The effects of glycosylation inhibitors on the proliferation of a spontaneously transformed cell line (3T6) \textit{in vitro}

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Summary

We have examined the effects of different inhibitors of glycosylation processing on the proliferation of a spontaneously transformed murine cell line (3T6) \textit{in vitro}. It was found that whereas two compounds that specifically inhibit distal steps in the glycosylation chain (swainsonine and castanospermine) only exerted marginal inhibitory effects on cell multiplication, a proximal inhibitor (tunicamycin) efficiently decreased the rate of DNA synthesis in a dose-dependent fashion. This tunicamycin-induced inhibitory effect on cell proliferation was cell cycle-specific, in the sense that cells in G\textsubscript{1} only were blocked in their cell cycle progression. Like others (Volpe & Goldberg, 1983), we found that tunicamycin inhibited the activity of 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG-CoA), which constitutes the rate-limiting step in the biosynthesis of cholesterol and isoprenoid derivatives, by catalysing the reduction of HMG-CoA to mevalonate, and it has been suggested that it plays a role in the control of cell proliferation and in tumour transformation. This raises the question as to whether tunicamycin exerts its inhibitory effects on cell proliferation via the isoprene-synthetic pathway in addition to its effects on asparagine-linked glycosylation. By adding exogenous mevalonate, the rate-limiting step at which HMG-CoA reductase converts HMG-CoA to mevalonate can be bypassed. We found that addition of mevalonate partially reverses the effects of tunicamycin on cell proliferation. This suggests that tunicamycin exerts different effects, which taken together lead to a cessation of cell proliferation. One of these effects is likely to be mediated via the mevalonate-synthetic pathway.

Key words: glycosylation, 3T6 cells, transformation.

Introduction

Normal, non-transformed cells grown under sub-optimal conditions, such as at high density or in low concentrations of serum, leave the cell cycle from G\textsubscript{1} and enter a reversible state of quiescence (G\textsubscript{0}) (Baserga, 1976). In contrast chemically or virally transformed cells as well as cells of tumour origin respond differently to such alterations in culture conditions. Although the rate of proliferation is gradually retarded, tumour cells are incapable of entering G\textsubscript{0}. Instead, transformed cells continue to traverse the cell cycle slowly until they finally die as a consequence of the environmental restraints (Engström & Zetterberg, 1983; Larsson & Zetterberg, 1986a). The ability or inability to enter a specific G\textsubscript{0} stage probably reflects one of the fundamental growth control mechanisms, which operates stringently in normal cells but is improperly relaxed in transformed cells. Therefore, studies of the molecular mechanisms that accompany growth-regulatory events in G\textsubscript{1} are of fundamental importance for understanding the cellular defects underlying the unrestricted growth of tumour cells.

It is generally believed that the glycosylation of critical molecules located on the cell surface have important implications for growth regulation. In this study we have used specific inhibitors that prevent glycosylation or interfere with the glycosylation reactions occurring on oligosaccharides after their attachment to protein molecules. In particular, we report that one compound, tunicamycin, inhibits cell proliferation in a dose-dependent manner. We also report that, along with its inhibitory effects on glycosylation, tunicamycin is a potent inhibitor of HMG-CoA reductase, which catalyses the reduction of HMG-CoA to mevalonate and is believed to be involved in growth regul-
different metabolic effects, which taken together result in a cessation of cell proliferation.

Materials and methods

Materials

Tunicamycin was purchased from Sigma AB, Sweden. Castanospermum and swainsonine were obtained from Boehringer-Mannheim (Sweden). The basal medium DME was purchased in liquid form from Flow Laboratories (Sweden). Fetal calf serum and trypsin were supplied by GIBCO (Sweden). All tissue-culture plastic was purchased from NUNC (through GIBCO, Sweden).

