Different receptors mediate the hepatic catabolism of tissue-type plasminogen activator and urokinase

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Tissue-type plasminogen activator (t-PA) and urokinase (u-PA) are proteins with partial structural similarity and which are of importance in the therapy of thrombotic diseases. Both are known to be cleared from the circulation *in vivo* by uptake in the liver. The present study investigated whether the hepatic catabolism of u-PA and t-PA is mediated by a common receptor system. Four experimental protocols of increasing complexity were used: hepatocyte plasma membranes, isolated primary hepatocytes, liver perfusion and whole animals. For t-PA, a specific high-affinity binding site to hepatocytes and plasma membranes could be defined with a mean K_d of 4 ± 3 nM, whereas the K_d for u-PA was < 300 nM. Binding of t-PA could not be competed for by u-PA, and vice versa. Furthermore, clearance of t-PA in isolated perfused rat livers and in rabbits *in vivo* was 3-fold higher than that of u-PA, and a 50–100-fold molar excess of u-PA failed to inhibit clearance of t-PA in either system, and vice versa. Taken together, the results imply that hepatic elimination of t-PA and u-PA is mediated by distinct receptor systems of differing affinity.

INTRODUCTION

The physiological plasminogen activators tissue-type plasminogen activator (t-PA) and urokinase (u-PA) are serine proteinases that convert the proenzyme plasminogen into plasmin. This trypsin-like proteinase has a broad specificity, with the predominant function of dissolving the fibrin network of thrombi in the vasculature [1]. Since t-PA exhibits fibrin specificity by activating plasminogen preferentially on the fibrin surface, it has gained considerable importance in the therapy of thromboembolic diseases [2].

t-PA and u-PA are glycoproteins encoded by single copies of different, but highly related, genes in the human genome [3]. t-PA is a major regulator of plasma fibrinolysis, whereas u-PA appears to be mainly involved in cellular functions and tissue remodelling. Both enzymes are organized into distinct structural domains and exhibit a high degree of amino acid sequence similarity [3]. The modules common to t-PA and u-PA are, in addition to the catalytic region, a growth-factor domain similar to that of mouse and human epidermal growth factors, and kringle domains (one in u-PA and two in t-PA) related to kringles 4 and 5 in the plasminogen molecule. In addition to these structures, u-PA contains a sequence called 'connecting peptide', whereas a finger domain, showing sequence similarity to the finger structure in fibronectin, is only found in t-PA.

In vivo both t-PA and u-PA have been shown to be removed from the circulation by the liver [4–7]. However, little information is available on the nature of the hepatic receptor systems for t-PA and u-PA, and it is not known whether a common receptor or cellular uptake system(s) might exist for both enzymes, in view of their structural and functional similarities. To address this question, we sequentially studied the binding and elimination characteristics of t-PA and u-PA in competition experiments employing liver cell membranes, primary hepatocytes, isolated liver perfusion and whole animals.

EXPERIMENTAL

Materials

Recombinant t-PA (Actilyse), female chinchilla rabbits (body wt. 2.3–2.5 kg) and male rats (chbb:Thom, outbred) weighing 250–300 g were supplied by Dr. Karl Thomae G.m.b.H. The rats were anaesthetized by using 3 ml/kg body wt. of a 1:1 mixture of Rompun (Bayer) and Ketavet (Parke-Davis). u-PA (Alphakinase 500,000) was purchased from Alpha Therapeutic G.m.b.H., Langen, Germany. Na^{125}I was obtained from Amersham Corp., Iodogen from Pierce and Sephadex G-25 and Q-Sepharose fast flow from Pharmacia. Collagenase type IV and test kits for alkaline phosphatase, 5'-nucleotidase and acid phosphatase were obtained from Sigma. L-Glutamylglycyl-L-arginylchloromethane (Glu-Gly-Arg- CH_2Cl) and D-phenylalanyl-L-prolyl-L-arginylchloromethane (Phe-Pro-Arg- CH_2Cl) were purchased from Calbiochem. All other chemicals were analytical-reagent grade.

