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AlaArg Motif in the Carboxyl Terminus of the $\gamma_1$34.5 Protein of Herpes Simplex Virus Type 1 Is Required for the Formation of a High-Molecular-Weight Complex That Dephosphorylates eIF-2α

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The $\gamma_1$34.5 protein of herpes simplex virus (HSV) type 1 functions to prevent the shutoff of protein synthesis mediated by the double-stranded-RNA-dependent protein kinase PKR. This is because $\gamma_1$34.5 associates with protein phosphatase 1 (PP1) through its carboxyl terminus, forming a high-molecular-weight complex that dephosphorylates the α subunit of translation initiation factor eIF-2 (eIF-2α). Here we show that Val^{193}Glu and Phe^{195}Leu substitutions in the PP1 signature motif of the $\gamma_1$34.5 protein abolished its ability to redirect PP1 to dephosphorylate eIF-2α and replication of mutant viruses was severely impaired. The $\gamma_1$34.5 protein, when expressed in Sf9 cells using a recombinant baculovirus, was capable of directing specific eIF-2α dephosphorylation. Deletions of amino acids 258 to 263 had no effect on activity of $\gamma_1$34.5. However, deletions of amino acids 238 to 258 abolished eIF-2α phosphatase activity but not PP1 binding activity. Interestingly, deletions in the AlaArg motif of the carboxyl terminus disrupted the high-molecular-weight complex that is required for dephosphorylation of eIF-2α. These results demonstrate that $\gamma_1$34.5 is functionally active in the absence of any other HSV proteins. In addition to a PP1 binding domain, the carboxyl terminus of $\gamma_1$34.5 contains an effector domain that is required to form a functional complex.

The cellular response to virus infection is a complex process involving different components. The double-stranded-RNA-dependent protein kinase (PKR) is one of the components that play a critical role in antiviral defense (16). In mammalian cells, PKR is induced by interferon, and it is activated by double-stranded RNA. Upon viral infection, PKR is activated to phosphorylate serine 51 on the α subunit of translation initiation factor eIF-2 (eIF-2α). Phosphorylation of eIF-2α increases its affinity for guanine nucleotide exchange factor eIF-2B, thus sequestering eIF-2B complex in an inactive complex with phosphorylated eIF-2 and GDP (12, 13). As a result, eIF-2B is not available to catalyze nucleotide exchange on nonphosphorylated eIF-2, which leads to inhibition of protein synthesis (23). Because viruses synthesize double-stranded RNA during their replication, many of them have evolved mechanisms to counteract PKR, such as blocking the activation of PKR, preventing the phosphorylation of eIF-2α, or promoting the degradation of PKR (16, 35). In addition to its role in antiviral defense, PKR has also been implicated in cellular functions such as growth regulation (4, 5, 33), differentiation (35), and apoptosis in uninfected cells (27, 36).

Previous studies demonstrated that PKR is activated in cells infected with wild-type or mutant herpes simplex virus type 1 (HSV-1). But only in cells infected with $\gamma_1$34.5 null mutants is eIF-2α phosphorylated (6, 18). Therefore, in cells infected with $\gamma_1$34.5 null mutants, initiation of DNA replication triggers the shutoff of total protein synthesis (6, 8, 9). Subsequent studies demonstrated that when human cells are infected with wild-type virus, the $\gamma_1$34.5 protein binds to protein phosphatase 1 (PP1), forming a high-molecular-weight complex that specifically dephosphorylates eIF-2α and thereby prevents the shutoff of protein synthesis (20, 21). Thus, unlike most viruses studied so far, HSV uses a unique strategy to evade the antiviral action of PKR (16).

The $\gamma_1$34.5 protein of HSV-1 consists of 263 amino acids with a large amino-terminal domain, a linker (or swivel) region containing repeats of three amino acids (AlaThrPro), and a carboxyl-terminal domain (10, 11). The triplet repeats are a constant feature of all strains, but the number of repeats varies from strain to strain (10). The carboxyl terminus of the $\gamma_1$34.5 protein is required to interact with PP1 and prevent translation shutoff during HSV-1 infection (9, 19, 21). A prominent feature of this domain is a 64-amino-acid region containing a PP1-interacting signature motif (Arg/Lys)(Val/Ile)XaaPhe (20), which is also present in a number of proteins that complex with PP1 (3, 14, 24, 37, 38, 40). These complexes are involved in diverse functions such as cell division, gene expression, glycogen metabolism, and neurotransmission.

The carboxyl terminus of the $\gamma_1$34.5 protein is homologous to a set of proteins known as GADD34 in human, hamster, and mouse (25, 30, 31, 41) (Fig. 1). GADD34 belongs to a family of proteins expressed under conditions of DNA damage, growth arrest, differentiation, and apoptosis (25, 41). Overexpression of GADD34 facilitates apoptosis induced by gamma radiation; however, its physiological role remains unknown (2, 25). Inter-

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   HSV-1    PARVRFSPHVRVRHLVVWAS AARLARRGSWARERADRARF RRRVAEAEAVIGPCLGPEAR  249
   HSV-2    RGKVCFSPRVQVRHLVAWET AARLARRGSWARERADRDRF RRRVAAAEAVIGPCLEPEAR  232
   Human    ARKVRFSEKVTVHFLAVWAG PAQAARQGPWEQLARDRSRF ARRITQAQEELSPCLTPAAR  622
   Mouse    ARKVHFAEKVTVHFLAVWAG PAQAARRGPWEQFARDRSRF ARRIAQAEEKLGPYLTPDSR  615
 Hamster    ARKVHFSENVTVHFLAVWAG PAQAARRGPWEQLARDRSRF ARRIAQAEEKLGPYLTPAFR  561

Consensus   --KV-FS--V-V--L-VWA- -A--ARRG-W-----DR-RF -RR---AE--LGP-L-P--R


   HSV-1    ARALARGAGPANSV------ -------------------- --------------------  263
   HSV-2    ARARARARAHEDGGPAEEEE AAAAARGSSAAAGPGRRAV- --------------------  261
   Human    ARAWARLRNPPLAPIPALTQ TLPSSSVPSSPVQTTPLSQA VATPSRSSAAAAAALDLSG-  671
   Mouse    ARAWARLRNPSLPQSEPRSS SEATPLTQDVTTPSPLPSET PSPSLYLGGRRG--------  657
 Hamster    ARAWARLGNPSLPLALEPIC DHTFFPSQ------------ --------------------  590

Consensus   ARA-AR-------------- -----------------------------------------
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FIG. 1. Amino acid sequence alignment of the carboxyl-terminal domains of the $\gamma_1$34.5 proteins of HSV-1 (10) and HSV-2 (32) and of GADD34 proteins of human (25), mouse (30), and hamster (41).

estingly, the carboxyl terminus of GADD34 functionally substitutes for the corresponding domain of the $\gamma_1$34.5 protein within the context of HSV-1 genome (19). A hypothesis derived from these studies is that the conserved carboxyl-terminal domain represents a functional module with a common role in virus infection and cellular processes.

