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Postentry Events Are Responsible for Restriction of Productive Varicella-Zoster Virus Infection in Chinese Hamster Ovary Cells

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Productive infection of varicella-zoster virus (VZV) in vitro is restricted almost exclusively to cells derived from humans and other primates. We demonstrate that the restriction of productive VZV infection in CHO-K1 cells occurs downstream of virus entry. Entry of VZV into CHO-K1 cells was characterized by utilizing an ICP4/β-galactosidase reporter gene that has been used previously to study herpes simplex virus type 1 entry. Entry of VZV into CHO-K1 cells involved cell surface interactions with heparan sulfate glycosaminoglycans and a cation-independent mannose-6-phosphate receptor. Lysosomotropic agents inhibited the entry of VZV into CHO-K1 cells, consistent with a low-pH-dependent endocytic mechanism of entry. Infection of CHO-K1 cells by VZV resulted in the production of both immediate early and late gene products, indicating that a block to progeny virus production occurs after the initiation of virus gene expression.

Varicella-zoster virus (VZV) is a human alphaherpesvirus that causes chicken pox (varicella) upon primary infection and shingles (zoster) upon reactivation of the virus from latently infected sensory neurons. In contrast to the broad host range of most alphaherpesviruses in cultured cells, productive VZV infection in vitro is restricted almost exclusively to cells derived from humans and other primates (64). Reported exceptions are guinea pig embryonic fibroblasts (62, 64), rabbit kidney cells (64, 68), and cotton rat fibroblasts (54). For many viruses, restriction in cultured cells is determined by specific cellular receptors that must be engaged for virus binding and entry. After successfully entering a cell, viruses can encounter other blocks to productive infection (20, 36). In studies using rats, cotton rats, or mice as in vivo models of VZV latency, viral DNA (1, 6, 27, 51–54, 69), viral transcripts (6, 27, 51, 54, 69), and immediate early viral proteins (18, 27, 51) were detected in infected cells, primarily neurons, indicating that VZV is capable of entering rodent cells and initiating early events in the infectious cycle. Cultured rat neurons and the mouse neuroblastoma cell line neuro-2A also permitted VZV entry, but productive infection was blocked in both cases (4, 37). Thus, the restriction of productive VZV infection in cultured nonprimate cells may be governed primarily by postentry events.

Much of our understanding of alphaherpesvirus entry comes from studies of herpes simplex virus type 1 (HSV-1) (60, 61). HSV-1 envelope glycoproteins gB and/or gC mediate binding of virus to cell surface glycosaminoglycans, predominantly heparan sulfate (59, 63). Following virus binding, gD engages one of several entry receptors (19, 40, 58, 60). Fusion of viral and cellular membranes then ensues, either directly at the cell surface (15, 16) or following endocytosis (38, 42, 43) by a mechanism that requires the participation of gB, gD, a heterodimer of gH and gL, and a gD entry receptor (5, 44, 47, 65). The emerging picture of entry from HSV-1 studies is one that involves multiple entry receptors and multiple entry pathways. This versatility likely contributes to the wide host range observed for HSV-1 in vitro and the success of this virus as a pathogen.

Much less is known about the binding and entry of VZV, largely due to the difficulty in obtaining high titers of cell-free (CF) virus. An entry process similar to that described for HSV-1 has been proposed for VZV, commencing with binding of virions to heparan sulfate via gB (25, 70), followed by interaction of viral glycoproteins with an entry receptor (7, 70). The interaction of mannose-6-phosphate (M6P) groups found on at least four VZV glycoproteins (gB, gE, gH, and gI) with the cation-independent mannose-6-phosphate receptor (CI-MPR) is believed to facilitate virus entry (7, 17, 70). While the exact consortium of VZV proteins needed for virus-cell fusion has not been defined, results from cell-cell fusion assays (9, 11, 12, 46), blocking assays with antiglycoprotein antibodies (9, 49), and competition assays with purified glycoproteins (57) implicate the involvement of gE, gB, and gH. It should be noted that the VZV genome does not encode a gD homolog, and thus VZV must utilize different entry receptors and/or utilize a different viral glycoprotein(s) to engage the entry receptors used by other alphaherpesviruses.

The discovery of HSV-1 entry receptors was facilitated by the prior identification of cell lines, such as Chinese hamster ovary cells (CHO-K1), that are particularly resistant to HSV-1 entry (40, 56). We sought to develop an analogous system to facilitate a methodical study of CF VZV entry by utilizing cell lines resistant to virus entry. To identify such cell lines, we focused our studies on VZV infection of cultured nonprimate cells. In this report, we present evidence that CHO-K1 cells permit VZV entry, characterize the pathway of VZV entry into these cells, and demonstrate that VZV restriction in CHO-K1 cells occurs after the initiation of virus gene expression.

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MATERIALS AND METHODS

Cells and viruses. MeWo cells, provided by R. Cohrs, University of Colorado, were propagated in Dulbecco modified Eagle medium (DMEM; Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT). NIH 3T3 cells were propagated in DMEM supplemented with 10% bovine serum (HyClone, Logan, UT). CHO-K1 cells and stable CHO-K1 transformants were propagated in F-12 medium (Life Technologies, Carlsbad, CA) supplemented with 10% FBS. Stable CHO-K1 transformants that produce nectin-1 (R3A) were propagated in medium supplemented with 250 μg of Geneticin (Life Technologies, Carlsbad, CA) per ml. Stable CHO-K1 transformants that express *Escherichia coli lacZ* from the HSV-1 ICP4 promoter (CHO-IEβ8) were propagated in medium supplemented with 150 μg of puromycin (A. G. Scientific, San Diego, CA) per ml. Stable CHO-IEβ8 transformants that produce herpesvirus entry mediator (HVEM), nectin-2, or nectin-1 (M1A, M2A, and M3A, respectively) were propagated in medium supplemented with 150 μg of puromycin and 250 μg of Geneticin per ml. CHO-K1, R3A, CHO-IEβ8, M1A, M2A, and M3A cells were all provided by G. Cohen, R. Eisenberg, and C. Krummenacher, University of Pennsylvania. All cells were maintained at 37°C in a 5% CO_2 environment.

