Varicella-Zoster Virus IE62 Protein Utilizes the Human Mediator Complex in Promoter Activation[▽]

Min Yang, John Hay, and William T. Ruyechan*

Department of Microbiology and Immunology and Witebsky Center for Microbial Pathogenesis and Immunology, University at Buffalo, Buffalo, New York

Received 8 August 2008/Accepted 29 September 2008

The varicella-zoster virus (VZV) major transactivator, IE62, is involved in the expression of all kinetic classes of VZV genes and can also activate cellular promoters, promoters from heterologous viruses, and artificial promoters containing only TATA elements. A key component of the mechanism of IE62 transactivation is an acidic activation domain comprising the N-terminal 86 amino acids of IE62. However, the cellular target of this N-terminal acidic activation is unknown. In the work presented here, we show that the IE62 activation domain targets the human Mediator complex via the Med25 (ARC92) subunit and that this interaction appears to be fundamental for transactivation by the IE62 activation domain. In contrast, the Med23 subunit (Sur2/TRAP150 β /DRIP130/CRSP130) of the Mediator complex is not essential for IE62-mediated activation. Further, the IE62 activation domain appears to selectively interact with a form of the Mediator complex lacking CDK8. Chromatin immunoprecipitation experiments showed that IE62 stimulates recruitment of Mediator to an IE62-responsive model promoter. Finally, immunofluorescence microscopy of VZV-infected cells demonstrated intranuclear translocation of the Mediator complex to viral replication compartments. These studies suggest that Mediator is an essential component for efficient VZV gene expression.

Varicella-zoster virus (VZV) is a member of the *Alphaherpesvirinae* and the causative agent of varicella (chickenpox) and zoster (shingles) (2). The VZV genome is a linear double-stranded DNA molecule that encodes approximately 70 proteins (18). The entire complement of VZV genes is believed to be expressed during lytic infection in three broad kinetic classes, immediate early (IE), early (E), and late. Transcription of VZV genes is performed by the host cell RNA polymerase II (RNAPII), as is the case with all other herpes viruses. Efficient expression of the VZV genome is driven by a small group of VZV gene products including those encoded by open reading frames (ORFs) 62, 4, 61, 63, and 10 (14). The major viral transactivator is the product of ORF62 and its complement, ORF71, which lie within the inverted repeats bracketing the unique short region of VZV DNA. This protein is commonly designated IE62 since it is synthesized in the immediate-early phase of lytic VZV gene expression. IE62 contains a potent N-terminal acidic transactivation domain (TAD) comprising minimally amino acids 1 to 86 of its 1,310-amino-acid sequence and can activate the expression of all three kinetic classes of VZV genes in the absence of other viral proteins (15, 54, 58).

IE62 is also capable of activating expression from model promoters containing only TATA elements and has been shown to be able to interact directly with TBP and TFIIB (53, 71). IE62 also interacts directly and synergizes with the ubiquitous cellular transcription factors Sp1 and upstream stimulatory factor (USF) (52, 71). In the case of USF this can result in levels of expression up to 20- to 30-fold greater than those predicted from the additive contributions of IE62 and USF

alone (71). The regions of IE62 required for interaction with these four cellular factors have been mapped, and all are located downstream of the acidic activation domain (52, 53, 71). Thus, to date, the target or targets of the IE62 TAD have not been identified.

The Mediator complex was first identified in *Saccharomyces cerevisiae* via biochemical and genetic screening as a multisubunit coactivator of RNAPII-directed transcription (37). Since then Mediator complexes have been identified in all eukaryotes thus far investigated (42). Mediator complexes can contain over 22 polypeptides, and some estimates of the total number of Mediator components range to over 30 (16). Mediator is believed to transduce signals between transcriptional activators such as Sp1, p53, the adenovirus E1A protein, and the VP-16 TAD and the general transcription apparatus. Each of these activators has been shown to have one (and in some cases two) Mediator subunits as binding targets (16, 42).

Mediator complexes can be divided into three portions: an extended core, a module of loosely or variably associated factors that interact with activators, and a module which is not always associated with the larger Mediator complexes and contains kinase activities. The presence of the kinase-containing module has been primarily associated with repression rather than activation of transcription (1, 20, 23, 29). Recent structural studies have shown that the core subunits form distinct submodules, designated the head, middle, and tail; the head submodule components are responsible for contact with RNAPII, the tail subunits are a platform for interactions with gene-specific regulators, and the middle is involved in transfer of positive and negative regulatory signal (12). In this classification scheme the kinase module remains separate and the Med24-Med27 subunits, some of which are known to interact with transcriptional activators (8, 46, 47, 63, 68), remain unassigned to any major structural submodule.

* Corresponding author. Mailing address: Department of Microbiology, 138 Farber Hall, University at Buffalo, SUNY, Buffalo, NY 14214. Phone: (716) 829-2312. Fax: (716) 829-2376. E-mail: ruyechan@buffalo.edu.

[▽] Published ahead of print on 8 October 2008.

In the work presented here, we have examined the physical and functional interactions of the IE62 TAD with the mammalian Mediator complex. Our data indicate that the presence of the Med25 subunit is required for IE62 activation and that, unlike the VP16 TAD, the IE62 TAD interacts with a form of Mediator that lacks cyclin-dependent kinase 8 (CDK8). Chromatin-immunoprecipitation (ChIP) assays indicate that IE62 increases recruitment of Mediator to a model promoter. Finally, we show that during VZV infection, Mediator is recruited to viral replication compartments.

