Deletion of the Carboxyl-Terminal Exons of K-sam/FGFR2 by Short Homology-mediated Recombination, Generating Preferential Expression of Specific Messenger RNAs

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Abstract

The K-sam gene was first identified as an amplified gene from human gastric cancer cell line KATOIII, and its product is identical to fibroblast growth factor receptor 2. The K-sam gene is located on human chromosome 10q26 and is preferentially amplified in the poorly differentiated types of gastric cancers. During the course of studies on the structural characterization of the amplification units, we found that the carboxyl-terminal exons of K-sam were deleted in three of four of the scirrhous type of gastric cancer cell lines. These deletions generate preferential expression of mRNAs encoding K-sam proteins lacking the carboxyl-terminal region containing the tyrosine residues at positions 780, 784, and 813. The carboxyl-terminal region has been reported to have a sequence required for the inhibition of NIH3T3 transformation, indicating that cells with amplification of the truncated K-sam gene have a growth advantage during the carcinogenic process for the scirrhous type of gastric cancers. This is the first report showing the deletion of the carboxyl-terminal exons of the receptor-type protein tyrosine kinase gene. Sequence analysis of the DNA sequences surrounding the deletion junctions shows the presence of unique sequences and indicates the involvement of short homology-mediated recombination in the generation of these deletions.

Introduction

Amplification of cancer-related genes during the carcinogenic process is one of the end points of genomic instability, and in some cases, a good correlation between oncogene amplification and the prognosis of the particular types of cancer has been demonstrated (1). We reported the structural characteristics of amplicons on 11q13 containing HST1, INT2, MB38, EXPI, and Cyclin D1 genes (2–6) and those on 17q12 containing c-ERBB-2, CAB1, GRB7, A39, and CS1 genes (7, 8). We and others have also reported their clinical implications (9, 10). There were reports from other laboratories on some structural characteristics of N-MYC and HuDbBP-RBidDX1 genes on 2p24 (11), CDK4, SAS, and MDM2 genes on 12q13–14 (12, 13) and BTK and AIB1 genes on 20q12–13 (14, 15).

K-sam was first identified as an amplified gene by the in-gel DNA renaturation method from human gastric cancer cell line KATOIII (16, 17), and its product was later found to be identical to the BEK or KGF3 receptor or FGFR2. The K-sam gene is located on human chromosome 10q26 and is preferentially amplified in the poorly differentiated types of gastric cancers. Expression of K-sam products has been correlated with a poor prognosis of gastric cancer (18). During the course of studies on structural characteristics of a core amplified region and on identification of end-joining sites between each amplification unit, we identified interstitial deletion of the carboxyl-terminal exons of the K-sam gene in three of four of the scirrhous type of gastric cancer cell lines with the K-sam gene amplification. Novel mRNAs encoding K-sam proteins without the carboxyl-terminal region containing the specific tyrosine residues at positions 780, 784, and 813 were preferentially expressed in these three scirrhous gastric cancers. This carboxyl-terminal region has a sequence required for inhibition of NIH3T3 transformation. It is very likely that the cells with amplification of the activated K-sam gene have a growth advantage during the carcinogenic process for the scirrhous type of gastric cancers. We further examined the DNA sequences surrounding the junctions of the deletion and found that these deletions might possibly be generated by short homology-mediated recombination.

Materials and Methods

Cell Lines and Tumor Tissues. Eight of the scirrhous type of gastric cancer cell lines, HSC39, HSC43, HSC44, HSC58, HSC59, HSC60, OCUMZM and KATO III, and a poorly differentiated type of gastric cancer cell line SNU16 were maintained in RPMI 1640 supplemented with 10% FCS, 0.15% sodium bicarbonate, 2 mm l-glutamine, and penicillin-streptomycin. All of the surgical specimens were frozen immediately in liquid nitrogen and stored at −80°C until use.

