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ETS2 is involved in protein kinase C-activated expression of granulocyte–macrophage colony-stimulating factor in human non-small lung carcinoma cell line, A549

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Abstract

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a cytokine expressed in the non-small lung carcinoma cells (NSCLC). However, transcriptional regulation of GM-CSF is not well characterized in NSCLC. In this study we found that two cis-acting ETS family consensus sites are important for transcriptional regulation of GM-CSF in A549 human lung carcinoma cells. These two sites are located separately at around −40 and −100 bp from the transcription start site. Results of transient transfection assays with A549 cells indicated that ETS2 had a strong positive effect on GM-CSF promoter activity. Furthermore, this activity was enhanced by protein kinase C activator, phorbol 12-myristate 13-acetate (PMA), in an ETS consensus-dependent manner, while PMA could also enhance the expression level of ETS2. The protein kinase C inhibitors decreased GM-CSF promoter activity induced by the protein kinase C activator PMA. We also found that antisense ETS2 mRNA decreased PMA-induced GM-CSF promoter activity, supporting the possibility that ETS2 is involved in protein kinase C-induced GM-CSF transcriptional function. Endogenous expression of GM-CSF mRNA was increased by ETS2 transfection and the increased expression was further enhanced by PMA. These data indicate that GM-CSF is up-regulated by ETS2, a target of protein kinase C.

Keywords: ETS2; GM-CSF; Protein kinase C; Lung adenocarcinoma

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a cytokine, which stimulates the proliferation, differentiation, and function of myeloid progenitor cells. Several clinical trials of anticancer chemotherapy combined with recombinant human GM-CSF have been performed [1–3]. Whilst GM-CSF can lead to rapid neutrophil recovery, which may promote tumor progression [4]. Autocrine or paracrine mechanisms of action of GM-CSF have been reported in hematological and non-hematological malignancies [5–8]. The production of GM-CSF is reported to be involved in both the in vitro invasiveness and the local progression of squamous cell carcinoma of the lung [9]. It has also been reported that patients with squamous cell carcinoma co-expressing GM-CSF and GM-CSF receptor showed significantly poorer prognosis than those expressing neither GM-CSF nor GM-CSF receptor, suggesting that GM-CSF can have a stimulatory effect on some human non-small cell lung cancer (NSCLC) [10]. Moreover, GM-CSF is involved in mobilization of endothelial progenitor cells (EPCs), so GM-CSF is likely a target for inhibition of tumor angiogenesis [11]. However, transcriptional regulation of GM-CSF in NSCLC remains unclear.

Previous studies of GM-CSF transcription control in T-lymphocytes have identified a number of regulatory

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elements. A conserved lymphokine element 0 (CLE0) (−54 to −31) and an upstream nuclear factor kappa-B (NF-κB) site (−85 to −76) have been identified on the proximal functional promoter region (−620 to +34) [12]. Mutation in either of these sites resulted in a decrease in PMA/Ca^{2+}-induced GM-CSF-luciferase reporter activation. It has also been confirmed that AP-1, NF-ATp, and a higher order NF-ATp/AP-1 complex all bind to the human CLE0 element. In addition, an enhancer region located 3.3 kb upstream containing functional NF-AT/AP-1 binding sites appears to act in conjunction with the proximal promoter in response to T-cell receptor activation [13,14].

In Jurkat T cells, ETS1 has been shown to transactivate human GM-CSF promoter stimulated with PMA and ionomycin [15,16]. The ETS family of transcription factors is characterized by an 85-amino-acid ETS domain that recognizes a core sequence GGAA or TTCC and thereby specifically binds to DNA. The ETS family of transcription factors plays important roles in the development and function of multiple mammalian cell types, including hematopoietic cells [17]. The human ETS2 gene is a homologue of the v-ets oncogene of the E26 virus, codes for a 56-kDa nuclear protein that can be phosphorylated, is turned over rapidly, and responds to protein kinase C [18,19]. Although previous studies have demonstrated that PKC activation of MAPKerk1/2 in epithelial cells is both associated with and required for phorbol 12-myristate 13-acetate (PMA)-induced GM-CSF production [20], the responsible transcription factors involved in this pathway have not yet been reported. The goal of this study was to identify the transcription factor that regulates PMA-induced GM-CSF expression in NSCLC.

