Amino Acid Substitutions in the Herpes Simplex Virus Transactivator VP16 Uncouple Direct Protein-Protein Interaction and DNA Binding from Complex Assembly and Transactivation*

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Peter Shaw, Jozo Knez, and John P. Capone‡

From the Department of Biochemistry, McMaster University, Hamilton, Ontario L8N 3S5, Canada

The herpes simplex virus transactivator VP16 directs the assembly of a multicomponent protein-DNA complex that requires the participation of two cellular factors, the POU homeodomain protein Oct-1, which binds independently to response elements, and VCAF-1 (VP16 complex assembly factor; also called HCF, C1), a factor that binds directly to VP16. A number of distinct properties of VP16 have been implicated in the assembly of the VP16-induced complex (VIC). These include its independent association with VCAF-1 and, under appropriate conditions, its ability to bind to DNA or to DNA-bound Oct-1 in the absence of VCAF-1. In order to probe the requirements of these individual interactions in the functional assembly of VIC, we mutated selected charged amino acids in two subdomains of VP16 previously shown to be important in protein-DNA complex formation. Purified VP16 proteins were analyzed for their ability to direct protein-DNA complex formation and to interact directly with VCAF-1. Several classes of mutants that were differentially compromised in VCAF-1 interaction, direct DNA binding, and/or association with DNA-bound Oct-1 were obtained. Interestingly, all of the derivatives were still capable of generating the VIC complex in vitro and activating transcription in vivo. Our findings indicate that the cooperative assembly of functional VP16-containing complexes can occur by pathways that do not necessarily require the prior interaction of VP16 with VCAF-1 or the ability of VP16 to bind directly to DNA or associate with DNA-bound Oct-1.

Transcriptional regulation of the herpes simplex virus immediate early (IE)¹ genes by the viral transactivator VP16 (also called Vmw65 or αTIF) has provided a valuable model system to investigate how multicomponent protein-protein and protein-DNA assemblies orchestrate specific gene regulatory patterns (reviewed in Refs. 1–3). VP16 is an abundant 490-amino acid-long structural phosphoprotein which, when delivered into the host cell by the infecting virus particle, strongly stimulates the transcription of the viral IE genes through recognition of cis-regulatory TAATGARAT (R = purine) target elements that are present in one or more copies in the upstream regions of responsive genes. VP16, however, has only weak intrinsic DNA binding activity and efficient binding to target sites requires the assembly of VP16 into a multicomponent complex along with at least two cellular factors, the ubiquitously expressed POU homeodomain protein Oct-1, and an additional cellular factor variously called VCAF-1, HCF, C1, or CCF (Refs. 4–14; referred to as VCAF-1 in this paper). The fully assembled functional complex, referred to as the VP16-induced complex (VIC), is thought to position the strong carboxyl-terminal acidic activation domain of VP16 in the correct spatial arrangement for functional interaction with downstream target proteins, among which include several basal transcription factors and adapter proteins (15, 16).

The ordered assembly of VP16-containing protein-DNA complexes involves cooperative sequence-specific protein-DNA interactions as well as specific and selective protein-protein associations with Oct-1 and VCAF-1. Oct-1 binds independently to TAATGARAT elements and directs the recruitment of VP16 via determinants present in the POU homeodomain (17–21). The prior binding of Oct-1 to TAATGARAT elements is a prerequisite to the formation of VIC; however, the efficient incorporation of VP16 into the complex requires the auxiliary component VCAF-1, a large cellular factor which can interact directly with VP16 in the absence of Oct-1 and DNA (5, 7, 8, 13, 14, 17). There is no evidence for the formation of Oct-1-VP16 or Oct-1-VCAF-1-VP16 heteromeric complexes in the absence of DNA; however, it has been demonstrated that VP16 is able to bind directly to DNA or to generate a complex with DNA-bound Oct-1 in the absence of VCAF-1 when high concentrations of VP16 are used in binding assays (17, 19, 22).

