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Localization of the Us Protein Kinase of Equine Herpesvirus Type 1 Is Affected by the Cytoplasmic Structures Formed by the Novel IR6 Protein

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Previous work revealed that the Us (unique short) segment of equine herpesvirus type-1 (EHV-1), like that of other alphaherpesviruses, encodes a serine/threonine protein kinase (PK). Experiments were carried out to identify the PK encoded by the EHV-1 EUS2 gene (ORF 69) and to ascertain its time course of synthesis and cellular localization. Western blot and immunoprecipitation analyses of EHV-1-infected cell extracts using a PK-specific polyclonal antibody generated against a bacterially expressed TrpE/PK fusion protein identified the Us PK as a 42- to 45-kDa phosphoprotein. The PK protein is first synthesized at 3 hr postinfection, is produced throughout the infection cycle, and is incorporated into EHV-1 virions. Interestingly, immunoprecipitation analyses revealed that the PK protein within the cytoplasm is associated with the 33-kDa IR6 novel protein of EHV-1, is expressed abundantly as an early protein, and is present in the large rod-like structures formed by the IR6 protein (ORF67 protein) within the cytoplasm of infected cells. Confocal microscopic examination of cells stained with fluorescein-labeled antibody clearly showed that the PK protein colocalized with the cytoplasmic IR6 rod-like structures and remained associated with these unique structures during infection. In contrast, in cells infected with the EHV-1 RacM strain in which the IR6 protein harbors four amino acid substitutions that prevent formation of the rod-like structures (Osterrieder *et al.,* 1996, *Virology* 217, 442–451), the PK protein localized predominantly to the nucleus. The possible significance of the association of the IR6 and PK proteins in EHV-1 replication is discussed.

INTRODUCTION

Equine herpesvirus type-1 (EHV-1) is a member of the Alphaherpesvirinae subfamily and a major pathogen that affects equine populations worldwide. EHV-1 infection manifests most often as an upper respiratory infection, but may also present as an abortagenic disease in pregnant mares or as an infection of the central nervous system that leads to paresis and ataxia (Allen and Bryans, 1986; Bryans and Allen, 1986; Crabb and Studdert, 1995; Colle *et al.,* 1996). The genome of EHV-1 has a two-isomer structure comprising a unique long (UL) region covalently attached to a short region (Henry *et al.,* 1981; Whalley *et al.,* 1981). The short region consists of a unique short (Us) segment bracketed by two inverted-repeat (IR) segments. Electron microscopic (Ruyechan *et al.,* 1982) and DNA sequence analyses have confirmed this arrangement (Grundy *et al.,* 1989; Flowers *et al.,* 1991, Flowers and O'Callaghan, 1992; Holden *et al.,* 1992a,b,c; Breeden *et al.,* 1992; Colle *et al.,* 1992; Telford *et al.,* 1992). Each IR segment was shown to contain six major open reading frames (ORF), IR1 through IR6, and three of these encode homologs of herpes simplex virus type-1 (HSV-1). The gene products of IR1 and IR4 are homologs of HSV-1 regulatory proteins ICP4 and ICP22, respectively, while the IR5 protein is a homolog of the Us10 virion protein of HSV-1. The IR6 (Breeden *et al.,* 1992), IR2 (Harty and O'Callaghan, 1991), and IR3 (Holden *et al.,* 1992a) gene products are not encoded by HSV or other human herpesviruses. The Us segment encodes nine major ORFs, four of which encode homologs of HSV glycoproteins gG, gD, gI, and gE (Colle *et al.,* 1992; Audonnet *et al.,* 1990; Elton *et al.,* 1991; Flowers *et al.,* 1991; Whalley *et al.,* 1991; Telford *et al.,* 1992). The Us segment also encodes structural protein homologs of HSV-1 Us2 and Us9 and two ORFs not present in HSV, a putative glycoprotein designated as EUS4 and an ORF encoding a 10-kDa protein (Breeden *et al.,* 1992; Colle *et al.,* 1992; Telford *et al.,* 1992; Audonnet *et al.,* 1990; Elton *et al.,* 1991). Analysis of the predicted amino acid sequence encoded by the EUS2 ORF revealed homology to the serine/threonine protein kinases encoded within the Us segments of other alphaherpesviruses (Colle *et al.,* 1992).

The HSV-1 protein kinase (PK) and pseudorabies virus protein kinase (PRV PK) are the most extensively characterized of the alphaherpesvirus Us protein kinases and have been shown to function as dimers, although the monomeric units of each PK differ considerably in size (HSV-1 is 68 kDa and PRV is 38 kDa). Each enzyme is able to use ATP as a phosphate donor, but not GTP (Purves *et al.,* 1987; Leader and Katan, 1988; Zhang *et al.,* 1990), and the intracellular localization of the PK proteins was shown to be restricted to the cytoplasm of

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infected cells (Purves *et al.,* 1986; Frame *et al.,* 1987; Leader and Purves, 1988). Several substrates for the Us PK have been identified during lytic infection. Purves *et al.* (1991) demonstrated that the HSV-1 PK was responsible for the posttranslational modification of the UL34 gene product and that the site of phosphorylation matched the ideal consensus site predicted from experiments employing synthetic peptides as substrates. The Us9 gene product has been reported as a target of the HSV-2 PK, and the major virion phosphoprotein of PRV has been shown to be phosphorylated by the PRV PK (Daikoku *et al.,* 1994; Zhang *et al.,* 1990). Despite the identification of several substrates, the function of the Us PK in viral infection is still not fully understood. Deletion of the Us PK has been demonstrated to affect the pathogenicity of HSV and PRV in experimental infections using animal models (Kimman *et al.,* 1992; Nishiyama *et al.,* 1992; Kurachi *et al.,* 1993).

The EHV-1 Us protein kinase is a putative 382-amino-acid protein product (42.5 kDa predicted molecular weight) encoded by the 1146-bp EUS2 gene as determined by DNA sequence analysis (Colle *et al.,* 1992; Telford *et al.,* 1992). The EUS2 protein possesses nine domains identified by Hanks *et al.* (1988) as being conserved in a comparison of 65 protein kinases of both cellular and viral origin as well as a diagnostic sequence (Smith and Smith, 1989) that indicates that the EUS2 protein is a member of the serine/threonine class of protein kinases. The EUS2 gene was shown to be transcribed as an early 2.9-kb messenger RNA beginning at approximately 2 hr postinfection and expressed throughout the infectious cycle (Colle and O'Callaghan, 1995). The Us PK gene of other alphaherpesviruses is often transcribed as a member of a 3′-coterminal family of messenger RNAs that includes the message for a downstream glycoprotein (Zhang *et al.,* 1990; McGeoch, 1991). This arrangement was demonstrated for EHV-1 Us transcripts as the 2.9-kb mRNA of the EHV-1 PK is 3′ coterminal with the 1.6-kb transcript that encodes the downstream putative glycoprotein EUS3, a homolog of HSV-2 gG and PRV gX (Colle and O'Callaghan, 1995).

