ABSTRACT

PARKS, KIMBERLY ANNE. The Innate Immune Response to Reovirus Infection: Repression of IFN Signaling and Hsp25. (Under the direction of Dr. Barbara Sherry).

The innate immune response is a critical first-line defense against viral infection. One aspect of this response to infection is the synthesis and secretion of Type I interferon (IFN). Secreted IFN initiates the Jak-STAT signal transduction pathway leading to the establishment of an antiviral state. Subverting this IFN response increases viral spread and replication, thus it is a common countermeasure employed by viruses. The host has evolved additional means of protection to bolster the innate response, making it more challenging for a virus to completely inhibit the antiviral state. Here, we investigate the cellular protein Hsp25 for potential antiviral activity and identify the mechanism of reovirus subversion of IFN signaling.

Previously, our laboratory used a proteomic approach to identify Hsp25 as a protein that is phosphorylated by a virus that does little damage to the heart but that is degraded by a virus that is potently myocarditic. To test the potential antiviral activity of Hsp25, a cell line was established that over-expresses Hsp25 together with GFP as a marker. When treated with H₂O₂ to mimic ischemic damage and then analyzed by flow cytometry, cells expressing the highest levels of GFP were best protected. Because the cells in the population expressed varying levels of Hsp25, the culture was sorted by FACS into four subpopulations based on their GFP intensities. After confirming by western blot that GFP levels correlated with those of Hsp25, with the most fluorescent cells containing the most Hsp25, the populations were tested for differences in infection frequencies, viral titer, and cell viability. No significant
differences were observed, suggesting that Hsp25 antiviral activity against reovirus infection is nonexistent or undetectable in within these Hsp25 over-expression cell lines.

Next, we identified the mechanism of reovirus subversion of IFN signaling. Our lab has previously shown that repression of IFN signaling is strain-specific. Though strain T1L can repress IFN signaling, strain T3D is unable to repress. Reovirus protein µ2 was identified as the determinant of the differences in the IFN response. Interestingly, T1L µ2 also alters the subcellular localization of IRF9, establishing a possible link to the Jak-STAT pathway. Here, we report µ2 represses of IFN signaling by binding to IRF9. As demonstrated using confocal microscopy, both reovirus strains T1L and T3D induced translocation of IRF9 to viral factories, though T1L was more effective than T3D. Furthermore, both T1L and T3D were co-immunoprecipitated with IRF9 when overexpressed. Results suggest that µ2 binds IRF9, directly or indirectly, and sequesters it in viral factories to repress IFN signaling.
The Innate Immune Response to Reovirus Infection: Repression of IFN Signaling and Hsp25

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CHAPTER 1

Literature Review
REOVIRUS

The mammalian *Respiratory Enteric Orphan* viruses (reoviruses) have a broad host range, with infection in humans almost ubiquitous (236, 245). Most infections either remain asymptomatic or result in the infrequent case of mild gastroenteritis or upper respiratory symptoms (101, 137, 273). There are three reovirus serotypes (type 1-3), each represented by a prototype strain isolated from a human host: type 1 Lang (T1L), type 2 Jones (T2J), and type 3 Dearing (T3D) (236). Though rarely associated with clinical manifestations except in the very young (168, 280), reovirus serves as a highly tractable model for viral pathogenesis and offers viable therapeutic potential as an oncolytic agent (163).

Reoviruses are noneveloped, icosahedral viruses with two concentric protein capsids surrounding a dsRNA genome comprised of ten discrete segments. The gene segments are grouped into large (L1, L2, and L3), medium (M1, M2, and M3), and small (S1, S2, S3, and S4) size classes according to their migration on a polyacrylamide gel (15, 254). During coinfection of multiple serotypes, the gene segments replicated from different parental strains are able to combine and form progeny viruses (245). These reassortant reoviruses are excellent tools to identify gene segments responsible for particular phenotypes. To control the exact combination of gene segments, a plasmid-based reverse genetics system was developed to generate recombinant viruses (118, 120). With this system, viruses can be engineered to contain mutations in specific reovirus genes. The twelve proteins encoded by the ten gene segments are also classified by size, with λ, µ, and σ designating large, medium, and small, respectively. There are eight structural proteins (λ1, λ2, λ3, µ1, µ2, σ1, σ2, and
and four nonstructural proteins (µNS, µNSC, σ1s, and σNS), with µNSC and σ1s
generated from an alternative start codon within their respective M3 and S1 gene segments.

Reovirus entry is mediated by the σ1 filamentous trimers that protrude from the virion
at the five-fold symmetry axes (11, 29, 136, 301). Virions employ a multi-step binding
process. Depending on the virus strain and cell type, they may first recognizing α-linked
sialic acid residues (10, 29), and then may bind junction adhesion molecule-A with higher
affinity (11). After attachment, virions are internalized by clathrin-dependent, receptor-
mediated endocytosis (18, 19, 54). Subsequent sorting into vacuoles resembling endosomes
and lysosomes is regulated by β1 integrin (161, 162). Within these endocytic compartments,
cathespins B, L, and S cleave outer capsid proteins σ3 and µ1 (53, 83). This partial
disassembly along with a concomitant conformational change in σ1 to a longer filamentous
extension from the particle, yields an intermediate subvirion particle (ISVP) (259, 268).
Following further proteolytic processing, the µ1 cleavage products µ1N and ϕ are responsible
for the formation of a pore in endocytic membrane (99). This allows the release of a
transcriptionally active core particle to the cytoplasm.

Primary transcription begins within the core as the viral RNA-dependent RNA
polymerase λ3 transcribes positive-sense, single-stranded RNA (ssRNA). Nascent ssRNA
exits through turret-like structures formed by pentameric λ2 proteins at the five-fold
symmetry axes (79). The transcripts are capped during exit by the methyltransferase and
guanylyltransferase activates of λ2, but they remains non-polyadenylated (158). Once
released into the cytosol, the positive-sense ssRNA can either be translated into reovirus
proteins or serve as a template for the generation of negative-sense ssRNA. Translation and
assembly are concentrated in structures known as viral factories that can differ in morphology between strains (22, 208, 253). Formation of viral factories is dependent on reovirus protein µNS, though other reovirus proteins can also contribute to the expansion and stabilization of these structures (28, 119, 208). The mechanism of release of viral progeny remains unclear, though evidence suggests that cellular apoptosis may aid viral spread (169).

**REOVIRUS PROTEIN µ2**

The M1-encoded µ2 protein (83 kDa, 736 amino acids) has multiple roles during infection. Though present in the core as a low-copy structural protein with only ~24 copies per virion, µ2 is expressed well during infection and is readily detected (39, 51, 208). Within the core, µ2 resides adjacent to the λ3 polymerase near the five-fold symmetry axes (316). This localization is thought to be significant for its role in transcription. By binding λ3, µ2 is a component of the transcriptase complex and serves as a cofactor for the polymerase (38, 303, 309). In addition to binding ssRNA and dsRNA (21), µ2 has NTPase and RNA 5’ triphosphatase activity made possible by an A-box NTP-binding motif within µ2 (114, 203). Given its key accessory role during reovirus replication, it is not surprising that siRNA knockdown of µ2 results in decreased RNA synthesis, thus limiting viral protein expression and production of progeny (28, 119).

Most µ2 expressed during infection localizes to viral factories (175) through its association with the factory protein µNS (22, 184). µ2 determines the morphology of viral factories in a temperature-sensitive, strain-specific manner (175, 208). A single amino acid polymorphism at µ2 amino acid 208 (proline in T1L, serine in T3D) determines the stability
of µ2 and by extension, its ability to function within viral factories. The ability of the stable T1L µ2 to bind microtubules results in viral factories forming an elongated, filamentous structure. Conversely, T3D µ2 is unable to bind microtubules and therefore generates globular, peri-nuclear viral factories. Further evidence of T3D µ2 instability is provided by the increased ubiquitination and degradation of T3D µ2 (185). The amino acid 208 polymorphism also determines the ability of µ2 to repress interferon (IFN) signaling (96). T1L µ2 is able to repress IFN signaling by affecting the localization of the cellular protein IRF9 (319). The continuation of this study of µ2 repression of IFN by Zurney et al is discussed in Chapter 3.

THE INTERFERON (IFN) FAMILY

IFNs were first identified more than 50 years ago as a secreted factor capable of interfering with viral replication (97). Further characterization revealed that interference is not virus specific and can effectively limit the spread and replication of a broad range of viruses. The heterogeneous family of IFN proteins is typically divided into three classes: Type I IFN, Type II IFN, and Type III IFN determined by the distinct cell-surface receptor complex they bind as well as their sequence homology and genetic locus. The use of a unique receptor complexes for a given IFN type undoubtedly reflects the limited homology between IFNs, grouped together only by an overall helical-bundle fold that classifies IFNs as class II alpha-helical cytokines (213). All IFNs utilize the JAK-STAT signaling pathway, initiated upon binding of extracellular IFN to its receptor complex (42, 246, 265). Initiation of this pathway results in the induction of interferon stimulated genes (ISGs) that collectively
generate an antiviral state. The ISGs identified as antiviral differ in their mechanism of action and can target viral entry, envelope uncoating, genome replication, assembly, and release of progeny (219). Antiviral activity extends beyond the direct-acting antivirals (DAA) that target specific stages of the viral life cycle. IFNs can additionally induce apoptosis to prevent viral spread and impact cells of the adaptive immune system to enhance viral clearance (219, 266). IFNs have been demonstrated to promote neutrophil survival (237), polarize CD4+ T cells toward a Th1 response (156), and activate macrophages (124), natural killers cells (172), dendritic cells (156), B cells (272), and CD8+ T cells (266). By stimulating both innate and adaptive immunity, IFNs successfully tailor the overall response to viral infection to include both an immediate block and prolonged protection.

The first IFN types identified were originally classified according to their acid stability. The Type I IFNs are acid stable, a useful feature for purification of these cytokines for therapeutic applications (212). IFN-γ, as the lone Type II IFN, is acid-labile and thus distinct from other known IFNs (63). The more recently discovered Type III IFNs (IFN-λ1, IFN-λ2, and IFN-λ3) were initially classified as interleukins (IL-29, IL-28A, and IL-28B, respectively) (63, 123, 255). Despite having higher structural similarity with IL-10/IL-22 rather than with other IFNs, the Type III IFNs have more amino-acid identity with other IFNs (74) and share similar modes of induction and antiviral activities (63, 123, 255).

Due to its earlier discovery than Type II and Type III IFN, the Type I IFN family has been comparatively well characterized. In mammals, it consists of 9 members, namely IFN-α, of which there are 13 known subtypes, and single forms of IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-δ, IFN-τ, and IFN-ζ (213). Research has focused primarily on the contribution of IFNs-
α/β as the critical effectors of the Type I response, though each subtype may have a unique and perhaps tissue-specific role. IFNs-α/β are produced by plasmacytoid dendritic cells (pDCs), macrophages, fibroblasts, and endothelial cells, thus establishing the potential for either a localized or systemic innate immune response (266). The final amount of IFN that is produced depends on two steps in the pathway: initial induction via a cellular receptor and amplification of this signal by a positive feedback loop from secreted IFN.

