JOURNAL OF VIROLOGY, Jan. 2002, p. 743–754 Vol. 76, No. 2
0022-538X/02/$04.00+0 DOI: 10.1128/JVI.76.2.743–754.2002

U_S3 Protein Kinase of Herpes Simplex Virus 1 Blocks Caspase 3 Activation Induced by the Products of $U_S1.5$ and U_L13 Genes and Modulates Expression of Transduced $U_S1.5$ Open Reading Frame in a Cell Type-Specific Manner

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Received 25 July 2001/Accepted 11 October 2001

The coding domain of the herpes simplex virus type 1 (HSV-1) α22 gene encodes two proteins, the 420-amino-acid infected-cell protein 22 (ICP22) and $U_S1.5$, a protein colinear with the carboxyl-terminal domain of ICP22. In HSV-1-infected cells, ICP22 and $U_S1.5$ are extensively modified by the U_L13 and U_S3 viral protein kinases. In this report, we show that in contrast to other viral proteins defined by their properties as α proteins, $U_S1.5$ becomes detectable and accumulated only at late times after infection. Moreover, significantly more $U_S1.5$ protein accumulated in cells infected with a mutant lacking the U_L13 gene than in cells infected with wild-type virus. To define the role of viral protein kinases on the accumulation of $U_S1.5$ protein, rabbit skin cells or Vero cells were exposed to recombinant baculoviruses that expressed $U_S1.5$, U_L13, or U_S3 proteins under a human cytomegalovirus immediate-early promoter. The results were as follows. (i) Accumulation of the $U_S1.5$ protein was reduced by concurrent expression of the U_L13 protein kinase and augmented by concurrent expression of the U_S3 protein kinase. The magnitude of the reduction or increase in the accumulation of the $U_S1.5$ protein was cell type dependent. The effect of U_L13 kinase appears to be specific inasmuch as it did not affect the accumulation of glycoprotein D in cells doubly infected by recombinant baculoviruses expressing these genes. (ii) The reduction in accumulation of the $U_S1.5$ protein was partially due to proteasome-dependent degradation. (iii) Both $U_S1.5$ and U_L13 proteins activated caspase 3, indicative of programmed cell death. (iv) Concurrent expression of the U_S3 protein kinase blocked activation of caspase 3. The results are concordant with those published elsewhere (J. Munger and B. Roizman, Proc. Natl. Acad. Sci. USA 98:10410–10415, 2001) that the U_S3 protein kinase can block apoptosis by degradation or posttranslational modification of BAD.

This report deals with the interaction of three herpes simplex virus (HSV) proteins designated $U_S1.5$, U_L13, and U_S3. These proteins are not essential for viral replication for most cells in culture but they appear to play a critical role in experimental animal systems (25, 33–35, 36, 41). Their properties and events that led to these studies were as follows.

The α22 gene contains two discrete transcriptional units, each with its own promoter. The α22 mRNA initiates upstream from the open reading frame (ORF) encoding the 420-amino-acid protein infected-cell protein 22 (ICP22) and is spliced such that the first exon is in the 5′ untranslated region (24, 39, 42). The $U_S1.5$ promoter and coding domain are contained entirely within the coding domain of the larger ORF encoding the ICP22 protein (9). The $U_S1.5$ ORF is colinear with the middle and carboxyl-terminal domains of the ICP22 ORF. The available evidence indicates that the $U_S1.5$ protein is translated from the methionine codon 171 of the ICP22 ORF (A. P. W. Poon, W. O. Ogle, and B. Roizman, unpublished results). Both ORFs are transcribed in cells infected and maintained in the presence of cycloheximide, and both proteins are made upon withdrawal of the drug. On that basis, they have been classified as α proteins (9, 16, 17). ICP22 is extensively posttranslationally modified by two viral protein kinases encoded by the U_S3 and U_L13 genes and by unidentified cellular kinases, and ICP22 is also nucleotidylated by casein kinase II (1, 23, 26, 27, 31, 35, 36). U_L13 has also been shown to modify the $U_S1.5$ protein (31). Both an intact α22 gene and the U_L13 protein kinase are required for the expression of a subset of γ_2 viral genes expressed late in infection exemplified by the products of the U_S11 and U_L38 ORFs (31, 35). The accumulation of U_S11 and U_L38 proteins in cells infected with a mutant virus expressing the $U_S1.5$ protein but not ICP22 is similar to that observed in wild-type virus infection, and posttranslational modification of $U_S1.5$ by U_L13 is required for this accumulation of γ_2 gene products (31). Additional evidence supporting the conclusion that $U_S1.5$ mediates the accumulation of this subset of γ_2 gene products is that posttranslational processing of ICP22 is not essential for accumulation of U_S11 or U_L38 proteins (32).

However, the mutant virus which expresses $U_S1.5$ protein but not ICP22 is highly attenuated in mice, suggesting that the sequences unique to ICP22 perform functions other than those of sequences shared by ICP22 and $U_S1.5$ (31). Furthermore, the insertion of a 20-codon linker at codon 200 or 240 of ICP22 had no apparent effect on functions associated with ICP22 and $U_S1.5$ (9).

As evidence has emerged that the function of these proteins is determined by the extent of posttranslational modification (31), one objective of the studies on these proteins was to map the sites of phosphorylation by both cellular and viral kinases.

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The phenotype of a mutant virus with a defective U_L13 gene is very similar to that of a virus with a defective α22 gene (35), suggesting that modification of ICP22 and/or $U_S1.5$ is an important function of U_L13 in the viral life cycle. Initial attempts to map these proteins by mutagenesis led to the discovery that amino acid substitution near the amino terminus of ICP22 can preclude the phosphorylation of ICP22 at sites near its carboxyl terminus (32). One alternative was to produce the proteins in isolation in mammalian cells and identify the sites by other means.

The U_L13 protein kinase targets a large number of both viral and cellular substrates (reviewed in reference 40). As noted above, U_L13 protein kinase appears to regulate the function of viral proteins. The protein kinase mediates the hyperphosphorylation of the translation initiation factor 1δ and the activation of cdc25C phosphatase (2, 20).

Until recently, little was known about U_S3 protein kinase other than a limited number of proteins whose posttranslational modification appears to be mediated by this enzyme (reviewed in reference 40). The connection to apoptosis was based on studies of the *d*120 mutant, in which both copies of the α4 gene encoding ICP4, the major regulatory protein of the virus, had been deleted (11). This mutant induced apoptosis in all cell lines tested (14, 15, 21). A recombinant virus derived by restoration of the α4 genes also induced programmed cell death, and only coexpression of a fragment encoding the U_S3 gene blocked apoptosis (22, 28). The apparent role of U_S3 in blocking apoptosis was confirmed in other studies. Whereas a wild-type virus blocked apoptosis induced by UV light irradiation or anti-fatty acid synthase (FAS) antibody, a mutant lacking the U_S3 gene failed to do so (19). In vivo, the U_S3 gene is required for the protection of corneal epithelial cells and primary afferent neurons from apoptosis in infected mice (3, 4). The mechanism by which U_S3 blocks apoptosis emerged in two recent studies. In the first, U_S3 provided in *trans* blocked apoptosis induced by the *d*120 mutant described above at a premitochondrial stage consistent with evidence that apoptosis was also blocked by overexpression of Bcl-2 (15, 28). The second and more recent study showed that the U_S3 protein kinase blocks apoptosis induced by BAD, a proapoptotic member of the Bcl-2 family of proteins (29).

To express the $U_S1.5$ protein in mammalian cells with high efficiency, we cloned the ORF in baculoviruses under the human cytomegalovirus immediate-early promoter. The objective of the studies was to isolate the unmodified forms as well as the forms posttranslationally modified by either the U_S3 or U_L13 protein kinases whose genes were also cloned in baculoviruses.

We report that whereas U_S3 augmented the amounts of $U_S1.5$ that accumulated in Vero cells exposed to recombinant baculoviruses expressing both U_S3 and $U_S1.5$, the accumulation of $U_S1.5$ protein was significantly reduced in cells doubly infected with baculoviruses expressing $U_S1.5$ and U_L13. The results indicate that cells infected with $U_S1.5$ or U_L13 contain activated caspase 3 characteristic of apoptosis and that the U_S3 protein kinase effectively blocks the activation of caspase 3, as would be predicted from its effectiveness in blocking apoptosis in infected cells.

MATERIALS AND METHODS

Cells and viruses. Vero cells were obtained from the American Type Culture Collection (Manassas, Va.), and rabbit skin cells were originally obtained from J. McClaren. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (rabbit skin cells) or 5% newborn calf serum (Vero cells). Insect cell line Sf9 (*Spodoptera frugiperda*) was obtained from PharMingen (San Diego, Calif.). HSV-1(F) is the prototype HSV-1 strain used in this laboratory (13). The construction of HSV-1 recombinant viruses R7041 (ΔU_S3), R7353 ($\Delta U_L13/\Delta U_S3$), and R7356 (ΔU_L13) was previously described (34–36). Wild-type baculovirus was obtained from PharMingen. The construction of recombinant baculoviruses Bac-glycoprotein D (Bac-gD) and Bac-U_S3 was described elsewhere (28, 29, 44).

Plasmids. pRB5252 contains the entire ICP22 ORF in vector pUC19 and thus contains $U_S1.5$ (32). The DNA fragment containing the putative $U_S1.5$ ORF (amino acids 171 to 420) was generated by PCR using pRB5252 as the template and primers AP1 (GGG AAT TCA TGC TAC GGC GCT CGG TG) and AP2 (ACG CGT CGA CCT ACG GCC GGA GAA A). AP1 included in the final product restriction site *Eco*RI (underlined) at the 5′ end of the $U_S1.5$ ORF, and AP2 incorporated a *Sal*I site (underlined) at the 3′ end. The *Eco*RI/*Sal*I-digested PCR product was purified and subcloned into the *Eco*RI-*Sal*I site of vector pGex 4T-1 (Amersham Pharmacia Biotech, Piscataway, N.J.). The resulting plasmid was designated pRB5450.

