Regulators of G protein signaling (RGS proteins) modulate G protein-mediated signaling pathways by acting as GTPase-activating proteins for $G_{i}$, $G_{q}$, and $G_{12}$ $\alpha$ subunits of heterotrimeric G proteins. Although it is known that membrane association is critical for the biological activities of many RGS proteins, the mechanism underlying this requirement remains unclear. We reported recently that the NH2 terminus of RGS16 is required for its function in vivo. In this study, we show that RGS16 lacking the NH2 terminus is no longer localized to the plasma membrane as is the wild type protein, suggesting that membrane association is important for biological function. The region of amino acids 7–32 is sufficient to confer the membrane-targeting activity, of which amino acids 12–30 are predicted to adopt an amphipathic $\alpha$-helix. Site-directed mutagenesis experiments showed that the hydrophobic residues of the nonpolar face of the helix and the strips of positively charged side chains positioned along the polar/nonpolar interface of the helix are crucial for membrane association. Subcellular fractionation by differential centrifugation followed by conditions that distinguish peripheral membrane proteins from integral ones indicate that RGS16 is a peripheral membrane protein. We show further that RGS16 membrane association does not require palmitoylation. Our results, together with other recent findings, have defined a unique membrane association domain with amphipathic features. We believe that these structural features and the mechanism of membrane association of RGS16 are likely to apply to the homologous domains in RGS4 and RGS5.

Regulators of G protein signaling (RGS1) proteins have emerged as major modulators of diverse aspects of biological activities (1–4). It has been well established that RGS proteins act as GTPase-activating factors for many of the heterotrimeric G protein $\alpha$ subunits (5–7). The core RGS domain is responsible for GTPase-activating protein activity, although it is not sufficient for biological function in vivo (8–12). The RGS4 NH2-terminal 33-amino acid domain is conserved in RGS5 and RGS16 and is predicted to adopt an amphipathic helix with clusters of basic residues. In this study, we demonstrate that the NH2-terminal domain of RGS16 is also required for membrane association and biological activity. Furthermore, with the aid of computer modeling, we found that the RGS16 membrane association domain has structural features most reminiscent of those found in the membrane intercalating domain of CTP:phosphocholine cytidylyltransferase (17). With that in perspective, we have carried out extensive mutational analysis to study the structure-function relationship of the RGS16 NH2-terminal domain. Our results fine map the core membrane association domain to the NH2-terminal amino acids 7–32 region. The bulk of this region consists of an amphipathic helix. The hydrophobic residues of the nonpolar face and the positive charge side chains positioned along the polar/nonpolar interface of the amphipathic helix are essential for membrane association. Moreover, we show that the conserved palmitoylation site in RGS16, Cys-12, which has been shown to be palmitoylated (12, 13), is also tightly membrane-bound and requires membrane association for its GTPase-activating protein activity toward $G_{i}$ (14, 15). It is unclear how they are targeted to the membrane. RET-RGS1, another potentially membrane-bound RGS member, contains a putative transmembrane domain and multiple cysteine residues for palmitoylation (9). GAIP and RGS4 also possess cysteine string motifs in their NH2 terminus and have been shown to be palmitoylated (12, 13). Although palmitoylated GAIP is found only in pellet fractions, the functional significance of palmitoylation has yet to be established. Surprisingly, palmitoylation of RGS4 is shown not to be required for membrane targeting (12). It is intriguing that a defective RGS4, which can no longer bind to G proteins, can be recruited to plasma membrane by a constitutively activating Ga subunit (16). These data suggest that RGS association with the plasma membrane may not be through simple binding to GTP-bound G proteins on the membrane but may rather involve an independent mechanism.