VP16 is a modular protein and contains separable domains that are important for complex formation and transactivation (19, 23–29). The carboxyl-terminal acidic activation domain is essential for transcriptional activation but not for complex assembly. The residual 400 amino-terminal amino acids are necessary and sufficient for VIC formation as well as for interaction with VCAF-1 and binding to DNA (14, 24, 25, 27). More recently, it has been demonstrated that this region also contains determinants for interaction with the virion host shutoff protein (Vhs), a viral structural protein that is responsible for the cessation of host protein synthesis following viral infection (30). At least two subregions within the amino-terminal domain are involved in complex assembly with mammalian factors in vitro; region 1, spanning residues 140–250, and region 2, encompassing residues 335–390 (19, 24, 25, 27). Both subregions are involved in VIC formation, binding to VCAF-1, interaction with DNA-bound Oct-1, and contribute to the weak DNA binding activity associated with VP16 (19, 24, 27, 31–33).

Despite a large number of mutational studies, it is not known
whether the individual interactions attributed to VP16 are essential for the assembly of transcriptionally active complexes. For instance, while VCAF-1 is essential for VIC formation and transcriptional activity, it is still not known whether the intrinsic ability of VP16 to bind independently to VCAF-1 is a prerequisite for multicomponent complex assembly. Also, the weak DNA binding activity associated with VP16, or VCAF-1-independent interaction with DNA-bound Oct-1, is only observed when very high concentrations of VP16 are used in binding assays (17, 19); thus, the physiological significance of these interactions is not clear. Most mutational studies of VP16 have been carried out using large deletions or linker insertions and these might be expected to cause conformational changes in VP16 that could have secondary effects on complex assembly and protein-protein interactions. The only point mutational analysis so far described assessed the overall effects of specific point mutants on VIC formation and transcriptional activation (27). In order to determine if individual protein-protein and protein-DNA interactions involving VP16 can be uncoupled from VP16-induced complex formation and transcription activation, we converted selected charged residues in regions 1 and 2 into alanine residues by site-directed mutagenesis. Mutant proteins were analyzed in vitro for protein-DNA complex formation with Oct-1 and interaction with VCAF-1 and in vivo for transcription of a VP16-responsive reporter gene. Among the mutants generated were those that were defective in direct VCAF-1 interaction and DNA binding but were still capable of generating the VIC complex and directing transcriptional activation in vivo. Our findings indicate that the assembly of functional VP16-containing multicomponent complexes does not necessarily require the prior interaction of VP16 with VCAF-1 or the capability of VP16 to bind directly to DNA or associate with DNA-bound Oct-1, suggesting that the cooperative assembly of VP16-dependent complexes can occur by different pathways.

MATERIALS AND METHODS

Site-directed Mutagenesis—Site-directed mutagenesis was carried out by the Kunkle method (34) using a commercially available kit (Bio-Rad). Single-stranded M13mp19 containing the antisense strand of the VP16 open reading frame was used as the template for mutagenesis. The following mutagenic oligonucleotides, designed to convert the indicated wild type amino acids into alanine residues, were used.

- R155A: 5’ GGCGCGGCGGGCGGGGC 3’
- R162A: 5’ GCAGCCGCGGGGGGGG 3’
- R164A: 5’ CACCCGCCGCGGGGGGG 3’
- R169A: 5’ GGCAGCTATACGCAACCTGTGTTG 3’
- C176A: 5’ GCCACCTCGCGCTCCTGGGTG 3’
- R360A: 5’ GCGCGTACGCGGCTCGATG 3’
- R366A: 5’ GCCTGACGGGCCTCGATG 3’
- R368A: 5’ CCAGCCGCCGCTCGCTAAATAC 3’
- K370A: 5’ GCGGCTACGCACAAATAGCGC 3’

The altered amino acid codons are underlined. The notation refers to the type and position of the wild type amino acid (single letter code) targeted for alteration to an alanine residue. Purified phage DNA containing the desired mutations were screened by DNA sequence analysis and the replicative form DNA was prepared (34).

