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BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Dissertation

RESPIRATORY SYNCYTIAL VIRUS INTERACTIONS WITH HOST-CELL RNA-PROCESSING STRUCTURES AND PROTEINS

by

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B.S., Utah State University, 2000

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Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

2013

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2013

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DEDICATION

For my children, who I hope will always believe in their abilities to do hard things.

ACKNOWLEDGEMENTS

I first wish to thank my mentor, Rachel Fearns, who allowed me to join her lab a little over five years ago when I knew almost nothing about viruses. She took the time to personally train me, not only in virology methods, but also in paper critique, grant-writing technique, paper writing and submission processes, and scientific ethics. In addition, she has been a role model for balancing the demands of becoming a mother with those of a career in academia.

I also wish to thank my committee members Ron Corley, Rahm Gumuluru, William Cruikshank, and Andy Henderson, who have been invaluable in suggesting experiments and critiquing my work. I especially thank Ron Corley for serving as my committee chair and Rahm Gumuluru for being my second reader.

I would like thank the director of graduate studies, Greg Viglianti, for teaching me the ropes of academic politics, for giving me invaluble professional advice on many occasions, and for doing much to create a collegial and uplifting environment at Boston University.

I wish to thank Linda Parlee and Kathy Furness for everything they have done to make my life run more smoothly as a graduate student, and for always being available for a chat, laugh, or complaint. I will miss them both terribly.

During my studies, I have cultivated many great friendships that I will cherish forever: I wish to thank Erin Hodges, Daniele Cary, Chadene Tremaglio, Nora Ramirez, Julie Duncan, Judy Yen, Claire-Marie Filone, Sarah Weber, Nathaniel Green, Dan Green, Sarah Noton, Laure Deflube, Robin Djang, Max Seaton, Wendy Puryear, Suzanne Geer,

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Tini Brauburger, Kristina Schmidt, Judith Olejnik, and Adam Hume, for much laughter, a few tears, and thousands of memories - in and out of the lab. I especially wish to thank Julie Duncan for her help during the inception of the dcp1 project. We spent a great deal of time discussing possible experiments, and she not only helped with many of the dcp1 phosphorylation experiments, but also reviewed the literature thoroughly in search of relevent findings.

I thank my sister Cami Dickey Jones and my mother-in-law Michele Hanley for helping to take care of my daughter Amelia during my schooling. There is no greater comfort than knowing that my children are well loved and well cared for in my absence.

I of course must thank my parents, David and Lynette Dickey, for encouraging me to do my best in all endeavors, for being constant sources of support during my education, and for teaching me from a young age that come what may, we must keep on keepin' on.

I wish to thank my husband, Tim Hanley, whom I love more than the moon (and that is saying a lot). He has stayed up many nights helping me study, is always enthusiastic about discussing science (even at 4:00 in the morning when I've suddenly had an idea), and has read every word of this dissertation. He is my best friend in every possible way.

Finally, I wish to thank two gorgeous little girls, Amelia and Lily Hanley. Although they have done everything in their power to thwart my academic goals, they are the source of my greatest joy and are surely the cutest distractions ever.

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RESPIRATORY SYNCYTIAL VIRUS INTERACTIONS WITH HOST-CELL RNA-PROCESSING STRUCTURES AND PROTEINS

(Order No.

)

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ABSTRACT

Respiratory syncytial virus (RSV) is a negative-strand RNA virus that causes significant pneumonia-related morbidity and mortality worldwide. There are currently neither vaccines nor effective therapies for RSV. As with other viruses, RSV mRNAs are translated using host-cell machinery, rendering the virus subject to cellular factors that regulate mRNA homeostasis. Stress granules (SGs) and processing bodies (p-bodies) are inter-dependent, stress-response cytoplasmic structures involved in mRNA triage and degradation, respectively. We hypothesized that RSV has evolved to manipulate cellular stress responses in order to facilitate optimal virus propagation. While wild-type (wt) RSV induced SGs in approximately 1% of infected cells, a mutant version of RSV whose Tr region was replaced with an inverted LeC sequence (LeC virus) induced SG formation in approximately 50% to 70% of infected cells. A 12U to A substitution relative to the 5' end of the LeC virus abrogated SG induction. Mixed-infection studies showed

that wt RSV was able to prevent LeC-mediated SG induction. Unlike Sendai virus. RSVmediated prevention of SG formation was independent of SG-associated t-cell intracellular antigen related (TIAR) protein. RSV infection altered neither the number nor distribution of p-bodies; however, p-body-associated decapping protein 1 (dcp1) was phosphorylated throughout RSV infection via the extracellular signal-regulated kinase (ERK) 1/2 pathway. RSV-mediated dcp1 phosphorylation was limited to serine 315, serine 319, and threonine 321. Dcp1 phosphorylation occurred in response to some, but not all, environmental stresses, and dcp1 was not phosphorylated during infection with HIV-1, measles, mumps, or canine distemper virus. Overexpression of dcp1 significantly attenuated RSV cytopathic effects, and preliminary data suggested that dcp1 phosphorylation regulated RSV-induced interleukin-8 production. Finally, an antibody toward cellular SG- and p-body-associated, RNA-binding protein p54 was able to recognize a subset of RSV nucleoprotein (N). p54 and RSV N contain a similar amino acid sequence motif, suggesting that they may have similar or competing activities that are important during RSV replication. Taken together, our results demonstrate that RSV can manipulate cellular RNA-processing structures and proteins to facilitate viral propagation.

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LIST OF ABREVIATIONS

μ	microliter
°C	degree(s) Celcius
5' RACE	5' rapid amplification of cDNA ends
A	alanine
Ab	antibody
Ago	argonaute
AKT	protein kinase B
APS	ammonium persulfate
ars.	arsenite
ATF2	activating transcription factor 2
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
BMV	Brome mosaic virus
CAT	chloramphenicol acetyl transferase
CCR	C-C chemokine receptor
CD	cluster of differentiation
cDNA	complementary DNA
CDV	canine distemper virus xxiii

CFP	cyan fluorescent protein
CGH-1	conserved germline helicase 1
CMV	cytomegalovirus
CPEB	cytoplasmic polyadenylation element binding protein
СХЗС	C-X-X-X-C chemokine
CTL	cytotoxic T-lymphocytes
CX3CR	C-X-X-C chemokine receptor
CXCR	C-X-C chemokine receptor
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA-binding domain
DC	dendritic cell
Dcp1	decapping protein 1a
DDX3	DEAD box protein 3, X chromosome
DDX6	DEAD box helicase 6
DEAD	aspartic acid-glutamic acid-alanine-aspartic acid
DHH1	DEAD box helicase homolog 1
DI	deionized
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide

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DNA	deoxyribonucleic acid
DOC	deoxychololate
dsRNA	double-stranded ribonucleic acid
DTT	dithiothreitol
DVP	defective virus particle
E. coli	Escherichia coli
EBOV	Ebola virus
edc	enhancer of mRNA decapping
EGFP	enhanced green fluorescent protein
elF	eukaryotic translation initiation factor
ELISA	enzyme-linked immunosorbent assay
Env	envelope
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
F	RSV fusion glycoprotein
FACS	fluorescence-activated cell scanning
FBS	fetal bovine serum
FDA	Federal Drug Administration
FITC	fluorescein isothiocyanate

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g	gram
G	RSV attachment glycoprotein
G3BP1	GTPase activating protein (SH3 domain) binding protein 1
GAG	glycosaminoglycans
Gag	group-specific antigen
GCN2	general control nonderepressible 2
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte/monocyte colony stimulating factor
h	hour
HAART	highly active antiretroviral therapy
HBSS	Hank's balanced salt solution
HBV	hepatitis B virus
HCF	host cell factor
HCV	hepatitis C virus
HEK	human embryonic kidney
HIV	human immunodeficiency virus
HIV-1	human immunodeficiency virus type 1
hpi	hour post infection
HRI	heme-regulated inhibitor

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	HRP	horseradish peroxidase
	HSP70	heat shock protein 70
	HSV	herpes simplex virus
	HTLV-1	human T-cell leukemia virus type 1
	ΙκΒ	inhibitor of NF-κB
	IFN	interferon
	IgA	immunoglobulin A
94.1 G	lgG	immunoglobulin G
	IL	interleukin
	IRAK	IL-1 receptor-associated kinase
	J	Joule
	JNK	c-Jun amino-terminal kinase
	kDa	kilodalton
	ко	knock-out
	L	RSV polymerase large subunit
	LB	Luria Broth
	LDH	lactate dehydrogenase
	Le	Leader sequence
	LeC	inverted complement of the Leader sequence
	LPS	lipopolysaccharide xxvii

Lsm1	Sm-like protein 1
LTR	long terminal repeat
LZIP	leucine-zipper protein
m	minute
М	molar
Μ	RSV matrix protein
M2-1	M2 ORF 1 protein
M2-1	RSV transcription processivity factor
M2-2	M2 ORF2 protein
MA	HIV-1 matrix protein p17
mAb	monoclonal antibody
MAGI	multinuclear activation of a galactosidase indicator
MAL	MyD88-adaptor-like
МАРК	mitogen-activated protein kinase
MDDC	monocyte-derived dendritic cell
MDM	monocyte-derived macrophage
Me31B	maternal expression at 31B
MEK	mitogen-activated protein kinase kinase
MIP	macrophage inflammatory protein

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miRNA	microRNA
MK2	MAPK-activated protein kinase 2
МКК	MAPK kinase
МККК	MAPK kinase kinase
ml	milliliter
mM	millimolar
MOI	multiplicity of infection
MPK6	mitogen-activated protein kinase 6
mRNA	messenger RNA
mRNP	mRNAs in ribonucleoprotein complexes
MV	measles virus
MVB	multivesicular body
MyD88	myeloid differentiation primary response gene 88
Ν	RSV nucleoprotein
NC	HIV-1 nucleocapsid protein p7
NCS	newborn calf serum
NF-ĸB	nuclear factor κ of B cells
NS-1	RSV nonstructural-1 protein
NS-2	RSV nonstructural-2 protein

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OGT	O-linked N-aceylglucosamine transferase
ORF	open reading frame
Р	RSV phosphoprotein
PABP	poly-A binding protein
PAMP	pathogen-associated molecular pattern
Pat1	protein associated with topoisomerase II 1
PatL1	Pat1-like protein 1
PBMC	peripheral blood mononuclear cell
p-bodies	processing bodies
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PERK	protein kinase RNA-like endoplasmic reticulum kinase
pfu	plaque forming unit
ЫЗК	phosphoinositide 3-kinase
PKR	protein kinase R
Pol	polymerase
PRR	pattern recognition receptor
PS	penicillin, streptomycin
PS/G	penicillin-streptomycin-L-glutamine
	xxx

РТВ	phosphotyrosine binding
PVDF	polyvinylidene difluoride
RANTES	regulated upon activation, normal T-cell expressed, and
	secreted
RFP	red fluorescent protein
RIPA	radioimmunoprecipitation assay
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
RNF8	RING finger protein 8
RNP	ribonucleoprotein
RSV	respiratory syncytial virus
rt	room temperature
RT-PCR	reverse transcriptase-polymerase chain reaction
RV	rotavirus
S	second
S	serine
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis xxxi

SFV	Semliki Forest virus
SG	stress granule
SH	RSV short hydrophobic protein
shRNA	short hairpin RNA
siRNA	small interfering RNA
SSC	saline-sodium citrate
т	threonine
TBK1	TANK-binding kinase 1
TBS	Tris-buffered saline
TEMED	tetramethylethylenediamine
TEV	tobacco etch virus
Th1	T helper cell type 1
Th2	T helper cell type 2
TIA-1	T-cell-restricted intracellular antigen 1
TIAR-1	T-cell-restricted intracellular antigen related protein
TICAM	Toll/IL-1R domain containing adaptor molecule
TIR	Toll-interleukin (IL)-1 receptor
TIRAP	Toll/IL-1R domain containing adaptor protein
TLR	Toll-like receptor

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TNF-α	tumor necrosis factor α
Tr	Trailer sequence
TRAM	Toll-receptor associated molecule
TrC	complement of Trailer sequence
TRIF	Toll/interleukin-1 receptor domain containing adaptor
	inducing interferon-β
UTR	untranslated region
UV	ultraviolet
VLP	virus-like particle
VSV	vesicular stomatitis virus
VSV-G	vesicular stomatitis virus G protein
w	vaccinia virus
WB	western blot
WNV	West Nile virus
wт	wild type
X-gal	5-bromo-4-chloro-3-indolyl-P-D-galactopyranoside
Xrn1	5'-3- exoribonuclease 1
YFP	yellow fluorescent protein

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Introduction

Respiratory syncytial virus

Overview

Respiratory syncytial virus (RSV) is a member of the family *Paramyxovirus* and the order *Mononegavirales*. RSV shares many genome, structure, and replication-strategy similarities with viruses within the same order, including measles, mumps, Sendai, Nipah, Ebola, Marburg, rabies, and vesicular stomatitis (VSV) viruses (263). It is hoped that studying the mechanisms by which RSV interacts with host cells will broaden our understanding of not only RSV, but also of related viruses.

RSV epidemiology

RSV is one of the leading causes of pneumonia-related deaths in infants, the elderly, and immuno-compromised persons worldwide. It is estimated that over 120,000 infants in the United States are hospitalized annually with RSV infection, accounting for approximately 25% of pediatric pneumonia and up to 70% of pediatric bronchiolitis hospitalizations (262). The global burden of RSV is approximately 64 million cases and 160,000 deaths per year (3). Unfortunately, there are currently neither vaccines nor effective treatments for the virus.

RSV morphology

RSV virions can acquire one of two forms: irregular spheres or long filaments. The virions are comprised of a nucleocapsid that is packaged in a lipid envelope, which is acquired from the cell membrane during virus budding. The envelope has three glycoproteins: the attachment glycoprotein (G), the fusion protein (F), and the short,

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hydrophobic protein (SH). The matrix protein (M) is believed to form a layer on the inner surface of the virion. The viral genome is associated with four proteins: the large polymerase subunit (L), nucleoprotein (N), phosphoprotein (P), and transcription processivity factor (M2-1).

RSV replication cycle

RSV enters host cells by fusing with the plasma membrane. The virus genome is released into the cytoplasm where it undergoes transcription to make viral mRNAs and replication to make antigenome and genome using the virus-encoded RNA-dependent, RNA polymerase. Viral mRNAs are translated using host-cell machinery, and virus particles are packaged and released by budding at the plasma membrane (Figure 1).


Figure 1. Overview of RSV replication. RSV enters host cells by fusing with the plasma membrane, a process mediated by envelope glycoproteins (red circles). The virus genome is released into the cytoplasm where it undergoes transcription to make viral mRNAs, which are capped (yellow circles) and polyadenylated (A's), and replication to make antigenome and genome, which are encapsidated by the RSV N protein (small blue circles) as they are synthesized. Transcription and replication are carried out by the virus-encoded RNA-dependent, RNA polymerase (purple ovals), which associates with nucleocapsids. Viral mRNAs are translated using host-cell machinery, and virus particles are packaged and released by budding at the plasma membrane.

There are eleven RSV proteins, whose roles in the RSV replication cycle are described in Table 1.

Protein	Apparent Molecular Weight (kDa)	Primary Function(s)
Nucleoprotein (N)	45	Binds tightly to RSV genome and antigenome as they are being synthesized to protect from cellular nucleases; may be part of polymerase complex
Phosphoprotein (P)	33	Major phosphoprotein; part of the polymerase complex; also forms complex with free N to maintain N solubility and availability for nucleocapsid assembly; phosphorylation associated with polymerase stabilization on the template; P association with M2-1 is essential for M2-1 function
M2 ORF 1 protein (M2-1)	22	Part of the polymerase complex; essential RSV transcription factor that

Table 1. RSV proteins and functions

		promotes transcription processivity
Large (L)	250	RNA-dependent, RNA polymerase; contains an active site for polymerization, and active sites for mRNA capping
Matrix (M)	25	Internal virus component that accumulates at the plasma membrane of host cells; important for viron morphogenesis and RNA synthesis regulation
Fusion (F)	50-70* (F ₁) 10-16* (F ₂) *differences due to variable glycosylation	Type-I transmembrane envelope glycoprotein that directs viral entry into host cells by mediating fusion between the viral envelope and plasma membrane; can mediate fusion with neighboring cells to form syncytia, synthesized as inactive precursor (F ₀) that is activated by cleavage that yields two disulfied-inked subunits (F ₁ and F ₂) plus a 27-nucleotide fragment.

Glycoprotein (G)	90	Type-II transmembrane, envelope glycoprotein; major virus attachment factor that binds host-cell membranes (receptor unknown); smaller, secreted form mimics the chemokine fractalkine to impair immune activation
Small hydrophobic (SH)	7.5 - 60	Envelope protein with unknown function, although there is evidence that it helps form an ion channel of unknown significance; reported to inhibit TNF-α signaling
M2 ORF2 protein (M2-2)	14.5	Nonessential accessory protein with unknown function(s); may play role in shifting balance of RNA synthesis from transcription to replication
Nonstructural-1 (NS1)	13.8	Nonstructural protein; inhibits IFN-α/β induction by inhibiting phosphorylation of interferon-regulatory factor 3 (IRF- 3).
Nonstructural-2 (NS2)	14.5	Nonstructural protein; inhibits IFN-α/β induction by inhibiting phosphorylation of interferon-regulatory factor 3 (IRF- 3); may target STAT2 for proteasome- mediated degradation

Cell entry

Cell entry is facilitated by RSV attachment to and fusion with the plasma membrane by the RSV glycoprotein (G) and fusion (F) proteins. Unlike other negative-strand viruses, G is not a strict requirement for cell entry; however, viral fitness *in vivo* is impaired in mutants lacking G (246). Initial interactions with host cells are thought to occur first via weak-affinity binding of F and G proteins to glycosaminoglycans (GAGs) (99) on the host cell surface, followed by a higher-affinity binding step; however, the host-cell receptor(s) for the second, higher-affinity attachment step have not been identified. The virus penetrates the cell by fusion of the envelope with the host-cell plasma membrane, which is mediated by the F protein.

Transcription and replication

Once cell entry has occurred, the RSV genome is uncoated and transcription and replication are initiated. RSV's 15.2 kb genome is comprised of a single strand of nonsegmented, negative-sense RNA that is tightly associated with virus nucleoprotein (N) in a helical nucleocapsid structure (148, 245). The ten RSV genes encode eleven proteins (M2-2 via an alternate translation start site in the M2 mRNA) and are flanked with transcription gene-start and gene-end sequences (56). As discussed in further detail in Chapter 1, the RSV genome includes two extragenic sequences termed leader (Le) and trailer (Tr) at the 3' and 5' termini, respectively. Le contains the promoter for both transcription of monocistronic mRNAs and for replication to produce the antigenome, while TrC (at the 3' end of the antigenome) contains the promoter for replication to produce the genome (Figure 2).





RSV RNA replication is dependent on the first 36 nts of the Le sequence. Nucleotides 1-11 recruit the viral polymerase to the template and signal RNA synthesis initiation, while nucleotides 12–36 increase transcript-encapsidation efficiency and promote the generation of full-length replication products (62, 63, 157). Genome transcription is dependent on Le-sequence nucleotides 1–11 and 37–44, in addition to the adjacent gene-start signal of the first gene. These sequences are important for the initiation of RNA transcription to produce viral monocistronic mRNAs, and replication. As with other negative-strand viruses, a transcription gradient is established, with genes at the 3' end being transcribed in the greatest quantities due to the polymerase falling off the template and differences in gene-end signal efficiency (176). RSV mRNA synthesis reaches a plateau at around 12-18 hours after infection (29). Some researchers theorize that M2-2 might affect the shift from transcription to replication (29). As antigenome and genome are produced, they are encapsidated with N protein, a process that protects the RNA from nucleases and is associated with increased polymerase processivity (157).

Next, the virus is assembled and packaged at the apical surface of polarized cells (203). Both actin (41) and tubulin (110) have demonstrated roles in RSV assembly and release. Virus is released by budding from the plasma membrane, a process that appears to be the reverse of cell entry (24) and requires the proteolytic activation of the F protein (14).

RSV pathogenesis and immune-system interactions

Humans are the only reservoirs for human RSV infection. The virus is spread primarily through large droplets from respiratory secretions; however, it is possible that the virus

can also be spread by fomites (98). The prodromal period from infection to onset of symptoms is approximately four to five days (111). RSV causes bronchiolitis in infants, during which necrosis of bronchiolar epithelial tissue and destruction of ciliated cells occurs (3, 183). Inflammatory responses cause a massive influx of immune cells to the airways, which results in edema in submucosal tissues, as well as excessive mucus production. Not surprisingly, immune-cell infiltratrion, excess fluids, mucus, and cellular debri can obstruct the bronchioles and alveoli, causing emphysema or the collapse of the airways. This is particularly dangerous in infants, whose bronchiole diameter is very small.

RSV tropism

RSV primarily infects the airway and lungs, with replication taking place in the superficial layer of respiratory epithelial cells. The virus is shed into the lumen from the apical surface of the cells. Infection is generally limited to ciliated cells at the luminal face (275). RSV can also infect many other cell types; however, infection is generally limited in non-epithelial cells. RSV infection in alveolar macrophages induces significant TNF- α , IL-8, and IL-6 production (26); however, infection of these cells results in drastically reduced virus production compared to infection of epithelial cells, and appears to be confined to initially-infected alveolar macrophages (i.e. no cell-to-cell spread) (27). RSV infection of monocyte-derived dendritic cells results in suppressed T-cell proliferation and cytokine production (70) through unknown mechanisms.

Immune responses to RSV

Both humoral and cellular immunity are important for RSV clearance and illness recovery. An underlying theme in RSV biology is that most aspects of the immune response that are beneficial in resolving RSV infection also contribute to disease development and pathogenesis. For example, decreased virus shedding in respiratory secretions is significantly associated with the appearance of RSV-specific IgA antibodies in patients with RSV disease (159), and the fact that fetuses do not begin to acquire significant levels of transplacental IgG antibodies until around 26-32 weeks of gestation likely helps explain why preterm infants are at increased risk for RSV infection. However, maternal antibodies have also been shown to suppress humoral immunity via unknown mechanisms (64). Th1 and Th2 CD4+ lymphocytes also are involved in RSV clearance from infected persons, and it is believed that the balance of these responses may play a role in determining whether cell-mediated immunity is protective against RSV disease or whether it exacerbates pathology of the virus. Th2 cytokines IL-5 and IL-13 are associated with hyper-responsiveness and airway eosinophilia in mouse models (107, 216). On the other hand, overexpression of Th2-cytokine IL-4 suppressed immune responses to RSV infection (22) and Th1-cytokine IL-12 has been shown to enhance cell-mediated immunity to RSV (244). Mouse models indicate that RSV G protein elicits a Th2-biased response, while RSV F protein elicits a Th1-biased response (13); however it is unclear if these findings are relevant in the context of infection with whole virus in human systems. These results suggest that the balance of Th1 and Th2 immune responses could influence disease outcome. RSV-specific CD8+ cytotoxic Tlymphocytes (CTLs) are generally believed to be important for virus clearance from airways (188); however, as with other immune mediators, an equilibrium seems to exist

that determines whether the cells contribute to exacerbated disease. Studies in mice show that adoptively transferred CTLs are able to improve RSV clearance from airways, but that the clearance is associated with severe (and often fatal) pulmonary disease (42). Taken together, these studies suggest that there is a delicate balance needed among immune mediators in resolving infection without exacerbating pathogenesis.

Toll-like receptors

Several Toll-like receptors (TLRs) are known to be activated by RSV infection. RSV F protein has been shown to bind TLR4 / CD14, and infection of TLR4-deficient mice demonstrated that TLR4-mediated immune responses are important for clearing virus from lungs (128). Two single nucleotide polymorphisms in the TLR4 gene have been linked to increased susceptibility to RSV infection in children (23). In addition, RSV signals through TLR3 late in infection to induce cytokine and chemokine production. Production of CXCL10 and CCL5 were linked to TLR3 activation. On the other hand, IRF3 was dispensable for CXCL8 production, which was dependent on myeloid differentiation primary response gene 88 (MyD88)/TLR4 signaling (204). RSV has been shown to activate TLR2 and TLR6 in mice (178) via unknown mechanisms.

Surfactants

Several studies have implicated a role for pulmonary surfactant proteins in limiting RSV infection. Researchers have shown that surfactant A binds RSV F protein to reduce RSV infectivity (91). RSV disease was more severe in mice with disrupted surfactant proteins (138), and reduced surfactant concentration and function has been correlated with severe disease in infants (117). Recent studies have shown that RSV decreases

surfactant levels in by interfering with surfactant mRNA translation (38). These findings suggest that surfactant proteins may be important in host-cell defenses against RSV.

Inflammation

As discussed above, inflammation during RSV infection can contribute to disease pathogenesis, particularly when bronchioles and airways become clogged with cells, debri, and fluids. RSV infection induces several chemokines, including RANTES, MIP-1 α , MIP-1 β , IL-8, and fractalkine (276), which recruit large numbers of immune cells to the sites of infection. IL-8 recruits neutrophils, which are the dominant cells found in the bronchial lavage and nasopharyngeal secretions of infants hospitalized for RSV disease (79). Neutrophils are important for RSV clearance, but they also cause cell damage (152, 259), and granulocytes recruited to airways can directly mediate the destruction of infected cells (259).

RSV immunomodulation

RSV has co-evolved with host-cell immune mediators. Not surprisingly, the virus is able to subvert and manipulate several aspects of the immune system. RSV F protein binds TLR4/ CD14 complexes, and thereby may alter trafficking, activation, and function of mononuclear cells (97). In addition, RSV has been shown to reduce interferon (IFN)- α production by plasmacytoid dendritic cells (214). As mentioned above, soluble RSV G protein mimics the chemokine fractalkine (251), and NS1 and NS2 proteins have been shown to inhibit interferon responses (58, 234, 250).

RNA granules

Eukaryotic cells display several types of nuclear and cytoplasmic granules containing mRNAs in ribonuceloprotein complexes (mRNPs). These granules have roles in mRNA triage, regulation, processing, storage, and turnover (202). Two inter-related cytoplamic mRNP-aggregate structures, stress granules and processing bodies (p-bodies), are highly conserved from yeast to mammals and have been implicated in host innate immune responses that are important during viral infections (15, 89, 118, 121, 122, 155, 205, 213).

Stress granules

Stress granules form in cells during environmental stress, including heat shock, endoplasmic reticulum (ER) stress, oxidative stress, nutrient deprivation, dehydration, cell crowding, and certain types of viral infections (16, 17, 113, 202). They contain stalled translation complexes (including translation factors, ribosome subunits, and mRNA), and are thought to be sites of mRNA storage and triage (16, 17, 113). The following figure shows a comparison of untreated cells with cells treated with sodium arsenite, which induces oxidative damage and stress granule formation. The cells are stained with an antibody toward eIF3, a stress-granule marker (red). In untreated cells (left panel), the eIF3 is diffuse in the cytoplasm. In arsenite-treated cells (right panel), eIF3 has a punctate distribution characteristic of stress-granule formation. Stress granules are discussed in further detail in Chapter 1.



Figure 3. Cytoplasmic stress granules. HEp-2 cells were untreated (left) or treated with sodium arsenite (right). The cells were immunostained for cellular eIF3, which condenses into cytoplasmic aggregates called stress granules during environmental stress.

Processing bodies

Unlike stress granules, p-bodies are constitutively present in cells. They are sites of mRNA degradation, and contain mRNAs, decapping enzymes, and exonucleases. P-bodies become larger and more numerous during stress conditions. The following pictures shows cells under steady-state conditions. The cells are stably transfected with red fluorescent protein (RFP)-tagged decapping protein 1 (dcp1), which is a protein found in p-bodies (red), and stained with DAPI to visualize the nuclei (blue). P-bodies are discussed in further detail in Chapter 2.





dcp1 (red) were stained with DAPI to visualize nuclei (blue). Dcp1 is concentrated in pbodies.

Stress granule and p-body associations

Stress granules and p-bodies are linked both functionally and spatially. Studies have demonstrated that during stress, there is an equilibrium among mRNAs undergoing active translation on free polysomes, translationally silent mRNAs in stress granules, and mRNAs undergoing degradation in p-bodies (48, 67, 169, 222). Stress granules are theorized to be intermediaries between mRNA translation and decay, and studies have shown that they can release mRNAs for resumed translation on polysomes (170) and they frequently form transient physical interactions with p-bodies whereby they may be able to exchange constituents (48, 115). Kedersha et al. proposed a model in which mRNA that is released from disrupted polysomes is sorted and remodeled in stress granules, and select transcripts are delivered to p-bodies for degradation (113, 115). This model is supported by evidence that the composition of both structures is highly dynamic, with rapid turnover of both proteins and mRNAs (115), as well as evidence that stress granules and p-bodies may exchange proteins and mRNAs (39, 115).

