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Identification and Characterization of a Small Modular Domain in the Herpes Simplex Virus Host Shutoff Protein Sufficient for Interaction with VP16

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The herpes simplex virus transactivator VP16 and the virion host shutoff protein vhs are viral structural components that direct the activation of immediate-early gene expression and the arrest of host protein synthesis, respectively, during an infection. Recent studies show that VP16 and vhs physically interact with each other in vitro and in infected cells, suggesting that their respective regulatory functions are coupled. In this report, we used the yeast two-hybrid system and affinity chromatography with purified VP16 fusion proteins to precisely map a region in vhs that directs interaction with VP16. Deletion analysis of vhs demonstrated that a 21-amino-acid-long domain spanning residues 310 to 330 (PAAGGTEMRVSWTEILTQQIA) was sufficient for directing complex formation with VP16 in vivo and in vitro when fused to a heterologous protein. Site-directed mutagenesis of this region identified tryptophan 321 as a crucial determinant for interaction with VP16 in vitro and in vivo and additional residues that are important for stable complex formation in vitro. These findings indicate that vhs residues 310 to 330 constitute an independent and modular binding interface that is recognized by VP16.

Herpes simplex virus (HSV) affords a useful model to investigate the mechanisms involved in the coordinated regulation of gene expression at both the transcriptional and posttranscriptional levels. The HSV type 1 (HSV-1) genome encodes more than 70 polypeptides that can be divided into at least three classes based on their kinetics of appearance during a lytic infection: immediate-early (IE, or α), early (E, or β), and late (L, or γ) (reviewed in references 43 and 53). The viral genes belonging to the different temporal classes are expressed in a tightly regulated cascade fashion during the course of an infection, mediated in part through combinatorial interactions among both viral and host regulatory factors.

At least two important viral regulatory proteins exist as preformed structural components of the herpes simplex virion and are delivered into the host cell by the infecting viral particle. The most prominent and best characterized of these virion-associated regulatory factors is VP16 (also called Vmw65 and αTIF), an abundant 490-amino-acid-long phosphoprotein that is present in the tegument of the virus (3). VP16 potently stimulates transcription of the viral IE genes by recognizing *cis*-regulatory consensus TAATGARAT (where R is a purine) target sites present in the promoters of the IE genes (reviewed in references 33, 44, and 52). VP16 does not bind to these sites directly but instead orchestrates the cooperative assembly of a multicomponent DNA-binding complex that includes at least two cellular factors, the octamer-binding protein Oct-1, which binds directly to TAATGARAT elements, and HCF (also called VCAF-1 and C1), which binds directly to VP16 and mediates its stable association with DNA-bound Oct-1 (18, 21, 23–25, 34, 40, 50, 56, 57). The spatial arrangement of the assembled complex positions the modular carboxyl-terminal acidic activation domain of VP16 for functional interaction with downstream targets, which include members of the basal transcription machinery (29, 51).

Transcriptional activation of IE genes by VP16 greatly increases the likelihood of productive infection; however, the activation function of VP16 is not absolutely essential for virus growth, since viruses that bear derivatives of VP16 compromised in protein-DNA complex assembly (1) or missing the acidic activation domain (28) remain viable in tissue culture when infections are carried out at high multiplicities. VP16 does however appear to play an essential structural role, since deletion of the VP16 open reading frame is lethal (54). The precise role of VP16 in virus morphogenesis remains to be established.

A second important regulatory event induced by one or more components of the infecting viral particle is the rapid cessation of host protein synthesis and degradation of host mRNAs soon after virus penetration and uncoating (12, 15, 16, 45). A large body of evidence indicates that this is mediated by the virion host shutoff protein vhs, a 489-amino-acid-long phosphoprotein which, like VP16, is a located in the viral tegument (13, 14, 27, 31, 35, 36, 41, 42, 47, 49). Vhs is less abundant than VP16 and is not essential for viral growth (42, 49). However, vhs null mutants display altered patterns of viral protein synthesis during infection, and virus yield is reduced approximately 10-fold, suggesting that vhs plays an important role in the lytic cycle (41, 49). vhs is sufficient for inhibiting protein synthesis and triggering mRNA turnover in the absence of other viral factors; however, its mechanism of action remains to be elucidated (22, 38). Recent in vitro studies suggest that vhs may promote RNA cleavage of 5′-capped mRNA substrates (10).

Viral mRNAs are also targets for vhs-mediated turnover; thus, activity from newly synthesized vhs must be downregulated during a lytic infection in order to sustain viral protein synthesis at late times (14–16, 26, 35, 36). Evidence suggests that vhs activity is downregulated by one or more newly synthesized viral proteins (13, 14, 16). Recently, we have demon-

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strated that vhs forms a complex with VP16 in vitro and in infected cells, suggesting that VP16 may serve to restrain vhs function (48). Consistent with this hypothesis, infection of nonpermissive cells with the VP16 null mutant 8MA (54) results in a precipitous decline in viral protein synthesis at early and late times, due in part to rapid degradation of viral mRNAs (28). Viral mRNA levels and protein synthesis are restored in virus expressing a transactivation defective, vhs-binding derivative of VP16 and in an 8MA derivative deleted in vhs, suggesting that rapid mRNA turnover in nonpermissive cells infected with 8MA is the result of unbridled vhs activity due to the absence of VP16 (28). These findings indicate that VP16 regulates viral gene expression at the posttranscriptional level by suppressing the activity of vhs at early and late times, thereby sparing viral mRNAs from destruction.

