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A Cellular Factor Binds to the Herpes Simplex Virus Type 1 Transactivator Vmw65 and Is Required for Vmw65-Dependent Protein-DNA Complex Assembly with Oct-1

PETER XIAO AND J. P. CAPONE*

Department of Biochemistry, McMaster University, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5

Received 15 May 1990/Accepted 25 June 1990

The herpes simplex virus transactivator Vmw65 assembles into a multicomponent protein-DNA complex along with the octamer binding protein Oct-1. Using affinity chromatography on columns conjugated with purified Vmw65 fusion protein expressed in *Escherichia coli*, we demonstrate that a cellular factor, distinct from Oct-1, binds to Vmw65 in the absence of target DNA and is necessary for Vmw65-mediated complex assembly with Oct-1.

The genes of herpes simplex virus (HSV) are expressed in a temporal fashion during the course of a lytic infection and are regulated by complex positive and negative control circuits mediated by combinatorial interactions of both viral and cellular factors. The immediate-early genes are the first to be expressed following infection, and their expression is strongly stimulated by a preformed virion phosphoprotein variously termed Vmw65, VF65, VP16, and αTIF (2, 5, 6, 22). Conserved *cis*-acting elements with a consensus sequence TAATGARAT (R = purine), present upstream of the immediate-early genes, are necessary for Vmw65 responsiveness; however, Vmw65 does not bind directly to these sites (4, 8, 13, 16, 18, 23). Instead, Vmw65 assembles into a multicomponent DNA binding complex along with cellular factors, one of which has been identified as the ubiquitous octamer binding protein Oct-1 (9, 14, 19–21, 24, 27). Many TAATGARAT elements contain overlapping octamer motifs (ATGCAAAT), and Oct-1 is able to bind these sites independently, while HSV-specific sequences (GARAT) downstream from the octamer binding site, though not necessary for Oct-1 binding, are essential for the formation of Vmw65-dependent complexes (1, 3, 9, 15, 21). Thus, Vmw65 achieves its specificity through protein-protein interactions with preexisting cellular factors and provides a valuable model system to investigate how eucaryotic transactivators that do not bind directly to DNA can alter patterns of gene expression (10).

Recent studies have shown that at least one other cellular factor, in addition to Oct-1, is necessary for the generation of Vmw65-dependent protein DNA complexes; however, there is no evidence demonstrating whether Oct-1 or other factors bind directly to Vmw65 (9, 12). To determine whether cells contain factors which interact directly with Vmw65, we fractionated HeLa cell extract by affinity chromatography on columns coupled with Vmw65 purified from *Escherichia coli* as a fusion protein with *Staphylococcus aureus* protein A. We demonstrate here that HeLa cells contain a factor(s) that interacts with Vmw65 and is required, along with Oct-1, for the generation of Vmw65-dependent protein-DNA complexes.

As described previously, the *E. coli* expression plasmid pRITsal directs the thermoinducible expression of amino acids 4 to 411 of Vmw65 fused to protein A (29). Vmw65 so synthesized lacks the acidic C-terminal transactivation domain but is fully capable of generating Vmw65-dependent complexes with Oct-1 and thus retains all the information necessary to direct protein-DNA complex assembly (11, 26, 28, 29). Protein A-Vmw65 fusion protein was expressed in *E. coli* N4830-1 and purified by affinity chromatography on immunoglobulin G-Sepharose 4B as described previously (29) and coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia). Briefly, cyanogen bromide-activated Sepharose was pretreated with 1 mM HCl according to the instructions of the manufacturer. Immediately before coupling, the gel was washed once with coupling buffer (CB) (0.1 M $NaHCO_3$ [pH 8.3], 0.5 M NaCl). To 1 ml of settled gel was added 5 mg of purified protein A-Vmw65 in 2.5 ml of CB, and the mixture was gently rotated overnight at 4°C. The settled gel was suspended in 5 volumes of 1 M glycine (pH 8.0)–1 mM dithiothreitol and incubated as described above for 8 h in order to block any remaining reactive groups. The gel was collected by brief centrifugation and washed sequentially with 10 volumes each of CB, 0.1 M acetate (pH 4)–0.5 M NaCl, and 0.1 M Tris hydrochloride (pH 8.0)–0.5 M NaCl. The above-described cycle was repeated twice more, and finally the gel was washed and suspended in affinity column buffer (AB) (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.9], 0.1 mM EDTA, 1 mM dithiothreitol, 50 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, 100 kU of apoprotein per ml, 10% glycerol) and stored at 4°C. As controls, protein A purified from *E. coli* harboring the parental expression vector pRIT2T was purified and coupled in parallel under identical conditions. Additional controls included cyanogen bromide-Sepharose coupled with bovine serum albumin (BSA) (Fraction V; Sigma Chemical Co.) or affinity gel that contained no immobilized ligand but in which reactive groups were neutralized with 1 M glycine (blank column). Protein coupling efficiency in all cases was greater than 95%.