**INDUCTION OF TYPE I IFN**

Numerous intracellular pathways lead to the induction of IFN following detection of viruses, bacteria, and fungi. The existence of a vast network of pathways using a multitude of receptors underscores the critical role of IFN in immune defense. Cellular receptors have been classified into groups according to their mechanism of pathogen recognition. Each type of pattern recognition receptor (PRR) recognizes a certain form of a pathogen-associated molecular pattern (PAMP). Detection of RNA viruses is mainly dependent on the RIG-I-like receptor (RLR) and Toll-like receptor (TLR) families (13). The RLR family is composed of the DExD/H RNA helicases retinoic acid-inducible gene (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and Laboratory of Genetic and Physiology 2 (LGP2) (195, 304). Though LGP2 has been identified as a regulator of RLR signaling, it cannot initiate the RLR cascade itself given its lack of the signaling effector domain shared by RIG-I and MDA5 (290). Both RIG-I and MDA5 contain two N-terminal caspase activation and recruitment domain (CARD) domains, a central ATP-dependent helicase domain, and a C-terminal RNA-binding/repressor domain. In uninfected cells, the CARD domain is extensively
phosphorylated, promoting a closed, inactive conformation (73, 202). Viral RNA recognition by the RNA-binding domain induces a conformational change that exposes the CARD domain, allowing for interaction with a homotypic CARD domain in an adaptor protein. The induction pathways of RIG-I and MDA5 converge at the adaptor protein mitochondrial antiviral signaling protein (MAVS) (251). Activated MAVS then relocalizes to form punctate aggregates on the mitochondrial membrane (92). Within these aggregates, MAVS activates the IRF3 kinases TANK-binding kinase 1 (TBK-1) and IκB kinase ε (IKKe) (109, 251). Phosphorylation of IRF3 causes a conformational change leading to formation of homodimers and the consequent exposure of a nuclear localization signal (NLS) (50, 147, 206). IRF3 homodimers translocate to the nucleus where they recruit transcriptional coactivators to induce IFN-α/β (90, 269).

The TLR family encompasses thirteen human and mouse type I transmembrane receptors, each binding a particular ligand (104, 195, 276). A leucine-rich repeat (LRR) is responsible for PAMP recognition and the cytoplasmic Toll/Interleukin (IL) 1-receptor homology (TIR) motif propagates the signal. Multiple TLRs play a central role in the response to viral infection: TLR3 recognizes dsRNA, TLR7 detects ssRNA, and TLR9 binds to hypermethylated CpG-rich DNA (108). The correct localization to encounter the given ligand is critical for detection. Much evidence indicates that the TLRs are compartmentalized to avoid an autoimmune response to host nucleotides (12). TLR3, TLR7, and TLR9 contain endoplasmic reticulum (ER) retention signals and thus remain in the ER until cell stimulation shuttles them to the surface plasma membrane or specific endosomal compartments. Each TLR activates a distinct signal cascade that can crosstalk with other pathways. TLR3 signals
through the adaptor protein TRIF, which creates two signaling branches by interacting with multiple proteins (110). The first arm of the pathway is activated when TRIF serves as a platform to recruit TRAF6 and the kinase RIP1, culminating in the activation of the transcription factors NFκB and AP-1 (141). Additionally, TRIF can bind to the E3 ubiquitin ligase TRAF3. Similar to RLR induce IFN-α/β, TRIF engagement of TRAF3 activates the kinases TBK1 and IKKε, resulting in the phosphorylation and dimerization of IRF3 and IRF7 to serve as IFN transcription factors (108, 231).

AMPLIFICATION OF TYPE I IFN INDUCTION: POSITIVE FEEDBACK LOOP

Production of IFN-β and the IFN-α subtypes following viral infection does not happen concomitantly. Instead, there is a defined timeline beginning with the direct induction of the immediate-early IFN genes (IFN-β and IFN-α4) (56, 170). These primary interferon genes can be induced independent of ongoing host protein synthesis and instead are contingent on the presence of preexisting proteins, mainly the constitutively expressed IRF3 (242, 244, 313). The secondary IFN genes (the remaining IFN-α subtypes) are delayed due to time required to transcribe additional transcription factors necessary for their induction. The production of secondary IFNs requires IRF7, which is expressed at low levels in most cells but is strongly induced by IFN-mediated signaling (240). This establishes a positive feedback model in which early IFN production induces the synthesis of IRF7, leading to the production of secondary IFNs as well as enhanced production of primary IFNs. In support of this model, IRF7, either as a homodimers or a IRF3-IRF7 heterodimer, was shown to be directly induced by ISGF3 (170, 240) and subsequently bind the IFN-α/β promoters to
dramatically enhance transcription (207, 297, 307, 308). Interestingly, IRF7 is prone to ubiquitination and degradation and has a short half-life of approximately 30 minutes (241, 315). Thus, this intricate control mechanism for IFN induction also serves as a means to render the induction of IFN transient in order to limit the potential harmful effects of overexpressed IFNs.

**JAK-STAT SIGNALING PATHWAY**

A common shared feature of most IFN-mediated signaling is the activation of the Janus family kinases (JAKs) that tyrosine phosphorylate the Signal Transducer and Activator of Transcription (STAT) family of latent cytoplasmic transcription factors. Together, activated JAKs and phosphorylated STATs are the main signaling proteins of the JAK-STAT pathway. Different combinations of interactions between the four JAK family members (JAK1-3 and Tyk2) and the seven STAT proteins (STAT1-4, 5a, 5b, and 6) are responsible for establishing a unique response to different cytokines. The generated response can be cell-type specific, with STAT4 and STAT6 functioning primarily in lymphocytes (64, 173). Different subsets of the JAK-STAT pathway are initiated by the three types of IFN and their unique receptor complex. The canonical Type I IFN signaling pathway is activated by the IFN ligand engaging a heterodimeric receptor comprised of two transmembrane subunits, IFNAR1 and IFNAR2 (64, 173, 204, 285). The central importance of this receptor in the immune response is demonstrated by IFN-α/β receptor-null mice, which are unable to establish an antiviral state (194). The IFNAR1 subunit has an elongated extracellular domain with affinity for IFN and an intracellular domain that is constitutively associated with Jak1
The smaller IFNAR2 receptor exists as three isoforms, though only isoform IFNAR2c is required to generate the signaling cascade (37, 191, 222). The intracellular domain of IFNAR2c is associated with the JAK family member Tyk2. The IFN affinity of the assembled heterodimeric IFNAR complex greatly exceeds that of the individual IFNAR1 or IFNAR2 subunit alone (37).

Engagement of IFN with its receptor complex results in a ligand-induced conformational change in IFNAR that is critical for signal propagation across the membrane (37, 216, 267). The intracellular IFNAR1-associate kinase Tyk2 is activated by the altered conformation and initiates a string of phosphorylation events on key tyrosine residues. Tyk2 first phosphorylates IFNAR1, creating a strong docking site for STAT2, then subsequently phosphorylates the anchored STAT2 (193, 216). STAT1 is phosphorylated by Jak1, enabling the formation of a stable heterodimer between the Src homology domains (SH2) domains of phosphorylated STAT1 and STAT2. The STAT1-STAT2 heterodimer binds IRF9, forming the transcription complex known as IFN-stimulated gene factor 3 (ISGF3) (70, 113). STAT1 homodimers can also form during Type I IFN signaling, though their contribution to the overall response is greatly overshadowed by ISGF3 formation and function. Assembled ISGF3 translocates to the nucleus and induces ISGs by binding IFN-stimulated response element (ISRE) sequences on ISG promoters (112, 227).

This initial intracellular signaling happens immediately after receptor detection of extracellular IFN. Time course analysis after IFN-α treatment revealed an increase in IFN signaling factors within 15 – 30 minutes (69, 129, 139). How is the signal cascade propagated so quickly? The cell is poised to respond by positioning necessary factors in the
vicinity of the IFN receptor before induction of IFN signaling. The Janus kinases are constitutively associated with their respective IFNAR subunits and STAT2 is also bound to both IFNAR2 and IRF9 (224, 238, 264). STAT1 is recruited by its weak association with STAT2, and this affinity is greatly augmented by the mutual phosphorylation of STAT1 and STAT2. Acetylation also aids in coordinated assembly at the receptor, though potentially only after IFN stimulation (278). During signaling, the transcriptional cofactor CBP catalyzes IFNAR2 acetylation, creating a docking site for IRF9. STAT1, STAT2, and IFR9 all become acetylated, a required step for both ISGF3 assembly and its subsequent ability to bind DNA.

There is also evidence to suggest that non-phosphorylated STATs are capable of serving as transcription factors in the absence of IFN. An increase in concentrations of STAT1 parallels an increase in expression of ISGs (32, 216). Moreover, STAT1 levels persist at elevated concentrations for days after a cell is exposed to IFN (102). STAT1 is found in the nucleus within these stimulated cells, suggesting it may serve as a long-term effector of IFN signaling.

The signaling response generated by Type II and Type III IFNs follows a similar pattern to that of Type I. Engagement of the Type II IFN receptor by IFN-λ results in the activation of JAK1 and JAK2, the kinases which phosphorylate STAT1 tyrosine residues, enabling STAT1 homodimerization (1, 17, 41, 265). The STAT1 homodimer translocates to the nucleus and binds the IFN-γ-activated site (GAS) promoter element. The Type III signaling pathway utilizes both STAT1 homodimers as well as ISGF3 and can therefore induce genes with either an ISRE or a GAS element (123, 255).
In addition to the JAK-STAT signaling pathways, IFNs can activate other non-classical signaling pathways. The p38 signaling pathway, a mitogen-activated protein kinase (MAPK) cascade, can generate an IFN-mediated signal (82, 94, 283) that contributes to the anti-proliferative effects of IFN (98, 174). The phosphoinositide 3-kinase (PI3K) pathway is also IFN-responsive and plays a role in the antiviral response by modulating apoptotic signals (9, 284, 292) and mRNA translation (45, 138).

INTERFERON REGULATORY FACTOR 9 (IRF9)

IRF9, also known as p48 or ISGF3γ, is one of nine members of the IRF family of transcription factors that serve to activate innate and adaptive immune responses to viral infection (243, 305). Though coded by distinct genes, these factors share a high degree of sequence homology. All IRFs have a highly conserved N-terminus that is characterized by a helix-loop-helix motif containing five tryptophan residues (57, 71, 200, 306). The less conserved C-terminal domain varies among the members and acts as a regulatory domain that, in conjunction with its binding partners, determines its transcriptional activity as an activator and/or repressor (275, 279). Most IRFs also have an IRF-associated domain (IAD) that mediates IRF dimerization and enables IRFs to bind DNA target sequences to activate gene transcription (182, 252). In IRF9, this region instead promotes association with STAT1 and STAT2 to form the ISGF3 heterotrimeric complex (289). Classically, it was believed that IRF9 associates with STAT1 and STAT2 only when both proteins are phosphorylated, but recent evidence suggests that the ISGF3 complex formed during IFN-γ signaling can contain unphosphorylated STAT2 (192). As the major DNA-binding element in ISGF3, IRF9 is a
critical component responsible for ISRE binding specificity and transcriptional activity (288). Accordingly, mouse embryonic fibroblasts (MEFs) harvested from IRF9 knockout mice fail to respond to IFN (88, 115). IRF9 is constitutively expressed in most cell types and can be induced further by IFN-γ production (140). High levels of IRF9 are critical for accelerating the IFN-α-induced early antiviral signaling, and increasing IRF9 expression correlates with higher levels of phosphorylated STAT and ISGs induction by IFN-α (164).

Through its integral role in IFN-mediated signaling, IRF9 is implicated in the regulation of oncogenesis. Functional ISGF3 stimulates the p53 pathway to suppress malignant cell transformation and enhance DNA damage-induced apoptosis (274, 306). The lack of ISGF3 in an IRF9 knockout cell line renders these cells more susceptible to cytotoxic chemotherapeutic drugs (299). Moreover, the Irf9 gene is directly activated by c-Myc, but further studies are needed to elucidate the exact role of IRF9 in oncogenesis.

