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African Swine Fever Virus Inhibits Induction of the Stress-Induced Proapoptotic Transcription Factor CHOP/GADD153

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Stress signaling from mitochondria and the endoplasmic reticulum (ER) leads to the induction of the proapoptotic transcription factor CHOP/GADD153. Many viruses use the ER as a site of replication and/or envelopment, and this activity can lead to the activation of ER stress and apoptosis. African swine fever virus (ASFV) is assembled on the cytoplasmic face of the ER and ultimately enveloped by ER membrane cisternae. The virus also recruits mitochondria to sites of viral replication and induces the mitochondrial stress protein hsp60. Here we studied the effects of ASFV on the induction of CHOP/GADD153 in infected cells. Interestingly, unlike other ER-tropic viruses, ASFV did not activate CHOP and was able to inhibit the induction of CHOP/GADD153 by a number of exogenous stimuli.

Protein misfolding in the endoplasmic reticulum (ER) or the mitochondrial matrix leads to ER or mitochondrial stress responses, respectively (15, 18, 34). These distinct pathways activate CHOP/GADD153 (CAATT enhancer-binding protein homologous protein/growth and DNA damage protein 153), a death-related transcription factor that facilitates the increased expression of cellular chaperones to counteract the buildup of misfolded protein in mitochondria (34). Interestingly, the overexpression of CHOP also leads to the downregulation of the antiapoptotic protein bcl-2 and to the induction of apoptosis and growth arrest (4, 19–21, 31, 35).

Many viruses use the ER as a site of replication and envelopment (ER-tropic viruses), and recent studies on members of the *Flaviviridae* showed that this activity can lead to the activation of CHOP and to increased susceptibility to apoptosis (17, 28). Cytopathic strains of bovine viral diarrhea virus, for example, activate ER stress pathways and induce the expression of CHOP, and this activity may contribute to the apoptosis seen in infected cells. Similarly, apoptosis induced by Japanese encephalitis virus correlates with the ability of the virus to induce CHOP in target cells (17). Furthermore, the accumulation of a misfolded envelope protein in the ER and the subsequent induction of CHOP have been linked to the neurovirulence of murine retrovirus CasBrE (9). Taken together, the data from this recent work suggest that the induction of CHOP and the consequent activation of apoptosis may be a general cellular response to viruses that interact with the ER.

African swine fever virus (ASFV) is a large cytoplasmic DNA virus that induces a mitochondrial stress response, as indicated by the increased expression of hsp60 (26). ASFV is also an ER-tropic virus with features expected to activate ER stress. The virus has a multigene family encoding cysteine-rich proteins with C-terminal luminal ER retention sequences that cause deformation of the ER and redistribution of luminal ER chaperones (1, 22, 27). ASFV also uses the cytosolic face of the ER as a site of assembly and is enveloped by ER membrane cisternae (2, 5, 27). The induction of mitochondrial stress, coupled with an ability to disrupt the ER, strongly suggested that ASFV infection would increase the expression of CHOP. Moreover, infected cells might be expected to be particularly sensitive to activators of CHOP because the virus encodes a dominant-negative inhibitor of NF-κB (25, 29), a transcription factor known to repress CHOP expression (23). Given these observations, the levels of CHOP in ASFV-infected cells were investigated.

CHOP was first induced by incubating Vero cells with tunicamycin to provoke protein misfolding in the ER (30). Cell lysates were analyzed for CHOP by immunoblotting (Fig. 1A), and the gel showed the expression of CHOP protein in response to tunicamycin (Fig. 1A, lane Tm). Lysates were then prepared from Vero cells that had been infected for increasing times with the Badajoz 1971 Vero cell-adapted (Ba71v) strain of ASFV (10). Infection was confirmed by blotting with anti-p30 and anti-p73 antibodies (Fig. 1A). Surprisingly, ASFV infection did not induce CHOP, and the transcription factor could not be detected in any of the samples.

