Characterization of the triplet repeats in the central domain of the γ134·5 protein of herpes simplex virus 1

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The γ134·5 protein of herpes simplex virus 1 (HSV-1) consists of an amino-terminal domain, a central domain with triplet repeats (Ala–Thr–Pro) and a carboxyl-terminal domain. The triplet repeats are a unique feature of the γ134·5 protein encoded by HSV-1, but the number of repeats varies among different strains. Notably, the central domain containing the triplet repeats is implicated in neuroinvasion. In this report, it has been shown that partial or full deletion of triplet repeats, i.e. from ten to either three or zero, in the γ134·5 protein has no effect on the virus response to interferon. The triplet deletion mutants replicate efficiently in CV-1 and mouse 10T1/2 cells. However, in mouse 3T6 cells, these mutants grow with delayed growth kinetics. This decrease in growth, compared with wild-type HSV-1(F), does not result from failure of the virus to suppress the RNA-dependent protein kinase response, but rather from a delay in virus release or egress. Accordingly, these mutant viruses are predominantly present within infected cells. These results indicate that deletions in the central domain of the γ134·5 protein impair virus egress, but not virus response to interferon.

INTRODUCTION

The γ134·5 protein of herpes simplex virus 1 (HSV-1; also known as Human herpesvirus 1) plays a pivotal role in virus virulence (Chou et al., 1990; MacLean et al., 1991; Whitley et al., 1993). Mutants that fail to express the γ134·5 protein are incapable of multiplying in the brain and cannot cause encephalitis in experimental animal models (Chou et al., 1990; Whitley et al., 1993). In HSV-infected cells, the γ134·5 protein inhibits the shut off of protein synthesis mediated by double-stranded RNA-dependent protein kinase (PKR) (Chou & Roizman, 1992; He et al., 1997a). In this process, the γ134·5 protein recruits protein phosphatase 1 (PP1), forming a high-molecular-mass complex that dephosphorylates eIF-2α and thereby prevents translation arrest (He et al., 1997b, 1998). This activity is linked to HSV resistance to interferon (Cheng et al., 2001a, b). Importantly, γ134·5 null mutants display a virulent phenotype in PKR knockout mice, but not in wild-type mice (Chou et al., 1990; Leib et al., 2000). Studies show that the γ134·5 protein is also involved in virus egress or release (Brown et al., 1994b). In mouse 3T6 cells, the γ134·5 mutants are defective in nuclear as well as cytoplasmic egress (Jing et al., 2004). Interestingly, the γ134·5 protein blocks surface expression of MHC class II molecules in virus-infected cells, which is believed to impair the functions of CD4+ T cells (Trgovcich et al., 2002). Recently, it has been reported that the γ134·5 protein interferes with autophagy (Tallóczy et al., 2002).

The γ134·5 protein of HSV-1 consists of a large amino-terminal domain, a linker region of triplet repeats (Ala–Thr–Pro) and a carboxyl-terminal domain (Chou & Roizman, 1990). The amino-terminal domain of the protein has 150 residues that facilitate virus egress, although the underlying mechanism remains unknown (Jing et al., 2004). The carboxyl-terminal domain functions to prevent the PKR response in virus infection (Cheng et al., 2001a; Chou & Roizman, 1994; He et al., 1996). This portion of the protein is similar to the corresponding domain of the cellular protein GADD34 expressed under conditions of DNA damage, growth arrest, differentiation and apoptosis (Hollander et al., 1997; Lord et al., 1990; Zhan et al., 1994). The linker region containing the triplet repeats is unique to the γ134·5 protein encoded by HSV-1, but the number of repeats varies from strain to strain (Bower et al., 1999; Chou & Roizman, 1990; Perng et al., 2002). Experiments suggest that the triplet repeats determine virus invasion of the central nervous system from the peripheral tissue (Bower et al., 1999; Mao & Rosenthal, 2003; Perng et al., 2002). These observations are consistent with the notion that variation in the triplet repeats serves to regulate the functions of the γ134·5 protein. However, the triplet repeats are not present in the γ134·5 protein encoded by HSV-2 (McGeoch et al., 1991).

