Formylpeptide receptor 1 mediates the tumorigenicity of human hepatocellular carcinoma cells

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ABSTRACT

G protein-coupled chemoattractant receptors (GPCRs) have been implicated in cancer progression. Formylpeptide receptor 1 (FPR1) was originally identified as a GPCR mediating anti-microbial host defense. However, the role of FPR1 in tumorigenesis remains poorly understood. The current study aims to investigate the potential of FPR1 to regulate human hepatoma growth and invasion. We found the FPR1 gene and protein expression in human intratumoral and peritumoral tissues of hepatocellular carcinoma (HCC) specimens and in human hepatoma cell lines. FPR1 activation mediated the migration, calcium mobilization and ERK-dependent IL-8 production by hepatic cancer cells. FPR1 knockdown substantially reduced the tumorigenicity of hepatoma cells in nude mice. Necrotic hepatic tumor cells released factor(s) that activated FPR1 in live tumor cells. Our results indicate a critical role of FPR1 in the progression of malignant human hepatic cancer. FPR1 thus may represent a molecular target for the development of novel anti-hepatoma therapeutics.

KEYWORDS

G protein-coupled receptor; HepG2; IL-8; liver

Introduction

HCC is the fifth most common cancer with increasing incidence worldwide. It is also one of the most lethal human malignancies due to the difficulty of early detection, rapid progression and chemoresistance. Hepatoma is characterized by vigorous angiogenesis and metastasis which account for high rate of postsurgical recurrence and extremely poor prognosis. HCC generally develops from chronic liver injury, leading to inflammation, matrix remodeling, fibrosis and cirrhosis. Chronic infections by hepatitis viruses such as hepatitis B virus (HBV) and hepatitis C virus (HCV) are major risk factors for HCC development.

Hepatocellular carcinoma cells

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FPR1 is a G protein-coupled 7 transmembrane cell surface receptor (GPCR), originally identified in phagocytic leukocytes, that mediates cell chemotaxis and activation in response to the bacterial formylated chemotactic peptides. FPR1 is involved in a broad spectrum of pathophysiologic processes including inflammation, wound healing, glioblastoma progression and the host defense against HCV infection.

As one of the most vascularized solid tumors, the HCC progression and prognosis correlate with the status of angiogenesis, due largely to elevated production of angiogenic factors, such as interleukin-8 (IL-8, CXCL8) by tumor cells. Previous reports have implicated IL-8 in the growth and angiogenesis of malignant tumors. In cancer models of pancreas, colorectum, melanoma and liver, IL-8 functions as an autocrine growth factor. In human hepatoma, clinical investigation has reported...
that high level of IL-8 is associated with higher frequency invasion of portal vein venous vessels and bile duct by tumor.\textsuperscript{13} IL-8 production was also associated with severe hepatitis and the development of hepatocellular carcinoma.\textsuperscript{14} Upregulation of the IL-8 receptor CXC chemokine receptor 2 (CXCR2) was found in HCC and was correlated with intrahepatic metastasis.\textsuperscript{15} In vitro experiments showed IL-8 production by HCC cell lines in response to tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)).\textsuperscript{16} Previous studies reported that N-formylmethionyl-leucyl-phenylalanine (fMLF), a bacterial chemotactic peptide activating FPR1, increased chemotaxis and production of angiogenic factor IL-8 by human glioblastoma.\textsuperscript{7,17} FPR1 in glioblastoma cells also interacts with agonists released by necrotic tumor cells,\textsuperscript{7} suggesting that tumor cells may utilize FPR1 to recognize agonists produced in the tumor microenvironment for their advantage. Since hepatocarcinogenesis involves a highly orchestrated interplay of injury, chronic inflammation and neovascularization,\textsuperscript{7} the multitude of FPR1 suggests that it may also play a role in the development of hepatic cancer.\textsuperscript{5-7, 9,17} In the present study, we report that FPR1 was expressed by HCC tissues from patients and the human hepatoma cell lines. Hepatoma cells responded to the FPR1 agonist fMLF by increased motility, proliferation and enhanced IL-8 production. FPR1 small hairpin RNA (shRNA) substantially reduced the tumorigenicity of hepatoma cells in nude mice. Our study thus demonstrates a significant role of FPR1 in the carcinogenesis of human hepatoma.

