Cyclophilin A Protects Peg3 from Hypermethylation and Inactive Histone Modification*  

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Imprinted genes are expressed from only one of the parental alleles and are marked epigenetically by DNA methylation and histone modifications. Disruption of normal imprinting leads to abnormal embryogenesis, certain inherited diseases, and is associated with various cancers. In the context of screening for the gene(s) responsible for the alteration of phenotype in cyclophilin A knockdown (CypA-KD) P19 cells, we observed a silent paternally expressed gene, Peg3. Treatment of CypA-KD P19 cells with the DNA demethylating agent 5-aza-dC reversed the silencing of Peg3 biallelically. Genomic bisulfite sequencing and methylation-specific PCR revealed DNA hypermethylation in CypA-KD P19 cells, as the normally unmethylated paternal allele acquired methylation that resulted in biallelic methylation of Peg3. Chromatin immunoprecipitation assays indicated a loss of acetylation and a gain of lysine 9 trimethylation in histone 3, as well as enhanced DNA methyltransferase 1 and MBD2 binding on the cytosine-guanine dinucleotide (CpG) islands of Peg3. Our results indicate that DNA hypermethylation on the paternal allele and allele-specific acquisition of histone methylation leads to silencing of Peg3 in CypA-KD P19 cells. This study is the first demonstration of the epigenetic function of CypA in protecting the paternal allele of Peg3 from DNA methylation and inactive histone modifications.

DNA methylation regulates a number of biological processes, including genomic imprinting, X chromosome inactivation, silencing of tumor suppressor genes, and repression of retroviral elements (1, 2). Genomic imprinting relies on establishing and maintaining the parent-specific methylation of DNA elements that control the differential expression of maternal and paternal alleles (3, 4). Although the essential DNA methyltransferases and methyl-CpG-binding proteins have been discovered (5), proteins that regulate the establishment and maintenance of allele-specific methylation of DNA have not been identified. Nevertheless, data for an active role of DNA methylation in gene silencing are both correlative and functional. In addition, DNA methylation may occur in conjunction with histone modification to play a critical role in biallelic silencing through chromatin remodeling (6).

A number of human-inherited diseases linked to faulty methylation pathways and exhibiting abnormal development include Rett, immunodeficiency, centromeric heterochromatin instability, and facial anomalies, and X-linked thalassemia/mental retardation syndromes (7–9). Moreover, aberrant methylation patterns are thought to be involved in tumorgenesis (10–13), causing genomic instability, abnormal imprinting, and deregulated expression of oncogenes or tumor suppressor genes.

The Peg3 gene is one of several genes identified in an imprinted region mapped to human chromosome 19q13.4 (14). The mouse homolog of Peg3 was the first imprinted gene identified from the proximal region of mouse chromosome 7 (15). Its high conservation between mice and humans suggests that it possesses critical cellular functions. Peg3 appears to be ubiquitous, but the highest mRNA levels are found in placenta, uterus, ovary, brain, and testis (16). In mice, targeted disruption of the paternally inherited copy of Peg3 eliminates Peg3 expression. Peg3-negative heterozygous mice suffer growth impairment. Females display compromised nurturing behavior, resulting in a high death rate in their offspring (17). In humans, Peg3 biallelic silencing has been observed in endometrial and cervical cancer cell lines and in a number of ovarian cancer and glioma cell lines (10, 18).

Cyclophilin A (CypA),2 a member of the immunophilin family of proteins, mediates inhibition of calcineurin by the immunosuppressive drug cyclosporine A (CsA), but the other cellular functions of CypA have remained elusive. Recently, different aspects of biological functions of CypA have emerged, suggesting that CypA is involved in multiple signaling events of eukaryotic cells. It might either act as a catalyst for prolyl bond isomerization or form stoichiometric complexes with target proteins. CypA possesses enzymatic peptidylprolyl isomerase activity, which is essential to protein folding in vivo. It promotes proper subcellular localization of Zpr1p, regulates interleukin-2 tyrosine kinase activity, and is required for retinoic acid-induced neuronal differentiation in P19 embryonal carcinoma (EC) cells (19–21).

