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Physical Interaction between Two Varicella Zoster Virus Gene Regulatory Proteins, IE4 and IE62

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Transfection assays demonstrate that the varicella zoster virus (VZV) immediate-early 62 (IE62) protein is a major transactivator of VZV gene expression, whereas a second immediate-early protein, IE4, can act as a major coactivator of transactivation mediated through IE62. To test whether IE62 and IE4 interact physically, we performed several protein–protein interaction assays. Coimmunoprecipitation analyses using VZV-infected cell lysates as well as purified protein mixtures demonstrate that IE62 and IE4 form stable complexes in solution under stringent salt conditions. Enzyme-linked immunosorbent assay protein–protein interaction assays and maltose-binding protein capture assays demonstrate that IE62 binds IE4 in a concentration- and dose-dependent manner. Far Western blot analyses show that IE4 binds to an undermodified form of IE62, and the use of calf intestinal phosphatase and protein kinases suggests that the interaction with IE4 is dependent on the phosphorylation state of IE62. An IE4 binding domain on IE62 has been mapped using a set of truncated IE62 fusion peptides. Collectively, these results imply a direct and specific physical interaction between IE4 and less-phosphorylated forms of IE62. These data have implications for virion assembly, as well as for the regulation of gene expression in VZV-infected cells.

INTRODUCTION

Varicella-zoster virus (VZV) is the infectious agent of chickenpox after primary infection and of shingles on reactivation of latent virus.

The coordinate and timely regulation of gene expression is an integral feature of the replication of VZV. Thus VZV genes contain complex regulatory regions with unique core promoter sequences and binding sites, which are targets for the combined controlling efforts of cellular and viral factors (Kantakamalakul *et al.,* 1995). The IE62 gene regulatory protein is capable of transactivation of all putative kinetic classes of VZV genes (Inschauspe *et al.,* 1989; Inschauspe and Ostrove, 1989; Perera *et al.,* 1992a; Baudoux *et al.,* 1995) and of autoregulation of its promoter (Disney *et al.,* 1990; Perera *et al.,* 1992b).

There are several mechanisms through which IE62 may be able to recognize and function at different promoter elements; one is to alter promoter binding capacity through interaction with other viral gene regulatory proteins such as the products of open reading frames (ORFs) 10, 61, 63, or 4 or with cellular proteins. Another potential mechanism is through posttranslational modification, such as phosphorylation.

Several lines of evidence suggest that functional interactions occur among VZV IE62, and IE4. First, IE62 and IE4 activate gene expression synergistically (Inschauspe *et al.,* 1989). Second, the intranuclear localization of IE4 has been reported to be affected by the presence of IE62 (Defechereux *et al.,* 1996). Third, the HSV ICP4 and ICP27 proteins (homologs to IE62 and IE4) have been shown to interact functionally and physically (Panagiotidis *et al.,* 1997). Finally, the IE62 and IE4 proteins are also both present in the VZ virion tegument, suggesting the potential for their intervention at the earliest stages of VZV replication (Kinchington *et al.,* 1995).

In an attempt to clarify the molecular mechanisms underlying the cooperative regulation of gene expression mediated by IE62 and IE4, as well as their presence in the virion tegument, we postulate that there is a direct physical interaction between them, and we have used a number of assay systems to demonstrate the interaction.

RESULTS

Given the ability of IE4 to assist IE62 in transactivation of viral promoters, we propose that there is a physical interaction between the two. Indirect evidence for this has already been reported (Defechereux *et al.,* 1996), showing that in the presence of IE62, IE4 is targeted to the cell nucleus. We used several assays to determine the existence of a physical interaction between IE62 and

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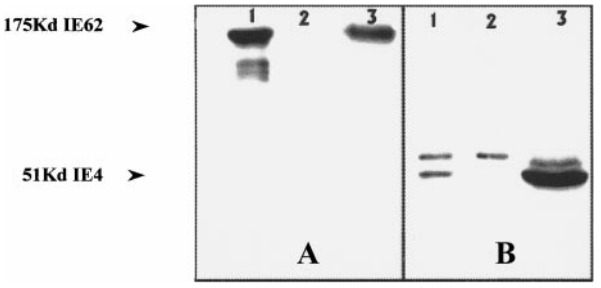

