HOST-MEDIATED ALTERATION OF MEASLES VIRUS POLYMERASE ACTIVITY: CONSEQUENCES FOR THE OUTCOME OF INFECTION

DISSERTATION

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By

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ABSTRACT

Measles Virus (MeV) has a negative-sense, single-stranded RNA genome that is encapsidated by the viral nucleocapsid (N) protein. This ribonucleoprotein complex (RNP) forms the template for virus transcription and genome replication. The virus encoded RNA-dependent RNA polymerase (RdRp) complex is composed of the monomeric viral large (L) protein and the tetrameric polymerase co-factor (P) protein. Interaction between the RNP and the RdRp, which acts as both a transcriptase and replicase, depends on transient contacts between P and the C-terminus of the N protein (N\textsubscript{TAIL}). The latter is an unstructured protein domain, with only limited conservation of amino acid sequence. Within this unstructured domain lie three conserved motifs that are enriched in amino acids with hydrophobic side groups. Two of these (Box-2 and Box-3) are responsible for interaction with P. The hallmark of P binding is the induced folding of N\textsubscript{TAIL}, which experiences a gain of $\alpha$-helicity following contact of the P protein X domain (XD) with Box-2. The proposed contribution of N\textsubscript{TAIL} Box-3 to P binding is that of a protein clamp, where induced folding gives rise to secondary contacts between Box-3 and XD that result in high affinity N\textsubscript{TAIL}/P complex formation. Transient cycles of binding and release between P and N allow the polymerase complex to advance along the RNP template. The critical factor that should thus determine the rate of polymerase elongation, either during transcription or replication, is the binding affinity
between N and P. This work addresses host-cell related factors that alter this affinity and thus influence the outcome of infection.

Hsp72 is the highly-inducible member of the 70-kDa heat shock protein family (HSP70). HSP70 family members play important roles in protein folding, either of newly-formed peptide chains or of those altered by a denaturing event. Hsp72 activity is typically associated with the latter, aiding in the renaturation of proteins damaged during periods of cellular stress, such as exposure to hyperthermia or cytotoxins. Chapter 1 of this work describes how hsp72, along with other heat shock proteins, are expressed in the central nervous system and how these protein chaperones act to maintain cellular homeostasis, protect cells from insult, and preserve the specialized function of nervous tissue (i.e., to initiate and propagate the electrical impulses that dictate the actions of the rest of the body). These same heat shock proteins can also enhance the gene expression of neurotropic viruses, and yet that stimulation of viral gene expression can enhance viral clearance by overcoming the host-restricted low levels of viral gene expression that otherwise confound adaptive antiviral immune responses. Thus, the stimulatory effect of hsp72 on viral gene expression remains consistent with its host protective functions – provided that the host is immune competent.

Chapter 2 focuses more specifically on the role of hsp72 on MeV gene expression. Hsp72, induced either by heat shock or directly expressed from a stably transfected plasmid, increases MeV virus transcription and genome replication both in
vitro and in vivo. Overexpression of hsp72 in neurons in susceptible mouse strains (i.e., those lacking effective antiviral immune responses) results in significantly increased virus-related mortality following intracranial inoculation. The functional effects of hsp72 on MeV infection have been mapped to its interaction with the MeV N protein, and indeed to the same domains of N_{TAIL} (Box-2 and Box-3) responsible for its association with MeV P. This finding suggests that hsp72 may act by altering the activity of the viral polymerase, either during transcription or genome replication, or both. Chapter 2 presents the results of experiments that delineated this effect. Using a reverse transcription-quantitative polymerase chain reaction (RT-qPCR) approach to monitor the kinetics of MeV transcription and genome replication, it was determined that the effect of hsp72 is to maintain nucleocapsid in a transcriptionally competent state during the late stages of infection. At these same times in cells lacking high levels of hsp72, viral transcriptional activity has declined, with low level replicase function being the predominant RdRp activity.

Chapter 3 describes a possible mechanism by which post-translational modification of Box-3 may alter the outcome of MeV infection in ways that mimic hsp72/Box-3 interaction. Here, the effect of tyrosine phosphorylation within N_{TAIL} on polymerase activity and infection phenotype is examined. Tyrosine phosphorylation of the MeV N protein is associated with viral persistence in neurons, and the persistent state is characterized by an abundance of defective interfering (DI) particles, truncated
genomic fragments that are replication competent in the presence of helper (i.e., parent) virus. Since DI genomes do not express significant levels of functional viral protein, if any at all, they may interfere with replication of parent virus by consuming proteins required to support genomic replication. Results of this chapter suggest a model connecting these findings. A tyrosine to aspartic substitution within the MeV NTAIL Box-3 at position 518 is used to mimic tyrosine phosphorylation. This substitution results in a reduced binding affinity for XD. This reduced affinity leads to elevated polymerase activity, an effect that can be attributed to enhanced cycles of RdRp binding and release from the RNP template during transcription and genome replication. This diminished affinity of P for the template also appears to promote template switching by the replicase during genome replication, as evidence by the dramatically enhanced production of copy-back DI particles. This observation provides a link between tyrosine phosphorylation and altered polymerase activity that is capable of promoting a persistent infection state.

Finally, chapter 4 describes an examination of the contribution of co-chaperone molecules to the high binding affinity between hsp72 and NTAIL. In mammals, co-chaperone functions are carried out by members of the 40-kDa heat shock protein family (HSP40). These proteins bind HSP70 family members and catalyze the rapid ATPase activity that is critical for high affinity substrate binding. Without co-chaperones, hsp72-target interactions can be transient and characterized by low affinity, insufficient for proper chaperoning of protein folding. Binding studies in this work show that HSP40 co-
chaperones are necessary for the well-described high affinity binding between hsp72 and \( N_{\text{TAIL}} \), making these co-chaperones an additional host factor capable of modulating viral gene expression.

Collectively, these studies delineate some of the effects that intracellular host-factors have on MeV infection. These findings help clarify the complex interactions that gives rise to the diverse outcomes of MeV infection.
For my dad
ACKNOWLEDGMENTS

I’d like to start out by thanking Dr. Michael Oglesbee for his kind attention to my career as a graduate student. His sharp intellect and insightful observations provided constant guidance, particularly during the times of confusion and disappointment that often mark this particular path. The atmosphere of cooperation and free expression of thought he promotes are vital to the success of all of us that have studied with him. These thoughts extend to all members of the lab, past and present, including Dr. Xinsheng Zhang, Dr. Tom Carsillo, Zachary Traylor, Yaoling Shu, Dr. Heather Miller, Dr. Macklin McCall and Dr. Pamela Lee and to the Niewiesk lab, especially Dr. Mary Carsillo. I’d particularly like to thank Xinsheng and Tom, who taught me how to do everything and kept me going for a lot of years.

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TABLE OF CONTENTS

Abstract ........................................................................................................................................ ii
Dedication ..................................................................................................................................... vii
Acknowledgments .................................................................................................................... viii
Vita ................................................................................................................................................ x
List of Tables ................................................................................................................................ xvii
List of Figures ............................................................................................................................ xviii
List of Abbreviations .................................................................................................................... xxi
Chapters:
1. Introduction ................................................................................................................................. 1
  1.1 Abstract ................................................................................................................................... 1
  1.2 Introduction ............................................................................................................................... 2
  1.3 Constitutive Expression of Heat Shock Proteins in the Brain ................................................ 9
    1.3.1 HSP70 Family .................................................................................................................. 9
    1.3.2 Hsc70 .............................................................................................................................. 11
    1.3.3 Hsp72 .............................................................................................................................. 12
    1.3.4 Hsp90 .............................................................................................................................. 13

xii
1.3.5 Low Molecular Weight Heat Shock Protein..............................14
1.4 Hyperthermic Induction of Heat Shock Proteins in the Brain..............14
  1.4.1 Hsp72.....................................................................................14
  1.4.2 Hsp90.....................................................................................26
  1.4.3 Low Molecular Weight Heat Shock Proteins..............................27
1.5 Hyperthermic Induction of Heat Shock Proteins and Viral Neurovirulence.................................................................................28
1.6 Conclusions....................................................................................32
1.7 Acknowledgments.............................................................................33

2. Hsp72 Sustains the Accumulation of Transcriptionally Active Nucleocapsid Templates from Measles Virus Edmonston Strain..............34
  2.1 Abstract.......................................................................................34
  2.2 Introduction..................................................................................35
  2.3 Material and Methods ..................................................................38
  2.4 Results..........................................................................................41
    2.4.1 Hsp72 Sustains Late Viral Transcription in Mouse Cells.............41
    2.4.2 Enhancing Effect of Hsp72 on Late Virus Transcription is Also Observed when Efficiency of Virus Entry into Mouse Cells is Optimized.........................................................46
    2.4.3 Hsp72 Enhancing Effect on Late Transcription is Also Observed
3. A Tyrosine Residue Within the Measles Virus N Protein C-Terminus Can Modulate Viral Polymerase Activity

3.1 Abstract

3.2 Introduction

3.3 Materials and Methods

3.3.1 Binding Reaction Analysis

3.3.2 XD-Induced Folding of NTAIL

3.3.3 Analysis of N Protein Function Using Minireplicons

3.3.4 Analysis of N Protein Function in Infectious Virus

3.4 Results

3.4.1 The Y518D Substitution Reduced XD Binding Affinity for NTAIL

3.4.2 The Y518D Substitution Does Not Alter the Ability of NTAIL to Undergo Induced Folding, Only the Folding that is Induced by XD

3.4.3 An N Protein Y518D Substitution Mutant Enhances MeV in Primate Cells
Minireplicon Reporter Gene Expression ........................................81

3.4.4 In Context of an Infectious Virus, N-Y518D is Associated with a
Marked Attenuation of Viral Gene Expression that is Attributed
to Increased DI Particle Formation................................................83

3.5 Discussion..................................................................................................88

3.6 Acknowledgments......................................................................................92

4. Hsp72 Co-Chaperones are Required for High Affinity Binding Between Hsp72
and the Measles Virus N Protein Unstructured Domain.................................93

4.1 Abstract......................................................................................................93

4.2 Introduction................................................................................................94

4.3 Materials and Methods...............................................................................96

4.3.1 Proteins ..................................................................................................96

4.3.2. Construction of Hsp72 Expression Plasmids.................................96

4.3.3 Construction of Hdj1 Expression Plasmids ...................................98

4.3.4 Expression of Recombinant Proteins.............................................99

4.3.5 Purification of Recombinant Proteins............................................99

4.3.6 Determination of Protein Concentration......................................101

4.3.7 Circular Dichroism.......................................................................101

4.3.8 Mass Spectrometry (MALDI TOF) .............................................101

4.3.9 Analysis of Commercial and Highly-Purified Hsp72 Samples ...102
4.3.10 Binding Reaction Analysis ................................................. 103

4.4 Results ....................................................................................... 104

4.4.1 Co-chaperones are Only Present in Commercially Prepared
Hsp72 ......................................................................................... 104

