The African Swine Fever Virus Virion Membrane Protein pE248R Is Required for Virus Infectivity and an Early Postentry Event[▽]

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The African swine fever virus (ASFV) protein pE248R, encoded by the gene E248R, is a late structural component of the virus particle. The protein contains intramolecular disulfide bonds and has been previously identified as a substrate of the ASFV-encoded redox system. Its amino acid sequence contains a putative myristoylation site and a hydrophobic transmembrane region near its carboxy terminus. We show here that the protein pE248R is myristoylated during infection and associates with the membrane fraction in infected cells, behaving as an integral membrane protein. Furthermore, the protein localizes at the inner envelope of the virus particles in the cytoplasmic factories. The function of the protein pE248R in ASFV replication was investigated by using a recombinant virus that inducibly expresses the gene E248R. Under repressive conditions, the ASFV polyproteins pp220 and pp62 are normally processed and virus particles with morphology indistinguishable from that of those produced in a wild-type infection or under permissive conditions are generated. Moreover, the mutant virus particles can exit the cell as does the parental virus. However, the infectivity of the pE248R-deficient virions was reduced at least 100-fold. An investigation of the defect of the mutant virus indicated that neither virus binding nor internalization was affected by the absence of the protein pE248R, but a cytopathic effect was not induced and early and late gene expression was impaired, indicating that the protein is required for some early postentry event.

African swine fever virus (ASFV) is a large enveloped deoxyvirus that causes a severe hemorrhagic disease in domestic pigs (38). The ASFV genome is a double-stranded DNA molecule of 170 to 190 kbp that encodes more than 150 polypeptides (47). The icosahedral virus particle contains more than 50 polypeptides and is composed of several concentric domains, including an internal DNA-containing nucleoid surrounded by a protein layer designated the core shell, an inner envelope, and an outer icosahedral capsid (8, 10, 20). An additional membrane acquired by budding through the plasma membrane envelops the extracellular virion (14).

The complex process of virus assembly occurs at specialized cytoplasmic sites, designated viral factories, and is initiated by the recruitment and modification of endoplasmic reticulum (ER) cisternae, which collapse to form the virus inner envelope, where the viral membrane proteins p54 and p17 are localized (8, 16, 21, 32, 37). This model, however, has been recently questioned, and based on data obtained using samples prepared by high-pressure freezing, it has been suggested that the inner envelope of ASFV consists of a single lipid bilayer (28). The icosahedral capsid layer, formed by protein p72, is then progressively assembled on one side of this envelope, while on the other side, the core shell domain, mainly constituted by the processing products of the polyproteins pp220 and pp62, is simultaneously constructed (6, 7, 20, 26). Finally, the viral DNA and nucleoproteins are packaged and condensed to form the nucleoid (15).

The functions of several virus proteins in the formation of the different domains of the virus particle have been investigated in recent years. Thus, the structural proteins p72 and pB438L and the nonstructural pB602L protein, described as a chaperone of p72 (22), have been shown to be required for the construction of the icosahedral capsid (24, 25, 26), while the polyprotein pp220 is essential for the formation of the inner core, constituted by the nucleoid and core shell domains (7). It has also been demonstrated that the processing of the polyproteins pp220 and pp62 by the virus-encoded protease is necessary for the assembly of a proper core (5). In addition, it is known that the transmembrane protein p54 is critical for the recruitment of envelope precursors to assembly sites (35), although the mechanisms underlying the conversion of ER cisternae into functional viral envelopes are mostly unknown. Studies of other transmembrane proteins detected as structural components of the virus particle could shed light on this matter. Some of the virion membrane proteins could also play a role in virus entry, as has been described for the proteins p12, identified as a viral attachment protein (11, 19), and p54, also involved in binding of virus to target cells (27).

The ASFV protein pE248R is a late structural component of the virus particle (33) that belongs to a class of myristoylated membrane proteins related to vaccinia virus L1 (30), one of the substrates of the pathway for the formation of disulfide bonds encoded by this virus (41). The protein pE248R also contains intramolecular disulfide bridges and has been recently identified as a possible final substrate of the ASFV-encoded redox system (33). In the present study, we investigated the membrane association, the localization in the virion, and the role of the protein pE248R in ASFV replication. Our results indicate that pE248R is a myristoylated integral membrane protein

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localized at the inner envelope of the virus particle. By using a conditional lethal ASFV mutant, vE248Ri, with an inducible copy of the gene E248R, we showed that the protein pE248R is required for virus infectivity and an early postentry event but not virus assembly.

MATERIALS AND METHODS

Cells and viruses. Vero and COS-7 cells were obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM) containing 5 and 10% fetal calf serum (FCS), respectively. The ASFV strain BA71V, adapted to grow in Vero cells, and vGUSREP, a BA71V-derived recombinant that expresses the *Escherichia coli lac* repressor, have been described before (23, 26).

Antibodies. Polyclonal antibodies against the structural proteins p72, pp220/p150, p37, pp62/p35, p54, pE120R, pE296R, and pE248R and the anti-p72 monoclonal antibody 17LD3 have been described previously (9, 29, 31, 33, 35, 39, 43, 44). The antibody against the structural membrane protein p17 will be described elsewhere (C. Suárez, M. L. Salas, and J. M. Rodríguez, unpublished data). Anti- β -actin monoclonal antibody AC-15 was purchased from Sigma.