Stress granule and p-body implications in virus biology

While the activities of stress granules and p-bodies have not been extensively characterized, both structures are associated with stalled translation - a condition that could prove deleterious to viruses dependent on host-cell machinery for their own translation. An emerging theme in the field of viral pathogenesis is the idea that cellular stress responses are involved in antiviral strategies; either by modulating viral RNA levels and/or regulating expression of proteins involved in the innate immune response.

This idea is given credence by a rapidly growing list of viruses that are able to subvert or manipulate RNA granule formation and/or activities as discussed in detail in Chapters 1 and 2, in addition to reports that stress responses can post-transcriptionally regulate cytokine production (15, 206).

Hypothesis

At the time this dissertation project commenced, very little was known about virus interactions with stress granules and p-bodies, and nothing had been reported with regard to RSV and these RNA-processing structures. We wished to investigate the relationships among RSV and stress granules, p-bodies, and their associated proteins. We hypothesized that RSV has evolved to manipulate stress responses in order to facilitate optimal virus propagation, and that cellular stress granules and p-bodies are part of a host antiviral response. We sought to determine whether i) RSV infection altered the distribution of stress granules or p-bodies, ii) RSV infection resulted in altered stress granule- or p-body-associated proteins, and iii) what impact such alterations had on RSV biology.

Materials and Methods

Buffers and reagents

General buffers and reagents

Phosphate-buffered saline (PBS): provided as powder (Sigma), dissolved in 1 L deionized (DI) H₂O to yield 0.01 M phosphate buffered saline (0.138 M NaCl; 0.0027 M KCl); pH 7.4, at 25 °C.

Tris-buffered saline (TBS): provided as powder (Sigma), dissolved in DI H₂O to yield 0.05 M Tris buffered saline (NaCl - 0.138 M; KCl - 0.0027 M); pH 8.0, at 25 °C.

RIPA buffer: 1X solution acquired from Boston Bioproducts. 50 mM Tris-HCL, pH 7.4, 150 mM NaCL, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS.

Cell culture buffers and reagents

Opti-MEM (Invitrogen)

Kaighn's Modification of Ham's F-12 Medium (F-12K) (ATCC)

Dulbecco's Modified Eagle's Medium (DMEM) containing high glucose: 4.5 g/L Dglucose and L-glutamine (GIBCO-Invitrogen catalog #11965-092)

Glasgow Minimum Essential Medium (GMEM) (Invitrogen)

RPMI 1640 medium containing L-glutamine (GIBCO-Invitrogen)

Fetal Bovine Serum (FBS) Qualified (GIBCO- Invitrogen); thawed at 37°C, heat inactivated for 30 min at 56°C; 25 ml aliquots stored at -20°C.

Newborn Calf Serum (NCS) heat inactivated (GIBCO- Invitrogen); thawed at 37°C, 50 ml aliquots stored at -20°C.

Penicillin-Streptomycin-L-glutamine (PS/G), 100x (GIBCO- Invitrogen); 5 ml added per 500 ml of media for a final concentration of 100U/ml penicillin, 100 μg/ml streptomycin, and 0.29 mg/ml L-glutamine; 5 ml aliquots PS/G stored at -20°C.

Phosphate-buffered saline (PBS) pH 7.4 (GIBCO-Invitrogen)

Hank's balanced salt solution (HBSS) (GIBCO-Invitrogen)

0.25% Trypsin, 1x (GIBCO-Invitrogen catalog)

Dimethyl sulphoxide (DMSO) (Sigma-Aldrich)

Ficoll-Paque PLUS (GE Healthcare)

Hygomycin B solution (GIBCO-Invitrogen); 50 mg/ml in PBS stored at 4°C

Gentamycin sulfate (G418) (GIBCO-Invitrogen): reconstituted in water to 500 mg/ml. Sterile filtered through 0.2 μm syringe filter. Aliquoted and stored at -20°C.

Puromycin (Sigma-Aldrich): reconstituted in water to 100 μ g/ml. Sterile filtered through 0.2 μ m syringe filter. Aliquoted and stored at -20°C.

Northern blot buffers and reagents

1.5% agarose gel: Gels were made by dissolving 1.5 g agarose (Sigma) in 86.5 ml DI H₂O and then immediately adding 10 ml 10X 3-(N-morpholino)propanesulfonic acid (MOPS) buffer and 3.6 ml 36.7% formaldehyde.

Running buffer. Made by diluting 10X MOPS buffer (Sigma) with DI H₂0.

Loading buffer: 50% glycerol, 1mM EDTA, and bromophenol blue tracking dye.

Transfer buffer. 175.32 g NaCl, 4 ml 2M NaOH, DI H₂O to 1 L, filter sterilized.

Neutralization buffer: 6X saline-sodium citrate (SSC), diluted in DI H₂0 from 20X SSC (American Bioanalytical).

Prehybridization buffer. Used 20 ml / membrane. For 20 ml, added 6 ml 20X SSC, 0.8 ml 2X Denhart's reagent (diluted from 50X solution provided by Sigma), 0.2 ml 10% SDS, and 13 ml DI H₂0.

Wash buffer: 2X SSC, 0.1% SDS. For 250 ml, add 25 ml 20X SSC and 2.5 ml 10% SDS to 222.5 ml DI H₂O.

Stringent wash buffer: 0.1X SSC, 0.1% SDS. For 150 ml, added 0.75 ml 20X SSC, 1.5 ml 10% SDS to 146.75 ml DI H₂O.

Film. Kodak BioMax MR 8" by 10".

Immunofluorescence buffers and reagents

Fixative: 5% formaldehyde with 2% sucrose in PBS.

Permeablilization buffer. 0.5% IGEPAL with 10% sucrose in PBS.

Wash buffer. 2% FBS in PBS.

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Antibody diluent: 2% FBS in PBS.

Western blot buffers and reagents

Loading buffer. 2X Laemmli sample buffer (BioRad) with dithiothreitol (DTT) added to a final concentration of 0.1M immediately before use.

Molecular-weight markers: Thermo Scientific* PageRuler* Plus Prestained Protein Ladder 10-250kDa (Fisher) (chemiluminescence) or Odyssey Protein Molecular Weight Marker (10-250 kDa) (0.5 mL) (Licor)

10% polyacrylamide gel: For 10 ml (2 gels), 2.82 ml H₂O, 3.75 ml 1 M Tris buffer pH 8.8, 100 μ l 10% SDS, 3.33 ml 30% (w/v) acrylamide (37.5:1) 0.8% bis-acrylamide (National Diagnostics), 50 μ l 10% ammonium persulfate (APS), and 10 μ l 1,2-

Bis(dimethylamino)ethane; N,N,N',N'-Tetramethyl-1,2-diaminoethane (TEMED).

4% polyacrylamide stacking gel: For 10 ml (4 gels): 7.3 ml H₂0, 1.25 ml 1 M Tris buffer pH 6.8, 100 μ l 10% SDS, 1.33 ml % (w/v) acrylamide (37.5:1) 0.8% bis-acrylamide (National Diagnostics), 50 μ l 10% APS, and 10 μ l TEMED.

Precast gels: 10%, 12-well Mini-Protean SDX precast gels were acquired from Biorad (catalog #456-1035).

10X running buffer. For 1 L, 30.3 g Trizma base (Sigma), 144 g glycine (Sigma), 10 g sodium dodecyl sulfate (SDS) (Sigma), add ultra-pure H₂O up to 1 L. Dilute 10x buffer to make 1x buffer in ultra-pure H₂O.

25X transfer buffer. For 500 ml, add 18.2 g Trizma base (Sigma) and 90 g glycine (Sigma) and add ultra-pure H_2O up to 500 ml. Let dissolve overnight. To make 1X transfer buffer, 10 ml 25X transfer buffer was diluted in 50 ml methanol (Sigma) with ultra-pure H_2O up to 250 ml.

Mason fractionation buffers and reagents

Tris-acetate buffer: 50 mM tris-acetate pH 8, 100 mM K-acetate, 1 mM DTT (added immediately before use), 1 mM ATP (added immediately before use).

Triton X-100 buffer. 10 mM tris-acetate pH 8.0, 10 mM K-acetate, 1.5 mM MgCl₂, and 0.1% triton X-100.

DOC-Tween 40 buffer. 10 mM tris-acetate pH 8.0, 10 mM K-acetate, 1.5 mM MgCl₂, 0.1% triton X-100, 0.5% deoxycholate (DOC), and 1% Tween 40.

Multinuclear activation of a galactosidase indicator (MAGI) assay buffers and reagents

MAGI fixative (500 ml): 1% formaldehyde/0.2% glutaraldehyde (13.5 ml 37% formaldehyde/4 ml gluteraldehyde/1 x PBS); stored in a glass bottle wrapped in aluminum foil at 4°C.

5-bromo-4-chloro-3-indolyl-p-D-galactopyranoside (X- gal) (EM Science catalog #9660); 400 mg of X-gal was reconstituted in DMSO to 40 mg/ml and stored in 1 ml aliquots at -20°C.

MAGI staining solution (for every 1 ml), 950 μl PBS/20 μl 0.2M potassium ferrocyanide/20 μl 0.2M potassium ferricyanide/1 μl 2M MgCl₂/10 μl 40mg/ml X-gal.

Cell lines

HEp-2 cells (ATCC) were cultured in Opti-MEM with 2% FBS (GIBCO-Invitrogen). WT (TIAR +/+) and TIAR knockout (TIAR -/-) mouse embryonic fibroblast lines were a generous gift from Dr. Paul Anderson and have been described previously (140). They were cultured in DMEM supplemented with 10% FBS. A549 cells (ATCC) were cultured in F-12K media supplemented with 10% FBS. Human embryonic kidney (HEK) 293T cells (ATCC #CRL-11268) were grown in complete DMEM High Glucose (+ 4.5 g/L D-glucose, + L-glutamine) supplemented with 10% FBS, 100 U/ml penicillin (GIBCO-

Invitrogen), 100 μ g/ml streptomycin (GIBCO-Invitrogen), and 0.29 mg/ml L-glutamine (GIBCO-Invitrogen). The HeLa (human cervical cancer) cell clone MAGI-CCR5 (obtained from the AIDS Research and Reference Reagent Program), which has been engineered to express high levels of human CD4, CXCR4, CCR5, and multiple copies of integrated HIV-LTR- β -gal reporter constructs driving the expression of β -galactosidase (β -gal) that has been modified with a nuclear localization signal at its N-terminus (123, 256) were propagated in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 500 μ g/ml G418, 1 μ g/ml puromycin, and 0.1 μ g/ml hygromycin B. All cells were incubated at 37°C with 5% CO₂.

Isolation and culture of primary MDMs

Human peripheral blood mononuclear cells (PBMCs) were isolated from commercially prepared leukopacks (New York Biologies Inc.) by Ficoll-Paque Plus (Amersham Biosciences) density gradient centrifugation. Peripheral blood was diluted with an equal volume of HBSS and 30 ml was overlayed onto 15 ml of Ficoll-Paque Plus in 50-ml conical tubes. The gradients were centrifuged at 400 x *g* for 30 minutes at room temperature (rt) with the centrifuge break turned off. PBMCs, appearing as a dense, white band above the red blood cell and granulocyte layer, were collected with a pipette, washed three times with 50 ml phosphate buffered saline (PBS) interspersed with centrifugation to pellet cells (5 min each at 300 x *g*) in order to remove residual Ficoll-Paque Plus, platelets, and red blood cells. PBMCs were used immediately for CD14+ monocyte isolation. Primary CD14+ monocytes were isolated from PBMCs using mouse anti-human CD14 monoclonal antibody (mAb)-conjugated magnetic beads and LS MACS cell separation columns (Miltenyi Biotec) according to the manufacturer's

protocol. CD14+ monocytes (6 x 10^5 cells/well in 12-well plates) were cultured in serumfree RPMI for 30 min to allow monocytes to adhere to tissue-culture plastic. Serum-free medium containing non-adherent cells was removed by aspiration and fresh RPMI supplemented with 10% normal human AB serum, 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.29 mg/ml L-glutamine was added to the cells. Monocytes were cultured for a period of eight days, during which they differentiated into MDMs. MDM phenotype was confirmed by flow cytometry.

RSV stocks

Wild-type (wt) RSV was strain A2, and all mutant viruses were based on this strain. To propagate virus, ~80% confluent HEp-2 cells in 150 cm² flasks were infected with RSV at a low multiplicity of infection (moi) of 0.01 in 10 ml Opti-MEM + 2% FBS. One to four hours post infection (pi), media was replaced with 20 ml fresh media. Media was changed every 36-48 hours. Once extensive syncytia formation was observed (approximately 3 days post infection for wt, Tr36, and 12 U/A viruses, and approximately five to six days post infection for LeC virus), cells and supernatant were collected in 50ml conical tubes. Cell suspension was vortexed for ten seconds, sonicated for 30 seconds, vortexed again for 10 seconds, and sonicated again for 30 seconds. Cell suspension was subjected to centrifugation at 300 x g at 4°C for 5 minutes. Supernatant was collected, aliquoted into sterile cryovial tubes, flash frozen in dry ice, and stored at -80°C. To sucrose purify RSV, the virus suspension was pelleted through 30% sucrose onto a 60% sucrose cushion (sucrose w/v in 10 mM Tris, pH 8.1) by centrifugation at 24,000 x g for 1 h at 4 °C. The white band at the 60/30% sucrose interface was collected, diluted approximately 1:10 in OptiMem supplemented with 50 mM HEPES and 100 mM MGSO4 * 7H2O, aliquoted, flash frozen in dry ice, and stored at -80 °C.

UV-inactivated virus was generated by exposing virus in 6-well plates to 200 J/m², approximately 15 cm from light source.

Plasmids

cDNAs encoding the RSV antigenome were constructed using plasmids that have been described in detail previously; the RSV/6120 cDNA includes a mutation in the SH gene that increases plasmid DNA stability without altering the protein coding region (40, 57, 109, 247). To facilitate manipulation of the RSV Tr region, single nt substitutions were made 10, 13 and 14 nts after the last U residue of the L gene end signal to introduce a BsiWI site. The Tr region was mutated by introducing PCR products, representing either the 5' terminal 57 or 36 nts of Tr. or the 44 nt LeC sequence between the BsiWI site and a delta ribozyme. All full-length clones were examined by multiple restriction digests to confirm their integrity, and the regions subjected to PCR amplification were sequenced to confirm that no spurious mutations had arisen. The plasmid pBruAEnvLuc2 is a single-round replication competent clone of pBru3ori (HIV-1) which contains a deletion in the env gene and luciferase in the nef ORF (272). The plasmid pHEF-VSV-G encodes for the envelope glycoprotein from vesicular stomatitis virus (VSV) (47). Plasmids expressing the minigenomes used in this study were based on the MP28 minigenome. described by Fearns et al. previously (82), and were designed to express the chloramphenicol acetyltransferase open reading frame (ORF) flanked by the following 3' cis-acting sequences: 1) RSV leader (Le) nucleotides 1-36, 2) RSV trailer complement (TrC) nucleotides 1-36, 3) RSV TrC nucleotides 1-57, or 4) RSV full-length TrC. All minigenomes were flanked with the 36 5'-terminal Tr-region nucleotides on the 5' end of the minigenome. pTM1 plasmids encoding RSV N, RSV P, RSV L, and RSV M2-1 under transcriptional control of the promoter for the T7 RNA polymerase have been

described previously (95). We acquired a myc-DDK-tagged open reading frame (ORF) DNA clone of *homo sapiens* dcp1a (accession number NM_018403) under the control of the pCMV6 promoter from Origene (RC201330).

RSV mutant virus rescue and growth

The recombinant viruses were rescued as described previously (57). Briefly, HEp-2 cells in 6-well plates were transfected with a mixture of T7 expression plasmids containing the RSV N, P, L, and M2-1 ORFs and a fifth T7-expression plasmid encoding the appropriate RSV antigenome. The cells were co-infected with the MVA strain of vaccinia virus expressing T7 RNA polymerase (269) and incubated in a CO₂ incubator at 32 °C. Three days post-transfection, cells were scraped into the medium, vortexed, sonicated, and then pelleted by centrifugation. The supernatant was passaged onto fresh HEp-2 cells in 25 cm² flasks and the flasks were incubated in a CO₂ incubator at 37 °C. Virus was passaged in this way every 4–7 days until each virus stock had reached a titer equaling or exceeding 2×10⁶ plaque forming units (pfu)/ml, or for the number of passages indicated in the figures and results text. Virus stocks were flash frozen in aliquots and stored at −70 °C. Following rescue, each recombinant virus was analyzed by reverse-transcription and PCR amplification of the region spanning the end of the L gene to the 5' end of the genome to confirm the presence of the mutations.

RSV multiple-step growth analysis

HEp-2 cell monolayers in six-well plates were infected with each indicated virus at an moi of 0.01 pfu/cell. An aliquot of the inoculum was titrated to confirm that equivalent

titers of each virus were used to infect the cells. The virus was allowed to adsorb for two hours, after which the cells were washed and the inoculum was replaced with 1 ml of Opti-MEM containing 2% FBS and antibiotics and incubated for the indicated periods of time. At each indicated time point, the entire medium overlay was removed, clarified by low-speed centrifugation, snap-frozen and stored at −70 °C until completion of the timecourse. The cell monolayers were washed twice with PBS, and fresh medium was added to the culture and incubation continued. The virus titers were determined by plaque assay.

RSV titration and plaque visualization

Virus was allowed to adsorb to cells for two hours, after which the cells were overlaid with Opti-MEM containing 2% FBS and 0.8% methylcellulose and incubated at 37 °C for 4–5 days. The cell monolayers were fixed with 80% methanol at 4 °C and then incubated with monoclonal antibodies specific to the RSV F or N protein (Serotec), followed by sheep anti-mouse immunoglobulin (Ig) G conjugated with horseradish peroxidase (HRP). Plaques were visualized by the addition of peroxidase solution substrate 4-CN (KPG).

Mouse studies

Balb/c female mice in groups of six (or five, where indicated in Figure 8) per time point, per virus were inoculated intranasally under light anesthesia with 0.1 ml of Opti-MEM containing 10^{5.5} pfu of Tr155, Tr57, Tr36, or LeC virus. At either 3, 4, or 5 days pi the mice were sacrificed and the nasal turbinates and lungs were harvested. The virus titers in these tissues were determined by plaque assay and the

mean log₁₀ pfu/g of tissue was calculated. The limit of detection in the upper and lower respiratory tracts was 2.0 and 1.7 log₁₀ pfu/g, respectively.

Northern blot analysis of viral RNAs

Cells were scraped into medium and collected by centrifugation. The resulting cell pellet was resuspended in Trizol reagent (Invitrogen) and total intracellular RNA was purified following the manufacturer's instructions, except that the RNAs were further purified by extraction with phenol–chloroform and ethanol precipitation. RNA samples representing one-tenth of a well were analyzed by electrophoresis in a 1.5% agarose gel containing 0.44 M formaldehyde, transferred to nitrocellulose and fixed by UV cross-linking (1200 J/m²). Duplicate blots were hybridized with a positive-sense probe toward CAT (minigenome study), or either negative-sense N-specific riboprobe or a positive-sense specific riboprobe (RSV Tr-mutant virus study), in a mixture of 6X SSC, 5X Denhardt's solution, 0.5% SDS, and 200 µg of sheared DNA per ml at 65 °C for 20 h. The blots were washed in 2X SSC–0.1% SDS at room temperature for 30 min, then at 65 °C for 2 h and then in 0.1X SSC–0.1% SDS for 15 to 30 min. RNA bands were quantitated by either phosphorimage quantitation using a Phosphor-Imager 445 SI (Molecular Dynamics) or by NIH ImageJ analysis of scanned autoradiograms.

5' RACE analysis of viral RNAs

HEp-2 cells in six-well dishes were infected with LeC isolates at the passage number indicated in the Results section. At 48 h pi, total intracellular RNA was isolated, as described above. RNA representing one-tenth of a well of cells was annealed to a

positive sense, L-specific primer (5' GAGTGTTGTTAGTGGAGATATACTATC) and used as a template for Sensiscript reverse transcriptase (Qiagen) according to the manufacturer's instructions. The cDNA was purified and tailed with dATP using terminal transferase. The tailed product was amplified by PCR using a nested L-specific primer (5' ACTTATAAATCATAAGCATATGAACATC) and a primer that annealed to the dATP tail (5' GACCACGCGTTCGATGTCGACTTTTTTTTTTTTTT). A second round of PCR was performed using a nested L-specific primer (5'

CAGATCAACAGAACTAAACTATAACCAT) to further amplify the product. The resulting DNA was sequenced using an L-specific primer.

Immunofluorescence microscopy

HEp-2 or TIAR knockout mouse embryonic fibroblasts seeded on coverslips in 12-well plates were either mock infected or infected with wt RSV or the Tr36, LeC or LeC 12U/A RSV mutant at the indicated moi. As a positive control for SG formation, mock infected cells were treated with 0.5 mM sodium arsenite for 30 min immediately prior to fixation. At 16.5 h pi, cells were fixed with 5% formaldehyde, 2% sucrose in PBS for 30 min, permeabilized with 0.5% Igepal, 10% sucrose in PBS for 20 min, and incubated with the indicated antibodies. Following washing in PBS, cells were incubated with isotype-specific secondary antibodies labeled with AlexaFluor 488 and AlexaFluor 633, as well as 4',6-diamidino-2-phenylindole (DAPI). Cells were analyzed by fluorescence microscopy.

Transfections

For plasmids expressing proteins and/or minigenomes under the control of the T7 promoter, monolayers of HEp-2 cells were transfected with Lipofectin reagent (Invitrogen) according to the manufacturer's instructions. Cells were concomitantly infected with the MVA strain of vaccinia virus expressing T7 RNA polymerase, except where noted. Six hours after transfection / infection, the media was replaced with Opti-MEM plus 2% FBS. For plasmids expressing proteins under the control of mammalian promoters, monolayers of HEp-2 cells were transfected using Lipofectamine 2000 (Invitrogen) according the manufacturer's instructions. Media was replaced 6 hours post transfection.

siRNA experiments

Mixtures of siRNAs toward dcp1 or p54 (siGENOME Set of 4 MQ-006371-00-0002, Human DDX6, NM_004397, 2nmol x 4) were obtained from Dharmacon, Inc. and transfected into monolayers of HEp-2 cells using Dharmafect transfection reagents according to the manufacturer's directions.

Antibodies

The antibodies used in the experiments described are presented in Table 2.

Table 2. List of antibodies.

Primary antibodies used with	h immunofluorescence	
mouse anti-RSV N	Serotec (MCA490)	

mouse anti-RSV F	Serotec (MCA491G)
goat anti elF3η	Santa Cruz (sc-16377)
goat anti-TIA-1	Santa Cruz (sc-1751)
rabbit anti-dcp1	Abcam (ab47811)
rabbit anti-p54	MBL (PD009)
mouse anti-DDX6/p54	Abcam (ab54611)
rabbit anti-Xrn1	Santa Cruz (SC-98459)
goat anti-CPEB	Abcam (ab73287)
Secondary antibodies used with immu	unofluorescence
Alexa Fluor® 488 chicken anti-goat IgG (H+L) *2 mg/mL*	Invitrogen (A-21467)
Alexa Fluor® 488 chicken anti-mouse IgG (H+L) *2 mg/mL*	Invitrogen (A-21200)
Alexa Fluor® 488 goat anti-rabbit IgG (H+L) *2 mg/mL*	Invitrogen (A-11008)
donkey anti-goat IgG Alexa Fluor 633	Invitrogen (A-21082)
Alexa Fluor® 647 Goat Anti-Rabbit IgG (H+L)	Invitrogen (A-21244)
donkey anti-goat IgG Alexa Fluor 633	Invitrogen (A-21082)
Primary antibodies used with western	-blot analysis
rabbit anti-dcp1	Abcam (ab47811)
rabbit anti-Xrn1	Santa Cruz (SC-98459)
goat anti-edc3	Santa Cruz

goat anti-edc4	Santa Cruz
rabbit anti-dcp2	Abcam
goat anti-RSV	Abcam
rabbit anti-p54	MBL (PD009)
mouse anti-p54	Abcam (ab54611)
anti-G3BP	BD Transduction (611126)
goat anti-TIAR	Santa Cruz (sc-1749)
goat anti-TIA-1	Santa Cruz (sc-1751)
goat anti elF3η	Santa Cruz (sc-16377)
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb	Cell Signaling Technology (4370P)
p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb	Cell Signaling Technology (4695P)
Phospho-p38 MAPK (Thr180/Tyr182)	Cell Signaling Technology
(D3F9) XP® Rabbit mAb	(4511P)
p38 MAPK (D13E1) XP® Rabbit mAb	Cell Signaling Technology (8690P)
Phospho-SAPK/JNK (Thr183/Tyr185)	Cell Signaling Technology
(81E11) Rabbit mAb	(4511P)
SAPK/JNK (56G8) Rabbit mAb	Cell Signaling Technology (4668P)
Phospho-Erk5 (Thr218/Tyr220)	Cell Signaling Technology

Antibody	(3371S)	
Erk5 (D23E9) Rabbit mAb	Cell Signaling Technology (3552S)	
Secondary antibodies used with we	stern-blot analysis	
HRP-conjugated anti-rabbit	Santa Cruz (sc-2004)	
HRP-conjugated anti-mouse	Santa Cruz (sc-2062)	
HRP-conjugated anti-goat	Santa Cruz (sc-2384)	
Anti-mouse IgG (H+L) (Dylight 800)	Cell Signaling Technology (5366S)	
Anti-rabbit IgG (H+L) (Dylight 680	Cell Signaling Technology	
conjugate)	(5257S)	
	1	

Cell harvesting and western-blot analysis

HEp-2 cells were lysed in 400 μ l (6-well plates) or 200 μ l (12-well plates) RIPA buffer (Boston Bioproducts) with protease inhibitors and HALT phosphatase inhibitors (Roche) according the manufacturer's instructions unless otherwise specified. Cells were incubated on ice for 10 minutes, after which supernatants were collected in microcentrifuge tubes, vortexed, and centrifuged at 16,000 x *g* for 10 minutes at 4°C. Unless otherwise specified, soluble fractions were collected and added to 2X Laemmli buffer (BioRad) with 0.1 M dithiothreitol (DTT).

Samples were separated on 10% SDS polyacrylamide gels at 120 Volts for approximately 90 minutes in Tris-glycine-SDS buffer, and transferred to nitrocellulose in tris-glycine buffer containing 20% methanol for 3 hours at 30 Volts. Membranes were incubated in either TBS plus 5% milk or Odyssey blocking buffer overnight at 4°C. Membranes were incubated with primary antibodies (1:1000 unless otherwise indicated) in either TBS with 0.1% Tween-20 (TBS-T) and 5% bovine serum albumin (BSA) (chemiluminescence) or Odyssey blocking buffer with 0.1% Tween-20 (Licor) overnight at 4°C on a nutator. Membranes were washed in TBS-T for four times for 5 minutes at room temperature and 60 rpm. Membranes were incubated with appropriate secondary antibodies (1:10,000) at room temperature for one hour at 60 rpm and then washed four times as above. For chemiluminescence analysis, membranes were blotted with Kimwipes and exposed to 3 ml Western Lighting Chemiluminescence Reagent (Perkin Elmer) for one minute. Blots were exposed to Blue Basic Autorad Film (ISC Bioexpress) for varying times (generally 30 - 90 seconds). For Licor analysis, membranes were rinsed in TBS and read with the Licor Odyssey reader. Protein bands were quantified by measuring fluorescence intensities.

Mason fractionation technique

HEp-2 or A549 cells were mock infected or infected with RSV (moi =5). Media was changed one hour pi. Eighteen hpi, cells were washed twice with PBS and lysed with Tris-acetate buffer. Cells were passed through an 18-guage needle 10 times, and incubated on ice for 10 minutes. Samples were centrifuged at 24,000 x g at 4°C for 10 minutes. Soluble fractions were collected (S1). Insoluble fractions (P1) were resuspended in Triton X-100 buffer and passed 10 times through an 18-guage needle. Samples were incubated on ice for 10 minutes and then centrifuged at 24,000 x g at 4°C for 10 minutes. Soluble fractions were collected (S2). Insoluble fractions (P2) were resuspended in DOC + Tween 40 buffer and passed 10 times through an 18-guage needle. Samples were incubated on ice for 10 minutes and then centrifuged at 24,000 x g at 4°C for 10 minutes. Soluble fractions were collected (S2). Insoluble fractions (P2) were resuspended in DOC + Tween 40 buffer and passed 10 times through an 18-guage needle. Samples were incubated on ice for 10 minutes and then centrifuged at 24,000 x g at 4°C for 10 minutes. Soluble (S3) and insoluble (P3) fractions were collected.

