Erschienen in: Journal of Virology ; 79 (2005), 14. - S. 9296-9300. - ISSN 0022-538X
https://dx.doi.org/10.1128/JVI.79.14.9296-9300.2005

Growth Suppression Induced by Downregulation of E6-AP Expression in Human Papillomavirus-Positive Cancer Cell Lines Depends on p53

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The ubiquitin-protein ligase E6-AP is utilized by the E6 oncoprotein of human papillomaviruses (HPVs) associated with cervical cancer to target the tumor suppressor p53 for degradation. Here, we report that downregulation of E6-AP expression by RNA interference results in both the accumulation of p53 and growth suppression of the HPV-positive cervical cancer cell lines HeLa and SiHa. In addition, HeLa cells, in which p53 expression was suppressed by RNA interference, are significantly less sensitive to the downregulation of E6-AP expression with respect to growth suppression than parental HeLa cells. These data indicate that the anti-growth-suppressive properties of E6-AP in HPV-positive cells depend on its ability to induce p53 degradation.

Modification of proteins by the covalent attachment of ubiquitin or ubiquitin-related proteins is involved in the regulation of many cellular and viral processes (3, 10, 23). Remarkably, not only do viral proteins represent substrates for such protein-protein modification systems, but some are also intrinsically involved in the conjugation of ubiquitin (ubiquitination) to cellular proteins, thereby redirecting the ubiquitin conjugation system for viral purposes (3). A prominent example for viral proteins associated with the ubiquitin-conjugation system is provided by the E6 oncoprotein of high-risk human papillomaviruses (HPVs) that are etiologically associated with cervical cancer and other malignant lesions of the anogenital tract. The E6 oncoprotein binds to the cellular ubiquitin-protein ligase E6-AP and utilizes E6-AP to target the tumor suppressor protein p53 for ubiquitination and subsequent proteasome-mediated degradation (13, 24). Furthermore, E6 has been reported to target additional proteins, including E6TP1, hScrib, hDlg, and Bak for ubiquitination and degradation in an E6-AP-dependent or E6-AP-independent manner (18, 25).

Continuous expression of E6 and E7, the two major HPV oncoproteins, is required for the maintenance of the transformed phenotype of cervical cancer cell lines (28). On the functional level, E6 has p53-dependent as well as p53-independent antiapoptotic properties (22). Indeed, interference with E6 expression or E6 activity results in the induction of apoptosis in HPV-positive cells, which is accompanied by a significant increase in p53 levels (6, 7). Similarly, the downregulation of E6-AP expression by antisense approaches or overexpression of a catalytically inactive E6-AP mutant results in the accumulation of p53 in HPV-positive cells but not in HPV-negative cells (4, 26). Moreover, ribozyme-mediated reduction of E6-AP expression enhances the apoptotic response of HeLa cells, an HPV-18-positive cell line, to the DNA damage-inducing drug mitomycin C (16). However, since E6-AP has been implicated in E6-mediated degradation of proteins other than p53 (e.g., E6TP1 and hScrib) (9, 21), it remains unclear if this apoptosis-enhancing effect is directly linked to the ability of E6-AP to target p53 for degradation in the presence of E6.

To determine if the presence of E6-AP contributes to the antiapoptotic function of E6, HPV-positive cells were transfected with small interfering (si) RNAs (8) directed against E6-AP, renilla luciferase, or Hdm2 and the effects of the different siRNAs on p53 levels and cell viability determined at various time points after transfection (Fig. 1). Treatment of SiHa cells (HPV-16 positive) and HeLa cells (HPV-18 positive) with siRNAs directed against either renilla luciferase or Hdm2 had no significant effect on either p53 protein levels or cell viability, supporting the notion that Hdm2 plays no role or only a minor role in p53 degradation in HPV-positive cells (12). In contrast, treatment of both cell lines with E6-AP-specific siRNAs targeting all known isoforms of E6-AP (27) resulted in p53 accumulation and had significant effects on cell viability starting at day 2 or 3 after transfection (Fig. 1A and C and data not shown). Treatment with E6-AP siRNA1 (directed against nucleotides 69 through 87 of the open reading frame; the numbering referring to E6-AP isoform 1, with nucleotide 1 referring to A of the start codon) resulted in efficient induction

