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The Rotavirus Interferon Antagonist NSP1: Many Targets, Many Questions

Michelle M. Arnold

Department of Microbiology and Immunology, Center for Molecular and Tumor Virology, Louisiana State University Health Sciences Center, Shreveport, Louisiana, USA

Rotavirus is a leading cause of death due to diarrhea among young children across the globe. Despite the limited coding capacity that is characteristic of RNA viruses, rotavirus dedicates substantial resources to avoiding the host innate immune response. Among these strategies is use of the interferon antagonist protein NSP1, which targets cellular proteins required for interferon production to be degraded by the proteasome. Although numerous cellular targets have been described, there remain many questions about the mechanism of NSP1 activity and its role in promoting replication in specific host species.

All viruses must evolve ways to replicate in the face of the host immune response. When a virus begins its replication cycle within a cell, viral RNA may be recognized as foreign by the host pattern recognition receptors (PRRs). A cascade of signaling events is then initiated that stimulates the transcription factors NF-κB and interferon (IFN) regulatory factors (IRFs) to translocate to the nucleus, bind to specific promoter sequences, and induce the transcription of type I IFN mRNA. After IFN is synthesized and secreted, it binds to IFN receptors to signal to the same or neighboring cells that an infection has occurred, triggering the production of IFN-stimulated genes (ISGs). ISGs have direct antiviral activities and thus are the effectors of the IFN response. Because IFN initiates a strong antiviral response in the infected host, viruses usually encode one or more ways to prevent induction of IFN or to interfere with the signaling cascade downstream of IFN receptors.

HOW DOES ROTAVIRUS AVOID DETECTION BY THE HOST PATHOGEN RECOGNITION MACHINERY?

Rotavirus is a member of the *Reoviridae* family. Although there are eight different species of rotaviruses (species A to H), *Rotavirus A* is responsible for the majority of life-threatening diarrhea in children (1, 2). The viral particle is nonenveloped and consists of three protein layers that surround the 11 segments of genomic double-stranded RNA (dsRNA). While synthetic dsRNA and *Reoviridae* genomes are commonly used to experimentally stimulate the IFN response, rotavirus takes many precautionary measures to protect itself from the innate host defenses encountered during infection.

In its natural host, rotavirus replicates in mature enterocytes at the tips of the small intestinal villi. Upon entering a cell, the virus sheds only the outermost layer of its protein shell, leaving a transcriptionally active double-layered particle to synthesize and extrude (+) RNAs for translation by host ribosomes. The virus encodes and packages its own RNA-dependent RNA polymerase (viral protein VP1) and capping enzyme (VP3) to synthesize (+) RNAs that have a 5′ cap structure equivalent to that of the host mRNA but that lack a polyadenylated tail. As viral proteins accumulate, they are concentrated in nonenveloped inclusions called viroplasms, which form in the cytoplasm and serve as the centers of viral replication. Within viroplasms, (+) RNAs can be used by newly forming viral particles to generate genomes for packaging. Replication to form new dsRNA genomes occurs only as particles are assembling, such that the genome should not be exposed to the cytoplasmic environment (2).

Seven of the 12 proteins encoded by rotavirus are RNA-binding proteins, and 5 of those are localized to the viroplasms in infected cells (2). One role of the viroplasm may be to serve as a reservoir or "sponge" that soaks up and sequesters viral RNAs (3). However, saturation of the "sponge" could lead to RNAs leaking out of the viroplasm, potentially alerting the host PRRs to the presence of virus. Another way in which the host could be alerted to a rotavirus infection is by the presence of small populations of uncapped or incompletely capped (+) RNA that arise because the VP3 capping enzyme is not absolutely efficient (4). The exposed 5′ phosphate groups signal the cytoplasmic PRRs retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein-5 (MDA5), leading to activation of the IFN response (5). In addition, uncapped rotavirus RNAs may activate other host antiviral proteins that bind to viral RNA, such as protein kinase R (PKR) or 2′-5′-oligoadenylate synthetase (OAS), but the role of these ISGs in inhibition of rotavirus is not well understood. Interestingly, the VP3 capping enzyme contains a phosphodiesterase domain that has been shown to degrade 2′,5′-oligoadenylates, thereby preventing the activation of RNase L, which would otherwise cleave viral and cellular single-stranded RNA (ssRNA) (6). Although rotavirus takes great steps to protect its genomic and (+) RNAs, there are inefficiencies in these processes that the virus must overcome in order to replicate. Therefore, rotavirus also encodes a nonstructural protein, NSP1, which primarily functions as an antagonist of the host IFN response to protect the virus from the innate immune response.

HOW DOES NSP1 PREVENT IFN INDUCTION AND SIGNALING?

