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The Varicella-Zoster Virus Immediate-Early Protein IE62 Is a Major Component of Virus Particles

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Varicella-zoster virus (VZV) open reading frame (ORF) 62 potentially encodes a protein with considerable amino acid homology to the herpes simplex virus (HSV) immediate-early regulatory polypeptide ICP4 (or IE3). To identify and characterize its protein product(s) (IE62), we used a rabbit antiserum prepared against a synthetic peptide corresponding to the C-terminal 13 amino acids of the predicted protein. This antiserum reacted with phosphorylated polypeptides of 175 to 180 kDa that were made in VZV-infected cells and in cells infected with a vaccinia virus recombinant expressing IE62, but not in control-infected cells, confirming its specificity and reactivity to the IE62 protein. The antiserum recognized a 175-kDa polypeptide in purified virions that comigrated with a major structural protein. Comparison of this reactivity with that of an antipeptide antiserum directed against the VZV ORF 10 product (homologous to the HSV major structural protein VP16) indicates similar levels of ORF 62 and ORF 10 polypeptides in VZV virions. In contrast, antipeptide antiserum directed against the VZV ORF 29 product, the homolog of the HSV major DNA-binding protein, failed to recognize any protein in our virion preparations. Treatment of virions with detergents that disrupt the virion envelope did not dissociate IE62 from the nucleocapsid-tegument structure of the virion. Differential sensitivity of VZV virion IE62 to trypsin digestion in the presence or absence of Triton X-100 indicates that IE62 is protected from trypsin degradation by the virus envelope; since it is not a nucleocapsid protein, we conclude that it is part of the tegument. Finally, we show that the virion 175-kDa protein either can autophosphorylate or is a major substrate in vitro for virion-associated protein kinase activity.

The human herpesvirus varicella-zoster virus (VZV) is the cause of two diseases: varicella, which results from a primary infection that usually occurs in childhood, and zoster, a disease caused by reactivated latent virus that occurs mostly in the elderly. Unfortunately, the poor growth characteristics of VZV in vitro have severely hindered efforts to study its biology. However, the generation of the nucleotide sequence of the genome (9) has markedly improved prospects for basic work on VZV, largely in the context of its organizational similarities to herpes simplex virus (HSV). The general layout of homologous genes in these two herpesviruses is virtually identical, particularly in the unique long regions of the genomes (9, 25). As a consequence, many of the functions of VZV gene products can be inferred from their HSV counterparts. Such assumptions must, however, be guarded, as functional interchange between VZV and HSV proteins appears to be limited (10, 12, 13, 37).

One successful case of genetic interchange has been demonstrated; proteins encoded by VZV open reading frame (ORF) 62 (IE62) can functionally replace HSV ICP4 (10, 12, 13, 37). It is therefore likely that many of the properties of these two proteins are similar. The genes for ICP4 and IE62 are both diploid and lie in the reiterated sequences bounding the short region of the respective genomes (8, 9, 25). Although little is known about IE62, HSV ICP4 has been well characterized and is a model for studies on IE62. ICP4 is an essential HSV phosphoprotein that is made under immediate-early (IE) conditions in HSV-infected cells (15, 34). Upon infection, it and other HSV IE polypeptides are transcriptionally activated in *trans* by a virion polypeptide designated VP16, α-TIF, or Vmw65 (5, 28, 31, 35). ICP4 migrates as a broad band on sodium dodecyl sulfate (SDS)-polyacrylamide gels, with an apparent molecular mass of 175 kDa (15, 32, 45). The protein has several forms; one is known to be stably phosphorylated throughout infection while others appear to cycle phosphate on and off (45). It is required (in part) for the expression of HSV early and late genes (15, 34). It localizes to discrete compartments of the infected cell nucleus (21) and is intrinsically able to bind specific DNA sequences (11, 22, 23) probably involved in the control of transcription through mechanisms not yet clear. Recently, small but detectable amounts of ICP4 have been shown to be associated specifically with HSV virions (47), and a form appears to be able to interact with the infected cell membranes (48).

IE62 appears to be similar but not identical to HSV ICP4. ORF 62 represents one of at least three VZV genes whose products act in *trans* to control the transcription of other VZV genes (4, 16, 17). IE62 appears to bind DNA sequences similar to those recognized by HSV ICP4 (46). The IE62 promoter has HSV-1 α-TIF-responsive elements (12, 27), but the VZV homolog to α-TIF (the product of ORF 10) is truncated, and evidence suggests that it is unable to enhance IE transcription (27). Recently, Forghani et al. (14) isolated a monoclonal antibody to IE62 and used it to characterize the protein as a true VZV IE phosphoprotein of 175 to 180 kDa. Several other reports describing VZV proteins with molecular masses of 175 to 180 kDa, including a 170- to 180-kDa VZV-induced phosphoprotein (36, 37, 40) and a

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VZV DNA-binding protein (36), have appeared in the literature. Earlier work defining VZV structural polypeptides noted the presence of a 175- to 180-kDa protein in partially purified virions but not in nucleocapsids (37, 39, 41).