4.4.2 Highly-Purified Hsp72 Binds $N_{TAIL}$ with Low Affinity ......... 106

4.4.3 Highly-Purified Hsp72 Supplemented with Hdj1 Binds $N_{TAIL}$
With High Affinity ........................................................................ 108

4.5 Discussion .................................................................................. 108

4.6 Acknowledgments ...................................................................... 110

Bibliography ..................................................................................... 111
**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Calculated equilibrium dissociation constants ($K_D$) for the binding of three Box-3 peptide variants with both XD and hsp72.</td>
<td>73</td>
</tr>
<tr>
<td>3.2</td>
<td>Calculated equilibrium dissociation constants ($K_D$) for the binding of four $N_{TAIL}$ protein variants with both XD and hsp72.</td>
<td>75</td>
</tr>
<tr>
<td>4.1</td>
<td>Calculated association and dissociation constants ($k_a$, $k_d$) and equilibrium dissociation constants ($K_D$) for the binding of $N_{TAIL}$ protein with commercial and gel purified hsp72.</td>
<td>107</td>
</tr>
<tr>
<td>4.2</td>
<td>Calculated association and dissociation constants ($k_a$, $k_d$) and equilibrium dissociation constants ($K_D$) for the binding of $N_{TAIL}$ protein with hsp72 alone, Hdj1 alone, or a combination of hsp72 and Hdj1.</td>
<td>107</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Extracorporeal blood heating used to measure the physiologic response to</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>hyperthermia in the canine brain.</td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>Hsp72 expression in the canine brain following the induction of whole body</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>hyperthermia by extracorporeal blood heating.</td>
<td></td>
</tr>
<tr>
<td>1.3</td>
<td>Constitutive expression of hsp72 in murine neuroblastoma cells mediates</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>cellular tolerance to subsequent heat stress.</td>
<td></td>
</tr>
<tr>
<td>1.4</td>
<td>Lithium chloride (LiCl) treatment induced neuronal differentiation in a murine</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>neuroblastoma cell line that constitutively expresses hsp72 (72-12) and in a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>vector-transfected control cell line (β-2).</td>
<td></td>
</tr>
<tr>
<td>2.1</td>
<td>Quantification of Ed-MeV N transcript and genome levels in neuroblastoma</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>cells (N2a) by RT-qPCR analysis of total cell RNA.</td>
<td></td>
</tr>
<tr>
<td>2.2</td>
<td>Nucleocapsid protein antigen expression in hsp72 overexpressing (N2a-HSP)</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>and control (N2a-V) neuroblastoma cells infected at an MOI = 1.0 with Ed-MeV</td>
<td></td>
</tr>
</tbody>
</table>

xviii
2.3 Nucleocapsid protein antigen expression in hsp72 overexpressing (N2a-HSP) and control (N2a-V) neuroblastoma cells infected at an MOI = 1.0 with Ed-MeV .................................................................45

2.4 Flow cytometric analysis of murine neuroblastoma cells stably transfected to express CD46..................................................................................................................47

2.5 MeV N transcript levels in CD46- and hsp72-expressing murine neuroblastoma cells (N2a-HSP-CD46) relative to CD46 expressing control neuroblastoma cells (N2a-V-CD46) ........................................................................48

2.6 Ratio of Ed-MeV N transcripts to full-length genomes in CD46 and hsp72 expressing murine neuroblastoma cells (N2a-HSP-CD46) relative to CD46 expressing control neuroblastoma cells (N2a-V-CD46)........................................................................50

2.7 Measles virus transcript production in Vero cells that were heat shocked to elevate hsp72 expression (HS), compared to non-shocked controls (NS).............52

2.8 Western blot analysis of purified Ed-MeV virion total protein showed the presence of hsp72..................................................................................................................55

3.1 Far-UV CD spectra and analysis of the α-helical propensities of $N_{TAIL}$ proteins ..................................................................................................................77

3.2 Induced folding of $N_{TAIL}$ proteins in the presence of XD..............................79

3.3 Luciferase reporter gene expression from MeV minireplicons using either parent N protein or N protein Y to D substitution mutants as template ..........82
3.4 Cell free infectious viral progeny release by Ed N, Ed N-Y451D, and Ed N-Y518D mutant following infection of Vero cells (MOI = 0.01) .......................84

3.5 Northern blot analysis of total RNA derived from Vero cells infected at an MOI = 1.0 with Ed N, Ed N-Y451D or Ed N-Y518D.............................................85

3.6 Serial low MOI (0.01) passage of Ed N-Y518D on Vero cells resulted in a progressive decrease in cell-free infectious viral progeny release, resulting in a greater than 250-fold loss in titer in just three passes.............................................87

4.1 Purification of recombinant hsp72 and Hdj1 from bacteria.............................................97

4.2 Protein expression in two preparations of human recombinant hsp72 ...............105
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-MoRE</td>
<td>α-Molecular Recognition Element</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BAEP</td>
<td>Brainstem Auditory Evoked Potential</td>
</tr>
<tr>
<td>Box-1</td>
<td>Measles Virus Nucleocapsid Protein (aa 401-420)</td>
</tr>
<tr>
<td>Box-2</td>
<td>Measles Virus Nucleocapsid Protein (aa 498-506)</td>
</tr>
<tr>
<td>Box-3</td>
<td>Measles Virus Nucleocapsid Protein (aa 517-525)</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>CD46</td>
<td>Preferred Cell Surface Receptor for the Edmonston Measles Virus Strain</td>
</tr>
<tr>
<td>CDV</td>
<td>Canine Distemper Virus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic Effect</td>
</tr>
<tr>
<td>CsCl</td>
<td>Cesium Chloride</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
</tr>
</tbody>
</table>
CSP ................................................................. Cysteine String Protein
CTL ................................................................. Cytotoxic T Lymphocyte
D ................................................................. The Amino Acid Aspartic Acid
DIC .......................................................... Disseminated Intravascular Coagulopathy
DNA ................................................................. Deoxyribonucleic Acid
DnaJ ...................................................... Bacterial Homologue of HSP40
DnaK ...................................................... Bacterial Homologue of HSP70
Ed-MeV ................................................ Edmonston Strain of the Measles Virus
EM ................................................................. Electron Microscopy
FITC ............................................................... Flourescein Isothiocyanate
GAPDH ................................................... Glyceraldehyde 3-Phosphate Dehydrogenase
Hdj1 .......................................................... Isoform of the 40-kDa Heat Shock Protein Family
h.p.i. ............................................................... Hours Post-Infection
hsc70 .............................................. Constitutive Isoform of the 70-kDa Heat Shock Protein Family
HSE ................................................................. Heat Shock Element
HSF ................................................................. Heat Shock Factor
HSP ................................................................. Heat Shock Protein
hsp27 .............................................. Isoform of the Small Molecular Weight Heat Shock Protein Family
hsp32 .............................................. Isoform of the Small Molecular Weight Heat Shock Protein Family
HSP40 ............................................................. 40-kDa Heat Shock Protein Family
hsp60 .......................................................... Isoform of the 60-kDa Heat Shock Protein Family
HSP70 .......................................................... 70-kDa Heat Shock Protein Family
hsp72 .......................................................... Inducible Isoform of the 70-kDa Heat Shock Protein Family
hsp90 .......................................................... Isoform of the 90-kDa Heat Shock Protein Family
K_D ............................................................ Equilibrium Dissociation Constant
kDa ............................................................ Kilodaltons
L ............................................................. Measles Virus RNA-dependent RNA Polymerase (Large Protein)
LSD ............................................................. Lysergic Acid Diethylamide
mAb ............................................................. Monoclonal Antibody
MeV ............................................................. Measles Virus
MHC ............................................................. Major Histocompatibility Complex
MIBE ............................................................. Measles Inclusion Body Encephalitis
MOI ............................................................. Multiplicity of Infection
mRNA ............................................................ Messenger Ribonucleic Acid
N2a-HSP ............................................. Murine Neuroblastoma Cells that Constitutively Express Human hsp72
N2a-HSP-CD46 ........................................ Murine Neuroblastoma Cells that Constitutively Express Human hsp72 and CD46
N2a-V ................................................. Vector Transfected (Control) Murine Neuroblastoma Cells
N2a-V-CD46 .......................................... Vector Transfected (Control) Murine Neuroblastoma Cells that Express CD46
N.......................... Measles Virus Nucleocapsid Protein
N-Y451D.............. Measles Virus Nucleocapsid Protein with a Tyrosine to Aspartic Acid Substitution at Position 451
N-Y518D.............. Measles Virus Nucleocapsid Protein with a Tyrosine to Aspartic Acid Substitution at Position 518
NBD ............................................ Nucleotide Binding Domain
NCORE.................. N-terminus of the Measles Virus Nucleocapsid Protein (aa 1-400)
NMR .................................................. Nuclear Magnetic Resonance
nt .......................................................... Nucleotide
NTAIL ...................... C-terminus of the Measles Virus Nucleocapsid Protein (aa 400-525)
NTAILΔ3 ................. C-terminus of the Measles Virus Nucleocapsid Protein (aa 400-525) with Box-3 deleted
NTAIL Y451D .......... C-terminus of the Measles Virus Nucleocapsid Protein (aa 400-525) with a Tyrosine to Aspartic Acid Substitution at Position 451
NTAIL Y518D ........... C-terminus of the Measles Virus Nucleocapsid Protein (aa 400-525) with a Tyrosine to Aspartic Acid Substitution at Position 518
P ......................... Measles Virus Phosphoprotein (Polymerase Co-factor Protein)
pAb.......................................................... Polyclonal Antibody
PBS .......................................................... Phosphate-Buffered Saline
PCR .......................................................... Polymerase Chain Reaction
RdRp ................................................................. RNA-dependent RNA polymerase
RNA ........................................................................................................... Ribonucleic Acid
RNP ............................................................................................................ Ribonucleoprotein Complex
RT-PCR ................................................................. Reverse Transcription Polymerase Chain Reaction
RT-qPCR .......................... Quantitative Reverse Transcription Polymerase Chain Reaction
RU ........................................................................................................ Response Units
SBD ........................................................................................................ Substrate Binding Domain
SDS-PAGE .................. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SEM ..................................................................................................... Standard Error of the Mean
SPR ........................................................................................................ Surface Plasmon Resonance
TCID$_{50}$ ................................................................. 50% Tissue Culture Infective Dose
TFE ........................................................................................................... 2,2,2-Trifluoroethanol
XD .............................................................. X Domain of the Measles Virus Polymerase Protein (aa 459-507)
Y ........................................................................................................ The Amino Acid Tyrosine
CHAPTER 1

INTRODUCTION

1.1 Abstract

Heat shock proteins (HSPs) play an important role in the maintenance of cellular homeostasis, particularly in response to stressful conditions that adversely affect normal cellular structure and function, such as hyperthermia. A remarkable intrinsic resistance of brain to hyperthermia reflects protection mediated by constitutive and induced expression of HSPs in both neurons and glia. Induced expression underlies the phenomenon of hyperthermic pre-reconditioning, where transient, low-intensity heating induces HSPs that protect brain from subsequent insult, reflecting the prolonged half-life of HSPs. The expression and activity of HSPs that is characteristic of nervous tissue plays a role not just in the maintenance and defense of cellular viability, but also in the preservation of neuron-specific luxury functions, particularly those that support synaptic activity. In response to hyperthermia, HSPs mediate preservation or rapid recovery of synaptic function up to the point where damage in other organ systems becomes evident and life threatening. Given the ability of HSPs to enhance gene expression by neurotropic viruses, the constitutive and inducible HSP expression profiles would seem to place nervous tissues at risk. However, we present evidence that the virus-HSP relationship can promote
viral clearance in animals capable of mounting effective virus-specific cell-mediated immune responses, potentially reflecting HSP-dependent increases in viral antigenic burden, immune adjuvant effects, and cross-presentation of viral antigen. Thus, the protective functions of HSPs span the well-characterized intracellular roles as chaperones to those that may directly or indirectly promote immune function.