MAGI Assay

Integration of HIV-1 into P4 or MAGI-CCR5 cells (327) leads to production of the viral transactivator, Tat, which binds the HIV-1 LTR and activates expression of the βgalactosidase gene in the nucleus. Individual infected cells or syncytia (cells that have fused and contain multiple nuclei) are counted in situ with a light microscope by virtue of their blue color after incubation with the β -gal substrate X-gal. The following describes the basic protocol performed in order to assay viral infectivity of viral stocks or experimental samples. MAGI-CCR5 cells were plated at 1 x 10⁴ cells per well of a 96well flat-bottom plate in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 500 µg/ml G418, 1 µg/ml puromycin, and 0.1 µg/ml hygromycin B. On the next day, cells were infected by removing the medium from each well and replacing it with virus containing sample in a total volume of 50 µl. After 4 h, medium was removed by aspiration, wells were washed twice with PBS, and 100 µl of medium was added. Two days later, the medium was removed and the monolayer was fixed at rt with a solution of 1% formaldehyde/0.2% glutaraldehyde in PBS for 5 min. The cells were then washed three times with 200 µl of PBS to remove the fixative and incubated for 1 h at 37°C in 100 µl of staining solution (4 mM potassium ferrocyanide,4 mM potassium ferricyanide, 2 mM MgCl2, and 0.4 mg X-gal per ml). The reaction was stopped by removing the staining solution and washing the cells twice with 100 µl of PBS. Cells were examined under the light microscope and those with dark blue nuclei were counted as positive for virus infection. The number of cells that stain positive for β -gal activity in situ is directly proportional to the number of infected cells.

Generation of HIV-1 stocks

Single-round replication competent HIV-1 stocks were generated via transient transfection of HEK293T cells with 3 μg (per well of a 6 well plate) of pBruΔEnvluc2 and 1 μg of envelope expression vector using the Lipofectamine 2000 (Invitrogen) method.

HIV-1 p249ag ELISA

HIV-1 capsid p24^{gag} in cell-free supernatants and cell lysates was measured using a laboratory-generated ELISA using the following protocol. A 96-well flat bottom Costar EIA plate (cat #3590) was coated with 100 µl human polyclonal HIV-1 immunoglobulin (HIV-lg; cat #3957; AIDS Research and Reference Reagent Program, Germantown, MD) diluted in PBS (4 µl in 10.5 ml PBS per plate). The plate was sealed with microplate adhesive film (USA Scientific) and incubated overnight at 37°C (the plate was sealed with microplate adhesive film at each subsequent step, prior to returning it to the incubator). The coating Ab was removed by rinsing the plate six times with PBS (300 ul/well) using an automated plate washer (Thermolab Systems Wellwash 4 MK-2). The plate was blocked with PBS/10% NCS (Gibco-Invitrogen) for 2 h at 37°C (200 µl/well). The plate was washed six times (300 µl/well) with ELISA washing solution (1X PBS with 0.2% Tween-20) using the plate washer. Plate washing was carried out in this same fashion throughout the rest of the protocol. Standards and samples were diluted in ELISA diluent (1X PBS containing 10% NCS and 0.5% Triton X-100) and added to the plate (100 µl/well). Two fold p24⁹⁹ standard dilutions (2 ng/ml to 0.03125 ng/ml) were prepared using commercially-available p24gag recombinant protein (ProSpec, East Brunswick, NJ) and were loaded on the plate. Sample dilutions varied from experiment

to experiment and were adjusted as necessary to lie within the linear range of detection. The plate was incubated 2 h at 37°C and washed six times with ELISA washing solution. Primary mouse monoclonal HIV-1 p24^{gag} Ab (clone 183-H12-5C; AIDS Research and Reference Reagent Program, Germantown, MD) was added to each well (100 μ l/well; 13.125 μ l Ab per 10.5 ml ELISA diluent; 1.25 μ g/ml final concentration) and the plate was incubated for 1 h at 37°C. The plate was washed six times with ELISA washing solution and 100 μ l of secondary Ab (goat anti-mouse horseradish peroxidase [HRP]; A2554; Sigma) in ELISA diluent (1:70,000 dilution) was added to each well. The plate was incubated for 1 h at 37°, washed six times with ELISA washing solution using the plate washer, and 100 μ l of detection solution (equal volumes of TMB Peroxidase Substrate and Peroxidase Solution; KPL, Gaithersburg, MD) was added to each well. The lowest standard dilutions were beginning to have a hint of blue, typically 20 m after addition of detection solution. The plate was read immediately at 450nm in a microplate reader.

Dcp1 mutagenesis

We acquired a myc-DDK-tagged open reading frame (ORF) DNA clone of *homo sapiens* dcp1a under the control of the pCMV6 promoter from Origene (RC201330). This construct was subjected to mutagenesis to generate constructs in which S315, S319, T321, or all three residues (Triple A) were replaced with alanine in order to prevent phosphorylation of the residues of interest using a Agilent Quickchange II site-directed mutagenesis kit according to the manufacturer's directions. The primers used were as follows (5' to 3'):
S315A forward: ctacacaatcccgttggcccctgttctcagtccc

S315A reverse: gggactgagaacaggggccaacgggattgtgtag

S319A forward: ttgagccctgttctcgctcccactctgccagc

S319A reverse: gctggcagagtgggagcgagaacagggctcaa

T321A forward: cctgttctcagtcccgctctgccagctgaag

T21A reverse: cttcagctggcagagcgggactgagaacagg

Triple A: cacaatcccgttggcccctgttctcgctcccgctctgccagctga

Triple A reverse: tcagctggcagagcgggggcgagaacaggggccaacgggattgtg

The resultant constructs were subcloned using digestion with XhoII and HindI and ligation into the original backbone using the NEB Quick Ligation Kit according to the manufacturer's directions, and sequenced to confirm amino-acid-sequence.

Chapter 1: The Relationship between RSV and Stress Granules

Chapter 1 Introduction

Stress granules

As described briefly in the Introduction, stress granules are cytoplasmic aggregates that form in response to several types of environmental stress. Canonical stress granules contain stable, translationally silent mRNAs, certain translation initiation factors (eIF4E, eIF4G, eIF4A, eIF4B, eIF3, eIF2, poly-A binding protein (PABP)), the small ribosome subunits (115, 116, 267), and key RNA-binding proteins (T-cell-restricted intracellular antigen 1 (TIA-1), T-cell-restricted intracellular antigen related protein (TIAR), and GTPase activating protein (SH3 domain) binding protein (G3BP1)) (116, 249). Many other proteins have been found in stress granules, and the composition of stress granules varies widely depending on the stressor, cell type, and environmental factors (92, 197). Stress granules are thought to be repositories for mRNAs and initiation complexes during times of stress where mRNAs can either be released for active translation on free polysomes once the stress is removed, or shuttled to degradation machinery (113).

Known stress-granule pathways

While the mechanisms of stress-granule assembly are largely unknown, several pathways directing stress-granule formation have been described. Typically, stress granules are assembled following phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) by protein kinase R (PKR), protein kinase RNA-like endoplasmic reticulum kinase (PERK), general control nonderepressible 2 (GCN2), or heme-regulated inhibitor (HRI) kinases (113, 116, 156); however, phosphorylation of eIF2α is

not a strict requirement for stress-granule induction. For example, stress-granule formation can result from the inhibition of eIF4A RNA helicase independently of eIF2α phosphorylation (20, 67, 122, 154, 158, 198). Of note, a number of viruses are known to activate PKR (generally by double-stranded RNA intermediates) and/or PERK during infection, which could induce stress-granule formation (51). Neither HRI nor GCN2 activation has been significantly associated with virus infection.

Stress granule relationships with other viruses

Stress responses play an important role in regulating mRNA fate during environmental stress and generally result in drastically reduced or altered host-gene expression, which would likely prove deleterious to viruses that depend on tightly regulated host-cell homeostasis and host-cell translation machinery in order to propagate. It therefore is not surprising that a large number of viruses have evolved to subvert or block certain aspects of the cellular stress response.

Several viruses (mammalian orthoreovirus, Semliki Forest virus, hepatitis C virus, and poliovirus) induce stress-granule formation early during infection, but inhibit stressgranule formation during intermediate and late phases of infection. Other viruses (Junin virus, rotavirus, cardiovirus, West Nile virus, dengue virus, cricket paralysis virus, herpes simplex virus 1, HIV-1, HTLV-1, and influenza A virus) inhibit stress-granule formation throughout infection. On the other hand, some viruses are reported to tolerate (mouse hepatitis coronavirus and vaccinia virus) or even benefit from (transmissible gastroenteritis virus) stress-granule formation during infection. Table 3 provides a summary of known mechanisms through which these viruses prevent, manipulate, or exploit host stress responses (267).

Table 3. Phenotypes of virus-stress granule interactions. Adapted from White etal., 2012 (267).

Virus	Virus Phenotype			
Viruses	that prevent stress-granule form	nation		
	throughout infection			
Junin virus	Nucleoprotein N and/or glycoprotein precursor GPC inhibit elF2α phosphorylation and arsenite-induced stress granules	(144)		
Sendai virus	Sendai virus Tr region binds TIAR and prevents stress- granule formation	(103)		
Rotavirus	RV infection induces extended eIF2α phosphorylation but not stress-granule formation; infection can prevent arsenite-induced stress granules	(172)		
Cardiovirus	(32)			

	exogenous stress	¥0
West Nile and dengue virus	TIA-1/TIAR interaction with viral 3'-terminal stem-loop structure inhibits stress- granule formation	(74)
Cricket paralysis virus	Prevents canonical stress granules by preventing Rox8 and Rin (homologues of TIA- 1 and G3BP, respectively) from aggregating in stress granules	(119)
Herpes simplex 1	Virion host shutoff protein mediates TIA-1 localization to cytoplasm from nucleus, but not to stress granules	(68)
HIV-1	HIV Gag interacts with stress-granule protein Staufen1 to form encapsidation-destined ribonucleoprotein complexes instead of translationally silenced ribonucleoprotein complexes	(1)

	Inhibits stress granules	
HTLV-1	through Tax protein	
	interaction with histone	(136)
	deacetylase 6 protein	
	(HDAC6)	
	Infection with VV mutants	
	that lack the dsRNA-binding	
	protein E3L form antiviral	
Vaccinia virus	stress-granule-like bodies	(224)
	associated with PKR	
1	activation and reduced viral	
	replication	
1-0	NS1 protein blocks eIF2a	
virue	phosphorylation and stress-	(118)
VIIUS	granule formation	
Virus that ind	uce stress granules early during	ng infection,
and sub	vert stress granules late in inf	ection
	Stress-granule formation	
	induced by cell entry;	
Mammalian	inhibited as infection	(198)
orthoreovirus	progresses; inhibits stress	(100)
	granules in response to	
	arsenite	

	Induces eIF2a-	
	phosphorylation-dependent	
	stress granules early in	
Comliki	infection; stress granules	
Semilki	correlate with host translation	(158)
Folest virus	shutoff; SFV infection	
	prevents formation of stress	
	granules by exogenous	
	stressors late in infection	
	Induces stress granules early	
	in infection; inhibits stress	
Hepatitis C	granules late in infection by	(20)
virus	recruiting stress granule	x=-7
	proteins to viral replication	
	factories	
	Stress granules induced early	
	in elF2α-phosphorylation-	
	independent manner,	and and installed
	correlated with translation	(154))
Poliovirus	shutoff; later stress-granule	(265) (197)
	prevention due to virus-	(266)
	mediated G3BP cleavage	
	that unlinks TIA-1 aggregates	
	from stalled initiation	

	complexes	
Viruses t	hat tolerate or exploit stress g	ranules
Mouse hepatitis coronavirus	stress-granule formation corresponds with translation inhibition and robust virus replication	(199)
Transmissible gastroenteritis virus	Virus-induced stress granules recruit nuclear protein PTB; formation of stress granules correlated with inhibition of viral RNA accumulation	(231)

RSV Le and Tr

As mentioned previously, RSV's genome encodes 11 genes and is flanked by two extragenic sequences: 44-nucleotide Le at the 3' end, and 155-nucleotide Tr at the 5' end. The Le and TrC sequences display extensive terminal complementarity. In fact, the sequences are identical for 10 of the 11 3' terminal nucleotides, corresponding to the polymerase-recruitment element, and the sequences are similar between nucleotides 12 and 36, the region required for efficient encapsidation of replication products.

It is unclear why TrC is more than three times longer than Le. A minigenome containing the terminal 36 nucleotides of Tr can replicate efficiently (60), indicating that these nucleotides contain the minimal replication promoter. Another study demonstrated that increasing the length of the TrC sequence increases promoter efficiency (81). It has not been determined whether the minimal replication promoter is sufficient to drive productive virus propagation in the context of RSV infection. It is possible that the Tr region beyond the minimal promoter is important for RSV replication. For example, TrC sequence between nucleotides 36 and 155 could augment genome production from antigenome, which would help explain the fact that during infection with negative-strand viruses, genome is produced in much greater quantities than antigenome. Alternatively, Tr region may be involved in other steps of the RSV replication cycle. One study demonstrated that the VSV Tr region contains a *cis*-acting sequence that is necessary for packaging of genome-sense nucleocapsids into virions (264). Another possibility is that RSV Tr could be involved in subverting host defense mechanisms. The Sendai virus (a related paramyxovirus) Tr region reportedly associates with cellular TIAR protein and thereby inhibits stress-granule formation and cellular apoptosis (103).

In these studies, we characterized RSV's Tr region activities in the context of both minigenome replicons and recombinant versions of RSV in which the Tr region was either truncated with deletion mutagenesis or substituted with inverted LeC sequence. Our goals were two-fold: first, to examine the relative strengths of the Le and TrC promoter regions, and second, to determine whether the Tr region is involved in subverting host defense mechanisms.

Note: While I have since used and propagated the viruses described below extensively, including rescuing the LeC virus from cDNA, the initial virus mutagenesis and experiments described in Sections 1.2 - 1.5 were performed several years ago by other researchers, as noted in the figure legends. They are discussed here as background information for my project.

Chapter 1 Results

1.1 Confirmation that full-length Tr is not necessary for efficient promoter activity in a minigenome system

We first wished to confirm the previously mentioned results demonstrating that full-length Tr sequence is not necessary for efficient promoter activity. To compare promoter activities of various sequences, we used a minigenome assay. Artificial template RNA was created in which the chloramphenicol acetyl transferase (CAT) ORF was flanked with the following RSV cis-acting sequences: 1) full-length Tr region (Tr155), 2) Tr nucleotides 1-55 relative to the 5' end (Tr55), 3) Tr nucleotides 1-36 (Tr36), or 4) an inverted LeC sequence (LeC).

HEp-2 cells were co-transfected with constructs encoding the minigenome of interest in addition to plasmids expressing RSV polymerase, L, and its associated proteins, N and P (see Figure 5). 24 hours after transfection, RNA was harvested and the replication product was detected using northern blot with a CAT-specific probe.



Minigenomes

Figure 5. Schematic diagrams of the RSV minigenomes. Plasmids expressing minigenomes encoding CAT (green box) flanked with various RSV promoter sequences (indicated, top right) were transfected into cells in addition to plasmids encoding RSV L, P, and N proteins under the control of the T7 promoter. Cells were concomitantly infected with vaccinia virus expressing the T7 polymerase. The replication product was detected using radiolabeled probes against CAT sequence.

As shown in Figure 6, the 57-nucleotide trailer showed slightly lower promoter activity for unknown reasons; however, the transcripts produced by the TrC-sequence-containing constructs were all very similar. Consistent with previous findings, the construct containing Le sequence produced less replication product. These data indicate that the region beyond 1-36 is not required for efficient replication.



Figure 6. Full-length RSV Tr sequence was not necessary for efficient promoter activity. HEp-2 cells were transiently transfected with plasmids expressing RSV N, P, and L (except where noted) proteins in addition to constructs encoding CAT under the control of RSV cis-acting sequences as indicated. Cells were concomitantly infected with vaccinia virus expressing the T7 polymerase. Twenty-four hours post transfection, RNA was harvested and replication product was detected using northern blot with a radio-labeled, CAT-specific probe.

1.2 Construction and recovery of mutant viruses

To investigate the functions of the RSV Tr region in the context of viral infection, several RSV mutant viruses were made. Mutations were introduced into a recombinant clone of RSV, called RSV/6120 (see Materials and Methods for details) (40). A unique BsiWI restriction site was introduced a few nucleotides downstream of the L gene-end signal (see Figure 7A and Materials and Methods section for details) and used to create deletions in the Tr region, such that the 5' terminal 36 nucleotides of the Tr were joined to the BsiWI site (Figure 7A). The viruses that were generated from these cDNAs are referred to as Tr155 and Tr36. It was expected that the Tr36 virus would be able to direct synthesis of genome RNA, and that a comparison of Tr155 and Tr36 would provide clues as to the role of nucleotides 37 to 155 of the Tr region.

In order to compare the strengths of the TrC and Le promoters and to determine whether the Tr or TrC sequences have additional roles during RSV replication, an RSV cDNA was created in which the Tr sequence downstream of the BsiWI site was replaced with the 44-nucleotide LeC sequence. This mutation was designed to place the Le promoter sequence at the 3' end of the antigenome, which should theoretically have allowed genome production; however, several attempts to rescue a virus with the authentic LeC sequence substitution were unsuccessful, indicating that placing LeC at this location was highly detrimental to virus replication. A LeC sequence was introduced that placed a C rather than G residue at position 4 of the Le promoter at the 3' end of the antigenome (Figure 7B). This nucleotide assignment has arisen spontaneously in the Le regions of RSV vaccine candidate viruses (85) and has been shown to direct significant replication

promoter activity (59, 82). This virus was successfully rescued and propagated, and is referred to as LeC.



LeC: 3' UCAAUAAUUUUUUAAUUUUUAGCAUGCUAAAAAAACCAAAUACGUUCAAACAACAUGCGUAAAAAAAGCGCA 5'

Figure 7. Mutations made in the RSV Tr region. A) A schematic diagram of the RSV genome with the 5' end, where mutations were made (enlarged). The positions of nucleotides 1, 36, and 155 relative to the 5' end of the genome, and the position of the BsiWI site, are indicated. The regions contained in viruses Tr155 and Tr36 are indicated by the lines below, with the deleted regions shown as dashed lines. B) Sequences at the 5' ends of the Tr36 and LeC virus genomes. The sequences are written as negative-sense RNA and the L gene-end signal is italicized. The substitutions that were introduced to create the BsiWI site are shown in boldface type. The sequences are aligned, with spaces introduced to maximize alignment (shown as dashes). Nucleotide 12 relative to the 5' terminus of the genome, which was found to be a key nucleotide in this study, is underlined. Identical nucleotides are shown in red. These viruses were made by Rachel Fearns.

1.3 LeC virus exhibited impaired growth and a small, faint plaque phenotype compared to Tr155 and Tr36 viruses

The fact that the LeC virus could be rescued and propagated demonstrated that the RSV Tr region does not contain a unique, essential, *cis*-acting packaging signal, contrary to what was described for VSV (264). However, during initial propagation of the mutant viruses, it was clear the LeC virus replicated with impaired efficiency compared to the other viruses. In addition, the LeC virus had a different plaque phenotype than the other mutants. As shown in Figure 8A, the plaques formed by the Tr155 and Tr36 viruses were similar to each other in size and were intensely immunostained, while those formed by the LeC virus were smaller and only faintly immunostained.

To ensure that the inefficient propagation of the LeC virus was due solely to the mutation at its genome 5' terminus, the LeC cDNA clone was further manipulated in a single cloning step to reintroduce the 155 nt Tr region. The virus produced from this clone, LeCR, is theoretically genetically identical to the Tr155 virus. Indeed, the two viruses exhibited similar plaque morphology and growth kinetics, confirming that the LeC phenotype was specific to the LeC mutation (data not shown). Several independent cDNA clones of the LeC virus were rescued and all shared the same growth phenotype. Furthermore, isolation of a revertant virus (LeC 12U/A; described below) confirmed that the poor growth phenotype of the LeC virus was determined by the genome 5' terminal region, and not a spurious mutation elsewhere.

To formally compare the growth kinetics of the mutant viruses, cell monolayers were infected at a multiplicity of infection (moi) of 0.01, and the levels of infectious virus present in the supernatant were measured at 24-hour intervals. All the viruses grew similarly over the first 24 hours, but their growth kinetics diverged thereafter. The Tr36 virus replicated slightly more slowly than the parental Tr155 virus; however, the differences between these viruses were very minor and they reached a similar maximum titer by five days post infection (Figure 8B). In contrast, the LeC virus grew more slowly and reached a maximum titer that was approximately 20-fold lower than the other viruses. These results demonstrate that the Tr region beyond the minimal promoter does have one or more functions during RSV replication, and that nucleotides 57-155 of Tr sequence are required for optimal replication; however, the results clearly show that the complete Tr region is not essential for efficient virus growth. The results also demonstrate that replacing the Tr promoter with LeC has a deleterious effect on virus propagation over a multi-cycle growth period, as well an effect on plaque phenotype.

To test whether the Tr mutations had a similar effect on virus growth *in vivo*, mice were inoculated with the viruses intranasally, and virus titers in the nasal and lung tissues were measured over a five-day period. The virus replication closely mirrored multi-cycle growth in cell culture and confirmed that while the Tr36 virus was able to propagate with similar kinetics to the Tr155 virus, the LeC virus had impaired growth in comparison (101). These results confirm that the Tr region beyond position 36 does not contain an unique sequence that is necessary for efficient RSV replication. The results also confirm that replacing the Tr region with the LeC sequence is injurious to virus propagation.



В



Note: this figure is continued on the next page.

С

	Day 3		Day 4		Day 5	
	Nasal turbinates	Lungs	Nasal turbinates	Lungs	Nasal turbinates	Lungs
LeC	3.1±0.15	2.6±0.21	2.9±0.16	3.0±0.17	2.9±0.19	3.8±0.05
Tr36	3.9±0.06	4.3±0.04	4.2±0.11	4.5±0.07	4.0±0.08	4.7±0.03
Tr155	4.4±0.05	4.3±0.09	4.5±0.08	4.6±0.15	4.1±0.12	4.7±0.02

Figure 8. Multi-cycle growth properties of the mutant viruses. A) Plaque

phenotypes of the mutant viruses. HEp-2 cells were infected with the indicated viruses at an moi of 0.01. Three days post infection, cells were fixed and stained with antibodies against the RSV F protein. The upper panel shows plaques within an entire well of a 6-well dish, and the lower panel shows representative images of plaques taken with 10× magnification; the LeC virus plaques are highlighted with arrows. B) HEp-2 cells were infected with the indicated viruses at an moi of 0.01 and cell medium supernatant fractions were collected at 24 h intervals until the cells were destroyed by infection. The mean average virus titers (with standard deviation) are shown for each virus at each time point (n=3). C) Mice in groups of six per virus per time point were sacrificed on the indicated days postinfection. The numbers show the mean virus titers±standard error mean (log₁₀ pfu/g tissue). Rachel Fearns perfomed the experiments show in Panels A and B. Michael Teng performed the experiments shown in Panel C.

1.4 The impaired growth and altered plaque phenotype of the LeC virus was not due to impaired promoter activity

To determine whether the impaired growth and altered plaque phenotype of the LeC virus were due to differences in the strength of the Le and TrC promoters that lie at the 3' ends of RSV mutant virus antigenome, the levels of viral RNA generated by the viruses were compared. HEp-2 cells were infected at a high moi (4 pfu / cell) and RNA accumulation was analyzed at various points during a single-cycle growth period. Northern blot analysis of genome-sense RNA demonstrated that at each time point tested, the LeC and Tr36 viruses produced approximately 34% of the RNA produced by the Tr155 virus (Figure 9, upper panel; quantification data not shown). These results indicate that while nucleotides 1-36 are sufficient for RNA replication to occur, full-length Tr region is necessary for maximum RNA production. Importantly, the LeC virus produced a similar amount of genome RNA as the Tr36 virus, which indicates that in the context of viral infection, the Le replication promoter is equivalent in strength to the minimal TrC promoter. Positive-sense RNAs were examined by hybridizing duplicate blots with a negative-sense riboprobe against the N gene. This probe detected monocistronic N mRNA, polycistronic readthrough mRNAs containing N sequence, and antigenome RNA. Importantly, there were no significant differences in the levels of mRNA or antigenome accumulation between the Tr36 and LeC viruses (Figure 9. lower panel). The fact that the RNA-accumulation profiles of the LeC and Tr36 viruses were almost identical suggests that the poor growth and altered plaque phenotype of the LeC virus compared to the Tr36 virus (Figure 8) was not due to a difference in their promoter strengths.





1.5 A 12U to A substitution relative to the 5' end of the LeC virus genome resulted in a rescued plaque phenotype

Experiments with the LeC virus demonstrated that its plaque phenotype was unstable, with occasional reversion to large, intensely stained plaques. To determine whether the reversions seen were the result of altered LeC genome sequence, the virus was passaged nine times to select for viruses with the revertant phenotype. Plague analysis of the passage nine (p9) viral stock showed that the virus population had evolved such that almost all the virus plaques had a phenotype similar to those of the Tr36 virus (Figure 10A). To characterize the 5' LeC sequence(s) in the revertant virus population, RSV genome RNA from cells infected with the p9 stock was amplified by 5' rapid amplification of cDNA ends (5' RACE). The resulting PCR products were cloned, and nine cDNA clones were sequenced. Several different DNA sequences were obtained. indicating that there was variation within the virus population; however, in 9/9 clones, position 12 relative to the 5' end of the genome had changed from a U to an A residue. Figure 10B shows typical sequence traces derived from LeC virus at p6 and p9, with the position 12 substitution indicated with an asterisk (note that the sequence is shown as positive-sense DNA so the change appears as an A to T substitution). As shown in Figure 7B, this substitution restored nucleotide 12 to the same assignment as in the wt RSV Tr region.

To determine whether the position-12 change was sufficient to confer the alteration in plaque phenotype that was observed in the p9 viruses, the LeC cDNA clone was mutated to substitute an A for the U at position 12 relative to the 5' end. This virus (LeC

12U/A) was rescued and passaged once (to remove residual vaccinia virus) and its plaque phenotype was compared to that of LeC and Tr36 virus stocks. As shown in Figure 10C, the plaques formed by the LeC virus were weakly stained with N and F antibodies, while the plaques formed by the LeC 12U/A virus were intensely stained and resembled the plaques formed by the Tr36 virus. These results demonstrate that the LeC region was under selective pressure to acquire a mutation that increased its similarity to the Tr sequence, and that this single-nucleotide substitution was sufficient to restore a strong, intensely stained plaque phenotype for to virus. In addition, we observed informally that the 12 U/A virus demonstrated similar growth kinetics and reached similar maximum viral titers compared to the Tr36 virus (data not shown). The LeC 12U/A virus was an important tool for investigating factors that could be responsible for the impaired growth and altered plaque phenotype of the LeC virus.



Figure 10. A substitution at position 12 relative to the 5' end of the LeC virus genome confered a plaque phenotype similar to that of Tr36 virus. A) Immunostained plaques of LeC virus at passages 6 (p. 6) and 9 (p. 9), as indicated. B) Sequence analysis of representative cDNA clones representing the 5' terminal region of the p. 6 and p. 9 LeC virus genomes, as determined by 5' RACE analysis of genome sense RNA. The sequence is shown as positive-sense DNA and positions 1 and 12 relative to the genome 5' terminus are indicated by asterisks. The poly A tract adjacent to position 1 represents the residues added to the cDNA by terminal transferase during the 5' RACE procedure. C) Example plaques from LeC, LeC 12U/A and Tr36 virus infections immunostained with anti-F or anti-N antibody, as indicated; the LeC virus plaques are highlighted with arrows. The experiments presented in this figure were performed by David McGivern and Robin Djang.