The pathogenic POka strain of VZV was provided by Ann Arvin, Stanford University. ROka-lacZ, a recombinant VZV derived from POka which contains the *E. coli lacZ* gene under control of the simian virus 40 early promoter (8), was provided by J. Cohen, NIAID, NIH. All VZV strains were propagated on MeWo cells. CF ROka-lacZ was prepared from infected MeWo cells showing 80% cytopathic effect (CPE) by lysing cells harvested in SPGA buffer (pH 8.0; 218 mM sucrose, 3.8 mM KH_2PO_4, 4.9 mM sodium glutamate, 1% [wt/vol] bovine serum albumin [BSA], 10% FBS) with glass beads (1-mm diameter) and centrifuging the lysate at 2,000 × *g* for 5 min. The resulting supernatant was used directly for infection of cells. Frozen stocks of CF POka (7.5×10^3 PFU/ml after thawing) and mock extract were provided by D. Krah, Merck & Co., Inc. Cell-associated (CA) POka was prepared by harvesting infected MeWo cells showing 80% CPE in trypsin–0.25% EDTA (2.5 ml per 150-mm dish of infected cells) and resuspending harvested cells in an equal volume of FBS containing 10% dimethyl sulfoxide. CF and CA stocks of POka and mock extract were all stored at −70°C and thawed immediately prior to use. Titers of CF and CA stocks and fresh CF preparations were determined by plaque assay on MeWo cells.

β-Gal detection assays. To detect beta-galactosidase (β-Gal) production in individual cells in a monolayer after infection with ROka-lacZ, cells were washed twice with phosphate-buffered saline, pH 7.4 (PBS), and then fixed in freshly prepared 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were washed twice in PBS, a substrate buffer (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40 in 100 mM PBS, pH 7.35) containing 1 mg/ml of Bluo-Gal (Sigma, St. Louis, MO) was applied, and cells were incubated at 37°C until blue colonies were visible by bright-field illumination. To detect β-Gal production in infected ICP4/β-Gal reporter cells, individual wells of cells in a 48-well dish were washed twice with 250 μl of PBS, and 30 μl of lysis buffer (Applied Biosystems, Bedford, MA) was applied. Lysates were transferred to microcentrifuge tubes and assayed immediately or stored at −70°C. Two to 20 μl of lysate was assayed, using a Galactostar chemiluminescent β-Gal detection kit (Applied Biosystems, Bedford, MA) according to the manufacturer's instructions.

Infection of ICP4/β-Gal reporter cells. ICP4/β-Gal reporter cells were placed into individual wells of a 48-well dish (5×10^3 cells per well) and infected 18 h after being plated with CF POka at a multiplicity of infection (MOI) of 0.05. Harvesting of infected cells for β-Gal assays was carried out at 18 to 21 h postinfection (hpi).

Removal of virus from CF inoculum. Virus was removed from the CF POka inoculum by centrifugation at 100,000 × *g* for 20 min at 4°C in a TLA 120.2 fixed-angle rotor. The supernatant was retained and used directly for infection and titration by plaque assay.

pH inactivation of CF virus. To test whether acidification of the CF POka inoculum would inactivate the virus, CF POka was combined with a 1/10 volume of 800 mM sodium citrate, pH 2.2, to reduce the pH of the virus suspension to 3.5 and was then incubated for 2 min at 37°C. The virus suspension was neutralized by adding 40 volumes of DMEM supplemented with 2% FBS and titrated by plaque assay on MeWo cells. To pH inactivate bound virus prior to infecting CHO-IEβ8 cells, chilled cells were inoculated with cold CF virus and incubated at 4°C for 1 h to allow the virus to bind to but not penetrate cells. Unbound inoculum was removed and replaced with warm pH-adjusted medium or sodium citrate buffer (40 mM sodium citrate, 10 mM KCl, 0.8% NaCl, pH 3.68). After 2 min at 37°C, the pH-adjusted medium or buffer was removed and replaced with regular medium.

TABLE 1. VZV infectivity in rodent cell lines in comparison to that in MeWo cells

Cell line	Source	No. of foci[a]
MeWo	Human melanoma	170, 165
NIH 3T3	Mouse fibroblasts	171, 178
CHO-K1	Chinese hamster ovary	132, 109
R3A	Derived from CHO-K1 cells; produces the HSV-1 receptor nectin-1	93, 111

[a] Foci comprised of blue-stained cells were counted 24 h after inoculation with 150 PFU of CF ROka-lacZ. Numbers obtained from duplicate inoculations in a single experiment are shown. The titer of the CF ROka-lacZ inoculum was determined by plaque assay on MeWo cells.

Indirect immunofluorescence microscopy. Cells growing on glass coverslips pretreated with 0.01% poly-L-lysine (Sigma, St. Louis, MO) were infected by applying 150 μl of CF VZV to coverslips and incubating them for 60 min at 37°C. Cells were fixed by incubation with freshly prepared 4% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were washed three times with PBS containing 0.5% BSA (PBS-BSA) and permeabilized for 3 min at room temperature with PBS-BSA containing 0.1% Triton X-100. Cells were then washed three times with PBS-BSA, and 150 μl of polyclonal antiserum or monoclonal antibodies diluted in PBS-BSA was applied for 45 min at 37°C. Polyclonal antisera against IE62 and gE (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted 1:200, and monoclonal antibodies against IE62 (provided by B. Forghani, Department of Health Services, Richmond, CA) were diluted 1:500 immediately prior to use. Cells were washed three times with PBS-BSA, and 150 μl of conjugated secondary antibody (Molecular Probes, Eugene, OR) diluted appropriately in PBS-BSA was applied for 30 min at 37°C. Cells were then washed three times with PBS-BSA. To visualize nuclei, cells were incubated with Hoechst 33342 (Sigma, St. Louis, MO) diluted to 0.5 μg/ml in PBS-BSA for 7 min at room temperature. Cells were washed three times with PBS and mounted in PBS containing 50% (vol/vol) glycerol onto glass slides. Images were captured using a Nikon TE200 inverted epifluorescence microscope equipped with a cooled charge-coupled device camera. Composites of representative images were prepared using Adobe Photoshop software.