Materials and methods

Cell culture. A549 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO_2 and 95% air atmosphere.

Transfection. Transient transfections were performed with Transfectam (Promega) according to the manufacturer's recommendations. Specifically, for one well, 1 μl transfectam reagent and 0.5 μg GM-CSF promoter in pGL2-basic plasmid were incubated for 10 min before application to subconfluent cells in 24-well plates. Co-transfection of various plasmids was performed with a mixture of 3 μl transfectam and 0.5 μg GM-CSF promoter in pGL2-basic plasmid, and 1 μg ETS factor in expression plasmid pCB6. Empty vector (pCB6) was added to ensure constant DNA input. Co-transfection with 10 ng per sample of the pRL-CMV vector, which expresses *Renilla* luciferase (Promega), ensured that differences in firefly luciferase reporter gene expression were not due to differences in transfection efficiency. Cells were incubated for 2 h with the DNA mixture, after which additional medium was added. Forty-eight hours after transfection, the medium was removed and cells were harvested. Phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12,13-didecanonate (4α-PDD), 4β-phorbol 12,13-dibutyrate (PDBu), sphingosine, staurosporine, Go6983, Go6976, or Ro-31-8220 (Sigma) was added to samples as indicated in each figure. Luciferase activity was measured using a dual-luciferase reporter assay system (Promega) and a luminometer (Lumat LB9507, EG & G Berthold). Absolute light emission generated from the luciferase activity was plotted and represents the fold induction of activity generated by experimental treatments with respect to the activity associated with basic vector alone. Values are shown as means ± SE ($n = 4$).

Plasmid constructs. The GMP(−120 bp)WT promoter was prepared by PCR using GMP(−629) in pCR2.1 vector as a template. We used the following oligonucleotide primers: 5′-primer, CCGCTCGAGCTG ATAAGGGCCAGGAGATTCC and 3′-primer, M13 (reverse): CAG GAAACAGCTATGAC. The PCR product was cloned into the *Xho*I and *Hin*dIII sites of pGL2-basic (Promega), a promoter-less luciferase expression plasmid.

The GMP(−69)WT promoter was also prepared by PCR using GMP(−629) in PCR2.1 vector as a template. The following primers were used: 5′-primer, CCGCTCGAGGCATTTTGTGGTCACCATT AATC and 3′-primer, M13 (reverse). It was also cloned into *Xho*I and *Hin*dIII sites of pGL2-basic.

The GMP(−120)MUT is the construct GMP(−120)WT with a mutated ETS binding site. It was prepared using a Transformer site-directed mutagenesis kit (Clontech) and a mutation primer. It contains a mutated ETS site as indicated in boldface letters, GGCCAGGAG AT**AT**CA CAGTTCAGG.

The GMP(−69)MUT is the construct GMP(−69)WT with a mutated ETS site. It was prepared using a Transformer site-directed mutagenesis kit (Clontech) and mutation primer: GGTCACCATTA ATCATT**AT**CTCTG TGTATTTAAGAG.

GM-CSF promoter GMP(−629) was obtained by PCR using a Genome Walker Kit (Clontech). The following primers were used: 5′-primer, GM-(−629 to 601), TTCTCAGAGTGGTGCAGTCTCGCT GCTG and 3′-primer, GM-(+58 to +29), AAGAGCAGCAGGCTCT GCAGCCACATCCTC. The PCR product was cloned into pCR2.1 vector using the Original TA cloning kit (Invitrogen). After confirming the sequence, it was cloned into the *Xho*I and *Hin*dIII sites of pGL2-basic vector. Full-length human ETS1 (1325 bp), ETS2 (1410 bp, a gift from Dr. D.K. Watson), Elf1 (1870 bp, cloned by us), PEA3 (a gift from Dr. J.A. Hassel), and ESE1 (a gift from Dr. T.A. Libermann) were cloned into the *Kpn*I–*Xba*I site of pCB6 downstream of the cytomegalovirus promoter. All constructs were verified by DNA sequence.