In this paper, we explore some characteristics of the product of VZV ORF 62 (IE62) and show that it is a phosphoprotein with an apparent molecular mass of 175 to 180 kDa. We also present evidence that, unlike ICP4 in HSV, IE62 in VZV appears to be a major component of the VZV virion and probably lies in the tegument of the virus.

MATERIALS AND METHODS

Cells and virus. VZV strain Scott and its growth on human foreskin fibroblasts (HFF) have been previously described (20). For virion purification, 175-cm^2 flasks at 75% confluency were each infected with 1/10 of the contents of a 175-cm^2 flask showing 50 to 80% cytopathic effect (CPE). At 24 h postinfection, the cells were trypsinized onto the same flask and the medium was replaced. Forty-eight hours later, attached cells were scraped from the flasks and put into ice-cold phosphate-buffered saline (PBS), and they were then processed for virions or extracts. Vaccinia virus (strain WR) and recombinants were grown as described previously (6). Vaccinia virus-IE62 recombinants were isolated from WR-infected cells transfected with the plasmid pKIP81 by methods described previously (6, 19). PKIP81 is a derivative of the vector pSC11 in which a blunt-ended *Sca*I (VZV coordinate 109226)-*Bgl*II (104169) (9) fragment containing the entire coding sequences of VZV gene 62 (109134 to 105201) has been cloned into the unique *Sma*I site of the vector pSC11 in the correct orientation to place the gene under the control of the vaccinia virus promoter p7.5. Recombinant virus was plaque purified and analyzed for correct insertion (data not shown). The KOS strain was used for HSV virion purification.

Antisera. The synthesis of peptides and the production of antisera against them were carried out as described previously (18). Antiserum specific for VZV IE62 was prepared by using the synthetic peptide NH_2-Tyr-Gly-Arg-Gly-Gly-Arg-Ser-Pro-Pro-Gln-Ser-Arg-Gly-Cys-COOH, corresponding to the C-terminal amino acids predicted from ORF 62 (8, 9), with additional C-terminal Cys and N-terminal Tyr residues added to facilitate coupling. Likewise, antiserum specific for the VZV ORF 10 product was prepared by using the peptide NH_2-Tyr-Met-Glu-Cys-Asn-Leu-Gly-Thr-Glu-His-Pro-Ser-Thr-Asp-COOH, corresponding to the predicted N terminus of ORF 10 (9), with an additional N-terminal Tyr residue. The characterization of antiserum against a VZV ORF 29 peptide has been described previously (18).

Purification and analysis of VZV virions. VZV was purified from infected cells by a procedure modified from methods described previously (33, 39, 41). VZV-infected HFF cytoplasmic extracts were prepared from 20 175-cm^2 flasks showing 75 to 90% CPE. PBS-washed cells were swollen for 30 min in 5 ml of TE buffer (10 mM Tris-HCl [pH 7.6], 1 mM EDTA) and Dounce homogenized in a rotary homogenizer for 20 strokes. The clarified cytoplasmic extract was centrifuged at 4°C through a linear 20 to 50% (wt/vol) sucrose gradient for 1 h at 17,000 rpm in a Beckman SW28 rotor. Gradient buffer consisted of 350 mM NaCl, 1 mM EDTA, and 10 mM sodium phosphate buffer, pH 7.4. The lower of two visible bands, containing enveloped virions (determined by electron microscopy), was harvested, diluted threefold in gradient buffer, pelleted, and allowed to resuspend overnight at 4°C. The resuspended virus was then layered on preformed linear 0 to 50% (wt/wt) potassium sodium tartrate–40 to 0% (vol/vol) reverse glycerol gradients prepared in gradient buffer and centrifuged at 15°C for 17 h at 20,000 rpm in the SW28 rotor. A broad diffuse band in the upper two-thirds of the tube containing nucleocapsids and membranous vesicles was discarded; a sharp, clumpy band one-third of the way from the bottom of the gradient containing enveloped virions was harvested, diluted, and pelleted as described above. Virions were resuspended in approximately 200 μl of PBS. Unless stated otherwise, all solutions were supplemented with 0.1 mM TLCK (*N*-tosyl-L-lysine-chloromethylketone) and 0.1 mM TPCK (*N*-tosyl-L-phenylalanine-chloromethylketone) (BMB Biochemicals, Indianapolis, Ind.). For trypsin treatment, virions were prepared as described above, except that the final gradient and pelleting of virus was performed in the absence of protease inhibitors. Trypsin (211 U/mg) was purchased from Worthington Biochemicals, Freehold, N.J., and was used at the concentrations described below. Incubation was performed in the absence or presence of 1% (vol/vol) Nonidet P-40 (NP-40) at room temperature for 10 min and was halted by the addition of 1 mM TLCK–1 mM TPCK–10 mM phenylmethylsulfonyl fluoride (PMSF), followed by solubilization in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) buffer and immediate boiling for 10 min prior to electrophoresis. Separated proteins were stained with silver by using a kit (Bio-Rad, Richmond, Calif.) or with Coomassie brilliant blue in a colloidal state (30).

