trans-Dominant Inhibition of Herpes Simplex Virus Transcriptional Regulatory Protein ICP4 by Heterodimer Formation

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Herpes simplex virus encodes a 175-kilodalton immediate-early transactivating protein referred to as ICP4. A mutant ICP4 molecule expressed from a stable transformed cell line lacks the sequences required for transactivation yet retains the ability to specifically associate with DNA and to form homodimers. Expression of the mutant ICP4 peptide from this cell line, designated X25, resulted in the inhibition of herpes simplex virus growth. Wild-type ICP4 homodimers were depleted in X25-infected cells by the formation of heterodimers containing the wild-type ICP4 molecule and the mutant peptide. While the ICP4 heterodimer retained DNA-binding activity, immunological studies suggest that the wild-type subunit of the heterodimer is conformationally altered in a region that serves as the antigenic epitope. Physical studies that determined the composition of the heterodimer and its native size and approximate shape support this observation. The structural change is in a region of ICP4 genetically implicated as important for transactivation and may result in an alteration in an interaction between ICP4 and a target protein essential to promote transcriptional activation. Sequestering wild-type monomers of a viral regulatory protein into heterodimers which are less proficient in transactivation may explain the dominant inhibitory activity of the X25 cells, resulting in attenuation of viral growth.

ICP4 is the major transcriptional regulatory protein expressed by herpes simplex virus type 1 (HSV-1). The 175-kilodalton (kDa) protein (7) induces the expression of many HSV genes during viral infection and also autoregulates its own expression (12, 34, 35). Clustered regions of ICP4 primary structure are conserved in the 140-kDa immediate-early (IE) protein of varicella-zoster virus (VZV) (24) and the 180-kDa IE protein of pseudorabies virus (PRV) (5), suggesting the presence of functionally important domains. The analysis of mutant polypeptides has identified several such regions of conserved primary sequence that are required for activity (11, 31, 38). One region specifies the primary structure important for specific DNA-binding activity (32, 38). ICP4 binds to DNA containing the consensus sequence ATCGTC (13). It has also been shown that ICP4 can bind to several sequences which differ from the ATCGTC motif (26). Within the defined DNA-binding domain are highly conserved sequences which are predicted to form a hydrophobic helix-turn-amphipathic helix structural motif, using the secondary structure models of Chou and Fasman (6). A second region of primary structure within the defined DNA-binding domain may confer DNA-binding sequence specificity, as mutations in the region can limit the range of sequences to which ICP4 can bind (18, 38). The two additional regions of primary sequence required for transactivation most probably reflect residues involved in interactions with target proteins involved in the process of transactivation. One of these regions (amino acids 143 to 210) (38) contains a highly conserved 24-amino-acid stretch containing 19 serine residues and was genetically implicated as a site of phosphorylation (11). The negative charge resulting from phosphorylation of these residues may serve as an activation domain in a manner analogous to that of other regulatory proteins (17, 22, 43). Alternatively, this region may function as a phos-

phate donor or acceptor for modification of target proteins. The second region of primary sequence important for transactivation is less well defined (located carboxy terminal to amino acid 774) and does not contain any obvious similarities to previously identified activation domains (11). The two regions somewhat functionally compensate for each other, as mutations affecting either domain alone only partially impair transactivity (11; N. DeLuca, unpublished data), while deletion of both regions completely abolishes ICP4 activity (38).

The impaired regulatory activity of several ICP4-deficient mutants can be restored by heterodimer formation (38). Cells coinfecting with pairs of viruses encoding ICP4 molecules altered in different regions of the molecule resulted in the partial reconstitution of ICP4 activity. ICP4 heterodimers were observed in the coinfecting cells. Thus, distinct functional domains of the ICP4 molecule may be assembled in heterodimers composed of mutant molecules, thereby resulting in intragenic complementation. The residues important for ICP4 multimerization have not yet been identified, but proposed dimerization interfaces such as the leucine zipper of the *fos-jun* heterocomplex (19, 30) or the helix-loop-helix motif of several regulatory proteins (28) are not evident in the primary sequence of ICP4.

This report describes the formation of an ICP4 heterodimer containing one wild-type and one mutant subunit. The mutant subunit lacks both transactivation domains yet retains DNA-binding and dimerization activities. The formation of the heterodimer resulted in a dominant inhibitory phenotype with regard to viral growth and ICP4 activities. A functionally important region of primary sequence that is deleted in the mutant peptide and hence is monomeric in the heterodimer appears to be conformationally altered in the heterodimer compared with its structure in the native homodimer. This observation suggests an explanation for the dominant inhibitory activity of the mutant protein.

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MATERIALS AND METHODS

Virus and cells. The procedures for the propagation and plaque assay of the wild-type strain of HSV-1 (KOS) on Vero cells were as described previously (8). The wild-type strain of PRV was propagated in the manner described for KOS. The HSV-1 ICP4-deficient virus *n*12 (11) and the ICP4 promoter mutant Δ AT (N. DeLuca, unpublished data) were propagated on E5 cells, a Vero-derived cell line which expresses complementing levels of wild-type ICP4. The X25 cell line, which is a stably transformed Vero cell line containing the mutant ICP4 plasmid pdi3-i11 (38), was constructed as described previously (9). Vero, E5, and X25 cells were maintained as described previously for cell lines hosting HSV infection (8).

Recombinant plasmids. The plasmid pdi3-i11 (38) encodes a mutated ICP4 polypeptide possessing a deletion of residues 32 to 275, inclusive, and a stop codon following residue 774. The plasmid pSV2neo (41), which encodes the bacterial neomycin resistance gene regulated by the simian virus 40 early promoter, was used to confer G418 resistance to transformed Vero cells. The *Eco*RI-to-*Bam*HI fragment of the plasmid p4 (11) encodes a portion of the ICP4 promoter from nucleotides -110 to +30 relative to the transcription initiation site containing a consensus ICP4-binding site near the start site of transcription (20). This fragment was end labeled with T4 polynucleotide kinase and [γ - 32 P]ATP (Dupont, NEN Research Products, Boston, Mass.) and used as a probe for gel retardation assays.