Reverse transcription-PCR. Cells were grown to about 70% confluency and were transfected with ETS2 in pCB6 using transfectam. After incubation for 24 h, cells were stimulated with PMA (50 nM). Following another 24 h incubation, cells were harvested and total RNA was extracted using Isogen reagent (NIPPON GENE). Reverse transcription-PCR experiments were performed using a RNA PCR kit (Takara) according to the manufacturer's instructions: 30 °C for 10 min, 42 °C for 60 min, 99 °C for 5 min, and 5 °C for 5 min for reverse transcription; 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s for 35 cycle. The following primers were used: for GM-CSF, 5′-primer, hGM-CSF-up, GGAGCATGTGAATGCCATC, and 3′-primer, hGM-CSF-down, GGATGACAAGCAGAAAGTC; and for glyceraldehyde-3-phosphate dehydrogenase, 5′-primer, GAPDH-up, CGG GAAGCTT GTGATCAATGG, and 3′-primer, GAPDH-down, GGCAGTGATGGCATGGACTG.

Western blot analysis. Whole cell lysates from A549 cells transfected with plasmid DNA or treated by PMA (50 nM) were subjected to SDS–PAGE using 10% gel and then the proteins were transferred to PVDF membrane. After blocking with 10% skim milk at 4 °C overnight, membranes were incubated with affinity-purified rabbit antisera to ETS2 (1/1000; Santa Cruz Biotechnology) at 4 °C for 2 h. The membranes were subsequently incubated with HRP-conjugated goat anti-rabbit IgG (1/10,000; Seikagaku Corporation) at room temperature for 1 h. Finally chemiluminescence was detected using the ECL kit (Amersham Life Science).

Results

PMA induces GM-CSF promoter activity in an ETS-binding site-dependent manner

Fig. 1A shows the constructs of GM-CSF promoter used in this study. We focused on 120 bp proximal GM-CSF promoter, because GMP(−120)WT possessed the strongest promoter activity compared to other longer promoter constructs (data not shown). We found one ETS consensus site at 40 bp and a second at 100 bp in the 120 bp GM-CSF promoter. The ETS consensus site was mutated from 5′-TTCC-3′ to 5′-TATC-3′ for further experiments. Promoter activity of GMP(−120)WT is about two times higher than that of GMP(−69)WT. These activities were reduced by the mutation of ETS consensus site. Especially, GMP(−69)MUT, which had no ETS binding site, showed almost no promoter activity. PMA (50 nM), a PKC activator, prominently enhanced promoter activities of GMP(−69)WT and GMP(−120)WT. These activities were also reduced by the mutation of ETS consensus sites (Fig. 1A). Because GMP(−120)WT showed the highest activity, we used it to investigate the effects of the other PKC activator and inhibitors. As shown in Fig. 1B, PDBu (50 nM), a protein PKC activator, also increased GMP(−120)WT activity to about the same extent as PMA, but 4α-PDD (50 nM), an inactive PMA analogue, did not. These data further indicate that PKC is involved in transcriptional activation of GMP(−120)WT. To confirm this conclusion, the effects of PKC inhibitors were examined. Here we used several PKC inhibitors: (1) PKC specific in-

