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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

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Herpes simplex virus 1 encodes a Fc receptor consisting of glycoproteins E (gE) and I (gI) and two protein kinases specified by U_L13 and U_S3, respectively. We report the following: (i) Antibody to U_L13 formed immune complexes containing gE and gI in addition to U_L13 protein. Immune complexes formed by monoclonal antibody to gE, but not those formed by monoclonal antibody to gI, also contained the U_L13 protein. This association may reflect direct interaction between gE and U_L13 inasmuch as IgG in preimmune rabbit serum and an antiserum made against another viral protein which does not react with the U_L13 protein directly also bound gE and U_L13. (ii) In cells infected with the wild-type virus, gE formed two sharp bands and a diffuse, slower migrating band. The slower sharp band was undetectable, and the diffuse slower migrating forms of gE were diminished in lysates of cells infected with a mutant virus lacking the U_L13 gene (ΔU_L13). (iii) Both gE and gI were labeled with ^{32}Pi in cells infected with wild-type or the ΔU_L13 virus, but the labeling was significantly stronger in cells infected with the wild-type virus than in those infected with the ΔU_L13 virus. (iv) In an *in vitro* protein kinase assay, U_L13 immunoprecipitated from cells infected with wild-type virus labeled gE in the presence of [γ-^{32}P]ATP. This activity was absent in precipitates from cells infected with ΔU_L13 virus. The labeled gE comigrated with the slower, sharp band of gE. (v) gI present in the U_L13 immune complex was also phosphorylated in the *in vitro* kinase assay. (vi) The cytoplasmic domain of gE contains recognition sequences for phosphorylation by casein kinase II (CKII). Exogenous CKII phosphorylated gE in immune complexes from lysates of cells infected with the ΔU_L13 mutant or in immune complexes from lysates of cells infected with wild-type virus that had been heated to inactivate all endogenous kinase activity including that of U_L13. In both instances, CKII phosphorylated gE in both the slow and fast migrating sharp bands. We conclude that U_L13 physically associates with gE and mediates the phosphorylation of gE and gI. U_L13 may also be a determinant in posttranslational processing of gE.

INTRODUCTION

Herpes simplex virus 1 (HSV-1) encodes two protein kinases specified by the U_L13 and U_S3 genes (Coulter *et al.,* 1993; Cunningham *et al.,* 1992; Daikoku *et al.,* 1997; Frame *et al.,* 1987; Overton *et al.,* 1992; Purves and Roizman, 1992; Purves *et al.,* 1986a,b, 1987). The kinase specified by the U_L13 gene is of particular interest since the protein is packaged in the tegument, a structural component of the virion located between the capsid and the envelope (Coulter *et al.,* 1993; Cunningham *et al.,* 1992; Overton *et al.,* 1992) and indeed, a protein kinase activity is associated with purified capsid–tegument structures (Lemaster and Roizman, 1980). Tegument proteins are generally released in the cytoplasm of newly infected cells and therefore U_L13 would be expected to phosphorylate viral proteins and, possibly, cellular proteins upon entry into cells. Genetically engineered viruses lacking the U_L13 gene show reduced capacity to replicate in nonhuman cells in culture (Purves *et al.,* 1993) and studies reported elsewhere indicate that U_L13 phosphorylates itself and at least two viral proteins, infected cell proteins 22 and 0 (ICP22 and ICP0) (Cunningham *et al.,* 1992, Ogle *et al.,* 1997; Purves and Roizman, 1992).

This report concerns the phosphorylation of HSV-1 Fc receptor by the U_L13 protein. Glycoprotein E (gE) acts as a Fc receptor by itself and more strongly in association with glycoprotein I (gI) (Dublin *et al.,* 1990; Johnson *et al.,* 1988). gE specified by HSV-1 and also by other alphaherpesviruses such as HSV-2, *Varicella zoster* virus (VZV), and pseudorabies virus are phosphorylated as well as glycosylated (Edson, 1993; Edson *et al.,* 1987; Montalvo and Grose, 1986; Olson *et al.,* 1997). The homolog of HSV-1 gI encoded by VZV has also been reported to be phosphorylated (Yao and Grose, 1994). The identities of the protein kinases responsible for the phosphorylation of these two glycoproteins are largely unknown, although the cytoplasmic domain of gE contains several casein kinase II (CKII) recognition sequences and CKII had been shown to phosphorylate VZV gE *in vitro* (Litwin *et al.,* 1992; Yao *et al.,* 1993).

The studies described in this report stemmed from the observation that antibody to U_L13 formed complexes with U_L13, gE, and gI and that in an appropriate buffer containing [γ-^{32}P]ATP, this complex phosphorylated the U_L13

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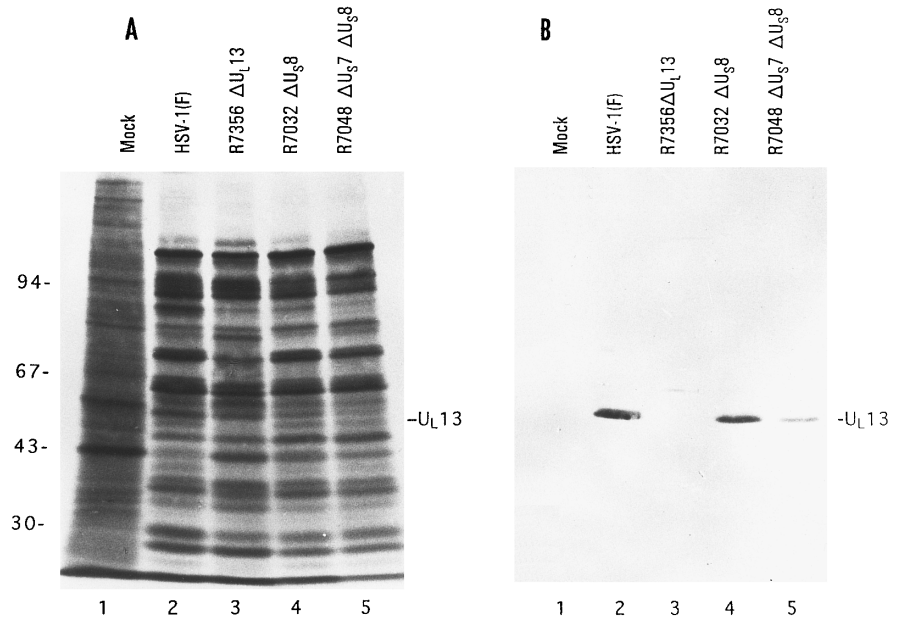

FIG. 1. (A) Autoradiographic image of ^{35}S-labeled cell lysates infected with parent and mutant viruses. Replicate cultures of Vero cells were either mock infected or infected with HSV-1(F), R7356 (ΔU_L13), R7032 (ΔgE), or R7048 (ΔgE, ΔgI) virus. At 12.5 h after infection, the cells were labeled with a mixture of [^{35}S]methionine and [^{35}S]cysteine for 6 h. Cells were then solubilized, electrophoretically separated in a 10% denaturing polyacrylamide gel, and electrically transferred to a nitrocellulose sheet. (B) The nitrocellulose sheet was reacted with U_L13 antiserum and subsequently with ECL immunoblotting reagents to detect the position of U_L13.

protein, gE, and gI. We report that (i) in cells infected with wild-type virus, gE forms two sharp bands and a diffuse band on top of the two sharp bands. The slower migrating sharp band of gE is diminished or undetectable, and the diffuse band is significantly reduced in lysate of cells infected with the ΔU_L13 mutant virus. The phosphorylation of gI is also reduced in lysate of cells infected with the ΔU_L13 mutant virus. (ii) U_L13 specifically associates with, and phosphorylates, gE *in vitro.* The labeled gE comigrates with the slower, sharp band of gE. (iii) gI, present in immune complexes containing U_L13, is also phosphorylated by U_L13 *in vitro.* (iv) Purified CKII added to immune complexes containing gE phosphorylates gE but the pattern of phosphorylation is different from that phosphorylated by U_L13.

