Characterization of African swine fever virion proteins j5R and j13L: immuno-localization in virus particles and assembly sites

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The j5R open reading frame (ORF) of the Malawi LIL 20/1 African swine fever virus (ASFV) isolate encodes a 111 amino acid protein with a putative transmembrane domain at the N terminus. Antisera raised against the predicted C-terminal peptide were used to identify the j5R protein by Western blotting in cells infected with ASFV or with recombinant vaccinia virus expressing the j5R ORF. This showed that the j5R protein migrates with an apparent molecular mass of 23–25 kDa, depending on the virus isolate, on SDS–PAGE and is expressed late during ASFV infection. The localization in infected cells and in virions of the j5R protein, and that of a previously characterized virion protein, j13L, which also contains a putative transmembrane domain, were studied by immunofluorescence and immuno-electron microscopy. Both proteins were expressed at 8–10 h post-infection (p.i.) as small multiple perinuclear foci which coalesced to a single area indicative of the virus factory at 18 h p.i. At the ultrastructural level j5R and j13L were detected mainly on membrane-like structures within the virus factory and on virus particles, suggesting that they may be involved in particle assembly. Negative contrast immuno-electron microscopy of mature extracellular virions confirmed that they are also integral structural proteins.

Introduction

African swine fever virus (ASFV) is the causative agent of a highly contagious swine disease characterized by haemorrhages and fever, which can range from unapparent to acute with high mortality (Moulton & Coggins, 1968). The virus is currently endemic in Sardinia and many African countries and represents a significant threat to the swine industry as there is no effective vaccine available (reviewed by Wilkinson, 1989).

Recently re-classified as the single member of the genus ‘African swine fever-like viruses’ (Dixon et al., 1995), this large (180–200 nm) icosahedral virus has morphological similarities with the iridoviruses whilst its genome and replicative strategies are similar to those of the poxviruses (reviewed by Vinuela, 1985; Murphy, 1995).

The virus particles are structurally complex, including a genome of 170–190 kbp containing about 150 open reading frames (ORFs) encoding approximately 50 structural proteins and in the region of 100 non-structural proteins (Dixon et al., 1994; Yanez et al., 1995). Each particle is composed of an 80 nm nucleoprotein core surrounded by a core shell, an icosahedral membrane–capsid complex and an outer envelope derived from the plasma membrane (Carrascosa et al., 1984; Vinuela, 1985; Cobbold et al., 1996; Andres et al., 1997; Rouiller et al., 1998). Replication of ASFV, including DNA synthesis (Vigario et al., 1967; Moulton & Coggins, 1968; Pan et al., 1980; Garcia-Beato et al., 1992) and particle assembly, is associated with perinuclear inclusions known as ‘virus factories’ (Moura-Nunes et al., 1975). The virus factory contains membrane elements of endoplasmic reticulum (ER) origin and virus particles at various stages of assembly (Moura-Nunes et al., 1975; Brookes et al., 1996; Rouiller et al., 1998). The particles leave the virus factory as a prelude to release from the cell by budding through the plasma membrane where the outer envelope is acquired (Breese & Deboer, 1966; Moura-Nunes et al., 1975).

Virus proteins inserted in these virion membranes are predicted to contain at least one transmembrane domain. Twenty-six ASFV ORFs have been shown to encode proteins which contain putative transmembrane domains (Yanez et al., 1995). Of these, five (p12, p17, p22, p54 also known as j13L, and j18L) have been found in purified virus particles (Camacho & Vinuela, 1991; Carrascosa et al., 1993; Simon-Mateo et al., 1993; Rodriguez et al., 1994, 1996; Sun et al., 1995, 1996).
general, virus transmembrane proteins play important roles in many aspects of virus replication and morphogenesis, and are often involved in eliciting immune responses. They may therefore contribute to a control strategy either by incorporation into a subunit vaccine or through the construction of replication deficient deletion mutants. A useful indication of their potential role in these events is often related to the location of the protein within the virus particle and virus associated structures in infected cells. This is an important aspect of the current study which focuses on two putative membrane associated viral proteins, j5R and j13L. We show that the protein encoded by the j5R ORF is incorporated into virions and has a similar location in membrane structures in virus factories, and in immature and mature virions, to that of the j13L protein.