**Results**

**The expression of FPR1 on human hepatocellular carcinoma tissues**

We performed histologic and immune fluorescence staining of FPR1 in tumor tissues from HCC patients. In surgical specimens, hematoxylin-eosin (H&E)-staining revealed poorly (Fig. 1A) and moderately (Fig. 1B) differentiated HCC with a trabecular pattern. In the high-grade intratumor specimens, multiple tumors of intrahepatic metastases and portal vein invasion were observed. The cellular and nuclear pleomorphism, intracellular vacuoles, mitotic patterns, vessel formation and the necrosis in central tumor tissues were also demonstrated (Fig. 1A, upper right panels). The peritumor (Fig. 1A and B, lower right panels) liver tissue showed a chronic inflammatory infiltration in the fibrous stroma, diagnosed as hepatic cirrhosis. Strong FPR1 signal was detected in grade III HCC specimens (Fig. 1A, left panels), and positive staining was enriched in intratumor area Fig. 1A, upper left panels). In contrast, the lesser aggressive grade II hepatoma specimens showed intermediate staining intensity of FPR1 in intratumoral tissues (Fig. 1B,
upper left panels) and very low FPR1 expression in peritumoral tissues (Fig. 1B, lower left panels). We then examined whether FPR1 expression is selectively enhanced in hepatocellular carcinoma. Fig. 1C shows that protein was detectable in human normal liver tissues adjacent to HCC. However, the levels were far lower than that in HCC tissues. Very few FPR1-positive cells were found in the adjacent normal liver tissues, demonstrating that FPR1 expression is selective in HCC and in particular in intratumor tissues.

We next measured FPR1 RNA in HCC tissues and found FPR1 mRNA was higher in grade III than in grade II carcinoma specimens. The highest mRNA expression was found in the poorly-differentiated intratumor samples (Fig. 1D and Fig. S1A and B), consistent with the results obtained with histology analysis. These results demonstrate the expression of FPR1 by human hepatocellular carcinoma.

The expression of FPR1 by human hepatoma cell lines

To further determine the biological function of FPR1 in hepatic cancer cells, we used established human hepatic cancer cell lines HepG2 and Hep3B. Fluorescence-activated cell sorting (FACS) analysis shows that both HepG2 and Hep3B cells expressed FPR1 (Fig. 2A). HepG2 cells expressed higher levels of FPR1 on cell surface than Hep3B cells (Fig. 2A). HepG2 and Hep3B cells also expressed FPR1 gene with higher levels in HepG2 cells (Fig. 2B and Fig. S1C and D).

FPR1 promotes human hepatoma cell chemotaxis and invasion

We next tested the capacity of the FPR1 ligand fMLF to induce directional migration of human hepatoma cell lines. Both HerG2 and Hep3B cells migrated in response to fMLF with a bell-shaped dose-response curve (Fig. 3A and C and Fig. S2A), typical of the cell response to chemoattractants. We also tested the capacity of Ac2–26, a specific cognate ligand for FPR1, to induce the migration of human hepatoma cell lines. Both HepG2 and Hep3B cells migrated in response to this FPR1 agonist (Fig. S2B-D). However, addition fo fMLF and Ac2–26 simultaneously to the cells in the upper wells of the chemotaxis chamber abrogated cell migration induced by equal concentrations of fMLF and Ac2–26 in the lower wells (data not shown). Thus, FPR1-induced migration of human hepatoma cell lines was based on chemotaxis rather than chemokinesis. Furthermore, the migration of HepG2 and Hep3B in response to fMLF was completely inhibited by pretreatment of the cells with the Gi protein inhibitor pertussis toxin (PT) and a FPR1-specific antagonist tBoc-MLF, but not cholera toxin (CT) or herbimycin A, a protein tyrosine kinase inhibitor (Fig. 3B and C and Fig. S2A-E), suggesting the involvement of a G-protein of the Gi-type coupled receptor.

