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Up-Regulation of Interleukin-4 Receptor Expression by Interleukin-4 and CD40 Ligation via Tyrosine Kinase-Dependent Pathway

Hyun-il Kim,[†] Eui-Young So,[†] Suk-Ran Yoon,[‡] Mi Young Han,[§] and Choong-Eun Lee[†]*

[†]Department of Biology, College of Natural Science, Sung Kyun Kwan University, Suwon, 440-746, Korea.
[‡]Immune Signal Transduction Reserach Unit. [§]Immune Regulation Research Unit,
Korea Research Institute of Bioscience and Biotechnology, Taejon, 305-600, Korea.

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Recently a B cell surface molecule, CD40, has emerged as a receptor mediating a co-stimulatory signal for B cell proliferation and differentiation. To investigate the mechanism of synergy between interleukin-4 (IL-4) and CD40 ligation in B cell activation, we have examined the effect of CD40 cross-linking on the IL-4 receptor expression in human B cells using anti-CD40 antibody. We observed that IL-4 and anti-CD40 both induce IL-4 receptor gene expression with a rapid kinetics resulting in a noticeable accumulation of IL-4 receptor mRNA within 4 h. While IL-4 caused a dose-dependent induction of surface IL-4 receptor expression, the inclusion of anti-CD40 in the IL-4-treated culture, further up-regulated the IL-4-induced IL-4 receptor expression as analyzed by flow cytometry. Pretreatment of B cells with inhibitors of protein tyrosine kinase (PTK) resulted in a significant inhibition of both the IL-4- and anti-CD40-induced IL-4 receptor mRNA levels, while protein kinase C (PKC) inhibitors had no effects. These results suggest that IL-4 and CD40 ligation generate B cell signals, which via PTK-dependent pathways, lead to the synergistic induction of IL-4 receptor gene expression. The rapid induction of IL-4 receptor gene expression through the tyrosine kinase-mediated signal transduction by B cell activating stimuli, would provide cells capacity for an efficient response to IL-4 in the early phase of IL-4 action, and may in part constitute the molecular basis of the reported anti-CD40 co-stimulatory effect on the IL-4-induced response.

* To whom correspondence should be addressed.
Tel: 82-331-290-7006; Fax: 82-331-290-7015; E-mail: celee@yurim.skku.ac.kr.

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Introduction

Cell–cell communication in the immune system is performed via two types of signals. One is the signal delivered by cell-secreted soluble factors, such as cytokines, and the other is the signal delivered via physical contact of cell-surface molecules expressed on the two interacting cells. Recently the role of B cell surface molecule CD40 and its interaction with CD40 ligand (CD40L) on T cells have been strongly implicated in T cell-dependent B cell activation, especially in proliferation, prolonged survival, and terminal differentiation of B cells (Hodgkin *et al.*, 1991; Banchereau *et al.*, 1994; Noelle *et al.*, 1994; Kato *et al.*, 1994). Most strikingly, the co-stimulatory action of CD40 in augmenting the IL-4-induced response has been observed with respect to B cell isotype switching to IgE and the subsequent production of IgE (Russet *et al.*, 1991; Shapira *et al.*, 1992; Splawski *et al.*, 1993). Moreover our recent study has shown that anti-CD40 can synergize with IL-4 to induce the expression of CD23, the type II IgE receptor in human primary B cells (Kim *et al.*, 1997), suggesting that there is a cross-talk in signaling generated by IL-4 and CD40, which in turn regulates IgE response. The signal transmission of IL-4 is mediated by the high affinity IL-4 receptors on the cell surface (Cabrillat *et al.*, 1987; Ohara and Paul, 1987), and the receptor modulation may be an important mechanism for the regulation of IL-4 action leading to B cell activation. Thus we have investigated the effect of CD40 ligation by anti-CD40 treatment on the IL-4 receptor expression and the signaling pathways involved in this process.

Our data demonstrate that not only can anti-CD40

induce IL-4 receptor in human primary B cells, it also works with IL-4 synergistically to enhance the IL-4-induced receptor expression. Such induction of IL-4 receptor by IL-4 and anti-CD40 stimulation occurs via PKC-independent and tyrosine-kinase-dependent signaling pathways, characteristic of cytokine-induced primary response gene induction. The rapid up-regulation of IL-4 receptor upon CD40 ligation may have implications in the role of CD40-CD40L interaction in amplifying the IL-4 response during the IL-4-induced B cell activation.