1.2 Introduction

A recent study involving whole body hyperthermia in dogs provides one of the first in-depth examinations of the intrinsic resistance of the brain to heat stress. In this case, the controlled induction of hyperthermia by heating blood (either directly or indirectly via peritoneal lavage) was used to analyze the effects of a thermal dosage that falls just short of the threshold at which systemic effects on hemostasis are realized (Fig. 1.1). Beyond this threshold, the effects of hyperthermia on the mammalian central nervous system become complicated by the onset of cerebral ischemia, edema and hemorrhage, although there are species-specific differences in the precise thermal dose at which such lesions occur. For dogs, the systemic heat stress threshold is surpassed when the average body temperature is maintained at 42.5°C for longer than 90 minutes. Beyond this range, the indicators of fulminant disseminated intravascular coagulopathy (DIC) become evident, characterized by severe thrombocytopenia, hypofibrinogenemia, elevated expression of fibrin degradation products and prolonged clotting times. Serum biochemistries and histopathological findings indicate that hepatocellular function defines the maximally tolerated thermal dose, and it is hepatocellular injury that triggers the onset
**Figure 1.1.** Extracorporeal blood heating used to measure the physiologic response to hyperthermia in the canine brain. (A) The extracorporeal blood circulation system allows for the application of a controlled thermal dose to the brain. In this schematic, the subject is shown in dorsal recumbency. Vascular access is gained at the left and right femoral veins, and the flow circuit between the pump/heating apparatus and the body is indicated by arrows. Open circles indicate the placement of thermisters (from rostral to caudal: tympanic membranes (2), esophagus, pulmonary artery, subcutaneous, bladder, deep rectum). Two thermisters also monitored temperature of blood entering and exiting the heating device, with global temperature monitoring used to regulate core body temperature. (B) Average core temperature +/- SD of a dog exposed to extracorporeal whole body hyperthermia and a euthermic extracorporeal circulation control dog. Panel (A) reprinted from Alterations in hemostasis associated with hyperthermia in a canine model, Diehl K.A. et al, © 2000 Wiley-Liss, Inc. with permission from Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc. Panel (B) reprinted from Veterinary Immunology and Immunopathology, Vol. 69, Oglesbee M.J. et al, Whole body hyperthermia: effects upon canine immune and hemostatic functions, Page No. 190, © 1999 Elsevier Science B.V. with permission from Elsevier.
Figure 1.1
of DIC\textsuperscript{50}. This coagulopathy leads to characteristic pathological consequences that culminate in multi-organ dysfunction and death, with the initial evidence of ischemic damage in brains of dogs being selective neuronal necrosis, particularly in cerebellar Purkinje cells.

The intrinsic thermostability of the brain was determined by analyzing its structural, biochemical and functional characteristics after being subjected to sub-threshold hyperthermia. There were little to no permanent, observable alterations in neurologic function, as measured by level of consciousness, motor function, brainstem reflexes and brainstem auditory evoked potential (BAEP) recordings. Although changes in BAEP recordings are reported to occur during heating\textsuperscript{168}, these findings suggest that such alterations are transient. Likewise, there was a lack of structural and biochemical changes in the brain that would indicate tissue injury. Of the variables measured, the only alteration was increased expression of the inducible 72-kDa heat shock protein (Fig. 1.2), a well-documented indicator of cellular stress response induction\textsuperscript{110}.

The stress response is well-conserved throughout evolution, and the genetic and protein constituents share significant homology in both prokaryotes and eukaryotes\textsuperscript{reviewed in 161}. In the first described example of a cellular reaction to an applied stress, Ritossa noted a characteristic “puffing” in the genes of \textit{Drosophila} salivary gland cells exposed to transient elevations in temperature that was shown to be accompanied by the elevated expression of a specific set of proteins\textsuperscript{144,171}. Further characterization of these and other related proteins has shown that this class of proteins, initially termed the heat shock proteins (HSPs), is in fact comprised of an array of molecules involved in the maintenance of normal cellular homeostasis as well as the response to and recovery from
Figure 1.2. Hsp72 expression in the canine brain following the induction of whole body hyperthermia by extracorporeal blood heating. (A) Western blot analysis of total protein from cerebellum using a monoclonal antibody recognizing a common epitope of hsc70 (denoted in this figure as hsp73) and hsp72. Hsp72 is elevated over time following hyperthermia in comparison with levels in euthermic control tissue (Eu), while hsc70 shows no evidence of induction. Note the prominent basal expression of hsp72 in control tissue. (B) Immunohistochemical staining of hsp72 in cerebellum 24 hours after whole body hyperthermia using an hsp72-specific monoclonal antibody. In the cortex (left panel), positive hsp72 staining was localized to both the nucleus and the cytoplasm of Purkinje cells and the nuclei of neurons and glial cells in the internal granular layer. In white matter regions (right panel), hsp72 expression is localized to cells with oligodendroglial morphologies (inset). Reprinted from Neuroscience, Vol. 113, Oglesbee M.J. et al, Intrinsic Thermal Resistance of the Canine Brain, Pages 60 (Panel A) and 62 (Panel B), © 2002 Elsevier Science Ltd. with permission from Elsevier.
all manner of stressful stimuli. The heat shock proteins are divided into families based upon molecular mass, such as the well-characterized 90-kDa, 70-kDa and small molecular mass groups, each of which has members that are expressed constitutively and/or in response to stress. Constitutively expressed HSPs are most often associated with functions required for normal cell growth and metabolism, including the regulation of protein maturation, folding and intracellular trafficking\textsuperscript{reviewed in 129}, while the inducible HSPs respond to conditions that promote protein denaturation and dysregulation of cellular metabolic pathways, a list of which includes (but is not limited to) thermal stress, oxidative injury, ischemia, chemical or heavy metal toxicity and viral infection\textsuperscript{reviewed in 129,154}. In the brain, as in other organs, induction of the cellular stress response results in a state of heightened resistance to a variety of insults in the post-stress interval. Exposure to transient hyperthermia is perhaps the best-known means of inducing this resistance phenotype, a treatment known as hyperthermic pre-conditioning\textsuperscript{89,182}.

In the central nervous system, there are distinct expression patterns for specific HSP isoforms, and in some cases there appears to be considerable functional overlap between the proteins involved. Studies have examined the effect of hyperthermia on the induction of a variety of HSP families within the brain. A large portion of this work focuses upon the constitutive and inducible 70-kDa family members under such conditions (i.e., hsc70 and hsp72, respectively), largely because elevated levels of hsp72 are a primary mediator of hyperthermic pre-conditioning, with temporal elevation of hsp72 levels corresponding to the window of cellular protection\textsuperscript{89}. Recently, other HSPs have also been shown to be important components in the integrated response of the brain to thermally induced changes in cellular homeostasis. The maintenance of normal clinical
neurologic status and electrophysiologic activity in dogs indicates that the responses of the central nervous system to hyperthermia include mechanisms by which synaptic activity is preserved in addition to those that promote cell viability, at least to a threshold thermal dosage where CNS injury occurs as a secondary event related to loss of function in other organs. The participation of heat shock proteins in the preservation of synaptic function in the face of cellular stress is supported by work in many in vivo and in vitro model systems. This review will focus on the potential role of constitutive and induced expression of 70-kDa, 90-kDa and low molecular mass HSPs in preservation of cell viability and synaptic performance in the CNS in the face of hyperthermia. Special consideration will also be given to the occurrence and probable significance of extracellular release of HSPs, specifically hsp72.

The protective roles of hsp72 will also be examined in the realm of infectious disease. Several neurotropic viruses respond to hyperthermic preconditioning of host cells by increasing viral gene expression and cytopathic effect. We will present evidence that supports a model describing how this virus-cell relationship can paradoxically promote viral clearance, reflecting the HSP-dependent increases in viral antigenic burden and the immunostimulatory roles of extracellular HSPs. From this perspective, HSPs can be viewed collectively as both supporting normal neurologic function during episodes of physiological hyperthermia (i.e., fever), and as components of the febrile response that ultimately provides protection against microbial invasion.
1.3 Constitutive Expression of Heat Shock Proteins in the Brain

**HSP70 Family:** Members of the 70-kDa HSP family (HSP70) are one of the most abundantly expressed classes of proteins in mammalian cells. They are involved in protein folding, the assembly of multimeric protein complexes and the trafficking of proteins between cellular compartments. Thus they are important for guiding and assisting protein precursors/subunits to their fully active structural state and preventing inappropriate aggregation. These actions are mediated by a conserved binding pocket which recognizes a broad array of protein substrates through the transient interaction with exposed hydrophobic domains, targeting linear sequences of 8 to 9 amino acids. The reversible nature of the affinity of these HSPs for their targets is mediated by the cyclic binding and hydrolysis of ATP and release of ADP by a nucleotide-binding domain within the molecule. For HSP70 bound to ATP, the peptide binding pocket is open, a conformation which promotes rapid association/dissociation with ligand. ATP hydrolysis to ADP causes closure of the binding pocket, thereby locking HSP70 onto the bound peptide. ADP-ATP nucleotide exchange allows for the repetitive cycle of binding and release. Thus by binding exposed hydrophobic domains and preventing the formation of insoluble multimeric complexes, HSPs provide nascent or denatured proteins with the opportunity to attain their normal structure and function. The chaperone functions of HSP70 involve more than just protein folding. HSP70 supports conformational changes that attend transmembrane protein transport, assembly and disassembly of multimeric protein complexes, and trafficking of binding partners into cell compartments such as the nucleus.
The constitutively expressed 73-kDa isoform (hsc70) carries out its functions by chaperoning newly-formed peptides during protein synthesis and maturation and by determining the assembly state of multimeric protein complexes within the cell. The highly-inducible 72-kDa HSP (hsp72, also known as hsp70) is typically expressed under varying conditions of cellular stress, including heat shock, ischemia, and toxin or heavy metal exposure, and is responsible for the rescue of denatured cytoplasmic proteins, allowing them to refold and return to their normal form and function. Studies demonstrate that HSP70 isoforms exhibit species-specific differences in both constitutive and induced expression in the brain. Since these isoforms exhibit functional similarities that reflect their high amino acid sequence homology, the presence of one may compensate for the absence of the other in any particular cell population, and their complimentary nature allows both to contribute to the intrinsic thermal resistance of the brain.