Methods

■ Cells and virus. IB-RS2, BSC40 and Hu TK^-143 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) growth medium or 2% (v/v) maintenance medium foetal calf serum (FCS). Pig bone marrow (PBM) cells were prepared from normal outbred pigs and grown in Eagle's medium supplemented with 12% (v/v) normal pig serum (Malmquist & Hay, 1960). Vaccinia virus (VV) Western Reserve (WR) strain was used to construct a recombinant VV. Two ASFV isolates were used predominantly: the virulent ASFV Malawi LIL 20/1 isolate (Haresnap et al., 1988) and the attenuated Uganda isolate (Hess et al., 1965). Other ASFV isolates used included Lisbon 57 (Lis 57), Lisbon 60 (Lis 60), Burundi 84/2 (Bur 84/2), Malawi Bongora 83 (Bon 83), which have been described elsewhere (Sun et al., 1995), and Tanzania 87 (Tanz 87), a gift from E. C. Anderson (Southern Highlands, Tanzania), and Mozambique 60 (Moz 60), a field isolate available at the IAH.

■ Plasmid and bacterial strains. Plasmid pGS53 (Chakrabarti et al., 1985) contains the VV p7.5 promoter within the VV thymidine kinase gene and was used to construct VV recombinants. DNA manipulations were performed using standard methods (Sambrook et al., 1989) and E. coli DH5α was used as the host for cloning.

■ PCR. PCR reactions were carried out using an annealing temperature of 55 °C and Taq DNA polymerase (Boehringer). DNA from ASFV purified from pigs infected with different ASFV strains (Wesley & Tuthill, 1984) or recombinant VV-infected cell lysates were used as the DNA template.

■ Preparation of infected cell lysates and of purified virus. PBM, IB-RS2 or BSC40 cells were infected with different ASFV strains or recombinant VV at an m.o.i. of 3–10. The infected cells were harvested at different times post-infection (p.i.) and lysed on ice with 50 mM PBM, IB-RS2 or BSC40 cells were infected with different ASFV strains or

■ Construction of a recombinant VV expressing j5R. The j5R ORF was amplified by PCR using clone LMW18 (Dixon, 1988), which contains the SalI j fragment of the Malawi LIL 20/1 isolate genome, as the DNA template, a forward primer (5'-GCGCCGATCCATGCTTTCCTTTTCTGT) containing a BamHI site and a reverse primer (5'-GGCGCGGTCGACCGCTTGACCGAGAGG-CTC) containing a SmaI site. The PCR product was cloned using BamHI and SmaI digestion and the pG535 plasmid. The recombinant plasmid pGSj5R was transfected into VV WR-infected Hu TK^-143 cells using lipofectin (GIBCO BRL) and thymidine kinase negative recombinant VV selected by growth in the presence of 5-bromo-2'-deoxyuridine (BrdU). The presence of the inserted j5R gene was confirmed by PCR using primers which flanked the insertion site in the VV genome and expression of the j5R protein was confirmed by Western blotting using rabbit antiserum raised against a synthetic peptide containing sequences from j5R (see below).

■ Peptide synthesis and antibody production. A 17 amino acid (LIKEANKYWWGNYDPYS) peptide containing sequences between amino acid residues 77 and 93 of the j5R protein was synthesized using the solid phase method (Merrifield et al., 1982). Rabbits were immunized intramuscularly with 500 µg of the synthetic peptide in Freund’s complete adjuvant and boosted subcutaneously with a half dose of the peptide in Freund’s incomplete adjuvant on days 21, 42 and 64. The antisera were collected on day 85 and the specific anti-peptide immunoglobulins were purified from the rabbit antisera by affinity chromatography using ProtOn columns to which j5R peptide had been coupled (Multiple Peptide Systems) according to the manufacturer’s instructions. The j13L peptide (SNEILKHYTNRQRLNEC) was synthesized and antiserum prepared as described by Sun et al. (1995).