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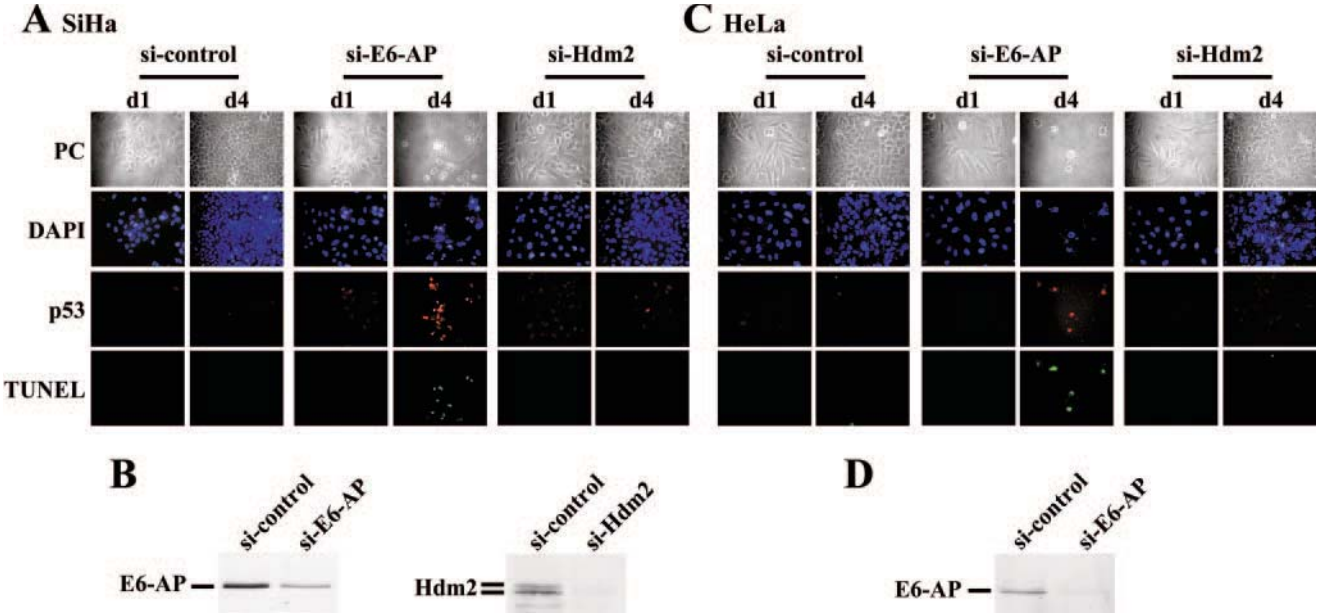

FIG. 1. Downregulation of E6-AP expression by RNA interference induces accumulation of p53 and interferes with the viability of HPV-positive cancer cell lines. Synthetic siRNAs specific for E6-AP (si-E6-AP), Hdm2 (si-Hdm2), or renilla luciferase (si-control) were transfected into the HPV16-positive cell line SiHa (A and B) and the HPV18-positive cell line HeLa (C and D) as described previously (7, 17). (A and C) Levels of p53 and induction of apoptosis (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling [TUNEL]) were determined 24 h (d1) and 96 h (d4) after transfection by fluorescence analysis (7, 17). In addition, cells were visualized by phase-contrast microscopy (PC) and nuclei by use of DAPI (4′,6′-diamidino-2-phenylindole). (B and D) Levels of E6-AP and Hdm2 were determined by Western blot analysis at 48 h posttransfection (11). Note that the levels of Hdm2 in HeLa cells are too low to be reproducibly detected under the conditions used.

of apoptosis (Fig. 1C), with only 3 to 5 percent of the cells remaining by 4 days posttransfection. Transfection of E6-AP siRNA2 (nucleotides 300 through 318) also interfered with cell viability of HPV-positive cells but slightly less efficiently than E6-AP siRNA1, with 15 to 20 percent of the cells remaining by 5 to 6 days posttransfection with no overt signs of apoptosis (data not shown). The reason for this difference in killing efficiency is presently unknown but may be explained by slightly different efficiencies of the siRNAs used in downregulating E6-AP levels. In this context, it should be noted that, while this work was under consideration, Kelley et al. (15) reported that downregulation of E6-AP results in the accumulation of p53 levels in HPV-positive cells and, under certain conditions, in the induction of apoptosis.

