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Mechanisms of regulation in the interferon factor 3 (IRF-3) pathway

A dissertation submitted in partial satisfaction of the requirements for the
Doctor of Philosophy

in

Biology

by

Kirsten Limmer

Committee in charge:

Professor Michael David, Chair

Professor Chris Glass

Professor Tracy Johnson

Professor Richard Kolodner

Professor Suresh Subramani

2008

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Chair

University of California San Diego

2008

DEDICATION

This dissertation is dedicated to the three most important people in my life: my mom, my dad, and my husband.

It was by a great stroke of luck that I was born to two such amazing individuals. My mother and father were not only wonderful and doting parents throughout my childhood, but they were also able to both pursue successful careers. Thus, they set an example for their children that it is possible to have it all: a rewarding career and a loving and fulfilling family life. To this day, my parents embody the ideal that I strive to achieve. I can only hope to one day become a fraction of the person that they both are.

My mother once told me that the most important decision that somebody can make in their life is in choosing who to marry. If this is true, I can confidently say that my most important decision was my best decision ever made. My husband Karl has been an enduring source of patience, love, and support throughout the years. Without him, this dissertation would never have reached completion. I often ask myself what I did to deserve such an amazing husband. I can only hope that he knows just how much I appreciate him. Karl, I love you with all my heart.

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The text of chapter two entitled 'the role of calcium in the activation of interferon regulatory factor 3 by lipopolysaccharide' represents a collaborative effort with Dr Edward Chiang. I am the co-primary author of this research project and co-directed and supervised the research which forms the basis of this chapter.

The text of chapter three entitled 'the role of the Schlafen protein family in the IRF-3 pathway' represents a collaborative effort with Dr Martina Severa. I am the primary author of this research project and directed and supervised the research which forms the basis of this chapter.

VITAE

EDUCATION:

2007-present	University of California, San Diego MD, in progress
2008	University of California, San Diego PhD, Biological Sciences
2001	University of British Columbia Bachelor of Science, Biological Sciences

PROFESSIONAL EXPERIENCE:

2002-Present:	Graduate Student Division of Biological Sciences University of California San Diego Principal Investigator: Michael David, PhD
2000-2002	Research Technician Division of Biological Sciences University of California San Diego Principal Investigator: Robert Rickert, PhD

TEACHING EXPERIENCE:

2006:	Teaching Assistant, UC San Diego, Molecular Basis of Disease
2005:	Teaching Assistant, UC San Diego, Lab in Molecular Medicine
2004:	Teaching assistant, UC San Diego, Biochemical Techniques Laboratory

HONORS, AWARDS:

1999-2000	UC San Diego Provost's Honors
1996-2000	University of British Columbia tuition scholarships: Provincial Examination Scholarship Provincial Government Scholarship Outstanding Student Initiative Scholarship Education Abroad Scholarship

ABSTRACT OF THE DISSERTATION

Mechanisms of regulation in the interferon factor 3 (IRF-3) pathway

by

Kirsten Limmer

Doctor of Philosophy in Biology

University of California San Diego, 2008

Professor Michael David, Chair

Interferon regulatory factor 3 (IRF-3) plays a critical role in the host cell response to both bacterial and viral infection. IRF-3 is activated by Toll-like receptors (TLRs) and cytoplasmic nucleic acid sensors, and serves to upregulate interferon beta and interferon stimulated genes (ISGs), thereby providing a quick and effective response to infection. In this work, two novel mechanisms of regulation in the IRF-3 pathway are revealed.

The first part of this thesis work shows that upon binding to lipopolysaccharide, TLR-4 signaling activates phospholipase C gamma 2 (PLCg2), which cleaves phosphatidylinositol (4,5)-bisphosphate (PIP₂) into Inositol 1,4,5-triphosphate (IP₃) and regulates IP₃R activity. Inositol 1,4,5-triphosphate receptors (IP₃Rs) mediate the release of calcium from the endoplasmic reticulum into the cytosol. The hypothesis presented here is that, upon calcium release, the calcium-dependent phosphatase calcineurin becomes activated and activates dynamin-dependent endocytosis of the LPS-TLR4 complex. Signaling occurs from the early endosomes via the TRAM-TRIF pathway, and induces the IRF3-dependent gene response. Our results suggest a novel means by which the IRF3 pathway is regulated.

The second part of this thesis work describes the Schlafen (Slfn) gene family and its complex role in the immune response. The family is comprised of nine murine members, which are divided into short (Slfn 1 and 2), medium (Slfn 3 and 4) and long (Slfn 5, 8, 9, 10,14) subtypes. The short subtype member, Slfn2, is a cytoplasmic protein that binds to foreign nucleic acids and inhibits protein translation, putatively as a host defense strategy. However, short and medium subtypes, Slfn2 and Slfn3, appear to *inhibit* Interferon stimulated gene (ISG) production via the IRF3 pathway, thus indicating a role in attenuating the immune response. Moreover,

Slfn8, a long subtype member, does not appear to have any effect on ISG induction, indicating divergent roles of different individual Slfn family members. We propose that the Slfn family is differentially regulated during a response to infection, thus initially mediating cellular defense and, later, downregulation of response through the varying participation of individual Slfn proteins within a complex.

Chapter 1: Background

Innate Immunity

The immune system is a collection of mechanisms within an organism that protects against disease by identifying and killing pathogens and tumor cells. Pathogenic infection of all types must be recognized, regardless of the route by which they enter the host. In multicellular organisms, a multi-layered faceted array of defense mechanisms exist, and in most, the first tier of defense is a physical barrier, such as skin, or mucous¹. If this layer of defense is breached, two levels of defense are responsible for the containment of infection: the adaptive immune response and the innate immune response. The cells of the innate system recognize, and respond to, pathogens in a “generic” way, but unlike cells of the adaptive immune system, do not confer long-lasting or protective immunity to the host². The innate immune system is often regarded as the cell’s “rapid response” defense, containing an infection until the adaptive branch of the immune response is adequately mounted. Many features of the innate defense have been conserved throughout evolution and are present in vertebrates, non-vertebrates, and plants¹. The effector mechanisms of innate immunity, which include antimicrobial peptides, phagocytosis, and the alternative complement pathway, are activated immediately after infection and rapidly control the replication of the infecting pathogen². Pattern recognition receptors

(PRRs) on antigen-presenting cells (APCs) of the innate response recognize pathogen-associated molecular patterns (PAMPs) on the invading pathogen. PRRs include the cell surface expressed toll like receptors (TLRs) -1, -2, -4, -5 and -6; the endosome expressed TLR-3, -7, -8, -9³; the cytosolic expressed nucleotide binding oligomerization domain (NOD) proteins⁴; Retinoic-acid-inducible protein I (RIG-I)⁵ and Melanoma differentiation associated gene 5 (MDA5)⁶. The signals induced upon recognition of a PAMP by a PRR control the activation of the body's next level of defense: the adaptive immune response. The adaptive immune system responds to a pathogen only after it has been recognized by the innate immune system⁷. T lymphocytes of the adaptive immune system use their antigen receptors to recognize peptides bound to MHC class II molecules on an APC². However, these peptides can be derived from self proteins or from microbial pathogens. Therefore, a co-stimulatory signal is needed to notify the T cell that a foreign peptide is being presented. The co-stimulatory molecules, CD80 and CD86, on the APC are upregulated upon recognition of a PAMP by a PRR. Binding of these molecules to a receptor on the T cell, CD28, leads to activation of the T cell⁸, thus signifying a bridge between innate and adaptive immunity.

Toll-like Receptor Family

Innate immune recognition is mediated by a system of germline-

encoded receptors (Toll receptors) that recognize conserved molecular patterns associated with microbial pathogens². These receptors, which are coupled to downstream signaling cascades that mediate the induction of immune-response genes, represent the most ancient host defense system found in mammals, insects and plants². Toll receptors, were first discovered in *Drosophila*⁹, and subsequent studies revealed that Toll also has an essential role in the insect innate immune response against fungal infection¹⁰. Human homologs, the Toll-like receptor (TLR) family, is comprised of nine members: TLR 1-9³. Based on their primary sequences, TLRs can be further divided into several subfamilies, each of which recognizes related PAMPs: TLR-2 heterodimerizes with either TLR-1 or TLR-6 and mediates the response to the bacterial and mycoplasma components, peptidoglycan (PGN) and lipoproteins; TLR-3, TLR-7 and TLR-9 recognize viral genetic material: double-stranded RNA (dsRNA), single-stranded RNA (ssRNA) and CpG islands, respectively³. However, the TLRs are unusual in that some can recognize several structurally unrelated ligands. TLR-4, for example, recognizes a divergent collection of ligands such as lipopolysaccharide (LPS), the plant diterpene, paclitaxel, the fusion protein of respiratory syncytial virus (RSV), fibronectin, and heat shock proteins^{8, 11, 12}. Specific PAMPs recognized by each TLR are outlined in Table 1-1¹³. The subcellular localization of different TLRs roughly

correlates with the molecular patterns of their ligands. TLR1, TLR2 and TLR4 are located on the cell surface and are recruited to endosomes after binding their respective ligands. In contrast, TLR3, TLR7 and TLR9 are expressed on endosomes, where they encounter their specific PAMPs and initiate a signaling cascade from this compartment³.

Toll-like receptor signaling through the MyD88-dependent pathway

The cytoplasmic tail of the Toll-like receptors is homologous to the intracellular region of the IL-1 receptor and is therefore referred to as the Toll/IL-1R homology (TIR) domain¹⁴. Toll like receptors directly interact with adaptor molecules, essential for downstream signaling of TLRs. The four adaptors identified, all containing TIR domains, are MyD88 (Myeloid differentiation factor 88)¹⁴, Mal (MyD88-adaptorlike)/TIRAP (TIR domain containing adaptor protein)¹⁵, TRIF/TICAM-1 (TIR containing adaptor molecule-1)¹⁶ and TRAM (TRIF-related adaptor molecule)¹⁷. TLR-4 is unique in its use of all four adaptors to activate two downstream pathways: the MyD88-dependent and –independent pathways.

The MyD88-dependent signaling pathways leading from TLR stimulation to NFkB activation are highly conserved between *Drosophila* and mammals. In mammalian cells, MyD88 functions as the adapter that facilitates binding of the serine/threonine kinase IRAK to the receptor(s)¹⁸. MyD88 or IRAK deficient mice are defective in LPS-mediated NFkB

activation. The TNF-receptor associated factor (TRAF) family member TRAF6 interacts with IRAK once the kinase has undergone phosphorylation. TRAF6 acts as an activator of the serine/threonine kinase TAK1, which in turn phosphorylates the NFkB inducing kinase NIK, a member of the MAPKKK (=MEKK) family ¹⁹. NIK then phosphorylates and activates the Ikb-kinases (IKKs). IKKa and IKKb participate in the formation of a large molecular weight kinase complex that can specifically phosphorylate two serines on the cytoplasmic NFkB inhibitor, Ikb ²⁰. Phosphorylation of Ikb causes its ubiquitin dependent degradation, leading finally to the activation and nuclear translocation of NFkB.

In addition to the NFkB pathway, TLRs also stimulate a signaling cascade that leads to the activation of the Stress Activated Protein Kinases (SAPK) such as SAPK1 (=JNK) and SAPK2 (p38) via MKK4 (=MEK4)²¹. Immediate downstream targets of this pathway are the MAPKAP kinases ²² and the related PRAK kinase ²³, the ribosomal protein kinases (Rsk) ²⁴⁻²⁶ and the Mnk kinases ²⁷, whose substrates in turn are the small heat shock protein Hsp27 ^{22, 23}, the ribosomal S6 protein ²⁴⁻²⁶ and the translation factor eIF-4E ²⁷, respectively.

Toll-like Receptor 4 Signaling

Upon binding to dsRNA and LPS respectively, TLR3 and TLR4 signal through a MyD88-independent pathway mediating the activation of

transcription factor Interferon Regulatory Factor 3 (IRF3). While TLR3 signals exclusively through this MyD88-independent pathway, TLR4 is unique in utilizing all four adaptor proteins in specifying downstream gene transcription. In addition to the MyD88-dependent pathway discussed above, TLR4 uses the TIR domain-containing adaptor inducing IFN-beta (TRIF)¹⁶, and TRAM (TRIF-related adaptor molecule). TRIF-dependent activation of the I κ B kinase epsilon (IKKe) and TANK-binding kinase 1 (TBK1) leading to the phosphorylation of IRF3, which dimerizes and translocates to the nucleus, binding to an Interferon stimulated response element (ISRE) to induce specific gene expression^{17, 28, 29}.

LPS-mediated TLR4 signaling leads to the induction of genes that are specific for either the MyD88-independent or dependent pathway, as well as genes that require both pathways to be intact. MyD88- or TIRAP- deficient mice have impaired pro-inflammatory cytokines (TNF-alpha, IL-1 beta and IL-6) production³⁰. Conversely, TRIF deficient and TRAM-deficient mice have impaired expression of IFN-beta and IFN inducible genes (ISGs) in response to LPS¹⁷.

Previously, our laboratory showed that ISG induction by LPS, which occurs in an IRF-3-dependent manner, requires the generation of Reactive Oxygen Species (ROS) by the NADPH-dependent oxidase NOX4³¹. Subsequent activation of ASK1 links LPS-induced ROS production

to the activation of MKK6 and p38, two kinases that we had previously identified as components of the LPS-induced IRF-3 activation cascade³².

LPS-induced internalization of TLR4 has conventionally been proposed to be solely for the purpose of cell desensitization to LPS to prevent excessive host responses, such as septic shock^{33, 34}. More recently, however, it has been demonstrated that endocytosis of the TLR4-LPS complex plays an important role in coordinating the activation of the two distinct pathways, TIRAP-MyD88 and TRAM-TRIF³⁵. Specifically, it was shown that TLR4 first induces TIRAP-MyD88 signaling at the plasma membrane and is then endocytosed and activates TRAM-TRIF signaling from early endosomes³⁵. Blocking endocytosis completely abolishes IRF3 phosphorylation and IRF3-dependent gene induction, whereas NF- κ B-dependent gene induction remains intact³⁵. Additionally, Kagan et al showed that LPS-TLR4 induced endocytosis is dependent on the GTPase dynamin, which plays a well-established role in clathrin-mediated endocytosis³⁶.

TLR-independent pathogen recognition

The conventional opinion has been that the TLR system is the main pathway in virus-induced innate immunity. However, it was demonstrated that IFN beta was induced even in TLR3-/- cells after viral infection or dsRNA transfection, indicating the involvement of a TLR3-independent

signaling pathway⁵. Previously, two proteins with roles in inflammation and apoptosis, NOD1 and NOD2, were identified as cytoplasmic bacterial sensors, which recognize bacterial cell wall components and induce the expression of proinflammatory cytokines through the NF- κ B pathway⁴, hinting at the possible existence of cytoplasmic sensors of viral infection as well. Certainly, a cytoplasmic sensor of viral components seemed only sensible, as the detection abilities of TLRs are limited by their locations on the cell membrane and endosomes. This notion was confirmed when the RNA helicase retinoic acid gene I (RIG-I) was identified as a cytoplasmic virus sensor⁵. More recently with the identification of RNA helicases, melanoma differentiation-associated gene-5 (MDA5) and laboratory of genetics and physiology-2 (LGP2), both of which can initiate and/or regulate RNA and virus-mediated type I IFN production and antiviral responses³⁷. The detection of viral cytosolic dsDNA has been demonstrated to be mediated by RIG-I as well³⁸. The more recently characterized DAI (DLM-1/ZBP1) is also a cytosolic DNA sensor and an activator of the innate immune response³⁹.

Engagement of the cytosolic RNA helicases RIG-I and MDA5 by dsRNA induces conformational changes that facilitates a CARD-mediated homotypic interaction between RIG-I/MDA5 and the mitochondrial adaptor MAVs (also known as IPS-1, VISA or CARDIF)⁴⁰. The ensuing

TBK1/IKKepsilon, IKK alpha beta gamma and MAPK signaling induces IRF3, AP-1 and NF-kappaB translocation, and IFN beta transcription³⁸. It has also been shown that signaling through the cytosolic DNA sensor, DAI, upon dsDNA stimulation mediates activation of TBK1/IKKepsilon and IKK alpha beta gamma, translocation of IRF3 and NF-kappaB, and expression of IFN-alpha/beta³⁸.

Interferon Regulatory Factor 3 (IRF-3)

The ubiquitously expressed interferon regulatory factor 3 (IRF3) is an important cellular response factor to host cell infection. Activation of IRF-3 requires phosphorylation of several serine residues located in two clusters at the carboxy-terminus of the protein. Interestingly, IRF3 activation by different pathways seems to be associated with phosphorylation of different residues^{41 42 43}. For example, Ser396 was reported as the minimal phosphoacceptor site for in vivo activation of IRF3 following Sendai virus infection or treatment with dsRNA ⁴¹. However, this serine residue is not subject to phosphorylation after LPS stimulation of cells suggesting that another major pathway is involved in IRF3 activation after TLR4 ligation ⁴¹.

IRF-3 contains a nuclear export sequence (NES) that maintains the protein in the cytoplasm until appropriately phosphorylated in response to infection. Once phosphorylated, IRF-3 dimerizes and translocates to the nucleus, where it interacts with the transcriptional co-activator,

CBP/p300⁴⁴, binding to Interferon stimulated response elements (ISRE) on promoters of Interferon stimulated genes (ISGs) and, most notably, promoting transcription of the IFN beta gene. Positive Regulatory Domains (PRDs) have been identified in the IFN beta promoter⁴⁵, and further characterization established PRD II as an NFkB binding element, whereas PRD IV is able to interact with ATF-2⁴⁶. The PRD I and PRD III regions serve as high affinity sites for the Interferon Regulatory Factors (IRF) 1, 2, 3 and 7.

Interferon

Interferons (IFNs) are a family of structurally related cytokines that are found only in vertebrates⁴⁷. IFNs exhibit a diversity of biological functions, as represented by three major biological activities: antiviral activity, antitumour activity and immunomodulatory effects. IFNs are divided into three classes on the basis of the receptor they bind. The type I IFNs consist of α , β , ω and ϵ . These members are induced in virally infected cells to confer an antiviral state on uninfected cells. In addition to their roles in innate immunity, IFN α/β have a variety of important immunomodulatory roles in adaptive immune responses as well as in tumor suppression^{48, 49}. Type II IFN consists solely of IFN- γ , which also has anti-viral activity but is strongly produced by activated T cells or NK cells and not by virus-infected cells⁴⁷. The more recently identified type III IFNs

include IFN λ 1-3 and also display anti-viral activity⁴⁷.

Type I interferon signaling

Induction of IFN- α/β genes is mediated by the activation of IRF-3 upon recognition of pathogen associated molecular patterns (PAMPs) by TLRs and cytosolic sensors, as discussed previously. Upon induction, IFN- α/β is secreted from the cell and initiates a cell signaling pathway in both in an autocrine and paracrine manner, resulting in the transcription of interferon stimulated genes (ISGs). IFN- α/β interact with the same receptor complex, termed the IFN- α/β receptor (IFNAR), which consists of at least two subunits, IFNAR-1 and IFNAR-2⁵⁰. Binding of the IFN- α/β to IFNAR1/2 activates associated Janus protein tyrosine kinases, Tyk2 and Jak1, which leads to the tyrosine phosphorylation and activation of several members of the Signal Transducer and Activator of Transcription (STAT) family of transcription factors⁵⁰⁻⁵³. Among those, STAT1, STAT2 and Interferon Regulatory Factor 9 (IRF9) cooperatively form the DNA binding protein complex ISGF3, which is required for upregulation of interferon stimulated genes (ISGs) through an Interferon Stimulated Response Element (ISRE). In cells lacking STAT1 or STAT2, type I interferons fail to mount an anti-viral state, or to elicit an anti-proliferative response⁵⁴.