FIG. 1. Coimmunoprecipitation *in vivo* of VZV-infected cell extracts with anti-IE62 antibody. MeWo cells were infected with VZV (Oka), and cell lysates were clarified and then reacted with or without a monoclonal antibody against IE62. Protein G–Sepharose was added to enhance immune precipitation. Western analysis was performed on the immunoprecipitates after SDS–PAGE. The blot was divided into two sections: section A was probed for IE62, and section B was probed for IE4. Each section contains the same three lanes: lane 1, infected cell lysate, protein G–Sepharose, and anti-IE62 antibody; lane 2, infected cell lysate and anti-IE62 antibody; and lane 3, infected cell lysate.

IE4, and demonstrate that this physical interaction is modified by protein kinase activity.

Coimmunoprecipitation of IE62 and IE4 using extracts of VZV-infected cells and VZV proteins synthesized in vitro

First, to determine whether these two proteins associate with each other in VZV-infected cells, we coimmunoprecipitated IE62 and IE4 from a VZV-infected MeWo cell lysate using a monoclonal antibody specific for IE62. The presence of VZV proteins in the immunoprecipitates was monitored by Western blot analyses. Clearly, in the immune precipitate collected using the anti-IE62 antibody, there is evidence of the presence of IE4, implying that IE62 and IE4 are associated in the infected cell (Fig. 1). The reverse experiment also was done, in which anti-IE4 antibody was used to precipitate infected cell extracts; in this case, IE62 was shown to be present in the precipitate (Fig. 2). Interestingly, the species of IE62 associating with IE4 in this assay is a faster migrating form.

To demonstrate a direct association between IE62 and

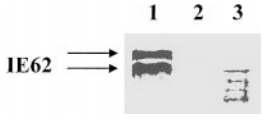

FIG. 2. Coimmunoprecipitation *in vivo* of VZV-infected cell extracts with anti-IE4 antibody. Cell extracts were made and reacted with polyclonal antibody against IE4 as described for Fig. 1. The figure shows a Western blot probed with anti-IE62 polyclonal antibody. Lane 1 is the VZV-infected cell lysate, lane 2 is infected cell lysate and protein G–Sepharose, and lane 3 is infected cell lysate, anti-IE4 antibody, and protein G–Sepharose. On this gel, IE62 has been resolved into two bands and IE4 seems to be associated with a faster-moving form.

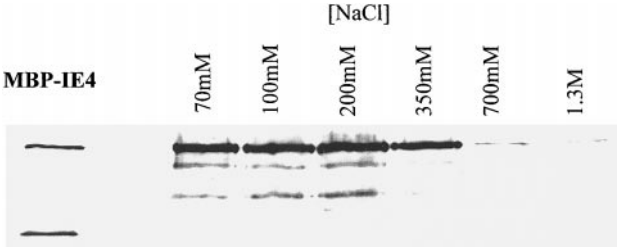

FIG. 3. Stability of the IE62–IE4 complex. Purified recombinant MBP4 and IE62 proteins were coimmunoprecipitated using a monoclonal antibody against IE62 in various salt concentrations ranging from 70 to 1300 mM NaCl. Washed immune precipitates were resolved by SDS–PAGE, followed by electroblotting onto nitrocellulose. A Western assay was performed using an antibody against MBP.

IE4, purified proteins were coimmunoprecipitated *in vitro*. IE62 protein was mixed with MBP4 or MBP, and monoclonal anti-IE62 antibody and protein G–Sepharose were added. The resulting immune precipitate contained both VZV proteins, demonstrating their complex formation in solution (not shown). To investigate the strength of this IE62–IE4 interaction, the salt sensitivity of the protein complex was analyzed. IE62 and IE4 were coimmunoprecipitated, as earlier, in solutions of increasing NaCl concentration. As shown in Fig. 3, the protein complex was stable in NaCl concentrations of up to 350 mM. These data argue against a weak or nonspecific ionic interaction. Note that the faster moving band in the left lane (MBP alone) does not appear to be involved in binding to IE62.