Baculovirus transfer vectors. pRB5450 was digested with *Eco*RI and *Not*I, and the resulting fragment containing the $U_S1.5$ ORF was purified and subcloned into the *Eco*RI-*Not*I site of the previously described baculovirus transfer vector pAc-CMV (44). The resulting plasmid was designated pAc-$U_S1.5$. pRB5151 contains the entire U_L13 coding sequence in the pGex 4T-1 vector (30). pRB5151 was digested with *Eco*RI and *Not*I, and the resulting fragment containing the U_L13 ORF was purified and subcloned into the *Eco*RI-*Not*I site of pAc-CMV. The resulting plasmid was designated pAc-U_L13.

Generation of recombinant baculovirus. Bac-$U_S1.5$ and Bac-U_L13 were generated using the PharMingen baculovirus expression vector system by cotransfecting transfer vectors pAC-$U_S1.5$ and pAC-U_L13, respectively, along with BaculoGold linearized baculovirus DNA (PharMingen) into Sf9 cells according to the manufacturer's instructions. Viruses were propagated in Sf9 cells grown in 20 ml of TNM-FH insect cell medium (PharMingen) in 150-cm^2 flasks. The supernatant containing virus was harvested and cleared by centrifugation at 1,000 rpm for 5 min at 4°C.

Preparation of cell lysates, electrophoretic separation, and immunoblotting of viral proteins from HSV-1-infected cells. Replicate cultures of Vero cells or rabbit skin cells in 25-cm^2 flasks were either mock infected or exposed to 5 PFU of virus per cell and maintained at 37°C in medium 199V (medium 199 supplemented with 1% calf serum). Cells were harvested at the indicated time after infection, washed three times with phosphate-buffered saline (PBS), and then solubilized in 200 μl of disruption buffer (50 mM Tris-HCl [pH 7], 2% sodium dodecyl sulfate [SDS], 710 mM β-mercaptoethanol, 3% sucrose). After 50-μl aliquots of lysate were boiled for 5 min, solubilized proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 11% bisacrylamide gels, transferred to nitrocellulose sheets, blocked with 5% nonfat milk, reacted with primary antibody followed by appropriate secondary antibody conjugated to alkaline phosphatase (Bio-Rad Laboratories, Hercules, Calif.), and visualized according to the manufacturer's instructions with BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium) (Sigma).

Exposure of mammalian cells to recombinant baculovirus. Subconfluent cultures of rabbit skin in 25-cm^2 flasks were incubated with baculovirus in a total volume of 1 ml for 1 h at 37°C. Then, 500 μl of baculovirus stock was used for each virus per infection unless otherwise indicated. For single infections, the volume was brought up to 1 ml with 199V. Culture medium was then replaced with fresh DMEM containing 5% newborn calf serum and 10 mM sodium butyrate unless otherwise indicated and was incubated at 37°C for 24 h. For experiments involving MG132 treatment, culture medium was replaced with fresh DMEM containing 5% newborn calf serum, 10 mM sodium butyrate, and 5 μM MG132 (BioMol, Plymouth Meeting, Pa.) at the indicated time before harvesting at 24 h after infection.

One-dimensional electrophoretic analysis of recombinant HSV-1 proteins. Cells infected with recombinant baculovirus were scraped, washed twice with PBS, and lysed in PBS-A* (1% Nonidet P-40 and 1% deoxycholate in PBS) containing 0.1 mM TLCK (*N*α-*p*-tosyl-L-lysine chloromethyl ketone), 0.1 mM TPCK (tosylsulfonyl phenylalanyl chloromethyl ketone), 1 mM phenylmethylsulfonyl fluoride, Pefabloc SC (1 mg/ml; Boehringer Mannheim, Mannheim, Germany), EDTA (0.5 mg/ml), leupeptin (10 μg/ml), pepstatin (10 μg/ml), aprotinin (1 μg/ml), and E-64 (10 μg/ml; Boehringer Mannheim) or PBS-A* in

which complete, Mini, EDTA-free protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany) had been dissolved according to the manufacturer's instructions. The amount of total protein in the lysate was quantified using the Bio-Rad Protein Assay according to the manufacturer's instructions. Solubilized proteins were subjected to SDS-PAGE on 12% bisacrylamide gels. Immunoblotting was performed as described above.

Two-dimensional electrophoretic analysis of recombinant HSV-1 proteins. Two-dimensional electrophoresis was performed using an immobilized pH gradient (IPG) for first-dimension isoelectric focusing (8). A protocol previously described (7) was modified. Briefly, rabbit skin cells infected with recombinant baculovirus were washed twice with PBS and lysed in 80 μl of lysis solution {8 M urea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 40 mM Tris base} containing 0.1 mM TLCK, 0.1 mM TPCK, 1 mM phenylmethylsulfonyl fluoride, Pefabloc SC (1 mg/ml; Boehringer Mannheim), EDTA (0.5 mg/ml), leupeptin (10 μg/ml), pepstatin (10 μg/ml), aprotinin (1 μg/ml), and E-64 (10 μg/ml, Boehringer Mannheim). The extract was kept on ice for 1 h and then sonicated, and the insoluble material was pelleted by centrifugation. The soluble fraction was transferred to a new tube, and 170 μl of rehydration stock solution (8 M urea, 2% CHAPS, 20 mM dithiothreitol [DTT], bromophenol blue) was added to the sample for a total volume of 250 μl. pH 3 to 10 L IPG Buffer (Amersham Pharmacia Biotech catalog no. 17-6000-87) was added for a final concentration of 0.5%. First-dimension isoelectric focusing (IEF) on 13-cm Immobiline DryStrip gels (linear pH 3 to 10 gradient) (Amersham Pharmacia Biotech catalog no. 17-6001-14) was performed using an IPGphor Isoelectric Focusing System (Amersham Pharmacia Biotech). IPG strips were rehydrated in the sample solution for 12 h and subjected to the following procedures: 500 V for 1 h, 1,000 V for 1 h, and 8,000 V for 2 h for a total of 17,500 V h at 20°C. IEF gels were then equilibrated for 15 min in SDS equilibration buffer (50 mM Tris-HCl [pH 8.8], 6 M urea, 30% glycerol, 2% SDS, 10 mg of DTT/ml, bromophenol blue). Equilibrated strips were overlaid onto 12% bisacrylamide gels and sealed with agarose sealing solution (0.5% agarose, 25 mM Tris base, 192 mM glycine, 0.1% SDS, bromophenol blue). Molecular weight markers were placed adjacent to the pH 10 end of the IPG strip on an IEF electrode strip (Amersham Pharmacia Biotech catalog no. 18-1004-40). Second-dimension SDS-PAGE gels were subjected to 20 mA for the first hour, followed by 30 mA. Immunoblotting of electrophoretically separated proteins was performed as described above.

Antibodies. Rabbit polyclonal antibody W2 made against the carboxyl terminus of ICP22/$U_S1.5$ was described elsewhere (23) and used at a 1:3,000 dilution for Western blotting. Mouse monoclonal antibody against gD (clone H170) purchased from the Rumbaugh-Goodwin Cancer Research Institute (Plantation, Fla.) was described elsewhere (44) and was used at a 1:2,500 dilution for Western blotting.

Measurement of DEVDase activity. Caspase 3 activity in cellular extracts was assayed using a tetrapeptide conjugated to phenylnitraniline (DEVD-pNA) (Bio-Mol). Rabbit skin cells grown in 25-cm^2 flasks were either mock infected or infected with the indicated recombinant baculovirus or combination of baculoviruses as described above. At 24 h after infection, the cells were scraped in their medium, washed twice with PBS, resuspended in 150 μl of lysis solution (0.1% CHAPS, 50 mM HEPES [pH 7.4], 1 mM DTT, 0.1 mM EDTA), and incubated on ice for 5 min. Lysates were then centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant was collected and analyzed for DEVDase activity according to the manufacturer's instructions. Chromophore release was quantified by measuring absorbance at 405 nm with a spectrophotometer after 2 h.

RESULTS

Kinetics of accumulation of $U_S1.5$ protein. Replicate cultures of Vero or rabbit skin cells were exposed to 5 PFU of HSV-1(F) per cell. In the experiment shown in Fig. 1, the cells were harvested at 2, 4, 6, 9, 12, 15, or 18 h after infection whereas the rabbit skin cells were harvested at 3, 6, 9, 11, or 24 h after infection. The harvested cells were solubilized, and the proteins were subjected to electrophoresis in denaturing gels and then reacted with rabbit polyclonal antibody directed to ICP22/$U_S1.5$ protein as described in Materials and Methods. The results were as follows: ICP22 appeared as a faint band in lysates of Vero cells harvested at 2 h after infection (Fig. 1, lane 2). Fully processed forms of ICP22 were detected in lysates of cells harvested at 4 h after infection (Fig. 1, lane 3). From 6 to 18 h after infection, the amounts of ICP22 present in cell lysates increased, but very gradually, suggesting that the peak rates of synthesis were between 2 and 6 h after infection (Fig. 1). In contrast, $U_S1.5$ was barely detectable at 2 h after infection and continued to increase in amount until 18 h after infection (Fig. 1, lane 8). In other experiments (data not shown), we found that $U_S1.5$ continued to increase for as long as 30 h after infection. In rabbit skin cells, both ICP22 and $U_S1.5$ protein was detected at 3 h after infection. In these cells, there was no significant increase in accumulation of either protein past 6 h after infection (Fig. 1, lanes 11 to 14).

Viral protein kinases negatively regulate $U_S1.5$ levels in infected cells. In this series of experiments, replicate cultures of Vero or rabbit skin cells were exposed to 5 PFU of wild-type HSV-1(F) or mutants lacking U_L13 (R7356), U_S3 (R7041), or both U_L13 and U_S3 ORFs (R7353) per cell. The cells were harvested at 18 h after infection and processed as described in Materials and Methods. The results were as follows. (i) As expected, both U_L13 and U_S3 protein kinases posttranslationally modify ICP22 (Fig. 2 and references 35 and 36). Furthermore, as expected on the basis of earlier reports from this laboratory, the presence and accumulation of slow-migrating forms of ICP22 were affected by the absence of one or both protein kinases (Fig. 2). As the absence of the U_L13 kinase has a more pronounced effect ablating slow-migrating forms than the absence of U_S3 alone and appears to be responsible for the loss of most of the forms when both kinases are absent (Fig. 2, compare lanes 3, 4, and 5 and lanes 8, 9, and 10), these data confirm previous results showing that the U_L13 protein kinase mediates more extensive modification of ICP22 in infected cells than the U_S3 protein kinase (Fig. 2 and references 35 and 36).

