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The IR4 auxiliary regulatory protein expands the *in vitro* host range of equine herpesvirus 1 and is essential for pathogenesis in the murine model

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Introduction

Six viral proteins play major roles in the regulation of EHV-1 gene expression: the sole immediate-early protein (IEP), four early proteins (IR2P, EICP0P, UL5P [EICP27], and IR4 [EICP22]), and the late tegument protein, α-*trans*-inducing factor (ETIF) (O'Callaghan and Osterrieder, 2008). The 1,487aa IEP is the major regulatory protein of EHV-1 and serves a bifunctional regulatory role by: i) independently *trans*-activating EHV-1 early promoters (Caughman et al., 1985; Harty et al., 1990; Smith et al., 1992, 1994); and ii) *trans*-repressing its own promoter (Harty and O'Callaghan, 1991; Smith et al., 1994) and some late promoters (Kim et al., 1999). The *IE* gene is further regulated by the IR2 protein, an amino-terminal truncated (323-1487aa) version of the IEP encoded within the *IE* gene (Harty and O'Callaghan, 1991; Caughman et al., 1995; Kim et al., 2006) and by the *IR3* RNA that is antisense to the *IE* transcript (Holden et al., 1992a; Ahn et al., 2007) The IEP also functions in concert with EHV-1 early regulatory proteins IR4 (Kim et al., 1997; Derbigny et al., 2000, 2002) and UL5P (Smith et al., 1993; Zhao et al., 1995; Albrecht et al., 2004, 2005) as well as with cellular transcription factors TATA box-binding protein (TBP) and TFIIB (Jang et al., 2001; Albrecht et al., 2003; Kim et al., 2003; Kim and O'Callaghan, unpublished results) and the nucleolar shuttle protein, EAP (Kim et al., 2001). Several IEP functional domains have been characterized, including the *trans*-activation domain (Smith et al., 1994; Buczynski et al., 1999), a site-specific DNA binding domain (Kim et al., 1995), a nuclear localization signal (NLS; Smith et al., 1995), a serine-rich tract (SRT; Kim et al., 2001), and a TFIIB-binding domain (Jang et al., 2001). Characterization of 17 EHV-1 *IE* mutants revealed that these functional domains are essential for virus replication (Buczynski et al., 2005).

IR4 is an early 293aa protein that in concert with the IE or the EICP0 protein significantly enhances the *trans*-activation of early viral promoters (Holden et al., 1992b, 1994, 1995; Kim

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et al., 1997). While not a DNA binding protein, IR4 greatly enhances the DNA-binding activity of the IEP, and is able to restore the DNA-binding ability of some IEP mutants (Kim et al., 1995). Additionally, IR4 interacts with itself to form dimers and higher-ordered complexes (Derbigny et al., 2000). The exact mechanisms by which IR4 regulates viral gene expression, however, remain poorly defined.

EHV-1 IR4 is unique in that it functions as a *trans*-activator during lytic infection yet may *trans*-repress EHV-1 genes during persistent infection mediated by defective interfering particles (DIP) (Ebner and O'Callaghan, 2006, 2008). The recombination events that generate DIP genomes produce one of two unique hybrid genes consisting of 5' sequences of *IR4* fused to 3' sequences of *UL5* (Baumann et al., 1987; Yalamanchili et al., 1990; Chen et al., 1996, 1999; Ebner and O'Callaghan, 2006, 2008). Both hybrid proteins (HYB1.0 and HYB2.0) are produced in large quantities during EHV-1 persistent infection (Chen et al., 1996, Ebner and O'Callaghan, 2006), and previous studies demonstrated that the HYB1.0 protein downregulates EHV-1 gene expression (Chen et al., 1999). Recent work revealed that IR4 residues within the HYB proteins are necessary for interference with standard virus replication, a hallmark of persistent infection (Ebner and O'Callaghan, 2008).

To examine the role of the IR4 protein during lytic and persistent infections, bacterial artificial chromosome (BAC) technology was used to delete both copies of the *IR4* gene from the highly pathogenic RacL11 strain of EHV-1. The resulting *IR4*-null virus (RacL11Δ*IR4*) was characterized with regard to replication in diverse cell cultures, pathogenesis in the murine model, and alterations in the replication cycle of EHV-1 at the levels of protein production and DNA replication. Overall, the results reveal that IR4 is essential for EHV-1 replication in the mouse as well as most cell types and for efficient replication in equine NBL-6 cells that allow limited growth of the *IR4*-null virus.