NUCLEAR TRANSPORT OF ISGF3

In order to prevent basal levels of STAT1, STAT2, and IRF9 from signaling in the absence of IFN, complex formation is regulated by inducible phosphorylation of the STATs and nuclear/cytoplasmic redistribution under the control of NLSs and nuclear export signals (NES). To date, the STAT proteins are the only documented transcription factors that are activated by tyrosine phosphorylation and subsequently interact through reciprocal SH2 domain interactions (229). STAT1 and STAT2 contain a nonclassical NLS that is nonfunctional in the monomeric state and only their heterodimerization during the formation of ISGF3 leads to the gain of a functional NLS (176, 250). The NLS revealed by
dimerization is bound by importin-α5, also known as karyopherin subunit alpha-1 (KPNA1) or nucleoprotein interactor 1 (NPI1), to mediate nuclear entry via the nuclear pore complex (NPC) (61, 179, 180, 183). A bipartite NLS within the DNA-binding domain of IRF9 was identified (132), but this finding seems incongruous with the fact that endogenous IRF9 localizes mainly to the cytoplasm. However, IRF9 association with the amino terminal coiled-coil region of STAT2 both before and after IFN stimulation serves to sequester IRF9 in a cytoplasmic complex (171, 228).

To transit through the NPC, importin-α5 binds not only the NLS of the ISGF3 cargo, but also the transporter protein importin-β1 that serves to shuttle the cargo along nucleoporins lining the channel of the NPC (14, 214, 235). The direction of nuclear transport is established by the ability of the transporter to bind to Ras-related nuclear protein (RAN) (84, 160). This GTPase exists as a gradient within the cell, with high concentrations of RAN-GTP beginning in the nucleus. Upon entry into the nucleus, importins bind RAN-GTP and undergo a conformational change that releases their cargo. Conversely, exportins initially bind RAN-GTP in the nucleus and release their cargo only when GTP is hydrolyzed in the cytoplasm. Most proteins that are exported from the nucleus contain a NES rich in leucine residues and hydrophobic amino acids that is recognized by the transporter chromosome region maintenance 1 (CRM1, also known as exportin-1) (67). CRM1 binds an NES in the STAT1 DNA-binding domain in a RAN-GTP dependent manner (177) as well as an NES in the C-terminus of STAT2 (8). Though IRF9 lacks a NES, it can be shuttled back to the cytoplasm through its association with STAT2.
INDUCTION OF IFN-STIMULATED GENES (ISGS)

To provide the first line of defense against viral pathogens, IFNs must induce a wide array of ISGs to serve as antiviral effectors. Recent genome-scale cataloging of ISGs by microarray data has vastly advanced the field since the initial discovery of ISGs more than 25 years ago (116, 130). These discovery-based screens have identified novel antiviral ISGs and many have been validated using multiple virus families. Most ISGs that have been identified to date were initially discovered due to their upregulation after IFN treatment and subsequently verified as antiviral by demonstrating a reduction in viral titer. Extensive profiling has determined that some ISGs have a broad activity against many viruses, while others are more restricted in specificity (249). Each type of IFN induces a unique and partially overlapping subset of the thousands of identified ISGs (46). The generated ISG response is cell-type specific and depends on the IFN dose and time of treatment, though typically it includes 200 – 500 proteins with deviations of as few as 50 or as many as 1000 proteins (43, 128, 239). Each virus tends to elicit a unique ISG profile, and within this response there are multiple redundant elements to ensure the generation of an antiviral state. As evidence that multiple factors contribute to protection, mice that are defective in one or more of the classical antiviral pathways still mount an antiviral response (318).

Not surprisingly, ISGs vary in their antiviral efficacy and many effectors work in combination to achieve a fully functional antiviral state (248, 249). The classical potent antiviral ISGs (MXA, PKR, OAS1, TRIM5, ZAP, APOBECC3G, and IFITM3) are complemented by a diverse range of weak effectors (for HUGO Gene Nomenclature Committee (HGNC)-approved designations of ISGs, please consult www.genenames.org).
The mechanisms of action of ISGs vary, with all steps of the virus life cycle (entry, uncoating, transcription, translation, genome replication, particle assembly, and egress) as potential targets for inhibition. Initial detection of virus is aided by the ISG IRF1, which has been demonstrated to induce an antiviral phenotype by affecting signaling from peroxisome-associated MAVS (49). Additional early post-entry events are subverted by an IFIT multi-protein complex that sequesters viral RNAs containing 5’ triphosphates (215). Viral trafficking within the cell is mediated by MxA localized near the ER, the ideal location to trap essential viral components (2). Replication is inhibited by RNA degradation of RNaseL after its activation by 2’, 5’ polymers of adenosine that are synthesized by OAS ISGs (35, 111, 226). Translation is effectively shut off by activating the antiviral protein PKR to phosphorylate the eukaryotic translation initiation factor eIF2α (233). Release of viral progeny can be specifically inhibited by viperin by its interaction with the host enzyme farnesyl diphosphate synthase to perturb lipid rafts and prevent egress of some viruses (295). Some pan-viral ISGs have broader effector mechanisms that target multiple cellular pathways. The ubiquitin homologue ISG15 can be covalently linked to hundreds of cellular proteins (78, 277, 317). Instead of promoting degradation, this reversible process parallels the activating effects of ubiquitin by increasing stability and modulating enzymes (157). When untethered, ISG15 can be secreted from the cell and act as a cytokine to extend and regulate the immune response (40).
REGULATION OF JAK-STAT SIGNALING

Though the pleotropic effects of Type I IFN can effectively limit viral spread and replication, prolonged or excessive IFN responses can be toxic to the cell. Mice deficient in negative regulators of IFN signaling suffer from multi-organ inflammation and drastically reduced lifespans (3). Similarly, enhanced IFN-α/β expression in humans is associated with inflammation and tissue damage, as evidenced by the disorders Systemic Lupus Erythematosus (SLE), dermatomyositis, and psoriasis (231). One means of limiting the negative effects of IFN is to generate a localized IFN response at the site of infection through the autocrine and paracrine IFN signaling. Widespread infection can lead to a systemic IFN response, exacerbated by the activation of pDCs, the ‘professional’ IFN-producers. Administration of IFN to patients is an approved treatment for many conditions, though it creates the systemic IFN response that may result in side effects (including flu-like symptoms, nausea, myalgia, leukopenia, and neurological effects) with increased severity correlating to higher dosage (105). The IFN response is therefore a critical balance between protection against pathogens and cellular toxicity.

In order to regulate Type I IFN signaling to prevent toxicity, multiple proteins target different steps of the signal transduction pathway to limit the strength and duration of signaling. At the receptor level, both subunits of IFNAR contribute to negative regulation. The gene encoding IFNAR2 can be alternatively spliced to generate either a decoy receptor with a truncated intracellular domain that lacks the capacity to signal (159) or a soluble receptor to restrict IFN from initiating a cascade through transmembrane receptors (204). The same effect can be achieved by the cleavage of the IFNAR2 intracellular domain by the
tumor necrosis factor-α-converting enzyme (TACE) protease (218). The IFNAR1 subunit is also actively degraded, though instead by ubiquitination of its negative regulatory domain (126). The receptor complex can easily be recycled for future signaling by returning it to its inactive conformation. This is achieved by protein phosphatases that are associated with both subunits (36, 188). Other additional negative regulators have been shown to modulate the IFN response through the intracellular domains of IFNAR. The ubiquitin-specific protease (UBP)43 interacts with IFNAR2 to inhibit signaling (166). The suppressor of cytokine signaling (SOCS) proteins constitute an entire family that functions to dampen the response to a range of cytokines and growth factors, with SOCS1 and SOCS3 both documented repressors of Type I IFN signaling (152). The SOCS1 mechanism has been identified and involves association with Tyk2 to target it for degradation by the proteasome (217). Induction of IFN can also be moderated by proteins that act in concert with transcription factors, including protein inhibitors of activated STATS (PIAS) (258), the activated form of the transcription regulator EMSY (60), members of the tripartite motif (TRIM) pathway (281), and IRFs (314). Recently, microRNA (miR) post-transcriptional regulators have also been discovered to have targets within the IFN signaling cascade, and further research is expected to yield additional targets (23, 178, 211). The negative regulators detailed here work in concert with other regulators effective against both IFN induction and the positive feedback loop to effectively discontinue IFN signaling.
VIRAL EVASION OF JAK-STAT SIGNALING

Viruses have the ability to prevent the formation of an antiviral state by subverting IFN signaling. The host can therefore most effectively limit viral spread and replication by establishing an IFN-mediated, paracrine induction of the antiviral state before the neighboring cells have been exposed to virus. In vivo, there remains a delicate balance between cellular detection of viruses to promote IFN production and viral replication to generate subversion proteins in concentrations high enough to dismantle cellular defenses. Even if a virion encounters a cell with a propagated antiviral state, it may retain the capacity to establish a productive infection. This is exemplified best by the IFN subversion of parainfluenza virus 5 (PIV5). Initially, PIV5 infects the cell but fails to replicate efficiently in cells that have mounted an antiviral state prior to infection (27, 47). The virion-associate V protein from incoming particles degrades STAT1 and, given that cells cannot perpetuate their antiviral state without continued IFN signaling, this disruption thereby facilitates subsequent viral replication (48). By concomitantly inhibiting both IFN production and IFN signaling, viruses successfully shift the balance to enable replication within cells.

The catalogue of evasive mechanisms that viruses have evolved to subvert IFN signaling is extensive. Examples of antagonism of the Type I IFN Jak-STAT signaling pathway are presented in Figure 1.1 and detailed in Table 1.1. Several key points are illustrated by the IFN antagonists listed here. Foremost, the entire spectrum of the IFN signaling response is open to subversion and more examples of antagonists are continuously being discovered. Despite limited genome space, a given virus may encode more than one protein to ensure effective inhibition of signaling. Lastly, a single viral protein often has
multiple functions during infection, and this can include inhibiting distinct factors of the induction and signaling cascade. Though costly for the virus to dedicate a percent of its genome to IFN subversion, these measures are critical for replication. Despite the likely need for multiple proteins to inhibit IFN signaling, these measures of subversion can block multiple signaling pathways simultaneously due to the common components between Type I, II, and III IFN.

HEAT SHOCK PROTEINS

The highly conserved family of heat shock proteins (Hsps) generally serves protective functions under stressful cellular conditions. These proteins were first identified by their increased expression in cells incubated at elevated temperatures (232) and subsequently shown to be responsive to a wide array of stimuli, including glucose deprivation, hypoxia, pathogen infection, and ultraviolet radiation. The main function ascribed to Hsps is their ability to chaperone nascent or aberrantly folded proteins, enabling them to fold into thermodynamically stable conformations (89, 263). Even a small increase in temperature can trigger protein unfolding and result in deleterious aggregation, thus Hsps are induced by an increase in temperature of just a few degrees (165, 190). The presence of Hsps during temperature shock and other cellular stresses prevents the accumulation of unfolded proteins that could otherwise damage the structure and function of the Golgi system, the endoplasmic reticulum (ER), mitochondria, and the overall internal organization of the cell (20, 209, 302). The protective effect of Hsps extends beyond the initial stress, as demonstrated by the
observation that levels of induced Hsps correlate with the degree of protection after a subsequent stress (151).

Mammalian Hsps are categorized into families according to their mass in kilodaltons: Hsp100, Hsp90, Hsp70, Hsp60, and the small Hsps (sHsps). Each family differs in its role during the stress response, with Hsp90 and Hsp70 having the most characterized mechanisms of protection. Hsp90 is constitutively expressed to high levels (approximate 1-2% of the total protein in unstressed cells) and is essential for cell viability (131, 262). In addition to its critical role in protein folding, Hsp90 associates with a number of signaling proteins and has been shown to promote the conformational maturation of the steroid receptor (199) and some serine/threonine kinases (296). Members of the well conserved Hsp70 family can assemble into multimeric complexes to assist in protein folding (247) that also function to transport proteins across cellular membranes (257). Hsp70 has been demonstrated to moderate caspase-independent apoptosis pathways (100, 225). Similarly, other Hsp family members have additional roles in response to shock, though all remain connected by their main function as molecular chaperones.