Retrospectively, one of the first indications that NSP1 played a role in avoiding host defenses came from studies that generated and/or

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Address correspondence to marno2@lsuhsc.edu.

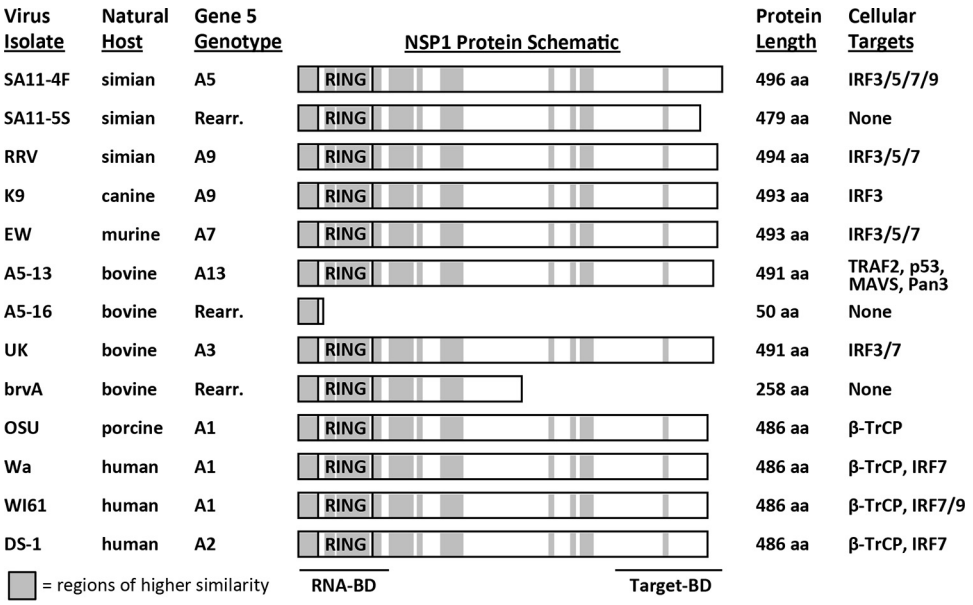

FIG 1 Summary of NSP1 proteins from different rotavirus isolates. Proteins commonly used in laboratory studies to investigate the role of NSP1 in rotavirus infections are included. Genotypes were determined using RotaC v2.11b (http://www.regatools.be/rota20/). Viruses with rearrangements in gene 5 are noted as "Rearr." and are listed directly underneath the corresponding parental virus isolate. Cellular targets include host proteins degraded in the presence of NSP1 but do not include proteins that bind to NSP1 without being degraded. Only IRF and β-TrCP targets have been validated in multiple studies. Approximate locations of the RNA-binding domain (RNA-BD) and the target-binding domain (Target-BD) are noted at the bottom, and regions of higher similarity are noted in gray. aa, amino acids.

isolated viruses containing rearrangements in genome segment 5, which encodes the NSP1 protein (7). Genome rearrangements can occur randomly during the replication process and typically consist of a partial sequence duplication that causes a premature stop codon to be inserted in the open reading frame. The result is a longer RNA that retains its packaging signals but also a truncated protein product. Rotaviruses with gene 5 rearrangements have NSP1 proteins with C-terminal truncations ranging from small (17-amino-acid) deletions to large deletions that eliminate nearly the entire NSP1 protein (Fig. 1). These viruses expressing truncated NSP1 proteins replicate to levels similar to the parental wild-type virus isolates in permissive cell culture, but the plaque sizes tend to be smaller. Given that IFN is important in limiting viral spread between cells, the reduced plaque size formed by rotaviruses with gene 5 rearrangements suggests a role for NSP1 in cell-to-cell spread (7, 8).

Comparison of rotaviruses expressing a C-truncated NSP1 with their wild-type parental counterparts was instrumental in identifying full-length NSP1 as a viral IFN antagonist that induces the proteasome-mediated degradation of host proteins required for IFN induction. IRF3, which is a transcription factor that binds to the IFN promoter to induce transcription, was the first host protein shown to be degraded when NSP1 was expressed in infected or transfected cells (8). IRF5, IRF7, and IRF9 are also targets of NSP1-mediated degradation, due to similarities with IRF3 in domain structure (9, 10). NSP1 specifically targets the IRF association domain of these proteins, which mediates IRF homo- or heterodimerization. The constitutively activated form of IRF3 is targeted by NSP1, as are forms of IRF3 that are unable to dimerize, suggesting that NSP1 broadly inhibits IFN production (10).

