

Effects of Insulin and LongR³ on Serum-Free Chinese Hamster Ovary Cell Cultures Expressing Two Recombinant Proteins

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Insulin is the most commonly used growth factor for sustaining cell growth and viability in serum-free Chinese hamster ovary (CHO) cell cultures. In the present study insulin and IGF-1 analogue (LongR³) were compared for their ability to support growth, viability, and production of two serum-free CHO cell lines expressing recombinant protein. The first cell line, VA12, expresses protein B, and the second cell line, CL23, expresses protein C. Both molecules are recombinant cytokine receptors. VA12 will grow in serum-free media lacking growth factor, while CL23 requires either insulin or LongR³ for cell growth. Both cell lines, however, require a growth factor for optimal performance under production conditions. In this study, LongR³ was better able to sustain the viability of both cell lines under production conditions than insulin. These data indicate that while insulin and LongR³ can both serve as growth and viability factors for CHO cells, LongR³ is the preferred growth factor for cell lines VA12 and CL23.

Introduction

Dihydrofolate reductase (DHFR)-deficient Chinese hamster ovary (CHO) cells are commonly used in the biotechnology industry for production of recombinant proteins. These cells are easy to grow, are genetically stable, and can express high levels of recombinant protein using the amplifiable selectable marker DHFR. To improve the safety of drugs produced with these cells, serum is removed from the growth and production media. To maintain the growth of serum-free DXBII cells in culture, insulin can be added to the media (1). IGF-1 will also serve as a growth and viability factor for these cells and can be either added to the media (2) or transfected into the cells for autocrine growth stimulation (3). The cells can also be adapted to grow in serum-free media without any growth factors (2, 4), but DHFR⁺ revertants have been observed (4).

LongR³ is a fusion protein derivative of human IGF-1 that binds with lower affinity to IGF-1 binding proteins and subsequently has much higher bioavailability than native IGF-1 (5). Specifically, LongR³ consists of the complete human IGF-1 sequence with the substitution of an Arg (R) for the Glu (E) at position 3. In addition there is a 13 amino acid peptide at the N-terminus (5).

In a previous report we have shown that LongR³ is able to substitute for insulin in serum-free production cultures (6). The cells used in that study required serum for growth and only used serum-free media in the production cultures. In the present study the effects of insulin and LongR³ on production cultures of two completely serum-free cell lines were investigated. We have shown that with the completely serum-free cells, LongR³ is better able to sustain higher cell viability than a recombinant form of insulin. The higher cell viability resulted in higher titers for the cultures maintained with LongR³.

Materials and Methods

Cell Lines. VA12 and CL23 cells were made by transfection of serum-free adapted DXBII cells (2) with

an expression vector containing cDNA encoding protein B or C, respectively. The host cells used were able to grow in serum-free media containing LongR³ (JRH, Kansas City, KS). The vector used for expression of both proteins was 2a5I (7). VA12 cells were adapted to grow in serum-free media without LongR³ after transfection with the expression plasmid. CL23 cells were grown in media containing either LongR³ or insulin as described below and in the results section.

Tissue Culture. VA12 cells were grown in selective serum-free IMX media (selective SF-IMX) (2) that did not contain LongR³ or transferrin. The VA12 growth media also included 150 nM methotrexate (Lederle Laboratories, Pearl River, NY) (MTX). CL23 cells were grown in the same base media with various amounts of insulin (Intergen, Ontario, Canada) or LongR³ as described in the results section. These cells were maintained in 300 nM MTX. In the CL23 growth maintenance study, cells were passed every 2-3 days and seeded by dilution at 5×10^5 cells/mL in a 125 mL shaker flask. The culture volume was 30 mL. Parallel cultures were maintained for at least 2 weeks in media containing various concentrations of insulin or LongR³ prior to production experiments.