It has been demonstrated that CypA specifically interacts with SIN3-Rpd3 histone deacetylase (HDAC) in vitro, suggest—

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2 The abbreviations used are: CypA, cyclophilin A; ChIP, chromatin immunoprecipitation; CsA, cyclosporine A; EC, embryonal carcinoma; HDAC, histone deacetylase; QRT, quantitative real-time; WT, wild type; KD, knockdown; KO, knockout; RNAi, RNA interference; Dnmt, DNA methyltransferase.

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* This work was supported by Grant CA66746 from the National Cancer Institute. In addition, DNA methylation may occur in conjunction with histone modification to play a critical role in biallelic silencing through chromatin remodeling (6).

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Role of Cyclophilin A in Epigenetics

ing that CypA affects gene expression by physically interacting with HDAC (22). In screening for the gene(s) responsible for the alteration of phenotype in CypA-KD P19 cells (21), we observed a silent paternally expressed gene, Peg3. Subsequently, we found an inverse relationship between mRNA expression and DNA hypermethylation as well as Peg3 reactivation from CypA-KD P19 cells by demethylation reagents and HDAC inhibitor, suggesting that epigenetic mechanisms play an important role in the regulating of Peg3 expression in CypA-KD P19 cells. Chromatin immunoprecipitation (ChIP) assays indicated a loss of acetylation and a gain of lysine 9 trimethylation in histone 3, as well as enhanced DNA methyltransferase 1 (Dnmt1) and MBD2 binding on the CpG islands of Peg3 in CypA-KD P19 cells. Our data demonstrate the epigenetic function of CypA, which protects the paternal allele of Peg3 from DNA methylation and inactive histone modifications.

MATERIALS AND METHODS

RNA Isolation, cDNA Synthesis, and Quantitative Real-time (QRT)-PCR—Total RNA samples were isolated from 3 × 10⁶ cells by using the RNeasy Mini Kit (Qiagen) with on-column DNase digestion according to the manufacturer’s protocols. Oligo(dT)-primed cDNA was synthesized from 3 µg of RNA using SuperScript™ (Invitrogen). The 20-µl products of reverse transcription were diluted to 40 µl, and 2 µl were used for each PCR reaction. PCR reactions were performed in a total volume of 25 µl containing 2 µM primers and 12.5 µl of the Power SYBR green PCR master mix (Applied Biosystems). The primers used in real-time PCR were cPeg3-F (5’-GGCTAACCAAGACAGTAGGGTAGT-3’) and cPeg3-R (5’-GTCTAGTGCACCCACACTGAAC-3’). As an input control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified using the following primers: GAPDH-F (5’-CAGTGCCAAAGTGG-AAGGAAAAATGC-3’) and GAPDH-R (5’-AATTTGCCGTG-TAGGTGGCTG-3’). QRT-PCR was performed under the following conditions: 95 °C for 10 min for the initial denaturing followed by 40 cycles of denaturing at 95 °C for 20 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. To monitor the efficiency of bisulfite treatment, the PCR products were subcloned into the TA cloning vector, and 15 different clones were sequenced individually. If >95% of cytosine was converted into thymidine, we selected those DNA samples for bisulfite sequencing analyses. Methylat-

