Vimentin Rearrangement during African Swine Fever Virus Infection Involves Retrograde Transport along Microtubules and Phosphorylation of Vimentin by Calcium Calmodulin Kinase II

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African swine fever virus (ASFV) infection leads to rearrangement of vimentin into a cage surrounding virus factories. Vimentin rearrangement in cells generally involves phosphorylation of N-terminal domains of vimentin by cellular kinases to facilitate disassembly and transport of vimentin filaments on microtubules. Here, we demonstrate that the first stage in vimentin rearrangement during ASFV infection involves a microtubule-dependent concentration of vimentin into an “aster” within virus assembly sites located close to the microtubule organizing center. The aster may play a structural role early during the formation of the factory. Conversion of the aster into a cage required ASFV DNA replication. Interestingly, viral DNA replication also resulted in the activation of calcium calmodulin-dependent protein kinase II (CaM kinase II) and phosphorylation of the N-terminal domain of vimentin on serine 82. Immunostaining showed that vimentin within the cage was phosphorylated on serine 82. Significantly, both viral DNA replication and Ser 82 phosphorylation were blocked by KN93, an inhibitor of CaM kinase II, suggesting a link between CaM kinase II activation, DNA replication, and late gene expression. Phosphorylation of vimentin on serine 82 may be necessary for cage formation or may simply be a consequence of activation of CaM kinase II by ASFV. The vimentin cage may serve a cytoprotective function and prevent movement of viral components into the cytoplasm and at the same time concentrate late structural proteins at sites of virus assembly.

Vimentin is a major component of type III intermediate filaments found in cells of mesenchymal origin and is also present in cells adapted to tissue culture and many transformed cell lines (6, 14, 16). Up until recently, intermediate filaments were generally thought to be static structures providing a rigid scaffold that is important for determining the shape and mechanical properties of cells. Major changes in the distribution of vimentin are observed, however, when cells move or divide. Vimentin filaments disassemble into aggregates and short filaments during metaphase (15, 37) and form motile “dots” and “squiggles” at the edge of the cell during cell spreading (35, 50). Surprisingly, vimentin is also redistributed in cells expressing misfolded proteins and during virus infections. These rearrangements do not appear to precede large changes in cell shape, but they may provide a protective function within the cytoplasm. In the case of some misfolded proteins, vimentin often forms a cage surrounding protein aggregates sequestered in aggresomes located at the microtubule-organizing center (MTOC). The vimentin cage may restrict the movement of potentially toxic protein aggregates into the cytoplasm (5, 25, 44, 45). The vimentin rearrangements observed during virus infection may also represent a protective response by the cell, since they occur primarily around sites of virus replication and assembly (28, 41).

African swine fever virus (ASFV) is a large cytoplasmic DNA virus that is assembled in viral factories in the cytoplasm. The factories contain viral structural proteins and viral DNA, and recent studies demonstrate that the factories resemble aggresomes, since they form at the MTOC and are surrounded by vimentin cages (19). The precise role played by the vimentin cage is unknown. By analogy with the rearrangements seen during the formation of aggresomes, the cage induced by ASFV may serve to protect the cell from a build up of viral proteins. The cage may also facilitate virus replication and assembly by preventing diffusion of viral components into the cytoplasm (19). Alternatively, since vimentin can confer rigidity to domains of the cytoplasm, the vimentin cage may provide a physical scaffold to facilitate the construction of the virus factory. Interestingly, profound changes in intermediate filament organization are not restricted to infection of cells by ASFV and have been reported for several viruses (30, 31, 33, 36, 40), yet very little is known about how these changes occur.

Vimentin rearrangements during cell spreading and cell division generally involve filament disassembly regulated by phosphorylation of N-terminal domains (2, 9, 10, 24), allowing the filaments and their precursors to move along microtubules (18, 21, 35, 50); in this study, we have investigated whether similar mechanisms operate during vimentin rearrangement in
The monoclonal antibodies recognizing the ASFV capsid protein p73 (4H3) and early ASFV protein p30 (C18) and the rabbit antibody recognizing vimentin (V9), mouse vimentin (3, 17, 34, 43, 47). Alexa Fluor conjugates were from Molecular Probes (The Netherlands). Antibodies recognizing vimentin (V9), α-tubulin (B-5-1-2) were from Sigma. Antibody to ERp57 has been described previously (38). The mouse monoclonal antibody recognizing CaM kinase II was purchased from BD Biosciences, and the rabbit polyclonal antibody recognizing phospho-threonine286 of CaM kinase II was purchased from Biosource International Inc. The mouse monoclonal antibody recognizing phospho-serine9 of CaM kinase II was purchased from Promega. 9E10 antibody was prepared from hybridoma cell line 9E10 obtained from the American Type Culture Collection.