1.6 Infection with wt RSV did not result in stress-granule formation

Previous results revealed that the altered plaque phenotype of LeC virus compared to the Tr36 virus was unrelated to promoter activity. In addition, the fact that the virus could be rescued and propagated demonstrated that LeC virus did not lack an essential cis-acting packaging signal. As described in the Introduction, a previous study implicated a role for the Sendai virus Tr transcript in subverting the cellular stress granule response (103, 208). We hypothesized that the impaired growth of the LeC virus may be due to stress responses. Because the relationship with RSV and stress granules had not been previously characterized, we first wished to determine whether RSV infection resulted in stress granules in infected cells using immunofluorescence. HEp-2 cells were mock infected or infected with RSV. After 16 hours, uninfected cells were treated with sodium arsenite for 30 minutes as a positive control for stress-granule formation. 16.5 hours after infection, cells were fixed and stained with antibodies toward RSV N protein to visualize RSV infection, and antibodies toward cellular eIF3 to visualize stress granules. The cells were examined by confocal microscopy. As shown in Figure 11A, eIF3 from mock-infected, untreated cells showed a diffuse cytoplasmic distribution, while eIF3 from arsenite-treated cells was condensed into cytoplasmic punctae characteristic of stress granules. In cells infected with wt RSV, eIF3 had a similar distribution as in mock-infected cells, suggesting that RSV has evolved a mechanism to either avoid inducing stress granules or to interfere with their assembly. The experiment described above was repeated staining for stress granule-associated protein TIA-1 instead of eIF3. As shown in Figure 11B, stress granules containing TIA-1 form in response to arsenite treatment, but not in response to infection with RSV, confirming that RSV infection does not result in significant stress-granule formation. Of note, stress

granules formed in approximately 0.5% of RSV-infected cells in both experiments (not shown).



Note: Figure 11 continues on the next page.



Figure 11. Infection with wt RSV did not result in stress-granule formation. HEp-2 cells were mock infected or infected (moi = 1). At 16 hpi, cells were treated with 0.5 mM sodium arsenite for 30 minutes. Cells were fixed and stained with antibodies toward RSV N protein (green) and either eIF3 (A) or TIA-1 (B).

1.7 Stress-granule formation was negatively associated with RSV inclusion bodies

As mentioned above, while the majority of RSV-infected cells did not show stressgranule formation, RSV infection did result in stress-granule formation in 0.5% - 5% of RSV-infected cells. We theorized that stress granules formation might limit RSV infection, and wished to assess whether there was a correlation between RSV-infected cells showing stress granules and RSV inclusion body size. HEp-2 cells were mock infected or infected with RSV. 16 hours post infection, cells were treated with sodium arsenite for 30 minutes, stress-granule formation was assessed using immunofluorescence and confocal microscopy. As demonstrated in Figure 12, we observed very few RSV inclusion bodies in areas where there was robust stress-granule formation. Inversely, we observed that in areas with large RSV inclusion bodies, stress granules were less numerous. These results were not quantified; however, they suggest proximity to prominent RSV inclusion bodies is correlated to impaired stress-granule formation.



Figure 12. Areas of cells with prominent RSV inclusion bodies demonstrated dispersed stress granules. HEp-2 cells were mock infected (right) or infected with RSV (left). 16 hpi, cells were treated with 0.5 mM sodium arsenite. Cells were fixed and stained for RSV N protein (green) and eIF3 (red), and examined by confocal microscopy. White arrows indicate areas with prominent RSV inclusion bodies.

1.8 RSV infection prevented stress-granule formation by arsenite, but not heat shock treatment

Our studies suggest that RSV infection did not result in significant stress-granule formation. There were two possibilities: 1) that RSV infection does not induce stress granules, or 2) that RSV infection activates stress pathways, but is able to prevent stress granules from forming. To help discern between these possibilities, we wished to assess whether RSV could prevent stress-granule formation induced by arsenite. HEp-2 cells were either mock infected or infected with wt RSV. Thirty-six hours post infection, cells were either treated with sodium arsenite for 30 minutes or incubated at 44°C for 60 minutes. Stress-granule formation was assessed using immunofluorescence. As demonstrated in Figure 13, cells that were mock infected and subsequently treated with sodium arsenite or heat shock showed classic stress-granule formation in 100% of the cells, as expected. Cells that were infected with RSV and then heat-shocked showed stress-granule formation in 100% of cells, similar to mock-infected, heat shocked cells. This result demonstrates that RSV infection is not able to prevent stress-granule formation induced by heat shock under the conditions tested. In contrast, RSV-infected cells that were treated with sodium arsenite showed stress-granule formation in only 15% of RSV-infected cells, indicating that RSV is able to prevent stress-granule formation by certain stressors.





Figure 13. RSV infection was able to prevent stress-granule formation by arsenite treatment, but not heat-shock treatment. Panel A) HEp-2 cells were mock infected or infected with wt RSV (moi =1). 36 hpi, cells were incubated at 44°C for 60 minutes or treated with 0.5 mM sodium arsenite for 30 minutes. Cells were fixed and stained with antibodies toward RSV N protein (first column) or cellular eIF3η (second column). Samples were analyzed using fluorescent microscopy. Panel B shows the quantification of the results shown in Panel A. Stress granules were assessed in at least 100 cells from each group.

1.9 RSV infection at 16 hpi did not prevent arsenite-induced stress-granule formation

RSV infection was able to prevent stress-granule formation by arsenite at 36 hpi. Because our previous results indicated that RSV infection did not result in stress-granule formation at 16.5 hpi, we wished to determine whether the virus was able to prevent arsenite-mediated stress-granule assembly at this time point. HEp-2 cells were mock infected, infected with RSV, and treated with sodium arsenite. 16.5 hours post infection, cells were fixed and assessed for stress-granule formation using immunofluorescence and confocal microscopy. As shown in Figure 14, cells that were mock infected or infected with RSV did not demonstrate significant stress-granule formation, while cells treated with sodium arsenite form stress granules as expected. Cells that were infected with RSV for 16 hours and then treated with sodium arsenite showed stress-granule formation similarly to arsenite-treated cells, indicating that RSV infection was not able to subvert arsenite-induced stress granules under the conditions tested. It is likely that the stoichiometry of stress-granule preventors vs. stress-granule inducers play a role in the outcome of stress granules, and it is possible that if we had used a larger moi of virus and/or a lower concentration of arsenite, RSV infection may have been able to prevent arsenite-induced stress granules at 16.5 hpi.


Figure 14. RSV did not prevent arsenite-induced stress granules at 16 hpi. HEp-2 cells were mock infected or infected with RSV (moi = 1). 16 hours post infection, the indicated samples were treated with 0.2 mM sodium arsenite as for 30 minutes. 16.5 hours post infection, cells were fixed and stained with DAPI (blue), or stained with antibodies toward RSV N protein (green) and eIF3 (red). Cells were examined by confocal microscopy.

1.10 Infection with LeC mutant resulted in significant stress-granule formation Our results demonstrated that infection with wt RSV did not result in significant stressgranule formation (Figure 11) and that RSV is able to prevent arsenite-induced stressgranule formation under certain conditions (Figure 13). These data suggested that RSV may have a mechanism to block stress-granule formation. We theorized that putative RSV-mediated, stress-granule subversion was due to Tr-region activity, as with the case of Sendai virus, and that the impaired growth of the LeC virus may be due to stressgranule induction. To determine whether LeC infection differed from other RSV viruses with regard to stress-granule formation, cells were mock infected, treated with sodium arsenite, or infected with wt RSV, Tr35, LeC or LeC 12U/A mutant viruses as above. 16.5 hours after infection, cells were fixed and stained with antibodies toward RSV N protein to visualize RSV infection, and cellular eIF3 to visualize stress granules. The cells were examined by confocal microscopy. Cells infected with the Tr36 or LeC 12U/A viruses showed very little stress-granule formation (Figure 15, panels D and F, respectively). In contrast, cells infected with LeC virus showed significantly more stressgranule formation (Figure 15E). These stress granules were not as discrete or as numerous as in the cells treated with arsenite; however, they were clearly discernible (Figure 16). Occasionally the stress granules were located directly adjacent to RSV inclusion bodies, suggesting the possibility of transient interaction, as has been shown previously (208). The percentages of infected cells showing stress granules were guantified and it was found that 53.1-64.7% of LeC virus infected cells showed stressgranule formation, compared to 0.4-1.8% of cells infected with LeC 12U/A virus (Figure 15). These results demonstrate that there is a relationship between the sequence at the

5' end of the RSV genome and stress-granule formation, and that the impaired growth and weak plaque phenotype of the LeC virus is correlated with stress-granule formation.





Panels A-F) Replicate cultures of HEp-2 cells were infected with indicated viruses at an moi of ~1. Cells were immunostained for RSV N (column i) and cellular eIF3 (columns ii and iii) at 16.5 h pi and examined by confocal fluorescence microscopy. The panels in

column iii are enlarged images of the top left quadrants of the images in column ii. At least 100 N expressing cells from each culture in two independent experiments were examined and the percentages of infected cells containing stress granules are indicated alongside the panels. Controls of mock infected cells and cells that were mock infected and then treated with 0.5 mM arsenite for 30 min immediately prior to fixation are shown in panels A and B, as indicated.



Figure 16. Localization of stress granules relative to RSV inclusion bodies in LeC virus infected cells. Cells were infected with LeC virus at an moi of 2. At 16.5 h pi the cells were immunostained for RSV N (column i) and cellular eIF3 (column ii) and examined by confocal microscopy. Column iii shows a merged image of N, eIF3 and DAPI stained cells.

1.9 Co-infection studies demonstrated that wt RSV could inhibit LeC-mediated stress-granule formation

There were two possible explanations for the differences among infections with wt. Tr36. LeC, and 12 U/A viruses with regard to stress-granule formation. The first was that the WT, Tr36, and LeC12 U/A viruses were able to actively subvert the stress-granule response, while the LeC virus was not. The second possibility was that a property of the LeC virus induced stress-granule formation, while the WT Tr155, Tr36 and LeC 12 U/A viruses did not. To help distinguish between these possibilities, a mixed-infection experiment was performed. HEp-2 cells were infected with wt RSV, LeC virus, or both LeC and wt RSV at an moi of 2 and 6, respectively. 16.5 hours post-infection, cells were fixed and stained for RSV N protein and eIF3, and stress-granule formation was assessed using confocal microscopy. As shown in Figure 17, stress-granule formation was present in 3.9 - 5.4 % of wt RSV-infected cells, while stress-granule formation was observed in 44.1 - 51.1 % of LeC-infected cells, consistent with previous results. Surprisingly, cells that had been infected with both wt RSV and LeC virus showed stress granules in 4.1 - 4.2 % of cells, similar to cells infected with only WT virus. These results suggest that wt RSV is able to actively disrupt or prevent LeC-mediated stress-granule formation.



Figure 17. wt RSV blocked stress-granule formation induced by the LeC virus. A) Replicate cultures of HEp-2 cells were infected with indicated viruses at an moi of 2 in the case of the LeC virus, and 6 in the case of wt RSV. Cells were immunostained for RSV N and cellular eIF3 at 16.5 h pi and examined by confocal microscopy. B) Bar graph illustrating proportions of cells that showed stress-granule formation. At least 100 cells from each culture were examined and the percentages of infected cells containing stress granules calculated. The data from two independent experiments are shown (represented by the black and grey bars).

1.12 The weak-plaque phenotype of LeC was not restored in TIAR knockout cells. As mentioned earlier, Iseni et al. reported that the Tr transcript of Sendai virus binds stress granule-associated protein TIAR, and proposed that this enables Sendai virus to subvert stress-granule assembly (103). We hypothesized that RSV utilizes a similar mechanism to prevent stress granules, and that the LeC virus displays impaired growth characteristics because it is unable to sequester TIAR and thereby prevent stressgranule assembly. If this model were correct, it would be expected that the LeC virus would have a plaque phenotype similar to wt RSV in cells lacking TIAR. To test this possibility, mouse embryo fibroblasts derived from TIAR-knockout mice were infected with wt RSV, or the Tr36, LeC, or LeC 12U/A viruses and assessed for plaque phenotype. The absence of TIAR expression was confirmed by western blot analysis (Figure 18A). Contrary to what we expected, compared to the other viruses, LeC virus displayed a small/faint plaque phenotype in TIAR knockout cells similar in size and intensity to LeC plaques in HEp-2 cells (Figure 18B). These data show that RSV subversion of stress-granule formation is independent of TIAR, and suggest that, unlike the situation with Sendai virus, the difference between LeC and wt RSV growth is not related to the ability of the Tr or TrC sequence to sequester TIAR.



Figure 18. The LeC virus phenotype was small and faint in TIAR knockout cells. A) Western blot analysis of total intracellular proteins from mouse embryonic stem cells derived from a WT (+/+) (lane 1), or TIAR knockout (-/-) mouse (lane 2). The 40 kDa TIAR protein was detected with a polyclonal antibody. B) Monolayers of TIAR-/-cells were infected with wt RSV, Tr36, LeC and LeC 12U/A viruses. Four days post-infection, virus plaques were immunostained with anti-F antibody. The pictures on the left are photographs of the infected wells. The pictures on the right are close-ups of the plaques, as visualized with microscopy.

1.13 Arsenite treatment and LeC infection induced stress-granule formation in TIAR KO cells

Because we had hypothesized that stress responses were responsible for LeC's impaired growth and weak plaque phenotype, we had expected that LeC virus would have a restored plaque morphology in TIAR KO cells, which we had assumed were deficient in stress-granule formation. To determine whether stress granules were able to form in these cells, we treated them with sodium arsenite or infected them with the LeC virus; two conditions that induced stress granules in other cells. As demonstrated in Figure 19, both arsenite treatment and LeC infection induced stress granules in TIAR knockout cells. These data indicate that TIAR is not necessary for LeC infection- or arsenite-induced stress granule-like formations. They are also consistent with previous results showing that the weak-plaque phenotype of the LeC virus correlates with stress-granule formation.



Figure 19. Arsenite treatment and LeC infection induced stress granules in TIAR KO cells. Monolayers of TIAR-/- cells were mock infected, mock infected and treated with arsenite for 30 min immediately prior to fixation, or infected with LeC virus at an moi of ~1. The cells were immunostained for eIF3 at 16.5 h pi and examined by fluorescence microscopy.

1.14 Transfected RSV proteins and minigenome prevented stress-granule formation.

Because the results shown in Figure 18 and Figure 19 indicated that RSV-mediated stress-granule subversion was not due to TIAR sequestration, we wished to further explore the conditions during which RSV infection is able to block stress-granule formation. Our results did not rule out the possibility of the RSV Tr region having a role in stress-granule prevention, however, we wished to examine whether expression of certain RSV proteins was sufficient to prevent stress-granule assembly. To investigate whether RSV proteins associated with RSV polymerase are able to prevent stress granules, we transiently expressed either N, P, M2-1, and L or N, P, L, M2-1, and the Tr155 minigenome in cells. The minigenome was added in case active replication was needed for stress-granule subversion. Twenty-four hours post infection, cells were treated with 0.5 mM sodium arsenite for 30 minutes, and stress-granule formation was assessed using immunofluorescence and confocal microscopy. As shown in Figure 20, cells that were mock transfected, arsenite-treated demonstrated robust stress-granule formation, as expected. Cells that were transfected with N, P, M2-1, and L formed stress granules in response to arsenite; however, there were very few cells that stained for N protein, indicating that the transfection efficiency was low. This was also the case for cells transfected with N,P, M2-1, L, and the minigenome; however, the cells that were transfected (as indicated by positive staining for N protein (green)) showed reduced stress granules compared to other cells. Because the transfection efficiency was so poor, no firm conclusions can be drawn from these results; however, it is possible that expressing N, P, M2-1, and L in setting in which the polymerase is active is sufficient to

prevent stress granules. These findings suggest that a minigenome system may be a useful tool for investigating RSV-mediated stress-granule subversion, and warrant further investigation.



Figure 20. Transfected proteins and minigenome may have been sufficient to prevent arsenite-induced stress-granule formation. HEp-2 cells were transiently transfected with plasmids expressing RSV N, P, L, and M2-1 proteins and /or the Tr155 minigenome in addition to a plasmid expressing the T7 polymerase. 24 hours post transfection, cells were treated with 0.5 mM sodium arsenite for 30 minutes. Cells were fixed and immunostained with antibodies toward RSV N protein (green) and eIF3 (red). Slides were examined using confocal microscopy.

1.15 RSV infection did not alter the electrophoretic profile of stress granuleassociated proteins.

We next wished to determine whether RSV infection altered key proteins associated with canonical stress granules. To address this question, HEp-2 cells were mock infected or infected with increasing levels of RSV. 18 hours post infection, cells were treated with sodium arsenite for 30 minutes as a positive control for stress. Cells were lysed, and stress-granule-associated proteins were detected using western-blot analysis. As shown in Figure 21, none of the proteins tested was altered in response to RSV infection; however, we did not test all proteins that have been associated with cellular stress responses, so it is possible that other stress granule-associated proteins are altered during RSV infection.



Figure 21. RSV infection did not alter the electrophoretic mobility of key stress granule-associated proteins. HEp-2 cells were mock infected or infected with increasing levels of RSV (the moi used is indicated). 18 hours post infection, cells were lysed with RIPA buffer containing protease inhibitors (but not phosphatase inhibitors). Proteins were assessed using SDS-PAGE and western blot analysis with antibodies toward the indicated proteins.

Chapter 1 Discussion

Summary of results

The goals of this study were to investigate possible roles of the RSV Tr region. In summary, our results demonstrated that replacing the RSV Tr region with the LeC sequence resulted in slower growth and lower maximum virus titer in both cell-culture systems and mice (Figure 8 and data not shown), as well as a small, weakly stained plaque phenotype. The impaired growth and weak plaque phenotype of RSV was not due to differences in promoter activity, since mRNA accumulation during LeC infection was similar to that of the Tr36 virus (101). However, infection with the LeC virus resulted in significant stress-granule formation in infected cells, while infection with wt RSV or other mutant viruses did not. Coinfection with wt RSV and the LeC virus demonstrated that wt RSV is able to block LeC-mediated stress-granule induction, and studies using TIAR knockout cells showed that unlike Sendai virus, RSV prevention of stress granules is independent of TIAR. These studies suggest that stress-granule formation is deleterious for RSV infection, and that RSV has evolved mechanisms for subverting host-cell stress responses.

Conflicting study

It should be noted that shortly after our results described above were published (101), a similar study was published that contradicted our results (143). In the paper, Lindquist et al. reported that RSV induces and benefits from significant stress-granule formation. In their hands, infection with RSV resulted in stress-granule formation in approximately 25% of infected cells at 16 hpi, and that cells with stress-granule formation contained larger RSV inclusion bodies than those without stress-granule formation. They further

reported that cells deficient in G3BP resulted in a 10-fold decrease in replication and concluded that stress-granule formation was beneficial for RSV replication.

It is unclear why they observe more stress granules in infected cells than we did. It is notable that their results demonstrated that only 25% of infected cells showed stressgranule formation. Thus, in both our study and theirs, the majority of wt RSV-infected cells do not form stress granules, which is consistent with the hypothesis that RSV has a mechanism to subvert the stress response. The authors concluded that because cells containing stress granules showed larger RSV inclusion bodies, it could be inferred that stress granules were beneficial to RSV biology; however, there is no formal data to suggest that RSV inclusion bodies are sites of RSV replication. Finally, the authors concluded that because RSV replicated less efficiently in cells lacking G3BP, an ATP-dependent RNA helicase, it could be inferred that stress granules are beneficial to RSV. It is possible, however, that G3BP is directly or indirectly important for RSV replication in a capacity separate from stress-granule formation. An exciting possibility is that interfering with stress granule assembly increases the availability of G3BP for participation in RSV replication.

In support of our conclusions regarding RSV's subversion of stress-granule formation, Fricke et al. recently published a study demonstrating that RSV is able to subvert stressgranule formation by sequestering phosphorylated p38 mitogen-activated protein kinase (MAPK) and O-linked N-acetylglucosamine transferase (OGT) into RSV inclusion bodies, thereby preventing the activation of MAPK-activated protein kinase 2 (MK2) (88). In addition, the group performed a well-controlled experiment in which they

demonstrated that in the small percentage of RSV-infected cells that showed stressgranule formation, the presence of small inclusion bodies was associated with the presence of stress granules. In addition, there was a significant negative association between cells showing large, prominent inclusion bodies and stress-granule presence. Taken together, their and our results demonstrate that infection with RSV does not result in significant stress-granule formation, and that the virus is able to actively subvert stress responses.

Possible explanations for LeC-mediated stress-granule induction: gain of function or loss of function?

There are at least three possible explanations for why the LeC virus induced stress granules in a significant proportion of infected cells whereas the LeC 12A/U, Tr36, and WT viruses did not. One possibility is that the Le promoter generated an RNA species that induced stress-granule formation (possibly because of the unnatural context of the Le promoter in the LeC virus). Another possibility is that the Tr36, LeC 12U/A, and WT viruses can actively inhibit stress-granule formation, while the LeC virus has lost its ability to subvert the stress response. The third possibility is a combination of these proposed mechanisms: it is possible that LeC is inherently more disposed to induce stress-granule formation, that all the viruses are able to prevent stress granules within certain limits, and that in the approximately 50% of LeC-infected cells demonstrating stress-granule formation, the virus was defeated in its anti-stress granule activities.

Regardless of whether LeC has a gain-of-function ability to induce stress granules, the data shown in Figure 17 provide evidence that wt RSV is able to actively prevent stress-granule formation. In this experiment, co-infection of cells with both LeC and WT viruses

resulted in a very low level of stress granule-containing cells, indicating that wt RSV was able to inhibit stress-granule formation by the LeC virus. The Tr36 virus infection did not induce stress granules, suggesting that the sequence required for stress-granule subversion may be located within the terminal 36 nucleotides of Tr, in a region that shares significant sequence similarity to LeC sequence (Fig. 1B). This is supported by the fact that a single point mutation in the LeC sequence (12 U/A) that increased the similarity of the LeC sequence to that of Tr was sufficient to enable the LeC virus to avoid stress-granule formation. This single nucleotide change might have restored a key sequence element or secondary structure to the LeC viral RNA. It is unclear if the sequence in question functions in the context of the encapsidated genome or antigenome RNA, or if it is present in an unencapsidated transcript generated from the TrC promoter. However, in a recent study we showed that the terminal 36 nts of the TrC promoter region could signal synthesis of an RNA transcript initiated at position 3 and extended approximately 25 nucleotides (185), and it is possible that this transcript is involved in subverting stress-granule formation.

Alternatively, it is possible that the LeC virus induces stress-granule formation because of its altered Tr sequence, but that RSV's ability to actively interfere with stress-granule formation is not directly related to Tr sequence. For example, LeC may form increased levels of dsRNA intermediates during replication that activate PKR signaling and thereby induce stress-granule formation that hampers its replication cycle. wt RSV is able to prevent LeC-mediated stress-granule formation, but it could be mediating this effect by sequestering p38 and OGT, as described by Fricke et al. An exciting possibility is that

the RSV Tr region binds and sequesters p38 or OGT in order to subvert stress responses.

Possible mechanisms for RSV-mediated stress-granule subversion

In light of the recent study by Fricke et al., we conclude that it is probable that RSVmediated stress-granule subversion is due to sequestration of p38 and OGT in viral inclusion bodies; however, the details of this mechanism have not been worked out, and it is possible that RSV has more than one mechanism for blocking the stress response.

Our studies demonstrate that RSV is able to prevent LeC-induced stress granules. Similarly to the results shown here, a mutant version of Sendai virus containing LeC in place of Tr induced stress granules to a greater extent than the WT virus (103). Sendai virus Tr-specific sequence was found to bind to TIAR, suggesting that it sequesters this protein to subvert stress-granule formation. However, RSV subversion of stress granules was independent of TIAR (Figure 18). It is possible that the RSV Tr sequence binds to an alternative stress-granule protein, such as TIA-1, a stress granule-associated scaffold protein that is closely related to TIAR (92, 114). Unfortunately, mouse embryo fibroblasts in which TIA-1 is knocked out did not support RSV infection, preventing us from performing an experiment similar to that shown in Figure 18 to test the importance of TIA-1. The deficiency in RSV growth was unrelated to the absence of TIA-1, as a "sister" control cell line also failed to support RSV cell-to-cell spread (data not shown). It is possible that RSV Tr might modulate the cellular stress-granule response due to a direct interaction with stress-granule proteins other than TIAR; however, a study by

Santangelo et al. showed no evidence of stress-granule protein sequestration by RSV RNAs (208).

Another possibility is that RSV inhibits stress-granule formation at a step upstream of stress-granule assembly. Santangelo et al. reported that wt RSV cannot inhibit the stress-granule response if infected cells are treated with arsenite prior to infection (208). This could either be because arsenite treatment overwhelms the stress granulesubverting activity of RSV, or because arsenite induces stress granules via an alternative pathway than RSV infection, and the virus is not able to inhibit the arsenitemediated pathway at the concentration used in the study. Another possibility is that RSV-mediated stress subversion requires the accumulation of a specific factor (for example, Tr RNA) before it is able to mediate stress-granule prevention, and that pretreatment with arsenite does not allow this to occur. Our results shown in Figure 13 demonstrating that a well established RSV infection can interfere with stress-granule formation induced by other stressors in certain cases indicate that the stochiometry of the factors involved (stress-granule inducers vs. stress-granule preventers) may determine the stress-granule formation outcome. Our results as well as others' suggest that stress-granule induction is not a Boolean phenomenon; rather, the degree of stressgranule formation by chemical stressors is commensurate with the concentration of stressor (data not shown). Given the study by Lindquist et al., in which conditions very similar to those used by our lab were used that yielded differing outcomes, it seems probable that RSV infection lingers near the interface of stress induction versus stress subversion, and that many factors can tip the balance in either direction. More studies will be needed to work out the mechanisms of RSV-mediated stress-granule subversion.

Evidence that stress granules are deleterious for RSV propagation

There is a growing body of evidence that stress granules are inherently antiviral. Our data suggest that stress granules may be responsible for the impaired growth and plaque phenotype of the LeC virus. Although the Tr36 and LeC viruses generated similar levels of RNA, they differed significantly with regard to virus growth and plaque phenotype (Figure 8), and their differences in plaque phenotype correlated with their propensity to elicit a cellular stress-granule response (Figure 15). Emerging evidence suggests that a number of viruses are restricted by the cellular stress-granule response (140, 199) and unsurprisingly, several viruses are known to either prevent or manipulate stress-granule formation (74, 75, 103, 158, 172, 198, 229, 265).

The correlation between stress-granule formation and RSV plaque phenotype suggests that stress granules were likely responsible for hindering LeC virus growth, but a mechanism for this inhibition has not been determined. Stress granules are involved in RNA translation and degradation control (16, 17), suggesting that they might inhibit LeC virus growth by limiting its protein expression. However, metabolic labeling analysis indicated that there was little, if any, difference in LeC viral protein synthesis compared to that of other viruses at a time (16 hours post infection) when stress granules were clearly detectable (data not shown).

One exciting possibility is that stress granules may limit growth of the LeC virus by manipulating cytokine production. This is supported by reports that stress granules can post-transcriptionally regulate cytokine expression (15). Preliminary studies have indicated that the LeC virus did not differ significantly from the Tr36 and LeC 12 U/A

viruses in the amount of interferon that was produced during infection (M. Galliano, R.E. Randall, and R. Fearns, unpublished data,); however, interferon is not a major player in RSV immune responses. Other cytokines and chemokines important during RSV infection, such as IL-8, IL-6, and RANTES, have not been examined in the context of LeC infection.

Future studies

An increasing number of scientists are reporting mechanisms by which viruses are able to overcome cellular stress responses, and an emerging theme is that viruses and cellular stress responses have co-evolved in such a way that there is a delicate equilibrium established between stress induction and stress subversion. We have demonstrated that RSV is able to prevent stress-granule formation in the majority of infected cells, but that occasionally fails in its stress-prevention activities. Furthermore, the virus can be modified in such a way (i.e. the LeC virus) that the balance is tipped in favor of stress-granule induction. Future studies are needed to determine the mechanism by which RSV is able to subvert stress-granule formation, the factors involved in determining the extent to which RSV can battle stress responses, and whether stress granules are definitively deleterious for RSV propagation.

Chapter 2: RSV Interactions with P-bodies and P-body-associated Proteins

Chapter 2 Introduction

Processing bodies (p-bodies)

As described briefly in the Introduction, p-bodies are constitutively present in the cytoplasm of most cells, and are believed to be sites of mRNA decapping and degradation. They increase in size and number during environmental stress when cellular translation is stalled. P-bodies contain decapping proteins 1 and 2 (dcp1 and dcp2), exonucleases such as Xrn1, deadenylases, and RNA-binding proteins with roles in nonsense-mediated mRNA decay pathways and micro-RNA gene silencing processes such as DDX3, Ago2, p54/DDX6, Lsm1, PatL1, and G3BP. While mRNA decay occurs inside p-body structures, p-bodies are not required for nonsense-mediated decay. The proportions of mRNA decay that take place inside vs. outside p-bodies has not been resolved (21).