Results

Construction of an EHV-1 *IR4* deletion mutant

Our previous studies revealed that IR4 is an auxiliary regulatory protein that physically interacts with the IE protein and enhances its ability to *trans*-activate EHV-1 promoters (Kim et al., 1995, 1997). We therefore hypothesized that the IR4 protein plays an essential role for viral replication. To address this hypothesis, the *IR4* genes contained in the terminal and internal repeats of the two isomeric EHV-1 genome were replaced with DNA cassettes harboring kanamycin (*KAN*) and zeocin (*ZEO*) antibiotic resistance genes using bacterial artificial chromosome techniques (Rudolph et al. 2002). Comparison of the *Bam*HI restriction enzyme digestion pattern of the RacL11 BAC to those of the single *IR4* knockout and the double *IR4* knockout mutant BAC revealed the appearance of a high molecular weight band and the loss of a lower molecular weight band in the *IR4* mutants, observations consistent with the predicted genomic changes resulting from *IR4* deletion as noted in Figure 1A. Southern blot analysis of the parent BAC genome and the single and double *IR4* knockout mutant BAC genomes indicated that a single copy of the *IR4* gene was successfully replaced by the *KAN* gene (Fig. 1B and C) and that the second copy of the *IR4* gene was successfully replaced by the *ZEO* gene. This was demonstrated by a failure of the *IR4* probe to hybridize to pRacL11Δ*IR4*(2x) DNA (Fig. 1B) and the appearance of bands of the expected size in samples incubated with *KAN* (Fig. 1C) and *ZEO* (Fig. 1D) specific probes. The *EUs4* gene, which encodes glycoprotein 2, was deleted in the construction of the BAC and was restored to RacL11*ΔIR4ΔEUs4* and RacL11BD*ΔEUs4*, creating RacL11*ΔIR4* and RacL11BD, respectively. PCR analysis (Fig. 1F) and DNA sequencing of the EHV-1/antibiotic resistance cassette junctions (data not shown) confirmed the insertion sites of the antibiotic resistance genes and the restoration of *EUs4* to both RacL11*ΔIR4* and RacL11BD. Growth kinetics of RacL11BD and wt RacL11

were monitored over a 72h time course and found to be indistinguishable (data not shown). Virus genotypes are summarized in Table 1.

Determination of the replication of RacL11Δ*IR4* in equine cells

Transfection of equine fibroblast-like NBL-6 cells with the RacL11*ΔIR4ΔEUs4* construct resulted in the production of viable virus. Western blot analysis performed using extracts of cells infected with RacL11*ΔIR4ΔEUs4* demonstrated that the IR4 protein was not produced by the *IR4*-null virus (Fig. 1E). Monolayers of NBL-6 cells were infected with RacL11*ΔIR4*, RacL11BD, or RacL11 and incubated to allow for plaque formation. Plaque morphology (Fig. 2A) and plaque size (Fig. 2B) were not altered in the absence of *IR4* in this equine cell type. Growth kinetics were analyzed by infecting NBL-6 cells with RacL11BD and RacL11*ΔIR4*, and virus production was measured at 3, 6, 12, 18, 24, 36, 48, and 72h post-infection. The replication of the *IR4* deleted virus was delayed and its titers were consistently one log or more below those of RacL11BD in the NBL-6 cells (Fig. 3). Interestingly, studies with equine ETCC cells revealed that this tumor cell line failed to support the replication of RacL11Δ*IR4*, whereas the parent virus replicated to high titers (Table 2).

EHV-1 RacL11*ΔIR4* is non-pathogenic in the CBA mouse

The effects of the deletion of *IR4* on EHV-1 pathogenesis were assessed by infecting groups of ten CBA mice with sterile medium, RacL11*ΔIR4*, or RacL11BD, and the percentage of body weight lost or gained in comparison to weight at the time of infection was determined at 24, 48, 72, and 96 hours post-infection. No statistical difference was observed between the mock group and the mice infected with RacL11*ΔIR4*, both of which gained close to 10% body weight by the termination of the experiment. Further, mice infected with the *IR4*-null virus failed to exhibit any clinical signs of infection. Consistent with our previous studies (Smith et al., 2005), mice infected with RacL11 derived from BAC DNA (RacL11BD) lost approximately one-fifth of their body weight by the termination of the experiment (Fig. 4). The *in vivo* replication capacity of the *IR4* deletion mutant was measured by quantifying the concentration of virus in lungs of infected mice. Two groups of 20 mice per group were infected intranasally with 5.0×10^5 pfu of RacL11*ΔIR4* or RacL11BD, and five mice from each group were sacrificed at 4, 48, 96, and 144h post-infection. Lungs were harvested from each mouse, and virus was quantitated by standard plaque assay on equine NBL-6 cells. RacL11*ΔIR4* was not recovered at any time point in any of the mice. In contrast, mice infected with RacL11BD produced an average of 4×10^6 pfu per lung at 48h post-infection and exhibited clinical signs of severe disease with mortality rates of 20%-40% (Table 3). Thus, the deletion of the *IR4* gene rendered EHV-1 non-pathogenic in the CBA mouse model of EHV-1 pathogenesis.

IR4 is essential for EHV-1 replication in non-equine cell types

EHV-1 can be propagated in a large number of cell types (O'Callaghan and Osterrieder, 2008). The finding that RacL11*ΔIR4* was not able to grow in either the mouse lung or in equine ETCC cells prompted the question as to whether the deletion of *IR4* prohibited EHV-1 replication in other cell types routinely used to cultivate this alphaherpesvirus. To address this question, assays examining the replication of RacL11BD and RacL11*ΔIR4* were performed in mammalian cell cultures representing a diversity of species, including mouse L-M, rabbit RK13, non-human primate Vero, and human HeLa cells. Whereas RacL11*ΔIR4* titers were lower than those that of RacL11BD in equine NBL-6 cells as shown above (Fig. 3), the *IR4* mutant virus failed to replicate in all other cell types tested (Table 2). Expression of *IR4* in RK13 cells by transient transfection resulted in the recovery of viable RacL11Δ*IR4* (Table 2), albeit at lower levels than that recovered from NBL-6 cells. These results indicated that replication of EHV-1 is severely restricted in the absence of IR4 in many cell types normally

permissive for EHV-1, and that ectopic expression of *IR4* in RK13 cells restores the ability of the *IR4*-null virus to replicate.

