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Molecules and Cells

Leukocyte Common Antigen-Related (LAR) Tyrosine Phosphatase Positively Regulates Osteoblast Differentiation by Modulating Extracellular Signal-Regulated Kinase (ERK) Activation

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Protein tyrosine phosphatases (PTPs) are pivotal regulators of key cellular functions, including cell growth, differentiation, and adhesion. Previously, we reported that leukocyte common antigen-related (LAR) tyrosine phosphatase promotes osteoblast differentiation in MC3T3-E1 preosteoblast cells. In the present study, the mechanism of the regulatory action of LAR on osteoblast differentiation was investigated. The mineralization of extracellular matrix and calcium accumulation in MC3T3-E1 cells were markedly enhanced by LAR overexpression, and these effects were further increased by treatment with an MEK inhibitor. In addition, LAR overexpression dramatically reduced extracellular signal-regulated kinase (Erk) activation during osteoblast differentiation. In contrast, a marginal effect of the inactive LAR mutant on Erk activation was detected. Expression of osteoblast-related genes such as ALP, BSP, DLX5, OCN, and RUNX2, was increased by LAR overexpression during osteoblast differentiation. On the basis of these results, we propose that LAR functions as a positive regulator of osteoblast differentiation by modulating ERK activation. Therefore, LAR phosphatase could be used as a novel regulatory target protein in many bone-associated diseases, including osteoporosis.

INTRODUCTION

Osteoblasts are mononucleated cells that are responsible for bone formation. Osteoblasts produce osteoid, which is composed of type I collagen. Osteoblasts are also responsible for mineralization of the osteoid matrix. Osteoblast lineage cells, such as osteoblasts and osteocytes, originate from mesenchymal stem cells (MSCs) and give rise to bone tissue (Ducy et al., 2000). This process results from the coordinated action of members of different signaling pathways, including several growth factors of bone morphogenic proteins (BMPs), fibroblast growth factors (FGFs), epidermal growth factors (EGFs) and insulin-like growth factors (IGFs). These growth factors play essential roles in the regulation of osteoblast differentiation (Canalis et al., 1993; Khan et al., 2000; Mundy, 1996). Most of the receptors of these growth factors belong to the receptor tyrosine kinase family, which can activate RAS-MAPK signaling via phosphorylation of tyrosine residues (Seger and Krebs, 1995; Zhang and Liu, 2002). The RAS-MAPK signaling pathway plays important roles in cell proliferation, differentiation, and survival (Mitin et al., 2005; Ory and Morrison, 2004). Many targets have been identified as downstream effectors of RAS, including the small GTPases Rac and Ral, Jun N-terminal kinase (JNK), phosphatidylinositol-3-phosphate kinase (PI3-K), p38 kinase (p38K), and NF-κB, but the classical target is the Raf-1/mitogen-activated, ERK-activating kinase (MEK)/MAPK axis (Campbell et al., 1998). Although RAS-MAPK signaling has marked effects on osteoblast differentiation, there have been contradictory reports (Chaudhary and Avioli, 2000; Higuchi et al., 2002; Hu et al., 2003; Kono et al., 2007; Kretzschmar et al., 1997; Xiao et al., 2000; 2002).

Reversible tyrosine phosphorylation, which is governed by the balanced activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs), regulates important signaling pathways involved in the control of proliferation, adhesion, and differentiation. Several PTPs, such as osteotesticular PTP (OST-PTP), low molecular weight PTP (LMW-PTP), and PTP receptor type-Z (PTP-RZ), have already been reported to play important roles in osteoblast differentiation (de Souza Malaspina et al., 2009; Schinke et al., 2009; Wheeler et al., 2002). Although several evidences suggest that these phosphatases could be regulators of signaling events during osteoblast differentiation, very little is known about the mecha-

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nism of regulation. Previously, we found that leukocyte common antigen-related (LAR) tyrosine phosphatase inhibited adipogeneic differentiation and promoted osteoblast differentiation (Kim et al., 2009). However, the mechanism of action of LAR on osteoblast differentiation has not been fully understood. It has been reported that LAR dramatically decrease EGF- and HGF-dependent MAPK activation (Kulas et al., 1996). Therefore, we speculated that LAR could be a key regulator of osteoblast differentiation by modulating ERK activation. The present study was conducted to determine the regulatory effects of LAR on the osteoblast differentiation of MC3T3-E1 preosteoblasts.