The mechanism(s) by which VP16 serves to dampen vhs function is not known. The simplest model is that binding of VP16 directly inactivates vhs. Alternatively, or in addition, VP16 may translocate vhs into the nucleus and/or into the viral assembly pathway. Identification of the determinants in the respective proteins that direct protein-protein interaction will contribute to understanding the functional significance of VP16-vhs complex formation in viral replication. In this study, we examined the sequence requirements in vhs that direct interaction with VP16. Using the two-hybrid system in *Saccharomyces cerevisiae* (17) and in vitro solid-phase capture assays with immobilized VP16, we demonstrate that a 21-amino-acid-long domain encompassing vhs residues 310 to 330 is sufficient for interaction with VP16.

MATERIALS AND METHODS

Plasmids. (i) Plasmids for two-hybrid analysis. DBD-vhs(179–344) is a yeast expression plasmid which expresses the GAL4 DNA-binding domain (DBD; amino acid residues 1 to 147) fused to the vhs *Apa*I-*Sma*I fragment (residues 179 to 344) and is suitable for two-hybrid analysis in *S. cerevisiae*; it has been described before (48). DBD-vhs(179–344)*in* is identical to DBD-vhs(179–344) except that it contains an in-frame *Xho*I linker (5′-CCCCTCGAGGGG) inserted between the codons for amino acid residues 330 and 331. It was constructed by substituting a fragment from a plasmid bearing a vhs gene that contained this linker insertion (pUC/CMV-vhsN331 [22]) for the corresponding fragment between the *Nru*I and *Sac*I sites in DBD-vhs(179–344). To generate DBD-vhs(179–330), the *Nru*I-*Eco*RV fragment from the in vitro expression vector pSPAS, which expresses vhs(179–344) from an SP6 promoter (48), was replaced with the *Nru*I-*Xho*I fragment from pUC/CMV-vhsN331 (after first filling in the *Xho*I overhang). This plasmid, designated pSPAX, was digested with *Nco*I (which provides the initiator methionine codon) and *Bgl*II (present downstream in the polylinker) and cloned in place of the *Nco*I-*Bgl*II fragment in DBD-vhs(179–344) to generate DBD-vhs(179–330). DBD-vhs(238–344) was constructed by cloning the *Nru*I-*Sac*I fragment from pSPAS into the corresponding sites of the low-copy parental GAL4 DBD expression vector pPC97(5). Similarly, DBD-vhs(238–310) was constructed by cloning the *Nru*I-*Xma*III fragment of vhs into the *Sma*I and *Not*I sites of pPC97. DBD-vhs(310–330) was constructed by cloning the *Xma*III-*Bgl*II fragment from pSPAX into the *Sma*I and *Bgl*II sites in the pPC97 polylinker after first filling in the *Xma*III end. DBD-vhsMBD contains the synthetic oligonucleotide 5′-GGCCGCCGGCGGTACCGAGATGCGCGTCAGC TGGACCGAAATATTAACCCAACAGATCGCCTA and its complement, 5′-GATCTAGGCGATCTGTTGGGTTAATATTTCGGTCCAGCTGACGCGC ATCTCGGTACCGCCGGC. This cassette encodes vhs residues 310 to 330, incorporates a TAG termination codon after codon 330, and contains *Xma*III- and *Bgl*II-compatible ends.

GAD-VP16$_{424}$, GAD-VP16$_{404}$, GAD-VP16$_{379}$, GAD-VP16$_{369}$, GAD-VP16$_{335}$, and GAD-VP16$_{\Delta 141-178}$ are yeast two-hybrid low-copy plasmids that express carboxyl-terminally truncated derivatives of VP16 (the number refers to the carboxyl-terminal deletion endpoint) or an internal deletion of VP16 residues 141 to 178 (derived from GAD-VP16$_{424}$) as fusions to the GAL4 acidic activation domain (GAD) and have been described previously (39, 48).

(ii) Plasmids for in vitro expression. pSPAS and pSPAX, the in vitro transcription-translation plasmids for vhs(179–344) and vhs(179–330), respectively, are described above. pSP(vhs) expresses full-length vhs, and pSP-vhsΔSma expresses vhs that bears an internal deletion of amino acids 149 to 344 (48). The in vitro expression vector for pSP-vhs(310–331) was constructed by cloning the *Xho*I-*Sac*I fragment from DBD-vhs(179–344) into the *Sal*I and *Sac*I sites of the in vitro expression plasmid pSPUTK (11). This plasmid expresses residues 310 to 330 of vhs linked to the GAL4 DBD. Similarly, pSP-vhsMBD expresses the synthetic oligonucleotide cassette encoding residues 310 to 330 described above linked to the GAL4 DBD. pSP-DBD(*Xma*) and pSP-DBD(*Cla*) are two vectors that express the GAL4 DBD and were constructed by subcloning the *Xho*I-*Sac*I and *Xho*I-*Cla*I fragments bearing the GAL4 DBD coding region from pPC97 and pPC62 (5), respectively, into pSPUTK. The accuracy of plasmid construction in each case was verified by dideoxy DNA sequence analysis with a commercially available kit (Sequenase; US Biochemical).

Yeast transformation and β-galactosidase assay. Two-hybrid expression plasmids were transformed into *S. cerevisiae* PCY2 (*MAT*α *Δgal4 Δgal80 URA3*::*GAL1-lacZ lys2-801*^{amber} *his3-Δ200 trp1-Δ63 ade2-101*^{ochre}) or Y190 (*MAT*α *gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,112 URA3*::*GAL lacZ*, *lys2*::*gal HIS3 cyh*^{r}) by the lithium acetate procedure (9). Leu$^+$, His$^+$, and Trp$^+$ transformants were selected as appropriate on synthetic complete medium plates lacking the corresponding amino acids. For liquid β-galactosidase assays, cells were grown in medium lacking appropriate amino acids and assayed for β-galactosidase activity by the sodium dodecyl sulfate (SDS)-chloroform permeabilization method (2). Filter assays were carried out by transferring colonies from plates to Whatman no. 3 filter paper. The filter was placed in liquid nitrogen to break open the cells and then incubated in 2 ml of buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0) supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 1 mg/ml). Filters were incubated at 37°C and observed for development of blue color. Strong two-hybrid interactions were detected within 30 min. Filters were incubated overnight in order to detect weak activity.

