The Q188R Mutation in Human Galactose-1-phosphate Uridylyltransferase Acts as a Partial Dominant Negative*

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A longstanding goal in the fields of molecular genetics and biochemistry has been to explain how naturally occurring mutations associated with human metabolic disease impair activity of the enzymes involved. This goal is particularly complex for enzymes composed of multiple subunits, because single mutations may exert both intra- and intersubunit effects on holoenzyme structure and function. We have previously applied a yeast coexpression system for human galactose-1-phosphate uridylyltransferase, a dimeric enzyme associated with galactosemia, to investigate the impact of naturally occurring mutations on subunit association and holoenzyme function (1). Here we describe the purification and characterization of two heterodimers, R333W/wild type (WT) and Q188R/WT, revealing that although the first exhibits ~50% wild-type activity, the second exhibits only ~15% wild-type activity. Neither heterodimer varied significantly from the wild type with regard to apparent \( K_m \) for either substrate, although Q188R/WT but not R333W/WT heterodimers demonstrated significantly increased thermal sensitivity relative to the wild-type enzyme. These results demonstrate for the first time a partial dominant negative effect caused by a naturally occurring mutation in human galactose-1-phosphate uridylyltransferase.

Impairment of the human enzyme galactose-1-phosphate uridylyltransferase (GALT)\(^1\) results in the potentially lethal inborn error of metabolism, galactosemia (2). Normal GALT catalyzes the second step of the Leloir pathway of galactose metabolism, as indicated: UDP-glucose + galactose-1-phosphate → UDP-galactose + glucose-1-phosphate (2). GALT enzymes from a variety of sources, including bacteria, yeast, and human tissues have been isolated and studied in great detail (2), demonstrating that this enzyme functions with ping-pong kinetics and a double displacement mechanism of catalysis proceeding through a uridyl-enzyme intermediate (3–6). The active site nucleophile for the bacterial enzyme has been identified as histidine 166 (7), which corresponds by homology to histidine 186 in the human sequence.

Purified GALT enzymes from bacteria (8), yeast (9), and humans (10) have been shown by a variety of methods to exist as dimers composed of identical subunits. Furthermore, as demonstrated by Frey and colleagues from their work with the Escherichia coli enzyme (11), each GALT subunit contains its own active site. Recently Wedekind and colleagues (12) confirmed this point when they reported the three-dimensional structure of the E. coli enzyme refined to 1.8 Å resolution.

That GALT functions as a dimer raises questions of both fundamental and clinical significance regarding the relationship between dimerization and activity and the impact of naturally occurring mutations on both. Indeed, the allelic heterogeneity observed in patient samples (13) demonstrates that many if not most patients with classic galactosemia are not true molecular homozygotes but rather compound heterozygotes. This observation raises the possibility that allelic combination and not just allelic constitution may play some role in determining GALT holoenzyme function and thereby patient outcome.

Previously, we have reported the development of a yeast expression system for human GALT and applied this system to the study of a handful of patient mutations in the homozygous, heterozygous, and compound heterozygous states (14–16). Recently, we have extended this system to include the coexpression of epitope-tagged alleles of GALT, thereby enabling both structural and functional studies of specific subunits in the context of their various dimer states (1). In particular, we have coexpressed wild-type human GALT with each of two naturally occurring mutant alleles, Q188R and R333W, and asked the questions 1) do heterodimers form? and 2) are these heterodimers active? Q188R, which accounts for 60–70% of the mutant GALT alleles identified in Caucasians with classic galactosemia (17, 18), affects a position predicted by homology with the E. coli enzyme to lie two residues from the active site nucleophile (His\(^{186}\)), and far from points of intersubunit contact (12). In contrast, R333W, which has been reported only in isolated cases of galactosemia (19), affects a residue predicted to lie very close to the subunit interface (12). We have demonstrated that both mutant subunits remain capable of forming abundant albeit inactive homodimers, as well as abundant and active heterodimers with the wild-type subunit (1).