IFN α/β -mediated induction of ISRE-containing genes thus represents a second wave of interferon stimulated gene (ISG) upregulation. As

discussed previously, the first wave of gene induction is initiated upon the initial recognition of pathogens by TLRs and cytosolic receptors, leading to IRF3 activation, ISRE binding, and activation of IFN α/β and ISGs. Signaling of IFN α/β through IFNAR1/2 leads to ISGF3 binding to an ISRE sequence as well, thus mediating the second wave of ISG induction. Numerous genes were identified that contain an ISRE. They represent components of the antiviral defense such as the 2'-5' poly-A-synthase (2'5'OAS) ^{55, 56} and the dsRNA activated protein kinase (PKR) ⁵⁷. PKR is a pivotal antiviral protein found in most human cells⁵⁸. Although not considered a “primary responder” of dsRNA, following activation by dsRNAs produced during viral replication, PKR phosphorylates the alpha-subunit of eukaryotic translation initiation factor (eIF2), causing a severe inhibition of cellular and viral protein synthesis⁵⁹. Additionally, 2'5'OAS, activates RNase L which, upon binding to dsRNA in the cytoplasm, cleaves both viral and cellular mRNAs to inhibit protein synthesis, thus providing a means of regulating translation in infected cells⁵⁸. In addition to antiviral proteins, ISRE-containing genes also include cell surface proteins such as ICAM ^{60, 61} or the MHC class I and II molecules ⁶², genes encoding chemokines such as the ISG15 and the IP10 gene ^{63, 64}, as well as many other genes with as of yet unknown functions such as ISG54, ISG56 ⁶⁵, GBP ⁶⁶ or 6-16 ⁶⁷

Regulation of the innate immune response

Paradoxically, type I IFNs are both pro- and anti-apoptotic in nature⁴⁹. For example, IFN β is an effective therapeutic for the autoimmune disease multiple sclerosis⁶⁸, but type IFN activity has been implicated in the pathogenesis of other autoimmune diseases such as systemic lupus erythematosus (SLE) and type-I diabetes⁴⁸. Dysregulation of IFN- α/β signaling may promote autoimmune responses by a multitude of effects on both the innate and adaptive immune systems. A major effect is dendritic cell activation and upregulation of MHC and costimulatory molecules (CD40, CD80, CD86), leading to efficient self-antigen presentation to previously quiescent low-affinity autoreactive T cells⁸. Thus, production of IFN and its downstream effects must be tightly regulated by the cell to prevent unwanted or inappropriate host responses. Accordingly, the cell places a great deal of emphasis on the negative regulation of type I IFN signaling, which is accomplished by mechanisms such as receptor internalization and degradation, dephosphorylation of Jaks and STATs by several phosphatases, induction of suppressors of cytokine signaling (SOCS), and repression of STAT-mediated gene activation by protein inhibitors of activated STATs (PIAS)⁴⁸.

Table 1-1. TLR recognition of microbial components (3).

MicrobialComponents	Species	TLR usage
Bacterial		
LPS	Gram-negative bacteria	TLR-4
Diacyl lipopeptides	<i>Mycoplasma</i>	TLR-6/TLR-2
Triacyl lipopeptides	Bacteria and mycobacteria	TLR-1/TLR-2
LTA	Group B <i>streptococcus</i>	TLR-6/TLR-2
PG	Gram-positive bacteria	TLR-2
Porins	<i>Neisseria</i>	TLR-2
Lipoarabinomannan	Mycobacteria	TLR-2
Flagellin	Flagellated bacteria	TLR-5
CpG-DNA	bacteria and mycobacteria	TLR-9
Fungus		
Zymosan	<i>Saccharomyces cerevisiae</i>	TLR-6/TLR-2
Phospholipomannan	<i>Candida albicans</i>	TLR-2
Mannan	<i>Candida albicans</i>	TLR-4
Glucuronoxylomannan	<i>Cryptococcus neoformans</i>	TLR-2 and TLR-4
Parasites:		
tGPI-mutin	<i>Trypanosoma</i>	TLR-2
Glycoinositolphospholipids	<i>Trypanosoma</i>	TLR-4
Hemozoin	<i>Plasmodium</i>	TLR-9
Profilin-like molecule	<i>Toxoplasma gondii</i>	TLR-11
Viruses		
DNA	Viruses	TLR-9
dsRNA	Viruses	TLR-3
ssRNA	RNA Viruses	TLR-7 and TLR-8
Envelope proteins	RSV, MMTV	TLR-4
Hemagglutinin protein	Measles virus	TLR-2
Host		
Heat-shock protein 60, 70		TLR-4
Fibrinogen		TLR-4

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Chapter 2:
The role of calcium in the activation of
Interferon Regulatory Factor 3 by
lipopolysaccharide

ABSTRACT

Toll-like receptor 4 (TLR4) induces two distinct signaling pathways controlled by TIRAP-MyD88 and TRAM-TRIF pairs of adaptor proteins, which elicit the transcription of pathway-specific or common genes. In this study, we report a novel mechanism by which the cell coordinates the activation of the two TLR4 pathways. Upon binding to LPS, TLR4 signaling activates phospholipase C gamma 2 (PLC γ 2), which cleaves phosphatidylinositol (4,5)-bisphosphate (PIP $_2$) into diacylglycerol and Inositol 1,4,5-triphosphate (IP $_3$) and regulates IP $_3$ R activity. Inositol 1,4,5-triphosphate receptors (IP $_3$ Rs) mediate the release of calcium from endoplasmic reticulum into the cytosol. We propose that, upon calcium release, the calcium-dependent phosphatase calcineurin becomes activated and promotes dynamin-dependent endocytosis of the LPS-TLR4 complex, whereby signaling occurs from early endosomes specifically via the TRAM-TRIF pathway and induces the IRF3-dependent gene response.

INTRODUCTION:

Toll like receptor 4 (TLR4) recognizes lipopolysaccharide (LPS) that is expressed on the cell wall of gram-negative bacteria.^{1, 2} TLR4 is unique in utilizing four adaptor proteins in specifying two distinct downstream transcription profiles. The MyD88-dependent pathway utilizes both MyD88 and TIRAP (TIR-associated protein) and leads to NF- κ B activation². This pathway is critical in mediating the induction of pro-inflammatory cytokines¹. In addition to this MyD88-dependent pathway, TLR4 uses a MyD88-independent signaling pathway. In this pathway, adaptor molecules TRIF and TRAM³ mediate downstream activation of IRF3, which binds to stimulated response element (ISRE) on the promoters of IFN α/β genes and interferon stimulated genes (ISGs)⁴⁻⁶.

Previously, our laboratory showed that ISG induction by LPS, which occurs in an IRF-3-dependent manner, requires the generation of Reactive Oxygen Species (ROS) by the NADPH-dependent oxidase NOX4⁷. ROS production often occurs simultaneously with elevated cytoplasmic Ca²⁺ concentrations, and there is evidence that calcium level oscillations and ROS generation/deactivation processes are intimately linked^{8, 9}. Calcium (Ca²⁺) is a ubiquitous intracellular signal responsible for controlling numerous cellular processes. Cells generate

their calcium signals by using both internal and external sources of calcium. The internal stores are held within the membrane systems of the endoplasmic reticulum (ER) or the equivalent organelle, the sarcoplasmic reticulum (SR) of muscle cells¹⁰. Release from these internal stores is by the inositol-1,4,5-trisphosphate receptor (IP3R) and ryanodine receptor (RYR) families (Figure 2-1). Activation of the IP3R is through binding of Inositol-1,4,5-trisphosphate (I(1,4,5)P3), generated by the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) by a family of phospholipase C enzymes (PLC beta, PLC gamma)⁹.

It was recently demonstrated that endocytosis of the TLR4-LPS complex plays an important role in coordinating the activation of the two distinct pathways, TIRAP-MyD88 and TRAM-TRIF¹¹. Specifically TLR4 first induces TIRAP-MyD88 signaling at the plasma membrane and is then endocytosed and activates TRAM-TRIF signaling from early endosomes¹¹. Additionally, Kagan et al showed that LPS-TLR4-induced endocytosis is dependent on the GTPase dynamin, which plays a well-established role in clathrin-mediated endocytosis¹². The regulation of dynamin in TLR4 endocytosis is not yet known. In neuronal cells, dynamin 1 binds to and is a substrate of, the calcium-dependent protein phosphatase calcineurin¹³. Dephosphorylation of dynamin 1 by calcineurin decreases its GTPase activity and enhances interaction with its partners, thus triggering clathrin-

coated endocytosis¹⁴. Calcineurin is a serine/threonine phosphatase that positively regulates the activation of NF-AT in T lymphocytes following an increase in cellular calcium concentration upon T cell receptor ligation. Although the activity of calcineurin increases upon LPS stimulation¹⁵, its role in inflammation is currently unknown. The importance of calcineurin in the immune response is underscored by the wide use of calcineurin inhibitors, immunosuppressive drugs FK506 (tacrolimus) and cyclosporin A (CsA) in post-allogeneic organ transplant; these drugs block calcineurin activity by forming inhibitory complexes with FK506-binding proteins and cyclophilins, respectively¹⁶.

Here we report that calcium is required for IRF-3 activation in response to LPS. Additionally, we propose a novel signaling pathway in which TLR4 binding to LPS leads to PLCgamma2 activation and IP3 production, increasing cytosolic calcium and activating calcineurin, leading to dynamin-induced endocytosis of the TLR-LPS complex, and thus initiating the TRAM/TRIF IRF3 signaling cascade from early endosomes.

MATERIAL AND METHODS:

Cells Lines – RAW264.7 cell line was purchased from American Type Culture Collection (ATCC). 293T cells expressing TLR4-CD14-MD2 were generously gifted by Dr. Katherine Fitzgerald (the University of

Massachusetts). Both RAW264.7 and 293T cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100ug/ml streptomycin and 2mM L-glutamine.

Reagents – 2-aminoethoxydiphenyl borate (2APB), Thapsigargin (Tg), were obtained from Calbiochem. Murine IFN- β was a generous gift from Biogen. Anti murine IRF-3 antibody was purchased from Invitrogen Life Technologies. Anti-Histone H3, PLC γ 1 and PLC γ 2 antibodies were purchased from Cell Signaling Technology. siRNA against PLC γ 1 and PLC γ 2 were purchased from Dharmacon with the catalogue number 040978 and 040979 respectively. Cyclosporin A was purchased from Sigma Aldrich.

Transfection – 1×10^5 RAW264.7 cells were seeded in a 24-well plate and transected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Rnase Protection Assay (RPA)- Total RNA was isolated using Trizol Reagent (Invitrogen). Ten micrograms of RNA and ^{32}P -labelled riboprobes for ISG54, RANTES, and GAPDH were incubated in buffer (4:1 formamide and 5x stock (200mM PIPES pH 6.4, 2M NaCl, and 5mM EDTA) overnight at 56C before digestion with T1 Rnase (Invitrogen). Protected fragments were solubilized in 98% formamide plus 10mM EDTA and subjected to electrophoresis on a 4.5% polyacrylamide/urea gel.

Western Blotting – Cells were lysed in RIPA buffer (50mM Tris-HCl pH8.0, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM sodium vanadate, and 1 mM PMSF). Cell lysates were resolved by SDS-PAGE, and blots were immunoblotted with the indicated Abs. All blots were developed with HRP-conjugated secondary Abs and ECL (Amersham Biosciences).

Electrophoretic Mobility Shift Assay (EMSA) - EMSAs were performed using a ³²P-labeled probe corresponding to the ISG15-ISRE (5'-GATCGGGAAAGGGAAACCGAAACTGAAGCC-3'). 5 ug of nuclear extract was incubated with poly-(dI-dC) and labeled probes in buffer (40 mM KCl, 20 mM Hepes, pH 7.0, 1 mM MgCl₂, 0.1 mM EGTA, 0.5 mM DTT, 4% Ficoll, 0.02% NP-40), and resolved on 6% TBE-PAGE.

Luciferase Assay – 4 x 10⁵ 293T cells were transfected in suspension in a 24 well plate by LF-2000. Cells were lysed by passive lysis buffer (Promega) 48 hours post transfection. The ISRE driven luciferase and SV-40 driven Renilla activities were quantified by the Dual Luciferase System (Promega) according to the manufacturer's protocol. The ISRE luciferase activity was normalized by the SV-40 driven Renilla activity to account for the differences in transfection efficiency between samples. Furthermore, the fold of expression was normalized against ISRE-Luciferase activity in GFP transfection cells.

Real Time PCR – Total RNA was extracted by using RNeasy Mini Kit according to the manufacture's instruction (Qiagen). 100ng of RNA sample was reverse transcribed using the Omniscript kit (Qiagen). PCR products were detected using the fluorescent dye SYBR green (Applied Biosystems). The sequences of the primer used in the assay are:

B-actin Forward, 5'-ACGGCCAGGTCATCACTATTG-3'

B-actin Reverse, 5'-CAAGAAGGAAGGCTGGAAAAGAG-3'

IkBalpha forward, 5'-GCGGGATGGCCTCAAGA-3'

IkBalpha Reverse, 5'-ATTGCTCGTACTCCTCGTCCTT-3'

ISG54 forward, 5'-TCTGATTCTGAGGCCTTGCA-3'

ISG54 reverse, 5'-CTTGCTGACCTCCTCCATTCTC-3'

RANTES forward, 5'-GCAGTCGTGTTGTCACCTCGAA-3'

RANTES reverse, 5'-GATGTATTCTGAACCCACTTCTTCTC-3'

TNF- α forward, 5'-TGGCCTCCCTCTCATCAGTT-3'

TNF- α reverse, 5'-GCTTGTCACCTCGAATTTGAGAAG-3'

TRAIL forward, 5'-AGACCTTAGGCCAGAAGATTGAATC-3'

TRAIL reverse, 5'-ATTCCTAAAGAGCACGTGGTTGAG-3'

TBP forward, 5'-CTTCGTGCAAGAAATGCTGAATAT-3'

TBP reverse, 5'-TGTCCGTGGCTCTCTTATTCTCA-3'

Formation of a unique DNA product was confirmed by verifying that products had a single melting temperature. Fluorescence-monitored PCR

values were normalized to B-actin or TBP values to account for any differences in cDNA recovery between samples.

RESULTS AND DISCUSSION:

Calcium released from intracellular stores is essential for IRF-3 mediated

LPS response:

Previous results from our laboratory demonstrated that reactive oxygen species (ROS) is required for IRF3-mediated signaling in response to LPS⁷. Cellular calcium concentrations and ROS production have been shown to be intimately linked¹⁰, thus we were interested in ascertaining whether calcium signaling plays a role in the LPS-stimulated IRF3 pathway. To investigate this question, we exploited the fact that the ISG54 gene is under the sole control of an ISRE in its promoter region; therefore, the induction of ISG54 by IFN α/β occurs strictly in an ISGF3-dependent manner whereas its activation as a consequence of viral infection or LPS stimulation requires the activation of IRF-3. We used the cell permeable intracellular calcium chelator, BAPTA, to pre-treat RAW264.7 murine macrophages and analyzed IRF3-mediated induction of the ISG54 gene. In the absence of free cytosolic calcium, ISG54 induction was blocked (Figure 2-2A). In contrast, depletion of extracellular calcium due to the presence of EGTA in the culture media had no effect on IRF-3 mediated

ISG54-induction (data not shown). After establishing that calcium is *required* for ISG54 induction, we sought to discern whether increased cellular calcium *augments* LPS-stimulated TLR4 signaling. We used the small molecules A23187 and Thapsigargin (Tg) to increase cytoplasmic calcium concentration. A23187 is a calcium ionophore, while Tg is a tight-binding inhibitor of the Sarco / Endoplasmic Reticulum Ca^{2+} ATPase (SERCA) (see Figure 2-1). Tg thus raises cytosolic calcium concentration by blocking the ability of the cell to pump calcium into the endoplasmic reticulum. Supplying either Tg or A23187 to the cell in combination with a low concentration (10ng/ml) of LPS enhances ISG54 induction in a dose dependent manner (Figure 2-2B). Since $\text{IFN}\beta$ treatment also leads to an increase in ISRE-containing gene induction through the ISGF transcriptional complex, $\text{IFN}\beta$ treatment was used to determine if the effect of calcium is specific for the TLR4 signaling pathway. Indeed, A23187 has no effect on $\text{IFN}\beta$ -mediated ISG54 induction (Figure 2-2B).

Calcium is released from endoplasmic reticulum through Inositol 1,4,5-triphosphate receptors:

Release of calcium from the endoplasmic reticulum can be mediated by either the inositol-1,4,5-trisphosphate receptor (IP3R) or ryanodine receptor (RyR). In order to determine which ER receptor is involved in LPS signaling, we employed cell permeable 2APB (2-

aminoethoxydiphenyl borate) or dantrolene, which antagonize the IP3R or RYR respectively. We observed that ISG54 induction by LPS was downregulated by 2APB, but unmodified by dantrolene (Figure 2-3A), thus indicating that the intracellular calcium increase upon LPS stimulation is due to calcium flux through IP3R. We observed that the induction of two other ISRE-containing ISGs, TRAIL and RANTES, were also dependent upon IP3R-mediated calcium release (Figure 2-3B).

TLR4 downstream signaling occurs via two separate pathways: MyD88-dependent and MyD88-independent. IRF3 activation is through the MyD88-independent, TRIF/TRAM pathway, while early NFkB activation is through the MyD88-dependent pathway. To determine whether the requirement of calcium is specific for the MyD88-independent pathway, we tested the effect of 2APB pretreatment on the transcriptional regulation of NF-kB dependent genes, TNF- α and I κ B α . Upregulation of these genes remained intact (Figure 2-3B).

We next sought to determine whether calcium is required for IRF3 activation or whether the effect of calcium on ISG54 induction is downstream of IRF3 activation. Transfection of cells with a constitutively active mutant of IRF3, IRF3-5D, induces LPS-independent ISG54 upregulation. IRF3-5D transfected cells pretreated with 2APB showed no downregulation of response, thus indicating that the calcium requirement

is upstream of IRF3 activation (data not shown). To confirm that blocking of calcium release has a direct effect on IRF3 activation, we analyzed IRF3 nuclear translocation upon LPS stimulation, both with and without 2APB pretreatment, by Western blotting nuclear extracts for IRF3. We saw that IRF3 nuclear translocation is dramatically decreased when InsP3R channels are blocked (Figure 2-3C). In corroboration, a reduction of IRF-3 DNA binding activity was also observed when RAW264.7 cells were treated with 2-APB (Figure 2-3D).

PLC gamma 2 mediates the IRF-3 activation:

Activation of the InsP3R is through binding of Inositol-1,4,5-trisphosphate (I(1,4,5)P3), generated by the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) by a family of phospholipase C enzymes (PLC beta, PLC gamma)⁹. To examine if PLC mediates the activation of IRF-3 in response to LPS, RAW264.7 cells were treated with U73122, a commonly employed general inhibitor of PLC, and its negative control, U73343. The ISG54 expression in response to LPS is inhibited by U73122 but not U73343, whereas ISG54 expression in response to IFN- β was affected neither by U73122 nor U73343 (Figure 2-4A).

Thus far, our findings indicate that the activity of a PLC enzyme is involved in LPS-induced ISG expression. PLC beta isoforms are typically associated with heterotrimeric G-proteins¹⁷ and as there is no evidence for

the involvement of a G-protein coupled receptor in LPS signaling, it is unlikely that PLC beta participates in TLR4 mediated signal transduction. On the other hand, PLC gamma isoforms have been reported to be activated after LPS stimulation of macrophages¹⁸⁻²⁰. PLCg2 has been to be tyrosine phosphorylated and activated by TLR ligands in murine macrophages²¹. Thus, it is reasonable to hypothesize that PLCg2 is the PLC isoform that mediates IRF3 activation in the LPS response. To test this hypothesis, RAW264.7 cells were transfected with either PLCg1 or PLCg2 siRNAs. Both PLCg1 and PLCg2 protein expression level was reduced by 60% after transfecting with the respective siRNAs (data not shown). Knocking down of PLCg2 but not PLCg1 reduced ISG54 mRNA level by 60% in response to LPS (Figure 2-4B). Similar results were observed with ISGs, TRAIL and RANTES (Figure 2-4B), while NF- κ B-mediated genes, TNFa and I κ Ba, were not affected (Figure 2-4C).

To confirm the role of PLCg2 in LPS-induced signaling, we obtained PLCg2 deficient mice from Dr James Ihle at the St. Jude Children's Research Hospital. Peritoneal macrophages from PLCg2^{-/-} mice displayed reduced ISG54 induction upon LPS stimulation compared to wild-type littermate controls (Figure 2-5A). Additionally, the ISRE-containing ISGs, TRAIL and RANTES, also exhibited decreased induction upon LPS stimulation (Figure 2-5A). In contrast, IFN-mediated ISG54 and

TRAIL induction was not decreased (Figure 2-5A). Additionally, induction of genes downstream of NF- κ B activation, TNF α and I κ B, was not affected (Figure 2-5B).