Oligonucleotide-directed cassette mutagenesis. The synthetic oligonucleotide encoding residues 310 to 330 of vhs was designed to incorporate unique *Xma*III, *Kpn*I, and *Pvu*II restriction sites (see Fig. 1) to facilitate cassette mutagenesis of this region. Short, double-stranded oligonucleotide cassettes designed to convert selected amino acids to alanine residues and containing appropriate ends for cloning into the above restriction sites were synthesized, phosphorylated at their 5′ ends with T4 polynucleotide kinase, and purified on 4% NuSieve agarose gels. Mutant oligonucleotide cassettes were used to replace the corresponding wild-type regions in the yeast and in vitro vhsMBD expression plasmids following digestion of these plasmids with the appropriate restriction enzymes and dephosphorylation with calf intestinal alkaline phosphatase. The point mutants generated by this procedure are listed in Fig. 3. A deletion derivative lacking codons for amino acids 321 to 330 was also constructed. The accuracy of mutagenesis in all cases was verified by dideoxy DNA sequence analysis.

In vitro transcription and translation. In vitro transcription with SP6 polymerase and translation in rabbit reticulocyte lysates were carried out with a commercially available coupled system (Promega) according to the manufacturer's protocol.

Purification of PA-VP16 and MBP-VP16 fusion proteins. The purification of protein A-VP16 (PA-VP16) and maltose-binding protein-VP16 (MBP-VP16) fusion proteins from induced bacterial cultures has been described before (39, 55, 57). Both MBP-VP16 and PA-VP16 contain residues 4 to 411 of VP16 and are thus missing the carboxyl-terminal acidic activation domain. Unfused protein A and maltose-binding protein were purified for use as controls in the binding assays.

Solid-phase capture assay. Purified PA-VP16 was covalently coupled to CNBr-activated Sepharose (Pharmacia) at 2 mg of protein per ml of settled beads as before (57). Beads were stored at 4°C as a 50% slurry in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9)–50 mM NaCl–1 mM dithiothreitol–10% glycerol–1 mM phenylmethylsulfonyl fluoride. [^{35}S]methionine-labeled proteins (5 μl of programmed rabbit reticulocyte lysate) were incubated with 45 μl of beads for 2.5 h at 4°C in 400 μl of buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.2), 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, and 2% bovine serum albumin (BSA). Beads were washed extensively with the same buffer followed by a final wash with buffer in the absence of BSA. Bound material was eluted by boiling the settled beads in an equal volume of twice-concentrated SDS-polyacrylamide gel electrophoresis sample buffer (2% SDS, 2% β-mercaptoethanol, 250 mM Tris-HCl, pH 6.8) and analyzed by SDS-polyacrylamide gel electrophoresis.

Solid-phase capture assays were also performed with immobilized MBP-VP16. Amylose beads coupled with MBP-VP16 were prepared as described previously (39). MBP-VP16 beads in 20 mM Tris-HCl (pH 7.4)–200 mM NaCl–1 mM EDTA–0.5 mM phenylmethylsulfonyl fluoride–1 mM dithiothreitol were incubated with labeled proteins as above and washed extensively in the above buffer followed with buffer supplemented with 0.01% BSA and 0.05% Nonidet P-40. Bound material was eluted from the beads and analyzed as above.

RESULTS

A 21-amino-acid-long region of vhs is sufficient for interaction with VP16 in the two-hybrid system. We have previously demonstrated that a derivative of vhs encompassing residues 238 to 344 was sufficient for interaction with VP16 in vitro (48). The yeast two-hybrid system was used to more precisely map the minimal region of vhs sufficient for stable interaction with

