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The Herpes Simplex Virus U_S11 Protein Effectively Compensates for the $\gamma_1$34.5 Gene if Present before Activation of Protein Kinase R by Precluding Its Phosphorylation and That of the α Subunit of Eukaryotic Translation Initiation Factor 2

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In herpes simplex virus-infected cells, viral $\gamma_1$34.5 protein blocks the shutoff of protein synthesis by activated protein kinase R (PKR) by directing the protein phosphatase 1α to dephosphorylate the α subunit of eukaryotic translation initiation factor 2 (eIF-2α). The amino acid sequence of the $\gamma_1$34.5 protein which interacts with the phosphatase has high homology to a domain of the eukaryotic protein GADD34. A class of compensatory mutants characterized by a deletion which results in the juxtaposition of the α47 promoter next to U_S11, a γ_2 (late) gene in wild-type virus-infected cells, has been described. In cells infected with these mutants, protein synthesis continues even in the absence of the $\gamma_1$34.5 gene. In these cells, PKR is activated but eIF-2α is not phosphorylated, and the phosphatase is not redirected to dephosphorylate eIF-2α. We report the following: (i) in cells infected with these mutants, U_S11 protein was made early in infection; (ii) U_S11 protein bound PKR and was phosphorylated; (iii) in in vitro assays, U_S11 blocked the phosphorylation of eIF-2α by PKR activated by poly(I-C); and (iv) U_S11 was more effective if present in the reaction mixture during the activation of PKR than if added after PKR had been activated by poly(I-C). We conclude the following: (i) in cells infected with the compensatory mutants, U_S11 made early in infection binds to PKR and precludes the phosphorylation of eIF-2α, whereas U_S11 driven by its natural promoter and expressed late in infection is ineffective; and (ii) activation of PKR by double-stranded RNA is a common impediment countered by most viruses by different mechanisms. The $\gamma_1$34.5 gene is not highly conserved among herpesviruses. A likely scenario is that acquisition by a progenitor of herpes simplex virus of a portion of the cellular GADD34 gene resulted in a more potent and reliable means of curbing the effects of activated PKR. U_S11 was retained as a γ_2 gene because, like many viral proteins, it has multiple functions.

The herpes simplex virus 1 (HSV-1) genome encodes two sets of functions. The first and paramount are functions related to viral gene expression, replication of viral DNA, synthesis of virion proteins, assembly, packaging, and egress of the virus from the infected cell. The second set of functions, no less important in the survival of the virus in the human population, is creation of the environment necessary to maximize the yield and spread of virus from cell to cell and from infected to uninfected individuals (reviewed in reference 38). Of these known genes, several play a significant role in abating or delaying a host response to infection. The earliest to be expressed is the U_L41 gene which encodes a protein that is introduced into the cell in virions during infection (26, 27). This protein reduces the synthesis of host proteins by causing the destruction of mRNA in a rather nonspecific manner and therefore could be expected to reduce the synthesis of cellular proteins deleterious to viral replication (26, 27, 44).

A second and very different approach to blocking host defense mechanisms is exemplified by infected cell protein 47 (ICP47). Proteosomal degradation of viral proteins could be expected to produce antigenic peptides which, if presented on the cell surface, could provoke a cytotoxic cell response early in infection and thus reduce viral yield. ICP47, an α protein made immediately after infection, blocks the presentation of antigenic peptides on the surface of the infected cells (20).

The focus of this laboratory has been on a third viral pathway designed to block cellular response to infection. In cells infected with most viruses, the synthesis of complementary mRNA leads to activation of double-stranded RNA-dependent protein kinase R (PKR). This enzyme phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF-2α) (23). A consequence of this phosphorylation is total shutoff of protein synthesis. This would be an example of a noble sacrifice of the infected cell for the sake of survival of the organism were it not for the fact that viruses, while activating the PKR kinase pathway by making double-stranded RNA, also express functions which block this host defense system (2–4, 6, 7, 10, 28, 30, 34). In the case of HSV-1, more than 50% of the viral DNA is represented late in infection in the form of cRNA (21, 25), and the gene whose product blocks the consequences of activation of PKR is $\gamma_1$34.5 (7). In the absence of the gene, eIF-2α is phosphorylated and protein synthesis is impaired beginning approximately 5 h after infection (7, 9). In its presence, protein synthesis continues unabated even though PKR is activated (9). Recent studies have shown that the carboxyl terminus of the $\gamma_1$34.5 gene binds to the protein phosphatase 1α (PP1) and redirects it to dephosphorylate eIF-2α (19). The effectiveness of the $\gamma_1$34.5-PP1 complex is apparent from the observation that the rate of dephosphorylation of eIF-2α in cells infected

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with wild-type virus is more than 1000 times that of uninfected cells or cells infected with the $\gamma_1 34.5^-$ virus (5, 19).

The studies described in this report concern another aspect of virus-induced block of the consequence of activation of PKR. Briefly, Mohr and Gluzman reported that serial passage of a $\gamma_1 34.5^-$ mutant resulted in the selection of a compensatory mutation capable of sustained protein synthesis (35). A characteristic of the compensatory mutants isolated by Mohr and Gluzman is a deletion in the α47 gene resulting in the juxtaposition of the promoter of the α47 gene next to the 5′ end of U_S11, a late (γ_2) viral gene. Preliminary studies of those mutants revealed that PKR was activated in cells infected with either the wild-type parent or the $\gamma_1 34.5^-$ virus, but protein synthesis was unaffected in cells infected with wild-type virus or the mutant carrying the compensatory mutations (5, 18).

