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Purification and Characterization of Arginine:Mono-ADP-Ribosylhydrolase from *Euglena gracilis* Z

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Arginine:mono-ADP-ribosylhydrolase was purified from a protozoan, *Euglena gracilis* Z, using [^{32}P]mono-ADP-ribosylated actin as a substrate. The enzyme showed molecular mass of 33 kDa both in SDS PAGE and gel filtration, indicating it to be a monomeric protein. It was strongly inhibited by ADP and ADP-ribose and activated by Mg^{2+}, DTT, and 2-mercaptoethanol. These results suggest that it recognizes the ADP-ribose moiety of the modified protein. Since the enzyme activity increased in S phase and late G_0 phase in a synchronous dividing culture, the enzyme may function in the regulation of the cell cycle.

Key words: ADP-ribosylhydrolase, cell cycle, *Euglena gracilis*, mono-ADP-ribosylation, NAD^+.

Mono ADP-ribosylation is involved in the posttranslational modification of cellular proteins during transfer of ADP-ribose from NAD^+ to various acceptor proteins (*1*). Some bacterial toxins, choleragen (*2*), botulinum C2 toxin (*3*), the heat-labile enterotoxin of *Escherichia coli* (*4*), and pertussis toxin (*5*), have catalytic activity for mono ADP-ribosylation of specific proteins of eukaryotic cells. The toxins modify different amino acids in the heterotrimeric GTP-binding and other proteins. For cholera and botulinum C2 toxin, pertussis toxin, botulinum C3 transferase, and diphtheria toxin, arginine (*4, 6*), cysteine (*7*), asparagine (*8*), and diphthamide (*9*), respectively, are modified with ADP-ribose. On the other hand, there are few reports on mono ADP-ribosylation in animals. NAD^+:arginine and NAD^+:cysteine ADP-ribosyltransferases modify arginine and cysteine residues in a target protein with ADP-ribose, respectively (*10*). ADP-ribosylation in animal cells is believed to be involved in regulation of some cell functions, although acceptor proteins have not been fully identified. Like other post-translational regulatory signals (*e.g.* phosphorylation), the modification of proteins appears to be reversible. ADP-ribosyl arginine hydrolase (*11*) and ADP-ribosyl cysteine hydrolase (*12*), which hydrolyze the bond between ADP-ribose and the amino acid residue, have also been identified in animal cells. An ADP-ribosylation cycle, composed of ADP-ribosyltransferase and hydrolase, has an important role in nitrogen fixation in the photosynthetic bacterium, *Rhodospirillum rubrum* (*13*). The dinitrogenase reductase which is involved in nitrogen fixation in the organism is controlled by reversible ADP-ribosylation. ADP-ribosylation inactivates the enzyme, and ADP-ribosylhydrolase regenerates it.

We have reported that *Euglena gracilis* Z exhibits two peaks of NAD^+: arginine mono ADP-ribosyltransferase activity during synchronous cell division; the first is in S phase and the later is in G_2-M transition (*14*). This means that reversible ADP-ribosylation has an important role in cell cycle progression in *E. gracilis*. In the present paper we describe the occurrence and characterization of ADP-ribosylhydrolase in *E. gracilis* and the physiological role of the enzyme in cell division.

MATERIALS AND METHODS

Organism and Culture—E. gracilis Z was grown on the medium of Koren and Hutner (*15*) at 26°C under illumination (6,000 lux). Late log phase cells were harvested by centrifugation at $3,000 \times g$ for 5 min. A synchronously dividing culture of *E. gracilis* was obtained at 20°C in 2 liters of Cramers and Myers' medium supplemented with vitamin B_1 (5 mg/liter medium) and B_{12} (5 μg/liter medium) (*16*). Illumination was provided by a cool-light fluorescent lamp (8,000 lux). Cell density was monitored every 2 h using a hemocytometer. A 24 h cell cycle could be entrained by imposing 14:10 light:dark cycles with a timer. Mitosis began at the onset of the dark and synchronous cell division was observed during the dark interval.