Having shown an interaction between IE62 and IE4 in the infected cell and in solution, an enzyme-linked immunosorbent assay was used to characterize the interaction. Wells were coated with either MBP or MBP4, and IE62 was added. The binding of IE62 was detected using

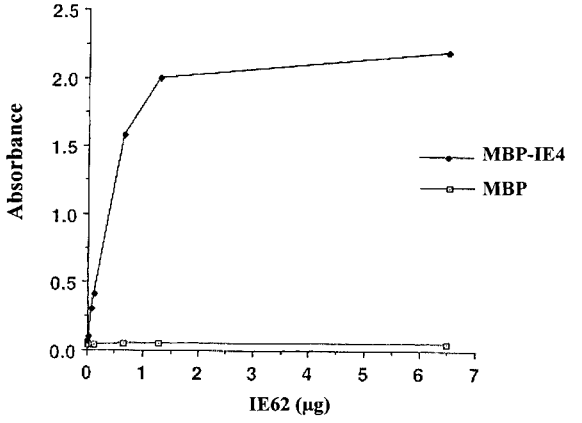

FIG. 4. Enzyme-linked immunosorbent assay of the IE62–IE4 interaction. The target proteins, MBP and MBP4, were bound to a 96-well microtiter plate and reacted with IE62. Bound IE62 was assayed using a monoclonal antibody against IE62 and a secondary antibody conjugated with horseradish peroxidase. Substrate color development was assayed at 450 nm.

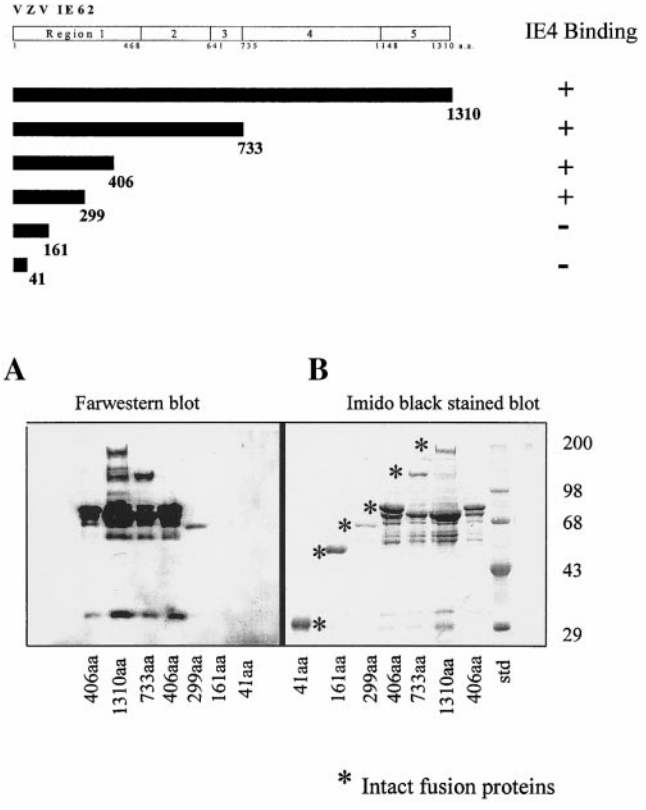

FIG. 5. Mapping an IE4 binding site on IE62. (Top) Structures of IE62 deletions fused to the C-terminus of GST, along with their binding capacity for IE4 (shown as + or −). The IE62 deletions were resolved on SDS–PAGE and electroblotted onto PVDF membranes. A far Western assay was then carried out using MBP4 as the interacting protein and anti-MBP antibody as the detector. (Bottom) IE4 binding detected by ECL immunodetection (A). (B) Imido black-stained blot, detecting all proteins.

the H6 anti-IE62 monoclonal antibody and a colorimetric sandwich assay. No IE62 bound to wells with MBP alone, but a saturable curve of binding to MBP4 was seen (Fig. 4).

Mapping an IE4 binding site on IE62

To determine which region or regions of IE62 are involved in interaction with IE4, we expressed various sequences from IE62 in bacteria as GST fusion proteins. Cleared bacterial lysates of each truncated fusion protein were resolved on SDS–PAGE and transferred to PVDF membranes. A far Western assay was performed using either MBP or MBP4 as the interacting protein. Visualization of MBP4 binding was accomplished with an antibody to MBP, followed by a conjugated second antibody for ECL detection. As demonstrated in Fig. 5, the minimal IE62 N-terminal region that binds IE4 consists of amino acids 1–299, whereas the sequence between amino acids 1 and 161 fails to bind. Therefore, amino acids 161–299 in IE62 contain sequences important for IE4 binding. This assay, however, does not rule out the possibility that other sequences in IE62 may also be involved in binding IE4.