Construction of plasmid pcDNA-E248R. To generate the plasmid pcDNA-E248R, the complete coding sequence of the gene E248R was excised from pRSETA-E248R (33) using the EcoRI and BamHI restriction enzymes and cloned into EcoRI- and BamHI-digested pcDNA 3.1 (Invitrogen).

Construction of plasmid pIND1.E248R. For the inducible expression of the gene E248R, we used the intermediate transfer vector pIND1 (9), containing the inducible promoter *p72.I**, which consists of the synthetic late promoter *p72.4* situated at a distance of 6 bp from the *E. coli lac* operator and the *lacZ* gene under the control of the late promoter *p72* (26).

For the generation of plasmid pIND1.E248R, a synthetic fragment of 760 bp containing the nucleotide sequence of the E248R open reading frame was obtained by PCR using the EcoRI E fragment as a template and the oligonucleotides 5'-GCGCCTGCAGATGGGAGGCTCTACAAGCAA and 5'-CGCGAAGCTTTTACGAAACGGCAGCATT, which contain PstI and HindIII restriction sites (underlined), respectively. Plasmid pIND1.E248R.FI was generated by inserting the PstI- and HindIII-digested PCR fragment into the PstI- and HindIII-linearized plasmid pIND1. A synthetic fragment of 540 bp containing the nucleotide sequence from the adjacent E165R open reading frame was obtained by PCR, using the EcoRI E fragment as a template and the oligonucleotides 5'-GCGCGCGGCCGCATGGCAACAAATTTTTT and 5'-GCCGGTACCTTAAGTTCTCATAATCCCGGCC, with NotI and KpnI restriction sites (underlined), respectively. The PCR fragment was inserted into the NotI and KpnI sites of plasmid pIND1.E248R.FI to obtain the final vector, pIND1.E248R.

Generation of recombinant virus vE248Ri. Recombinant virus was generated essentially as described previously (34) with minor modifications. Briefly, COS-7 cells were transfected with the linearized plasmid pIND1.E248R and infected with vGUSREP virus in the presence of different concentrations of isopropyl- β -D-thiogalactopyranoside (IPTG). At 72 h postinfection (hpi), the cells were harvested and the recombinant virus vE248Ri was isolated by sequential rounds of plaque purification in the presence of 250 μ M IPTG, a concentration that was found to be optimal for the production of the recombinant virus.

Plaque assay. Vero cell monolayers, in six-well plates, were infected with recombinant vE248Ri or parental BA71V. After 2 h, the inoculum was removed and the cells were overlaid with DMEM containing 0.55% Noble agar and 3% FCS in the presence or absence of 250 μ M IPTG. Five days later, the medium was removed and the monolayers were stained with 1% crystal violet.

One-step virus growth curves. COS-7 cell monolayers, in 24-well plates, were infected with 5 PFU of recombinant vE248Ri or parental BA71V per cell. After 2 h of adsorption, the cells were incubated in medium supplemented with 3% FCS. IPTG (250 μ M) was added, when appropriate, immediately after the adsorption period. Infected cells with their culture supernatants were harvested at different times postinfection, and titers were determined by plaque assay in the presence of 250 μ M IPTG.

Subcellular fractionation. Subcellular fractionation was performed as described previously (35). Briefly, Vero cells were mock infected or infected with 10 PFU/cell of the BA71V strain. At 20 hpi, the cells were resuspended at 10^7 cells/ml in homogenization buffer (20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 1 mM EDTA, 25 mM *N*-ethylmaleimide) supplemented with protease inhibitors (Complete EDTA-free cocktail; Roche) and passaged through a 25-gauge syringe 20 times. The homogenate was centrifuged at $750 \times g$ for 5 min to sediment nuclei and unbroken cells, and the postnuclear supernatant was subsequently

centrifuged at $100,000 \times g$ for 15 min to separate the cytosolic and membrane/particulate fractions. Membrane/particulate pellets were gently rinsed in homogenization buffer prior to resuspension in the appropriate buffer.

Triton X-114 and sodium carbonate extraction of membranes. Membrane/particulate cell fractions were treated with 2% Triton X-114 or sodium carbonate buffer (0.1 M sodium carbonate, pH 11.3) as described previously (35). Equivalent amounts of the resulting fractions were analyzed by Western immunoblotting.

In vitro transcription and translation. In vitro transcription-and-translation reactions were carried out using the TNT Quick Coupled transcription/translation system and canine pancreatic microsomal membranes from Promega with 0.5 μ g of pcDNA-E248R plasmid and 22 μ Ci of [35 S]methionine/cysteine (Perkin Elmer) in a final volume of 25 μ l. After 90 min at 30°C, the samples were treated with 4 μ g of pancreatic RNase and membranes were sedimented by centrifugation for 15 min at 30 lb/in² in an Airfuge ultracentrifuge. Treatment with proteinase K was performed for 1 h at 37°C with 0.5 mg/ml of the enzyme and 1% sodium dodecyl sulfate (SDS) in the presence or absence of 1% Triton X-100. All samples were analyzed by electrophoresis in SDS-12 to 20% polyacrylamide gels followed by fluorography. To determine the size of the protected fragment, unstained molecular size standards (6 to 200 kDa) from Bio-Rad and the 6.5-kDa p56 protein of phage ϕ 29 (kindly provided by M. Salas) (42) were used.

