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Phosphorylation of *Mycoplasma pneumoniae* Cytadherence-Accessory Proteins in Cell Extracts

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A cell-free system was used to characterize the phosphorylation of *Mycoplasma pneumoniae* proteins HMW1 and HMW2, which are involved in the adherence of this organism to human tracheal epithelium during infection. The pH and cation requirements for phosphorylation of HMW1 and HMW2 were determined, and the effects of glycolytic intermediates, cyclic AMP, and eukaryotic kinase-phosphatase inhibitors and stimulators on this process were examined. Phosphoamino acid analysis identified serine as the major phosphate acceptor for both HMW1 and HMW2 in this system.

While protein phosphorylation is widespread among numerous other prokaryotic species (3, 4, 20), little is known about phosphorylation in mycoplasmas. These organisms have very small genomes of 600 to 1,700 kbp (11) and are believed to have resulted from degenerate evolution of the *Lactobacillus* group of gram-positive bacteria (24, 25). Protein phosphorylation has been demonstrated in mycoplasmas (7, 16, 17), but it is not known how genome reduction may have affected genes encoding protein kinases and phosphatases or the role of protein phosphorylation in the regulation of cellular processes in these unusual prokaryotes.

We have begun to characterize protein phosphorylation in *Mycoplasma pneumoniae* (7), which causes bronchitis and atypical pneumonia in humans, particularly older children and young adults (5). We recently demonstrated (7) that *M. pneumoniae* cytadherence-associated proteins HMW1, HMW2, and HMW4 are phosphorylated at threonine and serine residues in an ATP-dependent manner, implying that a classical protein kinase-phosphatase system is functioning (HMW4 is a heat-modified form of HMW1 that appears during sample preparation [13] and is considered in this study combined with HMW1). The HMW proteins are required for localization of adhesin protein P1, and perhaps P30, to the *M. pneumoniae* attachment organelle (1, 12), a differentiated structure at one end of the mycoplasma cell (2). As components of a cytoskeleton-like structure (9, 15), the HMW proteins are thought to have an indirect scaffolding role in cytadherence (21, 22), and the phosphorylation state of HMW1 and HMW2 might regulate the dynamics of cytoskeleton interactions involving these proteins. Here we describe a cell-free technique that should allow us to characterize mycoplasma phosphorylation in a more manipulatable system, establishing pH, cation, and cofactor requirements and identifying potential allosteric regulators.

Virulent *M. pneumoniae* M129B17 or B18 and a noncytadhering derivative of M129 (12) lacking HMW1 and HMW2 (class I, isolate 2) were grown in Hayflick medium (10) and harvested in TN (0.15 M NaCl, 0.02 M Tris-HCl [pH 7.2]) as described previously (7). Cell pellets were used immediately or stored at −80°C. Several methods of obtaining cell lysates were compared, including Triton solubilization, digitonin treatment, osmotic shock, and freezing-thawing. The phosphorylation of mycoplasma proteins associated with cytadherence was generally very poor in all cases, with ^{32}P-phosphorylated HMW1 and HMW2 barely detectable. Rather, the radiolabel was associated at very high levels with three unidentified proteins of 54, 57, and 60 kDa (data not shown). This was surprising, given that the phosphoprotein pattern generated by metabolic labeling of *M. pneumoniae* with [^{32}P]H_3PO_4 is complex (Fig. 1).

Only when the Triton-soluble fraction was removed and phosphorylation was examined with the Triton-insoluble fraction was significantly greater labeling of additional proteins observed. Samples consisting of approximately 250 μg of cell protein (one-quarter of the yield from a 160-cm^2 flask) were extracted with Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) as described elsewhere (7). The Triton-insoluble fraction was suspended in 50 μl of 20 mM Tris-maleate (pH 6.0)–5 mM β-mercaptoethanol. [γ-^{32}P]ATP (30 μCi, 3,000 Ci/mmol; NEN Research Products, Boston, Mass.) was added. Some studies were also conducted with low-specific-activity [γ-^{32}P]ATP (30 Ci/mmol), as indicated below. Samples were incubated at 37°C, generally for 5 min but in some cases for periods ranging from 30 s to 15 min. Labeling was stopped by adding an equal volume of 2× gel loading buffer (200 mM Tris [pH 6.8], 4% sodium dodecyl sulfate [SDS], 40% glycerol, 8% β-mercaptoethanol, 0.04% bromophenol blue) and heating the mixture to 95°C for 5 min. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (14) and, in most cases, electroblotted (23) to polyvinylidene difluoride membranes (Bio-Rad, Hercules, Calif.) to reduce background levels of radioactivity (7). Blots or dried gels were exposed to Kodak X-AR film at −80°C for 2 to 7 days with an intensifier screen.

