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Interferon Regulatory Factor 3 Is a Cellular Partner of Rotavirus NSP1†

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The rotavirus nonstructural protein NSP1 is the least conserved protein in the rotavirus genome, and its function in the replication cycle is not known. We employed NSP1 as bait in the yeast two-hybrid interaction trap to identify candidate cellular partners of NSP1 that may provide clues to its function. Interferon regulatory factor 3 (IRF-3) was identified as an NSP1 interactor. NSP1 synthesized in rotavirus-infected cells bound IRF-3 in a glutathione *S*-transferase pull-down assay, indicating that the interaction was not unique to the two-hybrid system. NSP1 of murine rotavirus strain EW also interacted with IRF-3. NSP1 deletion and point mutants were constructed to map domains important in the interaction between NSP1 and IRF-3. The data suggest that a binding domain resides in the C terminus of NSP1 and that the N-terminal conserved zinc finger is important but not sufficient to mediate binding to IRF-3. We predict that a role for NSP1 in rotavirus-infected cells is to inhibit activation of IRF-3 and diminish the cellular interferon response.

Rotaviruses are the most important cause of severe, often life-threatening gastroenteritis in infants and children under 2 years of age (33). These viruses are ubiquitous in nature and are also responsible for a significant proportion of neonatal diarrheal illness in domestic animals, particularly in bovine and porcine species (18, 40). Substantial research efforts have thus focused on understanding the correlates of a protective immune response to rotavirus infection and the molecular mechanisms of virus replication so that efficacious vaccines can be developed.

The rotavirus segmented double-stranded RNA genome encodes six structural proteins (VP) and six nonstructural proteins (NSP) (reviewed in reference 17). The structural proteins VP1, VP2, VP3, VP4, VP6, and VP7 are well characterized in terms of their antigenic, structural, and biochemical properties. The functions of the rotavirus nonstructural proteins NSP1 to NSP6 are less well defined with regard to the roles that these proteins play in the rotavirus replication cycle. Intriguing functions have recently been described for some. NSP3 binds the 3′ consensus sequence of viral mRNAs (37) and acts as a functional analog of poly(A) binding protein through its interaction with eIF4GI (36). NSP4 is both an intracellular glycoprotein receptor for maturating rotavirus particles that bud through the endoplasmic reticulum (3, 32) and a viral enterotoxin that induces diarrhea in mice in the absence of any other viral protein (5). Functions of the remaining nonstructural proteins, NSP1, NSP2, NSP5, and NSP6, have been proposed based predominately on biochemical properties and activities of recombinant proteins (reviewed in reference 17).

NSP1 displays several interesting properties that warrant investigation. NSP1 has a calculated molecular weight of approximately 54,000 and is the least conserved protein encoded by the rotavirus genome when NSP1s of different strains are compared (23, 34). The N terminus contains a conserved zinc finger motif that binds zinc and viral mRNA in vitro (10, 22). Immunofluorescent staining showed NSP1 to be localized throughout the cytoplasm, in contrast to most other rotavirus proteins, which concentrate in viroplasms (24). NSP1 is also found associated with the cytoskeleton when analyzed by subcellular fractionation (24). NSP1 apparently is not required for rotavirus replication because strains with rearrangements in gene 5 that result in the synthesis of truncated NSP1 have been isolated from animals and from both immune-deficient and immune-competent children (1, 7, 15, 25, 35, 44, 45). Each of the described strains replicates in cell culture to titers close to those of their wild-type counterparts, but they yield small- to minute-plaque phenotypes. These observations suggest that NSP1 plays a role in regulating the efficiency of viral gene expression or in modulating host cell responses. We addressed the second possibility by constructing an MA104 cell cDNA library into the activation domain vector of the Matchmaker 3 yeast two-hybrid interaction trap (Clontech). We screened the library with NSP1 as bait to identify candidate partners of NSP1 that would provide clues to its function in rotavirus-infected cells.