Protein Purification—Plasmids expressing the various mutant derivatives of VP16 as fusion proteins to the maltoses binding protein (MBP) were constructed by subcloning the Sall fragment (corresponding to amino acids 4–411 of VP16) from the respective M13 replicative form plasmids into the Sall site of pMal-C (New England Biolabs). The MBP expression plasmid MBP-VP16, containing the wild type VP16 Sall fragment, has been described (33). Fusion proteins were purified from induced cultures of Escherichia coli DH5α by affinity chromatography on amylose resin according to the manufacturer’s instructions (New England Biolabs). Bound protein was eluted with column buffer (CB): 20 mM Tris-Cl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM diithothreitol, 0.5 mM phenylmethylsulfonyl fluoride) supplemented with 10 mM maito-ose. Peak fractions were pooled and exchanged into buffer D (20 mM HEPES, pH 7.9, 100 mM KCl, 0.5 mM diithothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 mM EDTA, 20%, v/v, glycerol) using PD10 gel filtration columns (Pharmacia Biotech Inc.). Fusion proteins were judged to be 60–70% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. GST-Oct-1 (from the pAGT-1 fragment) was covalently coupled PA-VP16 to the beads (Greiss and Westphal’s Staphylococcus aureus protein A) were purified by affinity chromatography on glutathione-Sepharose and IgG-Sepharose, respectively, as described previously (27, 31). VCAF-1 was purified from HeLa cell nuclear extracts by affinity chromatography on columns containing covalently coupled PA-VP16 fusion protein and suspended in buffer D as described previously (14, 30, 31).

Gel Retardation Analysis—Protein-DNA mobility shift assays were carried out as before (31). Briefly, standard 20-μl reactions contained 5 mM HEPES, pH 7.9, 0.1 mM diithothreitol, 0.5 mM EDTA, 25 mM KCl, 4 μg of nonspecific competitor DNA (a 1:2 mixture of salmon sperm DNA-poly(dI-dC)) and combinations of 0.05 μg of purified GST-Oct-1, 2–4 μl of affinity-purified VCAF-1, and various amounts of MBP-VP16 fusion proteins as described in the figure legends. Protein concentration in each reaction was normalized to 20 μg with BSA. Reactions were preincubated for 5 min prior to the addition of 20-end-labeled probe DNA corresponding to the promoter proximal TATAAG element from the HSV-1 ICPO gene. 5’ GATCCCGGCGAGATCGATGATGATTAG-3’ reactions were incubated for 20 min at 30°C. Binding reactions were run on 3.5% polyacrylamide gels run at 4°C in 0.25 × Tris borate-EDTA (34).

VCAF-1 Binding Assays—VCAF-1 binding assays were carried out essentially as described using purified MBP or MBP-VP16 fusion proteins adsorbed onto amylase resin (31). Briefly, 20 μl of settled beads coupled with the various fusion proteins (2.5 μg of protein/ml of settled beads) were incubated for 2 h at 4°C with an equal volume of affinity-purified VCAF-1 with constant rotation. After low speed centrifugation, the supernatant containing the nonbound material was collected, and the beads were washed three times with 10 volumes of buffer D containing 100 μg/ml BSA and 0.5% Nonidet P-40, followed by three washes with buffer D alone. Bound material was eluted from the settled beads by washing with 0.6 M KCl. Equivalent volumes of bound and nonbound material (6 μl) were assayed for VCAF-1 activity by mobility shift analysis with GST-Oct-1 and wild type MBP-VP16. Buffer components and protein concentration in the binding assays were normalized as appropriate.