**HEAT SHOCK PROTEIN 25 (HSP25)**

The murine Hsp25 and its human homologue Hsp27 belong to the family of sHsps that are widely expressed across species. Though detectable in most cell types, expression levels vary considerably (6, 134). Hsp25 contains a C-terminal α-crystalline domain that mediates interaction with actin, MAPK-activated protein kinase 2 (MK2), Akt, and other sHsp family members (25, 65, 127, 270). Oligomers comprised solely of Hsp25 form
aggregated of up to 800 kDa (122). Phosphorylation of Hsp25 at serine 15 and 86 can cause complex disruption and a subsequent loss of chaperone function (107, 234). Though its ability to bind denatured proteins is greatly diminished once phosphorylated, Hsp25 may retain additional cellular roles. Research has revealed Hsp25 to be highly multifunctional as a known regulator of actin polymerization, intermediate filament formations, cell migration, cell-cell adhesion, cell cycle progression, proinflammatory gene expression, mRNA stabilization, differentiation, and apoptosis (reviewed in (122)). Given its numerous and vital functions, it is not surprising that a marked increase in expression is observed in many cancer cell lines and aberrant expression of Hsp25 is associated with neurodegenerative disorders and cardiovascular disease (26, 31, 59, 93).

INTERPLAY BETWEEN HSPS AND VIRAL INFECTION

The first identified link between viruses and Hsps was established over thirty years ago in *Escherichia coli* when Hsp40 and Hsp70 homologues were demonstrated to be necessary for λ bacteriophage replication (310). As is the case for the λ bacteriophage, many viruses rely on the chaperone activity of host-encoded Hsps in order to properly fold viral proteins. Further, Hsps can affect viral trafficking, viral genome replication, and regulation of PKR activity (181, 198, 298). Through these and other mechanisms, Hsps have been shown to affect the spread and replication of numerous viruses, including Polyomavirus, Measles virus, Murine hepatitis virus, Human papilloma virus, Hepatitis B virus, Flock house virus, Porcine circovirus, Influenza virus, Human immunodeficiency virus, and Herpes simplex virus (reviewed in (117)). Certain viruses have evolved to directly counter the antiviral
activity of some Hsps. The Cap protein of Porcine circoviruses was shown to co-immunoprecipitate and co-localize with Hsp40, suggesting an interaction and possible viral evasion of the Hsp cellular defense (66). Similarly, Hepatitis B virus encodes a core protein that interacts with two Hsps, Hdj1 and hTid1 (261).

To date, Hsp25 has not been demonstrated to be antiviral, though there is mounting evidence to suggest that it is a factor in the virus life cycle. A study of the intracellular transport of adenovirus indicates that Hsp27 may affect viral trafficking by modulating microfilament dynamics (271). Multiple virus families induce Hsp25/27 expression or phosphorylation, including Retroviridae (144, 293), Togaviridae (197), Flaviviridae (33, 62, 145), Paramyxoviridae (260, 312), Hepadnaviridae (87, 146), Herpesviridae (68, 72, 80, 81), and Papillomaviridae (34, 155).
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elevated temperatures in Saccharomyces cerevisiae. Experimental cell research 190:57-64.


VP24 binds karyopherin alpha1 and blocks STAT1 nuclear accumulation. Journal of virology 80:5156-5167.


interferon regulatory factors and to bind DNA. The Journal of biological chemistry 272:9785-9792.


Figure 1.1. Viral evasion of the JAK-STAT signaling pathway. IFN-α/β binds to the IFNAR receptor, comprised of subunits IFNAR1 and IFNAR2, to initiate the signal transduction pathway. The Janus kinases JAK1 and TYK2 associated with the intracellular domains of IFNAR are phosphorylated and these activated JAKs then phosphorylate STAT1 and STAT2. Phosphorylated STAT1 and STAT2 heterodimerize and bind to IRF9 to form ISGF3, which translocates to the nucleus and binds to ISRE sequence to induce ISGs. Multiple viruses subvert specific steps of the JAK-STAT pathway, as indicated. See text and Table 1.1 for protein and virus abbreviations.
Table 1.1. Viral Evasion of the JAK-STAT signaling pathway. IFNAR, Jak1, Tyk2, STAT1, and STAT2 are all targets for viral subversion of IFN signaling. Multiple mechanisms are employed by different viruses to inhibit the pathway. The importance of deactivating the pathway is underscored by the viruses that, despite their limited genome size, encode multiple proteins to disrupt or prevent signaling.

<table>
<thead>
<tr>
<th>Target</th>
<th>Virus</th>
<th>Mechanism of Subversion</th>
<th>Viral Protein</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>IFNAR</td>
<td>West Nile Virus (WNV)</td>
<td>Depletes IFNAR1</td>
<td>Unknown</td>
<td>(58)</td>
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<tr>
<td></td>
<td>Severe Acute Respiratory Syndrome (SARS)</td>
<td>Phosphorylates IFNAR1, increases IFNAR1 ubiquitination</td>
<td>3a</td>
<td>(189)</td>
</tr>
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<td></td>
<td>Human Herpes Virus (HHV)-8</td>
<td>Downregulates expression</td>
<td>K3, K5</td>
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</tr>
<tr>
<td>Jak1</td>
<td>Human Cytomegalovirus (hCMV)</td>
<td>Reduces levels of Jak1</td>
<td>Unknown</td>
<td>(186, 187)</td>
</tr>
<tr>
<td></td>
<td>Herpes Simplex Virus (HSV)-1</td>
<td>Reduces levels of Jak1</td>
<td>Unknown</td>
<td>(30)</td>
</tr>
<tr>
<td></td>
<td>Murine Polyomavirus (MPyV)</td>
<td>Binds to Jak1 to prevent activation</td>
<td>T antigen</td>
<td>(300)</td>
</tr>
<tr>
<td></td>
<td>Measles Virus (MeV)</td>
<td>Prevents phosphorylation</td>
<td>C, V</td>
<td>(311)</td>
</tr>
<tr>
<td></td>
<td>Langat Virus (LGTV)</td>
<td>Prevents phosphorylation</td>
<td>Unknown Several nonstructural (NS) proteins</td>
<td>(16)</td>
</tr>
<tr>
<td></td>
<td>WNV</td>
<td>Prevents phosphorylation</td>
<td>VP40</td>
<td>(286, 287)</td>
</tr>
<tr>
<td></td>
<td>Marburg Virus (MARV)</td>
<td>Prevents phosphorylation</td>
<td>VP40</td>
<td>(286, 287)</td>
</tr>
<tr>
<td></td>
<td>Adenovirus 5 (AdV5)</td>
<td>Downregulates expression</td>
<td>Unknown</td>
<td>(256)</td>
</tr>
<tr>
<td>Tyk2</td>
<td>Human Papilloma Virus (HPV)-18</td>
<td>Attenuates phosphoryation</td>
<td>E6</td>
<td>(143)</td>
</tr>
<tr>
<td></td>
<td>Epstein-Barr Virus (EBV)</td>
<td>Prevents phosphorylation</td>
<td>LMP-1</td>
<td>(77)</td>
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### Table 1.1. Continued

<table>
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<th>Virus/Complement</th>
<th>Effect on STAT1</th>
<th>Proteins Responsible</th>
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<tr>
<td>Dengue Virus (DENV)</td>
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<tr>
<td>WNV</td>
<td>Prevents phosphorylation</td>
<td>Several NS proteins</td>
</tr>
<tr>
<td>LGTV</td>
<td>Prevents phosphorylation</td>
<td>Unknown</td>
</tr>
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<td>Japanese Encephalitis Virus (JEV)</td>
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<td>NS5</td>
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</table>

<table>
<thead>
<tr>
<th>STAT1</th>
<th>Effect on STAT1</th>
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</tr>
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<tr>
<td>HCMV</td>
<td>Sequesters STAT1</td>
<td>IE1 (210)</td>
</tr>
<tr>
<td>MeV, MuV</td>
<td>Dissociates STAT1 from IFNAR</td>
<td>(125)</td>
</tr>
<tr>
<td>Parainfluenza Virus (PIV)-5</td>
<td>Degrades STAT1</td>
<td>V (220, 221)</td>
</tr>
<tr>
<td>Hepatitis C Virus (HCV)</td>
<td>Degrades STAT1/Downregulates expression/Hypomethylation of STAT1</td>
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<tr>
<td>JEV</td>
<td>Prevents phosphorylation and nuclear translocation</td>
<td>NS5 (148, 149)</td>
</tr>
<tr>
<td>LGTV</td>
<td>Prevents phosphorylation</td>
<td>NS5 (16)</td>
</tr>
<tr>
<td>WNV</td>
<td>Prevents phosphorylation and nuclear translocation</td>
<td>Unknown (86, 133, 153)</td>
</tr>
<tr>
<td>DENV</td>
<td>Attenuates phosphorylation</td>
<td>NS5 (91)</td>
</tr>
<tr>
<td>Sendai Virus (SeV)</td>
<td>Prevents phosphorylation/dephosphorylates STAT1/</td>
<td>C (75, 76, 121)</td>
</tr>
<tr>
<td>Simian Virus (SIV) 5</td>
<td>Degrades STAT1</td>
<td>V (4)</td>
</tr>
<tr>
<td>MeV</td>
<td>Causes nuclear accumulation</td>
<td>V (205)</td>
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<tr>
<td>Influenza A Virus (IAV)</td>
<td>Attenuates phosphorylation</td>
<td>NS1 (103)</td>
</tr>
<tr>
<td>Ebola Virus (EBOV)</td>
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<td>VP24 (230)</td>
</tr>
<tr>
<td>MARV</td>
<td>Prevents phosphorylation</td>
<td>VP40 (223, 286)</td>
</tr>
<tr>
<td>Rabies Virus (RV)</td>
<td>Prevents nuclear translocation</td>
<td>P (24, 291)</td>
</tr>
<tr>
<td>MYXV</td>
<td>Prevents phosphorylation and</td>
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Table 1.1. Continued

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<th>Effect on STAT2</th>
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<td>AdV-5</td>
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<td>(256)</td>
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<tr>
<td></td>
<td></td>
<td>STAT2</td>
<td></td>
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<tr>
<td><strong>STAT2</strong></td>
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<td>HCMV</td>
<td>Sequesters of STAT2</td>
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<td>HSV-1</td>
<td>Reduces levels of STAT2</td>
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<td>Prevents phosphorylation and nuclear translocation</td>
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<td>EBV</td>
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</tr>
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<td>hCMV</td>
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<td></td>
</tr>
<tr>
<td>SeV</td>
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<td>Respiratory Syncytial Virus (RSV)</td>
<td>Downregulates expression/</td>
<td>NS1, NS2</td>
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<tr>
<td>MeV</td>
<td>Causes nuclear accumulation</td>
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<td></td>
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<td>Binding to IRF9 prevents signaling</td>
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CHAPTER 2

Over-Expression of Hsp25 to Assess Its Potential Role Against Reovirus Infection

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²Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC

Contributions to this were are as follows: RR cloned Hsp25 into a lentivirus construct, TK generated stably transformed cells overexpressing Hsp25, and RS/KP conducted all experiments together.
ABSTRACT

The heart is continuously exposed to viruses, leaving non-replenishable cardiac myocytes particularly vulnerable. The type I interferon response has been well-documented as a critical host defense against viral infection. However, it is unlikely that the heart would have evolved only one antiviral mechanism. Therefore, our laboratory previously used a proteomic approach to identify additional proteins involved in the cardiac antiviral response. Hsp25 was identified as a protein that is phosphorylated by a virus that does little damage to the heart but that is degraded by a virus that is potently myocarditic. To investigate Hsp25 as a potential innate antiviral protein, a cell line was established that over-expresses Hsp25 together with GFP as a marker. When treated with H$_2$O$_2$ to mimic ischemic damage and then analyzed by flow cytometry, cells expressing the highest levels of GFP were best protected. Because the cells in the population expressed varying levels of Hsp25, the culture was sorted by FACS into four subpopulations based on their GFP intensities. After confirming by western blot that GFP levels correlated with those of Hsp25, with the most fluorescent cells containing the most Hsp25, the populations were tested for differences in infection frequencies, viral titer, and cell viability. No significant differences were observed, suggesting that Hsp25 antiviral activity against reovirus infection is nonexistent or undetectable within these Hsp25 over-expression cell lines.