The VA12 production cultures were performed in SF-IMX (2), which is similar to the selective SF-IMX media and contains glycine, hypoxanthine, and thymidine and does not contain methotrexate. The production media used for the experiments with CL23, IMX7.1, is similar to SF-IMX and contains approximately 2× the amino acids and vitamins compared with SF-IMX. In the production experiments, shaker flasks were seeded with 30 mL of cell suspension at 2×10^6 cell/mL. The cells were suspended in production media supplemented with LongR³ or insulin at various concentrations or no growth factor, as described in the text. Stock solutions of insulin and LongR³ at 1 mg/mL were prepared in 10 mM HCL and dispensed to the cultures as required by the experiment. Cells were sampled from the cultures every 2-3

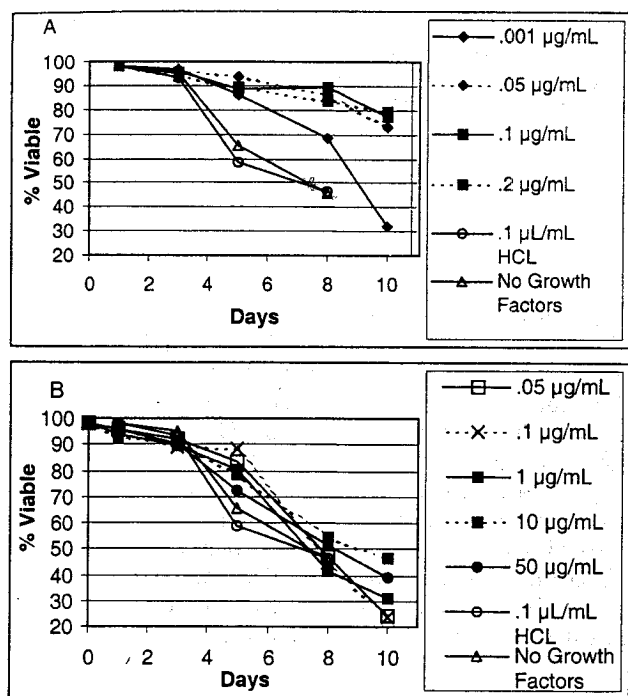


Figure 1. Viability of VA12 production cultures containing either LongR³ or insulin. (A) Percent viability of VA12 production cultures maintained in the presence of various concentrations of LongR³. (B) Percent viability of VA12 production cultures maintained in the presence of various concentrations of insulin. The concentration of each growth factor is presented in the graph legend on the right. Each point represents the average viability as determined by trypan blue staining from two independent cultures.

days, and viability, glucose consumption, pH, and lactate production were monitored. Glucose was added on demand. The pH of the production shake flask cultures ranged from about 6.8 to 7.2.

Results

Production Cultures using VA12 Cells. To compare the ability of LongR³ and insulin to maintain viability and support production from VA12 CHO cell cultures, a dose response to both growth factors was performed. Previous reports have shown that insulin at concentrations of 5–50 μg/mL are able to sustain CHO production cultures (6, 8, 9). In this study, insulin was tested at concentrations ranging from 0.001 to 0.2 μg/mL. LongR³ had previously been shown to be effective in sustaining CHO cell cultures at 0.1 μg/mL (6) and was tested at concentrations ranging from 0.001 to 0.2 μg/mL. Shake flask production cultures seeded with 2×10^6 cells/mL in SF-IMX production media were monitored. Initially, all of the cultures maintained similar viability, but after about 6 days, cultures supplemented with insulin lost viability more rapidly compared to LongR³-supplemented cultures. Growth-factor-free cultures began losing viability from the start of the production culture (Figure 1a and b). In general, cell viability was maintained to higher levels in the presence of LongR³ compared to insulin. Production cultures with >0.05 μg/mL LongR³ had final viability of 75–80% after 10 days in production (Figure 1a). At lower LongR³ concentrations, cell viability declined more rapidly. A dose effect of insulin on viability was observed with 10 or 50 μg resulting in highest cell viability; however, these numbers were still lower compared to the cases with >0.05 μg/mL LongR³ (Figure 1b).