In this report, we identify the EHV-1 EUS2 gene product, a homolog of the alphaherpesvirus Us protein kinase, as a 42- to 45-kDa phosphoprotein that is incorporated into virions, and we demonstrate that the EUS2 gene product associates with the EHV-1 IR6 protein shown previously to form rod-like structures in the cytoplasm of infected cells (Breeden *et al.,* 1992; O'Callaghan *et al.,* 1994; Sun *et al.,* 1995; Osterrieder *et al.,* 1996). The function of these large intracytoplasmic structures is not known; however, the findings presented here reveal that the PK protein readily associates with these structures. Furthermore, mutations within the IR6 protein that prevent the assembly of the rod-like structures (Osterrieder *et al.,* 1996) also affect the association of the PK protein with the IR6 protein and thereby change the localization of the PK protein from being predominantly cytoplasmic to intranuclear.

MATERIALS AND METHODS

Virus and cell culture

The Ab4p strain of EHV-1 was propagated in NBL-6 equine dermis cells and quantitated by plaque assay on RK-13 cells using established methods for EHV-1 propagation (O'Callaghan *et al.,* 1968; Perdue *et al.,* 1974). NBL-6 equine dermis cells were obtained from ATCC (CCL 57) and maintained in Eagle's minimal essential medium supplemented with 10% fetal calf serum. EHV-1 strain Ab4p was kindly provided by Dr. Elizabeth Telford (Institute of Virology, University of Glasgow, Glasgow, Scotland). EHV-1 strains RacL, RacM 24, and RacM 36 (Osterrieder *et al.,* 1996) were kindly provided by Dr. Anton Mayr (Institute for Medical Microbiology, Infectious and Epidemic Disease, Ludwig–Maximilian–Universität, Munich, Germany) and propagated in NBL-6 cells. The RacM strains encode an IR6 protein containing four point mutations, one or more of which prevent formation of the IR6 rod-like structures. These mutations map at residues 34 (Ala → Asp), 43 (Ala → Val), 110 (Val → Leu), and 134 (Leu → Pro) within the IR6 protein (Osterrieder *et al.,* 1996).

Generation of viral DNA clones

EHV-1 DNA subclones were cloned into vectors pUC18/19 or pBluescript (SK) (+ or −) (Stratagene, La Jolla, CA) and were propagated in either *Escherichia coli* strains DH5αF′ (Bethesda Research Laboratories, Gaithersburg, MD) or JM109 (Promega, Madison, WI). The generation and mapping of most EHV-1 Us clones were described previously (Colle *et al.,* 1992; Colle and O'Callaghan, 1995). Construction and cloning of the pATH/PK expression subclone were performed by standard techniques (Maniatis *et al.,* 1982). Briefly, clone pRH2, containing the majority of the EUS2 (PK) ORF, was digested with *Bam*HI and *Sma*I (Bethesda Research Laboratories) to release an 846-bp fragment that encodes 282 amino acids of the EUS2 protein product (residues 37–318). This fragment was then subcloned into unique *Bam*HI and *Hin*dIII sites of the pATH22 expression vector to create an in-frame fusion at the carboxy-terminus of the *trpE* gene. The *Hin*dIII site 5′ overhang was filled in by Klenow DNA polymerase to create a blunt end for ligation with the *Sma*I end of the EUS2 fragment. Thus, the pATH/PK clone contains a TrpE/PK fusion gene inserted downstream of the inducible promoter of the *trp* operon and encodes a fusion protein of approximately 68 kDa.

The pBluescript/PK (pBSPK) construct was created by digestion of the pPVUII-B clone, which encompasses the entire Us segment, with *Sal*I and *Pst*I to release a 1440-bp fragment containing the complete EUS2 (PK) ORF (Us

nucleotides 591 to 2031; see Colle *et al.,* 1992). This fragment was then cloned into pBluescript (SK) that had been digested with *Sal*I and *Pst*I to place the PK ORF under the transcriptional control of a T_7 DNA polymerase promoter.

In vitro transcription and translation

The pBSPK was *in vitro* transcribed and translated using the TnT coupled reticulocyte lysate system (Promega), following the manufacturer's protocol. Briefly, the PK mRNA was transcribed via the T_7 promoter by addition of the T_7 DNA polymerase and then translated in the presence of [^{35}S]methionine ([^{35}S]Met; New England Nuclear, Boston, MA). Labeled proteins were resolved by SDS–PAGE analysis through 4% stacking/12% separating polyacrylamide gels. Gels were dried on a Bio-Rad Model 583 vacuum drying unit (Bio-Rad, Hercules, CA) and exposed to XAR5 X-ray film (Kodak, Rochester, NY) for autoradiography.

Generation of the TrpE/PK fusion protein

The pATH/PK expression construct containing the *trpE*/EUS2 fusion gene was used to transform *E. coli* TB1 cells, and the fusion protein (TrpE/PK) was isolated as described by Koerner *et al.* (1991) with the modifications of McNabb and Courtney (1992) and Harty *et al.* (1993). The 68-kDa TrpE/PK fusion protein was resolved by SDS–PAGE in 4%/12% preparative gels, visualized by Coomassie brilliant blue staining, excised, and stored at −20°.

Preparation of anti-TrpE/PK serum

Gel slices containing approximately 100 μg of the TrpE/PK fusion protein were emulsified in equal volumes of either complete Freund's adjuvant (primary immunization only; Sigma Chemical Co., St. Louis, MO) or incomplete Freund's adjuvant (booster immunizations). A female New Zealand rabbit (1 kg) was immunized with 0.5-ml injections of this emulsion intramuscularly in each hindleg. Injections were carried out at 4-week intervals using procedures described elsewhere (Harlow and Lane, 1988; Harty *et al.,* 1993; O'Callaghan *et al.,* 1994). Preimmune serum was obtained prior to the beginning of the vaccination regime.

Radiolabeling and preparation of RK infected cell extracts

EHV-1 strains Ab4p, RacL, RacM 24, and RacM 36 were used to infect 5×10^6 RK cells at a multiplicity of infection (m.o.i.) of 10 PFU/cell, using procedures described elsewhere (Caughman *et al.,* 1985; O'Callaghan *et al.,* 1994). Briefly, infected cell polypeptides (ICPs) were steady-state labeled with [^{35}S]Met or [^{32}P]-orthophosphate ($^{32}PO_4$; Amersham, Arlington Heights, IL) at a concentration of 100 μCi/ml beginning at 1 hr postinfection, and extracts were prepared at various times postinfection by lysis of the infected cells in 1 ml RIPA buffer (0.1% SDS, 1% Triton X-100, 1% Na-deoxycholate, 0.15 *M* NaCl, 0.01 *M* Tris–HCl, pH 7.4, 1 m*M* EDTA) containing 2.5 m*M* aprotinin, 2.5 m*M* leupeptin, and 30 mg of phenylmethylsulfonyl fluoride per milliliter. Extracts of mock-infected cells were also prepared as above. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical Co., Rockford, IL). Unlabeled ICPs were prepared as above except radiolabeled isotopes were not added to the cultures.