MATERIALS AND METHODS

Cell culture and chemicals

MC3T3-E1 preosteoblasts were purchased from ATCC and cultured in maintenance medium [α-minimum essential medium (MEM) with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (Gibco-Invitrogen, USA)] at 37°C in a humidified atmosphere with 5% CO_2. MEK-inhibitor U0126 was purchased from Roche. A stock solution of U0126 was made at a concentration of 10 mM in dimethyl sulfoxide (Sigma Chemical, USA).

Osteoblast differentiation

To induce osteoblast differentiation, confluent MC3T3-E1 cells were switched to differentiation medium [maintenance medium plus 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate (Sigma Chemical, USA)], and the medium was changed every 2-3 days for 21 days.

Construction of human wild-type and mutant LAR vectors

To construct MC3T3-E1 cells stably expressing the Flag-tagged cytoplasmic domain of human LAR (spanning residues 1316-1897), the retrovirus-mediated infection system was used. For expression of cytoplasmic LAR, DNA encoding Flag-tagged cytoplasmic LAR was inserted into the multi-cloning site (MCS) of pRetroX-IRES-ZsGreen1 vector (Clontech, USA). Detailed methods of transfection and transduction were described in our previous reports (Jung et al., 2009; Kim et al., 2009). A cytoplasmic LAR mutant (D1-CS; inactive mutant; catalytic D1-Cys1522 was replaced with Ser) and a control vector were used as negative controls. The mutant construct was generated from the wild-type cytoplasmic LAR using the QuikChange™ site-directed mutagenesis kit (Stratagene, USA).

Immunoblot analysis

Cells were washed 3 times with ice-cold phosphate buffered saline (PBS) containing 1 mM sodium orthovanadate and harvested in ice-cold RIPA or NP-40 lysis buffer containing a protease inhibitor and phosphatase inhibitor cocktail (Roche, Switzerland). Protein concentrations were measured with the BCA protein assay kit (Pierce, USA). SDS-PAGE, western blot, and densitometry analyses were performed using standard protocols. The anti-ERK1/2 and phospho-ERK1/2 antibodies were purchased from Santacruz. The secondary antibodies (mouse and rabbit) were purchased from Abcam, and membranes were visualized using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce).

Quantitative RT-PCR

Total RNA was extracted from cultured cells with the RNeasy Mini kit (Qiagen, Germany), according to the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized using 1 μg of total RNA as the template, 500 ng of oligo (dT), and AccuPower™ RT-Premix (Bioneer, Korea) in a total volume of 20 μl, according to the manufacturer's recommendation. The targeted fragment of cDNA for each of the osteoblast differentiation-associated genes (Table 1) was amplified by PCR using 5 μl of the RT product, 10 pmoles of each of the primer pair and the PCR premix (Intron, Korea). The PCR products were separated by electrophoresis on 1.5% to 2% agarose gels and visualized by staining with ethidium bromide. The stained PCR band was scanned using a 1 D image analyzer (Kodak, USA) and the resulting densitometric numerical was normalized to levels of β-actin.

Table 1. Primer sequences

Gene	Primer sequences
CytoLAR	F: 5′-GTCAAGGCCTGTAACCCACT-3′
	R: 5′-CCGTCTTCTCGTGCTTCATA-3′
extra LAR	F: 5′-CCACATCTACCACGGAACTG-3′
	R: 5′-GGCCCAAGAGTGTAAGGTGT-3′
ALP	F: 5′-AACCCAGACACAAGCATTCC-3′
	R: 5′-ACTGGGCCTGGTAGTTGTTG-3′
BSP	F: 5′-AGGGGGCAGTGACTCTTCA-3′
	R: 5′-GTTCCTTCTGCACCTGCTTC-3′
DLX5	F: 5′-CCAGCCAGAGAAAGAAGTGG-3′
	R: 5′-CTGGTGACTGTGGCGAGTTA-3′
OCN	F: 5′-GCGCTCTGTCTCTCTGACCT-3′
	R: 5′-TTTGTAGGCGGTCTTCAAGC-3′
RUNX2	F: 5′-GCCGGGAATGATGAGAACTA-3′
	R: 5′-TATGGAGTGCTGCTGGTCTG-3′
β-actin	F: 5′-AGGCCCAGAGCAAGAGAGG-3′
	R: 5′-TACATGGCTGGGGTGTTGAA-3′

