The folate receptor, like many glycosylphosphatidylinositol-anchored proteins, is found associated with membrane domains that are insoluble in Triton X-100 at low temperature and that are enriched in cholesterol and sphingolipids. Depletion of cellular cholesterol has been shown to inhibit vitamin uptake by this receptor (Chang, W.-J., Rothberg, K. G., Kamen, B. A., and Anderson, R. G. W. (1993) J. Cell Biol. 118, 63–69), suggesting that these domains regulate this process. In this study, the importance of sphingolipids for folate receptor function was investigated in Caco-2 cells using fumonisin B₁, a mycotoxin that inhibits the biosynthesis of these lipids. The folate receptor-mediated transport of 5-methyltetrahydrofolate was almost completely blocked in cells in which sphingolipids had been reduced by ~40%. This inhibition was dependent on the concentration and duration of the treatment with the mycotoxin and was mediated by the sphingolipid decrease. Neither receptor-mediated nor facilitative transport was inhibited by fumonisin B₁ treatment, indicating that the effect of sphingolipid depletion was specific for folate receptor-mediated vitamin uptake. A concurrent loss in the total amount of folate binding capacity in the cells was seen as sphingolipids were depleted, suggesting a causal relationship between folate receptor number and vitamin uptake. These findings suggest that dietary exposure to fumonisin B₁ could adversely affect folate uptake and potentially compromise cellular processes dependent on this vitamin. Furthermore, because folate deficiency causes neural tube defects, some birth defects unexplained by other known risk factors may be caused by exposure to fumonisin B₁.

The folate vitamins play an essential role as cofactors in many biochemical reactions involving one-carbon metabolism. These include the biosynthesis of purines and thymidine, the regeneration of methionine from homocysteine, and histidine metabolism. Cellular processes dependent upon folate can be compromised if dietary levels of this vitamin are insufficient or if its transport into cells is affected. Two different systems are used for folate uptake into cells. The first uses a high capacity, low affinity transmembrane transporter known as the reduced folate carrier. The second involves a glycosylphosphatidylinositol (GPI)anchored protein referred to as the folate receptor (1, 2). This high affinity receptor is responsible for the transport of folate into cells of the placenta, kidney, breast, and other tissues with elevated requirements for this vitamin.

The mechanism by which the GPI-anchored folate receptor transports vitamin into the cytosol has received considerable attention in recent years. The immunocytochemical localization of several GPI-anchored proteins, including the folate receptor, to uncoated membrane invaginations called caveolae (3) led to the suggestion that the uptake of folate is mediated by these structures by a process termed endocytosis (4). In this and other studies, caveolae were equated with membrane domains that could be isolated based on their insolubility in Triton X-100 at 4°C (5, 6) and that are enriched in cholesterol and sphingolipids (7). More recent evidence has suggested that the Triton X-100-insoluble domains may include caveolae, but are primarily other membrane regions in which the GPI-anchored proteins (including the folate receptor) reside (8). Characterization of the protein components of caveolae isolated using new, detergent-free purification schemes has supported the conclusion that GPI-anchored proteins are not enriched in these structures (9, 10). Collectively, this evidence suggests that the folate receptor is not in caveolae, and therefore, endocytosis may not be the mechanism by which vitamin transport occurs.

Recent evidence suggests that uptake mediated by the folate receptor involves endocytosis (11, 12). However, the association of the folate receptor with Triton X-100-insoluble domains does appear to be important to its function. Depletion of cellular cholesterol through inhibition of its biosynthesis inhibited receptor-mediated folate uptake (13). Interpreting these results in the context of endocytosis, Rothberg et al. (14) suggested that this occurred because the clustering of the folate receptor in caveolae was disrupted. In terms of the effect on the endocytosis of the folate receptor, cholesterol depletion has been found to accelerate the rate at which this protein was recycled to the cell surface. How this results in an inhibition of folate uptake is unclear.