Materials and Methods

B cell purification and culture Human primary B cells were obtained by purification from freshly excised tonsils. Tonsils were gently teased apart and cells were released by grinding through a fine wire-mesh. Mononuclear cells were isolated by layering the cell suspension on Ficoll-Hypaque (d = 1.077; Sigma, St. Louis, USA) and centrifugation at 500 × *g* for 20 min. B cells were then purified by rosetting T cells twice with AET-treated sheep RBC. After adherent cells were removed by plate adherence, the purity of B cells was examined by staining with anti-CD20, anti-CD3, or anti-CD14 mAbs (Immunotech, Westbrook, USA) and found to be greater than 90%. Purified B cells were then cultured in RPMI media (GIBCO, Gaithersburg, USA) containing 10% FBS (GIBCO), 10 mM Hepes, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 50 μg/ml amphotericin B and 50 μg/ml gentamycin. Recombinant human IL-4 purchased from Genzyme (Cambridge, USA) or expressed in *E. coli* (Yang *et al.*, 1992), phorbol myristic acetate (PMA), or agonistic anti-CD40, 5C3 (Dr. Kikutani, Osaka Univ.), were treated to cells, which were then cultured for indicated periods in humidified 5% CO_2 at 37°C. Treatment of cells with protein kinase inhibitors were done for 1 h prior to the addition of IL-4, PMA, or anti-CD40.

IL-4 receptor mRNA analysis by Northern blot Total cytoplsmic RNAs from tonsillar B cells (1×10^8 cells) were isolated after treatment with IL-4 or anti-CD40 for 1–12 h, using 4 M guanidinium isothiocyanate and 5 M cesium chloride through ultracentrifugation (Chirgwin *et al.*, 1979). Five to ten micrograms of total RNAs were fractionated on a 1% agarose gel containing formaldehyde, transferred to nylon membrane, and hybridized with a [^{32}P]-labeled cDNA probe of IL-4 receptor (Dr. Gillis, Immunex Corp, Seattle, USA). RNA concentrations were determined by OD measurement and the amount of loaded RNA on the gel was confirmed by EtBr staining.

Flow cytometric analysis of surface IL-4 receptor expression B cells (1×10^6 cells) were cultured in the presence of IL-4 or anti-CD40 for 24 h. The cells were washed with PBS and recultured for 2 h in the fresh media according to Zuber *et al.* (1990). The levels of IL-4 receptor expression on cultured B cells were then analyzed by staining cells with mouse monoclonal anti-IL-4 receptor antibodies (M56 or M57, Immunex) as a primary antibody and anti-mouse IgG-FITC (Immunotech) as a secondary antibody in HBSS containig 3% FBS and 1% NaN_3 for 30 min at 4°C, using fluorescence-activated cell scanner (FACScan, Becton Dickinson, Mountain View, USA). An aliquot of each treated cell sample was stained with the secondary antibody alone to be used as control. Surface IL-4 receptor levels were expressed as the mean fluorescence intensity (MFI) for IL-4 receptor. ΔMFI was calculated as MFI of cells stained with anti-IL-4 receptor and anti-mouse IgG-FITC minus(−) MFI of cells stained with anti-mouse IgG-FITC alone. The values represent a mean of three independent determinations and S.D. is less than 10% of the mean.

Results and Discussion

Induction of IL-4 receptor gene expression by IL-4 and CD40 ligation Receptor modulation is considered as an important event for the ligand-induced signal transduction. Since there has been strong evidence indicating that CD40 stimulation provides a co-stimulatory signal for the IL-4-induced B cell activation response, we examined the effect of CD40 ligation on the IL-4 receptor gene expression and compared to that of IL-4 in human primary B cells. As shown in Fig. 1, the treatment of cells with IL-4 as well as anti-CD40 caused a noticeable induction of IL-4 receptor mRNA within 4 h. While the anti-CD40-induced IL-4 receptor mRNA expression is somewhat weaker than that induced by IL-4 (Fig. 1, Panel A), the addition of IL-4 to the anti-CD40 treated cells further enhanced the IL-4 receptor mRNA level (Fig. 1, Panel B). Such up-regulation of IL-4 receptor by its ligand IL-4 and anti-CD40 treatment has not been observed in certain EBV-transformed lymphoblastoid B cell lines which, despite the presence of a high number of IL-4 receptors, is shown to be unresponsive to the biological effects of IL-4, such as induction of IgE receptor and class II MHC expression (Russet *et al.*, 1988; Lee and Kim, 1995). This has been interpreted as that IL-4 receptor modulation may be required for the effective cellular response to IL-4 and the subsequent B cell activating stimuli, together which lead to the terminal differentiation of B cells.