Cell culture

Mouse Swiss 3T6 cells were purchased from Flow Laboratories (Stockholm) and maintained in monolayer cultures in tissue culture bottles. The stock cultures were grown in a humidified 5% CO2/95% air mixture in Dulbecco's modified Eagle's medium (DMEM) (Morton, 1970) supplemented with 10% (v/v) foetal calf serum, 50 units of penicillin per ml and 50 μg of streptomycin per ml. The cells were removed from the bottles for transfer by treatment with 0.25% (w/v) trypsin in Tris-buffered 0.9% (w/v) aqueous NaCl supplemented with 0.5 mM-EDTA at 4°C (McKeehan, 1977). The line was maintained by seeding 50,000 cells per 10 ml bottle and transferring them every third day. The cells were never allowed to reach confluency. Cells used for experimental purpose were grown in 50 mm plastic Petri dishes that had a glass coverslip on the bottom. The cell density at the initiation of each experiment was 5000-6000 cells cm⁻².

DNA synthesis

DNA synthesis was assayed by incorporating [³H]thymidine (3-5 μCi ml⁻¹, Amersham, 25 Ci mmol⁻¹) into acid-precipitable material. At the end of each experiment the glass coverslip was fixed in a 50:50 (v/v) mixture of ethanol and acetone, and processed for autoradiography as described above. The cells growing on the bottom of the dish were rinsed in prewarmed calcium/magnesium-free PBS (Dulbecco & Vogt, 1954) and thereafter lysed in 1 ml of 1 M NaOH. After 10 min the lysate was neutralized by adding 1 ml of 1 M HCl, mixed with an equal amount of ice-cold 10% (v/v) trichloroacetic acid and placed in a refrigerator overnight. The tubes containing acid-precipitable material were then spun at 180 g for 10 min and the supernatant was carefully removed by vacuum suctioning. The pellet was redissolved in 1 ml of 1 M NaOH and neutralized by addition of 1 ml of 1 M HCl, whereafter the absorbance at 260 nm, 280 nm and 310 nm was determined in a spectrophotometer. The sample that had been used for absorbance measurements was mixed with 10 ml Instagel and assayed for radioactivity in a Packard 3385 Tricarbo liquid scintillation counter.

Autoradiography

The glass coverslips were fixed in 50:50 (v/v) acetone:ethanol for at least 24 h and then hydrated in distilled water. Non-incorporated radioactive thymidine was removed by treating the slides in ice-cold 5% (w/v) trichloroacetic acid for 10 min. The preparations were washed in tap water for 10 min and finally air dried in a dust-free desiccator. To coat the slides, equal volumes of emulsion (K2 IIfor) and 2% (v/v) aqueous glycerol were thoroughly mixed at 45°C. The slides were coated in a glass-slide-dipping device and left to dry on a vertical rack. The autoradiographs were then placed in a sandwich box containing blue silica gel at 4°C for 5-10 days. Before development, the autoradiographs were left at room temperature for 2-3 h. The preparations were developed (8 min, Ilford Phenisol developer diluted 1:4 (v/v) with Analar water), fixed (10 min, Ilford 1F23 paper fixer diluted 1:1 (v/v) with Analar water), washed extensively in running water, air dried and finally stained in haematoxylin/eosin.

Protein synthesis

De novo protein synthesis was assayed according to a protocol devised by Larsson et al. (1986).

Time-lapse video recording

A 25 ml tissue-culture bottle containing exponentially growing 3T6 cells, which was previously kept in an incubator where it had been equilibrated in a 5% CO2/95% air mixture, was carefully sealed and then placed in an inverted microscope with an attached video camera system for time-lapse video recording. The temperature of the medium was carefully kept at 37°C. The recording apparatus comprised a Wild inverted phase-contrast microscope equipped with a video camera (National Panasonic VV-1350 AE/B), a time-lapse video recorder (National Panasonic VTR/NV 8800) and a monitor (Microochrome EVM 1710 R(N)). Light intensity at the camera during the recordings was measured at 0.1 lux. By using a crystal-controlled programmable timer (model KM 8200) one single picture was recorded every minute. The event time was denoted by a time-date generator (WJ 800) and thereafter taken from the tape revolutions and converted to real time. No significant cell death was observed during the recordings. The division time was taken when the division furrow in a pair of dividing cells was deemed to be complete. A field of 20-50 well-separated cells was chosen for recording in each experiment. All experiments were started when a cell density of 5000-6000 cells cm⁻² was reached.