Preventing IFN production is common among wild-type rotaviruses; however, not all rotaviruses induce IRF degradation (11). The production of IFN-β relies on the binding of both IRF and NF-κB transcription factors to the IFN promoter. NF-κB is activated when the inhibitory protein IκBα or IκBβ is phosphorylated and subsequently degraded by an Skp-Cul-F-box (SCF) E3 ubiquitin ligase complex containing the substrate binding protein β-TrCP. Some NSP1 proteins induce the proteasomal degradation of β-TrCP; in the absence of β-TrCP, NF-κB can no longer be activated and, thus, the production of IFN is prevented (12).

A phosphodegron-like motif, DSGΦS (where Φ is any hydrophobic residue), is located at the C terminus of NSP1 from human and porcine isolates of rotavirus (13). The same sequence is present in IκBα/IκBβ and is the motif responsible for binding to β-TrCP. Other host proteins known to contain this motif include β-catenin, nuclear factor, erythroid 2-like 2 (NRF2), and IFN alpha receptor 1 (IFNAR1). The SCF complex containing β-TrCP ubiquitinates each of these host proteins, targeting them to the proteasome for degradation. Signaling downstream of IFNAR1 requires the formation of a complex comprised of signal transducer and activator of transcription 1 (STAT1), STAT2, and IRF9. NSP1 has been shown to inhibit STAT1 phosphorylation and activation downstream of IFNAR1 signaling but not by inducing STAT1 degradation (14). Controlling signaling through STAT1 could potentially be related to an alteration in IFNAR1 levels that is caused by the NSP1-mediated loss of β-TrCP, but this idea has yet to be explored. NSP1 is thought to induce ubiquitination and degradation of β-TrCP in order to prevent it from acting on its normal cellular substrates, but, alternatively, it may function by binding to β-TrCP in order to disrupt its activity, which has been observed with several other viral proteins. Further studies are needed to learn how NSP1 induces the loss of critical proteins of the IFN induction cascade and how it blocks downstream IFN signaling events.

WHAT IS THE MECHANISM OF NSP1-MEDIATED PROTEIN DEGRADATION?

NSP1 is often referred to in general terms, so when the activity of an NSP1 from a specific virus isolate is described there is often an implication that the activity applies to NSP1 proteins from all rotaviruses. However, the capacity to induce degradation of a particular host target protein is not shared by all isolates of rotavirus (11). Sequence comparison of different rotaviruses reveals that the NSP1 protein is highly variable, more so than for any other rotavirus protein. The length of the NSP1 protein varies as well, ranging from 486 amino acids in human isolates to 496 amino acids in some animal isolates and to as much as 577 amino acids in avian isolates (15). Most of the sequence variability in NSP1 is localized in the C-terminal half of the protein, consistent with that region being responsible for the recognition of different target proteins (Fig. 1). In addition to IRF and β-TrCP, there have been reports of a number of other host proteins that are degraded by NSP1, including RIG-I, TNF receptor-associated factor 2 (TRAF2), mitochondrial antiviral signaling protein (MAVS), p53, and poly(A)-specific RNase subunit (Pan3) (reviewed in reference 16). Given the limited coding capacity of RNA viruses, it is expected that NSP1 has multiple functions, but how so many different host proteins can bind to the same site on the C terminus of NSP1 remains to be answered. Perhaps this domain is crucial for proper folding of NSP1, or perhaps it is posttranslationally modified in this region to stimulate a key activity.

The degradation of host proteins induced by NSP1 is dependent on the proteasome, and, coupled with the highly conserved RING domain localized to the N terminus, NSP1 has been described as an E3 ubiquitin ligase. However, there is a lack of direct evidence for NSP1 having ubiquitin ligase activity. None of the substrate proteins have been shown to be ubiquitinated or to experience an increase in ubiquitination in the presence of NSP1. Most of the substrate proteins have been shown to associate with NSP1, but only IRF3 has been identified as an interacting partner (17). Studies designed to show direct protein-protein interactions between NSP1 and its targets, or between NSP1 and an E2 ubiquitin-conjugating enzyme, which is needed to mediate ubiquitin transfer to the target substrate, would better support the idea of E3 ubiquitin ligase activity of NSP1. However, the experimental evidence that would best define NSP1 as an E3 ubiquitin ligase would consist of data from *in vitro* ubiquitination assays containing purified components required for ubiquitination, including the E1, E2, ATP, ubiquitin, NSP1, and target substrate proteins. Without additional data, there remains the possibility that NSP1 functions as a component of an E3 ubiquitin ligase complex or binds to proteins to destabilize them in another manner. Defining the activity of NSP1 will improve understanding of how viral IFN antagonists enhance viral spread and transmission. Additionally, viral IFN antagonist proteins are excellent targets for modification in vaccines. Because IFN helps to drive the development of an adaptive immune response, diminishing or eliminating the activity of a viral IFN antagonist might induce higher levels of IFN upon vaccination, which would contribute to a more effective adaptive immune response. Vaccine improvements should not be overlooked, as the efficacy of current vaccines in the underdeveloped countries that experience the most deaths due to rotavirus infections lags behind the efficacy in developed regions of the world. Rational and targeted approaches to improving vaccines would be aided by a more complete understanding of how NSP1 functions.