DNA-binding assays. Protein-DNA complexes were formed and electrophoretically separated as described previously (11, 18). To further reduce the electrophoretic mobility of complexes containing wild-type ICP4, the monoclonal antibody 58S (39) was added to the binding reactions as described previously (18).

Electrophoresis of viral polypeptides. Viral polypeptides extracted from infected cells were electrophoretically separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described previously (21, 23). Where appropriate, electrophoresed polypeptides were silver stained according to the protocol of the manufacturer (Bio-Rad Laboratories, Richmond, Calif.).

(i) Pulse-labeling of viral polypeptides. Approximately 5×10^5 infected Vero cells were labeled with [35 S]methionine (50 μ Ci/ml) (Dupont, NEN Research Products) for 1 h at the indicated time postinfection, harvested as described previously (11), and electrophoresed as described above.

(ii) Western immunoblot analysis. Electrophoresed polypeptides were electrophoretically transferred to nitrocellulose filters as previously described (9). ICP4 polypeptides were visualized by probing filters with either a rabbit polyclonal serum directed against ICP4 (1:500 dilution), obtained from Richard Courtney, Louisiana State University Medical Center, Shreveport, La., or the monoclonal antibody 58S (1:100 dilution [39]). The primary antibody was probed with either 125 I-protein A (Dupont, NEN Research Products) as described previously (9) or with the Protoblot secondary anti-rabbit or anti-mouse immunoglobulin G-alkaline phosphatase-conjugated antibodies as indicated by the manufacturer (Promega Biotec, Madison, Wis.).

Fractionation of infected cell extracts. Approximately 2×10^8 Vero or X25 cells were infected with wild-type HSV-1 strain KOS or the ICP4-deficient virus *n*12 at a multiplicity of infection (MOI) of 10 PFU per cell. At 12 h postinfection, the nuclei of infected cells were harvested, lysed, and clarified as described previously (18). The nuclear extract was ap-

plied to an S-300 Sephacryl gel filtration column (1.6 by 90 cm) equilibrated with CB0.5 (20 mM Tris hydrochloride [pH 8.0], 0.5 M KCl, 1 mM EDTA, 10 mM β -mercaptoethanol, 0.01% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate [CHAPS], 0.1 mM tolylsulfonyl chloramethyl ketone) at a rate of 12 ml/h. Fractions were assayed for ICP4 activity by gel retardation as described above. Material from the peak fractions was applied to a DNA affinity column constructed from complementary oligonucleotides corresponding to the ICP4-binding site used as a probe for gel retardation. The construction and procedure for the elution of the affinity column have been described previously (18).

Physical analyses of ICP4 native structure. (i) **Analytical gel filtration.** Approximately 5 μ g of affinity-purified ICP4 was applied as described above to the S-300 Sephacryl gel filtration column that previously had been calibrated with molecular weight standards (Pharmacia Inc., Piscataway, N.J.). The stokes radius (a) of the fractionated sample was determined from the average distribution coefficient, K_{ave} , which can be calculated from the equation $(V_e - V_o)/(V_t - V_o)$, in which the elution volume (V_e), the void volume (V_o), and the total column volume (V_t) can be measured. Given that the stokes radius is a function of the K_{ave} , the stokes radii of unknown proteins can be determined from that of standard proteins.

(ii) **Sedimentation velocity analysis.** Approximately 2 μ g of affinity-purified ICP4 was layered upon a 5-ml isokinetic gradient of 5% to 20% sucrose. Approximately 50 fractions (100 μ l) were collected following centrifugation at 34,000 rpm in a SW50.1 rotor (Beckman Instruments) for 20 h at 4°C. ICP4 was identified in the fractions by the Protoblot (Promega) immunoassay described above for Western blots. The sedimentation coefficients ($S_{20,w}$) were determined from analysis following cosedimentation of ICP4 samples with standards of known $S_{20,w}$ values. The standards were located in the fractions by a measurement of the total protein concentration in each fraction as measured by the Bio-Rad protein assay (Bio-Rad).

(iii) **Native molecular weight and frictional ratio determination.** The native molecular weight was calculated by using the Svedberg equation: $M = S_{20,w}RT/[D_{20,w}(1-\nu)]$, where $S_{20,w}$ was determined by sucrose gradient sedimentation, R is the gas constant, T is the temperature in degrees Kelvin, ν is the partial specific volume of ICP4 (the average value for proteins, 0.74 cm³/g, was used), ρ is the solvent density at 20°C, and $D_{20,w}$ is the diffusion coefficient. $D_{20,w}$ was calculated from the Stokes-Einstein equation: $D_{20,w} = RT/6\pi\eta aN_A$, where a is the stokes radius determined from gel filtration, η is the solvent viscosity at 20°C, and N_A is Avogadro's number. The frictional ratio (f/f_o), which yields a measure of the degree to which the shape of the molecule deviates from a sphere, was determined from the equation $f/f_o = a/(3\nu M/4\pi N_A)^{1/3}$, where M is the native molecular weight. An experimentally determined value of $f/f_o = 1$ indicates that the shape of the molecule is best represented by a sphere.