MATERIALS AND METHODS

Cells and viruses. MeWo cells, a human melanoma cell line that supports VZV replication, were grown in Eagle's minimal essential medium supplemented with 10% fetal bovine serum. VZV strain MSP was propagated in MeWo cell monolayers as described by Lynch et al. (41).

Plasmids. The pGEX-GST62AD and pGEX-GST16AD plasmids expressing glutathione *S*-transferase (GST) fusions containing the IE62 and VP16 activation domains, respectively, were derived from the pGEX-4T-3 plasmid (Amersham Biosciences, Piscataway, NJ). An N-terminal fragment of the IE62 protein (amino acids [aa] 1 to 107) which exhibited 3- to 10-fold higher levels of transactivation activity in a Gal4 fusion construct than the minimal IE62 TAD (aa 1 to 86) (53) was inserted between the BamHI and the SalI restriction sites in the parental plasmid to create pGEX-GST62AD. A second N-terminal IE62 fragment encoding aa 1 to 226 was inserted into the same sites to create pGEX-GST62(1-226). The VP16 TAD (aa 410 to 490) was inserted between the BamHI and the XhoI sites in the parental plasmid to create the pGEX-GST16AD plasmid. The pQE-Gal62AD and pQE-Gal16AD plasmids were constructed using the pQE-Tri expression vector (Qiagen, Inc. Valencia, CA). The DNA binding domain of the yeast Gal4 protein (aa 1 to 147) was inserted between the NcoI and the BamHI sites in the pQE-Tri vector followed by insertion of the IE62 TAD-containing N-terminal fragment (aa 1 to 107) yielding the pQE-Gal62AD. The VP16 TAD (aa 410 to 490) was inserted in a similar manner yielding the pQE-Gal16AD expression plasmid. All constructs were verified by sequencing at the Roswell Park Cancer Institute Biopolymer Facility.

The construction of the dual luciferase reporter vector pRFL/WT (where WT is wild type) containing the VZV bidirectional ORF28/29 promoter flanked by *Renilla* and firefly luciferase reporter genes was previously described by Yang et al. (70). The pUSFTALuc reporter vector described in Yang et al. (71) contains a minimal model IE62-responsive promoter consisting of a typical TATA element and an upstream consensus USF binding site. The cloning of pCMV62 plasmid expressing IE62 under the control of the cytomegalovirus immediate-early (CMV IE) promoter was described previously by Perera et al. (55). The Flag-Med25-expressing plasmid (pCDNA3FlagARC92) was provided by Anders Näär (Harvard University). The hemagglutinin (HA)-Med23-expressing plasmid (pCS2-hSur2-HA) was provided by Arnold Berk (University of California, Los Angeles). The pGalYY1N90 plasmid expressing the GalYY1AD was provided by Techung Lee (State University of New York, Buffalo, NY). The pFRLuc plasmid was purchased from Stratagene (La Jolla, CA).

GST tag protein affinity pull-down assays. Following induction with IPTG (isopropyl- β -D-thiogalactopyranoside), crude lysates of *Escherichia coli* expressing GST and GST62AD and GST16AD fusions were prepared and clarified as previously described (71). Aliquots (300 to 500 μ l) of the bacterial lysates were added to 50 μ l of glutathione-Sepharose beads and incubated for 1 h at 4°C. A total of 750 μ g of MeWo cell nuclear protein extract (52) in 300 μ l of buffer D (20 mM HEPES, pH 7.9, 100 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) was added to the protein-coupled beads and incubated overnight at 4°C. Following three washes with 0.1% Triton X-100 in buffer D, bound protein was eluted with 60 μ l of 2 $\times$ sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer by boiling and analyzed by SDS-PAGE and immunoblotting.

Transient transfection and reporter gene assays. Transient transfection assays in MeWo cells were performed in triplicate in 24-well plates as previously described (71). Briefly, cells were transfected at 90% confluence using 2 μ l of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) per μ g of total plasmid DNA transfected. Specific amounts of the plasmid DNA used in individual experiments are indicated in the figure legends. Cells were lysed at 48 h posttransfection in lysis buffer (50 mM HEPES, pH 7.4, 250 mM NaCl, 1% NP-40, 1 mM EDTA), followed by dual luciferase assays using a dual luciferase reporter assay system (Promega, Madison, WI). The pEF1 α -RL vector expressing *Renilla*

luciferase under the control of the EF1 α promoter (Promega, Madison, WI) was used as an internal normalization control reporter for transfections with reporter vectors expressing only the firefly luciferase reporter. The pCMV β -GalSport vector (Gibco, Carlsbad, CA) was used as an internal normalization control for assays performed with the dual luciferase pRFL/WT reporter. Each transfection experiment was repeated a minimum of three times. Average values of results from triplicate assays within a typical experiment are presented.

Immune capture and coimmunoprecipitation assays. MeWo cells in 10-cm cell culture dishes were transfected with 16 μ g of pCMV62 plasmid and/or 8 μ g of Flag-Med25 plasmid. In experiments where only one of the expression plasmids was used, pCDNA3.1(+) (Invitrogen, Carlsbad, CA) was added to maintain a constant amount of transfected plasmid DNA. At 48 h posttransfection, cell lysates were collected by adding 1 ml of Flag-lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 0.1% Triton X-100) supplemented with protease inhibitor cocktail prepared using protease inhibitor cocktail tablets (Roche Applied Science, Indianapolis, IN) to each dish. Clarified cell lysates were stored at -80°C prior to use in coimmunoprecipitation assays.