ChIP Analysis—ChIP assays were carried out using a kit from Upstate according to the manufacturer’s instructions. For Dnmt1 ChIP, cells were treated for 2 h with 5 µM 5-aza-dC to arrest the fleeting covalent association of methyltransferase with the DNA substrate. Briefly, 1 × 10⁷ cells were used per ChIP assay. After 10 min of 1% formaldehyde treatment, the cells were harvested and sonicated for 3 × 20 s using a Tekmar sonic disrupter set to 30% of maximum power to produce soluble chromatin, with average sizes between 300 and 1000 bp. The chromatin samples were then diluted 8-fold in the dilution buffer and precleared for 1 h using 75 µl of salmon sperm DNA/protein A- or G-agarose beads. Ten µg of antibodies were then added to each sample and incubated overnight at 4 °C. To collect the immunocomplex, 60 µl of salmon sperm DNA/protein A- or G-agarose beads were added to the samples for 1 h at 4 °C. The beads were washed once in each of the following buffers, in order: low salt, high salt, and LiCl immune complex wash buffer; they were then washed twice in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The bound protein-DNA immuno-complexes were eluted twice with 250 µl of elution buffer and subjected to reverse cross-linking at 65 °C for 6 h. The reverse cross-linked chromatin DNA was further purified by proteinase K digestion and phenol-chloroform extraction. DNA was then precipitated in ethanol and dissolved in 20 µl of TE buffer. Two microliters of DNA were used for each QRT-PCR with primers gPeg3-F (5’-ACCTGTAGAC-AAGGAGGTCC-3’) and gPeg3-R (5’-GTCTAGTGCACCCACACTGAAC-3’). For a positive control, DNA polymerase II antibody was used to immunoprecipitate actively expressed promoter, and mouse GAPDH promoter was amplified by using primers mGAPDH-F (5’-ACTCAGCAAGAGCCGCAGTGC-3’) and mGAPDH-R (5’-TGGGAAAGAGGAGGAGAG-3’). QRT-PCR was performed under the following conditions: 95 °C for 10 min for the initial denaturing followed by 40 cycles of denaturing at
Role of Cyclophilin A in Epigenetics

Cyclophilin A Knockdown (CypA-KD) Selectively Affects the Expression of Imprinted Genes—To identify genes that could be affected by the CypA-KD in P19 EC cells, we found repressed Peg3 in CypA-KD P19 EC cells (S1–7) by using subtraction-differential screening of the subtractive library. Undetectable Peg3 expression was also exhibited in the other stable CypA-KD clone S3-2, suggesting that suppression of Peg3 resulted from the knocking down of CypA (Fig. 1A).

To determine whether CypA-KD also affects the expression of other imprinted genes, we performed semiquantitative reverse transcription-PCR as well as QRT-PCR to detect Lsp29, Peg1/Mest, Igf2, H19, and Igf2r, using specific primers (Fig. 1). Again, we observed that Lsp29 expression levels were undetectable, suggesting that Lsp29 shares the same imprinting control region with Peg3 and coordinates silencing of the imprinted transcript Lsp29 with Peg3. H19 localized in a different locus was also undetectable, suggesting that cells lacking the CypA switch expressed H19 to silence. QRT-PCR analysis clearly demonstrated a 3–4-fold increase in Igf2, reduced Igf2r mRNA, but no effect upon Peg1/Mest expression in S1–7 and S3-2 cell lines (Fig. 1B). Collectively, these findings indicate that CypA-KD can selectively affect the expression of certain imprinted genes.

Peg3 Expression Is CypA-dependent—Results from the stable CypA-KD clones S1–7 and S3-2 clearly demonstrated that knockdown of CypA resulted in suppression of Peg3. To rule out the possibility that the observed changes resulted from clonal selection, we performed transient transfection of pshRNA-CypA into WT P19 EC and F9 EC cells (Fig. 2A). Non-specific short hairpin RNA (shRNA) and an empty vector were used as negative controls. Our results demonstrated that the mechanism may be a general phenomenon in EC cells.

To determine whether CypA-knockout (KO) also results in...
Role of Cyclophilin A in Epigenetics

repression of Peg3 expression, we measured Peg3 expression from CypA-KO Jurkat cells (a generous gift from Dr. Jeremy Luban) using semiquantitative reverse transcription-PCR. As shown in Fig. 2A, lane 9, we detected low levels of Peg3 as compared with those in wild type Jurkat cells. Together, these data clearly demonstrate that Peg3 expression is CypA-dependent.

CypA Isomerase Activity Is Required to Maintain Expression of Peg3—To determine whether CypA isomerase or FK506-binding protein isomerase activity is required to maintain expression of Peg3, WT P19 cells were treated with 1 μg/ml CsA and FK506 (100 ng/ml) or rapamycin (100 ng/ml), which inhibits the isomerase activity of CypA and FKBP51, respectively. After 72 h of CsA, FK506, or rapamycin treatment, the cells were harvested to prepare total RNAs. Two μg of total RNA were used to perform RT-PCR to detect Peg3 using a pair of specific primers. Only CsA-treated WT P19 cells had undetectable Peg3 (Fig. 2B), indicating that the isomerase enzymatic activity of CypA is necessary for Peg3 expression.