RESULTS

Expression of a mutant polypeptide of ICP4 is inhibitory to the growth of wild-type HSV-1. The ICP4 polypeptide of HSV-1 is composed of 1,298 amino acids (24). A truncated mutant possessing the amino-terminal 774 amino acids of the ICP4 polypeptide (*n*208) binds specifically to DNA, forms homodimers, and retains autoregulatory activity, but can transactivate early HSV genes only approximately half as

FIG. 1. Activities of the mutant and wild-type ICP4 molecules. The coding sequence of the truncated (n208) and the deleted (expressed from X25 cells) ICP4 molecules are shown relative to that of the wild-type (strain KOS). The regions of amino acid similarity between HSV ICP4 and that of the 140-kDa VZV analog are indicated by rectangles where sequences exhibiting the greatest identity are represented by filled rectangles (see McGeoch et al. [24]). The relative DNA-binding and regulatory activities of these ICP4 molecules are indicated. A plus in column a represents the ability of the ICP4 molecule to form a protein-DNA complex with DNA sequences containing a consensus binding site located in the ICP4 (IE3) promoter. Two pluses in column b reflect the wild-type level of transactivation measured by stimulation of tkCAT following transient expression, where the n208 ICP4 transactivates early genes approximately half as well as the wild-type ICP4 from strain KOS, and the X25-induced mutant fails to transinduce tkCAT. A plus in column c represents the wild-type autoregulatory activity of ICP4 in attenuating the expression of IE3CAT. This data is summarized from DeLuca and Schaffer (10) and Shepard et al. (38).

well as the wild-type ICP4 molecule (10). Due to its inability to express late viral genes, the mutant virus expressing the n208 protein can only be propagated on cell lines expressing the full-length ICP4 protein, indicative of its impaired function (11). Removing amino acids 32 to 275 from the n208 ICP4 molecule results in a mutant protein which retains DNA-binding, multimerization, and autoregulatory activities but is no longer able to transactivate in transient assays (38). This protein lacks a block of amino acids that is conserved in the VZV ICP4 counterpart and that consists of a tract of 19 serines out of 24 residues, followed by 7 acidic amino acids. This deletion mutant retains the large block of amino acid residues between 310 and 495 that are conserved in the VZV 140-kDa analog and that have been shown to be important for specific DNA-binding activity (32, 38). The activities of the truncated deletion mutant (designated X25) and those of the truncated (n208) and full-length ICP4 proteins (KOS) are described in Fig. 1 relative to the regions of conservation between HSV-1 ICP4 and the 140-kDa IE protein of VZV (24).

The truncated deletion mutant protein contains the ICP4 DNA-binding domain but is unable to transactivate in transient assays. In an effort to ascertain the effect of this ICP4 molecule on HSV gene expression and growth, Vero cell lines which contain stable copies of the gene expressing the mutant protein were constructed. Transformed Vero cells were generated by cotransfection of the plasmid encoding the ICP4 mutant (pdi3-i11) with pSV2neo and selection with the neomycin analog G418. G418-resistant cell lines were screened for the presence of the DNA specifying the mutant gene by Southern blot hybridization as described previously (40). Figure 2A shows the levels of DNA encoding the mutant ICP4 molecule in five positive cell lines. The A16 and X25 cell lines have on the order of 20 copies of the mutant ICP4 gene per haploid equivalent of cellular DNA. The resident mutant gene is under the control of the ICP4 IE promoter and thus is not constitutively expressed (N. DeLuca, unpublished data). The ICP4 promoter can be stimulated by a component of the HSV-1 virion, VP16 (1, 2, 33), and by the activity of another HSV IE gene, ICP0 (29). These proteins are supplied by infection with HSV. Infection of these positive cell lines with an HSV mutant (n12) that expresses an inactive ICP4 peptide corresponding to only the amino-terminal 251 amino acids (11) induces the expression of the resident ICP4 mutant gene without providing ICP4 function. The ICP4 peptides expressed in infected transformed cells were visualized by Western blot analysis

by using polyclonal ICP4 antibody and are shown in Fig. 2B. In addition to the 40-kDa n12 protein, the cell lines also expressed a 63-kDa immunoreactive peptide. The calculated molecular mass of the mutant translation product is 53.3 kDa. This value is reasonably close to the measured value, considering that wild-type ICP4 has an apparent molecular mass of 165 to 175 kDa on SDS gels (7), yet the primary translation product is predicted to be only 133 kDa (24). This difference is most probably due to extensive posttranslational modification and the atypical amino acid composition; the full-length protein and the X25 variant are 12.4 and 12.2% proline, respectively.

Figure 2B also demonstrates that the levels of the 63-kDa peptide roughly correlated with the copy number of the

FIG. 2. Cell lines expressing the ICP4 mutant polypeptide. (A) Southern blot depicting the levels of DNA encoding the mutant ICP4 protein in five positive cell lines relative to that in Vero cells. The adjacent lanes were loaded with plasmid DNA representing 0.5 to 40 copies of the gene per haploid equivalent of cellular DNA. (B) Western blot reflecting ICP4 polypeptides expressed in cell lines infected with an ICP4-deficient virus n12. The levels of the 63-kDa protein encoded by pdi3-i11 and the 40-kDa protein encoded by the n12 viral genome are a function of the number of copies of the resident gene, where clones A16 and X25 express the greatest amount of the 63-kDa polypeptide and also result in the greatest attenuation of n12 expression.

TABLE 1. Plaquing efficiency of HSV and PRV on transformed cell lines^a

Virus	A16	A23	Y21	X12	X25	Vero
HSV ^b	1.9×10^8	1.9×10^9	1.9×10^9	1.6×10^9	1.0×10^8	3.8×10^9
PRV					7.0×10^8	7.0×10^8
Δ AT ^c					1.4×10^8	3.5×10^9

^a A stock of virus was plated on each cell line, and the number of plaques were counted at 3 days postinfection.

^b Herpes simplex virus type 1, strain KOS.