In the immune capture assays, 50 μ l of EZview Red anti-Flag M2 affinity gel monoclonal anti-Flag antibody-coupled agarose beads (Sigma, St. Louis, MO) were incubated with 1 ml of cell lysate overnight. Following three 20-min washes with TBST buffer (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween-20), bound proteins were eluted by boiling with 50 μ l of SDS-PAGE sample buffer and analyzed by SDS-PAGE and immunoblotting. Coimmunoprecipitation experiments using the H6 monoclonal anti-IE62 antibody generated by Arvin et al. (3) using VZV-infected cell lysates were performed as described by Peng et al. (52) and Yang et al. (71). The antibodies used for immunoblot detection of the precipitated material included anti-Flag M2 antibody (Sigma, St. Louis, MO) and rabbit polyclonal anti-IE62 antibody raised against full-length IE62 purified from recombinant baculovirus-infected cells as described by Spengler et al. (62).

Med25 RNA interference. Predesigned silencer small interfering RNA (siRNA) targeting Med25 (ARC92) was obtained commercially (Ambion, Inc., Austin, TX) (5'-GGAUGGUCCAGUCCAUUUTT-3'). A scrambled control RNA (scRNA) (5'-UAUGCUCAGCGUCUAUGUGTT-3') was generated by Integrated DNA Technologies (Coralville, IA). A 10 nM concentration of Med25 siRNA or scRNA was transfected into MeWo cells at 30% confluence in 24-well plates using Lipofectamine 2000. For reverse transcription-PCR (RT-PCR) assays, cells were harvested 70 h posttransfection, and total cell RNA was isolated by using an RNAqueous kit (Ambion, Inc., Austin, TX) according to the manufacturer's instructions. For reporter gene assays, the pFRLuc reporter plasmids and plasmids expressing Gal4 fusion proteins containing TADs from viral and cellular transactivators were cotransfected into MeWo cells 48 h after the initial siRNA transfection. Cell lysates were collected 30 h after plasmid transfection, and the levels of the firefly luciferase were determined as described above.

RNA isolated from the cells in RNA interference experiments was treated with DNase I (Invitrogen, Carlsbad, CA) and purified using an RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Total RNA (2 μ g) was subjected to RT using oligo(dT)₂₀ as the primer and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). The primers used in the PCR to amplify the Med25 gene were 5'-GGTCTACGTGAATCATGGCGAGAA C-3' (sense) and 5'-AGAGACTCCAGGTCCTTGTGTGGTGA-3' (antisense). Use of these primers generated a PCR product of 159 bp. Quantum RNA β -actin internal standards (Ambion, Inc., Austin, TX) with a 1:9 ratio of primers and competitors were used in the reaction to amplify a 294-bp fragment of the β -actin gene as an internal control. The PCRs were run for 28 cycles, which was predetermined to be in the linear range of detection.

ChIP assays. ChIP assays were performed by using a ChIP assay kit (Upstate, Temecula, CA) according to the manufacturer's instructions. MeWo cells in 10-cm cell culture dishes (5×10^6 cells per dish) were cotransfected with 4 μ g of pUSFTALuc reporter vector and 1 μ g of the pCMV62 or pCDNA3.1 vectors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were fixed 42 h posttransfection with 1% formaldehyde in culture medium for 10 min at 37°C and lysed by addition of 200 μ l of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1). Cell lysates were sonicated on ice to shear DNA to lengths between 200 and 1,000 bp (four sets of 15-s pulses interspersed with 1-min breaks) using a 550 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) equipped with a 2-mm tip.

The sonicated cell supernatants were diluted 10-fold in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl) and precleared with 75 μ l of protein G agarose-salmon sperm DNA (50% slurry) for 2 h at 4°C. Twenty microliters of precleared lysate was saved for use as input controls. Immunoprecipitating antibodies were added to the remainder of the precleared lysate and the mixtures were incubated overnight at 4°C. The

antibodies and antiserum used included 5 μ g of normal rabbit immunoglobulin G (IgG) as antibody control (Santa Cruz Biotechnology, Santa Cruz, CA), 20 μ l of rabbit polyclonal anti-IE62 antiserum (62), 5 μ g of rabbit anti-Med23 antibody (Bethyl Laboratories, Inc., Montgomery, TX), and 5 μ g of monoclonal anti-CDK8 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

The immune complexes were collected by adding 60 μ l of protein G agarose-salmon sperm DNA (50% slurry). The agarose pellet was washed sequentially with 1 ml each of the low-salt wash buffer, high-salt wash buffer, LiCl wash buffer, and Tris-EDTA buffer included in the kit. The bound immune complexes were eluted with 2×250 μ l of elution buffer (1% SDS, 0.1 mM NaHCO₃). The eluates were heated at 65°C overnight to reverse the protein-DNA cross-link, and the protein was digested with proteinase K for 1 h at 45°C. The DNA was recovered by phenol-chloroform extraction and ethanol precipitation and subjected to PCR. The primers used to amplify the promoter region within the pUSFTALuc reporter vector were 5'-GCTAACATAACCCGGGAGGTACC-3' (forward) and 5'-GAGGATAGAATGGCGCCGCCCT-3' (reverse). PCR was performed with 22, 24, and 26 amplification cycles to confirm the linear range of the assays. The amplified fragments were resolved on a 2% agarose gel containing 0.5 μ g/ml ethidium bromide. Images and data quantification were obtained using Bio-Rad Quantity One software.