In the present study, we further examined the role of the carboxyl-terminal domain of the $\gamma_1$34.5 protein. We demonstrate that activation of eIF-2α phosphatase (PP1) by the $\gamma_1$34.5 protein is essential for replication of HSV-1 in infected cells. We also show that $\gamma_1$34.5 protein functions independently of other HSV proteins and provide evidence that the AlaArg motif in the carboxyl terminus of the $\gamma_1$34.5 protein is required to form a high-molecular-weight complex that dephosphorylates eIF-2α.

MATERIALS AND METHODS

Cells and viruses. The Vero, HeLa, and SK-N-SH cell lines were obtained from the American Type Culture Collection and propagated in Dulbecco's modified Eagle's medium supplemented with 5% (HeLa and Vero) or 10% (SK-N-SH) fetal bovine serum. Sf9 cells were purchased from GIBCO and grown in serum-free, Sf-900 SFM medium supplied by the manufacturer.

HSV-1(F) is a prototype HSV-1 strain used in these studies (15). In recombinant virus R3616, a 1-kb fragment from the coding region of the $\gamma_1$34.5 gene was deleted (7). In recombinant virus R8321 (20), codons encoding Val^{193} and Phe^{195} of the $\gamma_1$34.5 gene were replaced with those encoding Glu and Leu, respectively. In addition, this virus contained a 0.5-kb deletion in the thymidine kinase gene. Recombinant virus H9813 was constructed by cotransfection of viral DNA of R8321 (20) with plasmid pRB4867 on rabbit skin cells to restore the thymidine kinase gene. The recombinant progeny was selected and purified in hypoxanthine-aminopterin-thymidine medium. The construct was verified by hybridization of electrophoretically separated restriction enzyme digests with a ^{32}P-labeled *Bam*HI Q fragment as described previously (19). Preparation of viral stock and titration of infectivity were performed on Vero cells.

Recombinant baculoviruses were constructed as suggested by the manufacturer (GIBCO). Briefly, to construct GF9909, the donor plasmid pGF9907 was transformed into *Escherichia coli* DH10BAC cells, which contained the bacmid with a mini-attTn*7* target site and the helper plasmid. White colonies containing recombinant bacmids were selected on Luria-Bertani plates containing kanamycin (50 μg/ml), gentamicin (7 μg/ml), tetracycline (10 μg/ml), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside;100 μg/ml) and isopropyl-β-D-thiogalactopyranoside (40 μg/ml). Purified recombinant bacmid DNA was then used to transfect Sf9 cells using CellFectin reagent (GIBCO). Virus was harvested 3 days after transfection. In a similar way, plasmids pBH0010, pBH0011, pGF9908, pFastBacHT-gus, pGF2002, pGF2007, pGF2009, and pGF2011 were used to construct recombinant baculoviruses BH0010, BH0011, GF9910, GF9911, GF2003, GF2022, GF2023, and GF2024. Virus constructs were verified by PCR with primers OGF0019 (ATGCAGGATCCGCTAACCCCTCCCACCCCCCC TCACGCCCC) and OGF0013 (ATGCATCCGGATTACAGGGCCCGGGCA CGGGCCTCGGGCCCC), which are specific to the $\gamma_1$34.5 gene. Virus titers were determined by plaque assay on Sf9 cells.

Plasmids. Plasmid pRB4867 contains a *Bam*HI Q fragment of HSV-1(F) in the *Bam*HI site of pUC9 (19). pRB143 contains a *Bam*HI S fragment of HSV-1(F) in the *Bam*HI site of pBR322 (34). pRB4789 contains a *Bam*HI S fragment from pRB143 (26). pGEX-PKR contains cDNA encoding full-length PKR fused in frame to glutathione *S*-transferase (GST) (a gift from William G. Bryan). pRB4897 contains a *Bam*HI S fragment in which the codons for V^{193} and F^{195} in the $\gamma_1$34.5 gene were mutated to those for E and L, respectively (20). pRB4892 contains the coding domain of GST fused to the entire coding domain of PP1 except for the initiator methionine codon (21). pFastBacHTa and pFastBacHTb are expression vectors for making recombinant baculoviruses (GIBCO). pFastBac-gus contains the DNA encoding β-glucuronidase (GIBCO). pRB3027 contains approximately 100 bp of the 5′ untranslated region, the entire coding region of the $\gamma_1$34.5 gene, and the 3′ untranslated region derived from the *Bam*HI S fragment (19).

To construct pGF9908, an *Eco*RI-*Sal*I fragment, encoding PP1 from pRB4892, was cloned into the *Eco*RI and *Sal*I sites of pFastBacHTb. To construct pGF9907, a *Bam*HI-*Stu*I PCR fragment containing the entire coding region of the $\gamma_1$34.5 gene was ligated into the *Bam*HI and *Eco*RV sites of pBluescript II SK(+), resulting in plasmid pGF9901. An *Nco*I-*Hin*dIII fragment from pGF9901 was then ligated into the *Nco*I and *Hin*dIII sites of pFastBacHTb, resulting in plasmid pGF9907. To construct pGF2002, a *Bst*EII-*Dra*III fragment from pRB4897 was ligated into the *Bst*EII and *Dra*III sites of pRB3027, resulting in plasmid pBH9902. A *Bam*HI-*Stu*I fragment from pBH9902 was then cloned into the *Bam*HI and *Eco*RV sites of the pBluescript II SK(+), producing pGF2001. An *Nco*I-*Hin*dIII fragment from pGF2001 was cloned into the *Nco*I and *Hin*dIII sites of pFastBacHTb, yielding pGF2002. In this plasmid, Val^{193} and Phe^{195} in the $\gamma_1$34.5 gene were mutated to Glu and Leu, respectively. Cloning and deletions in the $\gamma_1$34.5 gene were done with PCR using pRB143 as the template. To construct pGF2007, a *Bst*EII-*Bsp*EI PCR fragment was amplified with oligoBH9716 (CATGGCCCGCCGCCGCCGCCATCGC) and OGF0013 and ligated into the *Bst*EII and *Bsp*EI sites of pGF9901, resulting in plasmid pGF2006. An *Nco*I-*Bsp*EI-Klenow fragment was then ligated into the *Nco*I and *Stu*I sites of pFastBacHTb, yielding pGF2007, which contains the region of the $\gamma_1$34.5 gene encoding amino acids 1 to 253. To construct pGF2009, a *Bst*EII-*Bsp*EI PCR fragment was amplified with oligoBH9716 and OGF0015 (ATGCATCCGGAT TAGCCGGCTCCGCGGGCCAGGGCCCGGGCA) and ligated into the *Bst*EII and *Bam*HI sites of pGF9901, resulting in plasmid pGF2008. A *Nco*I-*Bsp*EI-Klenow fragment was then ligated into the *Nco*I and *Stu*I sites of pFastBacHTb, yielding pGF2009, which contains the region of the $\gamma_1$34.5 gene encoding amino acids 1 to 258. To construct pGF2011, a *Bst*EII-*Bsp*EI PCR fragment was amplified with oligoBH9716 and OGF0016 (ATGCATCCGGATTACGCCGGGC CGGCTCCGCGGGCCAGGGCC) and ligated into the *Bst*EII and *Bsp*EI sites of pGF9901, resulting in plasmid pGF2010. A *Noc*I-*Bsp*EI-Klenow fragment was then ligated into the *Nco*I and *Stu*I sites of pFastBacHTb, yielding pGF2011, which contains the region of the $\gamma_1$34.5 gene encoding amino acids 1 to 260. To construct pBH0010, a *Bst*EII-*Bsp*EI PCR fragment encoding amino acids 28 to 238 was amplified and ligated into the *Bst*EII and *Bsp*EI sites of pRB4789, yielding plasmid pBH0002. The primers used were oligoBH9716 and oligoBH0005 (AGTCATCCGGATTAGCGGCGCGCCAGGCGGGCGGCCGA GGC). An *Nco*I-*Stu*I fragment from pBH0002 was then ligated into the *Nco*I and *Stu*I sites of pFastBacHTa, resulting in pBH0010, which contains the region of

