

# The Herpes Simplex Virus Type 1 U<sub>S</sub>11 Protein Interacts with Protein Kinase R in Infected Cells and Requires a 30-Amino-Acid Sequence Adjacent to a Kinase Substrate Domain

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**The herpes simplex virus type 1  $\gamma_1$ 34.5 gene product precludes the host-mediated protein shutoff response induced by activated protein kinase R (PKR). Earlier studies demonstrated that recombinant viruses lacking the  $\gamma_1$ 34.5 gene ( $\Delta\gamma_1$ 34.5) developed secondary mutations that allowed earlier U<sub>S</sub>11 expression and enabled continued protein synthesis. Further, *in vitro* studies demonstrated that a recombinant expressed U<sub>S</sub>11 protein binds PKR, blocks the phosphorylation of the  $\alpha$  subunit of eukaryotic initiation factor 2 (eIF-2 $\alpha$ ) by activated PKR, and, if provided prior to PKR activation, precluded PKR autophosphorylation. The present study furthers the hypothesis that early U<sub>S</sub>11 production precludes PKR-mediated host protein shutoff by demonstrating that (i) U<sub>S</sub>11 and PKR interact in the context of viral infection, (ii) this interaction is RNA dependent and requires a 30-amino-acid domain (amino acids 91 to 121) in the carboxyl domain of the U<sub>S</sub>11 protein, (iii) the proteins biochemically colocalize in the S100 ribosomal fraction, and (iv) there is a PKR substrate domain immediately adjacent to the binding domain. The results suggest that the U<sub>S</sub>11 interaction with PKR at the ribosome is RNA dependent and that the U<sub>S</sub>11 protein contains a substrate domain with homology to eIF-2 $\alpha$  in close proximity to an essential binding domain.**

Most viruses produce double-stranded RNA (dsRNA) during infection, activating interferon-inducible host protein kinase R (PKR). The activated PKR precludes protein synthesis in virus-infected cells by phosphorylating the  $\alpha$  subunit of eukaryotic initiation factor 2 (eIF-2 $\alpha$ ) (18). Protein synthesis is integral to productive viral infection, and viruses have evolved diverse strategies to counter PKR-mediated host protein shutoff (1–3, 10, 21, 22). Herpes simplex virus type 1 (HSV-1) is particularly susceptible to PKR-mediated protein shutoff as it encodes genes on both strands of the genome. Previous studies showed that dsRNA is readily produced in the course of HSV-1 infection and that half of the HSV genome is represented in dsRNA (17, 19). As a consequence HSV-1 carries a gene,  $\gamma_1$ 34.5, present in two copies flanking the unique long domain of the genome that subverts the PKR protein shutoff response (6). In HSV-1 infection, the  $\gamma_1$ 34.5 gene product recruits a host phosphatase and dephosphorylates eIF-2 $\alpha$ , thus maintaining protein synthesis (16). In contrast, viruses carrying  $\gamma_1$ 34.5 genes with deletions ( $\Delta\gamma_1$ 34.5) are incapable of maintaining late protein synthesis in human cells following PKR activation and thus display a  $\Delta\gamma_1$ 34.5 protein shutoff phenotype (7, 8).

Previous studies showed that serially passaged  $\Delta\gamma_1$ 34.5 viruses incur secondary mutations in the unique short (U<sub>S</sub>) domain that resurrect a cryptic mechanism to evade PKR (23). Studies using a recombinant  $\Delta\gamma_1$ 34.5 virus (R5104) demonstrated that this change in protein synthesis phenotype is associated with earlier kinetic expression of the U<sub>S</sub>11 gene

product and that a viral gene product precludes eIF-2 $\alpha$  phosphorylation by activated PKR (4). *In vitro* assays further demonstrated that recombinant U<sub>S</sub>11 protein physically interacted with PKR, was phosphorylated by activated PKR, and precluded eIF-2 $\alpha$  phosphorylation (5). Further biochemical assays also revealed that recombinant U<sub>S</sub>11 protein was especially effective at blocking PKR activation and subsequent eIF-2 $\alpha$  phosphorylation when added prior to dsRNA activation (5).

The goals of the present study were threefold: (i) to demonstrate a PKR-U<sub>S</sub>11 interaction in the context of viral infection, (ii) to define the binding and substrate domains of U<sub>S</sub>11 involved in PKR interaction, and (iii) to demonstrate colocalization of U<sub>S</sub>11 and PKR in infected-cell cultures.

## MATERIALS AND METHODS

**Cells and viruses.** The Vero, HeLa, rabbit skin cell, and SK-N-SH cell lines were obtained from the American Type Culture Collection. The cell lines were propagated in Dulbecco's modified essential medium supplemented with 5% newborn calf serum (Vero and rabbit skin cells) or 10 (SK-N-SH) or 5% (HeLa) fetal bovine serum. HSV-1 (F) is the prototype strain used in this laboratory (12). Recombinant viruses R5103 and R5104 are described elsewhere in detail (4). Briefly, recombinant virus R5103 lacks the native U<sub>S</sub>8 to U<sub>S</sub>12 genes and the  $\gamma_1$ 34.5, ORF O, and ORF P genes and maintains the remainder of the S component frozen in an inverted orientation (I<sub>s</sub> and I<sub>sl</sub>). The R5104 virus, derived from R5103, contains the sequence encoding the native U<sub>S</sub>10 gene and the U<sub>S</sub>11 gene fused to the  $\alpha$ 47 promoter inserted within and disrupting the U<sub>L</sub>23 locus.