Methods

Iodination of t-PA and u-PA. Recombinant t-PA and u-PA were labelled to high specific radioactivity by the Iodogen method [8] using Na^{125}I . The reaction mixture consisted of 2 mCi of Na^{125}I (50 μl), 8 μg of Iodogen, and 100 μl of t-PA or u-PA (1 mg/ml). After shaking for 20 min at room temperature, 400 μl of 50 mM-sodium phosphate buffer were added and the labelled protein was isolated on a Sephadex G-25 column. This iodination procedure resulted in specific radioactivities in the range of 1.5–2.5 Ci/ μg for t-PA and 0.25–0.4 Ci/ μg for u-PA.

Perfusion of isolated rat livers. Liver perfusions were performed on the isolated organ through the portal vein, essentially as described by Meijer *et al.* [9], using modified Hanks perfusion medium (total volume 115 ml [10]), containing 0.5% BSA and 1 mM- Ca^{2+} . The stock solution (80.0 g of NaCl, 4.0 g of KCl, 0.6 g of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.6 g of KH_2PO_4 and 2.0 g of

Abbreviations used: t-PA, tissue-type plasminogen activator; u-PA, urokinase; Glu-Gly-Arg- CH_2Cl , L-glutamylglycyl-L-arginylchloromethane ('GGACK'); Phe-Pro-Arg- CH_2Cl , D-phenylalanyl-L-prolyl-L-arginylchloromethane ('PPACK').

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MgSO₄·7H₂O/litre) was diluted 10-fold before the addition of 5 mM-NaHCO₃ and 10 mM-Hepes (final concns.). After gassing the diluted Hanks buffer for 30 min with O₂/CO₂ (19:1), the pH was adjusted to 7.4, and BSA and CaCl₂ were added. The perfusate flow rate was approx. 17 ml/min. Before perfusion with solutions containing t-PA either alone or in combination with u-PA, the livers were washed for 10 min with perfusion medium. In some experiments the active site of the plasminogen activator to be given in excess was blocked using Phe-Pro-Arg-CH₂Cl [11] for t-PA and Glu-Gly-Arg-CH₂Cl [12] for u-PA. After starting perfusion with the plasminogen activators, 0.5 ml samples were taken at multiple time points and immediately mixed with a 60-fold excess of the inhibitor. After completion of the perfusion experiments, the livers were weighed and the perfusate samples stored frozen at -20 °C until analysis of plasminogen activators by e.l.i.s.a. [13].

Isolation of hepatocytes. The perfusions for hepatocyte isolation were essentially performed as described above. Before isolation of the hepatocytes the livers were washed for 20 min with Mg²⁺- and Ca²⁺-free buffer containing 1% BSA. The isolation buffer in addition contained 5 mM-CaCl₂ and 240 units of collagenase/ml. After 20 min of recirculating perfusion, the liver was placed in a dish containing 40 ml each of preperfusion and perfusion buffer. The liver capsule was removed, and the cells were loosened by shaking for 15 min. The resulting suspension was filtered through four layers of surgical gauze and cells were collected by centrifugation (200 g, 5 min). The supernatant was removed and the pellet washed twice by resuspension in oxygenated Krebs-Henseleit buffer containing 2% BSA and 0.1% D-glucose. By using the Trypan Blue method, cell viability was found to be > 90% in all preparations. It has been shown that a period of 15–30 min at 37 °C is sufficient for hepatocytes to recover functional receptors after collagenase perfusion [14]. We therefore performed the binding assays after a 30 min regeneration period at 37 °C.

Isolation of hepatocyte plasma membranes. Rat liver plasma membranes were prepared as described in [15], and all steps were carried out at 4 °C. The livers were perfused for 5 min with physiological saline (0.9% NaCl) and then homogenized in 0.25 M-sucrose/1 mM-EGTA/5 mM-Tris/HCl, pH 7.2. The homogenate was loaded on top of 1.45 M-sucrose/20 mM-Tris/HCl, pH 7.4. After centrifugation at 35000 g for 30 min in a Sorvall TV 850 rotor, the interfacial layer, which resembled the crude plasma membrane, was collected, diluted 3-fold with homogenizing buffer and pelleted at 40000 g for 15 min. This pellet was washed again and stored at -70 °C. The protein concentration was determined as described in [16]. Plasma-membrane markers measured were alkaline phosphatase [17] and 5'-nucleotidase [18]. When acid phosphatase [19] was used as a marker enzyme, only a little lysosomal contamination could be detected in the membrane preparations.