The importance of sphingolipids, the other lipids enriched in Triton X-100-insoluble domains, for folate receptor function has not yet been investigated, although several studies have probed the importance of these lipids for other GPI-anchored proteins. Inhibition of sphingolipid biosynthesis influenced both the localization of GPI-anchored proteins to these Triton X-100-insoluble domains (15) and the transport of newly synthesized GPI-anchored proteins to the Golgi in yeast (16) and to the appropriate membrane surface in polarized epithelial cells (17). Therefore, the localization, transport, and targeting of these lipids and GPI-anchored proteins appear to be linked (18).
In this study, the effects of changes in the cellular levels of sphingolipids on folate receptor-mediated vitamin uptake were investigated in Caco-2 cells. Originally isolated from a human colon adenocarcinoma, Caco-2 cells were chosen for this study because their folate receptor (2) and the association of GPI-anchored proteins with Triton X-100-insoluble domains in these cells (19) have been characterized. Cellular sphingolipids were depleted using fumonisin B1, a mycotoxin produced by the fungus Fusarium moniliforme (20), fumonisin B1 blocks sphingolipid biosynthesis by inhibiting the reaction catalyzed by sphingosine N-acyltransferase (ceramide synthase) (21). The results presented here indicate that sphingolipids play an important role in folate receptor function and that fumonisin B1 could influence cellular folate status through its effects on these membrane lipids.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum was purchased from Atlanta Biologicals, Inc. RPMI 1640 medium and folate-free RPMI 1640 medium were from Life Technologies, Inc. [3,5,7,9-3H](Folic acid (25–30 µmol, 99% pure) was obtained from American Radiolabeled Chemicals. 5-[(3,5,7,9-
H)Methyltetrahydrofolate (30 µCi/mol, 97.5% pure) was purchased from Moravek Biochemicals, Inc. 125I-Labeled diferric transferrin was from NEN Life Science Products. Lovastatin was a generous gift from Merck. The high performance Silica Gel 60 TLC plates were obtained from Whatman. DEAE-cellulose, charcoal, all lipid standards, and other chemicals were from Sigma. The reagents for the bicinchoninic acid protein assay were purchased from Pierce.

Cell Culture—Caco-2 cells were purchased from the American Type Culture Collection and were routinely grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were plated at 2.5 x 10⁶ cells/cm² flask or at 8 x 10⁶ cells/150-mm flask/125-mm flask for quantitation of folate binding and uptake or for analysis of lipids, respectively. After 2 days of growth, the medium was changed to folate-free RPMI 1640 medium. RPMI 1640 medium was changed to folate-free RPMI 1640 medium and 10% fetal bovine serum such that the cells were maintained under these conditions for a total of 5 days. Treatments were done for the indicated times during this 5-day period such that they ended at the time of analysis.

Lovastatin-treated cells were grown in folate-free RPMI 1640 medium supplemented with lipoprotein-depleted fetal bovine serum prepared as described by Goldstein et al. (22). In all cases, the cells were confluent by the end of the experiment, but continued to divide at nearly the same rate as subconfluent cells. Neither the growth rate (as measured by cell counting) nor [3H]thymidine uptake nor the viability of the cells (as assessed by trypan blue exclusion) was affected by the treatments (data not shown).

Isolation of Triton X-100-insoluble Domains—Triton X-100-insoluble domains were isolated using the two-step sucrose gradient method described by Arregga et al. (23). Cells were washed twice with phosphate-buffered saline (8 µl/g NaCl, 0.2 µl/g KCl, 1.15 µl/g Na₂HPO₄, and 0.2 µl/g KH₂PO₄ and lysed by the addition of cold TNE buffer (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 5 mM EDTA) containing 1% Triton X-100 to the dish. After 20 min of incubation on ice, the cells were scraped from the dish with a rubber policeman and homogenized. The lysate was then adjusted to 40% sucrose with TNE buffer containing 80% sucrose, placed in an ultracentrifuge tube, and overlaid with 5.5 ml of TNE buffer containing 38% sucrose followed by 2 ml of TNE buffer containing 5% sucrose. The samples were ultracentrifuged at 120,000 x g at 37 °C for 15–20 h. The Triton X-100-insoluble membranes visible at the interface between the 5 and 38% sucrose layers were harvested with a syringe, diluted 5–fold with TNE buffer, and pelleted by ultracentrifugation (1 h at 120,000 x g at 4 °C) and quantitated by measuring 5-[(3,5,7,9-
H)Methyltetrahydrofolate (0.5 µCi) in folate-free medium for various amounts of time at 37 °C. The medium was then removed, and the cells were washed four times with cold phosphate-buffered saline before the addition of 1.5 ml of lysis buffer (1 µCi/ml Triton-HCl (pH 8.0), 20 µg/ml leupeptin, 20 µg/ml aprotinin, and 1 µl 5-methyltetrahydrofolate) to each flask. The cells were lysed by placing the flasks at ~80 °C for 15 min and thawed on ice. The cells were then collected and centrifuged for 20 min at 100,000 x g in an Optima TL ultracentrifuge to separate the membrane (pellet) and cytosolic (supernatant) fractions. The radioactivity in each fraction was quantitated by scintillation counting.