**Immunoblotting and generation of a U<sub>S</sub>11 polyclonal serum.** Nitrocellulose sheets containing the electrophoretically separated proteins were incubated in blocking solution–5% skim milk in phosphate-buffered saline lacking Ca and Mg (PBS-A) for at least 1 h, reacted with an antibody diluted in blocking solution for at least 4 h, and then washed five times with PBS-A containing 0.05% Tween. The nitrocellulose filter was next incubated with an appropriate alkaline phosphatase- or peroxidase-conjugated antibody diluted in blocking solution for approximately 90 min. The filter was next washed once in PBS-A–0.05% Tween and then four times in PBS-A. The alkaline phosphatase-stained immunoblots were developed with 150  $\mu$ g of 5-bromo-4-chloro-3-indolylphosphate (BCIP)/ml and

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300 µg of nitroblue tetrazolium/ml in AP buffer (100 mM Tris-HCl [pH 9.5], 5 mM MgCl<sub>2</sub>, 100 mM NaCl), whereas the peroxidase-stained immunoblots were developed using enhanced chemiluminescence as recommended by the manufacturer (Pierce) (31).

The antibodies used in these studies and their sources are as follows. The rabbit polyclonal antibody to PKR (K-17) was purchased from Santa Cruz Biotechnology, and the monoclonal antibody against U<sub>S</sub>11 was described previously (28). To generate polyclonal antibodies to full-length U<sub>S</sub>11, purified glutathione S-transferase (GST)-U<sub>S</sub>11 was generated from *Escherichia coli* transformed with plasmid pRB4776 (28). The antibody to full-length U<sub>S</sub>11 was generated using a standard protocol at Galena Biotechnology, Incorporated (Edmonton, Alberta, Canada) in accordance with National Institutes of Health (NIH) guidelines. Specifically, hens were initially immunized by using 100 to 125 µg of purified recombinant GST-U<sub>S</sub>11 with Freund's complete adjuvant followed 3 weeks later by three inoculations with GST-U<sub>S</sub>11 and Freund's incomplete adjuvant at 2-week intervals. The immunoglobulin Y antibody was purified from the eggs of the immunized hens 2 weeks after the last immunization.

**Coimmunoprecipitation of S10 fraction PKR with and without RNase treatment.** Replicate 25-cm<sup>2</sup> flask cultures of HeLa cells were incubated with 1 ml of 199V or 1 ml of 199V containing 10 PFU of HSV-1 (F), R5103, or R5104/cell. After a 2-h incubation, the inoculum was replaced with 5% newborn calf serum. At 6 h postinfection, medium was removed from mock- and virus-infected cells, ice-cold PBS-A was added and the cells were scraped, transferred to centrifuge tubes, and pelleted. Cytoplasmic S10 fractions were then generated as previously described (9, 15). After the addition of 1 µl of mammalian cell protease inhibitor cocktail (104 mM 4-[2-aminoethyl]benzenesulfonyl fluoride [AEBSF], 80 µM aprotinin, 2.5 mM leupeptin, 36 mM bestatin, 1.5 mM pepstatin A, 1.4 mM E-64) (Sigma P-8340), the lysates were shaken for 30 min at 4°C with 25 µl of protein A conjugated to Sepharose beads and centrifuged, and the supernatants were transferred to new tubes. The samples were then either mock treated or incubated with RNase A (40 µg/ml, final concentration) and RNase T<sub>1</sub> (300,000 U/ml, final concentration) for 30 min at 37°C. The mock- and RNase-treated samples were then reacted at 4°C with 1 µg of rabbit polyclonal immunoglobulin anti-PKR (K-17; Santa Cruz Biotechnology) overnight. The immunoprecipitate was incubated with protein A-Sepharose for 2 h, centrifuged, and washed three times in disruption buffer (50 mM Tris-HCl [pH 7.0], 2% sodium dodecyl sulfate [SDS], 700 mM β-mercaptoethanol, 2.75% sucrose), boiled for 5 min, electrophoretically separated on 13% (vol/vol) polyacrylamide gels cross-linked with *N,N'*-diallyl-L-tartardiamide containing 0.1% SDS, and electrically transferred to a nitrocellulose sheet, and immunoblotting was performed.

**Cosedimentation in S100 pellets and high-salt buffer elutions.** Replicate HeLa cells were mock infected or infected with 10 PFU of HSV-1 (F), R5103, and R5104 virus/cell and harvested at 6 h postinfection. S10 fractions were prepared as previously described using diethyl pyrocarbonate-treated reagents.

The S100 fractions and ribosomal pellet were prepared as described previously (20, 30). Briefly, the S10 equivalent from a 75-cm<sup>2</sup> culture was layered on a 3-ml isotonic S100 buffer solution (25 mM HEPES [pH 7.6], 115 mM KCl, 0.3 mM magnesium acetate {Mg[OAc]<sub>2</sub>}, 10% glycerol, 5 mM spermidine) and centrifuged using a TH-641 rotor in a Sorvall RC-60 ultracentrifuge at 150,000 × *g* for 2.5 h at 4°C. The ribosomal salt wash fraction, comprising the upper two-thirds of the ultracentrifuge supernatant fraction, was removed, passed over a Sephadex G-25 (Pharmacia) column, and stored. The lower one-third of the supernatant was discarded, and the S100 pellet was resuspended in 100 µl of isotonic buffer (25 mM HEPES, 115 mM KCl, 1 mM spermidine, 10% glycerol); 50 µl of threefold-concentrated 0.3 M salt wash buffer (25 mM HEPES, 785 mM KCl, 15 mM Mg[OAc]<sub>2</sub>, 1 mM spermidine, 3 mM dithiothreitol [DTT], 10% glycerol) was added, and the mixture was incubated with a stir bar overnight at 4°C. The sample was then loaded on 3 ml of isotonic S100 buffer solution and subjected to ultracentrifugation at 100,000 × *g* for 4 h at 4°C using a TH-641 rotor. Again the pellet and supernatant portions were divided. The supernatant salt wash fractions were dialyzed (1 ml:4,000 ml) with two buffer changes using 8,000-molecular-weight cutoff dialysis tubing at 4°C overnight. The volume of each dialysate was reduced using a rotary concentrator. The samples were then boiled in protein disruption buffer and stored at -20°C until all samples were prepared. The isotonic S100 pellet was resuspended in 100 µl of isotonic buffer (25 mM HEPES, 115 mM KCl, 1 mM spermidine, 10% glycerol), 50 µl of three-times-concentrated 0.8 M salt wash buffer (25 mM HEPES, 2.17 M KCl, 15 mM Mg[OAc]<sub>2</sub>, 1 mM spermidine, 3 mM DTT, 10% glycerol) was added, and the mixture was again incubated overnight and then subjected to centrifugation, separation, and concentration as described above for the isotonic fractions. The isotonic, 0.8 M salt wash, and pellet protein samples were then loaded and