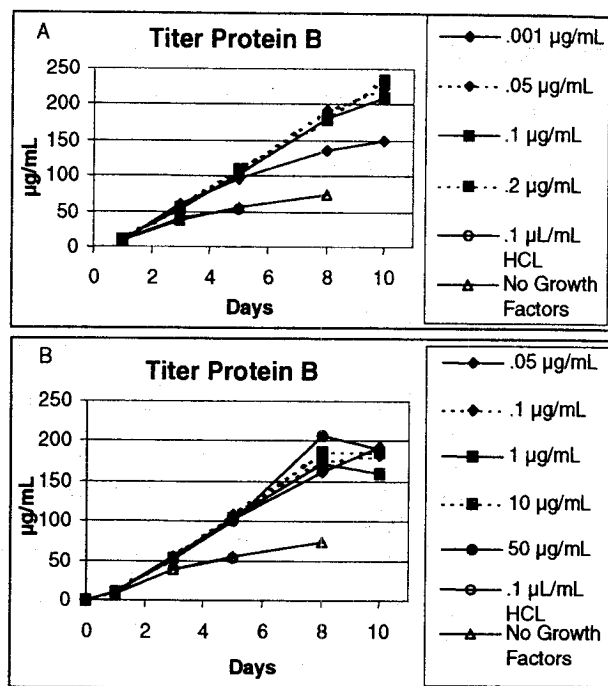


Figure 2. Titer of VA12 production cultures containing either LongR³ or insulin. (A) The titer of protein B from VA12 shake flask production cultures maintained with various concentrations of LongR³. (B) The titer of protein B from VA12 shake flask production cultures maintained with various concentrations of insulin. The concentration of each growth factor is presented in the graph legend on the right. Each point represents the average titer as determined by ELISA from two independent cultures.

After a 10 day production experiment, insulin-containing cultures had viability of less than 50%.

Production cultures containing LongR³ continued to produce protein B throughout the production run with final titers >200 mg/L (Figure 2a). Production in the insulin-containing cultures reached a plateau after 8 days, and the final titer was <200 mg/L (Figure 2b). The cultures that did not receive growth factors had substantially reduced viability, and titers compared to the cultures that received either LongR³ or insulin.

Glucose and lactate consumption from representative cultures from the production experiment are shown in Figure 3. The cultures containing the highest and lowest levels of growth factor had similar glucose and lactate profiles, but these differed from the cultures that did not contain growth factors. The cells, which did not receive growth factors, consumed less glucose and produced less lactate compared with the growth factor containing cultures (Figure 3 a and b). The cultures with intermediate levels of growth factor had lactate and glucose profiles similar to the cases with growth factors presented in Figure 3 (data not shown).

It has been shown that CHO cells contain an insulinase activity in their media and can degrade insulin, causing loss of viability in insulin-supplemented cultures (8). To determine whether feeding the cultures growth factors during the production runs could improve the culture performance, production cultures were run with addition of growth factor feeds (Figure 4a and b). These cultures were fed growth factors at the beginning of the run and on days 3 and 5. The cultures that received multiple insulin or LongR³ feeds did not perform differently than the cultures that were given growth factor at the beginning of the experiment. Similar to what was found for the cultures in Figures 1 and 2, the cultures containing

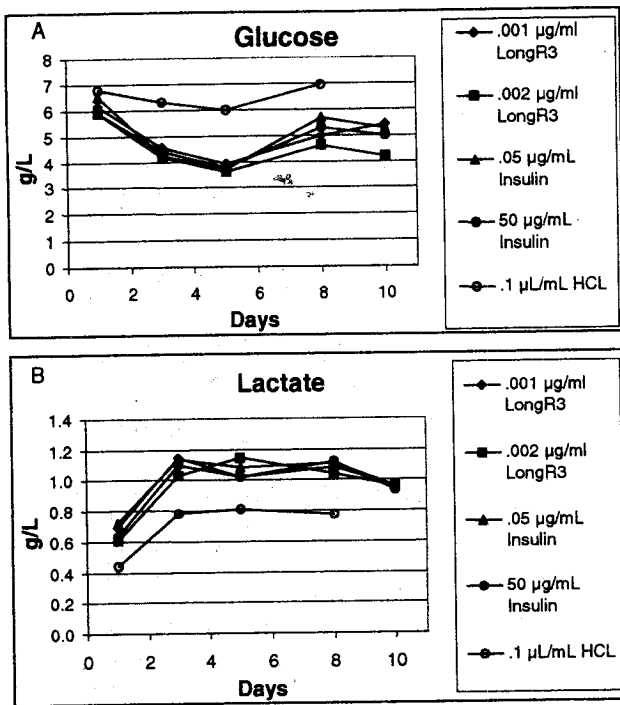


Figure 3. Glucose and lactate readings for production cultures. (A) Glucose measurements for VA12 shake flask production cultures. The concentration of growth factor in the production media is indicated in the figure legend on the right. (B) The concentration of lactate in VA12 shake flask production cultures containing various concentrations of growth factor. The concentration of growth factor is indicated in the legend on the right.