RESULTS

Cultured rodent cells permit early steps in the VZV life cycle. The infectivity of the *lacZ*-expressing reporter virus ROka-lacZ (8) in VZV-permissive MeWo cells was compared to its infectivity in the rodent cell lines NIH 3T3, CHO-K1, and R3A, a CHO-K1 derivative expressing the gD receptor nectin-1. Cell monolayers grown in six-well dishes were inoculated with freshly prepared CF ROka-lacZ and incubated with Bluo-Gal substrate at 24 hpi to detect β-Gal production. Foci comprised of blue-stained cells were observed for all cell lines tested, and the numbers of foci formed on rodent cell lines were comparable to the number formed on MeWo cells (Table 1). Although the majority of foci were comprised of one or two cells, some foci containing more than two cells were observed for all rodent cell lines tested (Fig. 1B to D). In inoculated CHO-K1 cells, >70% of foci were comprised of one or two blue-stained cells at 24 hpi; this value did not change at 48 or 72 hpi. Multiple-cell foci may arise as a result of division of the originally infected cells. In addition, the inoculum likely contains aggregates of virus that may infect a cluster of cells as opposed to a single cell. The VZV immediate early protein 62 (IE62) was also observed in NIH 3T3 cells that produced β-Gal (Fig. 1E to G). These observations suggested that cultured rodent cells permitted early steps in the VZV infectious cycle up to and including immediate early gene expression. Since similar numbers of foci were observed on CHO-K1 and

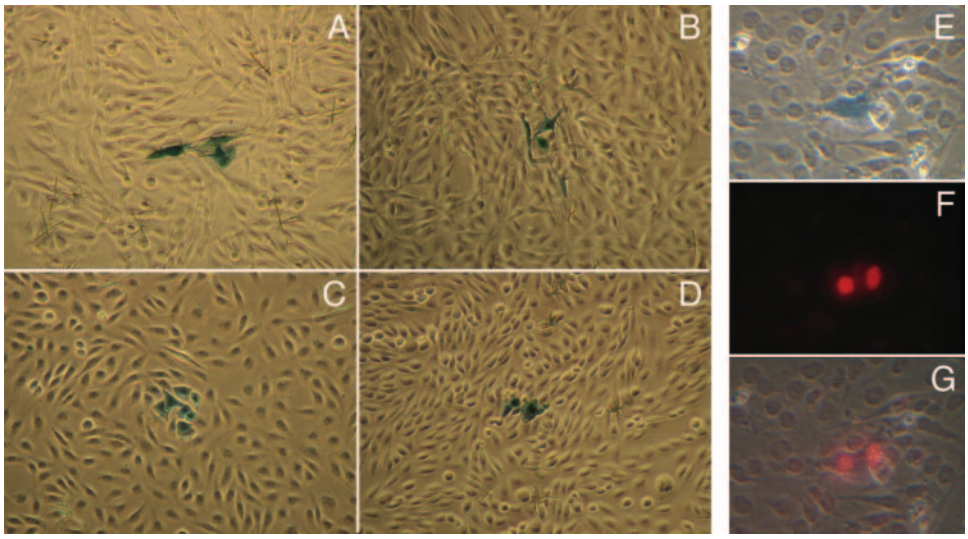

FIG. 1. Blue foci form on cultured rodent cells following inoculation with *lacZ*-expressing VZV. (A to D) Monolayers of fully permissive MeWo cells (A) and the rodent cell lines NIH 3T3 (B), CHO-K1 (C), and R3A (D) were inoculated with CF ROka-lacZ and stained at 24 hpi with Bluo-Gal. (E to G) Monolayers of NIH 3T3 cells grown in six-well dishes were inoculated with CF ROka-lacZ, stained at 72 hpi with Bluo-Gal (E), and subsequently stained with goat polyclonal antisera against VZV IE62 and an Alexa fluor 555-conjugated rabbit anti-goat secondary antibody (F). Merged signals are shown in panel G.

R3A cell monolayers, these initial studies also indicated that the expression of the alphaherpesvirus receptor nectin-1 did not appreciably enhance infection by CF VZV.

We next examined CF VZV infection of CHO-K1-derived reporter cells, CHO-IEβ8 cells, in which the HSV-1 immediate early ICP4 promoter drives the expression of an integrated *lacZ* reporter gene. Cohen and colleagues have demonstrated that the ICP4 promoter is activated by the VZV open reading frame (ORF) 10 gene product (41). We anticipated that increased β-Gal production would result if CF VZV could successfully enter CHO-IEβ8 cells and initiate immediate early gene expression. For these experiments, CF POka, a pathogenic VZV strain, was used, and cell extracts were assayed for β-Gal production using a chemiluminescence assay. Cells were inoculated with CF POka at an MOI of 0.05, and β-Gal production was assayed in cell extracts prepared at various times postinfection. A steady increase in β-Gal production was observed in cells inoculated with CF POka, resulting in a >10-fold increase from 0 to 18 hpi (Fig. 2). Comparable increases in β-Gal production were not observed in CHO-IEβ8 cells inoculated with extracts prepared from uninfected cells or with stocks of POka-infected MeWo cells (CA virus) at the same MOI (Fig. 2). As expected, inoculation of CHO-IEβ8 cells with HSV-1 at an MOI of 0.05 resulted in no detectable increase in β-Gal production over the background at 18 hpi (data not shown). Thus, the increase in β-Gal production correlated only with infection by CF VZV and not with cell debris, VZV-infected cells, or infection by HSV-1. In comparison to CF VZV, 20 times more CA VZV was required to evoke a 10-fold increase in β-Gal production by 18 hpi (data not shown). This observation may reflect differences in the mechanisms of infection by CA VZV and CF VZV. Infection of cells by CA VZV involves cell-cell fusion with uninfected cells (7). If infected MeWo cells were unable to fuse efficiently with CHO-K1 cells, this might explain why CA VZV was inferior to CF VZV in evoking β-Gal production in CHO-IEβ8 cells.

To further relate the increase in β-Gal production to CF VZV infection, control experiments aimed at removing virus from the CF inoculum or inactivating virus within the CF inoculum were performed. First, virus and residual cellular debris were removed from the CF preparation by ultracentrifugation, and the resulting supernatant, which contained no detectable virus by plaque assay, was used for inoculation.