A comparison of the labeling profiles obtained during culture with $^{32}P_i$ and in a cell lysate with [γ-^{32}P]ATP and only the Triton-insoluble fraction is shown in Fig. 1A, lanes a and b, respectively. The profiles were similar, with the phosphorylation in cell lysates generally representative of that seen with metabolic labeling (7). In particular, bands corresponding to HMW1 and HMW2 in electrophoretic mobility were radiolabeled in the cell extract but to a lesser extent than that obtained with phosphorylation during culture. These bands were not observed when a variant lacking the HMW proteins was analyzed the same way (data not shown).

When [α-^{32}P]ATP was substituted for [γ-^{32}P]ATP, radiolabeling was <10% of that obtained with [γ-^{32}P]ATP (Fig. 1B, lanes a and b, respectively), suggesting that phosphorylation is the result of kinase-specific labeling rather than ATP binding, ADP-ribosylation, or nonspecific association of phosphate

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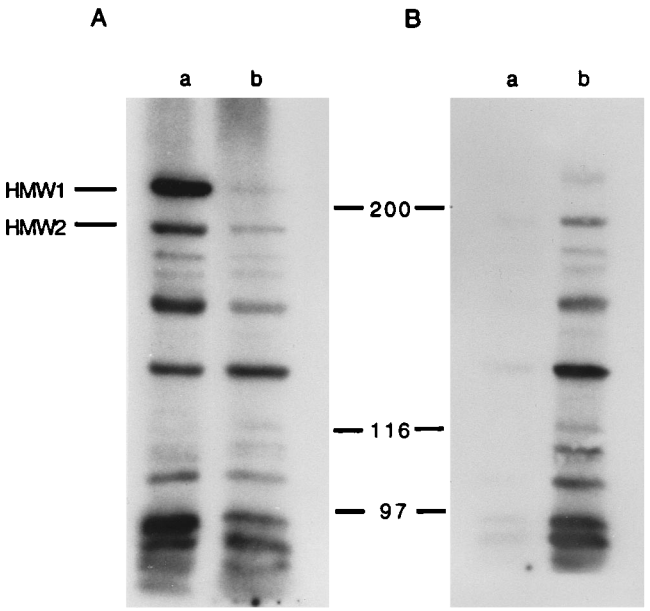

FIG. 1. Profile of Triton-insoluble *M. pneumoniae* proteins labeled in culture in the presence of [^{32}P]H_3PO_4 (lane a in panel A) or incubated in cell extracts with [γ-^{32}P]ATP (lane b in panels A and B) or [α-^{32}P]ATP (lane a in panel B). Proteins were separated by SDS-polyacrylamide gel electrophoresis, electroblotted to polyvinylidene difluoride membranes, and visualized by autoradiography. The positions of the protein standards are indicated in the center, and their molecular sizes are in kilodaltons.

with the proteins. Substitution with [γ-^{32}P]GTP as a phosphoryl donor yielded results similar to those obtained with [γ-^{32}P]ATP (data not shown). The phosphorylation patterns obtained with high- and low-specific-activity [γ-^{32}P]ATP were comparable, although the kinetics of radiolabeling was somewhat slower with the latter (data not shown).

M. pneumoniae protein phosphorylation patterns were similar between pHs 5.0 and 7.5 but decreased somewhat at pH 8.0, except for one unidentified protein of approximately 120 kDa that was phosphorylated only between pHs 6.5 and 7.5 (data not shown). On the one hand, the general lack of pH dependence was somewhat surprising, as Platt et al. (16, 17) reported a pH optimum of 5.5 to 6.0 for phosphorylation of 55- and 57-kDa proteins in *M. gallisepticum* and *Spiroplasma mellifera*, respectively, in cell lysates. However, Egan et al. have determined an intracellular pH of 7.1 for *M. gallisepticum* (8). In that study, the internal pH was maintained within 0.3 pH unit of 7.1 over an external pH range of 6.6 to 8.6. Thus, the pH range up to 7.5 reported here seems to be consistent with a neutral internal pH for mycoplasmas.

