Rotavirus inhibits IFN-induced STAT nuclear translocation by a mechanism that acts after STAT binding to importin-α

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The importance of innate immunity to rotaviruses is exemplified by the range of strategies evolved by rotaviruses to interfere with the IFN response. We showed previously that rotaviruses block gene expression induced by type I and II IFNs, through a mechanism allowing activation of signal transducer and activator of transcription (STAT) 1 and STAT2 but preventing their nuclear accumulation. This normally occurs through activated STAT1/2 dimerization, enabling an interaction with importin α that mediates transport into the nucleus. In rotavirus-infected cells, STAT1/2 inhibition may limit the antiviral actions of IFN produced early in infection. Here we further analysed the block to STAT1/2 nuclear accumulation, showing that activated STAT1 accumulates in the cytoplasm in rotavirus-infected cells. STAT1/2 nuclear accumulation was inhibited by rotavirus even in the presence of the nuclear export inhibitor Leptomycin B, demonstrating that enhanced nuclear export is not involved in STAT1/2 cytoplasmic retention. The ability to inhibit STAT nuclear translocation was completely conserved amongst the group A rotaviruses tested, including a divergent avian strain. Analysis of mutant rotaviruses indicated that residues after amino acid 47 of NSP1 are dispensable for STAT inhibition. Furthermore, expression of any of the 12 Rhesus monkey rotavirus proteins did not inhibit IFN-stimulated STAT1 nuclear translocation. Finally, co-immunoprecipitation experiments from transfected epithelial cells showed that STAT1/2 binds importin α normally following rotavirus infection. These findings demonstrate that rotavirus probably employs a novel strategy to inhibit IFN-induced STAT signalling, which acts after STAT activation and binding to the nuclear import machinery.

INTRODUCTION

Viral infection elicits a rapid host response, leading to the expression of type I and III IFNs. Type I IFNs include multiple IFN-α subtypes and IFN-β (IFN-α/β), while type III IFNs comprise IFN-λ1, IFN-λ2 and IFN-λ3 (IFN-λ). Induction of these antiviral and immunomodulatory molecules occurs following detection of viral nucleic acids by the host, and the triggering of signalling cascades leading to activation of the transcription factors NFκB, IFN response factor (IRF) 3 and IRF7 (Randall & Goodbourn, 2008). IFN-α/β and IFN-λ, secreted from infected cells, bind to their respective extracellular heterodimeric receptors, IFNAR1/2 and IL-28R/IL-10Rb, on neighbouring cells. This leads to formation of a signalling complex and phosphorylation of signal transducer and activator of transcription (STAT) 1 and STAT2 by JAK1 and TYK2 kinases. STAT1 and STAT2 heterodimer formation is followed by IRF9 association, nuclear translocation and gene transactivation. STAT1 also forms active homodimers upon IFN-α/β stimulation. During infection, IFN-γ secreted by activated T-cells plays an antiviral role by activating STAT1 through IFN-γ receptor engagement.

Nuclear import of STAT1/2 requires interaction with importin (IMP) α5 (Melen et al., 2003; Sekimoto et al., 1997). IMPα5 binds IMPβ1 and is transported through the nuclear pore complex to the nucleus, where STAT binds DNA and effects transcriptional outcomes. Nuclear export of STAT1/2 requires the nuclear export protein CRM1 (Banninger & Reich, 2004; McBride et al., 2000).

The importance of innate immunity in controlling virus replication means that most, if not all, viruses have evolved measures to counteract IFN-mediated innate responses (Randall & Goodbourn, 2008). These include interference with innate signalling components required for triggering IFN production, such as the pathogen-associated molecular pattern receptors RIG-I and MDA5, their adaptor protein MAVS, IRF3 and NFκB. Viruses also widely target the proteins required to transduce IFN signals,
including JAK1 and STAT1/2 (Randall & Goodbourn, 2008).