electrophoretically separated on denaturing polyacrylamide gels and electrically transferred to nitrocellulose filter, and immunoblotting was performed.

**Isolation of the tryptic peptide in GST-U<sub>S</sub>11 phosphorylated by PKR.** GST-U<sub>S</sub>11 (40 µg) was phosphorylated by incubation with 48 µg of partially purified, activated PKR as previously described in 30 mM Tris-HCl, pH 7.5–0.07 mM [γ-<sup>32</sup>P]ATP (13,000 Cerenkov cpm/pmol)–2.5 mM MgCl<sub>2</sub> in a final volume of 240 µl at 34°C (13). After 5 h of incubation, the reaction was stopped by the addition of SDS loading buffer, and the reaction mixture was boiled for 1.5 min, loaded into a 5-cm-wide lane of a 7% polyacrylamide–SDS gel, and electrophoretically separated for 20 h at 45 V (3 V/cm). After brief autoradiography of the wet gel, the major phosphorylated band, GST-U<sub>S</sub>11, was excised with reference to the autoradiogram, minced into 1-mm fragments, and digested with 4 µg of trypsin/ml in a final volume of 0.6 ml of gel plus 0.6 ml of 5 mM Tris-HCl, pH 8.0, for 18 h at 34°C. The liquid (containing 70% of the total <sup>32</sup>P radioactivity) was removed from the gel fragments and received trifluoroacetic acid at a final concentration of 0.05%. The gel fragments were extracted with 150 µl of 0.05% trifluoroacetic acid, and this extract (containing another 15% of the total <sup>32</sup>P radioactivity) was removed and combined with the initial liquid fraction. This pool was chromatographed on an Aquapore RP-300 reverse-phase column (4.6 by 250 mm; Brownlee Laboratories) as previously described (14). The <sup>32</sup>P-labeled material was initially eluted with the polypeptide material (as judged by the absorbance at 215 nm) at an acetonitrile concentration (43%) corresponding to partially digested or undigested protein (data not shown). This fraction was pooled, evaporated to dryness, and then redigested with 9 µg of trypsin/ml in 160 µl of 40 mM Tris-HCl, pH 8.1, for 18 h at 34°C. It was then rechromatographed on the reverse-phase column as described above. Most of the <sup>32</sup>P that bound to the column was recovered in a single phosphopeptide peak that eluted at 21% acetonitrile. The two (0.5-ml) fractions containing this phosphopeptide were subjected separately to automated amino acid sequencing in an Applied Biosystems sequencer with Cerenkov counting of the eluates from each cycle (14).

## RESULTS

**U<sub>S</sub>11 binds PKR in R5104-infected cells.** Previous studies have demonstrated that a recombinant expressed PKR protein binds U<sub>S</sub>11 from HSV-infected HeLa cell extracts and that a recombinant expressed U<sub>S</sub>11 protein binds PKR (5, 25). The goal of the first experiment was to determine if U<sub>S</sub>11 and PKR interact in the context of viral infection. To test this hypothesis, HeLa cell cytoplasmic S10 fractions were collected at 6 h postinfection from HeLa cells mock infected or infected with HSV-1 (F), R5103, and R5104 and immunoprecipitation of PKR was performed as described in Materials and Methods. The immunoprecipitated proteins were electrophoretically separated, transferred to nitrocellulose membranes, and probed with a monoclonal antibody to the HSV-1 U<sub>S</sub>11 protein as previously described (28, 31).

As demonstrated in Fig. 1A, immunoblotting of the starting supernatant samples with anti-U<sub>S</sub>11 serum demonstrated that equivalent quantities of U<sub>S</sub>11 were present in the R5104-infected cell lysate starting solutions used in the coimmunoprecipitation experiments. As expected, the monoclonal antibody to U<sub>S</sub>11 did not react with either the mock- or R5103-infected cell lysates. The R5104-infected cells produced greater amounts of U<sub>S</sub>11 protein than the wild-type virus at 6 h postinfection, consistent with the earlier kinetic expression of U<sub>S</sub>11 in the R5104 virus. The new and significant results of this experiment (Fig. 1B) indicate that U<sub>S</sub>11 and PKR coimmunoprecipitate in the R5104-infected-cell lysates with polyclonal serum against PKR (Santa Cruz Biotechnology; K-17) but not in the samples with rabbit preimmune serum.