Western blot analysis. Preconfluent Vero or COS-7 cell monolayers were either mock infected or infected with BA71V or recombinant vE248Ri virus in the presence or absence of 250 μ M IPTG at a multiplicity of infection of 10 PFU per cell. The cells were lysed at the appropriate times in Laemmli sample buffer, and equivalent amounts were electrophoresed in SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with antibodies as appropriate. Protein detection was carried out with peroxidase-conjugated antibodies and the ECL system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Radiolabeling and immunoprecipitation. Preconfluent monolayers of Vero cells were mock infected or infected with BA71V at 5 PFU per cell. At 16 hpi, cells were pulse-labeled for 3 h with 200 μ Ci/ml of [3 H]myristic acid (30 Ci/mmol; Perkin Elmer) in Opti-MEM (Invitrogen) without serum. At the end of the labeling period, the medium was removed and cells were washed with phosphate-buffered saline (PBS) and lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 1% Nonidet-P40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄, and protease inhibitor cocktail. Lysates were clarified by centrifugation at 14,000 rpm for 15 min at 4°C and incubated overnight at 4°C with 1:40 rabbit anti-E248R antibody or control preimmune serum. The antigen-antibody complexes were then rotated with 100 μ l of protein A-Sepharose beads for 1 h at 4°C. The immune complexes were washed three times with a buffer containing 50 mM Tris-HCl, pH 8.1, and 100 mM NaCl and finally resuspended in electrophoresis sample buffer. After reducing SDS-polyacrylamide gel electrophoresis (PAGE), radioactive proteins were detected by fluorography.

Localization of protein pE248R by immunofluorescence microscopy. Vero cells, grown on coverslips, were mock infected or infected with BA71V at a multiplicity of infection of 1 PFU/cell and fixed at 16 hpi with methanol at -20°C for 5 min. The cells were then incubated with rabbit anti-E248R antibody (1:1,000 dilution) and rat anti-p54 antibody (1:200 dilution) for 1 h at room temperature and then with the secondary antibodies (1:500 dilution) for 45 min at room temperature. The secondary antibodies used were goat anti-rabbit immunoglobulin G (IgG) coupled to Alexa 488 and goat anti-rat IgG coupled to Alexa 594, both from Molecular Probes. 4',6'-Diamidino-2-phenylindole was used along with the secondary antibodies to stain DNA in nuclei and viral factories. Coverslips were mounted with Mowiol/Dabco on glass slides, and the preparations were examined with a Zeiss Axioskop2 plus microscope coupled to a charge-coupled-device color camera. Images were processed using the Adobe Photoshop software program.

Electron microscopy. For conventional Epon section analysis, COS-7 cells were infected with BA71V or vE248Ri at 5 PFU per cell and fixed at the indicated times with 2% glutaraldehyde in PBS for 1 h at room temperature. Postfixation was carried out with 1% OsO₄ and 1.5% K₃Fe(CN)₆ in H₂O at 4°C for 30 min. Samples were dehydrated with acetone and embedded in Epon according to standard procedures. For immunoelectron microscopy, BA71V-infected Vero cells were fixed at the indicated time with 8% paraformaldehyde and 0.2% glutaraldehyde in 120 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), 50 mM HEPES, 4 mM MgCl₂, and 20 mM EGTA, pH 6.9, for 2 h at room temperature. The fixative was then removed, and a solution of 8% paraformaldehyde in 60 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), 25 mM HEPES, 2 mM MgCl₂, and 10 mM EGTA, pH 6.9, was added. Finally, the fixed cells were prepared for cryosectioning and immunogold labeling as described previously (7, 8). Specimens were examined at 80 kV in a Jeol 1010 electron microscope.

Infection of cells with vE248Ri(+) and vE248Ri(-) virus particles. COS-7 or Vero cells were infected with 1 PFU/cell of extracellular parental BA71V and with equivalent amounts of extracellular vE248Ri(+) or vE248Ri(-) virions (produced in the presence or absence of IPTG, respectively), as determined by Western blot quantification of the capsid protein p72 using the Quantity One software program (Bio-Rad) (not shown). For assessment of cytopathic effect, the cells were examined by phase-contrast microscopy at 48 hpi.

Virus binding and internalization assays. Vero cells in glass coverslips were infected with 5 PFU/cell of extracellular BA71V and equivalent amounts of extracellular vE248Ri(+) or vE248Ri(-) virions, determined as indicated above, in the presence of 100 μ g/ml of cycloheximide. After 2 h at 4°C in DMEM containing 25 mM HEPES, pH 7.4, and 2% FCS for virus binding, the cells were washed thoroughly with DMEM containing 2% FCS and shifted to 37°C for 4 h to allow virus internalization in the presence of cycloheximide. The cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min. The cells were then permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature, blocked with 1% bovine serum albumin in PBS at room temperature, and incubated with anti-p37 antibody (1:400 dilution) and anti-p72 monoclonal antibody (1:100 dilution) in blocking solution. After extensive washing with PBS, the cells were incubated for 1 h at room temperature with Alexa Fluor 555 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG antibodies, diluted 1:500 in blocking solution. TO-PRO-3 (1:500 dilution) was used along with the secondary antibodies to stain DNA. Coverslips were mounted on glass slides using Prolong gold. The preparations were examined with a confocal LSM510 Meta laser scanning microscope.