$\gamma_1$34.5 encoding amino acids 1 to 238. To construct pBH0003, a *Bst*EII-*Bsp*EI PCR fragment encoding amino acids 28 to 248 was amplified with oligoBH9716 and oligoBH0004 (AGTCATCCGGATTAGGCCTCCGCCACCCGGCGCCG GAACCG). The PCR fragment was ligated into the *Bst*EII and *Bsp*EI sites of pRB4789, yielding plasmid pBH0003. A *Nco*I-*Stu*I fragment from pBH0003 was then ligated into the *Nco*I and *Stu*I sites of pFastBacHTa, resulting in pBH0011, which contains the region encoding amino acids 1 to 248. For plasmids pBH0010, pBH0011, pGF9907, pGF9908, pGF2002, pGF2007, pGF2009, and pGF2011, a His tag was fused in frame to the initiator methionine codon of the $\gamma_1$34.5 gene.

GST pull-down assay. GST protein and a GST-PP1 fusion protein were induced by the addition of isopropyl-β-D-thiogalactoside to the medium with *E. coli* BL21 cells transformed with plasmid pGEX4T-1 or pRB4892, followed by affinity purification of the fusion protein from bacterial lysates on agarose beads conjugated with glutathione. Infected Sf9 cells were harvested, washed with cold phosphate-buffered saline, and lysed in buffer containing 50 mM HEPES (pH 7.6), 150 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, and 2 mM benzamidine. After 30 min on ice and centrifugation to remove nuclei, the supernatant was precleared with GST beads and then incubated with GST-PP1 fusion protein-bound beads at 4°C overnight. After three washes, the proteins bound to beads were resuspended in disruption buffer containing 50 mM Tris-HCl (pH 7.0), 5% 2-mercaptoethanol, 2% sodium dodecyl sulfate (SDS), and 2.75% sucrose. Samples were subjected to electrophoresis and processed for immunoblot analysis with anti-His tag antibody (1, 8).

eIF-2α phosphatase assays. Cells either mock infected or infected with viruses were harvested 15 h (HeLa cells) or 48 h (Sf9 cells) postinfection, rinsed with phosphate-buffered saline, resuspended in lysis buffer containing 10 mM HEPES (pH 7.6), 150 mM NaCl, 10 mM $MgCl_2$, 0.2% Triton X-100, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 2 mM benzamidine, placed on ice for 30 min, and subjected to centrifugation to remove nuclei. The supernatant fluids were saved for analysis. eIF-2 was purified from rabbit reticulocytes as previously described (17). GST-PKR fusion protein was expressed and purified from *E. coli* BL21 cells as described above. To prepare ^{32}P-labeled eIF-2α, eIF-2 was incubated with GST-PKR in buffer containing 20 mM Tris-HCl (pH 7.5), 40 mM KCl, 2.0 mM $MgCl_2$, and 0.17 mM [γ-^{32}P]ATP (10 Ci/mmol) for 30 min at 34°C. Aliquots of cell lysates were then incubated with phosphorylated eIF-2α in buffer containing 20 mM Tris-HCl (pH 7.5), 40 mM KCl, 2.0 mM $MgCl_2$, and 0.1 mM EDTA at 34°C for 2 min. The reaction was stopped by adding disruption buffer containing 50 mM Tris-HCl (pH 7.0), 5% 2-mercaptoethanol, 2% SDS, and 2.75% sucrose, followed by electrophoresis on a SDS–12% polyacrylamide gel, transferred onto a nitrocellulose membrane, and subjected to autoradiography (21). In addition, the nitrocellulose membrane was scanned by the PhosphorImage SI system, and the radioactivity of eIF-2α was quantitated using ImageQuant NT software (Molecular Dynamics Inc.).

Gel filtration chromatography. A Superdex 200 HR 10/30 column (1.0 by 30 cm; Amersham Pharmacia Biotech) was equilibrated with 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM EDTA and pumped at 0.5 ml/min, using a Amersham Pharmacia Biotech fast protein liquid chromatography system. Samples were injected, 0.5-ml fractions were collected on ice, and the absorbance at 280 nm was monitored (20).

Immunoblotting. Samples were solubilized in the disruption buffer described above, sonicated, boiled, subjected to electrophoresis on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, blocked with 5% nonfat milk, and reacted with anti-$\gamma_1$34.5 antibody (a gift from Bernard Roizman), anti-His tag antibody (Qiagen Inc.), or anti-eIF-2α antibody (a gift from Robert Schneider). The membranes were then rinsed in phosphate-buffered saline and reacted with either goat anti-rabbit or mouse immunoglobulin conjugated to alkaline phosphatase (Bio-Rad) or donkey anti-rabbit or anti-mouse immunoglobulin conjugated to horseradish peroxidase (Amersham Pharmacia Biotech. Inc.) (8, 19).

RESULTS

Activation of eIF-2α phosphatase by the $\gamma_1$34.5 protein is crucial for replication of HSV-1 in infected cells. The $\gamma_1$34.5 protein of HSV-1 prevents the total shutoff of protein synthesis in cells infected with HSV-1, which requires an interaction of PP1 with the PP1-interacting motif within the carboxyl terminus of the $\gamma_1$34.5 protein. To determine the contribution of this interaction to viral replication, we constructed a recombinant HSV-1, H9813, by cotransfection of the DNA of R8321 with plasmid pRB4867 to restore the thymidine kinase gene as described in Materials and Methods. In this virus, Val^{193} and Phe^{195} in the PP1 signature motif were mutated to Glu and Leu, respectively. The virus construct was verified by restriction digestion and Southern blot analysis (data not shown). Expression of the $\gamma_1$34.5 protein was detected by Western blot analysis with anti-$\gamma_1$34.5 antibody. Mutant H9813 expressed the $\gamma_1$34.5 protein to a level that is similar to that of wild-type HSV-1(F) (see Fig. 3A).