Vhs Binding Assays—Full-length [35S]methionine-labeled HSV-1 vhs protein was prepared by transcription and translation of the in vitro transcription plasmid pSPUTK vhs using rabbit reticulocyte lysates (Promega) as described previously (30). For binding assays, amylase beads adsorbed with equivalent amounts of MBP or the various MBP-VP16 fusion proteins were washed sequentially with 10 volumes of CB, CB + 0.2% BSA, and CB + 0.2% BSA + 0.05% Nonidet P-40. 50 μl of settled beads were recovered by low speed centrifugation and incubated with radiolabeled Vhs protein (200 μg of a 1:100 dilution of a 50 μg/ml solution of rabbit reticulocyte lysate). The beads were incubated for 1 h at 4°C with continuous rotation and recovered by centrifugation. Beads were washed once with 10 volumes of CB, three times with 10 volumes of CB + 0.2% BSA, and 3 times with 10 volumes of CB + 0.2% BSA + 0.5% Nonidet P-40. Bound material was eluted by boiling with an equal volume of twice concentrated sodium dodecyl sulfate sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Transfections and CAT Assays—The mammalian expression vector pEVR65 contains the full-length VP16 open reading frame under transcriptional control of the cytomegalovirus IE promoter. Expression vectors containing the various VP16 point mutants were constructed by TdT-mediated extension of the wild type SalI fragment in pEVR65 with the corresponding mutant fragments. Transfections were carried out in duplicate using 6-cm dishes of subconfluent Vero cells by the CaPO4 procedure as described previously (28). Transfections contained 5 μg of the p175cat reporter gene (contains the chloramphenicol acetyltransferase (cat) gene under the transcriptional control of the promoter/regulatory region of the HSV-1 ICPO gene) and with 0.2–2.5 μg of a replication deficient plasmid as indicated in the figure legend. Promoter dosage was normalized with appropriate amounts of empty vector and total DNA was kept constant (20 μg of DNA/dish) with sonicated salmon sperm DNA. Cell lysates were prepared 48 h post-transfection, and CAT activity was measured using the liquid scintillation method as described (35). Results were normalized to the value obtained with the reporter plasmid co-transfected with the empty expression vector and represent the average from at least three independent transfections carried out in duplicate.
Fig. 1. Amino acid substitutions in VP16. A, map of VP16 showing two subdomains important for protein-DNA complex assembly (Region 1 and Region 2). AAD (amino acids 420–490) represents the carboxyl-terminal acidic activation domain. The indicated amino acid residues were altered to alanine (A) residues by site-directed mutagenesis. The nomenclature refers to the amino acid residue and its position in the primary sequence. B, expression of MBP-VP16 fusion proteins in E. coli. The above mutant derivatives were cloned into the MBP expression vector pMal-C, expressed in E. coli, and purified by affinity chromatography. Shown is a Coomassie Blue-stained gel of the different purified MBP-VP16 fusion proteins (lanes c–l). Lane b is MBP purified from induced cultures in an identical manner. WT in this and subsequent figures (unless indicated otherwise) denotes the MBP-VP16 fusion protein containing wild type amino acids 5–411 of VP16. Molecular size markers, in kilodaltons, are shown in lane a.

RESULTS

Previous studies have shown that amino acids 140–250 and 335–390 of VP16 contain determinants that are important for VIC formation and VCAF-1 interaction (19, 24, 25, 27, 31). Both regions 1 and 2 are enriched in positively charged residues, relative to the whole protein. Since positively charged amino acids are important in both protein-protein and protein-DNA interactions, we decided to alter selected arginine residues to alanine residues (36). The introduced mutations are shown in Fig. 1A. These include alanine substitutions of arginines at positions 155, 162, 164, 169, 360, 366, and 368. Cys-176 was also mutated to examine the role of this sulfhydryl group. Also, Lys-379 was mutated, since the region surrounding this amino acid has been shown to be surface exposed and to be involved in protein-DNA complex formation (32).

VP16 and the various mutant derivatives were cloned into an MBP expression vector, and the proteins were purified by affinity chromatography on amylose resin (Fig. 1B; all MBP-VP16 derivatives encoded residues 4–411 and are thus missing the acidic activation domain). The purified proteins were used to monitor the following properties of VP16: direct DNA binding to the TAATGARAT elements, interaction with DNA-bound Oct-1, direct interaction with VCAF-1, VIC formation, and association with Vhs.