Polyadenylated mRNA was isolated from MA104 cells in exponential growth phase with TriZol reagent (Life Technologies) and purified by column chromatography with the mRNA purification system from Amersham Pharmacia. cDNA was synthesized according to the protocol supplied with reagents from Clontech, with modifications. Briefly, 5 μg of mRNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase and an oligo(dT)-*Xho*I linker primer. Double-stranded cDNA was generated with DNA polymerase I, and the ends were blunted with *Pfu* DNA polymerase (Stratagene). Double-stranded cDNA fragments ligated to *Eco*RI adapters were size fractionated with Chro-

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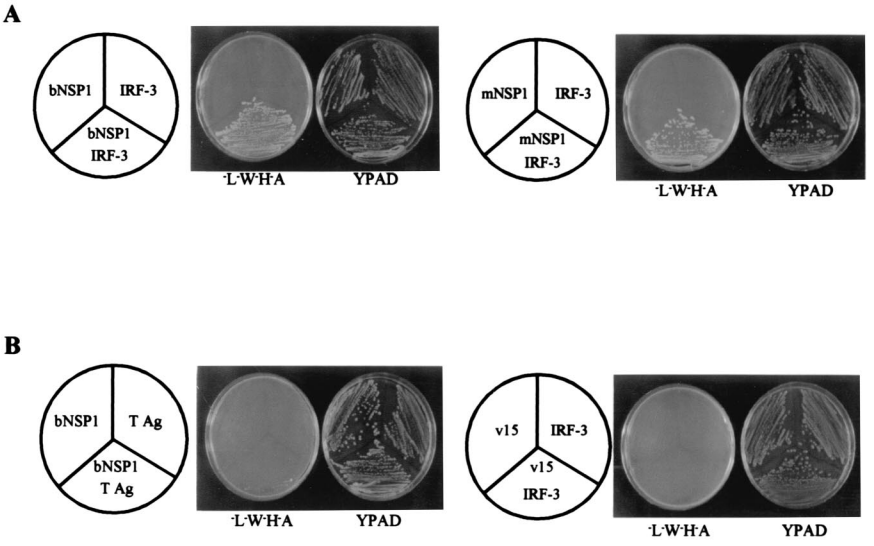

FIG. 1. NSP1 interacts with IRF-3. Bovine NSP1 (bNSP1) and murine NSP1 (mNSP1) in the yeast two-hybrid bait vector were tested for interaction with IRF-3 in the activation domain vector. Schematic diagrams indicate the quadrants of selection media where yeast containing the indicated plasmids were streaked. (A) Interaction between bovine NSP1 and IRF-3, or between murine NSP1 and IRF-3, on nutrient selection medium; (B) interaction of bovine NSP1 with simian virus 40 T antigen in the activation domain plasmid (left panel), and IRF-3 interaction with an irrelevant protein (v15) in the DNA binding domain plasmid (right panel). YPAD, yeast extract-peptone-adenine-dextrose.

maSpin 400 columns (Clontech) and ligated into the Matchmaker pGADT7 vector prepared by *Eco*RI-*Xho*I digestion. Transformations that were sufficient to obtain 2.5×10^6 CFU were performed. The colonies were collected into Luria broth, and DNA was purified by two sequential centrifugations through CsCl density gradients. The library was quantified by measuring the absorbance at 260 nm, aliquoted, and stored at −80°C.

Gene 5 encoding NSP1 of bovine rotavirus strain B641 was cloned by reverse transcriptase PCR (unpublished data). The nucleotide sequence of B641 gene 5 is 98% identical to that of gene 5 of bovine strain RF (9). A plasmid containing gene 5 of murine strain EW was a generous gift of Harry Greenberg, Stanford University School of Medicine. Gene 5 cDNAs from B641 and EW were cloned into pGBKT7 by standard cloning techniques to generate pGBK-bNSP1 (bovine) and pGBK-mNSP1 (murine), respectively. All yeast transformations followed the lithium acetate-polyethylene glycol (PEG) method described by Gietz and Woods (19). The DNA binding domain vector pGBKT7 carries the *TRP1* nutritional selection marker, and colonies containing bait plasmids were isolated by culture on synthetic complete medium lacking tryptophan (SC−W medium). The Matchmaker activation domain vector pGADT7 carries the *LEU2* nutritional marker for selection of yeast.