RESULTS

Accumulation of U_L13 protein is reduced in cells infected with ΔgE virus

The specificity of the rabbit polyclonal antiserum to GST–U_L13 fusion protein prepared as described under Materials and Methods was tested on electrophoretically separated proteins from ^{35}S-labeled cells that were mock infected or infected with wild-type virus HSV-1(F), recombinant viruses R7356 lacking the U_L13 gene (ΔU_L13), R7032 lacking the gene specifying glycoprotein gE (ΔU_S8), or R7048 lacking both the gE and gI genes ($\Delta U_S7/\Delta U_S8$). As shown in Fig. 1, the antiserum reacted with a M_r 57,000 protein that was present in lysates of wild-type virus-infected cells (Figs. 1A and 1B, lane 2) but absent in those of mock-infected cells (Fig. 1B, lane 1) or in lysates of cells infected with the ΔU_L13 mutant (Figs. 1A and 1B, lane 3). The apparent molecular weight of the protein corresponds to that predicted for U_L13 (McGeoch *et al.,* 1988). The antibody was further tested in different types of infected cells (Fig. 2). Again, the antiserum detected a protein with a M_r of 57,000 in electrophoretically separated proteins from lysate of wild-type [HSV-1(F)]-infected rabbit skin cells or Vero cells (Fig. 2B, lanes 2 and 6). The preimmune serum from the rabbit did not react specifically with any protein in the infected cells (Fig. 2A, lanes 2 and 3). The expression of U_L13 was also examined in the R7032 (ΔU_S8)-infected cells. We observed that the accumulation of U_L13 protein was decreased compared with that of the wild-type virus-infected cells (compare Fig. 2B, lanes 4 and 8 with lanes 2 and 6). In fact, the accumulation of U_L13 protein was lower in cells infected with mutants lacking gE or both gE and gI (Figs. 1A and 1B, lanes 4 and 5) than in cells infected with wild-type virus (Figs. 1A and 1B, lane 2). In Fig. 2, ICP27 served as an infectivity/loading control. As shown in Fig. 2B, the amounts of ICP27 were similar in all lysates of infected cells.

It has been observed that the expression of some viral genes, especially late genes, was delayed in cells infected with the ΔgE virus (unpublished studies from this laboratory). Since the accumulation of some late proteins in cells infected with the U_L13 deletion virus are also decreased due to the lack of the modification of ICP22 in

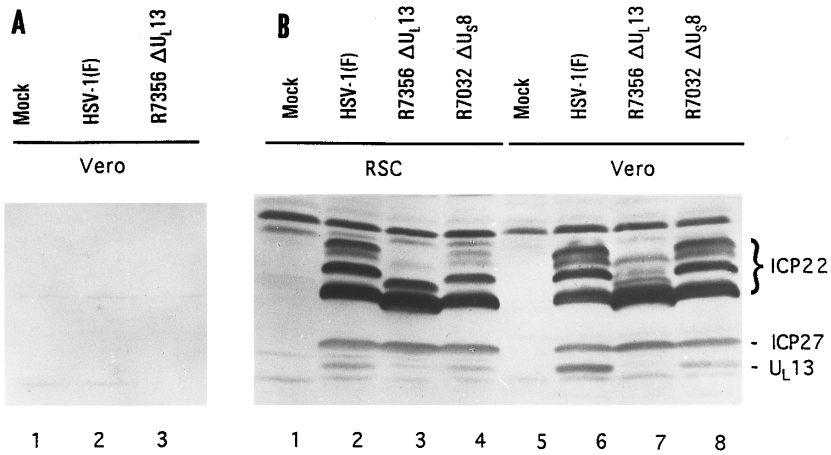

FIG. 2. Electrophoretic profiles of U_L13, ICP22, and ICP27 made in rabbit skin cells (RSC) or Vero cells infected with parent and mutant viruses. Replicate cell cultures were either mock infected or infected with HSV-1(F), R7356 (ΔU_L13), or R7032 (ΔgE) virus. The infected cells were harvested at 24 h after infection, solubilized, electrophoretically separated in a denaturing 8.5% polyacrylamide gel, electrically transferred to a nitrocellulose sheet, and reacted with preimmune serum (A) or U_L13 antiserum, R77 antiserum (ICP22), and monoclonal antibody H1117 (ICP27) (B).

the absence of U_L13 protein (Purves *et al.,* 1993), we examined the modification of ICP22 in cells infected with the gE deletion virus. Indeed, in rabbit skin cells infected with ΔU_S8 virus, the processing of ICP22 proteins is reduced relative to that of wild-type infected cells (compare Fig. 2B, lanes 2 and 4). However in Vero cells the processing of ICP22 in cells infected with the ΔU_S8 protein cannot be differentiated from that observed in wild-type virus-infected cells (compare Fig. 2B, lanes 6 and 8) and yet the synthesis of late proteins in these cells was delayed. We conclude therefore that the evidence available at this time does not relate the reduction in late protein synthesis in cells infected with the ΔU_S8 virus to altered processing of ICP22.

U_L13 forms a complex with the Fc receptor

The U_L13 antiserum was tested for its ability to immunoprecipitate U_L13 from infected cell lysate. In these series of experiments, immune complexes obtained from the reaction of antisera with lysates of ^{35}S-labeled infected cells were subjected to electrophoresis simultaneously and in the same apparatus as those shown in Fig. 1. The proteins separated by electrophoresis was electrically transferred to a nitrocellulose sheet. The key results of this experiment were as follows:

(i) The immune complexes from wild-type virus-infected cell lysate contained a protein band with an apparent M_r of 57,000 (Fig. 3A, lane 6). This protein band migrated to the same position as the U_L13 protein in the polyacrylamide gel shown in Figs. 1A and 1B, lane 2), but was absent from immune complexes obtained with lysate of cells infected with ΔU_L13 virus (Fig. 3A, lane 5). In an attempt to verify the identify of the M_r 57,000 protein, the nitrocellulose sheet was reacted with the U_L13 antiserum, but the protein migrated so close to the heavy chain of IgG brought down by immunoprecipitation that it was impossible to differentiate the protein from the IgG in this particular polyacrylamide gel (data not shown). As reported elsewhere, the anti-U_L13 serum does react with this protein, indicating that this protein is U_L13 (Ogle *et al.,* 1997).

(ii) The anti-U_L13 serum also precipitated several protein species with apparent M_rs ranging from 60,000 to 85,000. Additional studies revealed that these bands contained the Fc receptors encoded by HSV-1 gE and gI, inasmuch as they were not detected in immune complexes obtained with the anti-U_L13 antibody from lysate of cells infected with ΔU_S8 (ΔgE) or ΔU_S8/ΔU_S7 (ΔgE/ΔgI) mutants (Fig. 3A, lanes 3 and 4). gE has been reported to form several diffuse bands ranging in apparent M_rs from 80,000 to 85,000 (Para *et al.,* 1982), whereas the apparent M_r of gI has been reported to range from 60,000 to 70,000 (Johnson *et al.,* 1988). Separation of proteins in immune complexes from lysate of wild-type-infected cells on a lower percentage polyacrylamide gel and a shorter exposure of the autoradiograph yielded two major forms of gE (Fig. 3B, lane 2), whereas only the band with the faster mobility was apparent in precipitates from lysate of cells infected with the ΔU_L13 mutant (Fig. 3B, lane 3). The monoclonal antibody to gE reacts specifically with a double and single band in the immunoblot (Fig. 3C) that matches the bands seen in Fig. 3B, unambiguously identifying these bands as gE (Fig. 3C, lanes 2 and 3). We should stress that in these series of experiments, as shown in Fig. 3B, the data clearly relate the presence of the slow migrating band E with the presence of the U_L13 kinase.

