Bcl-2 Blocks Accretion or Depletion of Stored Calcium but Has No Effect on the Redistribution of IP3 Receptor I Mediated by Glycoprotein E of Herpes Simplex Virus 1[▽]

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We examined the status of stable, resting intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) and the calcium that can be released from intracellular stores in HEP-2 or VAX-3 cells overexpressing Bcl-2 after infection with wild-type or mutant herpes simplex viruses. The mutants included viruses lacking ICP4 or ICP27 and known to induce apoptosis. We report the following. Stable Ca^{2+} levels decrease after infection with wild-type or mutant viruses in both HEP-2 and VAX-3 cells. The histamine-sensitive calcium stores became depleted in wild-type and mutant virus-infected cells late in infection but increased significantly in ΔICP4 - or ΔICP27 -infected cells prior to depletion. In VAX-3 cells, the depletion in calcium stores did not take place as late as 24 h after infection, concomitant with lack of visually detectable cytopathic effects. Concurrent analyses showed that the amounts of IP3 Ca^{2+} -receptor type I (IP3R-I) remained stable throughout infection, but the intensity of the signal increased and intracellular distribution changed dramatically in both HEP-2 and VAX-3 cells infected with the wild-type and all mutant viruses, except for the mutant lacking glycoprotein E (ΔgE). In transfected HEP-2 cells, gE and gI were more effective at augmenting the signal intensity and redistribution of IP3R-I than gE or gI alone. We conclude the following. (i) Depleted histamine-sensitive calcium stores correlate with appearance of cytopathic effects. (ii) Apoptosis, the calcium stores, and cytopathic effects are regulated by Bcl-2. (iii) The changes in the distribution of IP3R-I are mediated by the viral Fc receptor complex, but the redistribution is not related to changes in stored calcium.

The studies described in this report centered on the status of stable Ca^{2+} and the Ca^{2+} that can be released from intracellular stores in the course of infection with herpes simplex virus type 1 (HSV-1). The impetus for these studies stemmed from three considerations.

Foremost, the proteins that regulate the amounts and distribution of intracellular calcium have a profound effect on virtually all cellular functions (34). Given the apparent strategy of HSV-1 to regulate all aspects of cellular metabolism (41), it seemed unlikely that at least some functions of HSV are not directed to control calcium metabolism.

A second and more focused reason stemmed from the accrued evidence from this and other laboratories that at least two replication-defective mutants of HSV-1—that is mutants lacking with the $\alpha 4$ or $\alpha 27$ genes encoding infected cell proteins no. 4 (ICP4) and 27 (ICP27), respectively—induce apoptosis in a cell-dependent fashion (1, 13, 14, 23). A voluminous literature has defined the role of calcium metabolism and apoptosis and in particularly the regulation of inositol triphosphate receptor type I (IP3R-I) and calcium stores in endoplasmic reticulum (ER) by pro- and antiapoptotic members of the Bcl-2 family. Thus, Bcl-2 has been reported to maintain the levels of calcium stored in endoplasmic reticulum in the face of fluctuations induced by proapoptotic members of the Bcl-2 family (12, 20, 30–33, 36).

Finally, the involvement of calcium metabolism in infected cells has already been demonstrated in herpesvirus-infected cells. Thus, Cheshenko et al. reported a rapid and transient increase of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) following exposure of cells to wild-type HSV-1 but not to a mutant incapable of virus entry by fusion of the envelope with the plasma membrane (5). The response was abrogated by drugs that block IP3R-dependent release of calcium from endoplasmic reticulum. In another report, Himpens et al. reported that human cytomegalovirus caused an increase of the free calcium and significantly interfered with Ca^{2+} homeostasis in human fibroblasts (17).

In this report, we examined the levels of stable resting Ca^{2+} and calcium that could be released from histamine-sensitive cytoplasmic Ca^{2+} stores in the course of infection of HEP-2 and VAX-3 cells with wild-type and mutant viruses. The VAX-3 cell line was derived from HEP-2 cells, which stably express high levels of Bcl-2. ΔICP4 mutant virus induces apoptosis in HEP-2 cells but not in VAX-3 cells (13). In addition, VAX-3 cells show no visual evidence of cytopathic effects at a time (24 h after infection) when the parental HEP-2 cells are totally decimated (13). A central question is whether the absence of apoptosis or cytopathic effects correlates with changes in Ca^{2+} homeostasis.

In addition to these studies, we have examined the status of IP3R-I in infected cells. We found that while the amount of receptor does not change after infection, it is significantly altered in distribution and appearance in both HEP-2 and VAX-3 cells infected with wild-type virus and all mutant viruses except the one lacking glycoprotein E (ΔgE). gE by itself acts as a weak Fc receptor. In a complex with glycoprotein I

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(gI), the Fc binding capacity significantly increases (41). One result reported here is that in transfected cells, gE, and to a greater extent gE plus gI, causes rearrangements in IP3R-I similar to those observed in (gE/gI)⁺ virus-infected cells.

MATERIALS AND METHODS

Cells and viruses. HEp-2 cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. The HEp-2 cell line stably expressing Bcl-2, VAX-3, has been described elsewhere (13). The cell line was maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 400 µg of G418 per ml. The cultures used in all experiments shown here were seeded less than 20 h prior to infection.