Preparation of [^{32}P]Mono-ADP-Ribosylated Actin—Actin was purified from rat brain (*17*) and its arginine residue was modified with [^{32}P]NAD^+ by mono-ADP-ribosyltransferase II purified by the method of Matsuyama and Tsuyama (*18*). The excess [^{32}P]NAD^+ was removed by gel filtration on a Bio-gel P2 column (1×50 cm) equilibrated with 10 mM imidazole-HCl (pH 7.4) containing 2 mM 2-mercaptoethanol and 1 mM $MgCl_2$. The modified protein was concentrated and used as a substrate for ADP-ribosylhydrolase. The specific radioactivity was about 15,000 cpm/μg actin (0.75-0.97 mol of ADP-ribose/mol of actin).

Preparation of Crude Enzyme—Cells were washed with

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10 mM imidazole-HCl buffer (pH 7.4) containing 10% propylene glycol and 2 mM dithiothreitol (DTT) and disrupted in the same volume of the buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM leupeptin, and 1 μM pepstatin A by sonication (10 kHz, 3 min). The supernatant obtained by centrifugation (10,000 $\times g$, 10 min) was used as a crude enzyme.

ADP-Ribosylhydrolase Assay—The reaction mixture (50 μl), containing 20 mM imidazole-HCl (pH 7.4), 2 μg of [^{32}P]mono-ADP-ribosylated actin, 1 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and enzyme, was incubated at 30°C for 30 min. After incubation the radioactivity that remained was measured by the method of Huang and Robinson (*19*) and released radioactive compounds were determined using high-performance liquid chromatography (HPLC). The reaction was stopped by the addition of the same volume of 5% perchloric acid and the mixture was centrifuged (10,000 $\times g$, 10 min). The supernatant was neutralized with 1 N KOH and centrifuged (10,000 $\times g$, 10 min). The supernatant was lyophilized and resuspended in 100 μl of 20 mM potassium phosphate buffer (pH 6.0) and subjected to HPLC analysis. The analytical column, Cosmosil $_5C_{18}$AR (4.6 $\times$ 150 mm) (Nacalai Tesque, Kyoto), was equilibrated with 20 mM potassium phosphate buffer (pH 6.0) at a flow rate of 0.5 ml/min, and then the concentration of acetonitrile was increased linearly to 30% over 30 min. The products of the mono-ADP-ribosylhydrolase reaction were quantified by absorbance measurement at 260 nm and by radioisotope detection (Beckman, Fullerton, CA, USA).

Purification of ADP-Ribosylhydrolase—Crude enzyme was loaded onto a DEAE Sepharose column (3 $\times$ 10 cm) equilibrated with 10 mM imidazole-HCl (pH 7.4) containing 10% propylene glycol, 2 mM DTT, and 1 mM PMSF (buffer A), and the column was eluted with 300 ml of a linear gradient of 0–1.5 M NaCl in the same buffer. The active fractions eluted at 1.2 M NaCl were collected and dialyzed against 2 liters of buffer A overnight. The fraction was loaded onto a DEAE Toyopearl 650 column (0.5 $\times$ 10 cm) equilibrated with buffer A and eluted with 10 ml of a linear gradient of 0–1.5 M NaCl in buffer A. The active fractions eluted at 1.2 M NaCl were collected and dialyzed against 500 ml of buffer A and loaded onto a AF-Blue Toyopearl 650 M column (0.5 $\times$ 5 cm) equilibrated with buffer A and eluted with 10 ml of a linear gradient of 0–2 M NaCl in buffer A. The activity was found in the fractions of 1.5 M NaCl. All operations were done at 4°C.

Determination of M_r by Gel Filtration and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—The M_r values of ADP-ribosylhydrolase were determined on a Superdex 200 HR 10/30 column equilibrated with 10 mM imidazole-HCl (pH 7.4) containing 200 mM NaCl, 2 mM DTT, and 1 mM PMSF. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (*20*).

Protein Determination—Protein concentration was determined using bovine serum albumin as a standard protein by the method of Bradford (*21*).

Reagent—[Adenylate-^{32}P]NAD$^+$ (800 Ci/mmol) was purchased from New England Nuclear. NAD$^+$ was from Oriental Yeast. Other reagents used were all of analytical grade.