DNA Preparation and Southern Blot Analysis. Genomic DNA was prepared from culture cells and tissues using a standard method (19). The extracted DNA was digested with EcoRI, and 10 μg of this digest was fractionated on 0.7% agarose gel and transferred to Hybond-N+ (Amersham). Hybridization was carried out in 50% formamide, 5× SSC (1× SSC: 150 mm NaCl, 15 mm sodium citrate), 5× Denhardt’s reagent, 5 mm EDTA, 0.1% SDS, 10% dextran sulfate, and 100 μg/ml of denatured salmon sperm DNA at 42°C for 14–16 h. All DNA probes were labeled with [α-32P]dCTP. The filter was washed twice in 0.1× SSC and 0.1% SDS at room temperature and twice at 65°C and exposed to Kodak XAR film at −70°C. The hybridization intensity in each sample was quantified by the BAS2000 bioimaging analyzer (Fuji Photo Film Co., Tokyo, Japan).

cDNA Selection. The DNA fragment within the second tyrosine kinase domain sequence was amplified by PCR with a biotinylated primer 5′-biotin-CTGCCAGGATATCAACAA-3′ and an unbiotinylated primer 5′-AC-CAACTGCTTGAACTTG-3′. Ten nanograms of the biotinylated DNA...
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Fig. 2. Identification of rearranged DNA fragments in the K-sam gene. EcoRI-digested DNAs of 5 scirrhous gastric cancer cell lines, HSC44, KATOIII, HSC39, HSC43, and OCUM2M cells and 13 primary scirrhous gastric cancer tissues, T3301 to T27, were hybridized with a TK-C3 intron probe as shown in Fig. 3B. Rearranged DNA fragments with the different sizes compared with a normal 9.9-kb EcoRI-DNA fragment were detected in KATOIII, HSC43, and OCUM2M cells.

NotI-cleaved pBluescriptII vector (Stratagene), and we sequenced 20 clones from each cell line.

Molecular Cloning of Rearranged DNA Fragments Containing the Breakpoints of the Deletion. EcoRI-digested DNA fragments of tumor DNAs with the sizes of 5 kb to 10 kb contained rearranged DNA sequences. These DNA fragments were extracted from agarose gel and then ligated to λZapII. A total of $1 \times 10^{6}$ plaques were screened with the TK-C3 intron probe under the same conditions for Southern blot hybridization and washed as described. Positive clones were isolated.

Probes for Southern Blot Hybridization. The following probes were used in Southern blot hybridization. C1 and C3 probes were prepared as described in our previous report (20). The TK-C3 intron probe DNA was amplified from genomic DNA by PCR. The specific primers were: 5'-TCACCTCTCACAACCAATGAG-3' and 5'-CATGATTTGAGAGGAACCCA-3'.

Results and Discussion

Identification of Deletions of the Carboxy-terminal Exons of the K-sam Gene. To identify a core amplified region of the chromosome 10q26 locus containing the K-sam gene, we performed Southern blot analysis on DNA samples of nine gastric cancer cell lines, including eight of the scirrhous type of gastric cancer cell lines, KATOIII, HSC39, HSC43, HSC44, HSC58, HSC59, HSC60, and OCUM2M, one poorly differentiated type of gastric cancer cell line SNU16, and 26 of the scirrhous type of primary gastric cancer tissues. Representative data are shown in Fig. 2. KATOIII, HSC39, HSC43, and OCUM2M cell lines had amplification of K-sam. By Southern blot analysis with an intron DNA probe between an exon encoding a portion of the second tyrosine kinase domain and a carboxyl-terminal exon, it was revealed that all of these cell lines except HSC39 had rearranged K-sam. T3301 DNA from a scirrhous type of gastric cancer tissue also contained the significantly amplified K-sam gene, but there was no detectable rearrangement of the gene (Fig. 2). It was likely that these rearranged DNA fragments were produced by deletion of the carboxyl-terminal exons of the K-sam gene. A minor band of the >9-kb DNA fragment was detected in both KATOIII and OCUM2M cells. It is possible that this minor band may be produced by minor rearrangement or by the RFLP.

