A single serine residue at position 375 of VP16 is critical for complex assembly with Oct-1 and HCF and is a target of phosphorylation by casein kinase II

Dawn O’Reilly, Olivia Hanscombe and Peter O’Hare

Marie Curie Research Institute, The Chart, Oxted, Surrey RH8 0TL, UK

We show that VP16 is phosphorylated by cellular kinases in vivo and in vitro and map the major sites of phosphorylation to be on serines towards the C-terminus, downstream of position 370 in both cases. Deletion of the acidic activation domain had no effect on phosphorylation, refining the sites to between position 370 and 411. Within VP16, the C-terminal boundary for complex formation with Oct-1 and HCF lies at position 388, and between 370 and 388 lies one serine, at position 375. This is a consensus casein kinase II (CKII) site and, using purified wild-type and mutant proteins, we show that it is the main CKII site in the body of the N-terminal complex-forming region. This site is also phosphorylated in nuclear extracts. Although other sites, mainly Ser411, are also phosphorylated by nuclear kinase(s), the single substitution of Ser375 to alanine abolishes CKII phosphorylation in vitro and virtually eliminates complex formation. This serine lies in a surface-exposed region of VP16 and, although complex formation is disrupted, other activities of the mutant are unaffected. Ser375 is also required in vivo where substitution to alanine abolishes transactivation, while replacement with threonine restores normal levels of activity.

Keywords: casein kinase/HSV-1/Oct-1/phosphorylation/VP16

Introduction

VP16, a structural component of herpes simplex virus (HSV) induces expression of the viral immediate-early (IE) genes by promoting the assembly of a multicomponent complex containing the cellular factors Oct-1 and HCF onto regulatory motifs present upstream of each of the IE genes (O’Hare, 1993). Oct-1 is a member of the POU domain protein family (Herr et al., 1988) whose members play a fundamental role in the regulation of transcription of related groups of genes in a wide array of cellular processes, and function not only by direct complex interactions with other members of the class, but also exhibit synergistic and antagonistic interactions with messenger signalling pathways (Rosenfeld, 1991; Ruvkun and Finney, 1991). Analysis of the involvement of one of the members of this family of proteins in HSV IE gene expression has illustrated how selective utilization of such factors can control the coordinate induction of a distinct set of genes.

The motifs responsible for VP16 induction do not bind VP16 directly (Marsden et al., 1987) but instead encompass specialized recognition sites for Oct-1, and VP16 is recruited onto these sites by interactions with Oct-1 and HCF to form the TRF-C complex (Kristie and Roisman, 1987; Gerster and Roeder, 1988; O’Hare and Goding, 1988; Stern et al., 1989; Katan et al., 1990; Kristie and Sharp, 1993; Wilson et al., 1993). VP16 exhibits stringent selectivity in the assembly of TRF-C at several levels. Although the POU domain of Oct-1 is sufficient for complex assembly, and the POU domain is relatively highly conserved within the family (e.g. almost 90% identity between Oct-1 and Oct-2), only Oct-1 has been shown to be competent for complex formation with VP16. Differences between the POU domains of Oct-1 and Oct-2 are mostly within the helix–turn–helix homeodomain, and transfer of helix two (of three helices) from Oct-2 into Oct-1 abolishes VP16 complex formation (Stern and Herr, 1991). More refined studies have demonstrated that selective recognition by VP16 is influenced by other homeodomain surface-exposed residues (Pomerantz et al., 1992) and dependent upon a critical glutamic acid residue within helix 2 of the Oct-1 homeodomain (Lai et al., 1992). The homeodomain is therefore not only involved in DNA binding but also in selective protein–protein interactions with other classes of regulatory factors.

Selectivity in complex assembly also operates at the level of the Oct-1 DNA-binding site. Only a subset of Oct-1-binding sites is competent to promote VP16 complex assembly. The critical feature of the competent sites is the conservation of a 3′ flanking motif, the GARAT motif, which is not necessary for Oct-1 binding per se, but is necessary for the recruitment of VP16 into the TRF-C complex (O’Hare et al., 1988). Recent evidence indicates that selectivity at the level of the DNA-binding site operates not by direct recognition of the flanking motif by VP16 but by an allosteric effect of the DNA sequence on Oct-1 such that it induces a particular conformation of the bound Oct-1 which is recognized selectively by VP16 (Walker et al., 1994). Thus, although octamer-binding sites are well conserved in genes which are clearly not coordinately regulated, selective involvement of the sites in differential transcriptional activation is likely to operate by site-specific recruitment of additional co-activators.