(iii) Binding of HSV-1 Fc receptor to an antigen–antibody complex would not per se evoke a great surprise. Additional experiments, however, led to the observation

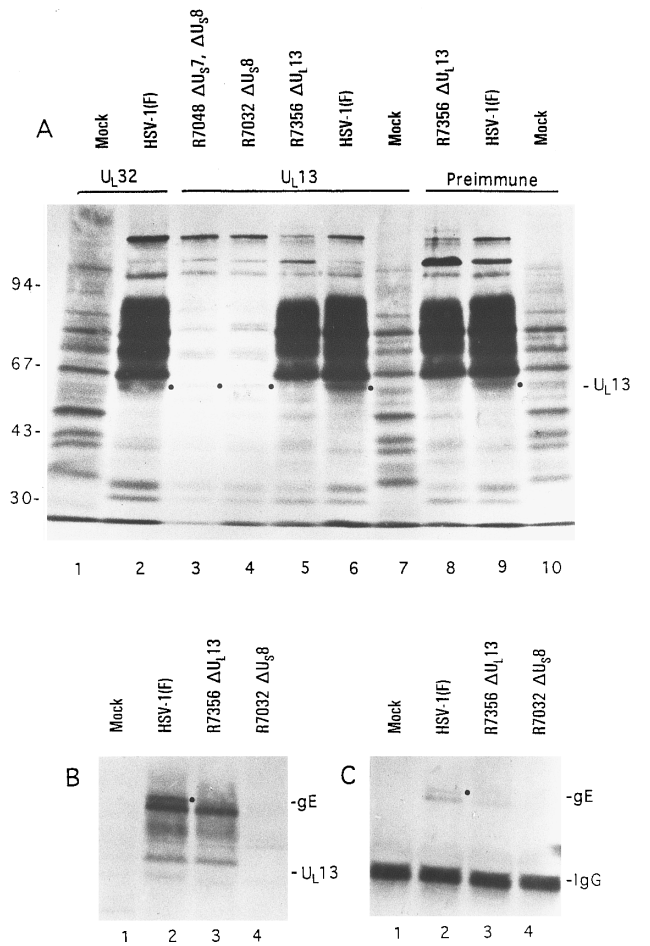

FIG. 3. (A) Autoradiographic image of ^{35}S-labeled infected cell lysate immunoprecipitated by U_L13, U_L32, or preimmune antiserum. Replicate cultures of Vero cells were either mock infected or infected with HSV-1(F), R7356 (ΔU_L13), R7032 (ΔgE), or R7048 (ΔgE, ΔgI) virus. At 12.5 h after infection, the cells were labeled by a mixture of [^{35}S]methionine and [^{35}S]cysteine for 6 h. Cells were then solubilized and immunoprecipitated with U_L32 polyclonal antiserum (lanes 1 and 2), U_L13 polyclonal antiserum (lanes 3–7), and preimmune serum (lanes 8–10). The precipitated proteins were eluted from protein A–Sepharose, electrophoretically separated in a 10% denaturing polyacrylamide gel, and electrically transferred to a nitrocellulose sheet. U_L13 is identified by a filled circle in A. (B) Autoradiographic image of ^{35}S-labeled infected cell lysate immunoprecipitated by U_L13 antiserum. Replicate cultures of Vero cells were either mock infected or infected with HSV-1(F), R7356 (ΔU_L13), or R7032 (ΔgE) virus. The cells were infected and processed in the same way as described in A except that the samples were electrophoretically separated in a 8.5% denaturing polyacrylamide gel and electrically transferred to a nitrocellulose sheet. (C) Photograph of the nitrocellulose sheet shown in B after immunoblotting with the gE-specific monoclonal antibody. The slower migrating sharp band of gE is identified by a filled circle in lane 2 of B and C.

that the Fc receptor may be binding to the U_L13 protein and not merely to IgG. Specifically, as shown in Fig. 3A, immune complexes obtained from wild-type virus-infected lysate with the U_L13 preimmune serum (lane 9) or with an anti-U_L32 polyclonal antibody (lane 2) contained a protein band with an apparent M_r identical to that of the U_L13 protein. Since neither the preimmune serum (Fig. 2A) nor the U_L32 antiserum reacted with U_L13 in previous assays (Chang *et al.,* 1996), one explanation for this observation is that U_L13 was bound to and precipitated with the Fc receptor complex.

Phosphorylation of gE and gI in infected cells is dependent on U_L13

In the next series of experiments we examined the proteins present in immune complexes captured by the anti-U_L13 polyclonal antibody from lysate of ^{32}P-labeled mock-infected and infected cells. Several phosphoproteins were captured in immune complexes by the U_L13 antiserum from cell lysate infected with wild-type virus or ΔU_L13 virus (Fig. 4A, lanes 7 and 8), but not from lysate of cells infected with ΔgE virus (lane 9) or the ΔgE/ΔgI virus (lane 10). Again, the profile of the phosphoproteins resembled those of gE and gI (Fig. 4A, lanes 2, 3, 7, and 8). As expected, both preimmune and immune sera captured gE and gI. Since HSV-1 gE and VZV gI had been reported to be phosphoproteins (Edson *et al.,* 1987; Yao and Grose, 1994), we verified the identities of these proteins by immunobloting with monoclonal antibodies specific to gE and gI. The gE antibody reacted with a doublet of sharp bands as well as a very broad band of diffuse protein above the doublet from cells infected with wild-type virus (Fig. 4B, lanes 2 and 7), but with a single sharp band and a much less diffuse, slow migrating band in cells infected with R7356 (ΔU_L13) virus (Fig. 4B, lanes 3 and 8). We should note that the slow and fast migrating band of gE were less well resolved in this gel than in the gel shown in Figs. 3B or 5C. The gI antibody reacted with a major broad band and a slower migrating band from lysate of cells infected with wild-type virus or the U_L13 deletion virus (Fig. 4C, lanes 2, 3, 7, and 8).

The gE and gI bands formed by immune complexes from cells infected with the R7356 (ΔU_L13) virus (Fig. 4A, lanes 3 and 8) exhibited substantially less phosphorylation than those of cells infected with the wild-type virus (Fig. 4A, lanes 2 and 7). The amounts of radioactivity quantified with the aid of a Betagen Betascope showed that the labeling of the individual bands of gE and gI in lanes 3 and 8 of Fig. 4A (ΔU_L13) constitute approximately 40% of the corresponding labeling of the glycoproteins from wild-type virus-infected cells shown in Fig. 4A (lanes 2 and 7). The immunoblot showed that the amount of the faster migrating sharp band of gE in the lysate of ΔU_L13 mutant virus (Fig. 4B, lanes 3 and 8) was comparable to the corresponding band in the lysates of cells infected with wild-type virus (Fig. 4B, lanes 2 and 7).

As noted above, the gE from lysate of wild-type virus infected cells formed a diffuse, heterogeneous mass of proteins migrating more slowly than the doublet of sharp bands (Figs. 4A and 4B, lanes 2 and 7). These heterogenous forms of gE were less abundant and migrated faster in cells infected with the ΔU_L13 mutant (Figs. 4A,