To more precisely examine the contribution of FPR1 to liver tumor cell motility and invasiveness, we used a wound-healing model by creating a gap in a confluent HepG2 cell monolayer. HepG2 cells treated with fMLF showed more rapid locomotion than vehicle-treated cells toward the center of the gap on cell monolayer (Fig. 3D and E). Addition of cyclosporin H (a FPR1 specific antagonist) and PT to the cells blocked the capacity of fMLF to induce human hepatoma cell migration in scratch wound-healing assays, confirming FPR1-mediated cell motility (Fig. S3A and B). Thus FPR1 activation enables HepG2 cells to exhibit higher motility.

In addition to promoting cell motility, fMLF elicited a potent dose-dependent and PT-sensitive Ca2+ mobilization in HepG2 and Hep3B cells (Fig. 4A and B and Fig. S4A and B). Sequential stimulation of HepG2 cells with fMLF at high and low concentrations or vice versa resulted in bidirectional desensitization (Fig. 4C and D). However, pretreatment with FPR1 antagonists cyclosporin H or tBoc-MLF reduced the response of HepG2 and Hep3B cells induced by fMLF (Fig. S4D-I). These results further support the specificity of FPR1 expressed by HCC cell lines in response to fMLF.

FPR1 mediates MAPK activation in hepatocellular carcinoma cells

Since FPR1 has been reported to mediate phosphorylation of MAP kinases in various cell types, we stimulated serum-starved HepG2 and Hep3B cells with 100 nM fMLF and determined MAPK phosphorylation at different time points. fMLF stimulated ERK1/2 phosphorylation with a maximum effect at 5 min. (Fig. 5A and Fig. S5A and B). Similar to fMLF, Ac2–26
Figure 3. Responsiveness of human hepatoma cell lines to fMLF. (A) Chemotaxis of HepG2 (upper panels) and Hep3B (lower panels) cells in response to 100 nM fMLF. (B) inhibition of HepG2 cell migration in response to 100 nM fMLF by pretreatment of the cells with PT (100 ng/mL) or the FPR1-specific antagonist tBoc-MLF (1 μM) for 30 min at 37°C. Representative results from three independent experiments are shown. (C) Percentage of HepG2 cell migration in response to different concentrations of fMLF (in the presence or absence of PT or tBoc-MLF) over total loading cells. (D) and (E), motility in wound-healing model. HepG2 cells grown to confluence on plastic were scratched to create a wound. p < 0.05, vehicle-treated vs. fMLF-treated cells. (D) Cells in 10% FCS/DMEM were photographed at 0 and 8 h. The results are representative of three independent experiments. (E) The mean distance (mm) of leading cells moving toward the ‘wound’ area was assessed. *Indicates significantly slower locomotion of HepG2 cells treated with vehicle (n = 3). † p < 0.05, vehicle-treated vs. fMLF-treated cells.

Figure 4. Calcium (Ca^{2+}) mobilization in human hepatoma cells induced by fMLF. Ca^{2+} mobilization in HepG2 cells was measured with a FlexStation II system using Fura-3 AM. Changes in intracellular calcium concentration in response to agonists were recorded as relative fluorescence units (RFUs). (A) Dose response of fMLF-induced Ca^{2+} flux in HepG2 cells. (B) Inhibition of HepG2 cell response to 10 nM fMLF by pretreatment of the cells with PT (100 ng/mL) but not CT (100 ng/mL). (C) and (D), cross-desensitization of Ca^{2+} flux in human hepatoma cells. (C), desensitization of 10 nM fMLF-induced Ca^{2+} flux by 1 μM fMLF in HepG2 cells. (D), 1 μM fMLF-treated HepG2 cell response to second challenge by 10 nM fMLF. Representative results from three independent experiments are shown.
induced the phosphorylation of ERK1/2 and MAPK in HepG2 and Hep3B cells (Fig. S5A–D). However, pre-treatment of the cells with FPR1 antagonists cyclosporin H or Boc markedly inhibited fMLF- or Ac2–26-stimulated ERK1/2 phosphorylation (Fig. S5A–D). Therefore, ERK1/2 MAPK is coupled to FPR1 in HCC cells.

