The classical swine fever virus Npro product is degraded by cellular proteasomes in a manner that does not require interaction with interferon regulatory factor 3

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Classical swine fever is a notifiable disease of pigs. The causative agent, classical swine fever virus (CSFV), is highly contagious and causes mild to severe haemorrhagic disease depending on the virulence of the strain. The RNA genome of CSFV is translated as a single polyprotein that is processed to yield 12 proteins. Like other pestiviruses, the first protein to be translated is the N-terminal autoprotease termed Npro. A novel pestiviral protein with no known cellular homologues, Npro antagonizes type I interferon (IFN) induction by binding and targeting the transcription factor IFN regulatory factor 3 (IRF-3) for ubiquitin-dependent proteasomal degradation. In this study, CSFV-infected PK-15 cells and stable cell lines were used to show that Npro is itself an unstable protein that is targeted for proteasomal degradation in a ubiquitin-dependent manner. In addition, Npro is not degraded as a direct consequence of its ability to interact with IRF-3 or to target IRF-3 for proteasomal degradation.

Pestiviruses such as classical swine fever virus (CSFV) encode a novel N-terminal autoprotease termed Npro (Lindenbach et al., 2007; Thiel et al., 1996). CSFV and the related bovine viral diarrhea virus (BVDV) prevent host production of type I interferon (IFN) (Baigent et al., 2002, 2004; Bensaude et al., 2004; Charleston et al., 2001; Ruggli et al., 2003; Schweizer & Peterhans, 2001). This is facilitated in part by the N-terminal protease (Npro) protein, which targets IFN regulatory factor 3 (IRF-3) for proteasomal degradation (Bauhofer et al., 2007; Chen et al., 2007; Hilton et al., 2006; Seago et al., 2007). In BVDV-infected cattle, the antagonism of the innate immune response by Npro, in combination with the E1 protein product, is essential in blocking type I IFN induction and establishing persistent infection in the natural host (Meyers et al., 2007).

The positive-stranded RNA genome of CSFV is translated as a polyprotein, from which Npro cleaves itself. However, the protease activity of Npro is not required for the inhibition of IFN-α/β induction or the targeting of IRF-3 for degradation (Gil et al., 2006; Hilton et al., 2006; Ruggli et al., 2009). The mechanism by which Npro orchestrates the proteasomal degradation of IRF-3 is as yet unknown, but involves the ubiquitination of IRF-3 (Chen et al., 2007; Hilton et al., 2006). Npro has been shown to interact with IRF-3; however, it is unclear whether this is a direct interaction or mediated through another protein (Bauhofer et al., 2007; Chen et al., 2007; Ruggli et al., 2009). Npro does not appear to inhibit the general phosphorylation of IRF-3 or interfere with the nuclear localization of a constitutively active form of IRF-3 (Bauhofer et al., 2007). To date, no cellular homologues of Npro have been identified and Npro does not appear to contain sequence similarities to other viral proteins that also bind IRF-3 and orchestrate its proteasomal degradation, such as rotavirus non-structural protein 1 (NSP1) (Bauhofer et al., 2007).

Here, we present data to show that the Npro product of CSFV is itself degraded rapidly by the proteasome in a manner that is dependent upon an intact ubiquitination system. Furthermore, Npro is not degraded as a direct consequence of its ability to interact with IRF-3 or to target IRF-3 for proteasomal degradation.

To generate PK-15 cells constitutively expressing the CSFV Npro product from a human cytomegalovirus (CMV) immediate-early promoter, we constructed plasmids encoding either His-tagged or non-tagged Npro (Alfort strain) and containing the woodchuck post-transcriptional regulatory element (WPRE) (Donello et al., 1998). WPRE has been shown to facilitate nucleocyttoplasmic transport of RNA and acts on additional post-transcriptional mechanisms to stimulate expression of heterologous cDNAs (Mähönen et al., 2007; Popa et al., 2002; Zufferey et al., 1999). Western blot analysis of cell extracts prepared from either stable cell line revealed barely detectable Npro protein levels. A previous study reported that PK-15 and SK-6 cell lines expressing an enhanced green fluorescent protein...
(EGFP)–Npro fusion protein under the control of the CMV promoter expressed 60 and 10 times less protein, respectively, than EGFP control lines (Ruggli et al., 2005). To investigate the stability of Npro, we treated two PK-15 single-cell lines with the proteasome inhibitor epoxomicin (Alexis Biochemicals) in the presence of the general caspase inhibitor ZVAD (BACHEM), the addition of which limited the apoptotic side effects of long-term epoxomicin treatment (Myung et al., 2001). As shown in Fig. 1(a), Npro and His-tagged Npro protein accumulated rapidly over a short period of time. Similar results were obtained using the proteasome inhibitor MG132 (Sigma) (see Fig. 1b–d) and suggest that the Npro protein of CSFV is targeted for proteasomal degradation in PK-15 cells (Myung et al., 2001). The proteasome inhibitors MG132 and epoxomicin were used interchangeably in this study because of their equal abilities to prevent Npro turnover.