Rotavirus, a member of the Reoviridae family, causes severe dehydrating diarrhea in infants. Innate immunity is important in controlling rotavirus infection, with rotaviruses counteracting innate responses via multiple strategies (Arnold et al., 2013b; Holloway & Coulson, 2013). Through the action of non-structural protein (NSP) 1, rotavirus inhibits IFN production by degrading IRAF (Barro & Patton, 2005). NSP1 from some rotaviruses also inhibits NFκB signalling by degrading the β-transducin repeat-containing protein, a molecule required for the degradation of IκB and subsequent release and nuclear transport of active NFκB (Graff et al., 2009; Holloway et al., 2009). NFκB inhibition by rotavirus probably limits IFN-β and cytokine production in infected cells. Rotavirus also inhibits IFN signalling by preventing the nuclear accumulation of STAT1/2 by an unknown mechanism (Holloway et al., 2009).

Here we analyse the mechanism behind rotavirus inhibition of STAT nuclear accumulation. It is demonstrated that rotavirus inhibits the IFN-stimulated nuclear translocation of activated STAT1, leading to its cytoplasmic accumulation. Rotaviruses encoding truncated NSP1 still efficiently block nuclear accumulation of activated STAT1, showing that residues after aa 47 of NSP1 play no role in this rotavirus property. Expression of each known rotavirus protein did not replicate the block to STAT nuclear translocation observed during infection. STAT1/2 appeared to associate normally with importin-α5 in rotavirus-infected cells. Thus, rotavirus inhibits IFN-induced STAT nuclear translocation by a novel mechanism that acts after STAT activation and binding to the nuclear import machinery.

RESULTS

Activated STAT1 accumulates in the cytoplasm of RRV-infected cells following IFN stimulation

We showed previously that STAT1 and STAT2 remain in the cytoplasm and are phosphorylated normally, on Tyr701 (p-STAT1) and Tyr690 (p-STAT2), respectively, following IFN-α or IFN-γ stimulation of rotavirus-infected cells (Holloway et al., 2009). The localization of p-STAT1 in cells infected with the rotavirus strain RRV following IFN-γ stimulation was further examined (Fig. 1a, b). A low m.o.i. of 0.5 was used to allow imaging of uninfected and infected cells in the same sample. Infected cells were identified by the presence of VP6 staining. In both uninfected and RRV-infected cells, p-STAT1 was detected only in the presence of IFN-γ (Fig. 1a). As expected, after 30 min of stimulation, 98% of uninfected cells showed strong p-STAT1 staining that was localized almost exclusively to the nucleus. In contrast, 96% of infected cells in IFN-γ-stimulated cultures showed p-STAT1 staining that was almost entirely cytoplasmic. This staining pattern was maintained after 2 h of stimulation with IFN-γ (Fig. 1a). The nuclear-to-cytoplasmic fluorescence ratio (Fn/c) of p-STAT1 in infected cells was substantially reduced over uninfected cells at 30 min and 2 h following stimulation with IFN-γ (Fig. 1b). In contrast, the nuclear accumulation of p38 following its activation with anisomycin was unaffected in RRV-infected cells (Fig. 1c), with the phospho-Thr180/ Tyr182-p38 (p-p38) Fn/c in infected cells unaltered over uninfected cells (Fig. 1d). These data imply that RRV inhibition of nuclear accumulation is specific for STAT1, and not due to a general effect on nuclear import.