Calcium-dependent phosphatase calcineurin mediates IRF3 signaling

The activation of the two distinct TLR4 pathways, TIRAP-MyD88 and TRAM-TRIF, is coordinated by endocytosis of the TLR4-LPS complex¹¹. TLR4 first induces TIRAP-MyD88 signaling at the plasma membrane and is then endocytosed and activates TRAM-TRIF signaling from early endosomes¹¹, a process that is dependent upon the GTPase dynamin. Although the regulation of dynamin in this context is not yet known, clathrin-coated endocytosis in neuronal cells is dependent upon the de-phosphorylation and activation of dynamin 1 by the calcium-dependent protein phosphatase calcineurin¹³. In effect, the calcineurin-dynamin complex function as a calcium sensor for endocytosis¹³. Calcineurin plays many functions in the immune response, and has been shown to be activated by LPS¹⁵, although its molecular mechanism in inflammation has not yet been revealed. The well-established role of the calcium-dependent phosphatase calcineurin in the immune response, and its positive regulation of dynamin in neuronal cells led us to hypothesize that calcineurin is the "calcium sensor" involved in responding to the increase in cytosolic calcium by LPS-induced PLC γ 2 activation. To test this

hypothesis, we used the calcineurin specific inhibitor, cyclosporin, to treat murine peritoneal macrophages and then tested their response to LPS. Strikingly, inhibition of calcineurin diminished ISG54 induction in a dose-dependent manner (Figure 2-6A). In contrast, we saw no effect of cyclosporin on IFN signaling (Figure 2-6A). In testing the LPS response of another IRF3-dependent gene, RANTES, we also observed a dose-dependent decrease of gene induction upon calcineurin inhibition (Figure 2-6A). To discern whether calcineurin activity is specific for the MyD88-independent IRF3 pathway, we tested the induction of the NF-kB-dependent gene, I κ B α , and observed no downregulation of LPS response (Figure 2-6B). Thus, calcineurin activity is required for TLR4 signaling specifically via the IRF3 pathway.

Conclusion

The host cell responds to bacterial component LPS through its TLR4 receptor on the cell membrane. Our results indicate that, once TLR4 has bound to LPS, PLC γ 2 is activated by an undefined signaling cascade. PLC γ 2 then cleaves PIP2 into IP3, which binds to and activates release of calcium through the IP3R on the endoplasmic reticulum. We propose that, upon increase of cytoplasmic calcium, the calcium-dependent phosphatase calcineurin is activated and dephosphorylates the GTPase dynamin, thus leading to endocytosis of the TLR4-LPS complex. Upon

translocation to the early endosome, TLR4 signaling through the MyD88-independent pathway commences and induces IRF3 activation and IRF3-dependent gene regulation. Our results illustrate a novel mechanism by which the cell coordinates the activation of the two distinct TLR4 signaling pathways.

The text of chapter two entitled 'the role of calcium in the activation of interferon regulatory factor 3 by lipopolysaccharide' was co-authored with Dr Edward Chiang. I am the co-primary author of this research project and co-directed and supervised the research which forms the basis of this chapter.

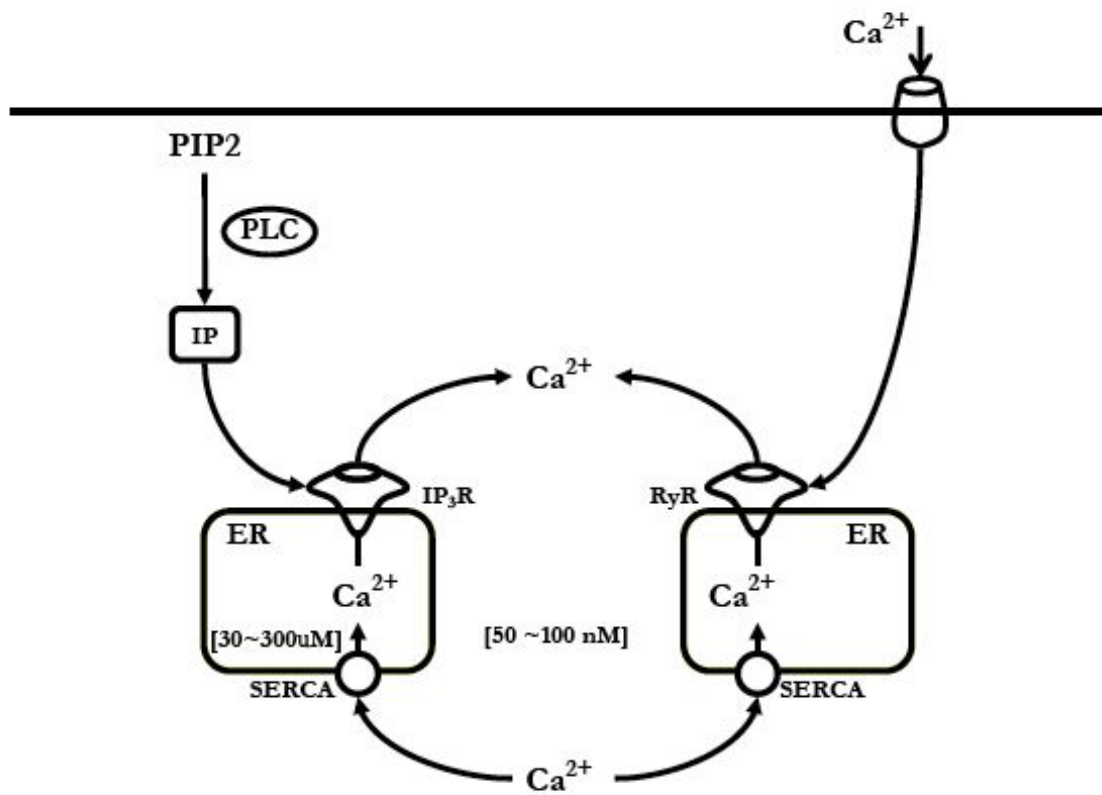


Figure 2-1. Calcium regulation in the cell (1).

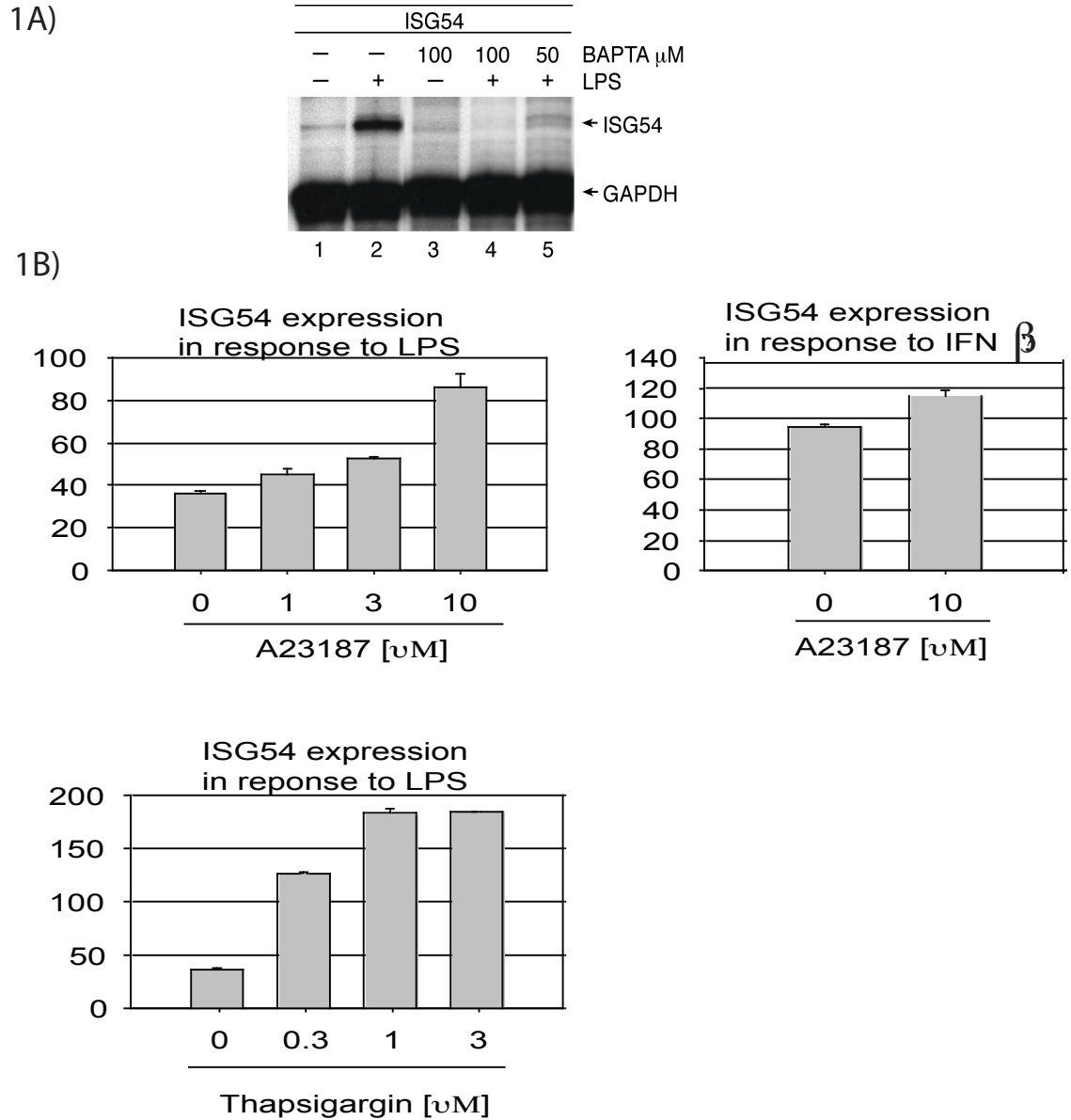


Figure 2-2. Intracellular calcium modulates IRF-3 mediated gene expression in response to LPS stimulation. (A) Total RNA was isolated from LPS stimulated U373 cells with or without preincubation with the intracellular calcium chelator, BAPTA. RNase protection assays were performed using a probe corresponding to the human ISG54 gene. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. (B) RAW 264.7 macrophages were pre-incubated for 1 hour with A23187 or Thapsigargin for the indicated concentration. Cells were subsequently induced with 10ng/ml LPS or 1000 IU/ml IFN beta for 5 hours. Total RNA was collected and ISG54 mRNA level was quantified by real-time PCR. Beta-actin was used as an internal standard. Y axis is fold induction over ISG54 expression in unstimulated and untreated cells.

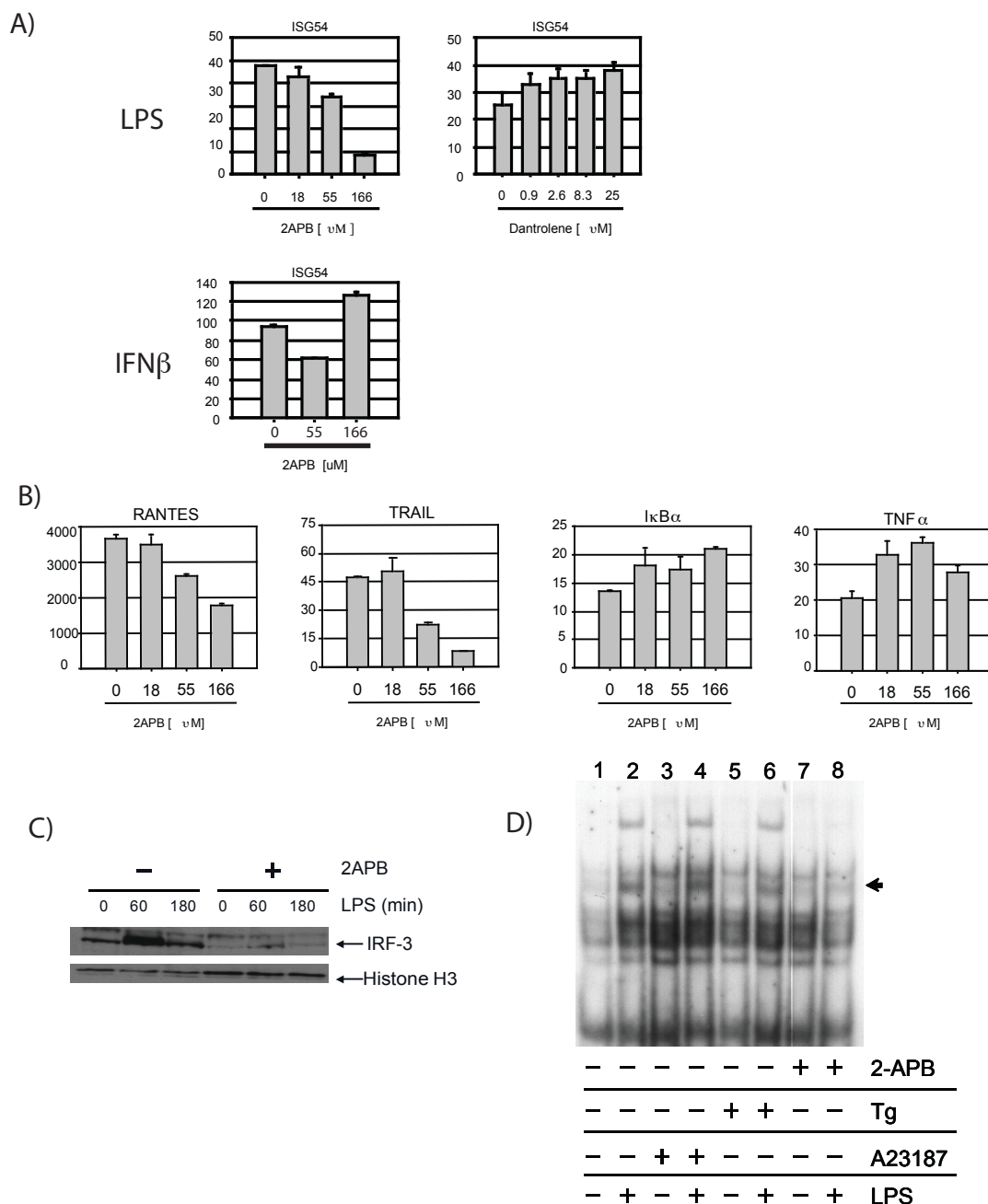


Figure 2-3. 2APB impairs IRF-3 activation in response to LPS stimulation. RAW 264.7 macrophages were pre-incubated with 2APB or dantrolene at the indicated concentration. Cells were subsequently induced with 10ng/ml LPS or 1000 IU/ml IFN β for 5 hours. Total RNA was collected and (A) ISG54 mRNA level or (B) RANTES, TRAIL, I κ B, and TNF α expression was induced by LPS and quantified by real-time PCR. B-actin was used as an internal standard. Y axis is fold induction over ISG54 expression in unstimulated, untreated cells. (C) The nuclear extracts were purified from untreated or LPS stimulated RAW264.7 cells with or without preincubation with 2-APB. The nuclear extracts were resolved by SDS-PAGE and then analyzed by immunoblotting with an IRF-3 specific antibody. Histone H3 was used as a loading control. (D) Electrophoretic mobility shift assay (EMSA) for ISRE DNA binding using nuclear extracts that were purified from unstimulated or LPS stimulated RAW264.7 cells with or without preincubation with 2-APB, Tg or A23187.

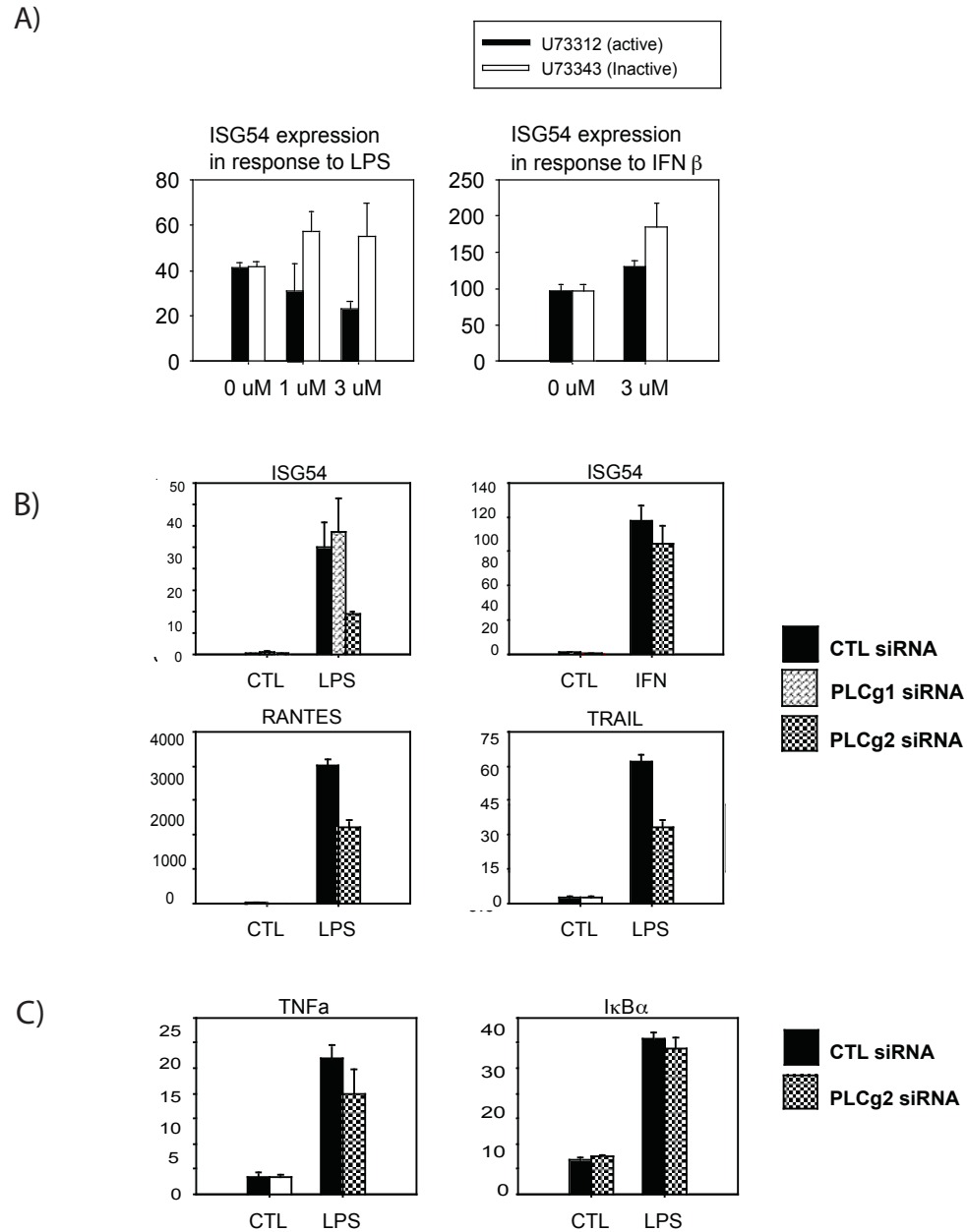


Figure 2-4. PLCg2 but not PLCg1 impairs IRF-3 dependent gene expression in response to LPS.

(A) RAW 264.7 macrophages were pre-incubated with U73312 or U73343 at the indicated concentration. Cells were subsequently induced with 10ng/ml LPS or 1000 IU/ml IFN β for 5 hours. Total RNA was collected and ISG54 mRNA level was quantified by real-time PCR. B-actin was used as an internal standard. (B and C) RAW 264.7 macrophages were transfected with siRNAs against either PLCg2 or PLCg1. Cells were stimulated with LPS or IFN β two days post transfection. Total RNA was isolated and (B) ISG54, RANTES, TRAIL and (C) I κ B α and TNF α mRNA levels were quantified by real-time qPCR. Beta actin was used as an internal standard. Y axis is fold induction over gene expression in untreated, unstimulated cells.