INTRODUCTION

The high redundancy in the pathways of innate immunity underscores the value of a first-line defense against viruses. This is particularly important in the heart given its unique
vulnerability to viral infection. It is estimated that between 5 - 20% of humans are affected by viral myocarditis (49). Though often fatal in infants, viral myocarditis usually resolves in adults, with rare cases progressing to chronic myocarditis or dilated cardiomyopathy (10, 24). Myocarditis can result in damage to the heart even in relatively healthy young adults, where it is the second leading cause of sudden unexpected death in young adults (12). Numerous viruses have been associated with myocarditis, but the majority of human cases are caused by adenoviruses and enteroviruses (5, 15, 25, 37).

Though accessed by all systemic viruses, the heart depends on the function of a cell type that is essentially non-replenishable. With the average human lifespan, only 1% of cardiac myocytes are renewed annually (3). Thus, the innate immune system must offer a formidable defense to prevent irrevocable damage. As the only barrier to immediate injury to the heart, the innate immune system is likely to have redundant mechanisms of defense in addition to the well-characterized Type I IFN system.

Previously, our laboratory used a proteomic approach to identify a novel protein in the cardiac antiviral response (29). Two-dimensional Differential Gel Electrophoresis (2D-DIGE) was used to examine proteins differently expressed between primary cardiac myocyte cultures infected with reovirus strains that vary in their myocarditic potential. Heat Shock Protein 25 (Hsp25) was identified as a protein that is degraded during infection with myocarditic reovirus strain 8B, but phosphorylated during infection with non-myocarditic strain T3D. Hsp25 phosphorylation has been established as a protective response in stressed cardiac myocytes. Hsp27, the human homologue of murine Hsp25, is phosphorylated above baseline levels in hearts from patients with ischemia or dilated cardiomyopathy (36).
Moreover, over-expressed Hsp25/27 is protective against ischemic damage in cardiac myocytes (13, 26, 28, 35, 45). While these studies suggest that phosphorylation of Hsp25 may be a factor in the stress response, there is no data to indicate direct antiviral activity. The degradation of Hsp25 by myocarditic reovirus strain 8B is the first evidence that suggests Hsp25 may be a viral target to circumvent potential Hsp25 antiviral activity.

Hsp25 belongs to the family of small heat shock proteins (Hsps) that are induced by various stressful stimuli, including heat shock, simulated ischemia, and viral infection (23, 27, 33). Though detectable in most cell types, Hsp25 is expressed at comparatively greater basal levels in the muscle tissues and the epithelial cells of the gastrointestinal and respiratory tracts (22, 44, 48). Hsp25 phosphorylation may alter its function and increase its cytoprotective ability, though conflicting results have been reported (39).

Here, we use a cell line stably over-expressing Hsp25 to assess its antiviral activity against reovirus infection. No change in infection frequencies, viral titer, and cell viability was observed with increasing levels of Hsp25. Over-expression of Hsp25 within the stably transformed cells may not be optimal for detection of antiviral effects and does not address Hsp25 function during viral pathogenesis.

**MATERIALS AND METHODS**

**Cells, Viruses, Infections.** All 293T cells were maintained in Dulbecco’s Minimal Essential Medium (DMEM) (Corning cellgro) supplemented to contain 5% fetal calf serum (FCS) (Atlanta Biologicals) and 2 mM sodium pyruvate (Corning cellgro). The 293T cell line that stably expresses Hsp25 was generated using a lentivirus vector in the laboratory of Tal
Kafri. Reovirus strains T1L and T3D were our laboratory stocks and were purified on CsCl gradients.

**Flow Cytometry.** 293T cells were plated in 6-well clusters and infected as indicated. After 24 hours, cells were collected using Trypsin-Versene in EDTA (VWR catalogue #12001-662) and fixed and permeabilized using Cytofix/Cytoperm (BD catalogue #554714) according to manufacturer’s instructions. A 1:1 mixture of α-T1L and α-T3D at a 1:500 dilution was used for reovirus probing for 20 minutes. Cells were washed 2x in BD Perm/Wash buffer before detection with a 1:500 dilution Goat α-Rabbit antibody conjugated to phycoerythrin for 20 minutes. After an additional two washes, cells were resuspended in 2% paraformaldehyde and analyzed on BD FACSCalibur cytometer. Summit and FloJo software was used for analysis. For FACS, the parental 293T population was collected using Trypsin-Versene and washed in PBS, but was not fixed and permeabilized.

**Plaque Assay.** 293T cells were plated in 96-wells clusters and infected as indicated. After 1 h incubation at 37°C, cells were overlaid with additional media and incubated for various intervals before freezing at -80°C. Cells were subjected to two additional freeze-thaw cycles and subsequently lysed in 0.5% NP-40. Viral titers were determined by plaque assay using L929 cells as previously described (41). Duplicate wells were averaged.

**Cytotoxic Effect.** 293T cells were plated in 96-well clusters. Cells were mock-infected or inoculated with T1L, T3D, or 8B diluted in media. The virus was allowed to adsorb for 1 hour at 37°C before overlay with fresh media. At 24, 48, or 72 hours PI, an MTT assay was performed as previously described (42) to quantify cell viability. A TECAN
Microplate Reader was used to determine the optical density at 570 nm and 650 nm. These readings were used to calculate absorbance, and replicate wells were averaged.

**RESULTS**

**Hsp25 protects against damage by H₂O₂.** To generate a cell line that over-expresses Hsp25, a lentivirus vector was utilized to stably transform HEK 293T cells. The delivered construct has Hsp25 cloned upstream of GFP, allowing for identification of cells expressing Hsp25 by fluorescence. Unlike the promoter-driven Hsp25, GFP is under the control of an internal ribosome entry site (IRES). This allows Hsp25 and GFP to be expressed as two independent proteins, thus avoiding any potential complications that could arise by tagging Hsp25 with GFP. Analysis of the stably transformed cell population by flow cytometry revealed that GFP was successfully expressed, though a range of intensities was observed (Figure 2.1). Using Summit software to create successive gating based on GFP intensities, it was determined that the intensity of GFP follows a bell-shaped curve distribution, with the majority of cells expressing intermediate levels of GFP.

Treating cells with hydrogen peroxide (H₂O₂) is a physiologically relevant way to induce oxidative stress (1, 2, 8, 11, 46). Following treatment with 600 µM, the distribution of GFP intensity shifted compared to the untreated control. Instead of an even bell-shaped distribution, there was a larger proportion of cells expressing higher levels of GFP. In mock-treated cells, the gates with the highest GFP intensity (P4 and P5) represented 32 percent of the population. When treated with H₂O₂, this increased to 51 percent. The observed increase in cells with a high GFP fluorescent intensity (larger proportion of the entire cell population)
likely reflects the loss of cells with a low GFP fluorescent intensity. Cells expressing higher levels of GFP preferentially survive after H$_2$O$_2$ treatment.

**GFP and Hsp25 expression levels are correlated in subpopulations.** To determine if GFP can be used as a marker of Hsp25 expression, 293T-Hsp25 cells were sorted by Fluorescent-Activated Cell Sorting (FACS) into subpopulations based on GFP expression and subsequently analyzed by western blot for Hsp25 and GFP expression (Figure 2.2). As expected, the cells retained their varied GFP expression even after multiple passages (Figure 2.2A). From the parental 293T-Hsp25 cells, four different cells populations were created to have increasing levels of GFP intensity, termed negative (Neg), 1+, 2+, and 3+. Cells expressing the lowest levels of GFP are represented by the Neg subpopulation and cells expressing the highest levels of GFP are represented by the 3+ subpopulation. GFP expression of subpopulations was confirmed by immunofluorescence.

To confirm cells expressing the highest levels of GFP also express the highest levels of Hsp25, whole cell lysates from 293T subpopulation were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted using anti-Hsp25 and anti-β-actin antibodies (Figure 2.2B). As expected, GFP and Hsp25 expression levels are correlated in subpopulations, with the parental 293T-Hsp25 cells expressing and intermediate level of both GFP and Hsp25.

**Cells over-expressing Hsp25 are not preferentially protected from reovirus infection.** Hsp25 could have any of the numerous antiviral mechanisms to limit viral spread
and replication. We first examined infection frequencies to determine if fewer cells within a given population were infected. 3+ and Neg cells were infected with reovirus strain T3D at a multiplicity of infection (MOI) of 10 or 2 plaque-forming units (PFU) per cell. At the indicated time post-infection (PI), cells were fixed for analysis by flow cytometry and probed using anti-reovirus protein μ2 polyclonal antibody. At 12 hours PI, most cells were uninfected and there was no difference in infection frequency between 3+ and Neg cells (Figure 2.3A). This serves as a control to demonstrate that the antibody does not detect input inoculum. Instead, detection requires replication, which takes time. More cells were infected by 24 hours PI, but the two subpopulations were infected at similar frequencies (Figure 2.3B). By 48 hours PI, virus-induced interferon(IFN)-β might mask Hsp25 antiviral effects. Therefore, 6 x 10^5 (neutralizing units) NU per cell anti-IFN was added to indicated samples at the time of infection. There was no significant difference in infection frequency between subpopulations, regardless of anti-IFN treatment (Figure 2.3C). Thus, cells over-expressing Hsp25 are not preferentially protected from reovirus infection.

**Reovirus replication is not decreased in cells over-expressing Hsp25.** Though cells in the 3+ population may be infected at similar frequency to the Neg population, it is possible the Hsp25 over-expression may decrease the viral load within the infected cells. To test this, viral titer in each subpopulation was examined by plaque assay. 3+ and Neg cells were infected at an MOI of 10 or 0.1 PFU per cell, as indicated (Figure 2.4). Cells were infected with a lower MOI to examine if overexpressed Hsp25 could affect cell-to-cell viral spread over a longer period of infection. A difference between subpopulations in inhibition of
viral release from the cell was tested by determining the viral titer in the cell supernatants at 72 hours post infection.

Total reovirus from whole cell lysates at 24 hours and 5 days PI and released reovirus in supernatants at 72 hrs PI were titered by plaque assay. There was no significant difference in titer between 3+ and Neg cells at any timepoint, indicating that reovirus replication is not decreased in cells over-expressing Hsp25.

Cell viability is similar between reovirus-infected 3+ and Neg subpopulations. Potential antiviral activity of Hsp25 could be manifested in its ability to promote cell viability in the context of infection. An MTT assay was used as a measure of cell viability. 3+ and Neg subpopulations were infected with reovirus strain 8B, T1L, or T3D at an MOI of 10 or 3 PFU per cell. Half the samples were treated with $6 \times 10^5$ NU per cell anti-IFN in order to avoid masking Hsp25 protective effects (Figure 2.5). At 24 hours (Figure 2.5A), 48 hours (Figure 2.5B), or 72 hours (Figure 2.5C) PI, cell viability was quantified by MTT assay. Given the lack of a significant difference between cell populations at any time point, cells over-expressing Hsp25 are not preferentially protected from reovirus-induced cytopathic effect.