Silencing of Peg3 Is Reactivated by Treating CypA-KD Cells with the DNA Methyltransferase Inhibitor—To determine whether DNA methylation is involved in silencing of Peg3 in CypA-KD P19 EC cells, we treated the S1–7 and S3-2 cell lines for 5 days with 1.0 μM 5-aza-dC, a DNA methyltransferase inhibitor, followed by amplification of the Peg3 129-bp fragment using semiquantitative reverse transcription-PCR and QRT-PCR. Fig. 3 shows that, upon 5-aza-dC treatment, the silent Peg3 gene in S1–7 and S3-2 was reactivated. These results demonstrate an inverse relationship between DNA methylation and Peg3 expression and support the hypothesis that Peg3 transcription is regulated by promoter methylation.

CypA-KD Resulted in Biallelic Methylation of CpG Islands Encoded within the Promoter, First Exon, and First Intron of the Peg3 Gene—To directly show methylation of the Peg3 gene, we used bisulfite modification and DNA sequencing to analyze the methylation status of 445-bp CpG islands encoded within the promoter, first exon, and first intron of this gene (Fig. 4). Analysis of 15 individual clones revealed that, in CypA-KD P19 cells, 26 CpG dinucleotides were 98% methylated, indicating biallelic methylation, whereas in WT P19 cells, the methylated CpG islands were at 42.3% frequency, which is consistent with monoallelic methylation. The transcription of each gene is indicated by an arrow. The positions of the CpG islands encoded within the promoter region, first exon, and first intron are indicated. Solid and open arrows within the indicated CpG islands are primers for bisulfite sequencing and PCR amplification of ChIP products, respectively.

Dnmt1 Is Responsible for Methylation of the Unmethylated Allele of Peg3 in the CypA-KD Cells—To determine whether Dnmt1 is responsible for the methylation of selected DNA targets, an in vivo complex of methylation analysis was used as described previously to detect and quantify the physical interaction of Dnmt1 with substrate genomic DNA in a physiological setting in chromatin (23). QRT-PCR analysis of expression of Peg3 and Usp29 in the presence or absence of 5-aza-dC treatment. Quantitation was achieved by measuring the increase in fluorescence during the exponential phase of PCR. The expression of WT was set as 1, whereas the expression levels of others were relative to the control, as indicated on the top of each bar graph.

FIGURE 3. Silent Peg3 is reactivated by treatment of cells with the DNA methyltransferase inhibitor. A, reactivation of Peg3 expression is shown by semiquantitative reverse transcription-PCR. Total RNA isolated from 5-aza-dC-treated or -untreated WT-P19, S1–7, and S3-2 cell lines was subjected to reverse transcription-PCR to amplify Peg3 and Usp29. Shown are WT P19 cells and S1–7 and S3-2 CypA-KD stable cell lines. B, QRT-PCR analysis of expression of Peg3 and Usp29 in the presence or absence of 5-aza-dC treatment. Quantitation was achieved by measuring the increase in fluorescence during the exponential phase of PCR. The expression of WT was set as 1, whereas the expression levels of others were relative to the control, as indicated on the top of each bar graph.

FIGURE 4. The schematic genomic structure of Usp29 and Peg3. Exons of these two genes are represented by black boxes. The transcription of each gene is indicated by an arrow. The positions of the CpG islands encoded within the promoter region, first exon, and first intron are indicated. Solid and open arrows within the indicated CpG islands are primers for bisulfite sequencing and PCR amplification of ChIP products, respectively.
positive control showed no significant difference between the two cell lines (Fig. 6). These results further substantiate the hypothesis that CypA-KD mediates biallelic methylation of the Peg3 promoter and the first exon.