Synthesis of some essential viral proteins is altered or abrogated in the absence of IR4

To identify the nature of the block in the replication of the *IR4*-null virus, RK13 cells infected with RacL11BD and RacL11Δ*IR4* (MOI=1) were examined at various times post infection by western blot analysis for the production of the IE protein and representative early and late proteins. Production of the IE protein in cells infected with the *IR4*-null mutant was indistinguishable from that observed in cells infected with the parent virus (Fig. 5A). The early essential regulatory protein UL5P (Fig. 5A) and the EHV-1 major DNA binding protein (UL53P, data not shown) were likewise produced independently of the presence of IR4 both in terms of kinetics and total protein levels. In the absence of IR4, the synthesis of the early EICP0 protein, a potent transcriptional *trans*-activator (Bowles et al., 1997), was found to be initially delayed but by 16 hours post-infection reached levels comparable to those in cells infected with the parent virus (Fig 5A). Late viral protein production was examined using antibodies specific for glycoproteins D and K (gD, gK) and *ORF12*, the EHV-1 *trans*-inducing factor (ETIF). Production of the essential ETIF protein, which is synthesized at the initiation of viral DNA replication, was both delayed and reduced in the absence of IR4 (Fig. 5A). Production of gD (Fig. 5A) and gK (data not shown), which are both essential envelope proteins, was reduced to undetectable levels in the non-permissive cell line, whereas gD production in NBL-6 cells infected with RacL11Δ*IR4* was detected, albeit at reduced levels, after an initial delay (Fig. 5A and B). Examination of the production of the EICP0 protein in infected NBL-6 cells at 2 and 8 hours post infection revealed no difference between RacL11BD and RacL11Δ*IR4* (Fig. 5B).

Viral DNA synthesis is inhibited in RK13 cells in the absence of IR4

While analysis of non-permissive RK13 cells infected with RacL11Δ*IR4* revealed impaired synthesis of a number of important viral proteins, the ability of the mutant virus to replicate DNA remained in question. To address whether IR4 production is required for viral DNA synthesis, real-time PCR was performed on samples containing total DNA harvested from RK13 cells infected with either RacL11BD or RacL11Δ*IR4* at 2, 8, 12, 24, and 48 hours post-infection. The results were normalized to the cellular *gapdh* gene as an internal control and expressed as the relative change in viral genomic copy number after an initial 2 hour time point. Compared to that of RacL11BD, the replication of RacL11Δ*IR4* DNA was inhibited in excess of 99.9%, and the earliest detection of viral DNA replication was delayed by 16 hours compared to RacL11BD (Fig. 6A). Interestingly, there was a 50% reduction in RacL11Δ*IR4* DNA copies detected at 8 hours post-infection which may indicate degradation of the mutant viral genome following entry. While RacL11Δ*IR4* also exhibited a lag in the synthesis of viral DNA and, ultimately, viral DNA failed to reach levels comparable to RacL11BD in permissive NBL-6 cells, the inhibition of viral DNA synthesis was far less pronounced at late times (Fig 6B). Severe cytopathology of cells infected with RacL11BD made reliable analysis of RacL11BD DNA replication at the 48h time point impractical.

Discussion

IR4 homologues have regulatory roles in many alphaherpesviruses. The HSV-1 homologue, *ICP22*, is thought to direct transcription away from cellular promoters to viral promoters *via* hyper-phosphorylation of RNA polymerase II (Rice et al., 1994, 1995; Leopardi et al., 1997). Likewise, the ORF63/70 protein is thought to have a role in regulating varicella-zoster virus (VZV) promoters, but its function is less clear (Jackers et al., 1992; Kost et al., 1995). Discerning the role of the IR4 protein of EHV-1 based on characterized functions of its homologues may be problematic, however, as the similarity among the different proteins is, in

many cases, quite low. Indeed, ICP22 of HSV-1 is a 420aa immediate-early protein, whereas IR4 is 293aa and is an early regulatory protein. Additionally, *IR4* is diploid in EHV-1, while its counterpart is haploid in HSV-1.

In HSV-1, *ICP22* is not essential for virus growth in some cell types (Post and Roizman 1981; Poffenberger *et al.*, 1993, 1994, Orlando et al., 2006). The VZV genome is structured more closely to that of EHV-1, and in VZV one copy of the diploid *IR4* homologue ORF63/70 is required for virus replication (Sommer et al., 2001). EHV-1 deleted for both copies of *IR4* was able to replicate in non-immortalized equine cells, although its growth kinetics were significantly retarded in comparison to those of the parent virus. It should be noted that while the diploid nature of *IR4* in EHV-1 made the generation of full *IR4* revertants unfeasible, the analysis of both BAC DNA and the properties of the progeny viruses demonstrated that any unintended alterations of the EHV-1 genome were unlikely, in agreement with the demonstration that IR4 produced *in trans* restored RacL11Δ*IR4* replication in RK13 cells. Additionally, recent experiments succeeded in generating an EHV-1 mutant virus devoid of the entire internal repeat of the S region and indicated that one copy of *IR4* is sufficient to support viral replication in RK13 cells that are non-permissive for RacL11Δ*IR4* (Ahn, Breitenbach, Zhang, and O'Callaghan, unpublished results).

