Local Hypomethylation in Atherosclerosis Found in Rabbit ec-sod Gene

Mikko O. Laukkanen, Sanna Mannermaa, Mikko O. Hiltunen, Saara Aittomäki, Kari Airenne, Juhani Jänne, Seppo Ylä-Herttuala

Abstract—Extracellular superoxide dismutase (EC-SOD) protects arteries against deleterious effects of superoxide anions and the development of atherosclerosis. In this study, we cloned and characterized rabbit ec-sod gene. We identified 6 rabbit C-elements and 5 CpG clusters in the cloned sequence. One of the CpG clusters is located on the coding sequence. Because CpG clusters are potential sites for methylation and may explain the occurrence of mutations, methylation status of each of the CpG dimers located in the coding sequence CpG cluster was characterized using direct genomic sequencing. Unexpectedly, a marked reduction in the amount of methylated CpG dinucleotides in ec-sod gene was detected in atherosclerotic aortas as compared with normal aortic intima-media. Although alterations in DNA methylation are well characterized in malignant tumors, the presence of methylation changes in atherosclerosis has not been studied even though both diseases are characterized by excess cellular proliferation and alterations in gene expression. Further analysis of the whole genomic methylation by high-pressure liquid chromatography in normal and atherosclerotic aortas revealed a tendency for a decreased 5-methylcytosine (5-mC) content in atherosclerotic aortas as compared with normal arteries. Hypomethylation in atherosclerotic aortas occurred at the same level as has been reported from malignant tumors. Although a causal relationship between the methylation level and expression of EC-SOD cannot be proven, our results show that ec-sod hypomethylation is associated with the development of atherosclerosis and suggest that it may affect structure and function of ec-sod and other genes possibly involved in the development of atherosclerotic lesions.

(E)xtracellular superoxide dismutase is an anti-oxidative enzyme that catalyzes dismutation of superoxide anion (O$_2^-$) to less reactive hydrogen peroxide. Biochemical and in situ hybridization studies have shown the presence of high amounts of ec-sod mRNA and activity in early atherosclerotic lesions where the enzyme is synthesized by smooth muscle cells (SMC) and macrophages. The high level of ec-sod expression in the arterial wall is physiologically important in maintaining low extracellular O$_2^-$ concentration, which prevents harmful effects of O$_2^-$, such as oxidation of cellular proteins and LDL and inactivation of endothelium-derived relaxing factor (NO).

DNA methylation, ie, formation of 5-methylcytosine (5-mC) from a cytosine residue by methyltransferase is an important factor regulating gene expression at different stages of development. DNA methylation, which is a form of epigenetic gene regulation leading to suppression of gene expression, is commonly concentrated to CpG clusters. 5-mC levels are not maintained during DNA replication, but methyl groups need to be added after each cycle of cell replication. In malignant tumors, genomic hypomethylation is associated to increased cellular proliferation, and oncogene and growth factor expression. Alterations in genome-wide- and gene-specific DNA methylation have been convincingly linked to the development of human cancers, inactivation of X chromosome, and genomic imprinting. However, there are no previous studies about the presence of hypomethylation in atherosclerotic lesions.

In this study, we have cloned rabbit ec-sod gene and identified 6 repeat elements and 5 CpG clusters in the cloned sequence. A marked hypomethylation of the ec-sod gene was found in atherosclerotic aortas. At the whole genomic level, a tendency for hypomethylation was detected in atherosclerotic lesions. The results suggest that hypomethylation may play a role in the proliferation of SMC and development of atherosclerotic lesions.

