Induction of Apoptosis in Human Esophageal Cancer Cells by Sequential Transfer of the Wild-Type p53 and E2F-1 Genes: Involvement of p53 Accumulation via ARF-mediated MDM2 Down-Regulation

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ABSTRACT

Transcriptional factor E2F-1 as well as tumor suppressor p53 have been shown to cause apoptosis independently in some types of human cancer cells when overexpressed. Here we report that sequential transfer of the wild-type p53 and E2F-1 genes efficiently induces apoptosis in human esophageal cancer cells and that E2F-1 overexpression directly activates expression of p14 (ARF), which inhibits MDM2-mediated p53 degradation, resulting in the stabilization of p53. Infection of human esophageal cancer cell lines T.Tn and TE8 with adenovirus vector-expressing E2F-1 (Ad-E2F-1) enhanced mRNA and protein expression of ARF and decreased MDM2 protein expression. Transfection of ARF plasmid decreased MDM2 protein expression, which in turn increased p53 protein expression. Infection of T.Tn and TE8 cells first with adenovirus-expressing wild-type p53 (Ad-p53) and then with Ad-E2F-1 resulted in rapid induction of apoptosis; in contrast, simultaneous infection with Ad-E2F-1 and Ad-p53 had no significant antitumor effect. As shown by Western blot analysis, infection with suboptimal concentrations of Ad-E2F-1 induced the accumulation of exogenous p53 transduced by suboptimal concentrations of Ad-p53. Moreover, Ad-E2F-1-mediated ARF expression inhibited the up-regulation of MDM2 by overexpressed p53 in TE8 cells. Thus, overexpression of ectopic E2F-1 protein may stabilize endogenous as well as ectopic p53 protein via the E2F-1/ARF/MDM2/p53 regulatory pathway and, in this way, render cells more sensitive to apoptosis, an outcome that has important implications for the treatment of human esophageal cancers.

INTRODUCTION

The E2F transcriptional factor family was first identified as a group of proteins with E1A-inducible cellular activity (1) and was later shown to transactivate their target genes by phosphorylating retinoblastoma protein (2). Expression of the E2F-1 product, which is the best characterized member of the E2F family, can promote cell-cycle progression through the G1 checkpoint by activating a series of genes that are critical for the G1 to S phase transition (3, 4). In addition, overexpression of E2F-1 can lead to transformation of an established rat embryo fibroblast cell line (5) or, in cooperation with an activated ras oncogene, to oncogenic transformation of rat embryo cells (6). These observations suggest that the E2F-1 gene is oncogenic; other evidence (i.e., E2F-1 knockout mice develop spontaneous tumors in several tissues) suggests that E2F-1 is also a tumor suppressor (7). Moreover, because the induction of S phase by E2F-1 is also accompanied by the characteristic effects of programmed cell death (8), it may also play a role not only in stimulating cell proliferation but also in coordinating apoptosis.

Overexpression of E2F-1 can activate CPP32, one of the most important apoptosis-inducing molecules, and can cause cleavage of the death substrate poly(ADP-ribose) polymerase, which suggests that activation of the caspase cascade may be a pivotal mechanism in E2F-1-mediated apoptosis (9). Moreover, this pathway seems separate from the p53-mediated pathway, because E2F-1 dose not increase the expression level of bax protein (a putative p53-mediated apoptotic pathway; Ref. 9). However, in light of our previous finding that overexpression of the wt-p533 gene by recombinant, replication-deficient viral
vectors induces apoptosis in a variety of human cancer cells of differing p53 status (10–14), we have hypothesized that the combined transfer of wt-p53 and E2F-1 genes into human cancer cells could be therapeutically advantageous because two different but major apoptotic pathways would be activated.

Recent studies have identified physical interactions between E2F-1 and ARF (human p14ARF and mouse p19ARF; Refs. 15, 16), which shares a portion of the p16INK4a coding region and has a unique first exon (termed exon 1β) originating approximately 20 kb centromeric to p16INK4a exon 1 (termed exon 1α; Ref. 17). Potential E2F-1 binding sites have also been detected in the sequence of the ARF promoter region (18). A proto-oncogene MDM2, which can be induced by wt-p53 activity, binds to p53 and masks the p53 transcriptional activation domain (19). MDM2 targets p53 protein for degradation in the ubiquitin pathway, which results in abrogation of its antiproliferative and apoptosis-promoting effects (20). In addition, it has been shown that ARF binds to and induces the degradation of the proto-oncogene MDM2, which results in stabilization of p53 (21). Furthermore, cell-cycle arrest mediated by ARF can be abolished in cells lacking functional p53 (22, 23), which indicates that ARF may act upstream of p53.

