

# Cultured Bovine Endothelial Cells Produce Both Urokinase and Tissue-type Plasminogen Activators

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**ABSTRACT** Cell extracts and conditioned media (CM) from cultured bovine aortic endothelial cells (BAEs) were fractionated by PAGE in the presence SDS, and plasminogen activator (PA) activity was localized by fibrin autography. Multiple molecular weight forms of PA were detected in both preparations. Cell-associated PAs had  $M_r$  of 48,000, 74,000, and 100,000 while secreted PAs showed  $M_r$  of 52,000, 74,000, and 100,000. A broad zone of activity ( $M_r$  80,000–100,000) also was present in both cellular fractions. In addition, PAs of  $M_r$  41,000 and 30,000 appeared upon prolonged incubation or repeated freezing and thawing of the samples, and probably represent degradation products of higher molecular weight forms. This complex lysis pattern was not observed when CM was subjected to isoelectric focusing. Instead, only two classes of activator were resolved, one at pH 8.5, the other at 7.6. Analysis of focused samples by SDS PAGE revealed that the activity at pH 8.5 resulted exclusively from the  $M_r$  52,000 form; all other forms were recovered at pH 7.6. The activity of the  $M_r$  52,000 form was neutralized by anti-urokinase IgG but was not affected by antitissue activator IgG indicating that it is a urokinaselike PA. The activities of the  $M_r$  74,000–100,000 forms were not affected by anti-urokinase. They were blocked by antitissue activator suggesting that all the forms in this group were tissue-type PAs. The multiple forms of PA were differentially sensitive to inactivation by diisopropylfluorophosphate (DFP). Treatment of CM with 10 mM DFP for 2 h at 37°C only partially inhibited the 52,000-dalton form. However, it completely inactivated the 74,000-dalton PA. The activity of the  $M_r$  100,000 form was not affected by this treatment, or by treatment with 40 mM DFP. Thus, cultured BAEs produce multiple, immunologically distinct forms of PA which differ in size, charge, and sensitivity to DFP. These forms include both urokinaselike and tissue-activator-like PAs. The possibility that one of these forms is a zymogen is discussed.

Plasminogen activator (PAs) convert the zymogen plasminogen into its proteolytically active form, plasmin (1). PAs are extremely specific serine proteinases found in a variety of cells, tissues, and body fluids (2, 3). They have been classified into two immunologically distinct groups, those that are neutralized by anti-urokinase antibodies (urokinaselike) and those that are neutralized by antitissue activator antibodies (tissue activator-like) (4). No cross reactivity has been reported between the two activator groups. Although these molecules activate plasminogen by cleavage of the same arginine-valine bond (5), the mechanism of activation differs considerably. Tissue activator, unlike urokinase, requires a cofactor before its PA activity can be expressed (6). Interestingly, this cofactor appears to be fibrin itself (7).

The plasma fibrinolytic system is responsible for the dissolution of blood clots, and thus promotes continued circulation

of blood through an unobstructed vascular bed. Unfortunately, the origin and characteristics of the PA in blood responsible for initiating and controlling this process remain unclear (5). The fibrin-dependency of tissue activators and their fibrin-binding properties guarantee their accumulation and activation on the surface of fibrin clots. These characteristics, together with the recent findings that these molecules are both efficient and specific thrombolytic agents (8) suggest that tissue activator, not urokinase, is the physiologically relevant vascular activator. We have begun to study the PAs produced by cultured endothelial cells since available information suggests that the vascular activator may originate from the endothelium itself (9, 10). Here we show that bovine aortic endothelial cells (BAEs) produce multiple molecular weight forms of PA; these forms include both urokinaselike and tissue-activator-like molecules.

## MATERIALS AND METHODS

### Cell Culture

BAEs were isolated from the aortae of freshly killed cows by the method of Booyse et al. (11) and cultured as indicated (12). The cells used for most of these studies had been passaged 5 to 20 times and were positive for Factor VIII antigen. Cloned BAEs were used in some experiments. One clone was isolated in our laboratory from a single cell that grew out of a primary cell preparation from bovine aorta. Four clones were isolated and kindly provided by Drs. C. Gajdusek and S. Schwartz (13), and the 6th clone was the generous gift of Dr. W. Laug (14). All cultures were grown to confluency in 60-mm dishes before use (15).