Methods

Library Screening

Approximately 500 000 plaques from a rabbit heart genomic library (Stratagene) were screened twice using random primed
Figure 1. Comparison of rabbit, human (U10116),^26^ rat (Z24721),^45^ and mouse (AF039602)^27^ DNA and amino acid sequences. A, DNA sequence. CpG dimers associated with the cluster are numbered and shadowed, translation initiation and stop codons are underlined, a black box above the sequence indicates the tetramerization site and asterixes glycosylation sites. Numbering is according to rabbit sequence. Mutations are shown by open boxes, in DNA they are also indicated by arrows and roman numbers (I to V). Five mutations are indicated in the sequences (see text for details). Mutation I is A to G substitution.^35^ Mutation II is A to C change in the rabbit gene at nucleotide^1^ 45. This mutation also creates a new CpG dimer. Mutation III is G to C change at nucleotide^1^ 141 in the rabbit gene. Mutation IV at nucleotide^1^ 115 is C to T deamination in the rabbit gene. Mutation V is C to G change in the human sequence^33,34^ causing disruption of a CpG dimer. Under the mouse sequence there are 4 bolded bases at nucleotides 31, 471, 526, and 672 showing the sequence difference in the mouse cDNA sequenced by Folz et al (U38261)^46^ and by Carlsson et al (X84940).^47^ These same
32P-labeled ec-sod probe (386 bp). Positive clones were isolated and subcloned into pBluescript SK vector (Stratagene). Sequencing of the clone SA1 and SA2 (Genbank accession No. Y13339 and AJ007044) was carried out using automated laser fluorescence sequencer (A.L.F., Pharmacia). Sequence analysis was done by Genetics Computer Group program package, Center for Scientific Calculation, Webgene launcher program package, Institute of Advanced Biomedical Technologies and Censor program.18

Experimental Animals

Tissue samples were isolated from a total of 38, 6- to 8-month-old New Zealand White (NZW) rabbits and 7, 1- to 3-year-old Watanabe heritable hyperlipidemic (WHHL) rabbits.19 Isolated samples were aortic intima-media, heart, liver, spleen, and sperm. WHHL rabbit lesions were used as a model of atherosclerosis because the composition of their lesions resembles human atherosclerotic lesions.20 Because it is difficult to find normal arterial wall in WHHL rabbits, normal NZW aortas were used as controls in the methylation analysis.

DNA Preparation

DNA was extracted (300 mg tissue wet weight) in 5 mL of digestion buffer containing 10 mmol/L Tris, 1 mmol/L EDTA, 0.3 mol/L NaAc, 1% SDS, and 100 μg proteinase K (pH 8.0). Tissue preparations were incubated overnight at 37°C, extracted once with phenol-chloroform and twice with chloroform. Isolated DNA was precipitated with ethanol and dissolved in Tris-EDTA-buffer (10 mmol/L Tris-HCL, 1 mmol/L EDTA, pH 8.0).

Screening of the Mutations

Mutations in the coding sequence were screened using polymerase chain reaction (PCR) techniques. The coding sequence was amplified in 2 segments. The first sequence was amplified using a primer pair 5’-GGGCGTGTTAAATCTGAGA-3’ and 5’-GCACGGCCAGGTTGATGAC-3’ and sequenced by primers 5’-GGATGTTGAATGACCCAGGCGG-3’ and 5’-GCCAGCGGTGTATGATCGG-3’. The second segment was amplified by a primer pair 5’-GGGCGTACTACCAACCCGCTG-3’ and 5’-CCGGGGCGCCGGCCGACTTTTC-3’ and sequenced by primers 5’-ACTACAAACCCGCTGGCCGTG-3’ and 5’-CTTTCGCCGCGCTCGCTTG-3’. The length of the amplified fragments were 593 and 331 bp, respectively. Tissue samples from the isolated tissues of 3 NZW rabbits were amplified in 5 duplicates, each of which was sequenced on both strands. The PCR product was sequenced by cycle sequencing employing automated sequencer.

Bisulphite Modifications

Reaction was done as described21 to convert nonmethylated cytosines to uracils. Two micrograms of DNA was dissolved in 30 μL of H2O, and 3 μL of 2 mol/L NaOH was added. Samples were incubated 10 minutes at room temperature, and 0.4 mL of a modification mixture was added to each sample. The modification mixture was done as follows: 1.9 g sodium metabisulfite (Sigma) was dissolved in 2 mL of H2O. NaOH (0.7 mL of 2 mol/L) and 0.5 mL of 1 mol/L hydroquinone (Fluka) were added. pH was adjusted to 5.0, and the volume was filled to 4 mL with H2O. Samples were then incubated at 50°C for 4 hours. DNA was purified with Wizard DNA Clean-Up System (Promega) according to the protocol of

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B

| Rabbit | MLALUCSLLL LAALQDTWS | II |
| Human | MLALLCSSL LAAGAATAW | I |
| Rat | NAALFLCNLL LVACGGSVT | III |
| Mouse | MLALFLYGLL LAACGSSV...T | I |