RRV inhibits nuclear translocation of STAT1/2

Although STAT1/2 clearly are prevented from accumulating in the nucleus in rotavirus-infected cells, it is unclear whether this is due to inhibition of STAT nuclear translocation, or reduced nuclear retention time of STATs before their export to the cytoplasm by CRM1. To differentiate between these possibilities, we analysed the effect of the CRM1-dependent nuclear export inhibitor Leptomycin B (LMB) on the fate of STAT2. Following IFN-α treatment of MA104 cells for 3 h in the absence of LMB, STAT2 was predominantly cytoplasmic (Fig. 2a). After IFN-α treatment for 3 h in the presence of LMB, STAT2 was predominantly nuclear, confirming that LMB inhibits STAT2 nuclear export over at least 3 h period under the conditions used (Fig. 2a). As previously observed (Holloway et al., 2009), STAT2 was mostly cytoplasmic in uninfected and RRV-infected cells in the absence of IFN-α and LMB (Fig. 2b). IFN-α induced the nuclear translocation of STAT2 in the absence of LMB, but this was blocked in RRV-infected cells, where STAT2 remained cytoplasmic. In the absence of IFN-α stimulation, LMB did not influence the localization of STAT2 in RRV-infected and uninfected cells, which remained largely cytoplasmic. In cells treated with LMB before the addition of IFN-α, STAT2 was observed in the nucleus of uninfected cells but was retained in the cytoplasm of RRV-infected cells (Fig. 2b). IFN-γ-activated STAT1 also remained in the cytoplasm of infected cells in the presence or absence of LMB, and LMB itself did not activate STAT1 (Fig. 2c). In cells stimulated with IFN-γ or IFN-α, p-STAT1 and STAT2 Fn/c values in infected cells were markedly reduced over uninfected cells, in the presence or absence of LMB (Fig. 2d). These data provide strong evidence that the inhibition of STAT1 and STAT2 nuclear accumulation in RRV-infected cells occurs through a block in nuclear import rather than enhanced nuclear export.

Amino acids after position 47 in NSP1 are not required for the inhibition of STAT1 nuclear translocation by rotavirus

Rotavirus NSP1 is implicated in antagonism of IFN-α/β induction. To test the possibility that NSP1 may be responsible for the inhibition of STAT nuclear import, we utilized several rotaviruses encoding truncated forms
of NSP1. SA11-4F, which encodes an intact NSP1, was included as a control. MA104 cells infected with SA11-5S and SA11-30-1A, which express NSP1 with 17 and 71 aa C-terminal deletions, respectively (Patton et al., 2001), showed inhibition of IFN-γ-stimulated p-STAT1 nuclear accumulation in >96% of infected cells (Fig. 3a). A5-16, which encodes only the first 47 aa of NSP1 plus three missense-encoded amino acids (Taniguchi et al., 1996), inhibited IFN-γ-stimulated p-STAT1 nuclear accumulation in 98% of infected cells (Fig. 3a). SA11-4F infection produced a similar inhibition of IFN-γ-stimulated p-STAT1 nuclear accumulation (Fig. 3a). These viruses also blocked IFN-γ-stimulated STAT2 nuclear translocation (data not shown). The p-STAT1 Fn/c in cells infected with each of the four rotaviruses was reduced compared with uninfected cells, and the p-STAT1 Fn/c in cells infected with SA11-4F was indistinguishable from the p-STAT1 Fn/c in cells infected with each rotavirus encoding a truncated NSP1 (Fig. 3b). These data indicate that residues after the first 47 aa of NSP1 have no role in the inhibition of STAT1/2 nuclear translocation by rotavirus.

**STAT inhibition by rotaviruses is highly conserved**

RRV, SA11, Wa and UK rotaviruses inhibit STAT2 nuclear accumulation in response to IFN (Holloway et al., 2009). We tested the ability of other rotaviruses to block p-STAT1 nuclear translocation. In 99% of mock-infected cells, p-STAT1 was largely nuclear following IFN-γ treatment, as before. In contrast, p-STAT1 was found predominantly in

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**Fig. 1.** Localization of activated STAT1 and p38 in rotavirus-infected cells. (a) MA104 cells infected with RRV (m.o.i. 0.5) for 6 h were stimulated with IFN-γ or left unstimulated for a further 30 or 120 min. Cells were fixed, permeabilized and stained for p-STAT1, rotavirus VP6 and nuclear DNA (DAPI). Images were obtained by confocal microscopy. (b) Nuclear to cytoplasmic ratios (Fn/c) of p-STAT1 were calculated from cell images produced as for (a). The mean p-STAT1 Fn/c ± SD (n ≥ 17) from a single experiment is shown, representing two to three independent experiments. ****, P < 0.0001. (c) Cells infected as for (a) were treated with anisomycin (Aniso) or left untreated for 30 min, then fixed, permeabilized and stained for phospho-Thr180/Tyr182-p38 (p-p38), VP6 and nuclear DNA. (d) Fn/c of p-p38 were calculated from images produced as for (c). The mean p-p38 Fn/c ± SD (n ≥ 15) from a single experiment is shown, representing two independent experiments.
the cytoplasm in 95–100 % of cells infected with B37, K8, M37, MDR-13, ST-3, TFR-41 or NCDV (Fig. 4a). Ty-1 rotavirus-infected cells could not be visualized using the anti-VP6 monoclonal antibody, and instead were detected using polyclonal antibodies to RRV. At an m.o.i. of 1, 69 % of cells were infected with Ty-1 (data not shown), with p-STAT1 blocked from entering the nucleus in 70 % of cells (Fig. 4b). Together with earlier findings (Holloway et al., 2009), these results show that the ability to block nuclear translocation of STAT1/2 is highly conserved amongst diverse group A rotaviruses.