### Preparation and Treatment of Cell Extracts and Conditioned Medium (CM)

To prepare cellular extracts, cultures were washed twice with cold phosphate buffered saline (PBS) (0.14 M NaCl, 0.01 M sodium phosphate, pH 7.2), extracted with 250  $\mu$ l of Triton X-100 (0.5% vol/vol in PBS; Sigma Chemical Co., St. Louis, MO) and removed with a rubber policeman. The culture dish was washed once with 750  $\mu$ l PBS, and the extract and wash were pooled. All samples were used immediately after preparation. Conditioned media was prepared by incubating washed monolayers in serum-free MEM for 24 h. This CM was collected, centrifuged at 1,000 g to remove cellular debris and stored in 0.5% Triton X-100 at  $-30^{\circ}\text{C}$  until used.

Inactivation studies with diisopropylfluorophosphate (DFP; Sigma Chemical Co.), were performed with Triton X-100-released cellular PA and with CM (in 0.2 M sodium phosphate, pH 7.2). Samples were treated with 1 mM or 10 mM DFP at  $37^{\circ}\text{C}$ . At 30 min, 1 h, and 2 h, 100- $\mu$ l aliquots were removed and quickly frozen in an ethanol dry ice bath. Samples were fractionated by SDS PAGE and analyzed for PA activity on fibrin-agar gels (fibrin autography). To prepare Triton X-100-released PA, cultures were washed twice with cold PBS, scraped into 1 ml of homogenization buffer (0.1 M Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM EDTA) and homogenized until 90–95% of the cells were disrupted. The nuclei were removed by centrifugation at 600 g for 10 min and then the membrane fraction was isolated by centrifugation for 90 min at 100,000 g. This microsomal pellet was resuspended in 0.5 ml of 0.1 M sodium phosphate, pH 7.2, an equal amount of Triton X-100 (wt/wt) was added, and the residual particulate matter was removed by centrifugation at 100,000 g for 90 min. This treatment resulted in the release of 85–95% of the PA activity from the membrane fraction.

### PAGE

SDS polyacrylamide slab gels and buffers were prepared as described by Laemmli (16) with resolving gels of 9% acrylamide and stacking gels of 4% acrylamide. Samples were applied to the gels and subjected to electrophoresis at 40 V at room temperature. Molecular weight standards included human transferrin (76,000), human serum albumin (65,000), ovalbumin (43,000), chymotrypsinogen (25,700), and soybean trypsin inhibitor (16,700). Portions of the gel containing these standards were removed and stained for 30 min with 1% Coomassie Blue and 50% trichloroacetic acid, and then destained in 10% acetic acid. The remaining portion of the gel was processed for fibrin autography to localize PA activity.

### Fibrin Autography

To prepare fibrin agar indicator gels (17), a 2% solution of agarose (LPG agarose, Miles Laboratories, Elkhart, IN) in water was boiled, cooled to  $47^{\circ}\text{C}$  and mixed with prewarmed PBS containing plasminogen (19.5  $\mu\text{g}/\text{ml}$ ) and thrombin (0.6 U/ml). Fibrinogen (10 mg/ml) in PBS ( $37^{\circ}\text{C}$ ) was added, the solution was mixed rapidly and poured on a glass plate. Final concentrations were 1% agarose, 5.8  $\mu\text{g}/\text{ml}$  plasminogen, 0.18 U/ml thrombin, and 2 mg/ml fibrinogen. The fibrin-agar gel was used immediately. After electrophoresis, the SDS gels were soaked in 2.5% Triton X-100 for 1.5 h to remove the SDS, patted dry with a paper towel, and applied to the surface of the fibrin-agar indicator gel. The indicator gel was allowed to develop at  $37^{\circ}\text{C}$  in a moist chamber, and then photographed. The dark areas of the indicator gel correspond to the lytic zones initiated by the interaction of PA in the SDS gel and plasminogen in the indicator gel. No lytic zones were detected in the indicator gel in the absence of plasminogen.

Activity neutralization studies were performed by incorporating rabbit anti-urokinase IgG (20  $\mu\text{g}/\text{ml}$ ) or rabbit antitissue activator IgG (10  $\mu\text{g}/\text{ml}$ ) into the fibrin-agar indicator gel mixture. A 9% SDS gel containing various PAs was placed on the antibody containing indicator gel and incubated at  $37^{\circ}\text{C}$ . Control fibrin agar plates containing normal rabbit IgG at the same concentrations were also tested.