Binding assays. All binding assays were done in triplicate. The final cell suspensions or plasma-membrane fractions were diluted to 10⁶ cells/ml or 1.5 mg of protein/ml with Krebs-Henseleit buffer containing 2% BSA and 0.1% D-glucose. A 50 µl portion of ¹²⁵I-t-PA or ¹²⁵I-u-PA in Krebs-Henseleit buffer (final concns. in the assay 0.25 nM) alone or in combination with increasing concentrations of unlabelled t-PA or u-PA (final concentrations in the assay 0.25 nM–1 µM) were added to polypropylene tubes. After addition of 200 µl of cell or membrane suspension and incubation with continuous shaking for 30 min at room temperature, the binding was terminated by a 2 min centrifugation at 15000 g. The supernatants were removed by

aspiration, and the pellets were washed with ice-cold buffer. The final pellets were counted for cell-associated radioactivity (γ radiation) and the protein from the combined supernatants was precipitated at 4 °C by addition of trichloroacetic acid to a final concentration of 10%. Trichloroacetic acid-soluble and -precipitable materials were counted for radioactivity separately. To determine the amount of t-PA irreversibly bound to hepatocytes at various temperatures, 200 µl of the cell suspension was mixed with 50 µl of Krebs-Henseleit buffer, pH 7.4, containing ¹²⁵I-t-PA (0.875 nM) and 2% (w/v) BSA and incubated for 30 min at 4, 20 or 37 °C. The cells were pelleted by centrifugation, washed twice, and resuspended in 100 µl of dissociation buffer. The three buffer systems employed were (i) Krebs-Henseleit buffer, pH 7.4, containing 100 mM unlabelled t-PA, (ii) Krebs-Henseleit buffer, pH 11, and (iii) 50 mM-glycine (pH 11)/0.15 M-NaCl. After incubation for 10 min at the respective temperature, the cells were pelleted and supernatants and pellets were counted in order to determine dissociable and cell-associated radioactivity.

Elimination of t-PA and u-PA from plasma in rabbits. Two groups of three rabbits each received 30 min infusions via an ear vein of t-PA alone (0.6 mg/kg) or in combination with u-PA (6.8 mg/kg; 14-fold molar excess over t-PA). An analogous experiment was performed with further groups of rabbits by administering u-PA (0.6 mg/kg) alone or together with t-PA (10 mg/kg; 14-fold molar excess over u-PA). Before dosing, u-PA was purified by column chromatography (Q-Sepharose fast flow, in phosphate buffer, pH 8.0) and inhibited by preincubation with a 10-fold molar excess of Glu-Gly-Arg-CH₂Cl [12]. Blood samples (0.5 ml), at multiple time points as shown in the Figures, were collected from a central artery of the contralateral ear into tubes containing final concentrations of 7.5 mM-EDTA and 2 µM-Phe-Pro-Arg-CH₂Cl [11] to inhibit t-PA. Plasma was prepared immediately by centrifugation, snap-frozen and stored at -25 °C until assayed. t-PA and u-PA antigen concentrations in plasma were analysed by an e.l.i.s.a. [13].

RESULTS

From competitive-binding assays we calculated *K_d* values (mean ± S.D.) of 1 ± 0.5 nM on binding of ¹²⁵I-t-PA to isolated hepatocytes and 2 ± 1.8 nM to purified hepatocyte plasma membranes. Saturation-binding studies on hepatocytes yielded both a high- (*K_d* 4 ± 3 nM; 2.2 × 10⁵ sites) and low- (*K_d* > 100 nM) affinity binding site for t-PA (Fig. 1). Next, the ability of unlabelled u-PA to compete for the binding of ¹²⁵I-t-PA and vice versa was investigated by using isolated liver cells and hepatic plasma membranes (Fig. 2). Whereas excess unlabelled t-PA in its native and deglycosylated (not shown) forms inhibited the binding of ¹²⁵I-t-PA in both systems, increasing amounts of u-PA remained without effect. As found in competition assays, binding of ¹²⁵I-u-PA to liver cells was not specific and could therefore not be competitively inhibited by a 4000-fold molar excess of either unlabelled u-PA or t-PA. The *K_d* for the binding of u-PA to hepatocytes was determined to be > 300 nM (results not shown).