Determination of HMG-CoA reductase activity

Cells in monolayer cultures were rinsed with calcium/magnesium-free phosphate-buffered saline (PBS) (Dulbecco & Vogt, 1954), removed from the dishes with a rubber policeman and collected by centrifugation at 180 g for 10 min. The supernatant was removed and 50 μl of a solution buffered at pH 7.35 containing 50 mM-K₂HPO₄, 5 mM-EDTA, 0.2 M-KCl and 5 mM-dithiothreitol was added to the cell pellets, which were stored at -20°C until the assay was continued.
Prior to analysis, 100 µl of a buffered solution containing 100 mM K2HPO4, 5 mM-dithiothreitol, 10 mM-glucose-6-phosphate, 0.7 unit of glucose-6-phosphate dehydrogenase ml⁻¹ and 2.5 mM-NADPH was added to the cell pellets. [¹⁴C]HMG-CoA (57 mCi mmol⁻¹, New England Nuclear) was added at a concentration of 0.9 nmol and the cell suspension was incubated at 37°C. Sixty minutes later the reaction was terminated by addition of 10 µl 1M-KOH; 0.4 pmol of [³H]mevalonate (1.28 Ci mmol⁻¹, New England Nuclear) was added as an internal standard. Twenty minutes later the solution was neutralized by the addition of 20 µl of 1M-HCl; 100 µl of the solution was used for ion-exchange chromatography and the remainder was used for protein determination using Biorad's protein assay, which contains an internal standard. After separation of [¹⁴C]mevalonolactone by ion-exchange chromatography, radioactivity was determined by assaying for ¹⁴C activity by means of liquid scintillation. The cts min⁻¹ values were corrected for background, spillover and quenching. The conversion activity of [¹⁴C]HMG-CoA to [¹⁴C]mevalonate was corrected for differences in protein content.

**Incorporation of [³H]glucosamine**

Cells in 50 mm Petri dishes were incubated with 10 µCi of [³H]glucosamine (10 mCi mmol⁻¹, Amersham)ml medium for 1 h. The cells were then rinsed in calcium/magnesium-free PBS, scraped off the dish with a rubber policeman. The cells were then washed with trichloroacetic acid and assayed for radioactivity as described above.

**Results**

Table 1 shows the effects of different inhibitors of glycosylation on DNA synthesis in proliferating 3T6 cells. It was found that a 24-h exposure to swainosmine had no significant effect on the rate of DNA synthesis as judged by a 1-h pulse labelling with tritiated thymidine. The swainosmine concentration used in this experiment was chosen on the basis of its inhibitory effects on growth of transformed NIH 3T3 cells in soft agar (de Santis et al. 1987). Other concentrations tested did not decrease the growth rate in our 3T6 cells (data not shown). Exposure to another glycosylation inhibitor, castanospermine, over a wide range of concentrations resulted in only a minor decrease in the rate of DNA synthesis. In marked contrast, tunicamycin decreased the rate of DNA synthesis in a dose-dependent fashion. At 0.4 µg ml⁻¹ the rate had decreased to a level below that following 24 h of serum starvation. It must be emphasized that we observed that tunicamycin addition resulted in an altered morphological appearance of the 3T6 cells. At 24 h after medium change the cells had rounded up and a significant proportion had detached from the cell surface (cf. Fig. 1A). However, each autoradiograph used in this study contained sufficient labelled and unlabelled cells to permit an accurate determination of the proportion cells having initiated DNA synthesis. The effect of tunicamycin on cell proliferation was reversible in the sense that tunicamycin-treated cells that were rinsed and subsequently exposed to fresh tunicamycin-free medium containing 10% serum resumed proliferation and grew to confluency over a 72-h interval (Fig. 1B).