Western blot and immunoprecipitation analyses

Western blot and immunoprecipitation analyses were performed as described previously (Flowers and O'Callaghan, 1992; Holden *et al.,* 1994; Smith *et al.,* 1992). For Western blot analyses, 20 μg of ICPs was resolved by SDS–PAGE, and the polypeptides were transferred via electroblotting to nitrocellulose filter paper (Schleicher & Schuell, Inc., Keene, NH). The nitrocellulose blots containing the denatured ICPs were blocked for nonspecific binding by incubation in either 0.5% gelatin or 2% nonfat dry milk for 30 min. The blots were then incubated with the rabbit anti-PK serum at a dilution of 1:1000 or anti-IR6 antibody (O'Callaghan *et al.,* 1994) at a dilution of 1:25,000. The specific protein bands were visualized using a secondary alkaline phosphatase-conjugated, goat anti-rabbit IgG (heavy plus light chains) antibody (Gibco BRL, Gaithersburg, MD). For immunoprecipitation analyses, 10–20 μg of ICP extracts were first precleared with preimmune rabbit serum (1:100) in a final volume of 100 μl, and the antibody protein complexes were pelleted using immobilized rProtein A agarose beads (RepliGen, Cambridge, MA). The EHV-1 PK protein or IR6 protein was immunoprecipitated from precleared radiolabeled ICP using either the anti-PK serum (1:200) or anti-IR6 (1:2500) serum, respectively. The immunoprecipitated proteins were resolved by SDS–PAGE in 4%/12% gels which were then dried and exposed to XAR5 X-ray film (Kodak, Rochester, NY) for autoradiography.

Purification of EHV-1 virions

Purified virions were prepared from the supernatant of Ab4p-infected NBL-6 cells collected at 72 hr postinfection as described previously (Perdue *et al.,* 1974). Briefly, virions in clarified extracellular supernatants were collected by polyethylene glycol precipitation and subjected to three cycles of rate velocity centrifugation in Dextran-10 gradients. The virion protein concentration was determined using the BCA protein reagent. In the Western blot analyses of virion protein, 5–10 μg protein was loaded in each lane.

Indirect immunofluorescence staining and laser-scanning confocal microscopy

NBL-6 cells were grown as semiconfluent monolayers in two section chamber slides (Nunc, Inc., Naperville, IL). Cells in one chamber were mock-infected and those in the other chamber were infected with EHV-1 (either Ab4p, RacL, RacM 24, or RacM 36) at an m.o.i. of 1 PFU/cell. The slides were harvested at 3, 6, 9, and 12 hr postinfection, washed in PBS, and then fixed in 100% acetone for 5 min at −20°. Immunofluorescence staining of infected cells was done as previously reported (O'Callaghan *et al.,* 1994). Briefly, slides were rehydrated in PBS for 10 min at room temperature and blocked with normal goat serum (NGS; 5% NGS, 0.1% bovine serum albumin in PBS) for 30 min in a humidified chamber at room temperature. Slides containing the fixed mock-infected and EHV-1-infected cells were then incubated with the primary antibody (either rabbit polyclonal anti-PK serum at a dilution of 1:1000 or anti-IR6 serum at a dilution of 1:5000) in 1% NGS, 0.1% BSA in PBS for 1 hr at room temperature, washed three times in PBS for 10 min each, and then incubated with a fluorescein-conjugated goat anti-rabbit immunoglobulin G (heavy plus light chains) $F(ab')_2$ fragments (Pierce). Following three washes in PBS for 10 min each, slides were overlaid with a mounting medium of 90% glycerol and 10% PBS containing 1 mg of *p*-phenylenediamine per milliliter to reduce fading. Slides were examined in a laser-scanning confocal microscope system (Model MRC1000; Bio-Rad Laboratories) attached to a Nikon Diaphot microscope and were illuminated with the 488-nm line of a krypton–argon laser. Images through the cell were taken at 1-μm intervals with a 60× objective (Nikon 60/1.3). Individual sections were Kalman averaged over three scans and stored on a computer. Images were projected using the Bio-Rad Laboratories COMOS software. A video printer (Sony UP-52000MD) was used to print final images.

RESULTS

Generation of a monospecific polyclonal antiserum against the Us protein kinase

A bacterially expressed TrpE/EUS2 fusion protein was used as the immunogen to generate rabbit monospecific polyclonal antibody to the EHV-1 EUS2 PK. EUS2 coding sequences from a *Bam*HI and *Sma*I fragment were inserted in frame with the *trpE* gene in the vector pATH22 (Fig. 1A). The fusion protein was predicted to have a molecular weight of 68 kDa and to express amino acids 37–318 of the EHV-1 PK (Fig. 1B). The pATH/PK plasmid was transformed into *E. coli* strain TB1, and the fusion protein was induced by tryptophan starvation. The TrpE/PK fusion protein was isolated from total cell lysates of induced and uninduced cultures that were fractionated into soluble and insoluble fractions, and the proteins were resolved by SDS–PAGE and visualized by Coomassie blue staining. As expected, TrpE induction produced an abundant 68-kDa protein in the pATH-PK-containing TB1 cells that was not present in the uninduced cells or in the soluble fraction of the induced cells (Fig. 1B). This protein was also not found in TB1 cells or in TB1 cells containing the pATH22 vector alone (data not shown). The proteins in the insoluble fraction were resolved by preparative SDS–PAGE, and the 68-kDa protein band was excised and used to produce a rabbit polyclonal antiserum against the EHV-1 PK.

To test the specificity of the anti-PK serum produced by immunization with the TrpE/PK fusion protein, the entire EUS2 ORF (1145 bp) was cloned into the plasmid pBluescript (SK) (Stratagene) under the control of the T_7 DNA polymerase promoter. This construct allowed the EUS2 protein product to be expressed using a coupled *in vitro* transcription and translation system (TnT; Promega) in the presence of [^{35}S]Met (40 μCi). SDS–PAGE analysis of the total protein translated in the *in vitro* reaction mixture revealed a predominant, labeled protein that migrates at approximately 42.5 kDa, the predicted size of the EUS2 protein product, as well as several other proteins of lesser amounts and various sizes (Fig. 2). The 42.5-kDa protein was not observed when the parental pBluescript (SK^+) plasmid was used as template in the *in vitro* TnT reaction. The anti-PK serum immunoprecipitated the 42.5-kDa protein from the *in vitro* TnT reaction mixture, indicating that the serum was specific for the EUS2 protein product. A slightly smaller protein species was immunoprecipitated by the anti-PK serum and is believed to result from initiation of translation at a second internal in-frame ATG in the PK ORF (Colle *et al.,* 1992). The anti-PK serum did not react with any protein from the *in vitro* translation of the pBSSK vector alone (Fig. 2). In addition, control experiments showed antisera to several other EHV-1 proteins failed to react with the EUS2 protein.

Analyses of the EHV-1 Us protein kinase in infected cells and virions

The molecular weight of the EHV-1 PK protein and the time course of its synthesis during a lytic infection were determined by Western blot analyses. NBL-6 equine dermis cells infected with EHV-1 strain Ab4p were harvested at 3, 6, 9, and 12 hr postinfection. The ICPs were resolved by SDS–PAGE, immobilized on nitrocellulose filter paper, and analyzed with the anti-PK serum. In Western blot analysis, the anti-PK serum reacted with a single protein that migrated with an apparent molecular weight of 42–45 kDa (Fig. 3A) which is in agreement with the predicted size of the 382-amino-acid polypeptide encoded by the EUS2 ORF (Colle *et al.,* 1992). Expression of this protein began at 3 hr postinfection, and the protein was readily detected until at least 24 hr postinfection (Fig. 3B). To determine if the PK protein is a component of the EHV-