While complex mechanisms of selectivity operate at several levels, to date no studies have examined whether, when the appropriate factors and sites are available, assembly of the complex may be regulated in some manner. Abundant evidence exists that phosphorylation of DNA-binding proteins, repressors or co-activators acts as a major control in the regulation of their function and thus of transcription pathways (Hunter and Karin, 1992; Karin and Hunter, 1995). Previous work has indicated that VP16 is phosphorylated in virus-infected cells (Wilcox...
et al., 1980; Moss, 1989), but additional studies have not reported any alteration in phosphorylation upon deletion of the virus-encoded kinases (Purves et al., 1991; Purves and Roizman, 1992; Coulter et al., 1993), indicating that cellular kinase(s) may be involved. In this work, we examine directly whether VP16 is phosphorylated by cellular kinase(s) in vivo where transactivation is assessed, and by nuclear extracts in vitro where functional activity in complex assembly is examined. We demonstrate that VP16 is phosphorylated in both situations, with the main region of phosphorylation mapping downstream of residue 370 in each case. We map the main sites involved in vitro and show that while other downstream sites are phosphorylated, Ser375 is the main site phosphorylated within the region of the protein previously shown to be critical for complex assembly. This site is a consensus casein kinase II (CKII) site, and we show that it is the only site phosphorylated by pure CKII in the N-terminus of the protein. Mutation of Ser375 to alanine virtually abolished CKII phosphorylation and complex formation in vitro and transcriptional activity in vivo. Substitution to a threonine at position 375 restores both functions. The results indicate that phosphorylation by cellular kinases may act as an important control of VP16 activity.

Results

A cellular kinase phosphorylates VP16 within the C-terminus

When transfected into cells, VP16 strongly induces the transcription of genes containing the appropriate upstream target signal. To examine whether VP16 was phosphorylated under such conditions, we transfected COS cells with a VP16 expression vector and 24 h later labelled the transfected cells with [32P]orthophosphate for 6 h. The cells were harvested and solubilized, and analysed for VP16 by Western blotting of the soluble extract, and by immunoprecipitation using the anti-VP16 monoclonal antibody LP1. The results demonstrate the presence of VP16 protein in the transfected but not the control cells (Figure 1a, lanes 1 and 2) and further demonstrate that VP16 was indeed phosphorylated (Figure 1, lanes 3 and 4).

To make a first approximation of the location of the phosphorylated sites in VP16, we took advantage of the fact that the protein contains only four lysine residues. Cleavage of VP16 with the lysine-specific protease EndoLysC will result in the production of five peptides with predicted mol wts of 28, 13, 8 and 3 kDa, as indicated in Figure 1. The in vivo 32P-labelled VP16 was first immunoprecipitated with LP1, the precipitate separated by SDS gel electrophoresis and then transferred to a nitrocellulose membrane. The VP16 band was excised, subjected to denaturation in 8 M urea and subsequently digested with EndoLysC as described in Materials and methods. Phosphopeptides were then separated by high resolution electrophoresis in urea-containing gels alongside a low molecular weight marker series. A doublet migrating at 16–17 kDa were the only bands observed (Figure 1b, arrowed). VP16 has a predicted mol. wt of 55 kDa but migrates during electrophoresis as 65 kDa. This anomalous migration is due to the C-terminal acidic domain, since deletion of this region results in a protein which migrates exactly as predicted (see e.g. Figure 2).

Fig. 1. VP16 is phosphorylated in vivo by cellular kinase(s). An expression vector for VP16 (VP16), or control vector (M) were transfected into COS cells which subsequently were radiolabelled with [32P]orthophosphate. Equal amounts of cell extracts were then analysed by Western blotting using LP1 for the expression of VP16 (lanes 1 and 2). Soluble extracts from the cells were incubated with the same antibody and 32P-radiolabelled VP16 immunoprecipitated from the test extract but not the control (lanes 3 and 4). After immunoprecipitation, the labelled VP16 band was transferred to a nitrocellulose membrane, the band excised and digested with the lysine-specific protease EndoLysC. Phosphopeptides were electrophoresed in high resolution urea-containing gels alongside low molecular weight markers (right hand panel). The lower schematic illustrates the position of lysine residues in VP16, (Ks), with numbers below indicating the predicted molecular weight of the peptide products. Also illustrated are the position of the antigenic regions recognized by a series of anti-VP16 antibodies as described in the text.