To determine the effects of tunicamycin on the cell cycle we studied exponentially growing 3T6 cells by time-lapse video recording. Fig. 2 shows the results of experiments in which cells were exposed to medium containing 10% serum (control) (A), or 0.4 µg tunicamycin ml⁻¹ serum-containing medium (B) for 24 h. Cell age, defined as time elapsed after the last mitosis, and intermitotic time for individual cells were determined from the video recordings. Postmitotic cells younger than 3-4 h were immediately forced to delay their cycle times and remained undivided after 36 h of exposure to tunicamycin, whereas older cells underwent mitosis on schedule. Since cell cycle progression and DNA replication is coupled to de novo protein synthesis under physiological growth conditions (Zetterberg & Larson, 1985) it was of interest to examine whether the tunicamycin treatment exerted any rapid effects on the rate of protein synthesis. Previous studies have shown that tunicamycin in lower concentrations causes only a limited inhibition of de novo protein synthesis (Hickmann et al. 1977; Surani, 1979; Larson, 1985). However, we found that the rate of overall protein synthesis was only marginally affected following a 4-h exposure to tunicamycin (0.1-0.4 µg ml⁻¹) (Table 2). Therefore, it is suggested that the immediate effects of tunicamycin on G1-traverse of 3T6 cells are mediated by other mechanisms.

In order to examine the effects of tunicamycin in N-linked glycosylation we examined the incorporation of [³H]glucosamine into trichloroacetic acid precipitable DNA synthesis in exponentially growing 3T6 cells

<table>
<thead>
<tr>
<th>Medium</th>
<th>Rate of DNA synthesis (cts/min unit DNA)</th>
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<tbody>
<tr>
<td>DMEM + 10% serum</td>
<td>100</td>
</tr>
<tr>
<td>DMEM + 0.1% serum</td>
<td>12 ± 7</td>
</tr>
<tr>
<td>DMEM + 10% serum + 5 µg Sw/m1⁻¹</td>
<td>109 ± 16</td>
</tr>
<tr>
<td>DMEM + 10% serum + 50 µg Ca/ml⁻¹</td>
<td>82 ± 17</td>
</tr>
<tr>
<td>DMEM + 10% serum + 0.4 µg Tu/ml⁻¹</td>
<td>4 ± 2</td>
</tr>
</tbody>
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The values represent means ± s.d. of three different experiments. Ca, castanospermine; Sw, swainosmine; Tu, tunicamycin.
Fig. 1. Morphological appearance of 3T6 cells exposed to 0.4 μg tunicamycin ml⁻¹ for 24 h (A). In one set of experiments the cells were rinsed four times in prewarmed DMEM and exposed to fresh 10% serum for 72 h (B).
Fig. 2. Relation between cell age and intermitotic time in 3T6 cells exposed to tunicamycin. Exponentially growing cells were exposed to 10% serum (A), or 10% serum + 0.4 μg tunicamycin ml⁻¹ (B), for 36 h. Cell age at medium change and intermitotic time was determined by time-lapse video recording.

Table 2. The effect of tunicamycin on protein synthesis in 3T6 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein synthesis (% of control)</th>
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<tbody>
<tr>
<td></td>
<td>4 h</td>
</tr>
<tr>
<td>10% serum</td>
<td>100</td>
</tr>
<tr>
<td>10% serum + 0.1 μg Tu ml⁻¹</td>
<td>82</td>
</tr>
<tr>
<td>10% serum + 0.2 μg Tu ml⁻¹</td>
<td>72</td>
</tr>
<tr>
<td>10% serum + 0.4 μg Tu ml⁻¹</td>
<td>83</td>
</tr>
</tbody>
</table>

Exponentially growing 3T6 cells were changed to medium containing 10% serum (control) or serum-containing medium supplemented with different concentrations of tunicamycin (Tu) for 4, 8 or 24 h. During the last 30 min [³H]leucine (20 μCi ml⁻¹) was added and the rate of protein synthesis was assayed by determining the incorporation of [³H]leucine per unit protein into trichloroacetic acid-precipitable material (see Materials and methods).

Fig. 3. The effects of N-linked tunicamycin on glycosylation in exponentially growing 3T6 cells. The cells were exposed to different concentrations of tunicamycin and the effects on incorporation of glucosamine into acid-precipitable material as measured by a 1-h pulse-labelling with 10 μCi of [³H]glucosamine ml⁻¹ in the medium. The cells were harvested and assayed for radioactivity as described in Materials and methods. In one set of experiments, the dose–response relationship of tunicamycin was titrated (A) and in another experiment the temporal response of exposure to 0.4 μg tunicamycin ml⁻¹ was determined (B).