Recent studies have demonstrated that variation in the triplet repeats seems to affect distribution of the γ134·5 protein...
The precise role of the triplet repeats in HSV infection is not well understood. In the present study, the triplet repeats in the \( \gamma_134 \)-5 protein encoded by HSV-1(F) in virus-infected cells were further examined. We show that deletions of the triplet repeats have no effect on the virus response to interferon involving PKR. When the number of triplet repeats is reduced to three or zero, virus egress is delayed or impaired compared with wild-type virus in mouse 3T6 cells. These results suggest that deletion of the triplet repeats in the \( \gamma_134 \)-5 protein of HSV-1 modulates virus egress, but not virus response to interferon.

**METHODS**

**Cells and viruses.** Vero, 143tk\(^{−}\), CV-1 and mouse embryo fibroblast (MEF) 3T6 cell lines were obtained from the ATCC and propagated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5 % (Vero) or 10 % (143tk\(^{−}\), CV-1 and MEF 3T6) fetal bovine serum. HSV-1(F) is a prototype HSV-1 strain used in these studies (Ejercito et al., 1998). In recombinant virus R3616, a 1 kb fragment from the coding region of the \( \gamma_134 \)-5 gene was deleted (Chou et al., 1990). To construct recombinant viruses JL0199R and JL0257R, plasmids pJL0106 and pJL0207 were transformed into an *Escherichia coli* RR1 strain that harbour wild-type HSV-BAC (bacterial artificial chromosome) as described previously (Cerveny et al., 2003). Positive clones were used to prepare HSV-BAC DNA with the Qiagen plasmid purification kit. Viral DNA was transfected into Vero cells using Lipofectamine reagent (Invitrogen). Virus was harvested and resuspended in ice-cold TE buffer (pH 7.8) containing NP-40 (0.5 %) and RNase A (50 \( \mu \)g ml\(^{−1}\)). The cytoplasmic fraction was collected and treated with proteinase K (0.5 mg ml\(^{−1}\)) for 30 min at 37 °C. Viral DNAs were prepared and subjected to restriction digests, electrophoretic separation in agarose gels, transfer to nitrocellulose membranes and hybridization with \(^{32}\)P-labelled DNA fragments as described previously (Chou & Roizman, 1994).

**Virus growth assay.** Monolayers of mouse 10T1/2, CV-1 or 3T6 cells were infected with viruses either at 0.01 or 10 p.f.u. per cell. After adsorption for 2 h, the monolayers were overlaid with DMEM and incubated at 37 °C. At 24, 48 and 72 h post-infection, samples were harvested and viruses, released by three cycles of freezing and thawing, were titrated on Vero cells.

**Immunoblotting.** Virus-infected cells were washed, harvested and solubilized in disruption buffer containing 50 mM Tris/HCl (pH 7.0), 5 % 2-mercaptoethanol, 2 % SDS and 2.5 % sucrose. Samples were then sonicated, boiled, subjected to electrophoresis on denaturing 12 % polyacrylamide gels, transferred to nitrocellulose membranes, blocked with 5 % non-fat milk and reacted with a selected primary antibody. The membranes were rinsed in PBS and reacted with donkey anti-rabbit immunoglobulin conjugated to horseradish peroxidase. Protein bands were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

**Interferon assay.** Monolayers of Vero cells grown to 80 % confluence were either untreated or pretreated with human leukocyte alpha interferon (IFN-α) (1000 U ml\(^{−1}\), Sigma) for 20 h. Cells were then infected with viruses at 0.05 p.f.u. per cell and incubated at 37 °C. At 48 h after infection, cells were harvested and virus yields were determined on Vero cells.