The 16–17 kDa doublet was too small to represent the largest 28 kDa EndoLysC product and, because of the anomalous migration of the acidic domain, it seemed reasonable to propose that the doublet represents the C-terminal EndoLysC product extending from residue 370 and encompassing the acidic domain. This proposal was confirmed by total protein staining combined with Western blot analysis of overexpressed and purified VP16 digested with EndoLysC, alongside analysis of the cleavage products from the in vivo labelled protein. These analyses showed that the largest 28 kDa product migrated as expected (i.e. not co-migrating with the 16–17 kDa doublet), and that the 16–17 kDa phosphorylated doublet co-migrated with the EndoLysC product specifically detected with an antibody (Figure 1, POS1) made to the C-terminal domain (not shown). The results indicate that the 16–17 kDa doublet represents a C-terminal phosphopeptide mapping between the lysine at 370 and the end of the protein (residue 490).

Formally it was possible that the upper band of the doublet represented a partial cleavage, i.e. 343–490 (see Figure 1c). However, we previously have demonstrated in limited proteolysis experiments that Lys370 is actually the most sensitive to cleavage by EndoLysC (Hayes and O’Hare, 1993). Furthermore, additional exhaustive
phosphorylated by the irregular line between S411 and T412) and shows
were purified after expression in bacteria (lanes 1 and 2 CBB staining) and
Residues within the complex-forming region are phosphorylated
EndoLysC cleavage did not alter the pattern, and anti-
peptide antibody (Figure 1, MCR9), which detects full-
length VP16 by recognition within the 343–370 region, did not detect either of the doublet bands. The simplest explanation for the doublet is, therefore, multiple phosphorylation sites in the C-terminal region leading to a shift in migration.
Together, these results indicate that VP16 is phosphorylated by a cellular kinase(s), that within the limits of detection the overwhelming majority of phosphorylation occurs downstream of residue 370, and that there may be multiple sites phosphorylated within this region.

**Identification of CKII sites within VP16**
To pursue further the identity of the kinase(s) involved in phosphorylation of VP16, we performed phosphoamino acid analysis of VP16 and VP16Δ immunoprecipitated from the NE kinase reactions. The results demonstrated that the majority of phosphorylation occurred on serines, with low but detectable phosphorylation on threonines (Figure 3a). Identical results were observed as expected for the truncated VP16 (Figure 3b). Figure 3c illustrates the region from Lys370 to the end of VP16Δ (indicated by the irregular line between S411 and T412) and shows that there are three candidate serines, at positions 375, 400 and 411. Two of these sites, 375 and 411, represent good consensus sites for CKII and protein kinase A (PKA) respectively. Apart from the serine at position 400, which does not conform to any consensus kinase site, positions 375 and 411 are the only serines present within the C-terminal phosphopeptide of VP16Δ. We were particularly interested in the potential site at residue 375 since it is located inside the C-terminal boundary of VP16 required for complex formation with Oct-1 and HCF (Figure 3c, indicated by the filled triangles at position 388) while the PKA site and a putative PKC site (on threonine) are located outside the boundary and are not required for TRF-C formation.
To explore phosphorylation by specific kinases further, we purified bacterially expressed VP16Δ or a variant of
VP16 phosphorylation at a critical serine residue

Fig. 3. VP16 is phosphorylated mainly on serines and Ser375 is a target for CKII. VP16 or VP16Δ were phosphorylated in vitro by the NE kinase(s), immunoprecipitated, transferred to PVDF and the isolated bands subjected to acid hydrolysis. Phosphoamino acids were separated by two-dimensional electrophoresis together with cold markers. Both VP16 (a) and VP16Δ (b) were phosphorylated mainly on serines. A minor but reproducible amount of phosphothreonine was detected for both species. The primary sequence of VP16 from Lys370 to the end of VP16Δ (irregular line between S411 and T412) is illustrated in (c). VP16Δ or VP16Δ containing a 375 serine to alanine substitution were expressed in bacteria and purified (d, lanes 1 and 2 CBB stain). Equal aliquots (100 ng) were then tested for phosphorylation by selected purified kinases as indicated. Asterisks indicate autophosphorylation of kinases. VP16Δ-phosphorylated by CKII was processed for determination of the EndoLysC phosphopeptide and produced the same phosphopeptide (e, lane 1) as the NE kinase(s); compare with Figure 2, lane 6. CKII-phosphorylated VP16Δ was also subject to phosphoamino acid analysis and only serine was detected (f).