RESULTS

Occurrence of ADP-Ribosylhydrolase in E. gracilis—To clarify the occurrence of ADP-ribosylhydrolase in *E. gracilis*, a cell homogenate was incubated with [^{32}P]mono ADP-ribosylated actin and the released radioactivity was analyzed by HPLC (Fig. 1). It was composed of two molecules, 5′-AMP and ADP-ribose. ADP-ribose was released by ADP-ribosylhydrolase and 5′-AMP was released by phosphodiesterase. The activities were disappeared when the homogenate was treated at 60°C for 5 min. These results indicate that ADP-ribosylhydrolase is present in *E. gracilis*.

Purification of ADP-Ribosylhydrolase from E. gracilis—It was necessary to separate ADP-ribosylhydrolase from phosphodiesterase to characterize its properties. These activities were found as two peaks on a DEAE Sepharose column chromatography (Fig. 2). The first peak was eluted at around 0.7 M NaCl and the second, at 1.2 M. The peaks were analyzed by HPLC to determine whether they contained ADP-ribosylhydrolase (Table I). The first contained 5′-AMP and the second contained ADP-ribose. These results mean that the first has phosphodiesterase activity (Peak I) and the second, ADP-ribosylhydrolase activity (Peak II), indicating that this column chromatography can separate ADP-ribosylhydrolase from phosphodiesterase.

The peak II fractions were collected and further purified

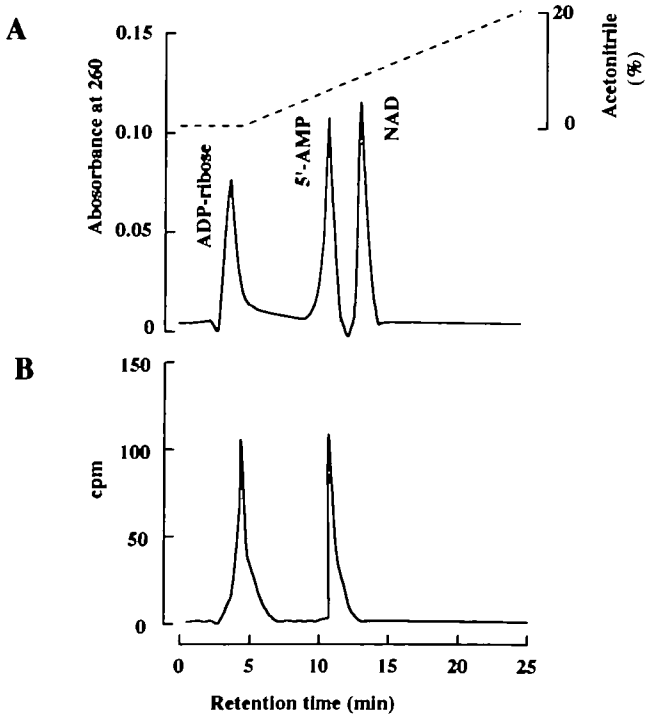

Fig. 1. **Determination of radioactive compounds released from [^{32}P]mono-ADP-ribosylated actin by HPLC.** The reaction was carried out and stopped by the addition of 5% PCA as described in "MATERIALS AND METHODS." The supernatant was lyophilized and then resuspended in 100 μl of potassium phosphate buffer. A 20 μl aliquot was analyzed by HPLC with a radioisotope detector. The elution profiles of authentic ADP-ribose, 5′ AMP and NAD were quantified by measuring the absorbance at 260 nm (A) and the elution of radioactivity was followed with a radioisotope detector (B).

by two steps of column chromatography as summarized in Table II. The enzyme was purified 3,300-fold over the crude extract of *E. gracilis* in a yield of 6.0%. SDS-PAGE of purified enzyme gave a single protein band of 33 kDa (Fig. 3). Gel filtration of ADP-ribosylhydrolase on a Superdex 200 10/30 column with several standard proteins indicated a molecular weight of 33,000 (data not shown), implying that the enzyme exists as a monomer in its native state.