It has been shown recently that RGS4 requires its NH2 terminus for membrane association and biological function as assessed by its ability to substitute the function of Sst2p, an RGS member in yeast (12). As noted by Linder and colleagues (12), the RGS4 NH2-terminal 33-amino acid domain is conserved in RGS5 and RGS16 and is predicted to adopt an amphipathic helix with clusters of basic residues. In this study, we demonstrate that the NH2-terminal domain of RGS16 is also required for membrane association and biological activity. Furthermore, with the aid of computer modeling, we found that the RGS16 membrane association domain has structural features most reminiscent of those found in the membrane intercalating domain of CTP:phosphocholine cytidylyltransferase (17). With that in perspective, we have carried out extensive mutational analysis to study the structure-function relationship of the RGS16 NH2-terminal domain. Our results fine map the core membrane association domain to the NH2-terminal amino acids 7–32 region. The bulk of this region consists of an amphipathic helix. The hydrophobic residues of the nonpolar face and the positive charge side chains positioned along the polar/nonpolar interface of the amphipathic helix are essential for membrane association. Moreover, we show that the conserved palmitoylation site in RGS16, Cys-12, which has been shown to be palmitoylated in RGS4, plays a minor role in membrane association. Taken together, our findings and work done by others have defined a unique amphipathic domain that will provide a structural basis for RGS membrane association and biological activities.

**EXPERIMENTAL PROCEDURES**

*Chimeric RGS16-GFP Plasmids—To visualize the subcellular localization of RGS16 protein in yeast cells, the coding region of RGS16 was fused to green fluorescent protein (GFP) cDNA and was expressed in yeast cells under the control of a galactose-inducible promoter in the pMW29 vector as described previously (18). Briefly, a BamHI site was introduced to RGS16 cDNA just before the stop codon by polymerase
chain reaction. The sense and the antisense oligonucleotide primers were CTCGAGATGTCGCGACCCCTACGGCCCTC and GGCTGACCTCGAGAAGTGTGTCGCTGCTATT, respectively. The polymerase chain reaction product was then treated with Klenow DNA polymerase and polynucleotide kinase and ligated to BamHI/Klenow-treated pBluescript II. The resultant Klenow termini were used to replace the wild type NH2 terminus of the above created pMW29-RGS16-GFP through the internal SmaI site, creating pMW29-(7–201)RGS16 and pMW29-(11–201)RGS16. To create RGS16 NH2-terminal-GFP fusion constructs, the NH2-terminal coding fragments for the wild type RGS16 and individual deletion mutants were digested with SacI, blunt ended with Klenow polymerase, and released from the pBluescript with XhoI. These fragments were then fused in frame to the start of the GFP coding region in the pEGFPN1 vector, which was blunt ended, and digested with XhoI and ligated to the SmaI site of pMW29, resulting in pMW29-(1–32)-GFP, pMW29-(7–32)-GFP, and pMW29-(11–32)-GFP.

Site-directed Mutagenesis of RGS16 cDNA—A standard polymerase chain reaction method was used to produce six point mutations near the 5′-end of RGS16, C2A, F8D, F8G, F8K, P9A, P9D, and P9G. The other mutants, C12A, R15A, R15E, K17A, K17E, F19A, F19D, F19E, F19N, F19T, K20A, K20E, R22A, R22E, L23D, F26D, L27D, H28E/K29E, R5A/K17A, R15A/K15E, K20A/R22A, and K20E/R22E, were created by site-directed mutagenesis using the Transformer™ site-directed mutagenesis kit (CLONTECH). Using the above generated 5′- and 3′-primers were CATATGACCTTCCCCAACACCTGCGCTGTGAGAGAGCTTAAG, respectively; the common 3′-primer was GGTTGAACTGTGGTGAAGGCTCAGCTGAGCC. The resultant NH2 termini of these three deletion mutants were used to replace their corresponding wild type sequences in pMW29. The sequences of the oligonucleotide primers for mutagenesis are available upon request.

Mechanism of RGS16 Membrane Association—Phosphoinositide hydrolysis in response to the yeast mating pheromone, a1-factor, can be monitored by yeast growth on a cell growth lawn on an agar plate. RGS16 can substitute for the yeast Sst2p in the attenuation of pheromone signaling (18). However, removal of amino acids 1–10 or 1–13 rendered RGS16 inactive in both membrane association and attenuation of pheromone signaling (Fig. 1B), indicating that membrane attachment of RGS16 is important for its function in the cell.

The Region of Amino Acids 7–32 Is Sufficient for RGS16 Membrane Association—Secondary structure prediction analyses predicts two helices (H1 and H2, as depicted in Fig. 1) within the NH2-terminal membrane association domain of RGS4 (19, 20). The region predicted to form an α-helix was aligned to the target sequence (the membrane intercalation domain of cytidylyltransferase, PDB accession number 1peh). The subcellular localization was subsequently modeled based on the algorithm of Lee and Subbiah (21). The algorithm uses self-consistent ensemble optimization to determine the global minimum structure resulting in the location of side chains with high accuracy.

