The Npro product of classical swine fever virus and bovine viral diarrhea virus uses a conserved mechanism to target interferon regulatory factor-3


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Classical swine fever virus (CSFV) is a member of the genus Pestivirus in the family Flaviviridae. The Npro product of CSFV targets the host’s innate immune response and can prevent the production of type I interferon (IFN). The mechanism by which CSFV orchestrates this inhibition was investigated and it is shown that, like the related pestivirus bovine viral diarrhea virus (BVDV), this involves the Npro protein targeting interferon regulatory factor-3 (IRF-3) for degradation by proteasomes and thus preventing IRF-3 from activating transcription from the IFN-β promoter. Like BVDV, the steady-state levels of IRF-3 mRNA are not reduced markedly by CSFV infection or Npro overexpression. Moreover, IFN-α stimulation of CSFV-infected cells induces the antiviral protein MxA, indicating that, as in BVDV-infected cells, the JAK/STAT pathway is not targeted for inhibition.

A major feature of most, if not all, viruses is their ability to evade the innate immune response of the host (reviewed by Goodbourn, 2000; Haller et al., 2006). The induction of type I interferon (IFN) is triggered by the recognition of viral nucleic acids, either presented to members of the Toll-like receptor (TLR) family in endosomes or generated during viral replication and recognized by the RNA helicases mda-5 and RIG-I (reviewed by Kawai & Akira, 2006). Whilst both mda-5 and RIG-I can recognize and respond to double-stranded RNA (dsRNA) (Andrejeva et al., 2004; Yoneyama et al., 2004, 2005; Yamashita et al., 2005; Kato et al., 2006), RIG-I can also recognize and respond to nucleic acids bearing an unprotected 5’ triphosphate (Hornung et al., 2006; Pichlmair et al., 2006). Activation of TLR3 and the RNA helicases by dsRNA indirectly permits the recruitment and activation of the kinases TBK1 and IKKε, which directly phosphorylate IRF-3 and IKKcomponents that activate nuclear factor κB (NF-κB). Activated IRF-3 and NF-κB translocate to the nucleus, where they form an ‘enhanceosome’ complex with other proteins, including activating transcription factor-2 (ATF-2), c-Jun, CREB-binding protein (CBP) and p300, leading to the transcriptional activation of the IFN-β promoter (reviewed by Merika & Thanos, 2001). IFN-β is subsequently secreted from the cell and stimulates an ‘antiviral state’ in neighbouring cells.

Recent studies have shown that both CSFV and the related pestivirus bovine viral diarrhea virus (BVDV) encode active blocks to type I IFN induction (Charleston et al., 2001; Schweizer & Peterhans, 2001; Baigent et al., 2002; Ruggli et al., 2003), as a consequence of blocking IRF-3 function (Baigent et al., 2002, 2004; Horscroft et al., 2005). This is a property of the Npro protein, a cysteine-like autoprotease that cleaves itself from the polyprotein (Rumenapf et al., 1998; Ruggli et al., 2003, 2005; La Rocca et al., 2005; Gil et al., 2006; Hilton et al., 2006; Bauhofer et al., 2007). However, there are reported mechanistic differences between the actions of the viral Npro proteins. Whilst CSFV Npro expression causes a reduction in cellular IRF-3 levels, it was suggested that this...
was a result of transcriptional inhibition of the IRF-3 promoter in CSFV-infected cells (La Rocca et al., 2005). In contrast, we reported recently that BVDV Npro prevents IRF-3 binding to DNA and targets its destruction by cellular proteasomes (Hilton et al., 2006); no effects on IRF-3 transcription were observed. We have therefore re-evaluated the properties of CSFV Npro and conclude that it behaves in a manner similar to the Npro protein of BVDV, targeting IRF-3 specifically for proteasome-dependent proteolysis, but is unable to affect IRF-3 transcription significantly. Independently, whilst this manuscript was in preparation, it was reported that CSFV Npro targets IRF-3 for degradation, with IRF-3 mRNA levels remaining unaffected (Bauhofer et al., 2007).

To confirm that the previously reported block to IFN production was operating at the level of IFN-β transcription, we examined the effect of infecting PK15 cells transiently transfected with an IFN-β reporter with CSFV. Fig. 1(a) shows that infection with CSFV (strains Brescia or Alfort) failed to activate the IFN-β promoter (lanes 1, 3 and 5), but both strains had the ability to block IFN-β induction when Sindbis virus was used as an inducer (Fig. 1a, lanes 2, 4 and 6). Moreover, a PK15 cell line expressing the CSFV Npro gene was unresponsive to Sindbis virus (Fig. 1a, lanes 7 and 8). To confirm that the CSFV Npro gene was sufficient to block IFN-β induction, we transfected plasmids capable of expressing the Npro gene of different CSFV strains into PK15 cells and examined the