Indicators of ER stress include induction of the chaperone BiP (32) and phosphorylation of double-stranded RNA-dependent protein kinase-like ER kinase (PERK) (13, 14). Therefore, these parameters of stress induction were investigated using ASFV-infected cells. Figure 1A shows that CHOP and BiP were induced by tunicamycin and that PERK was phosphorylated in response to dithiothreitol (DTT). Interestingly, ASFV infection did not induce the phosphorylation of PERK or the upregulation of BiP, demonstrating that ASFV did not induce an ER stress response. The lack of CHOP induction by ASFV was surprising, as previous studies had shown that ASFV induces mitochondrial stress. The absence of CHOP induction suggested that ASFV was able to block the activation of the transcription factor. CHOP is activated by several reagents, such as arsenite, which induces oxidative stress, and brefeldin A (BFA), DTT, thapsigargin, and tunicamycin, which induce protein misfolding in the ER (3, 4, 11, 12, 30). The ability of ASFV to block the activation of CHOP in response to

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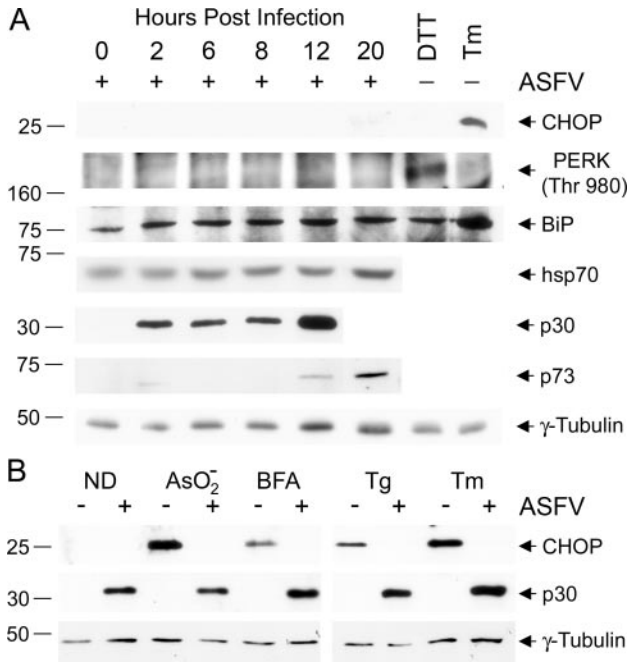


FIG. 1. CHOP upregulation is inhibited by ASFV. (A) Vero cells were infected with Ba71v for increasing times after infection (lanes 0 to 20), incubated with 10 mM DTT for 30 min (lane DTT), or incubated with 10 μg of tunicamycin/ml for 16 h (lane Tm). Lysates were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes prior to being blotted with B-3 (anti-CHOP), anti-phosphorylated PERK (Thr 980), 10C3 (anti-BiP), C92F3A-5 (anti-hsp70), C18 (anti-p30), 17LD3 (anti-p73), or GTU-88 (anti-γ-tubulin) antibodies. The positions of molecular mass markers are indicated to the left of the gel (in kilodaltons). (B) Vero cells 4 h after mock infection (lanes −) or Ba71v infection (lanes +) were incubated for a further 4 h with 50 mM sodium arsenite (AsO_2^-), 1 μg of BFA/ml, 500 μM thapsigargin (Tg), or 10 μg of tunicamycin (Tm)/ml or were left untreated (ND). Total protein (20 μg) from lysates was resolved by SDS-PAGE and transferred to nitrocellulose membranes prior to being blotted with either anti-CHOP or anti-p30 antibodies. The positions of molecular mass markers are indicated to the left of the gel (in kilodaltons). Images were resized and annotated by using Adobe Photoshop 7.0.

these stimuli was tested. The immunoblots in Fig. 1B show that incubation of cells with arsenite, BFA, thapsigargin, and tunicamycin induced CHOP. Significantly, CHOP was absent from lysates taken from cells that had been infected with ASFV (Fig. 1B) prior to the addition of the drugs. The results showed that infection with ASFV blocked CHOP activation in response to exogenous stimuli.