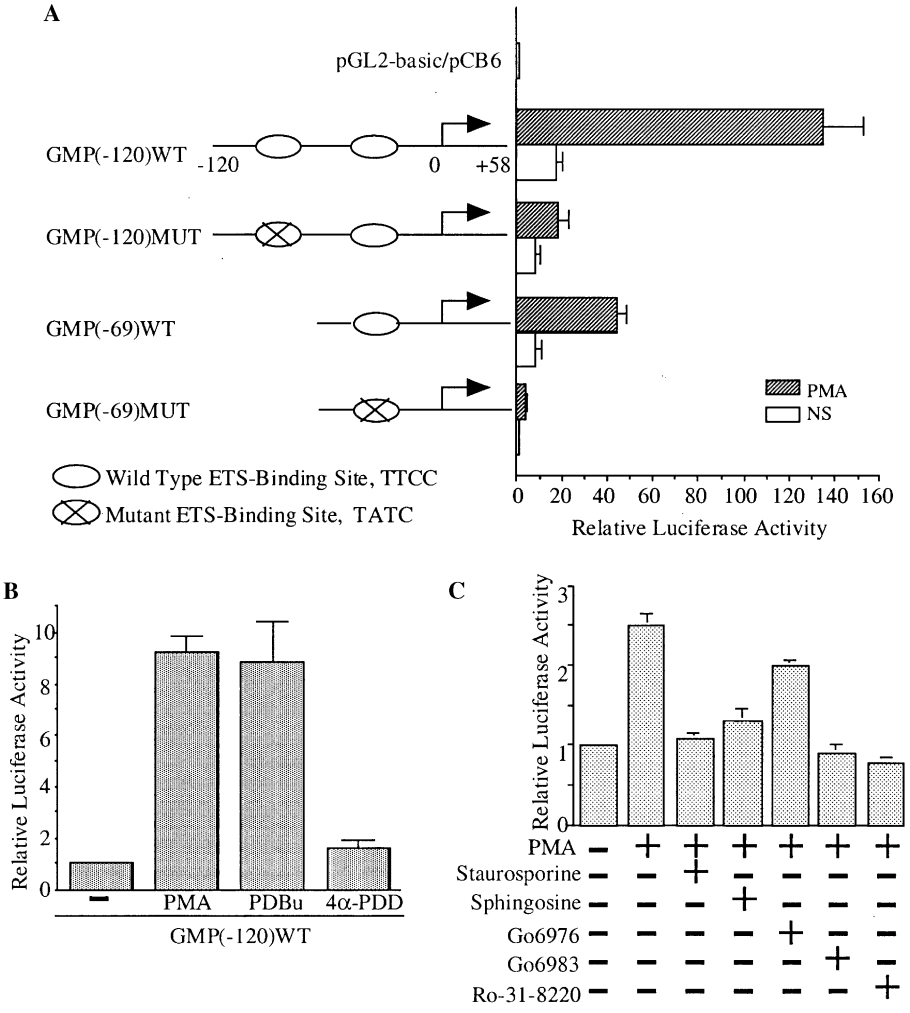

Fig. 1. Transcriptional activity of human GM-CSF promoter in A549 cells. (A) Structures of promoters with different ETS consensus elements are indicated in the left side. Twenty-four hours after transient transfection, A549 cells were cultured with PMA (50 nM) or NS (no stimulation) as indicated for about 20 h and then cells were harvested for luciferase assay. (B) Twenty-four hours after transient transfection with GMP(−120)WT, A549 cells were cultured with 50 nM PMA or PDBu or 4α-PDD as indicated for about 20 h and then cells were harvested for luciferase assay. (C) Forty hours after transient transfection with GMP(−120)WT, staurosporine (0.5 μM), sphingosine (2.5 μM), Go6976 (1 mM), Go6983 (1 mM), or Ro-31-8220 (1 mM) was added to cells and then 20 min later, PMA (50 nM) was added. Eight hours later, cells were harvested for luciferase assay. In (B) and (C), the fold activity of GMP(−120)WT in the absence of any stimulation was presented as 1.

hibitor, sphingosine; (2) different PKC isozyme inhibitors, staurosporine (inhibitor to PKC-$\alpha, \beta I, \beta II, \gamma, \delta$, and ε), Go6983 (inhibitor to PKC-$\alpha, \beta, \gamma, \delta$, and ξ), Go6976 (inhibitor to PKC-$\alpha, \beta I$, and μ), and Ro-31-8220 (inhibitor to PKC-$\alpha, \beta I, \beta II, \gamma$, and ε). Except for Go6976, all the other inhibitors strongly blocked PMA-induced GMP(−120)WT transactivity (Fig. 1C). Go6976 showed a weak inhibitory effect.

ETS2 is involved in GM-CSF promoter transactivation

To determine what ETS family factor(s) activate the promoter activity in A549, we screened the following ETS family factors, ETS1, ETS2, PEA3, ELF1, ESE1, and MEF in pCB6. Interestingly, only ETS2 obviously activated GMP(−120)WT (Fig. 2). ETS2 was also able to transactivate GMP(−120)WT in Hela cells (data not shown).