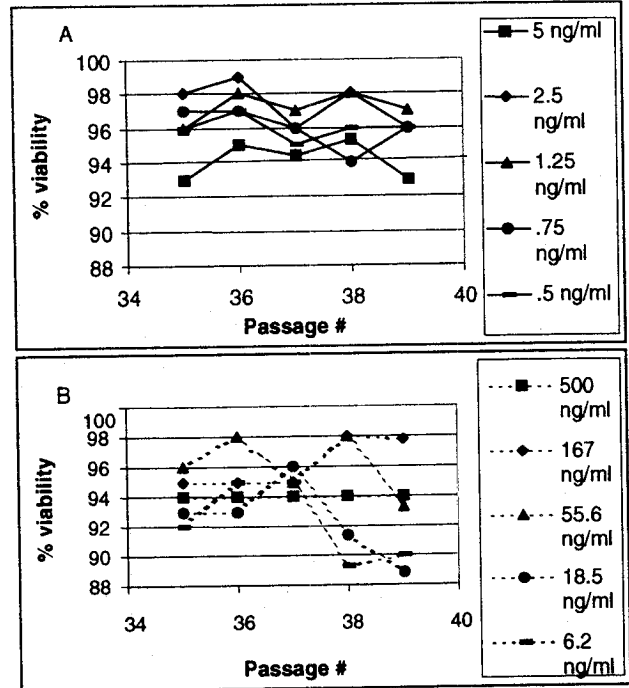


Figure 5. Viability of CL23 stock cultures grown in media containing LongR³ or insulin. (A) Percent viability of CL23 cells cultured in various concentrations of LongR³. Each point represents the average viability as determined by trypan blue for two independent cultures. (B) Percent viability of CL23 cells cultured in various concentrations of insulin. Each point represents the average viability as determined by trypan blue for two independent cultures. The LongR³ and insulin concentrations are indicated on the graph legend on the right.

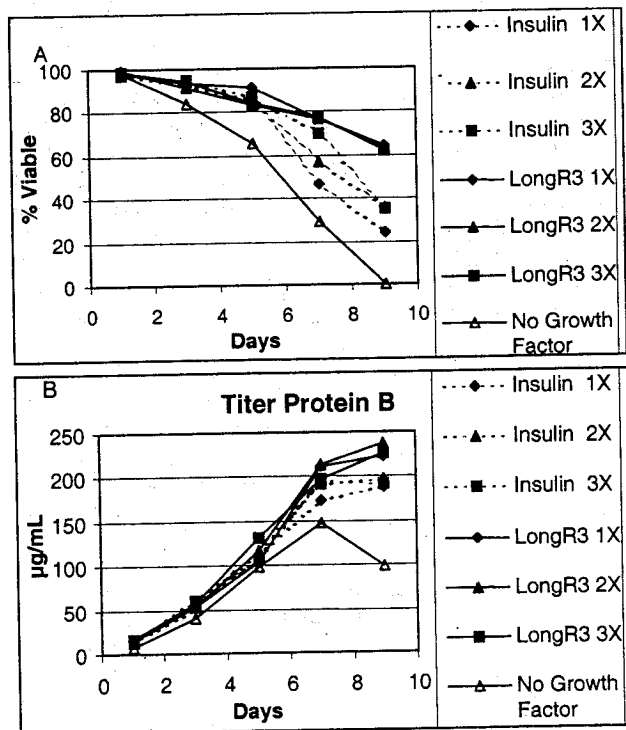


Figure 4. Viability and titer of VA12 production cultures fed with growth factors. (A) Percent viability of VA12 production cultures maintained with and without growth factor feeds. (B) Titer of VA12 cell production cultures maintained with and without growth factor feeds. In this experiment the cultures were fed growth factor at the start of the cultures (1×). Some cultures were fed again on day 3 (2×), and some were fed again on day 5 (3×). Each feed consisted of either 0.1 µg/mL of LongR³ or 10 µg/mL of insulin as indicated in the legend.

LongR³ had higher viability at the end of the production cycle than the cultures containing insulin (60% viable compared with 20–40% viable) (Figure 4a). The cultures containing LongR³ produced >200 mg/L protein B, and the insulin feed cultures produced <200 mg/L protein B (Figure 4b).