**The carboxyl-terminal U<sub>S</sub>11 RNA binding domain is required for high-level PKR binding in infected cells, and residues 91 to 121 are critical for PKR-U<sub>S</sub>11 interaction.** The previous experiments demonstrated that U<sub>S</sub>11 and PKR inter-

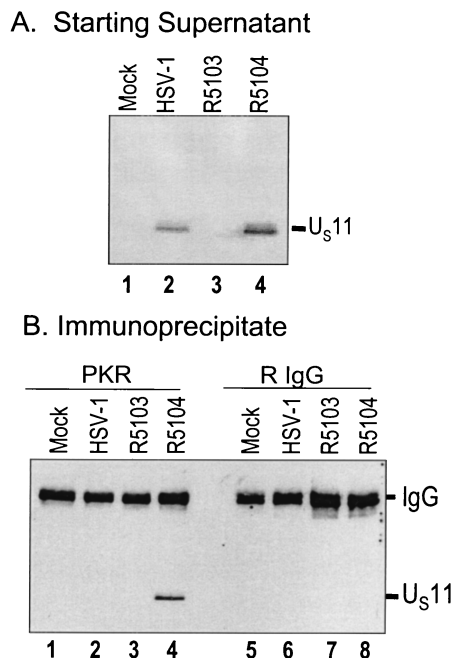


FIG. 1. U<sub>S</sub>11 protein coimmunoprecipitates with PKR in infected HeLa cell cytoplasmic fractions. Replicate cell cultures of HeLa cells were either mock infected or infected with HSV-1 (F), R5103, or R5104 virus, and S10 cytoplasmic fractions were generated. (A) Photograph of an immunoblot of the starting supernatant material. The electrophoretically separated proteins from mock-, HSV-1 (F)-, R5103-, and R5104-infected-HeLa cell S10 fractions (lanes 1 to 4, respectively) were probed with an anti-U<sub>S</sub>11 monoclonal antibody, and the relative amounts of U<sub>S</sub>11 in the starting lysates are shown. (B) Photographic image of electrophoretically separated proteins after immunoprecipitation from the above S10 fractions using 1  $\mu$ g of anti-PKR antiserum (K-17; Santa Cruz Biotechnology) or rabbit preimmune serum (R IgG; Southern Research Institute). S10 cytoplasmic fractions were pre-cleared with protein A and then incubated either with 1  $\mu$ g of anti-PKR antibody (K-17; Santa Cruz Biotechnology) or with 1  $\mu$ g of the rabbit preimmune serum antibody overnight. The complexes were precipitated with protein A-Sepharose and washed three times, and the immunoprecipitated proteins were electrophoretically separated, transferred to nitrocellulose filters, and probed with an antibody to U<sub>S</sub>11.

act in infected-cell lysates. The purpose of the next series of experiments was to determine the essential U<sub>S</sub>11 protein domain required for PKR interaction. To identify the minimum domain enabling interaction of PKR and U<sub>S</sub>11 in the context of HSV-1 infection, PKR was immunoprecipitated from cytoplasmic lysates prepared from mock- or recombinant-virus-infected HeLa cells. The recombinant viruses, created by Roller et al., contain deletions within the U<sub>S</sub>11 protein coding region (Fig. 2A) (29). The starting cytoplasmic lysate and immunoprecipitated proteins were then electrophoretically separated, transferred to nitrocellulose membranes, and stained using first a monoclonal antibody against U<sub>S</sub>11 and then a polyclonal serum against U<sub>S</sub>11.

Figure 2B, top, is an immunoblot of the starting supernatant S10 fraction and demonstrates the relative electrophoretic migration and quantities of the U<sub>S</sub>11 protein from the different recombinant viruses after probing with a mouse U<sub>S</sub>11 monoclonal antibody (27). The U<sub>S</sub>11 monoclonal antibody reacts

with an epitope within amino acids 5 to 35 of the U<sub>S</sub>11 protein and therefore cannot detect the U<sub>S</sub>11 protein produced by the R4779 recombinant viruses (Fig. : lane 7). Figure 2B, bottom, shows that the U<sub>S</sub>11 protein coimmunoprecipitated with PKR in the R4526- and R4778-infected cells and was easily detected (Fig. 2B, lanes 3 to 6). However, in the R4776- and R4777-infected cells little or no U<sub>S</sub>11 protein was seen in the immunoprecipitate. These data indicate that deletion of amino acids 88 to 152 of the U<sub>S</sub>11 protein completely eliminated the U<sub>S</sub>11-PKR interaction while deletion of amino acids 91 to 121 largely eliminated the U<sub>S</sub>11-PKR interaction. Disruption of amino acids 121 to 152 or amino acids 37 to 87 of the U<sub>S</sub>11 protein does not alter the interaction of U<sub>S</sub>11 with PKR (Fig. 2B, lanes 3 to 6).

To identify if deletion mutations in the amino-terminal region of U<sub>S</sub>11 interrupted U<sub>S</sub>11 coimmunoprecipitation with PKR, polyclonal serum against full-length U<sub>S</sub>11 was used to reprobe the membranes. Figure 2C, top, is an immunoblot of the starting cytoplasmic proteins and demonstrates that the new antiserum detected the U<sub>S</sub>11 protein in R4779-infected-cell lysates. Figure 2C, bottom, is an immunoblot of the immunoprecipitated proteins and shows that in the R4779-infected cells the U<sub>S</sub>11 protein coimmunoprecipitated with PKR, indicating that elimination of amino acids 5 to 35 of U<sub>S</sub>11 does not disrupt the U<sub>S</sub>11-PKR interaction (Fig. 2C, lane 7). These data indicate that amino acids 91 to 121 within the carboxyl-terminal RNA binding domain of U<sub>S</sub>11 are critical for coimmunoprecipitation with PKR in infected cells and that the amino domain of U<sub>S</sub>11 is not required for this interaction.