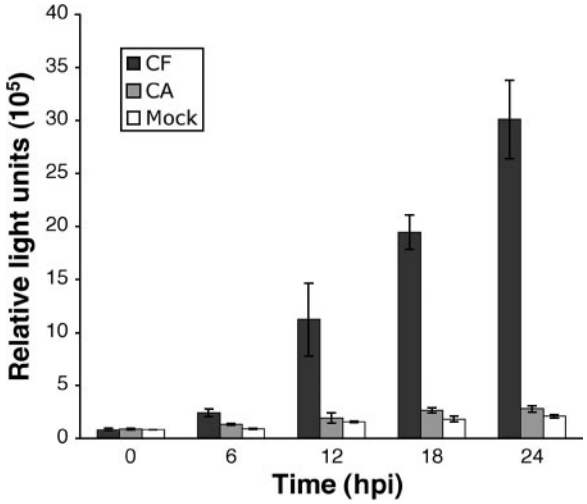

FIG. 2. Inoculation of CHO-IEβ8 cells with CF VZV results in increased β-Gal production. CHO-IEβ8 cells were inoculated with CF or CA POka at an MOI of 0.05. Mock-treated cells were inoculated with a lysate prepared from uninfected cells. Cell extracts were prepared at the indicated times postinfection and assayed for β-Gal production. The experiment was performed in triplicate.

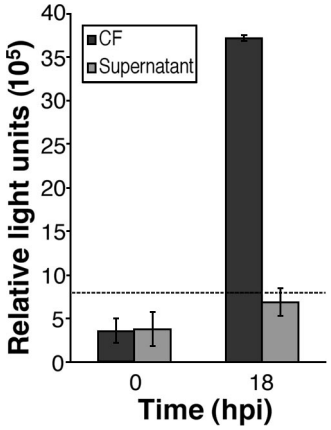

FIG. 3. Removal of virus from CF inoculum results in decreased β-Gal production. Virus was removed from the CF POka inoculum by centrifugation, and the supernatant was used to inoculate CHO-IEβ8 cells alongside the noncentrifuged CF inoculum. Cell extracts were prepared at 0 and 18 hpi and assayed for β-Gal production. The experiment was performed in triplicate. The dashed line indicates the increase in β-Gal production expected from cell growth alone.

Inoculation of CHO-IEβ8 cells with this supernatant reduced β-Gal production to background levels (Fig. 3). Second, virus within the CF inoculum was inactivated by a brief exposure to acidic pH. Brief exposure to acidic pH has been demonstrated to inactivate other alphaherpesviruses (24) and CA VZV (21), but this method of virus inactivation has not been described for CF VZV (48). To demonstrate that a brief exposure to acidic pH also inactivates CF VZV, CF POka was mixed with an acidic or neutral sodium citrate solution, incubated at 37°C for 2 min, neutralized by 40-fold dilution in medium, and titrated by plaque assay on MeWo cells. No plaques resulted from virus treated with acidic sodium citrate, while virus treated with neutral sodium citrate resulted in plaque numbers comparable to those for untreated virus (Fig. 4A). Treatment of CF POka prebound to CHO-IEβ8 cells at 4°C with medium buffered to pH 4.0 or less (Fig. 4B) or with sodium citrate buffer, pH 3.68 (Fig. 4C), resulted in a substantial decrease in β-Gal production. If acid treatment was delayed for 2 h, no reduction of β-Gal production was observed in comparison to untreated controls (data not shown). Intermediate levels of β-Gal production were observed if acid treatment was delayed for 30 min (Fig. 4C), suggesting that the virus became refractory to pH inactivation after entering cells. Acid treatment of bound virus did not reduce β-Gal production to background levels, which may reflect differences in pH sensitivity between free versus bound VZV virions. Because the removal of virus and inactivation of virus both resulted in decreased β-Gal production, we concluded that the increase in β-Gal production in VZV-inoculated CHO-IEβ8 cells arises from VZV-mediated activation of the resident ICP4 promoter following infection by free virions.

The ICP4/β-Gal reporter system enables the study of VZV entry. The data described above establish that CF VZV is able to enter rodent cells. We took advantage of the ICP4/β-Gal reporter system in order to characterize features of CF VZV entry into CHO-K1 cells. Heparan sulfate and CI-MPR have been implicated as important cell surface components for CF VZV binding and entry, respectively (7, 17, 25, 70). The capacity of heparin and free M6P to block infection of CHO-K1 cells was tested. CHO-IEβ8 cells were preincubated with heparin or M6P and then infected with CF POka in the continuous presence of heparin or M6P, and cell extracts prepared at 18 hpi were assayed for β-Gal production. Glucose-1-phosphate (G1P) was included as a specificity control in experiments with M6P. The presence of either heparin or M6P inhibited β-Gal production (Fig. 5A and B), indicating that infection was blocked by both heparin and M6P. Increasing concentrations of either heparin or M6P correlated with decreasing β-Gal

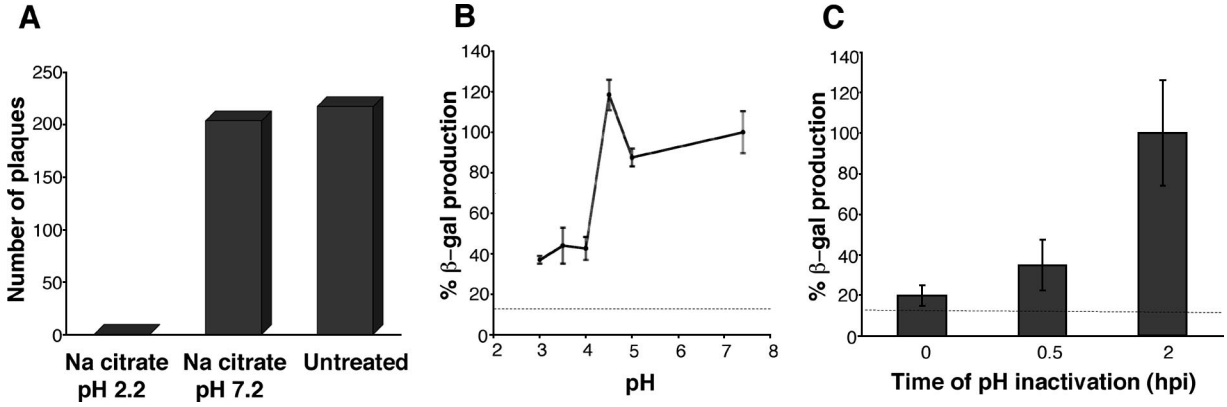

FIG. 4. Inactivation of CF VZV by brief treatment with acidic pH results in decreased β-Gal production. (A) CF POka was treated with 800 mM sodium citrate, pH 2.2 or 7.2, neutralized by dilution in medium, and then assayed for plaque production on MeWo cells. The average number of plaques for four separate wells was determined and scored relative to that for untreated, similarly diluted CF POka. (B) CF POka was allowed to bind to chilled CHO-IEβ8 cells at 4°C, treated briefly with warm medium at various pHs, and then incubated in regular medium. Cell extracts were prepared at 18 hpi and assayed for β-Gal production. The β-Gal production values were scored relative to those for control samples treated with unadjusted medium (set to 100%). The experiment was performed in triplicate. (C) CF POka was allowed to bind to chilled CHO-IEβ8 cells at 4°C and treated briefly with warm sodium citrate, pH 3.68, following a 0-, 0.5-, or 2-h incubation in regular medium at 37°C. Cell extracts were prepared at 18 hpi and assayed for β-Gal production. The β-Gal production values were scored relative to those for samples that were subjected to pH inactivation at 2 hpi (set to 100%). The experiment was performed in quadruplicate. Dashed lines in panels B and C indicate the increase in β-Gal production expected from cell growth alone.