RESULTS

The NH2 Terminus of RGS16 Is Required for Membrane Attachment—We have shown previously that removal of amino acids 1–12 renders RGS16 inactive in the yeast halo assay, and yet the core RGS domain retains all of the biochemical features of a GTPase-activating protein (18, 19). Most recently, Linder and colleagues (12) have shown that a similar domain in RGS4 serves as a membrane targeting sequence and is also required for RGS4 function. This prompted us to study if this membrane association is a general requirement for RGS function. We fused the RGS16 NH2 terminus to GFP to test if this region could confer membrane-targeting activity to a heterologous protein in yeast. As shown in Fig. 1A, the first 32 amino acids were sufficient to direct GFP to the plasma membrane.

We then tested if the dysfunction of the N-deletion mutants of RGS16 is correlated to its failure to localize to the plasma membrane using the halo assay and confocal microscopy. In the halo assay, yeast cells, in response to pheromone, are arrested in the G1 phase of cell cycle and hence form a clear area or halo on a cell growth lawn on an agar plate. RGS16 can substitute for the yeast Sst2p in the attenuation of pheromone signaling and prevents halo formation (18). Deletion of amino acids 1–6 did not affect RGS16 membrane association or biological activity (Fig. 1B). However, removal of amino acids 1–10 rendered RGS16 inactive in both membrane association and in attenuation of pheromone signaling (Fig. 1B), indicating that membrane attachment of RGS16 is important for its function in the cell.

RGS16 Membrane Association—In YPD medium, RGS16 forms a plasma membrane complex with Gαi1, Gα12/13, and Gα16 (3). Disruption of RGS16 affects the regulation of the non-adenyl cyclase gpa1 G-protein system. RGS16 co-localizes with Gαi1 and Gα12/13 proteins in the plasma membrane (3). RGS16 is involved in the regulation of the Gq/11 pathway (22). RGS16 is involved in the regulation of the Gq/11 pathway (22).

Modeling of structures was performed using LOOK (Molecular Application Group). The secondary structure prediction method was based on discrimination of protein secondary structure class (20). The region predicted to form an α-helix was aligned to the target sequence (the membrane intercalation domain of cytidylyltransferase, PDB accession number 1peh). The subcellular localization was subsequently modeled based on the algorithm of Lee and Subbiah (21). The algorithm uses self-consistent ensemble optimization to determine the global minimum structure resulting in the location of side chains with high accuracy.

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The Region of Amino Acids 7–32 Is Sufficient for RGS16 Membrane Association—Secondary structure prediction analyses predicts two helices (H1 and H2, as depicted in Fig. 1) within the NH2-terminal membrane association domain of RGS4, comprising approximately amino acids 1–5 and 12–30, respectively. To examine the role of these helices, three deletions, removing amino acids 1–6, 1–10, and 1–13, were made. As shown above (Fig. 1), deletion of 1–6 did not affect any membrane association or biological activity, whereas removal of 1–10 or 1–13 rendered RGS16 inactive in both membrane association and attenuation of pheromone signaling.

To fine map residues essential for membrane association, residues 8 and 9 of RGS16 were mutated because these amino acids are conserved in RGS4, RGS5, and RGS16 (see Fig. 3A). Point 8 is an amino acid with conserved hydrophobic side chain (leucine in RGS4 and 5 and phenylalanine in RGS16); position 9 is a conserved proline residue. In RGS16, removal of the hydrophobic side chain at position 8 (LSG, LSK) led to a loss of biological activity, correlating with a loss in membrane association (Fig. 2). Substitution of the conserved proline (P9A and P9G) had no effect on RGS16 function. At both positions,
mutations, which introduce negative charge (L8D and P9D), significantly diminished RGS16 membrane association. The requirement of residue 8 to be hydrophobic and the deleterious effect associated with introducing negative charges at positions 8 and 9 suggest that the side chains of these two residues may physically interact with the plasma membrane. The above results, in combination of the deletion, thus define amino acids 7–32 as the core membrane association region.