P-body assembly

As with stress granules, the mechanisms of p-body formation are largely uncharacterized. Some studies suggest that p-body formation involves mRNA and RNA-binding protein aggregation since cells treated with RNase show dispersed pbodies (78). There is some evidence that the DEAD-box helicase p54 (also called DDX6 and rck), which is discussed extensively in Chapter 3, is required to coat and relax mRNPs before their entry into p-bodies (77).

Nonsense-mediated decay pathway

Nonsense-mediated decay is a major mRNA degradation pathways that takes place in p-bodies. This process is initiated when the poly-A tail on capped and polyadenylated mRNAs is shortened by deadenylase enzymes. The mRNA is then decapped by the dcp1/dcp2 decapping complex. Cap removal renders the transcript susceptible to exonucleases such as Xrn1, which degrade RNA in a 5' to 3' direction (Figure 22).



Figure 22. Nonsense-mediated decay pathway. Nonsense-mediated decay is initiated when the poly-A tail (A's) on capped (red clouds) and polyadenylated mRNAs (blue wavy lines) is shortened by deadenylase enzymes. The mRNA is then decapped by the dcp1/dcp2 complex, which is stabilized by enhancers edc3, edc4, p54, and Xrn1. Cap removal renders the transcript susceptible to exonucleases such as Xrn1, which degrade RNA in a 5' to 3' direction.

Decapping proteins

As mentioned above, during nonsense-mediated decay, the 7-methylguanosine cap is cleaved from mRNAs by the dcp1/dcp2 complex. Studies in yeast have shown that dcp2 contains the catalytic activity necessary for the reaction, and that dcp1 is an essential cofactor that is believed to stabilize dcp2 in a closed, catalytically-active conformation necessary for decapping (220). The crystal structure of dcp1 in yeast has recently been characterized, and it revealed two important domains – one for binding dcp2, and another for binding decapping enhancers (219). In humans, dcp1 and dcp2 exist in a complex with enhancer proteins Edc3, Edc4 (also called Hedls), p54, and exonuclease Xrn1 ((35, 83). Edc4 is important for enhancing the catalytic activity of dcp2 and stabilizing the association between dcp1 and dcp2 (83). Our results have identified dcp1 as important during RSV infection, as discussed in this chapter.

Virus interactions with p-bodies

P-body-associated activities help regulate mRNA stability, metabolism, and expression. It therefore stands to reason that viruses could evolve to manipulate p-body formation and/or co-opt p-body proteins for use in viral replication cycles. Several viruses are reported to disperse p-bodies, degrade p-body proteins, and/or hijack p-body constituents to enhance replication. The following table summarizes such virus-p-body interactions:

Table 4: Virus interactions with cellular p-bodies

Virus	Mechanism of P-body Interaction	Ref
Virus	meenanism of 1-body interaction	itel.
Viruses that disperse of	or redistribute p-bodies during infection	on
Poliovirus and coxsackievirus	Disrupt p-bodies; mediate	(72)
	degradation of Pan3, Xrn1, and	
	Dcp1	
Adenovirus	Viral E4 11K protein disrupts p-	(94)
	bodies by redistributing p-body	
	components Lsm-1, Gee-1, Ago2,	
	and Xrn1 to aggresomes for	
	presumed proteolytic destruction	
Influenza virus	Viral protein NS1 associates with p-	(168)
	body protein Rap55; NS1-Rap55	
	complex prevents viral RNPs from	
	entering p-bodies	
Viruses that co-opt p-bo	ody components to augment replication	on
	Infection results in progressive	
West Nile virus	dispersion of p-bodies; virus directs	(46,
	relocalization of p-body components	
	Lsm1, DDX3, DDX6, and Srn1 to	74)
	virus replication factories for	

Yellow fever virus, dengue	Xrn1 is necessary for production of	(173,
virus, Kunjin virus, West Nile	sfRNA, which may be important for	215,
virus	virus replication	223)
HIV1	Conflicting reports: One study indicates viral RNAs are localized to p-bodies; another study contends they are not; Gag forms complexes with p54/DDX6 and Ago2 to promote virion assembly	(182, 196, 201)
Hepatitis C virus	HCV core proteins colocalize with DDX6 in p-body-like punctate complexes; DDX3 enhances HCV replication; re-distributes p-body components p54/DDX6, Lsm1, Wrn1, PATL1, and Ago2 into viral replication factories	(20)
Hantavirus	Viral nucleoprotein N binds to 5' cap of cellular mRNAs in p-bodies to prevent decapping and subsequent degradation; these complexes are released back into cytoplasm where the virus uses protected/"snatched" caps to prime virus mRNA synthesis	(167)

Brome mosaic virus	Requires p-body components Lsm1p-7p complexes, Pat1p, and Dhh1p required for efficient translation of viral transcripts and for viral RNA entry into replication complexes	(186)
Gamma herpes virus	Promote degradation of cellular RNAs to promote translation of viral mRNAs; viral protein SOX induces endonucleolytic cleavage of cellular RNAs for degradation by Xrn1	(61)

Hypothesis

In the previous chapter, we discussed our studies exploring the relationship that RSV has with cellular stress granules. We next wished to characterize the relationship the virus has with cellular p-bodies. We hypothesized that RSV infection altered either p-body-associated proteins or p-body structures.

Chapter 2 Results

2.1 RSV infection altered the electrophoretic mobility of dcp1, but not of other pbody-associated proteins.

Our studies discussed in Chapter 1 focused on the relationship between RSV infection and host-cell stress granules. We next wished to investigate RSV's relationship with pbody proteins and structures. To determine whether RSV infection results in altered pbody-associated proteins, HEp-2 cells in 6-well plates were mock infected or infected with increasing levels of RSV. After 17.5 hours, one well was treated with 0.5 mM sodium arsenite for 30 minutes. Eighteen hours after infection, samples were harvested and analyzed using SDS-PAGE and western blot analysis with antibodies toward a panel of p-body-associated proteins. As seen in Figure 23, neither the levels nor migration patterns of dcp2, edc3, edc4, eIF3, or Xrn1 were changed eighteen hours after infection. On the other hand, the electophoretic mobility of dcp1 was altered in RSVinfected cells. In mock-infected cells, dcp1 migrated as a doublet (Figure 23, lane 1); however, in arsenite-treated cells (lane 2) or cells infected with RSV at an moi of 1 or 5 (lanes 5 and 6), dcp1 migrated as only the upper band. These results indicate that dcp1 is altered in response to RSV infection, and that the effect is dose dependant.

To determine whether RSV infection results in an altered dcp1 migration pattern in a more authentic cell type, we repeated the experiment described above in A549 cells and obtained similar results, confirming that altered dcp1 migration profile seen during RSV infection was not cell specific (Figure 24). Figure 25 shows the RSV-mediated electrophoretic shift from a doublet to a single upper band in greater resolution than the previous figures.





arsenite for 30 minutes, or infected with increasing levels of RSV at the indicated moi. 18 hour post infection, cells were lysed with RIPA buffer. Soluble fractions of samples were assessed using western blot analysis and antibodies toward the indicated proteins.








2.2 The shift in dcp1 electrophoretic mobility observed during RSV infection was due to dcp1 phosphorylation.

Post-translational modifications of dcp1

Several studies have demonstrated that dcp1 can be post-translationally modified by phosphorylation. Blumenthal et al. found that dcp1 is phosphorylated during certain stages of neuronal development and in response to arsenite treatement (31). Xu et al. reported that dcp1 is phosphorylated in Arabidopsis plants during dehydration stress (271), and Rzeczkowski et al. discovered that dcp1 is phosphorylated in mammalian cells in response to treatment with IL-1a, anisomycin, or sorbitol (206). Ma et al. recently reported that dcp1 is phosphorylated during murine oocyte development (28), and Aizer et al. demonstrated that dcp1 is phosphorylated during mitosis (4). These studies suggest that dcp1 phosphorylation is important during development, stress, immune responses, and cell division. Most of these studies describing dcp1 phosphorylation had not been published at the time of our studies. Because Blumenthal et al, had reported dcp1 phosphorylation during arsenite stress and neuronal development, we hypothesized that the RSV-mediated shift in dcp1 migration was due to phosphorylation of dcp1. Cells were mock infected, treated with sodium arsenite (as a positive control for dcp1 phosphorylation), or infected with RSV. Eighteen hours after infection, cells were lysed and either mock treated or treated with λ -phosphatase, which dephosphorylates serine, threonine, and tyrosine residues. As shown in Figure 26, dcp1 from mockinfected cells migrated as a doublet (lane1), while dcp1 from arsenite-treated or RSVinfected cells migrated as only the upper band (lanes 3 and 5). In contrast, the samples from each group that were treated with phosphatase migrated as only the lower band (lanes 2, 4, and 6), indicating that the upper band results from dcp1 phosphorylation.



Figure 26. RSV-mediated change in dcp1 migration was due to phosphorylation. HEp-2 cells were mock infected, treated with sodium arsenite, or infected with RSV (moi = 5), and lysed as described, except that phosphatase inhibitors were not incuded in the RIPA buffer. Soluble fractions were either mock treated or treated with λ phosphatase (Santa Cruz, 100 U in a 50 µl reaction according to manufacturer's instructions) for 30 minutes. Samples were examined using western blot analysis with an antibody toward dcp1a.

2.3 RSV infection mediated dcp1 migration shift in all cell-lysate fractions.

To further explore RSV-mediated dcp1 phosphorylation, we wished to determine whether the phenomenon was unique to specific cellular fractions. Mason et. al. developed a protocol for fractionating RSV-infected cell lysates using increasingly stringent buffers alternated with centrifugation as depicted below:



Figure 27. RSV fractionation technique schematic.

Mason et al. used *in vitro* transcription assays to determine that the S3 fraction most active with regard to RSV transcription (149). We wished to determine whether phosphorylated versus unphosphorylated dcp1 accumulated in a specific RSV fraction.

HEp-2 cells were mock infected or infected with RSV (moi =5). Eighteen hours after infection, cells were lysed and fractionated using Mason's technique. Dcp1 phosphorylation was assessed using SDS-PAGE and western blot analysis. As shown in Figure 28, dcp1 was most concentrated in the S1 fraction, but was present in all fractions. In addition, dcp1 migrated as the upper band in all fractions from RSV-infected cells (lanes 2, 4, 6, and 8). These results indicate that accumulation of phosphorylated dcp1 was not specific to any RSV fraction. Dcp1 showed a slight increase in level in the P1 fraction, consistent with other data indicating that RSV infection may sometimes result in increased levels of dcp1. Future studies are needed to investigate this phenomenon.





2.4 Purified RSV was sufficient to mediate dcp1 phosphorylation.

As discussed in detail in the Materials and Methods section, the RSV stocks used in the majority of our experiments are derived from the clarified supernatant of RSV-infected cells. In brief, cells are infected with RSV at a very low moi. Three to five days after infection, the supernatants are collected and centrifuged to remove cells. Because the virus stocks contain cellular debris and proteins secreted by the infected cells including soluble RSV envelope glycoproteins and cytokines such as IL-1 (which has been reported to induce dcp1 phosphorylation), we wished to determine whether RSV itself and/or another factor in the supernatant-derived RSV stocks was responsible for inducing dcp1 phosphorylation.

To determine whether something in the supernatant other than RSV was able to induce dcp1 phosphorylation, the supernatant from RSV-infected cells was centrifuged at an ultra-high velocity in order to pellet the virus. Supernatant from above the pellet (which presumably contained little to no virus) was collected and a plaque assay was performed. The supernatant (sup) was found to have a negligible amount of virus. To address whether RSV was sufficient to mediate dcp1 phosphorylation, we purified RSV using a sucrose-cushion method (suc.) in which ultra-centrifuged RSV formed a band at the interface between 30% and 60% sucrose solutions. Because banded virus was diluted 1:10 in sterile media, the maximum percentage of sucrose in purified virus preparations was 6%. HEp-2 cells were mock infected, treated with supernatant from RSV-infected cells, treated with 6% sucrose (to determine whether sucrose in purified RSV preparations influenced dcp1 phosphorylation), or infected with sucrose-purified RSV. One hour after infection, cells were lysed and dcp1 phosphorylation was assessed

using SDS-PAGE and western blot analysis. As shown in Figure 29A, dcp1 from mockinfected cells (lane 1) or sucrose-treated cells (lane 2) migrated as a doublet. Dcp1 from cells treated with supernatant (lane 3) or infected with sucrose-purified RSV (lane 4) migrated as primarily the upper band. These results indicate that one or more factors in preparations of RSV that have not been purified is able to mediate dcp1 phosphorylation. Importantly, these results also demonstrate that purified RSV is sufficient to mediate dcp1 phosphorylation.

To determine whether infection with purified virus was able to mediate dcp1 phosphorylation over a longer period, HEp-2 cells were mock infected, infected with the supernatant of ultracentrifuged virus, treated with 6% sucrose, or infected with sucrosepurified virus. One hour post infection, the media was changed. Eighteen hours after infection, cells were lysed and dcp1 phosphorylation was assessed using SDS-PAGE and western blot analysis. As shown in Figure 29B, dcp1 from mock infected cells (lane 1), supernatant-treated cells (lane 2), or sucrose-treated cells (lane 3) migrated as a doublet, while dcp1 from sucrose-purified RSV (lane 4) migrated as only the upper band. These results indicate that treatment of cells with neither the supernatant from RSVinfected cells nor 6% sucrose are able to mediate dcp1 phosphorylation 18 hours after treatment (17 hours after removal of treatments). In addition, these results confirm that purified RSV is sufficient to mediate dcp1 phosphorylation late in RSV infection. Taken together, our results indicate that although one or more factors in unpurified virions is able to mediate a transient increase in dcp1 phosphorylation, purified RSV is sufficient to induce dcp1 phosphorylation.





lysed and examined using western blot analysis with an antibodies toward dcp1 or RSV proteins.

2.5. TNF-a and IL-1, but not IFN-a, mediated dcp1 phosphorylation

Several cytokines are induced during RSV infection. Kurt-Jones et al. showed that RSV F protein interacts with TLR-4 to induce proinflammatory cytokine production (128), and many cytokines are reportedly induced during RSV infection *in vitro*, including IL-6, TNF- α , IL-1 β , IFN- γ , IL-2, IL-12, IL-4, IL-5, IL-10, IL-13, G-CSF, and GM-CSF (218), as well as IFN- α and IFN β (187). Because our results suggested that a component in the supernatant of RSV preparations was able to transiently induce dcp1 phosphorylation, we wished to determine whether cytokines associated with RSV infection could mediate the effect. HEp-2 cells were treated with increasing concentrations of TNF- α , IL-1 β , or IFN- α for one hour. Cells were lysed and assessed for dcp1 phosphorylation using SDS-PAGE and western blot analysis. As shown in Figure 30, dcp1 was phosphorylated in response to all levels of TNF- α and IL-1 β . In contrast, treatment with IFN- α did not result in dcp1 phosphorylation at any of the concentrations tested. These results indicate that cytokines that are potentially present in RSV virion preparations that have not been purified may contribute to dcp1 phosphorylation.





2.6 Dcp1 was phosphorylated early during RSV infection, and remained phosphorylated throughout infection.

Our results from previous experiments indicated that dcp1 is phosphorylated both early during infection (1 hpi) and well after infection is established (16-18 hpi). To more extensively examine the kinetics of dcp1 phosphorylation during RSV infection, we performed a time course. HEp-2 cells were either mock infected, infected with regular RSV, infected with sucrose-purified RSV, or treated with sodium arsenite. At the indicated times post infection, cells were lysed and dcp1 phosphorylation was assessed using SDS-PAGE and western-blot analysis. As demonstrated in Figure 31, panels A and C, dcp1 was phosphorylated within 15 minutes of RSV infection, and remained phosphorylated throughout infection with either regular RSV (clarified supernatants from RSV-infected cells) or sucrose-purified RSV. These results suggest that dcp1 phosphorylation is triggered by an early event in RSV infection such as RSV-cell binding or fusion. In addition, these results indicate that one or more aspects of RSV infection mediates sustained dcp1 phosphorylation throughout infection.

2.6 UV-inactivated RSV mediated transient dcp1 phosphorylation.

To determine whether RSV replication is necessary in order for the virus to mediate dcp1 phosphorylation, the time course described above was also performed with both UV-inactivated regular RSV or UV-inactivated sucrose-purified RSV. RSV was irradiated at 200 J/m2. A plaque assay was performed to confirm that these conditions were sufficient to abrogate RSV replication (Figure 31, panel E). As shown in Figure 31 panels B and D, dcp1 from cells infected with UV-inactivated RSV showed a transient increase in dcp1 phosphorylation while virus was in contact with cells (lanes 2-4);

however, once the media was replaced, the increase in dcp1 phosphorylation began to reverse (lanes 5-7). By twenty-four hours post infection, dcp1 from cells infected with UV-inactivated RSV had a similar migration pattern to dcp1 from mock-infected cells (compare lanes 1 and 7). These results indicate that replication-competent RSV is not necessary for mediating dcp1 phosphorylation; however, replication-competent RSV is necessary for maintaining dcp1 phosphorylation throughout infection. Of note, the dcp1 phosphorylation profiles from samples infected with regular RSV preparations looked remarkably similar to those infected with sucrose-purified RSV (compare panels A and C, and panels B and D).



Figure 31. DCP1 was phosphorylated throughout RSV infection. HEp-2 cells were mock infected, infected with RSV (A), UV-inactivated RSV (B), sucrose-purified RSV (C), or UV-inactivated, sucrose-purified RSV (D), or treated with 0.5 mM sodium arsenite. For samples treated with UV-inactivated virus, RSV was subject to 200 J/m² irradiation.

Samples were lysed in RIPA buffer at the indicated times post infection and soluble fractions were analyzed using SDS-PAGE and western blot analysis with an antibody toward dcp1a.

2.7 RSV-mediated dcp1 phosphorylation correlated with ERK1/2 activation during infection.

Mitogen-activated protein kinases (MAPKs)

MAPKs are a family of serine/threonine-specific enzymes that regulate cellular responses to a number of stimuli, including environmental stress, DNA damage, proinflammatory cytokines, mitogens, and infections. MAPKs take part in phospho-relay signaling cascades, in which kinases are sequentially phosphorylated and are thereby rendered enzymatically active and able to phosphorylate the next substrate in the cascade:



Figure 32. Classical MAPK signaling pathway.

MAPKs are catalytically inactive under normal cellular conditions and require phosphorylation in order to become active. In a typical cascade, a stimulus induces phosphorylation of an activator protein, which in turn activates a MAP-kinase-kinase kinase (MKKK). MKKKs activate MAP-kinase kinases (MKKs), which activate MAPKs. MAPKs phosphorylate protein substrates on serine and/or threonine residues.



Figure 33: Mitogen-activated protein kinase cascades. Schematic showing the four major subfamilies of MAPK proteins, their cognate MAPKKs (MKKS), and MAPKKKs (MKKS), as well as general activators and outcomes of respective pathways.

Four important subfamilies of MAPKs have been identified: extracellular signal-regulated kinases (ERK1/2), c-Jun amino-terminal kinases (JNK), p38, and ERK5. It is important to note that a large number of stimuli can lead to activation of MAPKs; however, the MKKs that phosphorylate their cognate MAPKs are very specific for each subfamily (25, 139). For example, MEK 1 and MEK 2 (MKKs) phosphorylate ERK1/2 but do not phosphorylate JNK, p38, or ERK5. MAPKs are activated and deactivated by phosphorylation and de-phosphorylation, respectively. This process allows cells to regulate diverse cellular processes such as gene expression, movement, *cell division*,

proliferation, survival, metabolism, apoptosis, and host-defense mechanisms, including responses to infections (106).

Because dpc1 was phosphorylated so early during RSV infection, we speculated that RSV-mediated dcp1 phosphorylation was part of a MAPK signaling pathway. Of note, the studies above linking dcp1 phosphorylation to MAPK activation had not been published at the time of our investigation. To test the possibility of MAPK involvement in RSV-mediated dcp1 phosphorylation, we first sought to determine whether MAPK activation correlates temporally with dcp1 phosphorylation during RSV infection. HEp-2 cells were mock infected or infected with sucrose-purified RSV. At the indicated times post infection, cells were lysed. Dcp1 phosphorylation, ERK1/2 activation, JNK activation, and p38 activation were assessed using SDS-PAGE and western blot analysis. As shown in Figure 34, ERK1/2 activation occurred within 15 minutes of RSV infection and correlated with dcp1 phosphorylation throughout infection. JNK did not show increased levels of phosphorylation at any point during infection. P38 showed low levels of phosphorylation at 15 minutes and 24 hours post infection, but not at any other time points. Because dcp1 is phosphorylated throughout RSV infection, these results identified ERK1/2 as the most likely MAPK of those tested to be involved in RSVmediated dcp1 phosphorylation.



Figure 34. ERK1/2 activation correlated with dcp1 phosphorylation during RSV infection. HEp-2 cells were mock infected or infected with sucrose-purified RSV (moi = 5). Samples were collected at indicated times post infection. Media was changed after 60 minutes. Cells were harvested in RIPA buffer containing phosphatase and protease

inhibitors and analyzed using western blots and indicated antibodies. 24 hours post infection, one well was treated with sodium arsenite for 30 minutes. Dcp1, phospho-ERK1/2, phospho-JNK, phospho-p38, and RSV proteins were detected using SDS-PAGE and western blot analysis.

2.8. Inhibitors to ERK1/2 decreased RSV-mediated dcp1 phosphorylation

To further explore the possibility of MAPK involvement in RSV-mediated dcp1 phosphorylation, we used pharmacological MAPK inhibitors during RSV infection. The targets of the inhibitors used are shown in Table 5. While some inhibitors are highly specific, others inhibit more than one kinase. 5Z-7-oxozeanol is a chemical that has been demonstrated to strongly inhibit MKKK TAK1, and thereby repress ERK1/2, JNK. and p38 activation (73, 135, 211). U0126 and PD98059 inhibit MEK1 and thereby ERK1/2 activation (69), but also have been reported to inhibit MEK5 and thereby ERK5 activation. SB203580 specifically inhibits p38 activation (69). SP600125 strongly inhibits JNK activation; however its usefulness is limited as it can also inhibit activation of at least 20 other kinases, including PI3K and AKT (243), as well as p38, ERK1/2, and ATF2 (28). HEp-2 cells were mock treated with DMSO or treated with inhibitors that block ERK1/2, JNK, and/or p38 activation (table 2). One hour after treatment, cells were mock infected or infected with sucrose-purified RSV. One hour post infection, cells were lysed and dcp1 phosphorylation was assessed using SDS-PAGE and western blot analysis. In addition, total and phosphorylated ERK 1/2, ERK 5, JNK, and p38 were detected.

Table 5. Pharmacological MAP kinase inhibitors

Compound	Mechanism	ERK1/2	JNK	p38	ERK 5 inhibition
		inhibition	inhibition	inhibition	
			the second se		

5Z-7- oxozeanol	ATP-competitive irreversible inhibitor of ERK2, MEK1, and TAK1	strong	strong	strong	unknown
U0126	suppressor of MKK1 activation	strong	-	-	strong
PD98059	prevents activation of MEK1 and MEK5	strong	-	-	strong
PD184352	prevents MEK1	strong	-	-	none (2 μM) moderate (20 μM)
SP600125	reversible inhibitor of JNK	weak	strong	weak	unknown
SB203580	prevents phosphorylation of p38	290) 2	-	strong	unknown

As shown in Figure 35A, ERK 1/2, ERK 5, p38, and JNK were each phosphorylated in response to arsenite treatment (lane 2); however, only ERK 1/2 was phosphorylated in response to RSV infection (lane 3). This activation was blocked by inhibitors to ERK 1/2 (lanes 4-8). Dcp1 from cells that were mock infected migrated as a doublet (lane 1), while cells that were arsenite-treated or infected with RSV migrated as primarily the upper band (lanes 2 and 3). Treatment with 7-oxozaneal abrogated the RSV-mediated increase in phosphorylation, and resulted in even less dcp1 phosphorylation than is present in mock-infected cells (lane 4), indicating that MAPK signaling is likely involved

in RSV-mediated dcp1 phosphorylation. Dcp1 from RSV-infected cells pretreated with U0126 (lane 4), PD98059 (lane 5), 1 µM PD184352 (lane 6), or 10 µM PD184352 (lane 7) migrated as a doublet that appeared similar to dcp1 from mock-treated, mock-infected cells (lane 1). The ratio of phosphorylated to unphosphorylated dcp1 in these samples was similar to that of mock-infected cells (Figure 35B), suggesting that RSV-mediated dcp1 phosphorylation is part of the ERK1/2 signaling pathway. Dcp1 from RSV-infected cells pretreated with SP600125 (lane 8) or SB203580 (lane 7) migrated as primarily the upper band, indicating that neither JNK nor p38 is involved in RSV-mediated dcp1 phosphorylation. Consistently, pretreatment with the p38 inhibitor resulted in increased levels of phosphorylated dcp1, indicating that the p38 pathway might be involved in negatively regulating dcp1 phosphorylation. Because ERK 1/2 showed consistent activation that correlated with dcp1 phosphorylation during RSV infection, and because inhibitors to ERK 1/2 abrogated RSV-mediated dcp1 phosphorylation, we conclude that RSV-mediated dcp1 phosphorylation is part of the ERK 1/2 pathway; however, further studies are needed to determine whether dcp1 is a direct substrate for ERK 1/2 and whether other kinases are involved in the effect.

Of note, two groups have since reported MAPK involvement in dcp1 phosphorylation. Xu et al. reported that arabidopsis dcp1 is phosphorylated through the MPK6 pathway (271). In addition, Rzeczkowski et al. demonstrated that the MAPK JNK is able to directly phosphorylate dcp1. Their studies focused on JNK; however, the authors speculated that more than one MAPK could phosphorylate dcp1 based on the results of their experiments using non-specific MAPK inhibitors (206).



Α



Figure 35. RSV-mediated dcp1 phosphorylation was ERK1/2 dependent. A) HEp-2 cells were mock treated with DMSO or treated with indicated inhibitors for one hour. Samples were then mock infected or infected with RSV (moi = 5) for one hour, and harvested as above. Samples were examined using western blot analysis with the indicated antibodies. B) The fluorescent intensity of the dcp1 bands were calculated using Licor Odyssey software, and the proportions of upper band intensity (black, upper) and lower band intensity (gray, lower) were calculated. Representative of three independent experiments.

2.9 Investigation to determine whether RSV envelope proteins are necessary and/or sufficient to mediate dcp1 phosphorylation.

2.9.1 Dcp1 was phosphorylated by neither arsenite nor RSV at 4°C.

Our results demonstrated that dcp1 was phosphorylated very early during RSV infection. and our and others' experiments implicated MAPKs in dcp1 phosphorylation. We hypothesized that dcp1 phosphorylation occurred during a very early step of the RSV replication cycle such as virus attachment to and/or fusion with host cells. To determine whether RSV internalization is necessary for RSV-mediated dcp1 phosphorylation, we infected cells with RSV at 4°C in parallel with cells infected at 37°C. We used arsenite treatment as a positive control for dcp1 phosphorylation. One hour post infection, cells were lysed and dcp1 phosphorylation was assessed using SDS-PAGE and western blot analysis. As shown in Figure 36, dcp1 migrated as a doublet in mock infected cells incubated at 37°C and as only the upper band in arsenite-treated or RSV-infected cells incubated at 37°C. In contrast, dcp1 from all groups incubated at 4°C migrated as a doublet. Since dcp1 from cells treated with arsenite (which is internalized by passivetransport aquaglyceroporins (145, 258)) was not phosphorylated at 4°C, we cannot draw any conclusions regarding whether RSV internalization is necessary for dcp1 phosphorylation. It is possible that the kinases responsible for dcp1 phosphorylation are not enzymatically active at 4°C.





2.9.2 Transient overexpression of RSV F and G proteins was not sufficient to mediate dcp1 phosphorylation.

To further explore the possibility that RSV-mediated dcp1 phosphorylation is facilitated by attachment to and/or fusion with host cells, we transiently overexpressed RSV F, RSV G, or VSV G proteins in HEp-2 cells. 48 hours post transfection, cells were lysed and dcp1 phosphorylation was assessed using SDS-PAGE and western blot analysis. As shown in Figure 37, dcp1 from mock infected cells (lane 1) migrated as a doublet and dcp1 from RSV-infected cells (lane 2) migrated as only the upper band, consistent with previous results. Dcp1 from all transfected cells (lanes 3 through 11) migrated as a doublet. These results indicate that transient overexpression of RSV envelope proteins is not sufficient to mediate dcp1 phosphorylation. It is possible that expression of both RSV F and G are necessary for dcp1 phosphorylation or that a form of RSV F or G that is not present in transfected cells is necessary for dcp1 phosphorylation.