The results described above support the notion that E6-AP has anti-growth-suppressive properties in HPV-positive cell lines. However, since E6-AP is a cellular protein that is expressed in all cell lines tested regardless of their HPV status, the anti-growth-suppressive properties of E6-AP may not be related to its ability to interact with E6. To address this possibility, several HPV-negative cell lines expressing wild-type p53 (i.e., MCF-7, RKO, and U2OS) were treated with siRNAs against E6-AP or Hdm2. As reported previously (17), downregulation of Hdm2 resulted in p53 accumulation and induction of apoptosis in HPV-negative cells (Fig. 2A and data not shown), while no indication for p53 accumulation and/or interference with cell viability was observed upon treatment of the cells with E6-AP-specific siRNAs. The latter result, however, is difficult to interpret. In contrast to HPV-positive cells, in which E6-AP levels were significantly reduced at 2 days upon transfection of E6-AP-specific siRNAs (Fig. 1B and D), E6-AP levels were less significantly affected in HPV-negative cells even at day 3 or 4 posttransfection (Fig. 2B). However, E6-AP mRNA levels were downregulated to similar extents (approximately two- to threefold) in HPV-positive and HPV-negative cells by the siRNAs used (Fig. 2C). This apparent paradox (similar effects on RNA levels but different effects on protein levels) is probably explained by the notion that E6-AP has a rather long half-life in HPV-negative cells but is targeted for proteasome-mediated degradation by the E6 oncoprotein in HPV-positive cells (14). Thus, it is expected that transient siRNA treatment affects E6-AP levels in HPV-positive cells more significantly than in HPV-negative cells.

Taken together, the results described above corroborate previous data indicating that E6-AP is required for both p53 degradation and the viability of HPV-positive cell lines (4, 12, 15, 16, 27). However, they do not conclusively address the question as to whether the ability of E6-AP to facilitate p53 degradation in the presence of E6 is essential for the viability of HPV-positive cancer cells or whether other yet uncharacterized functions of E6-AP are involved. A possible way to address this issue is to generate HPV-positive cells, in which expression of endogenous wild-type p53 is abrogated. The rationale for such an approach is the assumption that if the anti-growth-suppressive activity of E6-AP is functionally linked to its ability to degrade p53, p53-null HPV-positive cells should be less sensitive towards siRNA-mediated downregulation of E6-AP expression. To generate p53-null cells, HeLa cells were transfected with pSUPER-p53, which allows the stable suppression of p53 expression by RNA interference (5). Upon selection, single-cell clones were established and the individual clones checked for p53 expression by Western blot analysis