Drugs. Calyculin A and KN-93 were from Calbiochem. 1-β-D-Arabinofuranosyl cytosine (Ara-C) and nocodazole were from Sigma.

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Indirect immunofluorescence. Cells grown on no. 1 glass coverslips (Merck, Dornset, England) were fixed with 100% methanol. When cells were arrested in mitosis, mitotic cells were lifted from the coverslips by mechanical shaking, fixed with 4% paraformaldehyde, and pelleted onto slides using a “Cytopsin 3” (Shandon, Runcorn, United Kingdom) at 1,000 × g for 10 min. Immunofluorescence was carried out as described previously (32). Digital images were captured with a Hamamatsu C-4746A DCC camera. In some cases, digital images of 2 μm of optical sections were deconvolved using Improvision Openlab 2.1.3 software (Improvision, United Kingdom).

Cell lysis, Western blotting, and immunoprecipitation. For whole-cell protein extraction, cells were lysed in denaturing buffer (20 mM Tris-HCl [pH 7.2], 5 mM EDTA, 5 mM EGTA, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 0.4% sodium dodecyl sulfate, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μM/ml antipain, and 1 μg/ml chymostatin) and passed through a 25-gauge syringe 20 times or sonicated for 5 seconds using a Microson Ultrasonic cell disruptor (Misonix). For cytoskeletal extraction, cells were lysed in PIPES buffer (10 mM PIPES [piperazine-N,N′-bis(2-ethanesulfonic acid); pH 6.8], 0.5% Triton X-100, 300 mM sucrose, 100 mM KCl, 3 mM MgCl2, 10 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 50 μM sodium vanadate, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml antipain, and 1 μg/ml chymostatin). The cytoskeletal fraction was pelleted at 14,000 × g at 4°C for 20 min, washed twice in PIPES buffer, and resuspended in denaturing buffer. Immunoprecipitation and Western blot analyses were carried out as described previously (32).

RESULTS

Examples of vimentin rearrangements produced by tissue culture-adapted and virulent isolates of ASFV. Previous studies of the effects of ASFV on vimentin monitored infection of Vero cells by Ba71v, a tissue culture-adapted strain of ASFV (19). Since vimentin is expressed predominantly in cells adapted to tissue culture (16), it was possible that virus-induced vimentin rearrangements may be restricted to tissue culture models. The distribution of vimentin in Vero cells infected with the Ba71v isolate was therefore compared with cells infected with ASFV. The results show that recruitment of vimentin into virus assembly sites occurs very early during infection. Recruitment was dependent on microtubules and occurred before viral DNA replication and late gene expression, suggesting that vimentin may serve a structural role early during the establishment of the virus factory. Once viral DNA replication was initiated, vimentin was phosphorylated by calcium calmodulin-dependent protein kinase II (CaM kinase II) and moved to the edge of the factory and formed a cage around the assembly site. At this later stage of infection, the cage may perform a protective role by preventing the diffusion of viral components into the cytoplasm.

MATERIALS AND METHODS

Cells and viruses. Culture of Vero cells (ECACC 84113001), African green monkey kidney cells, and porcine aortic endothelial cells (PAECs) and infection with Ba71v and Malawi Lil 20/1 strains of ASFV have been described elsewhere (19). Since vimentin is expressed predominantly in cells adapted to tissue culture (16), it was possible that virus-induced vimentin rearrangements may be restricted to tissue culture models. The distribution of vimentin in Vero cells infected with the Ba71v isolate was therefore compared with cells infected with ASFV. The results show that recruitment of vimentin into virus assembly sites occurs very early during infection. Recruitment was dependent on microtubules and occurred before viral DNA replication and late gene expression, suggesting that vimentin may serve a structural role early during the establishment of the virus factory. Once viral DNA replication was initiated, vimentin was phosphorylated by calcium calmodulin-dependent protein kinase II (CaM kinase II) and moved to the edge of the factory and formed a cage around the assembly site. At this later stage of infection, the cage may perform a protective role by preventing the diffusion of viral components into the cytoplasm.

