The purification and characterization of a glomerular-basement-membrane-degrading neutral proteinase from rat mesangial cells

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A neutral proteinase, capable of degrading gelatin, has been found in both an active and a latent form in the medium from the culture of rat mesangial cells. The latent form had an M_r of 80 000–100 000 and could be activated with either 4-aminophenylmercuric acetate or prolonged incubation at neutral pH. The active form of the enzyme was extensively purified. The estimated M_r of the purified enzyme on gel filtration was ~ 200 000, indicating that the active enzyme formed aggregates. However, analysis by SDS/polyacrylamide-gel electrophoresis under reducing conditions showed two protein bands, with M_r 68 000 and 66 000. Both proteins were found to contain proteolytic activity when run on SDS/substrate gels. The enzyme was inhibited by EDTA and 1,10-phenanthroline, but not by inhibitors for cysteine, serine or aspartic proteinases. The enzyme did not digest fibronectin, bovine serum albumin, proteoglycan or interstitial collagen. The enzyme degraded pepsin-solubilized placental type V collagen at 31 °C, whereas similarly solubilized type IV collagen was only degraded at higher temperatures. In addition, the neutral proteinase degraded native soluble type IV collagen. It also had activity on insoluble type IV collagen of glomerular basement membrane. The above properties suggest that the mesangial neutral proteinase belongs to the gelatinase group of metalloproteinases and that it may play a role in the normal turnover of extracellular glomerular matrix.

INTRODUCTION

It is now accepted that the turnover of tissue collagens and extracellular matrices is due to the combined actions of several enzymes [1,2]. In most connective tissues the initial cleavage of type I and III collagens is accomplished by a specific interstitial collagenase, and the fragments produced by these enzymes spontaneously denature at body temperature and are further degraded by enzymes with gelatinase activity [3].

Although there is evidence that the structural components of the renal glomerulus are turned over [4], the degradative mechanisms involved remain largely unknown. This is in part due to the fact that interstitial collagens are not found within normal glomeruli, which contain significant amounts of type IV and V collagen [5]. These collagen types are resistant to the action of interstitial collagenases [3]. There is evidence that specific gelatinases possess activity against these substrates [6–11]. In addition, there have been reports of cells that secrete enzymes which are capable of specifically degrading both type IV and V collagens [12,13]. Studies in our laboratories suggest that at least two distinct groups of endogenous proteinases may be involved in glomerular-basement-membrane (GBM) turnover (for review see [14]). The first consists of the lysosomal acid proteinases, of which cathepsins B and L have been isolated and characterized. The second is a neutral proteinase produced in serum-free culture by rat mesangial cells. Lovett *et al.* [15] have reported that a mesangial-cell-derived

proteinase degrades GBM, as demonstrated by the release of soluble hydroxyproline-containing peptides. The enzyme responsible appeared to be a metalloproteinase, but was not characterized in detail. In the present study we have purified to apparent homogeneity rat mesangial-cell neutral proteinase, with [14 C]gelatin as a substrate, and show that this enzyme closely resembles the gelatinase and collagenase(s) specific for types IV and V collagens derived from other cellular sources. The possible significance of this proteinase in the breakdown of GBM in the glomerulus is considered.

MATERIALS AND METHODS

Materials

[14 C]Acetic anhydride (19.7 mCi/mmol) and Amplify were from Amersham International (Amersham, Bucks., U.K.). Chromatographic media and calibration kits were obtained from Pharmacia (Uppsala, Sweden). Minimal essential medium was from Northumbria Biologicals Ltd. (Cramlington, Northumberland, U.K.). Rabbit anti-(human type IV collagen) IgG was generously given by Dr. G. Raugi, VA Medical Center, Seattle, WA, U.S.A. Rat serum fibronectin was kindly provided by Dr. R. Davies, Llandough Hospital, Cardiff, Wales, U.K.

Methods

The procedure used for establishing in culture and identifying homogeneous populations of rat glomerular

Abbreviation used: GBM, glomerular basement membrane.

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mesangial cells has been described by us in detail previously [15,16]. The cultures were maintained in minimal essential medium with 2 mM-L-glutamine, 10 μ g of transferrin/ml, 0.6 i.u. of insulin/ml, 100 i.u. of penicillin/ml, 100 μ g of streptomycin/ml and 20 % (v/v) foetal-calf serum.

