NSP1 of human rotaviruses commonly inhibits NF-κB signalling by inducing β-TrCP degradation

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Rotavirus is a leading cause of severe gastroenteritis in infants worldwide. Rotavirus nonstructural protein 1 (NSP1) is a virulence factor that inhibits innate host immune responses. NSP1 from some rotaviruses targets host interferon response factors (IRFs), leading to inhibition of type I interferon expression. A few rotaviruses encode an NSP1 that inhibits the NF-κB pathway by targeting β-TrCP, a protein required for IκB degradation and NF-κB activation. Available evidence suggests that these NSP1 properties involve proteosomal degradation of target proteins. We show here that NSP1 from several human rotaviruses and porcine rotavirus CRW-8 inhibits the NF-κB pathway, but cannot degrade IRF3. Furthermore, β-TrCP levels were much reduced in cells infected with these rotaviruses. This provides strong evidence that β-TrCP degradation is required for NF-κB pathway inhibition by NSP1 and demonstrates the relevance of β-TrCP degradation to rotavirus infection. C-terminal regions of NSP1, including a serine-containing motif resembling the β-TrCP recognition motif of IκB, were required for NF-κB inhibition. CRW-8 infection of HT-29 intestinal epithelial cells induced significant levels of IFN-β and CCL5 but not IL-8. This contrasts with monkey rotavirus SA11-4F, whose NSP1 inhibits IRF3 but not NF-κB. Substantial amounts of IL-8 but not IFN-β or CCL5 were secreted from HT-29 cells infected with SA11-4F. Our results show that human rotaviruses commonly inhibit the NF-κB pathway by degrading β-TrCP and thus stabilizing IκB. They suggest that NSP1 plays an important role during human rotavirus infection by inhibiting the expression of NF-κB-dependent cytokines, such as IL-8.

INTRODUCTION

Rotavirus is a leading cause of infantile gastroenteritis in humans and many animal species. Innate and adaptive immune responses play crucial roles in controlling rotavirus replication within the host (Angel et al., 2012; Holloway & Coulson, 2013). A vital arm of the innate response is the recognition of invading viruses by the host and the production of immunomodulatory and antiviral cytokines of the IFN family (Randall & Goodbourn, 2008). Rotavirus has evolved several mechanisms to counteract the host response, comprising interference with cellular proteins required for IFN induction, disruption of IFN signalling through its receptors and antagonism of specific cellular antiviral processes (Barro & Patton, 2005; Graff et al., 2009; Holloway et al., 2009, 2014; Sen et al., 2014; Zhang et al., 2013). A key factor in the evasion of host responses by rotavirus is its nonstructural protein 1 (NSP1), which is the least conserved rotavirus protein.

NSP1 is not required for rotavirus replication in cell culture, but enhances rotavirus replication in mice (Barro & Patton, 2005; Broquet et al., 2011; Feng et al., 2013).

Several studies show that NSP1 from some rotavirus strains can preferentially bind and degrade members of the IFN response factor (IRF) family including IRF3/5/7, key regulators of IFN induction (Barro & Patton, 2005, 2007; Graff et al., 2002). It has been proposed that NSP1 has E3 ubiquitin ligase activity and mediates degradation of its targets through the ubiquitin/proteasome pathway. NSP1 proteins from most human rotaviruses are not able to degrade IRF3, but may target IRF7 (Arnold & Patton, 2011). Additionally, some rotaviruses, including the porcine strain OSU, encode NSP1 proteins that bind and degrade the β-transducin repeat-containing protein (β-TrCP), a protein required for degradation of IκB (Graff et al., 2009). As IκB holds NF-κB inactive in the cytoplasm, NSP1 interference with β-TrCP inhibits the nuclear translocation of NF-κB. NF-κB is a key transcription factor required for induction of a range of cytokines in response to pathogens. It has been suggested that degradation of β-TrCP by NSP1 of human rotaviruses is

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The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences of NSP1 from rotaviruses B37 and CRW-8 are KM434193 and KM434194, respectively.
uncommon (Arnold & Patton, 2011), although inhibition of the NF-κB pathway by human rotaviruses has not been extensively studied.