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Antibodies. The monoclonal antibodies recognizing the ASFV capsid protein p73 (4H3) and early ASFV protein p30 (C18) and the rabbit antibody recognizing ASFV matrix protein p34 have been described previously (1, 11, 20). MO82, TM38, TM50, TM71, YT33, and 4A4 monoclonal antibodies were raised against synthetic peptides corresponding to specific serine phosphorylation sites of mouse vimentin (3, 17, 34, 43, 47). Alexa Fluor conjugates were from Molecular Probes (The Netherlands). Antibodies recognizing vimentin (V9), α-tubulin (B-5-1-2) were from Sigma. Antibody to ERp57 has been described previously (38). The mouse monoclonal antibody recognizing CaM kinase II was purchased from BD Biosciences, and the rabbit polyclonal antibody recognizing phospho-threonine286 of CaM kinase II was purchased from Promega. 9E10 antibody was prepared from hybridoma cell line 9E10 obtained from the American Type Culture Collection.

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the distribution of vimentin in primary PAECs infected with the virulent Malawi isolate. In each case, cells were fixed at 16 h postinfection and examined by immunofluorescence microscopy using antibodies specific for vimentin and the major ASFV capsid protein, p73. Extranuclear DAPI (4',6'-diamidino-2-phenylindole) staining was used to identify sites of viral DNA replication indicative of virus factories. Panels a to d of Fig. 1 show a Vero cell infected with Ba71v, and, as reported previously (19), vimentin (panel c) was rearranged into a cage surrounding the virus factory. Panels e to h show a similar experiment carried out for PAECs infected with the Malawi isolate. Two cells are shown, and one is infected, indicated by strong staining for the major capsid protein (e) and extranuclear DAPI staining (f, arrow). In the PAEC lacking viral markers, the vimentin was arranged in a filamentous network reaching the cell surface. In the cell infected with ASFV, the vimentin (g) was rearranged into a cage surrounding the assembly site. The results illustrate that rearrangement of vimentin is not restricted to virus adaptation to tissue culture but also occurs in primary cells infected with virulent isolates of ASFV.

**Vimentin is recruited into virus assembly sites very early after infection.** To study early events in vimentin rearrangement, it was necessary to identify virus assembly sites early during infection. ASFV factories are normally identified through perinuclear accumulation of extranuclear viral DNA and late gene products, such as virus structural proteins. These methods cannot be used at early times, because they require the onset of viral DNA replication and late gene expression. It is possible, however, to identify cells at early stages of infection by using antibodies to early proteins, such as p30. Interestingly, a time course study of ASFV infection (Fig. 2a to c) showed
that p30 was excluded from perinuclear sites as early as 6 h postinfection (arrow). This raised the possibility that perinuclear exclusion of p30 may indicate a very early stage of factory formation. ASFV factories invariably form close to the MTOC (19). Cells taken at 6 h were therefore counterstained with antibodies recognizing gamma-tubulin to identify centrosomes. Significantly, the areas that excluded p30 were located next to gamma-tubulin (panel d), and when the distribution of p30 was observed at 10 h, a time sufficient for the onset of viral DNA replication, viral DNA was observed in the area of the cytosol that contained gamma-tubulin and excluded p30 (panels e and f). The results suggested that the exclusion of p30 indicated an area of the cytoplasm close to the MTOC that would ultimately be used to assemble the virus factory. The distribution of vimentin in cells showing early exclusion of p30 from perinuclear sites was therefore studied (Fig. 2g to i). Interestingly, at 6 h, the vimentin was not rearranged into the cage seen at 16 h but, rather, was arranged into an “aster” to one side of the nucleus (h) and was located within the area of cytoplasm that excluded p30 (g). The locations of the vimentin aster and the MTOC were determined by triple staining using antibodies against gamma-tubulin. Panel i shows that the vimentin aster was located next to centrosomes containing gamma-tubulin, showing its location next to the MTOC. The results showed that vimentin was recruited into the virus assembly site next to the MTOC very early during infection, and in conjunction with clearing of p30 staining from this area, this was one of the earliest events observable during factory morphogenesis.