IE4 binds to differently modified forms of IE62

Based on its primary sequence, IE62 has a calculated molecular mass of 140 kDa, but migrates on SDS–PAGE as a protein of 175 kDa. The basis for this, as with its herpes simplex virus (HSV) relative, ICP4, is posttranslational modification, including phosphorylation. It is relatively straightforward to resolve IE62 on one-dimensional SDS–PAGE into several components, thus allowing an investigation into the binding of IE4 to different modified forms of IE62. IE62 polypeptides were separated by SDS–PAGE and electrotransferred to PVDF membranes in Tris–glycine buffer. An ECL far Western assay revealed the binding of MBP4 to faster migrating species of IE62 (Fig. 6). These results suggest that IE4 preferentially binds to less posttranslationally modified forms of IE62.

Interaction of IE4 with dephosphorylated IE62

In the above analysis, IE4 bound preferentially to less than fully modified forms of IE62. IE62 is phosphorylated (Roberts *et al.,* 1985), and at least part of the explanation for differential binding by IE4 may be the presence or absence of phosphate groups. Thus IE62 was dephosphorylated with calf intestinal alkaline phosphatase, and a far Western assay was performed, as above, with MBP4. The results of this assay (Fig. 7) demonstrate that

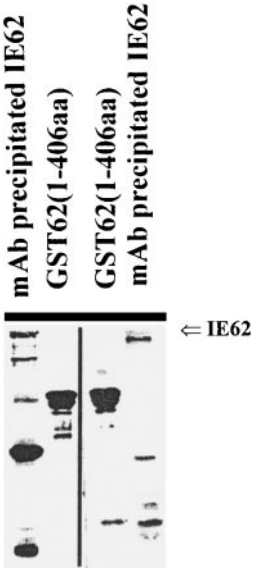

FIG. 6. IE4 binds to a less-modified form of IE62. An insect cell lysate containing IE62 was immunoprecipitated with anti-IE62 antibody, analyzed on SDS–PAGE, and electroblotted. GST62(1–406) was similarly analyzed, as a positive control. A far Western assay was carried out using MBP4 as interacting protein and anti-MBP antibody as the detecting antibody (right). (left) Imido black-stained proteins. The arrow points to the major imido black-stained IE62 polypeptide.

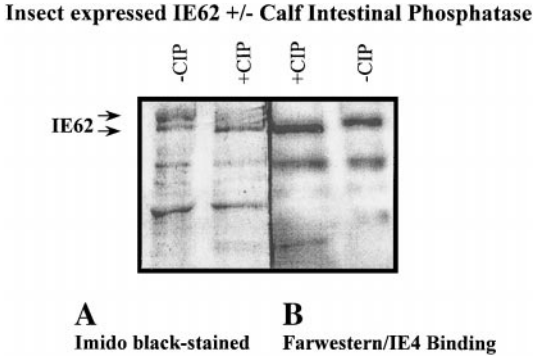

FIG. 7. IE4 binds to a less-phosphorylated form of IE62. IE62 purified from insect cells was treated with or without calf intestinal phosphatase, resolved by SDS–PAGE, and electroblotted. Affinity for IE4 was measured as before, using MBP4. (A) Imido black staining (B) Far Western blot. The arrows indicate greater (top) and lesser (bottom) phosphorylated forms of IE62.

IE4 is not binding to the most highly phosphorylated forms of IE62 but to a less phosphorylated form or forms. A Western blot was used to confirm that the faster migrating bands were authentic IE62 polypeptides (not shown).

Lack of interaction of IE4 with phosphorylated IE62

As a corollary to the previous experiment, GST62(1–299), a truncation mutant that binds to IE4 (see earlier), was treated with protein kinase C and protein kinase A in separate experiments, before carrying out far Western analyses with IE4. As anticipated (Figs. 8 and 9), phosphorylation of IE62 with either kinase removed the ability of IE4 to bind to IE62, supporting the previous result that IE4 binds to underphosphorylated IE62.

