Reduced redox potential of the cytosol is important for African swine fever virus capsid assembly and maturation

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INTRODUCTION

African swine fever virus (ASFV) is a large, icosahedral DNA virus that causes a lethal haemorrhagic disease in domestic pigs. At present, there are no control measures other than eradication by slaughter. The virus is endemic in Africa and some parts of south-western Europe, where it causes major economic problems (Almeida et al., 1967; Kelly & Robertson, 1973; Carrascosa et al., 1984). ASFV has recently been classified as the sole member of the family Asfarviridae and may be the missing evolutionary link between the large cytoplasmic DNA iridovirus and poxvirus groups.

ASFV assembles in cytoplasmic inclusions called virus factories (Nunes et al., 1975), which locate close to the microtubule-organizing centre and are surrounded by vimentin filaments and mitochondria (Carvalho et al., 1988; Rojo et al., 1998). In this way, ASFV factories resemble cellular inclusions called aggresomes (Heath et al., 2001; Stefanovic et al., 2005; Wileman, 2006), which form in response to protein aggregation. Electron micrographs of sections taken through virus factories reveal virions with concentric layers of differing electron density (Breese & Hess, 1966; Carrascosa et al., 1986; Arizuza et al., 1992). The outer capsid layer contains the major capsid protein, p73. This surrounds inner lipid envelopes derived from the endoplasmic reticulum (ER) (Cobbold et al., 1996; Andres et al., 1998; Rouiller et al., 1998), a matrix protein layer called the core shell and a central core structure containing the nucleoid (Andres et al., 1997). p73 is encoded by the B646L gene (Yanez et al., 1995) and provides 35% of the protein mass of the virus, whilst a further 25% is produced by proteolytic cleavage of the pp220 polyprotein (Andres et al., 1997, 2002a, b; Heath et al., 2003), which is incorporated into the matrix.

Assays that follow the biosynthesis and subcellular distribution of p73 have shown that p73 is synthesized in the cytosol, where it binds to a specific, virally encoded chaperone (CAP80) encoded by the B602L gene (Cobbold et al., 2001). When p73 is expressed in the absence of CAP80, the protein is folded incompletely and forms insoluble aggregates. In the presence of CAP80, the conformation of p73 changes, allowing it to remain soluble. During infection, p73 is released from CAP80 into the cytoplasm and then binds rapidly to the cytoplasmic face of ER-derived membranes (Cobbold et al., 2001), a process that may depend on virus membrane protein p54 (Rodriguez et al., 2004). Approximately 60% of this membrane-bound pool of p73...
becomes resistant to trypsin within 2 h and forms large, oligomeric complexes, suggesting assembly into the virus capsid (Cobbold et al., 1996; Cobbold & Wileman, 1998). This later stage requires continual protein synthesis, ATP and an intact ER calcium store (Cobbold et al., 2000). Release of ASFV from the cell involves transport from virus factories along microtubules to the cell surface (Jouvenet et al., 2004) and budding from the plasma membrane from projections that resemble filopodia (Jouvenet et al., 2006). At this point, ASFV leaves the reducing environment present in the cytosol and enters an extracellular space rich in oxygen. For small, enveloped viruses, preparation for entry into an oxidizing environment takes place in the lumen of the ER, where virus envelope proteins encounter an oxidizing environment and redox-sensitive chaperones that facilitate protein folding and disulphide-bond formation (Maggioni & Braakman, 2005). For large, cytoplasmic DNA viruses, such as poxviruses and ASFV, the situation is more complex because assembly takes place on the cytoplasmic face of membranes, which is reducing. In the case of poxviruses, a series of virally encoded thiol oxidoreductases are able to oxidize virus proteins exposed to the cytosol (Locker & Griffiths, 1999; Senkevich et al., 2000, 2002). Two of the proteins involved, A25 and G4, are thought to transfer electrons from thiol groups on virus proteins bound to the cytoplasmic face of the vaccinia virus (VACV) membrane to FAD bound within the VACV E10 protein.