^c A variant of HSV-1 deleted for the dinucleotide AT constituting part of the ICP4-binding site located at the start site of ICP4 transcription.

mutant gene shown in Fig. 2A. Moreover, in cell lines which expressed the most 63-kDa protein (X25 and A16), the levels of the 40-kDa *n*12 ICP4 mutant protein were diminished. This is consistent with previous results showing the ability of the ICP4 mutant protein to attenuate expression from the ICP4 promoter (38). The expression of the 63-kDa ICP4 mutant protein had little to no effect on the expression of the remainder of the *n*12 viral genome or on the growth properties of *n*12 (N. DeLuca, unpublished data). Therefore, consistent with previous results (38), the mutant peptide did not stimulate the expression of the HSV genome in the absence of wild-type ICP4.

The cell lines expressing the greatest amount of the 63-kDa protein were poor hosts for wild-type HSV growth, despite the presence of full-length ICP4 expressed from the viral genome (Table 1). The A16 and X25 cell lines exhibited an approximately 40-fold reduced plating efficiency of wild-type virus relative to that on Vero cells (0.026 PFU on X25 cells/PFU on Vero cells). The X25 cell line was also tested with another α herpesvirus, PRV, with respect to plaque reduction (Table 1). X25 cells were as efficient hosts for PRV as Vero cells were, supportive of a specific *trans*-dominant effect on HSV or HSV ICP4. In X25 cells, the 63-kDa mutant protein was induced by PRV to levels lower than those observed with the noninhibitory cell lines shown in Table 1 and Fig. 2B (N. DeLuca, unpublished data). Therefore, the inefficient induction of the mutant protein by PRV may be one reason for the lack of inhibition. This is consistent with the previous demonstration (3) that PRV does not possess a VP16 IE transactivating activity. The X25 cell line was chosen for a more detailed study of the inhibitory effect of the mutant ICP4 protein on wild-type HSV growth.

In transient assays, the X25 protein was unable by itself to significantly affect the expression of a tkCAT test gene (38). Therefore, the X25 protein may inhibit the activity of the wild-type protein or some other process during infection. To obtain an indication of the overall effect of the 63-kDa mutant ICP4 protein on viral gene expression during viral infection, Vero and X25 cells were infected with wild-type HSV-1 (strain KOS) and pulse-labeled with [³⁵S]methionine at different times after infection (Fig. 3). During the normal course of viral infection, early genes are expressed and their translation products are maximally synthesized between 4 and 6 h postinfection, after which time expression drops off (16). Late genes are maximally expressed from 12 to 15 h postinfection (16). In X25 cells, the expression of late genes was reduced compared with late-gene expression at the corresponding time postinfection in Vero cells. Reductions are evident in the amounts of ICPs 1, 2, 15, 48, and gB (Fig. 3). In addition, the levels of the late gC mRNA were reduced by a factor of 4 in X25 cells relative to that in Vero cells (N. DeLuca, unpublished data). Early genes are not shut off as efficiently in X25 cells as they are in Vero cells, as shown by the increased amounts of ICPs 6, 8, and tk (Fig. 3). Therefore, viral infection in X25 cells is interfered with during

events associated with late regulatory activities. Although not as pronounced, the observed impairment is similar to the defect observed for a virus (*n*208) which, like the 63-kDa protein, expresses an ICP4 molecule lacking the carboxy-terminal 524 amino acids (11).

The inhibition of wild-type virus by X25 cells was more pronounced at lower MOIs than at higher multiplicities. Figure 4 shows that virus yield from a single cycle of growth on X25 cells was 42% that from Vero cells at an MOI of 30 PFU per cell, whereas the yield from X25 cells was reduced to 6% of that from Vero cells at an MOI of 0.3 PFU per cell. This result is consistent with the observation that only the

FIG. 3. Polypeptide profile of wild-type HSV-infected Vero and X25 cells. Viral polypeptides expressed in Vero (V)- and X25 (X)-infected cells labeled at the indicated time (hours) postinfection (h.p.i.) with [³⁵S]methionine were resolved by SDS-polyacrylamide gel electrophoresis. Representative viral proteins are indicated by the ICP (infected cell polypeptide) designations and characterized as either immediate early (IE), early (E), or late (L) viral gene products. The 63-kDa polypeptide expressed from the resident gene in X25 cells is indicated with a bar to distinguish it from the 63-kDa IE protein ICP27 labeled with a dot.

FIG. 4. Growth curves for wild-type HSV strain KOS on Vero versus X25 cells. The cell lines were infected at various MOIs (PFU per cell), harvested after 18 h, and assayed on Vero cells for viral titer. Virus titer was plotted as log PFU per milliliter as a function of the log of the MOI.

cell lines expressing the greatest amount of the 63-kDa mutant protein (Fig. 2B) were inhibitory to virus growth (Table 1). Given that X25 cells contain approximately 40 copies per cell of the mutant gene (Fig. 2A) and each infecting virus delivers 2 copies of the full-length gene (27), then the average ratio of mutant to wild-type genes in infected cells would range from 20 (for an MOI of 0.3 and 1.0 PFU per cell) to 0.7 (for an MOI of 30 PFU per cell).

The inhibition of the wild-type virus by X25 cells is not a consequence of the 63-kDa mutant ICP4 protein attenuating the expression of the wild-type ICP4 molecule. While the 63-kDa protein retains the ability to attenuate ICP4 expression in the absence of wild-type ICP4 (Fig. 2B), Fig. 5 shows that the presence of the mutant ICP4 protein does not reduce the level of expression of the wild-type ICP4. The Western blots of Fig. 5 reveal that the quantity of wild-type ICP4 in X25 cells is equal to if not slightly elevated relative to that in Vero cells. Figure 5 also shows the relative levels of wild-type ICP4 in Vero and X25 cells expressed from the virus Δ AT, a deletion mutant lacking the dinucleotide AT in the ICP4 consensus binding site ATCGTC located at the start site of ICP4 transcription (11; N. DeLuca, unpublished data). This deletion impairs the association of ICP4 with the consensus sequence (11). The binding of ICP4 to this site has been implicated in transient assays as contributing to the attenuation of ICP4 expression (36). The Δ AT mutation did not alter the relative expression of ICP4 in Vero and X25 cells compared with the wild-type. In addition, the plating efficiency of the virus Δ AT is inhibited to the same extent as the wild-type virus by X25 cells (Table 1), further indicating