Analysis of mRNA by reverse transcription-PCR (RT-PCR). Total RNA was extracted from mock-infected or infected Vero cells by the TRIzol reagent (Invitrogen) and reverse transcribed into cDNA by using the RevertAid First Strand cDNA synthesis kit (MBI Fermentas). PCR amplification was performed with the addition of *Taq* DNA polymerase (Roche Applied Science) and the following primers: β -actin (forward, 5'-GAG AAG ATG ACC CAG ATC ATG-3'; reverse, 5'-TCA GGA GGA GCA ATG ATC TTG-3'), B646L (p72) (forward, 5'-ATG GCA TCA GGA GGA GCT TTT TGT CTT ATT-3'; reverse, 5'-TTA GGT ACT GCA ACG CAG CAC AGC TGA ACC G-3'), and E296R (pE296R) (forward, 5'-GCG CGC GAA TTC TTA TTT TAA TAA CGA TTT TAA TGT A-3'; reverse, 5'-CGC GCG CTC GAG ATG TTT GGA GCT TTT GTA AGC CAC C-3'). The PCRs were performed as follows: one cycle at 95°C for 1 min, three cycles at 95°C for 2 min, annealing at 45°C for 1.5 min, and extension at 72°C for 4 min. This was followed by 25 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 3 min and finally 1 cycle of extension at 72°C for 10 min. Amplified cDNAs were subjected to agarose gel electrophoresis, and bands were visualized by ethidium bromide staining.

RESULTS

The protein pE248R is a myristoylated and membrane-associated polypeptide. The amino acid sequence of the protein pE248R contains an N-terminal N myristoylation site (²GGSTSK⁷) (12) and a C-terminal putative transmembrane region (¹⁹⁴SAVFNIMVAAVVIVLIIVGFIA²¹⁶) (36). Therefore, we first examined whether the protein pE248R was myristoylated during ASFV infection by labeling the cells with [³H]myristic acid for 3 h at 16 hpi, followed by immunoprecipitation of cell extracts with anti-pE248R antibody. As shown in Fig. 1A and in agreement with previous results (1), three myristoylated bands of about 200, 90, and 28 kDa were detected in ASFV-infected cells. The 200-kDa band probably corresponds to the polyprotein pp220, which is known to be modified by myristoylation (44). The 28-kDa polypeptide was specifically immunoprecipitated with the anti-E248R antibody, indicating that the protein pE248R is myristoylated during the infection.

To determine whether the protein pE248R was associated with membranes, ASFV-infected cells, collected at 20 hpi, were subjected to subcellular fractionation as indicated in Materials and Methods, and the presence of the protein pE248R in the cytosolic and membrane fractions was analyzed by West-

FIG. 1. Myristoylation and membrane association of protein pE248R. (A) Mock-infected (M) and ASFV-infected (I) Vero cells were labeled with [³H]myristic acid for 3 h at 16 hpi, and cell extracts were analyzed by electrophoresis in SDS-12% polyacrylamide gels before (TOTAL) and after immunoprecipitation with anti-pE248R antibody (Δ E248R) or preimmune serum (PI). Molecular mass markers are indicated on the left (in kDa). The labeled bands are indicated by arrows. (B) Subcellular distribution of protein pE248R in ASFV-infected cells. Infected cells were disrupted at 20 hpi and fractionated into cytosolic (C) and membrane/particulate (M) fractions, as described in Materials and Methods. The membrane fraction was treated with sodium carbonate, and after centrifugation, equivalent amounts of the supernatant (S) and sediment (P), along with 2 μ g of purified ASFV particles (V), were analyzed by immunoblotting using the anti-pE248R antibody. Alternatively, the membrane fraction was extracted with Triton X-114 and subjected to temperature-induced phase separation. After centrifugation, the aqueous (A) and detergent-rich (D) phases were analyzed as before. The band corresponding to the protein pE248R is indicated by an arrow. Molecular mass markers are indicated on the left (in kDa).

ern blotting. As can be seen in Fig. 1B, the protein pE248R was detected only in the membrane fraction. To investigate the type of association of the protein with membranes, the membrane fraction was incubated with 100 mM sodium carbonate, pH 11.3, a treatment that dissociates peripheral proteins from membranes. As shown in Fig. 1B, the protein pE248R remained associated with the membrane sediment after such treatment. After treatment of the membrane fraction with Triton X-114 and temperature-induced phase separation, the protein pE248R partitioned entirely into the detergent phase (Fig. 1B). These results indicate that the protein pE248R behaves as an integral membrane protein, in agreement with its myristoylated state and the presence of a C-terminal hydrophobic region.

To investigate the topology of the protein pE248R, the protein was translated in vitro with or without membranes and then treated (or not) with proteinase K and analyzed by SDS-PAGE and fluorography. The transmembrane sequence of pE248R divides the protein into two unequal halves, a large

FIG. 2. Topology of membrane-associated pE248R. (A) The protein pE248R was translated in vitro as indicated in Materials and Methods in the absence (–) or presence of 2 and 4 μ l of canine microsomal membranes. Soluble (S) and membrane-associated (P) fractions were analyzed by electrophoresis in SDS-12 to 20% polyacrylamide gels under reducing conditions. (B) After in vitro translation, the membrane fraction was treated (+) or not (–) with proteinase K in the absence (–) or presence (+) of Triton X-100, and the samples were analyzed by SDS-PAGE under reducing conditions. The position of pE248R and that of the protected fragment are indicated by arrows. Molecular mass markers are indicated on the left (in kDa).