(ii) As described elsewhere, the U_L13 protein kinase also posttranslationally modifies $U_S1.5$ (31). In Vero cells infected with HSV-1(F), the $U_S1.5$ protein was detected as a faint, slow-migrating band with an apparent M_r of approximately 50,000 (Fig. 2, lane 3). The $U_S1.5$ protein was most abundant and formed numerous faster-migrating bands in cells infected with $\Delta U_L13/\Delta U_S3$ mutant virus (Fig. 2, lane 5), and it was intermediate in abundance and also in electrophoretic mobility in lysates of cells infected with the ΔU_S3 mutant virus (Fig. 2, lane 2). Cells infected with the ΔU_L13 mutant formed a somewhat abundant band with an M_r of approximately 43,000 and a small number of faint bands migrating more slowly (Fig. 2, lane 4). In Vero cells, rapidly migrating bands observed in lysates of cells infected with the ΔU_L13 virus which are not present in cells infected with wild-type virus (Fig. 2, compare lanes 3 and 4) reflect the lack of modification of $U_S1.5$ by U_L13. Such rapidly migrating bands are present to a lesser extent and do not migrate as rapidly in ΔU_S3 virus infection (Fig. 2, lane 2) and are more prominent in lysates of cells infected with the $\Delta U_L13/\Delta U_S3$ mutant virus than with the single ΔU_L13 kinase deletion mutant (Fig. 2, compare lanes 4 and 5). These data suggest that the U_S3 protein kinase mediates posttranslational modification of the $U_S1.5$ protein, albeit to a lesser extent than the U_L13 protein kinase.

(iii) The $U_S1.5$ protein was barely detectable in rabbit skin cells infected with the wild-type virus or ΔU_S3 mutant. In cells infected with the ΔU_L13 or $\Delta U_L13/\Delta U_S3$ mutant, $U_S1.5$

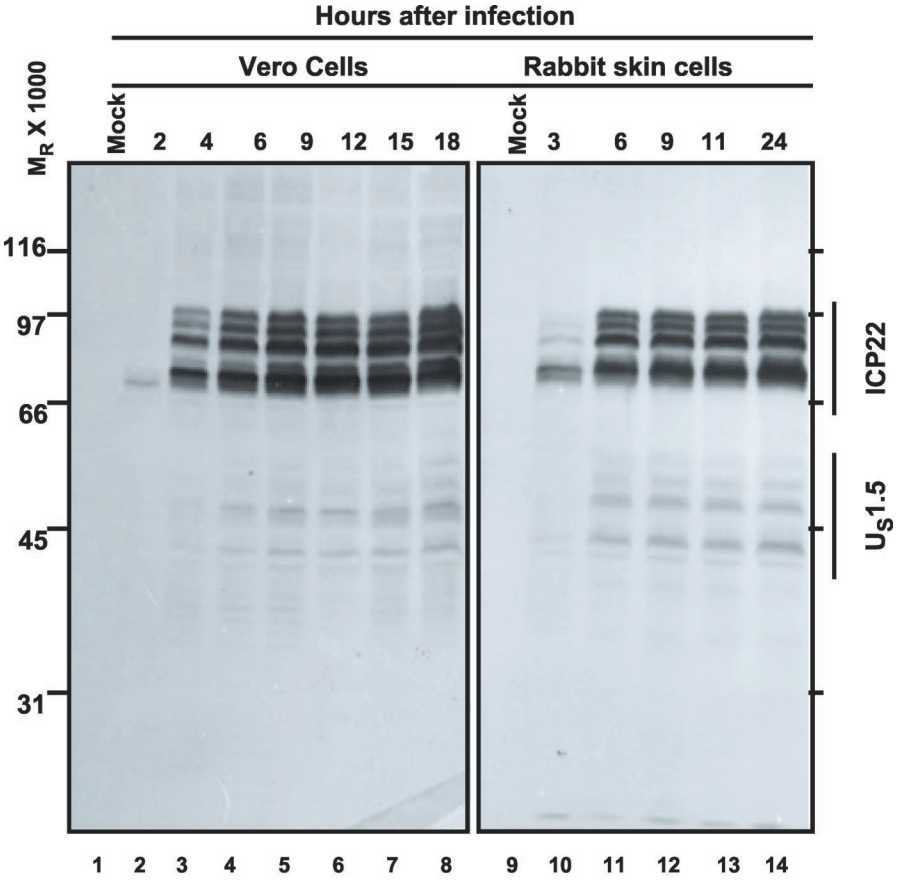

FIG. 1. Accumulation of ICP22/U_S1.5 proteins in HSV-1(F)-infected cells. Replicate cultures of Vero cells or rabbit skin cells were exposed to 5 PFU of HSV-1(F) per cell. Cells were harvested at the indicated times after infection, solubilized, subjected to SDS-PAGE, transferred to nitrocellulose sheets, and reacted with rabbit polyclonal antibody directed to the carboxyl-terminal domain of ICP22/U_S1.5 proteins as described in Materials and Methods.

formed a single band with an apparent M_r of 43,000 (Fig. 2, lanes 9 and 10).

We conclude from these studies that the accumulation of U_S1.5 protein is regulated directly or indirectly by viral protein kinases. The amounts of U_S1.5 were lowest in lysates of cells infected with wild-type virus. The effect of the kinases appeared to be cell type dependent. In infected Vero cells, both U_S3 and U_L13 kinases mediated an inhibitory effect on U_S1.5 accumulation which was additive, whereas in rabbit skin cells, the repressive effect appeared to be mediated primarily by the U_L13 protein kinase.

U_S1.5 protein is expressed in cells infected with recombinant baculovirus carrying U_S1.5 ORF. Both U_S1.5 and ICP22 have been shown to be phosphorylated by the HSV protein kinases. To investigate more closely the mechanisms by which viral protein kinases affect the accumulation of the U_S1.5 protein, it was necessary to express U_S1.5 and the protein kinases independently of other viral proteins. To this end, the sequence encoding the U_S1.5 ORF driven by the cytomegalovirus (CMV) immediate-early promoter was cloned into a baculovirus. In the experiments illustrated in Fig. 3, rabbit skin cells were exposed to approximately 10 PFU of baculovirus per cell and incubated in the presence or absence of sodium butyrate. Efficient expression of baculovirus genes in mammalian cells has previously been shown to require treatment with sodium butyrate, a histone deacetylase inhibitor (10). The cells were harvested 24 h after infection and solubilized, and the proteins were electrophoretically separated in a denaturing gel and probed with antibody to ICP22/U_S1.5 protein. The results shown in Fig. 3 indicate that the U_S1.5 protein readily accumulated in rabbit skin cells infected with the recombinant baculovirus and maintained in the presence of sodium butyrate. We should note, however, that the U_S1.5 expressed by baculoviruses in Vero cells or rabbit skin cells migrated significantly faster than that expressed in cells infected with wild-type or mutant viruses (compare Fig. 1 and 2 with Fig. 3). The results suggest that U_S1.5 protein made in HSV-1-infected cells undergoes posttranslational modifications other than those mediated by the two viral protein kinases and which were not observed in cells exposed to the recombinant baculoviruses expressing the U_S1.5 ORF. These modifications are likely due to modulation of the activity of cellular enzymes by HSV-1 infection which does not occur when U_S1.5 is transduced in the absence of other viral proteins.

Accumulation of U_S1.5 protein is differentially modulated by viral protein kinases in cells exposed to recombinant baculoviruses expressing U_S1.5 and each of the viral protein kinases. The purpose of this series of experiments was to determine the

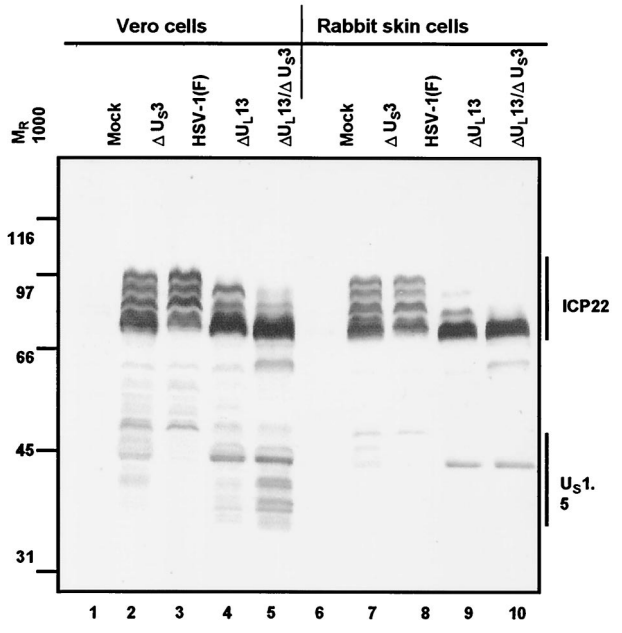

FIG. 2. Accumulation of ICP22/$U_S1.5$ proteins in cells infected with wild-type and mutant viruses. Replicate cultures of Vero cells or rabbit skin cells were exposed to 5 PFU of HSV-1(F) or mutants lacking U_S3 (R7041), or U_L13 (R7356) or both U_L13 and U_S3 (R7353) genes per cell. Cells were harvested at 18 h after infection and processed as described in the legend to Fig. 1.

effects of protein kinases on the accumulation of $U_S1.5$ protein in the absence of other viral proteins. To this end, replicate cultures of Vero cells or rabbit skin cells were exposed to approximately 10 PFU of baculovirus encoding $U_S1.5$ (Bac-$U_S1.5$) per cell alone or in combination with baculoviruses encoding U_S3 (Bac-U_S3) or U_L13 (Bac-U_L13) genes (10 PFU of each per cell) in the presence or absence of the proteasomal inhibitor MG132. Two series of experiments were done.

In the first series, cells were harvested at 24 h after infection and solubilized, and lysate containing 100 μg of total protein from each sample was subjected to electrophoresis in a denaturing polyacrylamide gel, transferred to nitrocellulose sheets, and reacted with antibody to the ICP22/$U_S1.5$ protein (Fig. 4). In the second series of experiments, the baculovirus-infected cell lysates were subjected to two-dimensional separation and reacted with the same antibody. The results shown in Fig. 5A (rabbit skin cells) and Fig. 5B (Vero cells) were as follows.