**IV**

| Rabbit | RDTNKhKTEI WAQATXQA QGEPAGALHA VCRQVPSATL DAAQVPSVOL |
| Human | RDMYAKTKEI WQEVQKQ....DDDYTLHA ACQVPSATL DAAQVPGTV |
| Rat | GJTHSKDELEI WMEIKQREA....DAREMLHACR VSQVPSAML PPPQQITGL |
| Mouse | GJTHKAKLIEI WMEIKQRREV....DAEMMLHICR VSQVPSATL PPPQQITGL |

**V**

| Rabbit | YNPRLQPGPA QLEAFPDOLEG PFPVEANLSER AIHVHCPFSDL SQCQDSTGAGH |
| Human | YNPRLQFAPRA KLDFAAELEG PFPENPISER AIHVHCFPSL SQCQDSTGPH |
| Rat | YNPRLQPSGS RLEAFPNSLEG PFAEQNSTMH AIHVHEFSDL SQCQDSTGPH |
| Mouse | YNPRLQFPGS RLEAYPSLEG PFAEQNASNR AIHVHEFSDL SQCQDSTGPH |

**VI**

| Rabbit | YNPLAQVHPQ AHPGDGFNPFAV RDGRLMKYRS GLAASLAGPH SIVGRVAVVH |
| Human | YNPLAVPBPQ AHPGDGFNPFAV RDGRLMKYRA GLAASLAGPH SIVGRVAVVH |
| Rat | YNPLGVHPQ AHPGDGFNFPP AHPGDGFKNV RDGRLMKHRM LGATLSAVPH SICQDSTGPH |
| Mouse | YNPMBVPHPQ AHPGDGFNVFA RNQGMLMKYVR GLTASLAGPH AILGSVAVV |

**VII**

| Rabbit | AGDEDLGRRG NAA5EVNGA GPRLACCVVG ASGPAPVVARQ AQEDAERKKR |
| Human | AGDEDLGRRG NAA5EVNGA GRRLACCVVG VCGGGLAQEQ ARESERKHK |
| Rat | AGDEDLGRRG NAA5EVNGA GRRLACCVVG TSNSEAERQ TK...ERKKR |
| Mouse | AGDEDLGRRG NAA5EVNGA GRRLACCVVG TSSSEAERQ TK...ERKKR |

**VIII**

| Rabbit | RRRESECKAR* 245 |
| Human | RRRESECKAR* |
| Rat | RRRESECKTT* |
| Mouse | RRRESECKTT* |

**IX**

Differences also exist between the mouse cDNA (U38261) and gene (A0039602).27 B, Amino acid comparison of the sequences shows the effect of point mutations on the amino acid sequence. The first mutation causes an amino acid change from Asn21→Asp in the mouse sequence. The second mutation in the rabbit leader sequence has no effect on the protein, whereas the third and fourth mutations in the rabbit sequence cause Glu26→Glu and Arg52→Trp changes, respectively. The fifth mutation is Arg213→Gly switch at the carboxyterminal end of the human EC-SOD monomer.33,34
manufacturer. The bisulphite reaction was completed by NaOH and DNA was precipitated with 5 mol/L ammonium acetate and ethanol.

PCR and Genomic Sequencing

One hundred ng of freshly modified DNA was used for the PCR amplification of the ec-sod gene CpG island. For the first PCR, primers (5'-GATTTGATTTGAATGGATT-3' and 5'-ACCCAGAAAAACCTCAAC-3') covered a 871 nucleotide region. PCR conditions were as follows: 96°C for 40 seconds, 52°C for 40 seconds, 72°C for 90 seconds, for 39 cycles. Two separate nested PCR were carried out after the first PCR to amplify the sequence containing all CpG sites within the ec-sod gene coding region (Figure 1). For the upstream PCR, primers (5'-GATTTGATTTGAATGGATT-3' and 5'-ACCCAGAAAAACCTCAAC-3') covered a 857 nucleotide region, whereas the downstream primers (5'-GATTTGATTTGAATGGATT-3' and 5'-ACCCAGAAAAACCTCAAC-3') covered a 446 nucleotide region. PCR conditions were as follows: 96°C for 3 minutes, 80°C for 3 seconds, followed by 96°C for 20 seconds, 52°C for 15 seconds, 72°C for 90 seconds, for 33 cycles for the upper 857 nucleotide region. The lower 446 nucleotide region was amplified with the same program having annealing step at 52°C for 15 seconds. PCR products were isolated with streptavidin-coated magnetic beads (Dynal AB) and sequencing reactions were carried out using the AutoRead kit (Pharmacia) with fluoroelabeled primers as described. Reaction products were analyzed on automated sequencer and the methylation status of each CpG doublet was determined in the following 5 levels: 0%, 25%, 50%, 75%, and 100%.22,23