Glucose and lactate were measured during the course of the production culture, and the cells that received growth factors had higher glucose consumption compared to the cases that did not receive growth factors (data not shown). The lactate levels in the cultures that received growth factor were similar to each other and greater than the control case, which did not receive growth factor (data not shown). These data were similar to what was found for glucose and lactate in VA12 cultures shown in Figure 3.

Production Cultures using CL23. To compare the effects of LongR³ and insulin with another serum-free cell line, experiments were performed using CL23, a serum-free CHO cell line expressing a second recombinant cytokine receptor (protein C). Unlike VA12, CL23 requires growth factor in its culture medium for cell growth. The first comparison was to determine whether insulin and LongR³ were both able to maintain stock cultures of these cells. CL23 cell cultures were maintained with either insulin or LongR³ for a 2-week period. In CL23 growth cultures, insulin and LongR³ could be titrated to ng levels and maintained cell viability at about 90% or higher (Figure 5a and b). Several attempts were made to culture CL23 cells in serum-free media without growth factors, but the cells lost viability after about 10 passages.

To compare LongR³ and insulin using CL23 production cultures, CL23 growth cultures maintained with LongR³ at 0.1 or 0.005 µg/mL were used in production cultures

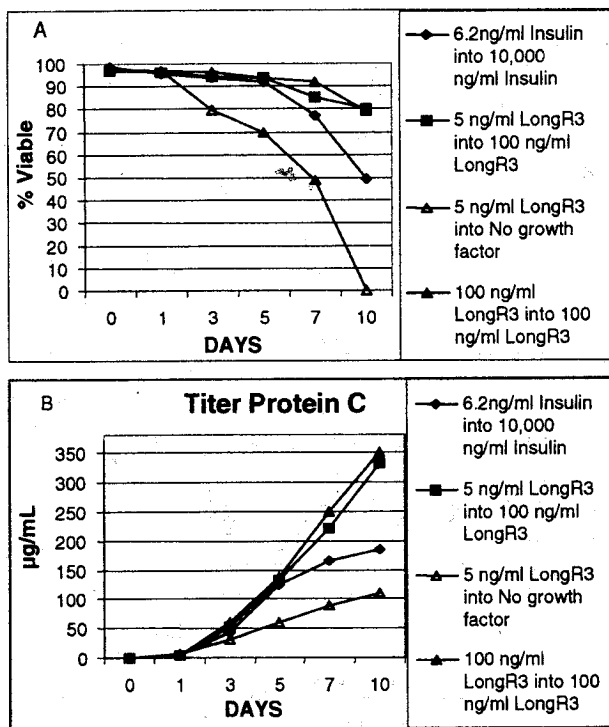


Figure 6. Viability and titer of CL23 production cultures taken from different stock cultures. (A) Percent viability of production cultures using various stocks of CL23 cells. (B) Titer values for the production cultures using CL23 cells. The different stock cultures of CL23 were cultivated as indicated in the graph legend. The insulin-containing production culture was set up from a stock culture containing 6.2 ng/mL insulin. Two of the LongR³-containing cultures were set up from stock cultures containing 5 ng/mL LongR³. The third LongR³-containing culture was taken from a culture containing 100 ng/mL LongR³. Each point represents the average from two independent cultures.

using media that contained 100 µg/mL LongR³. Since the viability of the LongR³ growth cultures was the same in high and low growth factor cultures, we wanted to assess if the level of growth factor in the growth media affected the outcome of the production culture. The CL23 cells maintained in 6.2 µg/mL insulin were used in production cultures that contained 10 µg/mL insulin. For this study, 2×10^6 cells/mL in IMX7.1 media were used. Similar to the results with the VA12 cells, the CL23 production cultures using LongR³ had significantly better viability than the insulin-containing cultures after day 5 of the production culture (Figure 6a). The titers in the LongR³ cultures were about 175% greater than those in the cultures containing insulin. In production cultures, cells grown in 0.005 µg/mL LongR³ performed similarly to cells grown in 0.1 µg/mL LongR³ (Figure 6b).