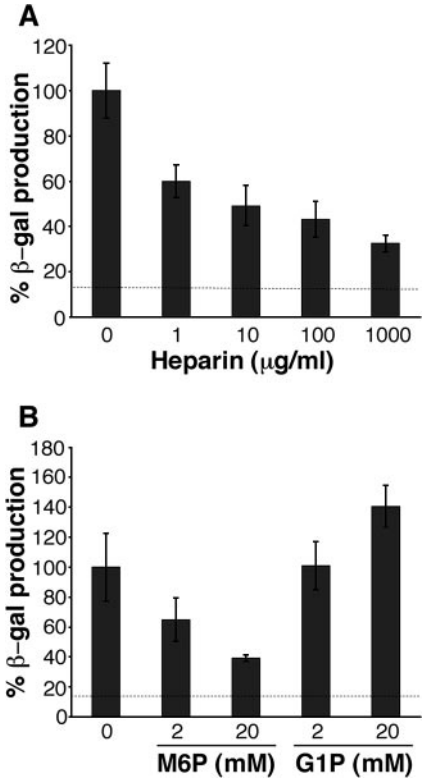

FIG. 5. Agents known to block VZV-cell surface interactions affect entry of VZV into CHO-K1 cells. CHO-IEβ8 cells were pretreated for 30 min with the indicated concentrations of heparin (A), M6P (B), and G1P (B) and then inoculated with CF POka. Infections were carried out in the continual presence of the agent under study. G1P was included as a specificity control for M6P blocking. Cell extracts were prepared at 18 hpi and assayed for β-Gal production. The β-Gal production values were scored relative to those for untreated control samples (set to 100%). Experiments for both panels were performed in triplicate. Dashed lines indicate the increase in β-Gal production expected from cell growth alone.

production, whereas increasing concentrations of G1P did not (Fig. 5B). A 20 mM concentration of M6P blocked infection of CHO-K1 cells with an efficiency comparable to that demonstrated by Gershon and colleagues with human embryonic lung fibroblasts (HELF cells) (70). This was not the case for blocking of infection by heparin. In experiments with HELF cells, the continuous presence of 10 μg/ml heparin was sufficient to block 90% of infection (70). In our experiments with CHO-K1 cells, the continuous presence of 1,000 μg/ml of heparin failed to reduce β-Gal production to background levels (Fig. 5B). This may indicate the presence of other cell surface molecules on CHO-K1 cells that are able to bind CF VZV which do not play a significant role in the infection of HELF cells. There is precedent for alphaherpesvirus binding to chondroitin sulfate (3) as well as evidence of an alphaherpesvirus entry pathway that is independent of proteoglycans (2, 26).

The involvement of gD receptors was tested by measuring the levels of β-Gal production in CHO-IEβ8 derivatives that produce the alphaherpesvirus gD receptors HVEM, nectin-2, and nectin-1 (M1A, M2A, and M3A, respectively) after infection with CF POka. These cell lines are known to carry sufficient numbers of entry receptor at the cell surface to allow alphaherpesvirus entry to be monitored by β-Gal production (31). Cells were infected at an MOI of 0.05, and cell extracts prepared at 0 and 18 hpi were assayed for β-Gal production. The presence of gD receptors resulted in at most a moderate (less than twofold) increase in β-Gal production (Table 2). These results indicate that gD receptors do not contribute appreciably to the entry of VZV into CHO-K1 and are consistent with our observation that the number of foci of infection found on ROka-lacZ-infected CHO-K1 cells was similar to the number found on infected CHO-K1 cells that produce nectin-1 (Table 1).

HSV-1 is known to utilize a low-pH-dependent endocytic pathway to enter CHO-K1 cells (43). The effect of lysosomotropic agents, which inhibit the acidification of endosomes, on CF VZV infection of CHO-K1 cells was therefore tested. The background level of β-Gal production in CHO-IEβ8 cells served as an indicator of cell viability as well as an indicator of nonspecific effects on β-Gal production caused by lysosomotropic agents. Background β-Gal production levels were not affected by the presence of either 50 mM ammonium chloride or 100 nM bafilomycin for 3 h (data not shown). To test the effects of these inhibitors on virus entry, CHO-IEβ8 cells were preincubated with medium containing 50 mM ammonium chloride or 100 nM bafilomycin for 30 min. Inhibitor-containing medium was prepared from fresh stock solutions of inhibitor and pH adjusted immediately prior to use. After preincubation, cells were chilled, and the inhibitor-containing medium was replaced with cold CF POka. After virus binding at 4°C for 1 h, warm inhibitor-containing medium was added, and cells were incubated for 2.5 h at 37°C. Cells were treated with citrate buffer, pH 3.68, for 2 min at 37°C to inactivate any virus that had not penetrated. Incubation was continued in medium without inhibitor, and cell extracts prepared at 21 hpi were assayed for β-Gal production. The presence of ammonium chloride and bafilomycin inhibited β-Gal production by 60% and 35%, respectively, relative to untreated, similarly processed controls (Fig. 6A), indicating that endosome acidification contributed to the entry of CF VZV into CHO-K1 cells. If the addition of ammonium chloride was delayed for 1 hour after the initiation of infection, less inhibition of β-Gal production resulted (Fig. 6B), suggesting that ammonium chloride exerts its effect at an early time following virus penetration.