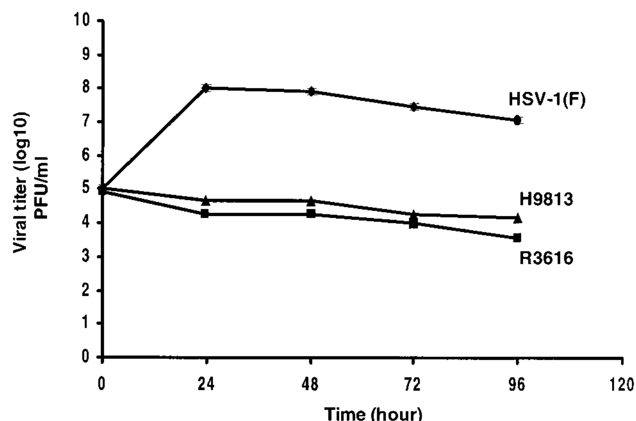


FIG. 2. Replication of wild-type HSV-1(F) and the $\gamma_1$34.5 mutants R3616 and H9813 in SK-N-SH cells. Confluent monolayers of cells were infected with viruses at 0.01 PFU per cell and incubated at 37°C. At various times postinfection, the cells were harvested, freeze-thawed three times, and titrated on Vero cells. Duplicate samples were analyzed in parallel at each time point, and the data represent assays from three experiments.

To examine the role of Val^{193} and Phe^{195} of the $\gamma_1$34.5 protein in viral replication, confluent human neuroblastoma cells (SK-N-SH) were infected with HSV-1(F), R3616, or H9813 at 0.01 PFU per cell. At different time points postinfection, the cells were harvested and virus yields were determined by plaque assay on Vero cells. As indicated in Fig. 2, in cells infected with HSV-1(F), there was an approximately 3-log increase in viral yield 24 h after infection, reaching a peak titer of 10^8 PFU/ml. The titer then dropped slightly 96 h postinfection. In contrast, in cells infected with the $\gamma_1$34.5 deletion mutant R3616, the viral yields did not increase after infection and the titer remained approximately 10^5 PFU/ml. Interestingly, in cells infected with H9813, viral replication resembled that of R3616, in which the $\gamma_1$34.5 gene has been deleted. The results suggest that Val^{193} and Phe^{195} in the PP1 interacting motif of the $\gamma_1$34.5 protein are crucial for virus replication.

Next, we sought to delineate whether viral replication is linked to eIF-2α phosphatase activity. In this experiment, cell extracts were prepared from HeLa cells either mock infected or infected with HSV-1(F), R3616, or H9813 and tested for their ability to dephosphorylate ^{32}P-labeled eIF-2α. Purified eIF-2 was phosphorylated with GST-PKR and [^{32}P]ATP in vitro and then incubated with cell lysates. As shown in Fig. 3B, the control reaction mixture lacking cell lysate contained two phosphorylated bands, one representing phosphorylated eIF-2α and one representing phosphorylated GST-PKR (lane 1). Incubation of HSV-1(F)-infected cell lysate with the eIF-2–GST-PKR reaction mixture resulted in dephosphorylation of [^{32}P]eIF-2α but not [^{32}P]GST-PKR (Fig. 3B, lane 3). In contrast, cell lysates from cells which were mock infected or infected

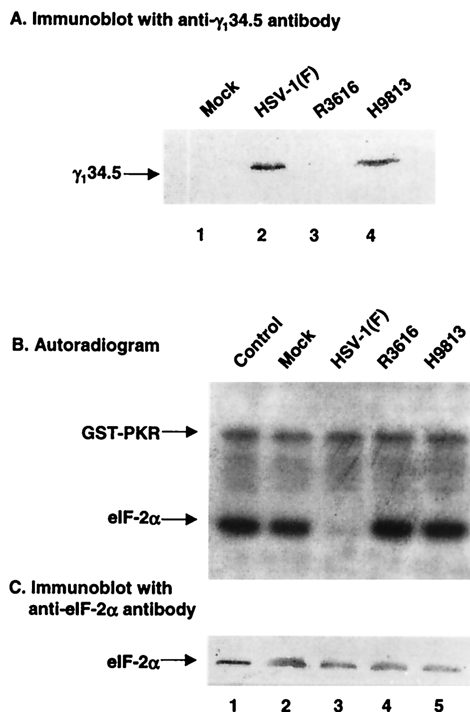


FIG. 3. (A) Expression of the $\gamma_1$34.5 protein. HeLa cells were mock infected or infected with HSV-1(F), R3616 (in which the coding region of the $\gamma_1$34.5 gene was deleted), or H9813 (in which Vla^{193}Glu and Phe^{195}Leu substitutions were made in the $\gamma_1$34.5 gene) at 10 PFU per cell). At 15 h postinfection, cells were harvested, washed with phosphate-buffered saline, and resuspended in disruption buffer containing 50 mM Tris-HCl (pH 7.0), 5% 2-mercaptoethanol, 2% SDS, and 2.75% sucrose. Samples were then electrophoretically separated on denaturing 12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot was probed with anti-$\gamma_1$34.5 antibody (1). (B) eIF-2α phosphatase activity in HeLa cells which were mock infected or infected with the indicated viruses. ^{32}P-labeled eIF-2α, prepared as described in Materials and Methods, was incubated with lysates of HeLa cells which were mock infected or infected with indicated viruses at 34°C. After incubation for 2 min, the reaction was stopped by the addition of disruption buffer, and samples were separated electrophoretically on a denaturing 12% polyacrylamide gel, transferred to a nitrocellulose membrane, and subjected to autoradiography (21). Lanes 1, ^{32}P-labeled eIF-2α and GST-PKR not reacted with cell lysates; lanes 2 to 5, ^{32}P-labeled eIF-2α reacted with lysates of cells which were mock infected or infected with the indicated viruses. (C) Immunoblot of the autoradiogram in panel B.