### Isoelectric Focusing and Two Dimensional Gel Electrophoresis

Isoelectric focusing of CM was performed in 5% polyacrylamide slab gels containing 6M urea (ultra pure, Schwartz-Mann, Orangeburg, NY), 0.5% Triton X-100, 10% glycerol, and 5% ampholytes (pH 3.5–10, 8–9.5, 9–11 in a ratio of 5:2:1; LKB Instruments, Inc., Rockville, MD). Conditioned media was dialyzed overnight in 0.5% Triton X-100, 6M urea to remove ions before application. The gels were electrofocused on an LKB multiphor system at 15 W for 3 h using electrode solutions of 1 M NaOH and 1 M  $\text{H}_3\text{PO}_4$ . After electrofocusing was completed, one lane was removed from the slab gel, sliced into 0.5-cm slices, and each slice was then soaked in 0.5 ml of 0.5% Triton X-100 for 30 min and the pH determined. The slices were allowed to continue soaking overnight and an aliquot assayed for PA activity on  $^{125}\text{I}$ -fibrin plates. Focused samples were also analyzed by SDS PAGE (2-dimensional gel electrophoresis). A duplicate lane was removed from the focusing gel and soaked for 60 min. in 0.05 M Tris-HCl pH 8, containing 10% glycerol, 0.025% bromphenol blue, and 1% SDS. It was then placed on top of an SDS gel and subjected to fractionation by SDS PAGE as described above. PA activity was localized by fibrin autography.

### Assay of Fibrinolytic Activity

PA activity was assayed on  $^{125}\text{I}$ -fibrin coated multiwell tissue culture dishes (24 wells, 16 mm; Costar Data Packaging, Cambridge, MA) as described (15). The standard cell-free assay contained in 1 ml: 4  $\mu\text{g}$  human plasminogen, 0.1% gelatin, 0.1 M Tris-HCl, pH 8.1, and a source of PA. Fibrinolysis was not observed when plasminogen or PA was omitted from the reaction mixture, indicating that hydrolysis of the  $^{125}\text{I}$ -fibrin resulted from conversion of plasminogen into plasmin by PA.

### Immunochemistry

Rabbit antiserum raised against human urokinase was a gift from Collaborative Research, Waltham, MA. To prepare IgG fractions, antiserum or nonimmune serum was combined with an equal volume of 0.15 M sodium chloride, and ammonium sulfate was added slowly to 45% saturation. This solution was stirred for 15 min and centrifuged at 20,000 g for 20 min. The pellet was resuspended in 0.15 M sodium chloride and the ammonium sulfate fractionation repeated two more times. The final precipitate was redissolved and dialyzed extensively against 0.07 M potassium phosphate, pH 7.2, and then chromatographed on a DEAE-cellulose column equilibrated in the same buffer. The unbound protein was collected, dialyzed against PBS and concentrated to 2 mg/ml of IgG. The rabbit antihuman tissue activator IgG (2 mg/ml) was isolated by adsorption onto Protein A-Sepharose columns as described (4) and was a generous gift from Dr. D. Collen, University of Leuven, Leuven, Belgium.

### Miscellaneous

Protein was determined by the method of Bradford (18) using bovine serum albumin as a standard. Radioactivity measurements of  $^{125}\text{I}$  were performed in a Micromedic gamma spectrometer (Micromedic Systems, Horsham, PA). Plasminogen was prepared by affinity chromatography on lysine-Sepharose as described (19). Bovine fibrinogen fraction 1 (CalBiochem-Behring Corp., San Diego, CA) was purified free of plasminogen by ethanol precipitation in the presence of lysine (20). Human  $\alpha$ -thrombin was a generous gift from Dr. Fenton (Albany, NY). Purified human urokinase (W.H.O. Standard) was supplied by Dr. A. Johnson, New York University. Human tissue plasminogen activator was purified from cultured melanoma cells and was a gift from Dr. D. Collen (21).