**Expression of rotavirus proteins does not inhibit STAT1 nuclear translocation**

Each of the 12 known RRV proteins was expressed with N-terminal FLAG tags in HEK293T cells. RRV protein expression was confirmed by Western blotting with anti-FLAG antibody (Fig. 5a). Proteins of the anticipated sizes were expressed from all 12 clones, except for FLAG-NSP4, which was smaller than expected (approximately 24 kDa). For this reason, expression from a construct encoding C-terminal FLAG-tagged NSP4 was also evaluated. This showed a product of 31 kDa, indicating that the N-terminal

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### Fig. 2. Effect of nuclear export inhibition on STAT localization in rotavirus-infected cells.

(a) MA104 cells stimulated with IFN-α for 3 h in the presence or absence of LMB were fixed, permeabilized and stained for STAT2 and nuclear DNA. Images were obtained by confocal microscopy. (b) At 5 h after RRV infection (m.o.i. 0.3), MA104 cells were treated with or without LMB for 1 h then stimulated with IFN-α or not for 30 min, with LMB remaining in the medium. Cells were stained for STAT2, VP6 and nuclear DNA. (c) Cells infected and treated as for (b) were stained for p-STAT1, VP6 and nuclear DNA. (d) Fn/c of STAT2 and p-STAT1 were calculated from images produced as for (b) and (c), respectively, from untreated (Untr.) and LMB-treated (LMB) cells. The mean Fn/c ± SD (n > 19) from a single experiment is shown, representing two independent experiments. ****, P < 0.0001.
FLAG tag may have inhibited NSP4 glycosylation. A fainter and slightly slower migrating additional band of unknown origin was also observed for NSP4-FLAG. Additional smaller proteins, probably signal peptide cleavage products, were detected in cells expressing FLAG-VP7 (Stirzaker et al., 1987). The extra higher-migrating band of FLAG-VP6 may be a multimer. Cells expressing FLAG-NSP5 contained potentially phosphorylated and dimeric forms (Torres-Vega et al., 2000; Welch et al., 1989).

STAT1 nuclear translocation was examined in cells expressing the individual RRV proteins. In all cases, >95% of cells expressing each RRV protein showed unimpeded p-STAT1 nuclear translocation following stimulation with IFN-γ (Fig. 5b). RRV infection of HEK293T cells also inhibited STAT1 nuclear translocation (Fig. 5c), demonstrating the suitability of these cells for determining the effects of RRV protein expression on STAT1. These data provide evidence that the known rotavirus proteins expressed in isolation are unable to inhibit IFN-stimulated STAT nuclear translocation.

**STAT1/2 bind IMPα5 normally in RRV-infected cells**

As STAT1/2 do not appear to enter the nucleus in rotavirus-infected cells, we hypothesized that the STAT dimers may be unable to interact correctly with the nuclear import machinery. Co- immunoprecipitation was used to assess the binding of endogenous STAT1/2 to transiently expressed IMPα5 following IFN-α stimulation of uninfected and RRV-infected MA104 cells (Fig. 6). In mock-infected cells, p-STAT1/2 were co-immunoprecipitated with IMPα5 only after IFN-α treatment. Two bands were observed for p-STAT1/2, which likely represent splice variants. In RRV-infected cells treated with IFN-α, p-STAT1/2 were co-immunoprecipitated with IMPα5 at levels similar to those in mock-infected cells. No p-STAT1/2 was co-immunoprecipitated with IMPβ1, either with or without IFN-α stimulation, consistent with previous observations that STAT1/2 do not use IMPβ1 for nuclear import (Melen et al., 2003). These results provide evidence that the interaction between IMPα5 and STAT1/2 following IFN-α stimulation is not blocked during RRV infection.