HSP70 also recognize small patches of surface hydrophobicity on native proteins, resulting in altered activity of the substrate, a function known as HSP-mediated activity control. Substrate activity may either be enhanced or diminished as a consequence of HSP70 binding, reflecting changes in conformation that influence interaction with binding partners or rate of degradation. As general examples of this capacity, HSP70 modulates cell cycle progression and induction of apoptosis by binding p53, kinases of the mitogen activated signal cascade, or apoptosis-inducing factor. As well, interactions with native proteins underlie some of the functions of hsc70/hsp72 in the maintenance and protection of synaptic signaling.
**Hsc70:** Hsc70, being the constitutively expressed member of the HSP70 family in the cytosol of most cells, is particularly prevalent in the brain. In fact, cellular levels of basal hsc70 expression in the brain exceeds that of most other organs\textsuperscript{13,15,114}. The reasons for such high basal levels have not been fully determined, but specific expression patterns may provide clues as to the requisite functions of this molecule in nervous tissue. A study of constitutive HSP70 expression (including both hsc70 and hsp72) during development of the rat CNS revealed that peak levels occur during the first 2-4 months of age, and its decline to typical adult levels correlates with the onset of physiologic maturity\textsuperscript{19}.

Immunohistochemical studies show hsc70 in both the cell bodies and dendrites/perikaryal extensions of neurons in the cortex, hippocampus and cerebellum. Electron microscopy and fractionation studies have been used to demonstrate hsc70 in and around synapses, in both pre- and post-synaptic compartments\textsuperscript{13,19,112,167}. Fractionation studies also show that hsc70 and other constitutively expressed heat shock proteins are localized to lipid rafts in the rat brain\textsuperscript{40}. Lipid rafts are membrane domains enriched in cholesterol and sphingolipids\textsuperscript{reviewed in 159}, and in neurons are proposed to be a critical component of the synapse\textsuperscript{reviewed in 166}. While the specific roles of hsc70 in synaptic transmission have not been completely elucidated, several functions of hsc70 either have been shown or would be predicted to support regulation of signaling between neurons. Its activity as a protein chaperone directing the proper folding of nascent polypeptides would be required during local protein production, which is necessary for the maintenance of synaptic plasticity\textsuperscript{102}. In addition to hsc70, a wide variety of mRNA transcripts and the protein complexes that are components of the cellular translational machinery have been demonstrated in post-synaptic densities\textsuperscript{4,169}. In this context, the role of hsc70 as a
chaperone for locally produced proteins is underscored by its co-localization with hsp40 at post-synaptic sites\textsuperscript{167}. The association with hsp40 boosts the ATPase activity of hsc70, regulating the peptide binding and release cycle that underlies its chaperone function\textsuperscript{108}.

Distinct from its function as a protein-folding chaperone, hsc70 is known to be operative in synaptic vesicle cycling, the basis for the rapid release and recovery of neurotransmitters by presynaptic neurons\textsuperscript{reviewed in 131}. Two other recognized hsc70 co-chaperones, cysteine string protein (CSP) and auxilin, are present in synaptic fractions and appear to mediate the separable activities of hsc70 in vesicular exo- and endocytosis, illustrating how specific collaborations between hsc70 and its partners determine its functional activity. CSP and hsc70 are required for normal, Ca\textsuperscript{2+}-dependent vesicular exocytosis and are components of a complex containing hsp90 that regulates recycling of Rab3A, a small G protein that mediates membrane fusion in the presynaptic terminal\textsuperscript{23,38,48,148}. Alternatively, hsc70 binding to auxilin is necessary for uncoating of clathrin-coated endocytotic vesicles in presynaptic nerve terminals. Inhibition of hsc70 interactions with auxilin results in a breakdown of synaptic vesicle trafficking, which eventually brings normal synaptic signaling to a halt\textsuperscript{113}.

**Hsp72:** A number of studies report no constitutive expression of hsp72 mRNA or protein in any region (i.e., forebrain, cerebellum or brainstem) of the adult rat brain in the absence of a clearly-defined stressor\textsuperscript{81}. Occasionally, low level constitutive protein expression is described, although often only slightly above background\textsuperscript{16,132}. It should be noted that the finding of low or no basal expression of hsp72 is in contrast to the significant, regional hsp72 levels described in both unstressed rabbits and dogs\textsuperscript{99,125} (Fig. 1.2) and in a group of elderly human patients that died acutely without evidence of agonal
stress or anti-mortem fever\textsuperscript{133}. Interestingly, constitutive hsp72 expression has been identified in post-synaptic fractions in both the cerebral cortex and cerebellum in tight association with synaptic elements\textsuperscript{112}. This example underscores the potential for functional overlap between the two cytosolic HSP70 isoforms in regulation of synaptic function in the broader sense, given that hsp72 may bind and be activated by both hsp40 and CSP in the same manner discussed previously for hsc70\textsuperscript{36,130}.

**Hsp90:** Also widely expressed in the brain is the constitutive member of the 90-kDa family, hsp90\textsuperscript{47,140}. Like hsc70, it is well-recognized as a chaperone involved in normal protein maturation, although hsp90 has a narrower range of substrates that includes regulatory kinases, steroid hormone receptors, and myosin. The hsp90 molecule is also involved in protein sorting to various organelles, including the proteosome\textsuperscript{184}. Although studies examining functions of hsp90 specific to the brain are few, those available do provide additional examples of the participation of heat shock proteins in activities critical to normal CNS activity. Relatively equivalent expression in the unstressed rat brain has been demonstrated in both cerebrum and cerebellum and, as with hsc70, its presence was noted in synaptosomes, specifically the pre-synaptic fraction\textsuperscript{13}. As stated previously, this localization has been linked to the requirement of hsp90, in complex with hsc70 and CSP, for efficient neurotransmitter release by the presynaptic neuron\textsuperscript{62,148}. A post-synaptic function, the regulation of continuous AMPA-type glutamate receptor cycling, has also been determined\textsuperscript{62}. Such constant cycling is proposed to account for the maintenance of synaptic glutamate receptor expression profiles in the face of receptor turnover, a phenomenon that may be the molecular basis for memory retention\textsuperscript{96}. 

13
Low Molecular Weight Heat Shock Proteins: The small HSPs (Hsp27 and Hsp32) have a restricted pattern of constitutive expression in the brain. Little to no demonstrable protein is present in the rat hippocampus, cerebellum or cerebral cortex, although expression of hsp27 in the brain stem is reported to be more robust\textsuperscript{11,12,88}. The latter finding is compatible with developmental studies in the mouse, where constitutive expression of hsp25 (the murine homologue of rat hsp27) is noted in a number of cranial nerves and their nuclei (i.e., cranial nerves V, VII, X and XII) just after birth and their levels remain constant into adulthood\textsuperscript{3}. The functional significance of this constitutive expression has yet to be elucidated.

1.4 Hyperthermic Induction of Heat Shock Proteins in the Brain

Hsp72: A common method of inducing a hyperthermic state in the central nervous system in various experimental settings has been the use of externally-applied whole body hyperthermia, either via extended exposure to elevated ambient temperature or the use of heating pads, although drug-induced hyperthermia, specifically treatment with lysergic acid diethylamide (LSD), is used for the study of heat shock protein expression in the brain in rabbits\textsuperscript{98}. The exact thermal dosage that is applied (i.e., the duration and degree to which measured core temperatures are elevated) can vary and this may account for minor differences in specific expression patterns that have been described within a particular animal species, in addition to contributing to the interspecies differences that have been noted. Because the method of heating can influence the precise thermal dose to the brain (and thus the hsp72 expression profile), monitored heating of the blood was chosen as an approach for induced hyperthermia in dogs\textsuperscript{125}. This method
resembles that used to deliver therapeutic doses of radiant energy in the treatment of human cancers, and is amenable to use in dogs, whose large size in comparison to other common laboratory animal species allows for constant, real-time monitoring of an array of specific physiologic parameters during the hyperthermic interval. When combined with concomitant fluid therapy and mechanical ventilation, which correct for the systemic effects of hyperthermia that may secondarily influence the central nervous system (i.e., reduced mean arterial pressure and respiratory alkalosis), this form of heating can be successfully used to measure the effects of sub-lethal hyperthermia without the interference of other heat-related physiologic responses.

Regardless of the specific method by which a thermal dosage is delivered, it has been well established that hsp72 is one of the main induced heat shock proteins in the mammalian brain. As discussed previously, basal hsp72 expression in the brain is species-dependent and often difficult to document. However, even mild hyperthermia within the physiological range of most febrile responses (i.e., up to a 3.5°C rise in body temperature for at least 60 min) results in measurable increases in hsp72 protein expression within 3-5 hours after the onset of temperature elevation, with the most striking expression in the cerebellum. The onset of stress-related transcriptional activity is regulated by cytosolic heat shock factors (HSF), particularly the HSF1 isoform in mammals. Under normal conditions, HSF is bound to HSPs in the cytosol. Following the onset of cellular stress, HSF1 is released and then activated by serine/threonine phosphorylation. This promotes homotrimerization, which is followed by translocation to the nucleus, where the HSF1 complex associates with heat-shock elements (HSE), DNA binding regions that promote transcription of stress-responsive
genes, like that of hsp72\textsuperscript{151}. The role of HSF1 in hsp72 induction is supported by the finding that hsp72 mRNA and protein expression patterns mimic those noted for HSF1 expression and DNA binding, which is more prominent in the cerebellum than in the cerebral cortex or hippocampus\textsuperscript{101,114}. There is a similar hsp72 expression gradient in dog brain following hyperthermia, with the greatest induction in the cerebellum, then decreasing sequentially from the hippocampus to the hypothalamus and frontal cortex, and finally becoming inapparent in the myelencephalon\textsuperscript{125}.