Fig. 4. The effects of tunicamycin on HMG-CoA reductase activity in exponentially growing 3T6 cells. The cells were exposed to 0.4 μg tunicamycin ml⁻¹ at the indicated time intervals, whereas the enzymic activity was determined as described in Materials and methods section.

We previously suggested that some critical event(s) in the cell cycle control may be regulated by the enzyme HMG-CoA reductase in 3T3 and 3T6 cells (Larsson & Zetterberg, 1986a,b; Larsson, 1987). Fig. 4 shows the effects of tunicamycin on the activity of this enzyme. We demonstrate that the HMG-CoA reductase activity is substantially and rapidly depressed by 0.4 μg tunicamycin ml⁻¹, indicating that this compound not only inhibits DNA synthesis and the rate of glycosylation, but also specifically decreases the activity of an enzyme that is believed to play a role in cell cycle control.

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(Brown & Goldstein, 1980; Larsson & Zetterberg, 1986a, b; Siperstein, 1984). This multifaceted spectrum of tunicamycin-induced effects, made it interesting to determine whether the inhibitory effects of tunicamycin on DNA replication could be reversed by supplementing some metabolite that appear distally to the rate-limiting HMG-CoA reductase step in the isoprene-synthetic pathway (Fig. 5).

Fig. 6 summarizes a set of experiments in which cells were simultaneously exposed to tunicamycin and mevalonate at concentrations normally required to reverse effects of other inhibitors of HMG-CoA reductase, such as mevinolin or compactin (Quesney Huneecas et al. 1979, 1980, 1983; Fairbanks et al. 1984; Habenicht et al. 1980). It was found that 0·1 mM mevalonate partially reversed the inhibitory effects of tunicamycin in the interval 0·4–1·2 µg ml⁻¹. The counteractive effect varied between 19 and 32% (Fig. 6, inset). A lesser counteractive effect (ranging from 6 to 22%) was achieved if the mevalonate concentration was increased to 1 mM.

Discussion

Transformed cells or cells of tumour origin express more highly branched oligosaccharides on their membrane glycoproteins than their normal counterparts (de Santis et al. 1987). However, in spite of considerable attention given to this issue, there has been no clear definition of the role of the carbohydrate change in the oncogenic cascade (de Santis et al. 1987). The present study has aimed at investigating the mechanism behind the inhibitory effects on cell proliferation exerted by different substances that prevent glycosylation or interfere with glycosylational processing. We used three inhibitors: namely, castanospermine, swainsonine and tunicamycin. Castanospermine inhibits the processing enzyme glucosidase I, resulting in glycoproteins with oligosaccharides containing one to three glucose residues and seven to nine mannose residues (Elbein 1984). In addition, castanospermine also exerts some effect on glucosidase II, but the major oligosaccharide accumulating in castanospermine-treated cells carries three glucose residues (Fuhrmann et al. 1985). Swainsonine on the other hand inhibits Golgi mannosidase II (Elbein, 1984), resulting in glycoproteins accumulating hybrid type oligosaccharides with one branch containing a high-mannose structure and another containing a complex type of structure. However, neither castanospermine nor swainsonine have total effects on protein glycosylation. Castanospermine inhibits the conversion of oligomannosidic glycans to complex type glycans only 40–70%, depending on the glycoprotein or cell type under study (Fuhrmann et al. 1985). Swainsonine is certainly an effective inhibitor of processing mannosidase II, but alternative pathways, independent of
mannosidase II, are known for complex assembly of complex type \(\alpha\)-glycans. In contrast, the action for the nucleoside antibiotic compound tunicamycin, which inhibits the formation of dolichyl-P-P-\(N\)-acetylglucosamine, results in the complete blockage of oligosaccharide chain addition to potential N-linked sites (Fig. 7).