Hydrophobic Residues in the \( \alpha \)-Helix of Amino Acids 12–30 Are Critical for Plasma Membrane Attachment—Previous studies by Linder and colleagues (12) suggest that the NH\(_2\)-terminal amino acids 1–33 sequence of RGS4 cannot be subdivided for membrane localization. This region is conserved among RGS4, RGS5, as well as RGS16. We therefore proceeded to study the role and structural features of the second helix (amino acids 12–30) in addition to residues at positions 8 and 9. We first predicted its three-dimensional protein structure via computer modeling and verified the reliability of the structure with structure-function analysis. The predicted three-dimensional structure of the helix is that of an amphipathic helix with a strip of hydrophobic residues on top and strips of positively charged residues along the polar/nonpolar interface of the amphipathic peptide (Fig. 3, B and C). Mutations were made for both the hydrophobic and basic residues to examine the contribution of these residues toward membrane targeting.

The hydrophobic strip consists of seven nonpolar residues. Single mutations of five of these nonpolar residues to an aspartate residue (A16D, F19D, L23D, F26D, and L27D) resulted in a loss of RGS16 biological activity (Fig. 4). Most notably, the A16D and F19D mutations completely abolished RGS16 function, as judged by its halo size close to that formed by cells

**FIG. 1.** Functional analysis of the RGS16 NH\(_2\) terminus. A, RGS16 NH\(_2\)-terminal amino acids 7–32 are sufficient to direct heterologous cytosolic protein GFP to plasma membrane. Constructs of the RGS16 NH\(_2\) terminus of various lengths with GFP at the COOH terminus in the yeast galactose-inducible expression vector pMW29 are diagrammed in the upper panel. The solid boxes indicate two putative \( \alpha \)-helices (H1 and H2) predicted by computer modeling. Numbers above the boxes indicate the corresponding amino acid residues in the RGS16 protein. Shown in the lower panel are confocal images indicating subcellular localization of each chimeric protein expressed in yeast cells (YDM400). B, RGS16 membrane association is correlated with its function. Constructs of RGS16 deleted of its NH\(_2\) terminus with GFP at the COOH terminus are shown in the top panel. Yeast transformants expressing individual GFP fusion proteins were subjected to halo assays and confocal microscope analysis. The ability of each protein to attenuate pheromone response and its localization in yeast are shown in the middle panel. Western blotting analysis indicating expression levels of these proteins is shown in the bottom panel. Numbers in parentheses in front of GFP represent the positions of amino acid residues in RGS16.
transformed with GFP alone. To establish the relevance of hydrophobicity in driving membrane targeting, Phe-19 was selected and was replaced by a series of residues with a decreasing degree of hydrophobicity (F19A, F19T, F19N, F19E, and F19D). As shown in Fig. 4B, the F19A substitution alone had little effect, whereas mutations leading to a decrease in the degree of hydrophobicity gave rise to a corresponding increase in the size of halo and a proportional increase in its distribution in the cytoplasm.

**Positively Charged Residues Are Augmentative to Membrane Attachment**—Based on the predicted model of the amphipathic helix (Fig. 3), the distribution of positively charged residues...
along the polar/nonpolar interface is reminiscent of those found in the amphipathic helices of phosphocholine cytidylyltransferase (17) and prostaglandin H synthase (22). In both enzymes, the helices serve as a membrane-targeting domain with positive charge residues positioned along the polar/nonpolar interface.

To study the role of these basic residues, a series of single and double mutations was generated. First, each basic residue was replaced with an alanine. The R22A mutation resulted in the loss of membrane targeting (Table I). R15A and K17A mutations had weak effects, whereas K20A had no effect. For double mutations, where a pair of basic residues was mutated to alanine residues, all resulted in the loss of membrane targeting (R15A/K17A, K20A/R22A, R15A/R22A, and K17A/K20A) (Table I). We repeated the alanine-scanning mutagenesis with a glutamate. Double mutations were generally more defective than the single mutations, all of which resulted in the loss of membrane targeting and biological activity. Among the single mutation series, the K20E replacement had little effect compared with other single mutations (R22E, R15E, and K17E). In the double mutation series, the H28E/K29E combination was less severe than others (R15E/K17E, K20E/R22E, R15E/R22E, K17E/K20E). The loss of function resulting from the replacement of the basic residues with either a nonpolar residue (Ala) or an acidic residue (Glu) demonstrates the importance of these basic residues. Because not all mutations on these basic residues have a similar effect on RGS16 biological activity, the role of these basic residues likely depends on their position along the amphipathic helix (see “Discussion”).