Direct Interaction of VP16 with DNA—Kristie and Sharp (17) first showed that if sufficiently large amounts of VP16 are used in DNA binding assays, VP16 can bind independently and sequence specifically to TAATGARAT elements. Fig. 2A shows the results of DNA binding assays with VP16 carried out in the absence of Oct-1 and VCAF-1 using the labeled ICP0 octa– TAATGARAT element. A 10–20-fold excess of VP16, compared with the amount sufficient to form VIC in the presence of Oct-1 and VCAF-1 (see below), was used in this case in order to observe direct DNA binding. As can be seen, MBP-VP16 (referred to as wild type protein unless noted otherwise), forms a distinct protein DNA complex with the probe (lane b). This interaction is sequence-specific, since it was not observed with mutant oligonucleotides that contained alterations in the GA- RAT portion (17, 37; data not presented). By way of comparison, a protein A-VP16 fusion protein (PA-VP16), which is larger than MBP-VP16, generates a slower migrating complex as expected (lane a). Mutant derivatives R155A, R164A, R169A, C176A, R368A, and K370A bound to DNA with efficiencies comparable with MBP-VP16 (compare lanes b with lanes c, e, f, g, j, and k, respectively). In contrast, R162A, R366A, and R368A were unable to bind to the probe (lanes d, h, and i, respectively), even when 3-fold higher concentrations of protein were used in the assays (not presented).

Interaction of VP16 with DNA-bound Oct-1—Oct-1 does not interact with VP16 in solution; however, under appropriate conditions, VP16 can interact weakly with DNA-bound Oct-1 in the absence of VCAF-1 (19, 22). In order to examine the effect of the introduced mutations on this interaction, mobility shift assays were carried out in the presence of purified GST-Oct-1 and the various VP16 derivatives. As shown in Fig. 2B, incubation of MBP-VP16 with GST-Oct-1 results in the formation of a weak Oct-1-MBP-VP16 complex that migrates slightly faster than the complex formed with MBP-VP16, which is also generated under these conditions with comparable efficiency (lane b). The reason this complex migrates slightly faster than the VP16-DNA complex is not clear; however, it may be due to conformation of the ternary complex that results in faster electrophoretic mobility compared with the VP16-DNA complex alone. A complex with similar mobility to wild type MBP-VP16-Oct-1 was observed with all the region 1 mutants (R155A,
prior interaction of VP16 with VCAF-1 is a prerequisite for VIC formation. If this were the case, all of the VP16 derivatives should interact with VCAF-1, since they were able to direct VIC formation. To examine this directly, VCAF-1 microaffinity assays were performed. The various MBP-VP16 mutant fusion proteins were linked to amylose resin and the beads were incubated with purified VCAF-1. In preparing the affinity matrix, care was taken to ensure that the concentration of the immobilized mutants was similar for each case. Bound and nonbound material was collected and assayed for VIC formation in the presence of wt MBP-VP16 and GST-Oct-1. As shown in Fig. 4, beads coupled with MBP-VP16, R155A, R162A, R164A, C176A, and R360A retained VCAF-1 activity (lanes c, d, e, f, h, and i, respectively), whereas VCAF-1 activity was not retained on beads coupled with MBP or to beads coupled with R169A, R366A, R368A, or K370A (lanes b, g, j, k, and l, respectively). These results demonstrate that prior and independent interaction between VP16 and VCAF-1 is not essential for VIC formation.

Interaction of VP16 with Vhs—We have recently identified the HSV-1 virion host shutoff protein vhs as a novel ligand for VP16 (30). Preliminary mapping studies indicated that stable interaction with Vhs requires several regions present in the amino-terminal 369 amino acids of VP16, suggesting that the overall conformation of VP16 is important for VP16-Vhs complex formation. We therefore determined whether the point mutant derivatives of VP16 were compromised in their ability to interact with Vhs. For this purpose, [35S]methionine-labeled Vhs was prepared by transcription and translation in vitro. The different MBP-VP16 point mutants were coupled to amylose beads as described above and incubated with labeled Vhs. The beads were recovered, washed extensively, and the bound material was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. As shown in Fig. 5, Vhs bound to all of the mutant VP16 derivatives as effectively as it did to MBP-VP16. Labeled Vhs did not bind to the beads alone (lane e) or to beads complexed with MBP (lane b). Thus, none of the VP16 point mutants were defective in interaction with Vhs. These findings show that the targeted mutations are not important for Vhs interaction and, moreover, that the introduced mutations did not grossly affect the overall conformation of VP16.