The findings that infection of mice with the *IR4*-null virus failed to cause clinical signs and to result in virus replication in the mouse lung were not unexpected given the observation that the *IR4*-null virus fails to replicate in murine cells in culture. The facts that the *IR4* mutant virus can be propagated in equine NBL-6 cells and is non pathogenic in the mouse model that mimics EHV-1 infection in the equine may be observations that could be exploited in future work to develop vaccines and gene delivery vehicles for the equine. In permissive NBL-6 cells, plaque morphology was indistinguishable in the absence of IR4, and average plaque size was not significantly different in the absence of IR4. It is likely that the production of late protein species, such as glycoprotein D, that mediate cell fusion and influence plaque morphology is sufficient by 72 hours post-infection in NBL-6 cells to yield similar plaque characteristics, even though overall virus production is reduced in this cell type.

A recent report (Orlando et al., 2006) showed that deletion of *ICP22* results in HSV-1 particles that contain greatly reduced quantities of the late proteins U_S11 and gC, produce increased amounts of ICP0 and ICP4, and exhibit biochemical and physical properties that are distinct from *wt* virus, resulting in a 500-fold decrease in infectivity. In the case of EHV-1, it remains to be determined whether non-infectious particles are produced in cells non-permissive for the *IR4*-null virus. However, in these cells, the IEP protein and early proteins UL5P and UL53P were produced in nearly identical amounts and with similar kinetics in the presence or absence of IR4, indicating that viral entry and the initiation of the viral replication cycle were not impaired. Also, the inhibition of the synthesis of ETIF, recently shown to be essential for EHV-1 secondary envelopment and virion maturation (von Einem et al., 2006), and the complete inhibition of synthesis of gD and gK, which are known to play essential roles in maturation, egress, and infectivity, argue that EHV-1 replication in the absence of IR4 is blocked at a stage prior to that of particle formation and release. Since EHV-1 DNA replication is impaired in excess of three logs in the absence of IR4 in non-permissive RK13 cells, future work will be required to determine whether IR4 plays a direct role in DNA replication and/or whether the IR4 protein is essential for transcribing early genes required for DNA synthesis

Materials and Methods

Virus and cells

Pathogenic EHV-1 strain RacL11 was propagated in equine NBL-6 cells. Mouse L-M, rabbit RK-13, equine NBL-6, equine ETCC (Allen and Bryans, 1974), primate Vero, and human

HeLa cells were propagated in Dulbecco's minimum essential medium (DMEM) supplemented with 5% or 10% fetal bovine serum (FBS), and selected cells were used in transfections and infections as indicated.

Plasmids

pGexEuS4 containing full length *EUs4* that encodes gp2 of EHV-1 (RacL11 strain) was generated by PCR amplification of *EUs4* and surrounding sequences with primers incorporating *Bam*HI and *Eco*RI restriction sites. pSVIR4 (formerly pSVEICP22) has been previously described. (Derbigny et al., 2000).

Generation and confirmation of *IR4*-null BAC

An EHV-1 RacL11 BAC (Rudolph et al., 2002) was deleted for a single copy of *IR4* by RED recombination as previously described (Yao et al., 2003). Briefly, a kanamycin (*KAN*) resistance marker generated by PCR using primers containing approximately 50bp of *IR4* flanking sequences in their 5' ends was electroporated into *E. coli* DY380 (containing the EHV-1 BAC) previously grown at 42°C to induce RED recombinases. Electroporated cultures were inoculated onto medium containing kanamycin (30μg/mL) to select for transformants. The second copy of *IR4* was deleted in the same manner employing a zeocin (*ZEO*) marker. Putative transformants were screened by: 1) PCR amplification of both EHV-1/antibiotic resistance marker junction sequences and *IR4* internal sequences; 2) Southern blot analysis using probes specific for *IR4* sequences, kanamycin (*KAN*) marker sequences, or zeocin (*ZEO*) marker sequences; and 3) sequencing of EHV-1/antibiotic resistance marker junctions.

Generation of *IR4* null virus

Purified BAC constructs were electroporated (Nucleofector, Amaxa Corp, Germany) into NBL-6 cells that were then incubated at 37°C for 5-7 days. Supernatant was transferred to fresh NBL-6 monolayers, and cells were observed for cytopathic effects. Green fluorescent plaques were selected and purified three times. *EUs4* revertant viruses were generated by co-electroporating BAC constructs with pGexEUs4. In the case of RacL11*ΔIR4*, cells transfected with the *EUs4* restoration plasmid were subsequently infected with the RacL11*ΔIR4ΔEUs4* virus. In both cases, non-fluorescing plaques were isolated and purified three times. DNA was isolated from each putative revertant and screened by PCR targeting full length *EUs4*.