A previous study in our laboratory showed that human rotavirus strain Wa strongly inhibits NF-κB nuclear entry in infected cells (Holloway et al., 2009). As the NSP1 of Wa, OSU and other human rotaviruses share sequence relatedness, here we investigated further the ability of several human rotaviruses and their NSP1 proteins to inhibit the NF-κB pathway. We found that a range of human rotaviruses could stabilize IκB and degrade β-TrCP during infection. NSP1 from these rotaviruses was able to block NF-κB-dependent reporter gene expression. In addition, a dual serine-containing motif in the C terminus of human rotavirus NSP1, similar to that found in IκB, was required for NF-κB inhibition. It was also shown that CRW-8, a rotavirus encoding an NF-κB pathway-targeting NSP1, preferentially inhibited the expression of IL-8 over IFN-β, suggesting that reducing the production of cytokines other than type I IFN may be particularly important for human rotavirus replication.

RESULTS

NSP1 sequence comparison amongst human and porcine rotaviruses

Several human rotaviruses were selected for evaluation of their ability to inhibit NFκB signalling. Those with known NSP1 genotypes A1 and A2 included Wa (A1), K8 (A1) and DS-1 (A2). The previously unsequenced NSP1 of strain B37 was found to be of genotype A2 and most closely related to the NSP1 of human rotavirus 69 M (99% nucleotide identity). The porcine strain CRW-8 was also analysed and found to have an NSP1 of genotype A8 that is most closely related to the NSP1 of human rotavirus strain GX54 (94% nucleotide identity) (Dong et al., 2013). GX54 NSP1 is similar to that of porcine YM and equine H1 rotaviruses. The amino acid sequences of NSP1 from Wa, K8, DS-1, B37, CRW-8, SA11-4F and OSU were compared by the maximum-likelihood method (Fig. 1). This revealed clustering of human rotavirus NSP1 proteins with those of OSU and CRW-8. SA11-4F NSP1 clustered separately, showing its divergence from the NSP1 of human and porcine rotaviruses.

Human rotaviruses and CRW-8 stabilize IκB and degrade β-TrCP during infection but do not target IRF3

Previously we demonstrated that infection with human Wa rotavirus strongly inhibits p65 nuclear entry (Holloway et al., 2009). Together with the sequence similarity of human and CRW-8 rotavirus NSP1 proteins to the β-TrCP-degrading NSP1 of OSU shown above and previously (Arnold & Patton, 2011; Graff et al., 2009), this Wa finding led us to examine the ability of additional human rotaviruses and CRW-8 to inhibit NF-κB signalling during MA104 cell infection. Mock-infected cells showed a substantial decrease in IκBα levels following treatment with TNF (Fig. 2a). In contrast, cells infected for 8 h with rotaviruses Wa, K8, DS-1, B37 or CRW-8 followed by TNF treatment all showed stabilization of IκBα. Cells infected with monkey rotaviruses SA11-4F and SA11-5S showed no IκBα stabilization following TNF treatment. In the absence of TNF stimulation, cells infected with SA11-4F or SA11-5S also showed decreased IκB indicating NFκB pathway activation by these viruses (Fig. 2a). VP6 expression was detected for all viruses, demonstrating efficient replication. Notably, all of the five rotaviruses that stabilized IκB were able to reduce endogenous β-TrCP to low (Wa, K8) or undetectable (DS-1, B37, CRW-8) levels in infected cells (Fig. 2b). Two faint non-specific bands were detected by the antibody to β-TrCP. As expected, infection by OSU but not SA11-4F also led to β-TrCP degradation. IRF3 degradation was examined to further characterize the phenotype of these rotaviruses. Wa, K8, DS-1, B37 and CRW-8 did not substantially reduce IRF3 levels at 4 or 8 h post-infection (p.i.) (Fig. 2c). As expected from previous studies (Barro & Patton, 2005), cells infected with SA11-4F but not SA11-5S showed decreased IRF3 levels. Lack of IRF3 degradation by SA11-5S was due to the inability of its truncated NSP1 to interact with IRF3. VP6 was detected for all viruses with substantially higher levels present at 8 h p.i. (Fig. 2c). An additional non-specific band below VP6 also was detected in some blots. Overall, these results indicate that human and porcine rotaviruses generally target the NF-κB pathway but not IRF3.