Many kinases require divalent cations for ATP binding. The effects of the cations Ca^{2+}, Mg^{2+}, and Mn^{2+} on incorporation of [γ-^{32}P]ATP into HMW1 and HMW2 were tested. $MgCl_2$ generally inhibited the phosphorylation of HMW1 and HMW2 in a concentration-dependent fashion over a range of 0.1 to 10 mM (Fig. 2A). Supplemental $MnCl_2$ also inhibited phosphorylation in this system (data not shown). The phosphorylation of most *M. pneumoniae* proteins in cell lysates was independent of the $CaCl_2$ concentration, although an increased radioactive background was often present and the electrophoretic mobility of some proteins, including HMW2, was altered in samples with $CaCl_2$ concentrations greater than 2 mM (Fig. 2B). Both HMW1 and HMW2 were phosphorylated at high levels without supplemental divalent cations. Maximum phosphorylation of HMW1 was observed with no Mg^{2+} and 10 mM Ca^{2+}. In contrast, HMW2 was phosphorylated at the highest levels when divalent cations were omitted. Addition of EDTA at concentrations ranging from 2 to 20 mM reduced but did not eliminate the phosphorylation of HMW1 and HMW2 (Fig. 3). In contrast, the phosphorylation of a 120-kDa protein was completely inhibited by 2 mM EDTA (Fig. 3). Addition of 20 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tet-