■ Sequential extraction of virion proteins. Percoll gradient purified extracellular ASFV (Uganda isolate) (Carrascosa et al., 1985) was treated first with 0.1% (v/v) n-octyl-β-D-glucopyranoside (NOG, Sigma) at 4 °C for 1 h in a Centrifloc-100 concentrator (Amicon). After centrifugation at 3500 r.p.m. for 10 min, solubilized proteins were collected and the virus was treated sequentially with 0.25, 0.5, 1.0 and 5.0% (w/v) NOG using the same conditions. The extracted proteins and the remaining virus were mixed with equal volumes of 2× SDS–PAGE loading buffer, separated by SDS–PAGE and proteins were detected by Coomassie blue or silver staining.

■ Detection of j5L and j13L antigens. For each immunolabelling application described below, the primary antibodies were used at approximately 50 µg/ml and 100 µg/ml, respectively. An anti-blue-tongue virus (BTB) antibody (A3) was used as an irrelevant antibody control, as were secondary antibody conjugates alone.

■ Immunoblotting. Proteins were separated by SDS–PAGE and transferred to Immobilon-P transfer membranes (Millipore). The membranes were blocked at 4 °C overnight or 37 °C for 1 h with PBS containing 10% (v/v) FCS or 1% (w/v) BSA (Sigma) and 0.1% (v/v) Tween 20. Blots were incubated with primary antibody followed by the appropriate horseradish peroxidase (HRP) coupled secondary antibody. Bound secondary antibodies were detected by enhanced chemiluminescence (ECL, Amersham).

■ Immunofluorescence. IB-RS2 cells were grown on glass coverslips in six-well plates and infected with the Uganda virus isolate for either 8 or 18 h at an approximate m.o.i. of 5×10³ TCID₅₀ units per cell. The cells were fixed in acetone–methanol (1:1) at −20 °C for 10 min, rinsed with PBS and blocked with PBS containing 3% (v/v) FCS. Cells were primary labelled with rabbit anti-j5R or j13L for 30 min at 37 °C, washed six times in PBS containing 1% (v/v) FCS and the antibody detected using an anti-rabbit immunoglobulin antibody conjugated to FITC (Southern Biotechnology) at 20 µg/ml. Samples were rinsed in PBS and mounted in ‘anti-fade’ (2.5%, w/v, 1.4-diazabicyclo[2.2.2]octane, Sigma) in 90% (v/v) glycerol in PBS.
Results

Predicted structure of ORF j5R

The j5R ORF is located 33 kbp from the right-hand DNA terminus of the Malawi LIL 20/1 ASFV isolate genome, is read towards the right DNA terminus and encodes a 111 amino acid protein with a predicted relative molecular mass of 12-9 kDa (Dixon et al., 1994). The homologous ORF on the BA71V isolate genome (H108R) (Yanez et al., 1995) also encodes a 111 amino acid protein which has 96% amino acid identity with the j5R sequence. Database searches did not find significant homology to other published protein sequences. Hydrophobic sequences were predicted at the N terminus between amino acids 1 and 20, and 25 to 40 (Kyte & Doolittle, 1982).

Expression of the ORF j5R in ASFV and recombinant VV-infected cells

A recombinant VV (VVj5R) was constructed which expressed the complete coding region of the j5R ORF from the Malawi LIL 20/1 isolate genome under control of the early/later VV 7:5 k promoter. To confirm that the recombinant virus contained the correct insert, primers which flanked the insertion site in the VV thymidine kinase gene were used to amplify the j5R insert and flanking sequences by PCR. The fragment was cloned and the sequence of the insert determined, confirming that one copy of the j5R gene had been inserted and that the coding region was not interrupted (data not shown).