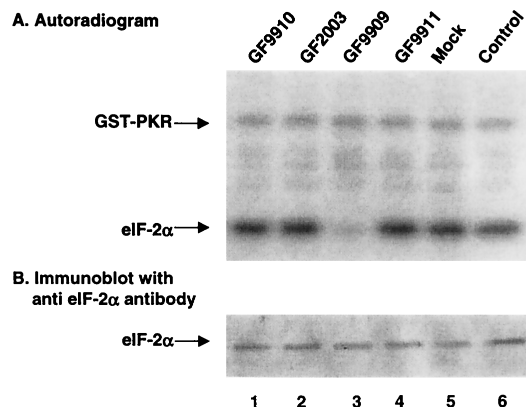


FIG. 4. (A) eIF-2α phosphatase activity in Sf9 cells which were mock infected or infected with recombinant baculoviruses. Sf9 cells (10^7 cells) were either mock infected or infected at 5 PFU per cell with GF9909, which expresses the wild-type $\gamma_1$34.5 protein, GF2003, which expresses the mutant $\gamma_1$34.5 protein with Val^{193}Glu and Phe^{195}Leu substitutions, GF9910, which expresses PP1, or GF9911, which expresses glucuronidase. At 48 h postinfection, cells were harvested and lysates were prepared as described in Materials and Methods. Aliquots of the lysates were then reacted with ^{32}P-labeled eIF-2 at 34°C. After a 2-min incubation, the reaction was stopped by adding disruption buffer, and samples were processed as described for Fig. 3B. Lane 6, ^{32}P-labeled eIF-2α and GST-PKR not reacted with cell lysates; lanes 1 to 5, ^{32}P-labeled eIF-2α and GST-PKR reacted with lysates of cells which were mock infected or infected with the indicated recombinant baculoviruses. (B) Immunoblot of the nitrocellulose membrane in panel A.

with R3616 exhibited little or no eIF-2α-specific phosphatase activity (Fig. 3B, lanes 2 and 4). Similarly, H9813-infected cell lysate did not exhibit eIF-2α phosphatase activity (Fig. 3B, lane 5). Western blot analysis with anti-eIF-2α showed that eIF-2α was present in all reaction mixtures (Fig. 3C), confirming that the disappearance of radioactive eIF-2α is due to dephosphorylation. The larger amount of eIF-2α in reaction mixture containing cell lysates is likely due to the presence of endogenous eIF-2α in the lysates (Fig. 3C, lanes 2 to 5). These results demonstrate that Val^{193} and Phe^{195} in the PP1 signature motif are critical for the $\gamma_1$34.5 protein to enhance eIF-2α dephosphorylation and that activation of PP1 by the $\gamma_1$34.5 protein is required for viral replication in infected cells.

The activity of the $\gamma_1$34.5 protein is independent of other HSV proteins. We investigated whether the $\gamma_1$34.5 protein was functionally active in the absence of other HSV proteins. We constructed the following recombinant baculoviruses: GF9909, expressing the wild-type $\gamma_1$34.5 protein; GF2003, expressing the $\gamma_1$34.5 protein with Val^{193}Glu and Phe^{195}Leu mutations in the PP1 binding motif; GF9911, expressing glucuronidase; and GF9910, expressing human PP1. Expression of the $\gamma_1$34.5 protein and PP1 was verified by Western blot with mouse monoclonal anti-His tag antibody, since these proteins were His tagged at the amino terminus (see Fig. 5; also data not shown). To assay for eIF-2α phosphatase activity, monolayers of Sf9 cells were mock infected or infected with GF9909, GF9910, GF9911, or GF2003 at 5 PFU per cell. Forty-eight hours after infection, cells were harvested and cell lysates were incubated with ^{32}P-labeled eIF-2α. Samples were then separated on an SDS–12% polyacrylamide gel, transferred to a nitrocellulose membrane, and subjected to autoradiography. Data in Fig. 4A indicate that lysate of mock infected cells (lane 5) or cells infected with recombinant baculovirus GF9911 expressing glucuronidase (lane 4) did not exhibit eIF-2α phosphatase activity. In contrast, the lysate of cells infected with GF9909 expressing the wild-type $\gamma_1$34.5 protein was able to dephosphorylate eIF-2α (Fig. 4A, lane 3), although it had no effect on phosphorylated GST-PKR. Lysate of cells infected with GF9910 expressing PP1 alone did not display any eIF-2α phosphatase activity. Interestingly, the lysate of cells infected with GF2003 lacked eIF-2α-specific phosphatase activity (Fig. 4A, lane 2). Since GF2003 expresses the $\gamma_1$34.5 protein with Val^{193}Glu and Phe^{195}Leu mutations in the PP1-interacting signature sequence, the result suggests that interaction of the $\gamma_1$34.5 protein with PP1 in Sf9 cells is essential to activate eIF-2α phos-

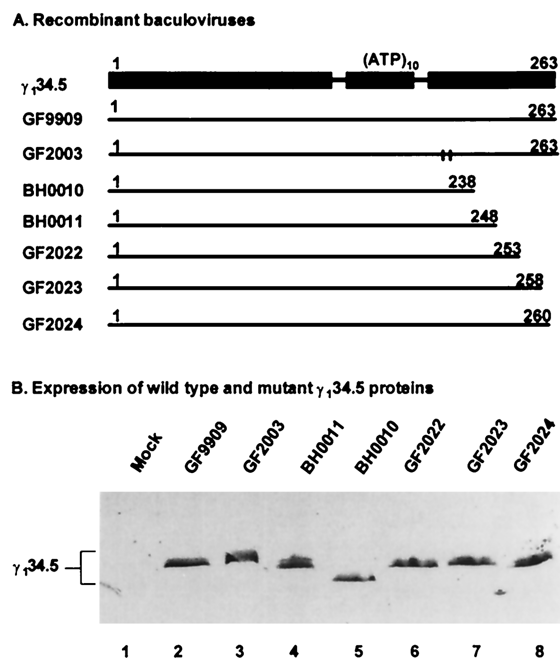
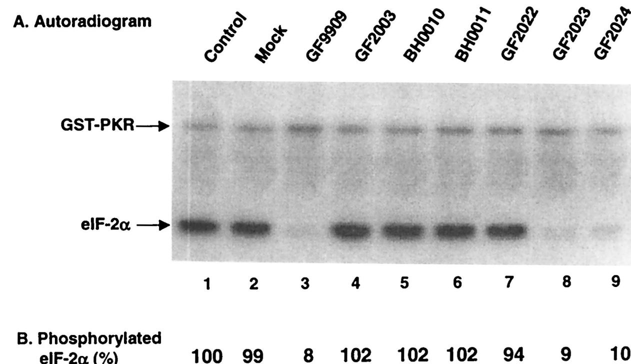


FIG. 5. (A) Schematic diagram of recombinant baculoviruses expressing wild-type and mutant forms of the $\gamma_1$34.5 protein. Line 1, domain structure of the wild-type $\gamma_1$34.5 protein. $(ATP)_{10}$ represents the triplet repeats of AlaThrPro, which connects the amino-terminal domain and the carboxyl-terminal domain. Numbers on the top denote the amino acid positions. Line 2, wild-type $\gamma_1$34.5 protein. Line 3, mutant $\gamma_1$34.5 protein with Val^{193}Glu and Phe^{195}Leu substitutions. Vertical lines indicate the positions of substitutions. Lines 4 to 8, deletion mutants expressing different-length segments of the $\gamma_1$34.5 protein. Numbers on the top of each line indicate the first and last amino acids contained in each construct. (B) Expression of wild-type and mutants of the $\gamma_1$34.5 protein. Cell lysates of Sf9 cells which were mock infected or infected with the indicated viruses were subjected to electrophoresis on a denaturing 12% polyacrylamide gel, transferred to a nitrocellulose membrane, and reacted with anti-His tag antibody (Qiagen Inc).