Immunofluorescence microscopy. MeWo cells were grown on four-well glass chamber slides (Nalge Nunc International Corp., Naperville, IL). Infections were initiated with VZV MSP strain-infected cell stocks (70). Cells were fixed at 12 and 24 h with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature and permeabilized with 1% Triton X-100 in PBS for 2 min. Cells were blocked with 10% goat serum for 10 min and incubated with primary antibody in 10% goat serum for 2 h at room temperature. Primary antibodies used included the monoclonal H6 anti-IE62 antibody (1:1,000 dilution) and rabbit polyclonal anti-Med23 antibody (1:500 dilution) (Bethyl Laboratories, Inc., Montgomery, TX). Fluorescently labeled secondary antibodies in 10% goat serum were added following triplicate washes, and incubation proceeded for 1 h. The secondary antibodies were Alexa Fluor 488 goat anti-mouse IgG(H+L) and Alexa Fluor 568 goat anti-rabbit IgG(H+L) (Molecular Probes, Inc., Eugene, OR), both at a dilution of 1:500. Nuclei were visualized by staining with DAPI ([4',6'-diamidino-2-phenylindole] 300 nM in PBS) (Invitrogen, Carlsbad, CA) for 3 min at room temperature.

The samples were viewed using a Zeiss Axiomager Z1 fluorescence microscope installed with filter sets suitable for viewing the Alexa Fluor 488 and 568 and DAPI dyes. Images were captured with a Zeiss MrM cooled charge-coupled-device camera controlled by AxioVision Rel, version 4.5, software at $\times 20$ magnification.

RESULTS

The IE62 acidic TAD targets a Mediator complex lacking CDK8. Based on the evidence that Mediator is a component of the RNAPII transcriptional apparatus and acts as a general coactivator for a variety of cellular and viral transactivators (8, 22, 27, 34, 46, 49, 56, 59, 63, 68), we hypothesized that Mediator interacts with the N-terminal IE62 TAD. The first set of experiments performed was aimed at determining if such an interaction could be demonstrated in protein pull-down assays. The N-terminal 107 amino acids of IE62 containing the acidic TAD (aa 1 to 86) and a second larger IE62 fragment (aa 1 to 226) were fused to the N terminus of GST and expressed in *E. Coli*. The VP16 acidic TAD (aa 410 to 490 fused to GST) and GST alone were used as positive and negative controls, respectively. The ability of the two TADs to interact with Mediator was assessed using nuclear extracts derived from MeWo cells that had been transfected with a plasmid expressing Flag-tagged Med25. The Med25 subunit interacts directly with the VP16 TAD and Yang et al. (68) showed that ectopically expressed Flag-Med25 is incorporated into the Mediator complex in cells. Eluates containing stably bound proteins were probed for the presence of Flag-Med25 and the endogenous Med23 and CDK8 Mediator subunits.

Both Med25 (Fig. 1A) and Med23 (Fig. 1B) were captured

FIG. 1. In vitro interaction of the IE62 TAD with the Mediator complex. (A) GST pull-down experiments showing that the IE62 TAD can capture ectopically expressed Med25. Bacterially expressed GST, a GST62AD fusion protein containing IE62 aa 1 to 107 which includes the TAD, a GST-fusion protein containing IE62 aa 1 to 226, and a GST16AD fusion protein containing the VP16 TAD (aa 410 to 490) were coupled to glutathione-Sepharose beads and incubated with nuclear extracts of MeWo cells transfected with the Flag-Med25-expressing plasmid. Flag-tagged Med25 was detected by immunoblotting using anti-Flag M2 antibody (upper panel). The lower panel is a Coomassie-stained gel showing the relative amounts of the four GST proteins coupled to the beads. (B) GST pull-down experiments with untransfected MeWo cell nuclear extracts examining the interaction of the IE62 TAD with endogenous Mediator subunits. The presence of Med23 and CDK8 was detected by immunoblotting as shown in the two upper panels. The lower panel is a Coomassie-stained gel showing the relative amounts of the three GST proteins coupled to the beads.

by the IE62 TAD suggesting that the IE62 activation domain, like the VP16 TAD, is capable of interacting with the Mediator complex. However, there was no evidence of CDK8 capture by the IE62 TAD in six repetitions of these experiments, whereas the presence of CDK8 was readily detectable in assays using the VP16 TAD. The VP16 TAD results are consistent with other studies investigating the interaction of this TAD with Mediator (25, 46, 68). The absence of CDK8 binding to the GST62AD fusion protein suggests that the IE62 activation domain targets a form of the Mediator complex lacking CDK8 or possibly causes dissociation of CDK8 upon its interaction with Mediator. The interaction between the Mediator complex and the IE62 TAD also appears to be considerably weaker than that involving the VP16 TAD. As indicated in the lower panels of Fig. 1A and B, despite the fact that the levels of the GST62TAD fusion protein present in the reaction were higher than those of the VP16 TAD fusion, the amounts of Med25 and Med23 captured by the IE62 TAD were lower than the amount captured by the VP16 TAD. Similar levels of capture of Med25 were observed with the GST fusion containing aa 1

of 226 of IE62 compared to that observed with the GST62TAD construct (Fig. 1A). Thus, additional IE62 flanking sequences do not increase this binding, further suggesting that the interaction of Med25 with the IE62 TAD is weaker than that with the VP16 TAD.