FIG. 5. Relative levels of ICP4 expression in Vero and X25 cells. (A) Western blot reflecting the ICP4 polypeptides identified by polyclonal antisera. Vero (V) and X25 (X) cells were infected with either mock (M), wild-type (wt), or Δ AT HSV (a mutant strain in which the dinucleotide AT of the ICP4 consensus binding site ATCGTC located at the ICP4 start site of transcription is deleted). The wild-type and 63-kDa ICP4 molecules are indicated. (B) The same SDS polypeptides described in panel A identified by reactivity with the ICP4 monoclonal antibody 58S, which does not recognize the truncated 63-kDa peptide expressed in X25 cells.

that the association of ICP4 with this binding site is not responsible for the growth inhibition. The two Western blots (Fig. 5) also reveal the differential immunoreactivity of the wild-type and mutant ICP4 molecules, in which the monoclonal antibody 58S (39) recognizes the wild-type ICP4 SDS peptide but not the mutant ICP4 SDS peptide (Fig. 5B). This differential immunoreactivity will be utilized in later experiments. The data described demonstrate that the *trans*-dominant effect of the 63-kDa mutant ICP4 protein upon the wild type is not a consequence of the mutant protein associating with the ICP4-binding site and down regulating wild-type ICP4 expression. Therefore, the observations and considerations described for Fig. 2 through 5 tend to indicate a mechanism for inhibition involving a nonproductive interaction between mutant and wild-type molecules and/or the competition of mutant and wild-type molecules for direct sites of action.

The 63-kDa mutant protein expressed from X25 cells forms heterodimers with the full-length ICP4 molecule. The plasmid used to construct X25 cells, pdi3-i11, transiently expresses an ICP4 mutant polypeptide in transfected Vero cells that is able to form a protein-DNA complex with a DNA probe containing a consensus ICP4-binding site (38). When X25 cells were infected with a mutant virus that does not express ICP4 DNA-binding activity (*n*12), the 63-kDa peptide was synthesized and was capable of binding to the specific DNA probe (Fig. 6A, lane 2, complex a). The binding activity was not present in uninfected X25 cells (Fig. 6A, lane 1), consistent with the requirement of HSV infection for induction of the resident gene. When wild-type virus expressing full-length ICP4 was used to infect Vero cells, a DNA-binding activity was expressed, which resulted in an ICP4 protein-DNA complex having a mobility much lower than that resulting from expression of the 63-kDa peptide from X25 cells (Fig. 6A, lane 3, complex b). Infection of X25 cells with the wild-type virus resulted in the diminution of the amount of complexes a and b and the formation of a novel complex (complex c), which had a mobility in between that of a and b (Fig. 6A, lane 4). The appearance of the novel complex could not be obtained by mixing the extracts used to generate complex a (lane 2) and complex b (lane 3) simultaneously in

FIG. 6. DNA-binding properties of the ICP4 molecules expressed in HSV-infected Vero and X25 cells. (A) Protein-DNA complexes formed with whole cell extracts prepared from mock-infected (—), η 12-infected (an ICP4-deficient virus labeled 4—), or wild-type HSV-infected (wt) Vero (V) or X25 (X) cells. The probe used in the gel retardation contains the consensus binding site located in the *Bam*HI to *Eco*RI fragment of the ICP4 promoter. Lane 5 depicts the complexes formed with a mixture of the extracts used from η 12-infected X25 cells and wild-type-infected Vero cells. Complexes labeled a, b, and c are described in the text. (B) Protein-DNA complexes in which purified ICP4 and the probe described for panel A were used. Lanes 1, 2, and 3 are described in the text.

a binding reaction (Fig. 6A, lane 5). This suggests that the proteins required to form complex c must be expressed in cells simultaneously.

The results of Fig. 6A indicate that the novel binding activity (complex c) is established prior to the preparation of HSV-infected X25 cell extracts rather than during the binding reaction itself. Therefore, it should be possible to independently isolate the proteins forming complexes a, b, and c and to determine their composition. Nuclear extracts from wild-type HSV-infected X25 cells were applied to a Sephacryl S-300 column to separate proteins on the basis of size. Following chromatography, the individual fractions were assayed by the mobility shift of a probe containing an ICP4-binding site (Fig. 7). Also subject to the same chromatographic separation and analysis was a mixture of extracts from wild-type HSV (KOS)-infected Vero cells and ICP4-deficient HSV (η 12)-infected X25 cells. The binding activities contributing to complexes a, b, and c were chromatographically separated such that those contributing to complex b eluted first, followed by those contributing to complex c, and lastly those contributing to complex a (Fig. 7A). As expected, chromatography of the *in vitro* mixture of extracts containing full-length ICP4 and the 63-kDa ICP4 mutant resulted in a profile similar to that obtained for

FIG. 7. Gel retardation assay of fractions obtained from gel filtration chromatography. (A) Protein-DNA complexes formed with fractionated wild-type HSV-infected X25 cells. The probe for the assay contained the ICP4 consensus binding site described in the legend for Fig. 6. Complexes a, b, and c are analogous to those in Fig. 6 and are discussed in the text. (B) Protein-DNA complexes formed with a mixture of extracts fractionated from wild-type-infected Vero cells and η 12 (ICP4-deficient virus)-infected X25 cells.

fractionation of KOS-infected X25 cells, except that the intermediate-sized complex c was absent (Fig. 7B). The higher-order protein-DNA complexes observed appear to form in the binding reaction, since the proteins making up these complexes are not size fractionated by gel filtration.