(i) Two-dimensional electrophoretic separations of lysates of rabbit skin cells and Vero cells infected with Bac-$U_S1.5$ show that $U_S1.5$ protein formed multiple isoforms with different isoelectric points (pI) (Fig. 5A and B, panel A). This observation indicates that $U_S1.5$ is posttranslationally modified (likely by phosphorylation) by cellular enzymes in the absence of any other viral protein expression.

(ii) The amounts of $U_S1.5$ protein expressed in Vero cells or in rabbit skin cells doubly infected with Bac-$U_S1.5$ and Bac-U_L13 were significantly smaller than those in cells infected with Bac-$U_S1.5$ only. This effect of U_L13 transduction on $U_S1.5$ protein expression was more pronounced in rabbit skin cells than in Vero cells and was observed in both one-dimensional separations (Fig. 4, compare lanes 2 and 4 and lanes 9 and 11) and two-dimensional separations (Fig. 5A and B, compare panels A and B).

(iii) A central question arising from the results described above was whether the U_L13 protein kinase mediates the proteasome-dependent degradation of the $U_S1.5$ protein. As shown in Fig. 4, the incubation of infected rabbit skin cells and Vero cells in medium containing the proteasome inhibitor MG132 for 6 h prior to harvesting increased the amounts of $U_S1.5$ protein in cells doubly infected with Bac-$U_S1.5$ and Bac-U_L13 compared to cells cultured in medium without MG132 (compare lanes 4 and 5 and lanes 11 and 12). The effect of MG132 was more pronounced in Vero cells than in rabbit skin cells but was apparent in rabbit skin cells, especially with regard to the low-molecular-weight isoform. The increase

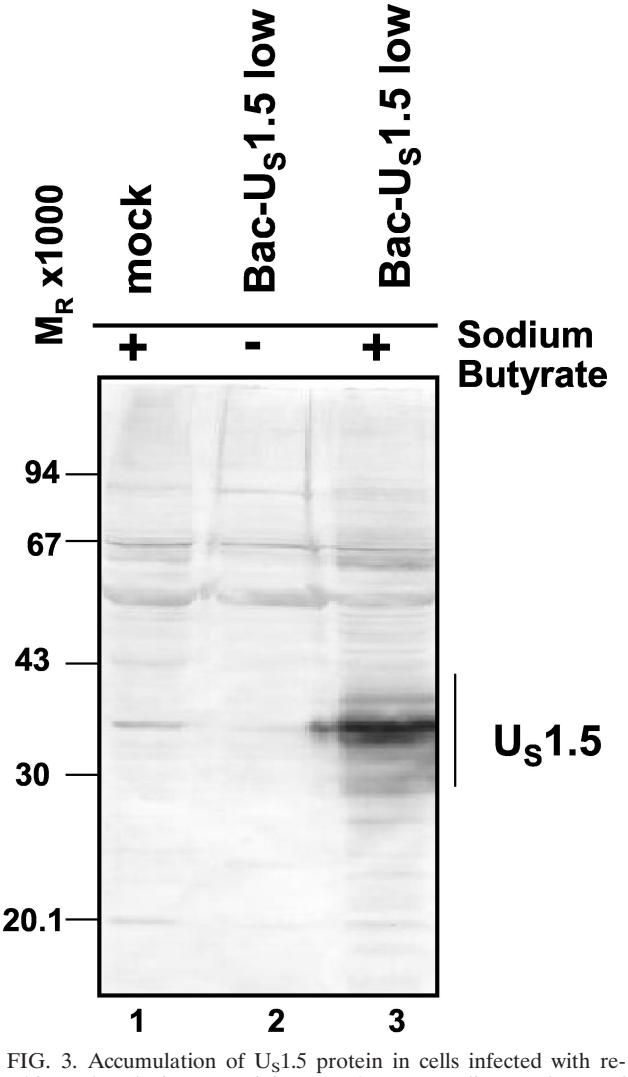

FIG. 3. Accumulation of $U_S1.5$ protein in cells infected with recombinant baculovirus containing $U_S1.5$ gene. Replicate cultures of rabbit skin cells were either mock infected or exposed to approximately 5 PFU of Bac-$U_S1.5$ per cell and cultured in the presence or absence of 10 mM sodium butyrate as indicated. Cells were harvested 24 h after baculovirus infection, solubilized, subjected to electrophoresis in denaturing polyacrylamide gels, transferred to nitrocellulose sheets, and reacted with rabbit polyclonal antibody directed against the carboxyl-terminal domain of ICP22/$U_S1.5$ proteins as described in Materials and Methods.

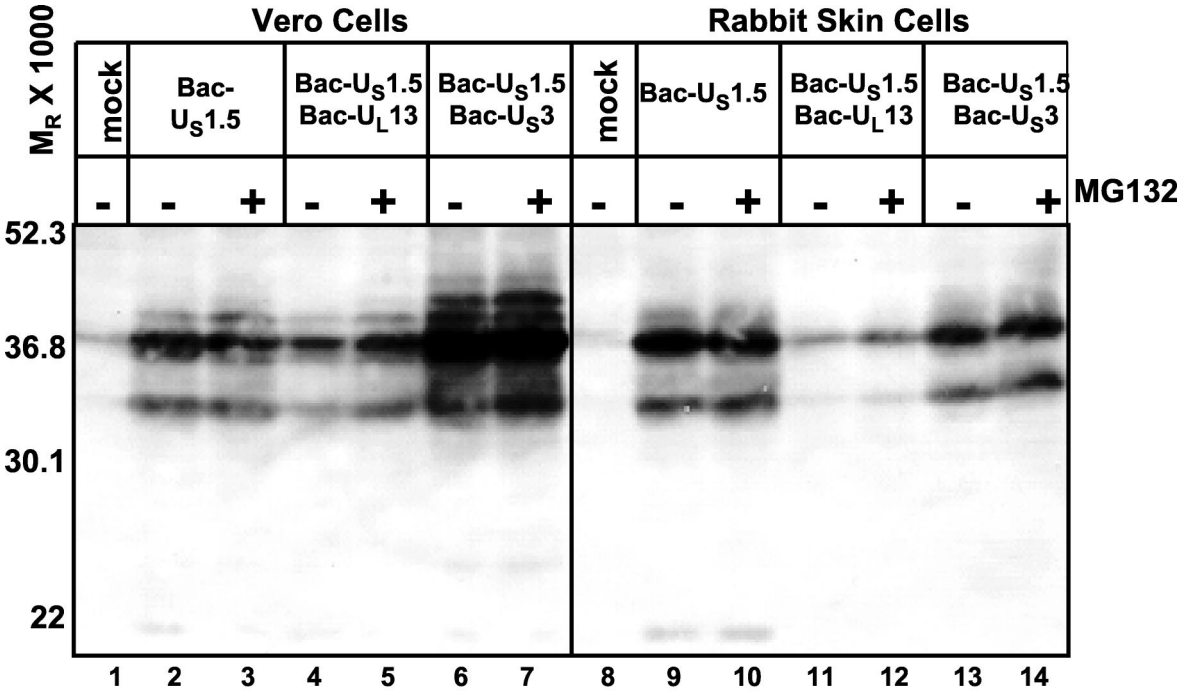

FIG. 4. Effect of viral kinase expression on accumulation of U_S1.5 protein expressed via recombinant baculovirus in Vero cells or rabbit skin cells. Replicate cultures were either mock infected or infected with approximately 10 PFU of the indicated baculovirus or baculoviruses and cultured in the presence of 10 mM sodium butyrate. Cells were harvested 24 h after baculovirus infection. Six hours prior to harvesting, the cultures were replenished with fresh medium containing 5 μM MG132 and 10 mM sodium butyrate where indicated. Cells were then solubilized, and lysates containing 100 μg of total protein were subjected to electrophoresis in denaturing polyacrylamide gels, transferred to nitrocellulose sheets, and reacted with rabbit polyclonal antibody directed against the carboxyl-terminal domain of ICP22/U_S1.5 proteins.

in the amounts of U_S1.5 protein in rabbit skin cells and Vero cells doubly infected with Bac-U_S1.5 and Bac-U_L13 was also apparent on two-dimensional separations of the cell lysates (Fig. 5A and B, compare panels B and C). This result indicates that U_L13 expression effects proteasome-dependent degradation of the U_S1.5 protein. If U_L13 targets U_S1.5 for proteasome-dependent degradation, U_S1.5 protein would have accumulated only in cells transduced with U_S1.5 and U_L13 for the period of MG132 treatment while the protein would have accumulated for the entire duration of baculovirus infection when cells were transduced with U_S1.5 alone. Therefore, the observation that MG132 treatment of cells transduced with U_S1.5 and U_L13 resulted in less U_S1.5 protein accumulation than transduction of U_S1.5 alone (Fig. 4, compare lanes 1 and 5 and lanes 9 and 12; Fig. 5A and B, compare panels A and C) is consistent with and supports this conclusion.

(iv) Treatment of rabbit skin cells coinfected with Bac-U_S1.5 and Bac-U_L13 with MG132 for 2 h prior to harvesting increased the accumulation of U_S1.5 compared to untreated coinfected cells, but less U_S1.5 was present compared to coinfected cells treated with MG132 for 6 h before harvesting (data not shown). This result suggests that U_S1.5 protein is constitutively synthesized and degraded in a proteasome-dependent manner in cells coinfected with Bac-U_S1.5 and Bac-U_L13 and accumulates when the proteasome-dependent degradation machinery is inactivated.

(v) Low levels of a highly electronegative form of U_S1.5 were present in rabbit skin cells coinfected with Bac-U_S1.5 and Bac-U_L13, a form that was not present in cells infected with Bac-U_S1.5 alone (Fig. 5A, compare panels A and B). This highly electronegative U_S1.5 isoform was very prominent in lysates of rabbit skin cells coinfected with Bac-U_S1.5 and Bac-U_L13 which were exposed to MG132 (Fig. 5A, panel C). This isoform was also faintly visible in two-dimensional separations of lysates of Vero cells coinfected with Bac-U_S1.5 and Bac-U_L13 and treated with MG132 but was not detectable in untreated coinfected cells (Fig. 5B, panels B and C). The appearance of a highly electronegative U_S1.5 isoform in lysates of treated cells when the U_L13 protein kinase is also transduced suggests more extensive phosphorylation of U_S1.5. It is of note that the observed effect of MG132 was more pronounced in two-dimensional separations than in one-dimensional separations because the electronegative isoform is not resolved by conventional one-dimensional SDS-PAGE. Accumulation of electronegative isoforms specifically when the proteasome is inactive indicates that the U_L13 protein kinase posttranslationally modifies the U_S1.5 protein and U_S1.5 isoforms modified by U_L13 are extensively degraded in a proteasome-dependent manner.

