Disruption of Protein Kinase Cη Results in Impairment of Wound Healing and Enhancement of Tumor Formation in Mouse Skin Carcinogenesis

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ABSTRACT

We have generated a mouse strain lacking protein kinase C (PKC) η to evaluate its significance in epithelial organization and tumor formation. The PKC-η-deficient mice exhibited increased susceptibility to tumor formation in two-stage skin carcinogenesis by single application of 7,12-dimethylbenz(a)anthracene (DMBA) for tumor initiation and repeated applications of 12-O-tetradecanoylphorbol-13-acetate (TPA) for tumor promotion. The tumor formation was not enhanced by DMBA or TPA treatment alone, suggesting that PKC-η suppresses tumor promotion. Epidermal hyperplasia induced by topical TPA treatment was prolonged in the mutant mice. The enhanced tumor formation may be closely associated with the prolonged hyperplasia induced by topical TPA treatment. In the mutant mice, after inflicting injury by punch biopsy, wound healing on the dorsal skin, particularly reepithelialization, was significantly delayed and impaired in structure. Impairment of epithelial regeneration in wound healing indicates a possibility that PKC-η plays a role in maintenance of epithelial architecture. Homeostasis in epithelial tissues mediated by PKC-η is important for tumor formation in vivo. We propose that PKC-η is involved in tumor formation modulated by regulation of proliferation and remodeling of epithelial cells in vivo.

INTRODUCTION

PKC3 is a family of phospholipid-dependent serine/threonine kinases that are activated by interactions with polar head groups of membrane lipids produced by various extracellular stimuli (reviewed in Refs. 1–3). The family consists of 10 isoforms, which are classified into three groups based on their structure and activation mechanisms. The conventional PKCα, β, βII, and γ isoforms are activated by intracellular transducers Ca2+ and DG, the latter of which is produced by phospholipase C. The novel PKCδ, ε, η, and θ isoforms are activated by DG in the absence of Ca2+. The atypical PKCζ and λ isoforms are not activated by Ca2+ or DG. The expression pattern of the isoforms depends on the tissue, cell type, and differentiation state. Potent mouse skin tumor promoters, such as phorbol diester TPA, strongly bind to and activate PKCs by substituting for DG, indicating that PKC isoforms may be causally involved in tumor formation.

Of these isoforms, PKCζ was originally cloned from skin cDNA libraries (4, 5) and is expressed predominantly in epithelial tissues (6). In the skin, it is localized in the epidermal granular layer (6, 7). Overexpression of PKCζ induces differentiation of human keratinocytes (8). PKCζ is activated specifically by CS that is produced in the granular layer of the epidermis (9). Furthermore, CS induces cell differentiation in mouse and human keratinocytes in culture (10, 11), induces scaling in the mouse epidermis (12, 13), and inhibits tumor promotion in mouse skin carcinogenesis (13). These observations imply that PKCζ plays roles in epidermal differentiation and tumor formation. However, multiple isoforms of PKC are expressed simultaneously in epithelial keratinocytes, and a specific function of these isoforms in vivo remains unclear.

To clarify functions of a protein in vivo, one of the promising strategies is gene targeting in ES cells and subsequent generation of knockout mice (14). Some PKC knockout mice exhibited impairment of a variety of cell functions, especially in the central nervous system and immune systems (15–21). PKC-γ-null mice show deficits in spatial and contextual learning (15). Disruption of PKCε induces super-sensitive γ-aminobutyric acid receptors to ethanol and allosteric modulators (16). PKCβ-null mice develop an immunodeficiency characterized by impaired humoral immune responses (17). PKCθ-null mice show deficits in T-cell activation (18). Disruption of PKCδ prevents B-cell tolerance and proliferation (19, 20). PKCζ-null mice show phenotypic alterations in secondary lymphoid organs (21). PKCε-null mice show enhanced insulin signaling through phosphatidylinositol 3'-kinase in muscles (22). However, no report is available on PKCζ or on tumor formation in these knockout mice with PKC isoforms.