Med25 is a functional target of the IE62 TAD. We next wished to determine if Med25 was a functional target of the IE62 TAD. Med25 has been shown to be a functional target of the VP16 TAD (46, 68). Both of the TADs from these two potent alpha herpesvirus transactivators are composed primarily of acidic and aliphatic amino acids. The two TADs are also similar in length to the IE62 TAD containing 86 amino acids and the VP16 TAD containing 81 residues (15, 54, 64). However, comparison of their sequences (54) indicates that they show very low levels of homology (3.9% identity and 5.9% homology). Further, the acidic activation domain of the cellular transactivator p53 interacts with Med17 as does the VP16 TAD but does not exhibit physical or functional interactions with Med25 (30, 68). Finally, aspects of IE62 function and mechanism are also reminiscent of the adenovirus E1A protein, which interacts with Med23 (4, 8, 13). Thus, there was no a priori reason for Med25 necessarily to be a functional target of the IE62 TAD.

Overexpression of Mediator subunits targeted by the VP16 TAD (Med25) and the adenovirus E1A protein (Med23) resulted in loss of activation in transfection assays due to competition between the free subunits and the Mediator complex for the specific activators (8, 72). Using this approach, both the IE62 and VP16 TADs were fused with the Gal4 DNA binding domain, and their activities were examined in the context of the pFRLuc (Stratagene, La Jolla, CA) reporter vector, which contains five Gal4 binding sites upstream of a consensus TATA element (Fig. 2A). A Gal4 fusion construct incorporating the N-terminal 90 amino acids containing the acidic TAD of the cellular transcription factor, YY1 (11), was also used in the transfection assays as a control since the amino acid composition of the YY1 TAD differs significantly from that of both of the two viral TADs. As shown in Fig. 2B, all three Gal4 fusion proteins exhibited significant and readily detectable levels of activity relative to the pQE-Tri DNA control, with the VP16 TAD being the strongest activator and the YY1 TAD the weakest.

Overexpression of Med25 virtually abolished the activity of the IE62 TAD under our experimental conditions. VP16 TAD activity was decreased to less than 40% of that observed in the absence of exogenously expressed Med25, in agreement with the results of Yang et al. (68). This differential inhibition correlates with the apparently weaker interaction observed with the IE62 TAD in Fig. 1A. Finally, transfection with the equivalent amount of the Med25-expressing plasmid resulted in little or no effect on the activity of the YY1 TAD (Fig. 2C). In contrast, transfection with a Med23-overexpressing plasmid (pCS2-hSur2-HA) whose use resulted in dramatic inhibition of the activity of a Gal4-E1A construct (8) exhibited little, if any, effect on the activities of the three TADs examined here. These results indicate that the Med25 subunit of Mediator is a direct target of the IE62 TAD.

Since the above experiments were performed with model promoters and Gal4 fusion proteins incorporating only the IE62 TAD, we wished to determine the effect of overexpres-

FIG. 2. Effects of ectopic overexpression of Med25 and Med23 on transcriptional activation by the IE62TAD. (A) Schematic depicting the pFRLuc reporter vector and the three effector plasmids expressing the Gal62AD, Gal16AD, and GalYY1AD fusion proteins. (B) Comparison of the transactivation activity of the three Gal4 fusion constructs. The plasmid pFRLuc (0.5 μ g) and 0.1 μ g of each of the plasmids expressing Gal62AD, Gal16AD, and GalYY1AD or the same amount of the parental pQE-Tri plasmid were cotransfected into MeWo cells. The transactivation activities of Gal62AD, Gal16AD, and GalYY1AD are presented as relative induction of the firefly luciferase activity in comparison to that observed with the control pQE-Tri plasmid. Error bars indicate the standard deviations derived from triplicate transfections. (C) Effects of ectopic overexpression of Flag-Med25 and HA-Med23 in transient transfection assays. MeWo cells were transfected with 0.3 μ g of pFRLuc reporter vector; 0.1 μ g of each of the plasmids expressing Gal62AD, Gal16AD, and GalYY1AD; and 0.4 μ g of each of the plasmids expressing pcDNA3.1, Flag-Med25, and HA-Med23. The activity of each transactivator in the absence of the overexpressed Mediator subunits was normalized to 100% (black bars). The levels of transactivation in the presence of overexpressed Flag-Med25 (open bars) and HA-Med23 (gray bars) are presented as the percentage of activation relative to that observed in the absence of ectopic expression of the Mediator subunits. Statistical significance was determined by a one-way analysis of variance followed by Tukey's posthoc test. (**, $P < 0.01$).

sion of Med25 on the activity of full-length IE62 in the context of a native viral regulatory element. The element chosen was the VZV ORF28/29 regulatory element, expression from which is activated by IE62. This element consists of two divergent overlapping promoters sharing an essential USF site and regulates expression of the viral DNA polymerase (ORF28) and the VZV major DNA-binding protein (ORF29) (70). The effect of ectopic expression of Med25 on this regulatory element was assessed using a dual luciferase reporter construct with *Renilla* luciferase in the position of the ORF28 gene and firefly luciferase in the presence of the ORF28 gene (Fig. 3A).