Partial Relief of the Repressed Peg3 in CypA-KD Cells by Treatment of Cells with the HDAC Inhibitor—It has been well established that the methyl-CpG-binding protein silences transcription by recruiting the HDAC-repressive machinery, which removes acetyl groups from histone, resulting in gene silencing (24, 25). To determine whether histone deacetylation is involved in silencing Peg3 in CypA-KD P19 EC cells, because HDAC binds to CypA (Fig. 7A), the S1-7 and S3-2 cell lines were treated for 3 days with 10 or 20 ng/ml trichostatin A, a histone deacetylase inhibitor, followed by amplification of the Peg3 129-bp fragment using semiquantitative reverse transcription-PCR. Our data demonstrated that trichostatin A only partially relieves CypA-KD-mediated Peg3 repression (Fig. 7B). This partial relief indicates that additional mechanisms of repression by methyl-CpG repressor complexes might exist in addition to the recruitment of histone deacetylation. We therefore examined various histone modifications in the Peg3 promoter of WT P19 and CypA-KD P19 cells by ChIP assays, using antibodies against modified histones (acetyl Lys-H3 and trimethyl Lys-9-H3) followed by QRT-PCR analysis.

Reciprocal Pattern of Acetyl Lys-H3 and Trimethyl Lys-9-H3 Was Enriched in the CypA-KD Cells—Histone acetylation was observed in both WT P19 and CypA-KD P19 cells but with a 33% weaker signal in CypA-KD P19 cells (Fig. 8A). Our results suggest that a less-acetylated histone binds to the Peg3 promoter in the CypA-KD P19 cell line S1-7, which correlates with repressed Peg3 expression in this cell line. The level of trimethyl Lys-9-H3 in the CypA-KD cells was ∼2.5× greater than that in WT P19 cells, which were set as 50/50 for two parental alleles (Fig. 8A), indicating a gain of histone methylation on the Peg3 promoter. A positive control, GAPDH promoter bound by RNA polymerase II, showed no significant difference between WT- and CypA-KD cell lines (Fig. 8A). In a pattern reciprocal to that of acetyl Lys-H3, trimethyl Lys-9-H3 was enriched exclusively in the CypA-KD P19 cell line. This predominant enrichment of the trimethylation of Lys-9-H3 correlated with the inverse relationship of paternally expressed Peg3 gene (Fig. 8B). It has been demonstrated that MBD2 is involved in the silencing of Peg3 expression in this cell line. The level of trimethyl Lys-9-H3 in the CypA-KD cells was ∼2.5× greater than that in WT P19 cells, which were set as 50/50 for two parental alleles (Fig. 8A), indicating a gain of histone methylation on the Peg3 promoter. A positive control, GAPDH promoter bound by RNA polymerase II, showed no significant difference between WT- and CypA-KD cell lines (Fig. 8A). In a pattern reciprocal to that of acetyl Lys-H3, trimethyl Lys-9-H3 was enriched exclusively in the CypA-KD P19 cell line. This predominant enrichment of the trimethylation of Lys-9-H3 correlated with the inverse relationship of paternally expressed Peg3 gene in the CypA-KD P19 cells. We conclude that silencing of Peg3 in the CypA-KD P19 cell line correlates with a gain of trimethyl Lys-9-H3 on the promoter region of the paternal allele.

MBD2 Is Involved in the Silencing of Peg3 Expression—Our data demonstrate that P19 EC cells have abundant MBD2, which also binds to CypA (Fig. 8B). It has been demonstrated that MBD2 is associated with HDACs in the MeCP1 repressor complex (26). To determine whether silencing of the hypermethylated Peg3 gene is consistent with a model involving methyl-CpG-binding proteins, ChIP analysis was used to study the occupancy of the methylated Peg3 promoter by MBD2 in the CypA-KD P19 cell line as compared with WT P19 EC cells. QRT-PCR analysis of ChIP products generated by immunoprecipitation with an antibody against MBD2 in the WT P19 and
Role of Cyclophilin A in Epigenetics

CypA-KD P19 cell lines revealed the MBD2-bound DNA fraction in the CypA-KD P19 cell lines was ~1.8-fold higher than that in WT-P19 cells (Fig. 8B), suggesting that MBD2 is involved in the silencing of Peg3 expression.

DISCUSSION

Using a revolutionary RNA interference (RNAi) technique, we have been able to analyze loss-of-function phenotypes for the first time to define the function of CypA, which is required to maintain the differential methylation of the CpG islands and histone modification in the promoter and its extended exonic region of the Peg3 gene. Although off-target effects have been documented during RNAi experiments and integration of an RNAi vector, this is not the case in our presented data. Based on the BLAST sequence data base, we did not find any other sequences identical to genomic promoter sequences in the BLAST sequence data base, we did not find any other sequences identical to genomic promoter sequences.