Quantitation of 5-Methyltetrahydrofolate Uptake—The internalization of the reduced folate derivative into the cytosol was quantitated as described by Smart et al. (24). Briefly, cells were incubated with 5 nM 5-[(3,5,7,9-
H)Methyltetrahydrofolate (0.5 µCi) in folate-free medium for various amounts of time at 37 °C. The medium was then removed, and the cells were washed four times with cold phosphate-buffered saline before the addition of 1.5 ml of lysis buffer (1 µCi/ml Triton-HCl (pH 8.0), 20 µg/ml leupeptin, 20 µg/ml aprotinin, and 1 µl 5-methyltetrahydrofolate) to each flask. The cells were lysed by placing the flasks at ~80 °C for 15 min and thawed on ice. The cells were then collected and centrifuged for 20 min at 100,000 x g in an Optima TL ultracentrifuge to separate the membrane (pellet) and cytosolic (supernatant) fractions. The radioactivity in each fraction was quantitated by scintillation counting.

RESULTS

Fumonisin B1-induced Reduction of Celluar Sphingolipids—Cells were treated with either fumonisin B1 (20 µg/ml (27.7 µM)
for 2 days) or lovastatin (25 μM for 3 days) to decrease the cellular sphingolipids or cholesterol, respectively. The specificity and effectiveness of these treatments were assessed by quantitating the major lipids both from whole Caco-2 cells and from the Triton X-100-insoluble domains. These results are shown in Table I. Consistent with the previous results of Brown and Rose (7), the Triton X-100-insoluble domains were found to be enriched in sphingolipids and cholesterol, containing ~95% of the former and 80% of the latter. Fumonisin B₁ treatment significantly reduced the levels of all the measured sphingolipids in both whole cells and the Triton X-100-insoluble domains. Lovastatin treatment specifically decreased cholesterol among the total lipids, but was found to affect the levels of several other lipids (both glycerolipids and sphingolipids) in the Triton X-100-insoluble domains. Overall, both treatments resulted in an ~20% decrease in the cholesterol/sphingolipid content of the Triton X-100-insoluble domains.

**Fumonisin B₁ Inhibition of 5-Methyltetrahydrofolate Uptake**—The consequences of these changes in membrane lipid composition on folate receptor function were evaluated by measuring the rate of uptake of 5-methyltetrahydrofolate by fumonisin B₁- and lovastatin-treated cells. As shown in Fig. 1, the rate of uptake of the vitamin was roughly linear in untreated Caco-2 cells grown in folate-free medium with either normal (Fig. 1, A and B, closed circles) or lipoprotein-depleted (Fig. 1B, triangles) serum. Uptake was inhibited by ~90% in the fumonisin B₁-treated cells (Fig. 1A). A similar level of inhibition, which has been reported previously by others (13), was observed in the lovastatin-treated cells (Fig. 1B). Uptake of the vitamin was unaffected in cells treated with fumonisin B₁ for only 1 h, indicating that the inhibition was not mediated by the mycoxin alone. Treatment with either various concentrations of fumonisin B₁ for 2 days (Fig. 2A) or 20 μg/ml for various amounts of time (Fig. 2B) demonstrated that the inhibition of 5-methyltetrahydrofolate uptake was both concentration- and time-dependent. The sphingolipid levels of these cells also decreased in a concentration- and time-dependent manner (data not shown), establishing that the inhibition of 5-methyltetrahydrofolate uptake in the fumonisin B₁-treated cells was mediated by the changes in the sphingolipid composition.