It has previously been shown that t-PA is internalized and degraded by liver cells [20–22]. We found that a substantial amount of ¹²⁵I-t-PA bound to hepatocytes could not be dissociated under various conditions (Table 1). The non-dissociable fraction increased with temperature and thus most likely represented internalized protein [23].

In order to exclude possible binding artefacts occurring as a result of the isolation procedures in studies employing purified cells and membranes, we then examined the uptake of t-PA and

Fig. 1. Specific binding of ^{125}I -t-PA to primary hepatocytes

The inset shows the Scatchard plot. Data were analysed by the TOPFIT program of Heinzel [40].

Fig. 2. Competition for ^{125}I -t-PA binding ($\pm$ S.D.) to primary hepatocytes and to hepatic plasma membranes by unlabelled t-PA and u-PA

Unlabelled t-PA (●), but not u-PA (■), could efficiently compete for the binding of ^{125}I -t-PA to primary hepatocytes (a) and to hepatic plasma membranes (b).

u-PA in intact liver, in which endothelial cells, Kupffer cells and parenchymal cells retain their morphological location and functional integrity. The clearance of t-PA from perfusate in isolated-liver-perfusion experiments was rapid and not significantly affected by the presence of a 100-fold molar excess of u-PA (Table 2; Fig. 3a), confirming the results of the previous binding assays. The hepatic elimination of u-PA, although already occurring at a more than 3-fold lower rate, was only slightly

reduced by addition of t-PA in 100-fold molar excess (Table 2; Fig. 3b). Inhibition of the active site as described under 'Methods', using Phe-Pro-Arg-CH₂Cl for t-PA and Glu-Gly-Arg-CH₂Cl for u-PA, had no influence on the results obtained, ruling out proteolytic degradation of t-PA or its binding sites by excess u-PA or vice versa.

To investigate whether the binding of t-PA and u-PA to hepatic cells or membranes and their catabolism in the isolated

Table 1. Temperature dependence of the non-dissociable fraction of cell-bound ^{125}I -t-PA

^{125}I -t-PA was incubated at a concentration of 22 fmol/ 10^6 cells for 30 min. The data are derived from an experiment in which the reversibly bound and not-dissociable fractions were determined by incubation with 50 mM-glycine (pH 11)/0.15 M-NaCl. Essentially identical results were found after incubation with a 57 000-fold excess of unlabelled t-PA or Krebs-Henseleit buffer, pH 11. The assays were performed three times as triplicate determinations.

Parameter	Temperature ($^{\circ}\text{C}$)...	4	20	37
Total bound (fmol/ 10^6 cells)		1.54 ± 0.17	1.80 ± 0.22	2.05 ± 0.24
Amount not dissociable (% of total bound)		39.6	44.7	61.8

Table 2. Clearance values (means $\pm$ S.D.) for the elimination of t-PA and u-PA

A, clearance (ml/min per g of liver wt.) of t-PA at an initial concentration of 0.1 $\mu\text{g}/\text{ml}$ from the perfusate of isolated rat liver, both alone and in the presence of a 100-fold molar excess of u-PA. B, clearance from the perfusate of an isolated rat liver of 0.1 μg of u-PA/ml alone and in the presence of a 100-fold molar excess of t-PA. C, clearance (ml/min per kg body wt.) from the circulation *in vivo* in rabbits of 0.6 mg of t-PA/kg alone and in the presence of 51-fold higher plasma levels of u-PA. D, clearance from the circulation *in vivo* in rabbits of 0.6 mg of u-PA/kg alone and in the presence of 33-fold higher plasma levels of t-PA.