Chromatography was carried out at 4°C using 2-ml disposable columns (Bio-Rad) packed with 300 μl of affinity gel. Columns were washed sequentially with 10 column volumes of AB, AB–100 μg of BSA per ml, and finally AB. Whole cell extracts were prepared from HeLa cells as described previously (17). The final buffer composition in the extract was 40 mM Tris (pH 7.9), 10 mM $MgCl_2$, 100 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10%

* Corresponding author.

glycerol. Prior to use, the extract was diluted with an equal volume of the above buffer without KCl to bring the salt concentration to 50 mM and 1 ml (20 to 25 mg of protein) of extract was applied to the Vmw65 and control columns at a flow rate of 2 ml/h. The column flowthrough was recycled twice and collected. The columns were washed sequentially with 10 column volumes of AB, AB–0.05% Tween 20, and finally AB. Bound material was eluted with 4 column volumes each of AB containing 0.1, 0.25, 0.4, and 1 M KCl. This was followed by 4 column volumes of 0.2 M acetic acid (pH 3.4). The fractions were desalted on PD10 columns (Pharmacia) with AB and concentrated to 200 μl by using Centricon 10 microconcentrators (molecular weight cutoff, 10,000; Amicon Corp.), and the protein concentration was determined.

On the premise that a cellular factor(s) which is required for Vmw65-mediated complex formation can bind directly to Vmw65, it was anticipated that cell extract chromatographed on a Vmw65-conjugated column might be depleted of such a factor and thus be unable to generate Vmw65-dependent complexes. To examine this hypothesis, mobility shift assays were carried out with a labeled oligonucleotide containing the promoter-proximal TAATGARAT element from the HSV-1 ICP0 gene as described previously except that whole-cell extracts (20 to 25 μg/20-μl reaction) rather than nuclear extracts were used (7, 29). Vmw65 was prepared from an Nonidet P-40 extract of purified HSV-1 virions (18) and was added, where indicated, at 0.05 μg per binding reaction.

Figure 1 shows the results of mobility shift assays using whole-cell extract that was subjected to chromatography on the various columns. In each case, an equivalent amount of protein (25 μg) present in the excluded fraction from each column was incubated with the labeled probe and 0.05 μg of Vmw65. Lane a is a control showing the major Oct-1-containing complex generated after incubating probe with whole-cell extract prior to affinity chromatography, whereas lane b demonstrates the formation of Vmw65-induced complex (VIC) after addition of Vmw65 to this extract. As demonstrated in the figure, the excluded fraction obtained following chromatography on the blank, BSA, or protein A-coupled columns was capable of generating both Oct-1 complex and VIC (lanes c to e, respectively). In contrast, the excluded fraction obtained following chromatography on the column containing immobilized Vmw65, while capable of forming the Oct-1 complex, was greatly reduced in its ability to generate VIC in the presence of Vmw65 (lane f). Thus, HeLa cell extract can be specifically depleted of its ability to generate Vmw65-dependent complexes by chromatography on columns containing immobilized Vmw65.

Material retained on the Vmw65-conjugated column was step eluted with increasing amounts of KCl as described above, and fractions were tested for their ability to reconstitute Vmw65-dependent complex formation in the depleted extract. As demonstrated in Fig. 2, material eluted with 0.1 mM KCl and 0.25 M KCl had little effect on VIC formation when added back to the deficient extract (lanes d and e). The 0.4 M KCl fraction was able to restore some VIC-forming ability to the extract, while the 1 M KCl extract was capable of significantly reconstituting activity (lanes f and g, respectively).