Here we report the purification and characterization of both the Q188R/WT and R333W/WT heterodimer pools. Our data demonstrate that despite their respective predicted locations, the R333W/WT heterodimer exhibits ~50% wild-type activity, whereas the Q188R/WT heterodimer exhibits only ~15% wild-type activity. In short, the Q188R mutation exerts a partial dominant negative effect on the adjacent subunit, whereas R333W does not. Neither heterodimer varied significantly from the wild type with regard to apparent \( K_m \) values for either subunit, although Q188R/WT but not R333W/WT heterodimers demonstrated significantly increased thermal sensitivity relative to the wild-type enzyme, presumably reflecting some perturbation of holoenzyme structure by the Q188R mu-

\(^1\) The abbreviations used are: GALT, galactose-1-phosphate uridylyltransferase; WT, wild type; UDPG, UDP-glucose.

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tation. These results are significant because they represent the first demonstration of a dominant negative interaction between subunits encoded by naturally occurring alleles of human GALT. These results also are of potential clinical interest because they add an additional level of complexity to the already long list of potential factors contributing to the heterogeneity of outcome observed for patients with galactosemia.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Plasmids, and Expression Studies**

All GALT expression studies were performed using yJK1, a haploid strain of Saccharomyces cerevisiae deficient in endogenous GALT due to a deletion in the GALT locus (14). All procedures including yeast transformations, culture manipulations, extract preparations, and GALT enzymatic assays were performed as described previously (1, 14). The Q188R and R333W mutations were introduced into the human GALT coding sequence as described previously (1, 14). Sequences encoding the FLAG epitope tag DYKDDDDK were introduced in frame following a start codon on the 5’ end of the human GALT coding sequence using the oligonucleotide 5’-GGCGATACCCTCTAAATTGGCTA-CAGGACGAGCAGCAGAGATGGCAGTTGGAACCCG-3’, as described previously for the hexahistidine tag (1). The plasmids employed in these studies, pFy3 (20) and pFy4 are both high copy number plasmids. pFy3 carries a TRP1 nutritional marker, whereas pFy4 carries a LEU2 nutritional marker, both of which complement auxotrophies in the host strain. pFy4 was constructed by ligating a ~2- kilobase pair Psil fragment (including yeast 2μ sequences) derived from pFy3 with a ~5.7-kilobase pair Psil backbone fragment derived from pFyE1 (16). All plasmid manipulations were performed using standard techniques (21) and the bacterial strain XL1-Blue (Stratagene).

Yeast extracts were prepared from 6-liter cultures grown to midlogarithmic phase in YPgal, harvested by centrifugation at 4°C, resuspended in 100 ml of lysis buffer (20 mM Hepes-KOH, 200 mM NaCl, plus protease inhibitors: pepstatin 1 μg/ml, aprotinin 2.1 μg/ml, leupeptin 0.5 μg/ml, antipain 2.5 μg/ml, phosphoramidon 0.6 μg/ml, E64 7.5 μg/ml, aprotinin 2.1 μg/ml, phosphoramidon 0.6 μg/ml, E64 7.5 μg/ml), and transferred to a Biospec Products bead beater containing 100 ml of prechilled acid-washed 0.5-mm glass beads. The suspension was vortexed-at 5°C for 4 hours each at high speed alternating with 4 min on ice. Each lysed cell suspension was then centrifuged at 4°C for 15 min at 15,000 × g to pellet insolubles. Protein concentrations of both crude yeast extracts and purified protein preparations were determined using the Bio-Rad protein assay kit using bovine serum albumin as the standard.

**Double Affinity Purification Procedure**

His6 Fusion Protein Purification—30 ml of lysed cell extract, prepared as described above, was brought to a final concentration of 25 mM imidazole mixed with 500 μl of FLAG purification buffer containing 37.5 μM FLAG peptide (NH2-Asp-Tyr-Lys-Asp-Asp-Asp-Lys-COOH) (IBI Inc.), as described above. The supernatant was collected as described above and dialyzed against standard buffer (20 mM Hepes, pH 7.5, 0.1 mM dithiothreitol). Each step of the purification procedure was monitored for total protein, for protein purity by SDS-polyacrylamide gel electrophoresis pattern visualized by silver staining, and by kinetic assays as described under “Experimental Procedures.”