Coupled with the previous reports (13, 14) of the effects of lowering cellular cholesterol on folate transport, the finding that depletion of cellular sphingolipids by fumonisin B₁ inhibited this process suggests that the cholesterol/sphingolipid-enriched domains are involved in this effect. To determine if fumonisin B₁ specifically inhibits processes dependent on these domains, the effect of this mycoxin on other types of uptake systems was assessed. Facilitative transport was measured by quantitating 2-deoxyglucose uptake. Treatment with fumonisin B₁ (20 μg/ml for 2 days) had no effect on the rate of uptake of this glucose analog by Caco-2 cells (data not shown). Receptor-mediated endocytosis was assessed by measuring transferrin uptake. Both the rates of internalization (Fig. 3) and externalization (data not shown) of 125I-transferrin were found to be very similar in control and fumonisin B₁-treated cells. The only difference found was in the amount of surface transferrin binding, which was 2.7 times more in the sphingolipid-depleted groups. While the reason for this difference is unclear, it is responsible for the line representing the fumonisin B₁-treated rate of uptake being offset from that of the control cells in Fig. 3. Therefore, transport processes not thought to involve sphingolipid-enriched domains (receptor-mediated endocytosis via clathrin-coated pits and facilitative transport, respectively) were not compromised by changes in the cellular sphingolipid levels.

**Lipid Depletion-induced Changes in Cellular Folic Acid Binding Capacity**—Previous studies of the effect of depletion of cellular cholesterol on folate uptake suggested that this process was compromised because the clustering of the folate receptor in cholesterol/sphingolipid-rich domains in the plasma membrane was disrupted (13, 14). To determine if the decrease in cellular sphingolipids caused by fumonisin B₁ affected the folate receptor in a similar manner, the amount of this protein localized in the Triton X-100-insoluble domains was determined. This was accomplished by quantitating the high affinity binding of folic acid in either solubilized whole Caco-2 cells or Triton X-100-insoluble domains. Because folic acid is essentially bound irreversibly by the folate receptor (Kᵣ = 0.4 nm (29)), the amount of this ligand bound is an approximate measure of the amount of this protein in the cell or fraction. As shown in Fig. 4, ~80% of the folate binding was localized to the Triton X-100-insoluble domains in untreated cells. Surprisingly, the total amount of folate receptor in the cell, but not its localization, was affected in both the fumonisin B₁- and lovastatin-treated cells. While ~50% of the total folate receptor was lost with either sphingolipid or cholesterol depletion, ~80% of

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**Table I**

| Lipid Composition of Control and Fumonisin B₁- and Lovastatin-treated Cells |
|-----------------------------|-------------------------------|
| Control | Fumonisin B₁ | Lovastatin |
| Cholesterol | 1727 ± 88 | 1829 ± 84 (0) | 1365 ± 60 (21) |
| TG | 475 ± 41 | 388 ± 15 (18) | 508 ± 16 (0) |
| FA | 1119 ± 101 | 1136 ± 23 (0) | 1022 ± 172 (9) |
| PC | 2762 ± 142 | 3129 ± 258 (0) | 2537 ± 110 (0) |
| PE | 1814 ± 50 | 1943 ± 49 (0) | 1777 ± 26 (2) |
| PI | 896 ± 53 | 934 ± 89 (0) | 945 ± 25 (0) |
| PS | 898 ± 59 | 850 ± 82 (5) | 1043 ± 25 (0) |
| CL | 429 ± 25 | 424 ± 45 (1) | 477 ± 25 (0) |
| SM | 1230 ± 38 | 809 ± 30 (34) | 1167 ± 26 (5) |
| Ceramides | 271 ± 19 | 117 ± 17 (57) | 254 ± 37 (6) |
| Sulfatides | 226 ± 28 | 91 ± 16 (60) | 284 ± 31 (0) |
| Gal-Cer | 100 ± 10 | 36 ± 10 (64) | 99 ± 8 (7) |
| Lac-Cer | 157 ± 25 | 119 ± 31 (24) | 144 ± 22 (8) |
| Gangliosides | 45 ± 4 | 23 ± 7 (49) | 52 ± 3 (0) |