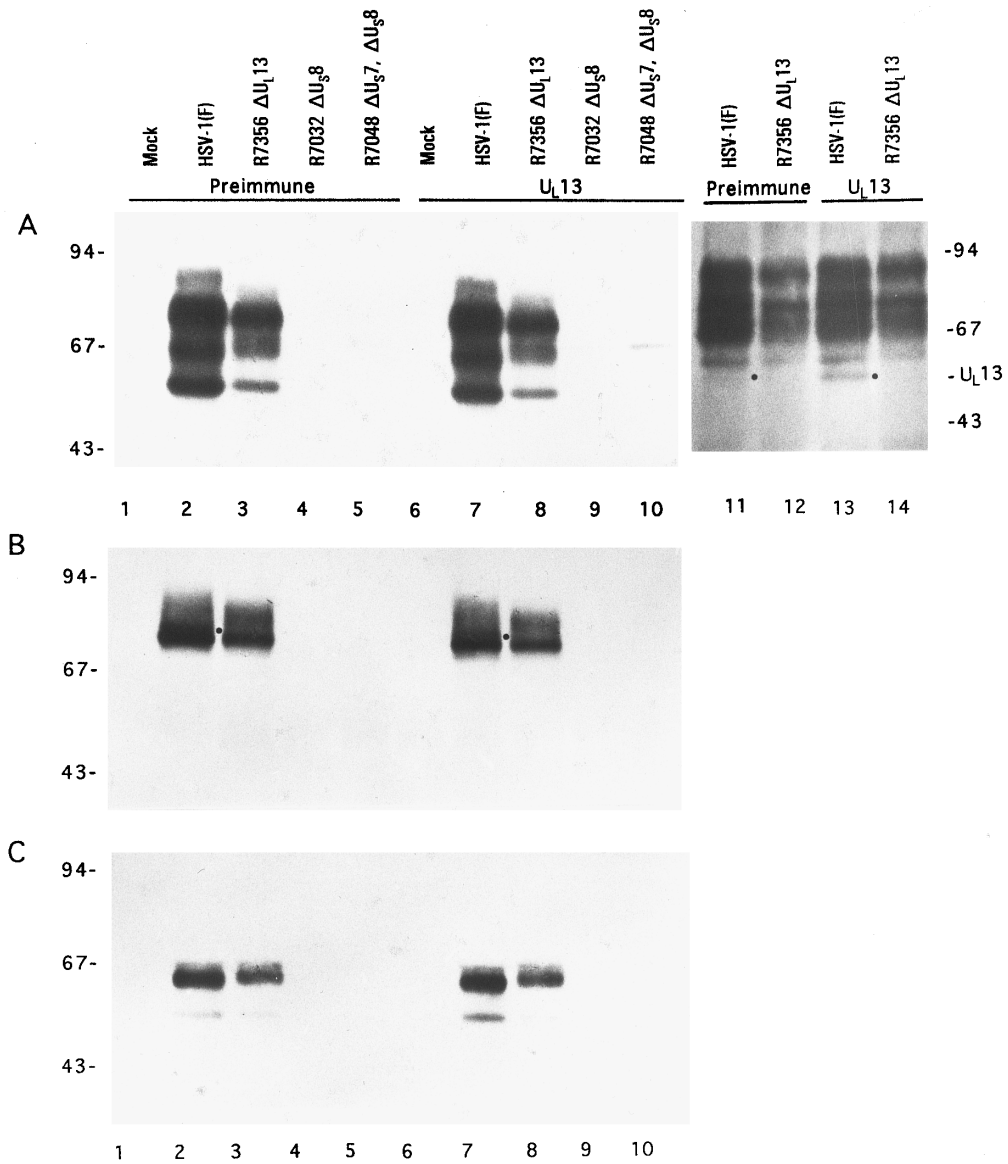

FIG. 4. (A) Autoradiographic image of [^{32}P]orthophoshate-labeled infected cell lysate immunoprecipitated by U_L13 or preimmune rabbit serum. (i) Lanes 1–10: replicate cultures of Vero cells were either mock infected or infected with HSV-1(F), R7356 (ΔU_L13), R7032 (ΔgE), or R7048 (ΔgE, ΔgI) virus; at 14 h after infection, the cells were labeled with ^{32}Pi for 4 h. (ii) Lanes 11–14: replicate cultures of Vero cells were infected with HSV-1(F) or R7356 (ΔU_L13) virus; at 6 h after infection, the cells were labeled with ^{32}Pi for 16 h. Cells were then solubilized and reacted with the preimmune serum (lanes 1–5, 11, 12) or the anti-U_L13 polyclonal serum (lanes 6–10, 13, 14). The complexes containing IgG were collected with protein A–Sepharose, eluted, electrophoretically separated in a denaturing 8.5% polyacrylamide gel, electrically transferred to a nitrocellulose sheet, and reacted with gE-specific monoclonal antibody (II481B2) (B) or monoclonal antibody specific for gI (Fd69) (C). In A, U_L13 is identified by a filled circle (lanes 11 and 13) and in B, the slower migrating sharp band of gE is identified by filled circles in lanes 2 and 7.

and 4B, lanes 3 and 8). This decrease in the amounts of the highly modified gE was also observed in ^{35}S-labeled cells (Fig. 3A, lane 5).

It had been reported that U_L13 is a phosphoprotein (Overton *et al.,* 1992). U_L13 in cells incubated with medium containing ^{32}Pi for 4 h was poorly labeled (Fig. 4A, lane 7). To improve the labeling of U_L13, the infected cells were labeled from 5 to 22 h after infection. This longer period of incubation with ^{32}Pi greatly improved the labeling of U_L13. We identified a labeled phosphoprotein of M_r 57,000, which was immunoprecipitated from lysate of cells infected with wild-type virus (Fig. 4A, lane 13), but not from that infected by the ΔU_L13 virus (Fig. 4A, lane 14). Again, a small amount of U_L13 was captured in complexes formed by IgG from preimmune serum of U_L13 (Fig. 4A, lane 11).

We conclude (i) that gE and gI found in immunoprecipitates are phosphorylated, (ii) that the extent of phosphorylation is higher in wild-type-infected cells than in cells infected with the ΔU_L13 mutant, and last (iii) that the slower migrating sharp band of gE is associated with the presence of a functional U_L13 gene. These results sug-

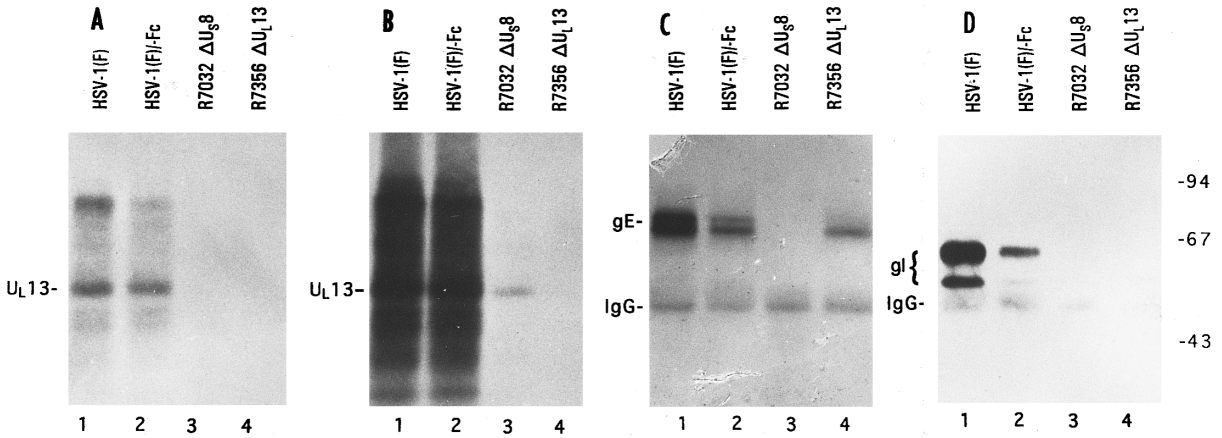

FIG. 5. (A and B) Autoradiographic images of electrophoretically separated proteins labeled with [^{32}P]ATP in *in vitro* protein kinase assays. Replicate cultures of Vero cells were infected with HSV-1(F), R7356 (ΔU_L13), or R7032 (ΔgE) virus. At 24 h after infection, the infected cells were solubilized. The cell lysates shown in lane 2 were reacted with preimmune serum for 2 h and then with protein A–Sepharose for 1.5 h to deplete the HSV-1 Fc receptor. All the other cell lysates were incubated with protein A for 1.5 h. At the end of the reaction, the precleared cell lysates were mixed with U_L13 antiserum and the immune complexes harvested with protein A beads. The complexes were rinsed extensively, reacted in the kinase buffer in the presence of [γ-^{32}P]ATP, rinsed again, eluted from protein A–Sepharose, electrophoretically separated in a denaturing polyacrylamide gel, electrically transferred to a nitrocellulose sheet, and exposed to X-ray film for 2 h (A) or 24 h. (B). (C) The nitrocellulose was reacted with gE-specific monoclonal antibody (II481B2). (D) The nitrocellulose was reacted with gI-specific monoclonal antibody (Fd69).