In an attempt to define the phenotype of the virus carrying the compensatory mutation, we constructed a mutant lacking the $\gamma_1 34.5$ and the U_S8 to -12 genes. This mutant, designated R5103, activated PKR and caused a shutoff of protein synthesis (5). We then inserted into the R5103 genome a DNA fragment consisting of the intact U_S10 gene and the U_S11 open reading frame fused to the α47 promoter. This virus, designated R5104, activated PKR but did not induce the shutoff of protein synthesis. Consistent with the conclusion of Mohr and Gluzman (35), the mutation maps in the domain inserted into the R5104 virus (5). Further studies yielded two significant observations. First, in stark contrast to lysates of cells infected with R5103 and other $\gamma_1 34.5^-$ mutants, the lysates of R5104 virus failed to phosphorylate the α subunit of eIF-2 (5). Second, in striking contrast to lysates of wild-type virus-infected cells, the phosphatase activity of lysates of R5104 virus-infected cells specific for eIF-2α could not be differentiated from that of mock-infected cells or those of cells infected with other $\gamma_1 34.5^-$ mutants (5). These results indicated that the compensatory mutation blocks PKR from phosphorylating eIF-2α.

The studies summarized in this report focused on U_S11 protein. We report that in cells infected with the R5104 recombinant the U_S11 protein is made early in infection, that U_S11 protein interacts with PKR and blocks the phosphorylation of eIF-2α by activated PKR in in vitro assays, and that the effectiveness of the U_S11 protein is greater if the protein is present in the reaction before activation of PKR than if it is after PKR has been activated by the addition of poly(I-C). We also found that U_S11 is phosphorylated in the presence of activated PKR but not in its absence. We conclude that U_S11 may have been an ancient mechanism for blocking the effects of activated PKR and that it has been supplanted by acquisition of the carboxyl-terminal domain of the $\gamma_1 34.5$ protein from a cellular gene. We also note that U_S11 protein made late in infection, after PKR has been activated, is ineffective.

Relevant to this report are some of the properties of the U_S11 protein. U_S11 is one of the most abundant viral proteins expressed at late times in viral infection (22, 31). It binds mRNA in a sequence- and conformation-specific fashion (39–41). In HSV-1-infected cells, U_S11 suppresses the synthesis of a truncated RNA colinear with the 5′ domain of the U_L34 mRNA (40). The protein accumulates in nucleoli, in the cytoplasm in association with the 60S ribosomal subunit, and it is also packaged in virions (31, 37, 41). In newly infected cells, the U_S11 protein has been found associated with ribosomes (41).

Recently a plethora of reports suggested that U_S11 may have novel functions not readily apparent from its localization in the infected cell. Thus, U_S11 protein has been reported to have functions similar to those of human immunodeficiency Tat and Rev proteins and has also been reported to complement Rev function in a Rev^- human immunodeficiency virus mutant (11). The U_S11 protein has been reported to confer thermotolerance and help restore protein synthesis in HeLa cells subjected to thermal injury (12).

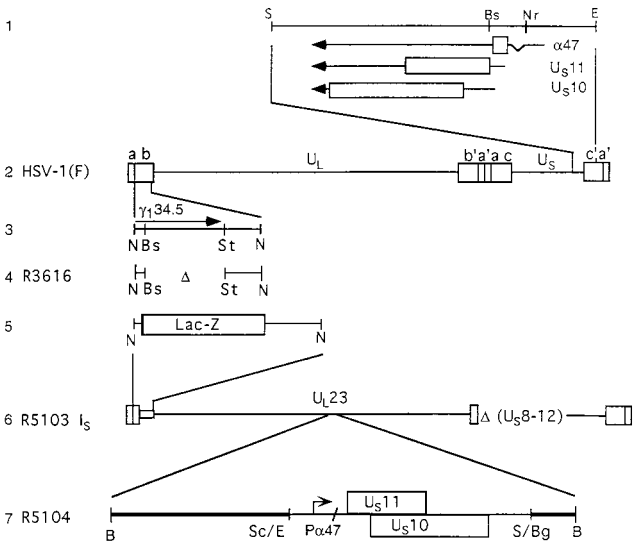

FIG. 1. Schematic representation of the HSV-1 genome and of genome domains relevant to this report. Line 1, sequence arrangement of the domains of the genes U_S10, U_S11, and α47 (U_S12) located at the terminus of unique short (U_S) sequence shown in the prototype (P) orientation. The coding sequences are shown as rectangular boxes; thin lines and arrows represent the transcriptional unit and polarity of the genes. Line 2, sequence arrangement of HSV-1 genome. The rectangular boxes represent the inverted repeat sequences *ab* and *b'a'* flanking the unique long (U_L) sequence and inverted repeats *c'a'* and *ca* flanking the U_S sequence. Line 3, map location of the $\Delta_1 34.5$ gene. In wild-type virus, the $\Delta_1 34.5$ gene is present in both copies of the inverted repeats. Line 4, representation of the structure of R3616 DNA in which both copies of the $\Delta_1 34.5$ had been deleted. Line 5, structure of the single *ab* sequence of the R5103 genome in which the $\Delta_1 34.5$ gene had been replaced with the *E. coli lacZ* gene. Line 6, sequence arrangement of the R5103 genome. In both the R5103 and parent R7023 genomes (29), the genes U_S8 to -12 as well as most of the internal inverted repeats had been deleted. The U_S sequence is in an inverted (I_S) arrangement. Line 7, sequence arrangement of DNA fragment inserted into the *Bgl*II site of the *Bam*HI Q fragment, resulting in construction of the R5104 recombinant virus. The thick line represents the *Bam*HI Q fragment disrupted by the insertion of the fragment consisting of the α47 gene fused to the coding domain of the U_S11 gene. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; E, *Eco*RI; N, *Nco*I; Nr, *Nru*I; S, *Sal*I; Sc, *Sac*II; St, *Stu*I.