**Formation of vimentin cages requires viral DNA replication.**

The above experiments showed that vimentin was first arranged as an aster at the MTOC and then formed a cage later during infection. The aster formed before DNA replication, however, and conversion into the cage was coincident with the appearance of late structural proteins, suggesting that late virus gene expression may be required for cage formation. To test this possibility, cells were incubated with AraC to prevent DNA replication and were observed at 16 h postinfection. Control experiments showed that AraC prevented expression of the late structural protein p73 and production of extranuclear DNA (data not shown). Panels a and c of Fig. 3 show that in the absence of AraC, 16 h was sufficient time for a vimentin cage (arrow) to form in control cells. Significantly, in the presence of AraC (panels b and d), vimentin redistributed to a perinuclear site but remained organized as an aster rather than a cage. The conversion of the aster of vimentin into a cage was therefore dependent on ASFV DNA replication.

**Rearrangement of vimentin by ASFV involves microtubules.**

Since the rearrangement of vimentin in cells often involves microtubules (7, 13), the distribution of microtubules in cells infected with ASFV was studied. Panels a to c of Fig. 4 show the distribution of alpha-tubulin in cells that were infected with ASFV for 6 h and stained with antibodies recognizing p30. Significantly, as seen for vimentin at this time point, alpha-tubulin (panel c) was concentrated close to the nucleus in the area of the cell that excluded p30. Since this area contains the centrosome, this probably represents the nucleation of microtubules at the MTOC. The experiment was repeated for cells infected with ASFV for 16 h, and panels d to f show the loss of the aster of tubulin and the rearrangement of microtubules into a cage surrounding the virus assembly site. Taken together, the above results showed that vimentin and tubulin were codistributed in cells infected with ASFV. Early during the formation of the virus assembly site, they were arranged in an aster at the MTOC, and both were rearranged into cages during the formation of the virus factory. This codistribution suggested that rearrangement of vimentin seen in infected cells may be dependent on microtubules. The ability of the virus to maintain a vimentin cage in the absence of intact microtubules was therefore tested by adding nocodazole to cells 14 h after infection. Cells were viewed 2 h later (Fig. 4g to i). Two cells are shown in detail, and one is infected with ASFV, as indicated by the presence of the viral matrix protein p34 (h) and extranuclear DAPI staining (g). As reported previously (19), viral proteins (h) and DNA (g) were dispersed by nocodazole. Interestingly, vimentin (panel i) was no longer arranged into a cage surrounding the assembly site but had collapsed into a ball next to the nucleus. A similar ball of disorganized vimentin was seen in the cell negative for viral markers. Taken together, the results showed that both vimentin and tubulin were arranged into a cage surrounding the virus factory and that the vimentin cage was maintained by microtubules.

Inward movement along microtubules is generally powered by a minus-end-directed motor protein, cytoplasmic dynein. The overexpression of p50/dynamitin leads to the inhibition of dynein-dependent transport and movement of vimentin to the cell periphery (21). We have reported previously that p50/dynamitin prevents late gene expression in cells infected with ASFV (19). Since redistribution of vimentin into a cage was