B. Phosphorylated eIF-2α (%)	100	99	8	102	102	102	94	9	10

FIG. 6. (A) Activity of the $\gamma_1$34.5 mutants in Sf9 cells. Sf9 cells (10^7 cells) were mock infected (lane 2) or infected with the indicated recombinant baculoviruses (lanes 3 to 9) at 5 PFU per cells at 27°C. At 48 h after infection, cells were harvested and lysates were prepared as described in Materials and Methods. Aliquots of each lysate were then incubated with ^{32}P-labeled eIF-2α, and samples were processed for autoradiography as described for Fig. 3B. (B) Quantitation of the phosphorylated eIF-2α. Phosphorylated eIF-2α in each lane in panel A was quantitated after eIF-2α phosphatase assays with a PhosphorImage SI system (ImageQuant software). The numbers indicate the percentages of phosphorylated eIF-2α remaining after incubation with the cell lysates relative to that of unreacted eIF-2α.

phatase. To confirm that there was no degradation of ^{32}P-labeled eIF-2α in the assays, Western blot analysis with anti-eIF-2α antibody (Fig. 4B) indicated that the level of eIF-2α is similar in all samples tested. These results demonstrate that the $\gamma_1$34.5 protein, when expressed in the absence of any other HSV protein, is still capable of redirecting endogenous protein phosphatase in Sf9 cells to dephosphorylate eIF-2α. Importantly, the activity of the wild-type and mutant $\gamma_1$34.5 proteins in Sf9 cells (Fig. 4A) closely reflects the activity seen in HeLa cells infected with HSV-1(F) and H9813 (Fig. 3B).

The ArgAla motif in the carboxyl terminus is critical for the function of the $\gamma_1$34.5 protein. Since the $\gamma_1$34.5 protein modulates eIF-2α phosphatase activity in Sf9 cells, we used this system to investigate the role of the carboxyl terminus of the $\gamma_1$34.5 protein by constructing a series of deletion mutants. Recombinant baculoviruses were constructed to express mutant forms of the $\gamma_1$34.5 protein with deletions from amino acids 238 to 263 (BH0010), 248 to 263 (BH0011), 253 to 263 (GF2022), 258 to 263 (GF2023), or 260 to 263 (GF2024) (Fig. 5A). These mutants, along with GF9909, expressing wild-type $\gamma_1$34.5 protein, and GF2003, expressing the $\gamma_1$34.5 protein with Val^{193}Glu and Phe^{195}Leu mutations in the PP1 signature motif, were used to infect Sf9 cells at 5 PFU per cell. Forty-eight hours after infection, cell lysates were prepared, subjected to electrophoresis on an SDS–12% polyacrylamide gel electrophoresis (PAGE) gel, and processed for immunoblot analysis with mouse monoclonal anti-His tag antibody. As shown in Fig. 5B, these mutants showed protein bands of the expected size and expressed the $\gamma_1$34.5 protein at a level comparable to that of GF9909 expressing wild-type $\gamma_1$34.5 protein.

We next tested the ability of these mutants to activate eIF-2α phosphatase in Sf9 cells. Aliquots of lysate from cells which were mock infected or infected with the $\gamma_1$34.5 expressing viruses were reacted with ^{32}P-labeled eIF2 and samples were processed for autoradiography as described above. As shown in Fig. 6, lysate of cells which were mock infected or infected with GF2003 showed no eIF-2α phosphatase activity (Fig. 6A, lanes 2 and 4). Lysates of cells infected with GF2023 or GF2024 displayed eIF-2α phosphatase activity comparable to that of wild-type $\gamma_1$34.5 protein (Fig. 6A, lanes 3, 8, and 9), indicating that deletion of the last five amino acids in the carboxyl terminus had no effect on the function of the $\gamma_1$34.5 protein. However, lysates of cells infected with GF2022, BH0010, or BH0011 failed to dephosphorylate eIF-2α. PhosphorImager analysis indicated that detectable ^{32}P-labeled eIF-2α was less than 10% after reaction with lysates of cells infected with GF9909, GF2023, and GF2024. In contrast, the level of ^{32}P-labeled eIF-2α remained unchanged for GF2022, BH0010, and BH0011 compared to that in the control reaction mixture (i.e., not reacted with infected cell lysates) (Fig. 6B, lanes 4 to 7 and 1). The data demonstrate that amino acids 238 to 258 of the $\gamma_1$34.5 protein are essential for dephosphorylation of eIF-2α.

Deletions of amino acids 238 to 263 from the carboxyl terminus do not affect the association of the $\gamma_1$34.5 protein with PP1. To address whether the deletions of amino acids 238 to 263 in the carboxyl terminus of the $\gamma_1$34.5 protein altered its

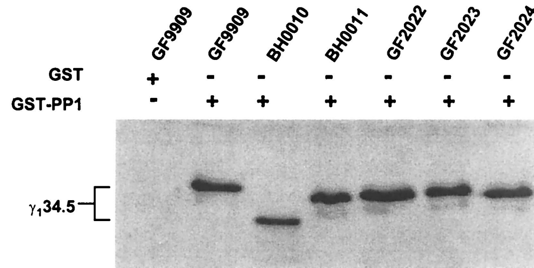
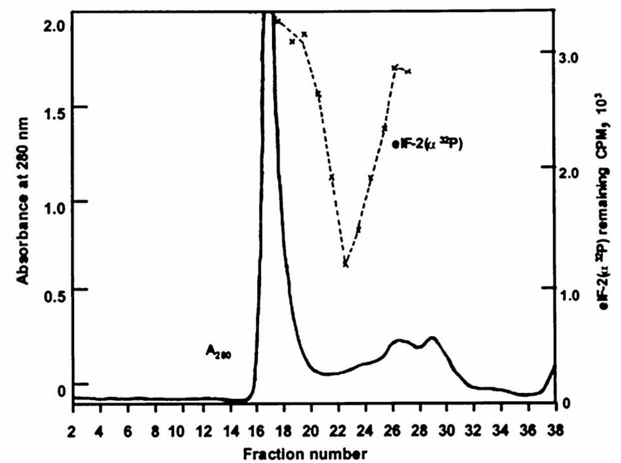


FIG. 7. Interaction of PP1 with the $\gamma_1$34.5 mutants. Sf9 cells infected with baculovirus expressing wild-type or mutant forms of the $\gamma_1$34.5 protein were harvested at 48 h postinfection and lysed in buffer containing 50 mM HEPES (pH 7.6), 150 mM NaCl, 10 mM $MgCl_2$, and 1% Triton X-100. After 30 min on ice, the lysates were precleared with GST beads and then incubated with GST-PP1 bound to beads at 4°C overnight. After washing three times, the protein complexes were solubilized in disruption buffer, electrophoretically separated on denaturing 12% polyacrylamide gel, and transferred to a nitrocellulose sheet. The blot was probed with anti-His tag antibody (Qiagen Inc).