Positive two-hybrid interactions were scored by determining colony growth on SC medium without leucine and tryptophan (SC−L−W medium) and activation of the reporter genes *HIS3*, *ADE2*, and *MEL1*. Reporter gene activation was indicated by growth in the absence of histidine and adenine and by the ability to metabolize the chromogenic substrate X-α-Gal (α-5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; ICN). pGBK-bNSP1 yeast cells (6×10^9) cultured in SC−W liquid medium were transformed with 120 μg of the cDNA library by the lithium acetate-PEG procedure. Transformants were plated on SC medium without leucine, tryptophan, histidine, and adenine (SC−L−W−H−A medium) and cultured for 2 to 4 days at 30°C. Colonies measuring 2 to 4 mm in diameter were restreaked on SC−L−W−H−A plus X-α-Gal medium and again incubated for 2 to 4 days at 30°C. Plasmid DNA was isolated by electroporation into DH10B cells (Life Technologies) and cultured on antibiotic selection media.

NSP1 interacts with IRF-3. We performed a two-hybrid interaction screen with bovine NSP1 as bait to identify candidate cellular partners of NSP1 that would shed light on the function of this protein in rotavirus-infected cells. We identified several colonies that grew on defined media that selected for the presence of both transcription activation and DNA binding domain plasmids and for activation of reporter gene expression. We isolated six library clones by transforming them into bacteria and sequenced the activation domain plasmid inserts. A BLASTX search with the nucleotide sequence of each of these clones yielded matches with human interferon (IFN) regulatory factor 3 (IRF-3). The cDNA clones ranged from 0.9 to 1.2 kb. The longest cDNA corresponded to a sequence from nucleotide 110 to the 3′ polyadenylate tail, according to the published sequence for human IRF-3 (4) (GenBank accession no. NM 001571). IRF-3 is a transcription factor that responds to virus infection by inducing the expression of the genes for IFN-α and IFN-β (4, 21, 26, 31). Subsequent IFN-stimulated gene products drive a cell to an antiviral state. Thus, identification of IRF-3 as a partner of NSP1 was relevant and warranted further study.

We performed a number of control experiments to ensure that the defined interaction was not an artifact or a false-positive interaction (Fig. 1). We retransformed pGAD–IRF-3 into yeast containing pGBK-bNSP1 and plated transformants on SC−L−W medium. Colonies capable of growth on this medium were then streaked onto SC−L−W−H−A medium containing X-α-Gal. Only yeast cells containing both plasmids

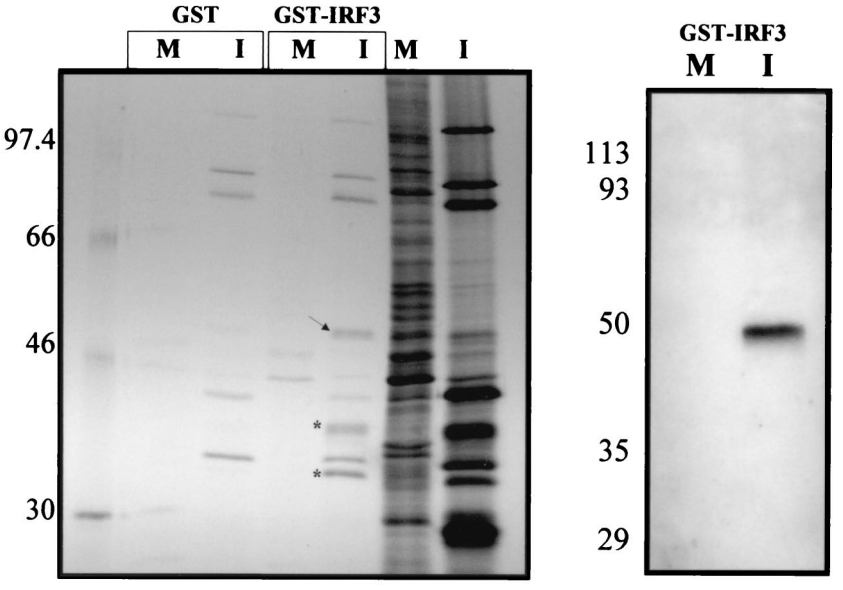

FIG. 2. NSP1 interacts with IRF-3 in a GST pull-down assay. IRF-3 was expressed in bacteria as a GST fusion protein. GST–IRF-3 or GST alone was bound to glutathione-Sepharose 4B, and radiolabeled mock-infected (M) or B641-infected (I) lysates were incubated with the beads. (Left panel) The arrow indicates an ~50-kDa NSP1 pulled down by GST–IRF-3. Asterisks indicate VP7 (~38 kDa) and NSP3 (~34 kDa). M and I lanes contain lysates not subjected to the pull-down assay. (Right panel) Western blot of GST–IRF-3 eluates with an anti-NSP1 monoclonal antibody (provided by R. L'Haridon and D. Poncet, INRA, Jouy-en-Josas, France). Molecular mass markers are noted at the left.