Exclusion of cellular proteins was assayed to monitor the purification procedure. Two flasks (175 cm^2) of uninfected cells were labelled by incubation in medium containing 1/10 the normal concentration of methionine plus 15 μCi of [^{35}S]methionine per ml for 48 h. The uniformly radiolabelled uninfected cells were added to the VZV-infected cells prior to the purification of VZV, as described above. At each stage of the purification, radiolabelled protein content was estimated by the 10% trichloroacetic acid precipitation of samples and scintillation counting, and protein content was assayed with a Bio-Rad protein determination kit as described by Bradford (3). Purity was assessed by expressing the percentage of counts remaining per microgram of protein.

Radiolabelling and immunological detection. All radiochemicals were purchased from New England Nuclear Corp., Boston, Mass. VZV-infected cells showing approximately 10 to 20% CPE were trypsinized, reseeded at monolayer density onto 75-cm^2 flasks in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered phosphate-free minimal essential medium with 2% (vol/vol) Serum Plus (JRH Biosciences, Rockville, Md.), and labelled at 4 h postreseeding with $^{32}P_i$ at 200 μCi/ml in phosphate-free medium containing 2% (vol/vol) Serum Plus. For infection with vaccinia virus, cells at monolayer density were infected at a multiplicity of infection of 1 PFU per cell, with labelling commencing at 6 h postinfection following a 2-h phosphate starvation. At 18 h postinfection, all cells were scraped from the flasks into the medium and pelleted; they were then washed with ice-cold PBS, lysed in 0.5% (vol/vol) Triton X-100–0.5 M NaCl–10 mM sodium phosphate (pH 7.6) at 500 μl per flask, and sonicated. Extracts were clarified by centrifugation at 100,000 × *g*. Extracts were diluted to 0.15 M NaCl, recentrifuged, mixed with 25 μl of rabbit antiserum, and incubated at 4°C overnight. Reactions were performed in the presence of 200 μg of peptide per ml, as described below. Immunoprecipitates were collected by using protein A-Sepharose (Sigma, St. Louis, Mo.), washed extensively in PBS,

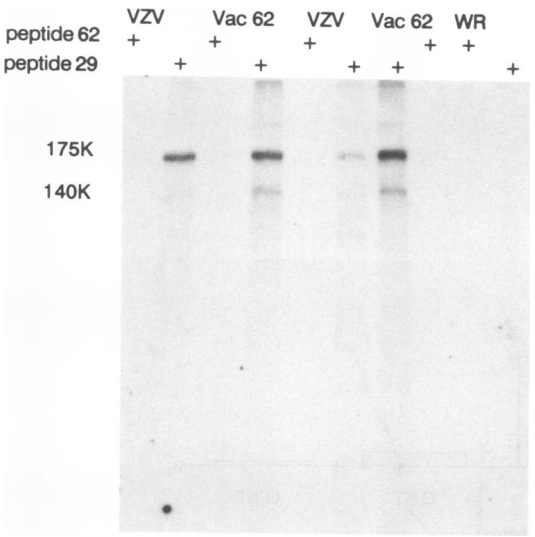

FIG. 1. Autoradiograph of $^{32}P_i$-labelled proteins immunoprecipitated from two preparations each of VZV- and Vac62-infected cell extracts and one preparation of wild-type vaccinia virus (WR)-infected cell extract. Samples were precipitated with anti-62 antipeptide antiserum. Reactions were performed in the presence (+) of either homologous peptide (peptide 62) or heterologous peptide (peptide 29) as described in the text. The approximate molecular weights (in thousands) of the labelled species are shown to the left of the figure.

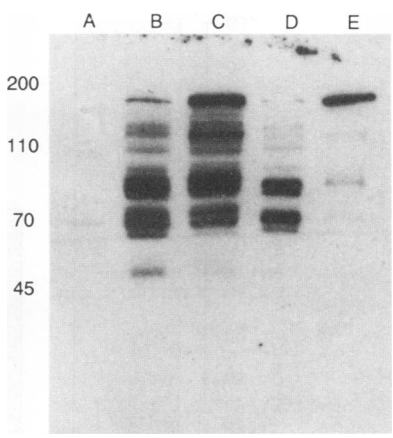

FIG. 2. Autoradiograph of a Western blot reacted with anti-IE62 serum, showing the polypeptides detected in samples prepared in the absence (lanes B and D) or presence (lanes C and E) of 0.1 mM TLCK and 0.1 mM TPCK. Samples are derived from uninfected cells (lane A) or cells infected with VZV (lanes B and C). Lanes D and E represent sucrose gradient-separated virus purified from the cytoplasm of infected cells. The approximate sizes (in kilodaltons) of marker proteins are shown to the left of the figure.

and subjected to SDS-PAGE and autoradiography. Western blot (immunoblot) analysis was performed as described previously (18, 19), using 1/100 dilutions of antisera and detection with ^{125}I-labelled protein A.