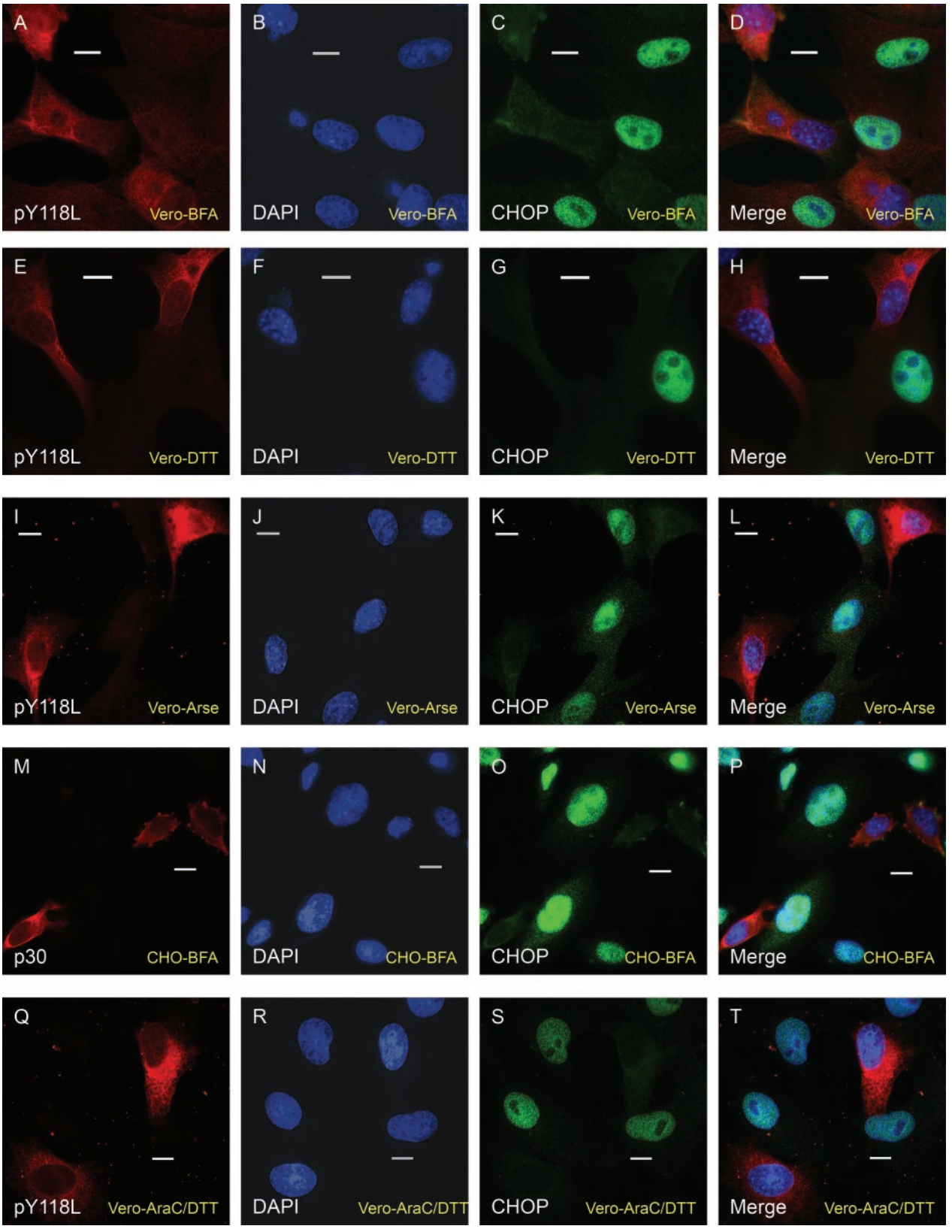
In the next experiment, the effects of ASFV on the location of CHOP in cells undergoing stress was examined. Fig. 2A to D show seven cells; three were infected, as indicated by the presence of the ASFV multigene family 110 protein pY118L (Fig. 2A) and extranuclear 4′,6′-diamidino-2-phenylindole (DAPI) staining of viral DNA (Fig. 2B). The merged image (Fig. 2D) indicates that CHOP was induced by BFA and was present in the nucleus of cells that were negative for viral markers. Significantly, CHOP was not present in cells infected with ASFV. Similar results were obtained after incubation with DTT (Fig. 2E to H) and arsenite (Fig. 2I to L). ASFV therefore inhibited the activation of CHOP and the subsequent accumulation of the protein in the nucleus.