The kinetic analysis of IL-4 receptor mRNA induction has revealed that IL-4 receptor gene activation is an early event during the IL-4 signal transduction, as shown by rapid accumulation of mRNA within 2–4 h of IL-4 stimulation, which soon reaches to the maximal level by 6 h (Fig. 2). Such rapid induction of IL-4 receptor gene expression suggests that it is, like the type II IgE receptor CD23, a primary response gene induced by IL-4, occurring in the absence of ongoing protein synthesis (Lee *et al.*, 1993) and may be responsible for the later expression of increased IL-4 receptor on the B cell surface observed at 24 h (Galizzi *et al.*, 1989). It should be noted that while the up-regulation of IL-4 receptor by IL-4 was more prominent with *in vitro* pre-activated human B cells compared to resting human B cells (Zuber *et al.*, 1990), anti-CD40 treatment alone has been shown rather ineffective in the induction of IL-4 receptor expression in resting as well as *in vitro* pre-activated B cells (Valle *et al.*, 1989). With

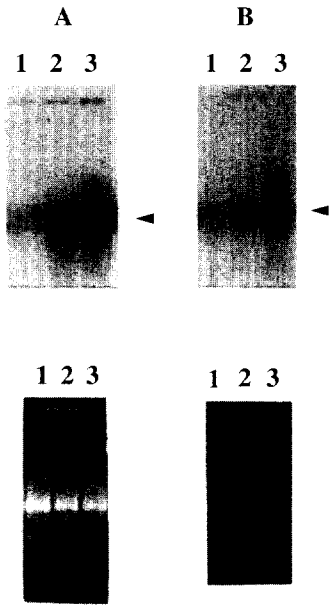

Fig. 1. Induction of IL-4R mRNA by IL-4 and anti-CD40 treatment.

Panel A: Tonsillar B cells were cultured in the presence of media alone (lane 1), 2 μg/ml anti-CD40 (lane 2) or 200 U/ml IL-4 (lane 3) for 4 h. The cells were harvested for RNA isolation after which Northern blot analysis was performed as in Materials and Methods.

Panel B: Purified B cells were cultured in the presence of media alone (lane 1), 2 μg/ml anti-CD40 (lane 2), or 2 μg/ml anti-CD40 plus 200 U/ml IL-4 (lane 3) for 4 h. mRNA analysis was then performed as in Panel A. Top: Northern blot autoradiogram; Bottom: EtBr-stained RNA gel.

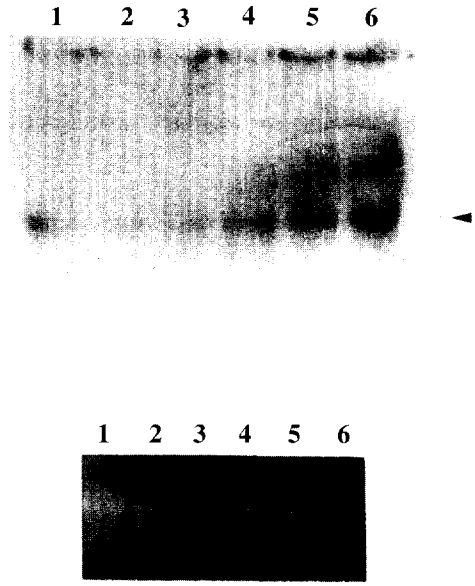

Fig. 2. Kinetics of IL-4 receptor mRNA induction. Purified B cells were treated with 200 U/ml IL-4 for various durations, after which RNAs were prepared for Northern blot analysis as in Fig. 1. Lane 1, media alone (0 h); lane 2, 1 h; lane 3, 2 h; lane 4, 4 h; lane 5, 6 h; lane 6, 12 h. Top: Northern blot autoradiogram; Bottom: EtBr-stained RNA gel.