**IMPα5 and IMPβ1 localization is unaffected by RRV infection**

To further examine any involvement of aberrant nuclear import in the RRV inhibition of STAT nuclear import, we monitored the localization of transiently expressed FLAG-IMPα5 and FLAG-IMPβ1 following infection. In the uninfected cells within RRV-infected MA104 cell cultures, FLAG-IMPα5 localized predominantly to the nucleus and nuclear membrane following IFN-α stimulation for 30 min (Fig. 7). The FLAG-IMPα5 localization pattern in the RRV-infected cells within these cultures was indistinguishable from that seen in uninfected cells (Fig. 7). This is consistent with the inability of rotavirus to reduce STAT1/2 binding to FLAG-IMPα5 demonstrated in Fig. 6. The location of FLAG-IMPβ1 in uninfected cells within the RRV-infected cultures following IFN-α stimulation was cytoplasmic and nuclear with a clear accumulation at the nuclear membrane. This FLAG-IMPβ1 distribution also was unaffected by RRV infection (Fig. 7). These findings do not support sequestration of IMPα5 or IMPβ1 as a mechanism for inhibition of STAT nuclear import by rotavirus.
DISCUSSION

Transcriptional activation mediated by STAT1 and STAT2 is widely targeted by viruses to evade innate immunity. Strategies used by viruses to inhibit STAT function include degradation of STATs, sequestration of active or inactive STATs, inhibition of upstream activation events and interference with STAT nuclear import (Randall & Goodbourn, 2008). The present study of events occurring early after rotavirus infection shows for the first time that although IFN-induced STAT activation is not inhibited early in rotavirus infection, and STAT1 and STAT2 bind IMPα, these STATs do not cross the nuclear membrane.

Following IFN-γ treatment of rotavirus-infected cells, p-STAT1 was produced but remained distributed throughout the cytoplasm. This confirms our previous Western blotting results, which showed normal activation of STAT1/2 in RRV-infected MA104 cells (Holloway et al., 2009). As further evidence of the specificity of STAT inhibition, the nuclear translocation of activated p38 was not blocked in cells infected with rotavirus. Other proteins such as IRF3 (Barro & Patton, 2005), at least when not targeted for degradation, also have been shown to pass unhindered into the nucleus. This provides evidence that the cellular nuclear import machinery generally remains intact during rotavirus infection.

Although it was found previously that STAT1/2 are prevented from accumulating in the nucleus of RRV-infected cells, it remained unclear whether they were stopped from entering the nucleus or were being more rapidly exported after entry (Holloway et al., 2009). DNA binding plays an important role in the retention of STAT1 in the nucleus (Meyer et al., 2003), so interference with STAT1/2 DNA binding during rotavirus infection was possible. Rabies virus employs this strategy to limit IFN-induced transcription, through the action of its P protein (Vidy et al., 2007). We showed here that despite enhanced STAT2 retention in the nucleus upon LMB treatment of uninfected cells, STAT2 and p-STAT1 remained cytoplasmic in rotavirus-infected cells treated with LMB prior to stimulation with IFN. This strongly suggests that rotavirus infection does not enhance STAT nuclear export, but rather inhibits STAT1/2 nuclear entry.