**Electron microscopy analysis.** Monolayers of MEF 3T6 cells were infected with viruses at 0.5 p.f.u. per cell in 35 mm dishes. At 24 h post-infection, samples were fixed in 4 % glutaraldehyde with 100 mM phosphate buffer (pH 6.8–7.2), fixed in 1 % osmium tetroxide in phosphate buffer, dehydrated in a series of ethanol concentrations (50, 70, 85, 95 and 100 % ethanol) and embedded in LX112 resin (Ladd Research Industries). Samples were removed from the Petri dishes and remounted on aluminium stubs. Ultrathin sections were cut with a Leica Urtacut UCT, placed on 200-mesh grids and contrast stained with uranyl acetate and lead citrate.
RESULTS

Construction of recombinant viruses with deletions in the triplet repeats (Ala–Thr–Pro) of the γ134-5 protein

As an initial step, two deletion mutants were constructed. In JL0109R, there was a complete deletion of ten triplet repeats in the γ134-5 protein. In JL0257R, only three triplet repeats were retained in the γ134-5 protein (Fig. 1a). The objective was to assess how triplet repeats affect virus replication during HSV infection. Recombinant viruses were constructed using the BAC system as described in Methods. The BAC plasmid inserted in the tk gene was removed by co-transfection of viral DNA and a plasmid containing the tk gene into Vero cells. To verify the virus constructs, Southern blot analysis was carried out after the tk gene into Vero cells. To verify the virus constructs, Southern blot analysis was carried out after digestion of viral DNAs (Fig. 1b). As expected, HSV-1(F), R3616, JL0109R and JL0257R yielded a 3 kb Dra III digestion of viral DNAs (Fig. 1b). As expected, HSV-1(F), R3616, JL0109R and JL0257R yielded a 3 kb Dra III fragment containing the tk gene (Fig. 1b, upper panel, lanes 2–5). In addition, HSV-1(F) yielded a 526 bp BstEII–DraIII fragment representing the wild-type γ134-5 gene. Due to deletions of the triplet repeats, JL0109R yielded a 436 bp BstEII–DraIII fragment and JL0257R yielded a 463 bp BstEII–DraIII fragment (Fig. 1b, lower panel, lanes 3 and 4, respectively). To examine protein expression, Western blot analysis was performed using anti-γ134-5 antibody and anti-Us11 antibody. The results in Fig. 1(c) show that, in virus-infected cells, HSV-1(F), JL0109R and JL0257R expressed γ134-5 proteins with different molecular masses (upper panel, lanes 2, 4 and 5), whereas the γ134-5 protein was not detected in cells infected with R3616, which lacks the γ134-5 gene (upper panel, lane 3). Notably, the γ134-5 variants were produced in the same abundance as the wild-type γ134-5 protein. Furthermore, comparable levels of Us11 were expressed in all virus-infected cells (Fig. 1c, lower panel, lanes 2–5).

Deletions in the triplet repeats of the γ134-5 gene do not affect virus response to IFN-α

To explore the role of the triplet repeats in virus response to interferon, virus growth was measured in Vero cells. In this experiment, monolayers of Vero cells were untreated or pretreated with IFN-α (1000 U ml⁻¹) to induce the antiviral state. Cells were then infected with the indicated viruses and virus yields were determined 48 h after infection. As seen in Fig. 2, in the absence of interferon, HSV-1(F) and R3616 reached titres of 4 × 10⁶ and 2.6 × 10⁶ p.f.u. ml⁻¹, respectively. Similarly, JL0109R and JL0257R replicated to titres of 2.0 × 10⁶ and 4.9 × 10⁵ p.f.u. ml⁻¹, respectively.

![Fig. 1.](image-url)
Fig. 2. Virus response to IFN-α. Monolayers of Vero cells were either untreated or pretreated with human leukocyte IFN-α (1000 U ml⁻¹; Sigma) for 20 h. Cells were then infected with viruses at 0.05 p.f.u. per cell and incubated at 37 °C. At 48 h post-infection, cells were harvested and virus yields were determined on Vero cells. Data are means from three independent experiments, with standard deviation indicated. Open bars, not IFN-α-pretreated; shaded bars, IFN-α-pretreated.