Western blot analysis

RK13 or NBL-6 cells were seeded onto 60mm tissue culture dishes (BD Biosciences, Durham, NC) and infected the subsequent day at a confluency of 80-90% with either RacL11BD or RacL11Δ*IR4* viruses. Protein extracts of infected cells were separated by SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane (Bio-Rad LSG, Hercules, CA), blocked in 1% skim milk, rinsed in Tris-Buffered Saline (pH 7.4) with 0.5% Tween-20 (TBST), and incubated with the indicated primary antibodies at a concentration of 1:2500 to 1:10,000. The membranes were washed again and incubated with a 1:10,000 dilution of the secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse antiserum; Sigma, St Louis, MO). Protein-antibody complexes were visualized by incubating the membranes in AP color reagent (Bio-Rad LSG, Hercules, CA) per the manufacturer's instructions.

Growth kinetics and plaque morphology

NBL-6, RK-13, L-M, Vero, HeLa, ETCC, and pSVIR4 tranfected RK13 cells were seeded to 80% confluence in 60mm dishes and infected at an MOI=1. Cells and supernatants were harvested at the indicated times post-infection. Serial dilutions of each sample were used to inoculate fresh NBL-6 monolayers. Monolayers were incubated in medium containing 1.5%

methylcellulose, and plaques were quantitated after 3 days by fixing with 10% formalin and staining with 0.5% crystal violet.

Animal experiments

Groups (n=10) of three-week-old CBA mice were infected intranasally with 5×10^5 pfu of RacL11*ΔIR4* or RacL11BD, or were mock-infected with sterile medium. Mice were weighed prior to inoculation and every 24h post-inoculation for five days. Differences in weight loss or gain over time were measured by comparing individual mouse weight at each interval to weight prior to inoculation. Virus replication in mice was measured by infecting groups of 20 three-week-old CBA mice as described above. Five mice from each group were sacrificed at 4, 48, 96, and 144h post-infection, and lungs were harvested. Whole lungs were combined in diluent and homogenized, and the virus concentration in each sample was quantitated as previously described (Smith et al., 2005).

Real-time PCR analysis of viral DNA replication

RK13 cells were infected with RacL11Δ*IR4* or RacL11BD as described, and monolayers were harvested at 2, 8, 12, 24, and 48 hours post-infection. Cell pellets were resuspended in 500μL of DNAzol (MRC, Inc., Cincinnati, OH) and incubated at 100°C for five minutes to ensure release of DNA. The samples were then diluted by a factor of 100 and stored at 20°C until ready for use. Primers specific for *gapdh* (forward) 5’ - TGC CCC CAT GTT TGT GAT G - 3’ and (reverse) 5’ - TGT GGT CAT GAG CCC TTC C - 3’ and primers specific for EHV-1 *UL5* (forward) 5’ - CAA CTG GAA GCA GCA ACA GC - 3’ and (reverse) 5’ - GCG GTG AAC TCT GGC CAC GC - 3’ were utilized to amplify viral and cellular DNA using an IQ5 real-time PCR instrument and IQ SYBR Green real-time PCR supermix (Bio-Rad LSG, Hercules, CA). The relative change in genomic copy number was extrapolated by determining threshold detection cycle differences in viral DNA after two hours and normalized against the cellular *gapdh* gene as an internal control for total sample DNA.

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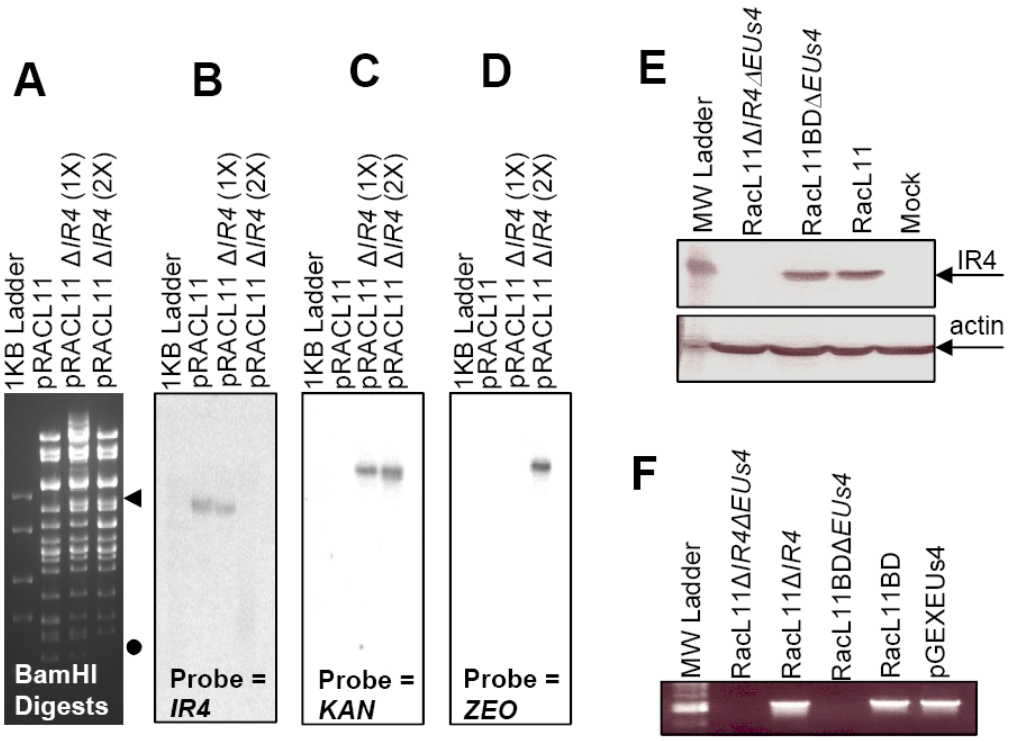

Fig. 1.