## RESULTS

### Fractionation of Cellular PAs by SDS PAGE

Cell extract and CM were fractionated by SDS PAGE and PA activity was localized by the development of lytic zones on fibrin indicator plates (Fig. 1). After 4 h of incubation, lysis zones corresponding to  $M_r$  of 48,000 in cell extracts (lane 1) and 52,000 and 74,000 in CM (lane 2) were apparent. The fractionation and analysis of urinary PA (urokinase) of  $M_r$  55,000 and 33,000 is shown in lane 3 for comparative purposes. Upon prolonged incubation of the cellular samples (8 h), an additional zone at  $M_r$  74,000 appeared in the cell extract sample (lane 4), while one at  $M_r$  100,000 appeared in the lane containing CM (lane 5). Cell extract also contained material at  $M_r$

100,000 but its detection required a longer incubation time (not shown). Characteristically, lysis was also observed in the area between the 74,000- and 100,000-dalton PAs but it appeared as an unresolved smear rather than as distinct bands (lanes 4 and 5). In a few preparations, additional forms of PA with molecular weights of 51,000 (extract) and 55,000 (CM) were also detected, although conditions under which they were generated have not been established. No activity was observed in the absence of plasminogen. These complex lysis patterns were not altered when samples were prepared in the presence of soybean trypsin inhibitor (50  $\mu\text{g}/\text{ml}$ ) and analyzed in the presence of 8 M urea (not shown). However, prolonged incubation of cell extract or CM at 37°C, or repeated freezing and thawing of the samples generated PAs of  $M_r$  41,000 and 30,000. These forms are probably degradation products of higher molecular weight forms. The molecular weights of the various cell associated and secreted PAs are summarized in Table I. Similar results were obtained when CM and extract from cloned BAEs was analyzed by these techniques (unpublished observation).

### Fractionation of Cellular PAs by Isoelectric Focusing

The isoelectric points (pI) of the various forms of PA in CM were determined (Fig. 2). Dialyzed CM was electrofocused as indicated. The gel was sliced into 5-mm sections and each slice was then tested for fibrinolytic activity on  $^{125}\text{I}$ -fibrin coated dishes. Two areas of activity were observed. The most active material accumulated between pH 8.4–8.6 with the peak at pH 8.5 (Fig. 2A). The less active material corresponded to a pI

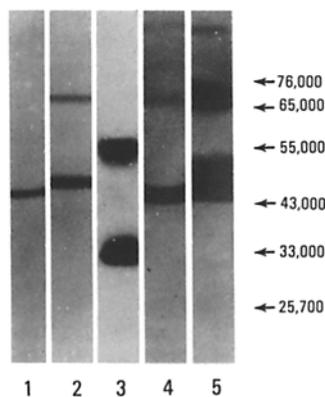


FIGURE 1 Fibrin autographs showing cell associated and secreted PAs fractionated by SDS PAGE. Conditioned media (100  $\mu\text{l}$ ), cell extract (150  $\mu\text{g}$ ), and urokinase (0.5 U) were fractionated by SDS PAGE and PA activity localized on fibrin-agar gels. The dark areas correspond to fibrin lysis zones. Cell extract (lanes 1, 4); CM (lanes 2, 5); urokinase (lane 3). Lanes 1–3 were allowed to develop for 4 h, lanes 4–5 for 8 h.

TABLE I  
Properties of Plasminogen Activators Produced by Bovine Aortic Endothelial Cells

Cell-associated		Secreted	pI
$M_r$	$M_r$	$M_r$	
48,000 $\pm$ 2,900	52,000 $\pm$ 2,200		8.5
74,600 $\pm$ 2,000	74,000 $\pm$ 4,600		7.6
80,000–100,000	80,000–100,000		7.6
100,000 $\pm$ 2,900	100,000 $\pm$ 3,300		7.6

Cell extract and CM were fractionated by SDS PAGE and PA activity localized on a fibrin-agar gel. Molecular weights are an average of at least five determinations. The pIs are calculated from experiments similar to those shown in Fig. 2 and are the result of two separate determinations.