In the present study, to obtain further insight into the role of PKCζ in tumorigenesis of epithelial cells, we generated mice that lack this enzyme and subjected them to tumor formation in two-stage skin carcinogenesis. Furthermore, we examined the wound healing process after skin injury to analyze maintenance of epithelial tissue architecture.

MATERIALS AND METHODS

Generation of PKCζ-deficient Mice. The mouse PKCζ gene (Prkcz) is a gene, containing 14 exons spanning 197 kb at 62 Mb distal from the centromere of chromosome 12 (Ensembl gene ID, ENSMUG21108), in which exon 9 encodes the ATP-binding site essential for enzymatic activity. Genomic DNA clones including exons 5–9 from nucleotide numbers 614-1279 of PKCζ cDNA were isolated previously (23). The targeting vector was constructed using the following fragments (Fig. 1A): a 4.3-kb BglII-SalI fragment located 5′ of exon 8; a 4.6-kb XbaI-EcoRV fragment 3′ of exon 9; a neomycin resistance gene (neo); and a diphtheria toxin A gene (DT-A) for negative selection. The linearized vector was electroporated into 129/SvJ-derived CCE ES cells (24, 25). DNA from G418-resistant ES clones was digested with KpnI or Sapi and hybridized with 5′- or 3′-probe (Fig. 1A). A targeted clone was injected into C57BL/6J blastocysts, and chimerical mice were generated. Males were mated with C57BL/6J females, and DNA from tail biopsies of the agouti offspring was analyzed to confirm the germ-line transmission of the mutant allele by Southern blot analysis and PCR. Heterozygous mutant (Prkcz+/−) mice with a genetic background of 129/SvJ and C57BL/6J were interbred to generate homozygous (Prkcz−/−) mice lacking PKCζ. Experiments were carried out with strict adherence to guidelines for minimizing distress in experimental animals.
**SKIN TUMOR PROMOTION IN PKC\textsubscript{\gamma}-DEFICIENT MICE**

The following primer sets were used, and the sizes of each amplicon are also indicated: (a) PKC\textsubscript{\gamma}, 5'-GTCTTACCCACCACCTTTG-3' and 5'-TACTG-CACCTTCTCGAGATGGGTCG-3' (GenBank accession number U43612, nucleotide numbers 1164-1336); and neo\textsuperscript{\gamma}, 5'-TCTCTGCCGAAAGATCCATCCA-3' and 5'-GTCGAGGAGGATGCTTTCGAGG-3' (accession number AF028009, nucleotide numbers 1922-2129). 428 bp; (b) PKC\textsubscript{\gamma}, 5'-AACGACAT-CATGGGAGGATGCTTTCGAGG-3' and 5'-AATTGCAGAGATGGTCTC-CAAATGGGAGG-3' (accession number X60304, nucleotide numbers 1733-2035) 303 bp; (c) PKCe, 5'-GACCTTTGAGTCTACACCAGACAGAATCC-3' and 5'-CAAGCCTTTGAGGCTGCGCTGCGACTCC-3' (accession number D28577, nucleotide numbers 720-1396), 677 bp.

**Immunoblotting.** The epidermis was prepared from frozen back skin (26). Epidermal extracts were subjected to SDS-PAGE, and the separated proteins were electrophoretically transferred onto nitrocellulose membranes. PKC\textsubscript{\gamma} was detected using a rabbit antibody that recognizes the NH\textsubscript{2} terminus (27) or the COOH terminus (Santa Cruz Biotechnology, Santa Cruz, CA) of PKC\textsubscript{\gamma}.

**Carcinogenesis Experiments.** Female 8-week-old mice were used. All animals were housed under a 12-h light/12-h dark cycle in a controlled atmosphere. The dorsal skin was shaved 1 week before DMBA treatment, and appropriate chemicals dissolved in 0.2 ml of acetone were applied to the shaved area. Eighteen mice from each group (Table 1) were used for the two-stage carcinogenesis protocol described previously (13). DMBA (100 \(\mu\)g) was applied topically as an initiator 1 week before promotion. TPA (10 \(\mu\)g) was repeatedly applied once a week for 20 weeks.