gest that U_L13 is mediating the posttranslational modification of gE and both the phosphorylation of gE and gI in infected cells.

gE is phosphorylated by U_L13 *in vitro*

The purpose of these series of experiments was to test the protein kinase activity of U_L13 protein present in immune complexes. Figures 5A and 5B show the immune complexes obtained with anti- U_L13 antibody, incubated in a protein kinase buffer containing [γ-^{32}P]ATP, electrophoretically separated on a denaturing gel, transferred electrically to a nitrocellulose membrane, and subjected to autoradiography for either 2 h (Fig. 5A) or 24 h (Fig. 5B), respectively. Figure 5C shows a photograph of the nitrocellulose membrane reacted with the monoclonal antibody to gE, and Fig. 5D shows the autoradiographic image of the membrane reacted with the gI monoclonal antibody and ECL detection reagents. The results were as follows:

(i) The immune complexes containing U_L13 phosphorylated a protein with the electrophoretic mobility of U_L13 protein and also mediated the phosphorylation of a slower migrating protein that coprecipitated with the U_L13 immune complex (Fig. 5A, lane 1). The phosphorylation of U_L13 was a result of autophosphorylation since phosphorylated proteins were not detected in assays of immune precipitates obtained from lysate of cells infected with R7356 (ΔU_L13) virus (Fig. 5A, lane 4), even after prolonged autoradiography (Fig. 5B, lane 4). Furthermore, U_L13 was the only protein labeled in immune complexes from lysate of cells infected with R7032 (ΔgE) virus (Fig. 5B, lane 3). As noted in Figs. 1 and 2, cells infected with ΔgE virus accumulated less U_L13 protein than cells infected with wild-type virus. Consistent with this finding, phosphorylated U_L13 protein in immune complexes obtained with U_L13 antibody from lysate of cells infected with ΔgE virus was observed only on prolonged autoradiography (Fig. 5B, lane 3). This decrease in the amount of U_L13 may also reflect the amount of U_L13 immunoprecipitated in the absence of the Fc receptor.

(ii) Two lines of evidence indicate that the slower migrating, phosphorylated protein was a component of the Fc receptor. First, Fig. 5C shows that the slow migrating ^{32}P-labeled band in Fig. 5A, lanes 1 and 2, corresponded to the slower migrating band of a doublet which reacted with the monoclonal antibody to gE. Second, we depleted the Fc receptor by reacting a mixture of infected cell lysate and preimmune serum with protein A before immunoprecipitation with U_L13-specific antiserum. This procedure resulted in the removal of a substantial amount of the more slowly migrating phosphoprotein and a slight decrease in the recovery of the autophosphorylated U_L13, indicating that the slower migrating form was a component of the Fc receptor (Figs. 5A and 5C, lane 2).

The specificity of the phosphorylation of gE was apparent from the absence of labeled gE in immune complexes from lysate of R7356 (ΔU_L13)-infected cells (Figs. 5A and 5B, lane 4) or those of R7032 (ΔgE)-infected cells (Figs. 5A and 5B, lane 3). We conclude from these series of experiments that gE was specifically phosphorylated by the precipitated U_L13.

(iii) In Fig. 4, gI was shown to be a phosphoprotein that coprecipitated with the U_L13 immune complex. It

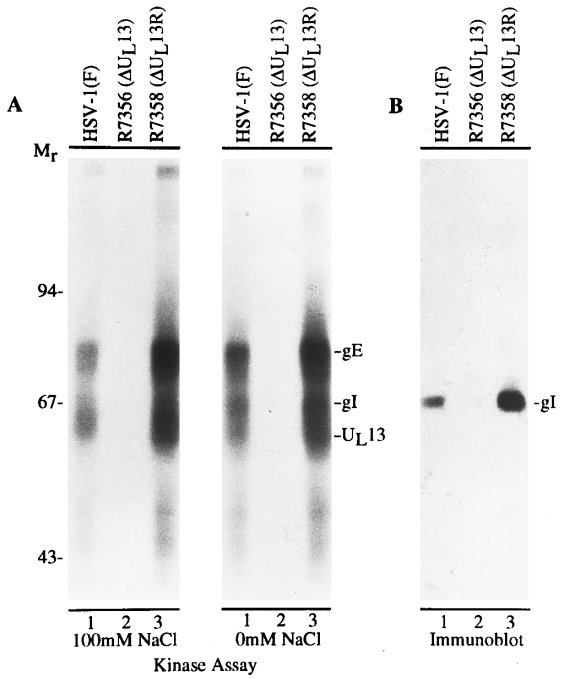

FIG. 6. (A) Autoradiographic images of proteins phosphorylated in an *in vitro* protein kinase assay. Vero cells were infected with HSV-1(F), R7356 (ΔU_L13), or R7358 (ΔU_L13 repair). At 24 h after infection the cells were solubilized. The cell lysates were precleared with protein A–Sepharose and then immunoprecipitated with the U_L13 polyclonal antibody. The kinase assay was carried out as described under Materials and Methods. The kinase reactions were electrophoretically separated in a denaturing polyacrylamide gel, electrically transferred to a nitrocellulose sheet, and exposed to X-ray film for 24 h. (B) Photograph of the nitrocellulose sheet after reaction with gI-specific monoclonal antibody (Fd69).

was of interest to determine whether U_L13 could mediate the phosphorylation of gI in the protein kinase assay. The nitrocellulose membrane shown in Fig. 5A was reacted with the monoclonal antibody to gI. The results, shown in Fig. 5D, indicate that gI migrated to locations intermediate between those of U_L13 protein and gE, and, as could be expected, the amounts of gI were decreased after depletion of Fc receptor from cell lysate (Fig. 5D, lane 2). The protein bands formed by gI did not correspond to any strong ^{32}P-labeled band in Fig. 5A.

gI is phosphorylated by U_L13 *in vitro*

Daikoku *et al.* reported that NaCl inhibited the activity of the U_L13 protein kinase. In an attempt to determine if NaCl inhibited the phosphorylation of gI shown in lane 1 of Fig. 5D, immune complexes of U_L13 was incubated in a protein kinase buffer containing NaCl concentration reduced from 200 mM to 100 mM NaCl (Fig. 6A, right) and 0 mM NaCl (Fig. 6A, left). The reactions were electrophoretically separated on a denaturing gel, transferred electrically to a nitrocellulose membrane, and subjected to autoradiography for 16 h (Fig. 6A). Figure 6B shows a photograph of the nitrocellulose membrane reacted with the monoclonal antibody to gI. The results were as follows:

The immune complexes containing U_L13 phosphorylated U_L13 and also mediated the phosphorylation of two slower migrating proteins that coprecipitated with the U_L13 immune complex (Fig. 6A, lanes 1 and 3). The upper band was identified as gE (data not shown). In Fig. 6A the middle ^{32}P-labeled band corresponded to a band which reacted with the monoclonal antibody to gI (Fig. 6B). We conclude from this experiment that gI was phosphorylated by the precipitated U_L13 *in vitro.*

U_L13 is coprecipitated with gE

Evidence from Figs. 3 and 4 suggested that U_L13 was captured in immune complexes with the Fc receptor. To determine which component of the Fc receptor U_L13 associates with, the immunoprecipitate of U_L13 was compared with those of monoclonal antibodies of gE or gI in a protein kinase assay. The U_L13 antiserum immunoprecipitated both U_L13 and a small amount of gE (Fig. 7A, lane 1), while the gE monoclonal antibody immunoprecipitated the gE protein and a small amount of a protein with an electrophoretic mobility characteristic of U_L13 protein (Fig. 7A, lane 2). The protein kinase assay done on the gE immunoprecipitate from R7356 (ΔU_L13) virus-infected cells failed to label gE due to the absence of U_L13 in the immunoprecipitate (Fig. 7A, lane 3). No phosphorylated proteins were detected in the kinase assays of immune complexes obtained with the gI mono-