Total 5-mC Analysis

Perkin-Elmer high-pressure liquid chromatography (HPLC) system was used to analyze cytosine methylation at the whole genomic level as described.24 The column was constructed from Beckman UltraSphere 5-μm particles.

Northern Blot Analysis

Tissue distribution of ec-sod mRNA was determined by Northern blot analysis. Total RNA was isolated from rabbit normal and atherosclerotic intima-media, heart, liver, and spleen by guanidinium isothiocyanate method and mRNA was isolated by Quick Prep mRNA isolation kit (Pharmacia). One μg of mRNA was separated by electrophoresis on a denaturing formaldehyde 1.5% agarose gel and transferred to HYBOND N nylon membrane (Amersham). The membranes were hybridized with a random-primed 32P-CTP-labeled rabbit ec-sod (386 bp) and human β-actin (1.1 kb) probes as described.17

Cell Culture Studies

The effect of ec-sod CpG cluster hypomethylation on the ec-sod expression was analyzed by culturing duplicate 10-cm plates (5 to 10×105 cells) of rabbit aortic SMC in the presence of 3 μmol/L 5-azacytidine (Sigma) for 1 month.26 As a control, we used SMC grown in the absence of 5-azacytidine to obtain the basic level of ec-sod expression. RNA and DNA were isolated from the cultured cells and the methylation level and ec-sod mRNA expression were determined as described above.

Results

Rabbit ec-sod Gene

We have cloned and characterized rabbit ec-sod gene, which has a 735 bp nonintronic coding sequence in the third exon similar to human and mouse ec-sod genes26,27 (Figure 2). Database analysis revealed 6 regions of rabbit C-repetitive sequences that represent typical short interspersed sequences (SINE) elements.28 These repeats have originated partly from tRNA genes29 and evolved by retrotransposition similar to Alu repeats30 found in human ec-sod gene26 or B repeats27 that are present in mouse gene. Human gene also contains a LINE 2B sequence in front of the coding sequence and 35-bp long poly-T after the coding sequence. LINE sequences are “long period interspersed sequences” in mammalian genomes that are retrovectors generated from RNA polymerase II transcripts.31

According to sequence analysis, rabbit ec-sod gene contains 5 CpG clusters, mainly located at the 5’ region of the cloned sequence. Similar CpG island patterns can also be seen on human and mouse genes (Figure 2). The first cluster is 365 bp long, 55% CG rich, with the observed/expected (O/E) value of 0.57 for CpG cluster. The second cluster is 254 bp long, 61% CG rich, and has the O/E value of 0.57. The length of the third cluster is 330 bp long, 65% CG rich, and has the O/E value of 0.55. These islands are separated by short sequences and may have arisen from a single CpG cluster. The fourth cluster is 338 bp long, 57% CG rich, with the O/E value of 0.5. The fifth and the largest (1067 bp) island is located on the coding sequence of the gene (Figure 1). Sequence in this island is 72% GC rich containing 121 CpG dinucleotides. The O/E ratio of the last CpG cluster (0.87) is well above the limit used to determine the presence of these clusters.32

Comparison of the DNA and amino acid sequences between the species is shown in Figure 1. Sequence similarity between rabbit and human, rat, or mouse DNA is 80%, 67%, or 63%, respectively (Figure 1A). CpG island comparison reveals a striking similarity among the species. Most of the conserved dimers are located in the region encoding for the active center. At nucleotide positions 74, 192, 345, 450, 471, and 612, 3 of the CpG dimers are conserved, whereas the fourth is a CpG dimer suggesting a possible deamination process. Independent dimers are well conserved between rabbit and human (69% similarity), whereas rabbit and rat or mouse show 33% and 31% similarity, respectively. Amino acid sequence is also well conserved between the species (Figure 1B). Sequence similarity between rabbit and human, rat and mouse amino acids is 80%, 71%, and 68%, respectively.

