Short communication

Characterization of the African swine fever virion protein j18L

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The African swine fever virus (ASFV) open reading frame (ORF) that is named j18L in the Malawi (LIL20/1) isolate and E199L in the Ba71V isolate encodes a cysteine rich protein of 195 amino acids with a predicted molecular mass of 21.7 kDa and a hydrophobic domain near the C terminus. There are several possible motifs for glycosylation, phosphorylation and myristoylation. Rabbit antisera and monoclonal antibodies raised against a recombinant ASFV j18L protein expressed as a fusion protein with glutathione S-transferase (GST) identified proteins of 19.0–20 kDa in cells infected with different ASFV strains and with a recombinant vaccinia virus expressing j18L. The monoclonal antibodies detected a protein of 20.0 kDa whereas rabbit antisera detected two proteins with relative molecular masses of 15.0 and 20.0 kDa in purified extracellular ASF virions. In ASFV-infected cells, the j18L protein was expressed late post-infection and was localized mainly in the viral factories.

African swine fever is an economically important swine disease caused by a large virus (ASFV) containing a long DNA genome of 170–190 kbp (for reviews see Vinuela, 1987; Costa, 1990). ASFV has similarities in genome structure and replication strategy to the poxviruses but is morphologically different and is classified separately. The complete nucleotide sequence of a non-virulent tissue culture adapted isolate (Ba71V) (Yanez et al., 1995) and more than 90 kbp from a virulent isolate (Malawi LIL20/1) have been determined (Dixon et al., 1994; Yozawa et al., 1994). The genome encodes more than 150 open reading frames (ORFs). Virions have a complex structure consisting of a nucleoprotein core surrounded by an internal lipid membrane and an icosahedral virus capsid. Extracellular virions have an additional lipid membrane (Carrascosa et al., 1984). Virion morphogenesis takes place in perinuclear factory areas. Genes encoding a number of structural proteins have been identified, including the major capsid protein p72 (Lopez-Otin et al., 1990), p10 (Munoz et al., 1993), p11.5 (Alcamí et al., 1993) and several proteins which are derived by proteolytic cleavage from a 220 kDa precursor (Simon-Mateo et al., 1993). A number of other virion proteins, p17 (Simon-Mateo et al., 1995), p12 (Alcamí et al., 1992), p54 or j13L (Rodriguez et al., 1994; Sun et al., 1995), contain putative transmembrane regions and are probably incorporated into virion lipid membranes. Of these, the putative virus attachment protein, p12, is in the external envelope (Carrascosa et al., 1993). A number of enzymes are also packaged into virions for use early during the next round of infection (Salas et al., 1981).

Pigs which survive ASFV infection are generally resistant to challenge with homologous but not heterologous virus strains. However, the mechanism(s) by which immune protection is mediated is poorly understood (see Vinuela, 1987). Identification of proteins involved in the immune response to the virus remains one of the most important tasks in ASFV research. Proteins which contain putative transmembrane regions are of particular interest since they may be incorporated into either the internal or external virion membranes and/or be expressed on the surface of infected cells. Here we report the identification of an ASF virion protein which is encoded by ORF j18L on the Malawi LIL20/1 isolate genome (E199L on the Ba71V genome) and contains a hydrophobic domain near the C terminus.

ORF j18L is 20 kbp from the right end of the Malawi (LIL20/1) isolate genome, is read towards the left DNA terminus and encodes a protein of 195 amino acids with a predicted molecular mass of 21.7 kDa (Dixon et al., 1994). Database searches did not reveal significant identity to other published protein sequences. The sequence is relatively cysteine rich and contains a total of 11 cysteines spaced throughout the first 140 amino acids. Between positions 142 and 171 there are 24 hydrophobic amino acids including an unbroken run between positions 151 and 171. The j18L protein has two potential Asn glycosylation sites at positions 50 and 131, two
casein kinase II (ck2) phosphorylation sites at positions 23 and 65, three protein kinase C (pkc) phosphorylation sites at positions 7, 41 and 109, and five myristoylation sites at positions 55, 94, 116, 127 and 178.

DNA encoding the N-terminal 153 amino acids of the jl8L protein was amplified by PCR from clone LMw18 (Dixon, 1988) (forward primer, 5′ CGCGGATCCATGTCATGCATGCAATTTCGACG; reverse primer, 5′ GCGCCCCGGGTTACATCATACATTAATTAAAAAGT), and cloned downstream of the glutathione S-transferase (GST) gene in the expression vector pGEX-2T (Pharmacia) using restriction sites incorporated into the primers. A protein of 41.0 kDa was detected in the IPTG-induced cell lysates, but not in uninduced cultures nor in cells transformed with the pGEX-2T vector without insert. The molecular mass of the expressed protein was about 3 kDa smaller than the predicted size.