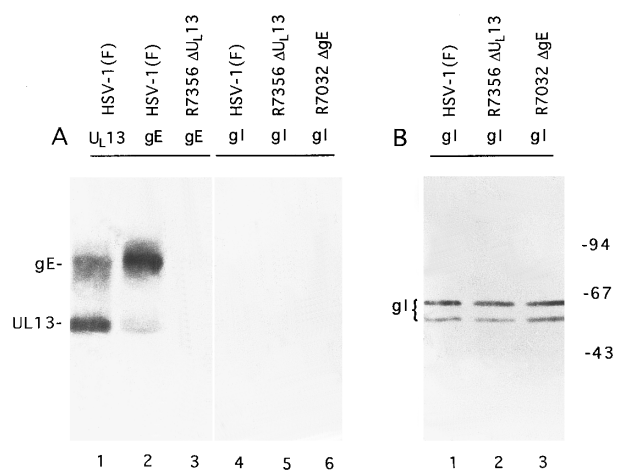

FIG. 7. (A) Autoradiographic image of proteins contained in immune complexes and labeled in an *in vitro* protein kinase reaction. Replicate cultures of Vero cells were infected with HSV-1(F), R7356 (ΔU_L13), or R7032 (ΔgE). The lysates of cells harvested at 24 h after infection were precleared with protein A and then reacted with anti-U_L13 polyclonal antiserum (lane 1), gE monoclonal antibody (H600) (lanes 2 and 3), or gI monoclonal antibody (Fd69) (lanes 4–6). Immune complexes were tested in the protein kinase assay as described in Fig. 5 and also under Materials and Methods, rinsed again, and processed for autoradiography as described in Fig. 5. (B) Photograph of the nitrocellulose membrane containing lanes 4–6 in A after reaction with the anti-gI monoclonal antibody (Fd69).

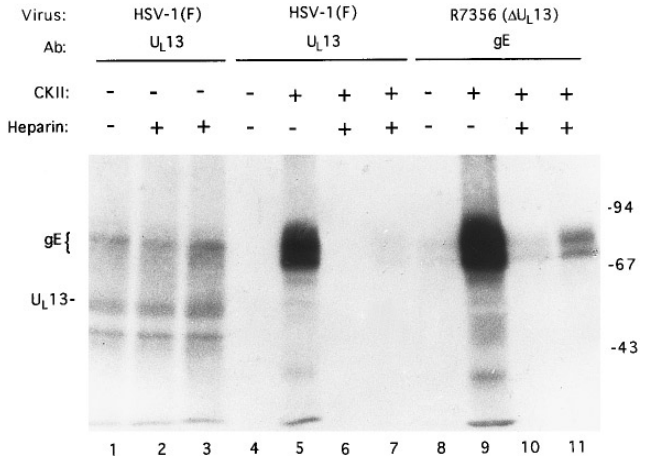

FIG. 8. Autoradiographic images of gE phosphorylated by purified CKII. Replicate cultures of Vero cells were infected with HSV-1(F) or R7356 (ΔU_L13). At 24 h after infection, the infected cells were solubilized. All the cell lysates were precleared with protein A and then immunoprecipitated with anti-U_L13 polyclonal antibody (lanes 1–7) or gE monoclonal antibody (H600) (lanes 8–11). Immune complexes in lanes 4–7 were heated-inactivated at 65°C for 10 min. All immune complexes were then reacted with [γ-^{32}P]ATP and U_L13 protein kinase buffer (lanes 1–3), or CKII protein kinase buffer (lanes 4 and 8), or a mixture of CKII protein kinase buffer containing exogenous, purified CKII (lanes 5–7 and 9–11). Heparin in final concentrations of 0.5 μg/ml (lanes 3, 7, and 11) or 5 μg/ml (lanes 2, 6, and 10) was added to the protein kinase buffer mixture to inhibit CKII kinase activity. On termination of the assay, the immune complexes were rinsed again and processed for autoradiography as described in the legend to Fig. 5 and under Materials and Methods.

clonal antibody and lysates of wild-type, R7356 (ΔU_L13), or R7032 (ΔgE) virus-infected cells (lanes 4–6). To confirm that gI was immunoprecipitated by the gI monoclonal antibody, the nitrocellulose membrane corresponding to lanes 4–6 in Fig. 7A was reacted with gI monoclonal antibody. As shown in Fig. 7B, lanes 1–3, gI was present in the immunoprecipitates, indicating that gI did not associate with the U_L13 protein under these experimental conditions.

Casein kinase II can phosphorylate gE *in vitro*

The homolog of HSV-1 gE in VZV was reported to be phosphorylated by CKII *in vitro* (Litwin *et al.,* 1992). Both the HSV-1 and VZV gE have CKII recognition sequences in the cytoplasmic domains of the proteins (Litwin *et al.,* 1992). In an attempt to determine if HSV-1 gE can be phosphorylated by CKII *in vitro,* gE immunoprecipitated from R7356 (ΔU_L13)-infected cells was reacted with CKII in a protein kinase assay *in vitro* in the presence of none or 0.5 or 5 μg/ml of heparin in the kinase buffer (Fig. 8, lanes 9–11). gE was phosphorylated by CKII *in vitro* (lane 9) and this phosphorylation was inhibited by heparin (lanes 10–11). In another part of the experiment, CKII was incubated with U_L13 immune complexes containing gE immunoprecipitated from cells infected with the wild-type virus. To inactivate all endogenous kinase activity, the immune complexes were heat-inactivated prior to the addition of CKII. This procedure inactivated all endogenous kinase activity (Fig. 8, lane 4), but did not affect the capacity of gE to be phosphorylated by CKII (Fig. 8, lane 5). As expected, the CKII activity was inhibited by heparin (lanes 7 and 8). Finally, heparin did not affect the activity of U_L13 kinase in assays with precipitates from wild-type-infected cells (Fig. 8, lanes 1–3). Interestingly, gE phosphorylated by CKII formed two bands (Fig. 8, lanes 5 and 9) whereas, as shown earlier, gE phosphorylated by U_L13 migrated as a slower migrating band.

Repair of the ΔU_L13 gene restores protein kinase activity and posttranslational processing of the Fc receptor

In an *in vitro* protein kinase assay with lysate of cells infected by the ΔU_L13 virus, the phosphorylation of the Fc receptor was completely abolished (Fig. 6A, lanes 2). Also, in immunoprecipitates from ΔU_L13 virus-infected cell lysates labeled *in vivo* with [^{32}P]orthophosphate using the anti-U_L13 antiserum, the posttranslational processing and overall phosphate labeling of gE and gI were diminished (Fig. 9B, lane 2). To verify that the

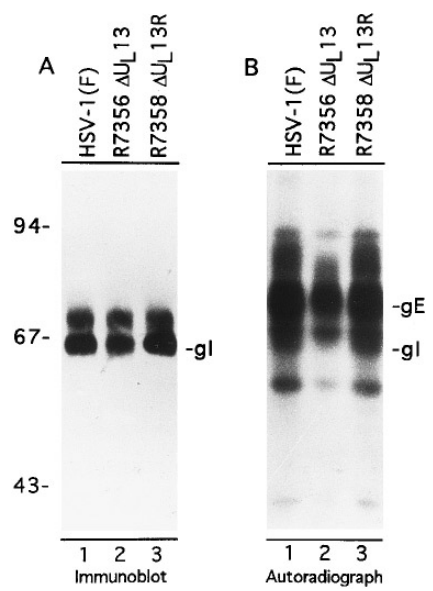

FIG. 9. Photograph and autoradiogram of an electrophoretically separated immune precipitate obtained from ^{32}P-labeled infected cell lysates with anti-U_L13 antibody. (A) Photograph of the electrophoretically separated preciptate after reaction with anti-gI-specific monoclonal antibody (Fd69). (B) Autoradiographic image of ^{32}P of the electrophoretically separated precipiated obtained with the anti-U_L13 antibody from lysates of Vero cells infected with HSV-1(F), R7356 (ΔU_L13), or R7358 (ΔU_L13 repair). The cells were labeled with [^{32}P]orthorphosphate at 14 h after infection for 4 h and then harvested, solubilized, immunoprecipitated with the anti-U_L13 rabbit polyclonal antiserum, and eluted from protein A–Sepharose. All samples were electrophoretically separated in an 8% denaturing polyacrylamide gel and transferred to a nitrocellulose sheet for autoradiography and reaction with the anti-gI antibody.

protein kinase activity and differential phosphorylation observed in this study was exclusively due to the U_L13 gene, R7358, the virus in which the U_L13 gene had been restored, was tested. Figure 6A shows the immune complexes obtained with the anti-U_L13 antibody, incubated in protein kinase buffer with different NaCl concentrations, electrophoretically separated on a denaturing gel, transferred to a nitrocellulose sheet, and subjected to autoradiograph for 16 h. In cells infected with the U_L13 repair virus (R7358), the specific kinase activity is restored, resulting in phosphorylation of U_L13, gE, and gI (Fig. 6A, compare lanes 2 and 3).