NSP1 from human rotaviruses and CRW-8 inhibits NF-κB-driven reporter gene expression

To test the function of NSP1 in isolation we utilized an NF-κB-driven luciferase reporter construct that showed robust activation when co-transfected with a vector expressing TRAF6. TRAF6 induces canonical NF-κB pathway activation upstream of TBK1 and IκK. Cellular expression of NSP1 from Wa, K8, DS-1, B37, CRW-8, SA11-4F and SA11-5S was confirmed by Western blotting (Fig. 3a). Expression of NSP1 from Wa, K8, DS-1, B37 or CRW-8

Fig. 1. Phylogenetic analysis of NSP1 from human (Hu), porcine (Po) and simian (Si) rotaviruses. NSP1 amino acid sequences were aligned and a phylogenetic tree created by the maximum-likelihood method using PhyML (v3.0 aLRT). The NSP1 (A) genotype assigned to each virus is indicated.
significantly inhibited TRAF6-stimulated reporter gene expression, compared with the empty vector (No NSP1) control ($P \leq 0.001$; Fig. 3b). In contrast, NSP1 from SA11-4F and SA11-5S did not inhibit NF-$\kappa$B signalling. These data provide evidence that NSP1 is responsible for the stabilization of I$\kappa$B observed during infection with human rotaviruses and CRW-8.

**Inhibition of NF-$\kappa$B requires C-terminal sequences of human rotavirus NSP1**

The C-terminal region of NSP1 from most human rotaviruses, OSU and CRW-8 contains a conserved sequence.
motif containing serine residues at positions 480 and 483 (DSGIS, Fig. 4a). It was previously identified (Arnold et al., 2013) that this motif resembles the sequence in IκBα that mediates binding to β-TrCP and subsequent degradation of IκB by the proteasome (Fig. 4a). To examine if the sequence in and around this motif is required for human rotavirus NSP1-mediated inhibition of NF-κB via β-TrCP binding, we constructed plasmids encoding Wa NSP1 lacking the last 10 C-terminal amino acids or with the two serine residues in the DSGIS motif substituted for alanine (S480/483A; Fig. 4a). Cellular expression of mutated NSP1 was detected by Western blotting at comparable levels to WT Wa NSP1 (Fig. 4b). Both the truncation and substitution mutations abolished the ability of Wa NSP1 to inhibit NF-κB-driven reporter gene expression (Fig. 4c), indicating a critical role for C-terminal sequences, and specifically Ser480 and Ser483, in this property of NSP1.

**NSP1 from human rotaviruses targets IRF7**

Previous studies showed that NSP1 from several human rotaviruses reduced cellular IRF7 levels (Arnold & Patton, 2011). To analyse this further, the effect of human rotavirus NSP1 on IRF7-stimulated IFNA1-Luc gene expression was determined. In the absence of NSP1, IRF7 expression strongly stimulated the IFNA1 promoter (Fig. 5a). In the presence of NSP1 from Wa, K8 or DS-1, this stimulation was inhibited by 52–63% (P<0.05). NSP1 from B37 or CRW-8 did not significantly reduce IRF7-stimulated reporter activity (P>0.05; Fig. 5a). As expected, SA11-4F NSP1 markedly inhibited promoter activity (P<0.001; Fig. 5a), while SA11-5S NSP1 had no effect (P>0.05; Fig. 5a). NSP1 inhibition of IκKe-stimulated IFNB-Luc gene expression also was examined. IκKe is an activator of IRF3 and acts downstream of virus-sensing innate immune activators including RIG-I and...
analysed as for panel (a). The mean ± SD of three independent experiments is shown. *, P<0.05; ***, P<0.001 compared with the No NSP1 control.