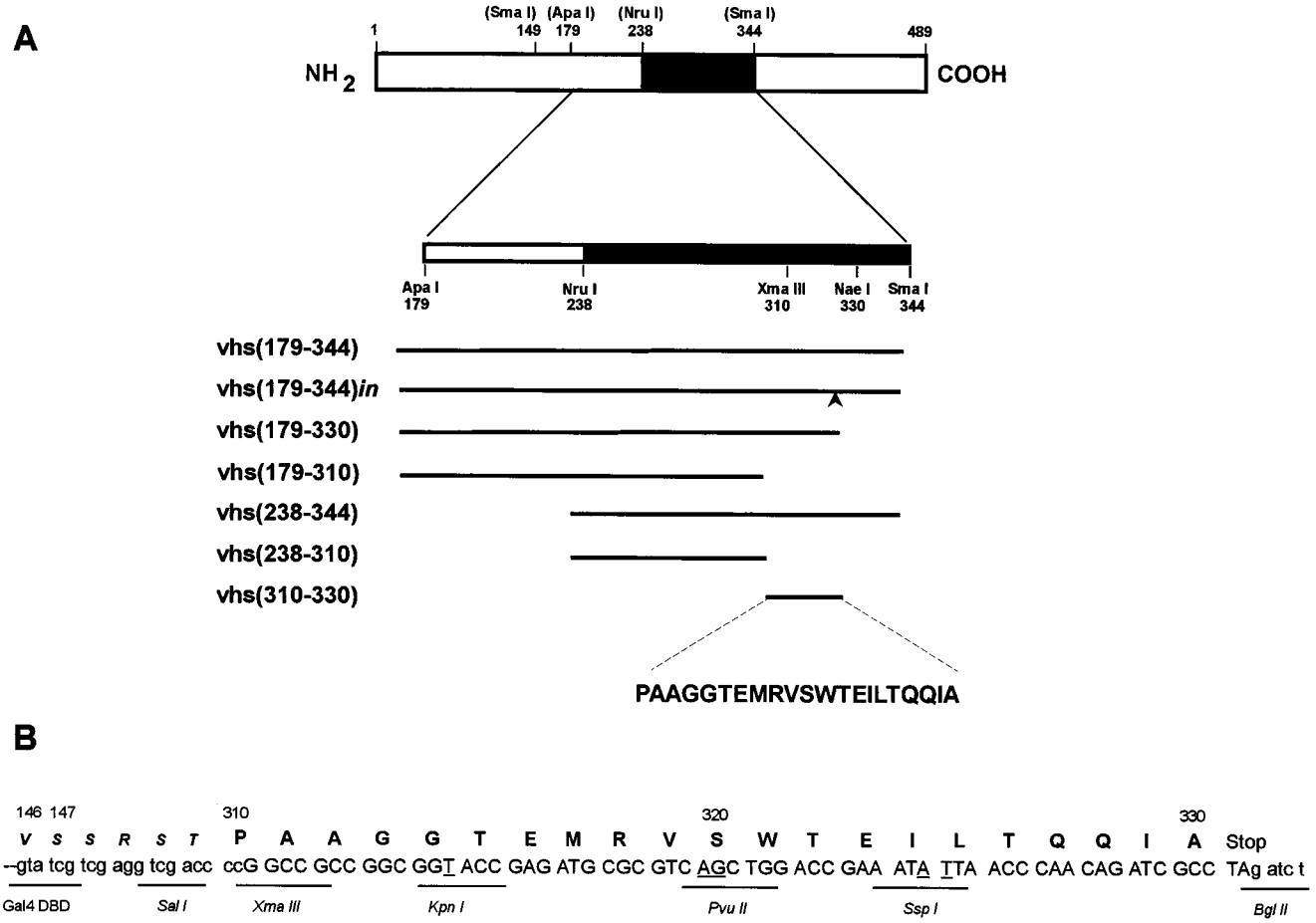

FIG. 1. (A) Schematic representation of the HSV-1 vhs gene and structure of deletion derivatives. The filled-in region between the *Nru*I and *Sma*I sites corresponds to the region of vhs previously shown to be sufficient for binding to VP16 in vitro (48). Deletions were constructed in the *Apa*I-*Sma*I fragment as shown and cloned into vectors suitable for two-hybrid analysis in *S. cerevisiae* or expression in vitro. The arrow in vhs(179–344)*in* indicates the position of an *Xho*I linker insertion. The primary amino acid sequence between residues 310 and 330 is shown at the bottom. The published sequence of vhs from HSV-1 strain 17 contains a threonine at position 317 in place of the methionine found in vhs from the KOS strain used in this study (30). (B) Structure of the synthetic oligonucleotide spanning residues 310 to 330. The top strand of the synthetic oligonucleotide is shown in capital letters. The underlined nucleotides are silent substitutions of the wild-type sequence that were incorporated in order to generate novel restriction sites. The TAG termination codon immediately following residue 330 is generated following cloning into the *Bgl*II site. The sequence of the end of the GAL4 DNA-binding domain coding region (encoding amino acids 1 to 147 of GAL4) and the junction to the vhs coding region are shown.

VP16 in vivo. A series of carboxyl- and amino-terminal deletions of the vhs *Apa*I-*Sma*I fragment (residues 179 to 344 [Fig. 1]) were generated and cloned into a GAL4 DBD expression vector. Plasmids encoding the DBD-vhs derivatives were cotransformed into yeast cells with GAD-$VP16_{404}$ (expresses VP16 residues 4 to 404 as a fusion to the GAL4 GAD), and transformants were assayed for expression of the resident GAL4-responsive *lacZ* reporter gene by measuring β-galactosidase activity by both liquid and filter assays. As demonstrated previously (48) and shown in Table 1, β-galactosidase activity was observed only when the DBD-vhs(179–344) and GAD-$VP16_{404}$ plasmids were cotransformed, indicative of functional interaction between the two expressed hybrid proteins. Interaction with $VP16_{404}$ was also observed with DBD-vhs(179–344)*in*, DBD-vhs(179–330), and DBD-vhs(238–344). Thus, residues 238 to 330 contain determinants necessary for interaction with VP16 in *S. cerevisiae*. Activity was not observed with DBD-vhs(179–310) or DBD-vhs(238–310). Western immunoblot analysis with antiserum directed against the GAL4 DBD demonstrated that the nonfunctional fusion proteins were expressed (46).

The above findings indicate that an important determinant for binding to VP16 lies between residues 310 and 330. To determine if this small region constitutes an independent and modular binding domain, DBD-vhs(310–330) was constructed and tested. This derivative activated expression of the *lacZ* gene in the presence of $VP16_{404}$ to a level of approximately 40 to 50% of that of DBD-vhs(179–344), indicating that this small region is sufficient for interaction with VP16. The higher enzymatic activity with the longer vhs fusion protein may indicate that the extended protein is more stable or that residues outside the minimal domain contribute to binding efficiency. Alternatively, there may be differences in the activation potential of the respective two-hybrid transactivator complexes.