The basis for regional differences in \textit{in vivo} expression of hsp72 following heat shock beyond differences in HSF1 expression are less clear. Studies of hippocampal neurons provide evidence of regional regulatory mechanisms of hsp72 expression. In the rat, hyperthermic treatment (core temperature of 42 – 42.5℃) showed significant elevations in hsp72-specific mRNA levels in neurons in the pyramidal and dentate granule cell layers, whereas protein expression was transient and of relatively low intensity when compared to that of local glial cells\textsuperscript{87}. The authors suggest that such findings demonstrate post-transcriptional control of hsp72 expression in these neuronal populations, although a specific mechanism has yet to be delineated. Should this be the case, there appears to be a threshold beyond which such control is relaxed. The effect of increased duration and intensity of hyperthermic treatment on hsp72 expression can be documented when comparing this study to one in which a longer exposure to higher elevations in ambient temperature (43.5℃ for 50 min.) was performed on hippocampal explant cultures. Here, neurons in the pyramidal layer and dentate gyrus demonstrate quite a robust pattern of hsp72 protein expression, on par with adjacent glial cell populations, and remain elevated at least five days post-shock. Regional differences in
hsp72 expression following heat shock may also reflect differences in the need for chaperone function during hyperthermia, where there is overlap in the contributions of hsp72 and hsc70, the latter being abundant in neurons\textsuperscript{167} and active in cells responding to hyperthermic stress\textsuperscript{25,46,53}. This view is supported by recent findings showing prominent basal hsc70 expression in neuronal populations that do not appear to express elevated levels of inducible hsp72, yet remain protected from apoptosis following hyperthermia\textsuperscript{15}.

In the cerebellum of adult rats, the use of light microscopy and immunocytochemistry detected robust expression only in the neurons of the granular cell layer following a physiologic increase in body temperature, while other neuronal populations, such as Purkinje cells, seemed non-responsive to heat stress\textsuperscript{11}. While there was no evidence of neuronal apoptosis in adult rats for this degree of CNS hyperthermia\textsuperscript{81}, a more profound, supraphysiologic elevation in temperature showed a correlation between hsp72 expression in neurons and resistance to apoptosis\textsuperscript{16}. Indeed, in a primary culture system, the induction of hsp72 by hyperthermic preconditioning was shown to prevent repolarization-induced apoptosis in granular layer neurons, confirming its protective effects in this cell population\textsuperscript{39}. In neonates (postnatal day 7), hyperthermia-induced apoptosis in the cerebellum was confined to the external granular cell layer, which at this point in development is comprised of actively dividing cells\textsuperscript{16}. Increased basal hsp72 expression was noted in mature, non-dividing cells but not in the external granular cell layer, indicating that the hsp72 expression pattern is inversely related to the susceptibility of heat shocked cells to apoptosis, reinforcing the general view that heat shock protein expression protects against apoptotic cell death\textsuperscript{reviewed in 163}. 
Our *in vitro* studies demonstrate a direct role for hsp72 in protecting neural cells against hyperthermic insult. Cell lines that were stably transfected with an hsp72 expression plasmid showed enhanced survival and colony forming ability following an otherwise lethal hyperthermic treatment (Fig. 1.3). These experiments further demonstrate that constitutive high-level expression of hsp72 does not perturb normal cellular metabolism, perhaps reflecting the cell’s ability to buffer the activity provided by overexpressed HSP. For example, hsp72 overexpression does not alter the chemical induction of neuroblastoma cell differentiation by lithium chloride treatment, as assessed by both morphologic and biochemical parameters (Fig. 1.4). In both control and hsp72 overexpressing cell populations, the degree of differentiation and the time to maximal effect was the same. Antisense oligonucleotide treatment was used to show that hsp72 in particular was responsible for the hyperthermia-related protection of CA1 neurons from the lethal effects of glutamate treatment. Such findings extend previous work documenting the protective effect of hsp72 on hippocampal neuron survival in culture to a form of injury specific to the central nervous system (i.e., glutamate toxicity).

Beyond the global ability of hsp72 to protect cells from the stress-related loss of maintenance functions, we see a role for hsp72 in preserving synaptic activity, a particularly important luxury function of neurons. Following a physiologic heating event, hsp72 expression was markedly up-regulated in both pre- and post-synaptic compartments derived from separate cerebellar and forebrain extracts, and was determined to be an integral component of the post-synaptic density, similar to that of its constitutive isoform described previously. These findings are mirrored by the rapid appearance of hsp72 in rat brain lipid raft extracts following a similar hyperthermic
Figure 1.3. Constitutive expression of hsp72 in murine neuroblastoma cells mediates cellular tolerance to subsequent heat stress. (A) Western blot analysis of total protein using a monoclonal antibody that recognizes a common epitope of hsc70 (denoted in this figure as hsp73) and hsp72. Murine neuroblastoma cell lines (N72-12, N72-16) are stably transfected with a plasmid that directs constitutive expression of hsp72 via the beta actin promoter. Vector transfected control cell lines (Nβ-1, Nβ-2) do not express hsp72, characteristic of murine cell lines. All cells express the normal constitutive HSP70 isoform (hsc70). (B) The cell lines expressing hsp72 constitutively from (A) show increased colony forming ability following severe heat shock compared with vector transfected control lines. Reprinted from A single codon in the nucleocapsid protein C terminus contributes to in vitro and in vivo fitness of Edmonston measles virus, Carsillo T. et al, © 2006, American Society for Microbiology with permission from the American Society for Microbiology.
Figure 1.4. Lithium chloride (LiCl) treatment induced neuronal differentiation in a murine neuroblastoma cell line that constitutively expresses hsp72 (72-12) and in a vector-transfected control cell line (β-2). (A) Morphologic differentiation in both cell lines was expressed as the development of axon-like processes greater than or equal to two cell diameters. (B) The number of cells categorized as differentiated based on morphologic criteria was increased to a similar degree in both cell lines following LiCl treatment. (C) Biochemical confirmation of cellular differentiation was based on the expression of neuron specific enolase (NSE) in lysates from LiCl treated cells, shown here by Western blot analysis.
Figure 1.4

(A) Images showing the effect of LiCl on 
- β-2
- 72-12

(B) Graph showing the percent differentiation over time (h) for
- NB-2 Untreated
- NB-2 Treated
- N72-12 Untreated
- N72-12 Treated

(C) Table showing the effect of LiCl on
- β-2
- 72-12
Studies using hippocampal neurons have also demonstrated the importance of such expression on the maintenance of synaptic function in injured neuronal populations. Expression of hsp72 in pyramidal layer neurons following hyperthermia has recently been shown to have protective effects upon synaptic plasticity. In a rat hippocampal explant model, hyperthermic treatment eliminated the suppression of long term potentiation in CA1 neurons induced by scopolamine. Involvement of hsp72 in this effect is supported by a similar time course of both hsp72 induction and retention of normal synaptic function in the face of drug-related muscarinic receptor blockade.

Further evidence of the protective effect on synaptic transmission is provided in work using Drosophila that overexpress native hsp72 in response to heat shock (i.e., more than normal reactive response levels). This increased expression promotes synaptic thermotolerance by enhancing presynaptic neuron performance, allowing for maintenance of signaling at elevated temperature, similar to results seen in another insect-based system. The effect of heat shock on maintenance of synaptic transmission appears to act via the modulation of presynaptic calcium levels at the nerve terminal and involves components of the cellular cytoskeleton, although the specific interactions involved have yet to be elucidated. Such findings have been extended in a mammalian system where, in the mouse medullary pre-Botzinger complex, thermal preconditioning showed an analogous preservative affect on synaptic activity in the face of subsequent hyperthermic challenge, again apparently through a specific modulation of presynaptic neurotransmitter release.

There are only a few studies that focus upon the in vivo expression of hsp72 in glial cells following hyperthermia relative to the number of reports on neuronal
expression. In the rabbit, the effect of brief, fever-range hyperthermia on cell-specific production of hsp72 messenger RNA has been examined in glia-rich regions of the forebrain, including the axon fiber tracts of the fimbria and corpus callosum, cortical layer 1 and the hippocampal fissure\textsuperscript{58}. The most abundant hsp72 mRNA levels were noted in all regional oligodendrocyte populations, while microglial transcript production varied depending on the anatomic region examined, ranging from 50% induction in cortical layer 1 to 90% in the fimbria. Astrocytes were not observed to express hsp72 transcripts in any region, supporting the finding that different glial cell types have different thresholds for the induction of the hsp72 response following heat shock. This pattern of hsp72-specific RNA expression in glial cells in the hyperthermic rabbit closely mirrors glial hsp72 protein localization in dogs\textsuperscript{125} and rats. In the latter, induction of hyperthermia in the physiologic range (41.5ºC) resulted in prominent, early hsp72 protein induction in oligodendrocytes within the white matter, scattered expression in microglia and little to no observable protein in astrocytes or neurons\textsuperscript{134}. The strong oligodendrogial up-regulation in hsp72 expression matches that seen in cultured rat oligodendrocytes following more intense hyperthermic treatment\textsuperscript{64}. Such induction was shown to be protective only against subsequent hyperthermic episodes, but not against insults promoting cellular oxidative damage.

A growing number of studies support the hypothesis that HSP70 (both constitutive hsc70 and induced hsp72) produced by glial cells may be transported to neurons and protect them from subsequent insult. An \textit{in vitro} study shows that cultured glioma cells release hsp72 into the extracellular milieu under normal, non-stressed conditions, and that such expression is increased following heat shock\textsuperscript{68}. This result is
consistent with reports in invertebrates which demonstrate transport of hsp72 from glial cells to neurons\textsuperscript{155,156,173}. Furthermore, exogenous HSP70 may be taken up by neurons and prevent cell death related to heat shock or the induction of apoptosis. In a differentiated neuroblastoma cell culture sensitive to the induction of cell death/apoptosis by heat shock or staurosporine, rapid uptake of a mixture of hsc70/hsp72 applied to the cells resulted in a phenotype that is much more resistant to the cytotoxic effects of either treatment\textsuperscript{68}. In another example of the protective effect of exogenous hsp72 on neuron function, the incubation of a mouse medullary slice preparation in a solution containing hsp72 prior to heat shock resulted in the preservation of synaptic transmission in comparison to non-supplemented control samples, mimicking the effects of hyperthermic preconditioning\textsuperscript{80}. Similarly, pre-treatment of slice preparations of rat olfactory cortex with hsp72 protects glutamatergic synaptic transmission from the excitotoxic effects of glutamate supplementation\textsuperscript{111}. A preliminary number of \textit{in vivo} studies in mammals, in addition to those in invertebrates mentioned above, have so far confirmed the ability of extracellular HSP70 supplementation to protect neurons against injurious stimuli. Labeled-hsc70/hsp72 injected intravitreally was found to be rapidly and widely distributed in neurons within the retina and protected photoreceptors from light-induced damage\textsuperscript{185}, mirroring the beneficial effect of hyperthermic preconditioning on retinal survival following a deleterious level of light exposure\textsuperscript{5}. A similar application of either mixed or individual HSP70 proteins increased the survival of damaged motor or sensory neurons, respectively, in the murine lumbar spinal cord following sciatic nerve axotomy\textsuperscript{71,170}. 

