Proenzyme to urokinase-type plasminogen activator in the mouse in vivo

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We have investigated whether urokinase-type plasminogen activator (u-PA) is present in the mouse in vivo as the proenzyme or as the active enzyme. u-PA in extracts of various murine tissues was of a one-polypeptide chain form with an electrophoretic mobility indistinguishable from purified proenzyme (pro-u-PA), as demonstrated by SDS-polyacrylamide gel electrophoresis under recucing conditions followed by immunoblotting. No 2-chain u-PA was detected in any of the extracts (detection limit 10% of that of one-chain u-PA). In bladder urine more than half of the u-PA was of the one-chain form. Together with previous immunocytochemical studies of the normal murine tissues and studies of the Lewis lung carcinoma, the present results indicate that in these tissues the one-chain proenzyme is the predominant form of u-PA in intracellular stores and for the first time demonstrates that at least in some cases the one-chain form constitutes a sizeable fraction of the u-AP in extracellular fluids in the intact organism.

Plasminogen activator *Urokinase-type* *Proenzyme* *Immunoblotting*

1. INTRODUCTION

Plasminogen activators catalyse the conversion of the inactive proenzyme plasminogen to the active protease plasmin, which in turn can degrade most proteins. Plasminogen is present in many extracellular body fluids and cellular release of plasminogen activators constitutes a means of generating localized extracellular proteolysis. Two types of vertebrate plasminogen activators can be distinguished based on M_r values, immunological reactivity, enzymatic properties, and distribution in the intact organism, namely tissue-type (t-PA) and urokinase-type (u-PA). Both are serine proteases, of ~70 and ~50 kDa, respectively. They seem to take part in different biological processes, t-PA being a key enzyme in thrombolysis, and u-PA among other functions being implicated in degradation of the extracellular matrix during tissue degradation in normal and pathological conditions, including cancer (reviews, [1–5]). Recently, the amino acid sequences of human u-PA [6] and human t-PA [7] were determined, demonstrating that these two enzymes are products of different genes.

We have previously reported that u-PA is released as an inactive proenzyme (pro-u-PA) from murine sarcoma virus-transformed fibroblasts cultured under serum-free conditions. The proenzyme was a one-polypeptide chain protein. By limited proteolysis with plasmin it was converted to active u-PA consisting of two polypeptide chains held together by one or more disulphide bonds [8]. Similar findings have been reported for u-PA released from cultured human cells of neoplastic origin [9–11]. SDS-PAGE under reducing conditions followed by immunoblotting allows discrimination between one- and two-chain u-PA

Abbreviations: u-PA, urokinase-type plasminogen activator; t-PA, tissue-type plasminogen activator; TBS-T, 0.05 M Tris-HCl (pH 7.4), 0.15 M NaCl, 1% (w/v) Triton X-100; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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in tissue extracts, and we have shown that extracts of Lewis lung carcinoma tissue contained one-chain u-PA but no detectable amounts of two-chain u-PA [12]. We now report that only one-chain u-PA is detectable in extracts of kidney, placenta, vas deferens, oxyntic mucosa of the stomach and the lung, which by immunocytochemical methods have been found to contain cells producing u-PA [13]. In addition, we report that more than half of the u-PA present in murine bladder urine was of the one-chain form, thus demonstrating unequivocally the extracellular presence of this form in the intact organism.

2. MATERIALS AND METHODS

Female and male 6–10 week old BALB/c mice were perfused and tissues extracted with 0.1 M Tris-HCl (pH 8.1), 0.5% Triton X-100 (10 μl/mg tissue, wet wt) as described [13,14]. Urine was collected directly from the bladder of diethylether-anaesthetized mice. u-PA enzyme activity was determined in the ^{125}I-fibrin plate assay, using inhibition with anti-u-PA IgG to determine the part of total plasminogen activator activity that was due to u-PA [13]. One unit of murine u-PA is defined in [15]. After SDS-PAGE proteins were transferred electrophoretically (10 V, 50 mA, 24 h) to nitrocellulose paper (Millipore GSWP 00010, 0.22 μm) in a buffer containing 0.125 M Tris, 0.2 M glycine (pH 8.6), 20% (v/v) methanol and 0.1% (w/v) SDS [16,17]. After transfer, the paper was air-dried. The dried paper could be stored at room temperature for several months without loss of immunoreactivity. Longitudinal lanes were cut out of the paper. The lanes were fixed with paraformaldehyde, incubated with human serum albumin in order to block remaining binding sites and incubated with anti-u-PA IgG (purified by affinity chromatography), non-immune IgG, or anti-u-PA preabsorbed with an excess of highly purified pro-u-PA [13]. In order to detect the sites of antigen-antibody reaction, the lanes were incubated for 45 min at room temperature with horseradish peroxidase-conjugated Protein A (Amersham), diluted 200-fold in 0.05 M Tris-HCl (pH 7.4), 0.15 M NaCl, 1% (w/v) Triton X-100 (TBS-T) with 0.25% (w/v) bovine serum albumin, followed by washing with TBS-T (5 × 5 min) and 0.05 M Tris-HCl (pH 7.6) (2 × 5 min). The peroxidase activity was demonstrated with diaminobenzidine-H_2O_2 [13]. After the peroxidase reaction, the lanes were rinsed shortly in 0.05 M Tris-HCl (pH 7.6), and photographed immediately afterwards. All other materials and procedures were as described [8,13,15,18,19].