Protein kinase activity. Approximately 1 μg of purified virus was assayed for protein kinase activity essentially as described previously (24, 43), but it was optimized for VZV. The reaction took place in 0.1% (vol/vol) NP-40–1.0 mM dithiothreitol–50 mM Tris-HCl (pH 7.6)–30 mM $MgCl_2$–15 μM ATP–5 μCi of [γ-^{32}P]ATP (New England Nuclear Corp.) and was allowed to proceed for 30 min at 37°C. Reactions were halted by the addition of SDS-PAGE buffer and boiling, and labelled proteins were detected by autoradiography following SDS-PAGE.

RESULTS

Identification of IE62 polypeptides and phosphopeptides. To identify and investigate the proteins encoded by VZV ORF 62, we developed two reagents. First, a specific antiserum was prepared against a synthetic peptide of the predicted C terminus of ORF 62. Second, we made and used a recombinant vaccinia virus (Vac62) in which ORF 62 is expressed independently of other VZV proteins to allow the verification of the specific reactivity of the antipeptide serum. We have designated the identified protein product of this VZV gene as IE62.

A 175- to 180-kDa IE phosphoprotein in VZV-infected cells has been previously described (40). We have used the anti-IE62 peptide serum to identify the products of VZV ORF 62 and determine whether they are phosphorylated. Figure 1 shows that the anti-IE62 antiserum immunoprecipitated 175- to 180-kDa phosphorylated polypeptides. Extracts of cells infected with VZV or with vaccinia viruses were labelled with $^{32}P_i$ and immunoprecipitated with the antiserum in the presence of heterologous peptide (200 μg/ml) derived from ORF 29 or the (homologous) peptide used to induce the antiserum. The radiolabelled, precipitated polypeptides from two preparations each of Vac62-infected and VZV-infected cell extracts and one preparation of wild-type vaccinia virus-infected cell extracts are shown in Fig. 1. A 175-kDa [^{32}P]phosphate-labelled polypeptide was precipitated from both VZV-infected and Vac62-infected cells but not from WR-infected cells. This immunoprecipitation was specific for IE62, since the reactivity could be blocked by homologous peptide (peptide 62) but not by heterologous peptide (peptide 29). In addition, a minor 140-kDa phosphorylated species is seen; this is likely to be either a breakdown product or a possible differentially phosphorylated form of IE62. The major polypeptide encoded by ORF 62, however, appears as a phosphoprotein of 175 kDa and is likely to be the protein of that molecular mass in VZV-infected cells that was described earlier by us and by others (36, 40).

In Western blots of VZV-infected cells using the antipeptide serum, numerous species of smaller size were detected (Fig. 2). Their abundance varied from preparation to preparation of VZV-infected cell extracts, but they tended to be prominent in extracts of harvested cells showing greater than 80% CPE (unpublished observations). Metzler and Wilcox (29) showed that HSV ICP4 was highly sensitive to protease activities which could be inhibited by the protease inhibitors TLCK and TPCK. We have also found that IE62 is susceptible to TLCK-TPCK-sensitive proteolytic activities. Duplicate VZV-infected cell preparations were harvested in the presence or absence of 0.1 mM TLCK and 0.1 mM TPCK and analyzed by Western blotting with our antipeptide serum (Fig. 2). In the presence of these inhibitors, (Fig. 2, lanes C and E), substantially more of the 175-kDa form is seen than in their absence (lanes B and D), and many of the smaller species are reduced in quantity. The presence of the smaller species even in the preparations harvested with the inhibitors implies that some of this proteolytic degradation occurs in infected cells before harvesting. As a consequence of