Incubation of the 1 M KCl eluent with probe in the absence of protein extract demonstrated that this fraction contained no Oct-1-binding activity (lane h). In addition, none of the other salt fractions contained detectable Oct-1 binding activity as assessed by gel retardation analysis (not shown). These data suggest that Oct-1 does not form a

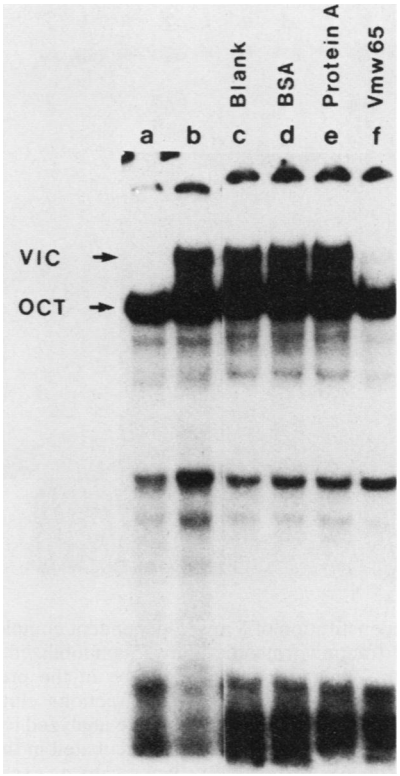

FIG. 1. Cell extracts chromatographed on columns containing immobilized Vmw65 are unable to form Vmw65-dependent protein-DNA complexes. HeLa whole cell extract was chromatographed on affinity columns containing no ligand (blank, lane c) or containing immobilized BSA, protein A, or protein A-Vmw65 (lanes d to f, respectively). An equivalent amount (20 μg) of the excluded fraction from each column was incubated with 0.05 μg of Vmw65 and the following labeled probe DNA.

5′-GATCCCGTGCATGCTAATGATATTCTTT
GGCACGTACGATTACTATAAGAAAGATC-3′

Protein-DNA complexes were resolved by electrophoresis on a 3.5% polyacrylamide gel (30:1 ratio of acrylamide to *N,N′*-bisacrylamide). Lanes a and b, Whole-cell extract, prior to chromatography, incubated in the absence or presence of Vmw65, respectively. OCT, Oct-1-containing complex. VIC, Vmw65-induced complex.

complex, directly or indirectly, with Vmw65 in the absence of target DNA or that such a complex, if formed, is unstable under the conditions used for chromatography. Addition of the 1 M KCl eluent to the deficient extract in the absence of Vmw65 had no effect on the mobility of Oct-1 (lanes k and l).

As mentioned above, sequences downstream from the octamer-binding site (GARAT) are necessary for VIC assembly; however, there is no evidence demonstrating whether Vmw65 interacts directly with GARAT sequences or whether this interaction is mediated by Oct-1 or another cellular factor. Interestingly, a fast-migrating complex is generated after the probe is incubated with the 1 M KCl eluent in the absence of protein extract (Fig. 2, lane h, band X′); however, this complex appears to be nonspecific on the basis of the following observations: (i) a complex of similar mobility was generated with the corresponding fractions from the control columns which were unable to reconstitute VIC formation (data not presented), and (ii) the addition of Vmw65 to the 1 M KCl eluent did not result in a change in the mobility of X′ as might be expected if X′ represented a