**FIG. 3. Formation of vimentin cages requires late viral gene expression.**

Vero cells were infected with the Ba71v strain of ASFV and incubated in the absence (a and c) or presence (b and d) of Ara-C (50 μg/ml). AraC was added 2 h postinfection. Cells were processed for immunofluorescence analysis at 16 h. Infection was indicated by the presence of the major capsid protein p73 (a) or early viral protein p30 (b) visualized by secondary antibodies conjugated to Alexa Fluor 488. Vimentin (c and d) was visualized by specific antibody and secondary antibodies conjugated to Alexa Fluor 594.
FIG. 4. Involvement of microtubules in rearrangement of vimentin. (a to f) Distribution of α-tubulin. Vero cells were infected with the Ba71v strain of ASFV and processed for immunofluorescence analysis at 6 h (a to c) and 16 h (d to f). Infection was indicated using antibodies against early protein p30 (b) or the capsid protein p73 (c) or extranuclear DAPI staining of viral DNA (d). The distribution of microtubules was visualized using antibodies specific for α-tubulin (c and f). (g to l) Effect of disruption of microtubule function on vimentin rearrangement. In panels g to i, Vero cells were infected with the Ba71v strain of ASFV for 14 h, incubated with nocodazole (10 μg/ml) for a further 2 h, and then processed for immunofluorescence analysis. Panel g, DAPI staining; panel h, viral matrix protein p34; panel i, vimentin. (j to l) Effect of p50/dynamitin. Vero cells expressing p50/dynamitin were infected with ASFV and viewed at 16 h. Infection was indicated using biotinylated antibody specific for early protein p30 (j), p50/dynamitin was located using antibodies specific for the c-myc epitope (k), and vimentin was detected using specific antibody (l). Protein distribution was visualized using appropriate secondary antibodies conjugated to Alexa Fluor 350, 488, or 594.
TABLE 1. Panel of antibodies recognizing serine phosphorylation sites at the N terminus of vimentin

<table>
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<td>50</td>
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<td>Rho kinase</td>
<td>38</td>
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<td>CaM kinase II</td>
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dependent on late gene expression (Fig. 3), it was not possible to use p50/dynamitin to study the role played by dynein motors during cage formation. The experiment therefore focused on the effects of p50/dynamitin on recruitment of vimentin to the MTOC early during infection. Cells expressing p50/dynamitin were infected with ASFV, and triple staining experiments (Fig. 4j to l) showed that in cells positive for p30 (j) and p50/dynamitin (k), vimentin was no longer recruited to the MTOC but was located at the cell periphery (l). The results suggest that recruitment of vimentin to the MTOC early during ASFV infection required cytoplasmic dynein.

Role played by phosphorylation during vimentin rearrangement by ASFV. Rearrangement of vimentin often involves phosphorylation of N-terminal domains by cellular kinases. This reduces interactions between N-terminal domains and facilitates disassembly and redistribution of filaments (2, 12, 23, 24). The possibility that ASFV caused phosphorylation of vimentin by tyrosine kinases was analyzed by Western blotting of a cytoskeletal fraction enriched for vimentin (35) with phosphorytrosine-specific antibodies. There was no evidence of phosphotyrosine residues in the fraction containing vimentin taken from cells infected with ASFV (data not shown), suggesting that vimentin was not phosphorylated on tyrosine residues during ASFV infection. The possibility that vimentin was phosphorylated on serine residues was tested using a panel of antibodies specific for phosphoserine residues present in the N-terminal domain of vimentin (Table 1). In each case, positive control experiments were conducted to show that when vimentin was phosphorylated in the Vero cells used in this study, the modification could be detected by the antibodies. Examples are shown in panel A of Fig. 5, where cells were either arrested in mitosis using the drug TN-16 (parts i and ii) or incubated with the phosphatase inhibitor calyculin A (iii and iv). Phosphorylation of serine 55 of vimentin was clearly visible in mitotic cells (i), indicating activation of cdc2 kinase. Similarly, phosphorylation of serine 38 by Rho kinase became evident in cells treated with phosphatase inhibitor (iii). Examples of the use of the antibodies in cells infected with ASFV are shown in parts v to vii. Parts v and vi show cells probed with the antibody specific for phosphoserine 55 (v) and the major capsid protein p73 (vi). The mitotic cell shows condensation of chromosomes and phosphorylation of vimentin, whereas the cell infected with ASFV is positive for capsid protein (arrow) and extranuclear DNA but negative for phosphoserine 55. The results demonstrated that ASFV does not induce phosphorylation of vimentin serine 55. When the remaining antibodies were tested in cells infected with ASFV, a positive signal was obtained only using antibody recognizing serine 82 (vii and viii), a substrate for CaM kinase II. Significantly, parts vii and viii of Fig. 5A show that phosphorylation at Ser 82 was detected on the cage of vimentin surrounding the virus assembly site. The results suggested that infection with ASFV activates CaM kinase II and that this may lead to phosphorylation of serine 82 of vimentin.