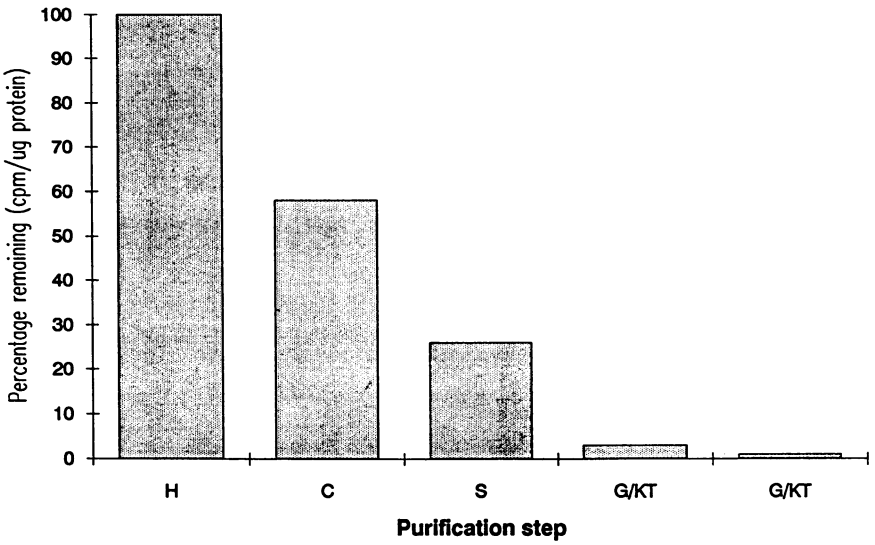

FIG. 3. Monitoring of VZV purification through specific loss of [^{35}S]methionine-labelled cellular proteins. Uninfected cells labelled as described in Materials and Methods were added to the VZV-infected cells prior to the purification of VZV. At each stage of the purification, radiolabelled protein content was estimated by 10% trichloroacetic acid precipitation and scintillation counting, and protein content was assayed as described in Materials and Methods. The percentage of counts remaining, expressed as counts per microgram of protein, is plotted for each fraction. H, whole cells; C, cytoplasmic extract; S, after sucrose gradient; G/KT, after glycerol-potassium sodium tartrate gradient centrifugation (performed twice).

these data, all experiments described here included these protease inhibitors.

Demonstration of IE62 in VZV virions. The identification of finite amounts of HSV ICP4 in the HSV virion (47) prompted us to examine whether IE62 was associated with the VZV virion. To obtain virions of sufficient purity, a double gradient procedure was employed, as outlined in Materials and Methods. The purification scheme was monitored by the loss of radiolabelled uninfected cell proteins during each step of the preparation. It was impossible to assess infectivity/protein ratios because the virion preparations were largely uninfectious after the preparation of the cytoplasmic extract. On the basis of the loss of radiolabelled cellular proteins from the virions, an overall purification of at least 135-fold was obtained (Fig. 3). Samples were also monitored for the exclusion of a nonstructural VZV protein, the ORF 29 product (see below), and by electron microscopy, which indicated that the purified virus preparation contained enveloped particles, with approximately 50 to 70% penetrated by stain (data not shown).

Virus structural polypeptides were then examined by SDS-PAGE and were stained with colloidal Coomassie brilliant blue (30). Figure 4 shows a stained gel of purified VZV virions, along with VZV-infected and uninfected cell proteins and two preparations of Vero cell-grown HSV-1 virions, one prepared by standard techniques of HSV purification from extracellular virus (33, 47) and a second prepared from the cytoplasm of HSV-1-infected cells by the methods described here. In the VZV particle profile, there are approximately 30 polypeptide species. The most abundant polypeptide in the virion preparations is the 155-kDa major capsid protein, which is the same size as the HSV major capsid protein found in nucleocapsids (37, 39, 41). Another very abundant polypeptide migrates at 175 kDa in the VZV virion profile. This polypeptide has no equivalent in abundance in either preparation of HSV, and it thus seemed to represent a structural component unique to VZV.

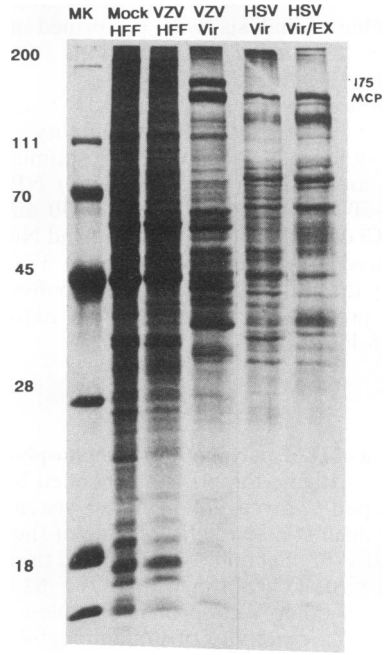

FIG. 4. Coomassie blue stain of electrophoretically separated proteins derived from VZV-infected (VZV HFF) or mock-infected (Mock HFF) cells or from VZV virions (VZV Vir). Proteins of HSV virions purified from the cytoplasm of HSV-infected cells (HSV Vir) or from extracellular virus (HSV Vir/EX) by the same technique were also electrophoresed. A set of marker proteins (MK), with approximate molecular masses in kilodaltons indicated to the left of the figure, is also shown. The proteins corresponding to the major capsid protein (MCP) and the 175-kDa species discussed in the text are indicated to the right of the figure. HFF, human foreskin fibroblasts.

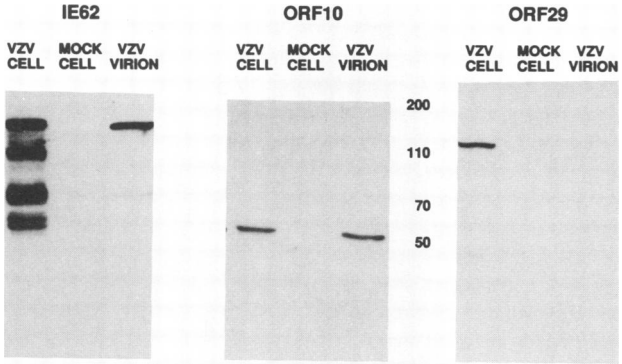

FIG. 5. Autoradiographs of triplicate Western blots of proteins derived from VZV-infected cells (VZV CELL), of mock-infected cells (MOCK CELL), or of VZV virions (VZV VIRION) probed with antiserum specific for ORF 62, ORF 10, or ORF 29. For VZV-infected cells and VZV virions, the amount of electrophoresed protein was adjusted to give approximately equal levels of reactivity with anti-ORF 10 antiserum. An amount of uninfected cells equal to that of VZV-infected cells was electrophoresed. Bound antibodies were detected with ^{125}I-labelled protein A. Molecular masses (in kilodaltons) are indicated.