unfractionated human primary B cells consisting of small resting and *in vivo* pre-activated B cells used in this study, both IL-4 and anti-CD40 were capable of inducing IL-4 receptor gene expression at a comparable level. The enhancement of the anti-CD40-induced IL-4 receptor mRNA level by IL-4 (Fig. 1B), suggests that two stimuli may work cooperatively to increase cellular response to IL-4.

Synergistic induction of surface IL-4 receptor expression by IL-4 and CD40 ligation As shown in Fig. 3 (Panel A), anti-CD40, at a concentration for the optimal B cell activation response (Gordon *et al.*, 1987), induced a marginal yet detectable level of surface IL-4 receptor expression as analyzed by flow cytometry using anti-IL-4 receptor antibody. Higher or lower concentrations of anti-CD40 were found to be less effective for IL-4 receptor induction (data not shown). The addition of IL-4 to the anti-CD40-treated B cell culture, however, induced a significant increase in the IL-4 receptor expression on the B cell surface (Fig. 3, Panel A), which is in a good agreement with the mRNA data in Fig. 1, Panel B. While IL-4 induced the receptor expression in a dose-dependent fashion leading to the saturation level at 100–200 U/ml of IL-4, addition of anti-CD40 to the IL-4-treated cells caused a striking up-regulation of IL-4 receptor (Fig. 3, Panel B). Similar effects of IL-4 and anti-CD40 in augmenting each other's response have been observed in our recent studies on the induction of CD23 (Kim *et al.*, 1997), a B cell differentiation marker molecule, whose expression is indicative of activated B cells (Gordon *et al.*, 1986; Yukawa *et al.*, 1987). Together, these data suggest that IL-4 and anti-CD40 each utilizes distinct signaling pathways to regulate IL-4 receptor and CD23 expression. The ability of anti-CD40 to up-regulate the IL-4-induced IL-4 receptor expression would further amplify the IL-4-induced cellular response including CD23 expression and other down-stream B cell activation events.

The IL-4 receptor gene expression is regulated by PTK-dependent pathways To investigate signaling pathways involved in the IL-4- or anti-CD40-induced IL-4 receptor expression, we have examined the effect of several protein kinase inhibitors on the IL-4 receptor gene expression upon IL-4 stimulation or CD40 ligation.

Although the IL-4-induced signal transduction pathways for B cell activation have not been elucidated in detail, our previous studies with quiescent human B cells have shown that, while PKC activators can effectively induce CD23 expression, IL-4 induces CD23 gene expression via pathways not involving PKC (Lee *et al.*, 1993). In the