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**Fig. 1.** The Npro polypeptide of CSFV is sufficient to block IFN-β induction and targets IRF-3-dependent transcription. (a) PK15 cells were mock-infected or infected overnight with CSFV strains Brescia or Alfort at an m.o.i. of 2 and then transfected with a reporter for IFN-β promoter activity, and the β-galactosidase reporter vector, pJATlac [for details of reporters, see Hilton et al. (2006)]. After a further 24 h, one set of a duplicate transfection was infected with Sindbis virus for 4 h before determining luciferase activity and normalizing results for β-galactosidase expression. The effect of Sindbis virus infection of a PK15 cell line expressing the Npro gene of CSFV strain Alfort is shown in lanes 7 and 8. (b, c) PK15 (b) or Vero (c) cells were transfected with the IFN-β reporter and pJATlac, and either a mammalian expression plasmid driving the overexpression of SV5 V, the Npro protein of BVDV (pe515 strain), the Npro proteins of CSFV strains Alfort, Brescia and Riems, or the control 'empty vector' pEF.plink2. Forty-eight hours after transfection, cells were either mock-treated or transfected with the synthetic dsRNA poly(I).poly(C), and cell extracts were prepared. (d, e) Vero cells were transfected with a reporter for IRF-3 activity (d) or NF-κB activity (e), pJATlac, and either a mammalian expression plasmid driving the overexpression of SV5 V, the Npro protein of BVDV, the Npro protein of CSFV strains Alfort, or pEF.plink2. Forty-eight hours after transfection, cells were either mock-treated or transfected with the synthetic dsRNA poly(I).poly(C), and cell extracts were prepared. (f, g) Vero cells were transfected with an IFN-β reporter (f) or an NF-κB-dependent reporter (g), pJATlac, either pEF.plink2 or the CSFV Alfort Npro-expressing plasmid pEF.Npro, and a mammalian expression plasmid driving the overexpression of TRIF, TBK1, mda-5 or RIG-I as indicated. All cell extracts were analysed for luciferase and β-galactosidase levels (King & Goodbourn, 1994). Relative expression values were calculated accordingly. A reference value of 100 has been assigned to the level of expression seen with dsRNA-treated or Sindbis virus-infected control cells (a–e) or the level of expression seen with each of the effectors in the absence of Npro (f, g). Values shown represent data from at least two independent experiments; error bars represent the standard error of the mean, or, for results from only two independent experiments, the range of values.
ability of these cells to induce IFN-β in response to the synthetic dsRNA poly(I).poly(C). The data in Fig. 1(b) show that all of these Npro genes were able to inhibit IFN-β induction to a considerable degree, and were comparable to the Npro product of BVDV. As seen for BVDV Npro, all of the CSFV Npro proteins that we examined (Brescia, Alfort and Riems strains) were also capable of inhibiting IFN-β induction in Vero cells, demonstrating that the species restrictions seen with CSFV are not due to a limitation in the block of IFN-β induction (Fig. 1c). Induction of the IFN-β promoter by dsRNA involves the activation from cytoplasmic pools and translocation to the nucleus of both NF-κB and IRF-3. Like the Npro product of BVDV (Hilton et al., 2006), CSFV Npro is able to block the activation of the IRF-3-dependent IFN-β (Fig. 1b, c) and IRF-3-dependent ISG54 (Fig. 1d) promoters efficiently, but unable to block the activation of an NF-κB-dependent promoter (Fig. 1e). This is in contrast to the properties of the V protein of the paramyxovirus parainfluenza virus 5/simian virus 5 (SV5), which acts as an inhibitor of the RNA helicase mda-5 (Andrejeva et al., 2004) and therefore blocks the activation of both IRF-3 and NF-κB (Fig. 1d, e).

Fig. 2. CSFV and the CSFV Npro product target IRF-3 for proteasome-mediated degradation. (a) Extracts from mock-infected or CSFV (Brescia strain)-infected cells PK15 cells (+) were examined by Western blotting for IRF-3 (Santa Cruz SC9082; top panel) or γ-tubulin (Sigma; bottom panel). (b) Extracts from CSFV-infected cells treated or not with 25 μM MG132 (Sigma) were examined by Western blotting for IRF-3 (top panel), Npro (middle panel) or tubulin (bottom panel). (c) PK15 cells were infected with CSFV for 16 h and then treated with MG132 for the indicated times before being analysed by Western blotting for IRF-3 (top panel), Npro (middle panel) or tubulin (bottom panel). (d) Nuclear extracts were prepared from PK15 cells infected with CSFV in the absence or presence of MG132 and were analysed by Western blotting for IRF-3 (top panel) and γ-tubulin (bottom panel). (e) Extracts of PK15 cells expressing or not the Npro gene of CSFV Alfort (PK15Npro) were examined by Western blotting for the Npro protein (top panel), IRF-3 (middle panel) and tubulin (bottom panel). (f) Total RNA from PK15 cells, PK15 cells infected with CSFV for 26 h or PK15 cells expressing the Npro gene of CSFV Alfort (PK15Npro) was analysed by an RNase-protection assay using probes specific for porcine IRF-3 (spanning the last 385 bp of the open reading frame; top panel) and β-actin (Enoch et al., 1986; bottom panel). Autoradiography was performed by using intensifying screens and film exposure at -80 °C. To quantify the RNase-protection signals, five exposures of the autoradiograph were obtained and two exposures containing non-saturated signals for each of the IRF-3 and actin signals were chosen for densitometry scanning using a Bio-Rad scanner and software. Equivalent data were obtained from a separate set of RNA preparations and analysis of both datasets shows that, when corrected to the actin mRNA levels, the mean ± SD IRF-3 mRNA levels in CSFV-infected cells are 86.1 ± 10.5 % and in Npro-expressing cells are 93.2 ± 15.2 % of those in mock-infected cells.
These data suggest that CSFV Npro targets IRF-3-dependent transcription specifically. Consistent with this, the block to transcriptional activation by IRF-3 was seen regardless of whether IRF-3 was activated by dsRNA (Fig. 1b–d) or by overexpression of the signal-transduction components TRIF, TBK-1, mda-5 or RIG-I (Fig. 1f); in contrast, activation of NF-κB in the presence of CSFV Npro by any of the signal-transduction components was either unaffected or even stimulated (Fig. 1g).