The fractions indicated by the bars in Fig. 7A and B were further purified by affinity to an oligonucleotide specifying a consensus ICP4-binding site, as described in Materials and Methods. The purified material resulted in gel shifts having the mobilities expected for complexes a, b, and c (Fig. 6B). The polypeptide composition of the purified material was determined by SDS-polyacrylamide gel electrophoresis (Fig. 8). The material giving rise to complex b (Fig. 6B, lane 3) was composed of a single 170-kDa protein that reacted with polyclonal ICP4 antibody; the material giving rise to complex a (Fig. 6B, lane 1) was composed of a single 63-kDa immunoreactive protein. Complex c (Fig. 6B, lane 2) is generated by material composed of both the 170- and 63-kDa immunoreactive species.

Native ICP4 has been shown to exist as a homodimer (25), raising the possibility that the protein-DNA complexes b and a are composed of homodimers of full-length and 63-kDa mutant protein, respectively, and that complex c is generated by the binding of a heterodimer of the full-length and the mutant proteins. This was shown to be correct by analytical gel filtration and sucrose velocity sedimentation analysis of

FIG. 8. Polypeptide composition of purified ICP4 molecules. (A) SDS-polyacrylamide gel electrophoresis of size-fractionated and affinity-purified material giving rise to the protein-DNA complexes a, b, and c in Fig. 6 and 7. The 170- and 63-kDa polypeptides of wild-type ICP4 and the X25-induced mutant are indicated, in addition to the exogenously added bovine serum albumin. (B) Western blot of resolved polypeptides shown in panel A. The first lane in A and B corresponds to the material giving rise to complex b, the second lane corresponds to the material giving rise to complex c, and the third lane corresponds to the material giving rise to complex a.

the purified proteins. Figures 9A and B show the elution profiles of the purified proteins from the analytical gel filtration experiment and the subsequent analysis determining the Stokes radii of the proteins, respectively. Figures 9C and D show the sucrose gradient profiles for the purified proteins and the subsequent analysis to determine the sedimentation coefficient (S), respectively. The experimentally determined Stokes radius and sedimentation coefficient ($S_{20,w}$) and the calculated values for diffusion coefficient ($D_{20,w}$), native molecular weight (M_w), and frictional ratio (f/f_o) are summarized in Table 2. Also shown are the sizes of the ICP4 polypeptides constituting the protein-DNA complexes a, b, and c. The values indicate that the novel complex c is generated by the DNA binding of a heterodimer composed of both the 170-kDa full-length and the 63-kDa mutant proteins. Likewise, complex b is generated by a homodimer of the full-length protein and a is generated by a homodimer of the 63-kDa mutant protein. As previously described for the wild-type protein by Metzler and Wilcox (25), the large frictional ratios (f/f_o) indicate that the molecules exist in an elongated conformation, having axial ratios which range from 20 to 1 for the full-length homodimer to 11 to 1 for the 63-kDa homodimer. The heterodimer assumes a conformation being more elongated than the 63-kDa dimer and more compact than the wild-type KOS dimer.

A functional region of the heterodimer is conformationally altered. Amino acids 775 to 1,298 of ICP4 have been implicated in the process of transactivation (8, 10, 11, 31). The mutant virus α 208 (Fig. 1) expresses the amino-terminal 774 amino acids of the ICP4 molecule and is deficient for late-gene expression and DNA synthesis. Early HSV genes are transcribed at approximately one-half the rate in the α 208

background compared with transcription in the wild-type background (11). The inhibition of viral late-gene expression in KOS-infected X25 cells, although not as pronounced as that reported for α 208-infected Vero cells (11), suggests that the single copy of the carboxy terminus within the heterocomplex is less active than that of the wild-type homodimer. In the following experiments, the monoclonal antibody 58S (39) was used as a probe to study the conformation of the native protein in the vicinity of the carboxy terminus. It previously has been shown that 58S reacts with an epitope on ICP4 which is, in part, determined by the primary sequence carboxy terminal to amino acid 1,030 (11). Moreover, the Western blots of Fig. 5A and B indicate that the monoclonal antibody 58S recognizes the wild-type ICP4 SDS peptide but does not react with the truncated 63-kDa mutant ICP4 SDS peptide expressed in X25 cells. Figure 10 shows the protein-DNA complexes a, b, and c observed in HSV-infected Vero and X25 cells, as described in Fig. 6A. Consistent with the absence of the antigenic epitope, the protein-DNA complex containing the homodimer of the 63-kDa mutant protein present in α 12-infected X25 cells is not affected by the addition of undiluted ascites fluid (Fig. 10A). However, a 1:1,000 dilution of the antibody is sufficient to further shift the protein-DNA complex containing the homodimer of the full-length protein present in wild-type virus-infected Vero cells (Fig. 10A). In wild-type virus-infected X25 cells, again the lowest-mobility complex containing the wild-type homodimer was further shifted with the most dilute concentration of antibody and the complex containing the mutant homodimer in the same extract was not shifted at all (Fig. 10A). Despite containing a single copy of the predetermined epitope, the protein-DNA complex containing the heterodimer present in the wild-type virus-infected X25-cell extract was shifted only after the addition of 100- to 1,000-fold more antibody than was required to shift the complex containing the wild-type homodimer. The observation that the mobilities of all the complexes with the exception of the heterocomplex are affected the same way at high and low antibody dilutions indicates that the reactivity of the antibody with the heterocomplex is specific and not due to general properties of the antibody preparation such as high salt or protein concentrations. The seemingly gradual shift of the heterodimer-DNA complex as a function of the antibody is consistent with the low affinity of the antibody for the complex, resulting in dissociation during electrophoresis. In addition, the reactivity of 58S with the wild-type protein in Western blots demonstrates that the antibody can recognize an epitope presented on an SDS peptide and doesn't require dimerization. However, the greater amount of antibody required to further shift the heterocomplex indicates that the epitope is presented differently in the heterocomplex compared with the wild-type homocomplex. This is consistent with the hypothesis that the region of ICP4 containing the epitope is structurally altered in the heterocomplex. The same patterns of reactivity were seen with purified homo- and heterodimers (Fig. 10B).