HSV-1(F) is the prototype HSV-1 strain used in this laboratory (11). The construction and properties of the ΔU_L41 (ΔVHS) mutant virus R2621 were reported elsewhere (39). R7032 is a recombinant virus derived from HSV-1(F) and contains a deletion in the gene encoding gE (27). The recombinant R7041 lacking the U_S3 gene has been described earlier (40). The $\Delta ICP27$ virus, a kind gift of R. Sandri-Goldin, carries a LacZ gene inserted into the ICP27 open reading frame (ORF) (42) and was propagated on an ICP27-complementing Vero-derived cell line (Vero 2.2) expressing ICP27 under its own promoter (42), kindly provided by S. Silverstein (Columbia University, New York, NY). The HSV-1 (KOS) $\Delta 120$ mutant, a kind gift of N. DeLuca, lacks both copies of the $\alpha 4$ gene and was grown in a Vero-derived cell line (E5) expressing the $\alpha 4$ gene. It also does not express the U_S3 gene (8, 23). The ICP0 null recombinant virus R7910 has been described before (19). Finally, the recombinant virus vC116G/C165A, encoding ICP0 C116G/C156A on an HSV-1 strain 17 [HSV-1(17)] background, was obtained from S. Silverstein, and its construction and phenotypic properties were described previously (19, 25).

Plasmid construction. For transient expression of gI in mammalian cells, a 1,172-bp EcoRI-PstI fragment containing the gI ORF was amplified by PCR from pRB123 with the primers 5' GCGAGGAATTCAGATGCCGTGCCGCC CGTTGCAG 3' (sense) and 5' GGCCTGCAGGCTATACCAACAGGGGAG CCGTTGGG 3' (antisense) and inserted into the EcoRI-PstI sites of the pAc-CMV polylinker (46), resulting in the pAc-gI transfer vector. For the expression of gE in mammalian cells, a 1,650-bp EcoRI-PstI fragment containing the gE ORF was amplified by PCR directly from the viral genome with the primers 5' GCGAGGAATTCACATGGATCGCGGGCGGTGGTG 3' (sense) and 5' GGCCTGCAGGTTACCAGAAGACGGACGAATCGG 3' (antisense) and inserted into the EcoRI-PstI sites of the pAc-CMV polylinker, resulting in the pAc-gE transfer vector.

Protein preparation, immunoblotting, and antibodies. Replicate cell cultures in six-well plates were either mock infected or exposed to 10 PFU of virus per cell for 2 h and maintained at 37°C in 199V medium consisting of mixture 199 (Sigma) supplemented with 1% calf serum. Cells were harvested at the indicated times after infection; rinsed three times with phosphate-buffered saline (PBS); solubilized in triple-detergent buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, 0.5% sodium deoxycholate, 100 µg ml⁻¹ of phenylmethylsulfonyl fluoride) supplemented with phosphatase inhibitors (10 mM NaF, 10 mM β -glycerophosphate, 0.1 mM sodium vanadate) and protease inhibitor cocktail (Sigma), as specified by the manufacturer; and briefly sonicated. The protein concentration in total lysates was determined with the aid of the Bio-Rad protein assay (Bio-Rad Laboratories), according to directions provided by the supplier. Approximately 60 µg of proteins per sample was subjected to further analysis.

Proteins were electrophoretically separated in a 7% denaturing polyacrylamide gel; electrically transferred to a nitrocellulose sheet; blocked with PBS supplemented with 0.02% (vol/vol) Tween 20 (PBST) and 5% (wt/vol) nonfat milk for 1 h at room temperature; and reacted with the proper primary antibodies, diluted in PBST-1% nonfat milk, overnight at 4°C. The rabbit polyclonal antibody for IP3R-I (H-80; Santa Cruz Biotechnology, Inc.) was used in a dilution of 1:500. The monoclonal antibody for calreticulin (Stressgen) was used in a dilution of 1:1,000. After several rinses with PBST-1% nonfat milk, the membranes were reacted with the appropriate secondary antibody conjugated to alkaline phosphatase (Bio-Rad) for 2 h at room temperature. Finally, following extensive washes, first with PBST-1% nonfat milk and then with PBS, the immunoblots were developed with 5-bromo-4-chloro-3-indolylphosphate (BCIP)-nitroblue tetrazolium (Denville Scientific, Inc.).