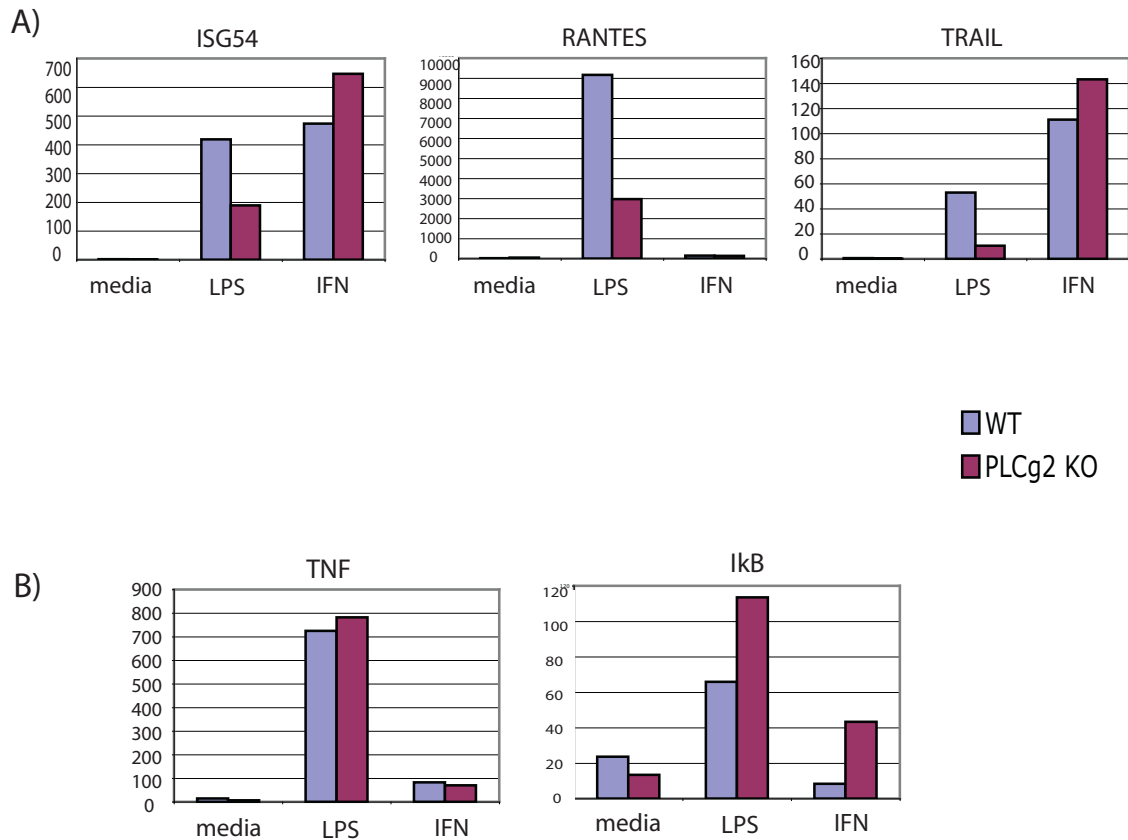


Figure 2-5. PLCg2^{-/-} mice show decreased induction of IRF3-dependent genes upon LPS stimulation. Peritoneal macrophages were harvested from PLCg2^{-/-} mice and WT littermates. Cells were subsequently induced with 100ng/ml LPS or 1000 IU/ml IFN β for 5 hours. Total RNA was collected and (A) ISG54, RANTES or TRAIL or (B) TNF or Ikb mRNA expression was quantified by quantitative real-time PCR. Results are normalized to TBP as an internal standard. Y axis is fold induction of gene expression in untreated, unstimulated cells.

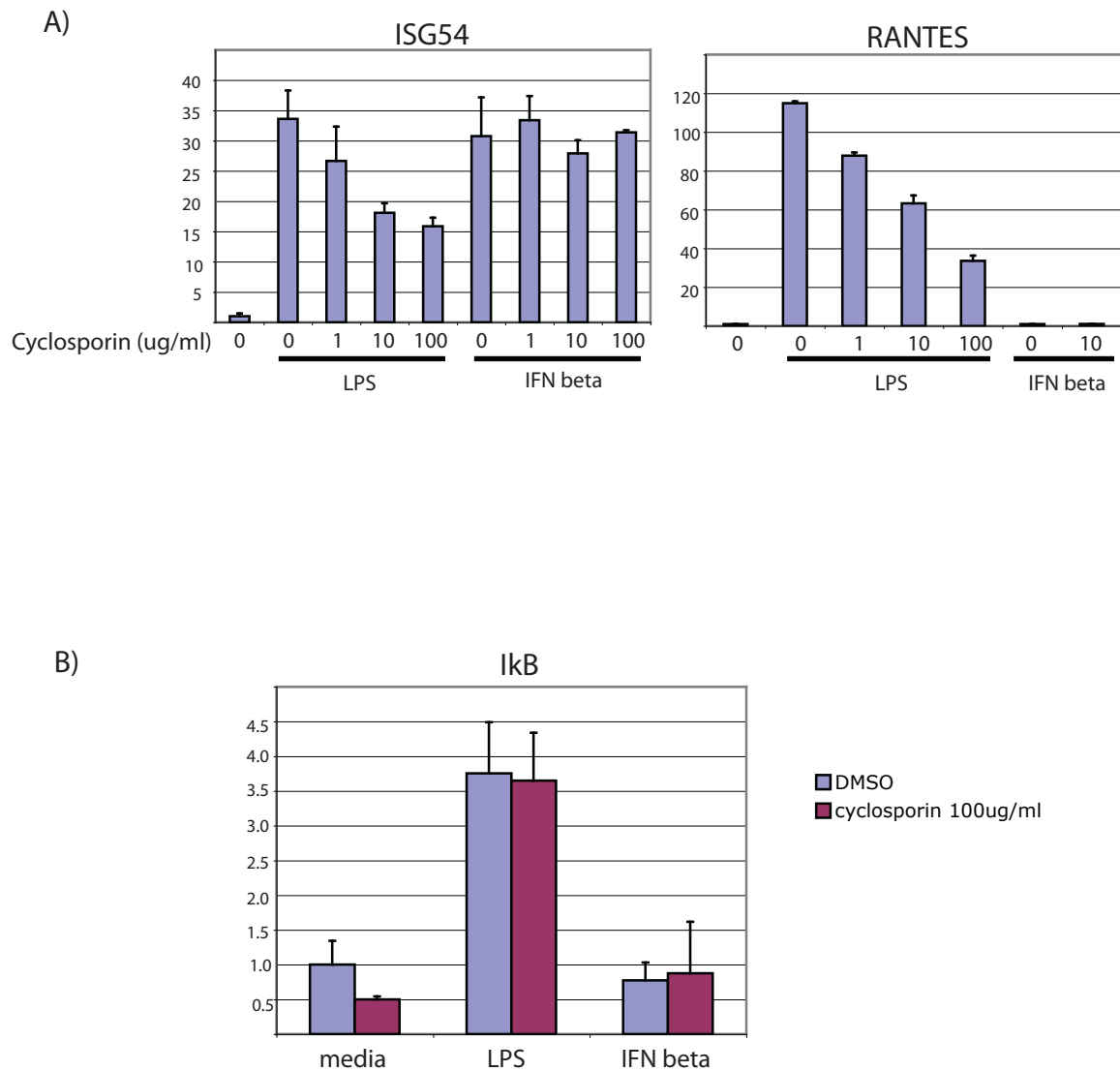


Figure 2-6. Calcium-dependent phosphatase calcineurin mediates induction of IRF3-dependent genes upon LPS stimulation. Peritoneal macrophages were harvested from WT B1/6 mice and pre-treated for one hour with increasing concentrations of cyclosporin, as indicated. Cells were subsequently stimulated with 100ng/ml LPS or 1000IU IFN β for 5 hours. Expression of (A) ISG54 or RANTES or (B) I κ B was analyzed by qPCR and normalized to TAT-BP gene expression.

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Chapter 3:

The role of the Schlafen protein family in the IRF3 pathway

ABSTRACT

The IRF3 pathway is involved in the cellular response to several types of pathogenic infection. IRF3 is activated by TLRs and cytoplasmic nucleic acid sensors and serves to upregulate IFN β and other ISGs, thereby providing a quick and effective response to infection. However, the dysregulation of IFN production may promote autoimmune responses by a multitude of effects on both the innate and adaptive immune systems. Thus, an infected cell walks a thin line between defending itself from infection and deleteriously overactivating its immune response. A balance is struck by an infected cell in which both immune-activating and immune-inhibitory genes are induced upon infection.

Here we show a previously uncharacterized protein family, Schlafen (Slfn), and its complex role in the immune response. The family is comprised of nine murine members, which are divided into short (Slfn 1 and 2), medium (Slfn 3 and 4) and long (Slfn 5, 8, 9, 10, 14) subtypes. Slfn2 is a cytoplasmic protein that binds to foreign nucleic acids and inhibits protein translation *in vitro*. However, Slfn 2 and 3 appear to *inhibit* Interferon stimulated gene (ISG) production, thus indicating a role in attenuating the immune response. Moreover, Slfn8 does not appear to have any effect on ISG induction, indicating divergent roles of different individual Slfn family members. We propose that the Slfn family is

differentially regulated during a response to infection, thus initially mediating cellular defense and, later, downregulation of response.

INTRODUCTION

Schlafen (Slfn) was discovered by Schwartz et al¹ as a family of genes that are differentially regulated during thymocyte maturation. Hematopoietic progenitors populate the thymus and expand by cell division to generate a large population of immature thymocytes, the earliest of which express neither CD4 nor CD8, and are therefore termed double-negative (DN) CD4-CD8- cells². As T cells progress through their development they become double-positive thymocytes (CD4+CD8+), and finally mature to single-positive (CD4+CD8- or CD4-CD8+) thymocytes that are then released from the thymus to peripheral tissues². In comparing the double positive (DP) and single positive (SP) stages of T cell development, Slfn1 and Slfn2 were found to be upregulated in SP CD4+ or CD8+ cells, whereas Slfn4 was found to be downregulated in SP cells. Since their discovery, the Slfn protein family has been shown to include nine murine members that can be divided into three subtypes, based upon their protein length (Figure 3-1).

Although Slfns represent a large family with orthologs in rats, humans, and non-human primates, no orthologs can be found in C.

elegans or Drosophila. Strikingly, the only non-mammalian 'organisms' that encode Slfn-like proteins are all members of the Pox-viruses. The large Pox virus genome is a linear, double-stranded segment of DNA³. After binding to its host cell, the Pox virus uncoats to release its core into the cytoplasm. Interestingly, the replication of this virus occurs entirely in the host cytoplasm, as it encodes all of its own replication machinery^{3, 4}.

The uniting structural feature of all Slfn members is an N-terminal divergent AAA domain. AAA domains are involved in ATP/GTP binding⁵ and proteins that contain AAA domains are ATPases Associated with various cellular Activities⁶. As the name implies, AAA proteins are associated with several different functions; they drive processes such as protein unfolding and degradation, aggregate disassembly, transcriptional activation, microtubule depolymerization, DNA helicase activity, DNA replication initiation, and cellular cargo transport⁷. The characteristic of AAA proteins is the coupling of ATP hydrolysis to mechanical force exerted on some structure. Many AAA proteins assemble into oligomeric ring structures with a central pore, through which macromolecule substrates are "threaded"⁸. The characterized AAA protein with the closest homology to the "short" subfamily I Slfns is RecG, a bacterial helicase that has been shown to unwind replication forks and catalyze their conversion to Holliday junctions⁹.

More recently, “long” subfamily III Slfn family members have been identified by Geserick et al¹⁰. These Slfn proteins contain C-terminal motifs characteristic of superfamily I RNA helicases, including Walker A (P-loop) and Walker B motifs, the latter containing a DEAQ box, as well as several other sequences conserved in RNA helicases. Additionally, most of the subfamily III Slfns also contain a C-terminal nuclear localization domain (Figure 3-1). RNA helicases perform essential functions in RNA metabolism, as modifiers of RNA secondary structures being involved in gene specific transcription, pre-mRNA processing, RNA export, translational initiation, or RNA degradation¹¹. RNA helicases such as RIG-I and Mda-5 have been shown to be important modulators of the immune response^{12, 13}.

During the course of this thesis work, it has been noted by other research labs expression levels of Slfns are elevated after infection with the intracellular pathogens *Brucella*¹⁴ and *Listeria*¹⁰. In vitro, Slfn1 has been shown to cause arrest of fibroblast growth by inhibition of cyclin D1^{1, 15}, and is generally believed to function as a pro-apoptotic, anti-proliferative protein. However, the mechanism of this speculated function has not been uncovered, and the characterization of Slfn1 as an anti-proliferative protein has recently been contested¹⁶.

Despite the fact that the Schlafen discovery is approaching its ten-year anniversary, strikingly little has been uncovered about the molecular

function of this large protein family. Here we uncover a role of Slfn2 in binding foreign oligonucleotides and inhibiting general cellular protein translation. Additionally, we have discovered that different Slfn subfamilies may play contradictory roles in the immune response by their dissimilar effects on type I IFN production.

MATERIALS AND METHODS

Cell Lines – RAW264.7 and 293T cell lines were purchased from American Type Culture Collection. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS), 100ug/ml streptomycin and 2mM L-glutamine.

Cloning- pCIN4-Slfn2 plasmid was a generous gift from Stephen Hedrick at the University of California, San Diego. Full-length Slfn1, Slfn3, and Slfn8 cDNAs were PCR cloned from RNA prepared from LPS-stimulated RAW264.7 cells and engineered to include 5' XhoI and 3' KpnI restriction sites. Viral Slfn cDNA was a generous gift from Zehua Feng at Saint Louis University Health Sciences Center, and was engineered to include 5' XhoI and 3' KpnI restriction sites. Slfn2 was cloned into bacterially-expressing N-terminal 6xHis tagged vector, pRSETC-His, using KpnI and XhoI sites. Slfn2 was cloned into the vector, pEGFP-C1, which expresses an enhanced green fluorescent protein at the N-terminus of the

protein. All Slfn cDNA was cloned (using KpnI and XhoI insertion sites) into a mammalian expressing vector, pcDNA3.1-myc-His, which expresses a Myc and 6Xhis tag at the C-terminus of the protein. All plasmids were sequenced to ensure absence of point mutations, and were checked for expression by Western blot for the myc tag.

Transfection- 293T cells were seeded at 10^5 cells/ml in a 96 well plate and transfected 12 hours later with 100ng of total plasmid and 7.7ul Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. Transfections were proportionately scaled up as appropriate.

Bacterially-Expressed Protein Purification- Competent BL21 (DE3) E.Coli cells (Stratagene) were transformed with appropriate pRSETC-His plasmid, as per manufacturer's protocol. Transformed cells were plated on carbenicillin-containing Luria Broth (LB) agar plates and grown at 30C for 24 hours. A single isolated colony was added to a large flask containing 100ml LB + 100ug/ml carbenicillin and grown for 12 hours at 37C. LB + 100ug/ml carbenicillin (900ml) was added to culture and grown at 30C until an OD600 of 0.4 was reached. To induce protein production, Isopropyl beta-D-1-thiogalactopyranoside (IPTG; EMD Chemicals) was added at a final concentration of 0.1mM and culture was grown at 30C for 4 hours. Cells were lysed with PBS + 0.1% triton and sonicated for 6 minutes on ice in the presence of a bacterial protease inhibitor cocktail

(Promega). 6XHis-tagged protein was purified with Probond nickel-charged agarose resin (Invitrogen) as per manufacturer's instructions. Purified protein was washed 6 times with PBS+0.1% triton, eluted with 200mM imidazole. Imidazole was removed from purified protein using ICON protein concentrator columns (Promega) and checked for expression and purity by Coomassie Blue stain of polyacrylamide gel.

Mammalian-expressed Protein Purification- pcDNA3.1-myc-His plasmids containing appropriate genes were transfected into 293T cells as mentioned previously, and harvested 24 hours post-transfection. Cells were lysed in buffer containing 50mM Tris-HCL pH 8.0, 150mM NaCl, 0.1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 1mM sodium vanadate, and 1mM PMSF. His-tagged protein was purified with Probond nickel-charged agarose resin and purified, as mentioned above.

Quantitative Realtime PCR- Total RNA was extracted using Rneasy Mini Kit (Qiagen) according to the manufacturer's instructions. 100ng of RNA sample was reverse transcribed using the Omniscript Kit (Qiagen). PCR products were detected using the fluorescent dye SYBR green (Applied Biosystems). The sequences of primers used in this assay are:

B-actin forward: 5'-ACGGCCAGGTCATCACTATTG-3'

B-actin reverse: 5'-CAAGAAGGAAGGAAGGCTGGAAAAGAG-3'

Slfn2 forward: 5'-TTCACGGAGAGCGAAGGCTA-3'

Slfn2 reverse: 5'-TTGGCTCCACGATTTCACG-3'

Plasmid Electrophoretic Mobility Shift Assay (EMSA)- Standard DNA binding reactions were performed for 30 min at 37C in shift buffer (20mM HEPES-KOH pH 7.4, 100mM NaCl, 2mM EDTA, 10% glycerol, 0.01% CHAPS) containing 12.5ng/ul plasmid DNA (pRH1712) and indicated amounts of proteins (see figures) in a final volume of 20ul. Aliquots of the binding reaction were transferred into shift loading buffer (75% sucrose, 0.1% bromophenol blue), loaded onto 0.8% agarose gels containing 0.05 ug/ml ethidium bromide and separated by electrophoresis in TBE-buffer (Tris-borate pH 8.3).

Synthesis of RNA and DNA for Binding Reactions-RNA was in vitro transcribed from plasmid pcDNA3 (Invitrogen; see figure 3-7A). Forward-strand single-stranded RNA (ssRNA) was synthesized by linearizing pcDNA3 plasmid with XhoI, and in vitro transcribed using the T7 polymerase from the Riboprobe In vitro Transcription Kit (Promega), as per manufacturer's protocol. Reverse-strand ssRNA was synthesized by linearizing pcDNA3 plasmid with HindIII, and in vitro transcribed using an SP6 polymerase. 32P-CTP was incorporated into the transcription mix at a ratio of 1:10 32P-labeled CTP to unlabeled CTP. RNA was purified of proteins and free nucleotides using a nucleotide removal column (Qiagen). Double-stranded RNA (dsRNA) was synthesized by mixing equimolar

concentrations of each ssRNA strand, heating the mixture to 90°C for 2 min and slowly cooling to room temperature to facilitate annealing of complementary sequences, and subjecting the mixture to RnaseA treatment to remove any ssRNA and RNA overhang. SsRNA transcripts containing modified nucleosides were transcribed as above, with either pseudouridine-5'-triphosphate, N6-methyladenosine-5'-triphosphate, or both (TriLink Biotechnologies, San Diego). The modified nucleoside was included in the in vitro transcription reaction mixture at a ratio of 1:10 with its respective unmodified nucleoside.

SsDNA for DNA-binding EMSAs was synthesized by single-primer PCR of the pcDNA3 MCS. PcDNA3 plasmid was linearized with HindIII and a PCR reaction was performed with a primer to the SP6 promoter region. DsDNA was synthesized by a standard PCR reaction of the pcDNA3 MCS, using primers to the T7 and SP6 promoter regions. 32P-dCTP was incorporated into PCR reactions at a ratio of 1:10 32P-labeled dCTP to unlabeled dCTP. DNA was purified of proteins and free nucleotides using a nucleotide removal column (Qiagen). RNA and DNA was quantified by spectrophotometry after removal of protein and free nucleotides.

Electrophoretic Mobility Shift Assays for RNA and DNA Binding-

Binding reactions were performed at 4°C for 30 min in EMSA buffer (43mM Tris-HCl pH 8.0, 50mM KCl, 8% glycerol, 2.5mM DTT, 50ug/ml tRNA (Sigma-

Aldrich) or 50ug/ml sonicated salmon DNA in the RNA or DNA EMSAs respectively, 0.5 units/ul Rnase inhibitor) with 12ng of DNA or RNA transcript and indicated amounts of purified protein in a final volume of 20ul. EMSA loading buffer (50% glycerol, 50ug/ml bromophenol blue, 50ug/ml xylene cyanol) was added to reactions and run at 150V at 4C for 3 hours on a 5% (79:1 acrylamide/bis) native gel (5% glycerol, 375mM Tris-HCl pH 8.3, 0.1% APS, 0.05% TEMED) with running buffer (25mM Tris-borate pH 8.3).