Confirmation of *IR4* deletion mutant BAC. Panel A: *Bam*HI restriction enzyme analysis of BAC DNA. ◀ = band added as result of *IR4* deletion; ● = band removed as result of *IR4* deletion. Panels B to D: Southern blot analyses of digested BAC DNAs. Panel E: western blot analysis of extracts of NBL-6 cells infected with EHV-1 *wt* (RacL11), EHV-1 RacL11 deleted of both the *IR4* and *EUs4* genes (RacL11Δ*IR4*Δ*EUs4*), or EHV-1 RacL11 deleted of *EUs4* (RacL11Δ*EUs4*). Panel F: PCR amplification using primers specific for *EUs4* of viral DNA lacking both *IR4* and *EUs4* genes (RacL11Δ*IR4*Δ*EUs4*), viral DNA lacking *IR4* with the *EUs4* gene restored (RacL11Δ*IR4*), viral DNA lacking *EUs4* gene (RacL11Δ*EUs4*), viral DNA with the *EUs4* gene restored (RacL11BD), and a control plasmid harboring the *EUs4* ORF (pGEX*EUs4*). Methods for restriction enzyme digestion, Southern blot analysis, and PCR protocols are described in the Materials and Methods.

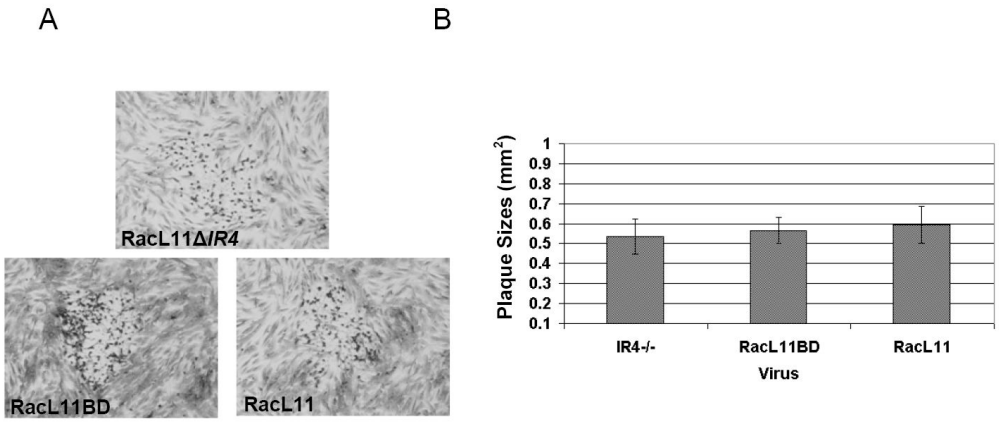

Fig. 2.

Plaques morphology and size of *wt* EHV-1 RacL11, BAC-derived EHV-1 deleted of the *IR4* gene with *EUs4* restored (RacL11Δ*IR4*), and BAC-derived EHV-1 RacL11 with the *EUs4* restored (RacL11BD). Panel A shows representative plaque morphology. Panel B shows plaque size. Bars represent means of 50 plaques of each virus; error bars represent standard deviation.

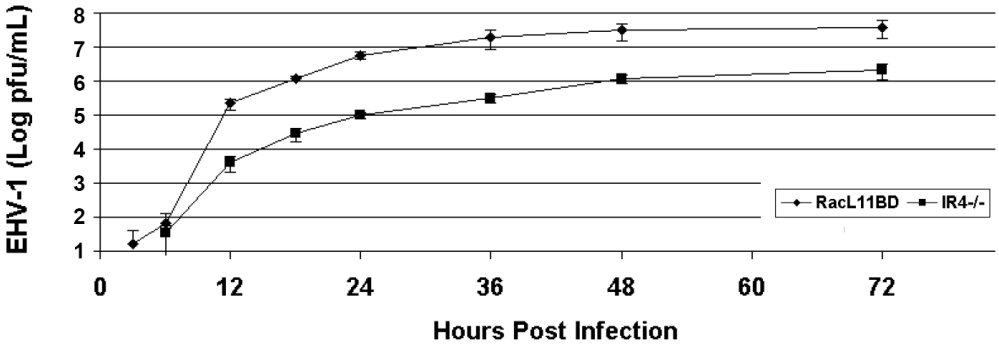

Fig. 3.

Growth kinetics of EHV-1 RacL11 derived from BAC DNA (RacL11BD) and RacL11 deleted of *IR4* (RacL11Δ*IR4*) in equine NBL-6 cells. Infected cells were harvested at 3, 6, 12, 18, 24, 36, and 72h post-infection, and virus was quantitated by standard plaque assay on NBL-6 cells.