(vi) In rabbit skin cells coinfected with Bac-U_S1.5 and Bac-gD, a recombinant baculovirus expressing the HSV-1 glycoprotein D (gD), U_S1.5 accumulated to the same level as in cells infected with Bac-U_S1.5 alone (data not shown), suggesting that the negative effect of coinfection of Bac-U_L13 with Bac-U_S1.5 on U_S1.5 expression is specifically mediated by the U_L13 gene and is not due to competition with Bac-U_S1.5 for cellular receptors during viral entry or for transcription factors which drive expression from the CMV promoter.

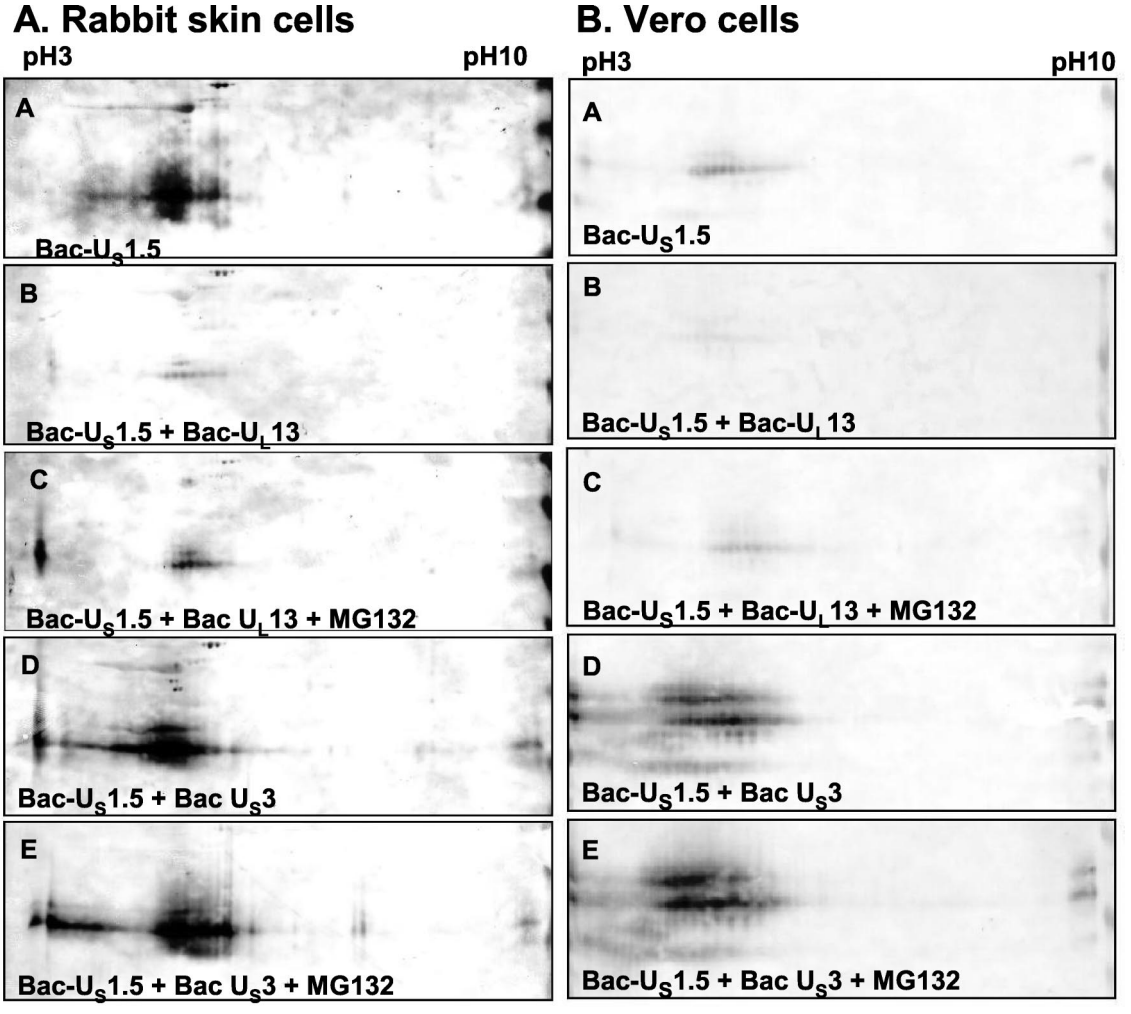

FIG. 5. Effect of viral protein kinase expression on posttranslational modification and accumulation of $U_S1.5$ protein expressed via recombinant baculovirus in rabbit skin cells (A) or Vero cells (B). Replicate cultures were either mock infected or infected with approximately 10 PFU of the indicated baculovirus or baculoviruses and maintained in the presence of 10 mM sodium butyrate. Cells were harvested 24 h after baculovirus infection. Six hours prior to harvesting, the cultures were replenished with fresh medium containing 5 μM MG132 and 10 mM sodium butyrate. Cells were solubilized in two-dimensional gel lysis solution, and two-dimensional electrophoretic separations were done as described in Materials and Methods. Electrophoretically separated proteins were transferred to nitrocellulose sheets and reacted with rabbit polyclonal antibody directed against the carboxyl-terminal domain of ICP22/$U_S1.5$ proteins.

(vii) Treatment with MG132 did not affect $U_S1.5$ accumulation in rabbit skin cells and Vero cells infected with Bac-$U_S1.5$ (Fig. 4, compare lanes 2 and 3 and lanes 9 and 10), suggesting that $U_S1.5$ is not constitutively degraded in a proteasome-dependent manner to a significant extent in the absence of U_L13 expression.

(viii) The amounts of $U_S1.5$ protein expressed in Vero cells doubly infected with Bac-$U_S1.5$ and Bac-U_S3 were vastly greater than those in cells infected with Bac-$U_S1.5$ only. This was observed in both one-dimensional separations (Fig. 4, compare lanes 2 and 6) and two-dimensional separations (Fig. 5B, compare panels A and D). It is noteworthy that in the lysates of cells doubly infected with Bac-$U_S1.5$ and Bac-U_S3, $U_S1.5$ protein formed multiple bands of differential electrophoretic mobilities indicative of posttranslational processing. In rabbit skin cells, the total amounts of $U_S1.5$ protein present in lysates of cells singly infected with Bac-$U_S1.5$ or both Bac-$U_S1.5$ and Bac-U_S3 were approximately the same (Fig. 4, compare lanes 9 and 13, and Fig. 5A, compare panels A and D). A notable difference is that in rabbit skin cells, the $U_S1.5$ protein formed multiple bands differing in electrophoretic mobility on two-dimensional separations, suggesting that it was posttranslationally modified in both the absence and presence of U_S3 protein kinase (Fig. 5A, panels A and D). However, highly electronegative forms of $U_S1.5$ which were not present in cells infected with Bac-$U_S1.5$ alone were observed in rabbit skin cells and Vero cells coinfected with Bac-$U_S1.5$ and Bac-U_S3 (Fig. 5A and B, compare panels A and D). The presence of these electronegative forms is indicative of a more extensive modification of $U_S1.5$, which is likely phosphorylation in cells coinfected with Bac-$U_S1.5$ and Bac-U_S3 compared to cells infected with Bac-$U_S1.5$ alone. Thus, these data suggest that U_S3 protein kinase mediates the posttranslational modification of transduced $U_S1.5$ protein in both rabbit skin cells and Vero cells.

(ix) MG132 treatment had no effect on the levels or post-

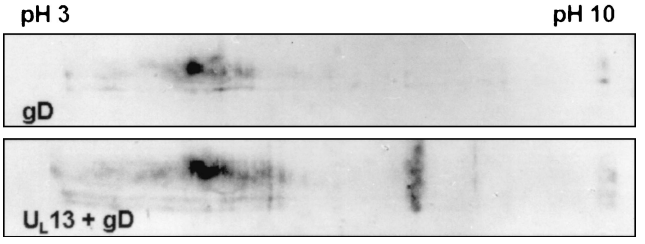

FIG. 6. Effect of U_L13 protein kinase expression on accumulation of gD expressed via recombinant baculovirus. Replicate cultures were either mock infected or infected with approximately 10 PFU of Bac-gD alone (top) or approximately 10 PFU each of Bac-gD and Bac-U_L13 (bottom) and were cultured in the presence of 10 mM sodium butyrate. Cells were harvested 24 h after baculovirus infection and solubilized in two-dimensional gel lysis solution. Two-dimensional electrophoretic separations were done as described in Materials and Methods. Electrophoretically separated proteins were transferred to nitrocellulose sheets and reacted with mouse monoclonal antibody directed against gD as described in Materials and Methods.

translational modification of $U_S1.5$ in rabbit skin cells and Vero cells coinfected with Bac-$U_S1.5$ and Bac-U_S3 (Fig. 4, compare lanes 6 and 7 and lanes 13 and 14; Fig. 5A and B, compare panels D and E). Thus, unlike that of U_L13, U_S3 function does not mediate proteasome-dependent degradation of the $U_S1.5$ protein.

(x) Treatment of rabbit skin cells and Vero cells infected with HSV-1(F) with MG132 during the course of infection caused increased accumulation of $U_S1.5$ compared to untreated infected cells (data not shown).

Therefore, these results indicate that both HSV-1 viral kinases, U_L13, and U_S3 mediate posttranslational modification of the $U_S1.5$ protein but with differential effects. Thus, modification of $U_S1.5$ by U_L13 targets $U_S1.5$ for proteasome-dependent degradation whereas U_S3 expression leads to extensive modification of $U_S1.5$ but does not cause the degradation of $U_S1.5$.