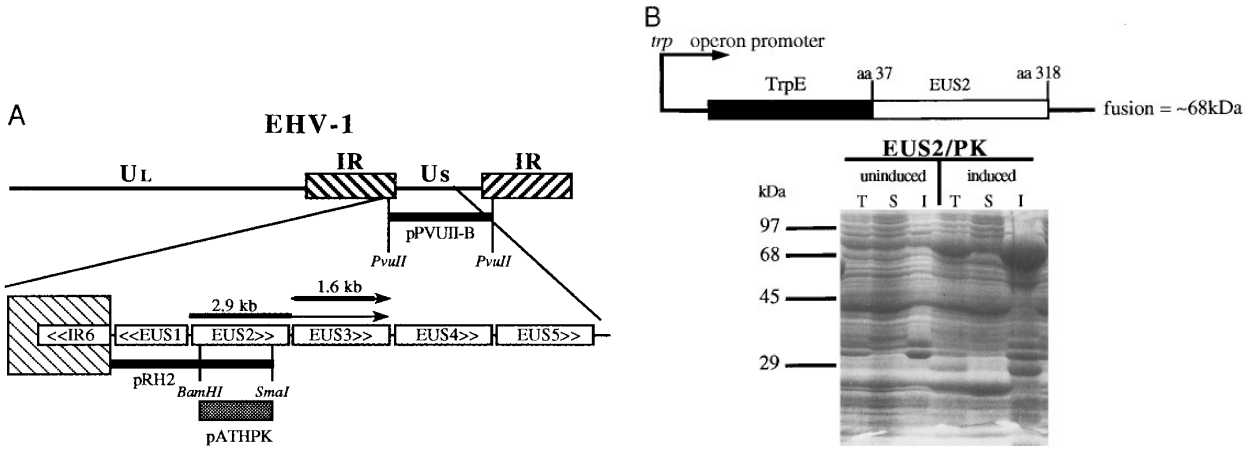


FIG. 1. Expression of the TrpE/PK fusion protein in *E. coli.* (A) Diagram of the EHV-1 genome structure. The open boxes show the location of the Us genes EUS1–EUS5 and the inverted-repeat gene IR6; the arrows (⩾ or ⩽) indicate the direction of the open reading frame. The location of the pRH2 and pPVUII-B clones is indicated by the heavy black lines, and the shaded box shows the portion of the EUS2 gene (Us base pairs 872–1718) that was fused to the *trpE* gene to generate the pATHPK construct. The 3′-coterminal 2.9-kb transcript, encoding the PK protein, and the 1.6-kb transcript, encoding the EHV-1 gG homolog of HSV-1, are indicated by the lines with arrowheads. (B) Identification of the TrpE/PK fusion protein expression in *E. coli* TB1 cells transformed with the pATHPK construct. Top shows the TrpE/PK fusion protein containing amino acids 37–318 of the EHV-1 PK and the predicted molecular weight of the fusion protein (68 kDa). T, total cell lysate; S, soluble fraction of total cells; I, insoluble fraction of the total cell lysate.

1 virion, virions were isolated, purified by established methods (see Materials and Methods), and analyzed by Western blot analysis using the anti-PK serum (Figs. 3A and 3B). The data revealed that the EHV-1 PK was associated with purified EHV-1 virions as a protein with a molecular size identical to that of the protein observed in infected cell lysates. Previous work showed that the IR6 protein is also a component of purified EHV-1 virions (O'Callaghan *et al.,* 1994).

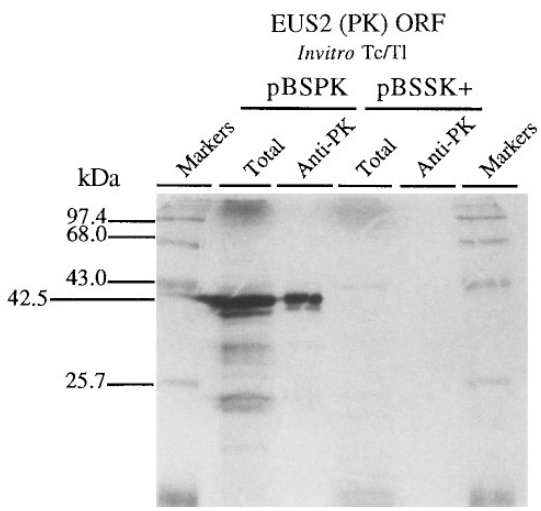


FIG. 2. *In vitro* expression of the EUS2 (PK) open reading frame. The EUS2 open reading frame was cloned into the pBluescript (SK) (pBSSK+) vector under control of a T7 DNA polymerase promoter to generate the pBSPK construct. Both the pBSSK+ vector and pBSPK construct were used as templates in an *in vitro* coupled transcription/translation reaction, and the protein produced was resolved by SDS–PAGE. The lanes marked Total show the total protein profile from the *in vitro* reactions mixture containing either pBSPK or pBSSK+. Lanes labeled Anti-PK are the protein immunoprecipitated from the total *in vitro* reaction by the anti-PK serum. Marker lanes show approximate molecular weights.

Confocal immunofluorescence analyses to determine the intracellular localization of the EHV-1 PK protein

To assess the intracellular localization of the EHV-1 PK within infected cells, laser-scanning confocal microscopy was employed. The EHV-1 Ab4p-infected NBL-6 cells harvested at 3, 6, 9, and 12 hr postinfection were acetone-fixed and stained using the anti-PK serum and a fluorescein-conjugated secondary antibody. Interestingly, the PK was observed to have a staining pattern identical to that previously reported for the EHV-1 IR6 protein (Fig. 4; O'Callaghan *et al.,* 1994). Fluorescence observed in cells fixed at 3 hr postinfection was very weak (data not shown). However, at 6 hr postinfection, the PK protein was found to be localized in rod-like structures that appear to extend from the nucleus to the ends of the fibroblast pseudopods in infected NBL-6 cells (Fig. 4B), as was also observed for the staining pattern of IR6 using the anti-IR6 serum (Fig. 4E). Coincident with the shrinking of the cytoplasm, by 9 hr postinfection, both the rod-like structures of the EHV-1 PK and IR6 proteins condensed around the nucleus to form the ring-like structure typical for the IR6 protein (O'Callaghan *et al.,* 1994) (Figs. 4C and 4F). The PK protein exhibited a distribution pattern identical to that of the IR6 protein until approximately 12 hr postinfection, the time of maximal PK protein accumulation, at which time some faint nuclear staining was observed for the PK (data not shown). Mock-infected cells exhibited no specific staining with either the anti-