**Epidermal Hyperplasia.** Female 8-week-old mice were used. The dorsal skin was shaved 1 week before TPA treatment. After topical application of TPA (10 \(\mu\)g) to the shaved area, the mouse was killed by cervical dislocation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Initiation</th>
<th>Promotion</th>
<th>PKC\textsubscript{\gamma}</th>
<th>Incidence (%)</th>
<th>Mean no. of tumors/mouse ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMBA</td>
<td>TPA</td>
<td>+/+/+</td>
<td>22</td>
<td>0.39 ± 0.78</td>
</tr>
<tr>
<td>2</td>
<td>DMBA</td>
<td>TPA</td>
<td>+/+/+</td>
<td>17</td>
<td>0.24 ± 0.67</td>
</tr>
<tr>
<td>3</td>
<td>DMBA</td>
<td>TPA</td>
<td>+/-/-</td>
<td>78</td>
<td>4.3 ± 6.7\textsuperscript{a}</td>
</tr>
<tr>
<td>4</td>
<td>DMBA</td>
<td>TPA</td>
<td>+/+/-</td>
<td>5.6</td>
<td>0.06 ± 0.24</td>
</tr>
<tr>
<td>5</td>
<td>DMBA</td>
<td>TPA</td>
<td>+/-/-</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>DMBA</td>
<td>TPA</td>
<td>+/-/-</td>
<td>5.6</td>
<td>0.11 ± 0.47\textsuperscript{b}</td>
</tr>
<tr>
<td>7</td>
<td>TPA</td>
<td>+/+/+</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>TPA</td>
<td>+/+/+</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>TPA</td>
<td>+/+/+</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>TPA</td>
<td>+/-/-</td>
<td></td>
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<td>0</td>
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<tr>
<td>11</td>
<td>TPA</td>
<td>+/-/-</td>
<td></td>
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</tr>
<tr>
<td>12</td>
<td>TPA</td>
<td>+/-/-</td>
<td></td>
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</tr>
</tbody>
</table>

\textsuperscript{a} Significantly different (\(P < 0.02\), \textit{t} test) from group 1 or 2.

\textsuperscript{b} Not significantly different (\(P > 0.6\)) from group 4 or 5.

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**PCR for Genotyping.** The allele-specific primer sets are as follows: \(Prkch\) exon 9, 5'-GTGATGCTTGCAGGATGAAAG-3' and 5'-GGAGCTCTGGAAGGCTG-3' (GenBank accession number D90402, nucleotide numbers 1164-1336); and neo\textsuperscript{\gamma}, 5'-TCTCTGCCGAAAGATCCATCCA-3' and 5'-GTCGAGGAGGATGCTTTCGAGG-3' (accession number AF028009, nucleotide numbers 1922-2129). 428 bp; (b) \(Prkch\), 5'-AACGACATCATGGGAGGATGCTTTCGAGG-3' and 5'-AATTGCAGAGATGGTCTC-CAAATGGGAGG-3' (accession number X60304, nucleotide numbers 1733-2035) 303 bp; (c) PKCe, 5'-GACCTTTGAGTCTACACCAGACAGAATCC-3' and 5'-CAAGCCTTTGAGGCTGCGCTGCGACTCC-3' (accession number D28577, nucleotide numbers 720-1396), 677 bp.

**RT-PCR.** RNAs containing poly(A) were isolated from the epidermis of newborn mice. Reverse transcription was performed using an oligo(dT) primer. The resulting cDNA was used as a template for PCR using 20 cycles.
were obtained from the back of each mouse using a punch biopsy instrument with 2-mm inner diameter by a previously reported method (28), with a slight modification. The wounds were left open and monitored daily. Complete healing was determined based on the following criteria: full closure; colorless, reepithelialization; and smooth surface of both healed wounds in the mouse.