MDA5. Like SA11-5S NSP1, human rotavirus NSP1 proteins and CRW-8 NSP1 did not inhibit IκBα-dependent IFNB-Luc reporter activity (Fig. 5b). In contrast, SA11-4F NSP1 strongly inhibited this pathway. These findings show a degree of inhibition of IRF7 signalling by NSP1 from some human rotaviruses. However, these NSP1 do not interfere with IRF3 signalling, in agreement with the data obtained for each virus strain in infected cells (Fig. 2c).

CRW-8 preferentially inhibits NF-κB-dependent cytokine expression in intestinal cells

Preliminary experiments showed that human rotaviruses did not efficiently replicate in HT-29 cells, so cytokine responses in HT-29 cells infected with CRW-8, SA11-4F and SA11-5S were analysed. Infectious titres of SA11-4F, SA11-5S and CRW-8 were approximately 1–2 logs lower in HT-29 than MA104 cells (data not shown).

Initially, the impact of rotavirus infection on IκBα and IRF3 levels in these cells was examined. Infection with CRW-8, but not SA11-4F or SA11-5S, stabilized IκB following TNF treatment (Fig. 6a), while IRF3 was degraded following infection with SA11-4F but not SA11-5S or CRW-8 (Fig. 6b). Thus, the phenotypes observed for these viruses in MA104 cells were preserved in HT-29 cells, allowing the impact of these rotaviruses on intestinal cell cytokine responses to be tested.

Levels of secreted IL-8, IFN-β and CCL5 were measured following rotavirus infection of HT-29 cells for 18 h (Fig. 7). TNF treatment was included as a control. IL-8 secretion was observed at very low levels in mock-infected cells and was strongly induced by TNF (Fig. 7a). IL-8 levels were significantly elevated following infection with SA11-4F and SA11-5S (P<0.001), whereas IL-8 secretion from cells infected with CRW-8 did not differ significantly from mock-infected cells (P>0.05). TNF did not induce secretion of IFN-β (Fig. 7b) or CCL5 (Fig. 7c). Compared with the low level in mock-treated cells, relatively high levels of IFN-β (P<0.001; Fig. 7b) and CCL5 (P<0.001; Fig. 7c) were secreted by CRW-8-infected cells. SA11-5S-infected cells also secreted substantial levels of IFN-β (P<0.001; Fig. 7b) and CCL5 (P<0.001; Fig. 7c). SA11-4F infection did not induce significant levels of IFN-β or CCL5 (P>0.05). These results indicate that CRW-8 inhibits production of IL-8 but not IFN-β or CCL5, whereas SA11-4F has the opposite effect by inhibiting IFN-β and CCL5 but not IL-8.

DISCUSSION

NSP1 is a major antagonist of innate immune responses to rotavirus and is a key determinant of rotavirus replication in animal models (Barro & Patton, 2005; Broquet et al., 2011; Feng et al., 2013; Graff et al., 2009). A limited number of rotaviruses have been shown previously to target the NF-κB pathway (Graff et al., 2009). Importantly, we demonstrate here that this targeting is a common feature of human rotavirus infection, and confirm that these rotaviruses do not generally target IRF3 (Arnold & Patton, 2011). Our findings also provide evidence that preferential targeting of NF-κB or IRF3 by NSP1 during rotavirus infection results in particular cytokine response profiles.