VP16Δ containing a single amino acid substitution, i.e. Ser375 for alanine (VP16Δ375SA), and tested phosphorylation by isolated CKII and PKA or PKC. Figure 3d illustrates total protein staining of the input preparation of the VP16 species. Equivalent amounts were added to the appropriate kinase reaction and phosphorylation assayed without LP1 immunoprecipitation. The results demonstrate that each of the kinases was capable of phosphorylating VP16 (Figure 3d, tracks 1). Substitution of Ser375 effectively abolished phosphorylation by CKII. This was a specific effect, with the Ser375 substitution having no detectable effect on phosphorylation by either of the other kinases. It was formerly possible that mutation of the site at 375 affected phosphorylation by CKII elsewhere in the protein. We view this as very unlikely since we show (Figure 3e, lane 1) that EndoLysC cleavage of CKII-phosphorylated VP16Δ also resulted in a single peptide migrating at ~6 kDa, which, from analysis of intact and truncated VP16, maps from 370 to 411 (see above) and which contains only one CKII site, i.e. Ser375. As expected, no peptide was observed for VP16Δ375SA (Figure 3e, lane 2). Furthermore phosphoamino acid analysis of CKII-phosphorylated VP16Δ demonstrated (Figure 3f) that the majority of phosphorylation took place on serine, while all consensus CKII sites apart from Ser375 and one other in the N-terminus of the protein are threonines (Figure 3f, schematic). Altogether, the results indicate that within the N-terminal region of VP16, Ser375 is indeed phosphorylated by CKII and, moreover, that it is the major single site for CKII.

Multiple kinases phosphorylate VP16 between residues 370 and 411

As shown above, NEs in which VP16 functions to recruit Oct-1 and HCF in TRF-C assembly also contain kinase(s) which phosphorylate VP16, mainly in the 370–411 region, encompassing the 375 CKII site. We next wished to examine whether Ser375 was also phosphorylated in the NEs, and whether CKII was the only kinase involved. VP16Δ and VP16Δ375SA were phosphorylated using
Fig. 4. Additional sites in VP16Δ phosphorylated by NE kinase(s). Equal aliquots of VP16Δ with no substitutions (wild-type) or the single 375 S→A substitution were phosphorylated in vitro by NE kinase(s) and immunoprecipitated (a, lanes 1 and 2). Little difference in total phosphorylation was detected. VP16Δ wild-type or 375SA were phosphorylated by CKII or the NE kinase(s), immunoprecipitated, transferred to nitrocellulose and cleaved with EndoLysC. The phosphopeptides were then separated in high resolution urea-containing gels (b) or in standard gels (c). Note that in (c) the lanes were run in parallel on the same gel and different exposures were then aligned. (d) VP16 and VP16375SA were phosphorylated by the NE kinase(s), immunoprecipitated and subject to phosphoamino acid analysis. Despite the lack of Ser375, serine remained the major phosphoamino acid, but its relative level was reduced.

CKII or NE, immunoprecipitated using LP1 and analysed, or further transferred to nitrocellulose, cleaved with EndoLysC and products separated in urea denaturing gels as described above. The results (Figure 4) demonstrate several points. Firstly, mutation of Ser375 which abolished phosphorylation by CKII had only a minor effect in absolute terms on phosphorylation by the NE (Figure 4a, lanes 1 and 2). Therefore, other sites must be targets for the NE kinase(s) but, from the results of Figure 2, lane 6, mutant containing the 375SA substitution was performed and, as predicted from the results above, serine remained these sites must also lie within 370–411.

Upon direct comparison of the EndoLysC cleavage products of CKII- versus NE-phosphorylated VP16Δ, it can be seen that the latter migrated at a significantly higher position in the gel (Figure 4b, cf. lanes 2 and 3). Furthermore, while the 375SA mutation had no perceptible effect on total NE phosphorylation, it resulted in a shift in migration of the 6 kDa peptide to a slightly faster migrating form. (Note again that quantitative differences in the intensity of the peptide cannot be attributed significance due to variations in recovery. However, the qualitative shift in migration was significant and reproducible.) This shift in migration of the 6 kDa peptide was also observed in conventional SDS gels (Figure 4c) between CKII and NE phosphorylation of VP16Δ (cf. lanes 1 and 2), and between NE phosphorylation for VP16Δ and VP16Δ-375SA (cf. lanes 2 and 3). We interpret these results as follows. Migration of the CKII peptide (and its complete loss in the mutant) is consistent with a single phosphorylation event at 375. The NE-phosphorylated peptide from VP16Δ375SA migrated higher than the CKII peptide, and the NE-phosphorylated peptide from VP16Δ higher again. This is consistent with the possibility of NE phosphorylation of three sites in the wild-type VP16Δ, and two in VP16Δ375SA (of necessity other than 375) while, as indicated above, CKII phosphorylation is at the single 375 site.