The phosphorylation of gE and gI in cells infected with the U_L13 repair virus was also examined (Fig. 9B). Immune complexes obtained from Vero cells infected with HSV-1(F), R7356(ΔU_L13), and R7358 (ΔU_L13 repair) and labeled with [^{32}P]orthophosphate from 14 to 18 h after infection were electrophoretically separated on a denaturing gel, transferred to a nitrocellulose sheet, and subjected to autoradiography for 2 h. (Fig. 9A shows the immunoblot of the nitrocellulose with gI monoclonal antibody to detect the amount of gI in each sample. The phosphorylation of gE and gI in cells infected with the repair virus (R7358) was restored to a level comparable to that of the wild-type virus (Fig. 9B, compare lanes 1 and 3). These results suggest that U_L13 is responsible for the phosphorylation of gE and gI in infected cells and the loss of this activity is not due to some unknown secondary mutation in the ΔU_L13 virus.

DISCUSSION

The salient features of the results presented in this report are as follows:

(i) We expected that complexes containing IgG, either bound to an antigen or free, would bind the HSV-1 Fc receptor glycoproteins, gE and gI. We did not expect, however, that complexes containing IgG directed to an irrelevant antigen would contain the U_L13 protein. Since U_L13 is captured by gE monoclonal antibody, but not by gI monoclonal antibody, these results suggest that the interaction is primarily between gE and U_L13 protein.

gE is a phosphoprotein located in the virion membrane and, by necessity, in the nuclear membrane for the preenvelopment of the capsid. The U_L13 protein is a tegument protein and partially responsible for the kinase activity demonstrated in purified capsid–tegument structures (Lemaster and Roizman, 1980, and unpublished data this laboratory). In principle, there are at least two explanations for the surprising affinity of the U_L13 protein for gE. The first and most straightforward explanation is that gE is a substrate for U_L13. The second and more complex explanation is that gE, as an envelope protein, serves as an anchor for the packaging of U_L13. In this report, we show that gE is in fact a substrate for U_L13, as discussed more fully below. We have not excluded a role for gE in the packaging of U_L13 protein since this would require an analysis of virions made from cells infected with the ΔgE virus.

(ii) gE from wild-type virus-infected cells forms two sharp bands and a diffuse, heterogeneous and slower migrating mass in denaturing polyacrylamide gels. In cells infected with ΔU_L13 virus, the slower migrating sharp band and the diffuse, heterogenous mass are grossly diminished. In *in vitro* protein kinase reactions containing U_L13 and gE, the ^{32}P label appears only in the slower migrating sharp band. These observations suggest that the U_L13 kinase is responsible for both the phosphorylation and, possibly, posttranslational processing of gE, but it remains to be seen whether the change in electrophoretic mobility is a direct effect of the phosphorylation of gE by U_L13.

(iii) The results of the experiments shown in Fig. 4 unambiguously show that U_L13 does not account for all of the phosphorylation of gE and gI. CKII is a serious candidate for a kinase responsible for the phosphorylation of gE. Specifically, gE has several CKII recognition sites in the endodomain accessible to the cytoplasm and nucleoplasm depending on the location of the membrane containing gE (Litwin *et al.,* 1992). In this study we showed that purified CKII added to immune complexes phosphorylated gE in the absence of any endogenous kinase activity. We also showed that the CKII activity is sensitive to heparin and that, conversely, the protein kinase activity in the *in vitro* reaction attributed to U_L13 is insensitive to heparin. It has recently been reported that the U_L13 kinase purified from HSV-2-infected cells is also insensitive to heparin inhibition (Daikoku *et al.,* 1997). The experimental results shown in Fig. 8 indicate, however, that CKII can account for some but not all of the phosphorylation of gE observed in the absence of U_L13. This conclusion is based on the observation that in the absence of U_L13, gE forms predominantly the faster migrating sharp band and a smaller amount of slower migrating diffuse, heterogeneous band (Fig. 3B, lane 3, and Figs. 4A and 4B, lanes 3 and 8). The gE phosphorylated *in vitro* by CKII forms two relatively sharp bands (Fig. 8, lanes 5 and 9). This suggests that if CKII is involved in phosphorylation of gE, it contributes primarily to the faster migrating band and to trace amounts of the slower migrating sharp band formed by gE contained in lysate of cells infected with wild-type virus (compare Fig. 4B lanes 3 and 8 with Fig. 8 lanes 5, 9, and 11).

(iv) In this study, we presented both *in vivo* (infected cells) and *in vitro* evidences that U_L13 can mediate the phosphorylation of gE and gI. We have not been able to demonstrate, however, an interaction between gI and U_L13. Immune complexes containing monoclonal antibody to gI did not capture U_L13 in detectable amounts.

(v) Last, the issue arises as to why gE and gI are phosphorylated by seemingly a myriad of protein kinases. By analogy with other viral proteins, we may

TABLE 1

Genotype and Phenotype of Recombinant Viruses Used in This Study

Virus	Genotype[a]	Phenotype
HSV-1(F)	Wild-type	Wild-type
R7356	ΔU_L13	ΔU_L13
R7358	ΔU_L13 repair	ΔTK
R7032	ΔU_S8	ΔgE
R7048	ΔU_S7, ΔU_S8	ΔgE, ΔgI, ΔTK

[a] (Δ) Gene containing deletion.

postulate that gE and possibly both gE and gI perform several functions and that the various protein kinases direct the functions of the glycoproteins by modifying them at different sites. In point of fact, gE and gI have been reported to play a role in blocking some host responses to infection (Dublin *et al.,* 1991), and to play a role in the spread of virus from cell to cell (Dingwell *et al.,* 1994, 1995); in psuedorabies virus the endodomain of the gE homologue has been shown to contribute to neurovirulence (Tirabassi *et al.,* 1997), and more recently evidence has accumulated that the expression of late genes is delayed in cells infected with ΔgE viruses (unpublished data from this laboratory). Identification of the various kinases and the sites they modify may help ultimately dissect the individual functions of these glycoproteins.

MATERIALS AND METHODS

Cells and viruses

Vero cells were obtained from the American Type Culture Collection. The rabbit skin cells were originally from John McClaren. HSV-1(F) is the prototype strain used in this laboratory (Ejercito *et al.,* 1968). The constructions of HSV-1 recombinant viruses R7032, R7048, R7356, and R7358 were reported elsewhere (Longnecker *et al.,* 1987; Meignier *et al.,* 1988; Purves and Roizman, 1992; Purves *et al.,* 1993). Table 1 lists the genotypes of all the viruses used in this study.