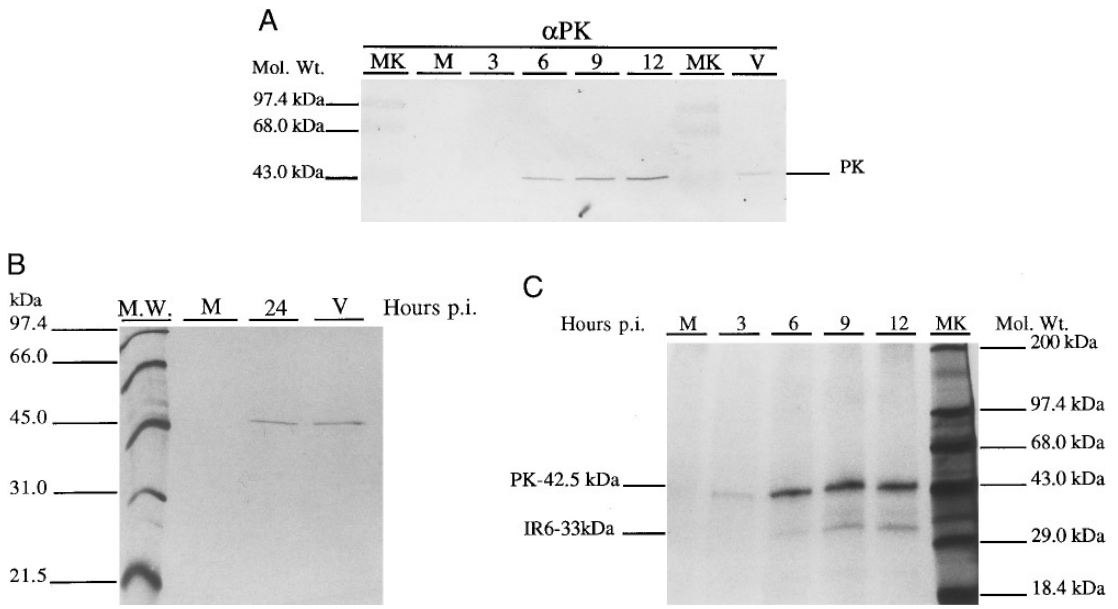


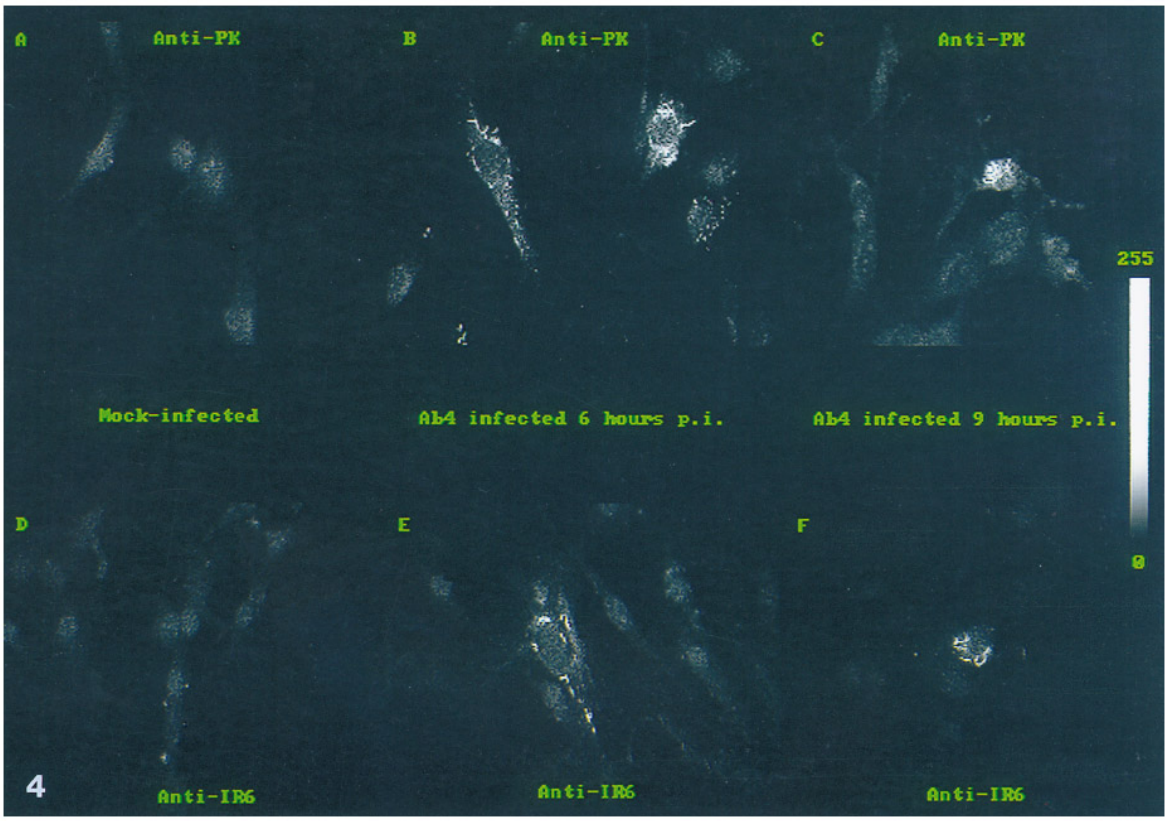
FIG. 3. Identification of the EHV-1 PK protein in infected cells. (A and B) Western blot analyses of proteins from EHV-1 Ab4p-infected RK cells harvested at 3, 6, 9, 12, and 24 hr postinfection, from mock-infected (M) cells, and from purified EHV-1 virions (V). Infected cell proteins resolved by SDS–PAGE were transferred to nitrocellulose then probed with anti-PK serum (1:1000 dilution), and reactive proteins were visualized using a secondary antibody conjugated to alkaline phosphatase. Marker lanes (MK) show approximate molecular weights. (C) Immunoprecipitation analysis demonstrating the phosphorylation of the EHV-1 PK protein in Ab4p-infected RK cells. The 42.5-kDa PK protein was immunoprecipitated with anti-PK serum from [^{32}P]orthophosphate-labeled infected cells harvested at 3, 6, 9, and 12 hr postinfection or from mock-infected cells (M). [^{32}P]Orthophosphate (100 mCi/ml) was added at 1 hr postinfection. The immunoprecipitates were resolved by SDS–PAGE and autoradiography. Marker lanes show molecular weights. The coimmunoprecipitated 33-kDa EHV-1 IR6 protein is also indicated.

PK serum or the anti-IR6 serum at any time postinfection (Figs. 4A and 4D).

Association of the PK and the IR6 phosphoproteins in EHV-1 infection

Since the HSV-1 Us3 protein kinase is a phosphoprotein (Frame *et al.,* 1987), the EHV-1 homolog was analyzed to determine if it shared this feature. Mock-infected and EHV-1 Ab4p-infected NBL-6 cells were radiolabeled at 1–2 hr postinfection with $^{32}PO_4$, and cellular extracts prepared at 3, 6, 9, and 12 hr postinfection were subjected to immunoprecipitation analyses using the anti-PK serum (Fig. 3C). The results demonstrated that the PK was indeed a phosphoprotein that was present in only small amounts at 3 hr postinfection; however, by 6 hr a much stronger signal from equivalent amounts of total protein (20 μg) was observed. A second phosphoprotein species of 33 kDa, first detected at 6 hr postinfection, was also observed to be immunoprecipitated using the anti-PK serum.