The VACV E10 protein is a member of the ERV1/ALR family of oxoreductases, and ERV1/ALR orthologues are also encoded by other cytoplasmic DNA viruses, including mimivirus (Raoult et al., 2004) and ASFV (Yanez et al., 1995). Disruption of the ERV1-ALR protein of ASFV results in slow replication in macrophages, small-plaque phenotype and defective morphogenesis (Lewis et al., 2000), suggesting that control of cytosolic redox potential may be important for ASFV assembly. Given these observations, we have asked whether agents that perturb the redox state of cells have an effect on the assembly of p73 into the virus capsid. The folding of p73 bound to CAP80 and the subsequent release and transfer of p73 to ER-derived membranes were inhibited when cells were oxidized, showing that a reduced cytosol was critical during early stages of ASFV assembly. Newly synthesized capsid precursors were disrupted by oxidizing conditions, suggesting that they would not survive in the extracellular milieu; however, they became resistant to oxidation as maturation progressed.

METHODS

Reagents, cells, viruses and antibodies. All reagents were from Sigma unless otherwise stated. Diamide was used at 0.5 mM and dithiothreitol (DTT), reduced glutathione and oxidized glutathione (GSSG) at 1 mM. A 5- and 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate ‘Image-iT LIVE Green Reactive Oxygen Species detection kit’ was obtained from Molecular Probes. The Vero-adapted ASFV strain BA71v was propagated and titrated by plaque assay on Vero cells as described by Enjuanes et al. (1976). Vero cells were infected at 10 p.f.u. per cell. After virus adsorption for 2 h, the supernatant was removed and cell cultures were incubated with fresh Eagle’s medium at 37°C for 16 h. Extracellular ASFV was purified as described by Black & Brown (1976). Monoclonal antibody (mAb) 4H3, to the major capsid protein, and a polyclonal serum raised to the N terminus of CAP80 have been described previously (Cobbold et al., 2001).

Metabolic labelling and immunoprecipitation. Cells infected with ASFV were preincubated with cysteine- and methionine-free medium for 15 min, and then medium was replaced with 1–2 MBq (35S)methionine and cysteine (35S-Express label; New England Nuclear) ml−1 in cysteine- and methionine-free medium. Cells were washed and chased in Dulbecco’s modified Eagle’s medium. In all experiments where chemicals were used, they were added after pulse labelling in the absence of chemical. At the appropriate time intervals, cells were washed once in PBS and released from the flask with EDTA/trypsin. Cells were lysed in 1% Brij: 35 immunoprecipitation buffer [10 mM Tris (pH 7.8), 150 mM NaCl, 10 mM iodoacetamide, 1 mM EDTA, 1 mM PMSF, 1 µg (each) of leupeptin, pepstatin, chymostatin and antipain ml−1] (Boehringer Mannheim) and immunoprecipitated as described previously (Wileman et al., 1993).

Preparation of cellular membrane fractions. ASFV-infected cells were resuspended in buffered sucrose [250 mM sucrose, 1 mM EDTA, 20 mM Tris (pH 7.5)] and homogenized by 20 passages through a 25-gauge needle. Whole cells and nuclei were removed by centrifugation at 6000 r.p.m. for 2 min in an Eppendorf 5415 centrifuge. Post-nuclear supernatants were pelleted at 14000 r.p.m. for 20 min at 4°C in an Eppendorf 5402 centrifuge to separate membranes (pellet) from cytosol (supernatant).

Trypsin-protection assays. Membrane fractions prepared from metabolically labelled cells were incubated with trypsin (0.4 mg ml−1) for 30 min at 37°C. Proteolysis was stopped by addition of 3 vols immunoprecipitation buffer containing 3% fetal calf serum and 10 mg hen egg white trypsin inhibitor ml−1 (Boehringer Mannheim). The amount of p73 remaining was determined by immunoprecipitation. For analysis of membrane-bound p73, membrane fractions were incubated with reduced or oxidized glutathione for 15 min and then incubated with trypsin as described above.

Sucrose-density sedimentation. Membrane fractions prepared from metabolically labelled cells infected with ASFV were solubilized in 1% Brij: 35 immunoprecipitation buffer and applied to the top of sucrose-step gradients containing 2 ml layers of 10, 20, 30, 35 and 40% sucrose with 1 ml of a 70% sucrose cushion, and left to equilibrate overnight at 4°C. After centrifugation at 40000 r.p.m. in a Beckman SW40 rotor, gradients were separated into 1.2 ml fractions and immunoprecipitated with mAb 4H3.