FIG. 3. Functional and physical interaction between Med25 and the full-length IE62 protein. (A) Schematic of the pRFL/WT dual luciferase reporter construct containing the VZV ORF28/29 regulatory element with important *cis*-acting elements indicated. The arrows indicate the transcription start sites and the direction of transcription for the ORF28 (left) and ORF29 (right) genes. RL, *Renilla* luciferase; FL, firefly luciferase. (B) Effect of ectopic overexpression of Flag-Med25 on IE62-mediated activation of the ORF28/29 regulatory element. MeWo cells were transfected with 0.5 μg of pRFL/WT vector, 0.01 μg pCMV62 plasmid, and increasing amounts of the Flag-Med25-expressing plasmid. The solid bar represents the *Renilla* luciferase activity controlled by the ORF28 promoter (28RL). The open bar represents the firefly luciferase activity controlled by the ORF29 promoter (29FL). The promoter activities in the absence of Flag-Med25 were normalized to 100%. (C) Effect of ectopic overexpression of Flag-Med25 on the basal activities of the ORF28/29 regulatory element. MeWo cells were transfected with 0.5 μg of p28RL/29FL vector and 0.1 μg of Flag-Med25-expressing plasmid or the same amount of pcDNA3.1 plasmid as control. (D) Immune capture experiments using anti-Flag M2 antibody. Whole-cell lysates of MeWo cells transfected with plasmids expressing IE62 alone or both Flag-Med25 and IE62 were incubated with anti-Flag M2 antibody-coupled agarose beads. Bead-bound proteins were resolved by SDS-PAGE and detected by immunoblotting. Lanes 1 and 2, 2% of the input whole-cell lysates, respectively; lanes 3 and 4, immunoprecipitates from the whole-cell lysates probed with anti-Flag M2 antibody (upper panel) and rabbit polyclonal anti-IE62 antibody (lower panel), respectively. (E) Whole-cell lysates of MeWo cells expressing Flag-Med25 alone or both IE62 and Flag-Med25 were incubated with monoclonal anti-IE62 antibody-coupled protein G-Sepharose (51, 70). Lanes 1 and 2, 1.5% of the whole-cell lysates, respectively; lanes 3 and 4, immunoprecipitates from the whole-cell lysates probed with rabbit polyclonal anti-IE62 antibody (upper panel) and anti-Flag M2 antibody (lower panel).

The results from transfection assays in the presence of increasing amounts of the Med25 expression plasmid are shown in Fig. 3. Exogenous Med25 inhibited IE62-mediated activation of the ORF28/29 promoter in a dose-dependent manner (Fig. 3B). In contrast, the basal promoter activity in the absence of IE62 was not altered in the presence of the highest amount of the Med25-expressing plasmid used in the presence of IE62 (Fig. 3C). Taken together, these results suggest that Med25 is a bona fide target of IE62 via the IE62 TAD and that this interaction is a key step in the mechanism of IE62 transactivation.

IE62 associates with Med25 in situ. We next wanted to confirm that the full-length IE62 protein associates with Med25 in situ. Immune capture and coimmunoprecipitation experiments were performed using nuclear extracts derived from MeWo cells in which IE62 and Flag-Med25 were coexpressed via transient transfection. IE62 was captured along with Flag-Med25 using anti-Flag antibody-coupled agarose beads, indicating that the two exogenously expressed proteins were associated in these extracts (Fig. 3D, lanes 3 and 4). In a reciprocal experiment using the monoclonal anti-IE62 antibody, Flag-Med25 was coprecipitated only from extracts con-

taining the IE62 protein (Fig. 3E, lanes 3 and 4). As indicated above, it has been shown that ectopically expressed Med25 can be incorporated into Mediator (68). Thus, the Med25 coprecipitated in these assays in association with IE62 likely represents a mixture of free Med25 and Med25 in the Mediator complex. The relatively small amounts of the coimmunoprecipitated proteins suggest, as did the data in Fig. 1 and 2, that the interaction between IE62 and Med25 is weak under our experimental conditions.

Med25 is required for full activation by the IE62 TAD in situ. Since all of the above data had been gathered using ectopically expressed Flag-tagged Med25, experiments were performed to examine the effects of knockdown of the expression of endogenous Med25 using siRNA. At 70 h posttransfection of Med25-specific siRNAs and scRNAs, total cellular RNA was isolated and examined by RT-PCR to assess the levels of Med25 message. Typical results are presented in Fig. 4A and show that Med25 message was significantly reduced in the presence of the Med25-specific siRNA but not in cells transfected with the scrambled control or in untransfected cells. The effect of the presence of Med25-specific siRNA on the activities of the Gal62TAD and Gal16TAD activator con-

FIG. 6. Intranuclear redistribution of Mediator in VZV-infected MeWo cells during the early phase of infection. Uninfected and VZV-infected MeWo cells were immunostained for Med23 (red) and IE62 (green) with rabbit polyclonal antibody and the H6 mouse monoclonal antibodies, respectively. Images show virtually complete overlap of the two signals in large foci in infected cells. The MeWo cell nuclei were counterstained with DAPI (blue). The cells were visualized using a Zeiss Axiomager Z1 fluorescence microscope. Images were recorded with a 20 $\times$ objective. Bar, 10 μ m.