To investigate the association of the PK and the 33-kDa phosphoprotein species, EHV-1 Ab4p-infected NBL-6 cells were steady-state labeled with [^{35}S]Met starting at 1 hr postinfection, and ICPs harvested at 3, 6, 9, and 12 hr postinfection were subjected to immunoprecipitation analyses as described above for the cells labeled with $^{32}PO_4$. The results were similar to those observed in the immunoprecipitation of the PK from the $^{32}PO_4$-labeled infected cell extracts. At 3 hr postinfection, the anti-PK serum immunoprecipitated only the 42- to 45-kDa PK protein; however, by 6 hr postinfection the 33-kDa protein species was readily coimmunoprecipitated by the anti-PK antibody (Fig. 5A). A more detailed time course analysis revealed that coimmunoprecipitation of the 33-kDa protein with the PK protein was first detected at 5 hr postinfection, but not at any earlier time points (data not shown). Since the intracellular staining pattern of the PK in the immunofluorescence analysis was characteristic of the EHV-1 IR6 rod-like structures (O'Callaghan *et al.,* 1994; Sun *et al.,* 1995) and the 33-kDa protein that associated with the PK protein was identical in size to the IR6 phosphoprotein (Breeden *et al.,* 1992; O'Callaghan *et al.,* 1994), it was believed that the 33-kDa protein was the EHV-1 IR6 protein. To determine whether the 33-kDa component was the IR6 protein, the EHV-1 PK/33-kDa complex was immunoprecipitated from Ab4p-infected cell extracts with the anti-PK serum, resolved by denaturing SDS–PAGE, and then blotted to nitrocellulose filter paper. The blot was then probed with anti-IR6 serum (Fig. 5B). Western blot analyses clearly identified the 33-kDa protein associated with the EHV-1 Us protein kinase protein as the IR6 protein since both proteins could be immunoprecipitated by the anti-PK serum. The anti-PK antibody, however, did not react directly with the IR6 protein in Western blot analyses (Fig. 3B), indicating that the coimmunoprecipitation was not due to cross-reactivity of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Anti-PK
B
Anti-PK
C
Anti-PK
255
0
Mock-infected
Ab4 infected 6 hours p.i.
Ab4 infected 9 hours p.i.
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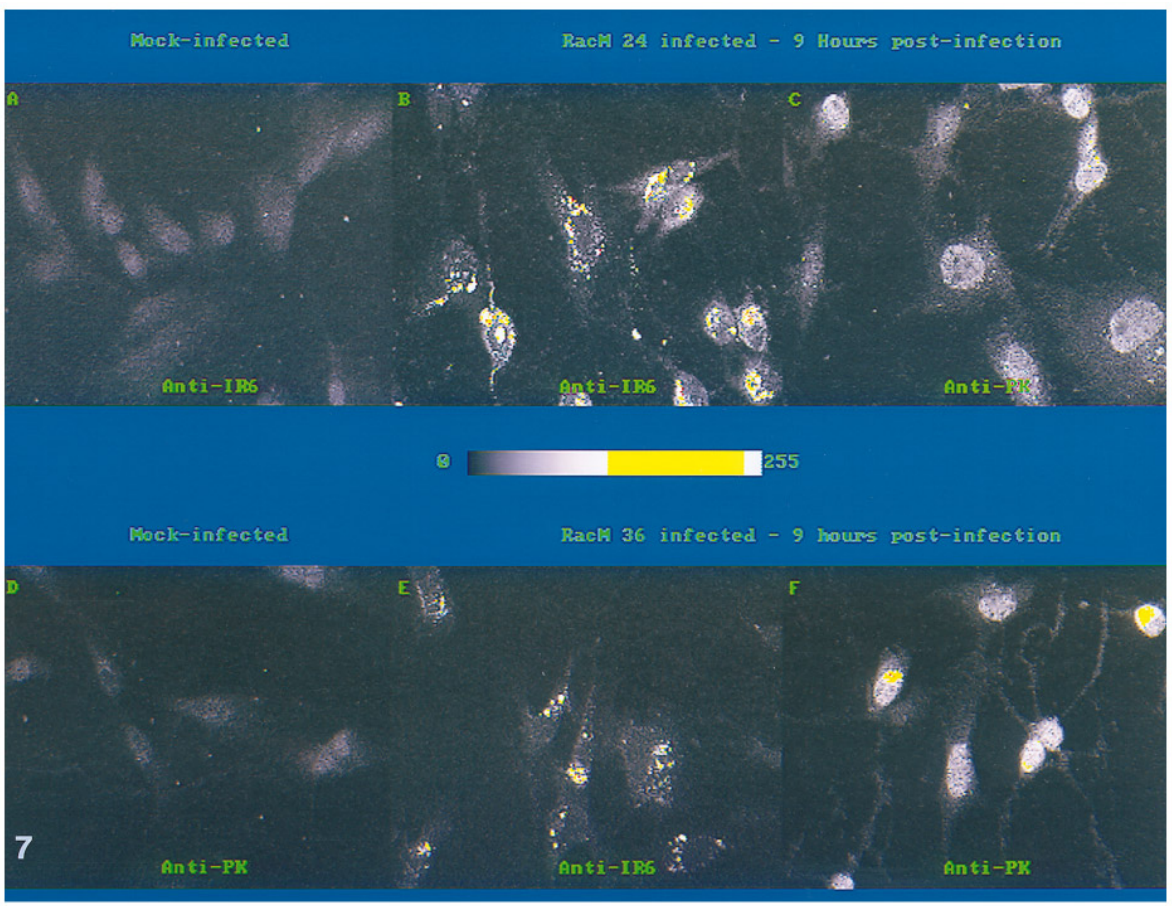
4
Anti-IR6
Anti-IR6
Anti-IR6


Mock-infected
RacM 24 infected - 9 Hours post-infection
A
B
C
Anti-IR6
Anti-IR6
Anti-PK
0
255
Mock-infected
RacM 36 infected - 9 hours post-infection
D
E
F
7
Anti-PK
Anti-IR6
Anti-PK

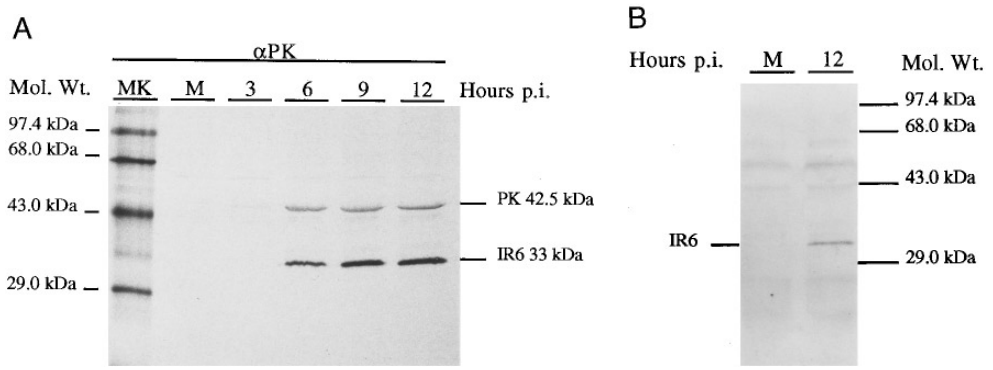


FIG. 5. Western blot analyses of proteins immunoprecipitated by the anti-PK serum demonstrating the association of the EHV-1 PK and IR6 proteins in Ab4p-infected RK cells. (A) Immunoprecipitation analysis demonstrating the coimmunoprecipitation of the EHV-1 PK and IR6 proteins. Proteins from [^{35}S]Met-labeled, infected cells harvested at 3, 6, 9, and 12 hr postinfection or from mock-infected cells (M) were immunoprecipitated with anti-PK serum (αPK), and the immunoprecipitates were resolved by SDS–PAGE and autoradiography. Marker lanes (MK) show approximate molecular weights. (B) Western blot analysis of proteins immunoprecipitated from 12 hr postinfection extracts by the anti-PK serum. The Western blot was probed with anti-IR6 serum (αIR6; 1:25,000), and reactive proteins were visualized using a secondary antibody conjugated to alkaline phosphatase. Approximate molecular weights are indicated at the right.

the serum. The anti-IR6 serum could readily coimmunoprecipitate the PK protein. However, the IR6 protein is made in large amounts throughout infection (Breeden *et al.,* 1992; O'Callaghan *et al.,* 1994), and thus most of the anti-IR6 antibody reacted with the IR6 protein not associated with the PK protein.