Activation of FPR1 promotes IL-8 production by human hepatoma HepG2 cells

Since activation of FPR1 induces the production of IL-8,11,25 we tested whether FPR1 also promotes IL-8 production by hepatoma cells. Untreated HepG2 and Hep3B cells produced low levels of IL-8 protein and treatment of fMLF and Ac2–26 increased IL-8 production (Fig. 5B and Fig. S5E and F), reaching a maximum at 48 h after stimulation with 100 nM fMLF (Fig. 5B). Addition of the ERK inhibitors PD98059, U0126 and CsH, but not p38 MAPK inhibitor SB203580, inhibited fMLF- and Ac2–26-stimulated IL-8 production by HepG2 cells (Fig. 5B and Fig. S5E and F). Thus, only the ERK1/2 MAPK pathway appears to be crucial for FPR1 agonist-induced IL-8 expression by hepatoma cells.

IL-8 contained in the culture medium of fMLF-stimulated HepG2 cells was biologically active. When cultured with conditioned medium from fMLF-stimulated HepG2 cells and fMLF-CsH-treated HepG2 cell, but not fMLF-U0126-treated HepG2 cells, HUVECs formed capillary-like structures on Matrigel surface (Fig. 5C and D), which was inhibited by the addition of a monoclonal antibody against human IL-8 (Fig. 5C and D). Thus, IL-8 in the conditioned medium from FPR1-activated HepG2 cells induces endothelial cells to form capillary-like structures, a key event associated with neovascularization.

**FPR1 knockdown by shRNA reduces the tumorigenicity of hepatic cancer cells**

To evaluate the role of FPR1 in hepatoma tumorigenicity, we used shRNA to delete FPR1 in HepG2 cells. After stable transfection of FPR1 shRNA of HepG2 cells, the expression of FPR1 mRNA (Fig. 6A) and fMLF-induced chemotaxis (Fig. 6B) were abolished. In addition, the ability of fMLF to induce IL-8 production by HepG2 cells (Fig. 6C) was abrogated. Further, the cells failed to respond to the proliferation stimulating activity of fMLF (Fig. 6D).

We then injected HepG2 cells transfected with FPR1 shRNA into the flanks of athymic mice. Tumor nodules formed by HepG2 cells transfected with FPR1 shRNA appeared later (Fig. 6E) and grew more slowly than those formed by wild-type HepG2 cells or by mock-transfected cells (Fig. 6F and G). By day 52, all mice implanted with wild-type or mock-transfected HepG2 cells were dead. In contrast, 90% of the mice bearing tumors formed by FPR1 shRNA-transfected HepG2 cells survived to the day 66 after implantation (Fig. 6H). To confirm the role of FPR1 in HCC progression by in vivo experiments using murine cancer cell lines in immunocompetent mice, we examined the effect of FPR1 tumorigenicity in H22 tumor model of BALB/c mice.
following the method described by another group.\textsuperscript{26} We show that FPR1-shRNA had similar antitumor effects in immunocompetent mice shown in immunocompromised mice with human cell line (Fig. S6A, B and C). These results indicate that depletion of FPR1 markedly reduced the ability of HepG2 cells to form tumors, confirming the contribution of FPR1 to the tumorigenicity of human HCC cells.

The production of FPR1 agonist activity by necrotic hepatoma cells

Necrotic cell death has been reported to promote hepatocarcinogenesis.\textsuperscript{27,28} and mitochondria of ruptured cells contain chemotactic formylpeptides that activate FPR1 in myeloid cells.\textsuperscript{29} We thus investigated whether necrotic hepatoma cells and tissues might

![Image of Figure 6](Image)