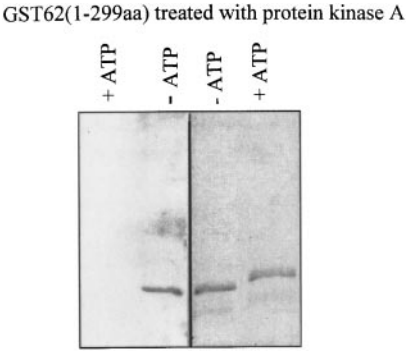

FIG. 9. Protein kinase A treatment of IE62 alters IE4 binding activity. GST62(1–299) was treated with protein kinase A in the presence and absence of ATP. Proteins were analyzed by SDS–PAGE before blotting, staining with imido black, and far Western analysis using IE4 binding.

Mapping a phosphorylated amino acid in IE62 involved in IE4 binding

Both protein kinase A and protein kinase C specifically phosphorylate amino acids in the primary structure of a protein based on consensus sequences. We examined the sequence or sequences in IE62 involved in IE4 binding (161–299) for serine and threonine residues that may be phosphorylated by the two kinases; Fig. 10 shows the analyses. The one residue that is commonly modified by both kinases is threonine 250; thus the phosphorylation state of this amino acid appears to be a factor in the ability of IE4 to bind to IE62.

DISCUSSION

We have demonstrated that the VZV gene regulatory proteins IE62 and IE4 exist in the infected cell as a

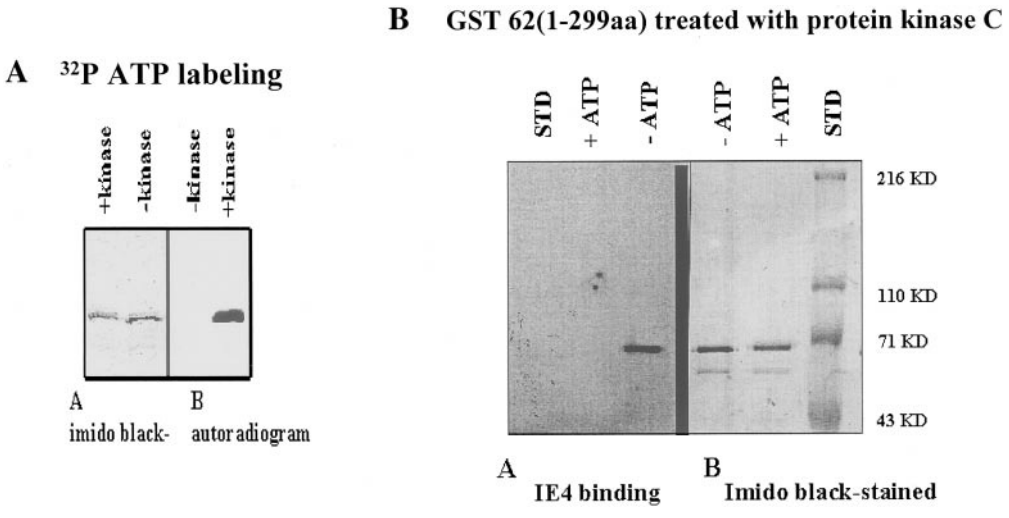

FIG. 8. Protein kinase C treatment of IE62 alters IE4 binding activity. GST62(1–299) was treated with protein kinase C. The proteins were analyzed by SDS–PAGE before blotting, imido black staining, autoradiography, or far Western analysis with MBP4 and anti-MBP antibody. (A) Samples were incubated with ^{32}P-ATP in the presence and absence of kinase, followed by autoradiography. (B) Far Western analysis using IE4 binding was performed on kinase-treated electroblotted proteins in the presence and absence of ATP.