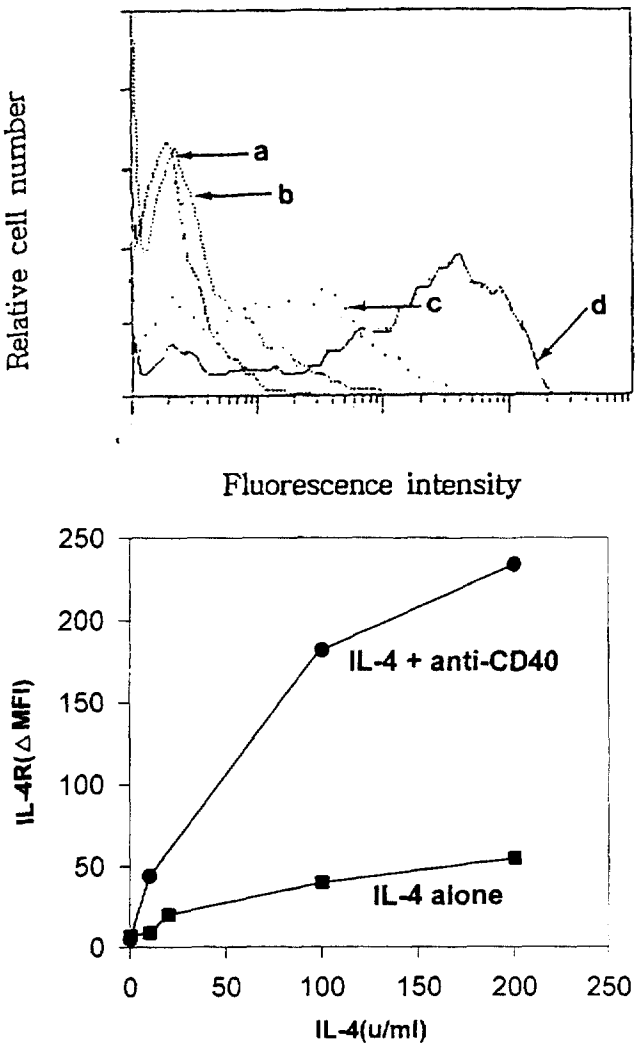

Fig. 3. Induction of surface IL-4 receptor expression by anti-CD40 and IL-4.

Panel A: Effect of IL-4 on the anti-CD40-induced IL-4 receptor expression. B cells were cultured in the presence of anti-CD40 (2 μg/ml) alone, or together with varying concentrations of IL-4 for 24 h, after which cells were harvested and stained with mouse anti-IL-4 receptor mAbs and anti-mouse IgG-FITC for FACScan analysis as described in Materials and Methods. A representative of FACScan histograms from several experiments is shown. a, anti-CD40; b, anti-CD40 + IL-4 10 U/ml; c, anti-CD40 + IL-4 100 U/ml; d, anti-CD40 + IL-4 1000 U/ml.

Panel B: Synergistic effect of anti-CD40 on the IL-4-induced IL-4 receptor expression. B cells were treated with varying doses of IL-4 alone, or together with anti-CD40 (2 μg/ml) for 24 h. Cell staining and FACScan analysis were done as in Panel A. Each point represents a mean of three independent determinations and S.D. is less than 10% of the mean.

present study we observed that the stimulation of B cells with PMA induced a noticeable induction of IL-4 receptor gene expression, which was completely abrogated upon the pretreatment of cells with a PKC inhibitor, H-7. The IL-4-induced IL-4 receptor mRNA levels were, however, not affected by H-7 (Fig. 4, Panel A). In contrast, the pretreatment of cells with tyrosine kinase inhibitors, tyrphostin, and genistein, caused almost complete inhibition of the IL-4-induced IL-4 receptor mRNA expression. Similar effects of protein kinase inhibitors were observed on the anti-CD40-induced IL-4 receptor mRNA levels, such that H-7 had no effects while tyrphostin and genistein completely abrogated the IL-4 receptor gene expression induced by anti-CD40 (Fig. 5). No apparent cytotoxic effects on B cells were noted for these inhibitors at the concentration used in this study. These data strongly indicate that both IL-4 and anti-CD40 activate distinct signaling pathways, each selectively involving PTK but not PKC, which again agrees well with the result of our previous study on the signaling mechanism of CD23 induction by IL-4 and anti-CD40 (Kim *et al.*, 1997).

The involvement of tyrosine kinases has been clearly demonstrated in the cytokine-induced primary resonse gene expression involving the JAK-STAT pathway (Darnell *et al.*, 1994; Schindler and Darnell, 1996) and anti-CD40 stimulation has been shown to evoke the modulation of tyrosine phosphorylation of various proteins (Faris *et al.*, 1994; Ren *et al.*, 1994). However, the role of tyrosine phosphorylation in the IL-4 receptor gene activation process by IL-4 or any other extracellular stimuli remains largely elucive, partly due to the unavailability of promoter structure of IL-4 receptor gene. Characterization of specific tyrosine kinases participating in the IL-4- or anti-CD40-induced IL-4 receptor gene expression together with the identification of *cis*- or *trans*-acting elements for IL-4 receptor gene activation would provide important clues for the IL-4 receptor gene regulation and the subsequent controling mechanism of the IL-4-mediated responses. Taken together, the results presented in this study strongly suggest that IL-4 receptor modulation, as a primary response induced by IL-4 and anti-CD40 via tyrosine kinase-dependent signaling process, may underlie the mechanism of co-stimulation for B cell activation and differentiation, such as IgE production and IgE receptor expression.