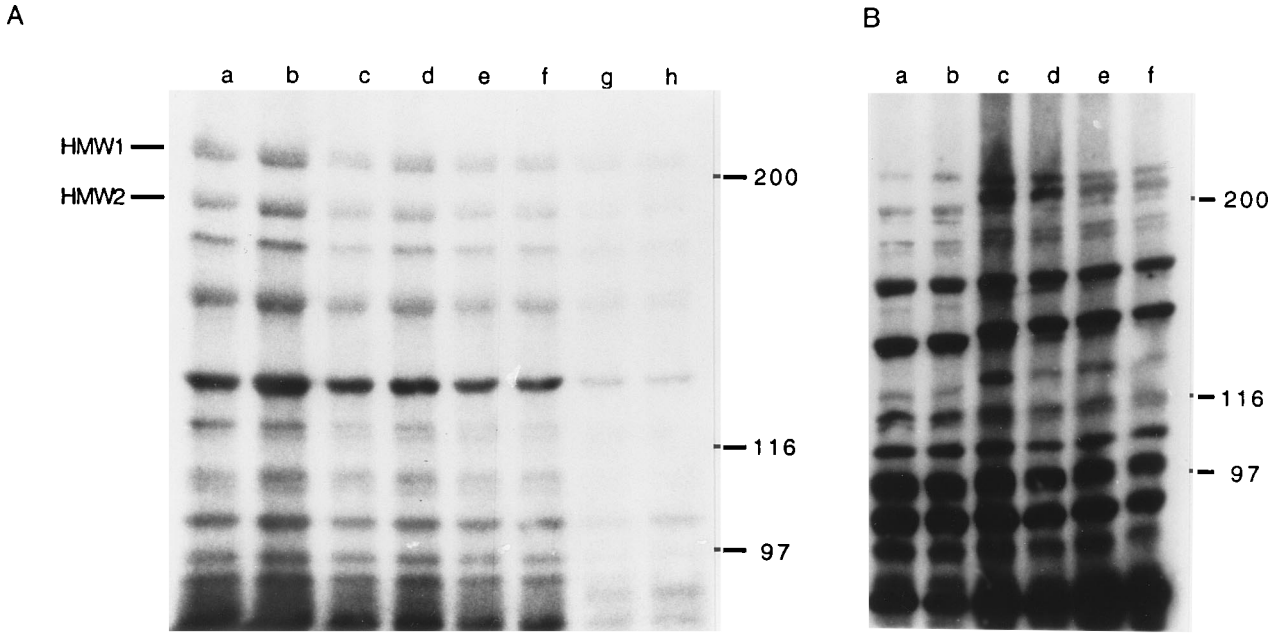

FIG. 2. (A) Profile of Triton-insoluble *M. pneumoniae* proteins incubated with [γ-^{32}P]ATP in cell extracts at various concentrations of $MgCl_2$ in the presence (lanes a, c, e, and g) or absence (lanes b, d, f, and h) of 100 μM cyclic AMP. Lanes: a and b, 0.1 mM $MgCl_2$; c and d, 0.5 mM $MgCl_2$; e and f, 1.0 mM $MgCl_2$; g and h, 10 mM $MgCl_2$. (B) Profile of Triton-insoluble *M. pneumoniae* proteins incubated with [γ-^{32}P]ATP in cell extracts at various concentrations of $CaCl_2$ with (lanes b, d, and f) or without (lanes a, c, and e) 0.1 mM $MgCl_2$. Lanes: a and b, 2 mM $CaCl_2$; c and d, 10 mM $CaCl_2$; e and f, 20 mM $CaCl_2$. Protein standards are indicated as in Fig. 1.

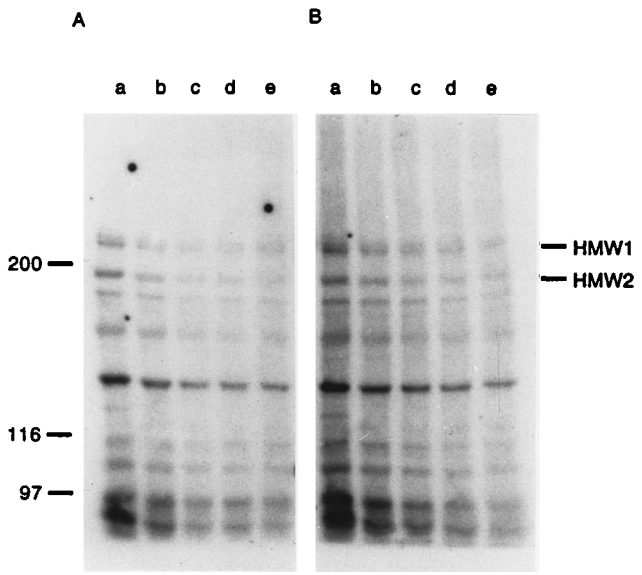

FIG. 3. Triton-insoluble *M. pneumoniae* proteins phosphorylated in cell extracts in the presence of increasing concentrations of EDTA. Thirty microcuries of [γ-^{32}P]ATP at 3,000 Ci/mmol (A) or 30 Ci/mmol (B) was used. Lanes: a, no EDTA; b, 2 mM EDTA; c, 5 mM EDTA; d, 10 mM EDTA; e, 20 mM EDTA. Protein standards are indicated as in Fig. 1.

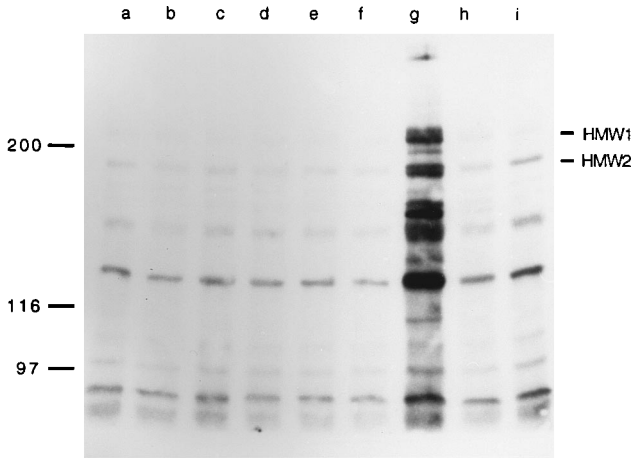

FIG. 4. Profile of Triton-insoluble *M. pneumoniae* proteins phosphorylated in cell extracts in the presence of glycolytic intermediates (each at 5 mM). Lanes: a, control; b, glucose 1-phosphate; c, glucose 6-phosphate; d, fructose 6-phosphate; e, fructose 1,6-diphosphate; f, fructose 2,6-diphosphate; g, 3-phosphoglycerate; h, 2-phosphoglycerate; i, phosphoenolpyruvate. Protein standards are indicated as in Fig. 1.