Properties of ADP-Ribosylhydrolase—ADP-ribosylhydrolase, when preincubated at various temperatures for 10 min, was stable up to 35°C and the activity was completely lost at 50°C in 10 mM imidazole-HCl (pH 7.4) containing 10% (v/v) propyleneglycol and 2 mM DTT. When the pH stability was determined by using the purified enzyme preincubated at various pH values for 10 min at 30°C, the enzyme retained full activity between pH 6.0 and 8.0. The optimum pH and temperature were 7.0 and 30°C, respectively.

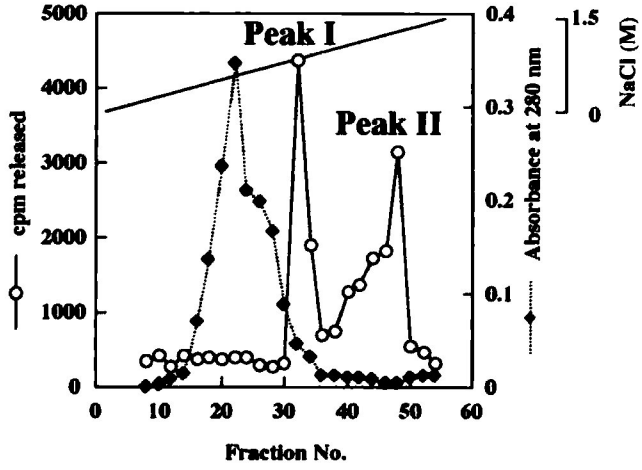

Fig. 2. **Elution profile of mono-ADP-ribosylhydrolase activity from a DEAE Sepharose column chromatography.** Homogenate of *E. gracilis* was applied to a DEAE Sepharose column equilibrated with buffer A. The column was washed with the same buffer and eluted with a linear gradient of NaCl. The gradient was started from fraction No. 1 and finished in fraction No. 50. Protein (◆) eluted was monitored by measuring absorbance at 280 nm and the radioactivity releasing activity (○) was measured as described in "MATERIALS AND METHODS."

TABLE I. **Determination of radioactive compounds released from [^{32}P]mono-ADP-ribosylated actin.** Ten microliters of each fraction was incubated with 2 μg of [^{32}P]mono-ADP-ribosylated actin in 100 μl of reaction mixture as described in "MATERIALS AND METHODS" for 30 min at 30°C. The reaction was terminated by adding the same volume of 5% perchloric acid. The supernatant was neutralized with KOH and subjected to HPLC. The number in parentheses shows a percentage of radioactivity recovered. This experiment was repeated three times with no significant differences. N.D., not detected (<30 cpm).

	Radioactivity (cpm)	
	5′ AMP	ADP-ribose
Peak I	4,708±231 (98)	N.D.
Peak II	N.D.	3,998±198 (95)

The reaction followed Michaelis-Menten kinetics toward mono ADP-ribosylated actin. The K_m value for ADP-ribosylated actin determined from double-reciprocal plots was 33 μM.

ADP-ribosylhydrolase was incubated with some metal ions for 10 min at 30°C and its activity was measured (Table III). At 1 mM, Mg^{2+} stimulated its activity 1.2-fold and Cu^{2+}, Co^{2+}, Ni^{2+}, and Ca^{2+} had no effect. Fe^{2+} inhibited

TABLE II. **Summary of the purification of mono-ADP-ribosylhydrolase from *E. gracilis*.** *E. gracilis* growing in late log phase was harvested and homogenized. The homogenate was centrifuged and the supernatant was used as the enzyme source. The purification was done as described in "MATERIALS AND METHODS" and specific activity was calculated by measuring the radioactivity of ADP-ribose released from the substrate using HPLC. In this table, a typical purification is shown.

	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Yield (%)	Purification (fold)
100,000×*g* sup	453.6	86	0.19	100	1
DEAE Sepharose	2.38	36	15.1	42.0	79
DEAE Toyopearl 650 M	0.205	20.4	99.5	23.8	523
AF-Blue Toyopearl	0.008	5.1	637.5	6.0	3,355

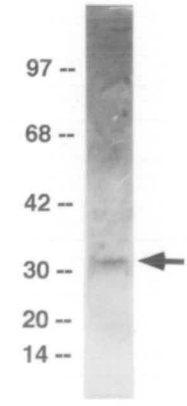

Fig. 3. **SDS PAGE of purified mono-ADP-ribosylhydrolase.** The purified enzyme (0.2 μg) was boiled with the same volume of SDS sample buffer for 2 min in boiling water and then loaded onto a 12.5% SDS polyacrylamide gel. After electrophoresis, the gel was stained with silver.