In order to determine how quickly CSFV-encoded Npro is turned over within infected PK-15 cells, we used the translation inhibitor cycloheximide (CHX) (Sigma) in conjunction with Western blot analysis. Fig. 1(b) shows that CSFV-encoded Npro is a short-lived protein in PK-15 cells, with a half-life of about 4 h. In agreement with the data obtained using stable cell lines, the proteasome inhibitor MG132, but not the cysteine protease inhibitor E64D (Sigma), was able to prevent Npro turnover.

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cells. Similarly, the serine protease inhibitor PMSF (Sigma) was unable to prevent Npro degradation (data not shown).

Next, we examined whether the presence of the other CSFV proteins could influence the level of Npro expressed in a stable cell line. PK-15 cells expressing His-tagged Npro were infected with CSFV and then left untreated or treated with proteasome inhibitor. Fig. 1(c) shows that CSFV infection led to only a small increase of His-tagged Npro protein in comparison to mock infection, suggesting that the other CSFV proteins do not modulate Npro stability significantly.

Components of the proteasome are localized throughout the cell in both the cytoplasm and the nucleus (Coux et al., 1996). We have shown recently that Npro also localizes to both the cytoplasm and the nucleus of CSFV-infected cells and cell lines stably expressing Npro (Doceul et al., 2008). To determine whether proteasome inhibitors lead to an accumulation of Npro in a particular compartment, cytoplasmic and nuclear extracts prepared from a His–Npro single-cell line treated with MG132 or epoxomicin were analysed by Western blot. Likewise, to determine whether Npro degradation leads to the depletion of Npro in either compartment, the same cell line was treated with CHX for different periods of time and extracts were analysed. Fig. 1(d) shows that Npro protein accumulated in both the cytoplasm and the nuclei of cells in which the proteasome activity had been inhibited. Fig. 1(e) shows that, after 2 h inhibition of protein translation by CHX, a comparable decrease of Npro levels in both compartments was observed. It is conceivable that Npro, an approximately 19 kDa protein, is degraded in the cytoplasm and not in the nucleus, but diffuses passively between compartments following its accumulation or degradation.

Most proteasomal substrates are ubiquitinated before being degraded. CSFV Npro (Alfort/187 strain, GenBank accession no. X87939) contains 13 lysine residues, one or more of which may be targeted for ubiquitination. To investigate whether Npro is ubiquitinated prior to its degradation, PK-15 cells stably expressing His–Npro were treated with MG132 before ubiquitinated proteins were precipitated from cell extracts by using a commercial kit (Ubiquitin Enrichment kit; Pierce). Analysis of the precipitated samples by Western blotting revealed a ladder of higher-molecular-mass forms of Npro from around 30 to 50 kDa, suggesting that Npro is indeed targeted for ubiquitination (Fig. 2a). Non-ubiquitinated Npro was also identified in the ubiquitin-enriched sample that was analysed and was presumably a consequence of co-precipitation with a ubiquitinated protein or the removal of ubiquitin by deubiquitinases. To provide more evidence that Npro turnover is reliant on an intact ubiquitination pathway, we used murine fibroblast ts20 cell lines stably expressing His-tagged or non-tagged Npro protein. The ts20 cell line [a gift from Sylvain Meloche (Institut de Recherche en Immunologie et Cancérologie, Université de Montréal, Montréal, Québec, Canada) with the consent of Harvey Ozer (New Jersey Medical School, Newark, NJ, USA)] expresses a temperature-sensitive E1 ubiquitin-activating enzyme (Bibeau-Poirier et al., 2006). When ts20 cells are shifted from a permissive temperature (34 °C) to a restrictive temperature (39 °C), the E1 ubiquitin ligase is

![Fig. 2.](http://vir.sgmjournals.org)
inactivated and proteins can no longer be degraded by the ubiquitin-dependent proteasome pathway. Fig. 2(b) shows that, in two independent ts20 cell lines stably expressing \( \text{N}^{\text{pro}} \), the inhibition of ubiquitin-dependent proteasomal decay, either by a shift in temperature or by the addition of epoxomicin, led to a dramatic increase in the amount of \( \text{N}^{\text{pro}} \) protein. These results confirm that an intact ubiquitin system is required for \( \text{N}^{\text{pro}} \) turnover.