When cells were pretreated with interferon, replication of HSV-1(F) decreased slightly (4-fold), with a titre of 8.2 × 10⁷ p.f.u. ml⁻¹. Because of the deletion of the γ₁34·5 gene, replication of R3616 decreased dramatically to a titre of 2.5 × 10⁴ p.f.u. ml⁻¹, exhibiting an interferon-sensitive phenotype. Under these conditions, JL0109R and JL0257R still replicated efficiently, reaching titres of 5.13 × 10⁷ and 2.5 × 10⁷ p.f.u. ml⁻¹, respectively. Thus, like wild-type HSV-1(F), recombinant viruses with deletions in the triplet repeats are capable of blocking the antiviral action of IFN-α. It is concluded from this experiment that the triplet repeats in the γ₁34·5 protein are not required to confer virus resistance to interferon.

Viruses with deletions in the triplet repeats of the γ₁34·5 gene exhibit differential growth properties in mammalian cell lines

Virus growth properties were further evaluated in CV-1 cells and mouse 10T1/2 cells. These cell lines are restrictive to the γ₁34·5 null mutant due to the shutoff of protein synthesis triggered by viral DNA synthesis. In this set of experiments, monolayers of cells were infected with viruses and virus yields were measured at different time points after infection. The results in Fig. 3(a) show that, in CV-1 cells, HSV-1(F) replicated to a titre of 2.0 × 10⁶ p.f.u. ml⁻¹ 24 h after infection. This virus maintained efficient growth, reaching a titre of 2.9 × 10⁷ p.f.u. ml⁻¹ at 72 h after infection. As expected, R3616 replicated poorly, with titres of 1.32 × 10³ and 2.6 × 10⁴ p.f.u. ml⁻¹ at 24 and 72 h, respectively. There was an approximately 1000-fold decrease in virus yield for R3616. Over the same growth period, JL0109R replicated as efficiently as wild-type HSV-1(F), with titres reaching 1.7 × 10⁶ and 2.8 × 10⁵ p.f.u. ml⁻¹ at 24 and 72 h, respectively. JL0257R displayed a similar growth pattern to JL0109R. A virtually identical situation was also observed for these viruses in mouse 10T1/2 cells (Fig. 3b). Therefore, the growth properties of the recombinant viruses JL0109R and JL0257R are indistinguishable from those of wild-type HSV-1 (F) in these cell lines.

Virus growth patterns were then assessed in MEF 3T6 cells, in which the γ₁34·5 null mutants exhibit growth defects (Brown et al., 1994a, b). Monolayers of cells were infected with the indicated viruses and virus yields were then determined at 6, 12, 24, 48 and 72 h post-infection. Fig. 4 shows that wild-type HSV-1(F) replicated to a titre of 2.02 × 10⁴ p.f.u. ml⁻¹ at 12 h and grew to 1.23 × 10⁵ p.f.u. ml⁻¹ at 24 h post-infection. It continued to maintain efficient replication throughout infection, with a titre of 4.13 × 10⁷ p.f.u. ml⁻¹ at 72 h after infection. In contrast, R3616 replicated poorly, with a titre of 2.6 × 10⁵ p.f.u. ml⁻¹ at 24 h post-infection, which increased to a titre of 1.7 × 10⁴ p.f.u. ml⁻¹ at 72 h post-infection.
Notably, JL0109R replicated to a titre of only $1.57 \times 10^6$ at 12 h. Although the titre of JL0109R increased to $3.74 \times 10^5$ p.f.u. ml$^{-1}$ at 24 h, virus yield was still 33-fold less than that of HSV-1(F). Similarly, JL0257R replicated to a titre of $3.33 \times 10^5$ p.f.u. ml$^{-1}$ at 12 h, with a titre of only $7.18 \times 10^4$ p.f.u. ml$^{-1}$ at 24 h. There was 171-fold decrease in virus replication for JL0257R compared with the wild-type. Interestingly, this reduction was not fully restored until 72 h after infection. Hence, alterations in the triplet repeat in the $\gamma_134$ protein delayed virus replication in MEF 3T6 cells. These results indicated that reduction of the triplet repeats from ten to three or zero impairs virus replication in MEF 3T6 cells.