Intranuclear translocation of Mediator in VZV-infected cells. IE62 is predominantly nuclear during early infection and localizes to viral replication compartments. At late times of infection in MeWo cells, IE62 is phosphorylated by a viral kinase, resulting in its translocation from the nucleus to the cytoplasm (19, 35, 36, 61). Confocal microscopy was employed to examine the cellular localization of Mediator in relation to that of IE62 in VZV-infected MeWo cells. Med23 was employed as the marker for Mediator-associated with IE62 based on the results obtained from the protein capture and ChIP assays.

In uninfected cells, Med23 appeared to be present exclusively in the nuclei and exhibited a diffuse punctate pattern throughout the nucleoplasm (Fig. 6, upper panel). This pattern is similar to that observed with TRAP220/Med1 in COS cells (51). In early-phase infected cells, however, the intranuclear distribution of Med23 was significantly altered, with the majority of the signal observed in large tight foci which completely overlapped with the presence of IE62 in viral replication compartments. These replication compartments are the major sites of viral transcription and DNA replication during alphaherpesvirus infection (40).

At late times during infection, transcription of the VZV genome begins to wane, IE62 is redistributed from the nucleus to the cytoplasm, and infected MeWo cells form large multinucleate syncytia (26, 35, 36, 41). Observation of late-phase infected cells (Fig. 7) showed the redistribution of the majority of IE62 from nuclear replication compartments to the cytoplasm, which now surrounds multiple nuclei within a syncytium. Med23, in contrast, returned to its diffuse punctate distribution within the nuclei. These results demonstrate that the subnuclear localization of Mediator is dynamically regulated in VZV-infected cells at different stages of infection and correlates with the presence of IE62 in viral replication compartments during the early phase of infection.

DISCUSSION

The IE62 protein is the major VZV transactivator and is capable of promiscuous activation of viral, cellular, and model

promoters (58). The transactivating activity of this 1,310-amino-acid polypeptide is associated with an acidic activation domain comprising the amino terminal 86 to 90 amino acids of the molecule (15, 54). While components of the general cellular transcription apparatus (TBP and TFIIB) and the cellular transcription factors Sp1 and USF have been shown to interact physically and functionally with IE62 (52, 53, 71), the target or targets of its N-terminal activation domain has/have remained unknown. In this study, we show for the first time that the IE62 TAD interacts with the mammalian Mediator complex. The component of Mediator with which the IE62 TAD interacts is Med25, based on evidence accumulated from protein pull-down assays, immune capture and coimmunoprecipitation of ectopically expressed IE62 and Med25, inhibition of IE62 TAD and full-length IE62 activity by ectopically expressed Med25, and Med25-specific siRNA experiments. Thus, Mediator and, more specifically,

FIG. 7. Redistribution of Mediator and IE62 at late times during infection. Infected MeWo cells are shown forming large syncytia characteristic of the late phase of VZV infection. The loss of colocalization of the intracellular distribution of Med23 (red) and IE62 (green) and the redistribution of Med23 resulting in the return to a more diffuse intranuclear signal are shown. The MeWo cell nuclei were counterstained with DAPI (blue). Bar, 10 μ m.

Med25 represent the first target(s) of the IE62 TAD to be identified with regard to transcriptional activation.

The multisubunit Mediator complex is believed to be required for activator-mediated transactivation of promoters via interactions of individual activators with specific Mediator subunits (42). Mediator can also interact with RNAPII (50). Thus, by inserting itself between the gene-specific activator and RNAPII, Mediator plays a fundamental role in orchestrating RNAPII activity in concert with specific transcriptional activators. Mediator subunits have been tentatively grouped into three categories. The majority make up the Mediator "core" that is present in all Mediator complexes isolated thus far. A second group is present in what has been designated the kinase or CDK module and includes CDK8, CDK11, cyclin C, Med12, and Med13 (12, 42). The presence of the kinase module has been associated with repression rather than activation of transcription in yeast; however, the presence of this module in a Mediator complex recruited to the promoter of the mouse *Egr1* gene has been associated with activation (67). A third loosely associated module has been designated the "activator" module since early studies showed interactions between several subunits of Mediator comprising this module and viral activators (8, 46, 63, 68).

Direct physical interaction with the Mediator complex in mammalian cells has been reported for a variety of transcriptional activators including nuclear receptors (10, 24, 43, 72), cellular transcription factors (32, 39, 47, 63, 69), and viral transactivators (8, 28, 46, 68). Specifically, the nuclear receptors interact with Med1 (10, 24, 43, 72), STAT2 interacts with Med14 (39), and the Smad2 (or Smad3)-Smad4 complex (32) and SREBP (69) interact with the Med15 subunit. The cellular factor p53 and the herpes simplex virus V16 TAD have been reported to interact with Med17 (30). All of these Mediator subunits are within the Mediator core. The Kaposi's sarcoma-associated herpesvirus Rta protein interacts with Med12, a component of the CDK module (28). Interactions involving Mediator subunits within the "activator module" include Med23 with C/EBP β (47), Elk1 (63), the adenovirus E1A protein (8), and Med25, which appears to be the primary target of the herpes simplex virus VP16 TAD (46, 68).