RESULTS

PKCγ Targeting. We constructed a targeting vector in which exon 9 of Prkch was replaced by neo' and obtained ES cell lines in which a single homologous recombination event had occurred at the Prkch locus (Fig. 1, A and B). Genotyping revealed generation of Prkch+/− mice (Fig. 1C). Thirteen pairs of Prkch+/− mice that were hybrids of 129/SvJ and C57BL/6J generated 870 offspring in 10 months, of which 226 were Prkch+/+, 437 were Prkch−/−, and 207 were Prkch+/−. These are consistent with the Mendelian inheritance pattern, indicating that mice homozygous for the disrupted Prkch allele are not subject to prenatal lethality.

By immunoblotting, an 80-kDa band corresponding to the native PKCγ was not detected in epidermal samples from wild-type and Prkch+/− mice, but not from Prkch−/− mice (Fig. 1D). In these mutant mice, significant accumulation of flanking molecules of PKCγ was not detected. In addition, expression levels of other PKC isoforms in the epidermis of Prkch−/− mice was not detected. In addition, expression levels of other PKC isoforms were comparable weight with wild-type littermates. However, 3.8% of 129/SvJ and C57BL/6J were alive, developed, appeared healthy, and more than 30 months of age and appeared indistinguishable from their wild-type littermates.

Tumor Formation. To examine the potential effect of PKCγ disruption on tumor formation, we performed two-stage skin carcinogenesis. In Prkch−/− mice, tumors appeared at 8 weeks of promotion and then increased continuously in number (Fig. 2). The incidence of tumor-bearing mice reached 78% and 4.3 tumors/mouse at 20 weeks of promotion (Table 1). In wild-type and Prkch+/− mice, tumors appeared around 10 weeks of promotion, and lower incidences (22% and 17%, respectively) and fewer tumors (0.39 and 0.28 tumor/mouse, respectively) were observed at 20 weeks. All tumors were papillomas. Evidently, Prkch−/− mice were significantly more susceptible to skin tumor formation than the wild-type and Prkch+/− mice (P < 0.02).

DMBA treatment alone without the promotion stage induced tumors at 5.6% incidence and development of 0.06 and 0.11 tumor/mouse in Prkch−/− and wild-type mice, respectively; no tumor developed in Prkch+/− mice (Table 1). No significant difference (P > 0.6) in the number of tumors formed among the three genotypes was observed. Repeated treatments with TPA or acetone as a solvent control did not induce any tumor formation in any of the genotypes. These results suggest that PKCγ functions as a negative regulator of the promotion stage.

Moreover, repeated treatments with DMBA (100 μg) at 4-week intervals induced tumor formation at 20 weeks at an incidence of 39% and 0.61 tumor/mouse in Prkch−/− mice, 44% and 0.67 tumor/mouse in Prkch+/− mice, and 39% and 1.1 tumor/mouse in wild-type mice. There were no significant differences (P > 0.3) among the three genotypes. These data suggest that PKCγ is not involved in mutational events in the epidermis.

Epidermal Hyperplasia. We examined epidermal hyperplasia to determine the potential effect of PKCγ disruption on keratinocyte proliferation in vivo. A single topical application of TPA at 10 μg induced formation of 40-μm-thick hyperplastic epidermis on day 2 in all genotypes (Fig. 3). The epidermal thickness returned to the basal level of 15 μm within 7 days in wild-type mice. However, in Prkch−/− mice, hyperplasia lasted up to 10 days. On day 7, basal layer cells highly expressed PCNA. These data suggest that PKCγ functions as a negative regulator of keratinocyte proliferation in the epidermis in vivo.

Wound Healing of Punched Skin. Effects of PKCγ disruption on the wound healing process were examined. In this study, we used congenic mice backcrossed with C57BL/6J for eight generations. In wild-type and Prkch+/− mice, contraction of the wound completed around day 5, reepithelialization proceeded, and complete healing with a smooth surface required around 11 and 9 days in the male and female groups, respectively (Table 2). In the Prkch+/− mice, healing was delayed for approximately 2 more days, although the contraction of the wound was completed in almost the same period of time as those of the wild-type and Prkch+/− mice.