In order for STAT1/2 heterodimers to enhance IFN-α/β-stimulated transcription, they must form a complex with IRF9. Recently it was reported that rotavirus NSP1 can degrade IRF9, and the authors speculated that reduced IRF9 might account for the block to STAT1/2 nuclear translocation in cells infected with rotavirus (Arnold et al., 2013a). Our results suggest that NSP1 is unlikely to be involved in inhibiting STAT translocation, as SA11-5S, SA11-30-1A and A5-16 rotaviruses that express NSP1 with varying degrees of C-terminal truncation efficiently blocked STAT nuclear translocation. This indicates that rotaviruses previously demonstrated to lack IRF degradative capacity maintain the ability to block IFN-α/β-stimulated STAT translocation (Barro & Patton, 2005, 2007; Graff et al., 2007). Clear evidence also exists that the presence of IRF9 is not required for the nuclear translocation of activated STAT2 (Banninger & Reich, 2004). However, rotaviruses block the translocation of both STAT1 and STAT2. In addition, the nuclear translocation of STAT1 homodimers, which are blocked...
from entering the nucleus in rotavirus-infected cells following stimulation with IFN-γ, is not known to involve IRF9. Thus, any known NSP1 function, including the ability to degrade IRF9, appears to be completely independent from the mechanism involved in prevention of STAT nuclear translocation by rotavirus. A recent study showed that IFN-β-stimulated STAT1 phosphorylation is inhibited at a late stage of rotavirus infection in HT-29 cells (Sen et al., 2014). NSP1 expression alone could mimic this effect, although NSP1-defective viruses and STAT1 nuclear translocation were not analysed. We have repeatedly shown here and previously (Holloway et al., 2009) that STAT nuclear translocation is strongly inhibited by rotavirus early (6 h) after infection. Consistent with our findings, the inhibition of STAT1 phosphorylation reported by Sen et al. (2014) was not observed at 6 h after rotavirus infection, and may represent an additional rotavirus strategy to limit IFN signalling later in the replicative cycle.

Viral inhibition of STAT signalling mostly requires the actions of one or more viral proteins, such as the V protein in the case of the paramyxoviruses (Ramachandran & Horvath, 2009). Individual expression of each of the 12 known rotavirus proteins did not inhibit IFN-induced STAT1 nuclear translocation, although it cannot be ruled out that the FLAG tag impeded function. It remains possible that a combination of rotavirus proteins and changed cellular conditions during infection may contribute to the block to STAT nuclear translocation. An as-yet-unidentified rotavirus protein also might be involved. The discovery of novel viral proteins that regulate host responses is not unprecedented. Examples include the influenza A proteins PA-X and PB1-F2, expressed through ribosomal frame shifting and leaky ribosomal scanning, respectively (Chen et al., 2006, 2007). Sequestration of IMPs by viral proteins can also prevent STAT nuclear import, as demonstrated for the SARS coronavirus (Frieman et al., 2007). We did not detect any inhibition of STAT1/2 binding to IMPx5 in RRV-infected cells following IFN-α stimulation. This suggests that STAT1/2 dimers form normally during rotavirus infection, and that these dimers can interact normally with IMPx. Therefore, it appears unlikely that a rotavirus protein can bind to either STAT1/2 or IMPx to prevent their association and subsequent passage through the nuclear pore. Due to difficulties in co-immunoprecipitating STAT1/2 with IMPβ1, we were unable to rule out any inhibition of STAT/IMPx5 binding to IMPβ1 during rotavirus infection. However, IMPx5 localization in the nucleus and at the nuclear membrane was unaltered in rotavirus-infected cells, suggesting that its ability to associate with IMPβ1 remained intact, at least for the majority of cargoes. The localization of IMPβ1 to the cytoplasm, nucleus and nuclear membrane also was unaltered in rotavirus-infected cells, ruling out gross changes to the nuclear import machinery as the cause of STAT nuclear translocation inhibition.

The distribution of p-STAT1, STAT1 and STAT2 throughout the cytoplasm in rotavirus-infected cells following stimulation with IFN provides evidence that STATs are not sequestered in distinct cellular compartments or viroplasms. The normal activation of STAT1 and the observed STAT distribution during rotavirus infection are similar to the effects induced by Mapuera virus V protein, which also inhibits STAT nuclear translocation (Hagmayr et al., 2007). It is unknown whether Mapuera V protein interferes with STAT1 binding to importins. Overall, it appears that rotaviruses block STAT1/2 nuclear translocation by a novel mechanism.