In the absence of IR6 rod structures, the Us PK localizes to the nucleus

The IR6 protein of the RacL, KyA, and Ab4p strains of EHV-1 readily form rod-like structures in EHV-1-infected cells (O'Callaghan *et al.,* 1994; Sun *et al.,* 1995). However, Osterrieder *et al.* (1996) demonstrated recently that the IR6 protein of the EHV-1 RacM strains (RacM 24 and RacM 36) harbors four point mutations [amino acid residues 34 (Ala → Asp), 43 (Ala → Val), 110 (Val → Leu), and 134 (Leu → Pro)] and that one or more of these four mutations prevents the formation of the IR6 protein rod-like structures in EHV-1-infected cells.

Using the RacM strains, it was possible to ascertain whether the EHV-1 PK protein could associate with the IR6 protein despite its inability to form the rod-like structures. The wild-type IR6 protein has been shown to form rod-like structure in the absence of other viral proteins, therefore the EHV-1 PK is not required for the assembly of the structures (Osterrieder *et al.,* 1996). Equine cells infected either with the RacM mutant virus or with RacL were labeled continuously from 1 hr postinfection with [^{35}S]Met and extracts prepared at various times were subjected to immunoprecipitation analyses with the anti-PK antibody followed by SDS–PAGE to identify the proteins in the immunoprecipitate. The anti-PK antibody immunoprecipitated both the PK protein and the IR6 protein from the RacL-infected cell extracts, indicating the association of these two proteins (Fig. 6A). These results were in agreement with those presented in Figs. 3C and 5A. In the case of the extracts of RacM-infected cells, in which the IR6 rod-like structures are not formed, the anti-PK antibody immunoprecipitated only the PK protein (Fig. 6B), indicating the lack of association of these two proteins in the RacM-infected cells. Importantly, immunoprecipitation analyses using the anti-IR6 antibody as a control demonstrated that the IR6 protein was synthesized in the RacM-infected cells and that the anti-IR6 antibody did not cross-react with the PK protein (Fig. 6B).

Interestingly, the failure of the Us PK protein to associate with the IR6 protein in RacM-infected cells radically altered the intracellular localization of the PK protein dur-

FIG. 4. Laser-scanning confocal analysis of the distribution of the EHV-1 PK and IR6 proteins in EHV-1-infected NBL-6 cells. Equine NBL-6 cells infected with EHV-1 strain Ab4p at an m.o.i. of 1 were fixed at the times indicated and then stained with either the anti-PK (1:1000) serum or the anti-IR6 (1:5000) serum. The bar at the right represents pixel intensity (brightness) ranging from none (0) to maximum (255). Details are explained under Materials and Methods. (A and D) Mock-infected cells fixed at 9 hr postinfection and stained with anti-PK or anti-IR6 serum as indicated. (B and E) Distribution of the EHV-1 PK and IR6 proteins within infected cells at 6 hr postinfection. (C and F) Distribution of the EHV-1 PK and IR6 proteins within infected cells at 9 hr postinfection.

FIG. 7. Laser-scanning confocal analysis of EHV-1 RacM 24- and RacM 36-infected NBL-6 cells at 9 hr postinfection, demonstrating the altered intracellular localization of the EHV-1 PK protein in the absence of its association with IR6. Equine NBL-6 cells were infected with either EHV-1 strain RacM 24 or RacM 36 at an m.o.i. of 1 and were fixed at 9 hr postinfection and then stained with either anti-IR6 serum (A, B, E; 1:5000) or anti-PK serum (C, D, F; 1:1000). The bar at the center represents pixel intensity (brightness) ranging from none (0) to maximum (255). Details are explained under Materials and Methods. Mock-infected cells were fixed at 9 hr postinfection and stained with either the anti-PK serum or the anti-IR6 serum as indicated.

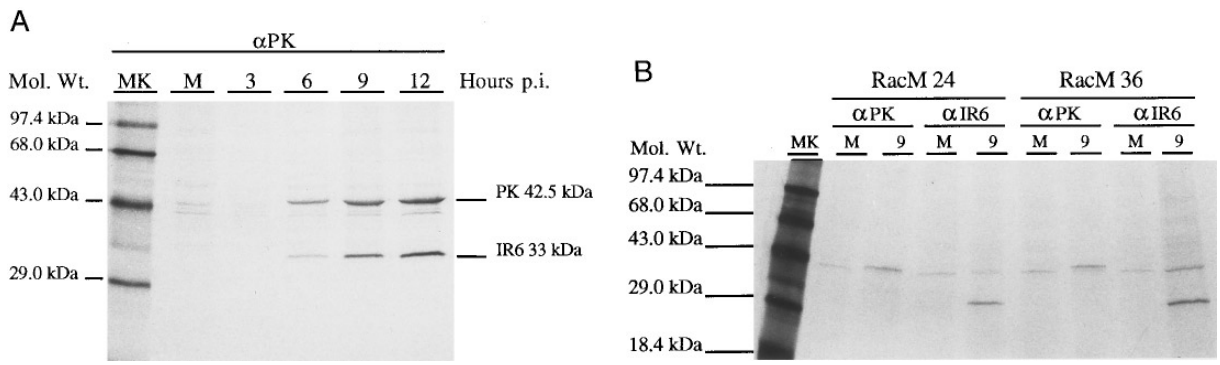


FIG. 6. Immunoprecipitation analysis of proteins by the anti-PK serum from EHV-1 strain RacL-, RacM 24-, or RacM 36-infected RK cells. (A) Coimmunoprecipitation of the EHV-1 PK and IR6 proteins with anti-PK serum (αPK) from [^{35}S]Met-labeled RacL-infected cells harvested at 3, 6, 9, and 12 hr postinfection or from mock-infected cells (M). The immunoprecipitates were resolved by SDS–PAGE and autoradiography. Marker lanes (MK) at left show approximate molecular weights. (B) Immunoprecipitation analysis of extracts harvested at 9 hr postinfection, demonstrating that in the EHV-1 RacM 24 and RacM 36 infections, αPK does not coimmunoprecipitate the EHV-1 IR6 protein that harbors specific amino acid substitutions; however, immunoprecipitation with anti-IR6 serum (αIR6) demonstrates that the IR6 protein is present in the infected cell. Mock-infected cell extracts (M) were also included as controls. The immunoprecipitates were resolved by SDS–PAGE and autoradiography. Marker lanes show approximate molecular weights. A nonspecific band present in faint amount was also detected in mock-infected cells.

ing lytic infection. Immunofluoresence analysis revealed that in the absence of PK/IR6 association, the PK protein localized to, and accumulated within, the nucleus in both RacM 24- and RacM 36-infected cells (Figs. 7C and 7F). The nuclear staining of the PK protein was first observed at 6 hr postinfection (data not shown). Figures 7B and 7E illustrate that the IR6 protein in cells infected with RacM strains does not form the rod-like structures, but exhibits a granular distribution in perinuclear vesicles which may be part of the endocytic degradation pathway (Osterrieder *et al.,* 1996).