In the present study, we demonstrated that: (a) E2F-1 gene transfer can down-regulate MDM2 protein levels through activation of ARF and thereby enhance apoptosis induced by exogenously transfected wt-p53; and (b) sequential transduction of p53 and E2F-1 promote apoptosis more efficiently than does simultaneous introduction of these genes. Together, our data indicate that this combination strategy may be useful in treating human squamous cell carcinoma of the esophagus.

MATERIALS AND METHODS

Cell Lines and Tissue Culture. The human esophageal cancer cell line T.Tn, which has a point mutation at codon 272 in p53 (mut-p53), was obtained from JCRB (Japanese Collection Research Bioresources, Japan) and maintained in DMEM/F-12 supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The human esophageal cancer cell line TE8, which contains wt-p53, was kindly provided by Dr. Nishihira (Tohoku University, Sendai, Japan) and maintained in RPMI 1640 supplemented with 10% FCS, 25 mM HEPES, and penicillin/streptomycin. A p53-expressing subline of HeLa cells, HeLa-S3, was maintained in DMEM supplemented with 10% FCS and penicillin/streptomycin.

Recombinant Adenoviruses. A recombinant, replication-deficient adenovirus vector that was capable of expressing either human p53 (24) or E2F-1 (25) was previously constructed and characterized. The resultant viruses were termed Ad5CMVp53 (Ad-p53; Refs. 10–14) and Ad5CMVE2F-1 (Ad-E2F-1; Ref. 25) and have been described therein. An E1A-deleted adenovirus vector lacking a cDNA insert (dl312) was used as a control vector.

RT-PCR. Total RNA was isolated using RNAZOL (Cinna/Bio Tecx, Friendswood, TX) in a single-step phenol-extraction method. One μg of total RNA was subjected to RT using an oligodeoxypentidylylamine primer and avian myeloblastosis virus polymerase (Promega) in a 40-μl reaction volume for 10 min at 22°C and then for 20 min at 42°C. Then, by using a PCR kit according to the manufacturer’s protocol (Perkin-Elmer Corp., Norwalk, CT), 2 μl of the RT reaction product was amplified by PCR in total volumes of 50 μl using the forward and reverse primer for exon 1β human p19ARF gene (p14ARF). This yielded a 188-bp fragment (26) that was run on a 1% agarose gel and visualized by ethidium bromide staining. The specific primer sequences for the exon 1β human ARF gene were as follows: forward primer (sense), 5′-TACTGAGGAGC-CAGGCCGT-CTA-3′; reverse primer (antisense), 5′-AGCAC-CACCACGGGTTC-3′. The amplification reaction involved 25 or 35 cycles of denaturation at 95°C for 1 min and annealing at 60°C for 1 min and at 72°C for 15 s in a Perkin-Elmer thermal cycler (Perkin-Elmer Corp., Foster City, CA). As a control, human β-actin was amplified as described previously (13).

Western Blot Analysis. For Western blot analysis, attached cells collected by trypsinization and cells floating in the culture medium were combined in a 10-ml conical tube and were washed twice in cold PBS. Cells were then lysed in SDS solubilization buffer [0.5 M Tris (pH 6.8), 10% SDS, and glycerc]. Equal amounts of proteins were boiled for 5 min and electrophoresed under reducing conditions on a 12.5% (w/v) polyacrylamide gel. Proteins were then electroblottransferred to a Hybond-polyvinylidene difluoride (PVDF) transfer membrane (Amersham, Arlington Heights, IL) and were incubated with primary antibodies against E2F-1 (KH95; Santa Cruz Biotechnology, Santa Cruz, CA), p53 (Ab-2; Oncogene Science, Manhasset, NY), MDM2 (SMP14; Santa Cruz Biotechnology), and ARF (C-18; Santa Cruz Biotechnology) and with mouse antihuman actin monoclonal antibody (Amersham). An Amersham enhanced chemiluminescence chemiluminescent system was used to detect secondary probes.