DISCUSSION

Though previous results suggest a strain specific difference in expression levels and phosphorylation state of Hsp25 after reovirus infection, this current study demonstrates that stably over-expressing Hsp25 in 293T cells reveals no antiviral activity. After sorting cells
according to their level of Hsp25 expression, there was no significant difference observed in infection frequency, viral titer, or cell viability. This could indicate a lack of Hsp25 antiviral activity against reovirus, or protection may be undetectable given the artificial cellular environment created by overexpression.

An increase in Hsp25 phosphorylation is detectable after certain stressful stimuli (36). Levels of Hsp25/27 levels or phosphorylation state have been observed after infection with members of multiple virus families, including Retroviridae (30, 47), Togaviridae (38), Flaviviridae (6, 14, 31), Paramyxoviridae (43, 50), Hepadnaviridae (21, 32), Herpesviridae (16, 17, 19, 20), and Papillomaviridae (7, 34). In conditions of oxidative stress, p38-MAPK is activated and this kinase phosphorylates and activates MK2, which then in turn phosphorylates Hsp25 in cardiac myocytes (4). Treatment with low concentrations of H$_2$O$_2$ can result in activation of p38-MAPK and lead to the phosphorylation of Hsp25 in myocytes (9, 18). Phosphorylation may be a necessary event to enable the antiviral activity of Hsp25. One possibility would be that phosphorylation of Hsp25 is required for its anti-apoptotic capacity. Some studies suggest phosphorylation disrupts the oligomerization state of Hsp25, therefore altering its function as well (22, 40). In Figure 2.1, treatment with H$_2$O$_2$ did result in preferential survival of cells expressing higher levels of Hsp25, the only hint of potential protective Hsp25 function within this study. The presence of H$_2$O$_2$ in this particular experiment may have provided the additional necessary stimulus for phosphorylating over-expressed Hsp25. If the baseline cellular machinery is unequipped to process and phosphorylate the drastically increased levels of Hsp25, this could explain the lack of observed antiviral activity in the subsequent experiments.
Overexpression of Hsp25 may mask any differences between the generated
subpopulations. The Neg subpopulation fails to overexpress Hsp25, but it still expresses the
normal amount of Hsp25 expressed from genomic DNA. Any antiviral activity of Hsp25
would be present at this level as this would be the biologically relevant amount during
infection. If additional levels of Hsp25 fail to increase the antiviral activity, no differences
would be observed between the Neg and the 3+ subpopulations. Moreover, there is always
the possibility that antiviral activity of Hsp25 is cell type specific, with no antiviral activity
possible in the 293Ts used in this study. Though the lack of significant difference between
our cell populations establishes that no antiviral activity can be detected in 293Ts with
overexpressed Hsp25, it remains to be determined whether Hsp25 could be antiviral in other
experimental conditions and cell types.
REFERENCES


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**Figure 2.1. Hsp25 protects against damage by H$_2$O$_2$.** When 293T cells stably expressing Hsp25 were analyzed by flow cytometry, a range of GFP intensities were observed. Successive gating from P4 to P8 revealed a bell-shaped curve distribution, with the majority of cells expressing intermediate levels of GFP. Treatment with 600 µM H$_2$O$_2$ to mimic ischemic damage shifted the distribution towards cells expressing higher levels of GFP. Gates P4 and P5 change the most and are highlighted in red. Results are representative of two experiments.
Figure 2.2. GFP and Hsp25 expression levels are correlated in subpopulations. (A) Parental 293T-Hsp25 cells were sorted by FACS into subpopulations (Neg, 1+, 2+, and 3+) based on GFP expression. GFP expression of subpopulations was confirmed by immunofluorescence. (B) Western blot analysis confirmed cells expressing the highest levels of GFP also express the highest levels of Hsp25. Though additional stocks of the subpopulations were frozen for later use, the parental 293T-Hsp25 population was sorted a second time to confirm freezing did not affect experimental results. The subsequent sorted yielded similar results.
Figure 2.3. Cells over-expressing Hsp25 are not preferentially protected from reovirus infection. 3+ Neg cells were infected with reovirus strain T3D at an MOI of 10 or 2 PFU per cell. At 12, 24, or 48 hours PI, cells were fixed for analysis by flow cytometry and probed using anti-reovirus protein µ2 polyclonal antibody. No difference in infection was observed at (A) 12 hours, (B) 24 hours, or (C) 48 hours. Changing the MOI or treating with α-IFN also had no effect on the percentage of uninfected cells. Representative histograms are shown with the black line indicating the mock-infected population. Results are representative of at least two independent experiments.
Figure 2.4. Reovirus replication is not decreased in cells over-expressing Hsp25. 3+ and Neg cells were infected at an MOI of 10 or 0.1 PFU per cell, as indicated. Total reovirus from whole cell lysates at 24 hrs and 5 days PI and released reovirus in supernatants at 72 hours PI were titered by plaque assay. There was no significant difference in titer between 3+ and Neg cells at any timepoint, thus reovirus replication is not decreased in cells over-expressing Hsp25. Vital titers were analyzed by plaque assay in two separate experiments with minor deviations in experimental setup. Both experiments resulted in no difference in titer between subpopulations.
Figure 2.5. Cells over-expressing Hsp25 are not preferentially protected from reovirus-induced cytopathic effect. 3+ and Neg subpopulations were infected with reovirus strains 8B, T1L, or T3D at an MOI of 10 or 3 PFU per cell. Half the samples were treated with 6 x 10^5 NU/cell anti-IFN in order to avoid masking Hsp25 protective effects. At 24 hours (A), 48 hours (B), or 72 hours (C) PI, cell viability was quantified by MTT assay. Cell viability did not differ between 3+ or Neg populations at any timepoint. Treatment with α-IFN did not result in a significant difference in 3+ or Neg cell viability. Results are representative of at least 3 independent experiments. Percent viability is calculated as the optical density relative to the corresponding control sample.
Infection
CHAPTER 3

Reovirus Protein μ2 Sequesters IRF9 in Viral Factories

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* The experiment for Figure 7 was completed in collaboration with Efraín Rivera Serrano.

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ABSTRACT

In response to viral infection, cells synthesize the cytokine interferon (IFN) to establish an innate protective response. Secreted IFN initiates the Jak-STAT signal transduction pathway leading to the establishment of an antiviral state. Subverting this Type I IFN response is a common measure employed by viruses, often resulting in increased spread and replication. Our lab has previously shown that repression of IFN signaling is strain-specific. Though strain T1L can repress IFN signaling, strain T3D is unable to repress. Reovirus protein µ2 was identified as the determinant of the differences in the IFN response: a proline at µ2 amino acid (aa)208 confers repression of IFN signaling, whereas a serine at aa208 results in an unstable µ2 protein that fails to prevent the establishment of an antiviral state. Interesting, the aa208 proline of T1L µ2 also alters the subcellular localization of IRF9, establishing a possible link to the Jak-STAT pathway. Here, we report the mechanism of µ2 repression of IFN signaling. As demonstrated using confocal microscopy, both reovirus strains T1L and T3D induced translocation of IRF9 to viral factories, though T1L was more effective than T3D. Furthermore, both T1L and T3D were co-immunoprecipitated with IRF9 when overexpressed. Results suggest that µ2 binds IRF9, directly or indirectly, and sequesters it in viral factories to repress IFN signaling.

INTRODUCTION

The Type I interferon response is a critical component of host innate immunity and offers an early defense against numerous pathogenic viruses. The production of IFN is triggered by viral infection and secreted IFN-α/β binds its receptor to initiate the Jak-STAT
pathway. The resulting signaling cascades begins with the phosphorylation and activation of the Janus kinases, Jak1 and Tyk2, which then phosphorylate the transcription factors STAT1 and STAT2 (7, 19, 23, 24). These activated STATs bind a third transcription factor interferon regulatory factor 9 (IRF9) to form the heterotrimeric transcription factor complex (ISGF3) that translocates to the nucleus and upregulates transcription of antiviral IFN-stimulated genes (ISGs) (6, 9). The significance of this pathway is underscored by the wide array of viruses that antagonize it. The mechanisms of viral modulation of IFN signaling vary greatly and target all steps of the pathway, from receptor degradation, to Janus kinase inhibition, to disassociation and regulation of ISGF3. In this study we report the mechanism of mammalian orthoreovirus (reovirus) subversion of IFN signaling.

Reoviruses are nonenveloped, iscosahedral particles containing a genome of 10 double-stranded DNA segments (22). Serological evidence of reovirus infection is prevalent in most mammalian hosts, including humans (25). Though almost ubiquitous, reovirus infection in humans is rarely associated with disease except in the very young (22). Reovirus offers a highly-tractable model for the study of viral pathogenesis, with neonatal mice serving as a prototypical experimental system. Following internalization of the virions through endocytic compartments, reovirus replicates and assembles in cytoplasmic inclusions called viral factories (VFs) (4). Studies of viral factories in infected cells indicate that the nonstructural protein μNS is sufficient to establish a structural inclusion similar to VFs (2, 4, 14, 27). This matrix serves as the foundation for VFs and recruits additional reovirus proteins, including the core component μ2, which determines the morphology of these structures (18). VFs are anchored to microtubules through μ2, resulting in a filamentous
structure that pervades the cytoplasm. The ability of \( \mu_2 \) to bind microtubules is dependent on the presence of a proline at \( \mu_2 \) residue 208 (30). Reovirus strain T1L contains a serine at \( \mu_2 \) aa208, thus enabling \( \mu_2 \) to bind microtubules and the form filamentous VF\(s \) (18).

Conversely, TD3 \( \mu_2 \) has a serine at this position and therefore forms globular, perinuclear VF\(s \) due to its inability to bind microtubules.

\( \mu_2 \) aa208 also determines the ability of a reovirus strain to repress IFN signaling (8, 31). T1L \( \mu_2 \) (proline at aa208) inhibits the IFN response and also alters IRF9 localization, causing it to co-segregate with the nuclear fraction during subcellular cytoplasmic-nuclear fractionation (31). T3D \( \mu_2 \) (serine at aa208) fails to prevent an antiviral state and does not interfere with IRF9 localization. Here, we report that reovirus \( \mu_2 \) represses IFN signaling by binding IRF9 and sequestering it in VF\(s \). Both T1L and T3D \( \mu_2 \) were able concentrate IRF9 in viral factories, though T1L \( \mu_2 \) was more effective. Binding of \( \mu_2 \) to IRF9, directly or indirectly, was confirmed by co-immunoprecipitation. Results suggest that when anchored in VF\(s \), IRF9 is unable to function as a component of ISGF3, thus inhibiting the Jak-STAT signaling cascade and the resulting antiviral state.

**MATERIAL AND METHODS**

**Cells, viruses, and plasmids.** Human AD293 cells were maintained in Dulbecco’s Minimal Essential Medium (DMEM) (Corning cellgro) supplemented to contain 5% fetal calf serum (FCS) (Atlanta Biologicals) and 2 mM sodium pyruvate (Corning cellgro). Mouse L929 cells were maintained in spinner culture in Minimal Essential Media containing 5% fetal calf serum and 2 mM L-glutamine (Corning cellgro). Reovirus strains T1L and T3D
were our laboratory stocks and rsT3D was generated by plasmids using reverse genetics {Kobayashi, 2007 #1997}. All virus stocks were purified on CsCl gradients. pCAGGs-M1-T1L and pCAGGs-M1-T3D were previously generated as described and express untagged µ2{Zurney, 2009 #2363}. pCAGGs-IRF9 was generated and donated by Curt Horvath’s laboratory and the expressed IRF9 contains an N-terminally His tag {Lau, 2000 #2488}. pcDNA-NS3/4A was a kind gift from Raymond Pickles’ laboratory.