The DBD-vhs(310–330) fusion protein contained three additional amino acids at the carboxyl terminus as a result of the cloning procedure. To ensure that these extra amino acids were inert in the two-hybrid assay, an oligonucleotide cassette spanning residues 310 to 330 and containing a termination codon was synthesized and cloned into the GAL4 DBD vector (see Fig. 1). This synthetic oligonucleotide is referred to as the vhs minimal binding domain (MBD). Table 1 shows that DBD-

TABLE 1. Interaction of vhs deletion mutants with VP16 in yeast cells[a]

GAL4 DBD hybrid	GAL4 GAD hybrid	β-Galactosidase activity	
		Filter assay (colony color)	Liquid assay (U ± SD)
DBD	None	White	<0.1
	GAD	White	<0.1
	$VP16_{404}$	White	<0.1
vhs(179–344)	None	White	<0.1
	GAD	White	<0.1
	$VP16_{404}$	Blue	80 ± 4
vhs(179–344)*in*	None	White	<0.1
	$VP16_{404}$	Blue	84 ± 7
vhs(179–330)	None	White	<0.1
	$VP16_{404}$	Blue	76 ± 9
vhs(179–310)	None	White	<0.1
	$VP16_{404}$	White	<0.1
vhs(238–344)	None	White	<0.1
	$VP16_{404}$	Blue	83 ± 6
vhs(238–310)	None	White	<0.1
	$VP16_{404}$	White	<0.1
vhs(310–330)	None	White	<0.1
	$VP16_{404}$	Blue	36 ± 3
vhsMBD	None	White	<0.1
	$VP16_{404}$	Blue	34 ± 4
vhsMBDΔ321–330	None	White	<0.1
	$VP16_{404}$	White	<0.1

[a] *S. cerevisiae* PCY2 was transformed with the indicated plasmids (DBD, GAL4 DNA-binding domain; GAD, GAL4 acidic activation domain) and assayed for β-galactosidase activity. For liquid assays, units of β-galactosidase activity are averages from at least three independent transformants assayed in duplicate and normalized to cell density (OD_{600}). For the filter assay, blue color, where indicated, was observed within 30 min following incubation of filters with buffer containing X-Gal. Where white is indicated, blue color was not observed even after overnight incubation.

vhsMBD interacted with $VP16_{404}$ as efficiently as did DBD-vhs(310–330), indicating that the appended extra amino acids did not influence interaction with VP16. A deletion derivative of this region, missing residues 321 to 330 (DBD-vhsMBDΔ 321–330), was inactive. Thus, a 21-amino-acid domain of vhs encompassing amino acid residues 310 to 330 is sufficient for interaction with VP16 in *S. cerevisiae.*

Specificity of interaction of the vhs minimal binding domain. In contrast to the small contiguous binding domain in vhs identified above, which suffices for interaction with VP16 in *S. cerevisiae*, the vhs binding interface in VP16 appears to consist of a large and perhaps noncontiguous domain. We previously demonstrated that the carboxyl-terminal end of VP16 that is necessary for interaction with vhs in vitro and in vivo mapped to amino acid 369 and that additional elements closer to the amino terminus were also required (48). In order to determine if the binding characteristics and specificity of the minimal region of vhs defined above were similar to those of the larger vhs protein, we compared the interaction of DBD-vhsMBD in yeast cells with a series of VP16 mutant derivatives. As shown in Table 2, DBD-vhsMBD interacted with VP16 truncated at amino acid 424, 404, 379, or 369 but not with VP16 truncated at amino acid 335 or with VP16 that contained an internal deletion of residues 141 to 178. Interaction was more efficient with GAD-$VP16_{404}$ and GAD-$VP16_{369}$ than with GAD-$VP16_{424}$ and GAD-$VP16_{379}$ if relative β-galactosidase activities are used as a measure. The same spectrum of interaction and relative activities was observed with DBD-vhs(179–344). Thus, the minimal peptide requires similar determinants in VP16 for interaction in vivo as does the larger vhs protein.

TABLE 2. Interaction of the minimal binding domain of vhs with VP16 deletion mutants in yeast cells

GAL4 DBD hybrid	GAL4 GAD hybrid	β-Galactosidase activity[a] (U ± SD)
vhs(179–344)	$VP16_{424}$	56 ± 10
	$VP16_{404}$	101 ± 2
	$VP16_{379}$	67 ± 6
	$VP16_{369}$	82 ± 6
	$VP16_{335}$	<0.1
	$VP16_{\Delta 141–178}$	<0.1
vhsMBD	$VP16_{424}$	13 ± 0.4
	$VP16_{404}$	34 ± 4
	$VP16_{379}$	21 ± 2
	$VP16_{369}$	48 ± 10
	$VP16_{335}$	<0.1
	$VP16_{\Delta 141–178}$	<0.1

[a] β-Galactosidase activity was measured as described in Table 1, footnote *a.*

Interaction of vhs with VP16 in vitro. To determine if the results obtained with the two-hybrid system reflected direct physical interaction between vhs and VP16, vhs derivatives were cloned into an in vitro transcription-translation vector, and the resultant [^{35}S]methionine-labeled polypeptides were tested for their ability to bind to a VP16 fusion protein (PA-VP16) that was covalently coupled to Sepharose beads. Full-length vhs, vhs(179–344), and vhs(Δ*Sma*) (a derivative missing residues 149 to 344) were synthesized in an unfused form; however, because of the small size of the minimal domain (2 kDa), derivatives encompassing this region were expressed in vitro as fusions to the GAL4 DBD. As controls, we also constructed two vectors that expressed the GAL4 DBD on its own [pSP-DBD(*Cla*) and pSP-DBD(*Xma*)].

The labeled vhs peptides were incubated with the VP16-coupled beads, and after extensive washing, the bound material was eluted by boiling in 2% SDS and analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 2A, vhs(179–344) bound specifically to PA-VP16 beads (compare lanes d and h; approximately 10 to 20% of the input vhs protein bound to the VP16 beads), while vhsΔ*Sma* (lane c) and the luciferase control (lane b) did not bind to either VP16 beads or control beads. Similarly, vhs(310–330) (Fig. 2A, lanes e, i, and n) and vhsMBD (Fig. 2B, lanes a and e) bound specifically to the PA-VP16 beads, whereas binding over background levels was not observed with the control GAL4 DBD proteins DBD(*Xma*) and DBD(*Cla*) (Fig. 2B, lanes g and h). Thus, amino acids 310 to 330 of vhs appended to a heterologous protein are sufficient for mediating direct protein-protein interaction with VP16 in vitro.