The expressed fusion protein was purified by affinity chromatography on glutathione-Sepharose columns and coated onto ELISA plates. These were incubated with normal pig serum, and antisera from pigs which had recovered from infection with different ASFV isolates (Malta, Belgium, Uganda and one pig infected with multiple strains). The expressed jl8L-GST fusion protein but not the GST protein alone was recognized by the antisera from infected but not from uninfected pigs (data not shown). This indicates that the jl8L protein is expressed during ASFV infection and antibodies against jl8L are induced in infected pigs.

To obtain sufficient protein for antibody production, insoluble expressed GST–jl8L fusion protein was separated by SDS-PAGE and purified by electroelution. The purified protein was used to immunize rabbits and mice to make polyclonal antiserum and monoclonal antibodies specific for the jl8L gene product. Monoclonal antibodies were screened by ELISA against extracts from E. coli expressing both GST alone or GST-jl8L fusion protein and those which reacted with only the fusion protein selected. Both the monoclonal and polyclonal antibodies reacted on Western blots with the GST–jl8L fusion protein (data not shown).

A recombinant vaccinia virus (VV), VVj18L, containing the complete jl8L ORF from the Malawi (LIL20/1) isolate downstream from the VV p7.5 promoter and within the thymidine kinase gene of VV WR strain was constructed. Cells were infected with either wild-type VV WR strain, recombinant VVj18L or the tissue culture-adapted ASFV strain Uganda. Cell extracts were prepared at 24 h post-infection and separated by SDS–PAGE followed by immunoblotting using either the monoclonal or polyclonal antibodies against the E. coli expressed jl8L protein. A protein of 190 kDa was detected in extracts of the VVj18L infected cells and of 200 kDa in extracts of Uganda ASFV-infected cells, but neither protein was detected in extracts of the mock infected or wild-type VV WR-infected cells. These proteins were not detected by normal rabbit serum or hybridoma cell medium (data not shown). These experiments demonstrated that both polyclonal and monoclonal antibodies were specific for the jl8L gene product, which was expressed as a protein with relative molecular mass of 190 or 200 kDa. The difference in size between the protein detected in VVj18L infected cells and in ASFV infected cells may reflect differences in size of the ORF in the Malawi and Uganda ASFV isolates. Alternatively, it may reflect differences due to expression of the protein in a heterologous vector. The molecular mass of the protein detected was about 1 kDa less than that predicted.

To investigate the temporal control of jl8L protein expression during ASFV infection, cells were infected with the tissue culture-adapted Uganda ASFV strain and harvested at different times post-infection. Cell extracts were separated by SDS–PAGE and the jl8L protein was detected by Western blotting using the monoclonal antibody against the jl8L protein (jl8L-14). As Fig.1(a) shows, the 200 kDa protein was detected from 10 h post-infection, suggesting that jl8L protein is expressed late during ASFV infection. To confirm this, cells were infected with the tissue culture-adapted Uganda ASFV strain in the absence or presence of cytosine arabinoside, which inhibits viral DNA replication and late gene expression. Cell extracts were separated by SDS–PAGE and the jl8L protein was detected as described above. Expression of the jl8L protein was inhibited by including 50 μg/ml cytosine arabinoside in the culture medium (Fig. 1b), confirming that jl8L is a late protein.

The jl8L protein sequence has two potential Asn glycosylation sites. However, growth of ASFV in the presence of 10 μg/ml tunicamycin or 13 pm-monomersin did not affect the mobility of the jl8L protein, suggesting that it is not modified by N- or O-linked glycosylation. Although we have not investigated whether the jl8L protein is modified by phosphorylation or myristoylation, it does not correspond in size to previously identified major phosphorylated or myristoylated proteins incorporated into virions (Salas et al., 1988; Aguado et al., 1991).

Pig bone marrow cells were infected with eight ASFV isolates from different locations in Europe (Lisbon 57, Tomera 83) and Africa (Malawi LIL20/1, Tanzania, Mozambique, Burundi, Republic of South Africa or Uganda). Cell extracts were separated by SDS–PAGE and the jl8L protein was detected by Western blotting using rabbit antisera against the jl8L protein. The jl8L protein varied in size between 190 and 200 kDa when extracts from cells infected with different isolates were
Fig. 1. Temporal expression of j18L during ASFV infection. (a) IBRS2 cells were either mock infected or infected with Uganda ASFV strain and cells extracted at 2, 4, 6, 10, or 24 h post-infection. Cell extracts were separated by SDS-PAGE and the j18L protein was detected by Western blotting using monoclonal antibody j18L-14 and horseradish peroxidase coupled anti-mouse secondary antibody. Bound antibodies were detected using ECL (Amersham). (b) Mock infected IBRS2 cells (lane 1) and IBRS2 cells infected with Uganda ASFV isolate in the absence (lane 2) or presence of cytosine arabinoside (50 μg/ml) (lane 3) were harvested at 24 post-infection. The j18L protein was detected as described for (a). The positions of molecular mass markers are indicated.

compared. Isolates from Malawi LIL20/1, Lisbon 57, Tomera 83 and Uganda expressed a 20.0 kDa protein and the other isolates expressed a smaller j18L product. The sequence of the j18L homologue encoded by ORF E199L on the Ba71V ASFV isolate genome (Yanez et al., 1995) is closely conserved in length (199 compared to 195 amino acids) and has 89% identical amino acids compared with the j18L encoded protein.