N-terminal region of about 22 kDa and a small C-terminal fragment of about 6.5 kDa. As shown in Fig. 2A, after centrifugation of the reaction mixture, the intensity of the 35 S-labeled protein, detected in the supernatant fraction, increased somewhat in the presence of membranes. The protein band was also detected in the pellet fraction when microsomes were present. After proteinase K treatment of the membrane fraction, a protected fragment of approximately 6.5 kDa was observed,

while no protected fragments were detected when the membranes were solubilized with Triton X-100 (Fig. 2B). These results suggest that the protein pE248R adopts a type II topology whereby its large N-terminal portion containing the four cysteine residues is oriented to the cytoplasm. **pE248R is present in the viral factory and localizes at the inner envelope of the viral particle.** As previously reported (33), the protein pE248R is a late structural component of the virus particle. According to this, an examination of cells infected with BA71V by immunofluorescence with anti-pE248R serum and with an antibody against the virion protein p54 showed the presence of the protein pE248R in the viral factories colocalizing with the p54 protein (Fig. 3). These results, together with the association of the pE248R protein with membranes in infected cells, suggested that the protein might be localized at the inner viral envelope, the only membranous structure of the intracellular virus. To assess this, we performed immunoelectron microscopy analysis of cells infected with ASFV. As can be seen in Fig. 4, the antibody against the protein pE248R labeled mainly the inner envelope layer of virus particles in the cytoplasmic factories.

Inducible expression of protein pE248R by recombinant virus vE248Ri. To investigate the role of the protein pE248R in ASFV replication, we generated a recombinant vE248Ri virus, in which the expression of the gene E248R is under the control of the *E. coli lac* operator/repressor system (Fig. 5A). For this, the BA71V-derived recombinant virus vGUSREP (26), constitutively expressing the *E. coli lac* repressor, was modified by replacing the original promoter of the gene E248R by an inducible promoter, *p72.I**, constituted by the late viral promoter *p72.4* and the operator sequence *O₁* (26).

The inducer dependence of recombinant vE248Ri was tested by performing a plaque assay in the presence and in the absence of 250 μ M IPTG. In the presence of the inducer, the lysis plaque numbers and the plaque sizes were similar for both parental and recombinant viruses, while in its absence, the plaque number for recombinant vE248Ri was drastically reduced (not shown).

FIG. 3. Immunofluorescence microscopy analysis of BA71V-infected Vero cells. Mock-infected (Mock) or BA71V-infected (BA71V) cells were fixed at 16 hpi and double labeled with anti-E248R antibody detected with Alexa 488 goat anti-rabbit IgG (pE248R) and with anti-p54 antibody detected with Alexa 594 goat anti-rat IgG (p54). Cells were counterstained with 4',6-diamidino-2-phenylindole to visualize cellular and viral DNA. Arrowheads indicate the positions of some viral factories.

FIG. 4. Localization of protein pE248R in virus particles. BA71V-infected cells were fixed at 24 hpi and processed for cryosectioning. Ultrathin sections were incubated with anti-pE248R antibody followed by protein A-gold (10-nm diameter). White arrows indicate labeling on the inner envelope of the virus particles. The capsid (c) and inner envelope (ie) layers are indicated by black arrows in the lower panel. Bars, 100 nm.

As can be seen in Fig. 5B, pE248R protein expression is regulated by the inducer in vE248Ri infections. Thus, the protein band was not detectable by immunoblotting in extracts from cells infected with recombinant vE248Ri in the absence of IPTG, while in the presence of the inducer, the expression of the protein was comparable to that with the parental virus.

We also examined the effect of pE248R protein repression on virus replication by performing one-step growth curve analyses. Figure 5C shows that under permissive conditions, the maximum virus titers of recombinant vE248Ri were close to those obtained with the parental BA71V. In contrast, under restrictive conditions, vE248Ri production was reduced by more than 2 log units at all postinfection times examined.

Polyprotein processing is not impaired in vE248Ri infections under restrictive conditions. As previously described, proteolytic processing of the two virus polyproteins pp220 and pp62 is a sensitive biochemical indicator of correct virus assembly, the processing being impaired when the normal morphogenetic processes are altered (7, 24, 25, 35). We therefore examined whether polyprotein processing was affected in infections with vE248Ri under restrictive conditions, as a first

FIG. 5. (A) Genomic structure of ASFV recombinant vE248Ri. The recombinant virus vE248Ri was obtained from vGUSREP, a BA71V-derived recombinant virus, which contains the *lac* repressor-encoding gene *lacI* inserted in the nonessential thymidine kinase locus (26). In the vE248Ri virus, the promoter of the gene *E248R* was replaced by an inducible promoter, *p72.1**, which is composed of a strong late promoter (*p72.4*) and the operator sequence O1 from the *E. coli lac* operon. (B) Inducible expression of protein pE248R. COS-7 cells were either mock infected (M) or infected with parental BA71V (WT) or recombinant vE248Ri virus in the presence (+) or absence (-) of 250 μ M IPTG. At 12 hpi, the cells were lysed and analyzed, along with 2 μ g of purified ASFV particles (V), by immunoblotting with antibodies against the proteins pE248R and p72 (loading control). The positions of the detected proteins are indicated. Molecular mass markers are indicated on the left (in kDa). (C) One-step growth curves of vE248Ri virus. COS-7 cells were infected with 5 PFU of vE248Ri virus per cell in the presence or absence of 250 μ M IPTG. At the indicated times of infection, the total virus titer of each sample was determined by plaque assay on Vero cells in the presence of the inducer. Parental BA71V infections were also titrated as a control.