**RGS16 Is a Peripheral Membrane Protein**—To confirm the membrane localization of RGS16 in yeast, we fractionated by differential centrifugation cell homogenates from yeast cells transformed with the wild type RGS16 and from mutant F19D yeast cells as a control. Different fractions were then subjected...
to Western blotting analysis using the RGS16-specific antibody. As expected, most of the RGS16 (wild type) was present in the crude membrane pellet (P3 fraction, 100,000 × g), with less than 20% in the cytosol (Fig. 5A). In contrast, the F19D mutant protein was exclusively cytosolic. To assess the physical nature of the RGS16 interaction with the plasma membrane, supernatants (S1 fractions, 375 × g) were treated with conditions that solubilize peripheral membrane proteins or integral membrane proteins. RGS16 was stripped off the membrane when incubated with 0.5 M NaCl (high salt), 0.1 M Na2CO3 (pH 11.0), or 1% Triton X-100 but was only partially released from the membrane by treatment with 0.8 M urea (Fig. 5B). These data indicate that RGS16 is a peripheral membrane protein.

**Mutations of the Putative Palmitoylation Cysteine Residues Had Minor Effects on Plasma Membrane Association—Cys-2 and Cys-12 of RGS4 have been shown to be palmitoylation sites (12). RGS16 contains cysteine residues at the same positions. In this study, we mutated Cys-2 and Cys-12 to Ala and examined the effect of these Cys residues on RGS16 activity and its membrane association. Although alteration of Cys-2 to alanine had very little effect on membrane association or on the attenuating activity of pheromone signaling, the C12A mutation was more detrimental to the functional properties of RGS16 (Fig. 6). This is consistent with the results obtained with (7–201)-GFP showing that the first 6 amino acids are not essential for RGS16 function (Fig. 1B).**

**DISCUSSION**

We have shown previously that the core RGS domain of RGS16 retains full activity in G protein binding and GTPase-activating protein activity in vitro but requires the NH2 terminus for biological activity in vivo as assayed by its ability to attenuate pheromone signaling in yeast. Our work in this study clearly shows that RGS16 NH2 terminus plays a critical role in membrane association and also shows that membrane association may be important for RGS biological function, in agreement with what has been shown with RGS4. The first 33 amino acids of RGS4, RGS5, and RGS16 are highly conserved. It was
suggested that the entire domain of amino acids 1–33 in RGS4 forms a continuous amphipathic helix and is required for membrane association (12). However, our secondary structure analysis suggests that there may be two putative α-helices in the NH$_2$-terminal amino acids 1–32. Our deletion experiments suggest that the sequence of amino acids 1–6, which includes the first α-helix, is not relevant for biological activity. The rest of the NH$_2$ terminus (amino acids 7–32), which consists of predominantly the second putative α-helix from amino acids 12–30, has been defined as core membrane association domain. Furthermore, through detailed mutational analysis, we show that the amphipathic features of the α-helix determine membrane-targeting activity for RGS16, and these are likely shared by the homologous RGS4 and by RGS5.

Analysis of the predicted model of the RGS16 amphipathic helix reveals many similar characteristics found in amphipathic α-helices involved in membrane intercalation, e.g. the membrane association domains of CTP:phosphocholine cytidylyltransferase and prostataglandin H synthase (17, 22). Typically, the nonpolar face of the amphipathic helices consists of a strip of hydrophobic residues, whereas strips of positively charged residues are positioned along the polar/nonpolar interface. The important factors contributing to membrane association are 2-fold. First, hydrophobic interactions of the nonpolar face of the helix and the lipid core increase the propensity of the helix. Second, electrostatic interactions between the side chains of arginine and lysine, which form strips of positive charges along the polar/nonpolar interface of the helix, and the negatively charged amphiphile of the anionic phospholipids in the membrane, are important. All mutations on hydrophobic residues on the nonpolar side of the putative amphipathic helix led to a loss of function. Among the hydrophobic residues, Ala-16 and Phe-19 seem to be the most crucial, consistent with their location in the middle of the core amphipathic helix. The requirement of hydrophobicity for membrane intercalation is supported further by a series of mutations on a selected residue Phe-19. As expected, replacing Phe-19 with alanine itself had no detectable effect. However, mutations leading to gradual reduction of hydrophobicity resulted in corresponding reductions in membrane association. However, it should be noted that none of the NH$_2$-terminal mutants attains the null biological activity seen with GFP alone. As shown previously, the RGS domain of RGS16 still retains full GTPase-activating protein activity compared with the wild type protein (10). Fortuitous interaction with the Gpa1p of the RGS16 NH$_2$-terminal mutants or and small portions of the mutant proteins which still remain on the membrane (seen with even the most cytosolic mutant F19D (Fig. 5A)) may contribute to the observed partial activities.