To address whether reduced virus replication in MEF 3T6 cells resulted from failure to counteract the PKR response, viral protein production was examined by Western blot analysis using anti-HSV antibodies. As indicated in Fig. 5(a), a high level of viral protein was detected in cells infected with HSV-1(F), JL0109R or JL0257R. In sharp contrast, little or no viral protein was detected in MEF 3T6 cells that were either mock-infected or infected with R3616. In parallel experiments, eIF-2$\alpha$ was analysed. Results in Fig. 5(b) show that eIF-2$\alpha$ was expressed in both mock-infected cells and cells infected with viruses. Phosphorylated eIF-2$\alpha$ was not detected in mock-infected cells or cells infected with HSV-1(F). However, a significant amount of phosphorylated eIF-2$\alpha$ was seen in cells infected with R3616 (Fig. 5b). In cells infected with the triplet deletion mutants, little or no phosphorylated eIF-2$\alpha$ was detected. The triplet deletion mutants were capable of preventing the translation shutoff mediated by PKR in 3T6 cells. Thus, the growth defect associated with JL0109R and JL0257R largely derived from a defect(s) after viral protein translation.

**Virus release is decreased in MEF 3T6 cells infected with the triplet repeat deletion mutants**

Based on the above analysis, levels of cell-associated and cell-free viruses were measured. Data in Table 1 show that, in HSV-1(F)-infected MEF 3T6 cells, total virus yield was $8.93 \times 10^7$ p.f.u. ml$^{-1}$; 63 % of the virus was found in the cell body and 37 % was present in the medium. As expected, in cells infected with R3616, the overall virus yield was low, with a titre of $1.73 \times 10^4$ p.f.u. ml$^{-1}$. There was an approximately 1000-fold drop in virus production compared with HSV-1(F). Moreover, a larger fraction (80 %) was associated with cells and a smaller fraction (20 %) was detected in the medium. Virus release into the medium was reduced by 17 % compared with HSV-1(F). Interestingly, in cells infected with JL0109R, the total virus yield was $8.77 \times 10^6$ p.f.u. ml$^{-1}$, which was close to that for HSV-1(F). However, a large fraction of the virus (70 %) was associated with cells and a smaller portion (30 %) was released into the medium. There was a 7 % decrease in virus release compared with
HSV-1(F). In addition, in cells infected with JL0257R, only 21.5% of total virus was released into the medium, which is around a 15% drop compared with HSV-1(F). Therefore, despite the quantitative difference, virus release was less efficient in cells infected with R3616, JL0109R and JL0257R. However, overall virus production for JL0109R and JL0257R was about 79- and 504-fold greater than that for R3616, respectively. This correlates with the ability of JL0109R and JL0257R to overcome the PKR response. Notably, these phenotypes were not apparent in CV-1 cells, where the relative distribution of cell-associated and cell-free viruses was similar for HSV-1(F), JL0109R and JL0257R.

The triplet repeats in the γ34.5 protein are required for efficient virus egress in MEF 3T6 cells

Localization of viruses in MEF 3T6 cells was further analysed. Cells were infected with viruses and processed for electron microscopic analysis 24 h after infection. As expected, in cells infected with HSV-1(F), virus particles were evident not only in the cytoplasm, but also in the extracellular space. A large number of vesicles containing virus particles was observed in the cytoplasm (Fig. 6a). In cells infected with R3616, there was a drastic decrease in overall virus particle production. In addition, virus particles were seen predominantly in the nucleus and perinuclear region. Vesicles containing virions in the cytoplasm were also absent under this condition (Fig. 6b). In cells infected with JL0109R and JL0257R, significant numbers of virus capsids and particles were present in infected cells. However, distribution of the virus particles and cell nucleus morphology were similar to those observed in R3616-infected cells (Fig. 6c, d). Large numbers of virus particles were in areas close to or associated with the outer nuclear membrane. Relatively low levels were seen in the nucleus or on the cell surface. It seems that JL0109R and JL0257R are capable of budding into the cytoplasm from the nucleus, but are defective in reaching the cell surface.