Tryptophan 321 is a critical residue for interaction. In order to identify individual residues in the minimal binding domain important for interaction with VP16, we converted specific amino acids to alanine residues by cassette mutagenesis. Alanine residues were chosen because they would not be expected to significantly affect the structure of the polypeptide backbone (6). A total of 12 alanine substitution mutants were created; T315A, E316A, R318A, S320A, W321A, T322A, E323A, I324A, T326A, Q327A, Q328A, and I329A (Fig. 3). The mutant oligonucleotides were cloned into the GAL4 DBD expression vector and tested for β-galactosidase activity following cotransformation of yeast cells with GAD-$VP16_{404}$ (Fig. 3). All of the derivatives, with one notable exception, remained capable of inducing β-galactosidase activity in the presence of $VP16_{404}$ and did so with an efficiency that was comparable to that of the

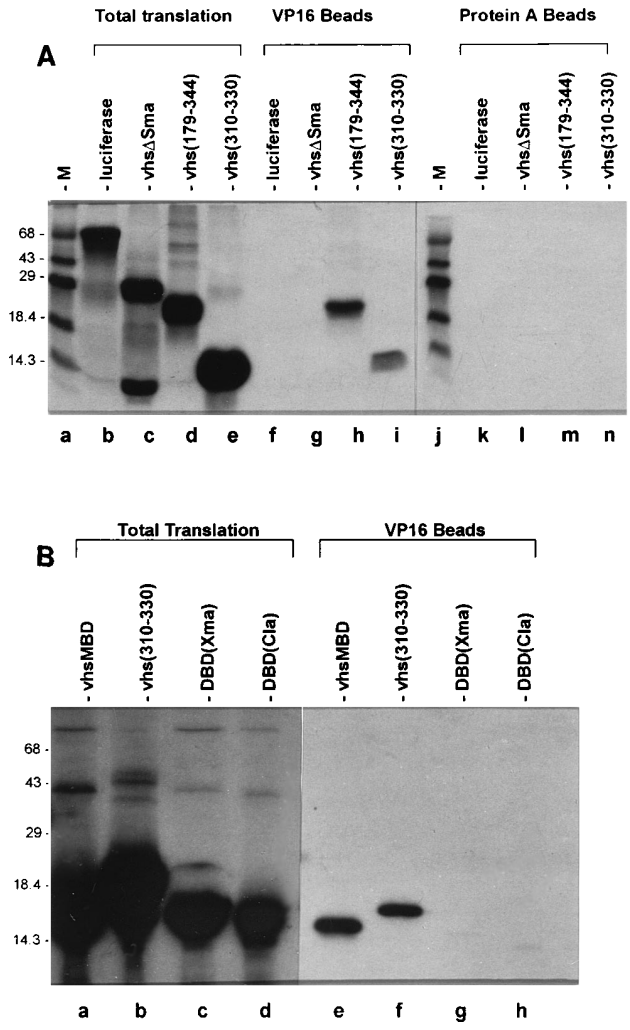

FIG. 2. Amino acids 310 to 330 of vhs are sufficient for interaction with VP16 in vitro. [^{35}S]methionine-labeled vhs derivatives were prepared in rabbit reticulocyte lysates programmed with the corresponding in vitro-transcribed RNAs. Vhs(310–330) and vhsMBD were expressed as fusions to the GAL4 DBD. Aliquots of the total translation mixture were incubated with PA-VP16 or protein A-coupled Sepharose, as indicated. The beads were washed extensively, and bound material was eluted by boiling in 2% SDS and analyzed by SDS-polyacrylamide gel electrophoresis. (A) Binding of vhs(330–331) to PA-VP16. vhs(179–344) was the positive control, while vhsΔ*Sma* was the negative control. (B) Binding of vhsMBD to PA-VP16. The GAL4 DBD derivatives DBD(*Xma*) and DBD(*Cla*) are negative controls. The positions of molecular size markers (lane M) are shown (in kilodaltons).

	310 315 320 325 330	β-galactosidase activity	
		- VP16$_{404}$	+ VP16$_{404}$
vhsMBD	PAAGGTEMRVSWTEILTQQIA	< 0.1	82 ± 5
T315A	PAAGGAEMRVSWTEILTQQIA	< 0.1	84 ± 8
E316A	PAAGGTAMRVSWTEILTQQIA	< 0.1	75 ± 7
R318A	PAAGGTEMAVSWTEILTQQIA	< 0.1	72 ± 10
S320A	PAAGGTEMRVAWTEILTQQIA	< 0.1	72 ± 14
W321A	PAAGGTEMRVSATEILTQQIA	< 0.1	< 0.1
T322A	PAAGGTEMRVSWAEILTQQIA	< 0.1	84 ± 6
E323A	PAAGGTEMRVSWTAILTQQIA	< 0.1	83 ± 6
I324A	PAAGGTEMRVSWTEALTQQIA	< 0.1	78 ± 4
T326A	PAAGGTEMRVSWTEILAQQIA	< 0.1	80 ± 3
Q327A	PAAGGTEMRVSWTEILTAQIA	< 0.1	81 ± 4
Q328A	PAAGGTEMRVSWTEILTQAIA	< 0.1	84 ± 5
I329A	PAAGGTEMRVSWTEILTQQAA	< 0.1	80 ± 6

FIG. 3. Structure of alanine substitution mutants of the vhs minimal binding domain and activity in the two-hybrid system. Specific amino acids in the 310 to 330 region of vhs were altered to alanine residues (underlined) by cassette mutagenesis of the oligonucleotide shown in Fig. 1 and tested for interaction with VP16 in the two-hybrid system. β-Galactosidase activity (units) was measured as described in Table 1, footnote *a*, except that yeast strain Y190 was used for transformations.

parental construct DBD-vhsMBD. The only point mutant that was defective in this assay was W321A. Similar results were obtained with filter assays; yeast cells transformed with W321A remained white, even after overnight incubation, whereas all of the other derivatives turned blue within 30 min. The alanine substitution derivatives were also tested for activity in the absence of cotransformed VP16$_{404}$ in case the introduced mutations fortuitously generated a transcription activation surface in vhs. As expected, all of the mutant derivatives were inactive in the absence of cotransformed GAD-VP16$_{404}$. These findings indicate that tryptophan 321 is crucial for interaction with VP16 in vivo.