ALP, alkaline phosphatase; BSP, bone sialoprotein; DLX5, distal-less homeobox 5; RUNX2, runt-related transcription factor 2; OCN, osteocalcin; OPN, osteopontin.

Alizarin Red S staining

Alizarin Red S staining was performed following methods previously described (Kim et al., 2009; Lee et al., 2008; Stanford et al., 1995). In brief, cultured cells were washed with calcium- and phosphate-free PBS, fixed for 30 min with 10% formalin and washed twice with distilled water prior to staining. Mineral levels within the cell were stained for 10 min using 40 mM Alizarin Red S solution (pH 4.2, Sigma Chemical), after which the cells were washed five times with distilled water and photographed under the microscopic field.

Calcium deposition assay

Accumulated calcium was measured using the calcium CPC Liquicolor kit (Stanbio Laboratory, USA). Briefly, the cells were washed with calcium- and phosphate-free saline solution and extracted with 0.5 N HCl. After centrifugation (500 × *g* for 2 min), the supernatant was collected and mixed with CPC liquicolor solution, and the amount of accumulated calcium was measured at 550 nm using a GeneQuant1300 spectrophotometer (GE Healthcare, UK).

Statistical analysis

All quantitative data were analyzed using an independent Student's *t*-test and were considered significant at $P < 0.05$.

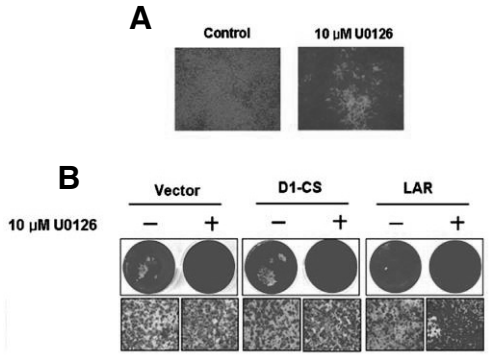

Fig. 1. Effect of MEK inhibitor (U0126) treatment on matrix mineralization in MC3T3-E1 cells. (A) Alizarin Red S staining after MEK inhibitor treatment. Cells were induced to differentiate into mature osteoblasts by treatment with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate, in the presence or absence of 10 μM U0126, and the medium was changed every 2-3 days. Twenty-one days later, cells were stained with Alizarin Red S to visualize the degree of bone mineralization. (B) Overexpression of LAR coupled with MEK inhibitor treatment promotes osteoblast differentiation of MC3T3-E1 cells. Cells were infected with a retrovirus containing the vector alone (pRetroX-IRES-ZsGreen1), cytoplasmic LAR or a LAR mutant (inactive mutant; catalytic domain Cys1522 replaced with Ser; D1-CS). Infected cells were selected by FACS sorting. Cells overexpressing cytoplasmic or mutant LAR were induced to differentiate into osteoblasts for 21 days in the presence of the MEK inhibitor (10 μM U0126), and cells were then stained with Alizarin Red S to visualize mineralization.

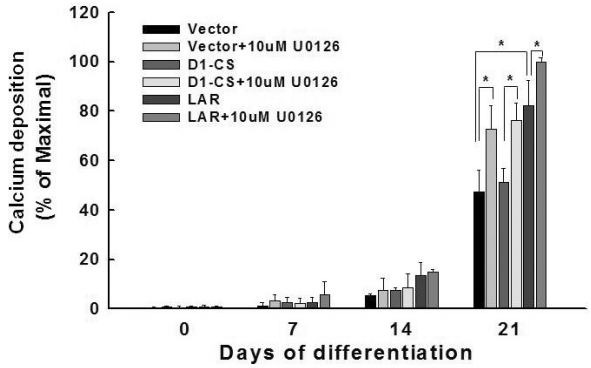

Fig. 2. Effect of LAR overexpression and MEK inhibitor treatment on calcium deposition during osteoblastic differentiation of MC3T3-E1 cells. In the presence or absence of 10 μM U0126, cells overexpressing LAR were induced to undergo osteoblastic differentiation for 21 days according to standard procedures. Calcium accumulation at the indicated days was monitored by measuring the calcium content of the cellular homogenate using commercially available assay kits. Data represent the mean percentage levels ± SD, compared with maximal data ($n = 3$; *, $P < 0.05$).