PBM cells were mock-infected or infected with the virulent Malawi LIL 20/1 isolate. BSC40 cells were mock-infected or infected with wild-type VV, the recombinant VVj5R, or the tissue culture adapted ASFV Uganda isolate. Cell extracts were separated by SDS–PAGE, blotted and reacted with antiserum raised against a C-terminal peptide from the j5R protein. A protein of 23 kDa was detected in extracts from cells infected with the Malawi LIL 20/1 isolate (Fig. 1A, lane b) and the VV recombinant VVj5R (lane d) and one of 25 kDa in extracts from cells infected with Uganda ASFV isolate (lane f). These 23 and 25 kDa proteins were not detected in uninfected cells (Fig. 1A, lanes a and e), nor were they detected in cells infected with wild-type VV (lane c) or in infected cells by reacting blots with the preimmune rabbit serum (data not shown). These results demonstrate that the antiserum is specific for the j5R product and suggest that there may be protein size variation. This was confirmed using a variety of ASFV isolates from different locations. It was found that Lis 57, Lis 60, Bur 84/2 and Moz 60 had a j5R protein of 25 kDa whilst Bon 83 and Tanz 87 had a j5R protein of 23 kDa (Fig. 1B). The protein detected by the antiserum against the j5R protein was considerably larger (23 or 25 kDa) than the size (12-9 kDa) predicted by computer analysis. Since the j5R sequence contains motifs for N-linked glycosylation and for phosphorylation, it may be post-translationally modified by either phosphorylation or glycosylation. BSC40 cells were infected with the tissue culture adapted Uganda ASFV strain in the absence and presence of tunicamycin, which inhibits N-linked glycosylation, or monensin, which inhibits O-linked glycosylation. No shift in size of the j5R protein was observed in the presence of either drug, indicating that the j5R protein is not modified by glycosylation (data not shown).

Temporal regulation of j5R expression

To determine the temporal control of j5R expression, Uganda virus-infected IB-RS2 cells were harvested at different times p.i. and extracted proteins separated by SDS–PAGE followed by immunoblotting using the rabbit anti-j5R antibodies. The 25 kDa protein was only detected at very low levels from 2 to 6 h p.i. in the cell lysates (Fig. 2, lanes b–d) and was expressed at a low level in the presence of cytosine arabinoside (lane i). However, abundant expression of the j5R protein was detected late (10–24 h p.i.) during ASFV infection (Fig. 2, lanes e and f).

Incorporation of j5R protein in extracellular ASF virions

To determine whether the j5R product is incorporated into ASF virions, the Uganda isolate was purified from the extracellular medium (Carrascosa et al., 1985). The virion proteins were separated by SDS–PAGE and detected by silver

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Immuno-electron microscopy (IEM)

Post-embedding labelling. Control and infected IB-RS2 cells, 18 h p.i., were harvested by centrifugation (800 g for 10 min) and fixed with 3% (w/v) freshly prepared paraformaldehyde in 0.1 M Sorenson’s phosphate buffer (pH 7.2) and processed to Lowicryl HM20 resin (Agar Scientific) by the progressive lowering of temperature method as described by Hyatt (1991). Cell sections were cut 70–90 nm thick and placed onto 700 mesh high transmission nickel grids. Sections were blocked in PBS containing 5% (w/v) BSA, 5% (v/v) normal goat serum and 50 mM glycine (5 × buffer) and immunolabelled with anti-j5R or anti-j13L antibody overnight at 4 °C. After washing, the antibodies were detected with anti-rabbit–gold (10 nm) conjugates (Biocell International) for 1 h at room temperature. The wash buffer and diluent was 1 × buffer. The sections were post-fixed in 2.5% (v/v) glutaraldehyde and contrasted with saturated uranyl acetate in 50% (v/v) ethanol and 3% (w/v) Reynolds’ lead citrate for 5 min and 1 min, respectively, and examined on a JEOL 1200ex microscope.