	Clearance			
	t-PA	t-PA + u-PA	u-PA	u-PA + t-PA
A	0.290 ± 0.025	0.256 ± 0.041		
B			0.127 ± 0.007	0.128 ± 0.021
C	15.5 ± 2.1	13.6 ± 1.4		
D			5.6 ± 0.8	5.8 ± 0.93

liver reflect the true mechanisms responsible *in vivo* for the degradation of these proteins, we further examined a possible competitive effect of u-PA in rabbits on the clearance of t-PA and vice versa. Fig. 4(a) shows the plasma concentration-time profiles of t-PA in rabbits after infusion of 0.6 mg of t-PA/kg alone or in combination with 6.8 mg of u-PA/kg (14-fold molar excess of dose), which resulted in 51-fold higher maximum plasma levels of u-PA over t-PA. As already observed in the isolated liver perfusion system, t-PA *in vivo* was again eliminated much faster than u-PA (Table 2), and its clearance was not affected by co-administration of a large molar excess of u-PA. The concentration-time profiles in Fig. 4(b) correspond to infusion of 0.6 mg of u-PA/kg alone or together with 10 mg of t-PA/kg (14-fold molar excess of dose). The co-administration of this excess of t-PA resulted in 33-fold higher maximum plasma levels for t-PA over u-PA, but no difference was found for clearance rates in animals receiving u-PA alone or in combination with excess t-PA.

DISCUSSION

Hepatic removal from the circulation has been previously shown for t-PA [7,24–27] and u-PA [4–6]. Accumulation of t-PA in the liver [27] and its binding to freshly isolated as well as to cultured primary rat hepatocytes [20,21] have been described. Moreover, both t-PA and u-PA bind to transformed liver cells [21,28–30] and to mouse liver cell membranes [31]. Endocytosis and lysosomal degradation of t-PA by liver cells have been described by Bakhit *et al.* [20], Einarsson *et al.* [21], Smedsrod *et al.* [22] and Kuiper *et al.* [24]. Protein degradation in intact

Fig. 3. Time course of mean ($\pm$ S.D.) t-PA and u-PA perfusate concentrations during isolated-liver-perfusion experiments

Concentration-time profiles: (a) ●, after addition of 0.1 μg of t-PA/ml to the perfusate ($n=3$); ■, after addition of 0.1 μg of t-PA/ml together with a 100-fold molar excess of u-PA ($n=5$); (b) ●, after the addition of 0.1 μg of u-PA/ml ($n=3$); ■, after addition of 0.1 μg of u-PA/ml together with a 100-fold molar excess of t-PA ($n=5$). There appears to be no significant effect of excess u-PA on the clearance of t-PA and vice versa.

hepatocytes is known to be highly temperature-dependent [23]. Therefore our finding that the t-PA fraction irreversibly bound to the cells increases with higher temperatures strongly argues for an internalization of bound t-PA (Table 1) and confirms the results of the other groups. In view of their structural similarities, one might expect a common hepatic receptor system for t-PA and u-PA. However, our competitive-binding studies revealed different and independent binding sites for ^{125}I -t-PA and ^{125}I -u-

Fig. 4. Time course of mean ($\pm$ S.D.) plasma concentrations in rabbits after infusion of t-PA or u-PA

(a) Competition of the t-PA clearance with excess u-PA: $\bullet$, plasma concentration-time profile after administration of t-PA alone ($n = 3$) or ($\square$) in the presence of 51-fold higher plasma levels of u-PA ($n = 3$); $\blacktriangle$, plasma levels observed for the excess u-PA. (b) Competition of the u-PA clearance with excess t-PA: $\bullet$, plasma concentration-time profile after administration of u-PA alone ($n = 3$) or ($\square$) in the presence of 33-fold higher plasma levels of t-PA ($n = 3$); $\blacktriangle$, plasma levels observed for the excess t-PA. No significant effect of excess u-PA on the clearance of t-PA and vice versa is apparent.

PA. Apparently the growth-factor and kringle structures, which are found with a high degree of sequence similarity in both t-PA and u-PA, do not represent common and/or sufficient recognition sites for the cellular uptake and degradation of these proteins in the liver.

Our results derived from the 'in vitro' systems were confirmed in intact livers and whole animals. u-PA has been reported to exhibit a longer half-life *in vivo* than does t-PA [5-7]. In agreement with these findings we observed much higher clearance rates for t-PA as compared with u-PA in isolated perfused rat livers and *in vivo* in rabbits. The lack of competition between the two proteins for removal from perfusate and plasma again implies the existence of different hepatic degradation mechanisms. From the data available at present, the high-affinity uptake system for t-PA might consist of one, or even different, type(s) of specific hepatic receptors. Fuchs *et al.* [4] indicated that,