We showed above (Figure 3a) that the major phosphorylation site by the NE kinase(s) was on serine. Comparative phosphoamino acid analysis of the NE-phosphorylated full-length wild-type VP16 or a full-length mutant containing the 375SA substitution was performed and, as predicted from the results above, serine remained the major phosphoamino acid (Figure 4d, cf. wild-type and 375SA). Absolute levels of phosphoserine cannot be used to assess the effect of the 375 substitution. However, since phosphothreonine was also detected, quantitative analysis of the phosphoserine:phosphothreonine ratios can be used. Quantitative estimates of the plates for Figure 4d (and similar additional samples) using PhosphorImager analysis demonstrated that the 375SA mutation resulted in a significant decrease in the relative phosphoserine content. The average of four independent estimates showed that the phosphoserine:phosphothreonine ratio for the 375SA mutant was ~70% that of the wild-type. (Note that if two sites were equally phosphorylated in a protein, complete loss of one site in a mutant would ideally result in a reduction to 50% of wild-type; if three sites are phosphorylated, loss of one site results in a reduction to ~66%). The reduction in ratio of the 375SA mutant to 70% was reproducible and significant. Together with the shift in migration of the 6 kDa peptide, these data indicate that 375 is a target for phosphorylation by the NE kinase(s), that an additional serine(s) is also a major target, and that lower efficiency phosphorylation at threonine(s) takes...
place. Since it is the only one within a consensus site, the additional serine phosphorylated by the NE kinase(s) is likely to be at position 411, a consensus PKA site (Figure 3c). Phosphorylation at this position has not been investigated further here.

Serine at 375 is essential for complex assembly

Of the sites identified in the analyses above, only Ser375 was contained within the region of VP16 involved in complex assembly with Oct-1 and HCF (see Figure 3c). We therefore wished to address the question of the relevance of this site to VP16 function. Full-length wild-type VP16 and the corresponding mutant with the single substitution at 375 were analysed for complex formation with Oct-1 and HCF. These assays utilize the same extracts as those shown above to phosphorylate VP16. Typical profiles of the purified proteins are shown in Figure 5a. Equivalent amounts were then assayed by gel retardation analysis in parallel dose–response experiments (Figure 5b) for the ability to be recruited into the Oct-1–HCF–VP16 DNA-binding complex TRF-C. The results were unambiguous and dramatic. The single S→A substitution at 375 had a major debilitating effect on VP16 complex formation and, while it was not abolished, the dose–response experiments indicate that specific activity of the mutant protein was at least 50- to 100-fold lower than wild-type.

Although infrequently addressed, it is important to rule out any major alteration in structure or folding of a protein as the cause of any particular phenotype. This seemed unlikely for several reasons. Firstly, the single serine to alanine mutation can be considered a relatively minor perturbation. Secondly, we have shown that the region encompassing residue 375 is present in a surface-exposed loop or domain and as such would be unlikely to have a major role in structure. Furthermore, we also subjected the wild-type and mutant proteins to limited proteolysis in protease dose–response experiments with a number of
site-specific enzymes and, in all cases, typified by Endo-
LysC (Figure 5c), not only the pattern of proteolytic
products (Figure 5c, open arrows) but also their appearance
at different doses of protease were virtually identical.
Similarly, in both quantitative and qualitative terms, the
patterns of proteolytic products using other proteases were
identical for the wild-type and mutant proteins. Since
limited proteolysis is used to analyse protein folding and
identify surface-exposed loops and linker regions, the
results indicate, within the limits of the technique, that
the S375 to alanine mutation did not induce any large
scale alteration in structure.

We also compared other activities of the wild-type and
mutant proteins. VP16 has weak intrinsic DNA-binding
activity and, although the role of this activity remains to
be determined, we wished to examine whether the mutant,
while unable to form a full complex, retained the weak
independent DNA-binding activity. Pure VP16 and
VP16375SA were therefore assayed for in gel retardation
assays in the absence of Oct-1 or HCF, and the results
(Figure 5d, arrowed) demonstrate that the mutant protein
retained the weak independent DNA-binding activity of
the wild-type protein. (Note, these results do not indicate
either way the relevance of VP16 DNA binding to complex
formation. We use the assay simply to demonstrate that
the S375 mutation, while abolishing complex formation,
did not affect all activities of VP16.)

Finally, we have shown previously that VP16 interacts
in vivo with a second HSV structural protein VP22 and
demonstrated specific requirements within VP16 for this
interaction (Elliott et al., 1995). Independent in vivo
localization and in vivo VP22 binding of VP16375SA
were indistinguishable from the wild-type protein (Gill
Elliott, personal communication).