**RNase A and T<sub>1</sub> treatment of R5104-infected-cell S10 fractions interrupts the U<sub>S</sub>11-PKR interaction.** Based on the previous experiments, we next examined whether PKR and U<sub>S</sub>11 interact directly or require an RNA intermediate. To address this question, the coimmunoprecipitation experiment was repeated using mock-infected, wild-type virus-infected, and recombinant R5103- and R5104-infected-HeLa cell lysates which were either mock treated or treated with RNases A and T<sub>1</sub> for 30 min at 37°C. The immunoprecipitated proteins were electrophoretically separated, electrically transferred to nitrocellulose membranes, and probed with the mouse monoclonal antibody to U<sub>S</sub>11. The results (Fig. 3) show that, in mock-treated R5104-infected-cell lysates, U<sub>S</sub>11 and PKR coimmunoprecipitated (lane 4) whereas RNase treatment disrupted the U<sub>S</sub>11-PKR interaction in R5104-infected cells (lane 8). These data indicate that PKR and U<sub>S</sub>11 binding in R5104-infected cells is RNase sensitive and support the hypothesis that the U<sub>S</sub>11-PKR interaction is RNA dependent.

**U<sub>S</sub>11 and PKR from infected-HeLa cell cytoplasmic fractions cosediment in the S100 ribosomal fraction and coelute when incubated at a high salt concentration.** Previous studies using cytoplasmic fractionation showed that PKR localizes at the ribosome and is present in the S100 pellet portion of disrupted cells (20, 30). Further biochemical analyses showed that PKR eluted from the ribosomal S100 pellet fraction in the presence of high-salt buffer (0.8 M KCl) but remained associated with the ribosomal pellet following incubation in less-stringent salt buffer (isotonic buffer) (20, 30). Additional studies demonstrated that U<sub>S</sub>11 localized to the ribosome (11, 28). To determine the stringency of the U<sub>S</sub>11-PKR interaction at the ribosome, biochemical analysis of the U<sub>S</sub>11-PKR interac-

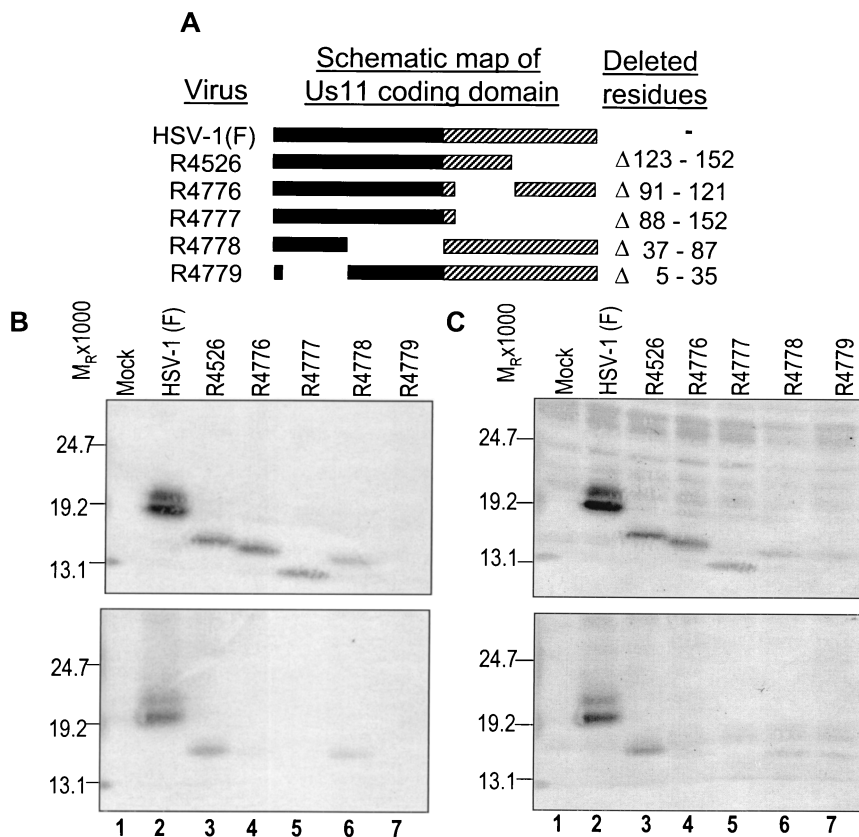


FIG. 2. Fine mapping of the  $U_s11$  protein domain required for PKR interaction using recombinant  $U_s11$  truncation virus-infected HeLa cell cytoplasmic S10 fractions and PKR immunoprecipitation. (A) Redrawing of Roller's schematic depiction of the  $U_s11$  truncation viruses and the amino acid deletions (29). Solid bars, N-terminal domains of the protein; hatched bars, C-terminal domains comprising tandemly repeated P-R-X amino acids. (B) Photographic depiction of immunoblots. (Top) Photograph of mock- and virus-infected HeLa cell cytoplasmic proteins after electrophoretic separation, transfer to a nitrocellulose membrane, and staining with a mouse anti- $U_s11$  monoclonal antibody. (Bottom) Immunoblot of the wild-type and mutant  $U_s11$  proteins coimmunoprecipitated with 1  $\mu$ g of rabbit anti-PKR antibody and then stained with an anti- $U_s11$  mouse monoclonal antibody. (C) Photograph of the immunoblots from panel B after they were reprobed with a chicken polyclonal antibody against  $U_s11$  and after the proteins were detected using the alkaline phosphatase-conjugated secondary antibody used for panel B. The new antiserum is capable of detecting the mutant  $U_s11$  from R4779 in which amino acids 5 to 35 have been deleted.

tion in the ribosome was performed using cosedimentation and high-salt-elution studies. The proteins present in the S100 pellet and salt wash samples were electrophoretically separated, transferred to nitrocellulose membranes, and immunoblotted using antibodies directed against  $U_s11$  and PKR.