We have previously identified at least four types of K-sam transcripts: from K-sam-I to K-sam-IV (21). The K-sam-I and K-sam-II cDNAs encode membrane-spanning forms, whereas the K-sam-III and K-sam-IV encode soluble forms lacking the transmembrane region. Comparison of the K-sam-I and K-sam-II cDNAs revealed different nucleotide sequences in the carboxyl half of the third immunoglobulin-like domain (17, 21). As shown in Fig. 3A, this carboxyl-half region of K-sam-II is identical to that of the KGF receptor, which shows high-affinity binding to KGF but not to the basic FGF, whereas the K-sam-I cDNA is identical to the cDNA of the BEK receptor, which provides high affinity to the basic FGF but not to KGF (22–26). Furthermore, K-sam generates several variant transcripts by alternative splicing (Fig. 3A), and the most abundant K-sam transcript in KATOIII cells was cloned as the K-sam-IIC3 cDNA, which has the

Fig. 1. Schematic diagrams of a probe and primers for enrichment of the K-sam cDNA sequences (upper) and vectors for cDNA cloning (lower) for the K-sam cDNA isolation method without the hybridization process by the vector ligation-mediated PCR after cDNA selection. E, EcoRI site; N, NotI site. The detailed isolation procedures are described in the “Materials and Methods” section.
KGF-binding motif and a short carboxyl terminus lacking the tyrosine residues at positions 770, 780, 784, and 813. The K-sam-IIC1 product contains a long carboxyl terminus with Tyr-770, -780, -784 and -813. K-sam-IIC3 showed greater transforming activity to NIH3T3 cells than did K-sam-IIC1 (20), and K-sam-IIC3-transfected L6 myoblast cells hardly differentiated, whereas K-sam-IIC1 transfectants showed a differentiated phenotype (27). The sequence surrounding Tyr-770 in K-sam-II corresponding to Tyr-769 in K-sam-I/BEK/FGFR2 is a potential binding site for the SH2 domain of phospholipase C-γ (28). However, mutation of the corresponding tyrosine in FGFR1 (Tyr-766) had no effect on mitogenic activity, cell proliferation, or cell cycle progression (29–31). It has been reported that transfection of deletion mutants of the carboxyl-terminal region of K-sam cDNA and mutants in Tyr-780, -784 and -813 showed ligand-independent activation by the carboxyl-terminal alterations and transforming activity to NIH3T3 cells (32). These results indicated that the carboxyl-terminal region containing Tyr-780, -784, and -813 had a sequence required for the inhibition of NIH3T3 transformation upon transfection.

To investigate structural alteration in the carboxyl-terminal exons, we performed Southern blot analysis on DNAs from the scirrhous gastric cancers with three probes designated as TK-C3 intron, C3, and C1 (Fig. 3B). C1- and C2-type carboxyl termini are encoded by a common exon with two different splice acceptor sites (20). A 9-kb EcoRI-DNA fragment was found to be detected in HSC44 cells without K-sam amplification as a normal DNA fragment by Southern blot analysis with these three probes. In KATOIII cells, both an amplified 9-kb DNA fragment and an amplified 8-kb DNA fragment were detected with a C3 probe corresponding to a portion of the C3

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**Fig. 3. Identification of deletions of the carboxyl-terminal exons of K-sam.** A, schematic presentation and predicted carboxyl-terminal amino acid sequences of the K-sam-I and -II transcripts. Immunoglobulin, TM, TK, and numbers on Y indicate extracellular immunoglobulin-like, transmembrane, tyrosine kinase domains, and positions of tyrosine residues, respectively. B, Southern blot analysis of DNA samples of HSC44, KATOIII, OCUM2M, and HSC43 cells. EcoRI-digested DNA was hybridized with C3, C1, and TK-C3 intron probes indicated by bold bars, which are shown on the map of the carboxyl-terminal exons of the K-sam gene (upper). Southern blot data are shown on the left. Schematic diagrams of deletion are shown on the right. Triangle/insert indicates the deleted region, and DNA segments with different lengths are represented by //. Different EcoRI sites are indicated by E(1) to E(4). Exons and introns are shown by boxes and thin lines, respectively. E, EcoRI site.
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Fig. 4. Schematic presentation and predicted carboxyl-terminal amino acid sequences of the newly identified K-sam transcripts. Immunoglobulin, TM, TK, and numbers on the Ys indicate extracellular immunoglobulin-like, transmembrane, tyrosine kinase domains, and positions of tyrosine residues, respectively. Larger Ys are tyrosine residues reported to be required for the inhibition of the transforming activity of K-sam (32). The numbers on the right indicate the number of clones obtained from cDNA libraries of OCUM2M and HSC43 cells. Data of the K-sam-Is in KATOIII cells are from our previous report (20). Novel mRNAs encoding K-sam proteins without the carboxyl-terminal region containing the specific tyrosine residues at positions 780, 784, and 813 were preferentially expressed in these three scirrhous gastric cancers. a.a., amino acid. ND, not determined.