Immunofluorescence- For direct visualization of transfected Slfn2, 293T cells on coverslips were transfected with Slfn2-pEGFP-C1. 24 hours post-transfection, coverslips were washed with PBS, fixed with 4% paraformaldehyde, and visualized by confocal microscopy. For indirect visualization of Slfn2, 293T cells on coverslips were transfected with Slfn2-pcDNA3.1-myc-His. 24 hours post-transfection, coverslips were washed with PBS, permeabilized with PBS/0.2% TX100 and blocked with 10% goat serum. After rinsing and incubation with anti-myc-biotin (9E10) in PBS/0.05% Tween20/3% BSA, cells were incubated with streptavidin-FitC and, after rinsing, overlaid with Vectashield (Vector labs). Protein localization was visualized by confocal microscopy.

In vitro translation Inhibition- In vitro translation reactions were performed using the Rabbit Reticulocyte Lysate System (Promega) with in

vitro transcribed GFP mRNA, as per manufacturer's protocol. To test the effects of Slfn2 on protein translation, Slfn2 or Stat3 protein purified from 293T cells was added to the in vitro translation reaction mixture in concentrations indicated (see figure).

Luciferase Assay- 293T cells were seeded at 10^5 cells/ml in a 96 well plate (200ul/well), with every condition in triplicate. Cells were transfected using Lipofectamine 2000 12 hours later with a mixture of plasmids: 40ng luciferase reporter plasmid, 40ng TK-renilla plasmid, 20 ng of each expression vector. If cells were to be stimulated with dsDNA then 100ng of polydA:dT (Amersham Biosciences) was added to the plasmid mix. An empty vector, pRH1712, was used to equalize the amount of DNA in each transfection condition. If cells were to be infected by Sendai virus (300HA U/ml), infection was performed 24 hours post-transfection and cells were harvested 12 hours post-infection. Cells were lysed by passive lysis buffer (Promega) 36 hours post-transfection. Luciferase reporter and SV40-renilla activities were quantified by the Dual Luciferase System (Promega) according to the manufacturer's protocol.

RESULTS AND DISCUSSION:

Schlafen 2 is upregulated in response to LPS and type I Interferon

Our interest in the IRF3 pathway instigated a search for other genes downstream of IRF3 activation, thus we performed a microarray expression profile on LPS-stimulated WT and IRF3^{-/-} peritoneal macrophages. This revealed several members of the poorly characterized Schlafen gene family to be downstream of IRF3 signaling. Our analysis by qPCR revealed that all Slfn genes tested (1-4) were upregulated by LPS and IFN β , with Slfn2 being the most highly induced. A timecourse of Slfn2 expression upon LPS and IFN β stimulation showed a strong, early response and a late, sustained response (Figure 3-2), indicating an important function of Slfn2 in the immune response. To confirm that Slfn2 expression is downstream of the IFN-mediated Jak/STAT signaling pathway, we used mice deficient in expression of IFNAR1/2-associated kinase tyk2 and transcription factor STAT1. A qPCR analysis of Tyk2^{-/-} and STAT1^{-/-} murine peritoneal macrophages stimulated with IFN β and revealed that Slfn2 expression was nearly ablated in these mice (Figure 3-3). Slfn2 gene expression downstream of LPS and IFN signaling has more recently been confirmed by Geserick, et al¹⁰.

Schlafen2 binds to plasmid DNA in vitro

The most revealing clue to the molecular function of Slfn lies in the divergent AAA domain possessed by all Slfn proteins. Many AAA proteins

have a role in DNA processes, such as replication, transcription and repair^{7, 8}. We found that the closest homolog to Slfn2 is a bacterial “putative regulator of transcription”, and the closest characterized AAA homolog to Slfn2, RecG, is involved in DNA recombination and repair in vivo⁹. Thus, we sought to detect whether Slfn2 functions in a DNA-related process by ascertaining its ability to bind to DNA. To do so, we performed a DNA gel retardation assay with bacterially-expressed His-tagged purified Slfn2 protein. Prmt1, a protein that does not bind DNA in vivo or in vitro, was used as a negative control and purified in an identical manner to Slfn2 protein. Binding reactions were performed with 250ng plasmid DNA (2.9kb) and varied concentrations of purified Slfn2 or Prmt1 protein. In the absence of MgCl₂, we observed no DNA gel retardation with Slfn2 protein (Figure 3-4A), whereas in the presence of 10mM MgCl₂, we saw a shift in plasmid mobility in a Slfn2 protein dose-dependent manner (Figure 3-4B). The molar ratio of plasmid DNA to protein is 1:200 and 1:1000 in the 1uM and 5uM protein conditions, respectively. We also observed binding when using Slfn2 protein purified from transfected human 293T cells (data not shown). Intriguingly, we consistently observed a characteristic DNA banding pattern over the course of several DNA binding assays. We set out to investigate whether the observed banding pattern is the result of Slfn2 binding, which would cause the DNA to be less mobile and therefore

migrate more slowly, or whether the addition of Slfn2 permanently alters the structure of plasmid DNA. To do so, we performed the DNA binding assay as described above, but then subjected the reaction to phenol chloroform extraction in order to remove any protein in the sample before loading onto the agarose gel. Once again, we observed the same banding pattern, indicating that the addition of Slfn2 permanently alters the plasmid DNA (data not shown). Although it is tempting to conclude that Slfn2 exhibits topoisomerase activity *in vitro*, more evidence is necessary to draw this conclusion as the permanent change in plasmid DNA may simply be due to Slfn2 binding to the DNA, making it more susceptible to trace nuclease contaminants. Our results show that Slfn2 protein facilitates a retardation of plasmid DNA at the most dilute protein concentration tested, which is a 200-fold excess of protein to DNA.

Schlafen 2 binds RNA and DNA *in vitro*

During the course of the DNA retardation experiments, we noticed that Slfn2 protein purified from BL21 E.Coli cells produced a nucleic acid smear that migrated faster than the plasmid DNA on an agarose gel (Figure 3-5). This nucleic acid smear was never seen with control protein Prmt1 and was seen when Slfn2 protein was electrophoresed in the absence of plasmid DNA (Figure 3-5). To determine the identity of the nucleic acid that Slfn2 protein binds to in bacteria, we initially treated Slfn2

purified protein with RnaseH, which degrades RNA in RNA:DNA hybrids, and to DNase. Upon these treatments, the nucleic acid persisted (data not shown). However, upon RNaseONE treatment, which degrades both single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA), we saw the nucleic acid disappear (Figure 3-6). Intriguingly, our results indicate that Slfn2 protein binds to bacterial RNA *in vitro*. Preparation of Slfn2 protein in mammalian cells, however, revealed no RNA binding, even under the least stringent lysis conditions (data not shown). To confirm the RNA-binding ability of Slfn2, we performed an electrophoretic mobility shift assay (EMSA) with mammalian-purified Slfn2 and ssRNA (12ng of a 150 base transcript) transcribed from the multiple cloning site (MCS) of pcDNA3 (Figure 3-7A). We witnessed binding of Slfn2 to ssRNA in the presence of $MgCl_2$ when protein was present at ~30 fold molar excess of protein (corresponding to 0.4uM Slfn2 protein), whereas in the absence of $MgCl_2$, Slfn2 binding was not observed until protein was present at ~60 fold excess (corresponding to 0.8uM Slfn2 protein) (Figure 3-7B). Isg15, a protein that does not have RNA binding ability, was used as a negative control and did not bind ssRNA at any concentration tested (Figure 3-7C). Additionally, an EMSA with 12ng of dsRNA (150bp double-stranded transcript) demonstrated that Slfn2 also binds to dsRNA, appearing to be less robustly than ssRNA, but at ~15-fold excess of Slfn2 protein

(corresponding to 0.4 μ M protein)(Figure 3-7D). An EMSA with 12ng of ssDNA or dsDNA was also performed. Slfn2 clearly binds both DNA structures beginning at 15-fold molar excess of Slfn2 protein for ssDNA binding (corresponding to 0.2 μ M protein) and 30-fold molar excess of Slfn2 protein for dsDNA binding (corresponding to 0.2 μ M protein) (Figure 3-8), thus corroborating our initial results.

Schlafen2 is exclusively cytoplasmic

Based on the nucleic acid binding capability activity of Slfn2, we hypothesized that Slfn2 may play a role in transcription, and we therefore sought to determine its cellular localization. Slfn2 cDNA was cloned into EGFP-C1 and the plasmid was transfected into 293T cells. Surprisingly, confocal microscopy showed that Slfn2 protein appears to be cytoplasmic in both unstimulated and LPS or IFN β stimulated cells. To exclude the possibility that nuclear translocation is impeded by the large GFP tag, we repeated the localization with myc-tagged pcDNA3.1-Slfn2, probed with anti-myc (9E10) antibody and again saw, by confocal microscopy, that Slfn2 is localized exclusively in the cytoplasm (Figure 3-9). The cytoplasmic localization of Slfn2 has recently been confirmed by Neumann et al¹⁷. Therefore, the cellular location of Slfn2 in unstimulated and LPS- and IFN-stimulated cells makes a direct role of Slfn2 in transcriptional processes unlikely under these conditions.

Eukaryotic-specific RNA modifications suppress the RNA-binding ability of Slfn2

Pathogenic infection is detected by cellular sensors as “foreign molecules” and initiates innate immune responses, including the induction of IFN alpha/beta and proinflammatory cytokines. Recent studies indicate at least two distinct pathways for the induction of type I IFN by viral infection¹⁸. Toll-like receptors (TLRs) are extracellular or endosomal PRRs for microbial pathogens, whereas retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) are novel intracellular PRRs for viral dsRNA. Recent advances in cytoplasmic virus sensors highlight their essential role in the induction of innate immunity and their discrimination between “self” and “non-self” nucleic acids¹⁸. Our discovery that Slfn2 is an exclusively cytoplasmic protein, upregulated by IRF3 and STATs, and able to bind DNA and bacterial (but not mammalian) RNA, led us to hypothesize that Slfn2 may function as a detector of foreign nucleic acid.

Eukaryotic RNA undergoes nearly one hundred different nucleoside modifications¹⁹, whereas bacterial mRNA undergoes no nucleoside modification, thus providing a feature for which mammalian cells to discriminate between host and pathogen RNA. In vitro transcripts and bacterial RNA, have been shown to have a stimulatory effect on the

innate immune system, while eukaryotic RNA does not²⁰. Kariko et al showed that incorporation of modified nucleosides, m5C, m6A, s2U, or pseudouridine ablates signaling activity through RNA-sensing Toll-like receptors, TLR3, TLR7 and TLR8²¹. Additionally, cytoplasmic RNA sensors PKR and RIG-I exhibit decreased binding and function when presented with modified RNA^{22, 23}. To test whether Slfn2 also exhibits specificity for unmodified nucleic acid, we incorporated modified nucleosides, m6A and pseudouridine, into in vitro transcribed ssRNA and performed an EMSA with increasing amounts of Slfn2 protein. The modified nucleosides were added at a 10-fold lower concentration than the analogous unmodified nucleoside. It has previously been shown in this system that the polymerase enzyme utilizes modified NTPs almost as efficiently as the basic NTPs, and that incorporation of modified nucleoside phosphates into RNA is proportional to the ratio contained in the transcription reaction²¹. Thus, we can reasonably assume that our m6A and pseudouridine -modified transcripts contain roughly 10% modified adenosine and 10% modified uridine, respectively. The amount of some modified nucleosides in mammalian mRNA has been estimated to be 3-5 nucleosides per mammalian mRNA molecule²⁴, therefore the extent of modification in our 150 base transcript should be roughly comparable to that of cellular mammalian mRNA. Strikingly, we found that Slfn2 binding

to either m⁶A or pseudouridine modified ssRNA is decreased, dramatically so when both modified nucleosides are incorporated into the in vitro transcript (Figure 3-10).

Schlafen 2 is a potent inhibitor of protein translation

Inhibition of protein translation has long been proposed as a defense strategy for the host cell. Several years ago, Hunt and Ehrenfeld demonstrated for the first time the inhibition of protein synthesis in cell-free rabbit reticulocyte lysates by dsRNA extracted from poliovirus-infected HeLa cells^{25, 26}. Around the same time, a similar type of inhibition of protein synthesis by dsRNA in cell-free systems prepared from IFN-treated cells was demonstrated^{27, 28}. Intriguingly, when cell-free systems were prepared from IFN-treated, vaccinia virus infected cells, translational inhibition occurred in the absence of dsRNA²⁹. More recently, some of the effectors of pathogen-mediated translation inhibition have been identified. Double-stranded RNA dependent PKR is upregulated by IFN, and functions to phosphorylate and inhibit EIF2 α , a eukaryotic translation initiation factor, thus halting protein translation and allowing the cell to reconfigure gene expression³⁰. Additionally, 2'5'-OAS inhibits protein synthesis through activation of the endoribonuclease, RnaseL, which degrades RNA³¹. Both nucleic-acid binding proteins, PKR and OAS/RnaseL, have been shown to halt protein translation in a cell-free

extract system³². In a series of luciferase assays to test Slfn2-mediated effects on specific gene expression, it was noted that Slfn2-transfected cells displayed an overall decrease in expression of the internal control plasmid. To test whether Slfn2 inhibits protein translation, we used the cell-free extract protein translation system that was used to characterize the function of PKR and RnaseL³². We reasoned that there may be a potential requirement of nucleic-acid binding on Slfn2 activity, hence we capitalized on the fact that bacterial preparations of Slfn2 protein bind tightly to RNA (Figure 3-5), and therefore used Slfn2 protein produced in this manner. Compared to the negative control Stat3, a protein that does not inhibit protein translation, the addition of Slfn2 purified protein to the assay resulted in significant translation inhibition in a dose-dependent fashion (Figure 3-11A). As mentioned, it was originally noticed that Slfn2 over-expressing cells displayed an inhibitory effect on protein expression. We saw a striking decrease of renilla-luciferase expression upon transient transfection of Slfn2, which is reversed upon the addition of viral Slfn-expressing plasmid to the transfection (Figure 3-11B).

Schlafen2 does not appear to function as an anti-proliferative protein

Upon detecting pathogenic infection, the host cell machinery launches a complex counter-attack, which serves to alert neighboring cells to infection, recruit immune cells to the site, and establish an

autonomous anti-viral state. Production of type I IFN by the infected cell allows the induction of hundreds of genes, all functioning with strikingly complex molecular mechanisms to mount an immune response. An important function of IFNs is to establish a pro-apoptotic state in target cells, helping to remove infected cells³⁴. Upon establishing that Slfn2 preferentially binds to (“senses”) foreign nucleic acids, we next directed our attention to uncovering the effector function of Slfn2 in the cell. Schlafens 1-3 have been implicated in cell cycle arrest^{1, 15}, thus we hypothesized that Slfn2 acts as a pathogenic sensor and sends a signal to the infected cell to stop proliferating. Contrary to initial reports on Slfn, we have never seen an appreciable decrease in cell number in Slfn2 transfected, overexpressing cells, including those that have been transfected with foreign nucleic acids. A recent report on Slfn1 and Slfn2 also claimed to be unable to reproduce the initial findings of an anti-proliferative function of these Slfn family members¹⁶. Taken together, at least in the contexts tested, the main function of Slfn2 in cellular defense does not appear to be anti-proliferative.

Slfns 2 and 3 attenuate the host response to cytoplasmic dsDNA and viral infection

In response to virus infections, viral components such as RNA and DNA are recognized by TLRs and cytoplasmic sensors, such as RIG-I and

Mda5, and cells are activated to produce type I interferons (IFNs). Upon binding to RNA, the interferon-inducible DEAD box-containing RNA helicases, RIG-I and Mda5, bind to MAVs and activate transcription factors IRF3 and NF- κ B, which then translocate to the nucleus where they promote transcription of interferon beta³⁵. To determine whether Slfn functions similarly to other nucleic acid sensors, we performed luciferase assays in collaboration with the Dr Kate Fitzgerald laboratory at the University of Massachusetts. At this point, we also decided to expand our focus to other Slfn family members. Therefore, we cloned Slfns 1,3,8, and viral Slfn into mammalian expression vectors with different tags and confirmed expression of all constructs. In order to test whether Slfn proteins facilitate transcriptional induction of IFN beta, we utilized a luciferase reporter spanning the entire IFNb promoter region, including all four Positive Regulatory Domains (PRDs) (Figure 3-12A), and co-transfected 293 cells with the luciferase reporter, renilla internal control plasmid, and different Slfn constructs. The specific effects of Slfns on IFN promoter stimulation were separated from their general translation inhibitory effects by normalizing IFN-luciferase activity to renilla-luciferase expression. Surprisingly, after normalizing luciferase expression, the short Slfn1 and 2, and the medium length Slfn3, as well as the viral Slfn *attenuated* the induction of the IFNb luciferase by transfected dsDNA

(Figure 3-12B). The longer form, Slfn8, seemed to have little effect (Figure 3-12A). Next we infected cells with Sendai virus, a single-stranded RNA virus that replicates in the host cytoplasm, and induces IFN in a TLR-independent, RIG-I mediated manner³⁶. Sendai virus infection produced similar results of IFN β promoter inhibition by Slfns 2 and 3 (Figure 3-12C). To test which PRD elements of the promoter were subject to inhibition by the Slfn proteins, we employed luciferase reporters representing the PRD regions individually. Neither the NF κ B responsive PRD-II luciferase (Figure 3-13A), nor the AP1-controlled PRD-IV reporter (not shown) were affected by any of these Slfn proteins, whereas the IRF3-dependent PRD-III/PRD-I luciferase reproduced the response pattern of the entire IFN β promoter (Figure 3-13B). When we performed the assay with different combinations of Slfn constructs, we saw that Slfn2 and Slfn3 synergistically inhibited IFN β upregulation, whereas when either Slfn2 or Slfn3 was combined with Slfn8, the inhibitory effect was reversed (Figure 3-13C).

To determine whether Slfn acts within the IRF3 pathway to modulate IFN β induction, the effect of Slfn expression in stimulation-independent activation of an ISRE luciferase, achieved through over-expression of the membrane proximal adaptor TRIF was tested. We found that TRIF-mediated activation of the ISRE luciferase was subject to inhibition by the Slfn 2+3 combination (Figure 3-14A). To determine if Slfn proteins act in the

IRF3 activation cascade rather than affecting transcriptional responses at the level of co-activator availability or enhancer region accessibility, the constitutively active form of IRF3, IRF3-5D, was used to stimulate ISRE-luciferase expression. We found that the IRF3-5D facilitated response was resistant to Slfn modulation (Figure 3-14B), leading us to the conclusion that Slfn proteins act upstream of IRF3 activation. This notion is further supported by the fact that induction of the NFkB luciferase via overexpression of MyD88 is not altered in the presence of Slfn proteins (Figure 3-14C).

Our results indicate that Slfn2 and Slfn3 downregulate the host cell response to pathogenic invasion by preventing overproduction of IFNs, which, if left uncontrolled, could have negative consequences for the host. There exists an important balance between host defense and overactivation resulting in chronic inflammation and autoimmune disease, and the presence of tightly controlled negative feedback mechanisms to maintain this balance has been well documented. Influenza virus NS1 protein has been shown to sequester dsRNA, thereby inhibiting the TLR-independent induction of IFN-alpha by influenza infection³⁷. Interestingly, a DEAD-box RNA helicase, Lgp2, binds to ssRNA and dsRNA in vitro and was recently shown to antagonize RIG-I- and MDA5-mediated signaling by forming heterodimeric complexes with these proteins³⁶.