step to investigate the role of the pE248R protein in virus replication. As shown in Fig. 6, in the absence of the inducer, the processing of the two virus polyproteins occurred to a similar extent to that in infections under permissive conditions with the recombinant virus or to that in control BA71V-infected cells, indicating that pE248R expression was not re-

FIG. 6. Polyprotein processing in cells infected with vE248Ri virus. COS-7 cells were mock infected (M) or infected with parental BA71V (WT) or recombinant vE248Ri virus in the presence (+) or absence (-) of IPTG. At 12 hpi, the cells were lysed and analyzed by immunoblotting with antibodies against the polyprotein pp220 and its mature products p150 and p37 or the polyprotein pp62 and its mature product p35. A sample of 2 μ g of highly purified ASFV (V) was also analyzed. The positions of the detected proteins are indicated. Molecular mass markers are indicated on the left (in kDa).

quired for this step. This result was somewhat surprising, since the protein pE248R is a structural protein of the virus particles, and provided the first indication that it might not be involved in virion assembly.

Normal virus particles are assembled in the absence of pE248R. To further investigate the phenotype of the recombinant vE248Ri virus, we examined by electron microscopy cells infected with vE248Ri virus in the presence or in the absence of IPTG for 24 h. As shown in Fig. 7, the viral factories in cells infected with the recombinant virus under restrictive conditions (panel C) were similar to those found in infections carried out in the presence of IPTG (panel B) or with the parental BA71V (panel A). In all cases, the factories contained abundant precursor membranes and similar numbers of immature and mature icosahedral virions. A more-detailed inspection of the vE248Ri “full” virions generated under restrictive (panel F) conditions did not reveal any significant ultrastructural difference with normal mature virus (panel D) or the virions generated under permissive (panel E) conditions, although the mutant particles must be defective, since they are not infectious. It can also be noticed that the virions produced in the absence of IPTG (panel H) are released from the cells by budding through the plasma membrane like those formed in the presence of the inducer (panel G). The exit of mutant particles from the viral factory is also observed by immunofluorescence microscopy (results not shown). Furthermore, the fact that the mutant vE248Ri virions can be obtained and purified from the extracellular medium, as indicated below, further supports the idea that the mutant virus can exit normally from the infected cells.

To further characterize the mutant vE248Ri particles, we purified them from the supernatants of infected cells by Percoll gradient centrifugation (18) to examine their protein composition. There was no apparent difference in the overall protein composition of 35 S-labeled BA71V and vE248Ri particles obtained in the absence [vE248Ri(-)] of IPTG as determined by SDS-PAGE (Fig. 8A). The two main labeled bands observed correspond to the core shell protein p150 and the capsid protein p72. In addition, Western blot analysis of extracellular

virions shows that the vE248Ri(-) particles contain the capsid protein p72, the inner envelope protein p17 (37), and the core shell p37 protein, product of polyprotein pp220, in amounts similar to those found in BA71V and vE248Ri(+) virus but, as expected, lack the protein pE248R, which is present in the wild-type and vE248Ri(+) virions (Fig. 8B).

Noninfectious vE248Ri(-) virus is unable to produce cytopathic effect. We investigated the capacity of noninfectious vE248Ri(-) particles to induce a cytopathic effect following infection of COS-7 cells. In agreement with results in previous studies, infection of cells with parental BA71V virus produced a strong cytopathic effect (Fig. 9), which is also observed in the presence of Ara C (not shown), indicating that this phenomenon is not dependent on late processes of the viral cycle. A similar effect, with extensive cell rounding, is found when cells are infected with vE248Ri(+), which had been produced in the presence of IPTG (Fig. 9). In contrast, vE248Ri(-) virions grown in the absence of IPTG were unable to cause a detectable cytopathic effect in either the absence (Fig. 9) or presence (not shown) of Ara C, with the infected cells resembling mock-infected cells.

pE248R is not required for virus binding or internalization. The results described above indicated that the protein pE248R was not required for morphogenesis and that the vE248Ri(-) virus produced in the absence of IPTG was unable to cause a cytopathic effect, raising the possibility that the defect of the mutant virus assembled in the absence of the pE248R protein could be related to an impairment of the virus entry process or some other early event in infection. To investigate this, we followed virus binding and penetration into cells by using immunofluorescence microscopy. For this, we used two antibodies. One of them recognizes the capsid protein p72, and the other one recognizes the core shell domain protein p37, one of the proteolytic products of the pp220 polyprotein. As can be seen in Fig. 10, after virus binding for 2 h at 4°C, the p72 antibody recognized a punctate signal, likely corresponding to virus particles bound to cells, in the infection with parental BA71V, vE248Ri(+), or vE248Ri(-) virus, while no signal was detected with the anti-p37 antibody. This result is in agreement with results in previous studies showing that at 4°C, virus binding but not internalization occurs (3).

