Purification and characterization of a plasminogen activator secreted by a pig kidney cell line

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The plasminogen activator secreted by calcitonin-treated pig kidney cells was purified, characterized and compared with human urinary urokinase. The purification procedure was based on the following steps: sulphopropyl-Sephadex chromatography, *p*-aminobenzamidine-Sepharose chromatography, preparative sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and isoelectrofocusing. The purified enzyme was obtained from the conditioned medium with a yield of 13% and a purification factor of 390-fold. Analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under non-reducing conditions showed one closely spaced doublet with an M_r of 50000; in the presence of reducing agents, two additional bands of M_r 30000 and 20000 appeared. The purified enzyme resembles the 53000- M_r components of human urinary urokinase in amino acid composition and two-dimensional tryptic peptide maps and in its catalytic properties, and the two enzymes cross-react immunologically with rabbit antibodies raised against either. The enzyme appears to be different from tissue plasminogen activator secreted by HeLa cells.

PAs are specific enzymes that catalyse the conversion of the zymogen plasminogen into the broad-spectrum trypsin-like enzyme plasmin. Plasmin has been considered to play a role in a variety of functions, including pro-hormone conversion (Virji *et al.*, 1980) and the generation of localized extracellular proteolysis associated with tissue remodelling and cell migration (Reich, 1978). Increased production of PAs has also been associated with neoplasia and with some cellular responses to tumour promoters (Wigler & Wilson, 1976), retinoids (Wilson & Reich, 1978) and hormones (Dayer *et al.*, 1981). PAs fall into two categories: one is the family of enzymes related to the human enzyme UK (White *et al.*, 1966), and the other to an activator isolated from uterine tissue (Rijken *et al.*, 1979), designated as tissue activator.

The LLC-PK₁ renal cell line responds to calcitonin by increasing cyclic AMP production (Goldring *et al.*, 1978), by activating cyclic AMP-dependent protein kinase (Ausello *et al.*, 1980) and by inducing PA synthesis (Dayer *et al.*, 1981). The

PA is induced by the process that requires transcription and translation (Dayer *et al.*, 1981; Sims *et al.*, 1981) and is secreted into the culture medium. Thus the LLC-PK₁ cell line can serve as a model for hormonal regulation of gene expression and as a potential source for defining the molecular aspects of PA induction. Isolation and characterization of the PA and molecular cloning of its DNA (Nagamine *et al.*, 1983) are basic requirements for such an analysis, and the present paper describes the isolation and characterization of a UK-like molecule from conditioned medium of calcitonin-stimulated LLC-PK₁ cells.

Materials

The reagents used in this work were obtained as follows: salmon calcitonin was Calcimar (5000 MRC units/mg) from Armour Pharmaceutical Co., Chicago, IL, U.S.A.; human plasma was from the New York Blood Center, New York, NY, U.S.A.; crude UK was from Leo Pharmaceuticals, Balleny, Denmark; purified UK and affinity-purified immunoglobulin G against UK were provided by Dr. T.-C. Wun, and antiserum against HeLa-cell PA and samples of tissue PA by Dr. W.-D. Schleuning, from our laboratory; *Erythrina* inhibitor was supplied by Dr. E. Dowdle, Univer-

Abbreviations used: PA(s), plasminogen activator(s); UK, human urinary urokinase; SDS, sodium dodecyl sulphate; PU, Ploug units (Ploug & Kjeldgaard, 1957).

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sity of Cape Town, South Africa; Trasylol was a gift from Dr. E. Truscheit, Bayer, Leverkusen, West Germany.

Methods and results

Cell culture and preparation of serum-free culture fluid

Cultures of pig kidney cell line LLC-PK₁ (Hull *et al.*, 1976) were grown in DME medium (Dulbecco & Freeman, 1959) with foetal bovine serum at a concentration of 5% or 10% (v/v). The cells were cloned, and one of the clones, C-4, which displays low basal intracellular and secreted PA

activity, and which responds to calcitonin (J. D. Vassalli & E. Reich, unpublished work) has been used in these studies. Confluent plates were washed (twice) with phosphate-buffered saline (0.14M-NaCl/0.009M-sodium phosphate buffer, pH 7.4) and incubated in serum-free medium containing 100 ng of calcitonin/ml. The medium was removed every 68 h for 6 or 9 days and was a source of PA designated here as the PK-cell PA.

Purification of the pig kidney PA

All isolation steps were performed without interruption to minimize the likelihood of activation or proteolytic degradation of the proteins; to

Table 1. Summary of the purification of PK-cell PA

For experimental details see the text. Activity was measured by the ¹²⁵I-fibrin-plate assay method as described by Unkeless *et al.* (1973).