Production of mesangial-cell serum-free culture medium

At confluence, the cells were washed extensively with Dulbecco's phosphate-buffered saline and further cultured for up to 5 days in serum-free minimum essential medium containing 0.2 % (w/v) lactalbumin hydrolysate. The conditioned medium was collected, centrifuged at 1000 *g* for 5 min, and the supernatant was used immediately or stored at -20°C until required. The adhered cells were lysed with 0.2 % (w/v) Triton X-100 for 10 min at room temperature and stored at -20°C .

Preparation of collagen types

Interstitial collagen (approx. 95 % type I and 5 % type III) was isolated from rat skin and radiolabelled with [^{14}C]acetic anhydride as described by Cawston & Barrett [17]. The labelled collagen was diluted with non-labelled collagen to give a final solution containing 1 mg/ml (sp. radioactivity 1×10^5 d.p.m./mg). Pepsin-solubilized type IV and type V collagens were prepared from human placenta by the methods of Sage *et al.* [18] and Madri [19] respectively. GBM was prepared as described previously [20]. Hydroxyproline was quantified by the method of Bergman & Loxley [21].

Column chromatography

Gel chromatography was carried out on a calibrated Sephacryl S-200 column equilibrated with 50 mM-Tris/HCl, pH 7.4, containing 50 mM-NaCl, 10 mM- CaCl_2 , 0.05 % (w/v) Brij 35 and 0.05 % (w/v) NaN_3 (buffer A). Ion-exchange chromatography was on DEAE-cellulose equilibrated in 10 mM-Tris/HCl, pH 8.8, containing 5 mM- CaCl_2 , 0.05 % Brij 35 and 0.05 % NaN_3 (buffer B).

Enzyme assays

The culture medium and purified preparations were examined for interstitial collagenase activity by using a diffuse-fibril assay [17]. Gelatinase activity was assayed essentially by the method described by Murphy *et al.* [22], except that [^{14}C]acetylated collagen prepared above was dialysed against 50 mM-Tris/HCl, pH 8.0, containing 200 mM-NaCl and 0.05 % NaN_3 for 24 h at 4°C and then denatured before its use by incubation at 60°C for 45 min. Where necessary, the gelatinase was fully activated by the inclusion of *p*-aminophenylmercuric acetate in the incubation mixture at a final concentration of 0.7 mM [23]. Casein-degrading activity was assayed with [^3H]acetylated casein [24]. One unit of enzyme activity is defined as that amount of enzyme which degrades 1 μ g of substrate/h under the given assay conditions.

Protein determination

Protein was estimated by the procedure of Lowry *et al.* [25], with bovine serum albumin as standard.

Breakdown of native type IV collagen

Conditioned medium from cultured human mesangial cells radiolabelled with [^3H]glycine and [^3H]proline was generously given by Dr. Paul Killen, NIH, Bethesda,

MD, U.S.A. SDS/polyacrylamide-gel electrophoresis and fluorography revealed the presence of pro- $\alpha 1$ and pro- $\alpha 2(\text{IV})$ collagens, in addition to several other matrix proteins. For assay, 40 μ l of labelled mesangial-cell supernatants (approx. 10000 c.p.m.) were incubated with the purified neutral proteinase and assay buffer (50 mM-Tris/HCl, pH 7.2, containing 100 mM-NaCl, 5 mM- CaCl_2 and 0.05 % NaN_3) to give a final volume of 80 μ l. The incubation was carried out at 35°C for 48 h, after which time 10 μ l of rabbit anti-(human type IV collagen) IgG was added to each tube. The samples were further incubated at 4°C for 16 h. To each tube was then added 20 μ l of packed Protein A-Sepharose beads, and the tubes were rotated for 2 h at 4°C . The beads were then washed (six times) with 50 mM-Tris/HCl, pH 7.2, containing 0.15 M-NaCl, 1 mM-EDTA, 1 % (w/v) SDS and 1 % Triton X-100, to remove all non-bound proteins. Thereafter, the beads were heated for 3 min at 95°C in double-strength SDS/polyacrylamide-gel-electrophoresis sample buffer and run on 6 %-acrylamide gels containing 2 M-urea. The gels were fixed, soaked with Amplify, and the ^3H -labelled proteins were detected by autoradiography by using pre-flashed Kodak X-O-Mat film with intensifying screens. Exposed autoradiograms were assessed by densitometry under linear conditions. The decrease in antibody-precipitable labelled type IV collagen was used to quantify the degree of neutral-proteinase-induced degradation.