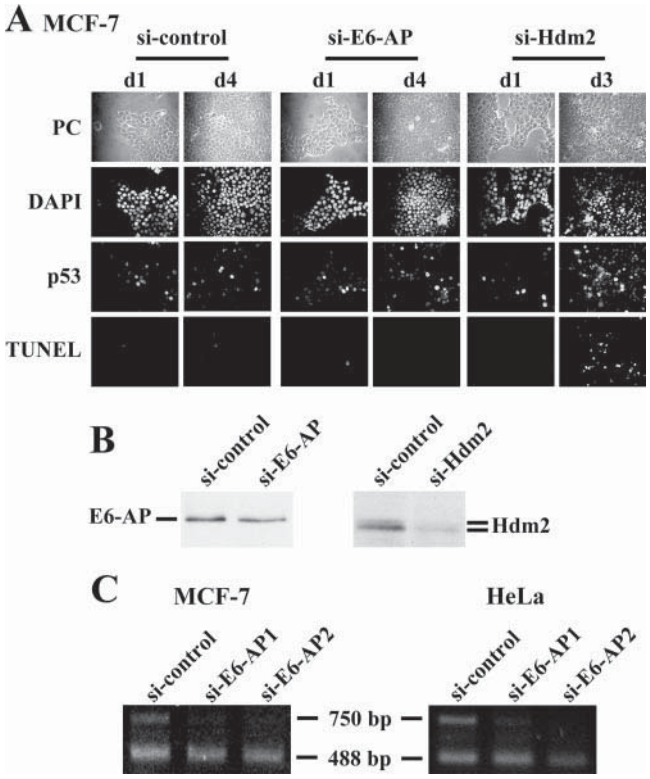

FIG. 2. siRNAs against E6-AP do not affect the growth of HPV-negative cells. Synthetic siRNAs specific for E6-AP (si-E6-AP), Hdm2 (si-Hdm2), or renilla luciferase (si-control) were transfected into the HPV-negative cell line MCF-7, which expresses endogenous wild-type p53. (A) Levels of p53 and induction of apoptosis (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling [TUNEL]) were determined 24 h (d1), 72 h (d3), or 96 h (d4) after transfection by fluorescence analysis (7, 17). In addition, cells were visualized by phase-contrast microscopy (PC) and nuclei by use of DAPI. (B) Levels of E6-AP and Hdm2 were determined by Western blot analysis at 48 h posttransfection (11). (C) E6-AP mRNA levels were determined at 48 h posttransfection by relative quantitative reverse transcription-PCR analysis (for further details, see reference 7). The primer pair used amplifies an mRNA region that is shared by all known E6-AP isoforms (27) and results in a 750-bp amplification product. As an internal standard, 18S rRNA was amplified (488 bp).

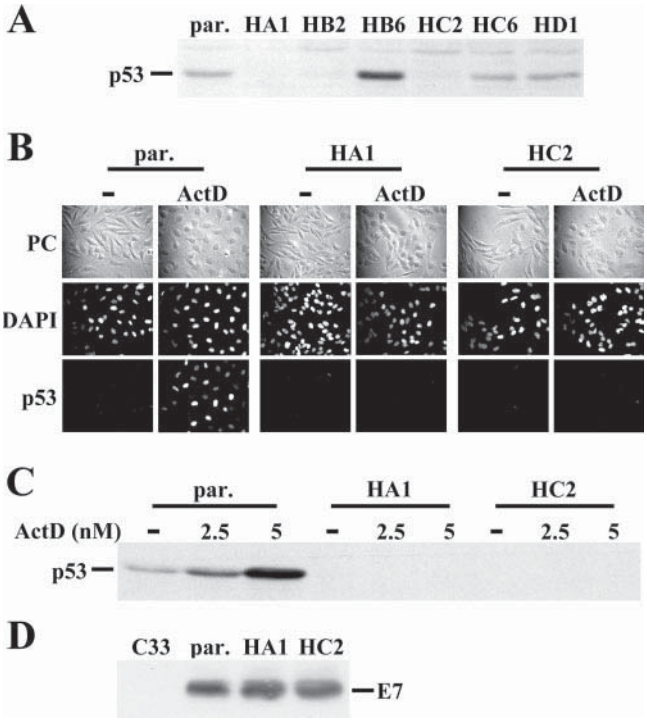

FIG. 3. Stable knockdown of p53 expression in HeLa cells by RNA interference. To generate HeLa cells in which p53 expression is stably suppressed by RNA interference, HeLa cells were transfected with pSUPER-p53 (5) together with a vector expressing the neomycin resistance gene. Cells stably containing the expression constructs were selected by resistance to neomycin and single-cell clones established. (A) Protein extracts were prepared (11) from various single-cell clones and p53 levels determined by Western blot analysis using mouse monoclonal DO-1. (B) Parental HeLa cells (par.) and the single-cell clones HA1 and HC2 were treated for 24 h with 5 nM actinomycin D (ActD) or, as a control, with dimethyl sulfoxide (−). Levels of p53 were determined by immunofluorescence (p53). In addition, cells were visualized by phase-contrast microscopy (PC) and nuclei by use of DAPI. (C) Parental HeLa cells (par.) and the clones HA1 and HC2 were treated for 24 h with 2.5 nM or 5 nM actinomycin D (ActD) as indicated or, as a control, with dimethyl sulfoxide (−). Levels of p53 were determined by Western blot analysis. (D) Levels of the HPV-18 E7 protein in parental HeLa cells (par.) and the clones HA1 and HC2 were determined with a polyclonal anti-E7 antibody by Western blot analysis as described previously (7). Extracts prepared from HPV-negative C33-A cells (C33) served as negative controls.