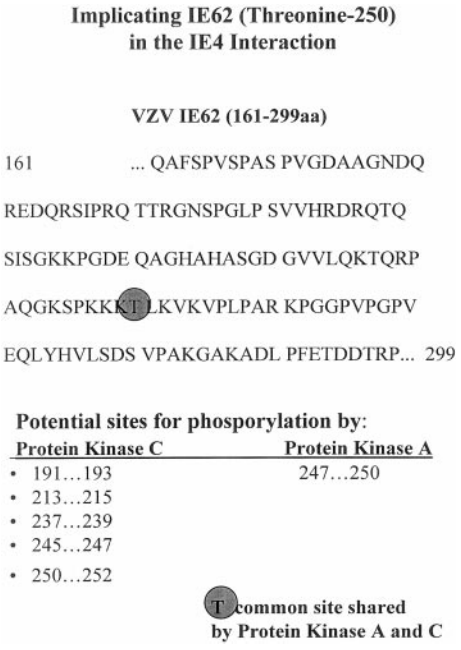

FIG. 10. Implicating IE62 threonine 250 in the IE4 interaction. The sequence of amino acids 161–299 of IE62, which we determined to be important for the IE62–IE4 interaction, is shown in the upper part of the figure. Potential sites for phosphorylation by protein kinase A and protein kinase C are listed in the lower half; the common site (T250) is circled.

complex and that the two proteins can form a stable interaction *in vitro* without the involvement of other proteins. Whether the *in vivo* complex contains additional viral or cellular proteins remains to be elucidated, but because formation of multicomponent complexes is the hallmark of eukaryotic gene regulation, it would be surprising if this were not the case. In recent experiments, we have been able to demonstrate that two other VZV proteins, IE63 and the ORF 9 product, can also be coimmunoprecipitated by anti-IE62 antibody from infected cell extracts. The IE62–IE4 interaction appears to involve a region of the IE62 molecule that lies between the acidic activation domain and the DNA-binding domain; we do not, however, rule out the involvement of other IE62 regions in IE4 binding.

Less-phosphorylated forms of IE62 appear to bind IE4 more effectively than more-phosphorylated forms, and thus phosphorylation seems to be involved in control of the IE62–IE4 interaction. A potentially important residue in this complex formation is threonine 250; a planned experiment is to mutate threonine 250 in viral variants and assess the role of this residue in the viral life cycle.

Because IE4 up-regulates the ability of IE62 to transactivate a variety of VZV promoters (Perera *et al.,* 1992), we assume that the physical interactions we have demonstrated are involved in this process. Presumably, IE4 aids IE62 transactivation through mechanisms that strengthen the interaction of IE62 with specific promoter sequences or with the preinitiation complex or through interaction with cellular factors that can enhance transcription. Interestingly, Michael *et al.* (1998) have shown that IE62 may also specifically interact with the IE4 promoter, potentially controlling IE4 production.

Phosphorylation and dephosphorylation of proteins are important control mechanisms of a variety of events that are crucial for growth and proliferation of cells. Many of these biological processes are regulated at the transcriptional level, which in turn is controlled by protein kinases (Hunter and Karin, 1992). Our investigation supports the concept that the differently phosphorylated forms of IE62 may play different roles in viral gene regulation. Indeed, as we (unpublished observations) and others (Defechereux *et al.,* 1996) have shown, IE4 is not always colocalized with IE62 in the VZV-infected cell, and it is quite possible that colocalization is also dependent on phosphorylation. Analagous to HSV ICP4 (Michael *et al.,* 1988) and to numerous transcription factors (Hunter and Karin, 1992), it may be that these phosphorylated forms vary in their ability to bind promoter sequences, resulting in differential viral gene expression. A further role for IE4 in binding to IE62 may be to "protect" it from phosphorylation through its binding to unmodified IE62, indirectly affecting its regulatory activity. Xia *et al.* (1996a,b) have shown that HSV ICP4 can be phosphorylated by protein kinase A and protein kinase C. The chief protein kinase A site or sites lie in a serine-rich region of ICP4, close to the N-terminus. There is an analogous serine-rich region in VZV IE62, but it is close to the C-terminus (aa 1285–1295) and is different from the phosphorylatable IE4-binding sequences described here.

Finally, as discussed earlier, both IE62 and IE4 are found in substantial amounts in the VZ virion tegument. Our data show (Spengler *et al.,* 2000) that although IE62 in the infected cell nucleus appears to be quite heavily phosphorylated and does not interact with IE4, the IE62 in purified virus particles migrates on SDS–PAGE as a less-phosphorylated species and interacts readily with IE4. Thus in addition to a potential role for the IE62–IE4 interaction in gene regulation, it is also possible that the modification state of these proteins controls the assembly (or disassembly or both) of virus particles in the infected cell.