Production of GST fusion protein to U_L13 and production of U_L13 rabbit antiserum

The entire coding domain of the U_L13 gene was amplified by PCR and fused to the glutathione *S*-transferase (GST) gene in pGEX-4T-1 plasmid (Pharmacia). PCR amplification was done with the PFU DNA Polymerase (Stratagene). The resultant plasmid was designated pRB5151. *Escherichia coli* BL21 cells transformed with pBR5151 were grown to an optical density of at least 0.5 at A_{600}, induced with 0.1 mM IPTG (isopropylthiogalactopyranoside) for 4 h and then harvested and lysed by sonication in phosphate-buffered saline (PBS-A: 0.14 M NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4). After addition of Triton X-100 to a final concentration of 1%, the GST–U_L13 fusion protein was absorbed to glutathione–agarose beads (Sigma), washed with PBS-A, and eluted with 10 mM reduced glutathione (Sigma) in 50 mM Tris–HCl (pH 8.0). The eluted protein was quantified by a protein assay (Bio-Rad). Two rabbits were injected twice subcutaneously with 0.5 mg of the purified GST–U_L13 fusion protein at Josman Laboratories (Napa, CA). After the initial immunization, the rabbits were boosted at day 21 and serum was collected at day 28 and used in these studies.

Preparation of radioactively labeled infected cell lysate

To prepare ^{35}S-labeled infected cell lysate, replicate Vero cell cultures were exposed to 20 PFU of parent or mutant viruses per cell. At 12.5 h after infection, the cells were overlaid with 1 ml of medium deficient in methionine and cysteine but supplemented with 50 μCi of [^{35}S]methionine and [^{35}S]cysteine (sp act >1000 Ci/mmol; Amersham, Arlington Heights, IL) for 6 h. The cells were rinsed and then solubilized in 500 μl of RIPA buffer [0.01 M Tris–HCl (pH 7.4), 0.15 M NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS] containing protease inhibitors [0.1 mM tosylsulfonylphenylalanyl-chloromethyl ketone (TPCK), 0.1 mM tosyl-*L*-phenylalanine chloromethyl ketone, (TLCK), 0.1 mM phenylmethylsulfonyl fluoride (PMSF)] and subjected to electrophoresis in denaturing polyacrylamide gels or to immunoprecipitation.

To prepare ^{32}P-labeled infected cell lysate, replicate 25-cm^2 Vero cell cultures were exposed to 20 PFU of parent or mutant viruses per cell. To label gE and gI, the cells were preincubated in Eagle's minimal essential medium containing no phosphate and supplemented with 1% dialyzed phosphate-free calf serum for 1 h at 13 h after infection. Cells were labeled with 100 to 200 μCi of ^{32}Pi (New England Nuclear) in 3 ml of medium for 4 h. To label U_L13 protein, infected cells were labeled from 6 to 22 h after infection. In both cases, cells were scraped and washed in PBS-A and resuspended in 500 μl of RIPA buffer containing protease inhibitors as described above.

Immunoprecipitation

Radiolabeled Vero cell lysate equivalent to half of a 25-cm^2 culture flask was first precleared by exposure to protein A-conjugated Sepharose beads (Sigma) at 4°C for 1 h. The precleared lysate was reacted with a 1:100 dilution of preimmune, U_L13, or U_L32 polyclonal antisera overnight at 4°C. The immune complex was then precipitated with protein A–Sepharose. After extensive washing of the Sepharose beads with PBS wash buffer (PBS-A with 0.5% NP-40 and 0.1% SDS), immune complexes were eluted from the beads by boiling for 5 min in disruption

buffer. The eluted proteins were electrophoretically separated in denaturing polyacrylamide gels. Anti-U_L32 polyclonal antiserum was described previously (Chang *et al.,* 1996).

Electrophoretic separation and immunoblotting of viral proteins

Replicate 25-cm^2 flask cultures of rabbit skin cells or Vero cells were exposed to 20 PFU parent or mutant viruses per cell and maintained at 37°C for 24 h. U_L13, ICP22, and ICP27 in infected cells were detected by immunoblotting as described previously (Ng *et al.,* 1996). Briefly, infected cells were pelleted by low-speed centrifugation, rinsed in PBS-A, and resuspended in 200 μl of disruption buffer [12.5 mM Tris–HCl (pH 6.8), 0.5% SDS, 2.5% glycerol, 5% β-mercaptoethanol]. Approximately half of each sample was loaded onto each lane of a denaturing 8.5% polyacrylamide gel. The electrophoretically separated polypeptides were electrically transferred to nitrocellulose membrane (Schleicher & Schuell) and reacted with the appropriate antibodies. The bound antibody was visualized with a second anti-mouse or anti-rabbit IgG antibody conjugated to alkaline phosphatase (Bio-Rad). The rabbit polyclonal antibody R77 was directed against ICP22 (Ackermann *et al.,* 1985). Monoclonal antibody to ICP27 (H1117) was described by Ackermann *et al.,* (1984) and was purchased from Goodwin Institute (Plantation, FL).

To detect U_L13 protein in the ^{35}S-labeled cell lysate, approximately 1/10 of a lysate of a 25-cm^2 culture flask was subjected to electrophoresis, electrically transferred to nitrocellulose membrane, and reacted with the U_L13 antiserum. The bound antibody was visualized with a second anti-rabbit IgG antibody conjugated to horseradish peroxidase and then to ECL detection reagents according to the manufacturer's instructions (Amersham). To detect gE in the ^{35}S- or ^{32}P-labeled cell lysates, the nitrocellulose was reacted with monoclonal antibody of gE, II481B2, and the bound antibody was visualized with a second anti-mouse antibody conjugated to alkaline phosphatase. Monoclonal antibody II481B2 was a kind gift of Patricia Spear (Para *et al.,* 1985). Glycoprotein gI was detected by immunoblotting with the monoclonal antibody, Fd69 (Longnecker *et al.,* 1987) and ECL detection reagents.

Protein kinase assay

U_L13 was immunoprecipitated from unlabeled infected Vero cell lysate as described above. To deplete the HSV-1 Fc receptor before the immunoprecipitation with U_L13 antibody, the infected cell lysate was first reacted with 10 μl of rabbit preimmune serum for 2 h and then with protein A–Sepharose for 15 h. The Fc-depleted lysate was then used for immunoprecipitation with the U_L13 antiserum as described above. The washed immune complex on the protein A-conjugated Sepharose beads was then washed once with protein kinase buffer [50 mM Tris (pH 8), 50 mM $MgCl_2$, 0.2 M NaCl, 0.1% NP-40, 1 mM DTT]. The protein kinase buffer was modified from the buffer described by Cunningham *et al.* (1992). Protein kinase assays were done as described elsewhere (Ng *et al.,* 1994). Briefly, the Sepharose beads were incubated with 70 μl protein kinase buffer and 50 μCi of [γ-^{32}P]ATP (sp act 6000 Ci/mmol, DuPont/NEN) at 37°C for 30 min. The beads were then washed extensively and the immune complexes were eluted from the protein A beads by boiling for 5 min in disruption buffer.

Phosphorylation of gE by CKII

Phosphorylation of HSV gE by CKII *in vitro* was carried out in a manner similar to the phosphorylation of VZV ORF62 by CKII as described elsewhere (Ng *et al.,* 1994). Briefly, gE was immunoprecipitated from R7356 (ΔU_L13)-infected cells by the gE monoclonal antibody (H600) (Longnecker and Roizman, 1986) or precipitated from HSV-1(F)-infected cells by the U_L13 antiserum as part of the Fc receptor. The precipitate from the HSV-1(F)-infected cells was heat-inactivated at 65°C for 10 min to inactivate any protein kinase activity associated with the precipitate. The protein kinase assay was initiated by reacting the gE bound on protein A–Sepharose with 5 enzyme μunits of CKII purified from sea star (Upstate Biotechnology Inc.) in a CKII buffer [50 mM Tris (pH 7.5), 150 mM KCl, and 10 mM $MgCl_2$] at 30°C for 30 min. To test for the specificity of the activity of CKII, heparin in concentrations of 0.5 or 5 μg/ml was added to individual reaction mixtures before the protein kinase assay (Mitchell *et al.,* 1994). After five washes with PBS-A wash buffer, gE was eluted from the protein A–Sepharose by boiling in disruption buffer.

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