(Fig. 3A). Some of the selected clones had p53 levels similar to those of the respective parental cells, while in other clones (i.e., HA1 and HC2), p53 could not be detected, suggesting that p53-specific siRNAs are expressed at different levels in the individual cell clones.

Upregulation of p53 levels in response to various stress stimuli is, at least in part, achieved by significantly attenuated degradation (2, 19, 20). To obtain further evidence that p53 expression in HA1 and HC2 cells is indeed abrogated, p53 levels in both clones were determined upon treatment with different concentrations of actinomycin D, a potent activator of p53 (1). While actinomycin D treatment induced significant p53 accumulation in parental HeLa cells (Fig. 3B and C), p53 in the HA1 and HC2 clones could not be detected by immunofluorescence or Western blot analysis (note that expression levels of the HPV E7 oncoprotein in the p53-null HeLa cell lines are not affected, demonstrating that they are indeed derived from HeLa cells) (Fig. 3D). In addition, similar results were obtained by treatment of the different cells with the proteasome inhibitor MG132 (i.e., significant upregulation of p53 levels in parental HeLa cells and no sign of p53 expression in HA1 and HC2) (data not shown). Thus, p53 expression is completely or almost completely abrogated in the clones HA1 and HC2.

The p53-null clones HA1 and HC2 were transfected with E6-AP-specific siRNAs and the effects of E6-AP downregulation on accumulation of p53 and on cell viability determined (Fig. 4). As expected, p53 was not detectable in the p53-null cells even 4 days after transfection (Fig. 4A). Remarkably, downregulation of E6-AP with E6-AP siRNA1 and E6-AP siRNA2 did not interfere with the viability of p53-null HeLa cells (Fig. 4A). Furthermore, no signs of apoptosis in the p53-null cells under conditions in which apoptosis in parental HeLa