24
Although *in vitro* studies demonstrate the capacity for neural cells to release hsp72 to the extracellular environment, there is a conspicuous absence of reports describing cerebrospinal fluid (CSF) levels of hsp72. Some cell types, such as hepatocytes, can release extracellular hsp72 in response to stress even in the absence of necrosis or apoptosis, resulting in elevated serum levels of hsp72\(^{54,57}\). Here, extracellular hsp72 invokes pro-inflammatory responses and may be viewed as an acute phase reactant\(^{31,32,107,129}\). Our studies on whole body hyperthermia in the dog reveal that CSF levels of hsp72, determined using a commercially available ELISA (StressGen Biotechnologies), are not significantly elevated despite the prominent neuronal and glial induction that is detected by both immunohistochemistry and Western blot analysis of brain total protein\(^{125}\) (Fig. 1.2). Dogs in which whole body hyperthermia was induced by peritoneal lavage did not show statistically significant changes from a pre-treatment value of 1.1 ± 0.5 (SD) ng/ml to 1.6 ± 1.1 ng/ml at time zero (i.e., the end of the hyperthermic treatment) and 2.1 ± 2.2 ng/ml at 24 h post treatment (n = 6 animals). In contrast, serum levels increased from a baseline of 0.6 ± 0.8 ng/ml to 6.1 ± 5.0 ng/ml at time zero and 4.9 ± 2.6 ng/ml at 24 h post treatment. Control animals received peritoneal lavage without heating and their serum and CSF hsp72 levels were within range of pre-treatment values. Sample analysis from dogs receiving their thermal dosage from heated blood (extracorporeal circulation or ECC whole body hyperthermia) revealed CSF levels of hsp72 that were comparable to the peritoneal lavage whole body hyperthermia treatment groups. Serum levels in control animals were within the reference range reported above, whereas serum levels in the hyperthermia group were much higher than in the peritoneal lavage groups. At 24 h post treatment, serum levels of hsp72 were 166.3 ± 163.0 ng/ml (n
= 4), declining to 10.6 ± 9.8 ng/ml at 3 d post treatment (n = 2) and returning to baseline by 8 d (0.8 ± 0.9 ng/ml, n = 4).

On the other hand, results from a separate body of ongoing work indicates that extracellular expression of hsp72 in the CNS may relate to the nature of the stress imparted to the tissue, as we have demonstrated significant elevations in CSF levels of hsp72 in dogs following ischemia/reperfusion injury of the spinal cord (unpublished observation). In this case, a transient aortic cross-clamp results in poliomyelomalacia affecting the lumbosacral spinal cord. Preliminary data show that CSF levels of hsp72 can rise to approximately 35 ng/ml, whereas serum levels of hsp72 are not affected. Elevated CSF levels of hsp72 are correlated to increased hsp72 expression in ependymal cells in addition to parenchymal glia and neurons. Based upon these two lines of experimentation, it appears that release of hsp72 into the CSF is restricted to events associated with tissue injury, where hsp72 may both act as a danger signal and passively impart injury tolerance to neurons. In the absence of tissue injury, there is an appropriate lack of such signaling, such that physiological episodes of hyperthermia do not, in and of themselves, precipitate inflammatory responses. Moreover, it is apparent that CSF and serum levels of hsp72 may change independently as a function of tissue source (i.e., brain versus liver).

**Hsp90:** While the inducible expression of hsp90 in the brain following hyperthermia has been examined, there is little evidence that protein levels change significantly\(^{13,139,140}\). This may reflect the relatively high basal levels in comparison to other organs, such as the kidney, where induction following hyperthermia is robust\(^{139}\). Although hsp90 does play a role in protecting neurons from thermal stress\(^{95,181}\), further
studies describing its neuron-specific functions are restricted to its activity in the unstressed state.

**Low Molecular Weight Heat Shock Proteins:** As opposed to the relatively low constitutive level of expression of small HSPs in the CNS, in particular hsp27 and hsp32, hyperthermia-induced expression appears to be robust and has a particular effect on the maintenance of synaptic activity, with a pattern distinct from that of hsp72. Adult rats subjected to a 41.5°C heat shock for 1 hr. results in elevated expression of these low mass HSPs in glial cells (Bergmann glia) in the cerebellum by 15 hours post-treatment, but not in neurons. By EM, hsp27 and 32 are localized to synaptic sites, particularly post-synaptic neurons and peri-synaptic glia. The authors suggest that these HSPs help preserve synaptic function and raise the possibility of secreted HSPs as mediators of recovery from injury\(^{11}\). Further work identified a similar pattern of hsp27 and 32 expression in glial cells (astrocytes and activated microglia) in the hippocampus, particularly in areas where a high number of synaptic connections are made\(^{12}\). In a separate study, transient (15 min.) 42-42.5°C heat shock was used to examine induced hsp27 expression throughout the brain. Western blot analysis showed high basal expression in brainstem, very weak expression in control cerebellum and hippocampus and none in cerebrum. Heat shock resulted in elevated expression in all regions, in similar proportions as the constitutively expressed molecule when measured at 24 hrs. Expression was strongly induced in glial cells, mostly astrocytes, in all regions studied by immunohistochemistry. Expression begins at 1.5-3 hrs, peaks at 24 hrs and remains present in some cells up to 6 days post-shock. Cerebellar Bergmann glia showed a pattern of hsp27 expression similar to that reported above. Constitutive expression was patchy in
ependyma of lateral, third and fourth ventricles, while there was strong induction in all ependyma and in nearby parenchymal cells. Maximal expression is observed at 24 hours, being largely maintained up to 6 days post-treatment. Cells of the choroid plexus showed a similar pattern. Various neuronal populations within a number of regions (hippocampus, circumventricular organs, hypothalamus and dorsal vagal complex) also express elevated hsp27 levels after heat shock. As with hsp90, the functional significance of these expression patterns in relation to preservation of synaptic function has yet to be described.

1.5 Hyperthermic Induction of Heat Shock Proteins and Viral Neurovirulence

The febrile state is a potent inducer of hsp72 expression and is a characteristic host response to infection by numerous microbial pathogens. At the same time, hyperthermic preconditioning has been shown to enhance gene expression of several neurotropic viruses, including human T lymphotropic virus type 1 (HTLV-1), herpes simplex virus type 1, measles virus (MeV) and canine distemper virus (CDV). The mechanism by which stress-conditioning enhances viral gene expression is best characterized for the latter two examples, which are closely related members of the morbillivirus genus. Here, we have shown that hsp72 binds the viral core particle, specifically the nucleocapsid protein that packages the viral RNA genome. Viral transcription and genome replication are enhanced through hsp72-dependent modifications of this ribonucleoprotein template, resulting in enhanced viral protein expression and virus-induced cytopathic effect. Results are identical to infection of cells pre-conditioned with transient hyperthermia or constitutively overexpressing an
hsp72 transgene, including the murine neuroblastoma cells illustrated in Figure 1.3. The question thus arises as to whether the febrile response and increased hsp72 expression in brain is detrimental or protective to the host in the face of such pathogens.

Using a mouse model of MeV CNS infection, we have shown that the induction of hsp72 following transient hyperthermia of neonatal Balb/c mice is correlated to an increased immune-mediated clearance of the Edmonston strain (Ed-MeV) from the brain following intracranial inoculation. Whole body hyperthermia was induced by placing animals in a heated chamber, and induction of hsp72 was demonstrated by Western blot analysis of brain total protein. Viral challenge groups included mice inoculated at the time of elevated hsp72 expression in brain and a control (non-heated) group. Forty seven percent of the non-heated animals supported persistent neuronal infection at 21 d post infection, based upon real time RT-PCR analysis of brain viral RNA burden, in contrast to only five per cent in the pre-conditioned animals (i.e., 95% clearance). The temporal onset and progression of clearance was associated with the development of virus-specific cell mediated immune responses known to be responsible for viral clearance from brain.

A model to explain the enhanced viral clearance in preconditioned tissues involves hsp72-dependent increases in viral antigenic burden and the extracellular release of hsp72 from virus-infected cells, where hsp72 can serve as an adjuvant and a mediator of antigenic cross-presentation, with both properties serving to enhance virus-specific cell mediated adaptive immune responses reviewed in. Key to viral persistence in multiple systems is the restricted antigen expression that confounds the immune-recognition of virus infected cells, particularly in the central nervous system. In this regard, hsp72-mediated increases in viral gene expression serve to unmask these viral reservoirs,
thereby promoting clearance at the expense of the initially infected cells. HSP release into
the extracellular environment has been shown to occur following cell necrosis, but not
after apoptosis, suggesting this release is restricted to times when cell death is
uncontrolled\(^9\). Thus, hsp72-dependent increases in MeV cytopathic effect, a reflection of
increased viral gene expression, are likely associated with the extracellular release of
HSPs. Stimulatory effects of extracellular HSPs on the immune response include
induction of inflammatory cytokine secretion (INF\(\alpha\), Il-1\(\beta\), IL-12 and GM-CSF), NO
production, chemokine production (MCP-1, MIP-2, RANTES) and dendritic cell
maturation with translocation of NF\(\kappa\)B to the nucleus\(^{164}\). The extracellular HSPs would
also include those associated with viral antigenic peptides. Peptides derived from a
number of viruses, including Vesicular Stomatitis Virus\(^{165}\), Herpes-Simplex Virus 2\(^{118}\),
Simian Virus 40\(^{18}\) and Hepatitis B Virus\(^{106}\), contain residues of defined antigenicity that
bind HSPs. HSP/antigen complexes have been shown to be potent immunogens against a
wide variety of viral, bacterial and neoplastic diseases, including UV-induced tumors in
C3H mice\(^{76}\) and \(M.\) tuberculosi\(s\)is and LCMV in Balb/c mice\(^{189}\). It is known that antigen
presenting cells recognize and bind HSP/antigen complexes via the CD91 membrane
receptor, resulting in re-presentation of HSP–associated antigens by either major
histocompatability (MHC) type I or type II molecules\(^8,17\). Some components of the
cytoplasmic processing mechanism for hsp72–associated antigens have been identified,
including HSP interaction with the transporter-associated with antigen processing\(^8\).
Cross-presentation is particularly attractive in the CNS in that it allows viral antigen
produced in a neuron to prime immune responses in uninfected antigen presenting cells.
CSF would serve as the probable vehicle of antigen transport to regional lymph nodes via
nerve sheaths, with priming of immune responses occurring in those regional lymphoid organs.

The hsp72-mediated enhancement of viral gene expression that promotes viral clearance would also appear to place nervous tissues at inherent risk in a host that is immunocompromised. For MeV, the resistance or susceptibility of inbred mice to viral encephalitis correlates with the strain-specific expression of the major histocompatibility complex (MHC) haplotype that in turn dictates the efficiency of MeV-specific adaptive T cell responses mediating clearance\textsuperscript{120}. Balb/c mice, which express the H-2\textsuperscript{d} haplotype, are inherently more resistant to MV-induced encephalitis and can generate a protective cytotoxic T lymphocyte (CTL) response in the face of intracerebral virus inoculation. It follows that the increase in hsp72 expression in the CNS following hyperthermia in this strain thus leads to more rapid clearance of the virus, given the ability of hsp72 to act as an endogenous adjuvant for bound antigens and promote cross-priming of CTL responses. In contrast, mice expressing the H-2\textsuperscript{b} haplotype (e.g., C57BL/6) are more susceptible to MeV-induced encephalitis due to deficient virus-specific CTL responses\textsuperscript{120}.