Figure 6. The effect of FPR1 shRNA on tumorigenicity of HepG2 cells in athymic mice. (A) the expression of FPR1 mRNA. The levels of FPR1 mRNA were examined by RT-PCR in HepG2 cells stably transfected with FPR1 shRNA. Wild-type (WT, nontransfected) and mock-transfected HepG2 cells were used as control. GAPDH PCR product was used as a loading control. Representative results from three independent experiments are shown. (B) migration of FPR1 shRNA-transfected HepG2 cells in response to fMLF. Percentage of FPR1-mediated cell migration in total loaded cells was calculated. Values represent the mean ± SEM (n = 3). * p < 0.05, compared with mock-transfected cells. (C) the effect of FPR1 shRNA on IL-8 production by HepG2 cells. IL-8 in the supernatants of HepG2 cells cultured in the presence of fMLF was measured by ELISA after 24 and 48 h. The experiments were repeated three times, and each time point consisted of three replicate samples. Values represent the mean ± SEM. * p < 0.05, mock-transfected vs. shRNA-transfected cells. (D) growth curves of mock- and shRNA-transfected HepG2 cells in response to 100 nM fMLF. Cell growth was measured by MTT assays and the results were expressed as the mean ± SEM OD values. Asterisk indicates significantly increased proliferation of HepG2 cells stimulated by fMLF, as compared to non-stimulated cells (p < 0.05). (E) tumor formation. HepG2 cells (at 1 × 10^6 cells in 100 μL of PBS per mouse) were injected subcutaneously into the flanks of athymic mice (10 mice per group). Mice were examined for tumor formation at indicated times. (F) representative images of xenograft tumors of each group at 45 d after HepG2 cell injection. * p < 0.05, tumors formed by mock- vs. FPR shRNA-transfected cells. (G) tumor growth. Tumor size 35 and 45 d after implantation of HepG2 cells is presented as the mean volume (mm³) of tumors from 10 mice per group. Values represent the mean ± SEM. * p < 0.05, compared with the tumors formed by mock-transfected cells. (H) survival rate of tumor-bearing mice was shown by Kaplan–Meier survival curves. * p < 0.05, mock- vs. FPR shRNA-transfected cells.
produce agonist(s) recognized by FPR1 on live hepatic cancer cells. HepG2 cells and HepG2 tumors formed in athymic mice released potent chemotactic activity for live HepG2 cells (Fig. 7A and B) and ETFR cells overexpressing FPR1 (data not shown). The FPR1 agonist activity released by necrotic HepG2 cells and tumor tissues was blocked by an anti-FPR1 antibody or by the FPR1-specific antagonist tBoc-MLF (Fig. 7B, and data not shown). Necrotic hepatoma cell supernatant also induced a robust intracellular Ca\textsuperscript{2+} mobilization in live HepG2 cells (Fig. 7C) which attenuated HepG2 cell to response to subsequently administered fMLF (Fig. 7D). These results suggest that the agonist(s) contained in the supernatants of necrotic HepG2 cells shares FPR1 with fMLF on HepG2.\textsuperscript{30} We additionally observed that necrotic hepatoma supernatants inhibited the expression of FPR1 on the surface of ETFR cells by necrotic tumor cell supernatants. The level of FPR1 on the surface of ETFR cells, treated as indicated, was measured by flow cytometry with an anti-FPR1 antibody or normal IgG. Representative results from three independent experiments are shown as a percentage of FPR1-positive cells.

**Figure 7.** FPR1 agonist activity in necrotic tumor supernatants. (A) chemotactic activity. Supernatants of HepG2 cells after three cycles of freezing and thawing (necrotic) were assayed for chemotactic activity on live HepG2 cells. Supernatants from apoptotic or live HepG2 cells were used as controls. Percentage of cell migration in response to supernatants in total loaded cells was calculated. Data are expressed as the mean ± SEM (n = 3). *p < 0.05, compared with control medium. (B) chemotactic activity of supernatants of tumor tissue extracts. The supernatants from necrotic HepG2 tumor tissues were assessed for chemotactic activity on live HepG2 cells, which were pre-incubated in the presence or absence of the FPR1-specific antagonist tBoc-MLF at 37°C for 30 min. Values represent the mean ± SEM (n = 3). *p < 0.05, compared with untreated (-) cells. (C), Ca\textsuperscript{2+} flux induced in HepG2 cells by necrotic tumor cell supernatants or fMLF. (D) desensitization of fMLF-induced Ca\textsuperscript{2+} flux by necrotic tumor cell supernatants in HepG2 cells. Representative results from three independent experiments are shown. (E) inhibition of FPR1 expression on ETFR cells by necrotic tumor cell supernatants. The level of FPR1 on the surface of ETFR cells, treated as indicated, was measured by flow cytometry with an anti-FPR1 antibody or normal IgG. Representative results from three independent experiments are shown as a percentage of FPR1-positive cells.