Immunofluorescence studies. For the infection experiments, HEp-2 cells grown on four-well confocal slides (ERIE Scientific) were exposed to 10 PFU of wild-type or mutant viruses per cell for 2 h. After incubation at 37°C for a total

of 9 h, the cells were fixed in 4% paraformaldehyde. The transient transfection experiments were performed in HEp-2 cells, seeded on four-well confocal slides at a confluence of 60 to 70%, by using the Lipofectamine Plus reagent (Invitrogen), as specified by the manufacturer. The cells were fixed in 4% paraformaldehyde 24 h posttransfection. After fixation, the cells were neutralized with 100 mM of glycine in PBS, permeabilized with 0.1% Triton X-100 in PBS in the presence of 2 mg/ml bovine serum albumin (PBS-TB), and reacted with primary antibodies, diluted in PBS-TB, for 2 h at room temperature. The IP3R-I rabbit polyclonal antibody (H-80; Santa Cruz, Inc.) was used in a dilution of 1:500, the ICP4 mouse monoclonal antibody (Goodwin Institute for Cancer Research, Plantation, FL) was used in a dilution of 1:1,000, the gE mouse monoclonal antibody (clone 3114), kindly provided by D. Johnson, and the gI mouse monoclonal antibody clone Fd69 (26) were used in a dilution of 1:500. Following this, the samples were rinsed several times with PBS-TB and reacted with Alexa Fluor 594-conjugated goat anti-rabbit and Alexa Fluor 488-conjugated goat anti-mouse secondary antibodies (Molecular Probes), diluted 1:1,000 in PBS-TB, for 40 min at room temperature. After several washes, first with PBS-TB and then with PBS, the samples were mounted in Vectashield mounting medium for fluorescence (Vector Laboratories) and examined in a Zeiss confocal microscope equipped with software provided by Zeiss.

Note that to obviate nonspecific binding of rabbit immunoglobulin G (IgG) to the HSV-1 Fc receptor, a critical issue in these studies, we tested a variety of blocking agents that included 10% horse serum, etc., as well as secondary antibodies alone, in the absence of primary antibodies. The immunofluorescence images obtained on cells infected with HSV-1(F) with other blocking reagents were identical to those reported here. Secondary antibody alone did not react with the wild-type virus-infected cells.

Ca²⁺ measurements. HEp-2 or VAX-3 cells (approximately 10⁵) were plated on 25-mm circle microscope coverslips (Fisher Scientific) inside wells of a six-well plate the day before the measurements. Cells were either mock infected or exposed to 10 PFU of virus per cell for 2 h and maintained at 37°C. Just before the measurements, the cells were rinsed with prewarmed KRB solution (125 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM Na₃PO₄, 20 mM HEPES [pH 7.4], 5.5 mM glucose) and loaded with 3 µM of Fura-2 AM (Molecular Probes), diluted in KRB, for 30 min at 37°C. Subsequently, the cells were rinsed three times with the KRB solution and were placed in a thermostatted (37°C) chamber on the stage of an inverted fluorescence microscope (Olympus IX81) connected to a chilled, high-quantum-efficiency Retiga EXi digital charge-coupled device camera. A 40×/1.35 oil UV-optimized objective was used. The samples were illuminated alternately at 340 and 380 nm, and the emitted light (filtered with an interference filter centered at 510 nm) was collected by the camera. Images at 340 nm and 380 nm were acquired using the Metafluor software at 2-s intervals for more than 1 min prior to stimulation with 100 µM histamine (Sigma). The stable resting [Ca²⁺]_i was calculated from those images. Capture continued at 2-s intervals following the stimulus for 4 to 5 min. Ratio analysis was performed offline. The [Ca²⁺]_i was determined by the equation $[Ca^{2+}]_i = K_d (S_{p2}/S_{h2}) [(R - R_{min})/(R_{max} - R)]$. R is the observed 340/380-nm fluorescence ratio (15). R_{min} is the 340/380 nm fluorescence ratio value determined in cells exposed to Ca²⁺-free KRB in the presence of 10 mM of the Ca²⁺ chelator EGTA and 30 µM of the Ca²⁺ ionophore ionomycin (Biomol). R_{max} is the 340/380-nm fluorescence ratio value determined in cells exposed to ionomycin in the presence of 10 mM CaCl₂. S_{p2} and S_{h2} are the proportionality coefficients of Ca²⁺-free Fura-2 and saturated Fura-2, respectively. The K_d of the Ca²⁺-Fura-2 interaction was assumed to be 225 nM in the cytosolic environment (15).

Approximately five individual fields in an equal number of coverslips (total of 250 cells per sample) were measured within the same experiment, and all of the experiments were performed at least three times. A total of over 800 mock- or virus-infected cells from randomly selected fields were analyzed. Representative traces showing the average [Ca²⁺]_i of 35 to 40 cells, measured from a single field of a sample, are depicted.

RESULTS

Changes in Ca²⁺ homeostasis after infection of cells with wild-type and mutant viruses. To evaluate the effect of HSV-1 on [Ca²⁺]_i, we monitored at different time intervals during the virus replicative cycle both the stable, resting [Ca²⁺]_i and the calcium that can be released from the major intracellular calcium stores upon stimulation with histamine, an IP3-generating agonist. As detailed in Materials and Methods, HEp-2 cells grown on 25-mm coverslips were mock infected or exposed to

FIG. 1. $[Ca^{2+}]_i$ levels in mock-infected or infected Hep-2 cells. Hep-2 cells were mock infected or exposed to 10 PFU of HSV-1(F), Δ ICP4, or Δ ICP27 mutant virus per cell. At 3, 8, 12, or 20 h after infection, the cells were loaded with 3 μ M of Fura-2 AM, as indicated in Materials and Methods, and images at 340 nm and 380 nm were acquired by using an Olympus IX81 inverted microscope equipped with the Metafluor software. The resting $[Ca^{2+}]_i$ was monitored at 2-s intervals for at least 1 min prior to the stimulation with 100 μ M histamine. The response to the histamine stimulus was monitored extensively. Representative traces, which correspond to the average $[Ca^{2+}]_i$ of 35 to 40 cells measured from a single field of a sample, are depicted.