When the cells are shifted to 37°C after 2 h at 4°C, which according to previous results allows virus internalization (3), and further incubated for 4 h at this temperature in the presence of cycloheximide, red punctate labeling can be observed with the anti-p37 antibody. Importantly, this signal is found in cells infected with the three viruses BA71V, vE248Ri(+), and vE248Ri(-) and does not colocalize with that of p72, strongly suggesting that the p37 signal corresponds to internalized subviral particles devoid of the capsid protein. Altogether, these results suggest that the absence of the pE248R protein does not impair the binding and penetration of the virus into cells.

pE248R is required for an early postentry event. The above findings suggested that the defect of the mutant vE248Ri(-) virus could be related to some postentry event, like early gene expression. To examine this possibility, cells were infected with 5 PFU/cell of parental BA71V virus or an equivalent amount of vE248Ri(+) or vE248Ri(-) virus in the presence of AraC to prevent DNA replication and late transcription. Parallel infections in the absence of AraC were carried out to follow

FIG. 7. Electron microscopy of vE248Ri-infected cells. COS-7 cells infected with parental BA71V (A) or with vE248Ri virus in the presence (B) or in the absence (C) of IPTG were fixed at 24 hpi and processed for conventional electron microscopy. The overall appearance of the viral factories is similar under all conditions, with the presence of numerous precursor viral membranes (arrowheads), as well as immature (open-head arrows) and mature (filled-head arrows) virions. As shown at a higher magnification, the morphology of the “full” vE248Ri virions generated in the absence of the inducer (F) is indistinguishable from that of the recombinant virus formed under permissive conditions (E) or of parental BA71V virus (D). Panels G and H show the budding and release of vE248Ri virus produced in the presence and absence of IPTG, respectively. Arrows indicate budding and released virus.

late protein expression. Western blot analysis was used to assess the expression of the early protein pE296R and the late capsid protein p72 (Fig. 11A). In cells infected with BA71V or vE248Ri(+) virus, the protein pE296R was detected in both the presence and absence of AraC, as expected of an early protein and as described previously (31). The protein p72 was also detected in these infections, only in the absence of AraC, in agreement with the late expression of this protein. In clear contrast, neither the pE296R nor the p72 protein was observed in the case of cells infected with the mutant vE248Ri(−) virus,

indicating that the infection with the pE248R-defective virus is arrested prior to early protein expression.

We also examined viral early and late RNA synthesis by RT-PCR analysis using specific primers for pE296R and p72 mRNAs, as well as for β -actin mRNA, used as a control for RNA loading and integrity. pE296R mRNA was seen in cells infected with BA71V and vE248Ri(+) virus in the presence and absence of AraC but not in the vE248Ri(−) infection (Fig. 11B). The p72 mRNA was detected in the absence of AraC in wild-type- and vE248Ri(+)-infected cells but not in cells in-

FIG. 8. Polypeptide composition of vE248Ri(–) virus. (A) ³⁵S-labeled BA71V and vE248Ri virus grown in the absence of IPTG [vE248Ri(–)] were purified by Percoll gradient centrifugation (18) and analyzed by SDS-PAGE followed by fluorography. The two main labeled bands, corresponding to the proteins p150 and p72, are indicated by arrows. Molecular mass markers (in kDa) are indicated on the left. (B) The presence of the proteins p72, p37, pE248R, and p17 was analyzed by Western blotting in BA71V, vE248Ri(+), and vE248Ri(–) virus. Molecular mass markers (in kDa) are indicated on the left.

fected with vE248Ri(–) virus. These results are in agreement with those obtained when examining viral protein expression and support the view that the block in gene expression observed in the absence of the protein pE248R occurs at the transcriptional step.

DISCUSSION

ASFV membrane proteins may play crucial roles in the pathways leading to the acquisition and modification of endoplasmic reticulum (ER) cisternae into the precursors of the viral envelope, as has been described for the membrane protein p54 (35). Additionally, some virion membrane proteins could function in virus entry into target cells, a process that has been reported to occur through a receptor-mediated endocytosis mechanism (2–4), with the participation of the proteins p12 and p54, which are involved in the attachment of virions to specific receptors in the host cell (11, 19, 27).

In this report, we have described the essential role of another ASFV structural transmembrane protein, pE248R, in the early steps of virus infection. The protein pE248R, which contains a hydrophobic C-terminal region and is myristoylated during the infection, behaves as an integral membrane protein and is localized in the inner envelope of mature virions as shown by immunoelectron microscopy. In vitro studies of its membrane topology indicate that the large N-terminal domain, containing four conserved cysteine residues, faces the cytosol, while the small C-terminal part resides inside the luminal space. This situation is similar to that observed with the protein p54, except that in this case, the small N terminus is the luminal part of the protein (35). The presence of a myristoyl group at the amino end suggests that the protein pE248R may be anchored to the inner envelope of the virus particle by both the myristoyl and the transmembrane region near the C terminus. The topology of the protein is consistent with previous

FIG. 9. Lack of cytopathic effect following infection with vE248Ri(–) virus produced in the absence of IPTG. COS-7 cells were mock infected (Mock) or infected with parental BA71V (BA71V), vE248Ri(+) virions grown in the presence of IPTG [vE248Ri(+)], or vE248Ri(–) virions grown in the absence of IPTG [vE248Ri(–)]. At 48 hpi, cells were examined by phase-contrast microscopy.

FIG. 10. Binding and internalization of vE248Ri virus. Vero cells were incubated with 5 PFU/cell of BA71V or equivalent amounts of vE248Ri(+) or vE248Ri(−) virus, as indicated. The cells were fixed after 2 h at 4°C (0 hpi) or after shifting to 37°C and further incubating for 4 h (4 hpi). The cells were double labeled with anti-p72 monoclonal antibody detected with Alexa Fluor 488 goat anti-mouse IgG (green) and anti-p37 detected with Alexa Fluor 594 goat anti-rabbit IgG (red). Cells were counterstained with TO-PRO-3 to visualize DNA. The arrows point to the red signal, corresponding to p37.

results suggesting that the cytoplasmic ASFV-encoded redox chain catalyzes the formation of the intramolecular disulfide bridges of pE248R (33).

