Hepatitis B Virus X Protein Modulates the Expression of PTEN by Inhibiting the Function of p53, a Transcriptional Activator in Liver Cells

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Abstract

The Hepatitis B Virus X (HBx) protein of hepatitis B virus plays a major role in hepatocellular carcinoma. It has been reported that the mutation and disruption of PTEN, a known tumor suppressor and a negative regulator of phosphatidylinositol 3'-kinase/AKT might be involved in tumor progression. However, the relationship between HBx and PTEN expression in hepatocellular carcinoma (HCC) development is not fully understood. This study reports on an investigation of whether PTEN expression in HBx-transfected cells is modulated by HBx or not. HBx decreased the expression of PTEN in HBx-transfected cells, as evidenced by EMSA. However, we confirmed that the binding of the p53 protein to p53 binding site-oligo of PTEN promoter is decreased in HBx-transfected liver cells by electrophoretic mobility shift analysis and, in addition, that HBx disrupts p53-mediated PTEN transcription, as evidenced by a PTEN promoter assay. Therefore, we conclude that HBx in liver cells down-regulates the expression of PTEN and activates AKT. This constitutes the first report to demonstrate that HBx has an effect on the p53-mediated transcription of PTEN, which, in turn, is associated with tumor suppression.

Introduction

PTEN, a tumor suppressor gene, is located on chromosome 10q23.3 (1) and is mutated at a high frequency in a variety of malignancies, including glioblastoma, melanoma, breast, prostate, and lung cancer (1, 2). The disruption of this gene in knockout mice results in the development of tumors (3). PTEN is a dual-specificity phosphatase and is able to act on both lipids as well as proteins. PTEN dephosphorylates PIP-3, which is produced by PI-3K, an important mediator of cell survival and proliferation (2, 4). The overexpression of PTEN suppresses cell growth and tumor formation in nude mice. In addition, the disruption and mutation of PTEN in tumor cells results in the activation of protein kinase B/AKT, which is activated via the PI-3K pathway cascade.

Human HBV induces acute and chronic hepatitis, and is closely associated with the incidence of human liver cancer (5). Among the four proteins that originate from the HBV genome, HBx is a multifunctional regulatory protein and has been reported to be associated with hepatocellular carcinogenesis. HBx induces liver cancer in transgenic mice (6). Although it does not bind directly to DNA, HBx affects transcriptional activation via its interaction with nuclear transcription factors and the cytoplasmic modulation of signal transduction pathways. It has also been reported that HBx interacts with transcription factors in the nucleus (7). Furthermore, an interaction between HBx and p53 inhibits p53 function (8), and several studies have demonstrated an inhibitory effect of HBx on DNA repair and apoptosis (9, 10). Cell signal transduction pathways, which are activated by HBx, include the Janus-activated kinase/signal transducers and activators of transcription and PI-3K pathways (11, 12). HBx also mediates the activation of signal transduction pathways such as the Ras/Raf/extracellular signal-regulated kinase and mitogen-activated protein kinase kinase kinase-1/c-Jun NH2-terminal kinase cascades, leading to the induction of activator protein-1 and nuclear factor NFκB (13, 14). Therefore, HBx is thought to be associated with the development of human hepatocellular carcinoma (HCC), but the precise function of HBx in the tumorigenic transformation of liver cells remains unclear.

Several studies have reported on the activation of the PI-3K/AKT pathway by HBx, which is associated with antiapoptosis activity and cell proliferation (12). On the basis of the findings reported herein, we provide evidence to indicate that the down-regulation of the PTEN gene by HBx results in an accelerated activation of the PI-3K/AKT pathway. The issues of whether HBx down-regulates the expression of the PTEN gene or not were investigated. Interestingly, HBx decreased the expression of PTEN in HBx-transfected cells, as evidenced by Northern and Western blot analysis. In addition, AKT was found to be activated by HBx, as evidenced by Western blot analysis and an AKT kinase assay. The findings herein also suggest that the p53-mediated transcription of PTEN gene is also regulated by HBx, as evidenced by a PTEN promoter assay and an EMSA. Lastly, the proliferation of liver cells that express HBx transcripts was higher than that of Chang and Chang-pEGFP cells. This constitutes the first report to demonstrate that HBx has an effect on the down-expression of PTEN, which is associated with tumor suppression.