In summary, our results suggest two different roles for Slfn2. On the one hand, Slfn2 acts a sensor of foreign nucleic acids, binding foreign RNA and DNA, and instigating the halt of protein translation *in vitro*, a strategy used by other host proteins to counter viral infection. On the other hand, Slfn2 seems to downregulate the immune response by inhibiting the transcription of Interferon stimulated genes (ISGs). However, these apparently incongruous functions may not be so paradoxical in the context of the immune response, as the host cell goes to great lengths to prevent overactivation of the defense response to pathogens^{34, 38, 39}. The observation that the “long” Slfn8 reverses the inhibitory activity of the shorter Slfn forms suggest that, perhaps, Slfns form complexes made up of different members, depending on the specific stimulus. Forming oligomers, in fact, is characteristic of AAA domain containing proteins⁵ and we have seen in preliminary experiments that Slfns 1,2,3, and 8 co-immunoprecipitate in various combinations (data not shown). It is possible that complexes with the longer Slfn members activate an immune response to pathogens, while complexes excluding these longer forms are the downregulators of the immune response. It is reasonable to presuppose that specific Slfns are up and downregulated at different time intervals, thus giving the cell time to respond to and counter infection and then turning the response off before it becomes damaging to the cell. As

we showed in Figure 3-2, Slfn2 expression in response to IFN and LPS is biphasic: an early robust response and a late, sustained response. It is possible that the initial response to infection stimulates the particular complex of Slfn family members that participate in host defense, while the second phase of gene expression stimulates the complex of Slfns that attenuate the response. Perhaps the inhibitory effect of Slfn2 on protein translation facilitates a reconfiguration of gene expression for the new phase of Slfns. A detailed temporal analysis of upregulation of all Slfn genes may provide insight into the role that different Slfn complexes play in the immune response.

Chapter three, entitled 'the role of the Schlafen protein family in the IRF-3 pathway', is a collaborative effort with Dr Martina Severa. I am the primary author of this research project and directed and supervised the research which forms the basis of this chapter.

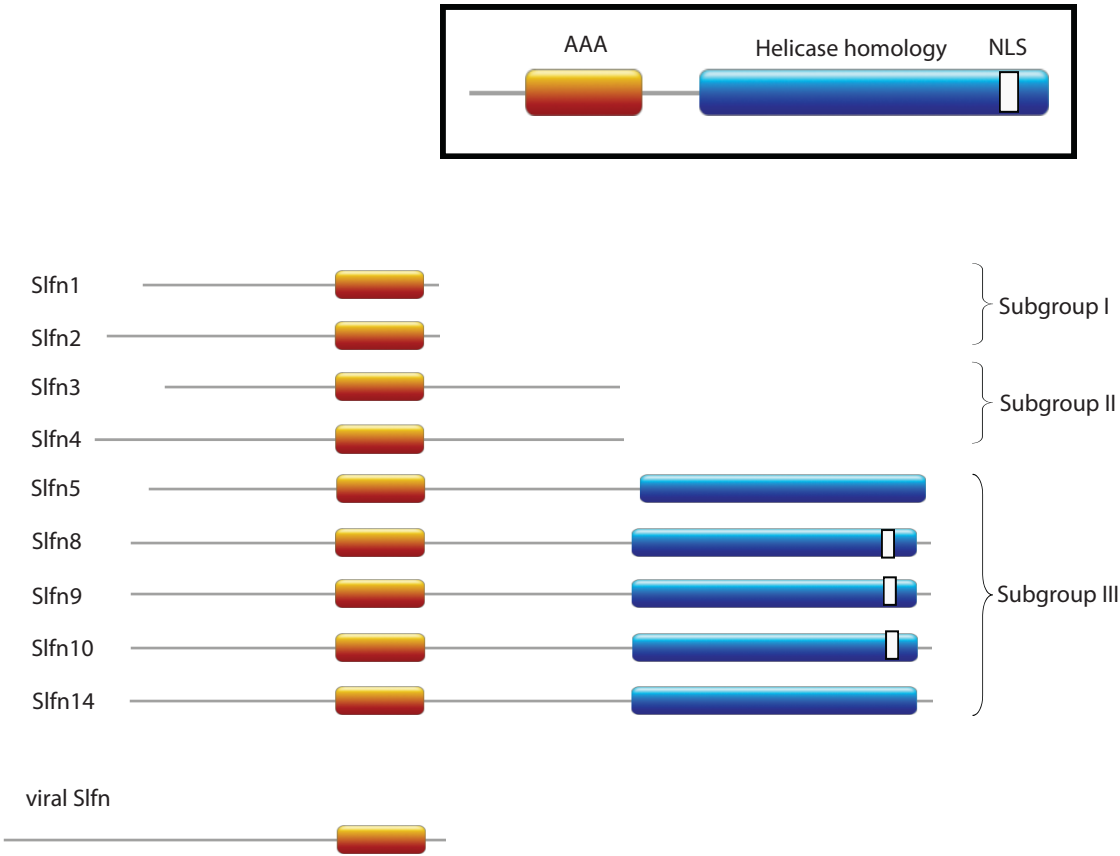


Figure 3-1. Schlafen family members and putative domains.

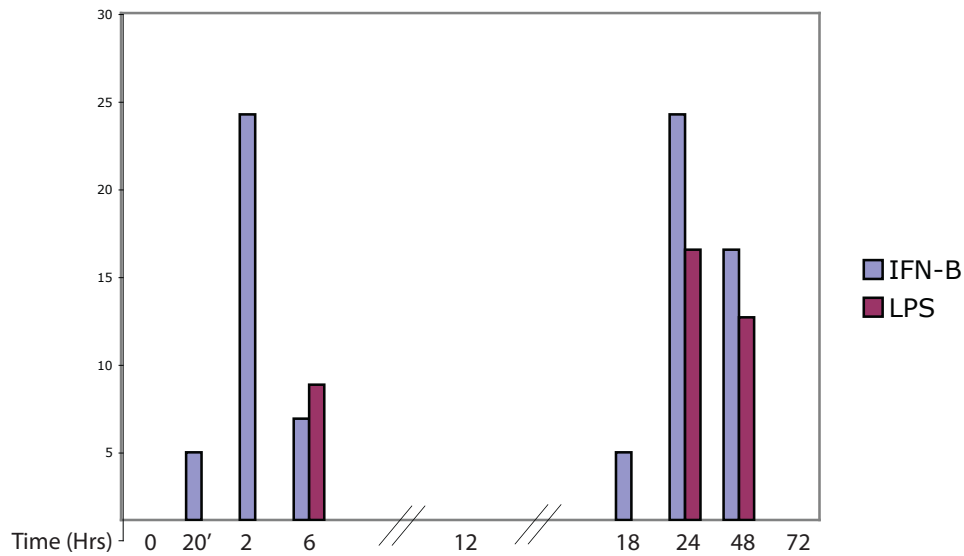


Figure 3-2. Slfn2 gene expression is induced by LPS and IFNbeta with a strong, early response and a late, sustained response. RAW 264.7 macrophages were stimulated with 100ng/ml LPS or 1000U IFNbeta for the indicated times. Total RNA was collected and Slfn2 mRNA level was measured by qPCR and is normalized to beta actin mRNA levels.

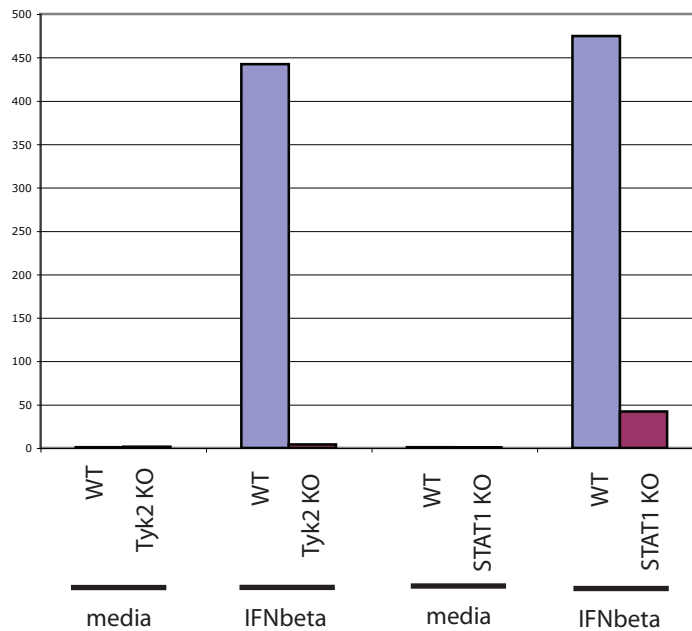


Figure 3-3. IFN-mediated Slfn2 gene induction is downstream of Tyk2 and STAT1. Peritoneal macrophages were harvested from Tyk2 or STAT1 knockout mice and their respective wild type littermate controls. Cells were stimulated with 1000U IFNbeta for 6 hours, total RNA was collected and Slfn2 mRNA levels measured by qPCR. Results are normalized to beta actin gene expression.

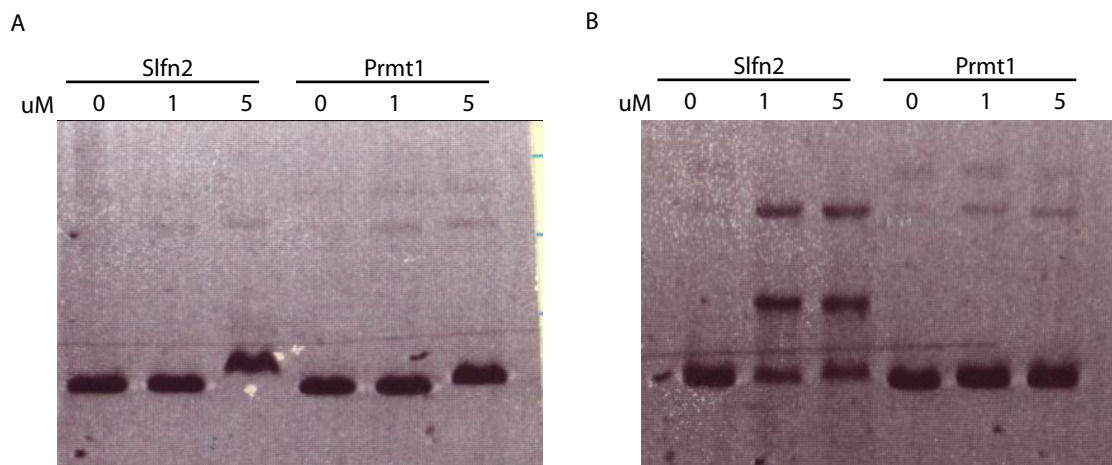


Figure 3-4. SLFN2 binds to plasmid DNA in the presence of Mg^{2+} *in vitro*. Slfn2 or Prmt1 protein was purified from BL21 E.Coli using a 6xHis-Nickel affinity resin system as described in 'materials and methods'. DNA binding reactions were performed for 30min at 37C in shift buffer (20mM HEPES-KOH pH 7.4, 100mM NaCl, 2mM EDTA, 10% glycerol, 0.01% CHAPS) containing 12.5ng/ul plasmid DNA (pRH1712) and in the (A) absence or (B) presence of 10mM $MgCl_2$. Protein was added at the indicated concentrations. The entire binding reaction was run on a 0.8% agarose gel containing 0.05ug/ml ethidium bromide. The calculated molar ratio of plasmid DNA molecules to protein molecules is ~1:200 and ~1:1000 in the 1uM and 5uM protein conditions, respectively.

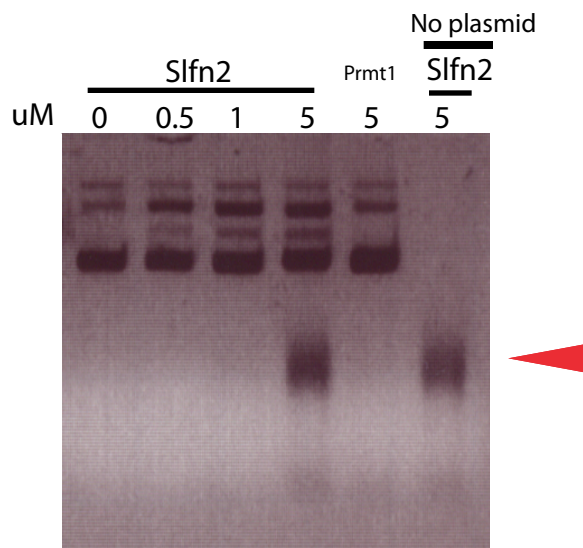


Figure 3-5. Purified Slfn2 binds to unidentified nucleic acid *in vitro*. Plasmid DNA binding assay was performed as above. Red arrow indicates unidentified nucleic acid bound by purified Slfn2 protein.

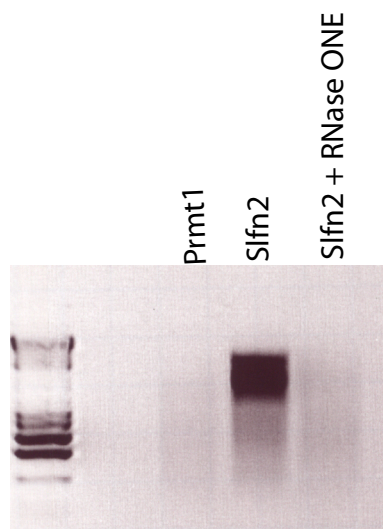


Figure 3-6. Slfn2 protein binds to bacterial RNA in vitro. Slfn2 protein was produced and purified from BL21 E.Coli cells using a 6xHis-Nickel affinity resin system as described in 'Materials and Methods'. Purified protein eluted from nickel resin was incubated with 20U RNaseONE (Promega) for 30minutes at room temperature. Products of the reaction were loaded onto a 0.8% agarose gel containing 0.05ug/ml ethidium bromide. DNase and RNaseH were ineffective at removing the bound nucleic acid (not shown).

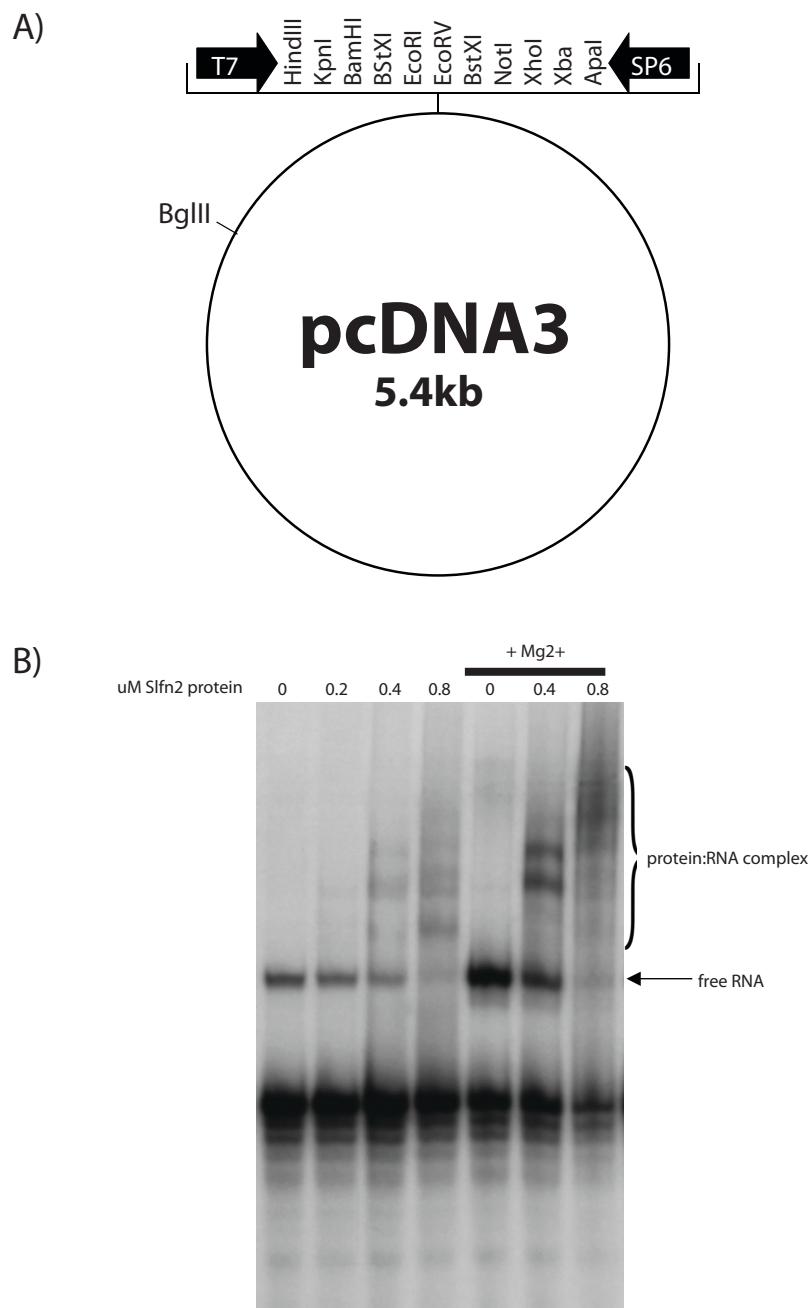


Figure 3-7. Slfn2 binds single stranded RNA (ssRNA) and double-stranded RNA (dsRNA).

Electrophilic mobility shift assays were performed with RNA in vitro transcribed from (A) plasmid pcDNA3 as described in Materials and Methods. Binding reactions were in standard shift buffer and were performed at 4C for 30 minutes. Proteins were purified from BL21 E.Coli using a 6XHis-nickel affinity resin system. B) 12ng of ssRNA (150 bases) and indicated concentrations of Slfn2 protein were added to the binding reaction in the presence or absence of 10mM MgCl₂. 15-fold, 30-fold, and 60-fold molar excess of Slfn2 protein to ssRNA was present in binding reactions with 0.2uM, 0.4uM and 0.8uM protein, respectively.

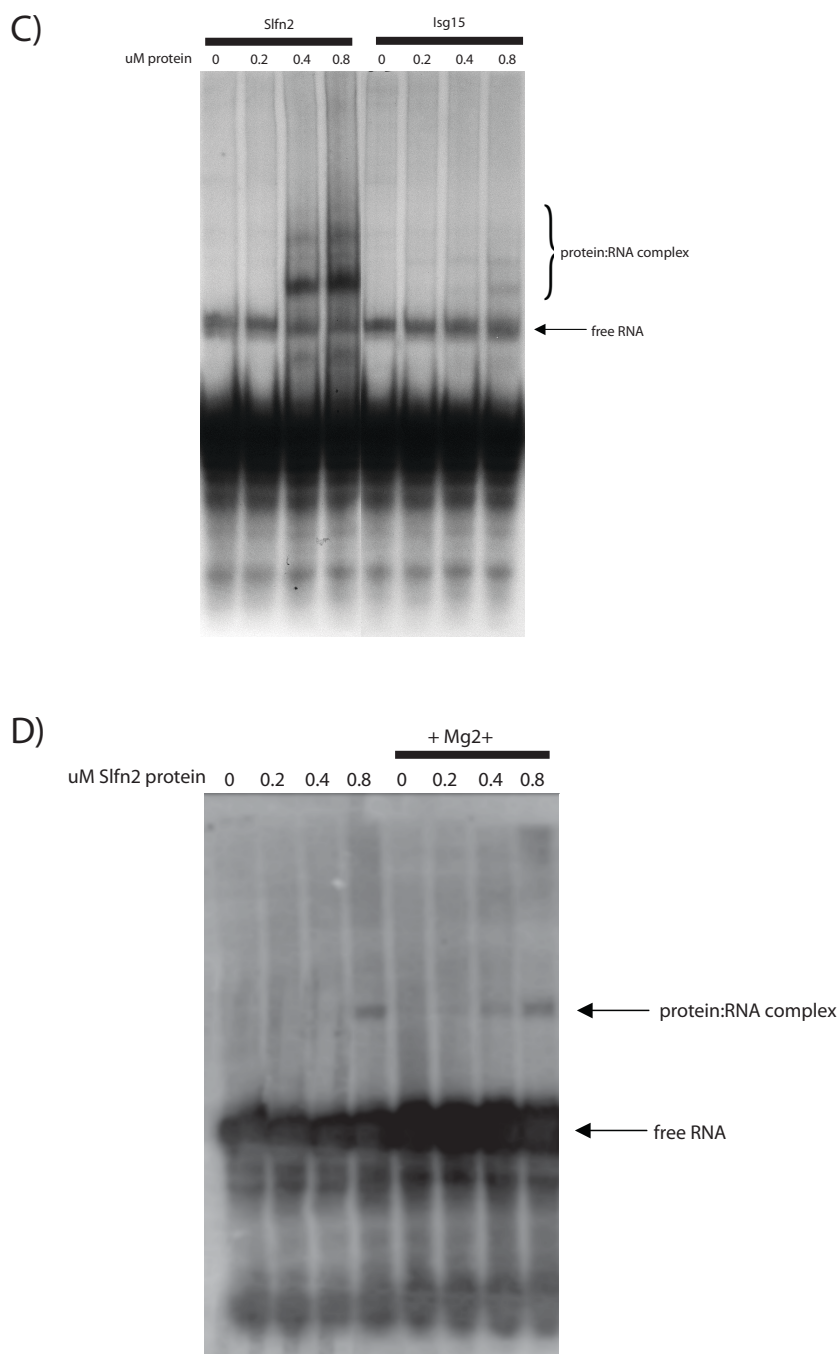


Figure 3-7. Slfn2 binds single stranded RNA (ssRNA) and double-stranded RNA (dsRNA), Continued. (C) 12ng of ssRNA (150 bases) and indicated concentrations of Slfn2 protein or control (Isg15) protein were added to the binding reaction in the presence 10mM MgCl₂. 15-fold, 30-fold, and 60-fold molar excess of Slfn2 or Isg15 protein to ssRNA was present in binding reactions with 0.2uM, 0.4uM and 0.8uM protein, respectively. (D) 12ng of 150 bp double-stranded RNA (dsRNA) and indicated concentrations of Slfn2 protein were added to the binding reaction in the presence 10mM MgCl₂. 8-fold, 15-fold, and 30-fold molar excess of Slfn2 protein to dsRNA was present in binding reactions with 0.2uM, 0.4uM and 0.8uM protein, respectively.