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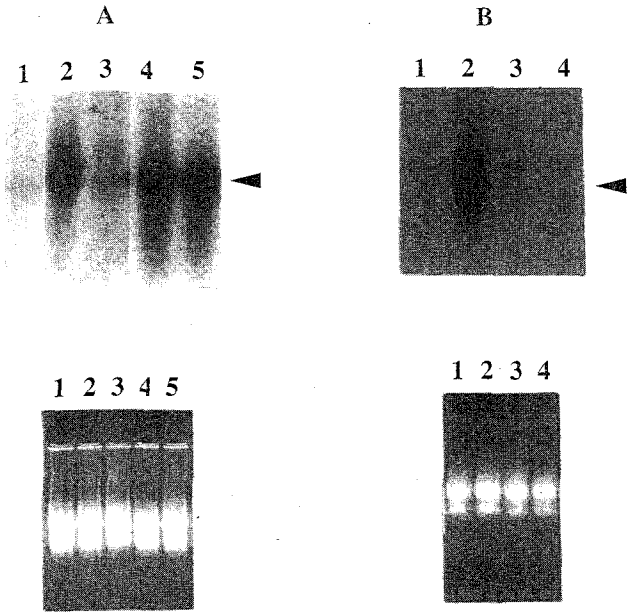

Fig. 4. Effects of protein kinase inhibitors on the IL-4-induced IL-4 receptor mRNA expression.
B cells were pretreated with media alone, or various protein kinase inhibitors for 1 h, after which cells were washed and cultured in the fresh media in the presence of PMA (40 ng/ml) or IL-4 (200 U/ml) for 12 h. Cells were then harvested for RNA isolation and Northern analysis was performed as in Materials and Methods.
Panel A: Effects of PK C inhibitors. Lane 1, media; lane 2, PMA; lane 3, PMA + H-7 (30 μM); lane 4, IL-4; lane 5, IL-4 + H-7 (30 μM)
Panel B: Effects of PTK inhibitors. Lane 1, media; lane 2, IL-4; lane 3, IL-4 + tyrphostin (50 μg/ml); lane 4, IL-4 + genistein (100 μg/ml).
Top: Northern blot autoradiogram; Bottom: EtBr-stained RNA gel.

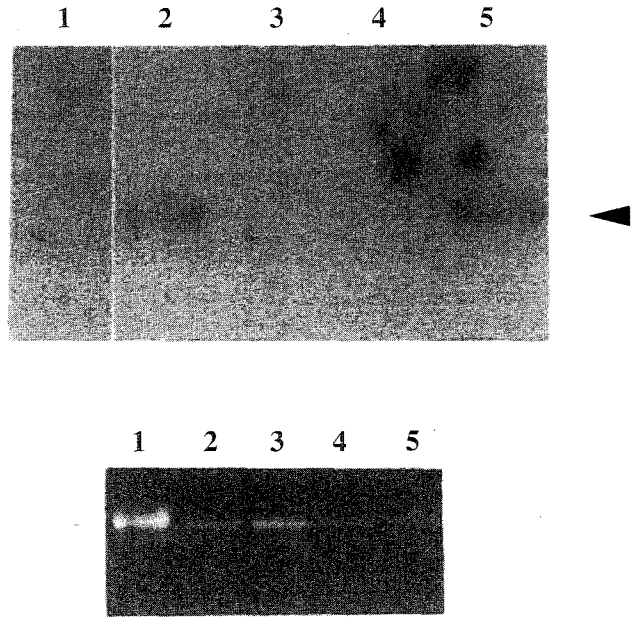

Fig. 5. Effects of protein kinase inhibitors on the anti-CD40-induced IL-4 receptor mRNA expression.
B cells were pre-treated with protein kinase inhibitors for 1 h and washed, after which cells were cultured in the presence of anti-CD40 (2 μg/ml) for 8 h. Northern analysis was performed as in Fig. 4. Lane 1, media; lane 2, anti-CD40; lane 3, anti-CD40 + tyrphostin (50 μg/ml); lane 4, anti-CD40 + genistein (100 μg/ml); lane 5, anti-CD40 + H-7 (30 μM). Top: Northern blot autoradiogram; Bottom: EtBr-stained RNA gel.

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