Materials and Methods

Construction of the Eukaryotic Expression Vector pEGFP-C2-X Carrying the HBx Gene.

For the construction of pEGFP-C2-X, the pHBV-315 plasmid, which contains the entire genome of HBV (adr subtype), was digested with BamHI and a BamHI fragment containing the HBx gene was then ligated to form a circular HBV DNA. The circular HBV DNA was used as a PCR template for amplifying the HBx gene. Oligonucleotides designed to encode the HBx protein were synthesized using standard phosphoramidite chemistry. The primer combination used to amplify the region encoding HBx protein was 5’-ACGGAATTCATGGCTGCTAGGGTGT-3’ (sense primer)
in 100

...Bio-Rad) on ice. Electroporation was carried out with a Bio-Rad

...30 min. The cells were lysed in a NP40 lysis buffer containing 20 m M Tris-HCl

...CO2-air. For HBx gene transfection, Chang cells were resus-

...Fluorescence Microscopy. After transfection, the cells were washed twice

...Proliferation Assay. Cell proliferation was investigated using a commercially available proliferation kit II (XTT, Boehringer Mannheim,

...Promoter Assay. To determine whether HBx has the ability

...Results

...embryonic kidney cell) genomic DNA using the PCR and primers

...Normal Blot Analysis. To detect the expression of HBx, PTEN mRNA levels were determined using Northern blot analysis, and total RNA was prepared from Chang, Chang-pEGFP, and Chang-pEGFP-X cells were subcultured into 96-well culture plates at a density of 10^5 cells/well in 100 µl of DMEM. After 24 h of incubation, the medium, in a 96-well plate, was discarded and replaced with 100 µl of new medium. The plates were incubated in a 37°C humidified incubator under an atmosphere of 5% CO2 for 12, 24, 48, and 72 h. At the end of the incubation, the medium was discarded, and the cells were washed with PBS. Fifty µl of XTT test solution prepared by mixing 5 µl of XTT-labeling reagent and 100 µl of electron coupling reagent was then added to each well. After 4 h of incubation in a 37°C and 5% CO2 incubator, the absorbance was measured on an ELISA reader (Molecular Devices) at a test wavelength of 490 nm.

...PBS, covered with a coverslip, and analyzed using an Olympus BX50 fluorescence microscope, with a U-MWB filter (Olympus), to visualize the expression of EGFP and HBx-EGFP Images were additionally processed using Adobe Photoshop 5.0.

...Northern blot analysis, total RNA was separated by electrophoresis in a 1.5%

...Hybridization was performed using the unlabeled wt oligo or a mutant oligo 5’-GGC AGT GGA CCC CAG GCA GCT ACA CTG GGA CTC CTC AGT AG-3’ (mutant oligo). Nuclear extract proteins (2 µg) were preincubated with the gel shift binding buffer [4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.05 mg/ml poly(deoxyinosine-deoxycytosine)] for 10 min and then incubated with the labeled probe for 20 min at room temperature. Each sample was electrophoresed in a 4% nonde-

...E. coli (human embryonic kidney cell) genomic DNA using the PCR and primers

...Northern Blot Analysis. To detect the expression of HBx, PTEN mRNA levels were determined using Northern blot analysis, and total RNA was prepared from Chang, Chang-pEGFP, and Chang-pEGFP-X cells using the RNAzol B reagent (Tel-test), according to the manufacturer’s instructions. For Northern blot analysis, total RNA was separated by electrophoresis in a 1.5% formaldehyde agarose gel. After electrophoresis, the gel was blotted onto Hybond-C membrane (Amersham, Buckinghamshire, United Kingdom). RNA was fixed to membrane by cross-linking for 3 min by UV irradiation. Hybrid-

...Fluorescence Microscopy. After transfection, the cells were washed twice with PBS, covered with a coverslip, and analyzed using an Olympus BX50 fluorescence microscope, with a U-MWB filter (Olympus), to visualize the expression of EGFP and HBx-EGFP Images were additionally processed using Adobe Photoshop 5.0.