U_L13 does not target gD for proteasome-dependent degradation. The results of the experiments described above suggest that expression of the U_L13 protein kinase targets $U_S1.5$ for proteasome-dependent degradation, and the question arose of whether this effect was specific to $U_S1.5$ or whether it also affected other viral proteins. To answer this question, we compared the expression of gD in cells infected with Bac-gD (44) alone or in the presence of the U_L13 protein kinase. In this experiment, rabbit skin cells were infected with 10 PFU of Bac-gD alone or 10 PFU each of Bac-gD and Bac-U_L13 per cell. The cultures were harvested 24 h after infection and processed as described above except that the cell lysates were probed with anti-gD monoclonal antibody. As shown in Fig. 6, simultaneous infection of cells with Bac-gD and Bac-U_L13 did not have a negative effect on the accumulation of gD in these cells, suggesting that U_L13 specifically targets $U_S1.5$ for proteasome-dependent degradation rather than generally activating the proteasome-dependent degradation pathway or mediating the general degradation of viral proteins.

Both $U_S1.5$ and U_L13 activate caspase 3 in rabbit skin cells. The experiments presented above indicated that HSV-1 specifically targets a viral regulatory protein, $U_S1.5$, for degradation via a viral protein kinase encoded by U_L13. Therefore, it was of interest to determine why this degradation event might be of advantage to the virus. As discussed in the Introduction, the *d*120 mutant predominately expresses α proteins and induces apoptosis (11, 21). As elimination of a cytotoxic pro-apoptotic factor would be advantageous and $U_S1.5$ was operationally defined as an α protein (9), the question arose of whether $U_S1.5$ induced apoptosis. To test this hypothesis, we measured DEVDase activity to assess caspase 3 activation, a seminal indicator of induction of apoptosis, in infected rabbit skin cells. The cells were harvested 24 h after infection with the indicated recombinant baculoviruses shown in Fig. 7. The results, normalized with respect to the level of caspase 3 activity in mock-infected cells, indicate that both $U_S1.5$ and U_L13 protein kinases induced caspase 3 activity in a dose-dependent manner. In cells infected with wild-type baculovirus, DEVDase activity was only slightly higher than that in mock-infected cells. Cells infected with Bac-U_S3, which carries a recombinant gene shown to have an antiapoptotic function (28, 29), showed no significant DEVDase activity above that of mock-infected cells. These results indicate that expression of $U_S1.5$ or U_L13 alone, in the absence of any other viral genes, induced apoptosis in rabbit skin cells.

U_S3 blocks caspase 3 activation induced by $U_S1.5$ and U_L13. Since U_S3 protein kinase provided in *trans* has been shown earlier to block apoptosis induced by the *d*120 mutant (28), it was of interest to determine whether coinfection of Bac-U_S3 with Bac-$U_S1.5$ or Bac-U_L13 blocked caspase 3 activation induced by $U_S1.5$ or U_L13. In this series of experiments, DEVDase activity was measured in rabbit skin cells harvested 24 h after infection with 7.5 PFU per cell of Bac-$U_S1.5$ or Bac-U_L13 and 7.5 PFU per cell of wild-type baculovirus or Bac-U_S3. The results shown in Fig. 8 were as follows. Cells coinfected with Bac-$U_S1.5$ and wild-type baculovirus exhibited an 8.4-fold increase in caspase 3 activity relative to that of mock-infected cells, while in cells coinfected with Bac-$U_S1.5$

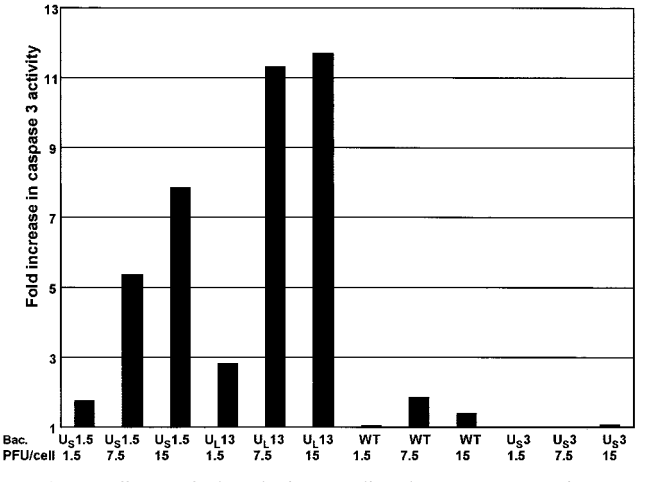

FIG. 7. Effect of baculovirus-mediated gene expression on DEVDase activity in cells. Replicate 25-cm^2 flask cultures of rabbit skin cells were either mock infected or infected with the indicated PFU of Bac-$U_S1.5$, Bac-U_L13, Bac-WT, or Bac-U_S3 per cell. The cells were harvested 24 h after baculovirus infection and assayed for DEVDase activity colorimetrically at 405 nm as described in Materials and Methods. The results are expressed as the fold increase in activity over that of mock-infected cells.

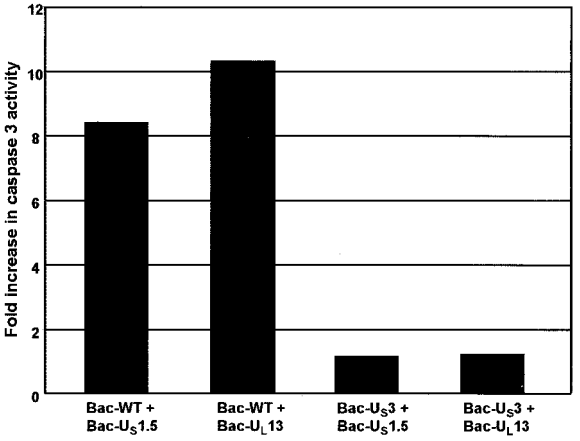

FIG. 8. Effect of baculovirus-mediated gene expression on DEVDase activity in cells. Replicate 25-cm^2 flask cultures of rabbit skin cells were either mock infected or infected with 15 PFU of the indicated baculoviruses per cell. The cells were harvested 24 h after baculovirus infection and assayed for DEVDase activity colorimetrically at 405 nm as described in Materials and Methods. The results are expressed as fold increase in activity over that of mock-infected cells.

and Bac-U_S3, the DEVDase activity was only slightly higher (1.2-fold) than that detected in mock-infected cells. Likewise, a 10-fold increase in caspase 3 activity was observed in cells coinfected with Bac-U_L13 and wild-type baculovirus compared to that observed in mock-infected cells while only a 1.2-fold increase was observed in cells coinfected with Bac-U_L13 and Bac-U_S3. These results indicate that expression of U_S3 blocks the activation of caspase 3 in rabbit skin cells mediated by either $U_S1.5$ or U_L13 proteins.

DISCUSSION

In the studies described in this report, we have examined the interplay of three HSV-1 proteins, $U_S1.5$, U_S3, and U_L13, in the context of mammalian cells transduced to express the proteins individually or in pairs by recombinant baculoviruses. The salient features of the results are as follows. (i) The accumulation of the $U_S1.5$ protein was reduced by concurrent expression of the U_L13 protein kinase and was augmented by concurrent expression of the U_S3 protein kinase. The magnitude of the reduction or increase in the accumulation of the $U_S1.5$ protein was cell type dependent. The effect of U_L13 kinase appears to be specific inasmuch as it did not affect the accumulation of gD in cells doubly infected by recombinant baculoviruses. (ii) The reduction in the accumulation of the $U_S1.5$ protein in the presence of U_L13 protein kinase was only partially due to proteasome-dependent degradation. (iii) Both $U_S1.5$ and U_L13 proteins activated caspase 3, indicative of programmed cell death. (iv) Concurrent expression of U_S3 protein kinase blocked activation of caspase 3 mediated by both $U_S1.5$ and U_L13. The interpretation of these results is tempered by the fact that in the course of viral infection there is a complex interplay between viral gene products. Although analyses of viral gene functions expressed in cells by isolated viral genes is extremely valuable, the functions expressed by isolated viral gene products may not reflect the events that occur in HSV-1-infected cells. The interpretation of the data must take into account what is actually confirmed in cells infected with wild-type or appropriate mutant viruses. A model of the functional interactions of these proteins interpreted in the context of HSV-1-infected cells is shown in Fig. 9. The key features of this model are discussed below.

The results obtained in the system described in this report and with HSV-1-infected cells suggest that $U_S1.5$ is posttranslationally processed in the absence of either U_L13 or U_S3 protein kinases. ICP22 and $U_S1.5$ are phosphorylated by cellular kinases (31), and at least ICP22 is also nucleotidylylated by casein kinase II (26, 27)

The results presented in this report indicate that $U_S1.5$ is in part degraded by the ubiquitin-proteasomal pathway and that it causes apoptosis in cells infected with a recombinant baculovirus expressing the protein. Partial degradation is deduced from the observation that the amounts of accumulating $U_S1.5$ protein increased in the presence of MG132, albeit not to the level observed in the presence of U_S3 protein kinase. A central question is the function of the two protein kinases in the accumulation of the $U_S1.5$ protein. One hypothesis that explains the data and is at least partially supported by the results of analyses of HSV-1-infected cells is that $U_S1.5$ signals the induction of apoptosis, that U_L13 protein kinase regulates the accumulation of $U_S1.5$ protein by targeting it for degradation and, in the context of baculovirus-transduced cells, it induces apoptosis, and that the U_S3 protein kinase blocks apoptosis. While U_S3 expression also promotes modification of $U_S1.5$, it does not target $U_S1.5$ for degradation, suggesting that modifications of $U_S1.5$ mediated by each viral kinase have discrete functions. Accordingly, the levels of accumulated $U_S1.5$ reflect both the decrease in degradation of the protein and the continued synthesis of $U_S1.5$ in the absence of induced programmed cell death. The data in support of the hypothesis are as follows. (i) The accumulation of HSV-1 progeny reaches completion between 18 and 24 h after infection depending on cell type and is accompanied by extensive cytopathic effects. An earlier report from this laboratory showed that in HEp-2 cells overexpressing Bcl-2, the development of cytopathic effects is delayed without significant effects on viral replication (15). These results suggest that cell death at the end of the replicative cycle reflects proapoptotic events blocked by Bcl-2. Since apoptosis is not induced in wild-type virus-infected cells during the replicative phase of infection, the data indicate that the cellular environment is tightly regulated by viral gene products.