RESULTS

Oxidizing agents prevent the release of p73 from CAP80

The effect of different redox conditions on dissociation of p73 from CAP80 was analysed by pulse–chase immunoprecipitation using an antibody specific for CAP80 (Fig. 1a). CAP80–p73 complexes were recovered from infected cells labelled for 2 min (lane 1). We have shown previously by quantitative immunoprecipitation using conformation-dependent and -independent antibodies that p73 is conformationally immature when bound to CAP80 at
2 min and is folded and then released from CAP80 during a 60 min chase (Cobbold et al., 2001). The loss of the lower band in lane 2 confirmed dissociation of p73 from the CAP80 protein after 60 min. In lanes 3 and 4, redox reagents were added after pulse labelling. Dissociation of p73 from CAP80 was unaffected by the reducing agent DTT (lane 4); however, the thiol-oxidizing agent diamide (O'Brien et al., 1970) (lane 3) inhibited CAP80–p73 dissociation.

To determine the effects of diamide on the rate of release of p73 from the CAP80 chaperone, infected cells were pulse-labelled for 2 min, to label nascent p73, and then chased for increasing times in the presence or absence of diamide (Fig. 1b). Diamide was added after pulse labelling. Under control conditions (top), CAP80–p73 complexes were recovered from cells labelled for 2 min, and p73 dissociated from CAP80 30 min into the chase [top of panel (b)]. A slight fall in levels of CAP80 recovered by immunoprecipitation was observed at the 30 and 60 min chase times in the presence or absence of diamide [bottom of panel (b)]; even so, it was clear that, in cells incubated with diamide during the chase, CAP80 and p73 remained associated, even after an extended 1 h chase. A densitometric analysis of autoradiographs (not shown) indicated that, in the presence of diamide, 80% of the bound p73 remained associated at 1 h, compared with 15% in control cells.

**Diamide slows the conformational maturation of p73**

The folding of newly synthesized p73 can be studied by following the appearance of epitopes recognized by a conformation-dependent mAb (4H3) and by analysing the production of trypsin-resistant fragments (Cobbold et al., 2001). The results of this type of experiment are shown in Fig. 1(c). Infected cells were pulse-labelled for 2 min to label nascent p73 and then chased for increasing times. After the 2 min pulse, low levels of the epitope recognized by 4H3 were detected (left lane of each time point); however, the epitope increased with the chase time, indicating conformational maturation of the protein. The right-hand lane shows the level of protease-resistant fragments obtained at each time point. These mirrored the increase in levels of p73 immunoprecipitated by 4H3 and showed that conformational maturation was rapid and essentially complete within 15 min of synthesis.
The effect of diamide present during the chase is shown in Fig. 1(d). Diamide was added after pulse labelling. Low levels of p73 were recognized by mAb 4H3 after a 2 min chase, and these increased slowly during the chase; moreover, production of protease-resistant fragments was also slowed in the presence of diamide. Densitometric analysis of autoradiographs demonstrated that, under control conditions, the conformational maturation of p73 was rapid between 2 and 5 min and peaked at 15 min. However, in the presence of diamide during the chase, the rate of maturation slowed and only 60% of p73 was recognized by 4H3 after 1 h (Cobbold et al., 2001). Addition of reducing agents such as DTT during the chase period had no effect on the rate of p73 folding (data not shown).

**Diamide inhibits the transfer of p73 onto the ER-derived membranes**

Once p73 molecules are released from CAP80, they are recruited rapidly onto ER-derived cisternae (Cobbold et al., 1996, 2001). We have shown previously that all of the p73 bound to ER-derived membranes is immunoprecipitatable with 4H3, showing that it undergoes conformational maturation before transfer to the membranes. The next experiments tested the effects of diamide on transfer of p73 to these ER-derived membranes. The pulse-labelling time was increased to 5 min to allow the initial folding of p73 into a conformation recognized by mAb 4H3, and diamide or DTT was added after pulse labelling. At this point, p73 is in the cytosol. Cells were then chased for 5, 15 and 30 min to follow transfer to ER-derived membranes (Cobbold et al., 2001). The levels of folded p73 present were determined by lysis and immunoprecipitation using the conformation-dependent antibody to p73. Fig. 2(a) shows that, under control conditions, p73 was detected in the membrane fraction (M) during the first 5 min of the chase, and levels reached a maximum at 30 min. This was largely unaffected when the cells were incubated with DTT during the chase. Less p73 was recovered during a chase in the presence of diamide. This is because diamide slows the folding of p73 into a conformation recognized by mAb 4H3 (Fig. 1d). The results also show that recruitment of p73 onto ER-derived membranes was inhibited when cells were incubated with diamide; densitometric analysis showed that only 5% of the pulse-labelled p73 protein was recovered from membranes after 30 min, compared with between 30 and 40% for control cells.