ASFV infection leads to activation of calcium calmodulin kinase II. CaM kinase II is a multisubunit enzyme consisting of catalytic, autoregulatory, and subunit assembly domains (22). Upon activation by calcium and calmodulin, CaM kinase II autophosphorylates threonine 286, leading to full activation of the enzyme (29, 46). This phosphorylation allows antibodies specific for threonine 286 to be used to detect the active form of the enzyme. CaM kinase II activation was analyzed by Western blotting of crude cytoskeletal fractions prepared from control cells and cells infected with ASFV. Figure 5B shows that the cytoskeletal pellet was enriched for tubulin (top) and vimentin (middle). Panel C (top) shows that CaM kinase II migrated as a 50-kDa protein and was found in both the cytosol and cytoskeleton fractions. The enzyme resolved as a doublet in the cytoskeletal fraction; the nature of the upper band is not known. The active form of CaM kinase II (bottom), recognized by the antibodies specific for threonine 286, was, however, recovered only from the cytoskeletal fraction. Significantly, levels of the enzyme increased after infection with ASFV. The blot also showed a band of 60 kDa in both pellet and supernatant; the nature of the protein is unknown. Taken together, the results suggest that CaM kinase II is activated during infection with ASFV, and this activation would explain the phosphorylation of vimentin at serine 82.

Phosphorylation of serine 82 is dependent on late gene expression. The time course of phosphorylation of serine 82 during ASFV infection was followed by Western blotting of lysates taken from cells at increasing times after infection. The top panel of Fig. 6 shows that there was a transient and low-level phosphorylation of vimentin at 4 h, but most phosphorylation was evident between 12 and 16 h postinfection, suggesting phosphorylation of serine 82 was associated with viral DNA replication and late gene expression. Cells were incubated with AraC at 2 h to prevent DNA replication and were examined 14 h later to provide a total of 16 h of infection. Images c to e in Fig. 6 show control cells with extranuclear viral DNA staining with DAPI (panel c, arrow) and the vimentin cage phosphorylated at serine 82 (e). Significantly, in cells incubated with AraC, serine 82 phosphorylation was not detected (h) even though the cell was infected, as indicated by the expression of the early protein p30 (g). The images are representative of many viewed by immunofluorescence, and in each case, there was no evidence for serine 82 phosphorylation in the presence of AraC. The results suggested that phosphor-
Inhibition of CaM kinase II slowed ASFV DNA replication and late gene expression. The results above showed that phosphorylation of vimentin by CaM kinase II was coincident with rearrangement of vimentin into a cage surrounding the virus assembly site. Moreover, both vimentin cage formation and vimentin phosphorylation required late gene expression. KN93...
is a specific inhibitor of CaM kinase II, and attempts were
made to see if inhibition of serine 82 phosphorylation would
affect the formation of a vimentin cage. Cells were infected for
4 h and incubated with the drug for a further 12 h and observed
by immunofluorescence. Parts i to iii of Fig. 7A show that in
the presence of KN93, there was no immunofluorescence sig-
nal for vimentin serine 82, indicating that the drug prevented
phosphorylation of vimentin by CaM kinase II. Parts iv to vi
showed that KN93 also prevented rearrangement of vimentin
into a cage in infected cells (vi). At the outset, this suggested
that phosphorylation of serine 82 was required for formation of
the vimentin cage. Interestingly, the DAPI staining in part iv
failed to show the extranuclear DAPI stain indicative of virus
replication. Cells were therefore incubated with H\textsuperscript{3} thymidine
and incorporation of the probe into viral DNA recovered from
a crude cytosolic fraction was tested (27). Figure 7B shows that
KN93 reduced incorporation of the probe, indicating again
that the inhibition of CaM kinase II slowed ASFV DNA rep-
lication. To assess the effects of the drug on viral gene expres-
sion, cell lysates were analyzed by immunoprecipitation for
expression of the early protein p30 and the late structural protein
p73 (right panel). Immunoprecipitates were resolved by sodium dode-
cyl sulfate-polyacrylamide gel electrophoresis and visualized by auto-
radiography. Bars, 10 μm.
ment, KN93 may act more specifically by preventing the phosphorylation of vimentin serine 82 that may be required to mobilize vimentin during cage formation.