ability to associate with PP1, we carried out a GST pull-down experiment. GST-PP1 was expressed, purified from *E. coli,* and incubated with cell extracts prepared from Sf9 cells that were either mock infected or infected with virus at 5 PFU per cell. The protein complexes bound to GST-PP1 were electrophoretically separated on an SDS-PAGE gel and processed for immunoblot analysis with anti-His tag antiserum. As shown in Fig. 7, GST-PP1, but not GST alone, pulled down wild-type $\gamma_1$34.5. In addition, GST-PP1 also pulled down mutant forms of the $\gamma_1$34.5 protein expressed from BH0010, BH0011, GF2022, GF2023, and GF2024. The results indicate that deletions of amino acids 238 to 263 from the carboxyl terminus of the $\gamma_1$34.5 protein do not affect the association of this protein with PP1, as measured under these experimental conditions.

Deletions in the AlaArg motif of the carboxyl terminus of the $\gamma_1$34.5 protein disrupt the formation of an eIF-2α-specific high-molecular-weight complex. Because binding of the $\gamma_1$34.5 protein to PP1 generates a high-molecular-weight complex that dephosphorylates eIF-2α in HeLa cells infected with HSV-1(F) (20), we investigated whether this complex is formed in Sf9 cells infected with baculoviruses expressing wild-type or mutant forms of the $\gamma_1$34.5 protein. Sf9 cells were first infected with recombinant baculovirus GF9909 expressing the wild-type $\gamma_1$34.5 at 5 PFU per cell, and the cells were harvested 48 h after infection. Lysate was then separated on a Superdex 200 column and fractions were collected. These fractions were assayed for their ability to dephosphorylate ^{32}P-labeled eIF2. The elution profile and eIF-2α phosphatase activity are shown in Fig. 8A. While the bulk of proteins eluted in fractions 15 to 20, eIF-2α phosphatase activity eluted as a single peak in fractions 23 to 24 with a relatively minimum level of protein. Based on calibration of the column with different-sized protein standards, eIF-2α phosphatase activity eluted at a position that corresponds to a molecular weight of 340,000, the same size as the complex containing the $\gamma_1$34.5 protein and PP1 eluted from lysates of HeLa cells infected with HSV-1(F) (20).

We also analyzed the distribution of the $\gamma_1$34.5 protein in the same Superdex column fractions derived from lysates of cells infected with GF9909. Aliquots of these fractions were subjected to electrophoresis in an SDS–12% PAGE gel and processed for immunoblot analysis with anti-$\gamma_1$34.5 antibody. As shown in Fig. 8B, for the lysate of cells infected with GF9909, a major portion of the $\gamma_1$34.5 protein was in fractions 16 to 19, coincident with a majority of the A_{280} and representing the void volume of the column. These fractions contain no eIF-2α phosphatase activity (Fig. 8A), and the nature of this $\gamma_1$34.5 peak remains unclear. However, a smaller, second peak of $\gamma_1$34.5 eluted in fractions 22 to 24, which is exactly coincident



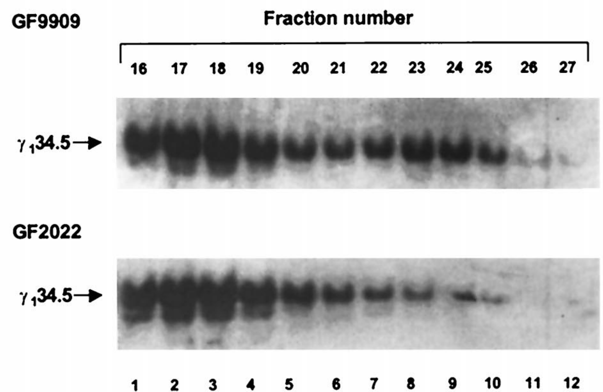


FIG. 8. (A) Superdex 200 gel filtration analysis of cytoplasmic extracts from Sf9 cells infected with GF9909, expressing the wild-type $\gamma_1$34.5 protein. Sf9 cells were infected with 5 PFU of GF9909 per cell at 27°C. At 48 h postinfection, cells were harvested and lysates were prepared and assayed for eIF-2α phosphatase activity as described in Materials and Methods. The dashed line represents ^{32}P-labeled eIF-2α remaining after incubation with aliquots of indicated fractions, measured as described in Materials and Methods. The size of the eIF-2α phosphatase complex (340,000) was estimated with reference to the elution position of the following protein size markers: horse spleen apoferritin (465,000), aldolase (150,000), bovine serum albumin (69,000), ovalbumin (45,000), and rabbit reticulocyte thioredoxin (11,600). (B) Immunoblot of fractions from Superdex 200 column chromatography with anti-$\gamma_1$34.5 antibody. Lysates of Sf9 cells infected with GF9909 or GF2022 were chromatographed as described for panel A. Aliquots of fractions 16 to 27 from each were separated on a SDS–12% polyacrylamide gel, transferred to a nitrocellulose membrane, and reacted with anti-$\gamma_1$34.5 antibody as described in Materials and Methods.

with the fractions containing eIF-2α phosphatase activity (Fig. 8A and B). We also analyzed lysates of Sf9 cells infected with GF2023 or GF2024 by chromatography on Superdex 200 and observed the same distribution of the $\gamma_1$34.5 protein as was observed from lysate of cells infected with GF9909 (data not shown). These results demonstrate that the $\gamma_1$34.5 protein is a component of a high-molecular-weight complex in Sf9 cells that is capable of dephosphorylating eIF-2α.

Since deletions of amino acids 238 to 258 in the carboxyl terminus of the $\gamma_1$34.5 protein abolished eIF-2α phosphatase activity (Fig. 5 and 6A), we next evaluated whether these deletions had any effect on the formation of a high-molecular-weight complex in Sf9 cells. Lysate of Sf9 cells infected with GF2022, which fails to activate eIF-2α phosphatase (Fig. 6), was chromatographed on the Superdex 200 column, and the distribution of the $\gamma_1$34.5 protein in column fractions was determined as described above. The elution profile is similar to that of GF9909 (data not shown). Results in Fig. 8B indicate that the complete absence of a $\gamma_1$34.5-containing peak eluting in fractions 22 to 24 that corresponds to activated eIF-2α phosphatase (Fig. 8A and B). In contrast, the large peak of $\gamma_1$34.5 not associated with eIF-2α phosphatase in fractions 16 to 19 was not diminished. When lysates of cells infected with BH0010 or BH0011, which also failed to undergo activation of eIF-2α phosphatase, were chromatographed on Superdex 200 and similarly analyzed, each also showed the complete absence of a $\gamma_1$34.5-containing complex in fractions 22 to 24 (data not shown). Collectively, these data indicate that deletions of amino acids 238 to 258 in the $\gamma_1$34.5 protein, notably deletions in the AlaArg motif, disrupted the formation of a high-molecular-weight complex that dephosphorylates the eIF-2α.