We next investigated whether the turnover of \( \text{N}^{\text{pro}} \) is related directly to its ability to interact with and to target IRF-3 for degradation. To do this, we utilized individual cell lines stably expressing \( \text{N}^{\text{pro}} \) point mutants: PK-15 cell lines expressing \( \text{N}^{\text{pro}} \) with either a cysteine to arginine mutation at position 112 (C\(_{112}\)R) or an aspartic acid to asparagine mutation at position 136 (D\(_{136}\)N), and a 3T3 cell line expressing \( \text{N}^{\text{pro}} \) with a leucine to proline mutation at position 8 (L\(_{8}\)P). The point mutations C\(_{112}\)R and D\(_{136}\)N in \( \text{N}^{\text{pro}} \) have recently been shown to abate its capacity to degrade IRF-3 and prevent IFN-\( \beta \) promoter activation, as well as prevent the interaction of \( \text{N}^{\text{pro}} \) with IRF-3 (Ruggli \textit{et al.}, 2009; Szymanski \textit{et al.}, 2009). Similarly, \( \text{N}^{\text{pro}} \) L\(_{8}\)P does not antagonize IFN-\( \alpha/\beta \) production (Gil \textit{et al.}, 2006).

Western blots of whole-cell extracts revealed that \( \text{N}^{\text{pro}} \) protein was barely detectable in the PK-15 cell lines individually expressing either the C\(_{112}\)R or D\(_{136}\)N point mutant or wild-type \( \text{N}^{\text{pro}} \) (Fig. 3a). Likewise, the level of L\(_{8}\)P \( \text{N}^{\text{pro}} \) and wild-type \( \text{N}^{\text{pro}} \) expressed in the 3T3 cell lines was low (Fig. 3b). However, treatment with proteasome inhibitor led to an accumulation of both mutant and wild-type \( \text{N}^{\text{pro}} \) in both cell types. In agreement, CHX assays using the PK-15 cell lines stably expressing C\(_{112}\)R or D\(_{136}\)N

**Fig. 3.** \( \text{N}^{\text{pro}} \) is not degraded as a direct consequence of its ability to interact with and to target IRF-3 for degradation. (a) PK-15 cell lines stably expressing \( \text{N}^{\text{pro}} \) or either the C\(_{112}\)R or D\(_{136}\)N \( \text{N}^{\text{pro}} \) point mutant were incubated in the presence or absence of epoxomicin and the caspase inhibitor ZVAD for 15 h. Whole-cell extracts were analysed by Western blotting for \( \text{N}^{\text{pro}} \) and \( \gamma \)-tubulin (loading control). (b) Mouse 3T3 cell lines stably expressing \( \text{N}^{\text{pro}} \) or the L\(_{8}\)P \( \text{N}^{\text{pro}} \) point mutant were incubated in the presence or absence of MG132 or epoxomicin and the caspase inhibitor ZVAD for 10 h. Whole-cell extracts were analysed by Western blotting for \( \text{N}^{\text{pro}} \) and \( \gamma \)-tubulin. (c) PK-15 cells stably expressing wild-type \( \text{N}^{\text{pro}} \) or the C\(_{112}\)R or D\(_{136}\)N \( \text{N}^{\text{pro}} \) point mutant were treated with CHX for the indicated periods of time and whole-cell extracts were analysed by Western blotting for \( \text{N}^{\text{pro}} \) and \( \gamma \)-tubulin. (d) Whole-cell extracts of IRF-3 knockout (\( \text{IRF-3} \)\(^{-/-}\)) and wild-type littermate (\( \text{IRF-3} \)\(^{+/+}\)) MEFs were analysed by Western blotting for IRF-3 and \( \gamma \)-tubulin. (e) \( \text{IRF-3} \)\(^{-/-}\) and \( \text{IRF-3} \)\(^{+/+}\) MEF cells stably expressing \( \text{N}^{\text{pro}} \) were incubated in the presence or absence of MG132 and ZVAD for 10 h. Whole-cell extracts were analysed by Western blotting for \( \text{N}^{\text{pro}} \) and \( \gamma \)-tubulin. (f) Whole-cell extracts from parental 3T3 or 3T3 \( \text{N}^{\text{pro}} \) cells stably expressing control or IRF-3 siRNA were analysed by Western blotting for IRF-3, green fluorescent protein (GFP) (a marker of the siRNA constructs), \( \gamma \)-tubulin and \( \text{N}^{\text{pro}} \).
N\textsuperscript{pro} turnover does not require interaction with IRF-3

N\textsuperscript{pro} confirmed that these proteins were degraded rapidly, like wild-type N\textsuperscript{pro}, and were not stabilized by their inability to interact with IRF-3 (Fig. 3c). In fact, C\textsubscript{112}R and D\textsubscript{126}N N\textsuperscript{pro}, particularly the former, appeared to be inherently less stable than wild-type N\textsuperscript{pro}. The reasons for this are unclear, but may involve different protein conformations (Szymanski et al., 2009).