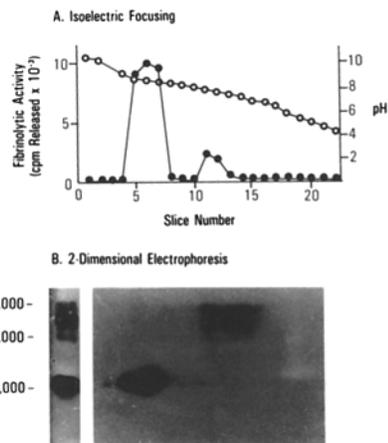


FIGURE 2 Analysis of cellular PAs by isoelectric focusing. Conditioned media was electrofocused on a 5% acrylamide slab gel as indicated in Materials and Methods. Lanes parallel to the direction of migration were cut out of the gel and treated as follows: (A) The gel strip was cut into 0.5-cm slices, each slice was then incubated in 0.5 ml of 0.5% Triton X-100 for 30 min at which time the pH of the sample was determined ( $\circ$ — $\circ$ ). Incubation was continued for 16 h at 4°C to allow PA to diffuse out of the gel slice. The PA activity of these samples was determined ( $\bullet$ — $\bullet$ ). (B) A duplicate lane from the isoelectric focusing gel was subjected to further fractionation by SDS PAGE. PA activity was localized by fibrin autography. The separate lane at the left shows CM fractionated only by SDS PAGE.

value of 7.4–7.8, with the peak of activity at pH 7.6. To relate these isoelectric forms to the molecular weight forms shown in Fig. 1, focused samples were subjected to additional fractionation by SDS PAGE (Fig. 2B). The PA form of pI 8.5 consisted of a single molecular species of  $M_r$  52,000. Unexpectedly, the smaller peak of activity (pI 7.5) contained all the higher molecular weight forms ( $M_r$  74,000–100,000). The pI of the various molecular weight forms of activator are summarized in Table I.

### Immunological Characterization of Cellular PAs

Urokinase and tissue activator were fractionated by SDS PAGE and then analyzed by fibrin autography. The fibrin indicator gels contained either anti-urokinase IgG or antitissue activator IgG. Nonimmune IgG was added to parallel fibrin indicator gels as controls. The presence of antitissue activator IgG blocked the development of tissue activator initiated lytic zones (Fig. 3) but did not affect the activity of purified urokinase. Conversely, the presence of antiurokinase IgG neutralized urokinase activity but was not effective against tissue activator. The activities of the multiple cellular PA forms also showed differential sensitivity to these IgG fractions (Fig. 4). Anti-urokinase completely neutralized the activity of the  $M_r$  52,000 form in CM (lane 2) but had no effect on the activity of the other forms ( $M_r$  74,000–100,000). In contrast, the presence of antitissue activator IgG (lane 3) blocked the activities of the high molecular weight forms but had little detectable effect on the activity of the lower molecular weight forms. These results were confirmed with CM prepared from cloned BAEs (unpublished observation). Fibrin-agarose plates containing nonimmune IgG did not affect the activity of any of the PA forms.

### Sensitivity of Cellular PAs to Inactivation by DFP

The relative sensitivity of the various PA forms to inactivation by DFP was assessed. CM was treated with DFP for

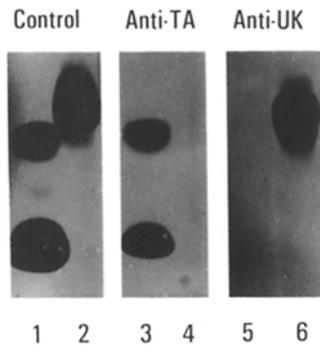


FIGURE 3 Neutralization of PAs by anti-urokinase and anti-tissue activator IgG. Urokinase (0.01 U; lanes 1, 3, 5) and tissue activator (0.01 U; lanes 2, 4, 6) were subjected to analysis by SDS PAGE and activity localized on fibrin-agar gels containing 80  $\mu$ g/ml of either nonimmune IgG (lanes 1 and 2), anti-tissue activator IgG (lanes 3 and 4) or anti-urokinase IgG (lanes 5 and 6). The gels were allowed to develop for 6 h.