DISCUSSION

Our previous work showed that the EUS2 gene of EHV-1 encodes a protein kinase with significant homology to the unique short protein kinases of other alphaherpesviruses, such as those of HSV-1 (26% identity) and PRV (45% identity) (Colle *et al.,* 1992). The 1146-bp ORF is transcribed to a 2.9-kb messenger RNA that appears by 2 hr postinfection (Colle and O'Callaghan, 1995), a finding that is consistent with the observation presented here that the EHV-1 Us PK protein is first detected at approximately 3 hr postinfection as a 42- to 45-kDa phosphoprotein that becomes incorporated into virions as demonstrated for the Us PK of both HSV-1 and PRV (Zhang *et al.,* 1990). The 33-kDa IR6 protein, which does not cross-react with the PK antibody, forms the rod-like structures and coimmunoprecipitates with the PK protein as shown by use of the anti-PK serum. Association of the PK and IR6 proteins was first detected between 4 and 5 hr postinfection, and these proteins were found to associate at late times after infection. The PK protein colocalizes only with the IR6 protein that forms rod-like structures in the cytoplasm of infected cells. No evidence has been found to indicate that IR6 associates or colocalizes with cytoskeletal elements such as actin, tubulin, vimentin, desmin, dynein, or kinesin (O'Callaghan *et al.,* 1994; Osterrieder *et al.,* 1996). These rod-like structures can be formed solely by the IR6 protein in the absence of other viral proteins (Osterrieder *et al.,* 1996) and therefore do not require the PK protein to initiate or maintain the formation of these structures.

However, in the EHV-1 RacM strain, specific amino acid substitutions in the IR6 protein prevent formation of the IR6 rod-like structures and also prevent the association of the PK and the IR6 proteins (Osterrieder *et al.,* 1996). The specific point mutation(s) that prevents the association of the PK and IR6 proteins is not known. Of the four point mutations identified in the IR6 gene of the RacM strains, it is possible that one or more prevents the association of the PK protein with the IR6 protein, while others affect formation of the rod-like structures. Identification of which one or more of the four single amino acid substitutions within the IR6 protein of the RacM virus prevents formation of the rod-like structures is under investigation. This information may reveal whether domains of the IR6 protein essential for formation of the cytoplasmic structures also mediate the interaction of the IR6 and PK proteins.

In the case of EHV-1 RacM-infected cells, it was observed that the intracellular localization of the EHV-1 PK protein was radically altered as the PK protein accumulated within the nucleus of infected cells. This observation was surprising because the Us protein kinases encoded by other alphaherpesviruses localize within the cytoplasm of infected cells (Purves *et al.,* 1987; Leader and Purves, 1988; Zhang *et al.,* 1990); however, none of the Us PK proteins of the other herpesviruses has been shown to be complexed with other viral or cellular proteins that may affect localization of the protein kinase. The possibility that the IR6 protein complexes were re-

acting with the rabbit anti-PK antibody directly, such as through Fc binding, was eliminated since the anti-PK antibody failed to react with the IR6 protein in Western blots or to immunoprecipitate the IR6 protein from the RacM-infected cell extracts.

The function of the Us protein kinases in herpesvirus infection remains to be defined. However, studies using synthetic peptide substrates have led to the identification of a putative target site of the PK action for both the HSV-1 and the PRV enzymes. The PK proteins of both of these herpesviruses prefer peptide substrates that contain several arginyl residues to the amino-terminal side of the phosphorylated serine or threonine reside. The ideal target sequence in the synthetic peptides was $(R)_n$-X-(S/T)VA (Purves *et al.,* 1986; Leader *et al.,* 1991). The UL34 gene product of HSV-1 has been shown to be posttranslationally modified by the HSV-1 Us PK during infection and to contain an ideal PK recognition sequence (Purves *et al.,* 1991). Daikoku *et al.* (1994) have demonstrated that the Us9 gene product, a tegument protein, is a target of the HSV-2 Us PK. Although the Us protein kinases of both HSV and PRV have been shown to be dispensable for growth in tissue culture (Longnecker and Roizman, 1987), several studies demonstrated a role for the Us PK in *in vivo* replication. Wagenaar *et al.* (1995) reported that infection of porcine nasal mucosa explant cultures with a PRV Us PK deletion mutant affected the ability of PRV virions to egress from the nucleus to the cytoplasm of infected epithelial cells. However, no such defect was observed in porcine lung alveolar macrophages infected with the same knockout virus. These findings suggest that the Us PK phosphorylation of a virion component may be required for proper transport of the virion through the infected cell, but that this function may be cell-type specific. In this regard, it is interesting that our ongoing studies suggest that the EHV-1 IR6 protein plays a role in virus egress from the nucleus (N. Osterrieder and D. J. O'Callaghan, in preparation). Phosphorylation of the IR6 protein, possibly by the associated Us PK, may be important for the IR6 protein to be functional. Interestingly, enzyme assays indicate that maximal protein kinase enzymatic activity is found when the PK protein is associated with the IR6 protein. The importance of the herpesviral protein kinase is not limited to tissue culture, as a reduction in virulence of both PRV and HSV-2 Us PK deletion mutants was noted in the experimental infection of pigs and mice, respectively (Kimman *et al.,* 1992; Kurachi *et al.,* 1993).

All herpesviruses examined to date encode a second protein kinase in the UL segment of the genome. The UL protein kinases, such as the HSV-1 UL13 protein, have been shown to localize to the nucleus of infected cells (Cunningham *et al.,* 1992) and may be involved in the virion host shutoff effect (Overton *et al.,* 1994). It has been shown that the UL13 protein kinase is responsible for the posttranslational modification of the HSV-1 immediate early protein ICP22 (α22) and that this modification affects the regulatory functions of the ICP22 protein (Purves and Roizman, 1992; Purves *et al.,* 1993; Carter and Roizman, 1996). ICP22-deficient and UL13-deficient HSV-1 mutants exhibit defects in viral replication that are cell-type specific and similar to that described above for the PRV Us PK deletion mutant. Recently, it has been suggested that the UL13 protein kinase is an ICP22-dependent C-terminal domain (CTD) kinase involved in the modification of the RNA polymerase II CTD during lytic HSV-1 infection (Rice *et al.,* 1995) and that this modification is required for proper regulation of viral gene expression. Although the EHV-1 Us PK protein has greater homology to the Us PK encoded by other alphaherpesviruses (see Colle *et al.,* 1992, Fig. 6) than to the UL13 protein kinases which localize to the nucleus, the EHV-1 Us PK localizes to the nucleus if its association with the IR6 protein does not occur as in the case of RacM-infected cells. The EHV-1 PK protein was shown to be incorporated into EHV-1 virions and likely encodes the serine/threonine protein kinase activity detected in EHV-1 virions (Randall *et al.,* 1972).

A common characteristic of protein kinases is the ability to autophosphorylate (Leader and Katan, 1988) which has been demonstrated for the HSV-1 and PRV Us PKs (Frame *et al.,* 1987; Purves *et al.,* 1987). The Us PKs of both HSV-1 and PRV have been shown to function as homodimers (Purves *et al.,* 1987; Zhang *et al.,* 1990). This may not be the case for the EHV-1 PK which may form a heterodimer with the unique IR6 protein. Heterodimer formation would be a unique finding for a herpesvirus protein kinase; however, no herpesvirus sequenced to date, other than equine herpesviruses 1 and 4 (Nagesha *et al.,* 1993) and bovine herpesvirus type 1 (Leung-Tack *et al.,* 1994), encodes a homolog of the IR6 protein. IR6 may function as a cofactor required for activation of the PK or may simply serve as an anchor to retain the EHV-1 Us PK protein in the cytoplasm.

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