**Transfections and infections.** Cells were seeded at a density of 2.0 x 10^5 (AD293 cells) or 1.0 x 10^6 (L929 cells) cells per well of a six-well plate the day before infection. For transfection, AD293 cells were plated at 1.0 x 10^5 cells per well 4 hours before transfection with 2 µg of DNA using 6 µl of FuGene (Promega) per manufacturer’s instructions. Infections were performed at the indicated multiplicities of infection (MOIs). Cells were inoculated with T1L, T3D, or rsT3D diluted in media. The virus was allowed to adsorb for 1 hour at 37° C before overlay with fresh media. For one experiment, cells were treated with 10 µM MG-132 (Calbiochem) diluted in media for 6 hours. Cells were harvested for whole cells lysates in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin) (Cell Signaling, Cat # 9803) supplemented with 100 mM protease and phosphatase inhibitors (Sigma) and 100 mM PMSF (Sigma). Lysates were sonicated for 15 s prior to centrifuging at 14,000 x g for 10 min at 4° C to remove cellular debris.
**Cytoplasmic/Nuclear Fractionation.** Cells were harvested in a hypotonic lysis buffer (10 mM HEPES pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 0.5% NP-40) containing 100 mM protease inhibitors and 100 mM PMSF. A slow centrifugation (500 × g for 5 min at 4°C) was used to pellet nuclei. The supernatant (cytoplasm) was removed and cellular debris was cleared by centrifugation at 14,000 × g for 10 min at 4°C. The nuclear pellet was washed once in hypotonic lysis buffer without added NP-40 and centrifuged at 500 × g for 5 min at 4°C. Nuclei were further disrupted by a high salt lysis buffer (20 mM HEPES pH 7.9, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 25% glycerol) containing 100 mM protease inhibitors and 100 mM PMSF combined with sonication for 15s. Remaining debris was removed by by centrifugation at 14,000 × g for 10 min at 4°C.

**Immunoprecipitation.** 100 µl of Protein A Agarose beads (50 µl packed beads) (EMD, Cat # 16-125) was washed and bound to 10 µl of anti-IRF9 (Abcam Cat # 109309) for 2 hours, rotating at room temperature. Excess antibody was removed by a wash in TBSX (20 mM Tris-HCl, 137 mM NaCl, 2 mM EDTA, 1% Triton). The protein A Agarose bead slurry was added to each immunoprecipitation sample and incubated with rotation overnight at 4°C. Samples were washed 3x in TBSX for 5 min with rotation, followed by centrifugation for 1 min at 8,200 x g. Immunoprecipitated proteins were eluted by boiling in 2x LSB for 5 min.

**Immunoblotting.** Protein content was determined by BCA protein assay (Pierce). 20 µg of protein from whole cells lysates was boiled in 2x LSB. Protein concentration from
cytoplasmic and nuclear fractions was determined by calculating equivalent cell numbers to account for the differences in yield between fractions. Samples were resolved using 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. Membranes were blocked for 1 hour in Tris-buffered saline (20 mM Tris [pH7.6], 137 mM NaCl) containing 0.1% Tween 20 (TBS-T) and 5% milk. Rabbit antibodies were added to TBS-T and 5% milk at the indicated concentrations and probed for 2 hours at room temperature or overnight at 4°C: Anti-IRF9 (1:500, Abcam Cat # 109309), rabbit polyclonal anti-μ2 (1:500; generated against two μ2 peptides by Open Biosystems), and anti-Reovirus (1:2500 of a 1:1 mix of anti-T1L and anti-T3D). For immunoprecipitation, a 1:1000 TrueBlot anti-Rabbit antibody conjugated to HRP (Rockland Cat # 18-8816-33) that does not detect the heavy chain of the immunoprecipitating antibody was used for detection. All other samples were probed with 1:2000 Goat anti-Rabbit HRP (Millipore Cat# AP132P). Bound antibodies were detected with ECL chemiluminescent reagent (GE).

**Confocal microscopy.** Cells were plated at a density of 4.0 x 10^4 cells per well in 8-well poly-D-Lysine chamber slides (VWR Cat # 2467) and infected as described. 24 hours post-infection, cells were fixed in 4% paraformaldehyde and permeabilized with 0.25% Triton X-100 in PBS. Slides were blocked in 5% goat serum in 1x PBS. After staining for DAPI, slides were probed for 1 hour with 1:2500 mouse anti-T1L σ1, 1:1000 rabbit anti-T3D σ1, and 1:100 anti-IRF9 (Abcam Cat # 109309), each diluted in 0.1% BSA in PBS. 1:1000 goat anti-rabbit conjugated to AlexaFluor 488 and 1:1000 goat anti-mouse conjugated to AlexaFluor 594 were used for detection of primary antibodies. Slides were treated with
ProLong Gold antifade reagent (Life Technologies) and imaged with a Zeiss LSM 710 laser scanning confocal microscope.

RESULTS

α-IRF9 does not cross react with reovirus protein µ1. µ1 is the most abundantly expressed reovirus protein expressed during infection. Previously, the vast amounts of µ1 have cross reacted with multiple different antibodies. To ensure the IRF9 antibody does not detect µ1, AD293 cells were either transfected with µ2 or infected with reovirus. Duplicate immunoblots were generated from lysates, one probed with α-IRF9 and the other with α-reovirus. Only the blot probed with α-reovirus detected a band in the location of µ1, which normally migrates around 75 kDa (Figure 3.1). Since µ1 and µ2 are a similar molecular weight, it is critical to have an antibody that does not cross react.

Transfected T1L and T3D µ2 co-immunoprecipitate with IRF9. The T1L M1 gene encoding the µ2 protein is both necessary and sufficient to cause IRF9 to co-segregate with the nuclear fraction in reovirus-infected cells (31). Further, T1L M1 is also associated with repression of IFN signaling. As an initial step to determine if µ2 is able to circumvent the innate immune response by interacting with IRF9, we investigated the ability of µ2 and IRF9 to interact by co-immunoprecipitation. AD293 cells were transfected with IRF9 and either T1L µ2, T3D µ2, or the irrelevant NS3/4A protein from Hepatitis C virus (HCV) as a control overexpressed protein. 24 hours post-transfection, whole cell lysates were harvested and an anti-IRF9 antibody or control anti-HA antibody was used for immunoprecipitation.
Both T1L and T3D μ2 were co-immunoprecipitated with IRF9, with no significant difference observed between strains in the levels of co-immunoprecipitated μ2 (Figure 3.2A). However, the level of μ2 in the lysates was slightly reduced for T3D given its instability, though this difference between strains was slightly masked due to overexpression by transfection. As expected, the control overexpressed HCV protein was not co-immunoprecipitated with IRF9. Due to the presence of the heavy chain of the anti-IRF9 migrating near IRF9, immunoblotting the immunoprecipitated samples for IRF9 did not yield interpretable results (Figure 3.2B). The band detected in untransfected cells is the heavy chain, thus obscuring immunoprecipitated IRF9. Therefore, the reverse experiment using anti-μ2 to co-immunoprecipitate IRF9 was not feasible.

**T1L μ2 may co-immunoprecipitate with cytoplasmic IRF9 during infection.** Co-immunoprecipitation of overexpressed T1L and T3D μ2 with IRF9 establishes the capacity of μ2 to interact with IRF9. We next address whether μ2 can function as an IRF9 binding partner during the natural course of infection. AD293 cells were mock-infected or infected with T1L or T3D and harvested 24 hours post-infection. Whole cell lysates were immunoprecipitated with either anti-HA as a control antibody or anti-IRF9. Though it appears as though T1L and T3D μ2 were co-immunoprecipitated with IRF9, these results cannot be interpreted given that the control anti-HA antibody also yielded a band at approximately the same molecular weight as μ2 (Figure 3.3). Due to the fact that an anti-HA antibody never resulted in the presence of this band when immunoprecipitating transfected μ2, as well as subsequent experiments using a different control antibody that were clean
negative controls (data not shown), the presence of a band likely reflects the anti-HA cross reacts with the highly-expressed µ1 protein that co-migrates with µ2. As common during infection, the difference in stability between T1L and T3D µ2 was apparent, with levels of T1L µ2 in the whole cells lysates markedly above those of T3D µ2.

T3D µ2 is not stabilized during infection by lowered temperature, proteasome inhibitors, or an increased MOI. To determine if more T3D µ2 could be co-immunoprecipitated if levels of T3D µ2 in the lysates were increased, multiple methods were employed to increase its stability. Unlike T1L µ2, T3D µ2 is poly-ubiquitinated and degraded by the proteasome (14). We therefore tested the ability of the proteasome inhibitor MG-132 to increase levels of T3D µ2 in the cytoplasm. No significance difference was observed in the amount of T3D µ2 in MG-132 samples compared to lysates from control DMSO-treated cells (Figure 3.4A).

Previous studies have indicated that T3D-infected cells that are incubated at 31°C (instead of the normal 37°C) decrease the amount of T3D µ2 co-localization with conjugated ubiquitin and also result in a higher percentage of cells showing filamentous inclusion bodies (14). Both results suggest an increased stability of T3D µ2 when incubated at a lower temperature. Incubating T1L- and T3D-infected cells 31°C failed to increase the level of T3D µ2 but did markedly decrease levels of T1L µ2, thus rendering a lower temperature impractical for co-immunoprecipitation studies (Figure 3.4B). Additionally, this experiment used a recombinant stock of T3D (rsT3D, generated by plasmid-based expression of all reovirus proteins) reported to express µ2 well, but this did not increase µ2 expression.
Finally, to equalize the levels of expression of T1L and T3D µ2, the MOI was adjusted such that µ2 expression during a lower MOI of T1L infection might parallel µ2 expression at a higher MOI of T3D infection. Though levels of µ2 could indeed be experimentally changed by altering the MOI, the vast difference in MOI (T1L MOI 1, T3D MOI 100) made any subsequent results biologically uninterpretable (Figure 3.4C).

Cytoplasmic and nuclear separation yields pure fractions. Having established an interaction between µ2 and IRF9, we next wanted to determine whether this interaction occurred in the cytoplasm or the nucleus. The N-terminus of µ2 contains a region that can function as a nuclear localization signal (10). When transfected alone, µ2 is not only bound to microtubules in the cytoplasm but is present in the nucleus as well (4). IRF9 contains a NLS in its DNA binding domain, but its association with STAT2 serves to retain IRF9 in the cytoplasm (11, 12, 21). It is therefore feasible that IRF9 and µ2 could interact in either the cytoplasm or the nucleus.

A subcellular fractionation protocol was developed to yield pure nuclear and cytoplasmic fractions (Figure 3.5) while still being compatible with co-immunoprecipitation (data not shown). To ensure clean separation of fractions without cross-contamination, L929 cells were harvested using a two-step procedure. First, a hypotonic buffer was used to lyse cells to obtain the cytoplasmic fraction. Intact nuclei were then separated by centrifugation, washed, and lysed by sonication in a high-salt buffer. Fractions were immunoblotted using antibodies to known cytoplasmic and nuclear markers to assess sample purity. Alpha tubulin, as a subunit of microtubules, serves as marker for cytoplasm (29). Sp1 is an apt nuclear
marker given its role as a transcription factor that remains bound to DNA and hence sequestered in the nucleus (28). After fractionation, alpha tubulin was detected in the cytoplasmic fraction and Sp1 only in the nuclear fraction, indicating the subcellular fractionation yields pure fractions.