MATERIALS AND METHODS

Cells and viruses

MeWo cells, a human melanoma cell line that supports the replication of VZV, were grown in Eagle's minimal essential medium with 10% fetal bovine serum. VZV strain Scott or Oka was propagated by mixing trypsinized VZV-infected cells with noninfected cells at a 1:4 ratio. Human foreskin fibroblast (HFF) cells were also used to generate VZV-infected cell extracts.

The baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) was grown in *Spodoptera frugiperda* (Sf21) cells as described in the Clontech (Palo Alto, CA) manual.

Construction of a recombinant baculovirus-expressing IE62 protein and its purification

A pGem plasmid containing the VZV *Eco*RIJ fragment (containing VZV ORF 62) was digested with *Sca*I and *Bgl*II and transferred into the Clontech transfer vector, which was digested with *Sma*I and *Bgl*II. Cotransfection of this transfer vector with Bac PAK 6 viral vector (Clontech) generated the recombinant baculovirus Bac62. Sf21 insect cells at 75% confluence were infected at an m.o.i. of 10 with Bac62 and expressed IE62 protein abundantly on day 2. This protein migrated on SDS–PAGE with VZV IE62 from VZV-infected HFF nuclear extracts and reacted with polyclonal and monoclonal anti-IE62 antibodies. To purify IE62, nuclear extracts of the insect cells were prepared as described by Lee *et al.* (1988). This extract was applied to a Pharmacia (Piscataway, NJ) Q Sepharose column, and fractions eluted with increasing NaCl concentrations; IE62 eluted with a peak at 400 mM. This peak fraction was then applied to an Sp Sepharose column and eluted at 300 mM NaCl. PAGE analyses and densitometry showed that this preparation of IE62 was approximately 90% pure.

Antibodies

Polyclonal antibodies to VZV IE4 were generously supplied by Dr. P. R. Kinchington (University of Pittsburgh). Monoclonal antibody to IE62 (H6) was purified from hybridoma cells (a gift of Dr. Ann Arvin, Stanford University), using the Pharmacia EZ Sep kit. Polyclonal antibodies to IE62 were made by resolving the chromatographically purified IE62 on 7% SDS–PAGE, excising 100 μg of the 175-kDa band, and emulsifying it in complete Freund's adjuvant for injection into rabbits. A histidine-tagged IE62 fusion protein containing the IE62 N-terminal 735 amino acids was purified over a nickel column (His Bind metal chelation resin; Novagen, Madison, WI) and similarly resolved and excised from an SDS–polyacrylamide gel. This was also injected into rabbits. An initial inoculation and two booster doses were sufficient to give high titers, as determined by enzyme immunoassay.

Construction of fusion proteins and their purification

To express the IE62 ORF as a fusion protein at the C-terminus of glutathione-*S*-transferase (GST), a two-step process was used. The Clontech transfer vector containing IE62 (used to construct the Bac62; see earlier) was digested with *Bam*HI and *Bgl*II liberating IE62 C-terminal (2.8-kb) sequences; this was ligated into pGEX (Pharmacia), which was digested with *Bam*HI, to give pGEX62 (C-terminal 2.8 kb). Next, a pET 28a vector (Novagen) with the IE62 N-terminal 2.2-kb sequence was digested with *Bam*HI, liberating a 2.2-kb fragment to be ligated into pGEX62, which was digested with *Bam*HI. The result was full-length IE62 in pGEX.

Truncated GST62 fusion proteins were constructed as follows. GST62 (1–735 aa) was generated by inserting the 2.2-kb N-terminus of the IE62 gene into pGEX digested with *Bam*HI. GST62 (1–406 aa), GST62 (1–299 aa), GST62 (1–161 aa), and GST62 (1–43 aa) were generated from pGEX62 (2.2 kb) using digestion with the following restriction enzymes: *Sal*I, *Sty*I, *Sph*I, *Stu*I, and *Xho*I, respectively. For growth, plasmids were transfected into DH5α cells by electroporation. Fusion proteins were expressed after isopropyl-β-D-thiogalactopyranoside (IPTG) induction from cultures of the transfected *Escherichia coli.* Extracts were prepared in PBS buffer and purified over glutathione–Sepharose 4B (Pharmacia Biotech).