RESULTS

Effect of MEK inhibitor treatment on osteoblast differentiation of MC3T3-E1 cells overexpressing LAR

To assess whether MEK inhibition can regulate osteoblast differentiation, we investigated the bone mineralization state during osteoblastic differentiation of MC3T3-E1 cells in the presence or absence of 10 μM U0126. Consistent with a previous report (Kono et al., 2007), mineralization of the extracellular matrix in MC3T3-E1 cells was markedly promoted with MEK inhibitor treatment (Fig. 1A).

Next, we examined the effect of LAR overexpression on the osteoblast differentiation when coupled with MEK inhibitor treatment. Analogous to our previous data (Kim et al., 2009), overexpression of LAR promoted osteoblast differentiation of MC3T3-E1 cells. Treatment of cells with a MEK inhibitor induced increased mineralization, as compared to cells not treated with the MEK inhibitor (Fig. 1B).

Effect of MEK inhibition on calcium deposition in MC3T3-E1 cells overexpressing LAR during osteoblast differentiation

To further clarify the effect of MEK inhibition on osteoblast differentiation, calcium deposition was analyzed during differentiation of MC3T3-E1 cells overexpressing LAR in the presence of 10 μM U0126. Accumulation of calcium was slightly increased up to 14 days and dramatically increased at 21 days after MEK inhibitor treatment. These data imply that treatment with a MEK inhibitor was effective at increasing calcium deposition. Furthermore, LAR overexpression significantly promoted calcium deposition in MC3T3-E1 cells during osteoblast differentiation, compared to cells treated with the LAR mutant or control vector (Fig. 2), which is similar to that observed in bone mineralization state.

Effect of ERK activation on osteoblast differentiation of MC3T3-E1 cells overexpressing LAR in the presence and absence of MEK inhibitor

On the basis of a report indicating that ERK activation plays a pivotal role in osteoblast differentiation (Kono et al., 2007), we investigated whether LAR overexpression had an effect on ERK activation during osteoblast differentiation of MC3T3-E1 cells. As shown in Figs. 3A and 3B, ERK activation was decreased after U0126 treatment in cells treated with the control vector and LAR overexpression vector. It is notable that overexpression of LAR significantly inhibited ERK phosphorylation (i.e., ERK activation) at both early phase of differentiation (Fig. 3A) and late stage of osteoblast differentiation (Fig. 3C), regardless of treatment with U0126. Compared with wild-type LAR, a marginal effect was shown on the activation of ERK in cells treated with the vector expressing the inactive mutant of LAR. In addition, the treatment of cells that overexpress LAR with U0126 had an additive effect on the inhibition of ERK phosphorylation (Fig. 3B). These results indicate that LAR regulates osteoblast differentiation via modulation of intracellular ERK activation.

Osteoblast gene expression in MC3T3-E1 cells overexpressing LAR in the presence and absence of MEK inhibitor

To investigate the molecular mechanism underlying the effect of LAR on osteoblast differentiation, the expression of several osteoblast-specific genes was analyzed by quantitative RT-PCR. A significant increase in alkaline phosphatase (ALP) gene expression was observed in cells overexpressing LAR, compared to expression in control cells (Fig. 4). Expression of other osteoblast-specific genes, such as bone sialoprotein (BSP), distal-less homeobox 5 (DLX5), runt-related transcription factor 2 (RUNX2) and osteocalcin (OCN), were also significantly increased in cells overexpressing LAR during osteoblast differentiation. On the basis of these results, we propose that LAR promotes osteoblast differentiation by the upregulation of