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A TG, triglyceride; FA, fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; SM, sphingomyelin; Cer, ceramide.
the remaining folate binding was found in the Triton X-100-insoluble domain. As with the inhibition of 5-methyltetrahydrofolate uptake, the decrease in total folate binding was dependent on both the duration of the treatment (Fig. 5A) and the concentration of fumonisin B₁ used (Fig. 5B). While these results indicate that the decrease in folate binding is mediated by the changes in lipid content in the cell, the possibility that fumonisin B₁ directly affected binding of the vitamin was further ruled out by the finding that the mycotoxin was unable to alter this parameter when added directly to the binding assay. Therefore, changes in the cholesterol or sphingolipid levels appear to decrease the total amount of folate receptor in the cells, but do not change its enrichment in Triton X-100-insoluble domains.

**Relationship between Folate Receptor Function and Number**—These results indicate that both the fumonisin B₁ and lovastatin treatments induce a loss or down-regulation of the folate receptor. The relationship between the change in folate receptor number and the inhibition of 5-methyltetrahydrofolate uptake was investigated by comparing these two parameters (Fig. 6). A linear relationship \( r = 0.967 \) between the amount of folate receptor in the cell and the ability to transport 5-methyltetrahydrofolate into the cytoplasm was found. Therefore, it seems more likely that the inhibition of vitamin uptake is caused by the loss of the folate receptor than by a change in the membrane localization of the protein.
FIG. 5. Time and concentration dependence of the fumonisin B₁-induced reduction in folic acid binding capacity. The [³H]folic acid binding activity was quantitated in cell lysates solubilized in Triton X-100-insoluble domains (Fig. 2A) or in Triton X-100-soluble domains (open circles) after treatment with 20 μg/ml of fumonisin B₁ for 0–3 days (A) or the indicated concentrations of fumonisin B₁ (FB₁) for 2 days (B). The data shown are the means ± S.E. of triplicate determinations.

FIG. 6. Correlation of 5-methyltetrahydrofolate uptake ability and folic acid binding capacity in Caco-2 cells. The values of these two parameters measured in whole cells for each of the treatments shown in Figs. 1, 2, 4, and 5 (control and fumonisin B₁- and lovastatin-treated) were compared and resulted in a correlation coefficient (r) of 0.967.

DISCUSSION

Treatment of Caco-2 cells with fumonisin B₁ resulted in almost complete inhibition of uptake of 5-methyltetrahydrofolate by the folate receptor. Consistent with it being mediated by the depletion of cellular sphingolipids, this inhibition was dependent on the concentration of mycotoxin used and the duration of the treatment. Fumonisin B₁ did not perturb either 2-deoxyglucose or transferrin uptake, indicating that the effect of sphingolipid depletion was specific for folate receptor-mediated transport. The inhibition caused by lowering the cellular sphingolipid levels was very similar to that previously observed when cellular cholesterol was depleted by inhibition of hydroxymethylglutaryl-CoA reductase (13). Therefore, it seems likely that decreases in the cellular levels of these two lipids perturb folate receptor function by the same mechanism and involve the Triton X-100-insoluble domain.

Quantitation of the amount of folic acid binding activity in the Triton X-100-insoluble domains in fumonisin B₁- and lovastatin-treated cells revealed that the lipid depletion did not cause redistribution of the folate receptor. Approximately 80% of the cellular folic acid binding capacity was localized to these cholesterol/sphingolipid-enriched domains in control and lipid-depleted cells. While localization was unaffected, the total amount of folic acid binding capacity was decreased by ~50% in fumonisin B₁- and lovastatin-treated cells. That folate receptor function and number decrease in parallel as sphingolipids are depleted from Caco-2 cells suggests that there is a causal relationship between these two parameters. Growth of cells in medium containing low concentrations of folate, which presumably increases the need to take up this vitamin, has been found to induce folate receptor function (29). Therefore, inhibition of folate uptake, or compromised folate receptor function, could lead to a drop in the amount of this protein in the cell. Alternatively, a decrease in the level of folate receptor in the cell could result in inhibition of vitamin uptake. The data presented here do not address the question of whether the decrease in folate receptor number caused inhibition of vitamin uptake or vice versa. Both parameters appear to decrease with roughly similar rates (Figs. 2B and 5A). Curiously, conditions that resulted in nearly complete inhibition of 5-methyltetrahydrofolate uptake (20 μg/ml fumonisin B₁ for 2 or 3 days) led to the loss of only half of the folate receptors in the cell. If vitamin uptake is compromised because of the decrease in the number of receptors, then why did the remaining folate receptors not support an intermediate level of folate transport into the cytosol? Perhaps the remaining folate receptors are not functional, or the altered lipid composition has affected some other critical component of the folate uptake pathway (e.g. folate polyglutamation).