3. RESULTS

In SDS-PAGE under non-reducing conditions, both murine pro-u-PA and active u-PA migrate as single bands of ~48 kDa; under reducing conditions, the pro-u-PA migrates at ~48 kDa, while active u-PA migrates as two bands of ~29 and ~18 kDa, respectively [8]. The immunoblotting procedure allowed the detection of as little as 10 U of purified pro-u-PA or active u-PA under non-reducing conditions. Under reducing conditions, the detection limit for pro-u-PA and the ~29-kDa band of active u-PA was 25 U, while the ~18-kDa band was visible only if more than 400 U was applied to the gel. Application of equal amounts of pro-u-PA and active u-PA to the gels always resulted in bands of approx. equal intensity at ~48 and ~29 kDa, respectively.

One-chain u-PA was found in extracts of kidney, placenta, vas deferens, oxyntic mucosa of the stomach and lung, while no two-chain u-PA was detected in any of these extracts (fig.1). This was true even when the gels were loaded with up to 75 μl of any of the extracts, corresponding to maximally 600 U of activator (not shown). These findings indicated that in all tissues less than 10% of the total u-PA content was present in the two-chain form.

The same conclusion was reached when the standard extraction buffer was replaced by 0.075 M potassium acetate, 0.1 M L-arginine (pH 4.2), 0.3 M NaCl, 10 mM EDTA, 0.25% (w/v) Triton X-100 [20] or by 0.1 M Tris-HCl (pH 7.4), 0.1% (w/v) SDS. There was no loss of either pro-u-PA or active u-PA during the extraction procedure, as judged by immunoblotting controls, in which purified preparations were added to the buffer before extraction (not shown).

Immunoblotting analysis of bladder urine showed that more than half of the u-PA immunoreactivity migrated as one-chain u-PA in SDS-PAGE under reducing conditions. Immunoblotting after SDS-PAGE under non-reducing condi-