The results shown in Fig. 4, lanes 1 to 8, demonstrate that PKR (top) and  $U_s11$  (bottom) remained associated with the pellet fraction and that neither protein dissociated into the isotonic salt buffer fraction. However, when the remaining proteins present in the pellet were incubated with 0.8 M KCl buffer, PKR dissociated from the ribosomal fractions and was detectable in the supernatant fraction (top, lanes 13 to 16) of the 0.8 M salt wash fraction of mock-, HSV-1 (F)-, R5103-, and R5104-infected-cell samples, consistent with data reported in the literature (20, 30). When the experiment was repeated and membranes were probed with an antibody directed against the HSV-1  $U_s11$  protein, the  $U_s11$  protein in the HSV-1 (F)- and R5104-infected-cell S100 pellet fraction also eluted (bottom, lanes 14 and 16). These data demonstrate that the  $U_s11$  protein and PKR protein cosedimented in the S100 ribosomal fraction and coeluted only after incubation at high salt con-

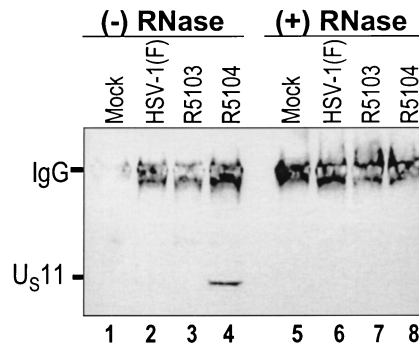


FIG. 3. RNase A and  $T_1$  incubation disrupts PKR- $U_s11$  interaction. Photograph of an immunoblot probed with an anti- $U_s11$  monoclonal antibody. PKR was immunoprecipitated from mock- and virus-infected-cell cytoplasmic lysates after either mock or RNase A and  $T_1$  treatment. The immunoprecipitated proteins were washed, disrupted, electrophoretically separated by SDS-PAGE, and transferred to nitrocellulose membranes. The membrane was then developed using an anti- $U_s11$  monoclonal antibody and an antimouse peroxidase-conjugated secondary antibody and developed by enhanced chemiluminescence. IgG, immunoglobulin G.

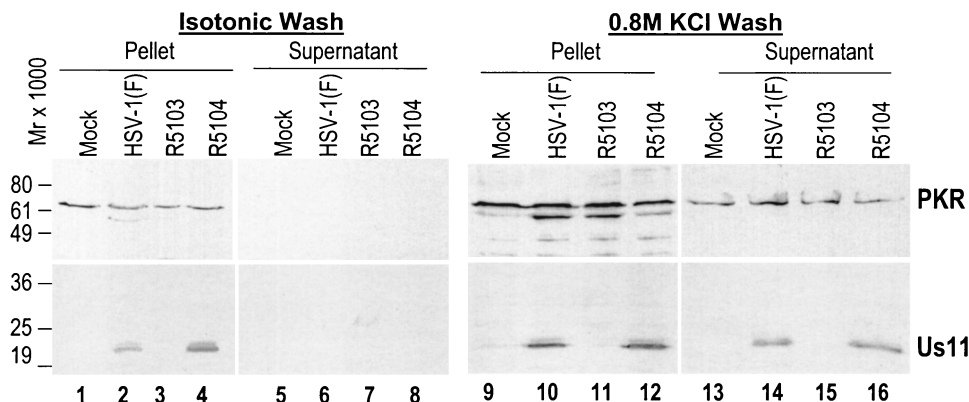


FIG. 4. Composite photograph of immunoblots of electrophoretically separated S100 ribosomal proteins from mock- or virus-infected HeLa cell after isotonic or high-salt (0.8 M KCl) washes. (Top) Demonstration of PKR in the S100 protein pellet following incubation in isotonic buffer (lanes 1 to 4) and the presence of PKR in the supernatant (lanes 9 to 12) following incubation in high-salt-concentration buffer. The U<sub>S</sub>11 protein is detectable only in the S100 ribosomal pellet (lanes 1 to 4) after incubation in isotonic buffer; however, following incubation in high-salt-concentration buffer U<sub>S</sub>11 is present in both the pellet (lanes 9 to 12) and supernatant (lanes 13 to 16).

centration, a characteristic of biochemically colocalizing proteins.

**U<sub>S</sub>11 protein is phosphorylated by activated PKR on three serine/threonine amino acids adjacent to the PKR binding site.** In R5104-infected cells, PKR is activated and an HSV gene precludes the activated PKR from phosphorylating eIF-2 $\alpha$  (4). Further in vitro experiments showed that the U<sub>S</sub>11 protein was phosphorylated by activated PKR in an inverse relationship to eIF-2 $\alpha$  phosphorylation (5). These findings suggested that U<sub>S</sub>11 inhibited activated PKR phosphorylation of eIF-2 $\alpha$  by providing an alternative substrate site.

To identify the amino acid domain in U<sub>S</sub>11 phosphorylated by activated PKR, a recombinant U<sub>S</sub>11 protein was incubated with purified activated PKR as well as nonactivated PKR in the presence of [ $\gamma$ -<sup>32</sup>P]ATP and isolated by SDS-polyacrylamide gel electrophoresis (PAGE). Only the U<sub>S</sub>11 incubated with activated PKR was radiolabeled. Following phosphorylation of a preparative amount of GST-U<sub>S</sub>11 by activated PKR in the presence of [ $\gamma$ -<sup>32</sup>P]ATP, the radiolabeled GST-U<sub>S</sub>11 was isolated by SDS-PAGE, digested with trypsin, run on a reverse-phase column, redigested with trypsin, and then refractionated by reverse-phase chromatography as described in Materials and Methods. Almost all of the <sup>32</sup>P that bound to the column was recovered in a single phosphopeptide peak and collected in two fractions, which were subjected separately to automated amino acid sequencing and Cerenkov counting of each Edman cycle. Each analysis gave the sequence VGADTTISKPSEA VRPPTI-R (the dash indicates an unidentified amino acid), demonstrating that the phosphopeptide corresponds to residues 66 to 86 in U<sub>S</sub>11 and that the site of phosphorylation occurs just before a long series of triplet (PRX) repeats that constitutes the carboxyl-terminal region (45%) of the protein. In addition, there were peaks of radioactivity associated with threonine 71, serine 76, and threonine 83. Serine 76 was the most prominently labeled, while threonine 70 and serine 73 were not radiolabeled (Fig. 5). The results indicate that activated PKR selectively phosphorylates 3 amino acids of the U<sub>S</sub>11 protein immediately adjacent to the 30-amino-acid PKR binding domain of PKR.