MATERIALS AND METHODS

Cells and viruses. All cell lines were obtained from the American Type Culture Collection. The properties of HSV-1(F), the prototype HSV-1 strain used in these studies, and the recombinant viruses R3616, R5103, and R5104 derived from it have been described elsewhere (5, 6, 13) and are represented in Fig. 1. The stocks of HSV-1(F) were prepared in HEp-2 cells. All recombinant virus stocks and all virus titrations were done in Vero cells. SK-N-SH and HeLa cells were used for protein radiolabeling studies and for the preparation of virus-infected cell lysates. Cells were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 5% newborn calf serum (Vero and HeLa) or with 5% fetal bovine serum (SK-N-SH).

Plasmids. Plasmid pRB4508, described previously (1), encodes the final 93 codons of the U_L10 open reading frame fused to the glutathione *S*-transferase (GST) coding sequence in plasmid pGEX-3x (Pharmacia). The U_L10 gene of HSV-1 encodes the viral glycoprotein gM, which is incorporated into the virion and cellular membranes of infected cells. Plasmid pRB4766 was prepared by inserting the U_S11 open reading frame contained within a Klenow-blunted *Eco*RI-*Xho*I fragment into blunted, *Hin*dIII- and *Xho*I-digested plasmid pGEX-KG such that the coding sequence of GST was fused in frame to the entire coding domain of the U_S11 gene (33, 41a). Plasmid pGEX-PKR(wt), kindly provided by Bryan R. G. Williams, encodes full-length PKR fused to GST and has been described previously (32). In addition, the control plasmid pGEX-3x (Pharmacia) was used to generate GST protein to serve as a control in appropriate experiments.

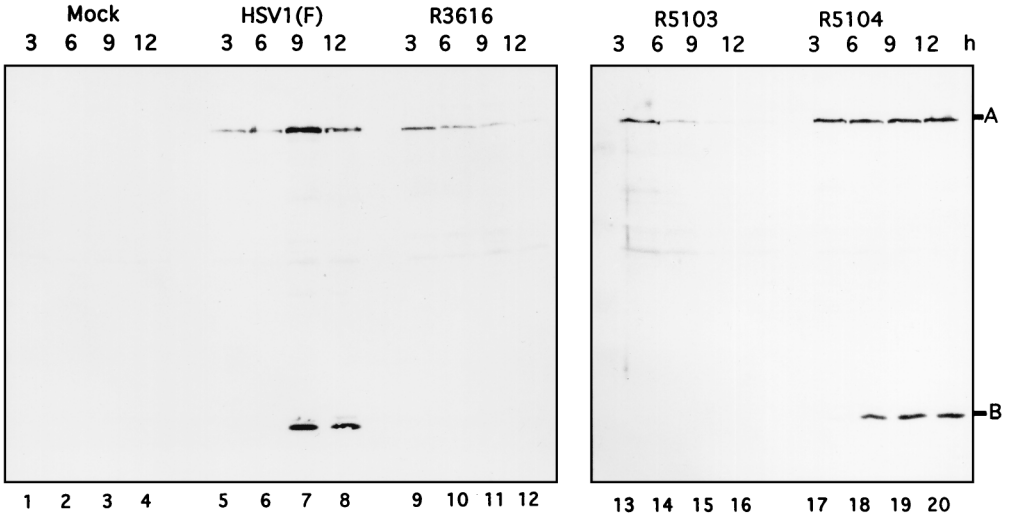

FIG. 2. Photograph of an immunoblot of lysates of wild-type and mutant viruses probed with U_S11 and ICP0 antibodies. Mock-infected SK-N-SH cells or SK-N-SH cells infected with HSV-1(F), R3616, R5103, or R5104 were harvested at 3, 6, 9, or 12 h after infection as described in Materials and Methods, lysed in disruption buffer, subjected to electrophoresis on an SDS–12.5% polyacrylamide gel, electrically transferred to a nitrocellulose sheet, and reacted with the anti-U_S11 or ICP0 monoclonal antibody as described in Materials and Methods. Positions of the U_S11 (labeled B) and ICP0 (labeled A) protein bands are indicated.

Labeling of proteins with [^{35}S]methionine and electrophoretic separation in denaturing gels. Protein labeling experiments were done as previously described (7, 36). Two hours before harvest, infected SK-N-SH cells in 25-cm^2 flasks were incubated in 1 ml of medium 199V (medium 199 supplemented with 1% calf serum) lacking methionine but supplemented with 50 μCi of [^{35}S]methionine (1,000 Ci/mmol; Amersham). The cells were then rinsed twice with PBS-A (phosphate-buffered saline [PBS]), scraped in 1 ml of ice-cold PBS-A, pelleted, solubilized in disruption buffer, boiled, electrophoretically separated on a denaturing 12.5% (vol/vol) polyacrylamide gel cross-linked with *N,N′*-diallyltartardiamide, electrically transferred to a nitrocellulose sheet, and subjected to autoradiography on Kodak XAR5 film or immunoblotting.