DISCUSSION

A transformed cell line (X25) containing stable copies of a plasmid encoding the DNA-binding domain of the herpes simplex viral transactivator, ICP4, was constructed. The expression of the mutant ICP4 gene in X25 cells is regulated by the ICP4 promoter and hence is not constitutively expressed but is induced only upon HSV infection. The mutant protein exhibited the ability to specifically associate with

FIG. 9. Analytical gel filtration and sucrose velocity sedimentation of purified ICP4 molecules. (A) Elution profile of purified ICP4 molecules from analytical gel filtration. (B) Analysis of data shown in panel A used to determine the Stokes radii of the ICP4 species. Protein standards and their respective Stokes radii in angstroms ($1 \text{ \AA} = 0.1 \text{ nm}$) used to calibrate the gel filtration column were as follows: 1, thyroglobulin, 85.0; 2, ferritin, 61.0; 3, aldolase, 48.1; 4, albumin, 35.5; 5, ovalbumin, 30.5; 6, chymotrypsinogen A, 20.9. (C) Sucrose gradient profiles for the purified ICP4 species. (D) Analysis of the sucrose gradient profiles used to determine the sedimentation coefficients (S) of the native ICP4 molecules. Standard 1 corresponds to catalase (11S), and standard 2 corresponds to bovine serum albumin (4.2S). X25:KOS corresponds to material giving rise to complex c, X25:X25 corresponds to material giving rise to complex a, and KOS:KOS corresponds to material giving rise to complex b.

TABLE 2. Physical properties of mutant ICP4 molecules

Complex	Stokes radius (Å) ^a	$D_{20,w}$ ^b	$S_{20,w}$ ^c	M_w (kDa) ^d	f/f_o ^e	Com-position ^f
KOS (b)	89	2.40×10^{-7}	7.8	304,000	1.99	170,000
X25:KOS (c)	73	2.93×10^{-7}	6.8	217,000	1.70	170,000 63,000
X25 (a)	54	3.96×10^{-7}	5.2	123,000	1.57	63,000

^a As determined by gel filtration chromatography (Fig. 9B).

^b As calculated from the Stokes-Einstein equation: $D_{20,w} = \frac{RT}{6\pi\eta a N_A}$

^c As determined from sucrose gradient centrifugation (Fig. 9D).

^d As calculated from the Svendberg equation: $M = \frac{SRT}{D(1-\nu\rho)}$

^e As determined from the frictional ratio: $\frac{a}{(3\nu M/4\pi N_A)^{1/3}}$

^f As determined from Fig. 8.

DNA and to dimerize. Consistent with earlier studies (38), the mutant ICP4 molecule was unable to transactivate viral gene expression. Despite the fact that the X25-induced ICP4 molecule is itself transcriptionally inactive, the expression of this mutant polypeptide resulted in dominant interference with the activity of wild-type ICP4. Upon infection of X25 cells with the wild-type HSV strain, viral growth was impaired as a function of the MOI, with the greatest inhibition occurring at the lowest multiplicities. While the induction of the resident ICP4 molecule from X25 cells is influenced by the MOI (N. DeLuca, unpublished data), the number of templates to be induced per infected cell remains constant. Thus, from consideration of relative gene copy numbers, at the lowest MOI (0.3 PFU per cell), the concentration of the X25-induced polypeptide is predicted to exceed that of the wild-type ICP4 introduced by the infecting virus. As the MOI of KOS increases, the ratio of mutant to wild-type ICP4 molecules decreases, consistent with the decrease in inhibition. Thus, the inhibition of viral growth was somewhat relieved by a greater production of the wild-type ICP4

FIG. 10. Immunoreactivity of protein-DNA complexes. (A) Gel retardation in which whole cell extracts prepared from *n12* (an ICP4-deficient virus)-infected X25 cells, wild-type HSV-infected Vero cells, and wild-type virus-infected X25 cells were used. The probe contained the ICP4 consensus binding site described in the legend of Fig. 6. Dilutions of ascites fluid containing the monoclonal antibody 58S were added to the binding reactions where indicated. (B) Gel retardation in which the size-fractionated and affinity-purified ICP4 molecules shown in Fig. 8 were used. Shown are the immunoreactivities of the protein-DNA complexes containing the homodimer (X25), the wild-type-mutant heterodimer (wt:X25), and the wild-type ICP4 homodimer (wt).

molecule relative to that induced in X25 cells. This data is consistent with the observation that the cell lines exhibiting the greatest quantity of mutant peptide (Fig. 2B) resulted in the highest level of inhibition (Table 1). In addition, it was found that the amount of wild-type ICP4 expressed from the infecting virus was similar in X25 cells and Vero cells. Therefore, it can be concluded that for a given amount of wild-type ICP4, its subsequent activity is either impaired or masked by the presence of the X25 peptide. X25 cells are not universally inhibitory to the growth of herpes viruses, as was demonstrated by the absence of inhibition observed for PRV-infected X25 cells.