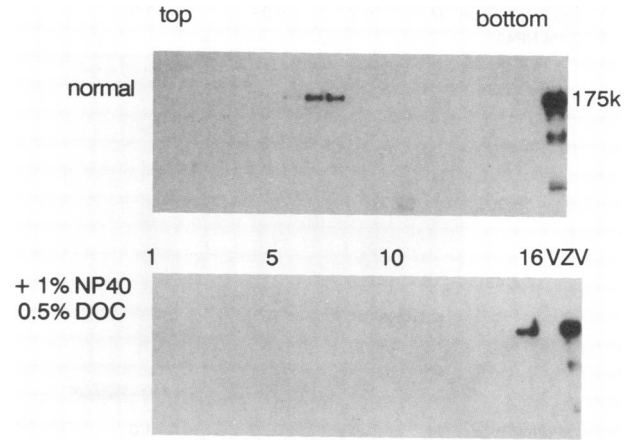

FIG. 6. Autoradiograph of Western blots showing migration of IE62-virion complexes on sucrose gradients. Purified virions were layered onto preformed 20 to 50% sucrose gradients made in the absence (top figure) or presence (bottom figure) of 1% NP-40 and 0.5% sodium deoxycholate. After centrifugation, gradients were fractionated into 16 fractions, with fractions 16 and 1 representing the bottom and the top of the gradient, respectively. Fractions were analyzed by Western blotting with antiserum specific to ORF 62. Sucrose was added to a sample of VZV-infected cell polypeptides (VZV) to mimic the final sucrose concentration in fraction 16. The 175-kDa protein (IE62) is indicated. DOC, sodium deoxycholate.

We then examined Western blots of VZV particle preparations for the presence of polypeptides derived from three VZV ORFs: 10, 29, and 62. ORF 29 is the VZV homolog of the HSV major DNA-binding protein ICP8, which is nonstructural. In contrast, anti-ORF 10 antiserum should react with a virion polypeptide, since its HSV homolog is α-TIF (Vmw65), a major HSV structural protein (1, 5). The anti-IE62 serum was used to determine whether any of the 175-kDa virion protein was derived from ORF 62, by analogy with results from HSV (47). Figure 5 shows these Western blots with extracts of VZV-infected cells, mock-infected cells, and VZV virions. Protein amounts in the lanes containing VZV-infected cells and VZV virions were adjusted to give approximately equal levels of reactivity with anti-ORF 10 antiserum, and an amount of mock-infected cell extract equal to that of the infected cell extract was electrophoresed. Anti-ORF 29 antiserum clearly reacts with a 130-kDa polypeptide present in VZV-infected cells only. In contrast, antiserum directed against ORF 10 recognizes a 47-kDa polypeptide in both infected cells and in virions, as expected. Anti-ORF 62 antiserum detected the 175-kDa polypeptide in substantial amounts both in infected cells and in virions. In addition, smaller species are also present in VZV-infected cell extracts. This result shows that IE62 is in the VZV virion and strongly implies that the abundant 175-kDa virion polypeptide is IE62. Interestingly, virions harvested in the absence of protease inhibitors occasionally show smaller species that are similar to those detected in infected cell extracts, indicating that the structural form of IE62 is also susceptible to TLCK-TPCK inhibited protease(s) (Fig. 2; compare lanes D and E).

Virion location of IE62. The two most likely reasons for the association of IE62 with purified VZV particles are that it is nonspecifically adhering to the outside of virions or it is actually incorporated into the virus particle. Previous studies indicated that there was no 175-kDa species present in purified VZV nucleocapsids (37, 39, 41, 49). Thus, if IE62 is part of the virion structure, it is likely to be in the envelope and/or the tegument. In order to address these issues, purified virions were subjected to a third sucrose gradient analysis in the absence or presence of 1% NP-40 and 0.5% sodium deoxycholate. If the polypeptide is in (or associated with) the virion envelope or in possible contaminating membrane vesicles, IE62 should be solubilized by these membrane-disrupting detergents and remain at the top of the gradient. Figure 6 shows the IE62 detected with the antiserum in fractions of such gradients. In the absence of detergent, IE62 is found in fractions 6, 7, 8, and 9, with the majority in fractions 7 and 8. These results correlate precisely with the presence of enveloped virions in the gradient, as detected by the silver staining of the virion proteins and by the location of the ORF 10 product (data not shown). In contrast, with virions centrifuged in gradients containing NP-40 and deoxycholate, IE62 is found at the bottom of the gradient. This fraction is the only fraction that contains the major capsid protein, the ORF 10 product, and other virion proteins (not shown). We interpret this to show that detergent treatment of VZV virions releases a nucleocapsid-tegument structure with a faster sedimentation rate than that of whole particles and that IE62 remains associated with this structure. This result implies that IE62 is not a membrane protein and does not adhere to the surface of purified virions but that it is closely associated with tegument-nucleocapsid structures.