Mutation Screening

Previously the following mutations have been identified: Arg311→Gly in the human eDNA region encoding the carboxyterminal end of the protein,33,34 Asn21→Asp in the amino-terminal portion of mouse eDNA and a 10-bp deletion in 3’ UTR of mouse eDNA35 (Figure 1A). Because CpG dimers are at high risk of mutations, we screened mutations from rabbit lung, kidney, spleen, liver, brain, and heart tissues. Three new mutations were identified in the coding sequence: 1) 45 A→C, 2) 76 G→C, and 3) 157 C→T (Figure 1A). The first mutation is located in a putative signal peptide, but it does not change the amino acid sequence, whereas the 2 latter point mutations caused changes in the amino acid chains Glu26→Gln and Arg37→Trp, respectively (Figure 1B). The first mutation created a new CpG dimer between dimers 6 and 7, whereas the third mutation disrupted CpG dimer 19. The third mutation is probably caused by deamination of the methylated CpG dimer. In 4 out of 5 animals, the new mutations caused allele variation. Mutations were also present in germ line.
Methylation of *ec-sod* CpG Cluster in Atherosclerotic Aorta and Other Rabbit Tissues

Because CpG clusters are potential sites for methylation and may explain the occurrence of mutations, the methylation status of 109 dimers (Figure 1A) of the *ec-sod* CpG cluster covering the coding sequence was determined using direct genomic sequencing. Methylation level of the dimers was analyzed from normal aortic intima-media, atherosclerotic intima-media, and from heart, liver, spleen, germ line cells (sperm), and cultured rabbit aortic SMC. According to preliminary analysis, no differences were found in the whole genomic methylation level between NZW and WHHL rabbit tissues, eg, kidney values in NZW versus WHHL are 3.85 versus 3.9, respectively (Laukkanen et al, unpublished data, 1999). The sequencing analysis of the CpG cluster showed higher 5-mC content in the island in all tissues studied as compared with atherosclerotic aorta and germ line, which was nonmethylated throughout the cluster (Figure 3). Based on the sequencing analysis, the 3’ end of the island was almost completely methylated in normal and atherosclerotic aorta, as well as in all tissues analyzed. A clear difference was seen in the 5’ end of the island between normal (n=4) and atherosclerotic aorta (n=4) in which the 5’ end of the island is almost completely nonmethylated. Also, the boundary between the hypomethylated and methylated region is well defined. The comparison of different rabbit tissues shows no common methylation pattern, but the distribution of 5-mC varies between tissues. The CpG island located on the coding sequence was completely nonmethylated in all germ line samples analyzed (n=3).

For the analysis of the whole genomic methylation, DNA was extracted, digested, and subjected for HPLC
Methylation of the CpG Island in Cultured SMC

To analyze the effect of hypomethylation on the expression of ec-sod, primary rabbit aortic SMC were cultured in the presence of 5-azacytidine, which is known to induce a general hypomethylation. Sequencing analysis revealed that the ec-sod CpG cluster was completely methylated in control cells (Figure 3). This supports earlier observations that have shown that in cell lines, genes associated with CpG clusters are methylated if they are not necessary for cell survival. It is interesting to note that the methylation pattern of the CpG cluster showed only minor changes (<10%) after induction of hypomethylation with 5 μmol/L 5-azacytidine (Figure 3). The decrease was random and did not resemble the hypomethylation pattern observed in atherosclerotic aorta. This implies that hypomethylation in ec-sod in vivo may have arisen by a specific mechanism. HPLC analysis was used to confirm the effect of 5-azacytidine treatment on the genome-wide methylation. The induction of hypomethylation caused a marked 52% reduction in the genome-wide methylation as compared with control cells (data not shown).

Northern Blot Analysis

Northern blot analysis of tissue distribution of ec-sod mRNA showed the highest expression in normal aortic intima-media, whereas atherosclerotic intima-media had decreased ec-sod mRNA synthesis, as shown in previous experiments. Heart showed much lower expression and liver and spleen very little, if any, expression (Figure 4). The distribution is comparable with reported enzyme activity in the same tissues. Northern blot analysis from cultured SMC (see above) showed that minor hypomethylation of the coding region did not increase mRNA expression in 5-azacytidine-treated SMC as compared with untreated control cells (Figure 4).