Polyacrylamide-gel electrophoresis

Electrophoresis was performed in SDS on vertical slab gels by the procedure of Laemmli [26]. For the separation of collagens 2 M-urea was included in both the gel and reservoir buffers, and the bands were detected by using Coomassie Brilliant Blue R-250 or with a silver stain [27]. For analytical SDS/polyacrylamide-gel electrophoresis of the purified neutral proteinase, the proteins were run on a continuous gradient gel of 7–16 % acrylamide, fixed, and stained with AgNO_3 . SDS/substrate gels were prepared by including type I gelatin in the standard Laemmli [26] acrylamide-polymerization mixture at a final concentration of 1 mg/ml. The samples were solubilized in non-reducing sample buffer and electrophoresed at 7°C , at 25 mA/gel. After electrophoresis the gels were washed in cold 50 mM-Tris/HCl buffer, pH 8.0, containing 50 mM-NaCl, 10 mM- CaCl_2 and 0.05 % Brij 35. The gels were then incubated overnight at 37°C in fresh Tris buffer. Zones of lysis were detected by using Coomassie Brilliant Blue R-250. Analytical isoelectric focusing was performed on 0.4 mm-thick polyacrylamide slabs [28], which were stained with AgNO_3 . pI values were determined by comparison with standard proteins.

RESULTS

Secretion of rat mesangial-cell neutral proteinase

The culture of rat mesangial cells in serum-free medium resulted in the appearance of gelatin-degrading activity in the supernatants. On day 1 of the culture, the total enzyme content (0.72 unit/ml; s.d. ± 0.51 ; $n = 4$) was present in a latent form. On further culture the proportion of active to latent enzyme increased, and, by day 5, 80 % of the total enzyme (7.91 units/ml; s.d. ± 0.79 ; $n = 4$) was present in an active form. The latent enzyme could be activated with either trypsin or *p*-amino-

Table 1. Purification of mesangial-cell gelatinase

Concentrated serum-free medium was incubated with 4-aminophenylmercuric acetate (0.7 mM) and chromatographed on different matrices as indicated in the Results section. Fractions were assayed for degradation of gelatin.

Stage	Gelatin-degrading activity (total units)	Sp. activity (units/mg of protein)	Recovery (%)	Purification (fold)
Crude starting material	9780	11.2	100	1
YM-10 filter	8160	55.9	83	5
Sephacryl S-200	4020	136.3	41	12.2
Concanavalin A-Sepharose	2329	233	24	20.5
DEAE-cellulose	1466	293	15	34.7
Sephacryl S-300	1022	444	10.4	39.7

Table 2. Effect of potential inhibitors on purified gelatinase activity of rat mesangial cells

Each potential inhibitor was dissolved in 50 mM-Tris/HCl/50 mM-NaCl/5 mM-CaCl₂ adjusted to pH 7.4 and incubated with purified enzyme for 5 min at 25 °C. Thereafter [¹⁴C]gelatin substrate was added and the remaining enzymic activity quantified. Results are shown as percentage of the activity in the absence of inhibitor. Each value represents the mean of triplicate determinations.

Compound	Final concn.	Activity (%)
None	—	100
1,10-Phenanthroline	2 mM	2
EDTA	10 mM	1
EGTA	10 mM	1
EDTA + Zn ²⁺ (10 mM)	10 mM	80
Cysteine	1 mM	67
	10 mM	27
Dithiothreitol	1 mM	54
	10 mM	4
N-Ethylmaleimide	10 mM	69
Phenylmethanesulphonyl fluoride	1 mM	100
Soya-bean trypsin inhibitor	1 mg/ml	100
Pepstatin	10 µg/ml	100
Leupeptin	1 µM	100

phenylmercuric acetate [23] and by prolonged incubation at 37 °C in assay buffer alone. The latter result may partially explain the predominance of active enzyme in the culture medium after 5 days. The cell lysates did not contain significant amounts of neutral proteinase activity.