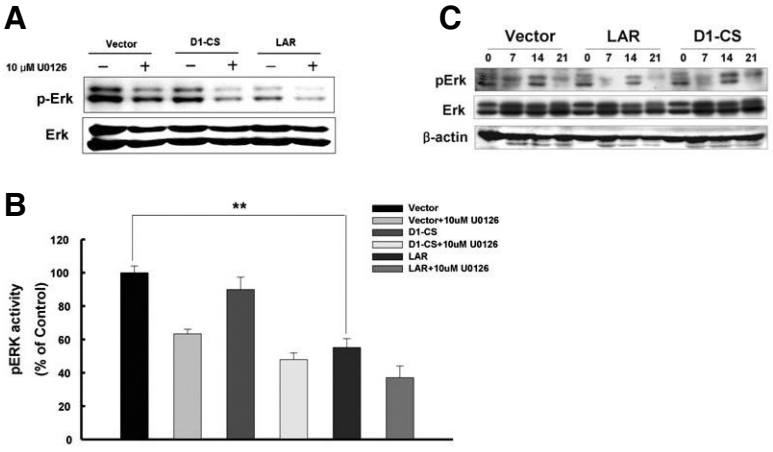

Fig. 3. LAR overexpression inhibits ERK activation during osteoblastic differentiation of MC3T3-E1 cells. (A) The phosphorylation level of ERK was assessed by Western blot analysis. Cells overexpressing LAR were grown to confluence and the MEK inhibitor U0126 (10 μM) was added to the medium for 4 h. After removal of the U0126-treated medium, the medium was changed into the osteoblast-inducing cocktail for 30 min, and cells were harvested with NP-40 lysis buffer. Reduced phosphorylation was observed in cells overexpressing cytoLAR, compared to cells treated with the control or LAR mutant vectors. (B) Graphical representation of the quantification of ERK phosphorylation (mean ± SD; $n = 3$; **, $P < 0.01$). (C) Analysis of ERK phosphorylation during fully differentiation process into osteoblast. Total cell lysates containing equal amounts of protein were measured with anti-phospho ERK1/2 antibody, and the same membrane was immunoblotted with anti-ERK1/2 antibody.

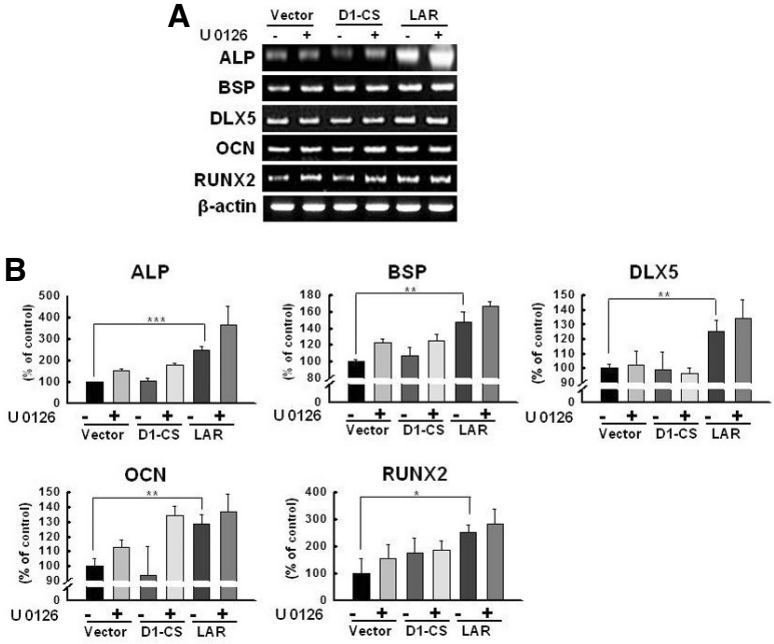

Fig. 4. Analysis of gene expression of differentiation-associated factors during osteoblast differentiation of MC3T3-E1 cells overexpressing LAR. (A) RT-PCR was performed on MC3T3-E1 cells grown for 21 days in the presence of osteoblast-inducing media. Where indicated by a "+" symbol, 10 μM U0126 was added to the culture conditions. Indicated genes were amplified using primers listed in Table 1. (B) Densitometry analysis of mRNA levels of each gene (mean ± SD; $n = 3$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

osteoblast-specific genes.