Immunoblotting. The nitrocellulose sheet containing the electrophoretically separated proteins was blocked with 5% skim milk in PBS-A (blocking solution) for at least 1 h, reacted with different antibodies diluted in blocking solution for at least 4 h, and rinsed five times in PBS-A–1% Tween for 15 min. The nitrocellulose filter was then reacted with an appropriate alkaline phosphatase-conjugated antibody diluted in blocking solution for approximately 90 min. The filter was then washed once in large volumes of PBS-A–1% Tween, washed four times in PBS-A, and developed by using 1× 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium in AP buffer (100 mM Tris-HCl [pH 9.5], 5 mM $MgCl_2$, 100 mM NaCl) (43).

The monoclonal antibodies against ICP0 (24) and U_S11 (41) have been described elsewhere.

GST protein affinity assay and immunoblotting. HeLa cells were mock infected or infected with HSV-1(F) for 14 h. During the last 2 h of infection, the infected cell proteins were labeled by incubation in medium 199V containing [^{35}S]methionine as described above. The cells were then rinsed twice with ice-cold PBS-A and resuspended in lysis solution (PBS-A containing 1% deoxycholate [DOC], 1% Nonidet P-40 [NP-40], and 5 mM sodium benzamidine) at a concentration of 4×10^6 cells/400 μl. The labeled infected cell lysates were then incubated with 20 μg of RNase A and 2 μg of RNase T_1 for 90 min at 37°C. The labeled infected cell lysates were sonicated by using a 3-mm probe for 10 s, and the insoluble material was removed by centrifugation. The soluble infected cell proteins were transferred to a new tube and used in the experiments described below.

GST and GST-PKR fusion proteins were expressed in *Escherichia coli* BL21 cells and induced by addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside. After a 90-min incubation, the bacteria were harvested. A 25-μl sample was then boiled, electrophoretically separated on a 10% (vol/vol) polyacrylamide minigel, and stained with Coomassie blue to quantify the relative amounts of GST and GST-PKR protein bound to glutathione-agarose beads (G-beads). Equivalent amounts of bound GST and GST-PKR protein were each reacted with 200 μl of the RNase-treated, labeled infected cell protein mixture described above for 12 h at 4°C. The beads were then harvested and washed seven times with PBS containing 1% DOC and 1% NP-40. The proteins bound to the G-beads were released by boiling in disruption buffer and electrophoretically separated on a sodium dodecyl sulfate (SDS)–denaturing 12.5% (vol/vol) polyacrylamide gel. The proteins were then electrically transferred to a nitrocellulose filter for autoradiography and immunoblotting.

In vitro phosphorylation of eIF-2α by PKR kinase. Phosphorylation reaction mixtures were incubated at 34°C in 10 mM Tris-HCl (pH 7.5)–20 mM KCl–2 mM $MgCl_2$ (TKM buffer) with the additions and for the times indicated in Results. The specific activity of the added [γ-^{32}P]ATP (purchased from ICN) was 2 to 10 Ci/mmol. Reactions were stopped by adding an SDS-containing, denaturing solution (16); the products were analyzed by electrophoresis for 20 h at 44 V (3 V/cm) on denaturing 7% polyacrylamide gels as described elsewhere (16), followed by silver staining, drying, and autoradiography.

Preparation of protein components and source of materials. Rabbit reticulocyte eIF-2 was highly purified from the ribosomal fraction as previously described (14). The nonactive form of PKR was partially purified free of eIF-2 from the same ribosome fraction by chromatography on DEAE-cellulose, ammonium sulfate fractionation, and then chromatography on phosphocellulose as described elsewhere (15). This preparation was added to phosphorylation reactions at a final concentration of 0.4 mg/ml. Recombinant chimeric proteins consisting of GST fused to either HSV-1 U_S11 or U_L10 (1) were prepared as described above. Partially purified nonactivated PKR from rabbit reticulocyte lysate was activated by incubation with 0.1 μg of poly(I-C) (P-L Biochemicals) per ml, 0.10 mM ATP, and 2.5 mM $MgCl_2$ as described previously (15).

RESULTS

R5104-infected cells produce the U_S11 protein earlier than HSV-1(F)-infected cells. In the context of the wild-type genome, U_S11 is expressed as a γ_2 gene. Since in R5104 the open reading frame of U_S11 was juxtaposed to the promoter of the α47 gene, it was necessary to verify that the U_S11 protein was made early rather than late in infection. Replicate 25-cm^2 flask cultures of SK-N-SH cells were mock infected or infected with 10 PFU of HSV-1(F), R3616, R5103, and R5104. At 3, 6, 9, and 12 h postinfection, the cells were harvested, rinsed twice with PBS-A, and resuspended and boiled in disruption buffer (50 mM Tris-HCl [pH 7.0], 2% SDS, 700 mM β-mercaptoethanol, 2.75% sucrose); the infected cell proteins were subjected to electrophoresis on a denaturing 12.5% polyacrylamide gel and transferred to a nitrocellulose sheet as described in Materials and Methods. Immunoblotting of nitrocellulose filters with the U_S11 and α0 antibodies (Fig. 2) revealed that the U_S11 protein, labeled B, is easily detectable within 6 h in the R5104-infected cells (Fig. 2, lane 18), whereas in the HSV-1(F) infected cells, equivalent amounts of U_S11 are not produced until at least 9 h postinfection (lane 7). In cells infected with R3616, protein synthesis shutoff occurs at the onset of viral DNA synthesis; consequently the U_S11 protein, a true late γ_2 protein, is not synthesized (lanes 9 to 12). The U_S11 protein is