10 PFU of wild-type or mutant viruses per cell. At 3, 8, 12, or 20 h after mock infection or infection, the wells were rinsed with calcium-free medium and then exposed to calcium-free medium containing Fura-2AM. Measurements in replicate samples were performed every 40 min for 3 to 4 h following the analysis of the initial sample. The stable, resting $[Ca^{2+}]_i$ was monitored every 2 s for at least 1 min. This was then followed by the addition of histamine, and the effect of the IP₃-generating agonist was monitored every 2 s for the next 4 to 5 min. The results shown in Fig. 1 and 2 were as follows.

The resting $[Ca^{2+}]_i$ in mock-infected cells was at approximately 200 nM during all of the experimental procedure. After histamine stimulation, the mean range increased between 500 and 600 nM (Fig. 1 and 2, column 1).

(i) As shown in column 2 of Fig. 1 and 2, a small reduction in the stable, resting $[Ca^{2+}]_i$ was observed as early as 3 h after infection with wild-type virus as compared to the level in mock-infected cells. The loss in stable, resting $[Ca^{2+}]_i$ became more pronounced in cells sampled between 8 and 12 h after infection. Since Fura-2AM was used to monitor the free calcium in the cytosol, these results suggest that there was a general reduction in the amount of the free cytosolic calcium in infected cells, in the Ca^{2+} -free environment in which the measurements were performed.

(ii) Histamine causes a release of Ca^{2+} from the IP₃R-containing intracellular calcium stores, such as ER and Golgi apparatus. Histamine caused no significant changes between 3 and 6 h after infection (Fig. 1, column 2). A small reproducible

increase of 100 nM above the mean value of mock-infected cells was noted in wild-type virus-infected cells between 8 and 12 h after infection. In cells sampled during the 12- to 16-h interval after infection, the free Ca^{2+} released from the stores increased from 220 to as high as 720 nM. These results indicate that some infected cells retain the amounts of free Ca^{2+} in their histamine-sensitive intracellular Ca^{2+} stores, whereas others contained considerably smaller amounts of stored Ca^{2+} available for release. Finally, at 20 to 24 h after infection, the cells responded poorly to histamine stimulation—an indication of a widespread depletion of major intracellular calcium stores in HSV-1(F)-infected cells. We conclude from these studies that HSV-1(F) infection decreases the levels of both the stable, resting $[Ca^{2+}]_i$ and the free Ca^{2+} that can be released from the histamine-sensitive intracellular calcium stores.

(iii) As shown in Fig. 1, columns 3 and 4, the levels of resting $[Ca^{2+}]_i$ in Δ ICP4 or Δ ICP27 mutant virus-infected cells could not be differentiated from those of mock-infected or wild-type virus-infected cells during the interval between 3 and 6 h after infection. As in the case of wild-type virus, the levels of stable free calcium decreased at later intervals. In the case of Δ ICP4 mutant virus-infected cells (Fig. 1, column 3), resting calcium levels in cells sampled between 20 and 24 h after infection, which remained attached to the well, were lower than those of wild-type virus-infected cells. In the case of Δ ICP27 mutant virus-infected cells, the mean levels of stable calcium in sampled cells were lower than those of wild-type virus-infected cells, starting as early as 12 h after infection. The results indi-

FIG. 2. $[Ca^{2+}]_i$ levels in mock-infected or infected HEP-2 cells. HEP-2 cells were mock infected or exposed to 10 PFU of HSV-1(F) or ΔgE mutant per cell. The cultures were processed as described in the legend to Fig. 1.

cate that the decrease in resting calcium levels in cells infected with the proapoptotic viruses was more dramatic than that observed in wild-type virus-infected cells.

Exposure to histamine between 8 and 12 h postinfection resulted in the release of much larger amounts of free calcium from the IP3R-containing intracellular calcium stores of $\Delta ICP4$ or $\Delta ICP27$ mutant virus-infected cells than from mock- or wild-type virus-infected cells. The peak response to histamine for both $\Delta ICP4$ - and $\Delta ICP27$ -infected cells was determined to have an average value of 800 nM $[Ca^{2+}]_i$, while many individual cells yielded peak values close to 1,000 nM $[Ca^{2+}]_i$. At the same time, the average peak values in mock-infected and wild-type virus-infected cells were calculated as 450 and 550 nM $[Ca^{2+}]_i$, respectively. In the latter cell populations, the peak values of individual cells did not exceed 700 nM $[Ca^{2+}]_i$. Between 12 and 16 h postinfection, only a small drop in mean peak value was observed in the $\Delta ICP4$ -infected cells as compared to the preceding time interval. In contrast, a wide range of peak values were obtained from the $\Delta ICP27$ virus-infected cells in response to the agonist, ranging from an average of 100 nM $[Ca^{2+}]_i$ (for the apoptotic cells) to 800 nM $[Ca^{2+}]_i$. As a consequence, the mean peak response to histamine dropped

significantly compared to that in the mock-infected cells. Finally, between 20 and 24 h after infection, the attached $\Delta ICP27$ -infected cells responded poorly to histamine, whereas the well-attached $\Delta ICP4$ -infected cells were still responding but appeared to be in the processing of losing Ca^{2+} from the intracellular calcium stores.