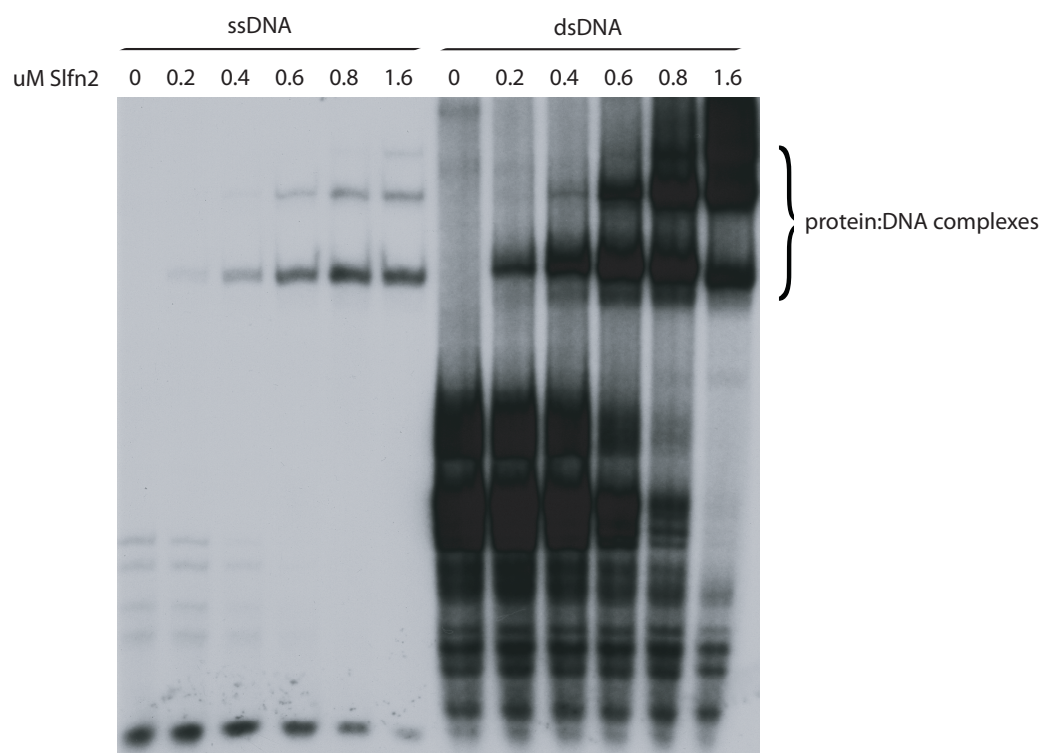


Figure 3-8. Slfn2 binds single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) in vitro. DNA was synthesized from the pcDNA3 multiple cloning site by single primer PCR (ssDNA) or two-primer PCR (dsDNA), as described in Materials and Methods. Binding reactions were performed as described with 12ng of either ssDNA (~150 bases) or dsDNA (~150bp) and indicated amounts of Slfn2 protein purified from transfected mammalian 293T cells using a 6XHis-nickel resin affinity system. Protein was present in approximately 15-fold, 30-fold, 45-fold, 60-fold, and 120-fold molar excess to ssDNA in reactions corresponding to 0.2uM, 0.4uM, 0.6uM, 0.8uM, and 1.6uM protein respectively. Protein was present in approximately 30-fold, 60-fold, 90-fold, 120-fold, and 240-fold molar excess to dsDNA in reactions corresponding to 0.2uM, 0.4uM, 0.6uM, 0.8uM, and 1.6uM protein respectively.

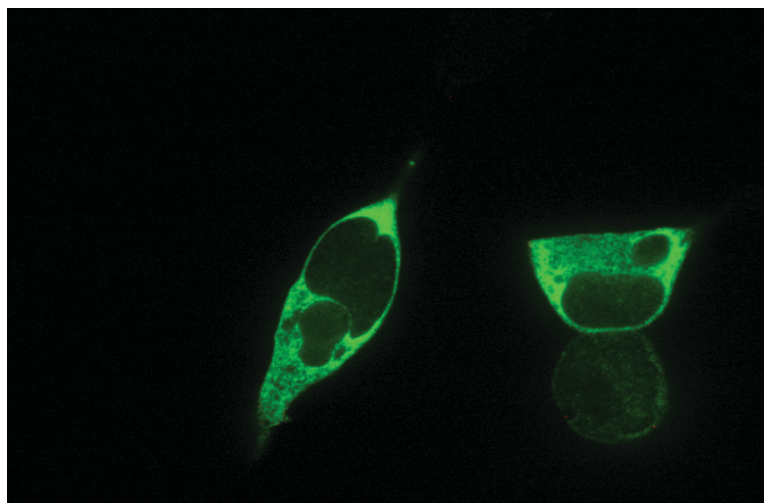


Figure 3-9. Slfn2 localizes exclusively to the cytoplasm. 293T cells were transfected with Slfn2-pcDNA3.1-myc-His. 24 hours post-transfection, coverslips were washed with PBS, permeabilized with PBS/0.2% TX100 and blocked with 10% goat serum. After rinsing and incubation with anti-myc-biotin (9E10) in PBS/0.05% Tween20/3% BSA, cells were incubated with streptavidin-FitC. Slfn2 protein localization was visualized by confocal microscopy.

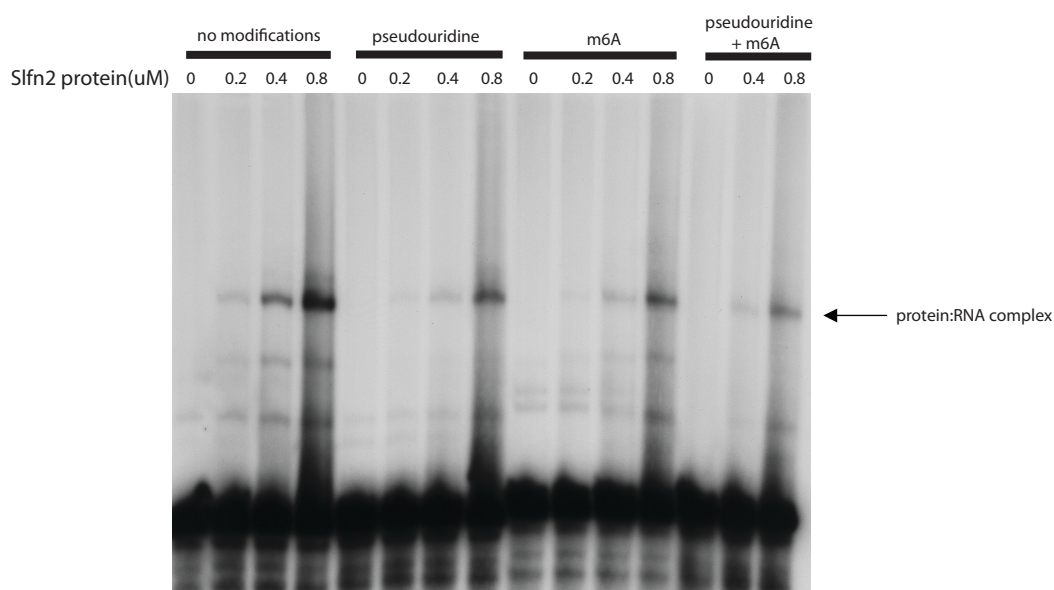


Figure 3-10. Nucleoside modifications of in vitro transcribed ssRNA decrease binding by Slfn2. ssRNA transcripts containing modified nucleosides were in vitro transcribed as described previously, with either pseudouridine-5'-triphosphate, N6-methyladenosine-5'-triphosphate (m6A), or both. The modified nucleoside was included in the in vitro transcription reaction mixture at a ratio of 1:10 with its respective unmodified nucleoside. Binding reactions were performed with 12ng ssRNA and indicated Slfn2 protein concentrations. Electrophoretic Mobility Shift Assay (EMSA) was performed as described previously.

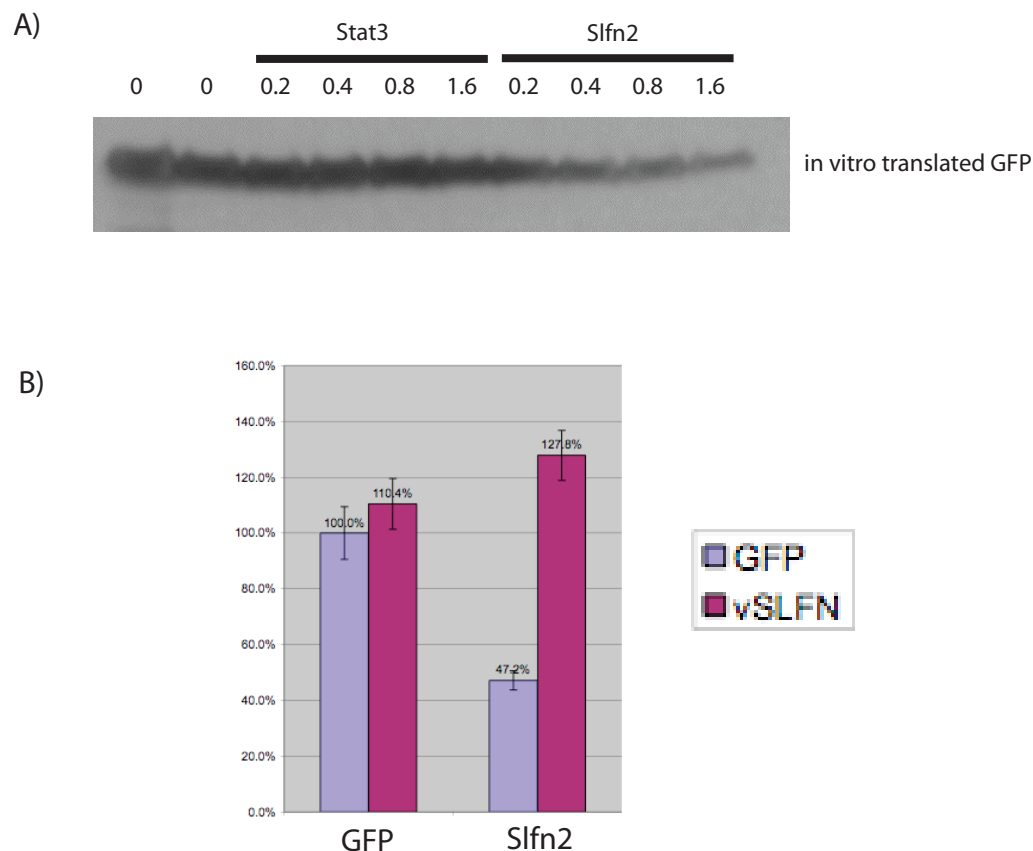


Figure 3-11. Slfn2 inhibits protein translation. (A) In vitro translation reactions were performed using the Rabbit Reticulocyte Lysate System (Promega) with in vitro transcribed GFP mRNA, as per manufacturer's protocol. To test the effects of Slfn2 on protein translation, Slfn2 or Stat3 protein purified from 293T cells was added to the in vitro translation reaction mixture in concentrations indicated. Slfn2 and control (Stat3) protein were purified from transfected 293T cells using a 6XHis-nickel resin affinity purification system, as described previously. (B) 293T cells were transfected with either pcDNA3.1-GFP or pcDNA3.1-Slfn2, in combination with either pcDNA3.1-GFP or pcDNA3.1-vSlfn (viral Slfn). Renilla-luciferase reporter plasmid was included in all transfections. Cells were lysed with passive lysis buffer (Promega Dual Luciferase Kit) 24 hours post-transfection and renilla-luciferase protein expression was quantified with a luminometer.

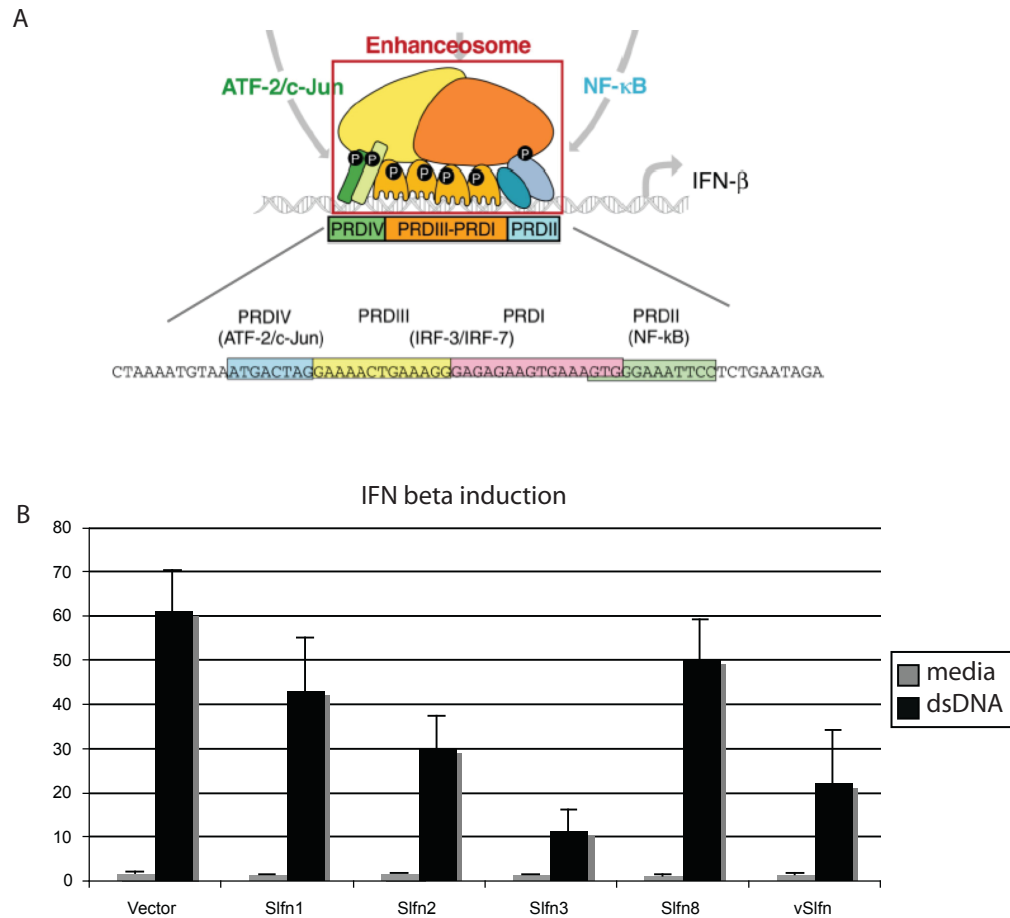


Figure 3-12. Slfn proteins modulate IFNbeta induction in response to dsDNA stimulation.

(A) The IFN beta promoter is composed of binding sites for multiple transcription factors. (B) 293T cells were seeded at 10^5 cells/ml in a 96 well plate. Cells were transfected using Lipofectamine 2000 12 hours later with a mixture of plasmids: 40ng luciferase reporter plasmid, 40ng TK-renilla plasmid, 20 ng of each expression vector. dsDNA-stimulation conditions included 100ng of polydA:dT (Amersham Biosciences) added to the plasmid mix. An empty vector, pRH1712, was used to equalize the amount of DNA in each transfection condition. Cells were lysed by passive lysis buffer (Promega) 36 hours post-transfection. Luciferase reporter and Tk-renilla activities were quantified by the Dual Luciferase System (Promega) according to the manufacturer's protocol.

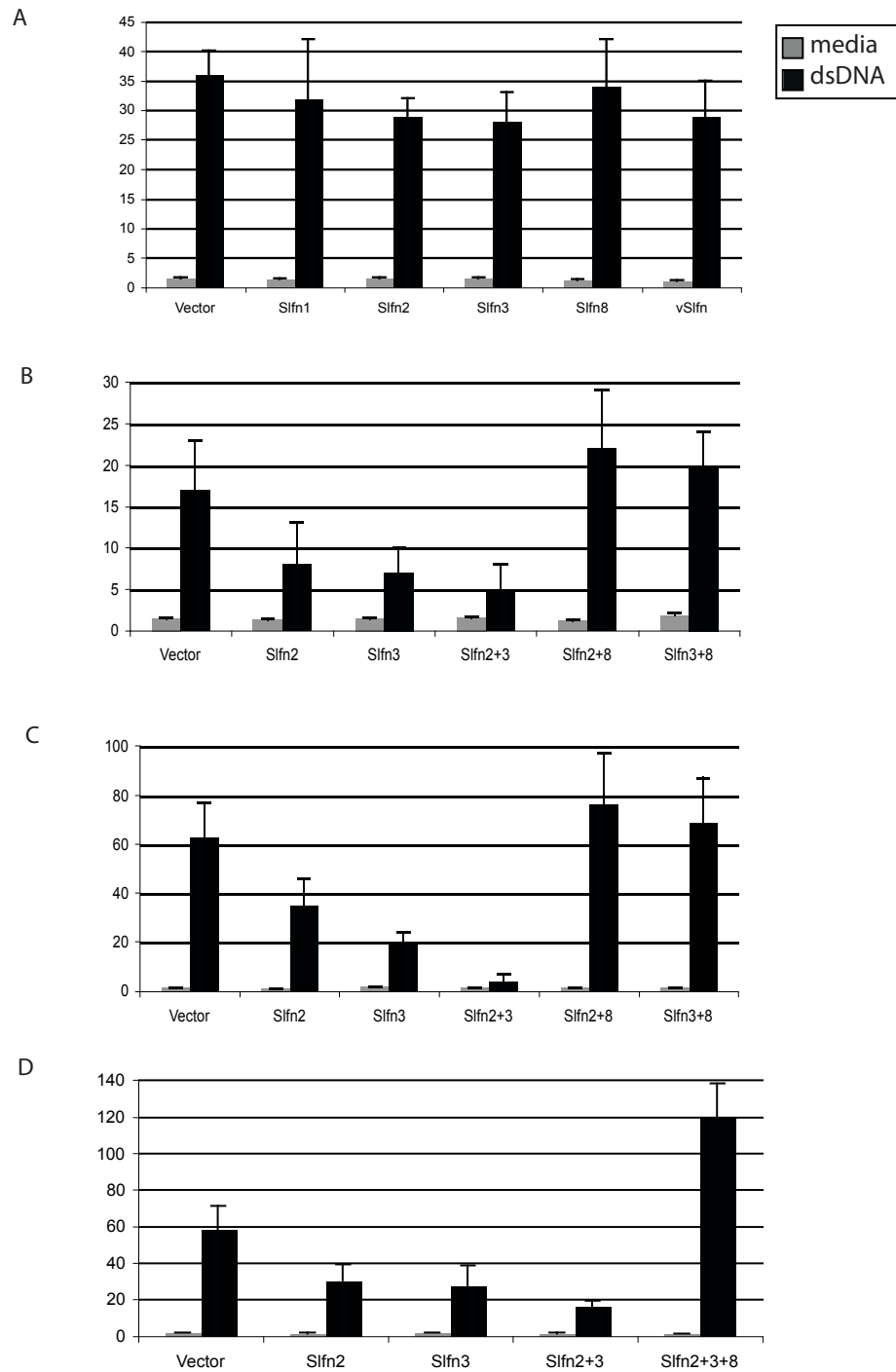


Figure 3-13. The modulating effect of Slfn 2 and 3 is unique to the PRDI/III site, and is rescued by combination with Slfn 8. Cells were transfected with individual Slfn constructs either with or without dsDNA stimulation and (A) PRDII-luciferase (B) PRD I/III luciferase (C) IFNbeta luciferase (D) ISRE-luciferase. Luciferase assays were performed as previously described.