In this report, results from the stable CypA-KD clones S1–7 and S3–2 clearly demonstrated that suppression of Peg3 resulted in cells lacking CypA. Silencing of Peg3 was also accompanied by transient knockdown CypA in both P19 and F9 embryonal carcinoma cells, indicating that the observed silent Peg3 does not result from clonal selection. Furthermore, treatment of P19 cells with CsA, an isomerase inhibitor, resulted in silencing of Peg3, suggesting that the isomerase enzymatic activity of CypA is necessary for Peg3 expression.

In contrast, treatment of P19 cells with FK506, rapamycin, FKBP isomerase blocking agents, did not affect Peg3 expression. These results further substantiate the notion that the isomerase enzymatic activity of CypA (and not FKBP or calcineurin phosphatase activity) is required for Peg3 expression. In addition, data obtained from CypA-KO Jurkat cells clearly demonstrated that Peg3 expression is CypA-dependent (Fig. 2A). An attempt to rescue the silent Peg3 with a CypA cDNA expression plasmid that is not targetable by the RNAi-CypA failed (data not shown). This failure to rescue implicated that the irreversible covalent bond modification of DNA methylation has been established.

The inverse relationship between mRNA expression and DNA hypermethylation as well as our findings of Peg3 reactivation by demethylating agents suggest that this epigenetic mechanism plays an important role in Peg3 regulation in CypA-KD P19 cells. Epigenetic switches consist of both DNA methylation and histone methylation (6). Bisulfite genomic sequencing and MSP analysis clearly demonstrated a biallelic methylation of CpG islands within the promoter region, first intron and first exon of the Peg3 gene in CypA-KD P19 cells, whereas methylation of this region in the WT P19 cells remains monoallelic (Fig. 5). CpG methylation at any critical site may increase the likelihood of binding of methylcytosine-binding proteins, which can recruit HDACs and H3-Lys-9 methyltransferase to mediate inactive chromatin remodeling. We hypothesized that MBD2 associates with methylated DNA within the promoter region of the repressed Peg3 gene. ChIP analysis with MBD2 antibody showed that more MBD2 was associated with the promoter region of Peg3 in the CypA-KD P19 cells (Fig. 8B). Additionally, a reciprocal pattern of acetyl Lys-H3 and trimethyl
Lys-9-H3 was enriched in the CypA-KD cells compared with WT P19 cells, as indicated by ChIP assays (Fig. 8A). Trimethylation of histone 3 on Lys-9 provides a histone code indicative of inactive chromatin structure. Therefore, DNA methylation is both a cause for and a result of heterochromatinization. Methylation patterns depend upon the activity of DNA methyltransferases. A Dnmt activity assay using a synthetic template, poly(dI-dC)-poly(dI-dC), showed no increase in global Dnmt activities (data not shown), whereas the Dnmt1-bound Peg3 promoter is 2.07-fold higher in the CypA-KD cells than in WT P19 cells (Fig. 6). Collectively, our data indicate that selective hypermethylation of DNA might have occurred in the CypA-KD cells, and Dnmt1 is involved in at least the maintenance of this hypermethylation. CypA may be necessary to retain a modulator, which is required for maintenance of imprinting gene expression, in the inactive cytoplasmic form as reported previously for the function of Hsp90 in chromatin remodeling (30, 31). The precise temporal and spatial control of imprinting gene expression may be altered when cells lack CypA.

It has been reported that DNA methylation patterns are remarkably stable and change little with the in vitro culture of cancer cell lines (32). In this study, we have demonstrated a simple epigenetic switch for Peg3 by knocking down CypA. The precise mechanism underlying this switch remains to be elucidated. It is possible that the lack of isomerase activity of CypA leads to a global redistribution of factors required for epigenetic modifications. Inactivation of Peg3 by hypermethylation likely confers a survival advantage, as Peg3 regulates the translocation of the proapoptotic Bax from cytoplasm to the mitochondria (33). CypA-KD P19 cells are indeed less sensitive to retinoic acid plus BMP4-induced apoptosis as compared with wild type laboratories for technical assistance and comments.

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