Overall, these results suggest that in contrast to the wild-type virus, the defective viruses $\Delta ICP4$ and $\Delta ICP27$ show significant accumulation of Ca^{2+} in the histamine-sensitive intracellular stores prior to induction of calcium depletion.

(iv) The stable free calcium levels in cells infected with the ΔgE virus were similar to those of wild-type virus-infected cells in cells sampled between 3 and 6, 8 and 12, or 12 and 16 h after infection (Fig. 2, column 3). At the later interval, the average value of stable free calcium measured in these experiments decreased below that of wild-type virus-infected cells. The response to histamine stimulation was very similar to that of wild-type virus-infected cells.

Bcl-2 blocks the changes in the Ca^{2+} homeostasis induced upon virus infection. The experiments described above were repeated on HEP-2 cells stably expressing Bcl-2 (VAX-3 cells) that were mock infected or exposed to 10 PFU of wild-type virus or $\Delta ICP4$ or $\Delta ICP27$ mutant virus per cell. Measurements were performed again during the time windows 3 to 6, 8 to 12, 12 to 16, and 20 to 24 h after infection in exactly the same manner as those performed in the parental HEP-2 infected cells, described above. The results (Fig. 3) were as follows.

As was the case in HEP-2 cells, the resting $[Ca^{2+}]_i$ in the mock-infected VAX-3 cells was calculated to be around 200 nM and the mean peak response to histamine was calculated to be around 450 nM (Fig. 3, column 1), implying that Bcl-2 by itself does not cause any obvious alteration in the intracellular Ca^{2+} levels in HEP-2 cells that could be detected by the experimental procedures employed in these studies. Interestingly, we noted a drop in the baseline of virus-infected cells and in particular in cells infected with the $\Delta ICP4$ or $\Delta ICP27$ mutants between 8 and 12 h after infection. These levels were restored at later time intervals. The noteworthy feature of VAX-3 cells is that they exhibited no changes in the free stored calcium as a consequence of viral infection even as late as 24 h after infection. In other experiments, no changes were observed as late as 36 h after infection (data not shown).

Overall, our data suggest that Bcl-2 blocks the calcium alterations induced upon virus infection and support the conclusion that Bcl-2 is a major Ca^{2+} regulator.

The redistribution of IP3R-I in infected cells. HSV-1 infection causes extensive remodeling in the organization of ER and cytoskeleton. In light of the observed changes in the stored calcium during infection, it was of interest to determine whether HSV-1 causes redistribution of IP3R, an intracellular Ca^{2+} channel. Our studies focused on IP3R-I, a ubiquitously expressed member of the family.

We report two series of experiments. In the first, we examined the levels of IP3R-I in HEP-2 or VAX-3 cells that were mock infected or exposed to 10 PFU of wild-type or $\Delta ICP4$ or $\Delta ICP27$ mutant virus per cell. The cells were harvested at 3, 8, 12, or 18 h postinfection, lysed, denatured, subjected to electrophoresis in denaturing gels, transferred to a nitrocellulose sheet, and probed with antibodies to IP3R-I or calreticulin. The latter served as a loading control. The results shown in

FIG. 3. $[Ca^{2+}]_i$ levels in mock-infected or infected VAX-3 cells. VAX-3 cells were mock infected or exposed to 10 PFU of HSV-1(F), Δ ICP4, or Δ ICP27 per cell. The cells were processed as described in the legend to Fig. 1.

Fig. 4 indicate that the IP3R-I protein levels remained stable throughout the 18-h interval after infection in both HEp-2 and VAX-3 cells. It is noteworthy that, in some instances and particularly in VAX-3 cells, we could resolve two closely migrating bands reacting with the anti-IP3R-I antibody.

FIG. 4. Endogenous levels of IP3R-I in virus-infected human epithelium cells. HEp-2 or VAX-3 cells were either mock infected (lanes 1, 5, 9, and 13) or exposed to 10 PFU of HSV-1(F) (lanes 2, 6, 10, and 14), Δ ICP4 (lanes 3, 7, 11, and 15), or Δ ICP27 (lanes 4, 8, 12, and 16) per cell. Cells were harvested at 3, 8, 12, or 18 h after infection and lysed, and equal amount of proteins were separated on a denaturing polyacrylamide gel. The electrophoretically separated proteins were transferred to a nitrocellulose sheet and reacted with rabbit polyclonal antibody to IP3R-I, as detailed in Materials and Methods. The bottom part of the nitrocellulose membrane from the HEp-2 experiment was analyzed for the expression of calreticulin, which served as a loading control.

In the second series of experiments, HEp-2 or VAX-3 cells grown on four-well slides were mock infected or exposed to 10 PFU of HSV-1(F) or various deletion mutants per cell. The cells were fixed between 9 and 10 h postinfection and reacted with rabbit polyclonal antibody to IP3R-I and a monoclonal antibody to ICP4 to identify infected cells. The secondary antibodies to rabbit IgG were conjugated to Alexa Fluor 594, whereas those directed to mouse IgG were conjugated to Alexa Fluor 488. Representative images collected at the same settings of a Zeiss confocal microscope that best illustrate our findings are shown in Fig. 5 and 6. The results may be summarized as follows.