exon sequence. Intensity of the 8-kb band was the same as that of the 9-kb band, whereas the 8-kb band was not detected with a C1 probe. These data clearly suggest that approximately half of amplified K-sam genes in KATOIII cells have deletion in the C1 or C2 carboxyl-terminal exon. Both a single copy of a 9-kb normal DNA fragment and an amplified 7.5-kb DNA fragment were detected with the C3 probe in OCUM2M cells. Therefore, the 3’ exon encoding the C1 or C2 carboxyl terminus was thought to be completely deleted in OCUM2M cells. In HSC43 cells, amplified DNA fragments were not detected with either the C3 probe or the C1 probe, whereas a 7-kb amplified band was detected with a TK-C3 intron probe, a part of the intron sequence between an exon encoding a part of the second tyrosine kinase domain and the C3 exon. These data suggest that a DNA region from C3 to C1 is completely deleted in HSC43 cells.

A deletion of exons 2–7 of the epidermal growth factor receptor has been reported to generate constitutive activation of phosphatidylinositol 3-kinase (33), whereas a splice site mutation in the androgen receptor gene was found to generate an exon 3 deleted splicing variant in breast cancer (34). Our finding in this study appears to be the first demonstration identifying a deletion of the carboxyl-terminal exons of the receptor-type of protein tyrosine kinase gene in cancer cells. In both OCUM2M cells and HSC43 cells, the rearranged band detected with the C3 probe or the TK-C3 intron probe was of the same intensity as that of the amplified bands commonly found with the 5’ exons of the K-sam gene as probes (data not shown), demonstrating that these tumors might have ampiclons quite homogeneous in the deleted regions, which suggests that the deletion may occur before the amplification. Deletion of the carboxyl-terminal exons of the K-sam gene was found in three of four of the scirrhous type of gastric cancer cell lines with the K-sam amplification, KATOIII, HSC39, HSC43, and OCUM2M. But the deletion was not found in four of the scirrhous type of gastric cancer cell lines without the K-sam amplification HSC44, HSC58, HSC59, and HSC60. As shown in the representative results of the Southern blot analysis (Fig. 2), the deletion was also not observed in three of the scirrhous type of primary gastric cancer tissues with the K-sam amplification and in 23 DNA samples of the scirrhous type of primary gastric cancer tissues without the amplification. These data suggest that the deletion may occur in cultured cells in association with the amplification. The reason that the deletion was not observed in primary tumors with the K-sam amplification remains unknown. It is possible that the sample number of the primary gastric cancer with the amplification is too small or that the deletion may occur by selection during culture.

Sequence Analysis of K-sam Transcripts of Cell Lines with the Deletion. We have previously reported that the most abundant K-sam transcript in KATOIII cells was cloned as the K-sam-IIC3 cDNA, which has the KGF-binding motif and a short carboxyl terminus lacking a transformation-inhibitory sequence containing Tyr-780, -784, and -813 (Fig. 3A; Refs. 20 and 32). To investigate what type of the K-sam transcripts is expressed preferentially in OCUM2M and HSC43 cells with deletion of the carboxyl-terminal exons of K-sam, we analyzed sequences of cDNA clones from these two cell lines. Our previously described PCR method was used to amplify cDNAs in proportion to the expression levels of the corresponding genes (8, 35, 36). To enrich cDNA fragments encoding the second tyrosine kinase domain of K-sam, we used our cDNA selection method by using the proportionally amplified cDNAs as described in the “Materials and Methods” section. This new procedure is based on the vector ligation-mediated PCR after cDNA selection by which we can obtain a specific cDNA sequence without the colony hybridization process. After a round of hybridization between a biotinylated DNA fragment encoding the second tyrosine kinase domain of K-sam and the random hexamer-primed cDNAs produced from mRNAs of these two cell lines, the cDNAs hybridized to the biotinylated DNA fragment encoding the second tyrosine kinase domain of K-sam were captured on avidin-coated magnetic beads and amplified by PCR. By this procedure, the cDNA clones containing a sequence of the second tyrosine