To express IE4 as a fusion protein at the C-terminus of the maltose-binding protein (MBP), the entire ORF 4 was excised from pGEX4 digested with *Bam*HI and ligated into pMalc2 (New England Biolabs, Beverly, MA) digested with *Bam*HI. Transfection and induction were conducted as above. The plasmid pGEX4 had been made by digestion of the cosmid pVFsp4 (a gift of Dr. G. Kemble, Aviron Inc.) with *Eco*RI. The largest fragment (14 kb) was digested with *Nco*I, and a 4-kb fragment was further digested with *Sal*I after blunting with Klenow fragments (GIBCO BRL, Grand Island, NY). The final fusion fragment was digested with *Sma*I and *Sal*I. Cell extracts carrying MBP4 were prepared in cell lysis (C) buffer (20 mM Tris–HCl, pH 7.4, 0.2 M NaCl, 1 mM EDTA). Protein purification was accomplished using amylose resin (New England Biolabs).

All DNA constructions were verified using restriction enzyme digestion, and all fusion proteins were analyzed by PAGE for purity and by Western blotting for specificity.

Coimmunoprecipitation assays

VZV-infected MeWo cells were harvested at 80% CPE and lysed in PBS by passage through a 25-gauge needle. Cleared lysates were mixed with anti-IE4 or anti-IE62 antibodies and protein G–Sepharose (Pharmacia) to facilitate immunoprecipitation. After mixing for 4 h at 4°C, immunoprecipitates were spun and washed three times in PBS with 0.1% Tween 80. Pellets were boiled in sample buffer and resolved on SDS–PAGE, followed by transfer to nitrocellulose. ECL Western analyses were performed on the blots. Similarly, purified recombinant proteins (from various constructs expressing IE62 or IE4) were added to PBS with 2% BSA and 0.1% Tween 80. Either anti-MBP or anti-IE62 antibodies were added with protein G–Sepharose as described, and material was resolved on SDS–PAGE followed by Western blotting.

Enzyme-linked immunosorbent assay

Target protein (500 ng), either MBP or MBP4, was bound overnight at 4°C onto 96-well plates and blocked for 2 h with 2% BSA before being reacted with IE62 protein for 1 h. After extensive washing, target proteins were reacted with the monoclonal antibody to IE62 for 1 h. After more extensive washing, a secondary antibody to mouse IgG conjugated with horseradish peroxidase was allowed to react for 1 h. After a final washing, the colorimetric substrate TMB was added, and plates were read at 450 nm after a 20-min reaction.

MBP fusion protein capture assays

Bacterial cell lysates expressing MBP and MBP4 were induced for 2 h with IPTG and harvested. Cell pellets were resuspended in lysis buffer, lysed by ultrasonic treatment, and centrifuged at 14,000 rpm for 20 min. Cleared lysates were applied to 50 μl of amylose resin and allowed to bind for 1 h at 4°C with gentle mixing. Bound resin was washed in lysis buffer and reacted with IE62 protein, which was diluted in lysis buffer with 2% BSA. After 1 h of gentle mixing, the resin was washed extensively, and pellets were boiled in sample buffer and resolved on SDS–PAGE. IE62 protein was detected by Western blotting, as discussed earlier.

Far western blots

Proteins were resolved on SDS–PAGE, electrophoretically transferred to Western PVDF membrane (Schleicher & Schuell, Keene, NH), and gradually renatured for 24 h in HEPES buffer (20 mM HEPES, pH 7, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 10% glycerol) with incubation at 4°C overnight in the presence of 5% nonfat dry milk. This buffer was then replaced with the probing proteins in a 2% BSA–PBS solution. After 1 h, blots were washed in PBS and reacted with anti-MBP antibody for 1 h, followed by 1 h with a donkey anti-rabbit antibody conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL). Purified IE62 was dialyzed in 50 mM Tris–HCl, pH 8.5, and 0.1 mM EDTA at 4°C overnight. The dialysate was divided in two, with one half receiving 0.5 μl of calf intestinal alkaline phosphatase (CIP) (26 U/μl; GIBCO BRL) and both incubated at 30°C for 30 min. These samples were then boiled in SDS buffer before SDS–PAGE analysis.

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