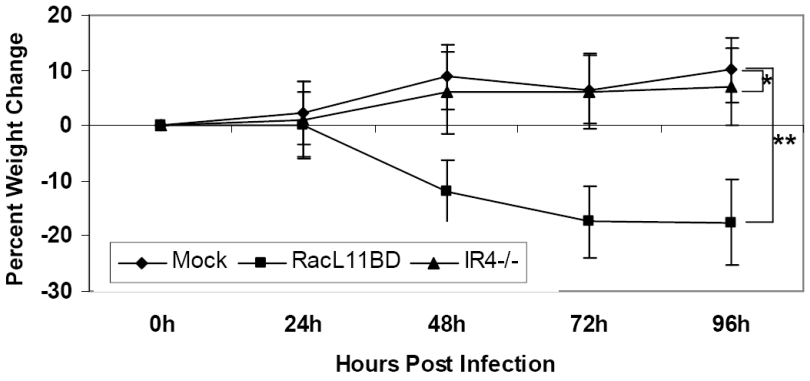

Fig. 4.

Weight loss or gain of CBA mice mock-infected or infected with EHV-1 RacL11BD or RacL11Δ*IR4* (IR4-/-). Mice were inoculated intranasally and weighed every 24h for 96h as described in the Materials and Methods. Data points represent means of ten mice; weight loss or gain was calculated in relation to the initial weight at the time of infection. Error bars represent standard deviation. (*) P = .44; (**) P < .001.

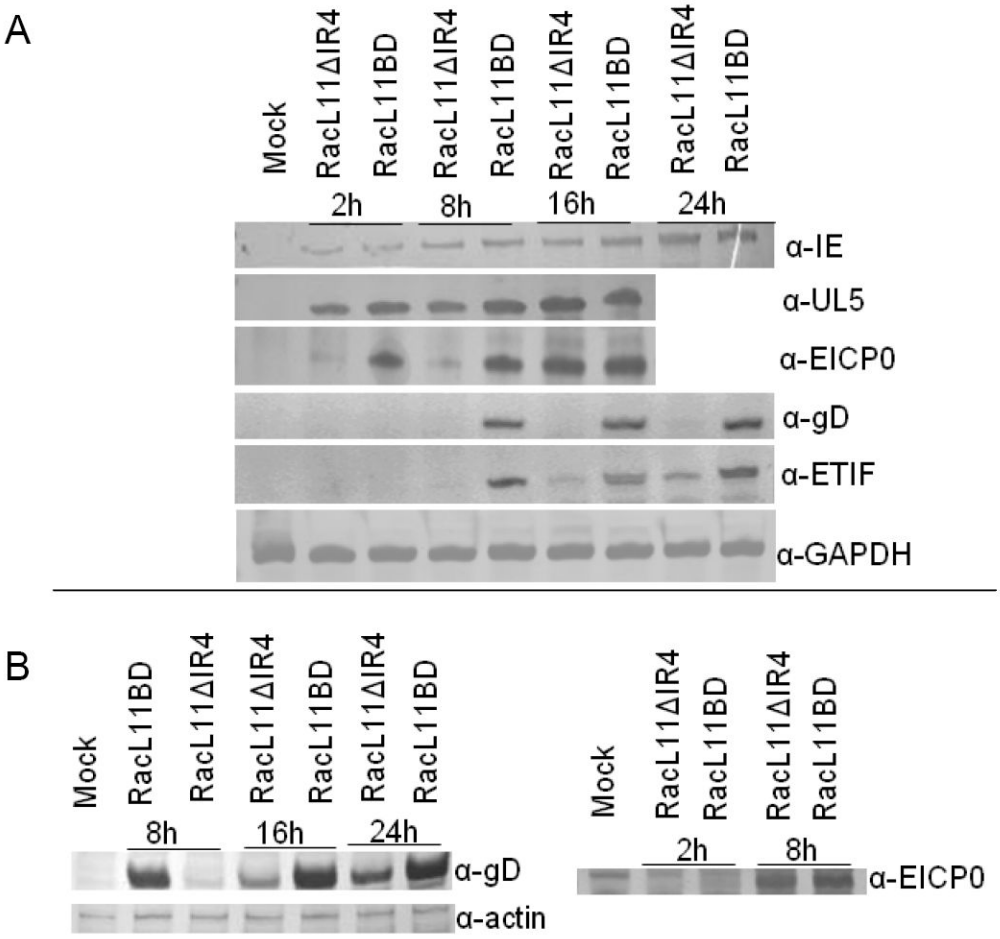

Fig. 5.

Western blot analysis of RK13 (A) and NBL-6 (B) cells infected with RacL11BD or RacL11Δ*IR4*. Infected cells (MOI=1) were harvested at indicated times and probed with antisera specific for the IE, UL5, EICP0, ETIF, and gD proteins. Rabbit gapdh or horse actin was detected as loading controls.