TABLE III. **Effect of divalent cations on the activity of purified mono-ADP-ribosylhydrolase.** The purified enzyme was incubated with 2 μg of [^{32}P]mono-ADP-ribosylated actin with or without various metal ions as described in "MATERIALS AND METHODS." The number in parentheses shows the percentage activity based on released radioactivity from the substrate. This experiment was repeated four times with similar results.

Metal ion	Radioactivity (cpm)
None	1,458±48 (100)
Ca^{2+}	1,385±37 (94)
Co^{2+}	1,447±24 (99)
Cu^{2+}	1,498±57 (102)
Fe^{2+}	1,207±75 (83)
Ni^{2+}	1,478±46 (101)
Mg^{2+}	1,844±44 (128)
Mn^{2+}	1,501±45 (103)
Zn^{2+}	1,419±58 (97)

TABLE IV. **Effect of some compounds on the activity of purified mono-ADP-ribosylhydrolase.** The purified enzyme was incubated with or without various compounds as described in "MATERIALS AND METHODS." The number in parentheses is the percentage activity based on radioactivity released from the substrate. This experiment was done in quadruplicate.

Compound	Radioactivity (cpm)
None	1,423±43 (100)
AMP	1,128±46 (79)
ADP	827±48 (58)
ATP	1,388±54 (97)
ADP-ribose	327±34 (23)
Ribose	1,406±53 (99)
NAD^+	1,421±65 (99)
Nicotinamide	1,434±32 (101)
Nicotinic acid	1,446±43 (102)

20% of the activity. Both 1 mM 2-mercaptoethanol and DTT increased the activity 1.3-fold, indicating that a sulfhydryl group is involved in the activity.

As shown in Table IV, ADP-ribose and ADP strongly inhibited its activity at 1 mM and the K_i values were 0.92 and 1.48 mM, respectively. AMP, ATP, and NAD^+ considerably inhibited the activity compared to ADP-ribose and ADP, indicating that the enzyme has affinity for ADP-ribose of modified protein.

Change of ADP-Ribosylhydrolase Activity during Synchronous Division of E. gracilis—The change of ADP-ribosylhydrolase activity was determined during synchronous cell division induced by a light-dark cycle. Homogenate prepared every 2 h was incubated with [^{32}P]mono ADP-ribosylated actin, and then [^{32}P]ADP-ribose released by the enzyme was measured by HPLC. During synchronous culture its activity increased in different phases, first at 10 h, corresponding to S phase, and secondly at 22 h, at the end of cell division (Fig. 4).

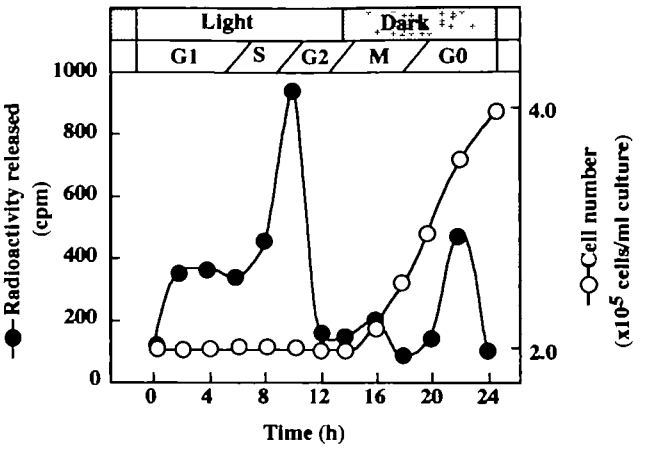

Fig. 4. **Change of mono-ADP-ribosylhydrolase activity in a synchronous culture induced by a light-dark cycle.** *E. gracilis* cultured autotrophically under continuous lighting was diluted 100 times with the same culture medium and exposed to a light:dark cycle of 14:10 (h : h). The synchronously dividing culture was maintained until the stationary phase was reached. The assay was carried at a cell density of around 1.0 to 2.0×10^5 cells/ml culture. The cell number (○) was monitored using a hemocytometer and the enzyme activity (●) was determined by measuring radioactivity of ADP-ribose released from the substrate using HPLC.