A block to productive VZV infection in CHO-K1 cells occurs late in the viral life cycle. The data described thus far indicate that early events in the VZV infectious cycle, up to and in-

TABLE 2. VZV infectivity in CHO-IEβ8 cells in comparison to that in CHO-IEβ8 derivatives expressing alphaherpesvirus gD receptors

Cell line	Alphaherpesvirus gD receptor present	Fold increase in β-Gal production[a]
CHO-IEβ8	None	19.7 ± 7.7, 26.6 ± 6.0
M1A	HVEM	15.8 ± 4.0, 48.3 ± 9.1
M2A	Nectin-2	31.9 ± 3.9, 46.0 ± 1.2*
M3A	Nectin-1	26.8 ± 2.6, 44.1 ± 7.1

[a] The values reported are the increases in β-Gal production derived by dividing the β-Gal production values measured at 18 hpi by the β-Gal production values measured at 0 hpi. Values from two independent experiments performed in triplicate or duplicate (*) are shown. Raw measurements of β-Gal production were used to generate the reported values. Data are means ± standard deviations.

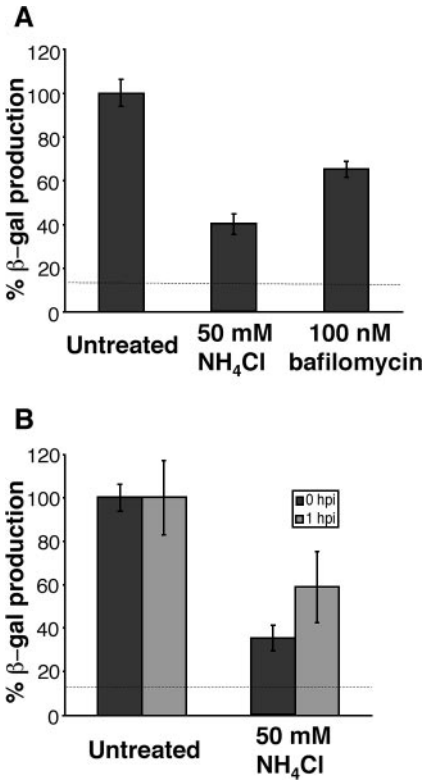

FIG. 6. Inhibitors of endosome acidification affect the entry of VZV into CHO-IEβ8 cells. CHO-IEβ8 cells were pretreated with an endosome acidification inhibitor at the indicated concentrations. CF POka was allowed to bind to chilled CHO-IEβ8 cells at 4°C, and warm inhibitor-containing medium was added following a 0- or 1-h incubation in regular medium at 37°C. Inhibitor-containing medium was removed at 2.5 hpi or 3.5 hpi (panel B, light gray bars), noninternalized virus was inactivated, and incubation was continued in regular medium. Cell extracts were prepared at 21 hpi and assayed for β-Gal production. The β-Gal production values were scored relative to those for untreated, similarly processed control samples (set to 100%). The results shown in both panels are for two independent experiments, each of which was performed in triplicate. Dashed lines indicate the increase in β-Gal production expected from cell growth alone.

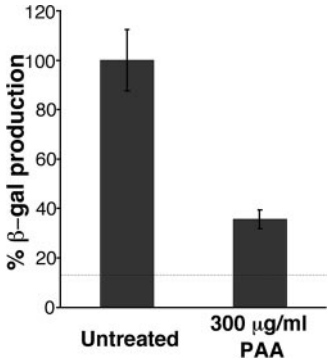

FIG. 7. Presence of PAA results in decreased β-Gal production. CHO-IEβ8 cells were pretreated with 300 μg/ml PAA and then inoculated with CF POka. Infections were carried out in the constant presence of PAA. Cell extracts were prepared at 18 hpi and assayed for β-Gal production. The β-Gal production values in the presence of PAA were scored relative to those for untreated, similarly processed controls (set to 100%). The experiment was performed in triplicate. The dashed line indicates the increase in β-Gal production expected from cell growth alone.

cluding transcription of the immediate early gene encoding IE62, can occur in CHO-K1 cells. However, infected CHO-K1 cells do not display the CPE typically seen in fully permissive host cells. Infected CHO-K1 cells and CF lysates prepared from infected CHO-K1 cells do not elicit plaque formation when seeded on MeWo cells. Since infectious progeny virions do not appear to be made in CHO-K1 cells, a block(s) to productive VZV infection must occur downstream of immediate early transcription. Alternatively, a block may be caused by a failure to produce an immediate early protein(s) other than IE62. To determine if replication of the VZV genome occurs in CHO-K1 cells, β-Gal production in VZV-infected CHO-IEβ8 cells was monitored in the presence of phosphonoacetic acid (PAA), a replication inhibitor that specifically blocks the elongation of template DNA by alphaherpesvirus DNA polymerases (34, 35). Control experiments established that background levels of β-Gal production in CHO-IEβ8 cells were not diminished by the continuous presence of 300 μg/ml PAA for 18 h, indicating that this concentration was not demonstrably toxic to cells (data not shown). In VZV-infected CHO-IEβ8 cells, a 65% decrease in β-Gal production relative to that in untreated controls was observed in the continuous presence of 300 μg/ml PAA (Fig. 7). These data suggest that the VZV genome is able to undergo replication in infected CHO-K1 cells. According to this interpretation, amplification of the viral genome is required for maximal β-Gal production in infected CHO-IEβ8 cells, and the β-Gal production that remains above background in the presence of PAA represents ICP4 activation caused by incoming tegument proteins and/or viral proteins produced prior to viral genome replication.

If replication of the VZV genome can occur in CHO-K1 cells, it is possible that the synthesis of viral late gene products could also occur. To address this possibility, CHO-K1 cells infected with CF VZV were examined for the presence of gE, a viral glycoprotein encoded by ORF68 that is known to be produced with late kinetics (23, 50, 66), by indirect immunofluorescence microscopy. Infected cells were also stained for IE62 to aid in identifying infected cells. Infected MeWo cells were stained in parallel as a positive control for IE62 and gE staining. Neither IE62 nor gE was detected in either MeWo cells or CHO-K1 cells at 0 hpi (data not shown). At 6 hpi, MeWo cells and CHO-K1 cells producing IE62 were readily detected, while cells producing both IE62 and gE were rarely detected (Fig. 8A to D and I to L). At 24 hpi, MeWo cells and CHO-K1 cells producing both IE62 and gE were readily detected (Fig. 8E to H and M to P). Using a complementary approach, the levels of IE62 and ORF68 transcripts in infected CHO-K1 cells were both observed to increase from 0 to 24 hpi (data not shown). The appearance of a late gene product in infected CHO-K1 cells at 24 hpi is consistent with the notion that replication of the VZV genome occurs in CHO-K1 cells and indicates that a block to productive VZV infection in these cells occurs late in the viral life cycle.