Figure 37. Transient overexpression of RSV envelope proteins was not sufficient to induce dcp1 phosphorylation. HEp-2 cells were mock transfected (lanes 1 and 2), mock infected (lane 1), infected with RSV (moi = 5, lane 2) or transfected with mammalian expression vectors encoding RSV F (lanes 3-5), RSV G (lanes 6-8), or VSV G (lanes 9-11) proteins (µg DNA are indicated). 48 hours post infection or transfection, cells were lysed with RIPA buffer and samples were centrifuged at 16,000 xg for ten minutes. Soluble fractions were assessed for dcp1 phosphorylation using SDS-PAGE and western blot analysis with an antibody toward dcp1a.

2.9.3 Unsuccessful incorporation of RSV envelope proteins into virus-likeparticles

Our results suggested that merely overexpressing RSV envelope proteins was not sufficient to mediate dcp1 phosphorylation, so we attempted to incorporate the proteins into lentivirus virus-like particles (VLPs) in order to isolate the RSV proteins without the context of an RSV infection, but in a manner that better simulates the mechanism by which the proteins encounter host cells during RSV infection. In brief, 293T cells were transfected with a plasmid encoding HIV-Gag proteins in addition to plasmids encoding RSV F, RSV G, or VSV G protein. 48 hours post transfection, supernatants were collected and an ELISA was performed to determine the p24 content of the VLP preparations. In a parallel experiment, pseudotyped defective virus particles (DVPs) capable of a single-round of infection were made. 293T cells were transfected with an HIV construct lacking envelope proteins (HIVAenv-luc) in addition to plasmids encoding RSV F, RSV G, or VSV G protein. 48 hours post transfection, supernatants were collected and an ELISA was performed to determine p24 concentration in particles. For DVPs, a multinuclear activation of a galactosidase indicator (MAGI) assay was performed to determine infectious titer. This experiment was performed to confirm that RSV envelope proteins had been incorporated into the DVPs since only particles with intact envelope proteins would be able to enter the cells. Next, HEp-2 cells were mock infected, infected with RSV, or infected with either VLPs (10 ng p24) or DVPs (10 ng p24). 24 hours post infection, cells were lysed and dcp1 phosphorylation was assessed using SDS-PAGE and western blot analysis. As shown in Figure 38, dcp1 from mockinfected cells migrated as a doublet (Panels A and B, lane 1), and dcp1 from RSVinfected cells migrated as only the upper band (Panels A and B, lane 2), as expected.

Dcp1 from cells infected with VLPs or DVPs migrated as a doublet (Panels A and B, lanes 2-8). The results of the MAGI assay (panel C) indicated that the DVPs in which we attempted to incorporate RSV envelope proteins F and/or G were not internalized. This likely suggests that the VLPs and DVPs did not properly incorporate the RSV envelope proteins. Because we were unable to confirm that the VLPs and DVPs used in the experiment incorporated the RSV envelope proteins, we cannot draw any conclusions from this experiment. We have since learned that the VLPs and DVPs would have a much greater chance of incorporating the RSV envelope proteins if the cytoplasmic tails were deleted from the envelope proteins using mutagenesis (Suryaram Gummuluru, personal communication). Future studies are needed to further investigate this.



С

Defective virus particle infection into cells				
Construct	DVPs / ml			
Mock transf.	0			
Gag	0			
Gag + F	0			
Gag + G	0			
Gag + F + G	0			
Gag + VSV G	4 x 10 ⁶			

Figure 38. Unsuccessful attempt to make virus-like particles. 293T cells were transfected with a plasmid encoding HIV Gag proteins (to make VLPs) or HIVΔEnv-luc (to make DVPs) and constructs encoding RSV F, RSV G, and/or VSV G protein. 48 hours post transfection, clarified supernatants were collected and p24 concentration was

determined using ELISA (data not shown). HEp-2 cells were mock infected, infected with RSV (moi =5), or infected with 10 ng p24/ml of indicated VLPs or DVPs. Twenty-four hours post infection, cells were lysed in RIPA buffer. Samples were centrifuged at 16,000 x g at 4°C for 10 minutes. Soluble fractions were assessed for dcp1 phosphorylation using SDS-PAGE and western blot analysis. C) Internalization of defective-virus particles was tested using a MAGI assay. In brief, MAGI-R5 cells were infected with indicated particles. One hour post infection, media was changed. Forty-eight hours post infection, cells were fixed and stained with X-gal to identify cells with successful virus internalization. Nora Ramirez did the p24 ELISAs for this experiment.

2.9.4 RSV G protein was not necessary for RSV-mediated dcp1 phosphorylation.

To further investigate the question of whether RSF envelope proteins are sufficient to mediate dcp1 phosphorylation, we tested recombinant viruses that lacked the RSV G protein in our dcp1 phosphorylation assay. As mentioned previously, RSV G is necessary for attachment to cells and has two forms: soluble and insoluble. The insoluble form is present on the surface of RSV virions, while the soluble form is secreted from RSV-infected cells and abundant in the supernatant of cells in late stages of infection. The soluble form is known to have immunomodulatory activities (251). Our previous results indicated that both RSV itself, as well as a factor in the supernatant of RSV-infected cells (Figure 29), were able to mediate dcp1 phosphorylation. Since RSV G protein is present both on virus particles (insoluble form) and as a secreted factor in RSV supernatants (soluble form), we wished to determine whether the protein is sufficient for RSV-mediated dcp1 phosphorylation. To test this possibility, HEp-2 cells were mock infected, infected with wt RSV grown in HEp-2 cells (as a positive control for dcp1 phosphorylation), infected with wt RSV grown in Vero cells, infected with UVinactivated wt RSV grown in Vero cells, infected with ΔG virus grown in Vero cells, or infected with UV-inactivated ΔG virus grown in Vero cells. Ninety minutes post infection. cells were lysed and assessed for dcp1 phosphorylation using SDS-PAGE and western blot analysis. As shown in Figure 39, dcp1 from mock-infected cells (lane 1) migrated as a doublet, while dcp1 from all infected cells (lanes 2 - 6) migrated as only the upper band. These results indicate that recombinant RSV grown in Vero cells was able to mediate phosphorylation, and that RSV G protein was not necessary for RSV-mediated dcp1 phosphorylation. Because it is possible that cytokines or another factor in the
virion preparations were able to mediate dcp1 phosphorylation, we cannot rule out the possibility that RSV G is able to mediate the effect.



Figure 39. Recombinant RSV was able to mediate dcp1 phosphorylation, but RSV G protein was not necessary for RSV-mediated dcp1 phosphorylation. HEp-2 cells were mock infected, infected with wt RSV grown in HEp-2 cells, wt RSV grown in Vero cells, UV-inactivated RSV grown in Vero cells, ΔG virus grown in Vero cells, or UV-inactivated ΔG virus grown in Vero cells. Ninety minutes post infection, cells were lysed in RIPA buffer and analyzed for dcp1 phosphorylation using SDS-PAGE and western blot with antibodies toward dcp1 and β -tubulin (loading control). Lysates from virus-infected cells were kindly provided to us by Michael Teng.

2.9.5 Experiments in which RSV was incubated with antibodies toward RSV F protein confirmed that the antibody was able to prevent RSV infection; however, conclusions could not be drawn regarding the role of the RSV F protein in mediating dcp1 phosphorylation.

To further investigate whether RSV F protein is the factor responsible for RSV-mediated dcp1 phosphorylation, we attempted to block the theoretical interaction between RSV F and its cognate MAPK-activator protein. HEp-2 cells were mock infected or infected in duplicate with RSV pre-incubated in 1.5-ml polypropylene tubes with increasing concentrations of a neutralizing antibody toward RSV F protein. Sixty minutes post infection, cells were lysed and dcp1 phosphorylation was assessed using SDS-PAGE and western blot analysis. A duplicate set of samples was incubated for 24 hours before cells were lysed and RSV infection was confirmed using SDS-PAGE and western blot analysis. A duplicate set of samples was incubated for 24 hours before cells were lysed and RSV infection was confirmed using SDS-PAGE and western blot using a polyclonal antibody toward RSV proteins. As shown in Figure 40A, incubating RSV with anti-F antibodies at either 10 µg/ml or 100 µg/ml prevented productive RSV infection (lower panel). Unfortunately, the conditions under which this experiment was performed prevented RSV from inducing robust dcp1 phosphorylation (lane 2), so conclusions could not be drawn regarding the antibody's effect on RSV-mediated dcp1 phosphorylation.

Another experiment was performed in which RSV was incubated with the antibody toward RSV F, antibodies toward various cytokines, or heparin at 37°C (as opposed to room temperature) for two hours before treatment of cells for one hour. As seen in Figure 40B, dcp1 from RSV-treated cells was phosphorylated (lane 2), indicating that these experimental conditions were more conducive to RSV-mediated dcp1 phosphorylation than those of the previous experiments. However, none of the pre-

treatments tested decreased RSV-mediated dcp1 phosphorylation (lanes 3-7), so no conclusions can be drawn from these experiments regarding the role of the RSV F protein in RSV-mediated dcp1 phosphorylation.





Figure 40. Experiments in which RSV was incubated with antibodies toward RSV F protein confirmed that the antibody was able to prevent RSV infection; however, conclusions could not be drawn regarding the role of the RSV F protein in mediating dcp1 phosphorylation. A) HEp-2 cells were mock infected with media (lane 1) or infected with RSV (lanes 2-6) that was pre-incubated on a nutator with increasing concentrations (µg/ml) of an antibody toward RSV F protein for one hour at room temperature in Opti-MEM + 2% FBS in polystyrene tubes. One set of cells was lysed one hour post infection to assess dcp1 phosphorylation (top panel). The other set of cells was lysed 24 hours post infection to assess RSV infection (bottom panel). Cells were lysed and soluble fractions were analyzed using SDS-PAGE and western blot with antibodies toward dcp1 or RSV proteins. B) RSV preparations (100 µl of virus diluted in 900 µl of Opti-MEM with 2% FBS) were treated with 20 µg/ml of mouse Ig (Southern Biotech), 20 μg/ml of anti-F antibody, 20 μg/ml of anti-IL-1β (clone 2805, R&D Systems catalog #MAB601), 20 µg/ml of anti-IL-6 (clone 6708, R&D Systems catalog #MAB206), 20 μ g/ml of anti-TNF- α (clone NF-7, Abcam catalog #ab9809), or 100 μ g of heparin (EMD Millipore catalog #375093) for 2 hours at 37°C with intermittent gentle vortexing. Treated virus preparations were added to HEp-2 cells and cells were incubated for 1 hour at 37°C. The cells were lysed and analyzed for dcp1 phosphorylation by western blot.

2.10 Dcp1 did not immunoprecipitate with RSV F protein during RSV infection.

Jining Lu's research group used bioinformatics to determine that RSV F protein shares a similar motif to p-body-associated, dcp1-binding-partner protein GW182 (unpublished data). We speculated that perhaps RSV F protein mimics GW182 in order to bind dcp1 and thereby interfere with dcp1 activities. To test this possibility, we used immunoprecipitation to determine whether RSV F protein binds to dcp1 during RSV infection. HEp-2 cells were either mock infected or infected with RSV (moi = 5). Eighteen hours post infection, cells were lysed with RIPA buffer. Samples were incubated with an antibody toward RSV F protein, and SDS-PAGE and western blot analysis were used to detect the presence of GW182, dcp1, and RSV proteins. As shown in Figure 41, RSV proteins were detected only in samples infected with RSV, as expected. Both GW182 and dcp1 were immunoprecipitated at very low levels. There were similar levels of GW182 and dcp1 proteins in samples from mock infected cells and RSV-infected cells, suggesting that the proteins were nonspecifically immunoprecipitated. These results indicate that under the conditions tested, RSV F protein does not bind to dcp1.



Figure 41. Dcp1 did not immunoprecipitate with RSV F protein. HEp-2 cells were mock infected or infected with RSV (moi = 5). Eighteen hours post infection, cells were lysed in RIPA buffer and centrifuged at 16,000 xg at 4°C. Samples were pre-cleared by incubation with sepharose beads at 4°C for one hour and then centrifugation at 2,400 xgat 4°C for 3 minutes. Samples were next incubated with an antibody toward RSV F protein at 4°C for one hour and then centrifuged at 2,400 xg at 4°C for 3 minutes. Samples were analyzed using SDS-PAGE and western blot with antibodies toward the indicated proteins.

2.11 Dcp1 phosphorylation occurred in response to some, but not all, environmental stresses.

As mentioned previously, dcp1 is phopshorylated in response to several types of stress. including arsenite treatment (31), dehydration stress (271), and treatment with IL-1a. anisomycin, or sorbitol (206). Because our lab is interested in cellular stress responses and pathways governing stress-granule formation, we wished to determine whether dcp1 was indeed phosphorylated by all forms of stress. We exposed cells to several forms of environmental stress known to induce stress-granule formation, including arsenite treatment (Panels A and B), thapsigargin treatment (Panels A and C), heat shock (Panel A), and UV-irradiation (Panel D). Dcp1 phosphorylation was assessed using SDS-PAGE and western blot analysis. As shown in Figure 42, dcp1 from untreated cells migrated as a doublet (lane 1 of Panels A-D). Dcp1 from cells treated with 0.25 mM or higher sodium arsenite migrated as primarily the upper band (Panel A, lane 4 and Panel B, lanes 4-6), confirming previous results indicating that sodium arsenite treatment induces dcp1 phosphorylation. There results also indicated that arsenite-mediated dcp1 phosphorylation is dose dependent. Dcp1 from thapsigargin-treated cells migrated as a doublet that favored the bottom band compared to mock-treated cells (Panel A, Janes 5-6 and Panel B, lanes 2-6). These results suggest that thapsigargin treatment not only does not result in increased dcp1 phosphorylation, but may even decrease levels of dcp1 phosphorylation. Dcp1 from heat-shocked proteins migrated as a doublet similar to dcp1 from mock-treated cells, indicating that heat shock does not result in increased dcp1 phosphorylation (Panel A, lane 7). Finally, dcp1 from cells exposed to all levels of UV-irradiation tested migrated as only the upper band. These results indicate that dcp1 is phosphorylated as a result of UV-irradiation (Panel D, lanes 2-6).

As discussed at length in Chapter 1, we created a mutant of RSV whose trailer region was replaced with the inverted complement of the Leader sequence (LeC). Infection with this virus resulted in significantly increased stress-granule formation compared to wt RSV. We speculated that perhaps dcp1 phosphorylation was part of a process employed by RSV to prevent stress-granule formation. To determine if there was a correlation between inhibition of stress-granule formation and dcp1 phosphorylation, we examined whether LeC infection resulted in dcp1 phosphorylation. HEp-2 cells were mock infected, treated with sodium arsenite, treated with thapsigargin, infected with wt RSV, or infected with LeC virus. Eighteen hours post infection, cells were lysed and dcp1 phosphorylation was assessed using SDS-PAGE and western blot analysis. As shown in Figure 43, dcp1 from cells that were mock infected, mock treated (lane 1) migrated as a doublet as expected. Dcp1 from cells treated with arsenite was phosphorylated, and dcp1 from cells treated with thapsigargin migrated as primarily the lower band, consistent with previous results. Dcp1 from cells infected with either wt RSV or LeC virus migrated as primarily the upper band, indicating that LeC infection mediates dcp1 phosphorylation.

Taken together, our results demonstrate that dcp1 is phosphorylated as a result of arsenite, UV-irradiation, and infection with LeC virus (all known to induce stress-granule formation), but not in response to thapsigargin or heat shock.

2





HEp-2 cells were treated with indicated concentrations of sodium arsenite. Panel C:

HEp-2 cells were incubated with indicated concentrations of thapsigargin. Panel D: HEp-2 cells were exposed to indicated levels of UV-irradiation and then incubated at 37°C for four hours. All cells were lysed in RIPA buffer and samples were centrifuged at 16,000 xg at 4°C for ten minutes. Soluble fractions were assessed for dcp1 phosphorylation using SDS-PAGE and western blot analysis.



Figure 43. Infection with LeC mediated dcp1 phosphorylation. HEp-2 cells were mock infected (lanes 1-3), treated with 0.5 mM sodium arsenite for 30 minutes (lane 2), treated with 10 µM thapsigargin for 30 minutes (lane 3), infected with RSV (moi = 5, lane 4), or infected with LeC (moi =3, lane 5). Eighteen hours post infection, cells were lysed and assessed for dcp1 phosphorylation using SDS-PAGE and western-blot analysis with an antibody toward dcp1a.

2.12 Dcp1 phosphorylation did not occur in response to infections with all viruses.
Dcp1 phosphorylation as a result of infection has not been reported for any other virus.
To determine whether other viruses are able to mediate dcp1 phosphorylation, we
collaborated with other laboratories in our department with access to various viruses.

The first virus we tested for ability to mediate dcp1 phosphorylation was HIV-1, a retrovirus. Primary human monocyte-derived macrophages were mock infected (lanes 1 and 2) or infected with HIV-1 (lanes 3 and 4). 72 hours post infection, samples from each group were treated with 1.0 mM sodium arsenite for 30 minutes (lanes 2 and 4). Cells were lysed and dcp1 phosphorylation was assessed using western blot analysis.

As shown in Figure 44, Panel A, dcp1 from mock infected cells (lane 1) migrated as a doublet , while dcp1 from arsenite-treated cells (lane 2) migrated as the upper band, confirming that dcp1 is likely able to be phosphorylated in primary monocyte-derived macrophages. Dcp1 from HIV-1-infected cells (lane 3) migrated as a doublet, while dcp1 from cells that were treated with arsenite (lane 2) or infected with HIV-1 and then treated with arsenite (lane 4) migrated as the upper, phosphorylated form. These results indicate that HIV-1 infection does not result in dcp1 phosphorylation.

We next wanted to assess dcp1 phosphorylation during infection with viruses that are more similar to RSV. We focused on measles virus (MV), mumps virus, and canine distemper virus (CDV). These viruses are negative-strand, enveloped RNA viruses that, like RSV, are in the family *Paramyxoviridae*. HEp-2 cells were mock infected, infected with MV, mumps virus, or CDV. Cells were treated with sodium arsenite or infected with RSV as positive controls for dcp1 phosphorylation. Twenty four hours post infection, cells were lysed and dcp1 phosphorylation was assessed using western blot analysis.

As seen in Figure 44 panel B, dcp1 from cells infected with MV (lanes 2 and 3), mumps virus (lanes 4 and 5) or CDV (lane 6) migrated as a doublet, while dcp1 in cells treated with arsenite (lane 7) or infected with RSV (lane 8) migrated as the upper band. These results suggest that MV, mumps virus, and CDV do not mediate dcp1 phosphorylation in HEp-2 cells.

Because HEp-2 cells are not as permissive to MV infection as other cell types, we wished to assess dcp1 phosphorylation during MV infection of a more authentic cell type. Immortalized B-cells were mock infected or infected with MV. Twenty-four hours post infection, cells were lysed and dcp1 phosphorylation was assessed. The results in Figure 44 panel C demonstrate that dcp1 in B-cells does not migrate as a doublet. It is possible that dcp1 is constitutively phosphorylated in immortalized B cells; however, because dcp1 does not migrate as a doublet in these cells, we cannot draw any conclusions from this experiment as to whether MV mediates dcp1 phosphorylation in B-cells. We observed an additional band between 175 and 250 kDa that appeared in samples infected with MV but not mock-infected cells. It is possible that our dcp1 antibody cross reacts with a viral protein. The only MV protein in this molecular-weight range is the L protein; however, we do not believe that it is the MV L protein, because this is expressed at a low level in infected cells. Alternatively, it is possible that dcp1 is modified with different post-translational modifications in B-cells infected with MV.





С



Figure 44. DCP1 was not phosphorylated during infection with HIV-1, measles virus, mumps virus, or canine-distemper virus. Panel A: Primary macrophages were mock infected (lanes 1 and 2) or infected with HIV-1_{ADA} (lanes 3 and 4). 72 hours post infection, samples from each group were treated with 1.0 mM sodium arsenite for 30 minutes (lanes 2 and 4). Cells were lysed with RIPA buffer, and samples were centrifuged at 16,000 xg at 4°C. Soluble fractions were analyzed using SDS-PAGE. Blots were probed using an antibody toward human dcp1a. Panel B: HEp-2 cells were mock infected (lane 1), infected with measles virus with an moi of 0.1 (lane 2) or 3 (lane infected with mumps virus at an moi of 0.1 (lane 4) or 3 (lane 5), infected with canine distemper virus at an moi of 0.1 (lane 6), treated with 0.5 mM sodium arsenite for 30 minutes (lane 7) or infected with RSV at an moi of 5 (lane 8). Twenty-four hours post infection, cells were lysed with RIPA buffer, and samples were centrifuged at 16,000 xa at 4°C. Soluble fractions were analyzed using SDS-PAGE. Blots were probed using an antibody toward human dcp1. Panel C: Epstein-Barr virus-transformed human Blymphoblastic cell s (B-LCL) were mock infected (lane 1) or infected with MV clone 3 (lane 2) or MV clone 6 (lane 3). Twenty-four (confirm) hours post infection, cells were lysed and soluble fractions were analyzed using SDS-PAGE and western blots with an antibody toward dcp1a.

Because our data suggested that RSV infection induced a change in the phosphorylation state of dcp1, we wished to determine whether dcp1 has a role in RSV's replication cycle. We first addressed this question by examining the effects of knocking down dcp1 on RSV protein expression. HEp-2 cells were mock transfected or transfected with an siRNA directed toward dcp1. Twenty-four hours later, cells were mock infected or infected with RSV. RSV protein levels were assessed by western blot analysis 24 and 48 hours post infection. As shown in Figure 45, siRNA treatment of cells caused a significant decrease in dcp1 levels; however, RSV protein expression was similar in control siRNA-transfected and dcp1 siRNA-transfected cells , indicating that dcp1 is likely not necessary for RSV protein production.

2.13 Dcp1 knockdown did not alter RSV protein expression during RSV infection.





2.14 Transient overexpression of dcp1 resulted in decreased RSV-mediated cytopathic effects.

To examine the impact of dcp1 overexpression on RSV infection, HEp-2 cells were either mock transfected or transfected with a plasmid encoding human dcp1a. 24 hours post transfection, cells were mock infected or infected with RSV. Cells were examined by microscopy 48 hours post infection (Panel A), and then lysed and examined using western blot analysis with antibodies toward dcp1a, RSV proteins, or β-tubulin. As shown in Figure 46, cells that were either mock transfected, mock infected, or dcp1-transfected and infected looked healthy and confluent. Cells that were mock transfected and cell death - *in vitro* hallmarks of productive RSV infection. In contrast, cells overexpressing dcp1 that were infected with RSV showed a drastic decrease in syncytia formation and cell death. These results indicate that dcp1 may play a role in host defenses against RSV infection. Figure 46B shows that oxerexpression of dcp1 did not affect significantly affect RSV protein production. These data suggest that dcp1 may have antiviral activities.



В



Figure 46. Dcp1 overexpression resulted in attenuated RSV-mediated cytopathic effects. HEp-2 cells were either mock transfected (only transfection reagents, no plasmids) or transfected with a plasmid encoding human dcp1a. 24 hours post transfection, cells were mock infected or infected with RSV (moi =0.5). Cells were examined by microscopy 48 hours post infection (Panel A), and then lysed and examined using western blot analysis with antibodies toward dcp1a, RSV proteins, and β -tubulin (Panel B).

2.15 A dcp1 mutant in which serine 315, serine 319, and threonine 321 were replaced with alanines abrogated RSV-mediated dcp1 phosphorylation.

In order to examine the role of dcp1 phosphorylation in RSV infection, we wished to create a dcp1 construct that was deficient in phosphorylation. Rzeczkowski (206) and Aizer (4) showed that dcp1 is phosphorylated at serine 315 and Blumenthal et al. showed that dcp1 was phosphorylated at serines 315 and 319, as well as thereonine 321 (31). We created mutant dcp1 constructs in which serine 315, serine 319, threonine 321, or all three residues were replaced with alanines in order to abrogate phosphorylation at those sites. Of note, these constructs are tagged with FLAG sequences and therefore migrate slightly more slowly than endogenous dcp1. To test whether these residues were phosphorylated during RSV infection, we transiently overexpressed WT or mutant dcp1 in cells. Twenty-four hours later, we infected cells with RSV. Dcp1 phosphorylation was assessed using SDS-PAGE and western blot analysis with antibodies toward dcp1. As shown in Figure 47, dcp1 migrated as a doublet (lane 1) in mock infected cells and as primarily the upper band in RSV-infected cells (lane 2), consistent with previous results. These bands are much fainter than bands from samples in which exogenous dcp1 was expressed, indicating that dcp1 was indeed overexpressed in transfected cells. Dcp1 from cells overexpressing the WT dcp1 construct migrated as a triplet in mock-infected cells. Recall that the transfected dcp1 is FLAG-tagged. Unphosphorylated dcp1 expressed from these constructs migrates at approximately the same rate as phoshorylated endogenous dcp1, so these bands are not distinguishable. The bottom-most band is unphosphorylated, exogenous dcp1. The center band is a combination of both phosphorylated, endogenous dcp1 and unphosphorylated, exogenous dcp1. The upper band is phosphorylated exogenous

dcp1. Dcp1 from wt-dcp1 transfected, RSV-infected cells migrated as a doublet, since both endogenous and exogenous dcp1 were phosphorylated. Dcp1 from mock-infected. S315A-transfected cells migrated as a doublet, while dcp1 from RSV-infected, S315-A transfected cells migrated as a triplet. The margin between the two upper bands was significantly narrower than the margin between the two upper bands in RSV-infected cells that were transfected with WT dcp1. This indicates that there is likely more than one phosphorylation event required for full dcp1 phosphorylation, and that S315 is one of the residues phosphorylated during RSV infection. Dcp1 from cells transfected with S319A or T321 migrated as a triplet, with significantly more exogenous dcp1 phosphorylated in response to RSV infection. Of particular interest, dcp1 from cells transfected with the triple-A mutant in which S315, S319, and T321 were all mutated to A residues migrated as a doublet in both mock-infected and RSV-infected cells. This indicates that dcp1 in which these residues are mutated cannot be phosphorylated in response to RSV infection. Taken together, these results suggest while there is likely more than one phosphorylation event that occurs during RSV infection, the events are limited to phosphorylation(s) of S315, S319, and T321. It is clear that S315 is one of the residues phosphorylated during RSV infection; however, it is unclear whether S319 or T321 is the other residue phosphorylated during infection since dcp1 from S319A and T321A have similar migration profiles. While we cannot make firm conclusions regarding the number and nature of RSV-mediated dcp1 phosphorylation events, the triple A mutant is an important tool for investigating the significance of RSV-mediated dcp1 phosphorylation since it is not phosphorylated during RSV infection.

Ø wt S315A S319A T321A TripleA mock RSV mock RSV mock RSV mock RSV mock RSV mock RSV



Figure 47. S315 was phosphorylated during RSV infection, and dcp1 phosphorylation was limited to S315, S319, and T321 during RSV infection. HEp-2 cells were mock infected or transiently transfected with constructs expressing wt dcp1, or dcp1 in which S315, S319, and/or T321 were mutated to alanine residues. Twentyfour hours post transfection, cells were infected with RSV (moi = 5). Twenty-four hpi, cells were lysed in RIPA buffer and dcp1 phosphorylation was assessed using SDS-PAGE and western blots with antibodies toward dcp1a.

Chapter 2 Discussion

Our investigation into the relationships that RSV has with cellular p-bodies and their associated proteins revealed that p-body-associated, decapping factor dcp1 is altered during RSV infection. This protein is important for decapping and mRNA regulatory activities in cells, and it is possible that these activities are important during viral infections as well. Doughterty et al. recently reported that dcp1 is rapidly phosphorylated during poliovirus infection (44, 72), a finding that lends evidence to the hypothesis that cellular mRNA decapping and degradation processes can interact with RNA viruses during replication.

Decapping and degradation processes have several implications during viral infection. Host-cell responses to viruses could rely heavily on these processes to regulate cellular mRNAs encoding antiviral mediators such as cytokines and chemokines. They could also potentially represent control points for host cells to regulated viral mRNAs. On the other hand, it stands to reason that viruses may have evolved to manipulate host-cell degradation processes in order to either facilitate an intracellular protein-expression milieu that is optimal for virus replication, or to prevent prospective host-cell-mediated virus-mRNA degradation.