Viral factories and IRF9 both co-segregate with the nuclear fraction. Though μ2 has a NLS and the potential to transit into the nucleus, during co-expression with μNS the two proteins interact and μ2 becomes anchored in VFs in the cytoplasm (4, 13). To assess the localization of IRF9 and viral proteins during infection, L929 cells were mock-infected or infected with reovirus strain T1L or T3D (MOI 25) and fractionated 24 hours post-infection. Cytoplasmic and nuclear fractions were resolved by SDS-PAGE and immunoblotted using either anti-IRF9 or anti-reovirus antibody. To account for the difference in protein yield between fractions, protein from equal numbers of cells was used to generate a biologically relevant comparison.

IRF9 was localized to the cytoplasm in all cases, but also to the nuclear fraction in T1L-infected cells (Figure 3.6). To determine whether cytoplasmic VFs co-segregate with the nucleus, fractions were immunoblotted using anti-reovirus antibody to detect μ1 and μ1C, both of which are known to localize to cytoplasmic VFs and not the nucleus (2, 3, 5, 17). Both proteins were abundant in the nuclear fractions of T1L- and T3D-infected samples. A different method of fractionation (using the Thermo NE-PER fractionation kit, catalogue # 78833) yielded similar results for both IRF9 and μ1/μ1C (data not shown). Therefore, presence of IRF9 in the nuclear fraction of T1L-infected cells likely reflects accumulation in
VFs, not the nucleus. The absence of IRF9 in the nuclear fraction of T3D-infected cells may result from the inherent instability of T3D μ2 leading to a disruption of IRF9-VF interactions by the harsher conditions of nuclear fractionation. Taken together, the co-segregation of VFs with the nuclear fraction and the disruption of T3D μ2-IRF9 binding during fractionation preclude using fractionation as a method to identify the subcellular compartment in which μ2 and IRF9 interact.

**IRF9 co-localizes with viral factories.** As the location of μ2-IRF9 interaction could not be investigated by fractionation, confocal microscopy was utilized to visualize VFs and IRF9 localization. AD293 cells were mock-infected or infected with T1L or T3D (MOI 10) and fixed 12 hours post-infection. Cells were probed with an anti-reovirus (to visualize VFs) or an anti-IRF9 antibody and imaged using a Zeiss LSM 710 laser scanning confocal microscope. Though both T1L and T3D μ2 co-localized with viral factories, a number of VFs failed to stain for IRF9 in T3D-infected cells (Figure 3.7). This finding verifies the western blot results that α-IRF9 does not cross react with μ1. Further, no viral factory proteins were detected in the nucleus, confirming that presence of viral factory proteins in the nuclear fraction must be due to VFs co-segregating with the nuclear fraction. Results suggest there may be differences in IRF9 localization to VFs between T1L and T3D infections.

**DISCUSSION**

To circumvent the innate immune response and prevent the establishment of an antiviral state, viruses have evolved strategies to suppress initial IFN induction or subsequent
IFN signaling. In this study, we report the mechanism of reovirus subversion of IFN signaling and propose a model for strain-specific differences in repression. Though both T1L and T3D μ2 are capable of binding IRF9 under conditions of overexpression, this finding does not necessarily extend to infection. Instead, the low level of expression of μ2 coupled with its inherent instability may result in low binding to IRF9. Confocal microscopy revealed that IRF9 co-localizes with viral factories, the putative site of IRF9-μ2 interaction. Further, neither VF nor IRF9 were observed in the nucleus, an interesting finding given the previous report of IRF9 co-segregation with the nuclear fraction. Evidence of viral factory co-segregation with the nucleus clarifies this discrepancy: IRF9 binds μ2 in VF and only the durable T1L μ2 interaction with IRF9 can tolerate the harsh disruption conditions of fractionation. Together, these data suggest T1L μ2 can effectively sequester IRF9 in viral factories and prevent IFN signaling, while the weaker interaction of T3D μ2 with IRF9 is easily disrupted, allowing for IRF9 to function in IFN signaling.

Inhibition of IRF9 or the ISGF3 complex is a common strategy to evade IFN signaling, though multiple mechanisms are employed to disrupt function. Human cytomegalovirus (hCMV) leads to the direct degradation of IRF9, preventing the formation of ISGF3 (15, 16). Similarly, multiple viruses degrade STAT1 or STAT2 or prevent their phosphorylation to disrupt ISGF3 formation (reviewed in (20, 26)). To date the only viral protein shown to directly bind IRF9 is the human papilloma virus (HPV) E7 (1). Here, we provide a novel mechanism of IRF9 subversion by sequestration in cytoplasmic VF.

The ability of reovirus protein μ2 to repress IFN signaling was previously published (31) though the method of repression and basis of differences in repression between T1L and
T3D strains remained unclear. Additional phenotypes are attributed to the differences in µ2. The stabilized form of µ2 that is expressed by T1L is able to bind microtubules resulting in a filamentous appearance of VF s (4, 18). T3D µ2 is instead polyubiquitinated and degraded (14, 18). Its aggregation in globular VF s and its inability to bind microtubules are attributed to its instability. These phenotypes were found to depend on the presence of either a serine or a proline at aa208, which also determines repression of IFN signaling (14, 18, 30). We propose based on the results in this study that the stability of µ2 also determines its ability to anchor IRF9 within VF s.

T1L or T3D µ2 each co-immunoprecipitated with IRF9 when overexpressed. This result could reflect two different situations. First, T1L and T3D µ2 could both effectively bind IRF9 and thus be co-immunoprecipitated at similar levels. Second, either T1L and T3D µ2 may be expressed in excess of IRF9 or anti-IRF9 is a limiting factor during immunoprecipitation. In this case only a fraction of the available µ2 would be captured by IRF9, masking any difference between strains. We suspect the latter, given that the level of expression achieved by T3D overexpression does not accurately reflect the levels present during infection. At 24 hours post-infection, levels of T1L µ2 in the lysates exceed that of T3D µ2. This may be partially due to an altered time course of infection between strains, as T3D µ2 is detectable earlier post-infection than T1L µ2 (data not shown). Attempts to stabilize T3D µ2 were unsuccessful (Figure 3.4). However, the comparatively low expression of T3D may be the main reason it is unable to repress IFN signaling during infection. Results from co-immunoprecipitation of transfected µ2 demonstrate it has the capacity to bind IRF9
and the data from infection reveal that T3D is unable to reach a level of expression necessary to effectively bind IRF9.

The co-localization of IRF9 and VFs demonstrated by confocal microscope also support this model of suppression. T1L and T3D were both shown to co-localize with VFs. Though all VFs present in T1L infection had some degree of co-localization with IRF9, many VFs in T3D infected cells did not co-localize with IRF9. The instability of T3D µ2 may fail to fully anchor IRF9 in VFs and therefore create a dynamic interaction. Though the unstable T3D µ2 retains the ability to bind IRF9, the interaction may be more transient, thus allowing IRF9 to escape VFs and function in IFN signaling before being recruited back to VFs by µ2. The data presented in this study suggest a novel mechanism for repression of IFN signaling by sequestration in of IRF9 in VFs and provide evidence for the strain-specific difference in repression.
REFERENCES


Figure 3.1. $\alpha$-IRF9 does not cross react with reovirus protein $\mu1$. AD293 cells were mock-infected or infected at an MOI of 10 or transfected with either T1L or T3D $\mu2$. 24 hours PI or post-transfection, cells were harvested and lysates were analyzed by SDS-PAGE and immunoblotting. When immunoblotting with $\alpha$-IRF9, no bands were detected (top panel). The presence of reovirus proteins was confirmed by immunoblotting additional lanes with an $\alpha$-reovirus antibody (bottom panel).
Figure 3.2. Transfected T1L and T3D μ2 co-immunoprecipitate with overexpressed IRF9. AD293 cells transfected with T1L or T3D μ2, or HCV NS3/4A to generate a control over-expressed protein. 24 hours post-transfection, cells were harvested and IRF9 was immunoprecipitated from whole cells lysates. (A) Anti-HA was used as a control antibody. HCV NS3/4A was not detected in the immunoprecipitation, but both T1L and T3D μ2 were co-immunoprecipitated with IRF9. No μ2 was immunoprecipitated with the control antibody. Results are representative of three independent experiments. (B) When immunoblotting for IRF9, there is a similar band between untransfected (UT) and IRF9-transfected cells.
Figure 3.3. T1L and T3D µ2 may co-immunoprecipitate with IRF9 during infection. AD293 cells were mock-infected or infected with T1L or T3D. 24 hours post-transfection, cells were harvested and IRF9 was immunoprecipitated from whole cells lysates. α-HA was used as a control antibody. Both T1L and T3D µ2 were co-immunoprecipitated with IRF9. However, since T1L µ2 was immunoprecipitated with the control antibody, results cannot be interpreted. It is possible that if α-HA cross reacts with µ1, then µ1 would co-immunoprecipitated both µNS and µ2, with µNS acting as the bridge between µ1 and µ2. The experiment was repeated with a change in the relative levels of T1L and T3D µ2 that was co-immunoprecipitated and additional optimization is necessary to generated reproducible co-immunoprecipitations during infection.
Figure 3.4. T3D μ2 is not stabilized during infection by lowered temperature, proteasome inhibitors, or an increased MOI. L929 cells were mock-infected or infected with an MOI of 1, 10, or 100 as indicated. Infected cells were either incubated at a lower temperature or treated with the proteasome inhibitor MG-132 to assess T3D μ2 stability. (A) A drastic difference in MOI did not result in equal levels of μ2 expression between T1L and T3D. (B) Incubation at a lowered temperature did not stabilize T3D μ2 and reduced expression of both T1L and T3D μ2. (C) Treatment with MG-132 did not result in increased levels of T3D μ2 in the lysates. The results in (A) were not repeated, though additional work at a range of MOIs suggests that changing the MOI cannot compensate for the instability of T3D μ2. The results in (B) and (C) are representative of two independent experiments.
Figure 3.5. Cytoplasmic and nuclear separation yields pure fractions. L929 cells were harvested using a two-step fractionation procedure to separate the cytoplasm and the nucleus. A hypotonic buffer was used to initially lyse cells and intact nuclei were separated from cytoplasm by centrifugation, washed, and lysed via sonication in a high-salinity buffer. Sample purity was assessed using known cytoplasmic and nuclear markers. Alpha tubulin, one subunit of microtubules, localizes only to the cytoplasm. Sp1 is a transcription factor that remains bound to DNA and hence sequestered in the nucleus.
Figure 3.6 IRF9 segregates with the nuclear fraction in T1L-infected cells. L929 cells were mock-infected or infected with reovirus strain T1L or T3D (MOI 25), and were fractionated 24 hours post-infection. Protein from equal numbers of cells was resolved by SDS-PAGE and immunoblotted using either anti-IRF9 or anti-reovirus antibody. IRF9 was localized to the cytoplasm in all cases, but also to the nuclear fraction in T1L-infected cells. When probed with an α-reovirus antibodies, reovirus proteins at the molecular weight of µ1 were observed in both the cytoplasm and the nucleus. µ1 forms a classic doublet as seen here, but it is known to only localize to the cytoplasm and viral factories. Results were confirmed in at least three different experiments.
Figure 3.7 IRF9 colocalizes with viral factories. AD293 cells were mock-infected or infected with reovirus strain T1L or T3D (MOI 10), and at 24 hours post-infection were fixed and stained for IRF9 (green), reovirus proteins (red), and DAPI (blue). Images were acquired with a Zeiss LSM 710 laser scanning confocal microscope. Both T1L and T3D μ2 co-localize with viral factories. In T3D-infected cells, a number of VFs fail to stain for IRF9.