Glucose and lactate were monitored in the CL23 production cultures, and it was found that the cultures that did not receive growth factor during the culture did not consume as much glucose compared with the cultures that did receive growth factor. The glucose consumption for cultures that received growth factor was similar (Figure 7a). The lactate profile of all the cultures was similar (Figure 7b).

Discussion and Conclusions

In this report we have compared the ability of LongR³ and insulin to maintain viability and production of two serum-free CHO cell lines, VA12 and CL23. VA12 stock cultures will grow without any growth factor, and the

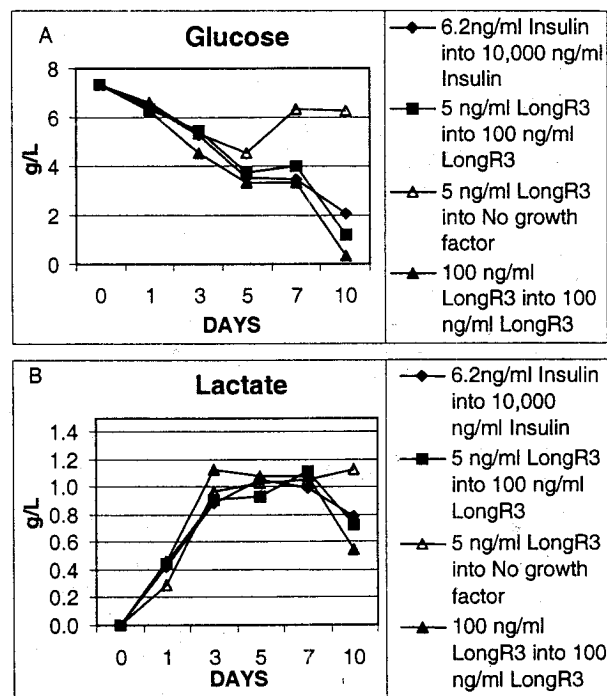


Figure 7. Glucose and lactate readings for CL23 production culture. (A) Glucose measurements from cultures presented in Figure 6. (B) Lactate measurements from cultures described in Figure 6. The different stock cultures of CL23 were cultivated as indicated in the graph legend. The insulin-containing production culture was set up from a stock culture containing 6.2 ng/mL insulin. Two of the LongR³-containing cultures were set up from stock cultures containing 5 ng/mL LongR³. The third LongR³-containing culture was taken from a culture containing 100 ng/mL LongR³. Each point represents the average from two independent cultures.

comparison was made in production cultures only. Here a consistent 15–20% improvement in culture titer was observed when using LongR³ compared with insulin. CL23 cell stocks were cultured with varying amounts of LongR³ or insulin, and it was found that both growth factors were able to maintain the cultures. As with the VA12 cells, CL23 production cultures containing LongR³ had better viability and titers than the production cultures maintained with insulin. We do not think the differences we see in the response to the growth factors are due to turnover or bioavailability of the ligands, because feeding during production did not change the outcome (Figure 4). The fact that the production cultures containing LongR³ continued to accumulate recombinant protein on day 10 of the culture suggests that the production culture time could be extended using LongR³.

CHO cells have relatively few insulin receptors (10), and the number of IGF-1 receptors fluctuates with growth conditions but is generally higher than the number of insulin receptors (11). Previous studies indicate that IGF-1 signals exclusively through its cognate receptor (12), and insulin is able to signal through the IGF-1 receptor only at supra-physiological concentrations. Since the number of insulin receptors is relatively low in CHO cells and insulin is required at high concentrations (10 µg/mL) in the experiments reported here, it seems likely that both insulin and LongR³ are signaling primarily through the IGF-1 receptor.

The differential response of the cultures to LongR³ and insulin could be due to several mechanisms. If both growth factors are signaling primarily through the IGF-1 receptor, it could be that the two ligands induce different conformational changes in the receptor upon signaling

that cause qualitative differences in the duration and nature of downstream events. It could be that the two ligands induce differences in the cycling of the receptor ligand complex, which could lead to differences in signal transduction. It has been shown that insulin and IGF-1 receptor ligand complexes have different rates of internalization and cycling (13). IGF-1 and insulin receptors are also known to form chimeric receptors on mammalian cells (14). Variability in the amount of chimeric receptors in the presence of each growth factor, as well as differences in the receptor ligand interactions, could also contribute to the observed differences in cell viability in the production cultures.

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