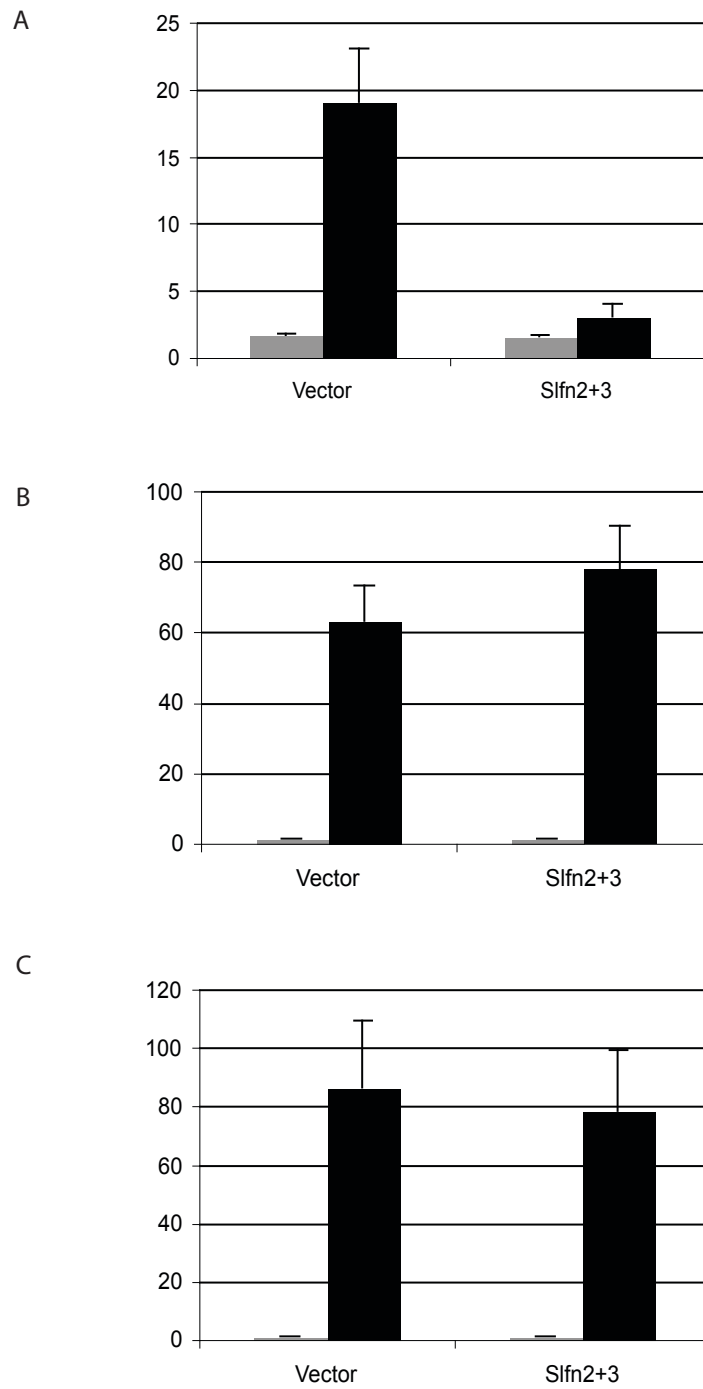


Figure 3-14. Slfn proteins act in the IRF3 activation cascade. 293 cells were transfected with (A and B) ISRE- or (C) NFkB-luciferase reporter and the His-tagged Slfn expression constructs as indicated. Transcriptional activation of the ISRE-luciferase was achieved through co-transfected (A)TRIF or (B)IRF3-5D, and NFkB-luciferase was induced through co-expression of (C)MyD88. Renilla-luciferase was used for normalization, and luciferase assays were performed as described previously.

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Chapter 4: Future Directions

SCHLAFEN DOMAINS WITH HOMOLOGY TO DEAD-BOX HELICASES

Distantly related helicase proteins share short, conserved amino acid sequence fingerprints, or motifs. Based upon consensus sequence homology, helicases are divided into “superfamilies”. All members contain a Walker A motif (P-loop) for ATP binding and a Mg²⁺-binding aspartic acid (Walker B motif)¹. Seven characteristic motifs were identified in superfamily I and II helicases, which encompass a large number of DNA and RNA helicases from archaea, eubacteria, eukaryotes and viruses¹. The solved crystal structures of proteins from various families show structurally conserved cores consisting of two domains, where the conserved motifs mostly face a cleft formed between the domains²⁻⁴. The Walker A and B motifs (motifs I and II) cooperatively bind the phosphates of an NTP, and they are thought to link hydrolysis of the beta–gamma phosphoanhydride bond with enzymatic activity of the helicase.

Sequence analysis of subgroup III Slfn proteins provides a clue as to the effector function(s) of these “long” Slfns. The C-termini of these proteins harbor typical Walker A and B motifs and other domains conserved in superfamily I RNA helicases⁵ (Figure 4-1). Additionally the N-terminus of Slfn 2 and 3 contain a divergent Walker A motif within their AAA domains. To obtain a more lucid picture of the molecular mechanism of the various members of the Slfn family, we will investigate

the functional implications of these domains in Slfn activity with a series of targeted mutations. Upon generation of the various mutants, we will investigate their effect upon Interferon gene expression, as performed previously with wild-type Slfn family members. It has been shown in DEAD-box helicases, that ATP binding enhances the binding of RNA and vice versa^{4, 6, 7}, thus we will test both nucleic acid binding ability and ATP hydrolysis in Slfn proteins with and without mutations in putative ATP hydrolysis and oligonucleotide-binding sites. To carry this out, we will employ electrophoretic mobility shift assays (previously described), as well measuring ATP hydrolysis using an assay as described in Oh et al⁸. Additionally, potential translation inhibitory effects of all wild-type and mutant Slfn proteins will be tested in the rabbit reticulocyte lysate system.

Walker A Motif:

A Walker A Motif, or P-loop, is an ATP/GTP binding site motif found in many nucleotide-binding proteins. It is a glycine-rich loop, preceded by a beta sheet and followed by an alpha helix⁹, and interacts with the alpha and beta phosphates of ATP or GTP. The classical consensus sequence is GxxxxGK-S/T.

Subgroup III Slfn proteins contain a Walker A motif in the C-terminal region. The sequence, GLPGSGKT, is conserved in all the subgroup III Slns. In other P-loop containing proteins it has been shown that the lysine is an

essential residue for ATP-binding and activity^{9, 10}. Hence, we theorize that this residue may be critical for ATP binding and effector function of Slfns 5, 8, 9, 10, and 14. Since we have already cloned Slfn 8, we propose to initially test the effects of a mutation in the Walker A domain using site-directed mutagenesis to change the critical lysine residue to an alanine (K606A).

Slfns 2 and 3, from subgroups I and II respectively, also contain a variant (GLDGKT) of the classical Walker A motif within their divergent AAA domain. This sequence is not present in the AAA domain of any other Slfn protein. To test the significance of this sequence in the function of Slfn2 and 3, we propose to generate a lysine→ arginine mutation (Slfn2: K274A; Slfn3: K210A) using site-directed mutagenesis.

Walker B Motif/DEAQ box:

The Walker B ATP-binding motif is classically composed of four residues, almost exclusively hydrophobic, that form a putative beta strand followed by an aspartate residue¹¹. A glutamate residue located next to the aspartate residue of the Walker B-motif in some AAA proteins points towards the gamma-phosphate of the bound nucleotide. This residue is conserved in the majority of helicases, in particular in certain RNA helicases in which it is part of a characteristic extension of the Walker B motif, called the DEAD box¹¹. The DEAD box family includes a large

number of proteins that play a role in RNA metabolism. Using energy from ATP hydrolysis, these proteins often serve to unwind RNA structures or dissociate RNA-protein complexes¹². Mutational analyses performed with other helicases have shown that the conserved aspartic and glutamic acid residues are necessary for ATP hydrolysis¹³. The carboxyl group of the aspartic acid coordinates the Mg²⁺ ion of MgATP/MgADP through outer sphere interactions, whereas the glutamic acid is suggested to act as a catalytic base in ATP hydrolysis¹.

Subgroup III Slfn proteins share a Walker B sequence with the DEAD box extension (I-I/V-DEAQ) in their C-terminus (Figure 4-1). We propose to test the role of the Walker B motif in Slfn function by mutating the conserved aspartate and glutamate of the DEAQ box. We will begin by site directed mutagenesis of Slfn8, in which we will replace both D670 and E671 with an alanine residue.

It has been shown in several DEAD-box containing proteins that blocking ATP hydrolysis by an E to Q mutation in the DEAx sequence holds the DEAD-box protein in an active confirmation and results in high-affinity interactions with their respective protein targets¹⁴⁻¹⁷. This suggests that unknown interacting partners of subgroup III Slfns might be identified by creating E-Q mutations in the Walker B motif and determining proteins that uniquely co-precipitate with ATP-locked Slfns.

Q-motif

The Q-motif is a more recently identified motif upstream of the P-loop that is unique to and characteristic of the DEAD box family of RNA helicases¹⁸. It consists of a 9 amino acid sequence containing an invariant glutamine and is often preceded by a phenylalanine roughly 17 amino acids upstream. The crystal structures of two DEAD box proteins show that the Q motif forms a binding pocket for the nucleotide base^{2, 19}. In the yeast translation-initiation factor eIF4A, sequence alignments, site-specific mutagenesis, and ATPase assays have shown that this motif and the upstream phenylalanine are highly conserved and that they control ATP binding and hydrolysis²⁰. Additionally, it has been speculated that the Q motif can directly regulate the affinity of the protein for the substrate through conformational changes associated with nucleotide binding, ultimately regulating helicase activity.

Subgroup III Slfn proteins contain a Q motif in their C-terminus with the sequence V/L-xLL-S/T-xQQY (Figure 4-1). Additionally, an upstream phenylalanine residue is conserved amongst these Slfn proteins. In the yeast translation-initiation factor Ded1, it was shown that the conserved glutamine of the Q-motif is essential for yeast cell viability²⁰. Thus, to further investigate the role of the Q-motif in Slfn function, we propose site-directed mutagenesis of both glutamines in the Slfn Q-motif. Initiating this

trial with Slfn8, we will make a double point mutation in residues Q580 and Q581, changing both to alanine residues.

Oligonucleotide binding motifs

Although our experiments have revealed nucleic acid binding capability of Slfn2, no consensus sequence implicated in DNA or RNA binding can be found within the protein sequence of Slfn2. Subgroup III Slfns, however, do contain sequences known to be involved in oligonucleotide binding. A conserved sequence fingerprint that is specific to the DEAD-box RNA helicases is a QxxR motif, which is found between motifs IV and V. Based upon the crystal structure of eIF4A, the arginine of this motif, which is well positioned for oligonucleotide binding, has been postulated to interact with RNA^{2,3}. Conserved across subgroup III Slfns is a QxxR motif. Mutating the arginine of this motif may provide insight into RNA binding, and thus we propose creating an R-A mutation in the subgroup III Slfns. Additionally, a conserved threonine that long Slfns possess in domain Ib has also been implicated in RNA binding of DEAD-box helicases²¹, which may also provide us with the opportunity to investigate RNA binding and downstream implications upon mutation of this residue.

Although motifs Ia, Ib, III, IV, V have been implicated in RNA binding, little is known about essential residues within the motifs. Additionally,

subgroup III Slfns appear to only have weak homology within these motifs. However, using site-directed mutagenesis to mutate entire motif segments at a time may provide us with more insight into the role of these motifs in Slfn function.

NUCLEAR LOCALIZATION SEQUENCE IN SUBGROUP III SLFNS

Previously, we observed that, while Slfn2 and 3 seem to downregulate the immune response by inhibiting the transcription of Interferon stimulated genes (ISGs), the “long” Slfn8 reverses the inhibitory activity of the shorter Slfn forms. It is reasonable to hypothesize that Slfns form complexes made up of different members, depending on the specific stimulus. It is possible that, in vivo, complexes with the longer Slfn members activate an immune response to pathogens, while complexes excluding these longer forms are downregulators of the immune response. Additional to the RNA Helicase homologous domains of subfamily III Slfns, Slfns 8, 9, and 10 have a bipartite nuclear localization sequence (NLS) in their C-terminus (Figure 4-1). A bipartite NLS is composed of two clusters of basic amino acids, separated by about ten amino acids²². The potential nuclear translocation ability of these longer Slfn family members may account for the divergence of Slfn effector function that we have observed in our luciferase assays. While the short Slfn complexes might stay in the cytoplasm, negatively regulating the IRF3 pathway, complexes

that include long Slfns might translocate to the nucleus. It is possible that nuclear translocation of Slfn complexes may serve to sequester shorter Slfns away from their target, or that long Slfns may have an effector function on gene transcription in the nucleus. Given our data, either explanation is plausible. We propose to make point mutations in all basic amino acids of the Slfn NLS. After verifying the elimination of nuclear translocation by immunofluorescence, we will look at interferon-beta expression in cells transfected with these NLS mutants, in combination with other WT Slfns.

KINASE ASSAYS

As we have shown in chapter 3, Slfn proteins modulate Interferon gene expression upon dsDNA transfection, asserting their effect within the IRF3 activation cascade. Although DNA-mediated signalling initiated by DAI and/or other cytoplasmic receptors is poorly understood, it is known that, similar to the other PRRs, signaling funnels into the TBK1/IKK-epsilon-IRF3 pathway to activate IFN gene expression²³. TBK1 is the kinase implicated in phosphorylation of IRF3, IRF7 and a newly identified IFN-inducing DEAD-box helicase, DDX3X²⁴. Although we have not yet been able to co-precipitate Slfn proteins with any proteins in the IRF3 pathway, it is possible that the Slfn interactions are too fleeting to be seen by this approach. Therefore, to investigate the possibility of a kinase-substrate

interaction of Slfns and TBK-1 in the IRF3 pathway, we propose an in vitro kinase assay as described in Soulat, et al²⁴ and mass spectrometry to identify phosphorylation site(s) on the Slfn peptides.

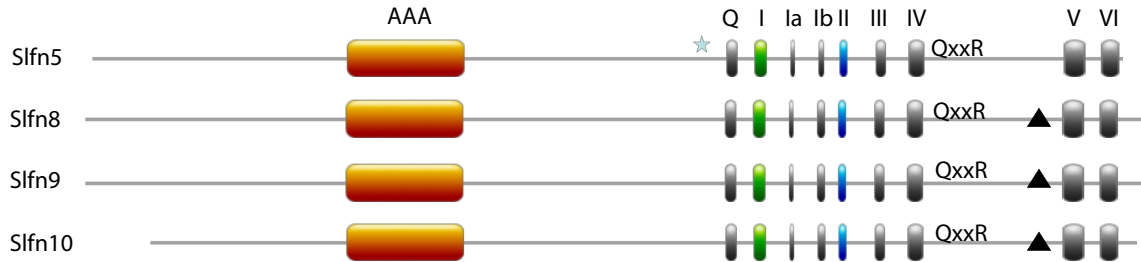
KNOCKDOWN OF SLFN PROTEINS

In the quest for the in vivo function of the Slfn family members, an siRNA knockdown approach will be advantageous. Previously, a Slfn1 knockout mouse was shown to have no phenotype²⁵, indicating a possible functional redundancy amongst Slfn proteins. Hence, we will take an approach that targets entire Slfn subfamilies at once. We have designed RNAi oligos to domains unique to different Slfn subfamilies, as well as shared sequences in subfamily I and II (Figure 4-2), and will use a systematic approach in knocking down gene expression of different Slfn combinations. To determine the effect of knocking down different Slfns in the immune response, we will look at downstream ISG expression by qPCR in response to different pathogenic stimuli, such as dsDNA, LPS, IFN, and polyI:C. Additionally, to examine the host response in cells depleted of the Slfn family, we will infect knock-down cells with VSV, CMV, and Sendai virus, and determine viral titer in these cells.

IDENTIFICATION OF SLFN-INTERACTING PROTEINS

We have generated stable cells lines expressing the different Slfn family members under a Tet-regulated promoter. Thus far, we have cell

lines for Slfns 1,2, 3 and 8, as well as v-Slfn. Each Slfn is equipped with a 6xHis and a CaM-BP tag for tandem-affinity purification, as well as additional Myc and Flag tags for identification. All constructs have already been tested in transient expression, and have been compared in functional assays (effects on dsDNA activated ISRE luciferase) to their untagged counterparts to exclude the possibility that these tags interfere with their function. We will use these Slfn-expressing cells to isolate cellular proteins that specifically interact with these distinct Slfn family members. Briefly, we will induce expression of the Slfn proteins with tetracycline in unstimulated, as well as IFN β or dsDNA stimulated cells, and purify the Slfn protein under low stringency conditions by sequential use of the two purification tags. The isolated immune-complex will be digested immediately (only a small portions will be reserved for silver-staining), in order to maximize the amount of protein available for HPLC/MALDI-TOF. This technical approach will be performed by the UCSD MS Core facility, who has extensive experience in these analyses. Once we identify a Slfn-interacting protein, we will map the interaction domain in order to generate Slfn or "protein X" mutants that are interaction deficient for further analysis in our functional assays.



Motif	Schlafen 5	Schlafen 8	Schlafen 9	Schlafen 10
Q	TVALLSDQQ	ILNLLTAQQ	ILNLLTAQQ	ILNLLTAQQ
I	GLPGSGKT	GLPGSGKT	GLPGSGKT	GLPGSGKT
Ia	DDIL	DRIL	DRIL	DRIL
Ib	VTRKT	VTRKT	VTRKT	VTRET
II	IIVDEAQ	IIVDEAQ	IIVDEAQ	IIVDEAQ
III	LYIFLDYFQ	FWIFLDYFQ	LWIFLDYFQ	LWIFLDYFQ
IV	LTRMLRSGDNIT	LTQVVRNGDKIA	LTQVVRNADKIA	LTQVVRNADKIA
V	SVHRFSGMERSIV	SIRRFSGLERSIV	SIRRFSGLERSIV	SIRRFSGLERSIV
VI	LLCLASRARTHLYIVK	LLCLASRIKTHLYILY	LLCLASRMKTHLYILY	LLCLASRMKTHLYILY
NLS		KKTYESMFLGEMRKRRR	KKAYEHMFLREMRKRRR	KKAYEHMFLREIRKRRR

Figure 4-1. Slfn subfamily III motifs with homology to SF-1/SF-2 helicases. Motif I and II are the WalkerA and B motifs respectively. ★ indicates conserved phenylalanine upstream of Q motif. ▲ indicates nuclear localization sequence (NLS) in Slfn 8, 9, and 10. Adapted from (5).

- A Subgroup I and II Schlafens
TAGAGAGTGAAATAGAAAA
CTGCAGAGCCCGAGTCCTG
GTGTGTGCGCTCAGAGTGG
- B Subgroup II Schlafens
GCGCCGAGTTAAAGGGCTA
ACAAATTGCCCTAACATTA
CAGTAAAGGACTTAGAAAA
- C Subgroup III Schlafens
TGAAGCAGAAGCTGGTGAA
AAGCAGAAGCTGGTGAACA
CTGAAGCAGAAGCTGGTGA
GAAGCAGAAGCTGGTGAAC

Figure 4-2. siRNA oligos specifically targeting (A) Slfn 1,2,3,4 (subgroup I and II), (B) Slfn 3 and 4 (subgroup II), and (C) Slfn 8,9,10 (subgroup III). SiRNA oligos were not able to be generated specifically against subgroup I Slfns, nor against all Slfns together (subgroup I, II, III). SiRNA oligos were generated using Dharmacon siDESIGN program.

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