DISCUSSION

This study has investigated how infection of cells with ASFV leads to the reorganization of vimentin into a cage surrounding the virus factory. The first experiments showed that production of the vimentin cage by ASFV was not restricted to tissue culture-adapted viruses and could be seen in primary PAECs infected with virulent isolates of ASFV. The subsequent experiments defined two stages in the rearrangement of vimentin. Vimentin was first arranged into an aster at the MTOC at the perinuclear site that would eventually become the virus factory. Following the onset of late gene expression, the vimentin aster was converted into a cage surrounding the virus factory. This rearrangement of vimentin was dependent on microtubules, since vimentin cages were lost in cells treated with nocodazole or expressing p50/dynamitin. Experiments using a panel of phosphoserine-specific antibodies showed that vimentin was phosphorylated on serine 82 during ASFV infection, suggesting that CaM kinase II was activated by ASFV. This was confirmed when increased levels of the activated form of CaM kinase II were observed following virus infection and when vimentin phosphorylation was blocked by KN93, an inhibitor of CaM kinase II. Attempts to demonstrate that vimentin cage formation was directly dependent on activation of CaM kinase II were made using KN93 to inhibit CaM kinase II during ASFV infection. KN93 prevented phosphorylation of vimentin on serine 82, but surprisingly, the drug also blocked ASFV DNA replication and late gene expression, showing that activation of CaM kinase II was required for late ASFV gene expression. It remains to be determined, however, if serine 82 phosphorylation by CaM kinase II was the sole activator of vimentin cage formation or whether phosphorylation was a bystander effect of activation of CaM kinase II during infection. Similarly, the role played by CaM kinase II in ASFV DNA replication is unknown. Studies on Rous sarcoma virus (26) have shown that CaM kinase II may be important during the transcriptional regulation of virus replication, and this may be true for ASFV. Alternatively, since the kinase is involved in signal transduction pathways, the effects of CaM kinase II may be indirect and involve phosphorylation of ASFV proteins by downstream kinases.

The vimentin rearrangements documented here could be useful during both early and late stages of virus replication. The early recruitment of vimentin to the MTOC would allow vimentin to play a structural role in the formation of the virus assembly site. Vimentin may, for example, provide a scaffold for recruitment of viral proteins necessary for virus DNA replication. Soon after the onset of viral DNA replication, vimentin is rearranged into a cage around the factory. Here, the cage may prevent the movement of viral components into the cytoplasm and concentrate structural proteins at sites of assembly, as suggested by Heath et al. (19). Serine phosphorylation is thought to reduce interactions between vimentin filaments and facilitate the redistribution of intermediate filaments. The serine phosphorylation seen in this study may similarly facilitate mobilization of vimentin during conversion of the vimen-

tin aster into a cage able to accommodate viral DNA and the virus factory. Whether these vimentin rearrangements are actively coordinated by the virus or are a bystander effect of viral infection remains to be resolved. We have suggested that virus factories resemble aggresomes formed in cells in response to an accumulation of misfolded protein. It is possible that an accumulation of viral proteins at the MTOC during the initial stages of infection may stimulate the aggresome response. The sequestration of protein aggregates in aggresomes is generally thought to serve a cytoprotective function. The rearrangements of vimentin observed in this study may indicate a use of the aggresome response as a cellular defense against the virus (28, 41); if this is the case, the present study shows this requires activation of CaM kinase II.

ASFV is closely related to the Iridoviridae, and a possible evolutionary link is supported by similarities in structure and morphogenesis (4, 38, 42) and sequence similarities between the major capsid proteins (39). Vimentin rearrangement around virus factories has been documented for the iridovirus frog virus 3 (30). Interestingly, vimentin is phosphorylated in cells infected by frog virus 3, and a temperature-sensitive mutant of frog virus 3 that is unable to phosphorylate vimentin is unable to rearrange vimentin or initiate late gene expression (8, 49). The results again point to a link between vimentin phosphorylation and rearrangement during virus infection and the control of late gene expression.

REFERENCES