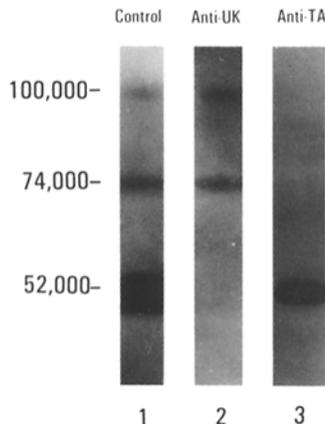


FIGURE 4 Neutralization of BAE PAs by anti-urokinase and anti-tissue activator IgG. CM was fractionated by SDS PAGE and analyzed on fibrin-agar indicator gels containing 20  $\mu$ g/ml nonimmune IgG (lane 1), 20  $\mu$ g/ml anti-urokinase IgG (lane 2) or 20  $\mu$ g/ml anti-tissue activator IgG (lane 3). The gels were allowed to develop for 6 h.

various times, fractionated by SDS PAGE, and residual PA activity was then localized by fibrin autography (Fig. 5). Although the 74,000 form was almost completely inactivated by treatment with 1 mM DFP (lanes A2 and B2), the 52,000-dalton form remained relatively active even after incubation with 10 mM DFP (lanes A3 and B3). The 100,000-dalton form was apparently refractory to DFP treatment since no change in the activity of this form was observed after 2 h of exposure to 10 mM DFP (lane B3). Treatment of CM with 40 mM DFP did not decrease the activity of this molecule (not shown). To insure that residual PA activity represented a DFP-insensitive molecule and was not simply due to hydrolysis and inactivation of the DFP during the incubation period, 0.5 U of urokinase was added to the CM sample that previously had been treated with 1 mM DFP for 2 h (the sample shown in lane A2). The mixture was incubated for an additional 30 min and then analyzed for urokinase activity. The urokinase was completely inactivated by this treatment (lane C2) even though cellular PA activity was still detected at  $M_r$  52,000. The DFP sensitivity of cell associated PAs was identical to their corresponding secreted forms (not shown).

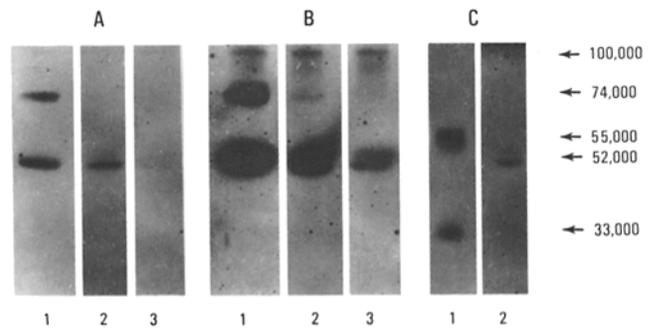


FIGURE 5 Inactivation of secreted PAs by DFP. Conditioned media (equivalent to 0.5 U of urokinase activity) was treated with 1 or 10 mM DFP for 2 h at 37°C. After incubation, the samples were frozen in a dry-ice ethanol bath, fractionated by SDS PAGE and residual PA activity determined on fibrin-agar gels. (A) Lane 1, untreated control; Lane 2, 1 mM DFP; Lane 3, 10 mM DFP, assayed for 4 h. (B) Same as (A) but after 8-h incubation. (C) Lane 1, urokinase (0.5 U); Lane 2, urokinase (0.5 U) was added to the sample treated for 2 h with 1 mM DFP (A, Lane 2) and then incubated for an additional 30 min. Assay was for 4 h.

## DISCUSSION

A variety of normal and transformed cells in culture have been shown to produce multiple, molecular weight forms of PA (22–25). These PAs were found to range in size from  $M_r$  36,000 to  $M_r$  100,000 and larger. The lower molecular weight PAs from several of these cell lines ( $M_r$  ~50,000) were neutralized by antiurokinase, but the activity of the larger molecular weight forms was apparently resistant to such treatment. Although this resistance suggests that the larger forms may be tissue-activator-like PAs, direct immunochemical and functional evidence for this conclusion is lacking. The relationship of the various forms to each other is not at all clear from these studies. Our studies with cultured endothelial cells resolve many of these uncertainties.