Separation of active and latent gelatinolytic activity

From a calibrated Sephacryl S-200 column, the latent enzyme was eluted with an apparent M_r of 80 000–100 000. The fractions containing the latent enzyme were subsequently pooled, activated with *p*-aminophenylmercuric acetate and rechromatographed on the same column. The majority of the activated enzyme was eluted with an M_r of 55 000–65 000. However, a smaller amount of the activated enzyme (15%) appeared in a high- M_r form. In contrast, when the medium from day 5 of the culture was concentrated and chromatographed on the same Sephacryl column, most of the enzyme was eluted with an apparent M_r in excess of 200 000. These

Fig. 1. Gelatin-substrate gel electrophoresis of purified mesangial neutral proteinase

Purified enzyme was electrophoresed under non-reducing conditions on SDS/7% polyacrylamide gels containing 1 mg of gelatin/ml as detailed in the Materials and methods section.

observations suggest that, once activated either in culture or with *p*-aminophenylmercuric acetate, the mesangial-cell gelatinase has a tendency to form higher- M_r aggregates.

Purification of the mesangial-cell gelatinase

Because the amounts of the wholly latent form of the gelatinase were low, the enzyme was purified from

Fig. 2. Analytical SDS/polyacrylamide-gel electrophoresis of purified mesangial neutral proteinase

The purified enzyme was electrophoresed on a 7–16% acrylamide continuous gradient gel, followed by silver staining. Lane (a), standard proteins; lane (b), mesangial neutral proteinase.

conditioned medium obtained from days 3–5. The enzyme was fully activated by treatment with *p*-aminophenylmercuric acetate, concentrated 50-fold by using an Amicon ultrafiltration cell fitted with a YM-10 membrane, and then chromatographed on a Sephacryl S-200 column equilibrated in buffer A. The fractions containing the gelatinolytic activity which was eluted with $M_r > 200\,000$ were pooled and applied to a column of concanavalin A–Sephrose equilibrated in buffer A. Most of the enzyme bound to this column, and was eluted with 300 mM- α -methyl mannoside. The eluted enzyme was dialysed against buffer B and chromatographed on DEAE-cellulose equilibrated with the same buffer. After removal of some unbound inactive protein, bound proteins were eluted with a linear gradient of 0–200 mM-NaCl in the start buffer. The gelatinolytic activity was eluted between 60 and 150 mM-NaCl. The enzyme was then dialysed against buffer A and re-chromatographed on a Sephacryl S-300 column in the same buffer. Table 1 summarizes the purification steps. The final material demonstrated a specific gelatinase activity of 444 units/mg of protein, with a recovery of approx. 10.4%. It also contained 19.7 units of casein-degrading activity/mg.

Inhibition of purified rat mesangial-cell gelatinase

The gelatinase was completely inhibited by EDTA or 1,10-phenanthroline (Table 2). Partial inhibition was

Table 3. Degradation of native type IV collagen by mesangial gelatinase

Details are given in the Materials and methods section. This Table shows the decrement in antibody precipitable type IV collagen at the end of a 48 h incubation period with increasing amounts of enzyme. The results are expressed in cm^2 , and were calculated from densitometric readings obtained from fluorograms. Data presented are means $\pm$ S.D. of triplicate determinations of single experiments.

Enzyme (unit)	Immunoprecipitable type IV collagen
–	4.86 ± 0.65
0.04	3.54 ± 0.42
0.08	2.64 ± 0.27
0.16	2.34 ± 0.32
0.32	1.80 ± 0.21

achieved with thiol compounds such as dithiothreitol and cysteine. The inhibitory effect of EDTA could be reversed by the inclusion of Zn^{2+} . Inhibitors of aspartate, cysteine or serine proteinases were without effect. These observations demonstrate that the purified gelatinase is a metalloproteinase.

Homogeneity and M_r determination

In SDS/gelatin substrate gel under non-reducing conditions as outlined in the Materials and methods section, two major zones of gelatin lysis were observed (Fig. 1), corresponding to M_r values of 68 000 and 66 000. In addition, the purified gelatinase when electrophoresed on a 7–16% acrylamide continuous gradient gel also demonstrated two closely migrating proteins, with apparent M_r values of 68 000 and 66 000 (Fig. 2). By using an antibody prepared against the M_r -68 000 enzyme, both the M_r -68 000 and -66 000 enzymes were specifically stained on a Western blot and recovered by specific immunoprecipitation techniques. In addition, the aggregated form of the purified enzyme was identified with the above antibody by using a dot-blot assay.