We anticipated that the 375 S→A substitution may
affect only one of the multiplicity of interactions involved
in complex formation and, therefore, that VP16375SA
may dominantly interfere with complex formation by
the wild-type protein. We titrated VP16375SA into reactions
containing a fixed amount of wild-type VP16. The results
(Figure 6) demonstrate that with the mutant protein some
interference in complex formation was observed even at
the lowest ratio tested (3:1) and that inclusion of
VP16375SA at a ratio of 15:1 almost completely abolished
TRF-C formation. This result necessitates that VP16375SA
interacts with a limiting factor in complex formation. The
DNA probe is present in excess, and our previous results
indicate that HCF is in considerably lower abundance
than Oct-1. Therefore, the data are consistent with the
interpretation that VP16375SA, while defective in some
aspect of complex assembly, can still interact with HCF.
The precise defect in VP16375SA remains to be investi-
gated (see Discussion) but, with reference to this analysis,
the results demonstrate that the mutant retains at least
some activity which enables it to interfere with the wild-
type complex formation. Altogether, the data provide
compelling evidence that the Ser375 residue which is
phosphorylated by CKII, and by a kinase in the NE, is also
functionally critically important for complex formation in
the same NE.

Serine but not aspartic or glutamic acid at 375 is
required for in vivo VP16 transactivation
To examine the involvement of Ser375 in vivo in transac-
tivation of target promoters, eukaryotic expression plasmids

![Fig. 6. VP16375SA dominantly interferes with complex formation by wild-type VP16. Control samples showing complex formation in the absence and presence of wild-type VP16 (0.01 μg) are shown in the left two tracks. Increasing doses of VP16375SA were pre-incubated with this constant dose of wild-type VP16 in nuclear extract, with the ratio of mutant to wild-type indicated at the bottom. Radiolabelled probe was then added after 5 min pre-incubation, and incubation continued for a further 20 min. Complex formation was then assessed by standard electrophoretic shift assay.](image-url)
the acidic domain as substrate, we show that the substituted threonine at position 375 also acts as an acceptor for phosphorylation by CKII in vitro (Figure 7e).

Discussion

In this work, we demonstrate that VP16 is phosphorylated in vivo by one or more cellular kinases, and that in vivo the vast majority of phosphorylation takes place at sites downstream of position 370. We show that the same region is the target for in vitro phosphorylation by kinases within NEs, and identify the major sites as Ser375, a consensus CKII site which we show is indeed the major N-terminal CKII site in the protein, Ser411, a consensus PKA site, and another minor threonine not identified definitively but also residing in the same peptide region 370–413 (see summary Figure 9). Mutation of Ser375 to alanine abolishes complex formation in the same NEs in vitro, and functional transactivation in vivo. We provide evidence that the mutant protein retains several other activities and has not undergone any major structural alteration. No other residue tested, including aspartic or glutamic acid, could functionally replace serine at 375, apart from threonine which restores both complex formation and transactivation to normal levels. We have as yet been unable to augment these data with the direct demonstration that CKII phosphorylation of VP16 induces complex-forming activity, since this requires purified kinase-free components, HCF, Oct-1 and VP16. However, in our hands, purified preparations of HCF from eukaryotic cells retain VP16 kinase activity and, to date, while we have pure forms of bacterially expressed HCF, none retain functional activity. Nevertheless, altogether the data above on phosphorylation and on the requirement for a serine or threonine at 375 provide strong evidence that phosphorylation of VP16 may play a major role in functional activity and that Ser375 is a critical target.

