoclonal antibody. *Biochemistry.* 21:6410.
 32. Andreasen, P. A., L. S. Nielsen, J. Grondahl-Hansen, L. Skriver, J. Zeuthen, R. W. Stephens, and K. Dano. 1984. Inactive proenzyme to tissue-type plasminogen activator from human melanoma cells identified after purification with monoclonal antibody. *EMBO (Eur. Mol. Biol. (Organ.)) J.* 3:51.
 33. Eaton, D. L., R. W. Scott, and J. B. Baker. 1984. Purification of human fibroblasts urokinase proenzyme and analysis of its regulation by proteases and protease nexin. *J. Biol. Chem.* 259:6241.
 34. Stump, D. C., H. R. Lijnen, and D. Collen. 1986. Purification and characterization of single-chain urokinase type plasminogen activator from human cell cultures. *J. Biol. Chem.* 261:1274.
 35. Mark, D. 1984. Immune complex interaction with neutrophils. PhD Thesis. Boston University, Boston, MA.
 36. Chapman, H. A., O. L. Stone, and Z. Vavrin. 1984. Degradation of fibrin by intact

840 TRANSLOCATION OF HUMAN NEUTROPHIL PLASMINOGEN ACTIVATOR

human alveolar macrophages in vitro. Characterization of a plasminogen activator and its role in matrix degradation. *J. Clin. Invest.* 73:806.

37. Lijnen, M. R., C. Zamarron, M. Blaber, M. E. Winkler, and D. Collen. 1986. Activation of plasminogen by pro-urokinase. I. Mechanism. *J. Biol. Chem.* 261:1253.
38. Baker, J. B., B. L. Bergman, A. Bajpai, and R. Gronke. 1986. Properties and action of protease nexin 1. *J. Cell. Biochem.* 10 (Suppl.):235. (Abstr.)