Past studies have implicated the cellular proteasome in the degradation of the cellular targets of NSP1 (Barro & Patton, 2005; Graff et al., 2009). The N terminus of NSP1 contains an atypical RING domain with conserved cysteine and histidine residues (Graff et al., 2007). RING domains are common in proteins with E3 ubiquitin ligase activity, adding weight to the notion that NSP1 is an E3 ligase. To date, however, ubiquitination of known NSP1 targets has not been reported. To our knowledge, our data are the first to indicate that β-TrCP is degraded during cellular
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A conserved motif containing two serine residues (DSGIS) is present in the C terminus of NSP1 of most human and some animal rotaviruses, including CRW-8 and OSU. This somewhat resembles the motif found in IκB (DSGIxS) that is phosphorylated on the serines by IκK, allowing binding and degradation of IκB by the E3-ubiquitin ligase β-TrCP.

Our data show that these serine residues in NSP1 are required for inhibition of NF-κB activity. It is not yet known if phosphorylation of these residues is required for targeting of β-TrCP, or alternatively whether mutation disrupts a structure required for β-TrCP binding. However, the strategy of mimicking the IκB serine motif in order to disrupt β-TrCP activity and NF-κB activation is employed by other viral proteins including vaccinia virus A49 and HIV-1 Vpu (Bour et al., 2001; Mansur et al., 2013). In contrast to NSP1 from the human and porcine rotaviruses analysed in the present study, A49 and Vpu do not target β-TrCP for degradation.

Particular cytokines are regulated by distinct groups of transcription factors. Thus, viral preference for the inhibition of a particular transcription factor may affect the spectrum of cytokine expression during infection. We compared cytokine expression in intestinal cells infected with the NF-κB-targeting CRW-8 and the IRF3-targeting SA11-4F. In the opposite pattern to SA11-4F, CRW-8 induces secretion of IFN-β and CCL5 but not IL-8. In agreement with these data, robust induction of IL-8 by rotaviruses that do not target NF-κB, including SA11-4F and RRV, has been described previously (Casola et al., 1998; Holloway & Coulson, 2006; Rollo et al., 1999). IFN-β and CCL5 are regulated by IRF3, and IL-8 is regulated largely by NF-κB. Thus, these data suggest that rotaviruses with NSP1 proteins that target NF-κB but not IRF3, including most human strains, may preferentially inhibit the expression of a distinct group of cytokines including IL-8. Although NF-κB can positively influence IFN-β and CCL5 expression, our data show that inhibition of NF-κB by CRW-8 does not strongly reduce expression of either of these cytokines, and that the role of IRF3 may be dominant. It cannot be ruled out that CRW-8 stimulates innate pathways more strongly than SA11-4F in HT-29 cells, and that IFN-β and CCL5 expression is in fact somewhat inhibited through NF-κB antagonism. On the other hand, it has been demonstrated that NF-κB is not essential for IFN-β expression during viral infection, although it may have a role in boosting IFN-β levels early in infection when IRF3 activation is low (Wang et al., 2007, 2010). It remains possible that inhibition of NF-κB by rotavirus more strongly influences IFN-β and CCL5 expression in other cell types or under different conditions. Rotaviruses that inhibit NF-κB but not IRF3 have been shown previously to reduce IFN-β expression (Arnold & Patton, 2011).

**Fig. 6.** Rotavirus stabilization of IκB and degradation of IRF3 in HT-29 cells. (a) Cells were mock-infected or infected with CRW-8, SA11-4F or SA11-5S at an m.o.i. of 3 for 8 h. After treatment without or with TNF (20 ng ml⁻¹) for 20 min, cells were lysed and analysed by Western blotting for IκBα, VP6 and β-actin. (b) HT-29 cells were mock-infected or infected with CRW8, SA11-4F or SA11-5S at an m.o.i. of 1 for 4 h and 8 h. Cells were lysed and analysed by Western blotting for IRF3, VP6 and β-actin.
In addition to IL-8, intestinal epithelial cells can produce a large range of cytokines and chemokines in response to pathogens, including IP-10, GRO-α, GM-CSF and CCL20 (Stadnyk, 2002). Many of these secreted factors are regulated partially or fully by NF-κB, so it is plausible that their expression would be inhibited by rotviruses that target NF-κB. Due to the roles of intestinal chemokines and cytokines in the attraction of multiple immune cells types, including neutrophils, T-cells and dendritic cells, inhibition of these factors may dampen immune responses to rotavirus.