The mutant vhsMBD derivatives were expressed in vitro and tested in the solid-phase capture assay as above. In these experiments, MBP-VP16 fusion protein was used as the affinity ligand in place of PA-VP16. Labeled proteins were incubated with MBP-VP16-coupled beads or control beads, and the bound material was analyzed by SDS-polyacrylamide gel electrophoresis. As expected, full-length vhs, vhs(179–344), and vhsMBD bound specifically to the VP16 beads (Fig. 4B) but not the control beads (Fig. 4C), whereas binding was not observed with vhs(Δ*Sma*) or the control GAL4 DBD proteins. T315A, T322A, E323A, I324A, and T326A bound specifically to the VP16 beads with efficiencies that were comparable to that of vhsMBD (see Fig. 5), while E316A, Q327A, Q328A, and I329A interacted with VP16 with a somewhat lower efficiency (20 to 50% of that of vhsMBD). R318A and S320A bound to VP16 with an efficiency of <5% (Fig. 4B, lanes h and i), and specific binding of W321A and vhs(Δ321–330) was not detected (lanes j and u). Identical results were obtained when PA-VP16 was used as the affinity ligand rather than MBP-VP16; thus, the nature of the fusion partner does not differentially affect the binding properties of the mutant vhs polypeptides (46).

The above findings demonstrate that tryptophan 321 is crucial for interaction with VP16 in vitro, consistent with the results obtained in the two-hybrid assay. Moreover, residues immediately upstream of tryptophan 321 (E-316, R-318, and S-320) and, to a lesser extent, downstream residues (Q327A, Q-328, and I329A) contribute to complex formation and/or stability in vitro.

DISCUSSION

We have recently demonstrated that VP16 interacts with vhs and downregulates vhs activity during a lytic infection, thereby allowing the accumulation and translation of late viral mRNAs (28, 48). In this report, we present evidence that a small modular domain in vhs encompassing amino acid residues 310 to 330 is sufficient for directing stable interaction with VP16. In contrast, VP16 requires a large domain or multiple domains within the amino-terminal 369 amino acid residues for interaction with vhs (48), implying that the overall conformation of VP16 or the juxtaposition of spatially separate binding determinants may be important for recognition of the small interaction domain in vhs. This situation is similar to the interaction of VP16 with DNA-bound Oct-1. In this case, the relatively

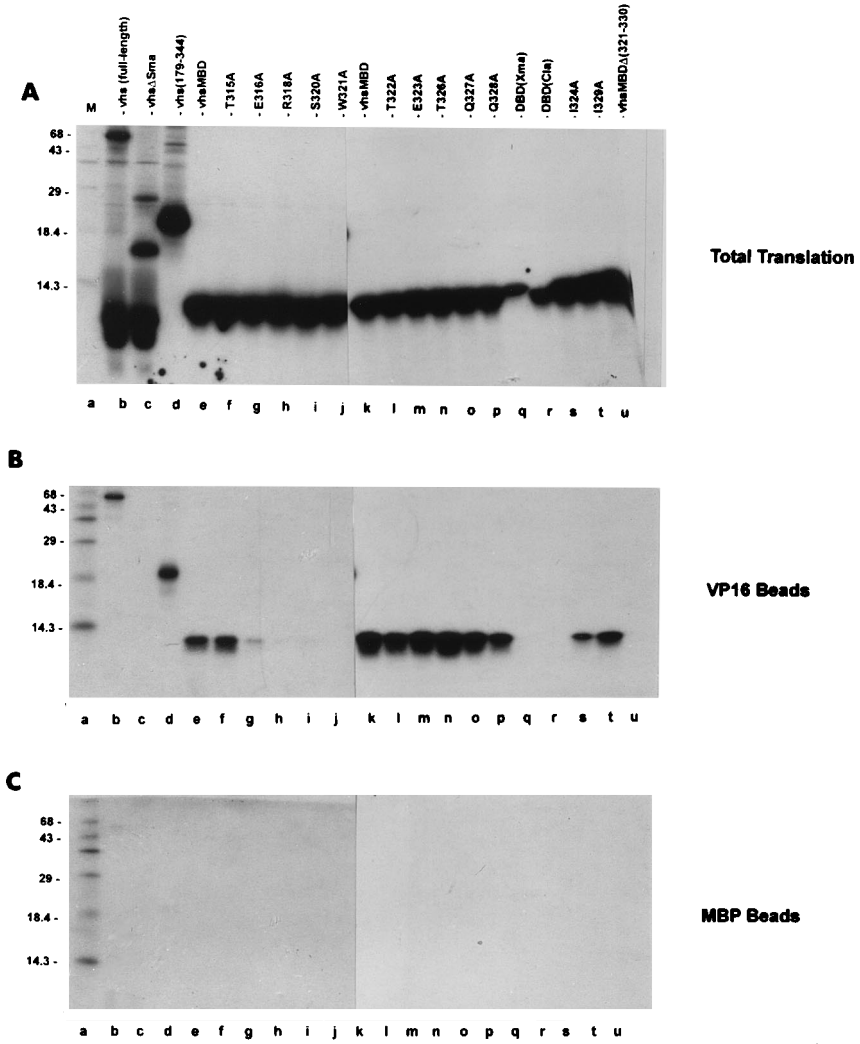

FIG. 4. Tryptophan 331 in the vhs minimal binding domain is essential for interaction with VP16 in vitro. Point mutant derivatives and a deletion derivative of the vhs minimal binding domain were transcribed and translated in vitro. Aliquots of the total translation reaction (A) were incubated with agarose beads coupled with MBP-VP16 (B) or control beads coupled with maltose-binding protein (C), and bound material was analyzed by polyacrylamide gel electrophoresis. Full-length vhs (lane b) and vhs(179–344) (lane d) were positive controls, while vhsΔ*Sma* (lane c), DBD(*Xma*) (lane q), and DBD(*Cla*) (lane r) were negative controls. The autoradiograph in panel A was exposed for 6 h, and the autoradiographs in panels B and C were exposed for 24 h. The positions of molecular size markers (lane m) are shown (in kilodaltons).