- (i) In both mock-infected HEp-2 and VAX-3 cells (Fig. 5 and 6, respectively), the IP3R-I signal was diffuse and barely detectable with the microscope settings employed in this study and designed to maximize the image resolution of infected cells. The diffuse IP3R-I protein signal was more readily detected under settings that distorted the images collected from cells infected with wild-type virus.
- (ii) In cultures of HEp-2 or VAX-3 cells exposed to wild-type virus (Fig. 5 and 6), we noted that the IP3R-I protein accumulated in a dense mass at one extremity of the cell close to the nucleus or dispersed around the perimeter of the nucleus.
- (iii) The images obtained in both cell lines exposed to the Δ ICP27 mutant showed low-level aggregation of IP3R-I around the perimeter of the cell. Most infected cells could not be differentiated from mock-infected cells with respect to the distribution of IP3R-I. It is also of interest that the levels of ICP4 detected by immunofluorescence were lower in Δ ICP27-infected cells than in cells infected with wild-type virus.

FIG. 5. Subcellular localization of the IP3R-I in mock-infected and infected HEP-2 cells. HEP-2 cells were mock infected or exposed (10 PFU/cell) to HSV-1(F), Δ ICP27, Δ ICP0, Δ gE, Δ US3, Δ RF, Δ UL41, or Δ ICP4 mutant viruses. At 9 h after infection, the cells were fixed in 4% paraformaldehyde and reacted with the mouse monoclonal antibody to ICP4 and the rabbit polyclonal antibody to IP3R-I, followed by reactions with the goat anti-mouse Alexa Fluor 488 and the goat anti-rabbit Alexa Fluor 594 antibodies, respectively, as detailed in Materials and Methods. All images were captured with the same settings of a Zeiss confocal microscope with the aid of software provided by the manufacturer.

(iv) In Δ ICP4 mutant virus-infected cells, the distribution of IP3R-I generally could not be differentiated from that in mock-infected cells, and only rarely could small aggregates dispersed in the cytoplasm be visualized in both HEP-2- and VAX-3-infected cells. In both cell lines, some round, smaller cells could be visualized with strong IP3R-I staining that was excluded only from the nucleus.

(v) The distribution of IP3R-I in HEP-2 or VAX-3 cells infected with either Δ ICP0 or the ICP0 ring finger mutant

could not be differentiated from those observed in wild-type virus-infected cells.

(vi) In this series of experiments, we included three additional mutants, Δ UL41, Δ US3, and Δ gE, described in Materials and Methods. The product of the UL41 gene is an RNase with the substrate specificity of RNase A (43). It causes selective degradation of mRNA. Δ UL41 virus was selected because of the possibility that the IP3R phenotype reflects the loss of a cellular protein, whose mRNA is degraded by the UL41 pro-

FIG. 6. Subcellular localization of IP3R-I in VAX-3 cells. The experiment described in the legend to Fig. 5 was duplicated with VAX-3 cells. The procedures employed in this experiment were identical to those described for Fig. 5.

tein. The distribution of IP3R-I in ΔU_L41 virus-infected cells could not be differentiated from those of wild-type virus-infected cells. We have noted, as will be reported elsewhere in detail, that ICP4 tended to accumulate in the cytoplasm of ΔU_L41 mutant virus-infected cells (M. Kalamvoki and B. Roizman, unpublished data). The protein kinase, encoded by the U_S3 ORF, phosphorylates a large number of cellular and viral proteins and also blocks cells from apoptosis induced by a wide variety of stimuli (2, 24, 28, 29). In cells infected with the mutant virus, the large majority of cells exhibited an IP3R-I distribution similar to that of wild-type virus-infected cells. A smaller fraction exhibited nuclei that were decreased in size.

IP3R-I was dispersed in the cytoplasm but also aggregated around the perinuclear space in both cell lines (Fig. 5 and 6). Finally, the cellular Fc receptor has been reported to regulate the distribution of membrane proteins in uninfected cells (21, 22). It was of interest, therefore, to determine if the viral Fc receptor—a function of glycoprotein E encoded by the U_S8 gene (41)—plays a similar role. As shown in both Fig. 5 and 6, IP3R-I was barely detectable in most ΔgE virus-infected cells. In a few cells, IP3R-I formed punctate structures dispersed in the cytoplasm.

We conclude from these studies the following.

- (i) The signal strength and distribution of IP3R-I in cells

infected with wild-type virus and all mutants, except the Δ gE mutant, were significantly stronger and differed from those of mock-infected cells. It must be emphasized that the IP3R-I distribution in mock-infected cells was barely visible under the settings used to obtain images of either viral protein or IP3R-I in cells infected with the wild-type or mutant viruses. Inasmuch as the total IP3R-I remained unchanged throughout infection, as illustrated in Fig. 4, we conclude that changes in conformation or aggregation of IP3R-I made it more accessible to the antibody. With the exception of mock-infected cells or cells infected with Δ gE mutant, IP3R-I was dispersed in the cytoplasm in the form of globs varying in size and shape. In some instances small globular aggregates appeared to be close to the nuclear membrane.