The reason for the apparent preference towards targeting NF-κB but not IRF3 by NSP1 of some rotaviruses, particularly human strains, is unclear. It may be that reduction of a broader range of cytokine responses is afforded by NF-κB targeting, in contrast to primarily limiting IFN expression. This strategy might be more effective in countering immune responses during human rotavirus infections. Rotavirus NSP1 gene selection in particular species also may be influenced by currently unknown targets of NSP1. Further analysis of the function of diverse NSP1 proteins will give greater insight into the counterbalances between rotavirus infection and the host response.

**METHODS**

**Cells and viruses.** The origins and propagation of MA104 cells, HT-29 cells, human embryonic kidney (HEK) 293T cells, human rotaviruses Wa, K8, DS-1 and B37, porcine rotaviruses CRW-8 and OSU and monkey rotaviruses SA11-4F and SA11-5S have been previously reported (Coulson & Kirkwood, 1991; Holloway & Coulson, 2006; Holloway et al., 2014; Londrigan et al., 2000; Patton et al., 2001; Theil et al., 1977). Wa, K8, DS-1 and CRW-8 were partially purified from infected MA104 cell cultures by sucrose-cushion ultracentrifugation (Arnold et al., 2009). SA11-4F and SA11-5S were purified from infected MA104 cells by glycerol gradient ultracentrifugation as described previously (Holloway & Coulson, 2006). OSU-infected MA104 cell culture harvest was clarified by low speed centrifugation. Virus titres after infectivity activation with porcine trypsin were determined by titration in MA104 or HT-29 cells as before (Londrigan et al., 2000; Sato et al., 1986).

**Plasmids and mutagenesis.** NSP1 genes from human rotaviruses and CRW-8 were amplified from viral dsRNA using reverse transcription (AffinityScript, Agilent) and PCR (Phusion, New England Biolabs). Initially, primers binding to regions outside each NSP1 ORF were used for PCR and sequencing (Big Dye 3.1, Applied Biosystems). This allowed the design of primers completely complementary to the ends of each NSP1 ORF, which were used for PCR amplification. NSP1 genes were inserted into pCMV-3Tag6 (Agilent) by standard methods, yielding plasmids encoding each NSP1 fused to 3 N-terminal FLAG tags. SA11-4F NSP1 cDNA (GenBank accession number AF290883), provided by John Patton, Laboratory of Infectious Diseases, NIAID, NIH, was used as a template to amplify the full-length NSP1 gene, which was inserted into pCMV-3Tag6. The SA11-5S NSP1 construct encodes a protein identical to SA11-4F NSP1 but truncated by 17 aa at the C terminus (Patton et al., 2001). The predicted amino acid sequence of NSP1 from Wa and K8 was identical to the published sequences (GenBank accession numbers JX406751 and D38152, respectively). Our DS-1 NSP1 protein sequence was identical to GenBank accession number HQ650120, except for substitution of Thr at position 331 with Ile. The GenBank accession numbers for the nucleotide sequences of NSP1 from rotaviruses B37 and CRW-8, which have not previously been sequenced, are KM434193 and KM434194, respectively. Wa NSP1 protein (S480/483A) was produced by site-directed mutagenesis of the Wa NSP1 plasmid (QuickChange II kit, Agilent). Plasmids encoding C-terminal truncated Wa and Sa NSP1 were constructed by standard PCR and