in mice, t-PA and u-PA are cleared by similar processes, but the hepatic clearance of ^{125}I -t-PA was not altered in the presence of a 10000-fold molar excess of unlabelled di-isopropylphospho-modified u-PA. Nevertheless, the possible existence of a common hepatic receptor for both t-PA and u-PA has so far only been investigated on Novikoff hepatoma cells, and a distinct receptor was found for each activator [28]. The K_d values reported for binding of t-PA to hepatoma cell lines were 10 nM for Novikoff cells [28] and 3.7 nM for Hep G2 cells [29]. Although these values seem to correlate well with our value calculated for the specific binding to primary hepatocytes, this comparison should be made with caution, since transformation of the cells may have altered the affinity or number of their receptors [32]. In contrast with our results with primary hepatocytes, in which the K_d was > 300 nM, Nguyen & Kruithof [28] found a significantly higher-affinity binding of ^{125}I -u-PA to Novikoff hepatoma cells (K_d 1 nM).

It recently became evident that fibroblasts, macrophages and spermatozoa, as well as a variety of transformed cells, express surface receptors for u-PA [32]. For human blood monocytes and cells of the monocyte line U937, these receptors were shown not to interact with t-PA [33]. Protein bound to these surface receptors was neither internalized nor degraded, and retained its enzymic activity, indicating that these u-PA receptors must be different from binding sites for t-PA or u-PA on hepatocytes and serve exclusively in providing the cells with a surface-associated proteolytic potential. This pericellular proteolytic activity derived from bound u-PA facilitates cell migration in general [33,34], which in the case of some tumour cells will result in a selective advantage for invasion of neighbouring tissues [33] and increase their metastatic potential [32,35]. Cultured endothelial cells (HUVEC) bind t-PA and thereby also acquire surface-located plasminogen-activator activity [36]. Upon binding of t-PA to HUVEC an increase in its catalytic efficiency, but no internalization was reported. Among a variety of proteins tested, u-PA competed specifically for this binding, indicating that the receptors involved in the hepatic catabolism of t-PA are not identical with its binding sites on HUVEC.

Both u-PA in its high-molecular-mass form [37] and the two naturally occurring glycosylation variants of t-PA [38] contain oligosaccharide structures, including glucosamine and galactosamine residues. t-PA in addition contains a high-mannose carbohydrate moiety that is responsible for part of the t-PA clearance via the mannose-specific receptor on liver endothelial cells [22,24]. Removal of the high-mannose oligosaccharides resulted in reduced clearance, whereas a variant with high mannose at all three glycosylation sites was cleared more rapidly than was native t-PA [25,39]. The exact role of the oligosaccharide structures at Asn¹⁸⁴ and Asn⁴⁴⁸ in t-PA and at Asn³¹² in u-PA in their hepatic clearance remains unclear. Smedsrod *et al.* [22], from competition experiments in cultures of separated liver parenchymal and endothelial cells, proposed that galactose-specific receptors are responsible for the uptake of t-PA in parenchymal cells. According to their hypothesis, u-PA, containing glucosamine and galactosamine residues, could compete for the binding of t-PA to parenchymal cells. Our experiments appear to rule out this possibility, since a high molar excess of u-PA failed to reduce the clearance of t-PA significantly. In agreement with these 'in vivo' data, we found no competition up to concentrations of $1\text{ }\mu\text{M}$ of the monosaccharides galactose and glucose in our binding experiments using primary hepatocytes and hepatocyte plasma membranes, whereas completely deglycosylated t-PA was able to compete efficiently (results not shown). The binding of t-PA and u-PA to Novikoff hepatoma cells was also reported to be unaffected by sugars [28].

Despite their structural similarity, t-PA and u-PA differ biochemically and immunochemically and fulfil different

physiological functions. A common receptor for both enzymes would remove t-PA and u-PA regardless of the differences in their physiological regulation and primary functions. The results obtained in the present study are the first conclusive evidence that their removal from circulation indeed involves different routes of hepatic catabolism. In conclusion, our data confirm the existence of a specific hepatic t-PA receptor which is responsible for uptake into parenchymal cells and is different from cellular sites involved in the physiological degradation of u-PA. This receptor-mediated uptake system constitutes the protein-structure-dependent part of the overall elimination of t-PA and must be different from the receptors on various cell types described for plasminogen activators so far.

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