Virus was purified from the extracellular medium of IBRS2 cells infected with Uganda isolate and virion proteins were analysed by SDS–PAGE and Western blotting (Carrascosa et al., 1984). Silver staining showed that virion proteins had a typical pattern for ASFV (Fig. 2c). The j18L protein was detected by immunoblotting using the rabbit antiserum (Fig. 2a) and monoclonal antibody (j18L-14) (Fig. 2b) specific for the j18L protein. Two proteins with relative molecular masses of 15.0 and 20.0 kDa were detected by the rabbit antiserum but not by the preimmune serum (Fig. 2a). The 20.0 kDa protein was the same size as the protein detected in infected cells. The 15.0 kDa protein could be a cleaved product of the 20.0 kDa protein or a cross-reactive protein. The failure to detect this 15.0 kDa protein in infected cells may be because it is present at much lower concentrations in extracts of infected cells than in purified virions. Interestingly, only the 20.0 kDa protein was detected in purified extracellular virions by the monoclonal antibody j18L-14 (Fig. 2b) indicating that this antibody is specific for an epitope present in the 20.0 kDa protein only. The proteins detected by immunoblotting did not co-migrate with any major bands detected by silver staining (Fig. 2c), suggesting that the j18L protein is a relatively minor component of virions.

To exclude possible non-specific association of the j18L protein with purified virions, sequential extraction of virions with different concentrations of the non-ionic detergent n-octylglucoside (NOG) was carried out at 4 °C for 1 h. The extracted virion proteins were separated from the treated virus using a Centricon 100 concentrator (Amicon), separated by SDS–PAGE and either detected by silver staining (Fig. 2c) or transferred to nitrocellulose membranes followed by immunoblotting using the j18L-specific monoclonal antibody j18L-14 (see Fig. 2b). The number of solubilized virion proteins detected by silver staining increased in parallel with increasing NOG concentration. Thus, treatment of virions with 0.1% NOG released five major proteins which ranged in molecular mass from 30 to 46 kDa. Small amounts of several more proteins were solubilized by 0.25% NOG, including a protein of molecular mass 72 kDa which is probably the major capsid protein. Most major virion proteins were solubilized by treatment with 0.5% NOG. These observations indicated that treatment of the virus sequentially with an incremental concentration range of NOG resulted, as expected, in the sequential liberation of virion proteins. The j18L protein was detected in proteins extracted from virions with 0.5–5% NOG (Fig. 2b), but was not among those proteins first extracted from virions.
Fig. 2. Presence of the J18L protein in extracellular virions. (a) Purified extracellular proteins from the Uganda ASFV strain separated by SDS-PAGE. Lane 1, proteins detected using preimmune serum; lane 2, proteins detected using polyclonal rabbit antiserum specific for J18L protein. Bound antibodies were detected using horseradish peroxidase coupled anti-rabbit serum and ECL. (b) Sequential extraction of purified ASFV particles with the non-ionic detergent NOG. Purified virions were sequentially treated for 1 h at 4 °C with 0.1, 0.25, 0.5, 1 and 5% NOG. Solubilized proteins were separated from non-solubilized proteins using Centricon 100 concentrators (Amicon). Solubilized proteins were separated by SDS-PAGE, blotted onto nitrocellulose membranes and J18L protein was detected using monoclonal antibody J18L-14, horseradish peroxidase coupled anti-mouse serum and ECL. Also shown are untreated virions (virus) and proteins remaining in virus preparations after sequential NOG treatment (treated virus). (c) Virus proteins treated in the same way but detected by silver staining. V, untreated virus; P, pellet remaining after NOG treatment.
Fig. 3. Immunofluorescence showing internal cell staining of the j18L protein. IBRS2 cells were infected with the Uganda ASFV strain, harvested at 16 h post-infection and permeabilized with methanol. (a) Cells stained with monoclonal antibody j18L-14 followed by FITC-conjugated goat anti-mouse IgG (× 40). (b) Cells stained with monoclonal antibody 4H3, which recognizes a 72 kDa major virion protein and stains virus factories.

Our results demonstrate that the j18L protein is incorporated into ASFV particles. The j18L protein has a hydrophobic region near the C terminus which may be inserted into membranes post-translationally in a similar manner to the C-terminal hydrophobic domains of synaptobrevin-like proteins (Kutay et al., 1995). Unlike the synaptobrevin-like proteins, which have their hydrophobic domain at the C terminus, in the j18L protein the hydrophobic domain is 24 amino acids from the C terminus. However, addition of an extra 13 amino acids downstream of the hydrophobic C-terminal region of synaptobrevin does not interfere with post-translational membrane insertion of synaptobrevin (Kutay et al., 1995). It is therefore possible that the C-terminal hydrophobic domain of j18L may function to anchor it in the membrane. If j18L is inserted into virion membranes it may have an important role in virion morphogenesis.

References


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