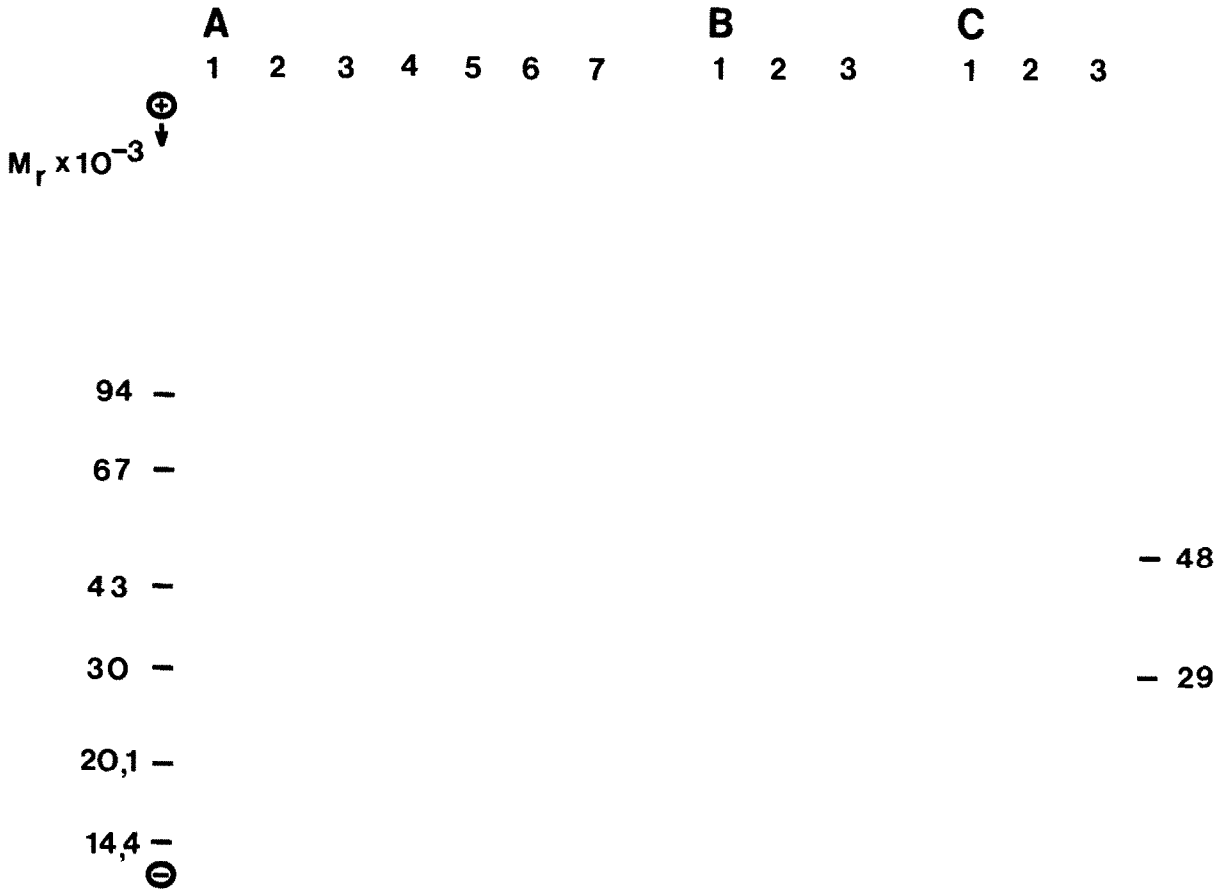

Fig.1. Immunoblotting analysis of u-PA in extracts of murine tissues. The following samples were separated by SDS-PAGE under reducing conditions: (1) 100 U purified active u-PA; (2) 100 U purified pro-u-PA; extracts of (3) kidney (25 μl, 200 μg protein, 195 U u-PA); (4) placenta (day 19 of pregnancy, 25 μl, 150 μg protein, 65 U u-PA); (5) vas deferens (25 μl, 75 μg protein, 70 U u-PA); (6) stomach (oxyntic mucosa, 50 μl, 250 μg protein, 60 U u-PA); (7) lung (50 μl, 250 μg protein, 200 U u-PA). Following SDS-PAGE proteins were transferred electrophoretically to nitrocellulose paper. The paper was stained immunochemically using either 5 μg/ml anti-u-PA IgG (A), 5 μg/ml anti-u-PA IgG preabsorbed with 42 kU/ml purified pro-u-PA (B), or 5 μg/ml nonimmune IgG (C). For all samples, the controls with preabsorbed anti-u-PA IgG and nonimmune IgG were negative. For clarity, only control stainings of 1–3 are shown. The localization of M_r-marker proteins [6] and the estimated M_r of the immunochemically stained band are indicated.

tions showed both ~48- and ~29-kDa forms of u-PA (fig.2). We have previously reported that the latter form has plasminogen activator activity. It is probably a conversion product of the ~48-kDa form [18,19], corresponding to the ~36-kDa, low molecular mass form of human u-PA [21].

4. DISCUSSION

The present findings demonstrate that more than 90% of the u-PA in extracts of a number of murine tissues is present in a one-chain form with a mobility in SDS-PAGE that is indistinguishable from that of pro-u-PA, and therefore presumably is identical to the inactive proenzyme form. In the tissues investigated in the present study, most u-PA immunoreactivity appeared to be located intracellularly as evaluated by immunocytochemistry with light microscopy. In kidneys and vas deferens, it was apparently located in secretion