...Promoter Assay. To determine whether HBx has the ability...
Several studies have shown that cell proliferation and growth are stimulated by HBx through the activation of signal transduction pathways. Therefore, the growth rate of Chang, Chang-pEGFP, and HBX-transfected cells was examined. The proliferation of Chang, Chang-pEGFP, and Chang-pEGFP-X cells was determined by XTT assay. As shown in Fig. 1D, the growth rate of HBX-transfected cells at 48 and 72 h, respectively, was approximately double that of Chang and Chang-pEGFP. This indicates that HBx modulates the proliferation of liver cells.

Increased Activity of AKT via the Down-Regulation of PTEN by HBX. PTEN, a known phosphatase and tumor suppressor, is frequently inactivated in several human cancers and in a series of related disorders that are characterized by a predisposition to cancer (4). We first examined the issue of whether PTEN expression is inhibited by HBx in Chang liver cells. As shown in Fig. 2A, in HBX-transfected liver cells, PTEN mRNA levels were reduced, as evidenced by Northern blot analysis.

Stambolic et al. (15) reported that the upstream portion of the PTEN gene contains a p53 binding element, that this element is required for the inducible transactivation of PTEN by p53, and that PTEN mRNA and protein levels are also elevated in response to p53 induction. Thus, the issue of whether HBX modulates the expression of PTEN through the down-expression of p53 or not was investigated. However, Han et al. (16) reported that p53 was not significantly affected by the HCV core, HBx, or HBX and the HCV core at the RNA and protein levels. Our data also showed that HBx had no effect on the expression of p53 protein, as determined by Northern and Western blot analyses (Fig. 2, A and B). On the other hand, in previous data,

EGFP, the fluorescence was distributed throughout the entire cell (Fig. 1A, panel d). In cells expressing EGFP-X, the fluorescence was observed in both the nucleus and cytosol (Fig. 1A, panel f). Therefore, we conclude that EGFP and EGFP-X were successfully transfected into Chang cells.

Chang-EGFP-HBX, which expresses human HBx protein, was constructed by transfecting the Chang cell line with the HBX expression vector (pEGFP-X) including the G418-resistant marker. After selection of the G418-resistant colonies, Chang-EGFP-HBX cells were observed with a phase contrast microscope, and EGFP fluorescence was observed with a fluorescence microscope: a and b. Chang liver cells: c and d. Chang cells transfected with pEGFP, e and f. Chang cells transfected with pEGFP-X (A). HBx m RNA levels were analyzed by Northern blot analysis. Total RNA (20 μg) was isolated from Chang, Chang-pEGFP, and Chang-pEGFP-X cells, and then hybridized to a probe specific for HBx (B). To detect the expression of HBX protein, 20 μg samples of total cell lysates were size fractionated by SDS-PAGE and transferred to nitrocellulose membranes, and then analyzed by Western blot analysis using a specific antibody for HBx. GAPDH was included as an internal control (C). To examine the effect of HBX on liver cell growth, Chang-pEGFP and Chang-pEGFP-X were subcultured into 96-well culture plates at a density of 10^3 cells/well in 100 μl of DMEM. The plates were incubated in a 37°C humidified incubator under an atmosphere of 5% CO₂ for 12, 24, 48, and 72 h. Cell proliferation was determined by an XTT assay. The absorbance was measured in an ELISA reader (Molecular Devices) at a wavelength of 490 nm. The results are expressed as the percentage of cell proliferation of the control at 0 h. The data shown are the mean from five independent experiments (D). bars, ±SD.

Fig. 1. Establishment of Chang liver cell lines with constitutive HBx expression and the effect of HBx on the growth of liver cells. Chang cells were transfected with cDNAs encoding EGFP and EGFP-X in the mammalian expression vector pEGFP-C2. After selection of the G418-resistant colonies, Chang, Chang-pEGFP, and Chang-pEGFP-HBX cells were observed with a phase contrast microscope, and EGFP fluorescence was observed with a fluorescence microscope: a and b. Chang liver cells: c and d. Chang cells transfected with pEGFP, e and f. Chang cells transfected with pEGFP-X (A). HBx m RNA levels were analyzed by Northern blot analysis. Total RNA (20 μg) was isolated from Chang, Chang-pEGFP, and Chang-pEGFP-X cells, and then hybridized to a probe specific for HBX (B). To detect the expression of HBX protein, 20 μg samples of total cell lysates were size fractionated by SDS-PAGE and transferred to nitrocellulose membranes, and then analyzed by Western blot analysis using a specific antibody for HBx. GAPDH was included as an internal control (C). To examine the effect of HBX on liver cell growth, Chang-pEGFP and Chang-pEGFP-X were subcultured into 96-well culture plates at a density of 10^3 cells/well in 100 μl of DMEM. The plates were incubated in a 37°C humidified incubator under an atmosphere of 5% CO₂ for 12, 24, 48, and 72 h. Cell proliferation was determined by an XTT assay. The absorbance was measured in an ELISA reader (Molecular Devices) at a wavelength of 490 nm. The results are expressed as the percentage of cell proliferation of the control at 0 h. The data shown are the mean from five independent experiments (D). bars, ±SD.