were capable of growth on selective medium (Fig. 1A). pGAD–IRF-3 was negative for autoactivation, as was evidenced by its failure to activate reporter gene expression in the absence of pGBK-bNSP1. We also tested potential interactions between (i) bovine NSP1 and an irrelevant protein (simian virus 40 T antigen) in the activation domain plasmid, (ii) IRF-3 and an irrelevant protein in the binding domain plasmid (v15), and (iii) IRF-3 with an empty bait plasmid (Fig. 1B and data not shown). These tests were also negative.

NSP1 of murine rotavirus strain EW (16) shares 37% amino acid identity with B641 NSP1 over the full-length protein. The N-terminal 230 amino acids are 51% identical, and this conservation includes the Zn^{2+} finger domain. The ability of EW NSP1 to bind IRF-3 was tested to determine if the observed interaction was limited to the bovine NSP1 used in the initial screen. EW NSP1 also interacted with IRF-3 (Fig. 1A). These data suggest that the NSP1–IRF-3 interaction is not limited only to bovine rotavirus NSP1, even though the sequence divergence between bovine NSP1 and murine NSP1 is relatively high.

NSP1 synthesized in rotavirus-infected cells binds IRF-3. The ability of NSP1 synthesized in infected cells to interact with IRF-3 was examined by a glutathione *S*-transferase (GST) pull-down assay as previously described (29). MA104 cells were infected with trypsin-activated B641 at a multiplicity of infection of 10 in the presence of 5 μg of actinomycin D per ml. Fifty microcuries of ^{35}S-trans label (>1,000 Ci/mmol; ICN) per ml was added 2 h postinfection, and the infection was allowed to proceed for an additional 4 h. Cells were harvested in lysis buffer (50 mM Tris-Cl [pH 8.0], 15 mM NaCl, 140 mM KCl, and 2% NP-40). Radiolabeled lysates from infected or mock-infected cells were then incubated with glutathione-Sepharose 4B beads bound to either GST or GST–IRF-3. Interacting proteins were eluted with 10 mM reduced glutathione, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and visualized by autoradiography. Figure 2 (left panel) shows that NSP1 bound GST–IRF-3 but not GST. The identity of the band migrating at the predicted molecular weight of NSP1 in the GST–IRF-3 eluate was confirmed by Western blotting with an anti-NSP1 monoclonal antibody (right panel). Rotavirus proteins VP7 and NSP3 were also retained by GST–IRF-3 but not GST. Whether these proteins are bound by IRF-3 or by NSP1 complexed to IRF-3 is not known. NSP1 binds the 5′ end of viral mRNA, and NSP3 binds the 3′ end (22, 37). NSP3 interacts with translation initiation factor eIF4GI, resulting in circularization of the viral mRNA in polysomes (36). A direct interaction between NSP1 and NSP3 was shown in a yeast two-hybrid assay (20) but thus far has not been reported to occur in infected cells. It is possible that NSP3 is brought down in this assay through interaction with NSP1. Alternatively, NSP3 may be pulled down through a viral mRNA bound to NSP1. The detection of VP7 in the GST–IRF-3 eluate is more difficult to interpret. It may be that this protein interacts with NSP1 in infected cells, but these interactions have not been investigated or described. Numerous attempts to coimmunoprecipitate NSP1 and IRF-3 were unsuccessful, indicating that the interaction may be transient or not sufficiently stable to survive the conditions of the immunoprecipitation. The data presented in Fig. 2 show that NSP1 synthesized in a native rotavirus infection interacted with IRF-3, and thus NSP1 and IRF-3 are not binding partners unique to the yeast two-hybrid system.