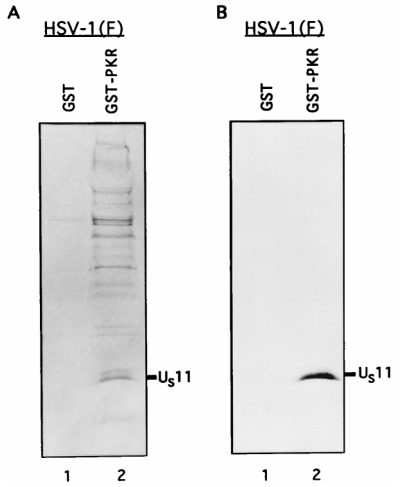

FIG. 3. Autoradiogram and photograph of proteins from infected cell lysates that were bound to GST or GST-PKR, subjected to electrophoretic separation and autoradiography, and reacted with the anti-U_S11 antibody. Equivalent quantities of GST or GST-PKR bound to beads were reacted overnight with the radiolabeled, RNase-treated, and sonicated lysates from 2×10^6 HeLa cells infected with HSV-1(F) as described in Materials and Methods. The beads were then washed seven times with PBS containing 1% NP-40 and 1% DOC, denatured in disruption buffer, boiled, subjected to electrophoresis in a denaturing 12.5% polyacrylamide gel, electrically transferred to a nitrocellulose sheet, reacted with the anti-U_S11 antibody, and subjected to autoradiography. The position of the U_S11 protein band is indicated. (A) Autoradiographic image of the electrophoretically separated proteins bound to beads; (B) photograph of the immunoblot.

also not produced in the R5103-infected cells, as R5103 lacks the domain encoding this protein (lanes 13 to 16). In addition to being probed for the U_S11 protein, the nitrocellulose filters were probed with antiserum directed against the α0 protein, labeled A (24), showing equivalent loading of protein in each lane of the gel (Fig. 2).

PKR binds the U_S11 protein in vitro. In an earlier publication, we reported that a viral or virus-induced factor blocked the phosphorylation of eIF-2α by activated PKR (5). The purpose of this series of experiments was to determine whether the U_S11 protein could be involved in this process by specifically interacting with PKR.

To test this hypothesis, we induced the expression of a GST-PKR fusion protein from a clone encoding the full-length PKR sequence fused to GST (32). The protein was bound to G-beads and rinsed four times with PBS-A–1% Triton X-100. The bound GST-PKR protein was then incubated for 12 h at 4°C with the [^{35}S]methionine-labeled cell lysates, either RNase digested or untreated as described in Materials and Methods. Experiments using untreated and RNase-treated samples yielded similar results. The photograph shown in Fig. 3 is from the experiments using RNase-treated lysates. An equivalent amount of GST protein bound to beads was also incubated with the labeled mock- and HSV-1(F)-infected cell lysates prepared from 2×10^6 HeLa cells to test the specificity of the PKR protein binding. Following the incubation, the GST and GST-PKR protein samples were washed nine times with wash buffer (PBS-A, 1% NP-40, 1% DOC), resuspended in disruption buffer, and boiled for 3 min. The proteins were then separated on a denaturing 12.5% polyacrylamide gel, transferred to a nitrocellulose sheet for autoradiography (Fig. 3A), then blocked with 5% skim milk, and probed with an antibody to the U_S11 protein (Fig. 3B).

As shown in Fig. 3, lanes 2, the GST-PKR protein bound the HSV-1 U_S11 protein. The results suggest that the chimeric protein preferentially bound the faster-migrating form of the protein (Fig. 3B, lane 2). This interaction was mediated by the PKR protein inasmuch as GST by itself failed to bind the U_S11 protein (Fig. 3, lanes 1).

U_S11 protein precludes the phosphorylation of eIF-2α by activated PKR in vitro. As reported in previously, the lysates of cells infected with the $\gamma_1 34.5^-$ mutant R3616 lack the specific eIF-2α phosphatase activity characteristic of wild-type virus-infected cells (5, 19). Since the $\gamma_1 34.5^-$ compensatory mutants do not phosphorylate eIF-2α (5), it was of interest to determine whether U_S11 played a role in this process. Two series of experiments were done to determine whether the U_S11 protein can suppress the phosphorylation of exogenously added eIF-2α by HSV-1-infected HeLa cell lysate in which PKR had been activated.