Interestingly, the staining pattern observed for IE62 in infected MeWo cells at 24 hpi (Fig. 8E) differed significantly from that observed in infected CHO-K1 cells at 24 hpi (Fig. 8M). In MeWo cells at 24 hpi, IE62 was located predominantly

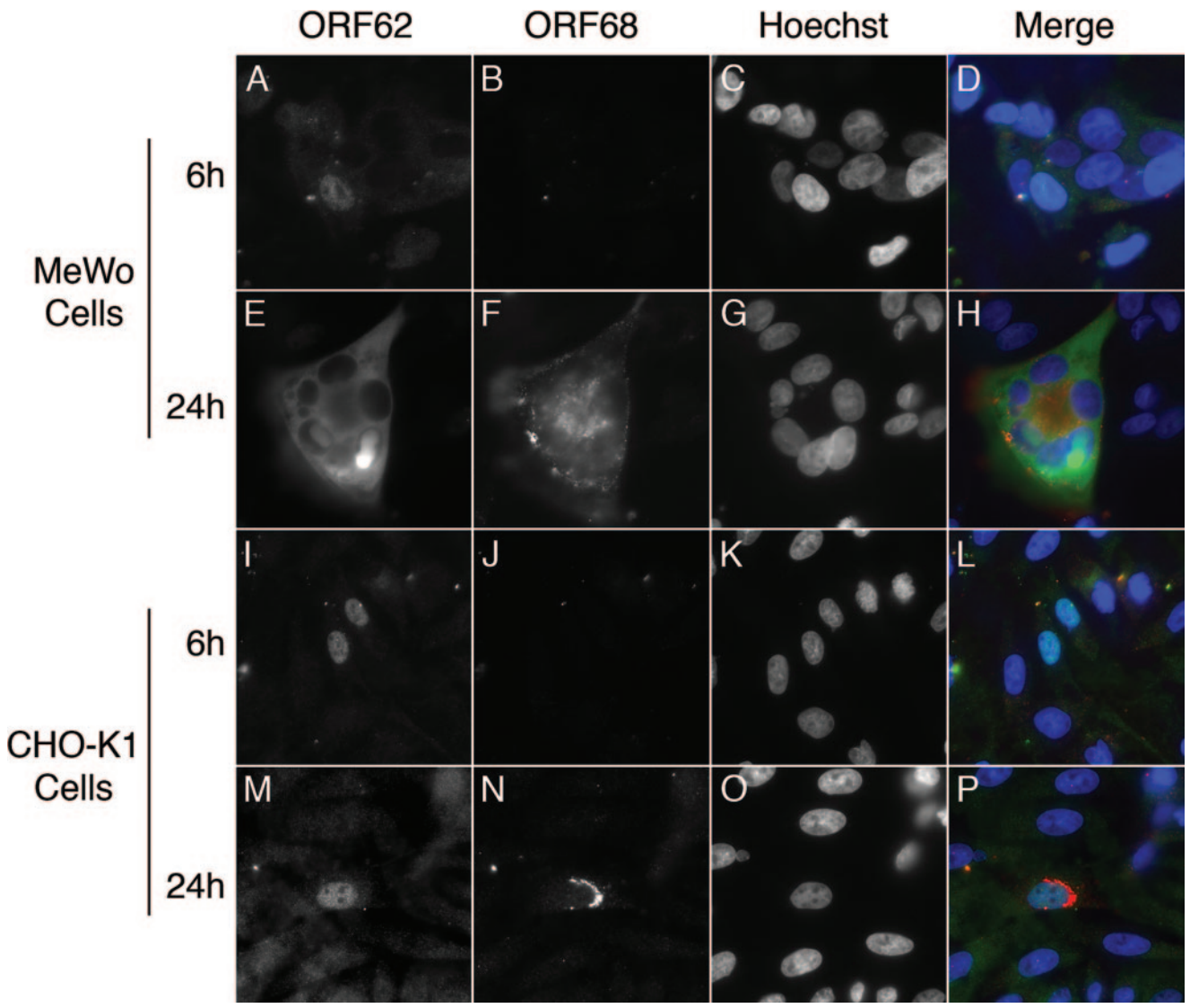

FIG. 8. VZV late protein gE is produced in infected CHO-K1 cells. Indirect immunofluorescence microscopy images of MeWo and CHO-K1 cells following infection with CF POka are shown. Cells were stained with mouse anti-IE62 monoclonal antibodies, goat anti-gE polyclonal antiserum, and conjugated secondary antisera (Alexa fluor 488-conjugated donkey anti-mouse for IE62 and Alexa fluor 555-conjugated rabbit anti-goat for gE); nuclei were stained with Hoechst reagent. Representative fields of infected MeWo cells at 6 hpi and 24 hpi are shown in panels A to D and E to H, respectively; representative fields of infected CHO-K1 cells at 6 hpi or 24 hpi are shown in panels I to L and M to P, respectively. Panels A, E, I, and M show IE62 (ORF62) staining, panels B, F, J, and N show gE (ORF68) staining, and panels C, G, K, and O show Hoechst staining. Merged signals are shown in panels D, H, L, and P.

in the cytoplasm and excluded from the nucleus, while at 6 hpi IE62 was located exclusively in the nucleus (Fig. 8A). The relocalization of IE62 from the nucleus to the cytoplasm late in the viral life cycle in fully permissive host cells is consistent with observations made by Kinchington and colleagues (13, 28–30). In CHO-K1 cells, IE62 was located exclusively in the nucleus at both 6 and 24 hpi (Fig. 8I and M). No evidence of cytoplasmic IE62 was observed either in infected CHO-K1 cells or in infected CHO-IEβ8 cells (data not shown).

DISCUSSION

One approach to identifying viral entry receptors is based on identifying cell lines that are resistant to viral entry. These resistant cell lines can be used for screening of cDNA libraries containing candidate viral receptor genes, as was done successfully for the identification of HSV-1 entry receptors (40, 67). In these studies, a reporting system consisting of a *lac*Z gene driven by the HSV-1 immediate early ICP4 promoter (ICP4/β-Gal) was employed so that HSV-1 entry could be measured by β-Gal production. We wished to test the feasibility of this approach for identifying VZV receptors by focusing our studies on cultured nonprimate cells, most of which do not support productive VZV infection. Restriction of productive VZV infection in the rodent cell lines studied here was found to occur downstream of virus entry, similar to previous observations made with cultured rat neurons and mouse neuroblastomas (4,

37). Inhibition of infectivity in CHO-K1 cells was observed in the presence of PAA, and both ORF68 and gE transcripts were detected in infected CHO-K1 cells. While these observations support the conclusions that replication of the VZV genome can take place in CHO-K1 cells and that a block to productive infection may occur late in the viral life cycle, we do not presently know if the levels of genome replication in CHO-K1 cells and fully permissive cells are equivalent. Thus, it is possible that a block to productive VZV infection occurs at the level of genome replication.