The precise mechanism of regulation of transcription by phosphorylation can operate at any of several levels, including, for example, regulation of nuclear translocation of the active components, structural or other alterations (e.g. dimerization) to stimulate or inhibit DNA binding, and direct effects on transcriptional activation domains through modulating co-activator interactions. The intracellular localization of the variant containing the 375 serine to alanine substitution is indistinguishable from wild-type VP16, and we demonstrate that the defect in in vivo transactivation can be explained by a defect in assembly of the TRF-C complex with Oct-1 and HCF. The independent DNA-binding activity of VP16375SA was unaffected, indicating that this is not the explanation for defective complex formation (and in any case there are no unambiguous results which demonstrate the relevance of VP16 DNA-binding activity). The results therefore indicate that Ser375 is involved in Oct-1 or HCF interaction. We previously showed that peptides corresponding to residues around position 375 were sufficient to interfere competitively with complex formation by intact VP16 (Haigh et al., 1990). However, the active inhibitory peptide was refined to residues 360–367 (Hayes and O’Hare, 1993), not requiring the 375 CKII site, and further results using different pairwise combinations of factors now indicate that the peptide inhibits interaction between HCF and VP16. Furthermore, we have established an assay
system which demonstrates direct, HCF-independent interaction between VP16 and intact Oct-1 in NEs. This interaction is efficient and takes place with amounts of VP16 similar to those required for full HCF-dependent complex. Ser375 is required for the direct interaction with Oct-1 while, for example residue 366, within the inhibitory peptide, is not required for the direct interaction but is required for the HCF-dependent complex. This is consistent with the results presented here that VP16375SSA is able to interfere competitively with complex formation by the wild-type protein, probably by retaining the ability to interact with HCF. Thus the requirement for serine or threonine at position 375 is likely to reflect specific involvement in the Oct-1 recognition step in complex assembly. Refined analyses have identified critical residues on the surface of the Oct-1 homeodomain (Stern and Herr, 1991; Pomerantz et al., 1992), and a single glutamic acid to alanine change within helix 2 substantially impairs VP16 binding (Lai et al., 1992). Ser375 is located within a surface-exposed region of VP16 which is masked after complex assembly (Hayes and O’Hare, 1993), and it is possible that Ser375 is involved in direct recognition of the critical helix 2 region of Oct-1. However, other scenarios for the detailed mechanism of the involvement in complex formation are equally valid at the moment. It may be that in the unphosphorylated state this region of the protein masks a domain required for Oct-1 binding and phosphorylation releases the inhibition. Furthermore, since current models of the pathway of TRF-C assembly indicate that HCF–VP16 interaction occurs prior to Oct-1 recognition, it is possible that HCF binding actually influences phosphorylation at the 375 site and subsequent Oct-1 interaction. These proposals are currently under investigation.

Phosphorylation of VP16 may act as a switch between functional and inactive states for transactivation, and this may be important in regulating VP16 activity during viral infection or in a cell type- or cell state-specific manner. Although previous results have indicated that VP16 is phosphorylated in virus-infected cells (Wilcox, 1980; Moss, 1989), it has not been demonstrated to be the target of viral-encoded kinases. This, combined with our results on the direct demonstration of VP16 phosphorylation by cellular kinases(s), indicates that the simplest explanation for phosphorylation in infected cells is via the action of the cellular enzymes. However, it is possible that subtle modification of VP16 phosphorylation by the viral-encoded kinase(s) may take place and/or that modulation of the cellular kinases throughout virus infection regulates VP16 transactivation function; for example, late in infection, switching off the transactivation function but maintaining the structural role will be important. Furthermore, CKII is present in the cytoplasm and nucleus. It will be interesting to examine whether VP16 phosphorylation at individual selected residues is altered during infection of cells and whether there is any correlation between cellular location and phosphorylation patterns.

However, while we have demonstrated that Ser375 can be phosphorylated by CKII, it is formally difficult to prove that this is the relevant kinase in vivo. Unlike other kinases, there are no good selective CKII inhibitors, and CKII is both ubiquitous and constitutively active (for a review, see Meisner and Czech, 1991). It is thus possible that the nuclear kinase responsible for 375 phosphorylation is not CKII despite the consensus site. We are examining CKII immunological depletion, conventional purification of the kinase(s) and generation of antibodies to phosphorylated and non-phosphorylated forms of peptides encompassing the 375 site to provide additional evidence on kinase identity. Nonetheless, CKII has been shown to phosphorylate several transcription factors, including for example SRF, Myb, Max, Fos and p53 and CREB, and CKII is itself a target for phosphorylation by cdc2 kinase (Meisner and Czech, 1991). Mitogenic stimulation has also been reported to induce CKII activity and, therefore, it is possible that cell state, or cell cycle controls of VP16 phosphorylation could operate through CKII. Our studies now add an additional level of complexity in the overall mechanism of action of VP16. Further studies of the modulation of VP16 phosphorylation in a cell type-specific manner or in response to messenger signalling pathways may provide a link between environmental stimuli and VP16 trans activation activity.

**Materials and methods**

**Plasmid construction**

The construction expression vectors pRG50 and pRG14 which contain respectively the complete coding sequence and that for the N-terminal region of VP16 from residues 1 to 411, each driven by the cytomegalovirus immediate early promoter, have been described previously (Meisner and Czech, 1991). VP16-375 and VP16-375SSA were subcloned into pRG50 and pRG14 between the EcoRI and BglII restriction sites. The position of the nucleotide changes within the coding sequence of VP16 were confirmed by DNA sequencing.
VP16 phosphorylation at a critical serine residue