Fig. 2. Activation of AKT via PTEN down-regulation in HBX-transfected liver cells. The p53 and PTEN mRNA levels in total RNA obtained from Chang, Chang-pEGFP, and Chang-pEGFP-X cells were assayed by Northern blot analysis. β-Actin was included as an internal control (A). PTEN and p53 protein levels in lysates obtained from Chang, Chang-pEGFP, and Chang-pEGFP-X cells were also detected by Western blot analysis. GAPDH was included as an internal control (B). Lysates of Chang, Chang-pEGFP, Chang-pEGFP-X, and Chang-pEGFP-X cells treated with or without 200 nM wortmannin for 4 h were prepared, and phospho-AKT (ser473) and total AKT were detected by Western blot analysis. GAPDH was included as an internal control (C). Lysates of Chang, Chang-pEGFP, Chang-pEGFP-X, and Chang-pEGFP-X cells treated with or without 200 nM wortmannin for 30 min were prepared. Kinase activity of AKT was subsequently determined by histone H2B phosphorylation in immunoprecipitates using an anti-AKT antibody. Total AKT was included as an internal control (D).
The transcriptional down-regulation of PTEN gene expression was clearly observed as assessed by Northern blot analysis (Fig. 2A), and the expression of the PTEN protein was decreased in Chang-pEGFP-X, as assayed by Western blot analysis (Fig. 2B). These results indicate that HBx has a suppressive effect on the expression of PTEN.

Several studies have reported that PTEN tumor suppressor inhibits the downstream function mediated by the PI-3K/AKT pathway through the dephosphorylation of phosphatidylinositol 3,4,5-triphosphate in vitro. Cells that are null for PTEN contain elevated levels of PIP-3 and active AKT when compared with matched cells expressing PTEN (2). We first found that HBx down-regulates PTEN mRNA and protein production in Chang liver cells. Thus, we examined the issue of whether AKT protein is activated by HBx or not. As shown in Fig. 2, C and D, the expression of AKT in Chang, Chang-pEGFP, and Chang-pEGFP-X cells was similar, but the activation of AKT was dramatically increased in Chang-pEGFP-X cells, as evidenced by Western blot analysis. Furthermore, AKT was found to be activated by HBx, as determined by a AKT kinase assay. On the other hand, the phosphorylation of AKT at serine 473 and the phosphorylation of the exogenous substrate histone H2B were decreased in Chang-pEGFP-X cells treated with Wortmannin (Calbiochem), a known PI-3K inhibitor. These results clearly show that HBx activates the AKT protein through the down-expression of PTEN.

Transactivation of the PTEN Promoter by HBx through the p53-Binding Site. To investigate whether the transcriptional activity of PTEN is regulated by HBx through the p53 binding site, the promoter activity of the PTEN gene was examined. As shown in Fig. 3A, a 621-bp genomic fragment corresponding to the region of the PTEN promoter between bases −1517 and −897 (pGL3-PTEN-pro621), a 448-bp fragment containing bases −1344 to −897 (pGL3-PTENpro448), and a 65-bp fragment containing bases −961 to −897 (pGL3-PTENpro65) were subcloned into a luciferase reporter plasmid. Both the pGL3-PTENpro621 and the pGL3-PTENpro448 contain the p53 binding site (−1190 and −1157) in the PTEN promoter region, but pGL3-PTENpro65 does not. These plasmids were cotransfected into Chang liver cells with the pEGFP or the pEGFP-HBX vectors. As shown in Fig. 3B, luciferase activity remained essentially unchanged, when the pGL3-basic and the pGL3-PTENpro65 were cotransfected with pEGFP-HBX, pEGFP, or without the vector into Chang cells, respectively. However, the luciferase activity of both the pGL3-PTENpro621 and pGL3-PTENpro448, which were cotransfected with pEGFP or without the vector, were ~10-fold higher than those for the pGL3-basic and the pGL3-PTENpro65 cotransfected with pEGFP-HBX, pEGFP, or without the vector. On the other hand, luciferase activities of the pGL3-PTENpro621 and pGL3-PTENpro448 cotransfected with pEGFP-HBX was ~5-fold lower than those for the pGL3-basic and the pGL3-PTENpro65 cotransfected with pEGFP or without the vector.