As shown in Fig. 4, a healed wound on day 12 in male wild-type and Prkch+/− mice showed full recovery of the tissue structure, which consisted of the dermal connective tissue and epidermis with a two-keratinocyte layer. In Prkch−/− mice, although the wound healed macroscopically on day 14, epidermal remodeling was still proceeding, with extensive hyperplasia and papilla structure of epithelial tissues. PCNA was mainly detected in basal cells but not in suprabasal cells, similar to the case of normal tissues. Inflammation was also proceeding. Many leukocytes and PCNA-positive fibroblasts were observed in the repaired dermis. However, no differences in repair of connective tissue were observed among the three genotypes of mice 5 days after injury (data not shown). These results suggest that PKCγ
PKC has been suggested to play essential roles in tumor formation in mouse multiple-stage skin carcinogenesis, which is one of the most studied in vivo approaches in experimental cancer research (reviewed in Refs. 30 and 31). The initiation is attributed to defined molecular events such as mutations of specific genes, e.g., c-H-ras (32, 33), whereas the promotion stage is attributed to long-term modulation of intracellular signal processing through activation of PKC by tumor promoters and progression to malignant conversion of tumors (31, 34). In transgenic mice with PKCβII and ε isoforms, sensitivities to colon carcinogenesis and epidermal carcinoma formation, respectively, increase (35, 36). However, transgenic mice with PKCδ are resistant to tumor promotion (37). These findings, together with those for the PKCζ knockout mice in this study, indicate that functions of PKC isoforms vary in tumor formation.

Previous reports suggest that PKCη is involved in differentiation of epithelial cells and that its activation is specifically mediated by CS in vitro and in vivo. Although repeated applications of CS to the skin stimulate terminal differentiation of the epidermis in vivo (12, 13), we found that the PKCη-deficient mice respond to CS in the form of scaling induction (data not shown), indicating that not only PKCη but also other isoforms respond to the differentiation-associated lipid. In fact, CS activates PKCe as well as PKCη in a cell-free system (10). In the PKCη knockout mice, signal transduction on terminal differentiation may be mediated by PKCe.

The prolonged epidermal hyperplasia in the PKCη-deficient mice induced by TPA treatment may be closely associated with increased tumor formation. Overexpression of PKCη inhibits cell growth in culture due to blockage of cell cycle progression from G1 to S phase (38, 39). Furthermore, PKCη phosphorylates p21, an inhibitor of cyclin-dependent protein kinase, and inhibits cyclin-dependent protein kinase 2, resulting in inhibition of cell cycle progression of keratinocytes in culture (40). However, in TPA-treated epidermis, lack of PKCη did not affect inductions of ornithine decarboxylase and c-Jun (data not shown), which are thought to be important in tumor promotion. In addition, there was no significant difference in phosphorylations of mitogen-activated protein kinases, extracellular signal-regulated kinase 1/2, and p38 (data not shown). PKCη might function as an inhibitor of cell cycle machinery in vivo, rather than as a signaling molecule in gene expression through the mitogen-activated protein kinase pathway.

Disruption of PKCη impaired epithelial regeneration during cutaneous wound repair. Wound healing is a complex process requiring the collaborative functions of many different tissues and cells, including those involved in inflammation and regeneration of defective cells, and finally resulting in remodeling (reviewed in Ref. 41). One of the mechanisms underlying all of these processes is the actions of PKC isoforms. The presence of PKCη has little effect on the viability, development, and fertility of a mouse, although a fatal abnormal phenotype is observed very rarely. In the epidermis, although the expression levels of other PKC isoforms are not changed, a possibility of compensation of PKCη functions by other isoforms cannot be excluded. Because gene targeting results in loss of function, the apparent resulting normal phenotype in a knockout mouse may imply that a backup system may function for homeostasis in an animal. Existence of multiple PKC isoforms within a cell could be important in regulation of a lipid-dependent signal transduction (29). This hypothesis may be tested by generating multiple deletions. For example, to study the epidermis, it is now possible to generate mice that are doubly or multiply deficient in PKCη, α, δ, ε, and ζ isoforms, which are expressed in squamous cells.