**Silver Staining Analysis**

16 μl of each diluted sample (total, 0.8 μg) were mixed with 4 μl of sample buffer (50% sucrose, 10% SDS, 321.5 mM Tris, pH 6.8, 77.5 mg/ml dithiothreitol, bromphenol blue), heated to 95°C for 5 min, chilled on ice, and then separated by SDS 10% polyacrylamide gel electrophoresis at 200 V for 45 min using a Life Technologies, Inc. Mini-V 8.10 vertical gel electrophoresis apparatus. Low prestained molecular weight markers (Bio-Rad) were included to provide an internal size standard. Following electrophoresis the separated proteins were detected by silver staining using Quick-silver (Amersham Corp.) according to the manufacturer’s protocols.

**Enzyme Assays and Kinetic Analysis**

Enzymatic analyses of purified proteins were performed using a spectrophotometric coupled assay as described previously (1) (20). The standard reaction was performed at 37°C and contained 0.1 mM glycine buffer, pH 8.7, 5 mM dithiothreitol, 0.5 mM glucose-1,6-diphosphate, 5 μM MgCl2, 0.8 mM NADP, 1.2 mM galactose-1-phosphate, 0.8 mM UDPG, 0.06 μg of glucose-6-phosphate dehydrogenase, and 0.1 μg of phosphoglucomutase in a total volume of 400 μl. Kinetic constants were assessed by the same way to detect the apparent Km, for UDPG the concentration of galactose-1-phosphate was held constant at 1.2 mM and the concentration of UDPG was varied from 0.02 to 1.0 mM and to determine the apparent Km for galactose-1-phosphate the concentration of UDPG was held constant at 0.6 mM and the concentration of galactose-1-phosphate was varied from 0.05 to 2.0 mM. Enzyme activity assay was carried out over at least six different substrate concentrations with the data point performed in duplicate. Controls were run either one or both substrates were routinely included and always gave the expected negative results. Apparent kinetic constants were calculated by fitting the data to the Michaelis-Menten equation using Sigma Plot. Kinetic assays on purified proteins were performed immediately following double affinity purification, and no loss of enzyme activity was observed over the course of the experiment. Apparent kinetic constants are reported as averages of three independent enzymatic assays involving preparations from three independent double affinity purifications.

**Effects of Temperature on Enzyme Activity**

GALT enzymatic assays were performed on purified proteins at different temperatures (see Figures 4 and 5) using a direct assay as described previously (1) (20). Activation energy was determined by fitting data points from the ascending slope of each profile to the Arrhenius equation (22) using Sigma Plot. GALT activity was assayed in three separate experiments at a total of ten different temperatures over a range from 22 to 37.5°C.

**RESULTS**

**Epitope-tagged Alleles of Human GALT**—To facilitate the recognition and isolation of subunits encoded by specific alleles of human GALT, we have introduced sequences encoding each of two small tags, FLAG (23) and His6 (24), onto the 5’ ends of both wild-type and mutant coding sequences of the human enzyme. The FLAG epitope, DYKDDDDK, is recognized by a high affinity, commercially available monoclonal antibody, enabling both detection and one-step affinity purification of fusion proteins (M2, IBI). The affinity of proteins containing a hexahistidine tag for a charged nickel resin (nickel-NTA, Qiagen Inc.) similarly enables one-step purification of His6-tagged proteins under nondenaturing conditions.

Previously we have shown by activity assays and Western blot analyses of extracts prepared from yeast expressing either untagged (native) or His6-tagged GALT proteins that the His6 tag does not impair either activity or abundance of human GALT expressed in yeast (1). Parallel studies were performed...
on extracts from yeast expressing FLAG-tagged GALT proteins; the ratio of activity to abundance for the FLAG-tagged protein relative to the native protein was 1.06, demonstrating that the FLAG tag, like His6, impairs neither activity nor abundance of human GALT expressed in yeast.

**Isolation of Defined GALT Heterodimer Pools**—Previously we have addressed questions of heterodimer formation and activity using coexpression of epitope-tagged alleles coupled with a nickel affinity purification procedure (1). We have demonstrated the specificity of the nickel-His6-GALT interaction and have further demonstrated that no detectable subunit exchange occurs in defined heterodimer preparations following isolation (1). Analyses of these samples provided qualitative answers to the questions addressed but could not provide true quantitative measures of heterodimer function because these preparations contained a mixture of heterodimers and His6-tagged homodimers.