It has recently been shown that castanospermine and swainsonine dramatically alter the growth phenotype of certain transformed cell types. In a recent study, Hadwiger et al. (1986) showed that NRK cells transformed with the McDonough strain of the feline sarcoma virus (SM-FeSV) reverted to a normal morphology in the presence of castanospermine. Furthermore, in the presence of this inhibitor the SM-FeSV-transformed cells became serum-dependent and incapable of growing in soft agar, which indicates that the transformed properties were altered. Similarly, NIH 3T3 fibroblasts transfected with human tumour DNA (al-1) lost their capacity to grow in soft agar after treatment with swainsonine (de Santis et al. 1987). However, we have examined the way swainsonine and castanospermine affect SV40-transformed Swiss 3T3 cells, but observed no inhibition of cell proliferation or any other alteration of the transformed phenotype (Larsson & Engström, unpublished data). The present study has been unable to demonstrate any significant inhibitory effect of castanospermine or swainsonine on the proliferation of 3T6 cells. In marked contrast, tunicamycin efficiently blocked DNA synthesis in this cell type. The magnitude of the effects on 3T6 cell growth was comparable to that observed with 3T3 cells (Jimenez de Asua et al. 1983, 1984; Larsson, 1985).

Furthermore, we demonstrate that tunicamycin exerts a cell cycle-specific effect on 3T6 cells, in the sense that only cells in early \(G_1\) were blocked in their cell cycle progression after tunicamycin treatment. Similar observations have been made on exponentially growing 3T3 cells in which tunicamycin exerts a \(G_1\)-specific effect (Larsson, 1985), as it also does in quiescent cells stimulated to resume proliferation by serum addition (Jimenez de Asua et al. 1983, 1984).

Our finding that tunicamycin's inhibitory action is limited to early \(G_1\) suggests that this compound acts via some stage-specific regulatory system(s). One such pathway that has previously attracted our attention is the biosynthesis of cholesterol and isoprenoid derivatives (Larsson & Zetterberg, 1986a,b). The rate-limiting step in this pathway is the conversion of HMG-CoA to mevalonate (Fig. 4); a step that is catalysed by the enzyme HMG-CoA reductase (Brown & Goldstein, 1980). If the activity of this enzyme is inhibited, e.g. by addition of 25-hydroxy-cholesterol, the cells become arrested in a cell cycle-specific manner (Larsson & Zetterberg, 1986b). Likewise with serum withdrawal, 25-hydroxy-cholesterol treatment forces the cells to leave the cell cycle from early \(G_1\). The effect is reversible and the cells return to the cell cycle when physiological growth conditions are restored (Larsson & Zetterberg, 1986b).

Volpe & Goldberg (1983) have demonstrated that one important action of tunicamycin is to specifically inhibit HMG-CoA reductase activity. As shown in the present study tunicamycin also inhibits the HMG-CoA reductase activity in 3T6 cells efficiently. Of special interest was the finding that the tunicamycin-induced inhibitions of both N-linked glycosylation and HMG-CoA reductase activity were attained within a few hours, whereas the inhibition of overall protein synthesis required a more prolonged treatment. Considered together with the finding of an immediate inhibitory effect on cell cycle progression, these data suggest that inhibition of either N-linked glycosylation or reductase activity, or both, may mediate the tunicamycin-induced growth inhibition. In order to clarify whether the inhibitory effects on 3T6 cell growth were in fact mediated via the mevalonate pathway we added mevalonate to tunicamycin-treated cells. We found that addition of mevalonate counteracts the tunicamycin-
induced inhibitory effect on cell proliferation, but only to a limited extent. Our finding that mevalonate exerted only a partial counteractive effect on DNA synthesis can be explained by intrinsic toxic effects of tunicamycin. In fact the cell numbers were significantly lower in the presence of tunicamycin, indicating that this compound seriously affects cell survival as well as blocking progression through the cell cycle. However, it is also conceivable that tunicamycin does act on different metabolic and regulatory levels, out of which one site of action is constituted by HMG-CoA reductase. HMG-CoA reductase is a transmembrane glycoprotein located at the endoplasmic reticulum with an active domain facing the cytosol and high-mannose asparagine-linked oligosaccharide chain-bearing domain oriented towards the lumen of the endoplasmic reticulum (Liscum et al. 1983; Chin et al. 1984). It is then considered that this oligosaccharide is of regulatory importance for the function of the enzyme, it is conceivable that inhibition of asparagine-linked glycosylation following treatment with tunicamycin would depress the enzyme activity.

The effects observed in our study can, however, only partly explain the inhibitory effects on DNA synthesis. It would therefore be interesting to examine other metabolic and regulatory effects of tunicamycin on this cell line. This work is in progress.

References


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