This study clearly demonstrates the importance of sphingolipids for folate receptor-mediated vitamin uptake. However, the mechanism by which this process is affected is not clear. The finding that depletion of cellular cholesterol also inhibits folate receptor function (13) suggests that this effect is mediated by changes in the membrane domains enriched in cholesterol and sphingolipids to which the GPI-anchored folate receptor has been localized. Alternatively, folate receptor number and function could be altered in response to changes in the level of one or more intermediates in the synthesis of these lipids that have a signaling role in the cell. Recent evidence has implicated several sphingolipids, including sphingosine, sphingosine 1-phosphate, ceramide, and sphingomyelin, in signal transduction (reviewed in Refs. 30–33). Fumonisin B₁ inhibition of sphingolipid biosynthesis should result in elevation of cellular long-chain bases and decreased levels of ceramide (34). In preliminary experiments, supplementation of fumonisin B₁-treated cells (20 μg/ml for 2 days) with 1 μM C₈-ceramide for 1 additional day (in the presence of fumonisin B₁) has been found to reverse the effects on the folate receptor (35). Because long-chain bases should still be elevated, this result suggests that increases in the levels of sphinganine and/or sphingosine are not responsible for the effect on the folate receptor. However, whether it is the loss of mature sphingolipids or ceramide, which has been suggested to play a role in the regulation of endocytosis (35), that mediates the effects of fumonisin B₁ cannot be determined from these experiments. Unraveling which of these is the critical factor will provide important information regarding the regulation of vitamin uptake mediated by the GPI-anchored folate receptor and will be addressed in future studies.

While fumonisin B₁ has proven to be a useful reagent for probing the role of sphingolipids in various cellular processes (34), the fact that it is a naturally occurring compound raises the possibility that these same events could be adversely affected by dietary exposure to this mycotoxin. F. moniliforme, the fungus that produces fumonisin B₁, is a common contaminant of corn. Exposure to fumonisin B₁ causes a variety of animal diseases and has been linked to an increased incidence of esophageal cancer in humans in areas of southern Africa and China (36–39). Investigation into the consequences of fetal

³ E. R. Smith and V. L. Stevens, unpublished results.
exposure to this mycotoxin using either mice or hamsters has shown that it causes developmental toxicity (40, 41). The mouse fetuses that survived to birth had gross skeletal and visceral abnormalities (42). Inhibition of folate uptake through fumonisin B₁-induced depletion of sphingolipids could lead to an intracellular deficiency in this vitamin. Since folate deficiency during the first trimester of pregnancy is associated with an increased risk of neural tube defects in the developing embryo, a deficiency in this vitamin. Since folate deficiency during the first trimester of pregnancy is associated with an increased risk of neural tube defects in the developing fetus (43–45), it is possible that some instances of high rates of occurrence of these birth defects unexplained by known causes might be linked to dietary exposure to fumonisin B₁. For instance, high rates of neural tube defects have been observed in Cameron County, TX from 1990 to 1991 (46, 47) and in Harris County, TX from 1989 to 1991 (48, 49). The prevalence of these birth defects was high among Hispanics (48), for whom corn and corn products are expected to represent a sizable portion of their diet. The occurrence of a high number of clusters of the fatal equine disease caused by fumonisin B₁ in Texas in 1989 has established that the corn crop was contaminated with this mycotoxin during this period. Coupled with the present finding that fumonisin B₁-induced depletion of cellular sphingolipids blocked folate uptake, this evidence suggests that there should be further investigation into the possibility that this mycotoxin may contribute to some birth defects not accounted for by other known risk factors.

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