(ii) As shown in the studies reported here, the accumulation of $U_S1.5$ is cell type dependent both in HSV-1-infected cells and in cells transduced with recombinant baculoviruses. Thus, $U_S1.5$ accumulates to different extents in HSV-1-infected or transduced Vero cells and in rabbit skin cells but is barely detectable in HEp-2 cells (Fig. 2 and 4; R. Hagglund, A. P. W. Poon, and B. Roizman, unpublished results). Curiously, although the $U_S1.5$ protein meets the definition of an α gene, in wild-type HSV-1-infected cells, it accumulates late in infection at times consistent with the development of cytopathic effects related to apoptosis. In cells infected at relatively high multiplicities of infection, the accumulation of the $U_S1.5$ protein continues unabated as late as 30 h after infection (A. P. W. Poon and B. Roizman, unpublished results). Although the results indicate that $U_S1.5$ mediates the activation of caspase 3,

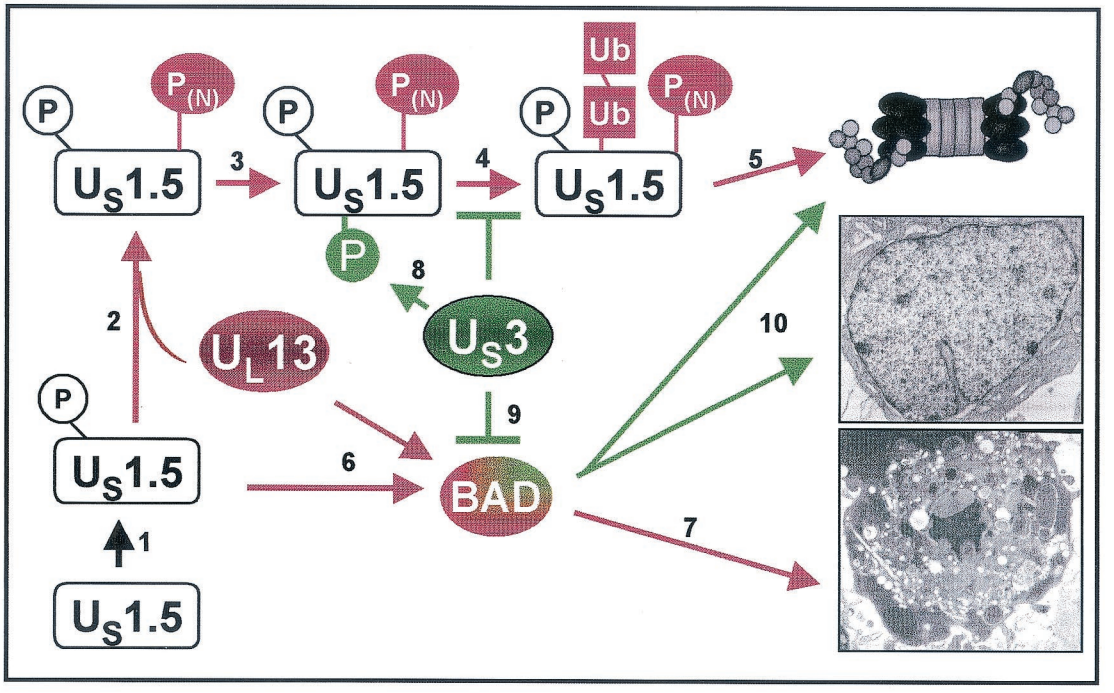

FIG. 9. Schematic representation of a model of the functions of viral proteins described in this report. The model consists of the following elements. $U_S1.5$ is rapidly phosphorylated by cellular kinases (step 1) to yield a product capable of inducing apoptosis possibly by activation of BAD (steps 6 and 7). In the presence of U_L13 protein kinase, $U_S1.5$ is phosphorylated and targeted for degradation by the ubiquitin proteasomal pathway (steps 3 and 4). In the presence of U_S3 protein kinase, $U_S1.5$ is additionally posttranslationally modified precluding its degradation. Results published elsewhere indicate that U_S3 protein kinase blocks apoptosis induced by BAD (step 10) and that in the presence of the protein kinase, BAD is in part degraded. The remaining BAD is posttranslationally processed (30).

the precise role of $U_S1.5$ in apoptosis in wild-type-virus-infected cells remains to be defined.

(iii) Both ICP22 and the $U_S1.5$ proteins are extensively posttranslationally modified by the U_L13 protein kinase (references 31, 35, and 36 and Fig. 2 and 5). Evidence supporting extensive posttranslational modification is reflected in the number and charges of isoforms made in HSV-1-infected cells and in cells transduced by recombinant baculoviruses expressing $U_S1.5$ and U_L13 proteins. As transduction of the viral kinases leads to the observation of highly electronegative $U_S1.5$ isoforms and addition of a phosphate group to a protein makes it more electronegative, it is likely that the viral kinases mediate the phosphorylation of the $U_S1.5$ protein. The evidence presented in Fig. 4 and 5 indicate that U_L13 function targets $U_S1.5$ protein for proteasomal degradation.

Phosphorylation is a common mechanism for targeting proteins for proteasome-dependent degradation in the cell. Proteins are targeted to the 26S proteasome for degradation by the covalent addition of a multiubiquitin chain. A cascade of ubiquitylation enzymes is responsible for the activation of ubiquitin and the assembly of the polyubiquitin chain (reviewed in reference 18). Briefly, ubiquitin is activated by the E1 ubiquitin-activating enzyme and trans-esterified to the E2 ubiquitin conjugating enzyme. The E3 ubiquitin ligase binds both the E2 and substrate proteins, facilitating their interaction and the assembly of the polyubiquitin chain. E3 enzyme complexes, such as $SCF^{\beta\text{-}TrCP}$, SCF^{Grr1}, SCF^{Skp2}, and SCF^{Cdc4}, include a subunit which contains a WD40 motif which specifically binds phosphorylated target proteins (reviewed in reference 12). Thus, only phosphorylated species of the target protein are ubiquitinated and targeted for degradation. We hypothesize that phosphorylation of $U_S1.5$ targets it for interaction with an E3 ubiqutin ligase which specifically binds phosphorylated targets. $U_S1.5$ is targeted for degradation by U_L13 in the absence of any other viral proteins.

(iv) Cells transduced with Bac-U_L13 activated caspase 3, a finding not observed with HSV-1(F)-infected cells. As noted in the Introduction, the U_L13 protein kinase has a wide substrate range which may include cellular protein essential for cellular survival. This substrate may be unavailable in HSV-1-infected cells or its modification by U_L13 or induction of apoptosis after such modification is blocked by other viral proteins, such as U_S3. Thus, the U_L13 protein kinase encodes functions which promote apoptosis and impede apoptosis induced by $U_S1.5$ by effecting its degradation. A possible reason that the dual mechanism of regulation of $U_S1.5$ activity might be advantageous for the virus is that U_S3 might not be as effective in inhibiting induction of apoptosis by $U_S1.5$ at high doses. While some degree of $U_S1.5$ expression may be necessary for functions such as the expression of a subset of γ_2 genes (31, 35), amounts of $U_S1.5$ protein above what is necessary for these functions would not be advantageous for the virus because of its ability to induce apoptosis. Thus, the virus tightly regulates $U_S1.5$ accumulation.

(v) As noted in the Introduction, U_S3 protein kinase mediates the phosphorylation of ICP22 and of $U_S1.5$, but the iden-

tity of its substrates is not known (reference 36 and Fig. 4 and 5). Recent studies have extended the early observations that U_S3 blocks apoptosis in *d*120 mutant-infected cells by demonstrating that it acts at a premitochondrial stage (28). Finally, still more recent studies demonstrated that U_S3 protein kinase blocks apoptosis induced by BAD. Thus, in cells transduced by BAD and U_S3 ORFs, BAD is partially degraded and what remains is posttranslationally modified (29). In this study, we show that U_S3 blocked apoptosis induced by the recombinant baculoviruses expressing either $U_S1.5$ or U_L13 proteins. As illustrated in Fig. 9, it is likely that both $U_S1.5$ and U_L13 proteins induce apoptosis at a similar, premitochondrial stage and that the U_S3 protein kinase blocks apoptosis by interfering with the activation of BAD protein.

To date, at least four different mutants have been shown to induce apoptosis. These are a mutant lacking gD (44), mutants lacking the regulatory proteins ICP4 (21) or ICP27 (5, 6), and a temperature-sensitive mutant unable to release viral DNA at nuclear pores from capsids at the nonpermissive temperature (14). The results presented in this and earlier reports from this laboratory would predict that $U_S1.5$ protein expressed with α kinetics may play a role in the induction of apoptosis in cells infected with mutants lacking regulatory proteins but not in cells exposed to the mutants lacking gD or that are incapable of releasing viral DNA from capsids. In the course of wild-type virus infection, induction of apoptosis by the $U_S1.5$ protein would be inhibited by U_L13 and U_S3 functions discussed above. Conversely, the absence of the two kinases would not automatically lead to apoptosis inasmuch as expression of the $U_S1.5$ gene appears to be cell type dependent and no evidence has emerged so far that indicates that $\Delta U_L13/\Delta U_S3$ or ΔU_L13 recombinant viruses induce programmed cell death. In essence, while we have demonstrated that $U_S1.5$ expression leads to apoptosis in rabbit skin cells in the absence of other viral proteins, cellular functions in response to stress may block this effect.

Apoptosis has been shown to be a common host cell response to viral infection (reviewed in reference 43). This response is advantageous to the host because the premature death of infected cells checks the production of progeny virus, thus impairing the spread of the virus to other cells. Many viruses encode functions which block apoptosis induced by replicative functions of the virus (21, 37, 38, 45). Current evidence indicates that HSV-1 has evolved multiple mechanisms to thwart this cellular response to its replicative functions.

ACKNOWLEDGMENTS

These studies were aided by grants from the National Cancer Institute (CA87761, CA83939, CA71933, and CA78766), the United States Public Health Service. R.H. is a Howard Hughes Medical Institute Predoctoral Fellow.