To additionally confirm that HBx is directly involved in the p53-mediated transcriptional activation of PTEN, we examined whether HBx is correlated to the binding of p53 to wt oligonucleotides that contain the sequence for the p53 binding site from the PTEN promoter by EMSA or not. As shown in Fig. 3, C and D, we confirmed that the nuclear lysates isolated from Chang, Chang-pEGFP, and Chang-pEGFP-X cells all induced an electromobility shift. In addition, the intensity levels of the shifted bands in the nuclear lysates from Chang and Chang-pEGFP were higher than those for the nuclear lysates from Chang-pEGFP-X cells. Moreover, the formation of an electrophoretically retarded complex was inhibited only when an unlabeled wt oligonucleotide (wt oligo) was introduced, but not when an oligonucleotide with mutations in each of the half-sites (mut oligo) was introduced.

A previous study reported that the PTEN promoter contains a unique p53 binding element and that the integrity of the p53 binding site in the PTEN promoter is essential for its transactivation by p53 (15). Several studies have reported that an interaction between HBx and p53 inhibits the binding of the p53 protein to the p53 binding element of several gene promoters (8, 17). Although HBx had no effect on p53 expression, as shown in Fig. 3, our data showed that the luciferase activity of the PTEN promoter containing the p53 binding element was significantly decreased in HBx-transfected cells, compared with Chang and Chang-pEGFP cells. Furthermore, the interaction of the p53 protein with oligonucleotides containing the p53 binding site in the PTEN promoter was modulated in HBx-transfected cells, as determined by EMSA. These results suggest that HBx regulates p53-mediated transcription of the PTEN gene.

Discussion

It is well known that HBx activates cell signal transduction pathways, such as the Janus-activated kinase/signal transducers and activators of transcription and PI-3K pathways (11, 12). HBx also medi-
PTEN DOWN-REGULATION BY HBX

Fig. 4. Cell survival and proliferation by activation of PI3K and AKT via PTEN suppression by HBx. HBx expression in HBx-transfected liver cells modulates the transcriptional activation of PTEN by inhibiting function of p53 as a transcriptional activator and also increase phosphorylation of AKT, which is known to be an important mediator of cell survival, proliferation, and growth.

states the activation of signal transduction pathways such as the Ras/Raf/extracellular signal-regulated kinase and mitogen-activated protein kinase kinase kinase kinase-1c-Jun NH2-terminal kinase cascades, leading to the induction of AP-1 and nuclear factor kB (13, 14). In terms of structural properties, PTEN contains a conserved catalytic motif found in multiple tyrosine phosphatases and has been shown to dephosphorylate a glutamine/tyrosine-rich peptide substrate, as well as the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate in vitro (4). Because missense mutations in the phosphatase domain often diminish its phosphatase activity, it has been suggested that the phosphatase activity of PTEN may play an essential role in tumor suppression. AKT, which is known to induce cellular transformations, may participate in cell growth, cell survival, and antiapoptotic function by growth factors (12, 18). In a recent study, PTEN was reported to inhibit downstream functions mediated by the PI-3K/AKT pathway, such as the activation of protein kinase B (also known as AKT), cell survival, and cell proliferation, presumably through the dephosphorylation of phosphatidylinositol 3,4,5-trisphosphate in vitro (2). Therefore, we assume that the activation of AKT for cell growth and survival could be enhanced through the down-expression of PTEN by HBx in liver cells.