In the first series, limiting amounts of lysates from HeLa cells infected with the $\gamma_1 34.5^-$ mutant R3616 were mixed with 5 pmol of purified eIF-2, [γ-^{32}P]ATP (2 to 10 Ci/mmol) and increasing amounts of either purified GST or the chimeric protein GST-U_S11 in 10 μl of TKM buffer as previously described (5). The results (Fig. 4) demonstrated a dose-dependent inhibition of the phosphorylation of eIF-2α by GST-U_S11 but not by GST, indicating that inhibition was mediated by the U_S11 component. No phosphorylation of eIF-2α occurred in the absence of added eIF-2 (Fig. 4, lane 1) or in reaction mixtures containing lysates of HeLa cells infected with the R5104 mutant. Two observations are particularly noteworthy. First, the GST-U_S11 chimeric protein precluded the phosphorylation of all eIF-2-associated proteins (e.g., eIF-2β and the M_r-39,000 protein) but had less effect on the cellular proteins which

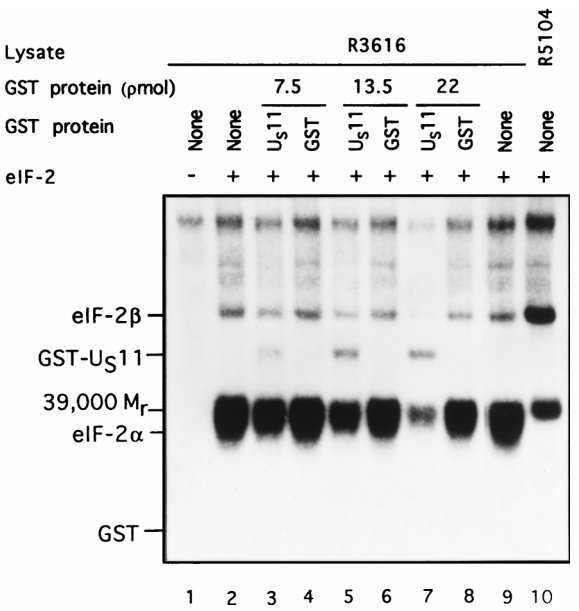

FIG. 4. Autoradiographic images of electrophoretically separated proteins from reaction mixtures containing lysates of cells infected with R3616 ($\gamma_1 34.5^-$) as a source of activated PKR, eIF-2, and either GST or GST-U_S11. Phosphorylation reactions contained the equivalent of 0.19 μl of lysates of R3616-infected cells (lanes 1 to 9) or R5104-infected cell lysate (lane 10), 5 pmol of eIF-2 (lanes 2 to 10), and GST-U_S11 (lanes 3, 5, and 7) or GST (lanes 4, 6, and 8) in concentrations shown in a final volume of 10 μl of TKM buffer. The mixtures were preincubated at 34°C for 30 s; the reaction was started by the addition of [γ-^{32}P]ATP (final concentration, 0.04 mM) and terminated by the addition of an SDS denaturing solution after 1 min of further incubation at 34°C. The mixtures were subjected to electrophoresis in denaturing polyacrylamide gels and autoradiography. The positions of eIF-2α, eIF-2β, the M_r-39,000 phosphoprotein, GST-U_S11, and GST are shown.

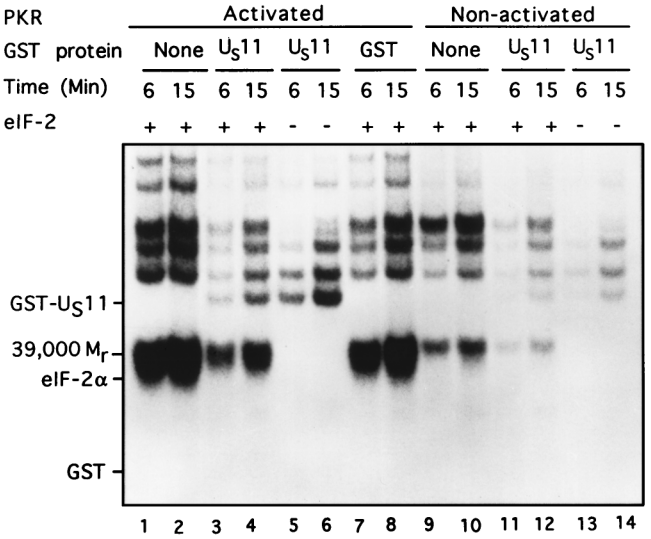

FIG. 5. Autoradiographic images of electrophoretically separated proteins from reaction mixtures containing activated or nonactivated purified PKR, eIF-2, and either GST or GST-U_S11. PKR, partially purified from rabbit reticulocyte ribosomes as indicated in Material and Methods, was preincubated with 0.10 mM ATP and 2.5 mM $MgCl_2$, either without (nonactivated PKR) or with poly(I-C) (0.1 μg/ml), for 20 min at 34°C. The contents of the reaction mixtures are given above the autoradiogram. The additions were 5 pmol of eIF-2, 22 pmol of GST-U_S11 or GST, and a final concentration of 0.09 mM [γ-^{32}P]ATP in a final volume of 10.5 μl of TKM buffer. The reaction was begun by addition of activated (lanes 1 to 8) or nonactivated (lanes 9 to 14) PKR and incubation at 34°C. Aliquots (5.0 μl) were removed from each reaction mixture and placed into disruption buffer after 6 and 15 min, boiled, and subjected to electrophoresis in denaturing gels and autoradiography. Positions of the U_S11 protein, M_r-39,000 phosphoprotein, and eIF-2α bands are shown. The position of GST is indicated, although it was not labeled.

migrated near the top of the gel. Second, GST-U_S11 but not GST was phosphorylated in this reaction. Unlike the incremental reduction in phosphorylation of eIF-2α, the phosphorylation of GST-U_S11 was not dose dependent and saturated at approximately 13.5 pmol of added GST-U_S11.