In order to characterize more precisely the biochemical properties of defined heterodimers of human GALT, we have developed and applied a sequential, two-step affinity purification procedure for heterodimers that exploits the specificities of available nickel and antibody affinity resins for fusion proteins carrying the His6 and FLAG tags, respectively (Fig. 1). Using these resins we have purified two GALT heterodimer species, as well as wild-type human GALT homodimers, to apparent chemical purity by SDS-polyacrylamide gel electrophoresis and visualization by silver staining. As a control for specificity of the isolation, equivalent (or greater) volumes of eluate from isolation procedures involving extracts including either His6-WT GALT homodimers alone, or FLAG-WT GALT homodimers alone, also were analyzed (first and second lanes). The doublet pattern apparent in the third, fourth, and fifth lanes presumably reflects differential impact of the two tags (His6 versus FLAG) on GALT mobility through the gel, although this point remains an assumption. Each His6-tagged subunit should include 386 amino acids, whereas each FLAG-tagged subunit should include 388 amino acids. Although the two subunit types should be present in equal amounts in purified heterodimer preparations, if the faster migrating band indeed represents the smaller His6-tagged species, these proteins may react differently with the silver stain than do the FLAG-tagged subunits, thereby perhaps accounting for the differential intensities of the bands in each doublet.

**Kinetic Analyses of Wild-type Homodimer and Mutant Heterodimer GALT Enzymes**—To investigate the possibility of intersubunit effects on activity by either the Q188R or R333W mutations, kinetic studies were performed comparing purified preparations of each heterodimer species with wild-type homodimers. Sample data are illustrated in Fig. 3. The compiled results of these studies (Table I) demonstrated that although each of the heterodimers is active, the Q188R/WT enzyme has an apparent specific activity that is only 12–16% that of the wild-type homodimer. In contrast, the R333W/WT enzyme has an apparent specific activity ~50% that of the wild-type homodimer, which is the value expected if there is neither positive nor negative interaction between the mutant and wild-type subunits in the heterodimeric holoenzyme. Mixing experiments involving individually purified preparations of WT/WT and WT/Q188R or Q188R/Q188R homodimers demonstrated no “trans-acting” negative impact of Q188R GALT on the wild type, thereby ruling out the possibility that Q188R was impacting heterodimer function via some mechanism other than intersubunit interaction (data not shown). Finally, apparent $K_m$ values for both galactose-1-phosphate and UDPG for each of the mutant/WT heterodimers were virtually indistinguishable from those obtained for the wild-type enzyme.

**Impact of Temperature on Activity of Wild-type Homodimer and Mutant Heterodimer GALT Enzymes**—As a final measure of GALT holoenzyme structure and function in the presence of the Q188R and R333W mutations, kinetic studies were performed testing each heterodimeric species over a range of temperatures from 22 to 60 °C. Purified wild-type homodimers were analyzed in parallel as a positive control. As illustrated in Fig. 4, activity associated with the wild-type homodimer rose steeply as the temperature increased from 22 to 43 °C, remaining fairly stable until about 47 °C, and then declined rapidly as temperature was increased further. Only about 7% of the maximum activity remained at 60 °C. A very similar profile was observed for the R333W/WT heterodimer, although peak activity was attained at a slightly lower temperature (~40 °C). However, the entire curve was shifted dramatically to the left for the Q188R/WT heterodimer, such that a temperature optimum was reached at 37.5 °C, and only ~50% maximal activity remained at 43 °C; the optimal temperature for the wild-type homodimer. The Q188R/WT heterodimer demonstrated no detectable activity at 60 °C.
Finally, activation energies of catalysis were estimated for the wild type and both heterodimer species by fitting those points derived from the ascending arms of each temperature profile to the Arrhenius equation (see “Experimental Procedures” and Fig. 5). By this method the activation energy for the wild-type homodimer was estimated to be 3.29 ± 0.18 kcal/mol. Similar results, 3.15 ± 0.34 and 3.02 ± 0.52 kcal/mol, were obtained for the Q188R/WT and R333W/WT heterodimers, respectively.