Transactivation Properties of VP16 Mutant Derivatives—There is a strict correlation with the ability of VP16 to assemble into the VIC complex with subsequent transactivation in vivo. It is thought that assembly of this multicomponent complex is required only to bring the activation domain of VP16 to the promoter; however, additional VP16-DNA, VP16-Oct-1 and VP16-VCAF-1 interactions in the full complex may influence the overall conformation of the complex and thus contribute directly to transactivation. We therefore cloned the different VP16 derivatives into a mammalian expression vector that restored the carboxy-terminal acidic activation domain and tested their abilities to transactivate gene expression in transient transfection assays using a CAT reporter gene that contained the promoter/regulatory region from the ICp4 gene. As shown in Fig. 6, co-transfection of Vero cells with the full-length wild type VP16 expression plasmid induced expression of the reporter gene approximately 30-fold when 0.5 μg of effector plasmid was used. Increasing the concentration of the wild type effector plasmid resulted in a reduction in activity as reported previously, likely as a result of squelching (24). Transfection of the mutant VP16 derivatives showed that all the mutants were capable of transactivating expression of the reporter gene, consistent with the observation that all of the mutant VP16 proteins remained capable of forming VIC. There were, however, variations in relative potency among the differ-
ent mutant proteins. At an effector plasmid dosage of 0.5 μg, R164A and R169A behaved as wild type, while the remaining derivatives activated expression at an efficiency of 30–40% compared with the wild type plasmid. R155A was only 20% as active as the wild type plasmid at 0.5 μg but was similar to wild type when 1 μg of plasmid was used. The reduced potency observed with some of the derivatives did not correlate with the ability of VP16 to bind independently to DNA or to interact with VCAF-1 and thus may be related to differences in the relative stability of the proteins in vivo.

**DISCUSSION**

In this study, we have analyzed the properties of specific point mutants of VP16 to examine the contributions of individual protein-protein and protein-DNA interactions of VP16 to the ordered assembly of VP16-containing complexes and VP16-mediated transcriptional activation. We showed that specific point mutations in two regions of VP16 selectively affected the ability of VP16 to directly bind to DNA, to associate with DNA-bound Oct-1, and to form a complex with VCAF-1 (summarized in Table I). All of the mutants tested remained capable
of forming VIC and transactivating gene expression in vivo, demonstrating that these individual interactions are not essential for the formation of a transcriptionally active VP16-containing protein-DNA complex.

VP16 derivatives in which arginines at position 162, 360, and 366 were altered failed to bind directly to DNA. This is consistent with previous mutational studies which showed that deletion of either region 1 or 2 destroyed direct DNA binding activity of VP16 (19). The putative DNA binding domain of VP16 has not been defined; however, Stern and Herr (19) showed that a synthetic peptide spanning a part of region 1 (amino acids residues 170–202) bound to DNA, albeit in a nonsequence-specific manner. All of the region 1 mutants were capable of forming a weak complex with DNA-bound Oct-1; however, the region 2 mutants were defective. This is consistent with previous findings that region 2 contains determinants for direct interaction of VP16 with DNA bound-Oct-1 (19, 24, 31). Our findings that the R368A and K370A mutants cannot recognize DNA-bound Oct-1, yet are still able to bind to DNA indicate that direct DNA binding activity of VP16, is not required for this interaction.

The functional relevance of direct DNA binding activity of VP16 or interaction with DNA-bound Oct-1 is not clear, since there is no evidence that the VP16-Oct-1 binary complex or VP16 on its own is transcriptionally active in vivo. As demonstrated previously by others (17, 19) and also shown here, the amount of VP16 required to observe these properties is significantly greater (10–100-fold) than that which is sufficient to generate the VIC complex. The high concentration of VP16 necessary to observe these interactions is probably not physiologically significant. Indeed, transactivation of viral IE genes is detrimentally affected by large amounts of VP16 (24). Moreover, the source of VP16 may have an effect on the efficiency of these interactions. For instance, PA-VP16 has a higher affinity for DNA compared with MBP-VP16 (Fig. 2A),2 whereas GST-VP16 has only very weak intrinsic DNA binding activity (22).