The effect of diamide on the oxidation state of living cells was analysed by using redox-sensitive dyes (Fig. 2b). Diamide caused a marked increase in reactive oxygen species, as shown by elevated green fluorescence in the middle panel. Following diamide wash-out for 1 h (lower panel), cells lost the strong fluorescent signal and could therefore recover a reduced cytosol. The effects of washing diamide from cells on the membrane binding of p73 were also studied. Cells pulse-labelled for 5 min and incubated for 30 min in the presence of diamide were transferred to complete medium for a further 60 min in the absence of (a) Diamide inhibits binding of p73 to membranes. Sixteen hours after infection with ASFV, cells were pulse-labelled for 5 min with [35S]methionine and cysteine in the absence of redox reagent. Medium containing diamide (0.5 mM) or DTT (1.0 mM) or control medium was added immediately after pulse labelling and then cells were chased for the indicated times. Cells were homogenized and post-nuclear membrane (M) and cytosolic (S) fractions were prepared and lysed in immunoprecipitation buffer. Levels of p73 present were determined by immunoprecipitation with mAb 4H3 followed by SDS-PAGE and autoradiography. (b) Vital dyes indicate that the effects of diamide on cellular redox potential are reversible. Vero cells were incubated with dye and cellular redox was monitored by fluorescence microscopy. Control cells incubated in the absence of diamide are shown in the top panel. Cells incubated in medium containing diamide (0.5 mM) for 2 h are shown in the middle panel. Cells incubated in medium containing diamide (0.5 mM) for 2 h, washed and viewed 1 h later are shown in the bottom panel. Exposure times were kept constant between observations. Bar, 10 µm. (c) The inhibition of membrane binding induced by diamide is reversible. Sixteen hours after infection with ASFV, cells were pulse-labelled for 5 min with [35S]methionine and cysteine in the absence of redox reagent (pulse). Medium containing diamide (0.5 mM) or DTT (1.0 mM) or control medium was added immediately after pulse labelling and cells were then chased as indicated. Cells chased in the presence of diamide (chase 30 min + diamide) were also washed and incubated for a further 1 h in the absence of diamide (chase 60 min – diamide). Cells taken at the end of the chase period were homogenized and post-nuclear membrane (M) and cytosolic (S) fractions were prepared and lysed in immunoprecipitation buffer. Levels of p73 present were determined by immunoprecipitation with mAb 4H3 followed by SDS-PAGE and autoradiography.
CAP80 undergoes a redox-sensitive conformational change, but does not form disulphide-linked complexes with p73

The CAP80 protein encoded by the BA71v ASFV isolate used in these studies has a central domain containing 15 repeats of a Cys–Ala–Ser–Thr (CAST) motif. The presence of a large number of cysteine residues in the centre of CAP80 prompted us to use non-reducing SDS-PAGE gels to search for disulphide bonds in the CAP80–p73 complex. CAP80 migrates at approximately 80 kDa on reducing SDS-PAGE gels. This is approximately 10 kDa larger than the size predicted from the B602L reading frame and is caused by the central cysteine-rich domain (Cobbold et al., 2001). Control cells or cells incubated with diamide after pulse labelling were chased for 30 min, the time taken for p73 to dissociate from CAP80. Fig. 3 shows that CAP80 migrated at approximately 70 kDa on non-reducing gels. The non-reduced protein therefore migrated faster than the reduced form, suggesting a more compact conformation, and implies that reduction of the cysteine-rich domain of CAP80 allowed CAP80 to adopt a more open conformation. The gel also shows that immunoprecipitates of control cells or cells incubated with diamide during the chase produced a single band for CAP80 at 70 kDa and did not show any bands migrating further up the gel, indicative of proteins interacting through disulphide bonds; this suggests that CAP80 does not form disulphide-linked complexes with itself, p73 or other proteins.