On analytical isoelectric focusing, the purified gelatinase displayed some expected charge microheterogeneity, with a prominent component at pI 8.1.

Substrate specificity

The purified enzyme showed minimal activity against casein and did not hydrolyse bovine serum albumin or labelled interstitial collagen in a diffuse-fibril assay. An important finding was that the purified enzyme did not degrade serum fibronectin or a ^{35}S -labelled chondroitin sulphate-rich proteoglycan isolated from cultured human glomerular epithelial cells incubated with $\text{Na}_2^{35}\text{SO}_4$ (M. Davies, G. Thomas & R. M. Mason, unpublished work).

Fig. 3 shows that pepsin-solubilized type IV collagen when incubated with the purified enzyme was extensively degraded at 37 °C, but not at temperatures below 34 °C. Similarly, the enzyme was capable of degrading pepsin-solubilized type V collagen at 37 °C. In addition, a small degree of degradation was noted at 31 °C. The action of the enzyme in each case was completely inhibited by the inclusion of either 10 mM-EDTA or 2 mM-1,10-phenanthroline.

Fig. 3. Degradation of soluble type IV and V basement-membrane collagens by mesangial neutral proteinase

(a) Soluble type IV collagen (50 µg), prepared by pepsin extraction of human placenta, was incubated in a total volume of 50 µl for 20 h: (i) with buffer alone; (ii)–(iv) with mesangial neutral proteinase (0.5 unit), (ii) at 37 °C and including 20 mM-EDTA, (iii) at 31 °C, (iv) at 37 °C.

Further experiments were carried out with bio-synthetically labelled pro- α 1 and pro- α 2(IV) collagen derived from cultured human mesangial cells. The results are shown in Table 3, and demonstrate that the mesangial neutral proteinase can degrade at 35 °C native type IV collagen in a concentration-dependent fashion.

The mesangial gelatinase at 37 °C also released soluble hydroxyproline-containing peptides from GBM in a time- and enzyme-concentration-dependent manner (3.5 ng/h per enzyme unit). As type IV is the major collagen constituent of GBM [29], this assay is essentially a measure of the degradation of an insoluble form of this collagen type. Analysis of the soluble products by Sephacryl S-200 gel chromatography showed two peaks of hydroxyproline; the first appeared in the void volume and the second with an apparent M_r of 25 000. This was taken as evidence that the collagen in the basement membrane was both solubilized and degraded by the action of the mesangial gelatinase.

DISCUSSION

This work establishes that cultured rat mesangial cells synthesize and secrete a neutral proteinase with gelatinase activity. The enzyme is secreted in a latent form and appears to undergo activation during prolonged culture. In addition to activity as a gelatinase, the neutral proteinase is capable of degrading insoluble type IV collagen from GBM as well as native type IV procollagens. Furthermore, pepsin-solubilized type IV and V collagens were also susceptible to degradation. The neutral proteinase has no demonstrable activity against interstitial collagens, proteoglycan or fibronectin.

Extensive purification of the mesangial neutral proteinase was achieved in relatively few steps. This was primarily due to the use of serum-free medium as the starting material. The purification protocol utilized two gel-permeation steps normally employed for the separation of globular proteins with relatively high M_r values. The elution pattern of the gelatinase activity suggests that the formation of higher- M_r aggregates can occur under the conditions of purification used in this study. Analytical SDS/polyacrylamide-gel electrophoresis under reducing conditions, in which these aggregates are broken up, allowed for estimation of the actual M_r of the gelatinase as 68 000 and 66 000. Immunological studies also indicated that the aggregated and the low- M_r forms are the same enzyme.