DISCUSSION

Several lines of evidence indicate that the $\gamma_1$34.5 protein of HSV-1 is crucial in counteracting the antiviral effect of PKR (6, 8, 9, 20, 21, 28, 29). HSV mutants that fail to express the $\gamma_1$34.5 protein induce a premature shutoff of protein synthesis in cell culture and are highly attenuated in experimental animal models (7, 39). Recent studies demonstrated that the $\gamma_1$34.5 deletion mutants replicate efficiently in $PKR^{-/-}$ knockout mice but not in $PKR^{+/+}$ mice (28, 29). We have recently found that in HSV-infected cells the $\gamma_1$34.5 protein binds to PP1, forming a high-molecular-weight complex that specifically dephosphorylates eIF-2α (20, 21), and that a PP1-interacting signature motif, (Arg/Lys)(Val/Ile)XaaPhe, in the carboxyl terminus of the protein is required to prevent shutoff of protein synthesis (20). These observations indicate that interaction between the $\gamma_1$34.5 protein and PP1 is critical for down-regulation of PKR activity.

To extend these studies, we further examined the role of the PP1-interacting motif in viral replication. We constructed recombinant virus H9813 and tested its ability to replicate in cell cultures. As shown in Fig. 2, Val^{193}Glu and Phe^{195}Leu substitutions in $\gamma_1$34.5 severely impaired replication of H9813 in SK-N-SH cells. Similar results were seen in mouse fibroblast line T101/2 (data not shown). Moreover, lysate of cells infected with H9813 did not exhibit eIF-2α-specific phosphatase activity. These observations strongly support the view that eIF-2α dephosphorylation mediated by the $\gamma_1$34.5 protein is essential for HSV-1 replication in infected cells. A linkage between viral replication, Val^{193}/Leu^{195} in the $\gamma_1$34.5 protein, and eIF-2α phosphatase activity underscores the importance of the PP1-interacting signature motif in HSV-1 infection. A number of cellular PP1 binding proteins have been reported to possess the signature sequence (Arg/Lys)(Val/Ile)XaaPhe (3, 14, 22, 24, 38). Among them are DARP-32 (dopamine- and cyclic AMP-regulated phosphoprotein) (40), NIPP1 (nuclear inhibitor of PP1) (38), G subunit (37), and splicing factor PSF (24). The mechanism of PP1 regulation by these proteins remains unclear. It is generally believed that these regulatory proteins either target PP1 to a particular subcellular location or modulate the PP1 catalytic activity towards a specific substrate.

An important finding emerged from our studies is that the $\gamma_1$34.5 protein activates eIF-2α phosphatase activity independent of any other HSV proteins. Significantly, the baculovirus system reproduces observation obtained from HeLa cells infected with HSV-1 (Fig. 3B). As shown in Fig. 4, recombinant baculovirus expressing the wild-type $\gamma_1$34.5 protein is capable of activating eIF-2α phosphatase in Sf9 cells. In addition, gel filtration analysis indicates that the $\gamma_1$34.5 protein is a component of a 340,000-molecular-weight complex that dephosphorylates eIF-2α in Sf9 cells (Fig. 8 and 9). These results parallel the previous findings obtained from HeLa cells infected with HSV-1(F) (20) and further demonstrate that the only HSV protein required for the formation of the high-molecular-weight complex is the $\gamma_1$34.5 protein. These studies also suggest that the $\gamma_1$34.5 protein is likely to interact with PP1 in Sf9 cells, since baculovirus expressing mutant $\gamma_1$34.5 protein, with Val^{193}Glu and Phe^{195}Leu substitutions in the PP1 signature motif, is inactive in Sf9 cells (Fig. 4, lane 2). The implications of these observations are twofold: first, the baculovirus system can be employed to evaluate domain functions of the $\gamma_1$34.5 protein efficiently. Second, it can be used as an alternative system to examine the molecular nature of the $\gamma_1$34.5-PP1 complex. Due to a low level of expression of the $\gamma_1$34.5 protein in HSV-infected cells, it has been difficult to obtain a sufficient amount of the $\gamma_1$34.5-PP1 complex (unpublished data). The use of the baculovirus system will help to resolve this problem.

Our studies support the notion that the carboxyl terminus of the $\gamma_1$34.5 protein consists of a PP1 binding domain and an effector domain. Data in Fig. 6 showed that deletions from amino acids 258 to 263 did not have any effect on the $\gamma_1$34.5 protein activity. Deletions from amino acids 238 to 258 in the carboxyl terminus, however, are deleterious. Although these mutants retain their ability to associate with PP1 (Fig. 7), they were unable to activate eIF-2α phosphatase (Fig. 6), indicating that besides a PP1 interacting domain, the carboxyl terminus of $\gamma_1$34.5 contains an effector domain. It is obvious that communication between the PP1-interacting motif and the extreme carboxyl terminus of $\gamma_1$34.5 determines eIF-2α dephosphorylation. These results defined a region containing amino acids 238 to 258, where a contiguous block of conserved amino acids is found not only in the $\gamma_1$34.5 proteins from HSV-1 and HSV-2 but also in GADD34 from mouse, hamster, and human (Fig. 1). While the roles of these amino acids remain to be elucidated, their involvement in the effector function of $\gamma_1$34.5 is intriguing. It is conceivable that they play similar roles in GADD34 under conditions of DNA damage, growth arrest, and differentiation (25, 30, 31, 41).

Of particular interest is the defective mutant GF2022 with a deletion of 10 amino acids from 253 to 263. This region contains a copy of the AlaArg motif (Fig. 1). Because amino acids 258 to 263 are dispensable (Fig. 5A and 6A), it is most likely that a deletion in AlaArg motif accounted for loss of the function. Although the exact role of AlaArg remains unknown, gel filtration analysis suggests that deletions in the AlaArg motif disrupted the formation of the active $\gamma_1$34.5-PP1 complex (Fig. 8B) and this is responsible for the failure of eIF2α phosphatase to become activated. These data do not eliminate the possibility that the AlaArg repeat may serve as a structural element in the $\gamma_1$34.5 protein. However, the fact that deletions in this motif did not affect the ability of $\gamma_1$34.5 to bind PP1 (Fig. 7) suggests that overall protein structure may not have been changed. It is possible that the AlaArg motif is involved in oligomerization of the $\gamma_1$34.5 protein and PP1. Alternatively, the AlaArg motif may be required for interaction with otherwise unknown components present in the $\gamma_1$34.5-PP1 complex. Work is in progress to address these possibilities.

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