**Virus IE enhancer/promoter, have been described previously (Greaves and O’Hare, 1989, 1990). SpHl and BglII sites were introduced at codons 361–363 and 381–383, respectively, by changes which did not affect the VP16 amino acid sequence. HindIII–Srrl fragments from these modified plasmids encompassing the introduced sites were then inserted into either pRG64 (Liu and O’hare, 1990) or pRG65 plus new SpHl and BglII sites. The 160 bp SpHl–BamHI fragment from pRG64 was subcloned into pTZ18U to give the vector pRG73 for further oligonucleotide-directed mutagenesis. Single-stranded DNA was prepared from pRG73, and most mutations in the VP16-coding sequence were generated with the Mutagene system by using 15mer oligonucleotides mismatched at the central nucleotide. Mutant clones were identified and reintroduced into SpHl- and BamHI-cut pRG64. The Srrl–BamHI fragment of pRG70 was introduced subsequently to restore the C-terminal activation domain and so produce the mutants in full-length form. Certain mutations of Ser375 were generated by insertion of an appropriate oligonucleotide pair into SpHl- and BglII-cut pRG64. All mutations were identified by sequence analysis of the resulting plasmids.

For large scale production of VP16 for proteolytic analysis and as substrate for in vitro phosphorylation assays, the VP16 open reading frame was fused to the GST reading frame contained in the plasmid pGEX2T (Pharmacia) to give pMK6 as described previously (Hayes and O’Hare, 1993). Derivatives of pMK6 for expressing mutant and truncated VP16 were made by subcloning the Sst fragment from the appropriate mutant in the pRG70 or pRG64 background.

**Cells, transfection procedures and transient expression assays**

HeLa cells and COS cells were cultured routinely in Dulbecco’s modified Eagle’s minimal essential medium containing 10% newborn calf serum. Transfections were performed in cells by using sodium-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES)-buffered saline as described previously (Greaves and O’Hare, 1989). For assays of transactivation, the target plasmid was pAB5, which contains the promoter from the IE 110 kDa gene of HSV-1 with a single octamer-GARAT motif from the HSV-1 VP16 amino acid sequence and VP16 expression and purification

VP16 constructs were made essentially as described previously (Greaves and O’Hare, 1989). COS cells (1 × 10⁶ cells) transfected with 2 μg of the expression vector were harvested at 42 h after transfection and prepared for electrophoresis by being boiled for 5 min in sample buffer. After electrophoresis and transfer to nitrocellulose, VP16 and variants were detected using LPI (1:2000) and, subsequently, goat anti-mouse immunoglobulin–horseradish peroxidase conjugate (1:4000). The filters were developed with diaminobenzidine and hydrogen peroxide as the substrate, with nickel enhancement or using the enhanced chemiluminescence system (ECL) according to the manufacturer’s instructions.

**Analysis of expression vector products**

Whole cell extracts of COS cells transfected with wild-type or mutant VP16 proteins in vivo were analysed by Western blotting of transfected cells with polyclonal antibody LP1 as previously described (Greaves and O’Hare, 1989). COS cells (1 × 10⁶ cells) transfected with 2 μg of the expression vector were harvested at 42 h after transfection and prepared for electrophoresis by being boiled for 5 min in sample buffer. After electrophoresis and transfer to nitrocellulose, VP16 and variants were detected using LPI (1:2000) and, subsequently, goat anti-mouse immunoglobulin–horseradish peroxidase conjugate (1:4000). The filters were developed with diaminobenzidine and hydrogen peroxide as the substrate, with nickel enhancement or using the enhanced chemiluminescence system (ECL) according to the manufacturer’s instructions.

**Extract preparation and gel retardation analysis**

Whole cell extracts of COS cells transfected with wild-type or mutant VP16 constructs were made essentially as described previously (Greaves and O’Hare, 1989). Cells (~10⁷ cells per dish) were transfected with requirement for immunoprecipitation. Whole cell extracts of COS cells transfected with wild-type or mutant...
Ation, VP16 was immunoprecipitated using LP1 separated by SDS gel electrophoresis and transferred in this case to nitrocellulose membranes (Luo et al., 1991). The phosphorylated VP16 band was excised and the slice was then blocked with 0.5 ml of 0.5% polyvinylpyrrolidone, 100 mM acetic acid for 30 min at 37°C, washed 10 times in H2O, and the protein denatured by heating at 50°C for 15 min in 30 ml of 8 M urea, 0.4 M NH4HCO3, 6.5 mM DTT. After cooling to 25°C, 5 µl of 100 mM iodoacetamide was added and the volume made to 100 µl with H2O, and the slice was incubated for 15 min prior to the addition of 1 µg of EndoLys.C. After overnight incubation at 37°C, a further 1 µg of the protease was added and incubation continued for another 6 h. The sample was then taken up in SDS sample buffer and electrophoresed in high resolution urea-containing gels with double stacking layers (Schagger and von Jagow, 1987).

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References


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