The NSP1 zinc finger is important but not sufficient to mediate binding to IRF-3. NSP1 shows considerable divergence in amino acid sequence among different rotavirus strains (23). The N-terminal 230 amino acids are more conserved than the C-terminal residues, yet the C terminus does retain some predicted structural conservation (M. E. Hardy et al., unpub-

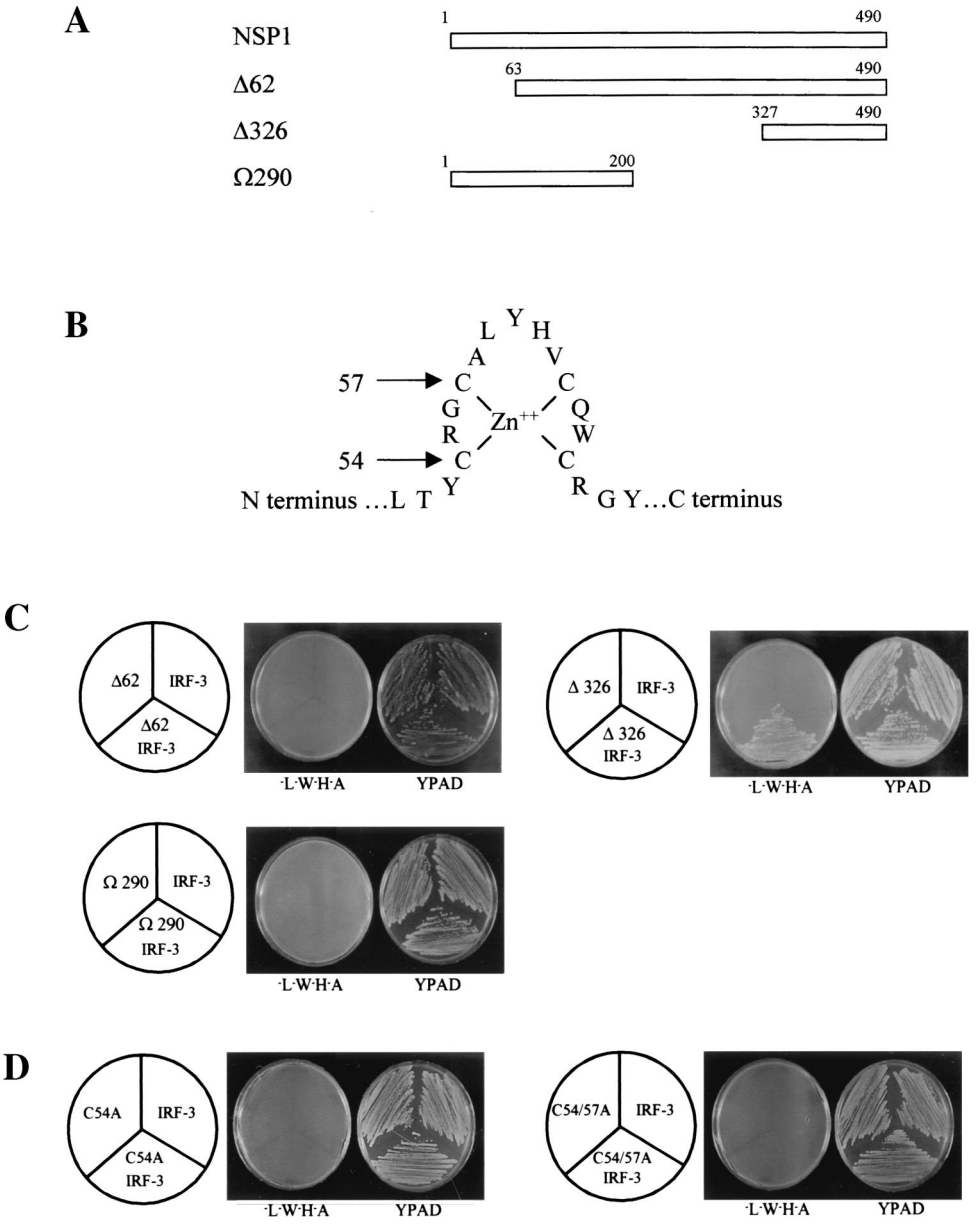

FIG. 3. Mutational analysis of NSP1 and the interaction with IRF-3. (A) Schematic diagram displaying deletion mutants constructed in the two-hybrid bait vector and analyzed for interaction in yeast. Amino acid numbers (relative to strain B641 numbering) are indicated. (B) Potential structure of the N-terminal conserved zinc finger with cysteine residues altered to alanines by site-directed mutagenesis as indicated. (C) Two-hybrid interaction analysis of the deletion mutants depicted in panel A. (D) Two-hybrid analysis of the cysteine mutants depicted in panel B. YPAD, yeast extract-peptone-adenine-dextrose.

lished observation). We made a series of mutations in NSP1 to define the domain(s) that mediates the interaction with IRF-3.