Fraction step	Volume (ml)	Activity (PU)	Protein (mg)	Specific activity (PU/mg)	Yield (%)	Purification factor
1. Harvest fluid	26000	1316900	2418	545	100	1
2. 1st SP-Sephadex chromatography	260	1098110	1937	567	83	1
3. 2nd SP-Sephadex chromatography	220	1079620	176	6134	82	11
4. Benzamidine-Sepharose chromatography	27	614925	7.30	84236	47	155
5. Preparative SDS/polyacrylamide-gel electrophoresis	3.5	447704	3.85	116287	34	213
6. Isoelectrofocusing	15	173430	0.81	214111	13	393

Fig. 1. Cation-exchange chromatography of partially purified PK-cell PA on SP-Sephadex C-25 (step 3). Proteins eluted from four SP-Sephadex columns were precipitated as described in the Methods and results section. The precipitate was dialysed and loaded on to a second column of SP-Sephadex. After the column had been washed with AA buffer, the proteins were eluted with a linear gradient of $(\text{NH}_4)_2\text{SO}_4$. Fractions of volume 10 ml were collected. Their ionic strength (---) was determined by conductance. Enzyme activity (○) was determined by the fibrin-plate assay, and protein (●) by the Bio-Rad assay.

this end acid pH and the presence of benzamidine were maintained wherever feasible. Aside from preparative SDS/polyacrylamide-gel electrophoresis and isoelectrofocusing, all steps of the procedure were performed at 4°C. A summary of purification of PK-cell PA is presented in Table 1.

Step 1 and 2: preparation of conditioned medium and first SP-Sephadex chromatography. The culture fluid was centrifuged to remove debris, adjusted to 10mM-benzamidine, acidified to pH 4.6 with acetic acid and loaded on to four SP- (sulphopropyl)-Sephadex columns equilibrated with 50mM-ammonium acetate buffer, pH 4.6, containing 10mM-benzamidine (AA buffer). After the column had been washed with 10mM-NaCl in AA buffer, the enzyme was eluted in a single step with 0.5M-(NH₄)₂SO₄ in AA buffer.

Step 3: second SP-Sephadex chromatography. Proteins eluted from the first SP-Sephadex columns were precipitated by dialysis against saturated (NH₄)₂SO₄ solution and collected by centrifugation at 6000g for 30min. The precipitate was dialysed against AA buffer and loaded on to a second SP-Sephadex column (52cm × 1.2cm). When the column was washed with buffered (NH₄)₂SO₄ solution of linearly increasing ionic strength (0.05M to 0.6M), the enzyme activity was eluted at a position remote from the bulk of protein (Fig. 1).

Step 4: benzamidine-Sepharose chromatography. The most-active fractions were pooled and concentrated, and were dialysed for 8h against phosphate-buffered saline and then applied to a benzamidine-Sepharose column (0.4cm × 5cm) at a flow rate of 5ml/h. The column was washed with 20ml of 0.5M-NaCl in phosphate-buffered saline and eluted with 27ml of 0.1M-glycine/HCl buffer, pH 2.2 (Table 1).

Step 5: preparative SDS/polyacrylamide-gel electrophoresis. Preparative SDS/polyacrylamide-gel electrophoresis was performed by the procedure of Hagen & Young (1975) as described by Schleuning *et al.* (1983). The purified enzyme analysed by SDS/polyacrylamide-gel electrophoresis migrated under non-reducing conditions as a single fuzzy band with an *M_r* of 50000 (Fig. 3, lane D), slightly below the 53000-*M_r* components of UK (Fig. 3, lane E).

Step 6: isoelectrofocusing. Preparative flat-bed isoelectrofocusing was performed in a Sephadex G-75 (superfine grade) gel with a 1:1 mixture of pH 7–10 and pH 8.5–9 Ampholines (Randola, 1973). The gel was equilibrated in 0.1% Nonidet P-40. Before application, the protein sample was dialysed against 0.1% Nonidet P-40 containing 2% (v/v) of the above mixture of Ampholines. Isoelectrofocusing proceeded for 14h at 10°C, at initial settings of 0.40kV and 22mA. The gel bed was

Fig. 2. Isoelectrofocusing of purified PK-cell PA (step 6). Preparative flat-bed isoelectrofocusing was performed in a Sephadex G-75 gel with a mixture of pH 7–10 and pH 8.5–9 Ampholines (see the Methods and results section). Enzyme activity (●) and protein (not shown) were measured as indicated in Fig. 1 legend. ○, pH.

sectioned with a grid and the fractions were eluted with 0.1% Nonidet P-40 (Fig. 2).