Physical analyses of the purified native ICP4 species in KOS-infected X25 cells showed that in addition to the presence of wild-type and X25-induced mutant homodimers, wild-type/mutant heterodimers formed in the infected cells. The heterodimer could not be formed *in vitro* by mixing extracts which contained both homodimers, indicating that dimerization may occur simultaneously with polypeptide folding. In support of this, the existence of monomeric ICP4 is not indicated by gel filtration analysis on ICP4 in crude cellular extracts (N. DeLuca, unpublished data). All three dimeric ICP4 species exhibited DNA-binding activity. Thus, if DNA binding is important in some capacity for transactivation, competition between the three ICP4 species for a limited number of DNA sequences would reduce the apparent activity of wild-type homodimers. While the specific association of ICP4 with DNA sequences flanking the promoter of an early gene transactivated by ICP4 does not appear to be necessary for transactivation of that viral gene (18), specific DNA-binding activity may be required directly for the stimulation of other viral genes (42). Therefore, a

consequence of sequestering wild-type polypeptides into heterodimers containing one wild-type and one mutant subunit is that the pool of wild-type homodimers is effectively reduced. The reduced number of wild-type homodimers present in infected X25 cells may compete with presumably less active heterodimers and X25 homodimers for binding to DNA sequences or other productive sites of action.

The physical analyses of the ICP4 species present in KOS-infected X25 cells yielded information regarding the shape of the dimeric complexes. These studies confirm earlier analyses of wild-type ICP4, which demonstrated that the native structure of the viral transactivator is homodimeric with an extended conformation (25). The frictional coefficient ratio of 1.99, which is a measure of how much the native structure deviates from a globular sphere having a ratio of 1.0, is similar to that of fibrous proteins. Human fibrinogen, a fibrous protein exhibiting similar molecular weight and sedimentation and diffusion coefficients to ICP4, has a frictional coefficient ratio of 2.3 (4). As was previously illustrated (25), if one assumes that the structure of native ICP4 is best approximated by a prolate ellipsoid, the axial ratio of this shape would approach 20:1. The same analysis applied to the mutant homodimer purified from X25 cells yields a native structure having a frictional coefficient of 1.57, which described in terms of a prolate ellipsoid corresponds to an axial ratio of roughly 11:1. The heterodimer is more compact than the wild-type homodimer and is more extended than the mutant homodimer. The more compact structure of the heterodimer relative to the wild-type homodimer indicates how the regions deleted from the 63-kDa mutant protein contribute to the extended conformation of the wild-type protein. It follows from this that the

conformation of the wild-type subunits in the heterodimer and the wild-type homodimer may be different.

The data discussed above indicate that the regions deleted in the 63-kDa mutant subunit of the heterodimer contribute to the extended conformation of the wild-type ICP4 homodimers and raise the possibility that the monomeric regions of the wild-type subunit in the heterodimer are conformationally altered. The observation that the wild-type/mutant heterodimers require 100- to 1,000-fold excess antibody relative to the wild-type homodimers in order to further retard the mobility of their respective protein-DNA complexes suggests that the heterodimers are structurally altered in the region of the molecule that serves as the antigenic epitope. As stated, the antigenic epitope has been mapped to a region of ICP4 which is deleted in the X25-induced mutant polypeptide (11), and therefore the epitope is monomeric in the heterocomplex but dimeric in the wild-type homodimer. Given that the antibody recognizes the monomeric SDS peptide, it is unlikely that the dimer contains a single epitope spanning an interface of the two subunits. However, maximal immunoreactivity may be dependent upon the dimeric structure of the antigenic epitope. The two epitopes may be juxtaposed in the native wild-type structure, making their interaction with the bivalent antibody potentially cooperative. Alternatively, the epitopes may be spatially separated in the native wild-type structure but their presentation to antibody may be dependent on the local conformation of the protein in the region containing the epitope. Both possibilities provide plausible explanations for the reduced affinity between the heterodimers and the monoclonal antibody. By using the reactivity of the monoclonal antibody as a paradigm for protein-protein interactions specified by the carboxy terminus of ICP4, it follows that a conformational change in this region of the wild-type subunit of the heterodimer may also result in an altered interaction between the heterodimer and target proteins necessary to activate transcription. We favor this hypothesis, since this region of the molecule is dispensable for DNA binding but has previously been genetically implicated as a domain contributing to the transactivation potential of ICP4 (11).

The inhibition of HSV-1 growth by the induced X25 ICP4 polypeptide reflects a dominant negative impairment of ICP4 activity. Polypeptide profiles of KOS-infected X25 cells indicate a transcriptional program consistent with that observed for other ICP4 mutants deleted for the carboxy terminus or possessing mutations specifying temperature sensitivity in the carboxy-terminal region (8, 11). Therefore, the presence of the mutant ICP4 molecules in X25 cells leads to an impairment of ICP4 activity analogous to that observed with an ICP4 mutant lacking one of the regions of primary sequence involved in transactivation.

Heterodimer formation between mutant subunits possessing different functional components might be expected to result in molecules of novel activity. We have previously described an example of this in which two different transcriptionally impaired ICP4 mutants possessing different subsets of the structural components necessary for regulatory activity are combined by heterodimer formation to result in a complex which exhibits greater transcriptional activity (37). Heterodimer formation effectively brought together subunits exhibiting different activities, resulting in complementation. An additional example of heterocomplex formation resulting in novel activity has been described for the interaction between the retinoic acid receptor and the thyroid hormone receptor. The regulatory activity in cells cotransfected with plasmids encoding these two steroid

hormone receptors varied depending on whether one or both of the receptors were expressed (15). Inhibition of activity leading to attenuated viral growth has also been reported for a truncated form of the HSV IE-specific transactivator, VP16, when expressed from transformed cells (14). Unlike the mechanism proposed herein, the mechanism of inhibition by the mutant VP16 probably does not involve alteration of the structure of the VP16 expressed from the infecting virus. The authors favor the hypothesis that the inhibition results from the formation of nonproductive VP16-cellular DNA-binding protein complexes which compete with wild-type VP16-cellular DNA-binding protein complexes for productive sites of action. Although the direct mechanisms of action of the mutant ICP4 and VP16 molecules may differ, both utilize a biological property normally required for productive function to interfere with the activity of their respective wild-type counterparts. Genetic approaches such as these will aid in the study of the molecular interactions involved in the function of essential viral proteins and potentially provide alternative antiviral strategies.

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