All of the results obtained so far were for the Ba71v strain of ASFV observed in monkey Vero cells. Figure 2M to P show the results of a similar experiment where CHO cells were infected with the Uganda isolate of ASFV (16) prior to the addition of BFA. Again, several cells are shown, and CHOP was expressed in uninfected cells in response to DTT but was absent from cells that were positive for the early ASFV protein p30. These data showed that the inhibition of CHOP activation was a general function of ASFV infection and was not constrained to Ba71v infection of Vero cells. The experiments were repeated with all of the stress inducers used in Fig. 1B for both CHO and Vero cells infected with the Uganda and Ba71v strains, respectively. Again, CHOP was localized to the nucleus in uninfected cells incubated with the drugs but not in cells infected with either strain of ASFV (data not shown). The experiments were repeated in the presence of cytosine β-D-arabinofuranoside (AraC) to block late ASFV gene expression. Figure 2Q to T show the induction of CHOP expression by DTT in the presence of AraC and Ba71v in Vero cells. The results indicated that AraC was unable to inhibit the ability of ASFV to prevent the expression of CHOP. This and similar experiments with arsenite, BFA, thapsigargin, and tunicamycin (data not shown) showed that inhibition of the transcription factor did not require viral DNA replication and/or the expression of late viral proteins.

Having shown a block in the activation of CHOP in response to oxidative and ER stresses, we investigated the ability of the virus to affect other cell stress pathways. Vero cells infected with ASFV for various times were analyzed by immunoblotting for the expression of cellular chaperones associated with the heat shock response. Figure 1A shows the effect of ASFV infection on stress-inducible hsp70 and indicates a slight increase in the expression of hsp70, with levels reaching a maximum at 20 h postinfection. The increased expression of hsp70, coupled with that previously observed for hsp60 (26), following infection shows that ASFV does not induce a general shutdown in stress response pathways and indicates selective inhibition of CHOP expression in response to cell stress.

Given that the first steps in the replication of ASFV include the activation of mitochondrial stress and the parallel recruitment of viral proteins to both the lumen and the cytosolic face

FIG. 2. Vero cells (A to L and Q to T) and CHO cells (M to P) were infected for 12 h with the Ba71v and Uganda strains of ASFV, respectively, and then incubated with 1 μg of BFA/ml (A to D and M to P), 1 mM DTT (E to H and Q to T), or 50 mM sodium arsenite (Arse) (I to L) for 4 h before fixation with 4% paraformaldehyde. The cells in panels Q to T were incubated throughout the experiment with 50 μg of AraC/ml to inhibit DNA synthesis. Cells were stained with either R30 (anti-pY118L) or biotinylated anti-p30 and anti-CHOP antibodies as well as DAPI dye. Primary antibodies were visualized with appropriate secondary reagents conjugated to $Alexa^{488}$ or $Alexa^{594}$. Digital sections (0.2 μm) were captured at a magnification of ×60 and digitally deconvolved by using Openlab 3.1. Bars, 10 μm. Merged images D, H, L, P and T were created digitally. Images were resized and annotated by using Adobe Photoshop 7.0.


A
pY118L
Vero-BFA
B
DAPI
Vero-BFA
C
CHOP
Vero-BFA
D
Merge
Vero-BFA
E
pY118L
Vero-DTT
F
DAPI
Vero-DTT
G
CHOP
Vero-DTT
H
Merge
Vero-DTT
I
pY118L
Vero-Arse
J
DAPI
Vero-Arse
K
CHOP
Vero-Arse
L
Merge
Vero-Arse
M
p30
CHO-BFA
N
DAPI
CHO-BFA
O
CHOP
CHO-BFA
P
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CHO-BFA
Q
pY118L
Vero-AraC/DTT
R
DAPI
Vero-AraC/DTT
S
CHOP
Vero-AraC/DTT
T
Merge
Vero-AraC/DTT

of the ER, an ability to block the activation of CHOP may prevent early apoptosis and ensure productive viral replication. Intriguingly, prolonged exposure to tunicamycin does eventually reduce the replication of ASFV (7), despite the lack of any major glycoproteins in mature virions (6). These observations imply that the prolonged activation of ER stress, possibly by misfolded cellular glycoproteins, can, in principal, be detrimental to ASFV replication. While the activation of CHOP is emerging as a general response to ER-tropic viruses, much less is known about how viruses inhibit the activation of CHOP. Viral inhibition of CHOP is implied because several ER-tropic viruses, including field strains of ASFV (8, 24, 33) and members of the *Flaviviridae*, are able to establish persistent infections, suggesting the suppression of apoptosis. It is anticipated that further studies on how ASFV inhibits the activation of CHOP will shed light on how other ER-tropic viruses may survive by inhibiting CHOP-induced apoptosis.

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