Homogeneity of the PK-cell PA

The purified enzyme was homogeneous by the criterion of analytical SDS/polyacrylamide-gel electrophoresis and it migrated, under non-reducing conditions, as a closely spaced doublet with an *M_r* of 50000 (Fig. 3, lane B). No additional bands were detected when the gel was overloaded with 80μg of the purified protein (Fig. 3, lane C). After reduction with 2-mercaptoethanol, the PK-cell PA showed two additional bands, with *M_r* 30000 and 20000 (Fig. 3, lane A), suggesting that the enzyme had been purified as a mixture of one-chain and two-chain forms. The 30000-*M_r* and 50000-*M_r* components were shown to be structurally related, since two-dimensional mapping of iodinated peptides showed that the 50000-*M_r* form contained most of the tryptic peptides present in the 30000-*M_r* component (Sudol, 1983). The 20000-*M_r* and 50000-*M_r* components were also shown to be structurally related by tryptic peptide mapping (results not shown). The two-chain form therefore does not appear to be an artifact arising during purification, and is more probably an activated product of a single-chain proenzyme.

The specific activity of the purified enzyme, 210kPU/mg, was comparable with that of the unpurified preparation (184 ± 35kPU/mg) the molarity of which was determined by an active-site

Fig. 3. SDS/polyacrylamide-gel electrophoresis of purified PK-cell PA and UK

Electrophoresis on 11% (lanes B, C and D) or 14% (lane A) polyacrylamide gels with 5% polyacrylamide stacking gels was as described by Laemmli (1970). Lane A, purified PK-cell PA analysed under reducing conditions; migrations of two small- M_r components M_r 30000 and 20000 are not consistent with the markers on the 14% gel; their relative M_r values were determined on 11% polyacrylamide gel. Lane B, purified PK-cell PA analysed under non-reducing conditions. Lane C, as in lane B, but overloaded with 80 μ g of the purified PK-cell PA. Lane D, PK-cell PA after preparative SDS/polyacrylamide-gel electrophoresis. Lane E, benzamidine-Sepharose-purified UK.

titration method (Jameson *et al.*, 1973) as described by Wun *et al.* (1982a).

Antibodies against PK-cell PA were raised and purified as described by Wun *et al.* (1982a). The immunoglobulin G isolated from antisera raised against PK-cell PA gave a single precipitin line with crude and purified PK-cell PA when Triton X-100 (1%) and benzamidine (50mM) were incorporated into the immunodiffusion gels (Ouchterlony & Nilson, 1973). No precipitation with tissue PA from HeLa cells was observed. Non-immune immunoglobulin G did not precipitate PK-cell PA (Sudol, 1983).

Determinations of M_r and pI of PK-cell PA

The M_r of the isolated enzyme was estimated by gel filtration on a Sephadex G-100 column. The peak of PA activity measured by the fibrin-plate method (Unkeless *et al.*, 1973) and the protein

profile of the eluted enzyme measured by the Bio-Rad assay (Bradford, 1976) were superimposable. The relative partition coefficient (Andrews, 1965) for the PK-cell PA was 1.46, corresponding to an M_r of 48000 ± 2100 ; this compares favourably with the value of 50000 determined by SDS/polyacrylamide-gel electrophoresis, by taking the average of the M_r values of the two closely spaced forms of the enzyme (Fig. 3, lane B).

PK-cell PA pI values of 8.20 ± 0.18 for the 50000- M_r form and 7.4 ± 0.2 for the 30000- M_r form were determined by zymography (Graneli-Piperno & Reich, 1978) after two-dimensional polyacrylamide-gel electrophoresis (O'Farrell, 1975) under non-reducing conditions. The results were confirmed by preparative isoelectrofocusing (Fig. 2).

Comparison of PK-cell PA with UK

The two enzymes were compared with respect to amino acid composition, tryptic peptide maps, immunological specificity and susceptibility to several inhibitors.

Amino acid analysis and peptide mapping. These two analyses show that PK-cell PA and UK are closely related. As shown in Table 2, with the exception of glutamic acid and cysteine, the amino acid compositions of the two enzymes are identical within the limits of experimental error. The patterns of iodinated tryptic peptides of PK-cell PA and UK shown in Fig. 4 indicate a high degree of structural similarity. Superimposable spots of tryptic peptides were found to co-migrate when equal amounts of radioiodinated tryptic-digestion products of both proteins were mixed and analysed on the same plate (Fig. 4c).