From this and the previous report, the ability to inhibit STAT nuclear translocation is conserved amongst 13 group A rotaviruses (Holloway et al., 2009). These include the avian rotavirus Ty-1, which is likely to be highly divergent in sequence compared to mammalian rotaviruses (Trojnar et al., 2009; R. Johnson, G. Holloway & B. S. Coulson, unpublished data). Thus, the ability to inhibit STAT nuclear translocation is expected to be essential for successful evasion of host responses by rotavirus. Further analysis of the mechanisms employed by rotavirus to inhibit STAT signalling, and how innate cellular responses are influenced, will enhance our understanding of rotavirus replication and survival within the host.

**METHODS**

**Cells and viruses.** The source and propagation of MA104 and 293T cells have been described (Holloway et al., 2009; Londrigan et al., 2000). HEK293T cells were obtained from the American Type Culture Collection and maintained as for MA104 cells. The sources, propagation in MA104 cells and infectivity titration of rotaviruses RRV, B223, NCDV, TFR-41, MDR-13, ST-3, B37, M37, K8 and Ty-1 were as before (Coulson & Kirkwood, 1991; Coulson et al., 1986; Graham et al., 2005; Huang et al., 1992; Kool et al., 1992). SA11-5S and SA11-30-1A rotaviruses were provided by John Patton, Laboratory of Infectious Diseases, NIH (Barno & Patton, 2005; Patton et al., 2001). A5-16 rotavirus was donated by Koki Taniguchi, Fujita Health University, Japan (Taniguchi et al., 1996). RRV and SA11-5S were purified by ultracentrifugation as described previously (Holloway & Coulson, 2006). All other viruses were used as infected MA104 cell lysates, produced by two cycles of freeze–thawing and clarification by low-speed centrifugation, as described previously (Coulson et al., 1986).

**Plasmids and transfection.** RRV genes were cloned into the pCMV-3Tag6 vector (Stratagene) to yield plasmids encoding RRV proteins fused to three C-terminal FLAG epitope tags (DYKDDDDK × 3) as described previously (Holloway et al., 2009). DNA from the pCMV-3Tag6 RRV NSP4 clone provided a template for PCR amplification of the NSP4 gene, which was inserted into a pCMV-3Tag6 vector (Stratagene) using standard techniques to yield a plasmid encoding RRV NSP4 protein fused to three C-terminal FLAG epitope tags, verified by DNA sequencing. Except where specified, deduced aa sequences were identical to those in GenBank: NSP1 (accession number AY117048, V50L i.e. valine at position 50 was substituted with leucine); NSP2 (EU636931); NSP3 (AY1065842); NSP4 (L41247); NSP5
Fig. 5. STAT1 localization in cells expressing individual RRV proteins. (a) HEK293T cells were transfected with plasmids encoding each RRV NSP or VP with N-terminal FLAG tags, or NSP4-FLAG. After 24 h cell lysates were analysed by Western blotting for proteins containing FLAG. Asterisks indicate bands of the approximate predicted molecular mass for each viral protein. Positions of molecular mass markers (kDa) are shown. (b) HEK293T cells were transfected as for (a), treated with IFN-γ.
Rotavirus inhibits STAT nuclear entry

Plasmids expressing human FLAG-IMP\(_1\) and FLAG-IMP\(_5\) were provided by Christopher Basler, Mount Sinai School of Medicine, NY, USA (Reid et al., 2006). The mouse IMP\(_\beta 1\) gene was subcloned into pCMV-3tag6 to produce the mouse FLAG-IMP\(_\beta 1\) plasmid. Transit LT-1 reagent (Mirus Bio) was employed for all plasmid transfections.