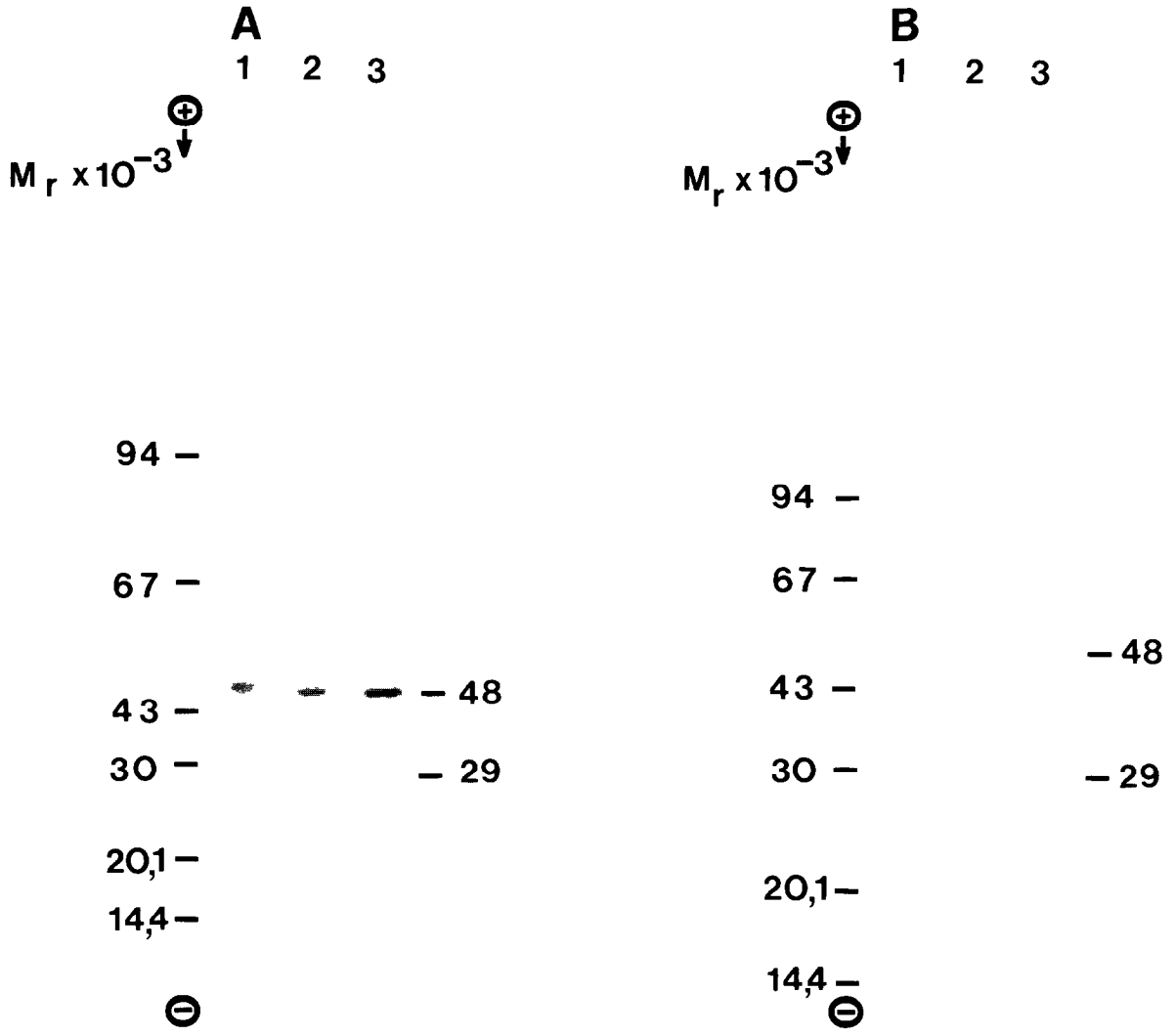

Fig.2. Immunoblotting analysis of u-PA in murine bladder urine. The following samples were separated by SDS-PAGE: (1) 100 U purified active u-PA; (2) 100 U purified pro-u-PA; (3) 2.5 μl (100 U) bladder urine. Experimental conditions were as described in the legend to fig.1 except that the SDS-PAGE was performed under both non-reducing (A) and reducing (B) conditions. Staining was performed with anti-u-PA IgG. Controls with nonimmune IgG or with anti-u-PA IgG preabsorbed with pro-u-PA were negative (not shown). Note that purified active u-PA migrates slightly slower than pro-u-PA in SDS-PAGE under non-reducing conditions.

granules in tubular and stereociliated cells, respectively [13]. This indicates that the one-chain proenzyme is the predominant form of u-PA in the intracellular stores.

We recently reported that more than 80% of the u-PA in extracts of Lewis lung carcinoma [12] was present in the one-chain form. In this carcinoma most of the u-PA immunoreactivity appeared to be located extracellularly or associated with the cell membrane [12]. These results suggested that one-chain pro-u-PA was present extracellularly in Lewis lung carcinoma. The finding here that more than half of the u-PA in bladder urine is present as the one-chain form unequivocally shows that this form can occur extracellularly under in vivo conditions, in agreement with findings in vitro [8–11].

Trace amounts of one-chain u-PA were found in freshly voided murine urine (not shown). This lat-

ter finding is in agreement with a previous study in which a one-chain form of u-PA was purified from human urine with a yield of 3% of the total plasminogen activator activity [22]. The mechanism of pro-u-PA activation in urine as well as the physiological role of this activation remains to be elucidated.