Flow Cytometric Analysis. For flow cytometric analysis, cells were collected, washed twice with cold PBS, and resuspended in PBS containing 0.1% Triton X-100 and 1 g/liter RNase for 5 min at room temperature. Samples were then stained with propidium iodide at 50 μg/ml and analyzed for DNA content in a cell sorter (FACScan; Becton Dickinson, Mountain View, CA).

Visualization of Apoptotic Nuclei. To visualize apoptotic nuclei, T.Tn and TE8 cells grown on coverslips were fixed with 1.0% glutaraldehyde and stained with DNA intercalating dye Hoechst 33342 (1 μM in PBS). Fluorescence was visualized using a fluorescence microscope (Zeiss, Oberkochen, Germany).

Plasmid Transfection. Cell transfections were carried out using the LIPOFECT ACE reagent according to the manufacturer’s instructions (Life Technologies, Inc.). Cells were plated on 6-well plates at a density of approximately 104 cells/well and allowed to grow to 70% confluence (24 h after seeding), at which time they were transfected using LIPOFECT ACE in a total volume of 1 ml OPTI-MENI (Life Technologies, Inc.). The cells were transiently or stably transfected with various amounts of ARF cDNA (pcDNA3-Myc-ARF; 21) as indicated in the figure legend for each experiment. After a 24-h incubation with the DNA/lipid mixture, the cells were replenished with growth media. Stably transfected T.Tn (T.Tn/ARF) and TE8 (TE8/ARF) cells were selected in medium supplemented with...
RESULTS

E2F-1 Overexpression Induces Endogenous ARF Expression and Promotes MDM2 Degradation. To examine whether overexpression of E2F-1 protein specifically affects the expression of other related genes, we first analyzed ARF expression by semiquantitative RT-PCR and Western blotting. Human esophageal cancer cell lines T.Tn, expressing mut-p53, and TE8, which contains wt-p53, were transduced with the E2F-1 gene by exposure to various concentrations of Ad-E2F-1. As revealed by the RT-PCR assay, ARF mRNA transcript expression was basal in parental T.Tn cells and very low in parental TE8 cells. However, when both cell lines were infected with Ad-E2F-1 at a MOI of 100, ARF mRNA expression was enhanced (Fig. 1). HeLa-S3 cells, which constitutively express ARF (27) as well as stabilized p53 (28), were used as a positive control for ARF mRNA detection. The amplified reactions were run for either 25 cycles (T.Tn cells) or 35 cycles (TE8 cells).

To determine whether E2F-1-mediated induction of ARF directly suppresses MDM2 expression, we transiently transfected T.Tn cells with a plasmid vector expressing human ARF cDNA. As shown in Fig. 2A, ectopically expressed ARF alone led to a dose-dependent reduction of the levels of MDM2 in T.Tn cells. We next asked whether ARF-promoted MDM2 degradation results in p53 stabilization and accumulation. In TE8 cells that were stably transfected with a plasmid that expresses ARF, there was a marked accumulation of endogenous wt-p53 (Fig. 2B). In contrast, the levels of p53 slightly increased in ARF-transfected T.Tn cells presumably because the T.Tn cells express mut-p53. These results suggest that overexpression of E2F-1 induced endogenous ARF expression, which in turn promoted MDM2 degradation, thereby leading to the stabilization of p53.

Effect of E2F-1 and p53 Overexpression on T.Tn and TE8 Cell Proliferation. A cell proliferation assay was performed to evaluate the effect of Ad-E2F-1 or Ad-p53 infection on T.Tn and TE8 cell growth in vitro. Cells were infected with either Ad-E2F-1 or Ad-p53 at various MOI; cells mock-infected
with culture medium were used as a control. Cells that remained attached to the culture plates as a monolayer were assessed for viability by measuring their trypan blue uptake. Infection with Ad-E2F-1 and Ad-p53 significantly suppressed the growth of both T.Tn and TE8 cells in a dose-dependent manner, although TE8 cells were more sensitive to E2F-1 and wt-p53 gene transfer when compared with T.Tn cells (Fig. 3). Control dl312 infection at 100 MOI had no effect on the growth of T.Tn and TE8 cells (data not shown).