The biochemical properties of the mesangial neutral proteinase have much in common with a group of previously described metalloproteinases that degrade basement membranes, but not interstitial collagens or casein. Some differences exist between these proteinases with regard to M_r , latency and substrate specificity. On the basis of substrate specificity, particularly towards

The products were analysed by electrophoresis on an SDS/polyacrylamide (6 %) gel under reducing conditions and the gel was stained with Coomassie Brilliant Blue. (b) Soluble type V collagen (20 µg) from human placenta was incubated in a total volume of 50 µl for 20 h: (i) with buffer alone; (ii)–(iv) with mesangial neutral proteinase (0.5 unit), (ii) at 37 °C and including 20 mM-EDTA, (iii) at 31 °C, (iv) at 37 °C. The products were analysed on a 6 %-polyacrylamide gel as above. The mobilities of type I collagen β - and α -chains are indicated as M_r markers.

type IV and type V collagens, these matrix-degrading enzymes fall into three groups. The first consists of enzymes with the ability to degrade both type IV and V collagens. These proteinases include enzymes isolated from human neutrophils [8,10], the culture medium of rabbit bone [9], human skin [7] and human synovium [6]. All are secreted in an inactive form. In addition, they readily digest gelatin and consequently are often referred to as gelatinases. The rabbit bone enzyme has been extensively purified; it has low activity towards casein and elastin and did not degrade proteoglycan [9]. The second enzyme group consists of metalloproteinases that digest specifically type IV collagen, and which have been identified in several tumour cell lines [11,30]. These enzymes do not cleave types I, II, III or V collagens or laminin. A similar enzyme is also secreted by human mononuclear phagocytes [12]. These enzymes cleave pro- $\alpha 1(\text{IV})$ and pro- $\alpha 2(\text{IV})$ collagen at a single point to form two pairs of degradation fragments, corresponding in M_r to about two-thirds and one-third of the original chains. The last group consists of collagen-type-V-degrading metalloproteinases identified in a murine sarcoma [31] and rabbit alveolar macrophages [13,32] and which do appear to degrade type IV collagen. It is not recorded whether these enzymes degrade gelatin.

The mesangial gelatinase digested two forms of soluble type IV collagen and was able to solubilize hydroxyproline from isolated GBM, which consists primarily of type IV collagen. The characteristics of the products of digestion released from all three collagen substrates did not suggest cleavage at a single point within the type IV chain, but rather suggest extensive degradation at multiple sites. Type IV collagen appears to be particularly susceptible to digestion by proteinases [33]. This has been attributed to the presence of non-collagenous sequences within the triple helix of the molecule. Thus pepsin [33], neutrophil lysosomal elastase [20], trypsin [33] and cathepsin B [20] all release similar-size hydroxyproline fragments from insoluble GBM substrates. On the basis of these prior observations, it would appear that the mesangial-cell neutral proteinase represents a gelatinase which closely resembles those enzymes described in the first group above.

Neutral metalloproteinases are usually secreted from cells in culture in a latent form. However, little is known about their activation *in vitro* or *in vivo*. Activators suggested to date include an endogenous activator of procollagenase present in rabbit synovial-fibroblast culture medium [34], plasminogen activator [35] and a low- M_r angiogenesis factor [36]. At present we have no information as to whether similar physiological activators exist for the mesangial-cell enzyme.

Degradation of the extracellular matrix is currently viewed as a multi-step process involving the co-ordinate action of a series of enzymes. This scheme details the initial cleavage of collagen substrate by highly specific enzymes, a process followed by the action of the less specific stromelysin and gelatinase enzymes [1,3]. The glomerulus has as its key extracellular matrix elements, the GBM and the mesangial matrix, and similar mechanisms presumably account for the normal catabolism of these structures [4,37]. Glomerular diseases associated with damage to the GBM or with excessive accumulation of matrix may be due to alterations in this normal matrix catabolic process. Mesangial cells are centrally involved in the pathogenesis of many forms of

glomerular disease, a fact attributable in part to the large number of inflammatory mediators secreted by this cell type [38]. The present work has provided evidence that cultured mesangial cells are capable of secreting an enzyme with substrate specificities and properties relevant to the catabolism of the glomerular extracellular matrix. Preliminary studies using specific antibodies have demonstrated the presence of the mesangial neutral proteinase within the mesangial area of normal glomeruli (J. Martin, M. Davies & D. H. Lovett, unpublished work), indicating that the present observations are not limited to cultured cells. Studies are needed to define the quantity and distribution of the mesangial neutral proteinase in various glomerular diseases, and may be expected to provide further insights into the mechanisms of glomerular extracellular-matrix catabolism.

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