small POU homeodomain of Oct-1 is sufficient to direct specific protein-DNA complex formation with VP16 (50), whereas a large domain within VP16 is necessary (19, 55).

There are no obvious features in the vhs minimal binding region that are common to other well-characterized protein-protein interaction motifs. Moreover, with the exception of the HSV-2 vhs homolog (see below), a related domain does not appear to exist in any other protein, including known VP16 ligands such as Oct-1 and HCF. Secondary-structure predictions indicate that the region spanning residues 310 to 330 exists predominantly in a β-sheet conformation, flanked by β-turns. The amino-terminal end is primarily hydrophilic in nature, while the carboxyl-terminal end is hydrophobic. Results from both the two-hybrid analysis and in vitro binding assays show that mutation of the tryptophan residue at position 321 or deletion of residues downstream from this amino acid abrogates interaction with VP16. Moreover, mutation of both charged and uncharged polar residues (e.g., E-316, R-318, and S-320) proximal to W-321 reduced the formation and/or sta-

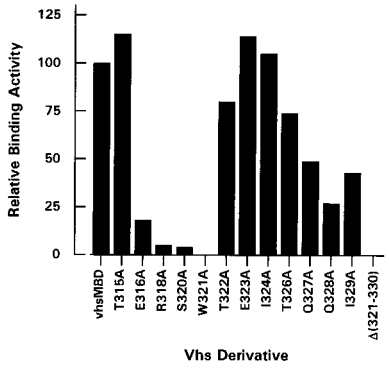

FIG. 5. Relative binding efficiency of vhs minimal binding domain mutants. The relative binding activity of the vhs point mutant derivatives was quantitated by phosphorimage analysis of the gels shown in Fig. 4. The values shown represent the amount of vhs protein bound to MBP-VP16 beads as a percentage of input protein, normalized to the value obtained with vhsMBD, which was taken as 100%. Binding over background was not detected with W321A or with vhsMBDΔ321–330.

bility of the vhs-VP16 complex in vitro, suggesting that these residues constitute part of a binding pocket. Tryptophan is principally hydrophobic in character, but the aromatic side chain can act as a donor in charge transfer interactions (8). Overall, the mutational sensitivity spectrum suggests that electrostatic, hydrogen-bonding, and possibly hydrophobic interactions may contribute to complex formation and stability. Whether other determinants outside this minimal region that contribute to complex formation and binding affinity exist in the intact molecule is currently under investigation.

Interestingly, with the exception of the tryptophan mutant, none of the alanine substitution derivatives were compromised in the two-hybrid assay. For instance, R318A and S320A, which interacted very poorly with VP16 in vitro, induced β-galactosidase activity as efficiently as vhsMBD in the two-hybrid assay. It is possible that complexes with these derivatives are less stable under the conditions employed in the solid-phase capture assay than in the more physiological conditions that exist in vivo in yeast cells. It should be noted that the two-hybrid assay is a sensitive but indirect measure of protein-protein interaction and may not necessarily measure of the stability of the vhs-VP16 complex. Weak and/or transient association between vhs and VP16 that may not be detectable in in vitro binding assays may suffice to efficiently activate the *lacZ* reporter gene in yeast cells.

Vhs homologs from other alphaherpesviruses, such as pseudorabies virus, equine herpesviruses, and varicella-zoster virus, have regions of sequence identity with HSV-1 vhs throughout their lengths (4); however, with the exception of vhs from HSV-2, the minimal VP16-binding region is not conserved. It is possible that HSV has evolved unique strategies and mechanisms of gene regulation that rely on a linkage between VP16 and vhs. Indeed, it is not known if vhs homologs participate in host shutoff activity, and moreover, the functional properties of VP16 differ in other viruses. For instance, transcription activation by VP16 homologs from other alphaherpesviruses appears to be mediated through distinct mechanisms (32), and at least for varicella-zoster virus, the VP16 homolog is not essential for virus growth (7).

Transient-transfection assays reveal that vhs functions in the absence of other viral proteins (22, 38). However, in infected cells, other viral proteins, such as the virion-associated protein kinase product of the UL13 gene, also play a role in shutoff activity (20, 37). It is possible that vhs activity or VP16-vhs association itself is regulated by additional factors, perhaps through phosphorylation events or additional levels of protein-protein interaction. In this regard, it is interesting that the minimal binding domain contains a consensus casein kinase II phosphorylation site (S/T-X-X-D/E). Further characterization of the vhs protein interaction surface and more precise identification of the binding determinants present in VP16 will help elucidate how combinatorial interactions between VP16, vhs, and other viral and cellular factors contribute to the multilevel regulation of viral gene expression.

Finally, it is conceivable that the vhs-VP16 complex is a novel antiviral target, since prevention or disruption of complex formation could lead to vhs-mediated degradation of viral mRNA and thus termination of productive infection. The vhs binding interface identified here provides a logical starting point for the development of potentially useful interventive strategies that target the VP16-vhs complex.

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