## DISCUSSION

The  $\gamma$ <sub>1</sub>34.5 gene encodes two distinct functions in HSV-1-infected cells. First, it enables efficient virus replication and spread in neuronal tissue. (7). Second, it precludes the PKR-mediated shutoff of protein synthesis in infected human cells (8). During serial passage in infected human cells, secondary compensatory mutations occur; these enable  $\Delta\gamma$ <sub>1</sub>34.5 recombinants to regain control over protein synthesis in the infected cells (23). These studies are extensions of previous work exploring the mechanism by which secondary mutations enable evasion of the host-mediated protein shutoff response. Prior studies have demonstrated that a viral factor precludes the protein shutoff response by inhibiting the phosphorylation of eIF-2 $\alpha$  by activated PKR in  $\Delta\gamma$ <sub>1</sub>34.5 virus-infected cells (4). Additional studies indicated that early expression of the U<sub>S</sub>11 protein in cells infected with a  $\Delta\gamma$ <sub>1</sub>34.5 recombinant virus enables viral evasion of the host protein shutoff response (5). Further biochemical studies showed that recombinant U<sub>S</sub>11 can bind PKR and prevent the phosphorylation of eIF-2 $\alpha$  by PKR (5).

In this report we determined that U<sub>S</sub>11 and PKR interact and colocalize in the context of virus-infected cells and characterized the binding and substrate domains of U<sub>S</sub>11 instrumental in the PKR interaction.

We first characterized the interaction of U<sub>S</sub>11 and PKR in virus-infected cells. Prior studies using recombinant expressed

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1  MSQTQPPAPV  GPGDPDVYLK  GVPSAGMHPR  GVHAPRGHPR
41 MISGPPQRGD  NDQAAGQCGD  SGLLRVGADT  IISKPSEAVR
81 PPIIPRTPRV  PREPRVPRPP  REPREPRVPR  APRDPRVPRD
121 RDRPRQPREP  RPPREPRTPR  TPREPRTARG  SV

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FIG. 5. Schematic representation of the HSV-1 (F) U<sub>S</sub>11 amino acid sequence in single-letter code. The sequenced tryptic peptide constituting the site of phosphorylation by activated PKR is in bold-face, and the residues phosphorylated by activated PKR are underlined. The boxed residues represent the required 30-amino-acid PKR binding domain (amino acids 91 to 121).

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US11 (residues 66-92)  V G A D T T I S K P S E A V R P P T I P R T P R V P R
                      . . . . .
eIF-2 $\alpha$  (residues 41-66) I E G M I L L S E L S R R - R I R S I N K L I R I G R

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FIG. 6. Homology between the substrate site of the HSV-1 U<sub>S</sub>11 protein and eIF-2 $\alpha$ . Solid line, identical residues; dots, semiconservative changes. Residues phosphorylated by PKR are in boldface.

proteins and infected-cell lysates have shown that PKR and U<sub>S</sub>11 can bind one another. In this report we demonstrated that U<sub>S</sub>11 and PKR interact in the context of virus-infected cells and that the PKR-U<sub>S</sub>11 interaction is dependent on RNA inasmuch as preincubation with RNase disrupts their coimmunoprecipitation. Finally, the proteins colocalized in the S100 ribosomal fraction and coeluted from the ribosomal fraction after incubation at a high salt concentration (0.8 M), indicating biochemical colocalization.

We next defined the binding and substrate domains of U<sub>S</sub>11 involved in the PKR interaction. Using a series of recombinant viruses carrying deletions and premature stop codons in the sequence encoding the U<sub>S</sub>11 protein, we identified a critical 30-amino-acid epitope at the beginning of the tandemly repeated 3-amino-acid arginine-X-proline domain of U<sub>S</sub>11 that enables the interaction with PKR. Further biochemical studies using recombinant U<sub>S</sub>11 and activated PKR demonstrated that a specific kinase substrate region (amino acids 66 to 92) with 22% amino acid identity and 44% amino acid homology with residues 41 to 66 of eIF-2 $\alpha$  (Fig. 6) exists adjacent to the PKR binding domain. Fine mapping of the adjacent binding and substrate domains introduces some interesting possibilities with regard to U<sub>S</sub>11-PKR functional interaction.

Previous work has demonstrated two possible mechanisms for U<sub>S</sub>11 inhibition of PKR. First, biochemical assays using a recombinant U<sub>S</sub>11 protein indicated that the protein can inhibit the phosphorylation of eIF-2 $\alpha$  by activated PKR (5). Second, similar biochemical assays demonstrated that when recombinant U<sub>S</sub>11 protein was added prior to poly(I)·poly(C), fractional amounts of the HSV protein blocked PKR activation and phosphorylation of eIF-2 $\alpha$  (5). The goal of this work was to extend the previous *in vitro* studies and analyze the structural and functional basis of U<sub>S</sub>11 in the preclusion of protein shut-off in virus-infected cells.