Next, to determine whether the degradation of N\textsuperscript{pro} is dependent on the level of IRF-3 within a cell, we utilized mouse embryonic fibroblasts (MEFs) in which the IRF-3 gene had been knocked out (−/− IRF-3) (a gift from Tadatsugu Taniguchi, Department of Immunology, Graduate School of Medicine and Faculty of Medicine, University of Tokyo, Tokyo, Japan) (Sato et al., 2000). The inability of the −/− IRF-3 MEFs to express IRF-3 was first confirmed by Western blotting (Fig. 3d). Stable transfections were then performed to generate −/+ − IRF-3 MEFs and control MEFs (derived from a +/+ IRF-3 littermate) that expressed N\textsuperscript{pro} (Alfort strain). These were treated with proteasome inhibitor and whole-cell extracts were analysed by Western blotting. Fig. 3(e) shows that N\textsuperscript{pro} was only detectable in both +/+ and −/− IRF-3 MEFs after inhibition of the proteasome, confirming that N\textsuperscript{pro} is still degraded rapidly in the absence of IRF-3. As the N\textsuperscript{pro}-expressing MEFs were non-clonal, the higher level of N\textsuperscript{pro} observed in the +/+ IRF-3 cells was most probably a result of variation in transfection efficiency. However, to further investigate the role of IRF-3 in N\textsuperscript{pro} degradation, an IRF-3 small interfering RNA (siRNA) construct (InvivoGen) was utilized to generate stable N\textsuperscript{pro}-expressing cell lines in which IRF-3 mRNA was targeted. A control siRNA construct (InvivoGen) was used as a negative control. Fig. 3(f) confirms that IRF-3 protein levels were reduced considerably by the IRF-3 siRNA construct. However, comparison of the N\textsuperscript{pro}-expressing control and IRF-3 siRNA cell lines revealed no observable difference in N\textsuperscript{pro} protein expression (Fig. 3f). These results confirm that the presence of IRF-3 does not stabilize N\textsuperscript{pro}.

Here, we have provided biochemical evidence that N\textsuperscript{pro} is an unstable protein that is degraded rapidly by the proteasome in a ubiquitin-dependent manner in PK-15, 3T3 and MEF stable cell lines and CSFV-infected PK-15 cells. CSFV translation produces a polyprotein and initially generates an equimolar quantity of viral proteins. It is feasible that N\textsuperscript{pro} is susceptible to rapid degradation in order to regulate its cellular levels in both the nuclear and cytoplasmic compartments. This may also be the case for the core product of the related hepatitis C virus, the turnover of which occurs via both ubiquitin-dependent and -independent proteasomal pathways (Shirakura et al., 2007; Suzuki et al., 2009).

Like N\textsuperscript{pro}, the rotavirus NSP1 product binds and targets IRF-3 for proteasomal degradation (Barro & Patton, 2005, 2007; Graff et al., 2002, 2007). NSP1 is also degraded in a proteasome-dependent manner, a property attributed to its proposed function as an E3 ubiquitin ligase (Graff et al., 2007). Furthermore, NSP1’s ability to degrade IRF-3 is independent of NSP1 proteasomal degradation (Sen et al., 2009). It remains to be determined whether the CSFV N\textsuperscript{pro} product targets IRF-3 via an intrinsic ubiquitin ligase activity or whether this function is mediated through a separate protein. N\textsuperscript{pro} has recently been shown to contain a zinc-binding TRASH motif, but the authors suggest that this is unlikely to confer E3 ubiquitin ligase activity (Szymanski et al., 2009). This study placed particular emphasis on investigating the correlation between N\textsuperscript{pro} and N\textsuperscript{pro}-mediated IRF-3 turnover. We show that N\textsuperscript{pro} is not degraded as a direct consequence of its ability to interact with and to target IRF-3 for ubiquitin-dependent proteasomal degradation. However, the data presented here show that an intact ubiquitin system is required for the turnover of N\textsuperscript{pro} per se.

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References


degradation of interferon regulatory factor-3 induced by N\textsuperscript{pro} from a cytopathic bovine viral diarrhea virus. Virolology 366, 277–292.