Using ASFV recombinant vE248Ri that inducibly expresses the gene E248R, we have demonstrated that the protein pE248R is essential for the formation of infectious progeny virus. However, repression of the protein pE248R did not block virus assembly, and mature virions formed in the absence of this protein were morphologically indistinguishable from wild-type virions at the electron-microscopic level. Also, no difference in overall polypeptide composition and in the amounts of the proteins p72, p17, and p37, representative of the capsid, inner envelope, and core shell domains, respectively, was observed between wild-type and mutant particles lacking the protein pE248R. Furthermore, processing of the virus polypeptides was not impaired in the absence of the protein pE248R, in keeping with the observation that proteolytic processing is a late maturational event related to the development of full icosahedral virions (7, 24, 25, 35). Nevertheless, although apparently normal, the ASFV particles lacking pE248R are defective, since their infectivity was at least 100-fold less than that of wild-type virus.

We have explored the defect of the mutant virus lacking the protein pE248R by examining the first stages of infection. Here we define virus entry as the steps leading to the appearance of subviral particles recognized by an antibody against the core shell protein p37 and lacking the capsid protein p72. According

to this, the entry of pE248R-deficient virions, including virus binding and internalization, is not affected by the absence of this protein. However, early and late gene expression is impaired, and no cytopathic effect is observed, in infection with the pE248R-defective mutant virus, indicating that the protein is required for some early postentry event.

Electron microscopic studies of the sequential events of the entry process first show virus particles adsorbed to the cell surface and in membrane invaginations. Soon after internalization, which is a temperature- and energy-dependent process, cytoplasmic vesicles containing a single virus particle are seen near the plasma membrane, while at later times large vacuoles with several particles are found. Thereafter, viral “cores” in the cytoplasm can be observed (3, 4). The mechanisms by which the virus is uncoated and exits the vacuolas are not well understood, although these steps are known to be inhibited by lysosomotropic agents, indicating the requirement of an acidic environment, and under these conditions, the virions are found retained in the intracellular vacuolas (3, 45). Although no evidence of fusion of viral and cell membrane has been found during the internalization step, membrane fusion might occur during the passage of virions through the cytoplasmic vacuolar membrane to deliver the viral “cores” into the cytoplasm (46).

We cannot ascertain at present whether the virus particles detected in the immunofluorescence microscopy assays after the binding step represent subviral particles within intracellu-

FIG. 11. Analysis of early and late gene expression in cells infected with parental BA71V and vE248Ri virus. Vero cells were mock infected (M) or infected with 5 PFU/cell of BA71V (WT) or equivalent amounts of vE248Ri(+) or vE248Ri(-) virus. AraC (40 μ g/ml) was added or not as indicated, and the cells were collected at 18 hpi. (A) Cell extracts were analyzed by Western blotting using antibodies against the early protein pE296R or the late protein p72. As a loading control, an anti- β -actin antibody was used. The sizes (in kDa) of molecular mass markers are indicated on the right. (B) Total RNA was extracted, and mRNA for p72, pE296R, and β -actin was analyzed by RT-PCR as indicated in Materials and Methods. Size markers (in nucleotides) are indicated on the right.

lar vacuolas or “cores” in the cytoplasm. In an attempt to clarify this issue, we used the lysosomotropic drug chloroquine to block the virus in the vacuoles. However, in the presence of the drug, we still can see the punctated signal with the anti-p37 antibody, which does not colocalize with the green puncta corresponding to p72 (results not shown), suggesting that at least some of the virions within the vacuoles are present as subviral particles devoid of the capsid layer. Nevertheless, the block in the first stages of infection observed in cells infected with pE248R-deficient virions would be consistent with virus retention in the intracellular vacuoles under these conditions, since early gene expression is thought to occur from viral “cores” in the cytoplasm with the transcriptional machinery packaged into the virions (38). Virus block within the vacuoles could also explain the absence of a cytopathic effect, which has been related to cell death by apoptosis, a process that is not triggered in the presence of chloroquine (17). Clearly, at present we cannot rule out the possibility that the protein pE248R might play a direct or indirect role in early transcription from cytoplasmic “cores” or in the induction of a cytopathic effect.

As mentioned in the introduction, the protein pE248R has been described as belonging to a group of myristoylated proteins related to vaccinia virus L1, which has been shown to be required for cell entry and membrane fusion and to associate with the viral entry fusion complex formed by at least eight proteins (13, 40). The properties of pE248R described here, its requirement for virus infectivity but not assembly, and its func-

tion in early steps of the infection further support this relationship, even though the entry and desencapsidation processes of ASFV may be quite different from those operating in the case of vaccinia virus. Thus, an attractive hypothesis would be that the protein pE248R is involved in the exit of the virus from intracellular vacuoles in a mechanism requiring membrane fusion. Further investigations of this protein, including studies of its possible association with other virion membrane proteins and fusogenic activity, will help to elucidate the mechanisms of ASFV entry and postentry pathways and contribute to a better knowledge of ASFV-cell early interactions.

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