The present findings together with the fact that plasminogen is present in many extracellular body fluids [23–28] make it likely that pro-u-PA and plasminogen often coexist in such fluids, and that as yet unknown initiating factors are crucial to the regulation of the cascade reaction that leads to plasmin-mediated extracellular proteolysis by this pathway. It may be speculated that such initiating factors are different in different biological processes that involve u-PA catalysed plasminogen activation, and that such initiators therefore contribute to the functional diversity of u-PA that is suggested by its occurrence in a variety of different cell types in the intact organism [12,13].

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REFERENCES

[1] Christman, J.K., Silverstein, S.C. and Acs, G. (1977) in: Proteinases in Mammalian Cells and Tissues (Barrett, A.J. ed.) pp.91–149, North-Holland Publishing Company, Amsterdam.
[2] Astrup, T. (1978) in: Progress in Chemical Fibrinolysis and Thrombolysis (Davidson, J.F. et al. eds) vol.3, pp.1–57, Raven, New York.
[3] Reich, E. (1978) in: Molecular Basis of Biological Degradative Processes (Berlin, R.D. et al. eds) pp.155–169, Academic Press, New York.
[4] Collen, D. (1980) Thromb. Haemost. 39, 616–623.
[5] Danø, K., Andreasen, P.A., Grøndahl-Hansen, J., Kristensen, P., Nielsen, L.S. and Skriver, L. (1985) Adv. Cancer Res., in press.
[6] Günzler, W.A., Steffens, G.J., Ötting, F., Kim, S.-M.A., Frankus, E. and Flohé, L. (1982) Hoppe-Zeyler's Z. Physiol. Chem. 363, 1155–1165.
[7] Pohl, G., Källström, M., Bergsdorf, N., Wallén, P. and Jörnvall, H. (1984) Biochemistry 23, 3701–3707.
[8] Skriver, L., Nielsen, L.S., Stephens, R. and Danø, K. (1982) Eur. J. Biochem. 124, 409–414.
[9] Wun, T.-C., Ossowski, L. and Reich, E. (1982) J. Biol. Chem. 257, 7262–7268.
[10] Nielsen, L.S., Hansen, J.G., Skriver, L., Wilson, E.L., Kaltoft, K., Zeuthen, J. and Danø, K. (1982) Biochemistry 24, 6410–6415.
[11] Eaton, S.L., Scott, R.W. and Baker, J.B. (1984) J. Biol. Chem. 259, 6241–6247.
[12] Skriver, L., Larsson, L.-I., Kielberg, V., Nielsen, L.S., Andresen, P.B., Kristensen, P. and Danø, K. (1984) J. Cell Biol., in press.
[13] Larsson, L.-I., Skriver, L., Nielsen, L.S., Grøndahl-Hansen, J., Kristensen, P. and Danø, K. (1984) J. Cell Biol. 98, 894–903.
[14] Unkeless, J., Danø, K., Kellerman, G.M. and Reich, E. (1974) J. Biol. Chem. 249, 4295–4305.
[15] Danø, K. and Reich, E. (1978) J. Exp. Med. 147, 745–757.
[16] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[17] Burnette, W.N. (1981) Anal. Biochem. 112, 195–203.
[18] Danø, K., Møller, V., Ossowski, L. and Nielsen, L.S. (1980) Biochim. Biophys. Acta 613, 542–555.
[19] Danø, K., Nielsen, L.S., Møller, V. and Engelhart, M. (1980) Biochim. Biophys. Acta 630, 146–151.
[20] Camiolo, S.M., Siuta, M.R. and Madeja, J.M. (1982) Prep. Biochem. 12, 297–305.
[21] Soberano, M.E., Ong, E.B., Johnson, A.J., Levy, M. and Shoellmann, G. (1976) Biochim. Biophys. Acta 445, 763–773.
[22] Husain, S.S., Gurewich, V. and Lipinski, B. (1983) Arch. Biochem. Biophys. 220, 31–38.
[23] Collen, D., Tytgat, G., Claeys, H., Verstraete, M. and Wallén, P. (1972) J. Clin. Invest. 51, 1310–1318.
[24] Beers, W.H. (1975) Cell 6, 379–386.
[25] LeBlanc, P.P. and Back, N. (1975) J. Natl. Cancer Inst. 54, 881–886.
[26] Casslén, B. and Ohlsson, K. (1981) Acta Obstet. Gynecol. Scand. 60, 97–101.
[27] Moody, G.H. (1982) Arch. Oral Biol. 27, 33–37.
[28] Fazleabas, A.T., Geisert, R.D., Bazer, F.W. and Roberts, R.M. (1983) Biol. Reprod. 29, 225–238.