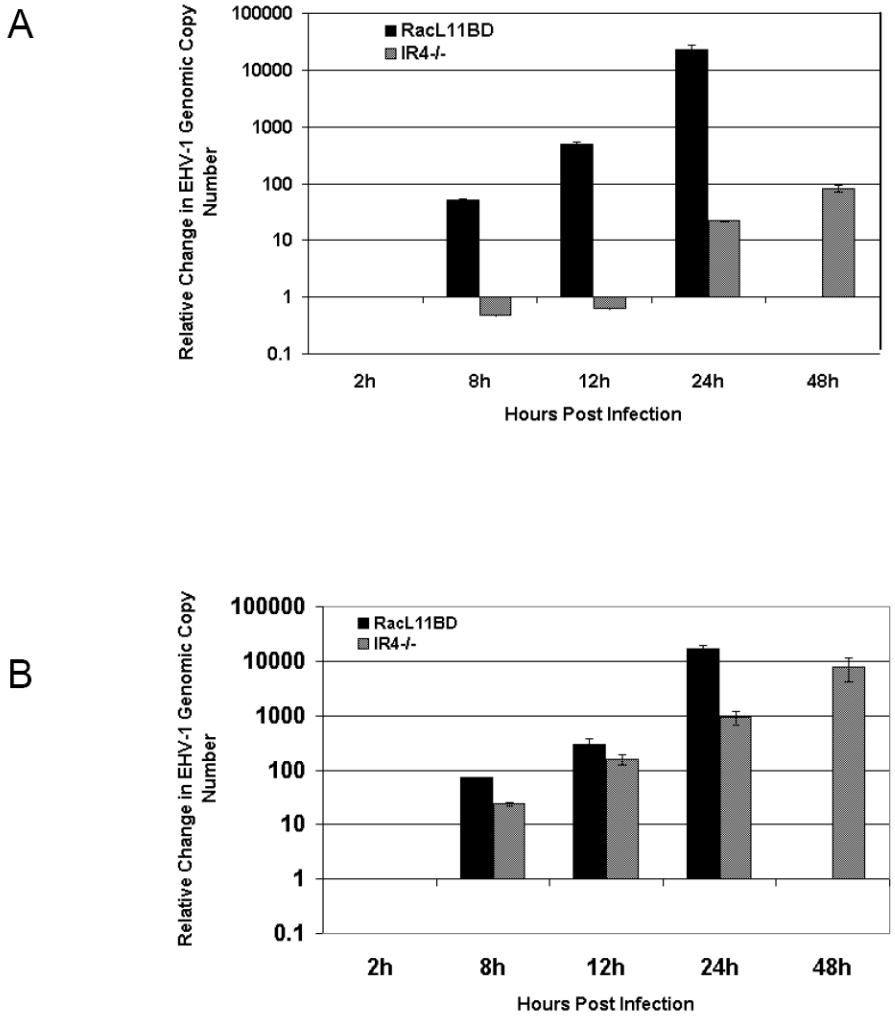

Fig. 6.

Comparison of viral DNA synthesis. RK13 (A) or NBL-6 (B) cells were infected with RacL11BD or RacL11Δ*IR4* and harvested at indicated times post-infection. Cell pellets were prepared as described in Materials and Methods, and the relative change in viral DNA copy number after two hours was measured using real-time PCR. Samples were tested in triplicate, and the results were normalized to total sample DNA. Extensive cytopathology made testing of RacL11BD infected cells at 48h impractical. Error bars indicate standard deviation.

Table 1

Virus Genotypes

Name	Genotype
RacL11	wild-type virus
RacL11BDΔEUs4	RacL11 derived from BAC DNA, EUs4 deleted in the construction of the BAC
RacL11BD	RacL11 derived from BAC DNA, EUs4 restored
RacL11ΔIR4ΔEUs4	RacL11 derived from BAC DNA, ΔIR4, EUs4 deleted in the construction of the BAC
RacL11ΔIR4	RacL11 derived from BAC DNA, ΔIR4, EUs4 restored

Table 2

Replication of RacL11ΔIR4 and RacL11BD in Diverse Cell Types

Cell Type	Virus Strain Tested	
	RacL11BD	RacL11ΔIR4
L-M	$3.83\times10^{6} \pm 4.71\times10^{5}$	< 10
RK13	$6.33\times10^{6} \pm 6.24\times10^{5}$	< 10
RK13+pSVIR4	ND	$7.7\times10^{4} \pm 1.04\times10^{4}$
ETCC	$2.33\times10^{6} \pm 8.50\times10^{5}$	< 100
NBL-6	$2.43\times10^{6} \pm 2.50\times10^{5}$	$1.05\times10^{5} \pm 1.32\times10^{4}$
Vero	$1.58\times10^{6} \pm 3.12\times10^{5}$	< 100
HeLa	$1.07\times10^{5} \pm 1.43\times10^{4}$	< 200

Cells were infected at a MOI of 1, and virus titers were determined at various times post infection by plaque assay on equine NBL-6 cell monolayers. Table shows results at 48 hours p.i.

Table 3

RacL11Δ*IR4* Fails To Replicate in the Lungs of CBA Mice.

	PFU Titer Determined in Equine NBL6 Cells			
Virus	**inoculum**†	**2 days pi**‡	**4 days pi**‡	**6 days pi**‡
RacL11ΔIR4	5×10^5	<50	<50	<50
RacL11BD	5×10^5	4.6×10^6	2.6×10^{5}*	4.7×10^{3}**

† = pfu;

‡ = pfu/lung;

n = 5 mice per group;

* one mouse succumbed to infection;

** two mice succumbed to infection. RacL11BD is parent RacL11 virus derived from BAC.