To further explore the association of IE62 with the VZV tegument, we performed experiments to determine the ability of the envelope to protect IE62 from proteases. Virions were treated with trypsin in the presence or absence of 1% NP-40, and the polypeptides were analyzed. The results are shown in Fig. 7, in which a silver stain of the proteins (top panel) and a Western blot to show IE62 (bottom panel) are presented. Comparison of the silver-stained proteins in the treated fractions (Fig. 7, top panel) indicated that many of the virion proteins, including the 175-kDa virion polypeptide, are more susceptible to trypsin digestion in the presence of NP-40 than in its absence. When the polypeptides in this experiment were Western blotted and probed with

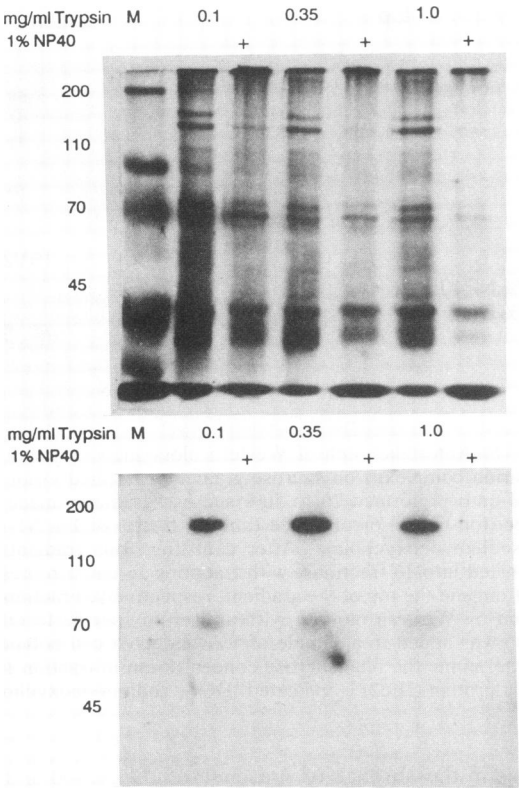

FIG. 7. Polyacrylamide gel and Western blot analyses showing the effect of NP-40 on the digestion of virion proteins by trypsin. Duplicate gels were prepared with marker proteins (M) or with proteins derived from virions treated with trypsin at the concentration indicated in the absence or presence (+) of 1% NP-40. After electrophoresis, one gel was silver stained (top panel) and the second was Western blotted with antiserum specific to ORF 62 (bottom panel). The approximate sizes (in kilodaltons) of the prestained marker proteins are shown to the left of each panel.

anti-IE62 serum (Fig. 7, bottom panel), it was clear that the 175-kDa IE62 polypeptide was much more resistant to trypsin treatment in the absence than in the presence of the detergent. Taken together, these data strongly suggest that IE62 is beneath the virion envelope and is probably a tegument protein.

Transfer of the [γ-^{32}P]phosphate of ATP to the 175-kDa virion protein. HSV virions have been shown to contain protein kinase activities, demonstrable by in vitro labelling of several polypeptides after incubation of detergent-treated virions with [γ-^{32}P]ATP (24). In light of the finding that VZV IE62 is phosphorylated, we examined VZV virions in the same way. VZV virions were permeabilized in an optimized buffer containing 0.1% NP-40 and [γ-^{32}P]ATP. The labelled species were then separated by SDS-PAGE and detected by autoradiography. Figure 8 shows that at least three major and several minor polypeptides are recipients of the radiolabelled [γ-^{32}P]phosphate of ATP. One of these corresponds to the major 175-kDa virion protein. In addition, two other major labelled species of 42 and 37 kDa are detected; at present, we are not certain of their identities. These labelled polypeptides reflect either an autophosphorylation activity of virion proteins or, more likely, their ability to be substrates of virion-associated protein kinase activity or activities. The profile and number of labelled VZV structural

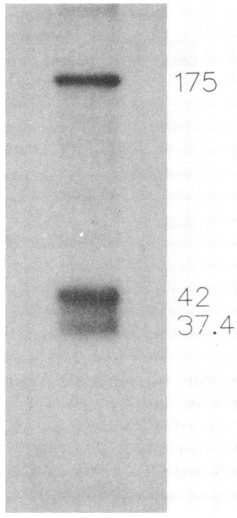

FIG. 8. Proteins labelled after the incubation of purified virions with [^{32}P]ATP in a buffer optimized for VZV virion protein kinase activity, as discussed in the text. After incubation, virions were subjected to SDS-PAGE and the radiolabelled proteins were detected by autoradiography. The approximate sizes (in kilodaltons) of the major species detected are indicated to the right of the figure.

phosphoproteins are quite different from those of HSV structural phosphoproteins (24).