**DISCUSSION**

The experiments reported here were designed to investigate intersubunit effects of naturally occurring mutations in human GALT. Our results clearly demonstrate that of the two catalytically null subunits studied, one (Q188R GALT) exerted a negative impact on heterodimer function; the other (R333W GALT) did not. These results, therefore, demonstrate for the first time a partial dominant negative effect caused by a naturally occurring mutation in human GALT. This work both extends and contrasts an earlier report by Nadler and colleagues (28), who were the first to observe interallelic complementation in human GALT. Those authors performed pairwise fusions of fibroblasts derived from galactosemic patients and detected GALT activity in 3 of 28 hybrids but in none of the original lines. The GALT activity produced by those hybrid cells was similar to that associated with the normal enzyme in terms of apparent \( K_m \) values, pH optima, and electrophoretic mobility on starch gels, but differed in terms of specific activity and thermal stability. It is interesting to note that the negative interactions reported here also impacted specific activity and thermal stability but not apparent \( K_m \) or activation energy of catalysis. Unlike the work of Nadler and colleagues, however, whose hybrid cells may have expressed GALT from as many as four different and unidentified alleles, the results reported here involved interaction of specified GALT subunits whose molecular defects were known.

**Q188R and R333W** — The observation that different mutations can exert differential impacts on holoenzyme function is not surprising. Studies of a variety of structural and catalytic proteins, including the human hemoglobins (reviewed in Ref. 29), collagens (reviewed in Ref. 29), and creatine kinase (30), have shown similar results. What is surprising, however, is that of the two mutations studied here, the one that demonstrated a partial dominant negative effect, Q188R, is predicted by homology with the *E. coli* GALT structure (12) to be distal to any points of subunit contact, whereas the mutation that demonstrated no detectable intersubunit effects, R333W, is located very close to the predicted subunit interface.

It may be relevant to note, however, that in *E. coli*, Gln\(^{168}\)His, which corresponds by homology to Gln\(^{333}\)His in humans, contributes to formation of one of the nine strands of an anti-parallel \( \beta \)-sheet that together form the “half-barrel” architecture of the subunit core. The substitution of arginine for glutamine at this position decreases the propensity of the region to form a \( \beta \)-sheet, as predicted by the method of Chou and Fasman (31). In contrast, *E. coli* residue Arg\(^{119}\), which corresponds to Arg\(^{333}\) in the human sequence, is not directly involved in the formation of any major elements of secondary structure predicted for the enzyme (12). In addition, the Q188R mutation affects a position only two residues from the presumed active site nucleophile, His\(^{184}\), and only four residues from a predicted zinc ligand, His\(^{184}\) (12). Considering both the catalytic and structural importance of the region, it is perhaps not surprising that the Q188R mutation results in functionally significant perturbations of both the subunit and the holoenzyme. It is interesting to note that a handful of other previously reported mutations in human GALT; V44M, F171S, S192N, H319Q, and A320T (13), all map to regions within the *E. coli* structure predicted to participate in formation of the anti-parallel \( \beta \)-strands of the subunit core. Further studies will be required to define those elements of sequence and structure most important for determining both the physical and functional parameters of subunit interaction in the GALT holoenzyme.

One potential complication to interpreting the data reported here stems from the fact that both mutant GALT subunits carried His\(_6\) tags. We have previously investigated potential impacts of the His\(_6\) tag on abundance and activity of the wild

![Diagram](https://example.com/diagram.png)

**Fig. 3.** Representative Eadie-Hofstee plots and activity curves (inset) for human GALT WT/WT homodimers and mutant/WT heterodimers purified from a yeast coexpression system by two-step, sequential affinity purification. Plots represent data sets obtained from kinetic assays in which the concentration of UDPG was varied while the concentration of galactose-1-phosphate was held constant at 1.2 mM (A) or the concentration of UDPG was held constant at 0.6 mM (B).