We next designed experiments to examine the mechanism of the Npro block to IRF-3 function, with the intention of distinguishing between the proposed mechanism for CSFV of inhibiting IRF-3 transcription (La Rocca et al., 2005) and the degradation of IRF-3 by proteasomes, as reported for BVDV (Hilton et al., 2006). Initially, we infected PK15 cells and analysed cell extracts by Western blotting. Fig. 2(a) shows that, 24 h after CSFV infection, IRF-3 had disappeared almost completely, and had not returned by 72 h. When we performed infections in the presence of MG132, a specific inhibitor of proteasome function, we observed that loss of IRF-3 in response to CSFV infection was overcome (Fig. 2b). In order to demonstrate that IRF-3 is turned over continuously during CSFV infection, we allowed cells to accumulate viral gene products and then treated them with MG132. Fig. 2(c) shows that IRF-3 levels are undetectable after infection with CSFV, consistent with viral-specific loss of IRF-3, but rise to levels equivalent to those seen in uninfected cells within 20 h of MG132 treatment; importantly, levels of CSFV Npro are unaffected by the MG132 treatment. These results suggested that, as in BVDV infections, IRF-3 is targeted for proteasomal degradation in CSFV-infected cells. Similar to our previous observations on BVDV, we note that IRF-3 accumulates in the nucleus in CSFV-infected cells if the degradation is prevented (Fig. 2d), indicating that CSFV infection is also able to activate IRF-3 nuclear translocation. To extend these observations, we compared the levels of IRF-3 in parental PK15 cells or in PK15 cells stably expressing the Npro product of the Alfort strain of CSFV. Fig. 2(e) shows that IRF-3 levels are considerably lower in the latter cell type. The above data indicate that the CSFV Npro protein targets IRF-3 for degradation.

To investigate whether IRF-3 mRNA was also targeted, we examined its fate in PK15 cells infected with CSFV. Total RNA was extracted from PK15 cells that had been infected with CSFV for 26 h, from uninfected PK15 cells or from the PK15 Npro cell line and analysed for the presence of porcine IRF-3 mRNA by using an RNase-protection assay. Fig. 2(f) shows that, in contrast to the effects on IRF-3 protein level, IRF-3 mRNA showed no marked reduction in levels following CSFV infection. Furthermore, IRF-3 mRNA levels in the PK15 line expressing the Alfort Npro product were comparable with those seen in the parental PK15 line.

To complement the similarities between CSFV and BVDV, we investigated whether CSFV targets the JAK/STAT pathway that is responsible for amplification of the initial IFN response. Fig. 3 shows that the response of a typical IFN-responsive gene, MxA, is unaffected by CSFV infection, in contrast to the equivalent response of this gene to dsRNA, which is inhibited completely. The presence of an intact JAK/STAT pathway during CSFV infection is consistent with non-cytopathic BVDV infections (Baigent et al., 2002; Schweizer et al., 2006) and confirms that the related viruses use similar evasion strategies.

These conclusions are in agreement with the results of a recent publication (Bauhofer et al., 2007) that reported, whilst our manuscript was in preparation, that the CSFV Npro product targets cellular IRF-3 for degradation without affecting IRF-3 mRNA levels or IRF-3 transcription. The basis for the discrepancy with the observations made by La Rocca et al. (2005), who observed a decrease in IRF-3 promoter activity, remains unclear; it should be stressed that these authors examined the effects of CSFV infection, rather than the effect of Npro protein alone, and indeed the data shown in Fig. 2(f) suggest that CSFV infection can cause a small decrease in IRF-3 mRNA levels. However, this may not be a function of the Npro protein, as IRF-3 mRNA levels were less affected in a cell line expressing Npro (Fig. 2f). We also note that, unlike Bauhofer et al. (2007), La Rocca et al. (2005) did not investigate the effects of CSFV infection on control promoters, and it remains possible that their observations reflect some general effect of CSFV on cellular transcription; it is interesting to note that recombinant CSFV nucleocapsid protein has been reported to have the ability to regulate transcription of some genes (Liu et al., 1998).

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References