Elusive mechanism of RSV-mediated dcp1 phosphorylation

Our results showed that dcp1 is phosphorylated within 15 minutes of RSV infection. The results shown in Figure 31 demonstrate that infection with sucrose-purified RSV resulted in sustained dcp1 phosphorylation over a twenty-four hour period. In contrast, infection with UV-inactivated RSV resulted in transient dcp1 phosphorylation only while the virus was in contact with the cells, as the effect began to reverse after the virus was removed

at one hour post infection. These results provide evidence that dcp1 phosphorylation is mediated by early events in the RSV replication cycle such as attachment, fusion, or cell entry, and maintained subsequently upon infection and *de novo* RSV- protein synthesis.

Our data indicated that one or more factors in the supernatants of RSV-infected cells was able to mediate dcp1 phosphorylation (Figure 29), and that the cytokines TNF- α and/or IL-1 β could have been responsible for this effect. In addition, our data showed that purified RSV was able to mediate dcp1 phosphorylation (Figure 29); however, we were not able to determine the mechanism by which this occured. Our initial hypothesis was that binding of the RSV attachment protein G to cell surface molecules was responsible for inducing dcp1 phosphorylation since G is known to interact with ERK 1/2; however, experiments using a mutant virus in which the G protein was deleted showed that G is dispensable for RSV-mediated dcp1 phosphorylation, as shown in Figure 39. It is possible, however, that dcp1 was phosphorylated by cytokines in the RSV Δ G virion preparations, so our data did not rule out the possibility that RSV G can mediate dcp1 phosphorylation.

We next shifted our focus to determine whether RSV F protein could mediate the effect, since F protein is involved in cell binding and has been shown to activate TLR4 signaling, which is involved in inflammatory cytokine production. Unfortunately, attempts to determine the role of F protein in dcp1 phosphorylation by overexpressing F (Figure 37), incorporating RSV F into VLPs (Figure 38), or using neutralizing antibodies against F (Figure 40) were not successful. As mentioned previously, personal communication with Rahm Gumuluru and colleagues revealed that deleting the cytoplasmic tail of the F protein would likely improve its incorporation into lentivirus-based VLPs.

Further studies are needed to discern the roles of RSV G and F proteins in dcp1 phosphorylation. For example VLPs incorporating cytoplasmic-tail-deficient G and/or F proteins could be used to assess their abilities to mediate dcp1 phosphorylation. In addition, sucrose-purified viruses in which F or G have been deleted could be used to assess their role(s) in this effect.

MAPK involvement in RSV-mediated dcp1 phosphorylation

Our data showed that ERK1/2 was activated shortly after exposure of the cells to RSV, and that this correlated with dcp1 phosphorylation (Figure 34). In addition, we found that inhibiting ERK1/2 with four separate inhibitors known to prevent ERK1/2 activation abrogated RSV-mediated dcp1 phosphorylation (Figure 35), suggesting dcp1 phosphorylation is a downstream effect of RSV-induced ERK1/2 activation. While RSV infection has been reported to activate the MAPKs p38 (193, 225, 252) and JNK (252), these kinases were not activated in our experiments (possibly because of differences in types of cells used in experiments), and inhibiting these enzymes did not decrease RSV-mediated dcp1 phosphorylation (Figure 35).

Several studies have shown that ERK1/2 activation is important during RSV infection. Kong et al. showed that RSV attachment to cells activated ERK1/2 pathways within 5 minutes, and that inhibition of ERK1/2 activation impaired RSV replication (125). Pazdrak and colleagues showed that ERK1/2 activation is required for maximum induction of RANTES production during RSV infection in alveolar epithelial cells using a posttranscriptional mechanism (193), and ERK1/2, p38, and JNK activation have been correlated with RSV-mediated cytopathic effects related to inflammation (112). Monick et al. reported that RSV-mediated ERK1/2 activation is required for both RSV-induced

inflammation and extended survival of cells (171). Importantly, Chen et al. confirmed that ERK1/2 activation occurred within 5 minutes of RSV infection and found that ERK1/2 was required for RSV-induced IL-8 production (50). In addition, they showed that inhibitors of ERK1/2 decreased IL-8 protein levels by 50% but had little effect on IL-8 mRNA levels, suggesting that the effect was post-transcriptional. These findings are consistent with a model in which dcp1 phosphorylation is involved in posttranscriptionally regulating IL-8 production.

Dcp1 residues phosphorylated during RSV infection

Our results indicated that mutating serine 315, serine 319, and threonine 321 completely abrogated RSV-mediated dcp1 phosphorylation (Figure 47); however, teasing out the individual contributions has proven to be more complicated. Serine 315 is clearly involved in dcp1 phosphorylation, as phosphorylation in response to RSV infection was significantly reduced in cells overexpressing S315A. This is consistent with studies by Blumenthal, Rzeczkowski, and Ma, which have shown that S315 is the most important residue involved in dcp1 phosphorylation. Of note, RSV infection of cells overexpressing S315A resulted in a dcp1 phosphorylation intermediate that migrated more quickly than dcp1 in cells overexpressing WT dcp1, but more slowly than unphosphorylated dcp1 (Figure 47). This indicates that there is more than one phosphorylation event. Data using the triple mutant indicate that the phosphorylation events are limited to S315. S319, and T321; however, because the dcp1 profiles from cells overexpressing S319A or T321A look similar, it is difficult to discern the contributions of these residues to dcp1 phosphorylation. It is possible that they are both phosphorylated during RSV infection. Alternatively, it is possible that only one of them is normally phosphorylated during infection, but that phosphorylation of other residues can occur in the event that

phosphorylation at one site is blocked. Future studies could involve using double mutants to tease out the contributions of individual amino acids to dcp1 phosphorylation during RSV infection.

Significance of dcp1 phosphorylation during RSV infection

Our findings indicated that decapping protein dcp1 is rapidly phosphorylated during RSV infection. Many studies have shown putative roles for dcp1 phosphorylation. Blumenthal and colleagues reported a correlation between dcp1 phosphorylation and neuronal development (31) and Aizer et al. demonstrated that dcp1 was phosphorylated at S315 and during mitosis (4). Ma et al. showed that phosphorylated dcp1 important for decapping-mediated degradation of maternal mRNAs during oocyte maturation (147), and Rzeczkowski and coworkers showed that dcp1 phosphorylation was important in mediating p-body distribution (206). Importantly, this group also demonstrated that dcp1 phosphorylated dcp1 regulated IL-8 production. Studies in yeast have shown that dcp1 phosphorylation is associated with decreased decapping activity (129), however the biological effect of dcp1 phosphorylation in mammalian cells has yet to be fully elucidated.

Phosphorylation is a post-translational modification that can alter protein function in many ways. As discussed previously, phosphorylation can act as a molecular switch to turn proteins "on" or "off" by mediating conformational changes. It is possible that dcp1 phosphorylation is important in the protein's ability to interact with dcp2 or other proteins important during mRNA decapping, and that dcp1 is phosphorylated as part of a host-cell response to orchestrate optimal expression of proteins involved in antiviral pathways such as cytokines and chemokines. Another possibility is that RSV-mediated dcp1

phosphorylation aids RSV propagation, for example by down-regulating certain cytokines. Another possibility is that dcp1 phosphorylation during RSV infection is significant for another, hitherto undefined function of the protein. Future studies are needed to determine the significance of dcp1 phosphorylation during RSV infection.

Conclusions

In conclusion, we have demonstrated that sucrose-purified RSV infection results in dcp1 phosphorylation within 15 minutes of infection and that this effect is mediated, at least in part, by ERK1/2 activation. We have preliminary data suggesting that overexpression of a dcp1 mutant (Triple A) in which RSV-mediated dcp1 phosphorylated was abrogated nearly doubled RSV-induced IL-8 production (data not shown), indicating that phosphorylated dcp1 is important for regulating IL-8 production during RSV infection. We propose a model in which RSV attachment to and/or fusion with moieties in the host-cell plasma membrane activates ERK1/2-mediated dcp1 phosphorylation, and that phosphorylated dcp1 regulates one or more effector molecules important in host antiviral defense processes such as cytokine production.

Chapter 3: Investigation into a case of possible structural mimicry of cellular p54 by RSV nucleoprotein

Chapter 3 Introduction

RSV inclusion bodies

When RSV infects cells, RSV N, P, L, and M2-1 proteins (43, 90) localize in cytoplasmic aggregates known as inclusion bodies as early as four hours after infection (unpublished data). Garcia et. al. used transient transfections to demonstrate that RSV N and P proteins are the minimal requirements for inclusion body formation (90). The role of inclusion bodies during infection has not been characterized. They have long been thought to be sites of RSV replication; however, this has not been proven and recent studies suggest that they may be sites where host-cell proteins are sequestered to avoid stress responses (88).

The following picture (Figure 48) shows cells that have been infected with RSV for 16 hours at an moi of 0.5 in order to visualize both infected and uninfected cells. The cells have been stained with DAPI (blue) to visualize the nucleus, and for RSV N protein (green) to visualize RSV infection (they are also stained for cellular protein dcp1 (red)). The cell just left of center is undergoing productive infection, and the green structures are RSV inclusion bodies. RSV N protein is most concentrated around the periphery of the inclusion bodies in an O-ring configuration, but is also present inside the inclusion bodies and diffusely throughout the cytoplasm. In addition, RSV N protein is seen at the plasma membrane of adjacent cells, which are possibly being infected.



Figure 48. RSV inclusion bodies. Cells are stained with DAPI (blue) to visualize the nucleus, with antibodies toward RSV N protein (green) to visualize inclusion bodies, and with antibodies toward dcp1 (red).

Brown et al. showed that host factors heat shock protein 70 (HSP 70) and actin localize to RSV inclusion bodies during infection (37), although a role for these proteins in the RSV replication cycle has yet to be elucidated. Recently, Fricke et al. demonstrated that cellular p38 and OGT proteins are sequestered in RSV inclusion bodies, thereby inhibiting MK2 activities and stress-granule formation (88). It is possible that identifying additional cellular factors that are recruited to RSV inclusion bodies will provide important clues to elucidating aspects of the RSV replication cycle and/or RSV interactions with host defense mechanisms.

Cellular DEAD-box protein p54

Human p54 (also referred to as rck and DDX6) is a cellular, DEAD-box RNA helicase that is generally associated with negatively regulating translation. p54 homologues are found in *Saccharomyces cerevisiae* (DHH1), *Caenorhabditis elegans* (CGH-1), *Xenopus* (Xp54), and *Drosophila* (Me31B). Although its activities have not been extensively characterized, several putative functions have been reported, including translational repression (52, 54, 164-166, 181), decapping enhancement (55), mRNA shuttling (226), RNA unwinding (5), pre-mRNP assembly (232, 236), and ribonucleoprotein (RNP) remodeling (80, 105, 217). Not surprisingly, p54 has been shown to be important in the development of mammals (153); however, its role in development has not been fully elucidated. p54 is included in some stress granules, though it is not a requirement for canonical stress-granule composition. It is found in all p-bodies, and the protein has recently been reported to be required for p-body formation (18, 84, 163). However, its specific activities in these structures are unknown. Minshall et al. proposed a model in which one domain (D2) of p54 acts as a protein binding platform, while the ATP/helicase domain mediates ribonucleoprotein (RNP) remodeling and thereby regulates the balance

between translational repression and activation (163). Of particular interest, Chu and Rana demonstrated that p54 interacts with argonaute proteins Ago1 and Ago2 in active RNA-induced silencing complexes (RISCs) in the context of both siRNA and miRNA, and showed that p54 is the effector molecule in miRISC that represses translation. They proposed that the specificity of the repression may be determined by the sequence of miRNA binding to complementary sites in the 3' UTR of target mRNA (52).

p54 implicated in viral replication cycles

Given its role in translational regulation, it is not surprising that p54 has been increasingly recognized as important for virus biology. Jangra et al. found that p54 helicase activity is essential for efficient Hepatitis C virus (HCV) replication (104). The group later showed that PatL1, LSm1, and p54 localization to p-bodies was not required for p54's role in HCV infection (195). Ariumi et al. showed that the virus sequesters p54 and other p-body components to HCV replication factories (20). p54 also has roles in HIV infection. Chable-Bessia et al. reported that p54, GW182, LSm-1, and Xrn1 (all pbody components) repressed HIV-1 gene expression by preventing viral transcripts from associating with polysomes. In addition, they found that knocking down p54 resulted in HIV-1 reactivation in PBMCs isolated from HAART-treated HIV-infected persons (45). In seeming contrast, another group showed that p54 aids in HIV capsid assembly (201). thereby facilitating viral replication. Several other viruses have reported relationships with p54. Yu et al. demonstrated that p54 is required for efficient genome packaging of prototype foamy virus (PFV) (274), and p54 has been shown to bind the 3' UTR of Dengue virus and to facilitate Dengue virus replication (260). The adenovirus 11k protein binds and escorts p54 to aggresomes to be degraded (94), and p54 has been

demonstrated to play an important role in the replication cycle of Brome mosaic virus (BMV) (12).

Hypothesis

The studies discussed in Chapters 1 and 2 of this dissertation focused on RSV's relationship with stress granules, p-bodies, and p-body-associated protein dcp1. During the course of these studies, we identified p54 as a protein that might be important during RSV replication because it appeared to localize to RSV inclusion bodies during infection. We hypothesized that p54 was important either for the RSV replication cycle or for host antiviral activities toward RSV.

Results

3.1 A polyclonal antibody toward cellular p54 recognized a component within RSV inclusion bodies.

While investigating RSV's relationship with cellular stress granules, we sought to determine whether RSV proteins colocalize with stress granule- and p-body-associated protein p54. To determine whether this was the case, cells were mock infected or infected with RSV. 16 hours post infection, cells were fixed and immunostained with antibodies toward RSV N and cellular p54.

In mock infected cells, p54 had a punctate distribution consistent with p-bodies that are constitutively present under steady-state conditions (Figure 49). In RSV-infected cells,
the p54 antibody stained both punctate dots characteristic of p-bodies, as well as regions inside RSV inclusion bodies. p54 did not appear to be part of stress granules, which are larger than p-bodies, under either condition. Interestingly, the molecule recognized by the p54 antibody didn't colocalize perfectly with RSV N. As shown in Figure 49B, the bulk of RSV N protein formed O-rings around the inclusion bodies, while the component recognized by the p54 antibody localized to the inside core of inclusion bodies. These results indicate that the RSV N protein partially colocalizes to RSV inclusion bodies with a molecule recognized by the p54 polyclonal antibody.



Figure 49. Cellular p54 protein appeared to localize to RSV inclusion bodies.

HEp-2 cells were either mock infected or infected with RSV (moi = 1). After 16 hours, cells were fixed and immunostained with DAPI (blue), RSV N protein (green), and human p54/rck (red). Slides were examined by confocal microscopy. Panel B shows an enlargement of the merged image of RSV-infected cells.

3.2 Monoclonal antibody toward p54 did not stain components inside RSV inclusion bodies.

We next wished to determine whether a different antibody toward p54 yielded the same result. HEp-2 cells were mock infected or infected with RSV. 16 hours post infection, cells were fixed and immunostained using the same antibody toward RSV N that was used in the previous experiment to visualize RSV inclusion bodies; however, a monoclonal antibody toward p54 was used to stain for p54 instead of the polyclonal antibody used previously. As demonstrated in Figure 50, p54 appeared in puncate dots in mock infected cells as before. However, in RSV-infected cells, the monoclonal antibody toward p54 did not stain components inside RSV inclusion bodies as the polyclonal antibody had. Instead, the p54 distribution was altered in cells demonstrating prominent RSV inclusion bodies. In these cells, p54 appeared in large, amorphous aggregations that were not characteristic of either stress granules or p-bodies.



Figure 50. Monoclonal antibody toward p54 did not recognize p54 in RSV inclusion bodies. HEp-2 cells were either mock infected (a and b) or infected with RSV (moi = 1) (c). After 14 hours, some cells (b) were treated with 0.5 mM sodium arsenite for 30 minutes as a positive control for cellular stress, and all cells were fixed and stained with RSV N protein (green) and human p54 (red). Slides were examined by fluorescent microscopy.

3.3 RSV infection resulted in a second, faster-migrating band recognized by polyclonal p54 antibody.

Because our immunofluorescence results indicated that p54 might colocalize with RSV proteins, we theorized that perhaps p54 had a role in the RSV replication cycle. We next wished to assess whether RSV infection altered the elecrophoretic mobility of cellular p54. HEp-2 cells were mock infected, infected with RSV, or treated with sodium arsenite. 16 hours post infection, cells were lysed and p54 in soluble and insoluble (pellet) fractions was assessed using SDS-PAGE and western blot analysis with either our polyclonal or monoclonal antibodies toward p54. As shown in Figure 51, both the polyclonal and monoclonal antibodies recognized a protein that migrated as a ~54 kDa band, as expected. In RSV infected cells, the polyclonal p54 antibody recognized an additional, faster-migrating protein (Figure 51, hereafter referred to as "protein X"); however, this protein was not detected by the monoclonal p54 antibody. Taken together, our results indicated that the polyclonal p54 antibody recognized a protein in RSV inclusion bodies that migrated as a ~40-45 kDa band.

The polyclonal antibody was raised against a 5' terminal peptide of p54. The monoclonal antibody was raised against recombinant, full-length protein corresponding to amino acids 1-484 of human p54. We theorized that RSV infection resulted in an alternate, possibly truncated, form of p54.





3.4. Protein X was present in cell fractions corresponding to high RSV transcription activity.

As mentioned in Chapter 2, Mason et al. developed a technique for concentrating viral RNPs using serial fractionation (see Chapter 2, Figure 27). To assess whether protein X was present in the fraction corresponding to high RSV transcriptional activity, HEp-2 cells were mock infected or infected with RSV. 16 hours post infection, cells were lysed and analyzed using SDS-PAGE and western-blot analysis with the polyclonal antibody toward p54. As shown in Figure 52A, the faster-migrating protein recognized by the polyclonal p54 antibody migrated in the S3 fraction of RSV-infected cells, which correlates with the highest RSV *in vitro* transcription activity. To determine whether protein X was recognized by the polyclonal p54 antibody in a more authentic cell line, the experiment was repeated in A549 cells. As seen in Figure 52B, the faster-migrating protein was present in the S3 fraction of A549 cells.



Figure 52. Protein X was found in RSV cell fraction corresponding to highest RSV *in vitro* transcription activity. HEp-2 (A) or A549 (B) cells were mock infected or infected with RSV (moi =5). 18 hours post infection, cells were fractionated according to the method described by Mason et al. Samples were analyzed using SDS-PAGE and western blot with a polyclonal antibody toward cellular p54.

3.5 Protein X was not present in RSV virions

Because the results above indicated that Protein X was only recognized in samples from RSV-infected cells, we theorized that perhaps the protein was a viral component of RSV. Protein X was approximately 43 kDa, which corresponded to the size of RSV N protein; however, Figure 52 demonstrated that while N was found in all RSV fractions, protein X was found primarily in the S3 fraction. To test the possibility that protein X was a viral protein, we used SDS-PAGE and western blot analysis to determine whether p54 recognized a factor in either wt RSV or LeC virions. We included samples from RSV-infected cells as positive controls. As demonstrated in Figure 53, the polyclonal antibody toward p54 recognized p54 in the S1 and S3 fractions of RSV-infected cells, as well as in LeC virus preparations. The antibody recognized Protein X in both the S1 and S3 fractions of RSV-infected cells, but it did not recognize Protein X in virions. These data suggested that Protein X was not a viral structural protein.



Figure 53. Protein X was not recognized in RSV virions. HEp-2 cells were infected with RSV and fractionated according to the method described by Mason et al. Samples from the S1 and S3 fractions, as well as virions from wt RSV and LeC virus preparations, were analyzed using SDS-PAGE and western blot with a polyclonal antibody toward p54.

3.6 siRNA toward p54 did not affect RSV protein production

Because our results suggested that RSV infection may result in a truncated form of p54, we wished to determine whether p54 is important in the RSV replication cycle. HEp-2 cells were transfected with siRNA against p54. Twenty-four hours post transfection, cells were infected with RSV. Twenty-four hpi, cells were lysed and analyzed using SDS-PAGE and western blot analysis with antibodies toward p54 and RSV proteins. As shown in Figure 54, p54 was reduced in cells transfected with siRNA toward p54 (lanes 4 and 5 (duplicate), top panel). RSV protein production was similar in siRNA-treated cells (lanes 4 and 5, bottom panel) to that of mock-transfected cells (lanes 2 and 3 (duplicate), bottom panel). These data suggest that p54 may not be necessary for efficient RSV protein production, but it is difficult to draw firm conclusions since the siRNA knockdown was not efficient.



Figure 54. siRNA toward p54 did not affect RSV protein production. HEp-2 cells were mock transfected (lanes 1-3) or transfected with siRNA toward p54. 24 hours post transfection, cells were mock infected (lane 1) or infected with RSV (lanes 2-5). 24 hpi, cells were lysed and assessed using SDS-PAGE and western blot analysis with polyclonal antibodies toward p54 (top) or RSV proteins (bottom).

3.7 Protein X was identified as RSV nucleoprotein.

We next sought to determine whether the faster-migrating protein X recognized by the polyclonal p54 antibody was indeed a truncated or alternatively spliced version of p54, or whether it was another protein entirely using mass spectometry analysis. HEp-2 cells were mock infected or infected with RSV. 16 hours post infection, cell were lysed and protein was immunoprecipitated with the polyclonal p54 antibody. Samples were separated on SDS-PAGE gels and stained with Coomassie Blue. The band corresponding to protein X was excised and analyzed by mass spectometry. As shown in Figure 55, protein X was present in only RSV infected cells, as was the case in earlier experiments. The mass-spectometry results indicated that protein X showed substantial similarity with RSV N protein. These surprising results demonstrated that the polyclonal antibody toward p54 likely recognized RSV N in addition to cellular p54.



Figure 55. Protein X was identified as RSV N protein. HEp-2 cells were mock infected or infected with RSV (moi =5). 18 hours post infection, cells were fractionated according to the method described by Mason et al. Samples from the S3 fractions were immunoprecipitated with the polyclonal p54 antibody, and samples analyzed using SDS-PAGE and Coomassie Blue staining. The band indicated by the arrow was excised and subject to mass-spectometry analysis.

3.8 The polyclonal p54 antibody recognized purified N protein.

To confirm that the polyclonal p54 antibody was able to recognize RSV N protein, an experiment was performed in which lysates from RSV-infected cells, RSV N protein purified from baculovirus, or RSV P protein purified from baculovirus were analyzed using SDS-PAGE and western blot with the polyclonal p54 antibody.

As shown in Figure 56A, the purified preparations of P and N were very clean, with no other prominent bands. Figure 56B demonstrates that the polyclonal antibody toward p54 recognizes baculovirus-purified N protein (lane 3), which runs slightly more slowly than N protein from RSV-infected cells (lane 4) due to the addition of a histidine tag and tobacco etch virus protease cleavage site. The antibody did not recognize P protein. Taken together, these results indicate that the polyclonal antibody toward p54 is cross-reactive with RSV N protein.



Figure 56. The polyclonal p54 antibody recognized purified N protein. A) RSV proteins P and N were purified from baculovirus as described in the Materials and Methods section. Samples were run on 10% SDS gels and stained with Coomassie Blue. B) HEp-2 cells were either mock infected (lane 1) or infected with RSV (lane 4). 18 hours post infection, cells were lysed. Samples from these cells and samples from the baculovirus-purified N and P shown in panel A were analyzed using SDS-PAGE and western blot with the polyclonal antibody toward p54. The experiment in this figure was performed by Robin Djang.

3.9 The polyclonal p54 antibody recognized RSV N in inclusion bodies after approximately 12 hours of infection.

Although the results of previous experiments indicated that the polyclonal p54 antibody recognized RSV N, we believe that the antibody toward p54 and the antibody toward RSV N recognize two antigenically different populations of the viral protein. Recall that in immunofluorescence experiments shown in Figure 57, the antibody toward RSV N bound to N in O-rings around inclusion bodies, while the antibody toward p54 recognized N protein in the "core" of inclusion bodies. The results suggest that there are two conformationally distinct populations of RSV N. It is possible that N protein on the periphery of or inside inclusion bodies incorporates one or more post-translational modifications, such as phosphorylation, methylation, and/or acetylation that creates an antigenically distinct subpopulation of the protein.

To determine whether there was a temporal relationship between the two forms of RSV N we performed a time course to assess at what point during RSV infection the p54 antibody would recognize viral protein. HEp-2 cells were mock infected or infected with RSV. At various points post infection, cells were fixed and analyzed using immunofluorescence. As shown in Figure 48, RSV N protein was not present in mock infected cells, but steadily increased as RSV infection progressed. From 8 hpi onward, RSV N was present in small, yet distinct, inclusion bodies in addition to being diffusely present in the cytoplasm. The p54 antibody stained punctate dots characteristic of pbodies in mock infected cells and at 4 and 8 hpi; however, from 12 hpi onward, the antibody primarily stained RSV N protein inside RSV inclusion bodies. Of particular interest is the fact that the p54 antibody did not recognize RSV N at 8 hpi, when inclusion bodies were apparent, but did recognize N at 12 hpi. Studies have shown that

after RSV entry into a cell, the virus genome primarily undergoes transcription until 12-15 hpi, at which point the balance of polymerase activity shifts from transcription to genome replication (29). Alternatively, it is possible that the p54 antibody didn't recognize N protein until a certain accumulation threshold had been reached. These results indicate that that the p54 antibody recognizes an alternative population of RSV N protein that might be concomitant with a shift from RSV transcription to replication.



Figure 57. The p54 antibody recognized N 12 hours post RSV infection. HEp-2 cells were mock infected or infected with RSV. At the indicated times post infection, cells were fixed and stained with DAPI (blue) and antibodies toward RSV N (green) or p54 (red). Cells were examined using confocal microscopy.

3.10 The polyclonal p54 antibody recognized transfected RSV N protein

To gain more information about the population of RSV N recognized by the p54 antibody, we wished to determine whether the p54 antibody recognized RSV N that was transiently expressed in HEp-2 cells by a plasmid. HEp-2 cells transiently transfected with an empty vector or plasmids expressing RSV N, RSV N and P, or RSV N, P, and L. Twenty-four hours post transfection, cells were either lysed or fixed, and anti-p54antibody recognition of RSV N was examined using western blot analysis or immunofluorescence, respectively.

As shown in Figure 58A, the polyclonal p54 antibody recognized N in lysates from RSV infected cells, as well as N in cells transfected with N or N and P. It is unclear why N was not recognized in cells transfected with N, P, and L, although p54 in that lane appears very weak, indicating that there may have been less protein loaded. In cells transfected with empty vector, p54 appears in enlarged p-bodies or stress granules. It is possible that the transfection process or vaccinia virus in the sample elicited a stress response and that transfected N protein blocked this response; however, this has not been confirmed.

As shown in Figure 58B, the p54 antibody recognized RSV N in the context of RSV inclusion bodies in cells transfected with N and P, which have been shown to be the minimum RSV protein requirements for inclusion body formation (90). These results suggest that the form of RSV N recognized by the polyclonal p54 antibody is present in cells transfected with the protein.





Figure 58. The polyclonal p54 antibody recognized transfected N. HEp-2 cells were mock infected or infected with RSV, or transfected with empty vector, or RSV N, RSV N and P. Twenty four hours later, cells were lysed and analyzed using SDS-PAGE and western blot analysis with a polyclonal antibody toward p54 (A) or fixed and analyzed using immunofluorescence with antibodies toward RSV N (green) or cellular p54 (red)(B). Samples were examined using confocal microscopy. Michael Allen helped with the experiments presented in this figure.

3.11 RSV N protein showed similarity to cellular p54

The fact that the polyclonal p54 antibody recognizes RSV nucleoprotein, but does not recognize other proteins present in the samples tested suggests that p54 and RSV N might have an antigenically similar motif. We used BLAST bioinformatics to examine whether RSV N protein shared amino-acid similarity with with the p54 peptide sequence used to raise the polyclonal p54 antibody. As demonstrated in Figure 59, RSV N and the p54 peptide share sequence similarity at a motif corresponding to the area adjacent to the RNA-binding residues of RSV N. This result indicates p54 and RSV N share similar primary structure that may translate to structural similarities between the two proteins.



Figure 59. RSV N protein motif shows similarity to cellular p54. The amino-acid sequence of RSV N protein was compared to the cellular p54 sequence using bioinformatic BLAST analysis. Black arrows indicate sequence identity, grey arrows indicate similarity, and the blue arrow indicates a difference in the peptide to which the p54 antibody was raised and the human p54 sequence. In human p54, this amino acid is K, which is more similar to the RSV sequence.