**Discussion**

In this article, we showed that the classic leukocyte chemoattractant receptor FPR1 is expressed by human HCC tissues and hepatoma cell lines. To our knowledge, this is the first demonstration that FPR1 may contribute to the progression of HCC by mediating tumor cell chemotaxis, proliferation, and production of IL-8 in response to endogenous agonist(s). A growing body of evidence has suggested important roles of chemoattractant GPCRs in cancer initiation and progression by influencing aberrant cell growth and survival.\textsuperscript{31} GPCRs are also implicated in the invasion and metastasis of cancer cells and contribute to the establishment and maintenance of a permissive tumor microenvironment.\textsuperscript{31} The FPR1 involvement in cancer malignancy has been shown by its roles in glioblastoma tumorigenicity.\textsuperscript{7} FPR1 expressed by highly malignant human glioma cells promoted the motility, growth, and the production of
The glioblastoma-promoting activity of FPR1 is mediated in part by transactivation of the epidermal growth factor receptor (EGFR), associated with increased methylation of p53 gene. FPR1 in glioblastoma cells is stimulated by Annexin 1 released by necrotic tumor cells. Our present study extended the functional scope of FPR1 to its potential role in promoting the growth of malignant human hepatoma.

As a primary malignancy that emerges on a background of chronic liver diseases, HCC is the fifth most common cancer worldwide with an extremely poor prognosis. Chronic inflammation and improper wound healing following hepatic injury are crucial causative factors for cancer in the liver. The link between an inflammatory state and cancer can be viewed from an extrinsic perspective for which infection and subsequent chronic inflammation drive oncogenesis. In that case, cancers associated with inflammation are generally aggressive. Functional FPR1 has reportedly been detected in hepatocytes, and it promoted the production of acute phase proteins in response to fMLF. Thus, FPR1 expressed on hepatocytes, and it promoted the production of acute phase proteins in human hepatoma cells supports the adverse effect of this chemokine on hepatoma growth and angiogenesis. In vivo mitochondrial proteins from necrotic HepG2 cells stimulate FPR1 in monocyte to release IL-8. Thus, the current study provides evidence for IL-8 as a molecule down-stream of FPR1 signaling pathway to amplify the effect of FPR1 on hepatic tumor growth.

Materials and methods
Patients and specimens
Tumor samples were obtained from patients with pathologically confirmed HCC at the First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China. All samples were coded anonymously in accordance with local ethical guidelines (as stipulated by the Declaration of Helsinki), and written informed consent was obtained. The protocol was approved by
the Review Board of the First Affiliated Hospital, Sun Yat-sen University. Detailed information is described in Supplementary Materials and Methods.

Cells

Detailed information about cell lines including human hepatic cancer cells HepG2 and Hep3B, rat basophilic leukemia cells stably transfected with epitope-tagged FPR (ETFR), human umbilical cord vein endothelial cells (HUVECs), human peripheral blood mononuclear cells (PBMC) and monocytes is described in Supplementary Materials and Methods.

Chemotaxis and Ca²⁺ flux

Assays for tumor cell chemotaxis and Ca²⁺ mobilization were performed according to the described procedures. Details can be obtained from Supplementary Materials and Methods.

Tumorigenesis

To generate xenografts with hepatoma cells transfected with FPR1 shRNA, 1 × 10⁶ cells were injected into the flank of each 4-week-old female athymic Ncr-nu/nu mouse. Animal care was provided in accordance with the Guide for the Care and Use of Laboratory Animals. Detailed information about FPR1 knockdown in HepG2 cells and tumor implantation is described in Supplementary Materials and Methods.

Additional Materials and Methods

A full description of the methods including histology and immunofluorescence staining, RT-PCR, flow cytometry analysis, wound-healing assays, Western blotting, ELISA, formation of capillary-like structures, MTT assay, generation of tumor cell supernatant and tissue extracts, and statistical analyses can be found in Supplementary Materials and Methods.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgment

The authors thank Dr. Xiwu Bian (Institute of Pathology and Southwest Cancer Center, Southwest Hospital, Third Military Medical University, Chongqing, China) for his helpful critique of the manuscript.

Funding

This work is supported by the grants from National Nature Science Foundation of China 81200674 (QL) and 31170861 (SBS), Chen K and JM Wang were funded in part by Federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. HHSN261200800001E and were supported in part by the Intramural Research Program of the NCI, NIH, United States of America.

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