In addition to the two species (mouse and hamster) used for the experiments reported here, we have observed that cultured chicken embryo fibroblasts also form blue foci after infection with ROka-lacZ (data not shown). Thus, VZV may be able to utilize an entry receptor that is conserved across many species. In this respect, it is worth noting that the M6P-binding domains of CI-MPR, which are implicated to be important for VZV entry (7, 17, 70), are conserved across many species (10). The ability of CF VZV to enter CHO-K1 cells is a significant distinction from HSV-1 and implies that VZV is able to utilize an entry receptor that is unique from those used by HSV-1. The existence of novel alphaherpesvirus entry receptors on CHO-K1 cells has also been postulated for equine herpesvirus 1 (EHV-1) (14) and pseudorabies virus (PRV) (45).

Our findings are consistent with a pathway of CF VZV entry into CHO-K1 cells that involves cell surface interactions with heparan sulfate and CI-MPR and that can proceed by a low-pH-dependent endocytic pathway. Receptors for gD do not appear to play a role in CF VZV entry. This result must be interpreted with caution because the numbers of surface-localized gD receptors in the CHO-K1-based cell lines utilized for our analyses are known to be low and to vary between lines (31). Nevertheless, our results are consistent with the notion that unique receptors are required for CF VZV entry. It will be pertinent to determine whether VZV, like HSV-1, utilizes multiple entry receptors and/or multiple entry mechanisms to gain entry to the different cell types encountered during infection of the host. The entry of free virions into host cells is relevant for at least three steps in the most recently proposed model of primary VZV pathogenesis (32). Infection of a new host is initiated primarily by airborne free virions released from cutaneous lesions, which invade epithelial cells in the upper respiratory tract. The virus subsequently infects T cells within tonsillar lymphoid tissues; infected T cells then enter the circulation and transport virus to the skin. Infected T cells do not undergo fusion with adjacent cells (39, 55), and infected T cells can produce abundant amounts of complete, enveloped virions (55). Consequently, the initial infection of T cells and the spread of virus from T cells to other target cells are both thought to be mediated by free virions.

We have demonstrated that the ICP4/β-Gal reporter gene, combined with a sensitive chemiluminescence-based β-Gal detection assay, can monitor VZV entry into small numbers of target cells at a low MOI. The ICP4/β-Gal reporter system should therefore provide a useful tool for further investigations into VZV entry, including both entry mediated by CF virus and that mediated by CA virus. Understanding the fundamentals of CF VZV entry into relevant target cell types, such as human respiratory epithelial cells and T cells, and how this process is distinct from entry mediated by CA virus should provide insight into the pathogenesis of VZV. Entry studies may also uncover new means for blocking VZV infection of target cells and for improving the infectivity of live attenuated varicella-zoster vaccines.

This study indicates that restriction of productive VZV infection in CHO-K1 cells occurs after the initiation of virus gene expression, likely late in the viral life cycle. Even though at least one late structural protein, gE, is produced in VZV-infected CHO-K1 cells, no evidence of progeny virion production was detected. For CHO-K1 cells infected with PRV, a 10,000-fold reduction in progeny virion production was noted in comparison to that in fully permissive RK13 cells (45). This defect could not be corrected by expression of nectin-1, prompting speculation that the restriction of PRV in CHO-K1 cells occurs during entry as well as at a step(s) downstream. The postentry block to PRV infection is believed to occur after early gene expression but has not been characterized further. In contrast, more modest decreases in progeny virion production were noted in CHO-K1 cells infected with EHV-1 (14) and in HVEM-expressing CHO-K1 cells infected with HSV-1 (40). Thus, CHO-K1-derived cells are generally considered to be fully permissive for both EHV-1 and HSV-1.

One striking characteristic of VZV-infected CHO-K1 cells is the strictly nuclear localization of IE62. IE62 staining in NIH 3T3 cells also appeared to be nuclear (Fig. 1). Phosphorylation of IE62 by a viral protein kinase encoded by ORF66 is required in order for IE62 to relocate from the nucleus to the cytoplasm (13, 28–30). Relocation of phosphorylated IE62 to the cytoplasm enables its incorporation into the tegument of progeny virions. The nuclear confinement of IE62 and the lack of virion production that we observed in infected CHO-K1 cells bear resemblance to observations made with a POka mutant in which ORF66 production was prevented by stop codon insertion (POka66S). IE62 was observed to be strictly nuclear in POka66S-infected MeWo cells, and severe defects in progeny virion formation were also observed in POka66S-infected T cells (55). The failure of IE62 to reach the cytoplasm may be one plausible explanation for the failure of progeny virion production in VZV-infected CHO-K1 cells. While we do not yet know whether VZV virion formation in CHO-K1 cells is directly dependent on proper IE62 localization, we speculate that restriction of VZV in CHO-K1 cells could arise from a defect in the production of ORF66 and/or its ability to phosphorylate IE62. This defect would prevent IE62 from escaping the nucleus, which in turn could impair virion assembly. In contrast to our observations with infected CHO-K1 cells, IE62 staining in rat neurons infected in vivo was observed only in the cytoplasm, not in the nucleus (18). This pattern of IE62 staining is consistent with that observed in latently infected human neurons (22, 33). The dissimilarity in IE62 staining patterns observed for rodent cell lines infected in vitro versus rat neurons infected in vivo lends credence to the notion that infections established in the rat model of VZV latency are not simply abortive infections. A comprehensive study of viral gene expression and viral protein localization in a variety of cultured rodent cells may help to clarify whether rodent models of VZV latency represent truly latent infections capable of reactivation or abortive infections. Further studies to elucidate the mechanism of postentry restriction of VZV in nonprimate cells may uncover new cellular targets for antiviral intervention and lead

to the development of suitable nonprimate models for studying VZV pathogenesis and latency.

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