Negative contrast immuno-gold labelling. Either purified Uganda or Malawi virus (5 µl at approximately 10⁶ TCID₅₀/ml) was adsorbed directly to Formvar–carbon coated gold grids until almost dry and excess was removed on blotting paper. Adsorbed virus was treated with NOG at 0, 0.25, 0.5 or 1.0% (v/v) for 5 min at room temperature prior to washing in PBS. Specimens were blocked by floating the grids on PBS containing 5% (w/v) BSA and primary labelled with anti-j5R or anti-j13L antibody for 1 h at room temperature. After washing, the antibodies were detected as described above. The wash buffer and diluent were PBS containing 1% (w/v) BSA. The grids were post-fixed in 2.5% (v/v) glutaraldehyde and contrasted with 2% (w/v) phosphotungstic acid (buffered to pH 7.2). For further details refer to Hyatt (1991).
staining and immunoblotting. The preparation had a typical ASF virion protein profile containing the previously reported major structural proteins (Carrascosa et al., 1985) (Fig. 3, lane a). Immunoblotting using the anti-j5R antiserum, but not the preimmune serum, identified a protein of 25 kDa in purified extracellular ASF virions (Fig. 3, lanes c and b, respectively); this was confirmed by electron microscopy (see below). The molecular mass of this protein was the same as that of the protein detected in the Uganda ASFV isolate-infected cell lysates (Figs 1 and 2). The j5R protein migrated with a similar mobility to a minor virion protein detected by silver staining (Fig. 3, lanes a, c, f and g). To exclude non-specific association of the j5R protein with ASF virions, the purified extracellular ASFV was treated sequentially with increasing concentrations of the non-ionic detergent NOG and the liberated proteins were separated by SDS–PAGE followed by immunoblotting (Fig. 3, lanes d–g). The 25 kDa protein was not among those proteins first extracted from virions by lower concentrations of NOG, but was extracted by treatment with 0±5% NOG (Fig. 3, lanes f and g). This suggests that the j5R protein is incorporated into virions as a structural protein and not as a loosely associated contaminant. As no protein was extracted at 0±25% NOG, but detection was maximal at 0±5%, it is suggested that j5R is not an outer virion component but perhaps luminal between two membrane/structural layers and maximally released after the outer layer has been removed.
Similar extraction profiles have been observed for other ASFV proteins (Hingamp et al., 1995).

**Immuno-localization of j5R and j13L at the cellular level**

The major location of both j5R and j13L in ASFV-infected cells was the virus assembly site, known as the virus factory. The antigens were detected as multiple small perinuclear foci at 8 h.p.i. (Fig. 4a,c) but not before. At 18 h.p.i. the antigens were present in one large perinuclear area (Fig. 4b,d). No fluorescent signal was observed in uninfected cells with either anti-j5R or anti-j13L antibodies (data not shown).

**Immuno-localization of j5R and j13L at the intracellular level**

The localization of j5R and j13L in the virus factory was confirmed by IEM. The major components within the virus factories that were labelled with the anti-j5R antibodies were the membrane elements and viral structural intermediates; a smaller amount of probe was detected on the virus particles themselves. In the average virus factory (n = 10) 10% (12/120) of the virus particles were labelled with one or two gold particles each. Within a given factory the distribution of gold probe was 81% (n = 223/277) on the membrane-like structures, 3% (n = 8/277) on the particles and 16% (n = 46/277) on the virus factory matrix (Fig. 5a). On mature virus particles, as they budded from the plasma membrane of infected cells, both the pre-embedding extraction technique (not shown) and post-embedding methods found that the anti-j5R probe was mainly located on the icosaedron viral capsid (Fig. 5b). Gold-labelled antibody was observed both on the inside and the outside of the icosaedron; however, due to the resolution (± approximately 25 nm) of the indirect immuno-labelling technique, it was not possible to determine the exact location of the protein with reference to the inner or outer aspects of the capsid. j5R was also detected on particles without a nucleoprotein core, ‘empty’ capsids, indicating that the presence of j5R is not dependent on the presence of the viral core (Fig. 5b, insert).