Capsid assembly and maturation require a reducing redox potential

We have described a protease-protection assay to follow the kinetics of p73 oligomerization and assembly on ER-derived membranes. During the assay, trypsin is added to membrane fractions isolated from infected cells at increasing times and capsid maturation is indicated by an increase in the resistance of p73 to protease digestion. The increase in protease resistance is lost in the presence of mild detergents, suggesting that it is related to capsid assembly on cellular membranes. p73 binds to membranes 5 min after synthesis; capsid maturation begins 1 h later and is completed by 2–3 h (Cobbold et al., 1996). At the same time, p73 oligomerizes from a 150–200 kDa homodimer/trimer (Cobbold et al., 2001), seen in the centre of the gradient (fraction 6), into a large complex of approximately 50 000 kDa, which migrates in the bottom fractions of a 10–40 % sucrose gradient (Cobbold & Wileman, 1998). This is approximately 10 % of the size of ASFV particles (555 000 kDa; Andres et al., 1997). The complex may represent virus capsids partially dissociated by detergent or very large assembly intermediates. Protease-protection and sucrose-centrifugation assays were used to determine the effect of DTT and diamide on the assembly and conformational maturation of p73 within these structures. Infected cells were pulse-labelled for 20 min, which allowed binding of p73 to ER-derived cisternae, and then chased for 2 h in complete medium. Post-nuclear membrane fractions were divided into two aliquots; one was incubated with trypsin, whilst the other was solubilized and centrifuged on 10–40 % sucrose gradients. A comparison of the autoradiographs on the right of Fig. 4(a) showed that the majority of membrane-associated p73 was resistant to trypsin after 2 h, indicating conformational maturation of p73 on ER-derived membranes; during this period, the majority of the solubilized membrane-associated p73 [lanes 1–10 of Fig. 4(a)] migrated towards the bottom of the gradient, indicating incorporation into a large complex. Addition of DTT during the 2 h chase had no effect on the maturation of p73 (Fig. 4b). Fig. 4(c) shows cells incubated with diamide during the 2 h chase. This time period was sufficient for p73 to reach a conformation recognized by the p73 antibody (Fig. 1d) and to bind membranes. In the presence of diamide, the quantity of p73 protected from trypsin and the formation of large complexes containing p73 migrating at the bottom of the sucrose gradient were reduced substantially. Capsid maturation on membranes was therefore inhibited when cells were oxidized by diamide, indicating that the reduced redox potential maintained in the cytosol is important for both p73 recruitment onto membranes and subsequent assembly into capsid and capsid precursors.
Newly assembled ASFV virions are destabilized when incubated with oxidized glutathione

Next, we tested whether oligomerization and capsid precursor assembly would be reversed under oxidizing conditions. Infected cells were pulse-labelled and chased for 2 h to allow capsid assembly and maturation, as indicated by the protease-protection and oligomerization assays. Cells were then homogenized and crude post-nuclear membrane fractions were incubated in the presence or absence of oxidized glutathione (GSSG) and trypsin prior to sucrose-density centrifugation. GSSG was chosen because GSSG and reduced glutathione form the main redox buffer of the cytosol. Under control conditions (Fig. 4d), approximately 70% of the p73 complex present in the membrane fraction migrated towards the bottom of the sucrose gradient and was resistant to trypsin. In contrast, when membranes were treated with GSSG and trypsin, only a small proportion of p73 was observed in fractions 1–3 of the sucrose gradient (Fig. 4e). In addition, tryptic fragments of p73 were observed running in fractions 5–8 of the gradient (representing complexes of 150–250 kDa), suggesting that addition of GSSG to membranes caused disassembly of the virus capsid and/or capsid precursors and exposed them to trypsin.

The above experiment showed that GSSG destabilized the membrane-bound p73 complex; even so, some material resistant to GSSG was detected at the bottom of the sucrose gradient. To see whether the proportion of protected material would increase with time, post-nuclear membrane fractions were taken from infected cells at increasing times and incubated in the absence or presence of GSSG and trypsin and then immunoprecipitated by using mAb 4H3 (Fig. 5a). A comparison of lanes 1 and 2 at each time point shows that, following the 10 min pulse, very little p73 was resistant to protease digestion; however, trypsin resistance increased during the chase. Interestingly, the proportion of p73 protected from trypsin at each time point decreased following addition of GSSG (lane 3), showing that GSSG increased sensitivity to the protease. Interestingly, after an extended 3 h chase, almost 70% of p73 molecules remained resistant to protease in the presence of GSSG, showing that complexes containing the p73 protein became progressively more resistant to oxidation with time (Fig. 5b). This may be important to prepare the virus for release from the cells. When the experiment was repeated for virions purified from medium taken from labelled cells, virus particles were
completely resistant to the combined effects of trypsin and GSSG (Fig. 5c). It is important to note that increased resistance of viruses released from cells to GSSG may also be due to the fact that extracellular viruses gain an additional outer envelope when they are released from the plasma membrane, and that this envelope could be responsible for a proportion of the increased resistance observed.