![Fig. 7. Cytokine responses during infection of HT-29 cells with rotaviruses that differ in their abilities to stabilize IκB and degrade IRFs. Levels of secreted IL-8 (a), IFN-β (b) and CCL5 (c) were determined in the supernatant fluids of cell cultures after mock-infection or infection with CRW-8, SA11-4F or SA11-5S at an m.o.i. of 0.3 for 18 h. Cytokines secreted from cells treated with TNF were analysed as controls. The mean±SD of three independent experiments is shown. **, P<0.01; ***, P<0.001 compared with the Mock control.](image-url)
cloning methods. Plasmids expressing human IRF7 and IκKc (Liang et al., 2012), and the pGL3-basic reporter constructs containing the firefly luciferase gene under control of the human IFNAR1 (IFNA1-Luc) and IFNβ (IFNB-Luc) promoters were provided by Fanxiu Zhu, Department of Biological Science, Florida State University (Zhu et al., 2002). The plasmid expressing TRAF6 was from Luke O’Neill, Trinity College Dublin (Harte et al., 2003). pNF-kB-Luc (Clontech) and pRL-TK (Promega) have been described previously (Holloway et al., 2009).

**Sequence alignment and phylogenetic analysis.** NSP1 amino acid sequences were analysed at [www.phylogeny.fr](http://www.phylogeny.fr) (Dereeper et al., 2008). Sequences were aligned using **MUSCLE** (Edgar, 2004) and phylogenetic trees created by the maximum-likelihood method using **PhyML** (v3.0 aLRT) with the approximate likelihood ratio test (Guindon et al., 2010). Graphical representation of the phylogenetic tree was created with **TreeDyn** (v198.3) (Chevenet 2010). Graphical representation of the phylogenetic tree was created by normalizing activity: 0.09 mIU Fugene HD transfection reagent as suggested by the manufacturer (Promega). Plasmids were included as follows – NF-κB activity: 0.09 μg NF-κB-Luc, 0.01 μg pRL-TK, 0.1 μg TRAF6 or an empty control vector and 0.3 μg empty vector or NSP1 plasmid. IRF7-driven *IFNA1* promoter activity: 0.1125 μg *IFNA1*-Luc, 0.0125 μg pRL-TK and 0.025 μg IκKc, together with 0.33 μg of an empty vector or NSP1 plasmid and 0.02 μg of the IRF7 plasmid or an empty control vector. IκKc-driven IFNA1 promoter activity: 0.1125 μg IFNB-Luc, 0.0125 μg pRL-TK, 0.075 μg IκKc or an empty control vector and 0.3 μg empty vector or NSP1 plasmid. At 24 h after transfection cell lysates were analysed for *Renilla* and firefly luciferase expression using the Dual Luciferase assay system (Promega) and a Fluostar Omega microplate reader (BMG Labtech). Results were calculated by normalizing *Renilla* luciferase levels to firefly luciferase levels in each sample.

**Cytokine assays.** HT-29 cells were mock-infected, infected with the indicated rotaviruses at m.o.i. 5 (MA104) or m.o.i. 1 (HT-29) for 4 h or 8 h. For analysis of IκBz levels, cells were mock-infected or infected with the indicated rotaviruses at m.o.i. 5 (MA104) or m.o.i. 3 (HT-29) for 7 h 40 min then treated for 20 min with or without TNF (20 ng ml⁻¹; Cell Signalling). β-TrCP levels were determined in MA104 cells infected for 8 h without TNF treatment. Cell lysis, SDS-PAGE and Western blotting were performed as previously described (Holloway & Coulson, 2006). Rabbit polyclonal antibodies to IκBz and β-actin, and rabbit mAbs to IRF3 and β-TrCP (clone D13F10), were from Cell Signalling. Mouse monoclonal anti-FLAG-M2 antibody was from Sigma. Polyclonal rabbit antibodies to RRV were used to detect VP6 (Halasz et al., 2008).

**Reporter assays.** HEK 293T cells in 24-well trays were used. Each well was transfected with a total of 0.5 μg plasmid DNA combined with 1.5 μl Fugene HD transfection reagent as suggested by the manufacturer (Promega). The plasmid expressing TRAF6 was from Luke O’Neill, Trinity College Dublin (Harte et al., 2003). pNF-kB-Luc (Clontech) and pRL-TK (Promega) have been described previously (Holloway et al., 2009).

**Statistics.** Data were analysed by one-way ANOVA with Dunnett’s multiple comparison test.

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