The distribution of j13L was similar to that previously described (as p54; Rodriguez et al., 1996) and was also very similar to that of j5R (Fig. 5c, d, compared with a, b, respectively). Quantitative data indicated that in the average virus factory (n = 10) 20% (30/150) of the virus particles were labelled with one or two gold particles each. Within a given factory the distribution of gold probe was 71% (n = 189/267) on the membrane-like structures, 3% (n = 8/267) on the particles and 26% (n = 70/267) on the virus factory substrate material (Fig. 5c). In addition, j13L was found on mature virus particles as they budded from the plasma membrane of infected cells (Fig. 5d); this had not been previously described (Rodriguez et al., 1996). Again the anti-j13L probe was located on both the inner and outer aspect of the viral capsid (Fig. 5d). No anti-j5R or -j13L antibodies were observed on non-viral structures in infected cells or on uninfected cells. Similarly, no signal was observed using an irrelevant (anti-BTV) antibody (data not shown).

**Immuno-localization of j5R and j13L in purified virus particles**

The j5R and j13L proteins were detected on both avirulent Uganda and virulent Malawi virus particles purified from the extracellular supernatant by negative contrast IEM (NCIEM) (Fig. 6). The treatment of particles with NOG detergent was effective in revealing more of the antigens as the NOG concentration was increased. The optimal concentrations for the detection of j5R and j13L were 0.25 and 0.5% NOG, respectively (Fig. 6a, b). Only 4–6% of the untreated particles were labelled with either anti-j5R or anti-j13L probe; at 0.25% this increased to 16–20% and at 0.5% approximately 20–25% of the particles were labelled (n = 56). However, particles treated with more than 0.5% NOG were prone to collapse and disintegrate, leaving a mass of indistinguishable protein/DNA material, some of which was labelled (Fig. 6c, j13L). Controls using irrelevant antibody (BTV) or secondary antibody alone were negative during all of the above investigations (data not shown). There was little change in the number of gold-labelled antibodies per virus particle (2–6) as the concentration of NOG detergent was increased; this may have been a result of antigen masking by outer membrane components at low concentrations and antigen loss at higher concentrations if the protein
was not tightly anchored in the particle substructure. The latter was observed as an increase in specific labelling on the grid substrate as the detergent concentration was raised (data not shown).

**Discussion**

We have identified a novel ASFV encoded virion protein, j5R, which contains a putative transmembrane domain. The j5R protein expressed in cells infected with different ASFV isolates varies in apparent molecular mass between 23 and 25 kDa. This is considerably larger than the size predicted by computer analysis of the j5R ORF (12–9 kDa). The reason for this size discrepancy is not clear. Our results, however, indicate that the j5R protein is not glycosylated and in previous studies no virion proteins of similar molecular mass to the j5R protein were labelled with either $^{32}$P, [$^{3}H$]palmitic or [$^{3}H$]myristic acid (Salas et al., 1988; Aguado et al., 1991). The fact that the protein detected by SDS–PAGE is about twice the size predicted from the sequence suggests that the 23–25 kDa protein may be a dimer. However, as the j5R protein sequence does not contain cysteine residues, and the samples were boiled in the presence of SDS and mercaptoethanol prior to electrophoresis, it is unlikely that j5R forms SDS-resistant dimers. Protein size anomalies have been documented for other integral membrane proteins such as glycophorin A (Lemmon et al., 1992) and bacterial porins (Yu et al., 1979) and this has been attributed to interactions between $\alpha$-helices of the transmembrane domain. A further, perhaps simpler, explanation for the anomalous migration of j5R may involve the presence of both proline and/or acidic and basic amino acids as has been described for other viral proteins (Ferguson et al., 1984; Gordon et al., 1988; Kovacs & Moss, 1996). The central region of the j5R protein was found to encode a 35 amino acid...
sequence containing 7 basic and 4 acidic residues plus 3 prolines.