WHAT IS THE ROLE OF NSP1 IN DETERMINING THE HOST RANGE OF ROTAVIRUS?

Within the *Rotavirus A* species, there are a vast array of different isolates (or strains) that infect different hosts. Isolates are typically adapted to replicate to high titers, and thus cause disease, in a specific host species. In addition to this host range restriction, rotaviruses experience cell and tissue tropism, as infections are normally localized to mature enterocytes of the small intestines. The natural attenuation of animal rotaviruses in humans is the basis for some rotavirus vaccines. An animal rotavirus isolate serves as the backbone of the vaccine strain, which limits replication in the inoculated infant, but the outer capsid contains viral proteins from human virus isolates, allowing a protective immune response to develop. Discovering how replication of an animal rotavirus is restricted in humans may also help our understanding of the innate immune response to other live-attenuated vaccines.

The neonatal mouse model of rotavirus infection, where homologous (murine) virus isolates replicate in the intestines of mice significantly better than heterologous (nonmurine) virus isolates, has been instrumental in experimentally demonstrating host restriction. This model has been used to identify several viral proteins that play a role in host range restriction, including outer capsid protein VP4, which is necessary to mediate efficient viral entry, and NSP1, which is an essential factor that strongly influences replication (18). Heterologous rotaviruses replicated poorly in wild-type mice, but when STAT1 knockout mice were used, the restriction on replication was lifted. These results indicate that the IFN response regulates rotavirus replication *in vivo*. The homologous (murine) isolate of rotavirus efficiently suppressed the IFN response by inducing the degradation of IRF3 in mouse embryonic fibroblasts, whereas one of the poorly replicating heterologous (bovine) isolates did not induce IRF3 degradation or block IFN induction (19). The inability of the heterologous rotavirus to induce IRF3 degradation was not due a failure to recognize and bind to the murine IRF3 (20), suggesting that there is another cellular factor that confers a species-specific effect on replication but that has not yet been described.

The host restriction of replication is common among many viruses when infection occurs in a nonnative host, and the link to IFN-mediated control of replication in heterologous hosts has been demonstrated in several cases (as one example, the induction of IFN in mouse cells restricts myxoma virus replication). Species-specific replication of viruses goes beyond the entry steps, which require the viral receptor protein to be present, to the expression of IFN antagonist proteins that are important for evading the host immune response. One might predict that a rotavirus isolate that has evolved to prevent IFN induction in one host species might not be as efficient at inhibiting IFN in a different host species, thereby limiting replication. A better understanding of the required interactions between IFN antagonists and host proteins, and how these interactions allow species-specific replication, is needed for many viruses, including rotavirus.

PERSPECTIVES

The role of NSP1 in antagonism of the IFN response is the subject of much study, but many of the mechanistic details needed to define the activity of NSP1 are not known. How does NSP1 induce

degradation of host proteins? Does it do so by acting directly as an E3 ubiquitin ligase to induce ubiquitination, by usurping other cellular ubiquitin ligase complexes, or by another mechanism that destabilizes proteins in the IFN induction pathway? The answer to these questions might provide insight into why there seems to be a broad range of substrate proteins targeted for degradation by NSP1. Although there appears to be an evolutionary division between NSP1 proteins that target IRFs and those that target β-TrCP for degradation (13), some target both substrates (11), and the understanding of how this occurs is incomplete. There also may be other target proteins that have not yet been identified that tie these divergent substrates together. Even though there are different host targets of NSP1, all wild-type rotaviruses appear to target the IFN response for inhibition, which reinforces the importance of this innate immune response pathway in fighting viral infections.

There are many additional questions that remain about NSP1. Do NSP1 proteins that induce the degradation of β-TrCP stabilize the levels of other proteins with the DSGΦS motif (such as β-catenin or IFNAR1), and, if so, does the stabilization impact the production or signaling of IFN? Is the key function of NSP1 to induce degradation of host proteins, or are there other activities that prevent IFN induction or signaling? NSP1 might impact host pathways other than IFN induction, a conjecture which is also in need of further exploration. RNA viruses typically utilize their protein coding capacity very efficiently; therefore, NSP1 is expected to be a multifunctional protein. For instance, the RNA-binding activity of NSP1 might serve to sequester viral RNAs from host PRRs or might have an entirely different role. Ultimately, the continued study of NSP1 will answer these and other important questions about the function of viral IFN antagonist proteins and lead to a better understanding of how the host IFN system can prevent viruses from spreading efficiently from one host species to another.

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