(ii) The results indicate that the changes in the levels of Ca^{2+} in infected cells illustrated in Fig. 1 and 2 are not due to changes in the distribution of IP3R-I. As noted earlier in the text, in wild-type or mutant virus-infected VAX-3 cells overexpressing Bcl-2, the changes in the basal and stored calcium levels were largely suppressed compared to those observed in the parental, HEp-2 cells. In contrast, the distributions of IP3R-I receptor in infected HEp-2 or VAX-3 cells were largely identical.

The changes in the distribution and intensity of IP3R-I correlate with the expression of gE or gE and gI. The distribution of IP3R-I shown in Fig. 5 and 6 showed that the distribution and signal intensity of IP3R-I in cells infected with Δ gE mutant virus resembled those of mock-infected cells rather than wild-type virus-infected cells. To test the hypothesis that the changes in the distribution and signal intensity of IP3R-I correlate with the expression of gE, four-well slide cultures of HEp-2 cells were transfected with the empty vector MTS, a plasmid encoding gE, gI, or a mixture of gE and gI. The cultures were fixed in 4% paraformaldehyde 24 h after transfection and reacted with antibody to IP3R-I (Fig. 7, all panels), gE (A to F), or gI (G and H), as described in the legend to Fig. 7. The results were as follows.

Under the setting designed to obtain high-resolution images, IP3R-I was barely visible in mock-transfected cells (Fig. 7A). To visualize IP3R-I, the image in panel A was manipulated with the auto-contrast feature of the Photoshop software. This image, shown in panel B, indicates that the IP3R-I was in the form of small, relatively uniform evenly distributed aggregates. The images of cells transfected with gE alone (E and F), gI alone (G), or the mixture of gE and gI (C and D) were captured with the same settings as that shown in panel A. In cells transfected with gE alone, most cells exhibited Alexa Fluor 488 fluorescence (green) around the nucleus (F). In some cells, there was also accumulation of IP3R-I in the form of readily detectable globular structures in the vicinity of the nucleus and in part coincident with gE (E). In cells transfected with the plasmid encoding gI, the gI signal was weak and the IP3R-I was barely visible (G). Application of the auto-contrast feature of Photoshop software (H) showed that gI was expressed and that at least some of IP3R-I was partially aggregated but not to the level seen in cells transfected with the plasmid encoding gE. The most striking redistribution of IP3R-I was observed in cells cotransfected with plasmids encoding gE and gI. In these cells, gE was readily observed in or abutting the nuclear membrane and in association with globular material containing

FIG. 7. Subcellular localization of IP3R-I in HEp-2 cells transiently expressing gE, gI, or gE and gI. HEp-2 cells were either transfected with the empty vector MTS (pAc-CMV) (A), pAc-gE (E and F), or pAc-gI (G) or cotransfected with pAc-gE and pAc-gI (C and D), as detailed in Materials and Methods. At 24 h after transfection, the cells were fixed in 4% paraformaldehyde and doubly stained either with the mouse monoclonal antibody to gE and the rabbit polyclonal antibody to IP3R-I (A, C, D, E, and F) or with the mouse monoclonal antibody to gI and the rabbit polyclonal antibody to IP3R-I (G). All of the images were captured with a Zeiss confocal microscope, with the aid of the software provided by the manufacturer. The images in panels B and H are identical to those shown in panels A and G, except that they were manipulated with the auto-contrast feature of Photoshop software.

IP3R-I. A large number of globular structures containing IP3R-I, dispersed in the cytoplasm, did not overlap with gE.

We conclude that the redistribution of the IP3R-I correlated with the expression of gE and in particular in cells transfected with both gE and gI. Since gE alone and especially gE and gI function as Fc receptors, the results suggest that the redistribution of IP3R-I is linked to the expression of Fc receptor.

Finally, the observation that structures reacting with the IP3R-I antibody are distributed independently of gE indicates that these structures are not artifacts due to accumulations of Fc receptors.

DISCUSSION

The studies described in this report yielded two significant observations.

The first concerns both stable free calcium and histamine-sensitive calcium released from histamine-sensitive cytoplasmic stores. The stable, resting calcium levels decreased in wild-type virus-infected cells and in cells infected with most mutants between 8 and 12 h after infection. The decrease was less dramatic in VAX-3 cells than in HEp-2 cells. We cannot identify a specific viral function as a determinant of the levels of stable Ca^{2+} in infected cells.

Histamine induces the release of drug-sensitive calcium from IP3R containing cytoplasmic calcium stores by generating IP3, which binds and causes the opening of IP3R channels. The average peak calcium levels observed after histamine stimulation in HEp-2 cells between 3 and 16 h after infection were elevated relative to those of mock-infected cells. The increase was particularly striking in cells infected with ΔICP4 or ΔICP27 mutants. While they remained high in cells infected with ΔICP4 (Fig. 1) and to a lesser extent in the ΔgE mutant (Fig. 2), the responses to histamine stimulation in all other virus-infected cells were generally very low, indicative of depletion of histamine-sensitive calcium stores at late times (20 to 24 h) after infection. At this time, the cells exhibited widespread cytopathic effects.