Using sequential viral vector infection, we next examined the ability of transduced E2F-1 genes to cooperate with transduced ectopic wt-p53 genes in T.Tn and TE8 cells. T.Tn cells were infected with Ad-E2F-1 at a MOI of 20 or Ad-p53 at a MOI of 10, further exposed to Ad-p53 (10 MOI) or Ad-E2F-1 (20 MOI) 24 h after the first infection, and then assessed for cell viability 72 h after the second infection. TE8 cells were doubly infected with Ad-E2F-1 (20 MOI) and Ad-p53 (10 MOI). These MOI of Ad-E2F-1 and Ad-p53 were considered suboptimal, as determined by cell growth assays, and neither dose by itself could induce death of the entire cell population.

Infection first with Ad-E2F-1 and then with Ad-p53 had no effect on the growth of T.Tn and TE8 cells, whereas the reverse caused significant loss of viability in both cell lines (Fig. 4A, left panel, and data not shown). In addition, because TE-8 cells are more sensitive than T.Tn cells to this combination therapy, lower MOI (5 MOI) of Ad-p53 plus Ad-E2F-1 showed a significant antitumor effect (Fig. 4A, right panel). As revealed by phase-contrast photomicrographs, T.Tn cells infected with Ad-p53/Ad-E2F-1 suffered a rapid loss of viability attributable to massive cell death, as evidenced by floating, highly light-refractile cells; Ad-E2F-1/Ad-p53-infected cells suffered no such fate (Fig. 4B). Simultaneous Ad-p53 and Ad-E2F-1 infection as well as infection with either vector alone induced no growth inhibition. The total MOI used for Ad-p53 or Ad-E2F-1 infection alone was adjusted to 30 MOI with dl312 infection. To obtain more details about the cell death observed in T.Tn cells that were infected first with Ad-p53 and then with Ad-E2F-1, cells were stained with the DNA-intercalating dye Hoechst 33342 and then were examined by fluorescence microscopy. As this analysis showed, Ad-p53/Ad-E2F-1-infected cells underwent apoptosis characterized by the extreme condensation and partial fragmentation of nuclei (Fig. 4B).

To confirm the induction of apoptosis of TE8 cells by sequential infection, cells were harvested 72 h after the Ad-E2F-1 infection and then were assayed for DNA content by flow cytometric analysis. Ad-p53/Ad-E2F-1-infected TE8 cells exhibited a less-than-expected DNA content in the diploid G0-G1 peak, indicating apoptosis-specific nuclear fragmentation; Ad-E2F-1/Ad-p53 infection, however, did not enhance apoptosis (Fig. 4C).

**Blockage of MDM2 Up-Regulation and Induction of p53 Accumulation by Sequential Transfer of wt-p53 and E2F-1 Genes.** To further study the mechanism of apoptosis induced by sequential infection with Ad-p53 and Ad-E2F-1, we determined the expression levels of various regulatory proteins after transfer of p53 and/or E2F-1 genes. We used higher MOI of Ad-p53 and Ad-E2F-1 for these experiments, because detection of suboptimal p53 and E2F-1 proteins was difficult, presumably because of the low sensitivity of the assay. When T.Tn and TE8 cells were infected with Ad-p53 at 50 MOI, a substantial increase in the level of exogenous p53 was observed; however, when the cells were coinfected instead with Ad-p53 and Ad-E2F-1 or sequentially infected with Ad-E2F-1 first and Ad-p53 second, a decrease in the level of exogenous p53 gene product was seen. In contrast, the level of p53 was clearly enhanced by sequential infection with Ad-p53 first and Ad-E2F-1 second (Fig. 5A and B), a result presumably attributable to a dramatic reduction in Ad-p53-induced MDM2 protein expression (Fig. 5A).

Finally, to explore further the dependence of rapid MDM2 degradation on E2F-1, TE8 cells were infected first with Ad-p53 at 50 MOI and then with Ad-E2F-1 at various MOI. As shown in Fig. 5C, ectopic overexpression of E2F-1 activated endogenous ARF expression in a dose-dependent manner, resulting in markedly decreased expression of MDM2, which was up-regulated by the feedback loop of p53.