While prior *in vitro* data demonstrated that U<sub>S</sub>11 could preclude PKR activation, in HSV-1-infected cells, PKR is activated (4, 9, 15). In R5104-infected cells, early U<sub>S</sub>11 expression only partially restores the wild-type protein synthesis phenotype. The level of protein synthesis, while dramatically greater than that in cells infected with virus carrying  $\Delta\gamma_134.5$ , is still reduced relative to that in wild-type virus-infected cells. The *in vitro* assays demonstrate that higher levels of recombinant U<sub>S</sub>11 were required to preclude the activated-PKR phosphorylation of eIF-2 $\alpha$ . In contrast, when U<sub>S</sub>11 was added prior to PKR activation, the inhibition of eIF-2 $\alpha$  phosphorylation was complete and extremely sensitive to small amounts of U<sub>S</sub>11. In the event that inhibition of PKR activation was the principal mechanism, one would suspect a more prominent protein synthesis in the second-site mutant viruses. Furthermore, in the  $\Delta\gamma_134.5$  suppressor mutant viruses, repair of the  $\gamma_134.5$  locus restores the higher wild-type protein synthesis levels (24). This indicates that some eIF-2 $\alpha$  must be phosphorylated in the second-site mutant viruses.

The RNA used in the biochemical studies, poly(I)·poly(C) creates dsRNA of sufficient length to bind and activate PKR. Possible interpretations of the *in vitro* data are that the recombinant U<sub>S</sub>11 protein sequesters the poly(I)·poly(C) and that U<sub>S</sub>11 acts in a dominant-negative manner precluding PKR dimerization. Based on the data in this report, amino acids 91 to 121 of U<sub>S</sub>11 (within the RNA binding domain) and RNA are required for PKR binding, suggesting that the U<sub>S</sub>11 protein either sequesters RNA or binds PKR and precludes PKR dimerization. What is uncertain, however, is if the poly(I)·poly(C) used in an *in vitro* experiment reflects the heterogeneity of dsRNA in virus-infected cells. Unlike PKR, the U<sub>S</sub>11 protein does not contain a classic dsRNA binding motif. It binds RNA in a site- and conformation-specific manner and has only been demonstrated to bind two specific viral transcripts, U<sub>L</sub>34 and a synthetic transcript running antisense to the U<sub>S</sub>11 coding domain, despite >40% of the HSV genome being represented in dsRNA. The poly(I)·poly(C) used in biochemical assays, however, may not reflect the levels of RNA with the correct sequence and conformation compatibility for U<sub>S</sub>11 binding in virus-infected cells.

The alternative mechanism is that U<sub>S</sub>11 inhibits the activated PKR from selectively phosphorylating eIF-2 $\alpha$ . While both mechanisms have been demonstrated *in vitro*, studies using cells infected with a recombinant virus that expresses U<sub>S</sub>11 earlier in infection have demonstrated that a viral factor precludes the phosphorylation of eIF-2 $\alpha$  by activated PKR. This suggests that the inhibition of activated PKR is important in cellular infection. Recently a recombinant virus that lacks amino acids 5 to 87 ( $\Delta 5-87$ ) of U<sub>S</sub>11 was tested, and reduced levels of late viral protein synthesis were found (26). It was proposed that a reduced quantity of  $\Delta 5-87$  U<sub>S</sub>11 protein in infected cells was responsible for the significantly lower level of protein synthesis. However, based on *in vitro* studies extremely low levels (0.8 pmol) of U<sub>S</sub>11 inhibit PKR activation and enable continued protein synthesis (5). Based on the data in this report it is equally plausible that, by deleting the kinase substrate domain, the investigators created a U<sub>S</sub>11 mutant incapable of precluding host protein shutoff effectively. Based on the previous data as well as the data in this report, U<sub>S</sub>11 preclusion of activated PKR from phosphorylating eIF-2 $\alpha$  appears a tenable hypothesis.

One of the puzzles regarding the role of U<sub>S</sub>11 in restoring the wild-type protein synthesis phenotype is that the protein is carried in with the virus but appears incapable of precluding host protein shutoff. The U<sub>S</sub>11 protein has been demonstrated by SDS-PAGE analysis as well as two-dimensional electrophoresis to exist in phosphorylated and unphosphorylated forms with different electrophoretic mobilities. Interestingly, in infection with wild-type virus, the virion-associated U<sub>S</sub>11 exists in the phosphorylated form and localizes to the ribosome early in infection (11). Phosphorylation may alter the conformation of U<sub>S</sub>11, making the protein a poor substrate, or may reduce its ability to interact with PKR. Another possibility is that the U<sub>S</sub>11 protein requires a specific RNA for PKR association and that the virion-associated U<sub>S</sub>11 lacks this moiety. The data in this report show that RNase treatment disrupts the PKR-U<sub>S</sub>11 interaction in infected-cell lysates but do not determine if the proteins interact through an RNA bridge. As discussed above U<sub>S</sub>11 binds RNA in a conformation- and sequence-specific

manner. It is possible that the RNase preincubation cleaves single-stranded domains and disrupts a complementary stem-loop conformation. These changes could disrupt a specific RNA bridge tethering U<sub>S</sub>11 and PKR or could alter the structure of U<sub>S</sub>11, reducing its ability to bind PKR. Prior studies, however, showed that activated PKR could phosphorylate U<sub>S</sub>11 and that recombinant U<sub>S</sub>11 could preclude eIF-2 $\alpha$  phosphorylation independent of infected-cell RNA (5). Ongoing experiments examining specific RNA species in recombinant virus infected cells should clarify these issues.

In summary this work provides additional evidence supporting the hypothesis that U<sub>S</sub>11 inhibits the PKR-mediated host protein shutoff response by demonstrating protein interaction and biochemical colocalization in the context of cellular infection. In addition, fine mapping of the U<sub>S</sub>11 protein demonstrates that RNA and a 30-amino-acid epitope (residues 91 to 121) are necessary for PKR interaction in infected cells. Finally, a PKR kinase substrate site adjacent to the PKR binding site is defined; this site may be important for dissecting the mechanism by which U<sub>S</sub>11 precludes host protein shutoff in cells infected with virus carrying  $\Delta\gamma_134.5$ . Future work will explore the mechanism by which U<sub>S</sub>11 precludes protein shutoff in infected cells, further defining the protein complex and ascribing functional relevance in a cell-based system.

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