Thus, the carrier protein may have some influence on binding properties, perhaps by masking or exposing cryptic binding interfaces (17). It has been proposed that VP16 binds to the GARAT portion of the target site which, while not required for Oct-1 binding, is necessary for VIC assembly (5, 17). More recent evidence indicates that the GARAT subregion serves to alter the conformation of the POU homeodomain and that this is necessary for subsequent recognition by VP16 (22). Thus, the carrier protein may have some influence on binding properties, perhaps by masking or exposing cryptic binding interfaces (17). It has been proposed that VP16 binds to the GARAT portion of the target site which, while not required for Oct-1 binding, is necessary for VIC assembly (5, 17).

Region 2 is essential for VIC formation and also contains critical determinants for direct interaction with VCAF-1 (19, 24, 31). We, and others, have shown that the requirements for interaction with VCAF-1 can be uncoupled from VIC formation (19, 31). Thus, VIC formation requires residues up to amino acid 388, whereas VCAF-1 can still interact with VP16 that is truncated to amino acid 379 (24, 31). In addition, a linker insertion at position 379 abolished VIC formation but not interaction with VCAF-1 (19). Hayes and O'Hare (32) have shown
previously, using limited proteolysis of VP16 and of VP16-containing protein-DNA complexes, that the region surrounding Lys-370 is exposed on the surface of the protein and thus likely to be important for protein-protein interactions. Moreover, using peptide competition, these authors showed that a synthetic peptide encompassing amino acids 360–373 (REHAYSRARTKNNY) or a truncated variant containing amino acids 360–367 was able to inhibit VIC formation. The simplest conclusion was that this peptide interfered with the ability of VP16 to bind to VCAF-1, although this was not directly demonstrated. Our findings that mutation of arginine residues 366, 368, and lysine 370 disrupt VCAF-1 interaction is consistent with the conclusion that this subregion represents an important interface for VCAF-1 interaction. Furthermore, the finding that mutation of arginine 169 (this work) or a linker insertion at amino acid 177 (19) also disrupts association with VCAF-1 indicates that region 1 contributes to this interaction, further emphasizing that the overall conformation of VP16 is important. More significantly, all of the VP16 derivatives were capable of generating VIC in vitro and of transactivating expression of a VP16-responsive CAT reporter gene in vivo. Thus, the direct and independent interaction of VP16 with VCAF-1 is not absolutely required for the assembly of functionally active VIC complexes, suggesting that complex assembly can occur by different pathways. It is possible that multiple and additional layers of cooperativity occurring between VCAF-1, VP16, and Oct-1 in the full complex may compensate for deficiencies in individual interactions. The fact that determinants important for VP16 interaction with VCAF-1 and Oct-1 overlap, or are in close proximity to each other, suggests that VCAF-1 also contacts Oct-1, as implied previously by mutation and peptide competition studies (19, 32). Similarly, the observation that the intrinsic DNA binding activity of VP16 is dispensable for VIC formation does not necessarily mean that this interaction does not occur in the fully assembled complex.

In summary, our findings suggest versatility and flexibility in the assembly of VP16-containing complexes, whereby complexes can assemble by different pathways. This is compatible with recent findings that demonstrate that the conformation of Oct-1 POU homodomain, and possibly the VP16-induced complex itself, is different on distinct TAATGARAT elements and that diverse response elements may have different functional properties in vivo (22, 38, 40–42). This flexibility could provide a mechanism by which VP16 function can adapt to different physiological conditions in the host cell and thus modulate progression of the lytic cycle. For instance, the surprising observation that direct interaction of VCAF-1 with VP16 can be uncoupled from subsequent higher order protein-DNA complex assembly and transactivation does not necessarily mean that prior interaction between VCAF-1 and VP16 is dispensable for VIC formation under all conditions. Thus, in cells where VCAF-1 concentration is low, prior assembly of the VCAF-1-
VP16 binary complex may be a necessary prerequisite for efficient VFC formation and subsequent transcriptional activation. Elucidation of the basis and physiological relevance for flexibility in the assembly and function of VP16 multicomponent complexes will contribute to our understanding of specificity and selectivity in viral and cellular gene regulation.

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