**DISCUSSION**

The tumor suppressor protein p53 functions as a transcriptional factor that regulates cell-cycle arrest and apoptosis in normal as well as in cancer cells (29). Transfer of the wt-p53 gene by an adenovirus vector has proved effective in inducing apoptosis in some types of human cancer cells (10–13), and its therapeutic efficacy is being assessed in Phase I/II clinical trials in human non-small cell lung cancer (30) and SCCHN (31).
Adenovirus vectors made replication-defective have been used successfully for efficient gene transfer into various types of human tissues. However, because high doses of adenovirus alone may also affect the normal cells surrounding the tumor, the amount of the vector must be minimized. Thus, strategies designed to effectively induce apoptosis in cancer cells may be critical to improving the therapeutic potential of $p53$ gene therapy. In the present study, we demonstrated that sequential transfer of the wt-$p53$ gene first and the $E2F-1$ gene second effectively caused the apoptotic death of human esophageal cancer cells.

Normally, steady-state levels of $p53$ protein are regulated by complexing the proteins with MDM2, which in turn promotes the rapid degradation of $p53$. Thus, the cellular concentration of $p53$ protein is kept low by MDM2, even in the absence of genotoxic stress. Interestingly, the $MDM2$ gene, a proto-oncogene that is amplified in 30–40% of human sarcomas, is known to be a transcriptional target of $p53$, and the stress-induced up-regulation of endogenous $p53$ protein is known to enhance $MDM2$ protein expression through a feedback regulatory loop (32). Consistent with these observations, we observed that adenovirus-mediated overexpression of exogenous wt-$p53$ induced $MDM2$ protein accumulation (Fig. 5, A and C), which in turn may have inhibited the ability of $p53$ to induce apoptosis.

**Fig. 3** Effects of Ad-E2F-1 and Ad-p53 on the growth of T.Tn (A) and TE8 (B) human esophageal cancer cells. Cells were plated in triplicate in 24-well culture plates and infected with either Ad-E2F-1 or Ad-p53 at indicated MOI. Mock-infected cells were used as a control. Cell viability was determined by trypan blue staining on a daily basis. Each point, the mean ± SD of triplicate experiments.
Induction of apoptosis by sequential infection with Ad-p53 first and Ad-E2F-1 second. In A, T.Tn and TE8 cells were cultured as monolayers in triplicate in 24-well or 6-well culture plates and then infected with Ad-E2F-1 (20 MOI for T.Tn, 5 MOI for TE8) or Ad-p53 (10 MOI for T.Tn, 5 MOI for TE8); they were further exposed to Ad-p53 (10 MOI for T.Tn, 5 MOI for TE8) or Ad-E2F-1 (20 MOI for T.Tn, 5 MOI for TE8) 24 h after the first infection and were finally assessed for cell viability. ■, mock-infected; □, Ad-E2F-1/Ad-p53-infected; □, Ad-p53/Ad-E2F-1-infected. Results were expressed as the mean ± SD of triplicate experiments. Student’s t test was used to compare viability between the treatment groups.*, statistical significance (P < 0.05). B, phase-contrast photomicrographs of T.Tn cells infected with indicated vectors (Ad-E2F-1, 20 MOI; Ad-p53, 10 MOI). Cell morphology was evaluated 48 h after the second infection. Bottom row, right, T.Tn cells infected first with Ad-p53 at 10 MOI and then with Ad-E2F-1 at 20 MOI were stained with Hoechst 33342 and analyzed under a fluorescence microscope. C, TE8 cells exposed first to Ad-p53 at 10 MOI and then to Ad-E2F-1 at 20 MOI were harvested at 72 h posttreatment and then were assayed for DNA content by propidium iodine staining and subsequent flow cytometric analysis. Induction of apoptosis in these cells and cells infected with the vectors in the reverse order was then compared.
Indeed, it has been reported that p53 degradation is accelerated when MDM2 cDNA is transfected into human cells (33). Together, these results suggest that disruption of the feedback regulatory loop between p53 and MDM2 may enhance the cytocidal effect of wt-p53 gene transfer.