**DISCUSSION**

The absence of PKCη has little effect on the viability, development, and fertility of a mouse, although a fatal abnormal phenotype is observed very rarely. In the epidermis, although the expression levels of other PKC isoforms are not changed, a possibility of compensation of PKCη functions by other isoforms cannot be excluded. Because gene targeting results in loss of function, the apparent resulting normal phenotype in a knockout mouse may imply that a backup system may function for homeostasis in an animal. Existence of multiple PKC isoforms within a cell could be important in regulation of a lipid-dependent signal transduction (29). This hypothesis may be tested by generating multiple deletions. For example, to study the epidermis, it is now possible to generate mice that are doubly or multiply deficient in PKCη, α, δ, ε, and ζ isoforms, which are expressed in squamous cells.

**Table 2** Wound healing in PKCη-deficient mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex</th>
<th>Prkch</th>
<th>No.</th>
<th>Healing duration (days)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Male</td>
<td>+/+</td>
<td>16</td>
<td>11.1 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Male</td>
<td>+/−</td>
<td>27</td>
<td>11.0 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Male</td>
<td>−/−</td>
<td>17</td>
<td>13.5 ± 2.0a</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Female</td>
<td>+/+</td>
<td>14</td>
<td>9.4 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>Female</td>
<td>+/−</td>
<td>25</td>
<td>9.2 ± 1.6</td>
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<tr>
<td>F</td>
<td>Female</td>
<td>−/−</td>
<td>16</td>
<td>11.1 ± 1.9b</td>
<td></td>
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</tbody>
</table>

*a* Significantly different (*P* < 0.01, t test) from group A or B.

*b* Significantly different (*P* < 0.05) from group D or E.

Regulates growth of keratinocytes in remodeling of epithelial tissues during the wound-healing process.

**Fig. 3.** Epidermal hyperplasia induced by topical treatment with TPA (10 μg). Wild-type mice, A–F, Prkch−/− mice, G–L. H&E staining (A–C and G–I) and immunostaining with anti-PCNA antibody (D–F and J–L) are shown. Representative images on day 0 (A, D, G, and J), day 2 (B, E, H, and K), and day 7 (C, F, I, and L) after treatment are shown. Thickened epidermis was observed in B, H, and I. Cells strongly stained by the anti-PCNA antibody were observed in E, K, and L. Bar, 50 μm.

**Fig. 4.** Wound healing in male mice of wild-type (A), Prkch−/− (B), and Prkch−/− (C) with C57BL/6J background. Skin wound healing on day 12 (A and B) or day 14 (C–E) after infliction of a 2-mm-diameter wound with a punch biopsy instrument. H&E staining (A–D) and anti-PCNA antibody staining (E) are shown. Boxed area in C is enlarged in D and E. Bar, 100 μm.
various growth factors. Epidermal growth factor is known to stimulate migration and proliferation of keratinocytes, whereas transforming growth factor β inhibits their growth. However, no significant differences in mRNA levels of epidermal growth factor and transforming growth factor β1 in the skin were observed between wild-type and PKCγ mutant mice (data not shown). Some recent works using knockout mice clearly showed importance of inflammation in wound healing (42–44). Additional studies are required to clarify whether PKCγ is involved in the production of and/or response to inflammatory cytokines in epithelial regeneration.

Most human cancers originate from epithelial tissues. Studies using epithelial tissues of animal models are important in understanding human carcinogenesis. PKCγ knockout mice can be useful tools for this purpose. Additional studies are necessary to elucidate molecular and cellular mechanisms by which PKCγ, as well as other PKC isoforms, proliferation, differentiation, and carcinogenesis of epithelial cells are regulated.

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