Discussion

Ec-sod gene has so far been cloned from human and mouse. Each of these genes contains a nonintronic coding sequence located in the third exon and species characteristic SINE elements that are known to be retroposons. Rabbit C-element is a characteristic SINE element with defined structural features. In human and mouse ec-sod genes, corresponding LINE elements can be found in the 5' region of the sequence. The importance of these elements for the function of ec-sod gene is unknown but may reflect the evolutionary origin of the gene.

Mutation screening of the coding sequence revealed 3 new mutations. Two of these point mutations also changed the amino acid sequence. In principle, mutations could be at least partially explained by methylation because methylated cytosines in CpG dimers are frequently deaminated and mutated to thymidines, which has lead to rarity of these nucleotides because the mutation pressure on methylated CpG is 40 times higher than on nonmethylated dinucleotides. In spite of the high mutation pressure, we found only 1 dimer that was deaminated and mutated to TpG. This suggests a strong protection of the methylated dimers to keep cytosines in the ec-sod sequence. This is also supported by the similarity of the CpG pattern on the coding sequence of the cloned genes. Deamination and mutation of dimers would change the amino acid composition, which could lead to disappearance of the functional gene from the genome.

We characterized the methylation level of the CpG island located on the coding sequence of the gene. CpG islands are normally found in nonmethylated motifs in germ line and in tissues where the gene associated to the island is expressed. However, ec-sod CpG island located on the coding sequence shows a high degree of methylation in all adult tissues studied (Figure 3), whereas in germ line it was completely nonmethylated, revealing that the island is not protected against methylation and that the methylation occurs during embryogenesis. The distinct hypomethylation in ec-sod 5' region in atherosclerotic aorta clearly differs from other analyzed tissues. In SMC, where artificial hypomethylation was produced by 5-azacytidine, the local methylation status of the ec-sod CpG cluster remained almost unchanged. This is indicative for independent regulation and maintenance of the methylation pattern of the ec-sod CpG cluster, which is also supported by the random decrease of methylation in 5-azacytidine-treated SMC as compared with the well defined decrease in atherosclerotic aorta.

Northern blot analysis of mRNA isolated from different tissues indicated that arterial wall is one of the major sites of ec-sod expression. Variation between tissues clearly demonstrates that ec-sod is not a housekeeping gene but is strongly induced in certain physiological environments. This is supported by our previous findings showing that the expression of ec-sod is increased in the early phase of atherogenesis and decreased in complicated and connective tissue-rich human and rabbit lesions.

It is not known whether hypomethylation of ec-sod in atherosclerotic lesions contributes to lesion formation or whether it occurs as a consequence of the pathological
process. Also, as has been reported earlier, our results do not indicate a causal relationship between methylation pattern of the CpG island and ec-sod expression. However, it is conceivable that hypomethylation has at least partly developed through increased cell proliferation, which is a typical feature in atherogenesis. Because ec-sod methylation level in normal arterial wall was already lower than in other tissues, we cannot exclude a possibility that specific conditions in the vessel wall, such as deficiency in cytokine methyl transferase activity, may contribute to the hypomethylation of the CpG island. It is also possible that, similar to cancer cells, hypermethylation of genes involved in the inhibition of cell growth or apoptosis may be involved.

To our knowledge this is the first demonstration of hypomethylation in atherosclerotic lesions. The reduction observed in the methylation status of atherosclerotic lesions as compared with normal aortic intima-media is similar to the hypomethylation level reported in malignant tumors. According to preliminary results, similar hypomethylation can be found in human atherosclerotic plaques and in SMC isolated from intima of balloon-denudated rabbit arteries (Hiltunen et al, unpublished observation, 1999). These findings also support the hypothesis that lesion SMC may differ from normal arterial SMC, which might be reflected as differences in gene expression and potential for gene regulation.

Normally, a genome-wide hypomethylation in malignant tumors is associated with increased expression of oncogenes and growth factors that leads to excess cell proliferation. Because SMC proliferation is an important factor in the development of atherosclerotic lesions, it is possible that similar mechanisms may at least partially account for the progression of atherosclerotic lesions in rabbit arteries.

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