Further, the activation by ETS2 was obviously enhanced by PMA treatment (Fig. 3A). Both the individual and combined effects of ETS2 and PMA were ETS binding-site-dependent. These data indicated the possibility that ETS2 transactivated GM-CSF promoter through the PKC pathway. Further, the involvement of ETS2 was confirmed by the introduction of antisense mRNA, which blocked the promoter activity (Fig. 3B). Then we examined the protein level change of ETS2 after plasmid DNA transfection and PMA treatment (Fig. 3C). The results proved that transfection of ETS2 and antisense ETS2 plasmids into A549 cells indeed induced the protein expression change. The upper protein (54 kDa) is supposed to be the full-length isoform of ETS2 and the lower one (52 kDa) may be a phosphorylated form of ETS2 [21]. PMA treatment enhanced the expression levels of both, which is consistent with previous report that PKC enhances the stability of ETS2.

Additionally, in response to the introduction of ETS2 into cells, the level of endogenous GM-CSF mRNA increased and a further increase was seen upon addition of PMA (Fig. 4). The expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as a control, was not changed by any treatment.

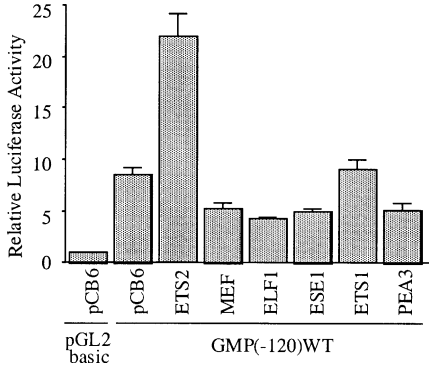

Fig. 2. Effects of several ETS transcription factors on GM-CSF promoter activity. A549 cells were transiently co-tranfected with the indicated ETS transcription factor described in "Materials and methods" and GMP(−120)WT. Forty-eight hours after transfection, cells were harvested for luciferase assay.

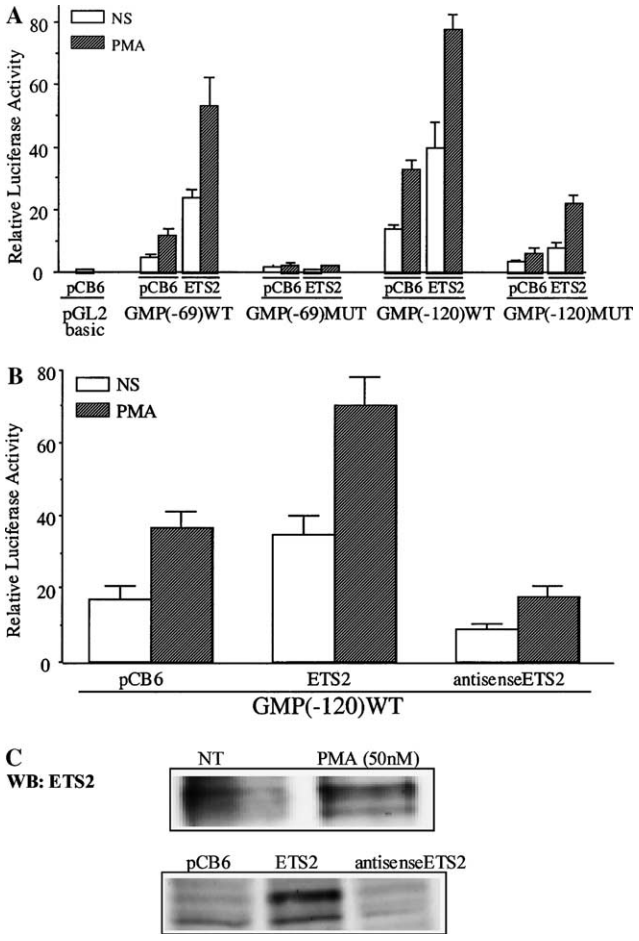

Fig. 3. PMA and ETS2 activate GM-CSF promoter activity. (A) A549 cells were transiently co-transfected with ETS2-expressing vectors and GM-CSF promoters. Twenty-four hours after transfection, cells were stimulated with PMA (50 nM) for about 20 h and then harvested for luciferase assay. (B) Effect of antisense mRNA of ETS2. A549 cells were transiently transfected with sense or antisense ETS2 in pCB6 as indicated in the figure. Twenty-four hours after transfection, cells were stimulated with PMA (50 nM) for about 20 h and then harvested for luciferase assay. (C) Forty-eight hours after DNA transfection or 20 h after treatment with PMA (50 nM), whole cell lysates were harvested for Western blotting.