raacetic acid (EGTA) had no effect on the phosphorylation of HMW1 and HMW2 (data not shown). The reason for incomplete inhibition of the kinase by EDTA is not clear. Cation-independent kinases have been reported in bacteria (19), but none has been characterized in detail. Perhaps the kinase responsible for phosphorylation of HMW1 and HMW2 covalently incorporates a metallo cofactor, thereby accounting for the incomplete sensitivity to chelators.

We tested the ability of cyclic AMP, a stimulator of cyclic AMP-dependent kinases; staurosporin, a general eukaryotic protein kinase inhibitor; and okadaic acid, a general eukaryotic phosphatase inhibitor, to stimulate or inhibit the phosphorylation of HMW1 and HMW2. Addition of cyclic AMP resulted in a slight decrease in the intensity of the phosphorylation profiles (Fig. 2A). Okadaic acid and staurosporin had no identifiable effect on the levels of phosphorylation of these proteins or any other *M. pneumoniae* proteins resolved under our experimental conditions (data not shown).

Many protein kinases are allosterically regulated by cellular metabolites. For example, certain glycolytic intermediates are known to activate the kinase that phosphorylates seryl residue 46 of HPr, the phosphocarrier protein in the phosphoenolpyruvate-dependent glucose transferase system (6, 18). As shown in Fig. 4, little change in radiolabeling was observed with HMW1 or HMW2 in the presence of most of the glycolytic intermediates tested. Radiolabeling patterns changed only nominally with glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, 2-phosphoglycerate, and phosphoenolpyruvate (Fig. 4, lanes b, c, d, h, and i, respectively). However, approximately two- and threefold reductions were seen with fructose 1,6-diphosphate and fructose 2,6-diphosphate, respectively (Fig. 4, lanes e and f, respectively). In contrast, 3-phosphoglycerate stimulated the phosphorylation of HMW1 and HMW2 nine- and sevenfold, respectively (Fig. 4, lane g).

At the low concentration of ATP used in these studies, changes in ATP availability, for example, due to ATPase activity, could have a significant impact on the extent of protein phosphorylation. When we repeated the study with 30 μCi of low-specific-activity [γ-^{32}P]ATP (i.e., 100-fold more ATP), fructose diphosphate still inhibited labeling of HMW1 and HMW2 approximately two- to threefold. However, an only two- to threefold increase in phosphorylation was observed with 3-phosphoglycerate (data not shown). Thus, the increased labeling in the presence of 3-phosphoglycerate appears to be due to inhibition of an ATPase. It is not clear if the effects of the diphosphofructose were due to inhibition of the kinase or activation of a phosphatase. However, it is noteworthy that under glycolytic conditions, high levels of fructose 1,6-diphosphate have been detected in *M. gallisepticum* by nuclear magnetic resonance analysis (8). Phosphoenolpyruvate and glucose 6-phosphate were present at much lower levels, while other intermediates of the Embden-Meyerhoff pathway were not identified. The authors of that study speculated that fructose 1,6-diphosphate may play a key role in the regulation of metabolism in *M. gallisepticum.*

Phosphoamino acid analysis following metabolic labeling with $^{32}P_i$ phosphate during culture has revealed mostly phosphothreonine and some phosphoserine in HMW1 and mostly phosphoserine with a trace of phosphothreonine in HMW2 (7). Phosphoamino acid analysis was performed here on HMW1 and HMW2 to determine if phosphorylation in cell extracts yields the same pattern. Samples were processed as described previously (7), except that the bands of interest containing HMW1 or HMW2 were first identified by autoradiography and then localized by overlaying the blot with the autoradiograph. The correct alignment was determined by using GLOGOS stickers (Stratagene, La Jolla, Calif.). Serine was identified as the major phosphate acceptor for both HMW1 and HMW2, respectively, with no detectable phosphothreonine (data not shown). Addition of 3-phosphoglycerate to the labeling reaction resulted in an increase in the intensity of the phosphoserine spot for both HMW1 and HMW2. The lack of phosphothreonine raises the possibility that HMW1 and HMW2 may be phosphorylated by more than one kinase.