<table>
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kinase domain were successfully obtained from the enriched cDNA libraries produced from mRNAs of these two cell lines. Twenty cDNA clones in the HSC43 cells and 18 cDNA clones in the OCUM2M cells were sequenced. Schematic presentation and predicted carboxyl-terminal amino acid sequences of the newly identified K-sam transcripts were shown in Fig. 4. In the HSC43 cells, 17 of 20 clones were K-sam-IIH1; 2 clones were K-sam-IIH2, and the other two K-sam-IIH3. Two K-sam-IIH2 cDNA clones had two tyrosine residues at positions 802 and 817, whereas the other 18 of 20 clones had no putative transformation-inhibitory sequence containing Tyr-780, -784, and -813. In the OCUM2M cells, all clones (8 K-sam-IIO1, 7 K-sam-IIO2, 2 K-sam-IIO3, and one K-sam-IIO4) had no transformation-inhibitory sequence. These results demonstrated that deletions of the carboxyl-terminal exons of K-sam resulted in utilization of K-sam-IIH3. Two K-sam-IIH2 cDNA clones had two tyrosine residues at positions 802 and 817, whereas the other 18 of 20 clones had no putative transformation-inhibitory sequence containing Tyr-780, -784, and -813. In the OCUM2M cells, all clones (8 K-sam-IIO1, 7 K-sam-IIO2, 2 K-sam-IIO3, and one K-sam-IIO4) had no transformation-inhibitory sequence. These results demonstrated that deletions of the carboxyl-terminal exons of K-sam resulted in utilization of

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4 The nucleotide sequences reported in this paper will appear in the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank nucleotide sequence database with the following accession numbers: AB030073 for K-sam-IIH1; AB030074 for K-sam-IIH2; AB030075 for K-sam-IIH3/O4; AB030076 for K-sam-IIO1; AB030077 for K-sam-IIO2; and AB030078 for K-sam-IIO3.

Fig. 5. Short homology-mediated recombination may generate the deletion of the carboxyl-terminal exons of the K-sam gene. A. Schematic deletion map of the K-sam gene in the three scirrhous gastric cancer cell lines. The 5' and 3' breakpoints of the K-sam deletion in KATOII, OCUM2M, and HSC43 are indicated by arrows. Exons are shown by boxes. The distances among the 5' breakpoints and between the 5' EcoRI site and the 3' breakpoint in HSC43 are shown in kilobases. These 5' breakpoints were present in a quite closely located region. E. EcoRI site. B. Alignment of the DNA sequence surrounding the deletion junction in KATOII and OCUM2M and the germ-line DNA sequence surrounding the 5' and 3' breakpoints in KATOII and OCUM2M. +, identical nucleotides. Underlined nucleotide sequences are short homologous sequences. These data clearly indicate that deletion of the carboxyl-terminal exons of K-sam in KATOII and OCUM2M cells during gene amplification occurs through short homology-mediated recombination. C. Alignment of the DNA sequence surrounding the deletion junction and the germ-line DNA sequence surrounding the 5' breakpoints in HSC43 cells. +, identical nucleotides. Underlined nucleotide sequences are the perfect 7-bp direct repeat (ATATTTA). We could not obtain the 5'-flanking sequence of the 3' breakpoint in HSC43 cells in direct sequencing analysis of the BAC clone. Therefore, we compared the DNA sequence surrounding the deletion junction in HSC43 cells and the germ-line DNA sequence surrounding only the 5' breakpoint in this cell line. Interestingly, an ATATTTA sequence was found at the recombination site, and the perfect 7-bp direct repeat (ATATTTA) was also present in the 3'-flanking sequence of the 3' breakpoint in HSC43 cells, suggesting that an ATATTTA sequence may present in the 3'-flanking sequence of the 3' breakpoint in HSC43 cells. These data suggest that short homology-mediated recombination generates the K-sam deletion also in HSC43 cells.
novel carboxyl-terminal exons and generated preferential expression of K-sam mRNAs encoding novel proteins lacking the transformation-inhibitory sequence. It is very likely that the cells with amplification of the activated K-sam gene have growth advantage during the carcinogenic process for the scirrhous type of gastric cancers.