### DISCUSSION

In this study, we have investigated whether the reducing environment of the cytosol is important for the assembly of the capsid of ASFV. Our results show that, when cells are oxidized by diamide, the conformational change observed in p73 when p73 binds to CAP80 is slowed and p73 remains bound to the viral chaperone. Diamide also prevented transfer of p73 to cellular membranes and slowed capsid assembly. We were also unable to detect binding of the CAP80-p73 complex arrested by diamide to membranes. This means that release from the chaperone is required for transfer of p73 to the ER-derived membranes or that recruitment of p73 onto these membranes requires other redox-sensitive factors that may facilitate transfer of p73. Candidates include ASFV-encoded Erv1p/Alrp proteins, described below. Under control conditions, membrane-associated p73 assembles into a large capsid precursor and these later stages of capsid assembly were also inhibited by diamide. The CAP80 protein encoded by the ASFV isolate used in this study has a central domain containing 15 repeats of Cys-Ala-Ser-Thr (CAST). The conformation of CAP80 became more compact when analysed on non-reducing SDS-PAGE gels. This raises the possibility that the reducing conditions that normally prevail in the cytosol reduce cysteine residues in the CAST repeat, allowing CAP80 to maintain an open conformation. Oxidation of cells by diamide may inhibit this conformational change and prevent dissociation of p73 and CAP80. It is unlikely that diamide disrupts the conformation of p73 unduly, or generates aberrant disulphide bonds with CAP80. We have demonstrated that binding of p73 by conformation-dependent antibodies is not affected if oxidizing agents are added to cell lysates prior to or during immunoprecipitation (Cobbold et al., 1996), and non-reducing SDS-PAGE gels failed to detect p73–CAP80 complexes in control cells or cells incubated with diamide.

Having shown that capsid assembly required reducing conditions, the assay was modified to determine whether the stability of the pre-assembled capsid and/or large capsid precursors was also dependent on reducing conditions. Interestingly, large complexes containing p73 isolated from membrane fractions were unstable in the presence of GSSG, despite being resistant to protease digestion (Fig. 5a, 120 min chase). This result was unexpected because it implied that the large capsid complexes, which we believe are late assembly intermediates, would be unstable once they reached an oxidizing environment following release from the cell. The capsid precursors became progressively more resistant to GSSG with time and, 3 h following transfer of p73 to the ER-derived membranes, the capsid and/or capsid precursors were resistant to GSSG. This time course was

![Fig. 5. ASFV capsids show time-dependent increases in stability towards oxidized glutathione. (a) Sixteen hours after infection with ASFV, Vero cells were pulse-labelled for 10 min with [35S]methionine and cysteine and chased for increasing times as indicated. Cells were homogenized and post-nuclear membranes were prepared. Capsid stability was tested by incubation in the absence or presence of GSSG (1.0 mM) and trypsin as indicated. Levels of p73 that are resistant to trypsin in the presence of GSSG increase with time of maturation. (b) The percentage of p73 resistant to trypsin alone (labelled 'control') or the combination of GSSG and trypsin (labelled 'GSSG') were calculated from densitometric analysis of autoradiographs. (c) Purified viruses are resistant to oxidized glutathione. ASFV-infected cells were labelled metabolically for 6 h and then chased for a further 16 h. The supernatant was collected and extracellular ASFV virions were purified and then incubated in the absence (lane 1) or presence of GSSG and trypsin (lane 2) or reduced glutathione (GSH) and trypsin (lane 3) prior to solubilization, immunoprecipitation with mAb 4H3, SDS-PAGE and autoradiography.](http://vir.sgmjournals.org/83)
consistent with our previous results showing that ASFV is released from cells between 3 and 4 h following synthesis of p73 (Cobbold et al., 1996) and the observation that viruses isolated from culture supernatants were also resistant to GSSG. The mechanism that provides a progressive increase in stability to oxidizing conditions is unknown, but may involve structural protein pE248R, which has two intra-molecular disulphide bonds. pE248R is associated with the inner virus envelope and exposed to the cytosol, where it binds to the ASFV-encoded Erv1p/Alrp family thiol oxidase (pB119L) (Rodriguez et al., 2006). pB119L is a late protein that localizes to virus factories, making it possible that pB119L oxidizes pE248R during assembly; this may stabilize ASFV during capsid maturation.

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REFERENCES