The findings presented here and published previously (7) suggest that an ATP-dependent serine-threonine protein kinase(s) is responsible, at least in part, for the phosphorylation of HMW1 and HMW2. Therefore, one would predict that phosphorylation of these proteins during culture would be dependent upon mycoplasma ATP pools. To test this predic-

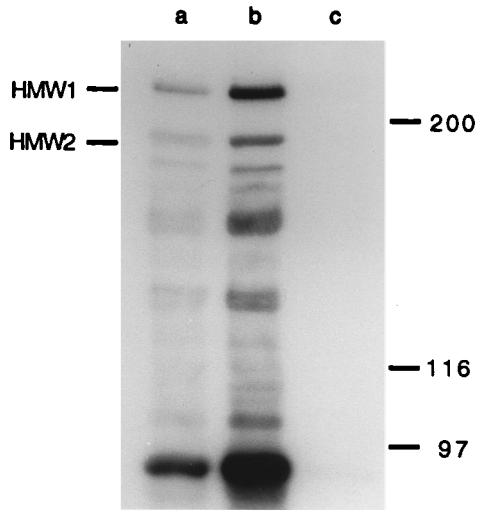

FIG. 5. Profile of Triton-insoluble *M. pneumoniae* proteins labeled metabolically in the presence of $[^{32}P]H_3PO_4$ with or without glucose. Lanes: a, glucose present during both preincubation and labeling periods; b, glucose omitted during preincubation but present during labeling period; c, glucose omitted from both preincubation and labeling periods. Protein standards are indicated as in Fig. 1.

tion, we starved mycoplasma cultures for glucose for 2 h and then labeled them with $[^{32}P]H_3PO_4$ for 2 h in the presence or absence of glucose. Virtually no labeling occurred when glucose was absent during both the preincubation and labeling periods (Fig. 5, lane c). The same was true when nonmetabolizable 2-deoxyglucose was substituted for glucose (data not shown). When the cells were starved of glucose during preincubation but glucose was added during labeling, phosphorylation was even greater than when glucose was present for the entire 4 h (Fig. 5, lanes b and a, respectively). The transient enhancement with omission of glucose seems consistent with the inhibition by diphosphofructose in cell extracts. Levels of diphosphofructose should drop in the absence of glucose. On the basis of the findings shown in Fig. 4, this would result in an increase in phosphorylation.

To test if glucose starvation resulted in a decline in the ATP pool, we measured the ATP content with a somatic cell bioluminescence assay kit (Sigma) before and after glucose starvation. Mycoplasma cultures in the log phase were incubated in phosphate-deficient Dulbecco's modified Eagle's medium (Sigma) with or without glucose. After 4 h at 37°C, corresponding to the total time for preincubation and labeling, the cells were harvested in 5 ml of TN. A 0.1-ml volume of ATP Assay Mix solution (Sigma) was added to a reaction vial and incubated at room temperature for approximately 3 min. A 0.05-ml cell sample volume was added to a separate vial containing 0.1 ml of somatic cell ATP releasing agent and 0.05 ml of an ATP standard (diluted in TN). A 0.1-ml volume of this solution was transferred to the reaction vial, and the amount of light emitted was immediately measured with a model 2000 ATP Integrating Photometer (Biospherical Instruments, Inc., San Diego, Calif.). Total protein concentration was determined with the bicinchoninic acid assay (Pierce, Rockford, Ill.). Glucose starvation for 4 h was accompanied by a drop in the ATP content from 2.75×10^{-11} to 7.62×10^{-12} mol of ATP per μg of protein.

In summary, we have described and characterized phosphorylation of *M. pneumoniae* cytoskeletal proteins HMW1 and HMW2 in a cell-free system. Our findings suggest that the phosphorylation of these proteins under the conditions described is physiologically relevant on the basis of a comparison with phosphorylation patterns seen with metabolic labeling in culture. Establishment of a stricter correlation awaits analysis of phosphopeptide mapping patterns for HMW1 and HMW2 radiolabeled in culture and in vitro.

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