Similarly, most mutations on the basic residues expectedly led to a loss of function. A single replacement of basic residues with an alanine generally had lesser effect on RGS16 compared with double mutants. This observation is consistent with the fact that multiple basic residues are positioned at the polar/nonpolar interface. The lesser effect of removal of a single positive charge may be caused by redundancy as its loss can be countered by neighboring basic residues. However, similar replacement with a glutamate will significantly introduce electrostatic repulsion at the polar/nonpolar interface, leading to reduction of helical stabilization and hence membrane intercalation. This possibility is supported by the observation that (Glu) mutations on the basic residues have much dramatic effects than their (Ala) mutants. Another interesting observation is that the K20A mutation has no effect on biological activity of RGS16. This is consistent with the predicted structure of the amphipathic helix, where the side chain of Lys-20 is oriented toward the lipid core and away from the polar/nonpolar interface. This deviation explains why the loss of the positive side chain of Lys-20 has no effect on the helical stabilization.

Many membrane association mechanisms commonly involve cooperative interaction of two components: hydrophobic components and a cluster of basic residues. In MARCKS (myristoylated alanine-rich C kinase substrate) proteins or p60Src, the hydrophobic component is a myristoyl group (23, 24); in CTP:phosphocholine cytidylyltransferase, it is the hydrophobic side chains in the nonpolar face of the amphipathic α-helix (17). In the case of RGS16 and most probably RGS4 and RGS5, the mechanism used may resemble that of CTP:phosphocholine cytidylyltransferase. However, there are noticeable differences. The length of the predicted amphipathic α-helix of RGS16 is about half the size of CTP:phosphocholine cytidylyltransferase. Second, unlike the latter, RGS16 has the conserved palmitoylation site within the amphipathic helix. Although studies on RGS4 and RGS16 in yeast do not demonstrate significant relevance of palmitoylation in membrane targeting, it is difficult to rule out its role in mammalian cells. It is of great interest to understand the role of palmitoylation in RGS proteins.

As a first approximation, our findings suggest that RGS16 interacts with the plasma membrane through hydrophobic interactions between nonpolar residues of RGS16 and membrane lipids and through electrostatic interactions between the basic residues and the anionic phospholipids in the membrane. However, the mechanism of RGS membrane association is complicated by several reported observations. First, RGS4 and RGS16 appear to be largely present in the membrane in yeast cells, but these proteins are largely located in the cytosol in mammalian cells (16). Second, cotransfection of a constitutively activating Gαq can bring a G protein-binding-defective RGS4 protein to the plasma membrane (16). Furthermore, a recent report shows that the calcium ionophore A23187 mimics agonist-induced redistribution of RGS3 (25). These observations all point to a possibility that there may be a complex mechanism regulating the subcellular redistribution of RGS proteins which involves downstream events of G protein activation. One possibility is that G protein activation somehow triggers binding of a cellular factor to the membrane-targeting region of the RGS proteins for translocation to the membrane. In fact, we have identified a membrane protein that binds strongly to the exact region required for membrane association. The biological significance of this interaction remains to be seen. Nevertheless, our extensive mutational analysis and computer modeling have defined a unique membrane association domain that is shared by RGS4, RGS5, and RGS16. Understanding the structural basis of the RGS membrane targeting domains will definitely help understand how RGS proteins function to attenuate signaling pathways by G protein-coupled receptors.

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The Membrane Association Domain of RGS16 Contains Unique Amphipathic Features That Are Conserved in RGS4 and RGS5
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