We have recently generated transgenic C57BL/6 mice that constitutively overexpress hsp72 in neurons\textsuperscript{34}. In these animals, intracerebral inoculation with the non-rodent-adapted Ed-MeV strain results not in more rapid clearance, as in the heat-shocked Balb/c mice, but in widely disseminated infection and a six-fold increase in mortality. Death is associated with increased brain viral RNA burdens that are almost two orders of magnitude greater than that of wild type C57BL/6 mice infected with the same virus. The outcome mimics the characteristic pathological features of Measles Virus Inclusion Body
Encephalitis (MIBE), a fulminant infection of the CNS of immune compromised humans that can be caused by either wild type or vaccine strains of MeV. When the same hsp72 construct is expressed in H-2\textsuperscript{d} (resistant) mice, viral RNA burdens are significantly reduced relative to inoculated non-transgenic control mice and mortality is not observed. These results support the importance of H-2 haplotype, and thus the capacity for a robust antiviral immune response, in determining whether elevated hsp72 levels are host-protective or a host determinant that promotes viral neurovirulence.

A recombinant infectious MeV variant has also been generated that exhibits an attenuated response to hsp72-dependent increases in viral transcription\textsuperscript{187}, and this virus fails to exhibit enhanced mortality in the transgenic hsp72 overexpressing H-2\textsuperscript{b} mice relative to the wild type controls. Although less neuroviraluent, this viral variant would also be more likely to persist in the face of a competent immune response despite elevations in hsp72. Thus we are beginning to see that the fever-induced expression of hsp72 in the CNS may have disparate effects on the outcome of infection in the brain that reflects the nature of the interaction between hsp72 and the inciting agent as well as the ability of the immune system to respond to the biological effect of such an interaction.

1.6 Conclusions

Synaptic activity might be considered a dispensable luxury function, yet to lose it would constitute central nervous system failure. Profiles of heat shock protein expression in the brain appear to have evolved in such a way as to preserve this function in addition to promoting cell survival in the face of hyperthermia and other stimuli that otherwise
result in a loss of cellular homeostasis. So far, the HSP70 family provides the best example of this protective effect and the disparate expression patterns of hsc70 and hsp72 reflect the functional overlap between the two molecules and emphasize their complimentary roles. Here, hsp72 may again play a protective role by promoting clearance of neurotropic viruses. Our model for this clearance involves the hsp72-mediated increases in antigenic burden and the extracellular release of HSPs, a phenomenon documented in the CNS but restricted to instances where cell injury is involved. Hsp72-mediated increases in viral gene expression may drive the extracellular release of HSPs by promoting viral cytopathic effect, and both would enhance virus-specific adaptive immune responses leading to clearance. There appears to be a liability associated with this protective mechanism, in that enhanced neurovirulence can be the outcome in the face of an immune deficiency, and that viral variants can emerge that are capable of establishing persistent infection by becoming non-hsp72 responsive.

1.7 Acknowledgements

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CHAPTER 2

HSP72 SUSTAINS THE ACCUMULATION OF TRANSCRIPTIONALLY ACTIVE NUCLEOCAPSID TEMPLATES FROM MEASLES VIRUS EDMONSTON STRAIN

2.1 Abstract

The C-terminus of the measles virus (MeV) nucleocapsid protein (NTAIL) mediates binding of nucleocapsid to the viral polymerase co-factor protein (P) and the major inducible 70-kDa heat shock protein isoform (hsp72). P binding to NTAIL links the nucleocapsid to the viral RNA-dependent RNA polymerase. The stimulatory effects of hsp72 on viral transcription have been proposed to be the result of destabilizing the P/NTAIL interaction, thereby enhancing cycles of binding and release required for transcript elongation. The present work addressed this hypothesis by examining the kinetics of transcription and replication by Edmonston MeV (Ed-MeV) in murine neuroblastoma cells that either lack basal hsp72 expression or constitutively overexpress hsp72. Expression of the CD46 receptor for Ed-MeV enhanced both the synchronicity and the efficiency of the virus infection cycle, although hsp72 overexpression did not affect rates of MeV primary transcription. Results were confirmed in Vero cells where hsp72 was induced by heat shock. Instead, effects of hsp72 were manifest during tertiary transcription when new transcriptases use newly formed nucleocapsid templates. Hsp72
was found to sustain transcriptase activity at late times post-infection when the predominant viral polymerase activity in non-hsp72 overexpressing cells was that of a replicase. Because the hsp72 effect was not detected during the secondary transcription phase where newly synthesized transcriptases are recruited by the incoming nucleocapsid template, we propose that the major function of hsp72 is to sustain the accumulation of transcriptionally active nucleocapsid templates.

2.2 Introduction

The highly-inducible isoform of the 70-kDa HSP family (hsp72) is typically expressed under varying conditions of cellular stress that may lead to protein denaturation, including hyperthermia\textsuperscript{90}, ischemia\textsuperscript{41}, and toxin or heavy metal exposure\textsuperscript{79}. This induced expression of heat shock proteins (HSPs) is the hallmark of the cellular stress response\textsuperscript{110}. In addition to facilitating proper folding and assembly of protein substrates\textsuperscript{70}, hsp72 can also affect function of native protein targets, a phenomenon known as HSP-mediated activity control\textsuperscript{60}. Both of these functions have been incriminated in support of both DNA and RNA virus replication by hsp72 (see reviews\textsuperscript{103,121}). Selective overexpression of hsp72 during measles virus (MeV) infection results in elevated viral transcription and genome replication in multiple cell lines \textit{in vitro}\textsuperscript{35,176}, replicating the effect of transient heat stress on MeV infection of Vero cells\textsuperscript{175,186} and leading to increased viral protein expression and cytopathic effect. \textit{In vivo} constitutive overexpression of hsp72 in murine neurons has the same effect\textsuperscript{34}. The intracranial MeV inoculation of neonatal transgenic C57BL/6 mice that constitutively express hsp72 in neurons leads to significantly increased viral transcript levels in brain,
resulting in increased viral membrane glycoprotein expression, increased viral cytopathic effects, and increased virus-induced mortality compared to non-transgenic mice.

The stimulatory effect of hsp72 on MeV transcription and/or replication is mediated through the binding of the viral nucleocapsid protein (N)\textsuperscript{186}, the structural protein that packages the RNA viral genome to form the nucleocapsid. It is the complex of RNA genome and N protein that acts as the template for transcription and genome replication by the virus encoded RNA-dependent RNA polymerase (RdRp). Catalytic activity of the RdRp is attributed to the MeV L protein. The L protein binds a polymerase cofactor (P) protein, and it is P binding to N that links the polymerase to the nucleocapsid template for transcription and genome replication. Docking sites for the P protein (and thus the polymerase complex) are found on the C-terminus of N protein, within a 125 amino acid intrinsically disordered domain known as N\textsubscript{TAIL}\textsuperscript{21,22}. These same N\textsubscript{TAIL} docking sites are also recognized by hsp72\textsuperscript{186}. The docking sites are short conserved sequences that are enriched in amino acids with hydrophobic side groups, and are referred to as Box-2 and the more C-terminal Box-3. Box-2 is bound by the X domain (XD) of the polymerase cofactor P protein. This binding induces a conformational change in N\textsubscript{TAIL} that results in secondary contacts between Box-3 and XD, forming a high affinity N\textsubscript{TAIL}/XD complex (K\textsubscript{D} = 80 nM)\textsuperscript{22}. This high affinity binding is predicted to hinder the processive movement of the polymerase complex along its nucleocapsid template\textsuperscript{20}, thus requiring the action of a cofactor that would promote cycles of binding and release by relaxing the affinity of P for N\textsubscript{TAIL}. Hsp72 binds Box-2 with an affinity comparable to XD, and is capable of competitively inhibiting XD/Box-2 interaction\textsuperscript{186}. Hsp72 is also capable of directly binding Box-3, and this has been shown to be a basis for
hsp72-dependent stimulation of MeV transcription\textsuperscript{186,187}. Hsp72 thus emerges as a candidate transcriptional cofactor for MeV, just as hsp60 is a probable cofactor for the vesicular stomatitis virus transcriptase, of which it is an integral component\textsuperscript{138}.

The current work was designed to test and/or refine this model by defining the kinetics of hsp72-dependent stimulation of MeV transcription and replication. Primary transcription is the period defined by the linear accumulation of viral transcripts due only to the activity of the input RdRp complex and template (Phase I). If hsp72 enhances polymerase elongation rate, then infecting cells containing elevated hsp72 levels should increase the rate of primary transcript accumulation, provided that hsp72 can function in trans with the transcriptase. Secondary transcription occurs in phase II when translation of viral protein provides additional polymerases, and the rate of transcript accumulation is exponential. In phase III, once genome replication commences, the transcription rate further increases since new polymerase complexes are now acting on new templates\textsuperscript{136}. At later times (i.e., phase IV and V), the rate of transcription declines while genome levels continue to rise. Hsp72-dependent changes in secondary and/or late transcription, in the absence of any changes in the rate of primary transcription, would suggest that hsp72 influences gene expression through assembly of transcriptional complexes (i.e., transcriptase and nucleocapsid template) or by maintaining nucleocapsid templates in a transcriptionally competent state. The approach to measuring the influence of hsp72 on the kinetics of MeV transcription and genome replication used reverse transcription - quantitative PCR (RT-qPCR) as previously described\textsuperscript{136,137}. Murine neuroblastoma cells were selected as a cell system because they express no basal levels of hsp72\textsuperscript{35}, unlike other cells of primate origin\textsuperscript{110,175,176}. Analysis of infected neuroblastoma cells thus
accentuates the potential difference between basal and hsp72-dependent MeV transcription and genome replication.

2.3 Materials and Methods

Generation of murine neuroblastoma cells that constitutively express human hsp72 (N2a-HSP) has been described previously\textsuperscript{35}. Non-hsp72-expressing cells are transfected with plasmid vector alone to act as controls (N2a-V). CD46-expressing cells (both N2a-HSP and N2a-V) were established by stable transfection with a CD46 expression plasmid (Apex3p CD46-C2). The construct contains a puromycin resistance gene and cells transfected with the CD46 expression plasmid were selected with 1 µg/µL puromycin (Invivogen). CD46 expression was analyzed by flow cytometry using Flourescein Isothiocyanate (FITC)-conjugated mouse anti-human CD46 monoclonal antibody (BD Biosciences). Resulting polyclonal cell lines were designated N2a-HSP-CD46 and N2a-V-CD46.