In contrast to the events noted in infected HEp-2 cells, the histamine-sensitive calcium levels in infected VAX-3 cells did not differ in a measurable manner from those of mock-infected cells throughout the 24-h interval of infection.

The significance of these studies stems from the published evidence cited in the introduction that Bcl-2 maintains the levels of calcium stored in endoplasmic reticulum, in the face of fluctuation induced by proapoptotic members of the Bcl-2 family. Our results are consistent with these findings. Thus, the histamine-sensitive calcium pool is higher in HEp-2 cells infected with the proapoptotic HSV mutant ΔICP4 or ΔICP27 than in wild-type virus-infected cells, at the time when apoptosis is readily observed: that is, between 8 and 16 h after infection. Ultimately, however, the histamine-sensitive stored Ca^{2+} levels decreased in infected HEp-2 cells concomitant with the appearance of cytopathic effects. In VAX-3 cells, there was no increase or decrease in the levels of histamine-sensitive stored Ca^{2+} and no evidence of cytopathic effects even late in infection.

Two different pathways have been ascribed to Bcl-2 (10, 37). According to the first, Bcl-2 lowers the total endoplasmic reticulum Ca^{2+} pool so there is less Ca^{2+} to be released (12, 33, 35). The second pathway is that Bcl-2 does not affect the ER Ca^{2+} pool but instead does not allow as much Ca^{2+} to be released through the IP3R (4). Our results lead us to the conclusion that Bcl-2 blocks both the increase and depletion of stored calcium. To accomplish both events, Bcl-2 must regulate histamine-sensitive Ca^{2+} pools both positively and negatively: that is, Bcl-2 must maintain the structure and activity of cal-

cium channels and pumps irrespective of the signals that impinge on these calcium channels and pumps. We should also note that markers associated with apoptosis are not apparent even late in infection with wild-type virus. Our data are consistent with the hypothesis that the cytopathic effects observed late in infection that are also blocked by Bcl-2 are not caused by classical activation of procaspases.

The second significant observation concerned the distribution of IP3R-I. IP3R-I is a widely distributed member of the family of IP3 receptors (44). Its function is regulated by Ca^{2+} levels, by phosphorylation by a large number of protein kinases, and by its interactions with numerous proteins. Among them are the cytoskeletal proteins ankyrin, actin, talin, vinculin, myosin, and 4.1N; calcium-binding proteins calmodulin, CaBP/calendrin, and chromogranins; apoptosis-related proteins cytochrome *c*, caspase 3, and calpain; calcium channel proteins TRPC3, RhoA-TRPC1, and Na/K-ATPase; and cytosolic proteins IRBIT, Bcl-2, Bcl-X(L), Cdc2, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), etc. (3, 6, 34, 45). Our studies showed that IP3R-I protein changes its appearance and distribution as visualized by its interaction with antibody in HEp-2 or VAX-3 cells infected with wild-type virus. Briefly, the amount of fluorescent antigen increases and the protein appears to aggregate into relatively large amorphous masses, globular structures dispersed in the cytoplasm or abutting the nuclear membrane. Under a confocal microscope setting that allowed maximum resolution of these structures, the IP3R-I was barely visible. Since the total amount of IP3R-I protein remained unchanged throughout infection, the necessary conclusion is that in uninfected cells IP3R-I is in dense structures that limit accessibility of the antibody to the protein. In infected cells, the protein is likely to be dispersed or in loose aggregates and hence readily accessible to cognate antibody. An exception to these findings was cells infected with the ΔgE mutant: the vast majority of these cells resembled mock-infected cells.

In recent years, a large number of functions have been attributed to gE (7, 9, 16, 18, 38). The foremost property of gE, however, is that of an Fc receptor. While it acts as a receptor by itself, its affinity for IgG is much higher when it is complexed with gI. The ΔgE mutant was included in this study primarily on the basis of reports that in uninfected cells native Fc receptors affect the trafficking of membrane proteins (21, 22). To test the hypothesis that gE, and more specifically the combination of gE and gI required to form potent Fc receptors, can more efficiently restructure IP3R-I, cells were transfected with gE, gI, or gE plus gI. The results showed that gE induced redistribution of IP3R-I in some cells but that the combination of gE and gI was most effective in redistributing IP3R-I. The results indicate that gE play a key role in this redistribution and support the hypothesis that the redistribution is associated with the Fc functions of gE and gI.

Although the findings indicate a novel function of gE, the significance of the results remains to be defined. Thus, the redistribution of IP3R-I took place in both HEp-2 and VAX-3 cells and, therefore, it is not involved in apoptosis or development of cytopathic effects. However, while the stable resting calcium levels in cells infected with ΔgE mutant virus late in infection are lower than those observed in wild-type virus-infected cells, histamine-sensitive stored calcium is remarkably similar to that of wild-type virus-infected cells (Fig. 2, column

3). The results, therefore, would suggest that the redistribution of IP3R-I protein in infected cells enables fluctuation of the stored calcium, but further tests will be necessary to determine whether this is the case.

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