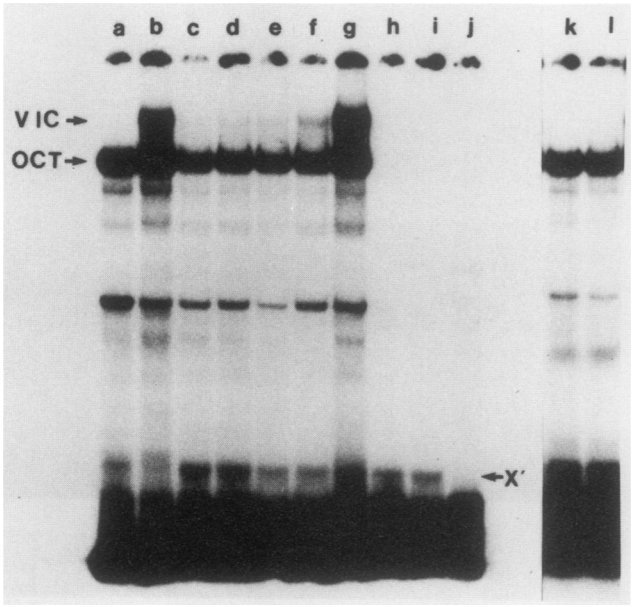

FIG. 2. Reconstitution of Vmw65-dependent complex assembly. The excluded fraction from the Vmw65-immobilized column was incubated with Vmw65 and labeled probe in the presence of an equivalent volume of each of the salt fractions eluted from the column, and protein-DNA complexes were analyzed by gel retardation. Lanes a and b, Whole-cell extract incubated in the absence or presence of Vmw65, respectively; lanes c to g, excluded extract incubated with Vmw65 and no further addition (lane c) or containing 5 μl of the 0.1, 0.25, 0.6, or 1 M KCl fraction (lanes d to g, respectively); lanes h and i, 10 μl of the 1 M KCl fraction incubated alone or in the presence of Vmw65, respectively; lane j, Vmw65 incubated alone. Lanes k and l are the same as lanes c and g, respectively, except that Vmw65 was not included in the reactions. X', Nonspecific protein-DNA complex generated after the 1 M KCl fraction was incubated with probe.

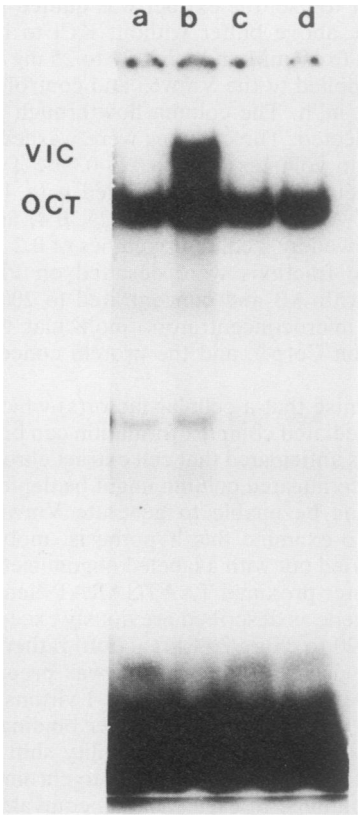

FIG. 3. The cellular factor interacting with Vmw65 is heat sensitive. The excluded fraction from the Vmw65-immobilized column was incubated with Vmw65 and probe DNA in the absence (lane a) or presence of 5 μl of the 1 M KCl fraction that was untreated (lane b) or that was heated for 2 min at 80°C (lane c) or 100 °C (lane d).

DNA binding activity that could specifically interact with Vmw65 (Fig. 2, lane i). X' most likely represents a DNA binding protein which nonspecifically interacts with the column and that has a higher affinity for the probe DNA than for the competitor DNA [poly(dI-dC)].

We have tentatively designated the Vmw65-interacting factor as VCAF-1 (for Vmw65 complex assembly factor), although we do not know at present whether the reconstituting activity represents one or several factors. VCAF-1 has a molecular weight greater than 10,000 as determined by gel exclusion chromatography using Sephadex G25 and by the fact that it is retained following ultrafiltration using membranes with a molecular weight cutoff of 10,000. VCAF-1 is probably proteinaceous in nature since it is completely inactivated by heat treatment (Fig. 3, lanes c and d).

In summary, at least one other cellular factor in addition to Oct-1 is required to generate Vmw65-dependent protein-DNA complexes and this factor can interact directly with Vmw65 in the absence of target DNA. Vmw65 is capable of discriminating among different DNA-bound octamer binding proteins by virtue of subtle differences in the POU-homeodomains; however, there is no evidence that Oct-1 and Vmw65 interact directly with each other (12, 25). On the contrary, as described above, we have not been able to detect Oct-1 binding to a Vmw65-conjugated column in the absence of target DNA. It is intriguing to speculate that cellular factors such as VCAF-1 may mediate interaction of Vmw65 with Oct-1 and confer selectivity among different octamer binding proteins. Recently, Kristie et al. have shown, using Oct-1 depleted nuclear extracts or Oct-1 expressed in vitro, that two cellular factors, termed C1 and C2, are involved in Vmw65-dependent complex assembly with Oct-1 (12). It remains to be established whether one or both of these factors are similar to VCAF-1.

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