REFERENCES

1. **Ackermann, M., M. Sarmiento, and B. Roizman.** 1985. Application of antibody to synthetic peptides for characterization of the intact and truncated α22 protein specified by herpes simplex virus 1 and the R325 $\alpha22^-$ deletion mutant. J. Virol. **56:**207–215.
2. **Advani, S. J., R. Brandimarti, R. R. Weichselbaum, and B. Roizman.** 2000. The disappearance of cyclins A and B and the increase in the activity of the G_2/M phase cellular kinase cdc2 in herpes simplex virus 1-infected cells requires the expression of $\alpha22/U_S1.5$ and U_L13 viral genes. J. Virol. **74:**8–15.
3. **Asano, S., T. Honda, F. Goshima, D. Wantanbe, Y. Miyake, Y. Sugiura, and Y. Nishiyama.** 1999. U_S3 protein kinase of herpes simplex virus type 2 plays a role in protecting corneal epithelial cells from apoptosis in infected mice. J. Gen. Virol. **80:**51–56.
4. **Asano, S., T. Honda, F. Goshima, Y. Nishiyama, and Y. Sugiura.** 2000. U_S3 protein kinase of herpes simplex virus protects primary afferent neurons from virus-induced apoptosis in ICR mice. Neurosci. Lett. **294:**105–108.
5. **Aubert, M., and J. A. Blaho.** 1999. The herpes simplex virus type 1 regulatory protein ICP27 is required for prevention of apoptosis in infected human cells. J. Virol. **73:**2803–2813.
6. **Aubert, M., J. O'Toole, and J. A. Blaho.** 1999. Induction and prevention of apoptosis in human HEp-2 cells by herpes simplex virus type 1. J. Virol. **73:**10359–10370.
7. **Berkelman, T., and T. Stenstedt.** 1998. 2-D electrophoresis. Using immobilized pH gradients. Principles and methods. Amersham Pharmacia Biotech, Piscataway, N.J.
8. **Bjellqvist, B., K. Ek, P. G. Righetti, E. Gianazza, A. Görg, R. Westermeier, and W. Postel.** 1982. Isoelectric focusing in immobilized pH gradients: principle, methodology, and some applications. J. Biochem. Biophys. Methods **6:**317–339.
9. **Carter, K. L., and B. Roizman.** 1996. The promoter and transcriptional unit of a novel herpes simplex virus 1 α gene are contained in, and encode a protein in frame with, the open reading frame of the α22 gene. J. Virol. **70:**172–178.
10. **Condreay, J. P., S. M. Witherspoon, W. C. Clay, and T. A. Kost.** 1999. Transient and stable gene expression in mammalian cells transduced with a recombinant baculovirus vector. Proc. Natl. Acad. Sci. USA **96:**127–132.
11. **DeLuca, N. A., A. M. McCarthy, and P. A. Schaffer.** 1985. Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. J. Virol. **56:**558–570.
12. **Deshaies, R. J.** 1999. SCF and cullin/ring H2-based ubiquitin ligases. Annu. Rev. Cell Dev. Biol. **15:**435–467.
13. **Ejercito, P. M., E. D. Kieff, and B. Roizman.** 1968. Characterization of herpes simplex virus strains differing in their effects on social behavior of infected cells. J. Gen. Virol. **2:**357–364.
14. **Galvan, V., and B. Roizman.** 1998. Herpes simplex virus 1 induces and blocks apoptosis at multiple steps during infection and protects cells from exogenous inducers in a cell-type-dependent manner. Proc. Natl. Acad. Sci. USA **95:**3931–3936.
15. **Galvan, V., R. Brandimarti, J. Munger, and B. Roizman.** 2000. Bcl-2 blocks a caspase-dependent pathway of apoptosis activated by herpes simplex virus 1 infection in HEp-2 cells. J. Virol. **74:**1931–1938.
16. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. **14:**8–19.
17. **Honess, R. W., and B. Roizman.** 1975. Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. Proc. Natl. Acad. Sci. USA **72:**1276–1280.
18. **Jackson, P. K., A. G. Eldridge, E. Freed, L. Furstenthal, J. Y. Hsu, B. K. Kaiser, and J. D. R. Reimann.** 2000. The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases. Trends Cell Biol. **10:**429–439.
19. **Jerome, K. R., R. Fox, Z. Chen, A. E. Sears, H.-Y. Lee, and L. Corey.** 1999. Herpes simplex virus inhibits apoptosis through the action of two genes, U_S5 and U_S3. J. Virol. **73:**8950–8957.
20. **Kawaguchi, Y., C. Van Sant, and B. Roizman.** 1998. Eukaryotic elongation factor 1δ is hyper-phosphorylated by the protein kinase encoded by the U_L13 gene of herpes simplex virus 1. J. Virol. **72:**1731–1736.
21. **Leopardi, R., and B. Roizman.** 1996. The herpes simplex virus major regulatory protein ICP4 blocks apoptosis induced by the virus or by hyperthermia. Proc. Natl. Acad. Sci. USA **93:**9583–9587.
22. **Leopardi, R., C. Van Sant, and B. Roizman.** 1997. The herpes simplex virus 1 protein kinase U_S3 is required for protection from apoptosis induced by the virus. Proc. Natl. Acad. Sci. USA **94:**7891–7896.
23. **Leopardi, R., P. L. Ward, W. O. Ogle, and B. Roizman.** 1997. Association of herpes simplex virus regulatory protein ICP22 with transcriptional complexes containing EAP, ICP4, RNA polymerase II, and viral DNA requires posttranslational modification by the U_L13 protein kinase. J. Virol. **71:**1133–1139.
24. **Mackem, S., and B. Roizman.** 1980. Regulation of herpesvirus macromolecular synthesis: transcription-initiation sites and domains of α genes. Proc. Natl. Acad. Sci. USA **77:**7122–7126.
25. **Meignier, B., R. Longnecker, P. Mavromara-Nazos, A. E. Sears, and B. Roizman.** 1988. Virulence of and establishment of latency by genetically engineered deletion mutants of herpes simplex virus 1. Virology **162:**251–254.
26. **Mitchell, C., J. A. Blaho, and B. Roizman.** 1994. Casein kinase II specifically nucleotidylylates in vitro the amino acid sequence of the protein encoded by the α22 gene of herpes simplex virus 1. Proc. Natl. Acad. Sci. USA **91:**11864–11868.
27. **Mitchell, C., J. A. Blaho, L. McCormick, and B. Roizman.** 1997. The nucleotidylylation of herpes simplex virus 1 regulatory protein α22 by human casein kinase II. J. Biol. Chem. **272:**25394–25400.
28. **Munger, J., A. V. Chee, and B. Roizman.** 2001. The U_S3 protein kinase

blocks apoptosis induced by the *d*120 mutant of herpes simplex virus 1 at a premitochondrial stage. J. Virol. **75:**5491–5497.
29. **Munger, J., and B. Roizman.** 2001. The U_S3 protein kinase of herpes simplex virus 1 mediates the posttranslational modification of BAD and prevents BAD-induced programmed cell death in the absence of other viral proteins. Proc. Natl. Acad. Sci. USA **98:**10410–10415.
30. **Ng, T. I., W. O. Ogle, and B. Roizman.** 1998. U_L13 protein kinase of herpes simplex virus 1 complexes with glycoprotein E and mediates the phosphorylation of the viral Fc receptor: glycoproteins E and I. Virology **241:**37–48.
31. **Ogle, W. O., and B. Roizman.** 1999. Functional anatomy of herpes simplex virus 1 overlapping genes encoding infected-cell protein 22 and $U_S1.5$ protein. J. Virol. **73:**4305–4315.
32. **Poon, A. P. W., W. O. Ogle, and B. Roizman.** 2000. Posttranslational processing of infected cell protein 22 mediated by viral protein kinases is sensitive to amino acid substitutions at distant sites and can be cell-type specific. J. Virol. **74:**11210–11214.
33. **Post, L. E., and B. Roizman.** 1981. A generalized technique for deletion of specific genes in large genomes: α gene 22 of herpes simplex virus 1 is not essential for growth. Cell **25:**227–232.
34. **Purves, F. C., R. M. Longnecker, D. P. Leader, and B. Roizman.** 1987. Herpes simplex virus 1 protein kinase is encoded by open reading frame U_S3 which is not essential for growth in cell culture. J. Virol. **61:**2896–2901.
35. **Purves, F. C., W. O. Ogle, and B. Roizman.** 1993. Processing of the herpes simplex virus regulatory protein α22 meditated by the U_L13 protein kinase determines the accumulation of a subset of α and γ mRNAs and proteins in infected cells. Proc. Natl. Acad. Sci. USA **90:**6701–6705.
36. **Purves, F. C., and B. Roizman.** 1992. The U_L13 gene of herpes simplex virus 1 encodes the functions for posttranslational processing associated with phosphorylation of the regulatory protein α22. Proc. Natl. Acad. Sci. USA **89:**7310–7314.
37. **Rao, L., M. Debbas, P. Sabbatini, D. Hockenbery, S. Korsmeyer, and E. White.** 1992. The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. Proc. Natl. Acad. Sci. USA **89:**7742–7746.
38. **Ray, C. A., R. A. Black, S. R. Kronheim, T. A. Greenstreet, P. R. Sleath, G. S. Salvesen, and D. J. Pickup.** 1992. Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1 beta converting enzyme. Cell **69:**597–604.
39. **Rixon, F. J., and J. B. Clements.** 1982. Detailed structural analysis of two spliced HSV-1 immediate-early mRNAs. Nucleic Acids Res. **10:**2241–2256.
40. **Roizman, B., and D. M. Knipe.** 2001. The replication of herpes simplex viruses, p. 2399–2459. *In* D. M. Knipe, P. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott-Williams and Wilkins, New York, N.Y.
41. **Sears, A. E., I. W. Halliburton, B. Meignier, S. Silver, and B. Roizman.** 1985. Herpes simplex virus 1 mutant deleted in the α22 gene: growth and gene expression in permissive and restrictive cell lines and establishment of latency in mice. J. Virol. **55:**338–346.
42. **Watson, R. J., M. Sullivan, and G. F. Vande Woude.** 1981. Structures of two spliced herpes simplex virus type 1 immediate-early mRNAs which map at the junctions of the unique and reiterated regions of the virus DNA S component. J. Virol. **37:**431–444.
43. **Young, L. S., C. W. Dawson, and A. G. Eliopoulos.** 1997. Viruses and apoptosis. Br. Med. Bull. **53:**509–521.
44. **Zhou, G., V. Galvan, G. Campadelli-Fiume, and B. Roizman.** 2000. Glycoprotein D or J delivered in *trans* blocks apoptosis in SK-N-SH cells induced by a herpes simplex virus 1 mutant lacking intact genes expressing both glycoproteins. J. Virol. **74:**11782–11791.
45. **Zhu, H., Y. Shen, and T. Shenk.** 1995. Human cytomegalovirus IE1 and IE2 proteins block apoptosis. J. Virol. **69:**7960–7970.