The findings presented here clearly show that HBx down-regulates PTEN expression without having an effect on the expression of p53, a known transcriptional activator of PTEN in liver Chang cells that express HBx transcripts (Fig. 2, A and B), and that HBx results in AKT activation as well (Fig. 2, C and D). Moreover, HBx modulates the expression of PTEN by inhibiting the function of p53, although HBx had no effect on p53 expression (Fig. 3). Furthermore, among Chang, Chang-pEGFP, and Chang-pEGFP-X cells, the growth rate of HBx-transfected cells is significantly higher than the others (Fig. 1). As illustrated in Fig. 4, because HBx binds directly to p53 and also inhibits the transcriptional activity of the p53-transactivated PTEN promoter, HBx expression in HBx-transfected cells decreases the expression of PTEN, which is able to dephosphorylate PIP-3 to phosphatidylinositol 4,5-bisphosphate. In addition, a reduction in PTEN expression by HBx results not only in the elevation of PIP-3 levels, but the activation of AKT protein as well. Phosphorylated AKT regulates a number of biological processes, such as cell proliferation, apoptosis, and growth, by serving as a positive modulating transcription factor.

Wt-p53 is a nuclear phosphoprotein that acts as a tumor suppressor. It has been implicated in multiple cellular processes, including the inhibition of proliferation of transformed cells, the suppression of oncogenic transformation, and the mediation of cell cycle arrest and apoptosis (19). Among these functions, the mechanism of cell cycle control is well documented. At the molecular level, p53 functions as a transcription factor. Wt-p53 stimulates the expression of genes of which the promoters contain p53-binding sites, whereas some mutants of p53 appear to have lost this activity (20). Wt-p53 also represses a wide variety of cellular and viral promoters (21), in particular some promoters that contain a TATA sequence (22). Transcriptional repression has been shown to be involved in the induction of apoptosis by p53. Some mutant forms of p53 that are incapable of inducing apoptosis are defective in repression but not in their ability to activate transcription (23). Evidence for the central role of p53 as a tumor suppressor arises from genetic studies showing that mice that are homozygous for a deletion in the p53 gene develop tumors with a high frequency (24).

Stambolic et al. (15) reported that the DNA sequence between bases −1190 and −1157 in the PTEN promoter constitutes a p53 binding site, and that the PTEN promoter-derived oligonucleotide can specifically bind to p53, as assayed by EMSA. In addition, the removal of the DNA sequences either upstream or downstream from the p53 binding element of the PTEN promoter has no effect on transactivation by p53, but the deletion of the region encompassing the p53 binding site results in the loss of p53 responsiveness, as measured by a luciferase reporter assay. Furthermore, PTEN mRNA and protein levels are elevated in response to p53 induction, and the activation of AKT is reduced at time points coincident with maximal PTEN induction. Thus, we examined the issue of whether the expression of PTEN is regulated via a reduction in p53 expression by HBx. However, as shown in Fig. 2, A and B, HBx had no effect on p53 expression. On the other hand, in HBx-transfected liver cells, the expression of PTEN mRNA and protein was down-regulated.

The binding region of p53 is within the transactivation domain of HBx. A direct interaction between p53 and HBx has been documented by several groups (8). In the case of transgenic mice that express HBx from integrated HBV-DNA, it has been shown that tumor development correlates precisely with the binding of p53 to HBx in the cytoplasm and the complete blockage of the translocation of p53 to the nucleus (17). As shown in Fig. 3, our data show that p53-mediated transcription of the PTEN gene is modulated by HBx, as determined by a PTEN promoter assay and EMSA. On the basis of these previous reports, it is reasonable to assume that interactions between p53 and the HBx protein in HBx-transfected liver cells may inhibit the binding p53 to a p53 binding site of the PTEN promoter, thus modulating the expression of PTEN in HBx-transfected liver cells that are modulated by HBx.

In conclusion, HBx expression in HBx-transfected liver cells modulates the transcriptional activation of PTEN and also increase the phosphorylation of AKT. Therefore, this is, to the best of our knowledge, the first report to confirm that the inhibition of p53 transcriptional activity by HBx is related to the activation of AKT, which is known to be an important mediator of cell survival, proliferation, and growth, via the down-expression of the tumor suppressor PTEN gene.

References
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