In seeking a strategy to specifically inhibit p53-mediated MDM2 up-regulation, we focused on ARF, a protein that can interact directly with the MDM2 protein (21). ARF reportedly inhibits the ubiquitin ligase activity of MDM2 (33) and promotes MDM2 degradation (21). Although the biochemical mechanism underlying ARF-mediated MDM2 degradation remains unclear, our present data show that transfection of human
esophageal cancer cells with a plasmid designed to express an entire sequence of human ARF reduced the levels of constitutive MDM2 expression (Fig. 2A) and induced p53 accumulation (Fig. 2B). The mutant form of p53 in these cells was also stabilized by ectopic ARF expression, consistent with a previous report that a mutation of p53 did not affect the association with MDM2 (34); mut-p53, however, could not activate the transcription, which indicated that ARF function is dependent on the status of the endogenous p53. Recently, it was shown that the ARF promoter is highly responsive to E2F-1 overexpression and that E2F-1 directly activates expression of the human ARF in a p53-null human osteosarcoma cell line (16), which is similar to our finding in human esophageal cancer cells infected with Ad-E2F-1 of both the mRNA and protein levels (Fig. 1, A and B). In addition, we showed the existence of an intact autoregulatory loop between ARF, MDM2, and p53 in those same cells (Fig. 1B).

Together, these results suggest that the possible interaction of p53 and E2F-1 may enhance growth inhibition and/or apoptosis induction, although our finding that simultaneous infection with suboptimal concentrations of Ad-p53 and Ad-E2F-1 could not lead to cell death (Fig. 4B), suggesting that this effect is not so unambiguous. In fact, our finding is consistent with the finding by Frank et al. that coexpression of wt-p53 and E2F-1 did not enhance the growth-inhibitory effect in human SCCHN (35). It is likely that the physical interaction of the p53 and E2F-1 proteins suppresses the antitumor effect of coinfection of Ad-p53 and Ad-E2F-1 in SCCHN. In addition, as our data suggest, sequential infection with Ad-E2F-1 followed by Ad-p53 infection apparently induces no apoptotic cell death in human esophageal cancer cells (Fig. 4B), whereas suboptimal Ad-E2F-1 infection 24 h after suboptimal Ad-p53 infection induces an accumulation of p53 protein (Fig. 5, A and B), and the subsequent rapid induction of apoptotic cell death (Fig. 4). Thus, our data clearly suggest that the order of gene transfer may be important for mediating p53 accumulation as well as for causing apoptosis in human esophageal cancer cell lines.

Although the mechanisms by which Ad-p53/Ad-E2F-1 infection selectively induces apoptosis are not completely understood, the possible existence of a regulatory feedback cycle controlling the level of each protein has important implications. Although Ad-E2F-1 infection promotes endogenous ARF up-regulation, the amount of constitutive MDM2 is very low in the absence of exogenous wt-p53; thus, the level of ARF may be rapidly suppressed through the feedback mechanism. In contrast, when Ad-p53 infection precedes Ad-E2F-1 infection, Ad-p53 activates the transcription of MDM2, which leads to the enhanced synthesis of MDM2 protein, which might in turn be effectively degraded by ARF protein induced by the later Ad-E2F-1 infection. Alternatively, the localization of each protein in the subnuclear compartments may be essential for the responses (36). When Ad-E2F-1 infection precedes Ad-p53 infection, Ad-E2F-1 can induce expression of ARF, which may in turn sequester constitutive MDM2 in the nucleolus; however, the increased amounts of MDM2 resulting from the Ad-p53 infection may be sequestered in the nucleolus because of the localization of antagonistic ARF in the nucleolus, thereby preventing the transactivation in the nucleolus. But as our results show that this is not the case. When Ad-p53-infected cells were subsequently transduced with Ad-E2F-1, up-regulated nucleoplasmic MDM2 protein could be moved to the nucleolus by E2F-1-induced ARF, thus allowing the activation of p53 in the nucleolus. The precise mechanisms that mediate this cooperation, however, remain to be elucidated.

In light of our data, we conclude that wt-p53 and E2F-1 genes, sequentially transferred into human esophageal cancer cells, can cooperate to efficiently induce apoptosis by stabilizing p53 via ARF-mediated MDM2 degradation. We also conclude that, because combination treatment with suboptimal doses of adenovirus vectors is sufficient to achieve antitumor effects, this strategy may also minimize the risk of adenovirus toxicity. Thus, the sequential combination of Ad-p53 followed by Ad-E2F-1 as gene therapy may lead to the development of molecular therapies for human esophageal carcinoma.

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