DISCUSSION

PTPs are enzymes that modulate signaling pathways by removing phosphoryl groups from tyrosine residues of specific cellular proteins (Zhang, 2002). These enzymes can act as biochemical "on" or "off" switches in differentiation-associated signaling pathways (Hunter, 2000). Several PTPs have shown to be strongly involved in the regulation of osteoblast cell growth and differentiation (de Souza Malaspina et al., 2009; Schinke et al., 2009; Wheeler et al., 2002). Recently, we demonstrated that LAR phosphatase has a pro-osteoblastic effect in MC3T3-E1 cells (Kim et al., 2009). However, its mechanism of action on osteoblast differentiation remains poorly understood. Here, we investigated the regulatory roles of LAR on the osteoblast differentiation of MC3T3-E1 cells.

The RAS-MAPK signaling pathway plays important roles in not only cell survival but also proliferation and differentiation (Mitin et al., 2005; Ory and Morrison, 2004). Several previous studies have investigated the role of the MAPK pathway on osteoblast differentiation. It was reported that MAPK signaling inhibited BMP signaling and downregulated type I collagen gene expression in MC3T3-E1 cells (Chaudhary and Avioli, 2000; Kretzschmar et al., 1997). Additionally, it was demonstrated that treatment with the ERK inhibitor PD98059 promoted early osteoblast differentiation and mineralization in C2C12 and MC3T3-E1 cells (Higuchi et al., 2002). However, contradictory data about the effect of MAPK signaling on osteoblast differentiation have also been published. Several groups have published that ERK activation promoted osteoblastic differentiation and mineralization, as well as ALP activity and the deposition of bone matrix proteins (Jaiswal et al., 2000; Lai et al., 2001). Under our experimental conditions,

however, activation of the ERK pathway showed a negative effect on matrix mineralization both *in vitro* and *in vivo*, indicating a negative regulatory role for ERK activation on osteoblast differentiation (Kono et al., 2007). Interestingly, overexpression of LAR increased the terminal differentiation and mineralization of osteoblast MC3T3-E1 cells. In addition, overexpression of LAR, but not the LAR D1-CS inactive mutant, induced a dramatic decrease in ERK activation during osteoblast differentiation (Fig. 3). These findings indicate that the pro-osteoblastic effect of LAR may occur via modulation of ERK activity during osteoblast differentiation. Furthermore, the marginal effect of the inactive LAR mutant on ERK activation strongly supports the importance of phosphatase activity in controlling osteoblast differentiation. However, more studies are necessary to clarify the detailed mechanism of LAR on ERK activation.

The transcriptional regulation of osteoblast differentiation has been relatively well characterized, with Runx2 as the master regulator that controls expression of osteoblast-specific genes and bone formation (Franceschi and Xiao, 2003; Karsenty, 2008). Another transcription factor, Dlx5, is an indispensable regulator of osteoblast differentiation because it induces expression of Runx2 during BMP signaling. In addition, Dlx5 plays an important role in promoting expression of the mature bone cell phenotype (Ryoo et al., 1997). Expression of osteoblast-related genes during osteoblast differentiation is also well documented. Expression of the ALP gene increased in a time-dependent manner during osteoblast differentiation. BSP and OCN genes were expressed in cells that had reached the mineralized tissue-formation stage (Mizuno and Kuboki, 2001). BSP may serve as a direct matrix-associated signal in promoting osteoblast differentiation, which results in the increased production of a mineralized matrix (Gordon et al., 2007). Consistent with these data, the present study demonstrated that cells showing increased mineralization with LAR overexpression had higher mRNA expression of osteoblast-specific genes, compared with those carrying the control vector or the inactive LAR mutant (Fig. 4).

In conclusion, the present study clearly demonstrated that LAR positively regulates osteoblast differentiation by modulating ERK activation, which appears to be mediated by differentiation-associated gene expression. Collectively, results obtained from the present study indicate that osteoblastic differentiation may be controlled by LAR phosphatase, suggesting that it could be used as a novel, regulatory target protein in bone-related diseases, such as osteoporosis.

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