3.12 Dcp1 antibody recognized a component inside RSV inclusion bodies: a cautionary tale.

Because our immunofluorescence and western blot experiments demonstrated that p54 and RSV N shared an antigenically similar motif, and our BLAST analysis showed that the two proteins share sequence similarity, we hypothesized that RSV N protein may mimic p54 during infection. It seemed possible that given their amino-acid similarity, RSV N might be able to bind p54 interacting partners and thereby manipulate cellular responses to viral infection such as stress-granule or p-body formation. To explore this possibility, we screened a number of known p54 interacting partners to determine whether they were altered during RSV infection. As discussed at length in Chapter 2, we identified dcp1 as a protein whose migration on SDS gels was altered as a result of RSV infection. We next used immunofluorescence to determine whether the cytoplasmic distribution of dcp1 was changed during RSV infection. HEp-2 cells were mock infected or infected with RSV. 18 hours post infection, cells were fixed and immunostained to visualize RSV infection and dcp1 distribution.

As shown in Figure 60A, samples from mock-infected cells showed dcp1 in p-bodies, as expected. In RSV-infected cells, the antibody used stained protein inside RSV inclusion bodies, similar to the staining seen with the polyclonal p54 antibody. As presented in Chapter 2, there is no strong evidence for dcp1 colocalizing with RSV inclusions; however, the specificity of this antibody was lot dependent, as shown in Figure 60A. Because of our experience with the cross-reactive p54 antibody, we wished to confirm the apparent dcp1 / RSV N colocalization was real using a method not reliant upon potentially cross-reactive antibodies. U20S cells stably expressing red fluorescent

protein (RFP)-tagged dcp1 were mock infected or infected with RSV. 18 hours post infection, cells were fixed and stained with antibodies toward RSV N.

Figure 60B demonstrates that in mock infected cells, dcp1 was present in p-bodies as expected. In RSV-infected cells, dcp1 was in p-bodies, but was excluded from RSV inclusion bodies. Taken together, these results indicate that the antibody used in the experiment shown in Figure 60A likely recognized a component other than dcp1, and that dcp1 does not localize to RSV inclusion bodies.





Figure 60. Dcp1 distribution during RSV infection. HEp-2 cells (A) or U2OS cells stably expressing RFP-tagged dcp1 (B) were mock infected or infected with RSV (moi = 1, Panel A, or moi = 0.5, Panel B). 18 hours post infection, cells were fixed and stained for DAPI (blue) (A and B), RSV nucleoprotein (green), (A and B) and p54 (red)(A). Cells were examined using fluorescent microscopy.

3.13 Dcp1 antibody recognized RSV P protein.

Because the antibody toward dcp1 recognized a component inside RSV inclusion bodies, we thought it likely that the antibody was recognizing an RSV protein, similarly to the p54 antibody. To determine whether this was the case, we performed an experiment in which HEp-2 cells were transiently transfected with vectors expressing His-tagged RSV N or P proteins. They were then immunoprecipitated using antibodies toward the His tag, and analyzed using western blot and the antibody toward dcp1 that recognized protein inside RSV inclusion bodies. Samples were also probed with the cross-reactive polyclonal antibody to p54. As shown in Figure 61, both dcp1 and p54 were immunoprecipitated nonspecifically for unknown reasons. However, consistent with previous results, the p54 antibody recognized RSV N protein, and the antibody toward dcp1 recognized a protein that migrated at the same size as RSV P protein in the sample from cells overexpressing RSV P. These results indicate that the dcp1 antibody is able to recognize RSV P.





Chapter 3 Discussion

In summary, our results demonstrated that a polyclonal antibody toward cellular p54 cross-reacted with RSV N protein, that the two proteins share amino-acid similarity, and that there are two antigenically-distinct populations of RSV N protein. In addition, an antibody toward dcp1 recognized RSV P protein.

A cautionary tale

Because of our experience with two cross-reacting antibodies, we recommend that great care be taken when interpreting co-localization data with RSV proteins. While the polyclonal antibody that recognized RSV N was very specific in that it seemed to recognized only p54 and RSV N, the dcp1 antibody was not as clean and at times recognized RSV P, N, and G proteins (data not shown). It seems likely that because certain RSV proteins are highly expressed compared to host proteins, they have a high propensity for nonspecifically interacting with antibodies. This indicates that several methods should be employed when determining the authenticity of colocalization observations regarding the viral proteins.

p54 and N similarity

Our results demonstrating that an antibody toward p54 can recognize RSV N in a number of contexts suggests that they share a similar epitope. Bioinformatic analysis confirmed that the proteins share a sequence with similar primary amino-acid structure. This lends credence to the possibility that they may be structurally similar and might share or interfere with each others' activities.

Viral mimicry?

It is possible that our results with the cross-reactive antibody toward p54 (which also recognizes RSV N) reveal an unfortunate artifact; however, it is possible that this phenomenon has significant implications for the RSV replication cycle and is not merely a coincidence. One possibility, mentioned earlier, is that RSV N protein mimics p54. The idea that a viral protein may mimic a cellular factor is not without precedent, as myriad examples of structural, molecular, and epitope mimicry by viruses have been previously described. Molecular mimicry is a phenomenon in which pathogens have "captured" host genes during evolution to encode proteins with primary amino-acid sequences and structures that are similar to cellular proteins (7, 179). Structural mimicry occurs when pathogens encode proteins that display no obvious amino-acid sequence similarity to host factors, but are structurally similar enough to their homologs that they can be recognized by host immune effectors, as described by Stebbins et al. (238)

Several viruses are known to mimic host proteins. Poxviruses and herpesviruses, which are very large DNA viruses with between 100-200 genes, encode numerous homologues to cytokines, chemokines, and cytokine receptors. Herpesviruses are especially notorious for producing cytokine homologues (7, 19, 34, 108, 126, 160, 212, 235, 237, 248, 268, 273) and chemokine homologues (33, 65, 66, 76, 86, 124, 127, 146, 190, 194, 207, 233, 239, 261, 277), most likely to either suppress the host immune response to viral infection or to recruit potential target cells to the site of infection. For example, Kaposi's sarcoma herpes virus (KSHV/HHV-8) encodes the viral protein vMIP-3, a homologue of human CCL4, which recruits CCR4+ Th2 cells to the site of infection. Poxviruses, on the other hand, specialize in producing cytokine-receptor homologues (8,

9, 11, 53, 71, 102, 150, 175, 189, 200, 209, 210, 227, 230, 240, 270), chemokinereceptor homologues (2, 131-134, 161, 180, 192, 253, 257), and cytokine- and chemokine-binding proteins (10, 36, 93, 130, 174, 184, 191, 228, 230) in order to suppress the antiviral immune response. For example, vaccinia virus encodes a protein, B18R, which acts as a soluble interferon receptor capable of binding interferon- α and - β . thereby disrupting antiviral immune responses (53). Specific roles for theses immunomodulatory proteins vary widely; however, a unifying theme is manipulating host immune responses to facilitate viral propagation. Smaller viruses have also been reported to encode cytokine homologues. HIV tat protein mimics chemokines to competitively displace beta-chemokine receptors CCR2 and CCR3 from binding their cognate chemokines (6). Of particular interest, RSV G protein mimics the chemokine CS3C to bind the CS3C-recptor CX3CR1 in order to induce leukocyte chemotaxis and facilitate infection (251). Several viruses have evolved mimicry mechanisms for manipulating other aspects of the immune system. Poxviruses, herpesviruses, retroviruses, paramyxoviruses and picornaviruses are reported to express homologues of proteins that regulate the complement system and/or homologues of proteins that bind complement receptors and aid in host-cell entry (reviewed by Bernet et al. (30) and Mastellos et al. (151). Some viral expression of proteins with epitopes that are similar to host molecules contributes to autoimmune disease. For example, dengue virus proteins NS1, p4M, and E are antigenically similar to epitopes on platelets, endothelial cells, and coagulatory molecules, and cross-reactive antibodies to the viral proteins facilitate autoimmune disease that contributes to the dengue virus pathogenesis (137, 141, 142). In addition, some studies suggest that human coronoavirus 229 may mimic myelin basic protein and interact with autoreactive T cells, thereby contributing to multiple sclerosis

(242). It is undetermined whether this mimicry benefits the virus. Other viruses mimic host proteins in order to manipulate cell-cycle progression and proliferation. Human cytomegalovirus (CMV)-encoded protein kinase UL97 has been shown to mimic cdc2/Cyclin-dependent kinase 1 in order to promote nuclear egress coincident with viral arrest of the cell cycle, as described by Hamirally et al. (100). Herpes simplex virus (HSV) VP16 has been shown to mimic the human basic leucine-zipper protein (LZIP) in order to associate with host cell factor (HCF) in order to control cell-cycle progression (87). HSV-1-encoded viral protein ICP0 is a functional mimic of host E3 ubiquitin ligases capable of degrading the cellular DNA damage response E3 ligase RNF8, thereby promoting viral propagation (49). Finally, there is evidence that Hepatitis B virus (HBV) surface antigen (HBsAg) mimics components of the apoptotic cell clearance pathway in order to subvert the adaptive immune response (255).

These examples demonstrate that viruses have evolved elegant mechanisms to coexist, subvert, and manipulate host defense processes. It is possible that RSV N mimics p54 in order to interfere with one or more of its activities, including translational repression, p-body formation, or RNP remodeling. Alternatively, perhaps RSV N mimics p54 inside inclusion bodies in order to recruit RNP-remodeling proteins and facilitate replication. It is, of course, possible that p54 is important during RSV replication in a manner that is independent from viral mimicry. Further studies are needed to elucidate the relationship between RSV biology and host protein p54.

Two populations of N

Our results suggest that there are two antigenically distinct populations of RSV N protein (Figure 49): one population that is concentrated at the periphery of inclusion bodies, and

another that comprises the core of inclusion bodies. In addition, while antibodies toward RSV proteins recognize N in all cell fractions, remarkably, the antibody toward p54 recognized only N in the S3 fraction of the same lysates (Figure 52).

It is possible that the two forms of N could have different post-translational modifications. The possibility of an alternative population of RSV N protein that may be concomitant with a shift from RSV transcription to replication lends credence to an exciting hypothesis that post-translationally-modified RSV N protein might have an epigenetic role in the RSV replication cycle. In eukaryotes, DNA is wound tightly around proteins called histones, which act to compact nucleic acid. Histone modifications can include lysine methylation, arginine methylation, lysine acetylation, and serine/threonine/tyrosine phosphorylation (96, 241, 254). The cellular machinery responsible for regulating gene transcription is extremely sensitive to these changes, For example, acetylation of lysine serves to neutralize the positive charge of the amino acid, thereby reducing the attraction between the histone and negatively charged DNA. This results in a loosened chromatin structure, which gives polymerases access to the nucleic acid. In general, histone acetylation is associated with active transcription, although the two are not obligatorily linked (120).

Examples of post-translational modifications to viral nucleoproteins have previously been described. Mass-spectometry analysis showed that Ser-2 of Menangle virus (a porcine paramyxovirus) is acetylated during infection, although no functional role for the modification has been reported (221). Hepatitis delta virus (HDV) encodes two proteins, small and large delta antigens, which are involved in viral replication and assembly, respectively. Both viral proteins are acetylated *in vivo*. The small delta antigen of HDV is
acetylated at lysine 72 and mutation of this residue causes a cellular redistribution of the protein from the nucleus to the cytoplasm. This suggests that acetylation of the small delta antigen may be required for efficient viral RNA replication (177).

Post-translationally modified RSV N could serve a role similar to that of histones, and modified N protein could be either more or less tightly associated with RSV genomic and antigenomic RNA during transcription and replication. Perhaps RSV replication takes place in the core of inclusion bodies, and packaging takes place at the periphery. Future studies are needed to characterize the nature and significance of the two populations of RSV N protein.

Conclusions

Our data provide evidence that RSV N protein may mimic cellular p54. It is possible that RSV N interferes with one or more p54 activities, such as recruiting binding factors, remodeling mRNP complexes, or repressing translation. In addition, our data support the hypothesis that there are two antigenically distinct populations of N. Future studies are needed to elucidate any mechanisms by which N may mimic p54, as well as to reveal the differences in the two N populations.

Overview Discussion

Our studies indicate that RSV interacts with many aspects of host stress responses. We have shown that RSV blocks stress-granules from forming in the majority of infected cells, that RSV infection results in the alteration of a protein associated with mRNA degradation, and that RSV N protein shares antigenic simialrity to a protein involved in regulating mRNA homeostasis. These studies not only advance our understanding of RSV-host interactions, but also provide a launching pad to more thoroughly investigate how RSV and hosts intermingle to promote propagation and survival, respectively.

Future Directions: stress-granule project

The studies presented in Chapter 1 revealed two important findings. First, RSV has a mechanism for subverting stress responses. This is confirmed by a recent report by Fricke et al. that RSV blocks stress-granule formation by sequestering p38 and OGT in viral inclusion bodies (88). Second, a mutant RSV virus that induces stress granules during infection was correlated with impaired growth and a weak plaque phenotype. This lends support to a growing body of evidence that cellular stress responses may be linked to primordial immune responses, and that stress granules may have antiviral activity.

Additional studies are needed to determine the mechanism of RSV-mediated stressgranule subversion. Preliminary evidence suggested that an RSV minigenome expressing full-length RSV Tr in addition to RSV polymerase-associated proteins might have been sufficient to block stress granules (Figure 20). These minigenomes could be used to determine whether RSV Tr is involved in subverting stress responses, and whether RSV Tr interacts with host-cell proteins involved in stress-granule formation.

While stress granules are associated with stalled translation and are thought to direct select transcripts to p-bodies for degradation, studies are needed to determine the mechanisms by which stress granules may combat RSV infection. This could be accomplished by inducing stress-granules in cells (using heat, chemicals, or overexpression of G3BP), and examining the effects these treatments have on RSV replication, mRNA and protein production, plaque phenotype, and / or cytotoxicity.

Future Directions: dcp1 project

The studies in chapter two focused on the relationship between RSV and p-bodyassociated decapping factor dcp1, and revealed that dcp1 is phosphorylated throughout RSV infection via the ERK 1/2 pathway. These results are significant for several reasons. First, dcp1 phosphorylation has not been described for any other virus. Second, preliminary data (not shown) indicated that dcp1 phosphorylation is important in regulating cytokine responses to viral infection, revealing dcp1 as an important molecule in innate immune response. Third, these findings add to the increasing evidence that cellular RNA-processing structures and proteins are important for cellular antiviral responses.

Future studies are needed to elucidate the mechanism of RSV-mediated dcp1 phosphorylation and to determine how dcp1 phosphorylation might communicate with innate immune pathways. Our results suggest that dcp1 is phosphorylated very early during RSV infection, likely before the virus is internalized. We believe that it is probable that RSV mediates dcp1 phosphorylation during initial interactions between receptors on

the host cell membrane and the RSV F and/or G protein(s). Studies using purified F and/or G could help determine whether these proteins are sufficient to mediate dcp1 phosphorylation. Alternatively, mutant RSV viruses lacking RSV F or G could provide valuable insights into whether these proteins are necessary for the effect.

Rzeckowski et al. demonstrated that the phosphorylation status of dcp1 could influence IL-1-induced IL-8 production (206), and our preliminary data suggested that cells overexpressing a phospho-deficient dcp1 mutant resulted in higher levels of IL-8 production in response to RSV infection at 24 hpi than cells overexpressing wt dcp1. Additional studies are needed to confirm this effect and to determine whether dcp1 phosphorylation is important for regulating other proinflammatory cytokines such as IL-6 and/or TNF-α or other aspects of host antiviral responses such as surfactant production.

Our results indicated that RSV-mediated dcp1 phosphorylation was part of the ERK 1/2 pathway, however, further experiments are needed to determine whether dcp1 is a direct substrate for ERK 1/2. Kinase assays could be used to explore this possibility. In addition, studies could be conducted to determine whether dcp1 is phosphorylated obligatorily during ERK 1/2 activation.

Finally, future studies are needed to determine whether dcp1 phosphorylation is important for decapping activities and/or binding interacting partners. As mentioned previously, dcp1 is an essential cofactor for the catalytically active decapping protein dcp2. The mutants we generated for this study are tagged with FLAG. Cells overexpressing wt or phospho-deficient dcp1 could be used to immunoprecipitate dcp1 (with an antibody toward ddk or FLAG) and any bound proteins. Mass-spec analysis could be used to determine any differences in proteins bound by the two versions of

dcp1, which would indicate whether dcp1 phosphorylation is important for binding or recruiting interacting proteins. Decapping assays could be performed with lysates from mock-infected cells and RSV-infected cells to determine whether RSV-mediated dcp1 phosphorylation is important for decapping functions.

Future Directions: RSV N biomimicry project

Chapter 3 discussed our investigation into RSV's relationship with cellular p54. These studies provide a cautionary tale against interpreting colocalization results, since two separate antibodies were able to recognize RSV proteins. In addition, they provide evidence for two antigenically-separate populations of N, and a possible role for N in mimicking p54.

Future studies are needed to identify how the N populations differ. As discussed earlier, it is possible that the two forms of N differ in their post-translational modifications. Immunoprecipitation experiments could be performed in which RSV N pulled down with the polyclonal antibody toward RSV could be compared to RSV N pulled down with the polyclonal antibody toward p54 using mass-spectometry analysis.

Additional studies are also needed to determine whether RSV N mimics cellular p54. Structural analysis using bioinformatics may reveal important similarities between the proteins that could provide clues as to the functional significance of such similarity.

Overarching theme: stress responses as part of a cellular antiviral campaign

While the studies discussed in Chapters 1, 2, and 3 are fairly distinct, a unifying theme is that viruses and eukaryotes have co-evolved to establish a complex equilibrium between cellular stress-response structures and proteins involved in mediating antiviral responses and virus strategies for evading, manipulating, and combating these responses. Stress granules have the potential to limit viral infection by regulating translation, RNP structures, and/or targeting mRNAs to p-bodies for degradation. It is therefore not surprising that RSV has evolved to prevent stress granules from assembling during infection. Similarly, p-bodies and p-body-associated proteins have obvious potential for interfering with virus replication by either regulating viral RNAs or by regulating factors such as cytokines and chemokines involved in antiviral defenses. Finally, p54, which is associated with both stress granules and p-bodies, is linked to translational repression, mRNP remodeling, and p-body assembly - all of which could be important during RSV replication.

Model

Our studies have helped to characterize the complex relationships among RSV, stress granules, p-bodies, and some of their associated proteins. We propose a model in which RSV subverts the deleterious effects of stress responses by interfering with stress-granule formation, possibly by Tr-region-dependent sequestration of p38, OGN, or other SG-associated proteins. In addition, we believe that p-body protein dcp1 is phosphorylated during RSV infection as part of a host-response to regulate immune factors and the nature of immune responses. We posit that phosphorylated dcp1 is

necessary for interacting with dcp2 in the decapping complex, and that the dcp1-dcp2 complex is important for regulating mRNAs, including those that encode cytokines involved in combating RSV infection. Finally, we propose that RSV N protein may mimic p54 to interfere with stress-granule and/or p-body antiviral activities. For example, N may mimic p54 in order to interfere with potential p54-mediated recruitment of viral mRNAs to p-bodies, and thereby prevent viral transcript degradation. Together, our data provide evidence that RSV has evolved to interact with and, in some cases, subvert, elements of host-cell stress responses, and establish a foundation for future studies regarding RSV interactions with host-cell RNA-processing structures and proteins.

LIST OF ABBREVIATIONS - JOURNAL TITLES

Adv Cancer Res	Advances in Cancer Research
Am J Hyg	The American Journal of Hygeine
Am J Physiol Lung Cell Mol Physiol	American Journal of Physiology – Lung Cellular and Molecular Physiology
Am J Respir Cell Mol Biol	American Journal of Respiratory Cell and Molecular Biology
Am J Respir Crit Care Med	American Journal of Respiratory and Critical Care Medicine
Ann Neurol	Annals of Neurology
Annu Rev Immunol	Annual Review of Immunology
Arch Dis Child	Archives of Disease in Childhood
Arch Virol	Archives of Virology
Biochem J	Biochemical Journal
Biochem Soc Trans	Biochemical Society Transactions
Bioessays	BioEssays
Biol Reprod	Biology of Reproduction
Cell Host Microbe	Cell Host & Microbe
Cell Microbiol	Cellular Microbiology
Curr Biol	Current Biology
Curr Opin Immunol	Current Opinion in Immunology
Curr Top Microbiol Immunol	Current Topics in Microbiology and Immunology
Cytokine Growth Factor Rev	Cytokine & Growth Factor Reviews
Dev Dyn	Developmental Dynamics
EMBO J	The EMBO journal
Eur J Immunol	European Journal of Immunology
Eur Respir J	European Respiratory Journal
Exp Biol Med (Maywood)	Experimental Biology and Medicine
FASEB J	The Journal of the Federation of American Societies for Experimental Biology
FEBS Lett	Federation of European Biochemical Societies Letters

Genes Cells Genes Dev Immunol Res Int Arch Allergy Immunol J Biochem J Biol Chem J Biomed Biotechnol J Biomed Sci J Biosci J Cell Biol J Cell Sci J Clin Invest J Clin Pathol J Exp Med J Gen Virol J Immunol J Infect Dis J Leukoc Biol J Mol Biol J Pediatr J Virol J Virol Methods Mol Cell Biol Mol Cell Mol Reprod Dev Nat Immunol Nat Methods Nat Rev Immunol Nat Rev Mol Cell Biol Nat Struct Biol Nat Struct Mol Biol Nucleic Acids Res Pediatr Pathol

Genes to Cells Genes & Development Immunologic Research International Archives of Allergy and Immunology The Journal of Biochemistry The Journal of Biological Chemistry Journal of Biomedicine and Biotechnology Journal of Biomedical Science Journal of Biosciences Journal of Cell Biology Journal of Cell Science The Journal of Clinical Investigation Journal of Clinical Pathology The Journal of Experimental Medicine Journal of General Virology The Journal of Immunology Journal of Infectious Diseases Journal of Leukocyte Biology Journal of Molecular Biology Journal of Pediatrics The Journal of Virology Journal of Virological Methods Molecular and Cellular Biology Molecular Cell Molecular Reproduction and Development Nature Immunology Nature Methods Nature Reviews Immunology Nature Reviews Molecular Cell Biology Nature Structural & Molecular Biology Nature Structural & Molecular Biology Nucleic Acids Research Pediatric Pathology

PLoS Biol PLoS One PLoS Pathog Proc Natl Acad Sci U S A Public library of science (PLoS) Biology PLoS ONE PLoS Pathogens Proceedings of the National Academy of Sciences of the United States of America RNA Biology Trends in Immunology Trends in Microbiology Viral Immunology Virus Research

RNA Biol Trends Immunol Trends Microbiol Viral Immunol Virus Res

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Curriculum Vitae

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Education

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Publications

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Hanley LL, McGivern DR, Teng MN, Djang R, Collins PL, Fearns R. Roles of the respiratory syncytial virus trailer region: effects of mutations on genome production and stress granule formation. Virology. 2010 Oct 25;406(2):241-52.

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Laura L. Dickey, Bethany Neilson, Jeffery S. Horsburgh, Daniel P. Ames, and David K. Stevens. BASINS (Better Assessment Science Integrating point & Non-point Sources). 2001, 2003, and 2010. Environmental Protection Agency. EPA Publication # 823B01001.

Awards

- Russek Student Achievment Award, 1st place, Boston University 2012
- Laurence Corwin Award, Boston University, 2011
- Russek Student Achievement Award, 2nd place, Boston University 2011
- Russek Student Achievement Award, 2nd place, Boston University 2010
- Boston University School of Medicine Department of Microbiology Travel Award, Fall 2010
- Boston University School of Medicine Division of Graduate Sciences Travel Award, Fall 2010
- Best Presentation, Intermountain regional ASM meeting, March 2005
- Best Poster Award, BYU Microbiology departmental retreat 2004
- International Rotary Club Award for Aid in Developing Country Dominican Republic, 2000
- Honors at Entrance scholarship, 1996-2000
- Coolidge scholarship, Utah State University, 1999-2000
- Corbett Award for Women in Science, Utah State University 2000
- Outstanding Senior, USU Department of Biological Engineering, 2000
- · Outstanding Junior, USU Department of Biological Engineering, 1999
Research Experience

2006-2013 Boston University School of Medicine Department of Microbiology, Immunology Training Program

 Graduate student in the lab of Rachel Fearns, Ph.D: Examined significance of RSV trailer length and sequence on RSV promoter activity and replication, and characterized RSV's relationship with stress granules, and determined that RSV Trailer region is involved in subversion of cellular stress responses. Also determined that RSV infection mediates the phosphorylation of cellular RNA-processing protein dcp1 via the ERK 1/2 pathway, and that dcp1 phosphorylation is limited to residues S315, S319, and T321.

2003-2006 Brigham Young University Department of Microbiology and Molecular Biology

 Graduate student in the lab of Richard Robison, PhD: Characterized differences in cytokine responses in *B. pseudomallei*-infected primary cells from normal and diabetic subjects

1999-2003 Utah State University Environmental Management Research Group, Utah Water Research Laboratory

 Research Assistant in lab of David K. Stevens, PhD: Compiled data sets for and wrote training manual for riparian-zone watershed software (BASINS), published by the EPA; Developed complete set of training materials for EPA-sponsored, graduate-level course at USU: BASINS: Watershed Modeling; Collected water samples and assessed dissolved oxygen, pollutant, coliform, and algae levels for Teton River in Driggs, ID; Compiled Geographical Information Systems (GIS) data sets for Virgin River (UT) and developed documentation for MapWindow; an open-source program that allows manipulation of geospatial data

1998-2000 Utah State University Department of Biological Engineering

 Research assistant in lab of Wynn Walker, Ph.D., Senior Associate Dean: Assessed moisture levels and chemical content of fertilizer-treated soil in Honeyville, UT; Used watershed modeling software to predict soil moisture needs based on weather, geography, and local crops in Nephi, UT; Helped design and install remote water-quality assessment system in rural Dominican Republic community; trained local workers in operating procedures

Teaching Experience

 Teaching assistant for Fundamentals in Biological Sciences, Genetic and Microbiology Modules, Boston University Division of Graduate Medical Sciences, Fall 2011; Disease and Therapy, Boston University School of Medicine, Fall 2010 and Fall 2011; Medical Microbiology, Boston University School of Medicine, Fall 2007; Advanced Techniques in Molecular Biology, Brigham Young University Department of Microbiology and Molecular Biology, Spring 2005; Introduction to Microbiology, Brigham Young University Department of Microbiology and Molecular Biology, Fall 2005; Microbial Physiology Brigham Young University Department of Microbiology and Molecular Biology, Fall 2004 and Fall 2005

- Instructor for BASINS Watershed Modeling graduate-level, EPA-sponsored continuing education course, Utah State University Department of Environmental Engineering (quarterly from 1999 - 2003)
- Kindergarten Teacher, Meta International School Nakkornpattom, Thailand, 2003
- English Teacher, Westgate Academy Gotemba, Japan, 2001-2002.

Formal Data Presentations:

- Laura Dickey and Rachel Fearns. RSV Trailer Region May Prevent Stress-Granule Formation via a TIAR-Independent Mechanism. Boston University Science and Engineering Day, March 2012 (poster presentation), and Boston University Russek Student Achievement Day, May 2012 (oral presentation).
- Laura Dickey and Rachel Fearns. RSV Trailer Region May Prevent Stress Granule Formation. Talk given at Respiratory Syncytial Virus Symposium, December 2010 (oral presentation).
- Laura Dickey, Robin Djang, and Rachel Fearns The complicated relationship between respiratory syncytial virus and cellular stress granules and processing bodies. Boston University Infectious Disease Seminar, February 2010 (oral presentation).
- Laura L. Dickey, Justin C. Hoopes, Bruce C. Scalje, Kim L. O'Neill, and Richard A. Robison. An Infection Model for Examining the Effects of Gender and Diabetic State on Proinflammatory Cytokine Secretion by Phagocytic Cells in Response to Infection with Burkholderia pseudomallei. 2006 General ASM meeting, Toronto (poster presentation).
- Laura Dickey, Spencer Pruitt, and Richard Robison. An in vitro Macrophage Infection Model for Determining Virulence in Burkholderia pseudomallei. Intermountain ASM meeting, Salt Lake City, March 2005 (oral presentation).
- Laura Dickey, Spencer Pruitt, and Richard Robison. An in vitro Macrophage Infection Model for Determining Virulence in Burkholderia pseudomallei. General ASM meeting, Atlanta. July 2005 (poster presentation).

Volunteer Service

- Department of Microbiology Student Representative Boston University Department of Microbiology (2009-2011)
- Committee for Improving Graduate Student and Postdoctoral Training Boston University Department of Microbiology (2010-2011)
- Journal Club Organization Committee Boston University Department of Microbiology (2010-2012)
- Student Representative for Department of Microbiology and Molecular Biology Brigham Young University Graduate Student Association (GSA) (2004-2006)