DISCUSSION

We have described the isolation and characterization of arginine:mono-ADP-ribosylhydrolase from a protozoan, *E. gracilis Z*. This enzyme is involved in a reversible mono-ADP-ribosylation system. We have reported that arginine:mono-ADP-ribosyltransferase activity is distributed in chloroplasts, mitochondria, microsomes, and cytosol and that the activity changes during the cell cycle progression (*14*). ADP-ribosylhydrolase was suggested to occur in the cell and to be heat-labile. *E. gracilis* contained two hydrolyzing activities as judged from the reaction products; one was phosphodiesterase and the other, mono-ADP-ribosylhydrolase (Fig. 1). The two enzymes were separated by a DEAE Sepharose column chromatography (Fig. 4 and Table I). It was evident that the activity eluted later was mono-ADP-ribosylhydrolase which was involved in the mono ADP-ribosylation cycle. Although mono-ADP-ribosyltransferase isozymes existed in each subcellular fraction, only one hydrolase was found in *E. gracilis* with a K_m value of 33 μM for ADP-ribosylated actin. Here it was shown that phosphodiesterase affected ADP-ribosyl actin as well as mono-ADP-ribosylhydrolase (Figs. 1 and 2 and Table I). This suggests that some proteins modified with ADP-ribose are cleaved by phosphodiesterase in the cell and that phosphoribose is left on them. If phosphodiesterase participates in intracellular events, another enzyme may remove phosphoribose on the modified protein for the reversible mono-ADP-ribosylation cycle.

It was reported that mono-ADP-ribosylhydrolase was purified from adrenal glands using mono-ADP-ribosylated actin and that it had a molecular mass of 61 kDa (*22*). Moss *et al.* also reported that two types of arginine:mono ADP-ribosylhydrolase occurred in mammalian tissues (*23*). Both types of enzyme showed the same molecular mass of 39 kDa in SDS-PAGE, but one showed a dependency on Mg^{2+} and DTT and the other did not. The enzyme purified from *E. gracilis* showed a molecular mass of 33 kDa in SDS-PAGE (Fig. 3). Although ADP-ribosylhydrolase from turkey erythrocytes, and rat and mouse brains was activated 5-fold or more by Mg^{2+} and DTT, their effect on the activity of the enzyme from *E. gracilis* was relatively small. These findings suggest that there are different families of mono-ADP-ribosylhydrolase.

As shown in Table IV, the purified hydrolase was strongly inhibited by 1 mM ADP-ribose and ADP, but AMP, ATP, NAD^+ had no effect on the hydrolase reaction. Inhibition of the enzyme activity was similar to that of mono-ADP-ribosylhydrolase of adrenal glands (*22*). These results indicate that this enzyme recognizes the ADP-ribose moiety of modified protein. Accordingly it is evident that the enzyme contains a binding site for ADP-ribose. The K_m value for ADP-ribosylated actin suggests that the enzyme has another mechanism to recognize acceptor protein modified with ADP-ribose, because the K_m value was 5 times higher than that of rat brain (*24*) and adrenal glands (*22*).

E. gracilis is convenient as a model organism to examine changes of biological substances during cell division (*25*). We have reported that mono-ADP-ribosylation in *E. gracilis* is found in the S phase and G_2-M transition, and mono-ADP ribosylation is arginine-specific (*14*). ADP-ribosylhydrolase exhibited two different peaks of activity

in a synchronous cell division cycle: the first corresponded to the S phase, and the other to the G_0 phase (Fig. 4). These results suggest that a mono-ADP-ribosylated protein modified in the S phase is turned over rapidly. In contrast, a protein modified in the G_2-M transition should be retained during cell division and then cleaved by the hydrolase. However, the physiological acceptor protein and role of mono ADP-ribosylation in *E. gracilis* are still unknown. Further examination is needed to establish the role of mono ADP-ribosylation in living cells and we propose that *E. gracilis* is a suitable model organism for this purpose.

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