Sequence Analysis of the DNA Sequences Surrounding the Deletion Junctions of the K-sam Gene. Gene amplification is one of the basic mechanisms. It lead to the overexpression of oncogenes, whereas homozygous deletion resulted in functional loss of tumor suppressor genes. These two types of alterations might be caused in part by common pathways including induction of a double-strand break (37, 38), and there have been a few reports on the cloning of interamplification junctions (39, 40) and junctions of homozygous deletion (41–44). We examined the DNA sequences surrounding the deletion junctions of the K-sam gene in the three cell lines, KATOIII, OCUM2M, and HSC43. By plaque hybridization with the TK-C3 intron probe, these three rearranged DNA fragments were isolated from the λZapII library, which was produced from EcoRI-digested DNA fragments of these three cell lines. We also subcloned a 9-kb normal DNA fragment containing the 5′ deletion breakpoints in these three cell lines from a BAC clone isolated in our laboratory (Fig. 3B and Fig. 5A). Sequence analysis of these three rearranged DNA fragments and the normal DNA fragment was performed to determine the 5′ deletion breakpoints in the three cell lines, KATOIII, OCUM2M, and HSC43 (Fig. 5A). We next isolated other BAC clones containing the 3′ deletion breakpoints in these 3 cell lines (data not shown) and obtained the DNA sequences surrounding the 3′ deletion breakpoints in these 3 cell lines except HSC43. Schematic deletion map of the K-sam gene in these 3 cell lines is shown in Fig. 5A. It is noted that the 5′ breakpoints of the K-sam deletion in these 3 different cell lines were localized to a quite closely located region (Fig. 3B and Fig. 5A), suggesting that this region might contain a recombination hot spot, which frequently induces a double-strand break.

Alignment of the DNA sequence surrounding the deletion junction in KATOIII and OCUM2M and the germ-line DNA sequence surrounding the 5′ and 3′ breakpoints in KATOIII and OCUM2M is shown in Fig. 5B. Interestingly, we found the presence of a perfect 6-bp direct repeat (TGCGCTG) in KATOIII cells and that of a perfect 4-bp direct repeat (GGTT) in OCUM2M cells at the recombination site. Therefore, we compared the DNA sequence surrounding the deletion junction in HSC43 cells and the germ-line DNA sequence surrounding only the 5′ breakpoint in this cell line (Fig. 5C). Interestingly, an ATATTTA sequence was found at the recombination site, and the perfect 7-bp direct repeat (ATATTTA) was also present in the 3′-flanking sequence of the 3′ breakpoint in HSC43 cells (Fig. 5C), suggesting that an ATATTTA sequence may present in the 5′-flanking sequence of the 3′ breakpoint in HSC43 cells. These findings indicate that deletion of the carboxyl-terminal exons of K-sam in HSC43 cells as well as KATOIII and OCUM2M cells during gene amplification occurred through short homology-mediated recombination.

Neither one nor two nucleotide overlaps, which have been found at breakpoint junctions in the interstitial deletion and in the translocation (41, 42), were detected in this study. It has been reported that the FRA3B/FHIT locus is a frequent target of homologous recombination between long interspersed nuclear elements resulting in FHIT gene deletion by sequence analysis of this locus and FHIT gene deletion junctions in its associated cancer cell lines (43). Although Alu-mediated homologous recombination in germ-line cells is thought to be responsible for partial gene duplications or deletions in many inherited diseases, the Alu-mediated homologous recombination in somatic cells has only been reported in the tandem duplication of the ALL/MLL gene in acute myeloid leukemia (45). There is one report that short direct repeats ranging in size from 4 to 7 bp were found in the breakpoints of homozygous deletions of the Rb gene in two retinoblastomas and in one osteosarcoma (44). Our finding appears to be the first demonstration showing that interstitial deletion during gene amplification may occur through short homology-mediated recombination.

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References

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