Immunoassays and immunological properties. The cross-reactivity between PK-cell PA and UK was determined by quantitative immunoprecipitation. Immunocomplexes were precipitated as described by Kessler (1975, 1976). Residual enzyme activities in supernatants were determined by the fibrinolytic assay and expressed as the percentage of the enzyme activity in the control sample without antibody. To calculate the residual activity (RA) the following formula was used:

$$RA = 100[1 - (C_n - D) \times (B - D) - 1] \times 100$$

where B is an average of fibrinolytic activity in the sample where antibody was replaced by buffer, C_n are average values of fibrinolytic activities in duplicate samples with n dilutions of antibody, and D is an average value of fibrinolytic activity in the sample where both antibody and enzyme have been replaced by the equivalent volume of buffer.

Both enzymes share some common antigenic determinants: UK was precipitated by antibodies raised against PK-cell PA, and anti-UK recog-

Table 2. *Amino acid compositions of PK-cell PA and UK*
Abbreviations: N.I., not included in calculations; N.D., not determined.

Amino acid	Composition (residues/molecule)				
	50000- M_r PK-cell PA		53000- M_r UK		
	Present paper*		Wun <i>et al.</i> (1982a)	Johnson <i>et al.</i> (1977)	White <i>et al.</i> (1966)
	(incomplete)	(complete)	(incomplete)	(incomplete)	(complete)
Asp	46	45	45	43	40
Thr	27	27	29	28	32
Ser	36	36	34	35	35
Glu	62	61	49	48	44
Pro	26	26	29	30	29
Gly	43	42	46	45	41
Ala	20	20	24	23	20
Cys	37†	36†	26	20	19
Val	24	24	23	22	23
Met	9	8	6	8	8
Ile	18	18	19	18	20
Leu	35	34	37	33	34
Tyr	21	20	18	19	20
Phe	17	17	15	14	14
His	18	17	17	23	20
Lys	31	30	27	35	31
Arg	29	29	23	25	22
Trp	N.I.	9‡	N.D.	N.D.	10

* Values obtained from 24 h hydrolysates.

† Determined as cysteic acid by the method of Moore (1963); expressed relatively to aspartic acid.

‡ Estimated spectrophotometrically by the method of Bencze & Schmid (1957).

nized and precipitated PK-cell PA. Homologous reactions of the antibodies with their relevant antigens differed quantitatively from the heterologous ones. Antibodies used in the homologous reactions and diluted two orders of magnitude precipitated approximately the same amounts of the enzymes as undiluted antibodies in the heterologous reactions (Fig. 5).

Susceptibility to proteinase inhibitors. The catalytic activity of UK and analogous PAs of mouse origin is resistant to most macromolecular proteinase inhibitors (Dano & Reich, 1975). We compared the effects of three inhibitors on PK-cell PA and 53000- M_r UK, by using the amidolytic assay with compound S-2444 (KABI, Stockholm, Sweden) as substrate. Enzyme assays were conducted as specified by Cleason *et al.* (1978). The results were analysed by using a linear plot of $[S]/v$ against $[S]$ (Engel, 1981).

Both enzymes were not inhibited by soya-bean trypsin inhibitor, *Erythrina* inhibitor and one preparation of Trasylol. It is worth noting that both enzymes were inhibited by a second Trasylol preparation, with $K_i = 1.5 \times 10^{-5}$ M for UK and 3.2×10^{-5} M for PK-cell PA. *Erythrina* inhibitor inhibited tissue PA from HeLa cells with $K_i = 0.9 \times 10^{-7}$ M, as reported by Heussen (1982).

K_m values for compound S-2444 hydrolysis by UK, PK-cells PA and HeLa-cell PA were 8.3×10^{-5} M, 1.8×10^{-4} M and 4.7×10^{-4} M respectively.

Discussion

UK-like PAs have been isolated from the conditioned media of several types of cultured cells, e.g. human pancreatic carcinoma (Wu *et al.*, 1977), MSV-transformed 3T3 cells (Dano *et al.*, 1980) and human epidermoid carcinoma cells (Wun *et al.*, 1982b). In the present study, a method was developed for the purification of the PA that is secreted by pig kidney cells in response to calcitonin. The purification method was based on cation-exchange and benzamidine-Sepharose chromatography and preparative SDS/polyacrylamide-gel electrophoresis; it has proved to be convenient for the isolation of apparently homogeneous preparations of the enzyme from substantial volumes of cell-culture medium.

The following aspects of the work and properties of the enzyme deserve brief comment.