**Immunofluorescence microscopy.** Cells grown on glass coverslips were transfected with plasmids for 20–24 h in medium containing FBS and/or infected with rotavirus for given times and m.o.i as described previously (Holloway et al., 2009). Infected cells were maintained in serum-free medium. Cells were treated with IFN-\(\alpha\) (1000U ml\(^{-1}\); Merck), IFN-\(\gamma\) (50 ng ml\(^{-1}\); Becton Dickinson) or anisomycin (5 \(\mu\)g ml\(^{-1}\); Sigma) as indicated. When used, Leptomycin B (LMB; 2.8 ng ml\(^{-1}\), Sigma) was added to culture medium 1 h before IFN or control treatments. Cells were fixed, permeabilized, blocked and stained and mounted as before (Holloway et al., 2009), using rabbit monoclonal antibodies to phospho-Tyr\(\alpha\)701-STAT1 (p-STAT1) and phospho-Thr\(\alpha\)180/Thr\(\alpha\)182-p38 (p-p38; Cell Signalling Technologies), mouse monoclonal anti-FLAG antibody M2 (Sigma), mouse monoclonal antibody RVA to rotavirus VP6 (Coulson et al., 1987) and rabbit polyclonal antibodies to RRV (Coulson et al., 1987) and STAT2 (Santa Cruz Biotechnology). Images were analysed using Image J version 1.47 software (Schneider et al., 2012), whereby the nuclear to cytoplasmic ratio (Fn/c) was calculated from nuclear and cytoplasmic fluorescence levels minus background. For visual analysis of FLAG-IMP\(_5\)/\(\beta 1\) localization, 20 uninfected and 20 infected cells expressing FLAG-antigen were compared.

**Immunoprecipitation.** Co-immunoprecipitation of STAT1/2 with IMP\(_5\) was performed as before (Reid et al., 2007) with modifications. MA104 cells cultured in medium containing 10% (v/v) FBS in 80 cm\(^2\) flasks were transfected with plasmids expressing FLAG-IMP\(_1\) or FLAG-IMP\(_5\). After 40 h, cells were mock-infected or infected with RRV at an m.o.i of 5 for 7 h, then left untreated or treated with IFN-\(\alpha\) (1000U ml\(^{-1}\) for 30 min. Cells were lysed in IP buffer consisting of 50 mM Tris/HCl (pH 7.5), 280 mM NaCl, 0.5% (v/v) NP-40, 0.2 mM EDTA, 2 mM EGTA, 10% (v/v) glycerol, 1 mM DTT, 1 mM Na\(_3\)VO\(_4\) and complete protease inhibitor cocktail (Roche). Clarified cell lysates were gently mixed at 4°C with anti-FLAG-M2 affinity gel (Sigma) for 4 h. Following washing of beads three times with cold IP buffer, bound proteins were eluted by incubation at 95°C for 10 min in SDS-PAGE loading buffer and analysed by SDS-PAGE and Western

**Immunofluorescence microscopy.** Cells were transfected with plasmids expressing FLAG-IMP\(_5\) or FLAG-IMP\(_1\), mock-infected or infected with RRV (m.o.i. 5) for 7 h then treated without or with IFN-\(\alpha\) for 30 min. Cell lysates were immunoprecipitated with anti-FLAG antibody. Total cell lysates (Input) and precipitated proteins (IP) were analysed by Western blotting for proteins containing FLAG, p-STAT1 and p-STAT2. Data are representative of three independent experiments.

**Fig. 6.** IFN-induced recognition of STAT by IMP\(_5\) is maintained in RRV-infected cells. MA104 cells were transfected with plasmids expressing FLAG-IMP\(_5\) or FLAG-IMP\(_1\), mock-infected or infected with RRV (m.o.i. 5) for 7 h then treated without or with IFN-\(\alpha\) for 30 min. Cell lysates were immunoprecipitated with anti-FLAG antibody. Total cell lysates (Input) and precipitated proteins (IP) were analysed by Western blotting for proteins containing FLAG, p-STAT1 and p-STAT2. Data are representative of three independent experiments.

**Fig. 7.** IMP\(_5\) and IMP\(_\beta 1\) localization in RRV-infected cells. MA104 cells were transfected with plasmids expressing FLAG-IMP\(_5\) or FLAG-IMP\(_\beta 1\), infected with RRV (m.o.i. 0.5) for 6 h then treated with IFN-\(\alpha\) for 30 min. Cells were fixed, permeabilized and stained for the FLAG epitope, rotavirus antigen (Rotavirus) and nuclear DNA. Images were obtained by confocal microscopy.
blotting, along with the original cell lysates, for p-STAT1, p-STAT2 and FLAG as described previously (Holloway & Coulson, 2006; Holloway et al., 2009).

**Statistics.** The t-test was used unless otherwise stated.

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