Schematic diagrams of the bovine NSP1 mutants used in the following analyses are shown in Fig. 3A. NSP1 deletion mutants were constructed by PCR and by standard cloning techniques. Deletion mutant Δ62 is missing the first 62 amino acids. The fragment was amplified by PCR with oligonucleotides 5′-CG CGGATCCAGATCAATAACCATTACAAAGTGTGG-3′ (positive) and 5′-CCGCTCGAGGGTTCAACATCTGAAAG TTC-3′ (negative) and the full-length B641 gene 5 clone as the template. *Bam*HI and *Xho*I sites for cloning are underlined. Deletion mutant Δ326 is missing the N-terminal 326 amino acids. This mutant was constructed by PCR amplification with the negative-sense primer described above and with the positive-sense primer 5′-CGCGGATCCAGATCAATAACCAT TACAAAGTGTGG-3′. Deletion mutant Ω290 contains only the N-terminal 200 amino acids and was constructed by PCR amplification with primers 5′-CGCGGATCCTGATGGCGA CTTTTAAGGAC-3′ (positive) and 5′-CCGCTCGAGTATT ACTGGTAAGTGTGACAT-3′ (negative). PCR fragments were cloned into the pGBKT7 vector. All constructs were sequenced prior to transformation into yeast to ensure that the correct reading frame had been maintained. Each mutant

NSP1 bait construct was transformed into AH109 cells alone or into AH109 cells containing pGAD-IRF-3. Transformants were then scored for interaction based on the ability to grow on nutrient-deficient medium.

The zinc finger domain present between amino acids 42 and 72 (numbering according to that of B641 NSP1) in NSP1 is completely conserved among rotavirus strains. Mutant Δ62 was constructed to ascertain the contribution of the N-terminal zinc finger to the interaction between NSP1 and IRF-3. Figure 3C shows that deletion of the N-terminal 62 amino acids abolished the interaction with IRF-3, as evidenced by the inability of yeast cotransformed with Δ62 and IRF-3 to grow on selective medium. A second construct (Δ326) contained only the C-terminal 164 amino acids. This mutant scored positive for interaction with IRF-3, though the colonies consistently grew more slowly than those with full-length NSP1 (Fig. 3C). A construct containing the analogous C-terminal residues of EW NSP1 (Δ327) also interacted with IRF-3 (data not shown). A final deletion mutant, Ω290, consisted of the N-terminal 200 amino acids and contained the zinc finger. Surprisingly, this mutant failed to interact with IRF-3.

The results of the deletion analyses were not straightforward, and they suggested that structure played a significant role in the IRF-3 interaction. Therefore, we performed oligonucleotide-directed site mutagenesis (QuikChange; Stratagene) to disrupt the N-terminal zinc finger so that the overall structure of the protein would be maintained as much as possible. In one mutant the cysteine at position 54 was replaced with alanine (C54A mutant). In a second mutant, the cysteines at both position 54 and position 57 were replaced with alanines (C54A/57A mutant) (Fig. 3B). Neither of these mutants interacted with IRF-3 (Fig. 3D).

Taken together, the mutagenesis data suggest that proper folding and the structure of NSP1 are important for interaction with IRF-3. The inability of mutant Δ62, the C54A mutant, and the C54A/57A mutant to interact with IRF-3 and the reduced growth rate of Δ326 suggested that the zinc finger was important for binding. However, this domain was not sufficient for binding because the Ω290 mutant, which contained the entire zinc finger, did not interact with IRF-3. The interaction of the C-terminal 164 amino acids of NSP1 with IRF-3 (Δ326 and EW Δ327) and the lack of interaction between Ω290 and IRF-3 suggest that the interaction is not an artifact mediated simply by a zinc finger domain that may be present in a wide variety of proteins.