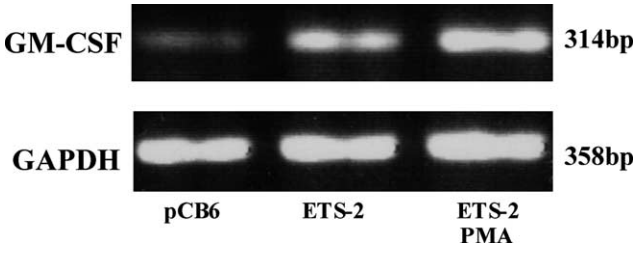

Fig. 4. PMA and ETS2 increase GM-CSF mRNA expression in A549. Reverse transcription-PCR for GM-CSF and GAPDH transcripts was performed as described in "Materials and methods."

Discussion

The production of GM-CSF by squamous cell carcinoma cell lines is closely related to the in vitro invasiveness and MMP activity of the cancer cells [9]. In addition, recombinant GM-CSF stimulates the invasiveness of less invasive LK-2 and LC-1 cells, and this stimulation is abrogated by the neutralizing anti-GM-CSF antibody. Furthermore, anti-GM-CSF antibody decreases the invasiveness of highly invasive EBC-1 and NCI-H157 cells. GM-CSF also increases the MMP activity of LK-2 and LC-1 cells [2,9]. Considering the fact that GM-CSF is actively involved in cancer progression as proven by previous studies, it is important to know how the GM-CSF expression is regulated at transcriptional levels. So, we investigated how the GM-CSF expression is controlled in A549 cells.

Our study indicates that ETS2 and PMA up-regulated not only the activity of a transiently transfected GM-CSF promoter, but also the transcription of the endogenous GM-CSF gene in A549 cells. It seems that ETS2 and PMA activated GM-CSF promoter through the two ETS-binding sites. Moreover, antisense experiments showed that inhibition of endogenous ETS2 in A549 cells attenuated not only the base-line level of promoter activity, but also the activity activated by PMA. Taken together, these results indicate that ETS2 is a crucial transcription factor, which regulates GM-CSF expression in A549 cells. Compared to the proximal GM-CSF promoter (−629 to +58), and to other truncated mutant promoters with different lengths and ETS consensus sites, GMP(−120)WT produced the strongest transactivation activity (data not shown). Therefore, in this study, we focused on two proximal ETS sites on the promoter. It has also been reported that neither the upstream enhancer region nor other regions outside the proximal promoter are involved in the activation of GM-CSF transcription in epithelial cells [22]. However, the possibility still exists that some other elements on this promoter, such as the NF-κB binding region, can respond to PMA stimulation and account for the total observed activity of PMA-induced GMP(−120)WT in A549 cells. In T cells, both the proximal (−85 to −76) and distal (−2002 to −1984) NF-κB binding sites have been reported to be involved in PMA induction of the GM-CSF promoter [23]. In Jurkat T cells, ETS1 has been shown to transactivate human GM-CSF promoter stimulated with PMA and ionomycin [15,16]. ETS1 and ETS2 interact with the same DNA-recognition sites and may function antagonistically, either activating or repressing transcription of the same target gene, depending on promoter context, cell-type, or stage of differentiation [24,25]. Cell-specific regulation of GM-CSF by different transcription factors may be an interesting subject for future studies. ETS1 and ETS2 have a mitogenic and transforming activity when over-expressed in NIH3T3 cells [26]. It was also reported that the oncogene PyMT-positive mice having heterozygous ETS2 allele developed smaller size tumor than those having two wild type ETS2 alleles [27]. Therefore, our studies indicate that ETS2 is a key transcription factor in the protein kinase C pathway that transactivates the GM-CSF promoter related to tumor progression in A549 cells.

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