By light microscopy, both proteins j5R and j13L were associated with the sites of ASFV particle assembly known as the virus factories (Fig. 4). At the ultrastructural level, j5R protein was detected mainly on the outer face of the viral capsid when immunolabelled using the pre-embedding technique but mainly on the inside using the post-embedding technique (Fig. 5). The difference between the pre- and post-embedding techniques may result from the inability of the antibodies to penetrate the virus particles using the former method. In contrast, the j13L protein was detected mainly on

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**Fig. 5.** Intracellular immuno-localization of ASFV j5RL and j13L on virus factories and particles. Detection of j5R on ASF virus factories (a), particles (b) and 'empty' capsids (insert) was by the IEM post-embedding technique. Detection of j13L on these structures is shown in (c) and (d), respectively. Labelled structures include virus particles with ('full', F) and without ('empty', E) the nucleoprotein core (NC), viral capsid (C), membrane-like structures (M) and labelled virus factory matrix (arrow). Bars, 200 nm.
Fig. 6. Immuno-localization of ASFV j5R and j13L on purified mature extracellular virus particles. Detection of j5R by NCIEM on virus particles followed treatment with 0–25% NOG (a); detection of j13L followed treatment with 0–5% (b) or 1–0% (c) NOG. Note the deterioration of the inside of the viral capsid using the pre-embedding technique but both inside and outside the capsid using the post-embedding technique (Fig. 5). These data must be used only as a guide, however, as the resolution of indirect immunolabelling is not sufficient to differentiate quantitatively between localization of the protein on the inside or outside of the capsid structure.

In the absence of NOG detergent, both j5R and j13L were infrequently detected on whole extracellular virus particles by NCIEM, suggesting that the peptide epitopes recognized by the antisera are not generally exposed on the outer envelope of mature infectious particles. Similarly, sequential extraction of virions with NOG suggests that the majority of both proteins are not located close to the virion surface since treatment of virions with relatively high concentrations of NOG (0–25–0–5%) was required for detection of the j5R and j13L proteins by NCIEM and for solubilization from virions for Western blot analyses. Together, the pre- and post-embedding technique and NCIEM results, for both j5R and j13L, are consistent with a location both on the inner and outer faces of the ER-derived viral capsid membrane complex below the outer envelope.

Although the immuno-localization studies demonstrated that j5R and j13L are integral viral structural proteins (Figs 3 and 6), their predominant localization in infected cells is in the virus factory (Figs 2 and 5). These factories consist of three main features: particles with (‘full’) and without (‘empty’) the nucleoprotein core, and membrane-like structures. The latter represent early stages in virus particle assembly (Brookes et al., 1996; Cobbold et al., 1996; Rouiller et al., 1998). Our data agree with the previously reported localization of p54 (Rodriguez et al., 1996), the homologue of j13L in a Spanish virus isolate, and indicate that the j5R protein has a similar location.

Previous studies have shown that the p54 protein (j13L) is essential for ASFV viability (Rodriguez et al., 1996) and that antisera against this protein neutralize virus infectivity by more than 70%. The mechanism of this neutralization has been shown to be by inhibition of virus attachment to cells (Gomez-Puertas et al., 1996). These results suggest that the j13L (p54) protein may play an important role in virus attachment or entry into cells, which would be consistent with a location on the viral capsid (Fig. 5 c, d).

The selective association of anti-j5R and anti-j13L probes with the virus factory membrane-like structures (70–80% of the total observed label; Fig. 5 a, c) contrasts with their relative absence in other areas, such as the factory matrix (10–26%) and virus particles (3%). The specific localization suggests that these proteins may play a defined role in virus assembly, for example in the acquisition of the lipoprotein ER-derived membranes. The mechanism of ASFV morphogenesis is not fully understood. In VV, the virus particles acquire their structural detail as the concentration of detergent increases from 0–25 to 1–0% (a, b, c). Bar, 100 nm.
membranes by a wrapping process involving the trans-Golgi network (Sodeik et al., 1993; Schmelz et al., 1994). Recently, a similar model for ASFV morphogenesis has been proposed which suggests that the viral capsid is formed by association of the major capsid proteins with the ER and a progressive formation of icosahedral particles (Rouiller et al., 1998). A prediction of this model is that virion proteins incorporated into the ER may be exposed both on the inner and outer faces of the capsid (Cobbold et al., 1996). As both j5R and j13L demonstrated this type of distribution, it is suggested that they may play an important role in the early stages of virus morphogenesis.

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