To quantify the observed differences, subcellular distribution of virions was counted in 15–20 cells for each virus. As summarized in Table 2, in the absence of the γ34.5 gene, a large fraction (30%) of virus particles was trapped in the nucleus and 61% were in the area around the nuclear membrane. In cells infected with JL0109R, around 73% of virus particles accumulated in the outer nuclear membrane region, whereas 14% were on the cell surface. Furthermore, in cells infected with JL0257R, roughly only 5.5% of virus particles were on the cell surface and 88% accumulated in the outer nuclear membrane region. JL0257R was more defective in cytoplasmic egress than JL0109R. These results correlate with a delay in virus growth kinetics and a decrease in release. Collectively, these data suggest that the triplet repeats of the γ34.5 protein play an important role in facilitating virus cytoplasmic egress.

DISCUSSION

Several lines of evidence have indicated that the γ34.5 protein is a multifunctional protein, which consists of an amino-terminal domain, a central domain with triplet repeats (Ala–Thr–Pro) and a carboxyl-terminal domain (Bower et al., 1997, 1999; Cheng et al., 2003; Chou & Roizman, 1990, 1992; Jing et al., 2004; Tallozcy et al., 2002; Trgovcich et al., 2002). The carboxyl-terminal domain acts to prevent the interferon response, involving PKR (Cheng et al., 2001a; Chou & Roizman, 1994). It has recently been found that, in MEF 3T6 cells, the amino-terminal domain is required for virus egress (Jing et al., 2004). The central domain in the γ34.5 protein encoded by HSV-1 is implicated in neuroinvasion (Bower et al., 1999; Mao & Rosenthal, 2003; Perng et al., 2002). To extend these studies, the triplet repeats in the central domain of the γ34.5 protein encoded by HSV-1(F) were further examined. These data demonstrate that the triplet repeats in the central domain of the γ34.5 protein facilitate virus egress but not interferon response in infected cells.

Previous studies have demonstrated that the γ34.5 protein inhibits the interferon response through the carboxyl- but not the amino-terminal domains (Cheng et al., 2001a; Chou & Roizman, 1994). The role of the triplet repeats has not been investigated. The data presented in this study show that, when the triplet repeats were reduced to three or zero, the mutant viruses were still resistant to interferon, like...
wild-type virus. These mutants replicated efficiently in CV-1 and mouse 10T1/2 cells, which are non-permissive to the $\gamma_{134.5}$ null mutants due to the PKR response. Furthermore, these triplet deletion mutants were capable of blocking the shutoff of protein synthesis and virus-induced eIF-2α phosphorylation was inhibited or reduced. As the $\gamma_{134.5}$ protein recruits PP1 to form a high-molecular-mass complex that dephosphorylates eIF-2α (Cerveny et al., 2003; He et al., 1997b), these results suggest that the triplet repeats are not essential for the functional interaction between the $\gamma_{134.5}$ protein, PP1 and eIF-2α in HSV-infected cells. This is consistent with the model that the carboxyl terminus of the $\gamma_{134.5}$ protein is a functional module that dictates virus response to interferon.

Although they replicated efficiently in CV-1 and 10T1/2 cells, the triplet deletion mutants grew with delayed kinetics in MEF 3T6 cells and did not replicate as well as wild-type virus. The different phenotypes associated with these

### Table 2. Distribution of virions in MEF 3T6 cells

Virus particles present in the nucleus, perinuclear region and cytoplasm and on the cell surface were counted in electron micrographs of at least 15–20 randomly sampled MEF 3T6 cells infected with the indicated viruses. The mean numbers of virus particles per cellular compartment are shown and the numbers in parentheses denote percentages of virus particles in the different sections of a cell.

<table>
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<tr>
<th>Virus</th>
<th>Mean no. of particles per cell (%)</th>
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<tbody>
<tr>
<td></td>
<td>Nucleus</td>
</tr>
<tr>
<td>HSV-1(F)</td>
<td>48 (21%)</td>
</tr>
<tr>
<td>R3616</td>
<td>16 (30%)</td>
</tr>
<tr>
<td>JL0109R</td>
<td>18 (12.5%)</td>
</tr>
<tr>
<td>JL0257R</td>
<td>10 (6%)</td>
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![Cellular distribution of viruses in MEF 3T6 cells.](image)
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