In the second series, we determined the effect of GST-U_S11 on the activity of PKR partially purified from rabbit reticulocyte ribosomes. Specifically, the reaction mixtures consisted of activated and nonactivated PKR, [γ-^{32}P]ATP (2 to 10 Ci/mmol), 0.09 mM ATP, 2.5 mM $MgCl_2$, and, where added, 5 pmol of eIF-2 and 22 pmol of GST-U_S11 or GST. On the basis of previous experiments involving infected cell lysates depicted in Fig. 4 and kinase reactions using serial dilutions of GST-U_S11 (not shown), we calculated the concentration of U_S11 protein which effectively blocked the phosphorylation of eIF-2α by the activated PKR. The results (Fig. 5) were as follows.

(i) PKR activated by preincubation with poly(I-C) phosphorylated eIF-2α (Fig. 5, lanes 1 and 2), whereas nonactivated PKR did not (lanes 9 and 10). (ii) GST-U_S11, added at a level found to be just saturating in Fig. 4 and on the basis on serial dilution experiments (not shown), blocked the phosphorylation of eIF-2α by activated PKR (lanes 3 and 4), whereas GST had no effect (lanes 7 and 8). (iii) GST-U_S11 more effectively inhibited the phosphorylation of eIF-2α than that of the M_r-39,000 protein and that of other proteins present in the reaction mixture, suggesting that under the conditions used, the inhibitory effects of U_S11 were more specific than those shown in Fig. 4. (iv) GST-U_S11 (lanes 3 to 6) but not GST (lanes 7 and 8) was phosphorylated in the presence of activated PKR. The levels of phosphorylated U_S11 were significantly reduced and barely detectable in the presence of nonactivated PKR (lanes 11 to 14). Moreover, the phosphorylation of GST-U_S11 by PKR was considerably greater in the absence (lanes 5 and 6) than in the presence (lanes 3 and 4) of eIF-2. This observation suggests that U_S11 protein and eIF-2α may be competing substrates for the activated PKR.

U_S11 is more effective in blocking the phosphorylation of PKR and of eIF-2α if present in the reaction mixture before rather than after the activation of PKR by poly(I-C). The purpose of this series of experiments was to test the effectiveness of GST-U_S11 in blocking the phosphorylation of eIF-2α before and after activation of PKR. In these experiments, the reaction mixtures contained identical amounts of reactants but the order of addition differed.

In the first part of experiment, the rabbit reticulocyte PKR was reacted with poly(I-C) after addition of GST or GST fusion proteins, and purified eIF-2. In the second part of the experiment, poly(I-C) was added first. The results (Fig. 6) indicate that GST-U_S11 was far more effective in blocking the phosphorylation of eIF-2α when added prior to activation of PKR than when added after PKR had already been exposed to poly(I-C). Specifically, the phosphorylation of eIF-2α was completely suppressed by 0.8 pmol of GST-U_S11 added before activation of PKR (Fig. 6, lane 5). In contrast, significant amounts of eIF-2α were phosphorylated in the presence of much higher concentrations of GST-U_S11 (e.g., 3.2 pmol [lane 10]) added after activation of PKR. In these experiments, GST alone and the GST-U_L10 fusion protein had no effect on the phosphorylation of eIF-2α (lanes 6, 7, 13, and 14).

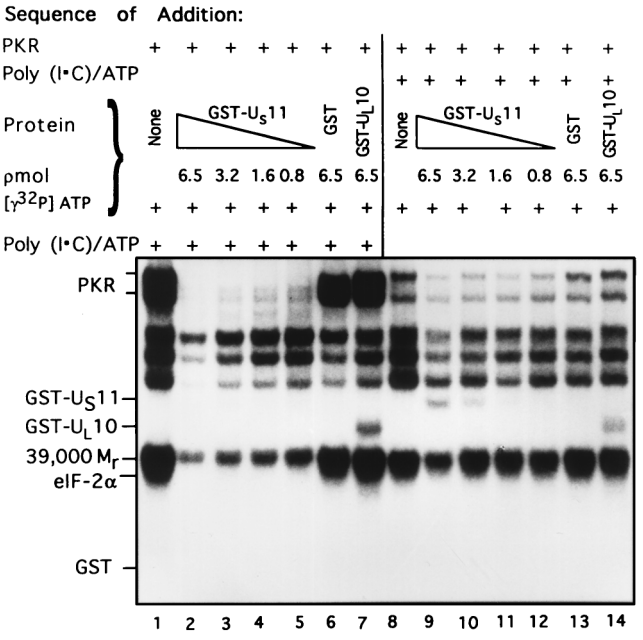

FIG. 6. Autoradiographic images of electrophoretically separated proteins from reaction mixtures in which PKR was activated before or after addition of the GST-U_S11 chimeric protein. The order of addition of the reactants in mixtures 1 through 14 is shown at the top. Phosphorylation reaction mixtures contained of eIF-2 (2.5 pmol) and PKR; poly(I-C) (0.1 μg/ml) and 0.09 mM [γ-^{32}P]ATP were added after (lanes 1 to 7) or before (lanes 8 to 14) addition of GST, GST-U_S11, or GST-U_L10 in the concentrations shown. All components were in TKM buffer, and the final volume was 5.25 μl. The reaction mixtures were incubated at 34°C for 15 min. The reaction was stopped by the addition of disruption buffer, and the proteins were separated on denaturing gels and subjected to autoradiography. Positions of the reacting proteins are shown.