**Table I**

Kinetic parameters of human GALT WT/WT homodimers and mutant/WT heterodimers isolated from yeast

| Dimer                | Specific activity | Apparent \( K_m \) galactose-1-phosphate | Apparent \( K_m \) UDPG |
|----------------------|-------------------|------------------------------------------|------------------------|
|                      | \( \mu \text{mol/g/} \text{min} \) | (mol/mg/min) | (mol/mg/min) |
| Wild type/Wild type  | 123.75 ± 21.41 (100%) | 0.73 ± 0.02 | 0.13 ± 0.00 |
| Q188R/Wild type      | 17.15 ± 2.83 (13.86%) | 0.60 ± 0.06 | 0.17 ± 0.04 |
| R333W/Wild type      | 55.48 ± 8.75 (44.83%) | 0.81 ± 0.09 | 0.16 ± 0.02 |
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**FIG. 4.** Effects of temperature on activity of human GALT WT/WT homodimers and mutant/WT heterodimers isolated from yeast. WT/WT, Q188R/WT, and R333W/WT human GALT dimers were analyzed for activity at each of the temperatures indicated, as described under "Experimental Procedures." Values showing error bars represent averages of at least three separate assays; those without error bars represent the results of single assays.

**FIG. 5.** Energy of activation for human GALT WT/WT homodimers and mutant/WT heterodimers isolated from yeast. WT/WT, Q188R/WT, and R333W/WT human GALT dimers were analyzed for activity over a range of temperatures from 23 to 37.5 °C. The energy of activation was determined by fitting data points derived from the ascending slope of each profile to the Arrhenius equation using the Sigma Plot program. The plots illustrated represent data from single experiments for each dimer; activation energies listed represent averages (± S.D.) of data from three separate experiments.

Type and both mutant forms of GALT and observed no significant effects (1). Nonetheless, at least in theory, the partial dominant negative effects that we have attributed here to the Q188R mutation could reflect some combination of effects of the Q188R mutation together with the His6 tag. Two previous observations argue against this possibility: 1) coexpression studies involving untagged wild-type and Q188R GALT subunits showed heterozygote activities depressed relative to those involving other mutations (15 and 2) coexpression studies involving overexpression of untagged Q188R GALT subunits, but not R333W GALT subunits, relative to the wild type resulted in depressed levels of GALT activity in crude extracts (data not shown). Although both of these observations suggest that the His6 tag was not functionally significant to the dominant negative effects associated with the Q188R mutation in the study reported here, we cannot formally rule out this possibility.

**Impact of Temperature on Activity**

The data reported here suggest that heterodimers involving the Q188R mutation lose activity at significantly lower temperatures than do either wild-type homodimers or heterodimers involving the R333W mutation. Although the data presented here do not distinguish between kinetic and thermodynamic events, they do demonstrate clear differences between the dimer populations investigated. Preliminary attempts at strictly kinetic studies of thermal denaturation of the wild-type homodimer suggested a bi- or multiphasic denaturation profile, significantly complicating these studies.

**Dimer Stability and Subunit Exchange**

To investigate stability of the homo- and heterodimers included in this study, a number of experiments were performed to reveal potential subunit dissociation in the wild-type enzyme under a variety of conditions. Purified dimers were subjected to conditions ranging from nondenaturing to 1 M NaCl and 5 M urea, with incubation times ranging from 0 to 48 h. Under none of these conditions was any evidence of subunit dissociation observed (data not shown). These results, demonstrating an extremely stable GALT dimer conformation, are fully consistent with previous reports involving both the human (1) and bacterial (32) enzymes.

**Heterodimers and Heterozygotes**

Finally, the results reported here raise the obvious question: do human carriers of the Q188R mutation exhibit less detectable GALT activity than do carriers of the R333W mutation or perhaps other mutations in GALT? Previously we have observed and reported subtle evidence from coexpression studies in yeast consistent with this hypothesis (15). The situation in humans may be more complex, however. In theory, assuming no differences between wild-type and Q188R or R333W GALT subunits in terms of synthesis, association, or stability, if Q188R/WT heterodimers exhibit 15% wild-type activity and R333W/WT heterodimers exhibit 50% wild-type activity, one would expect to see 32.5% wild-type activity in whole cell lysates from carriers of the Q188R allele and 50% wild-type activity in carriers of the R333W allele. Considering the broad range of activities observed even in the control population, a very large data set of genotyped and biochemically characterized galactosemia carriers will be required to resolve such a subtle distinction. Nonetheless, the results reported here, coupled with clinical reports of potential ophthalmologic (33–35) and gynecologic (36) risk factors identified for some galactosemia carriers, reinforce the importance of furthering our understanding not only of GALT mutations and their effects on homodimer function, but of their effects on subunit interaction and heterodimer function, as well.

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