DISCUSSION

By using specific antiserum, the major product of VZV ORF 62 (IE62) has been confirmed to be a 175-kDa phosphorylated species. This protein is identical, we believe, to the 175- to 180-kDa phosphorylated polypeptide described by us (36) and others (40). Recently, a monoclonal antibody which reacts with a similar-sized IE phosphoprotein from this open reading frame has been described (14).

Several properties of IE62 described here are very reminiscent of the properties of HSV ICP4. Both proteins are apparently much larger, as determined by SDS-PAGE, than are predicted from the ORFs (140 kDa for IE62 and 132 kDa for ICP4). The atypical amino acid composition of both proteins and their posttranslational modifications likely account for these differences (29, 45). Both proteins are clearly phosphoproteins, although we have not observed multiple phosphorylated species of IE62, as have been detected for ICP4 in HSV-infected cells (45). It may be that IE62 is phosphorylated to a different extent than ICP4. Interestingly, we also show that IE62 is phosphorylated in Vac62-infected cells, suggesting that at least part of its phosphorylation is independent of other VZV proteins and can be catalyzed by cellular protein kinase(s) or by itself. A further similarity between IE62 and HSV ICP4 is susceptibility to protease activities, and we have thus found it necessary to use protease inhibitors in our studies on IE62. However, we have not obtained VZV-infected cell extracts devoid of these smaller species and thus cannot eliminate the possibility that some are specifically processed or modified forms of the larger protein.

The most surprising and interesting finding with IE62 is the detection of substantial amounts of the protein associated with purified virions, and its abundance in the virion is clearly a major difference between VZV and HSV. We believe our virions are of sufficient purity to exclude the

possibility that the major 175-kDa protein species detected is a nonspecific contaminant, since an expected nonstructural protein (from ORF 29) is not detected in the virions. Furthermore, the continued association of IE62 with the nucleocapsid in gradients, even after the virion envelope has been solubilized, is taken as further proof that it is not a contaminant. We also consider it unlikely that the 175-kDa protein is encoded by a VZV gene other than ORF 62. First, there are very few VZV genes of sufficient coding capacity for a polypeptide of 175 kDa, even with extensive modification (9). Secondly, the levels of IE62 detected with specific antisera are similar to the levels of ORF 10, which is the homolog of a major HSV structural protein. However, to confirm that the virion 175-kDa polypeptide is only IE62, tryptic digest experiments comparing the virion 175-kDa form and IE62 (as obtained by immunoprecipitation) are currently in progress.

The question remains as to the reason for the apparent abundance of IE62 in VZV virions. One possibility is that virion IE62 functions during the IE events of infection. In HSV, IE gene transcription is activated by the virion protein α-TIF, acting in conjunction with cellular factors (1, 5, 28). Although VZV contains an equivalent to α-TIF (Fig. 5), this protein lacks the acidic carboxyl tail considered crucial in the recognition of the cellular factor OCT-1 and the subsequent interaction of this complex with the octamer consensus sequence in IE promoters (7, 27, 38, 44). In light of this lack of an IE transactivator function for ORF 10, VZV probably uses alternative mechanisms to initiate infection efficiently. One way of achieving this would be to incorporate a significant quantity of IE protein(s) into the virion, thereby bypassing the need for an α-TIF activity. Furthermore, virion IE62 may act to stimulate transcription from its own promoter or that of other VZV IE genes during the initial events of the infectious cycle. We are currently investigating the activity of IE62 on the promoters of other putative VZV IE genes to explore this possibility.

Virions contain a phosphoprotein of 175 kDa (37), and it is clear from the results presented here that the virion 175-kDa protein (likely IE62) phosphorylation state can be altered in vitro. This occurs either through autophosphorylation or through the activities of protein kinases. We do not know the role of protein kinase activity associated with the VZV virion or its relationship to the functions of IE62, if any, but genes for two protein kinase activities in the VZV genome and in other herpesviruses have been identified (26, 42). In addition, both herpesvirus-specific and cellular protein kinases in other herpesvirus particles have been described (43). Given the potentially important role of protein kinases in the regulation of virus (and cellular) gene functions, it may not be coincidental that VZV IE62 is a substrate for phosphorylation in the virion. It is not unreasonable to propose that virion protein kinase(s) may function to regulate the activity of IE62 in the virion and in the early stages of the infectious cycle.

Finally, as we have observed elsewhere (2), VZV IE62 is a major VZV antigen in the human immune response, recognized by both major histocompatibility complex class I- and class II-restricted human T lymphocytes. Both helper and cytotoxic T-cell responses to this VZV tegument protein can be detected in human subjects for more than 20 years following primary VZV infection. Perhaps part of the explanation for the broad nature of this cellular immune response to IE62 is that it is a major component in VZV particles.

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