DISCUSSION

In an earlier report, we showed that a DNA fragment containing the U_S10 gene in its natural environment and the U_S11 gene driven by the α47 promoter imparted on the recipient $\gamma_1 34.5^-$ virus (R5104) the phenotype of sustained protein synthesis, whereas in the absence of the inserted DNA fragment the parent virus (R5103) lacked this capacity. We reported also that the phenotype of R5104 was different from that of the wild-type virus in two important respects even though both viruses were capable of sustained protein synthesis. Specifically, unlike the wild-type virus, R5104 did not induce the phosphorylation of eIF-2α in infected cells by activated PKR. Moreover, the lysates of cells infected with the R5104 virus lacked the eIF-2α-specific phosphatase activity which is highly elevated and readily demonstrable in wild-type virus-infected cells. We concluded that the gene affected by the second site mutation which compensates for the absence of the $\gamma_1 34.5$ gene operates by a mechanism different from that of the $\gamma_1 34.5$ gene. The suspicion also focused on the U_S11 gene as the possible bearer of the compensatory mutation. But in fact, the only mutation that we could identify with certainty is the apparent replacement of the γ_2 promoter with that of the α47 promoter.

In this article, we report the results of studies designed to test the hypothesis that the compensatory mutation is in fact the expression of the U_S11 as an early protein. The report consists of three parts. In the first, we showed that in cells infected with R5104, U_S11 is made earlier in infection. In the second series of experiments, we showed a physical interaction between PKR and U_S11 protein. In the third, we showed that in in vitro assays, in the presence of U_S11 the phosphorylation of eIF-2α is impaired. The key experiment in this series dealt with the order of addition of reagents. If U_S11 was added to the reaction mixture before PKR was activated, both phosphorylation of PKR and the phosphorylation of eIF-2α were blocked at lower concentrations of U_S11 protein than if U_S11 was added after PKR had been activated. The effect of U_S11 protein was dose dependent.

In essence, the conclusion to be reached from these studies is that U_S11 can substitute for the $\gamma_1 34.5$ protein if it is present in appropriate amounts or form early in infection and that U_S11 protein blocks the shutoff of protein synthesis by binding to PKR and preventing it from phosphorylating eIF-2α. The results also raise three very interesting questions.

First and foremost, the mechanism by which U_S11 protein blocks PKR is not known. There are two clues, however. The first is that U_S11 is phosphorylated in the presence of PKR. There are, however, no data to unambiguously determine whether U_S11 irreversibly blocks PKR or whether it merely competes with eIF-2α for PKR. The second clue stems from the observation that U_S11 is less effective if added to the reaction mixture after activation of PKR. This observation suggests that activated PKR has a much lower affinity for U_S11 than the preactivated protein. If U_S11 binds to the activation site, it may be expected that bound U_S11 interferes with access to the PKR activation site by a third molecule.

The second question relates to the fact that U_S11 is packaged into the virion and upon entry of the virus into infected cells becomes associated with ribosomes. The question is why the U_S11 brought into the cell by the virus does not preclude the shutoff of protein synthesis by activated PKR. Indeed, in cells infected with R3616, which contain virion-associated U_S11, protein synthesis is shut off as early as in cells infected with the R5103 recombinant, which lacks the U_S11 gene (data not shown). These results suggest that U_S11 introduced into cells by the infecting virus may have a function other than that of precluding the shutoff of protein synthesis.

Finally, the question arises as to why $\gamma_1 34.5$ protein evolved and supplanted U_S11 in precluding the shutoff of protein synthesis. The $\gamma_1 34.5$ protein expresses at least two functions. The entire gene is required for viral replication in the central nervous system and only the 3′-terminal 70 codons are required to block the shutoff of protein synthesis by activated PKR. This domain of the gene is homologous to the corresponding domain of GADD34, a conserved mammalian gene expressed during growth arrest, during differentiation, or after DNA damage (8, 17).

We may speculate that the progenitor of HSV-1, HSV-2, and the simian B virus acquired this domain from the corresponding cellular gene and that in time, U_S11 acquired additional functions and may have evolved a late promoter. The selective pressure is not difficult to appreciate: whereas the modern U_S11 even under the best circumstances precludes the phosphorylation of eIF-2α, the virus carrying the $\gamma_1 34.5$ gene renders the activated PKR totally impotent by dephosphorylating eIF-2α as rapidly as it is formed, since none is detected.

Viruses appear to have evolved myriad mechanisms designed to defeat the shutoff of proteins synthesis resulting from the activation of PKR by double-stranded RNA. These range from selective destruction or inactivation of PKR to precluding double-stranded RNA from activating PKR (2–4, 6, 7, 10, 28, 30, 34). In the case of poxviruses, two viral protein employing different pathways are dedicated to the task of nullifying PKR as a threat to viral replication (4, 10). HSV is unique in two respects. First, it has sequestered a cellular function to perform this task, and second, it may have retained the vestiges of an older, now cryptic mechanism to block this host response to infection.

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