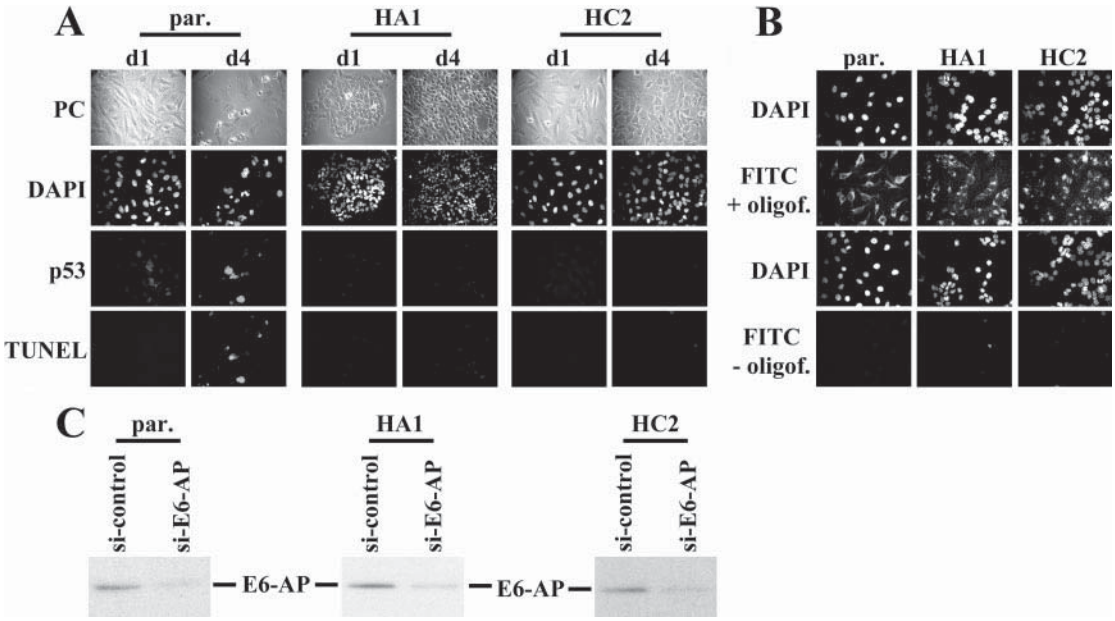

FIG. 4. Downregulation of E6-AP expression does not interfere with the growth of p53-null HeLa cells. (A and C) E6-AP-specific synthetic siRNAs were transfected into HeLa cells (par.) and the p53-null clones HA1 and HC2. Levels of p53 and induction of apoptosis (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling [TUNEL]) were determined 24 h (d1) and 96 h (d4) after transfection by fluorescence analysis. (A) In addition, cells were visualized by phase-contrast microscopy (PC) and nuclei by DAPI. (B) As a measure of transfection efficiency, HeLa cells (par.) and the HeLa derived p53-null clones HA1 and HC2 were incubated with fluorescein isothiocyanate (FITC)-dextran in the presence (FITC + oligof.) or absence (FITC − oligof.) of Oligofectamine (Invitrogen) as indicated. In addition, nuclei were visualized by use of DAPI. (C) E6-AP levels were determined by Western blot analysis using a mouse monoclonal antibody.

cells was significantly induced were observed. Both the transfection efficiencies (Fig. 4B) and the efficiencies of siRNA-mediated downregulation of E6-AP levels (Fig. 4C) were similar for p53-null cells and parental HeLa cells.

Similar to the case with E6-AP, downregulation of expression of the HPV E6 oncoprotein results in p53 accumulation and apoptosis in HeLa cells (7). Since E6 is known to have both p53-dependent and p53-independent antiapoptotic properties (22), the effects of siRNAs directed against E6 mRNA were tested in the p53-null HeLa clones HA1 and HC2. In comparison to that of parental HeLa cells, the sensitivities of the HA1 and HC2 clones towards E6-specific siRNAs were significantly reduced with respect to cell viability and induction of apoptosis (Fig. 5).

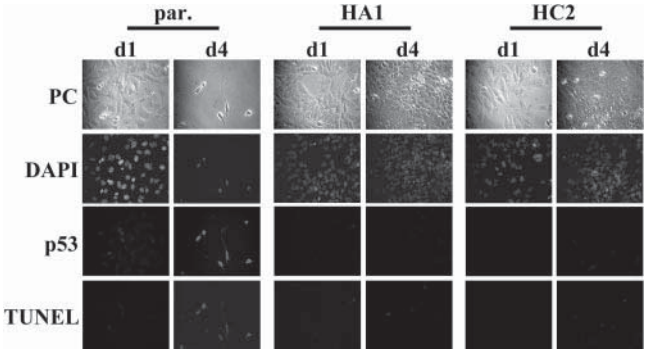

FIG. 5. Downregulation of HPV E6 expression does not interfere with the growth of p53-null HeLa cells. E6-specific synthetic siRNAs (7) were transfected into HeLa cells (par.) and the p53-null clones HA1 and HC2. Levels of p53 and induction of apoptosis (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling [TUNEL]) were determined 24 h (d1) and 96 h (d4) after transfection by fluorescence analysis. In addition, cells were visualized by phase-contrast microscopy (PC) and nuclei by use of DAPI.

The data presented above indicate that, under cell culture conditions, p53 represents the main target for the anti-growth-suppressive activities of both E6-AP and E6 in HeLa cells. However, since levels of E6-AP (and presumably E6; note that the levels of endogenous E6 in HeLa cells cannot be detected due to the lack of appropriate antibodies) were significantly but not completely downregulated upon transfection of siRNAs directed against E6-AP in the p53-null HeLa clones (Fig. 4C), the data do not exclude the possibility that E6-AP and/or E6 has additional p53-independent functions that are required for the viability of HeLa cells. If this is the case, however, it can be postulated that the threshold level of E6-AP and/or of E6 required to perform these p53-independent functions is lower than the one required for E6/E6-AP-mediated degradation of p53.

We are grateful to Noel Whitaker for critical reading of the manuscript.

M.A.D. is a fellow of the International Graduate School in Genetics and Functional Genomics at the University of Cologne. This work was supported by the European Commission (QLGI-CT-2001-02026) (M.S.), the Deutsche Forschungsgemeinschaft (M.S.), the Deutsche Krebshilfe (F.H.-S.), and the Wilhelm-Sander-Stiftung (F.H.-S.).

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