Analysis of cell extracts and conditioned media from cultured BAEs by SDS PAGE revealed the presence of at least three distinct molecules with PA activity, and a number of others not clearly resolved by this approach (Fig. 1). That these activities result from the presence of only two classes of activator can be argued from the following observations. First, the complex molecular weight pattern observed by SDS PAGE reduced to a rather simple, two component pattern upon isoelectric focusing (Fig. 2A). Symmetrical peaks of PA activity were detected at pH 8.5 and at pH 7.6. Subsequent fractionation of the focused samples by SDS PAGE (Fig. 2B) revealed that the activity recovered at pH 8.5 contained only one molecular species of activator, that of  $M_r$  52,000. This form was not detected at the lower isoelectric point. The size and charge properties of the  $M_r$  52,000 form are similar to those reported for urokinase (26). All of the higher molecular weight forms ( $M_r$  74,000–100,000) migrated to one position in the gel, at pH 7.5. The higher molecular weight, lower pI of these other forms is consistent with the properties of tissue-activator-like PAs (21, 27). Second, and in agreement with these conclusions, are the antibody neutralization experiments. Only the activity of the  $M_r$  52,000 form was blocked by antiurokinase (Fig. 3); it was not affected by antitissue activator (Fig. 4). The activity of all the high molecular weight forms ( $M_r$  74,000–100,000) was neutralized by antitissue activator (Fig. 4) but was not affected by anti-urokinase (Fig. 3). Third, the high molecular weight forms behaved as a single class of biochemically similar mole-

cules with respect to their interaction with fibrin (Loskutoff and Mussoni, unpublished observations). All of these forms not only bound to fibrin, but also appeared to require fibrin for their activity. This fibrin cofactor requirement is the primary functional distinction between tissue-type and urinary PAs (6). The  $M_r$  52,000 form resembled urokinase in that it showed no association with fibrin.

Thus, it is clear from the above considerations that the  $M_r$  52,000 form is a urokinaselike PA, while the  $M_r$  74,000–100,000 forms behave in all respects like tissue-type PAs. Whether these two classes of PA are actually products of two separate genes (22) remains to be determined. Similarly, the exact relationship among the multiple forms of tissue-type PA ( $M_r$  74,000–100,000) remains unclear. Although it is possible that they are products of multiple genes, the fact that they demonstrate similar pIs, are sensitive to the same antisera, and interact similarly with fibrin, suggests a common origin from a single gene. The DFP-inactivation studies (Fig. 5) would seem to suggest a precursor-product relationship. That is, the relative insensitivity to DFP of the  $M_r$  100,000 form is consistent with the behavior of a proenzyme. Like other zymogen activation reactions (28), activation in this case would be expected to be a proteolytic process resulting in the formation of a lower molecular weight, DFP-sensitive molecule. The  $M_r$  74,000 form has these properties. The relative insensitivity of the unresolved activity between  $M_r$  80,000–100,000 suggests that this material may represent inactive degradation products of the larger form. The ability to detect PA activity at all in DFP-resistant material seems at first glance, to be inconsistent with this model. However, this activity may arise after treatment with DFP, perhaps during SDS PAGE, and may only reflect a small percentage of the potential activity of the molecule. Conclusive support for these speculations awaits purification of the relevant molecules and specific activity determinations.

It is intriguing that cells of the vascular wall should be associated with both tissue-type (fibrin-dependent) and urokinaselike (fibrin-independent) PA activity. It may be that expression of both activities is critical for the role of the endothelium in maintaining vessel patency. On the other hand, recent observations indicate that urokinase is a relatively ineffective thrombolytic agent when compared to tissue-type PAs (8), suggesting that it may be important for processes other than fibrinolysis (29). Rijken et al (4) detected only tissuelike PA activity in sections of the vascular wall. This finding differs from the results presented here and may reflect species or tissue specificity, or changes that occur in cultured cells. It is also possible that the presence of both types of activator reflects a heterogeneity of cells in our cultures. If so, it is unlikely that such heterogeneity is due to the presence of a subpopulation of nonendothelial vascular cells (i.e., smooth muscle cells or fibroblasts) since the morphology of cultures maintained at confluency for 2 wk and fed every 3 d, did not change from the "cobblestone" appearance of the original cultures. If the other cells were present, the cultures would quickly convert to a multilayered population of elongated cells under these conditions (30). Laug (14) recently observed multiple molecular weight forms of PA in a cloned isolate of BAEs. We also have confirmed our results with six separate clonal isolates, indicating directly that BAEs produce both types of PA. Because BAEs may be phenotypically unstable (31), it is possible that the cultures consisted of two subpopulations of endothelial cells with one producing only the urokinaselike molecule and the other the tissue-type PAs. Satisfactory resolution of this possibility will depend upon development of techniques to

detect both activities in single cells. In any case, it is clear that endothelium has the potential to produce both classes of activator. How expression of each is regulated, and the relative importance of each in *in vivo* fibrinolysis will await further studies.

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