Our hypothesis was that IE62 specifically targets Mediator via specific interactions between its TAD and one or more Mediator subunits. These interactions would then aid in stabilizing the presence of Mediator at promoters, resulting in the increased activation observed in the presence of IE62. Confirmation of this hypothesis would be congruent with the fact that the majority of VZV promoters show low levels of basal activity in the absence of IE62 (14, 58) and with the results of Fan et al. (21) showing that Mediator is recruited in an activator-specific manner and does not associate with many highly active RNAPII promoters in yeast. The GST pull-down experiments presented in Fig. 1 indicated that the IE62 TAD, like the already well-characterized VP16 acidic activation domain, is capable of capturing the Mediator complex. There were, however, significant differences between the results obtained with the two TADs. Much less signal was observed for the Flag-tagged Med25 and endogenous Med23 subunits in pull-downs with the IE62 TAD than with the VP16 TAD. This was true despite the fact that larger amounts of the IE62 TAD were present on the glutathione-Sepharose beads. These results sug-

gest that the interaction of the IE62 TAD with Mediator is weaker than that of the VP16 TAD.

A second important difference consistently observed between the two TADs was that the CDK8 subunit of Mediator was not present in pull-downs performed with the IE62 TAD but was present, as previously reported (25, 68), in pull-downs with the VP16 TAD. Thus, the IE62 TAD may interact only with a form of Mediator lacking CDK8. Mammalian Mediator complexes containing all or some of the CDK module components have been reported (60). Alternatively, the interaction of the IE62 TAD with Mediator may result in the dissociation of the CDK8 subunit and possibly other subunits within the kinase module. It is known that the kinase module can interact with the RNAPII C-terminal domain (CTD) (29) and that herpes simplex virus infection results in alteration of CTD phosphorylation (31, 57). Thus, it is possible that VZV infection may result in specific alteration of the RNAPII CTD that does not require CDK8.

Our results indicate that the activity of the IE62 TAD is inhibited by the ectopic overexpression of Med25 but not Med23. Further, the inhibition of IE62 TAD activity is significantly greater than that observed here and by others (68) for the VP16 TAD. The more extensive inhibitory effect of the exogenous Med25 on the IE62 TAD activity than on the VP16 TAD activity is consistent with the apparently lower level of affinity of the IE62 TAD for Mediator observed in the GST pull-down assays. Based on this differential affinity, the ectopically overexpressed Med25 would be expected to more efficiently displace the IE62 TAD interacting with Med25 in the endogenous Mediator complex.

The requirement for Med25 in IE62 TAD-mediated activation was corroborated by siRNA experiments which showed that Med25-specific siRNA significantly reduced the activity of the IE62 TAD. The residual activity present for both the IE62 and VP16 TADs is most likely due to the presence of Med25 protein pools remaining in the cell (68). Taken together, these results suggest at least partial conservation of a transcriptional activation mechanism between two herpesviruses that are believed to have diverged some 65 million years ago (44).

The involvement of Mediator in the context of full-length IE62-activated transcription was confirmed by the ChIP assay data demonstrating increased recruitment of Mediator, as monitored by the increase in the presence of Med23, to the model USFTALuc promoter upon cotransfection of pCMV62 with the reporter plasmid. The ChIP assay data also support the results from the protein pull-down experiments showing no increase in recruitment of CDK8 to the promoter. This suggests that the IE62 TAD not only alone but also in the context of the full-length IE62 molecule may interact with or recruit a form of Mediator lacking the CDK8 subunit.

Several lines of evidence indicate that Mediator is required for VZV replication during infection. First, ectopically expressed Med25 inhibited the IE62-mediated expression of the complex native VZV ORF28/29 bidirectional regulatory element but not basal level expression in transient transfection assays. ORF28 and ORF29 encode the catalytic subunits of the viral DNA polymerase and the major DNA-binding protein, respectively. High levels of expression of both proteins are required during productive infection (45). Second, we observed a remarkable redistribution of Mediator during the

early phase of viral infection, resulting in virtually total overlap with the IE62 signal present in large foci reminiscent of viral replication compartments in herpes simplex virus infection (40). To the best of our knowledge, this is the first demonstration of Mediator redistribution in response to viral infection. Third, during the late phase of viral infection, when IE62 redistributes from the nucleus to the cytoplasm, we observed that Mediator returned to its punctate, generalized nuclear distribution, suggesting that interaction with IE62 is required for the alterations observed during the early phase of infection.

Med1/TRAP220 has been shown to be required for peroxisome proliferator-activated receptor γ adipogenesis in mouse embryo fibroblasts and for Aurora-A kinase expression but not for MyoD-stimulated myogenesis, indicating that this subunit regulates cell-specific transcription and differentiation (24, 65). VZV can proliferate in skin, epithelial cells, T cells, and neurons during productive infection, with various requirements for the expression of specific genes (5, 6, 33, 48, 61). Thus, it is possible that interactions with other Mediator subunits either via the IE62 TAD or other IE62 domains or with other VZV transcriptional activators such as the viral IE4 and ORF61 proteins (14) may be required for the correct expression of specific VZV genes. The demonstration of an interaction between Mediator and IE62 presented here will, in the future, both allow the examination of the potential role of Mediator in the complex tissue tropism of VZV in its human host and lead to new insights into the mechanism of Mediator action in general.

ACKNOWLEDGMENTS

We thank Arnold Berk, Te-Chung Lee, and Anders Naar for providing plasmids expressing Med23, the YY1 TAD, and Med25, respectively, and Shinobu Yamamoto for the comparison of the TAD sequences.

This work was supported by grant AI18449 from the National Institute of Allergy and Infectious Diseases.

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