The data lead us to propose that at least one point of contact with IRF-3 resides in the C-terminal domain of NSP1. The inability of mutant Δ62 or the Zn^{2+} finger point mutants to interact with IRF-3 in yeast might be explained by a structural inhibition of C-terminal binding in the absence of the Zn^{2+} finger. Alternatively, proper folding of full-length NSP1 may require formation of the zinc finger, and thus the authentic conformation may be disrupted even by the point mutations. Computer predictions of the secondary structures of the C-terminal ~164 amino acids of B641 and EW NSP1 show conserved regions of α-helices connected by random coils. Consistent with the mutagenesis data, it is likely that this structure is critical, and the interaction between IRF-3 and NSP1 may be driven primarily by the α-helical content of the C terminus of NSP1. Secondary-structure analysis of three viral proteins known to interact with or modulate the function of IRF-3 (see below), influenza virus NS1 (43), adenovirus E1A (26), and human papillomavirus E6 (39) also predicts ~40% α-helices and 40% random coils, suggesting that these structures may be important. Further mutational analyses that disrupt discrete structures in the C terminus are required to refine domains important in the interaction.

Role of NSP1–IRF-3 interaction in rotavirus-infected cells. The role of IFN in rotavirus infection is unclear, as the data are conflicting and dependent on the species studied. Both humans and animals infected with rotavirus have serum IFN-α and excrete IFN-α in stool (11, 14, 27). Calves treated with recombinant human IFN-α2 were protected from diarrhea induced by tissue culture-adapted bovine rotavirus (42), whereas piglets were not (28). The colon adenocarcinoma cell line HT-29 shows weak or no IFN-β response following rotavirus infection, although evidence for IFN-induced gene expression was apparent (38). HT-29 and CaCo-2 cells become resistant to rotavirus infection when they are pretreated with IFN-α (6). Despite evidence for a protective effect of IFN, studies in mice which were administered exogenous IFN-α/β or mice lacking the IFN receptor (IFN-α/β receptor$^{-/-}$) showed no differences in disease severity or duration compared to controls (2). These data suggested that the IFN response was not an important nonimmune mediator of protection from rotavirus disease in mice. Collective interpretation of these data is difficult because of the wide range of rotavirus strains, cell lines, species of virus, means of cell isolation, methods of virus adaptation, and animal models used. Given that the cellular IFN response is promiscuous in reaction to virus infection or exposure to extracellular pathogens, it is difficult to accept the idea that the IFN response is not important in rotavirus infection. In fact, a recent analysis of global gene expression in rotavirus-infected CaCo-2 cells showed up-regulation of IFN-stimulated genes 16 h postinfection (12). It is therefore very likely that viral interference with the cellular IFN response is not absolute and that it primarily serves to diminish the magnitude or effectiveness of the response. There are no reports of mechanisms that rotaviruses may have evolved to ameliorate the IFN response to promote infection and spread. The data presented here suggest that NSP1 may fulfill such a function in the rotavirus replication cycle. In support of this hypothesis, the small-plaque phenotype displayed by rotavirus strains that do not encode NSP1 may be explained if the IFN response in cells infected with these viruses is not down-regulated.

We identified IRF-3 as a cellular partner of NSP1. IRF-3 is a 427-amino-acid transcription factor that is constitutively expressed in all cell types (4). IRF-3 resides latently in the cytoplasm, and upon infection by a virus or exposure of cells to double-stranded RNA, IRF-3 is phosphorylated, dimerizes, and translocates to the nucleus, where it complexes with transcription coactivators such as CBP/p300 (8, 30, 41). We predict that the consequence of the NSP1–IRF-3 interaction in rotavirus-infected cells is the functional inhibition of IRF-3 and down-regulation of the innate cellular IFN response. Precedent for this prediction is provided by several viral systems. The adenovirus E1A protein down-regulates IRF-3-induced transcription by competing with IRF-3 for binding to CBP/p300 (13, 26). A second mechanism of IRF-3 inhibition is displayed by human papillomavirus type 16. The E6 protein of

human papillomavirus type 16 binds IRF-3 directly and downregulates induction of IFN-β (39). Finally, the NS1 protein of influenza virus inhibits nuclear translocation of IRF-3 in infected cells (43). Clearly, IRF-3-mediated gene induction is an important cellular response to infection, and viruses apparently have evolved mechanisms to thwart such a defensive response. Ongoing studies are directed toward identification of mechanisms by which NSP1 may inhibit the action of IRF-3.

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