(1) The present paper describes a purification method of PA from pig kidney cell cultures that yields homogeneous preparation of enzyme by several criteria (listed below), including SDS/

Fig. 4. Comparison of iodinated peptides of PK-cell PA and UK

The radioiodination and performic acid oxidation of proteins in single polyacrylamide-gel slices, tryptic digestion and peptide mapping were performed as described by Elder *et al.* (1977) and by Karess & Hanafusa (1981). (a)–(c) are autoradiograms of ^{125}I -labelled peptides of the indicated proteins: (a) PK-cell PA; (b) 53000-M, UK; (c) PK-cell PA and UK. (d) is a drawing that shows the differences between the ^{125}I -labelled peptides of PK-cell PA and UK: ●, spots common to both proteins; ⊗, spots unique to the PK-cell PA; ○, spots unique to UK.

Fig. 5. *Immuno-cross-reactivity of PK-cell PA and UK*

(a) Immunoprecipitation of UK with anti-UK (○) and anti-(PK-cell PA) (●) antibodies. (b) Immunoprecipitation of PK-cell PA with anti-UK (●) and anti-(PK-cell PA) (○) antibodies. Antibody against tissue PA from HeLa cells (■) and immunoglobulin G from non-immune rabbit serum (□) were used as controls. The same amounts of enzyme were treated with antibodies, incubated and immunoprecipitated. Residual enzyme activities in supernatants were determined by the fibrinolytic assay and calculated as described in the Methods and results section.

polyacrylamide-gel electrophoresis. Partial purification of PA from LLC-PK₁ cells treated with colchicine was reported by Bobbitt *et al.* (1980). The procedure previously reported results in enzyme preparations that on SDS/polyacrylamide-gel electrophoresis under non-reducing conditions showed multiple protein bands. Our studies also show the structural and immunological relation of PK-cell PA to UK, and do not agree with the immunological data of Bobbitt *et al.* (1980), who reported that both enzymes are immunologically distinct.

Since the PA species purified by Bobbitt *et al.* (1980) were obtained from cultures exposed to colchicine and incubated for long periods, as opposed to the calcitonin-treated cells reported in the present paper, there is a possibility, although it seems unlikely, that different PAs are secreted in response to those two inducers. Another possibility is that the PA isolated by Bobbitt *et al.* (1980) had undergone partial proteolytic degradation during incubation and/or purification.

(2) The purification scheme described in the present paper is reproducible, and 1 mg of purified PK-cell PA was routinely obtained from 30 litres of

conditioned medium. Although the moderate recovery remains to be improved, the procedure yields highly purified enzyme that has retained a high specific catalytic activity.

(3) The purity of the enzyme was established by the following criteria. (a) The protein migrated on SDS/polyacrylamide-gel electrophoresis as a Coomassie Blue-stainable closely spaced doublet. Overloaded gels did not show any additional bands. (b) A single precipitin line was obtained in Ouchterlony double-immunodiffusion with rabbit antibodies. (c) Specific catalytic activity of the purified enzyme was comparable with that measured for the crude enzyme and calculated from molarity determined by active-site titration.

(4) The PK-cell PA is closely related to the 53000- M_r form of UK. The two proteins are similar, but not identical, by the following criteria: amino acid analysis, tryptic peptide mapping, immunochemical properties and catalytic properties.

(5) Our values for the M_r of PK-cell PA, namely 50000 and 48000 ± 2100 determined by SDS/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969; Laemmli, 1970) and by Sephadex-gel

filtration (Andrews, 1965) respectively, differ slightly from that reported by Sims *et al.* (1981), who obtained an estimate of 45000 from SDS/polyacrylamide-gel electrophoresis.

(6) The final preparation of PA partially purified by Bobbitt *et al.* (1980) comprised two fractions, each with a variety of M_r species. The pI values of the different M_r species were reported to be in the pH range 6.8–7.2, values that differ from the pI values reported in the present paper: 8.2 ± 0.2 for 50000- M_r PK-cell PA and 7.4 ± 0.2 for 30000- M_r PK-cell PA. The absence of detergents from the buffers of the reported isoelectrofocusing procedures might have been responsible for different migration of the PA species, in view of their tendency to associate with other proteins and bind to different supports (Bobbitt *et al.*, 1980).

(7) Our data on the susceptibility of PK-cell PA and UK to some macromolecular inhibitors supplement and confirm the results obtained by Bobbitt *et al.* (1980) indicating that the enzymic activity of PK-cell PA more closely resembles that of the UK than tissue PAs.

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