Viral N mRNA and genome production was defined using a previously described two-step SYBR green RT-qPCR assay\textsuperscript{136,137}. In brief, Edmonston B MeV (Ed-MeV) was propagated on Vero cell monolayers and cell-free virus was harvested and purified from the supernatant by sedimentation on a discontinuous sucrose gradient. For both murine neuroblastoma and Vero cells, monolayers were infected at an MOI = 1.0, and total RNA isolated hourly from 0-12 h.p.i. and every six hours from 18-60 h.p.i. (RNeasy, QIAGEN), followed by DNase I treatment (TURBO DNA-free\textsuperscript{TM}, Ambion). One µg of total RNA was denatured at 70°C for 5 min and annealed to either 0.4 µg of oligo dT primers (for viral N transcript RT-PCR reactions) or random hexamer primers (for viral
genome and 18S RNA RT-PCR reactions). Primer-annealed RNA was added to reverse transcription reaction mixtures that also included 25 nmol of dNTP and 1 µl of AffinityScript Multiple Temperature Reverse Transcriptase (Stratagene) in a final volume of 20 µl. Reactions were incubated for 90 min at 48°C. SYBR green quantitative PCR was performed in a Roche 480 LightCycler using previously reported N gene, L-Trailer and 18S RNA specific primers and PCR cycle conditions. Serial dilutions of p(+)MV were used as the PCR standard. Calculated values for MeV transcripts and genomes were adjusted for variation in 18S RNA levels.

Viral N antigen expression in cell monolayers was defined by confocal microscopy and Western blot analysis of total cellular protein. For confocal microscopy, murine neuroblastoma cell monolayers were grown on glass coverslips and infected at an MOI = 1.0. Cells were fixed with 4% paraformaldehyde at 24, 48 and 72 h.p.i. Prior to fixation, cells were stained with Hoechst 33342 dye (Molecular Probes) for nuclear visualization. After fixation, cells were pre-incubated separately with 0.1% Triton X-100, 10% normal goat serum in 1x PBS, and Image-iT FX signal enhancer, with PBS washes in between. After the final wash, fixed cells were incubated for 1 hour with 100 µL anti-MeV N mouse monoclonal antibody (mAb) (Abcam) diluted 1:200 in PBS with 1% normal goat serum, washed, then incubated 45 minutes with 100 µL Alexa Fluor 546 goat anti-mouse mAb (Molecular Probes) diluted 1:2000 in PBS with 1% normal goat serum. Coverslips were mounted to glass slides with Vectashield® mounting media (Vector laboratories, Inc.) and visualized using a Leica TCS SP2 AOBS Confocal Laser Scanning Microscope.
For Western blot analysis, subconfluent murine neuroblastoma monolayers (N2a-HSP and N2a-V) were infected with Ed-MeV at an MOI = 1.0. Cells were lysed in RIPA buffer (Santa Cruz Biotechnology, Inc.) and total protein concentration was determined with the Pierce Micro BCA protein assay kit. Seven μg of total protein were loaded onto pre-cast 10% Bis-Tris polyacrylamide gels (Invitrogen) for electrophoresis, then transferred to nitrocellulose membranes. Membranes were blocked with 5% bovine serum albumin (BSA) in 1x PBS with 0.01% Tween-20. Membranes were then incubated with anti-MeV N mouse mAb (2D7, Abcam Inc.) diluted 1:500 in 1x PBS with 1% BSA and 0.01% Tween-20 for 1 hour, followed by incubation with horseradish peroxidase-labeled anti-mouse polyclonal antibody (pAb) (BD Biosciences) diluted 1:2000 in 1x PBS with 1% BSA and 0.01% Tween-20. Signal was detected by chemiluminescence (Superwest pico chemiluminescence detection kit, Pierce, Inc.). The same procedure was followed for detection of hsp72 (mouse mAb C92, Assay Designs, diluted 1:2000) and β-actin (rabbit mAb 13E5, Cell Signaling Technology, Inc., diluted 1:400).

Northern blot analysis of viral transcript levels was performed as previously described\(^1\). Briefly, Vero cells were infected at an MOI = 1.0 and total RNA was harvested at 24 h.p.i. Ed-MeV N gene cDNAs were used to prepare virus strand-specific \([\alpha^32P]UTP\)-labeled N gene riboprobes. The pTRI-GAPDH-Mouse riboprobe (Ambion) was used to detect cellular GAPDH transcripts as an RNA loading control. Phosphorimager signal intensities were quantified using Image J software.
2.4 Results

Hsp72 sustains late viral transcription in mouse cells

When cell monolayers were infected with the Edmonston (B) strain of MeV at an MOI = 1.0, N2a-HSP cells had a significantly increased N transcript and genome expression level relative to N2a-V cells at > 12 h.p.i. (Figures 2.1A and B). This difference was not apparent at earlier post-infection intervals that would include the period of primary transcription, prior to the recruitment of new polymerases onto the incoming nucleocapsid templates, when only input viral polymerase is active. Results described here are representative of two separate experimental trials.

Linear increases in transcript levels, corresponding to primary transcription, was documented between 6 and 10 h.p.i. in N2a-HSP and N2a-V cells (Figure 2.1C and D). Signal above background was not detected prior to 6 h.p.i. There was a 2.7-fold increase in the slope of the line describing the accumulation of primary transcripts in N2a-HSP relative to N2a-V, but this resulted in similar apparent polymerase elongation rates (0.05 nt/s and 0.07 nt/s, respectively) and non-parametric repeated measures ANOVA of the transcript levels showed no significant difference between the cell types. The increase in transcripts became non-linear with similar kinetics in both N2a-HSP and N2a-V after 10 h.p.i., corresponding to what has previously been described as phase II of MeV RdRp activity during infection, when newly formed transcriptases act on pre-existing nucleocapsid template\textsuperscript{136}. The rate of increase in both transcript and genome levels was further increased after 20 h.p.i. in both N2a-V and N2a-HSP cell lines, corresponding to phase III of RdRp activity that reflects both newly-formed transcriptase and replicase
FIGURE 2.1. Quantification of Ed-MeV N transcript (A) and genome (B) levels in neuroblastoma cells (N2a) by RT-qPCR analysis of total cell RNA. Cells were infected at an MOI = 1.0. N2a-HSP cells are stably transfected with an hsp72 expression plasmid under the control of the β-actin promoter, while the N2a-V line is transfected with an empty plasmid vector for use as a non-hsp72 expressing control. Total RNA was harvested hourly for the first 12 h.p.i., then every six hours until 60 h.p.i. Infections were performed in duplicate and RNA copy number expressed as a mean ± standard error of the mean (SEM). The phases of viral polymerase activity are indicated at the top of each panel. Hsp72 containing cells showed significant increases in both N transcript levels (p < 0.001) and genome levels (p < 0.01) (nonparametric repeated measures ANOVA). In both cell lines, the increase in transcript levels is exponential at ≥ 12 h.p.i. (C) and linear between 6-10 h.p.i. (D); transcript signal above background was not detected at < 6 h.p.i. Linear fitting of the change in transcript levels between 6 and 10 h.p.i. is consistent with primary transcript accumulation. Non-parametric repeated measures ANOVA of primary transcript levels during Phase I did not reveal significant differences between N2a-V and N2a-HSP cells.
action. Levels of viral transcripts and genome were significantly greater in N2a-HSP relative to N2a-V cells during this and subsequent PI intervals (Figure 2.1A and B).

Qualitative differences in the kinetics of viral transcript production were observed between N2a-V and N2a-HSP infected cells at these late PI time points. For infected N2a-V cells, viral transcript levels became relatively constant after 42 h.p.i., whereas genome levels continued to rise exponentially. This corresponds to what has previously been described as phase IV and V RdRp activity, in which the replicase function increases in the face of declining transcriptase function\textsuperscript{136}. In contrast, exponential increases in both viral transcripts and genomes were observed throughout the 60 h infection interval in N2a-HSP cells, with no evidence of declining transcriptase activity.

The protein levels based upon Western blot analysis of total cell protein were proportionate to the transcript levels (Figure 2.2). Infected N2a-HSP and N2a-V cell monolayers were harvested at 24, 48 and 72 h.p.i. N protein antigen was readily detected in N2a-HSP cells at 24 h.p.i. whereas levels were below the threshold of detection in N2a-V cells. Viral N antigen was not detected in the latter until 48 h.p.i. Confocal immunofluorescence analysis of N protein expression supported the increased magnitude of viral gene expression in N2a-HSP versus N2a-V infected cells, but also allowed an evaluation of the uniformity of antigen expression within the cell populations (Figure 2.3). Overall, the N protein was expressed in relatively few N2a-V and N2a-HSP cells at 24 h.p.i. Such low level infection is consistent with our inability to plot primary transcript accumulation prior to 6 h.p.i., and raises the possibility that differences between N2a-HSP and N2a-V cells reflect, in part, an enhanced viral spread between infected and uninfected cells in N2a-HSP. Viral infectivity was based upon titers using
FIGURE 2.2. Nucleocapsid protein antigen expression in hsp72 overexpressing (N2a-HSP) and control (N2a-V) neuroblastoma cells infected at an MOI = 1.0 with Ed-MeV. Total cell protein was harvested at 24, 48 and 72 h.p.i., resolved by 10% SDS-PAGE, transferred to nylon membranes, and probed with antibodies specific to MeV N protein, hsp72, or β-actin (as a protein loading control). Levels of N protein reflect differences in late transcripts that were documented by RT-qPCR.
FIGURE 2.3. Nucleocapsid protein antigen expression in hsp72 overexpressing (N2a-HSP) and control (N2a-V) neuroblastoma cells infected at an MOI = 1.0 with Ed-MeV. Cells were fixed at 24, 48 and 72 h.p.i. and signal detected by indirect immunofluorescence for MeV N protein (Alexa Fluor secondary antibody, Hoechst counterstain for nuclei). Confocal microscopic images show a low and heterogeneous level of antigen expression at 24 h.p.i., suggesting inefficient viral initiation of infection. Increased viral N protein expression and the expression of CPE in N2a-HSP cells, including formation of syncytia, is apparent at 48 and 72 h.p.i.
CD46 expressing cells (i.e., Vero cells), a preferred receptor of Ed-MeV. Receptor utilization in N2a cells is unknown and our results suggest that the receptor is less efficient in mediating viral attachment and penetration than CD46.

Enhancing effect of hsp72 on late virus transcription is also observed when efficiency of virus entry into mouse cells is optimized

In order to alleviate potential restrictions to infectivity at the level of receptor utilization, N2a-HSP and N2a-V cells were transfected to constitutively express CD46. Surface expression of CD46 was comparable for N2a-HSP-CD46 and N2a-V-CD46 cells, with 82.6% and 74.9% labeled cells, respectively (Figure 2.4A). Confocal microscopic analysis of MeV-infected N2a-HSP-CD46 and N2a-V-CD46 shows widespread MeV-N antigen expression at 24 h.p.i. in both cell lines (greater than 80% antigen positive cells in all examined fields), consistent with a more synchronous and widespread infection (Figure 2.4B). Receptor expression is thus a significant determinant of murine neuroblastoma cell permissiveness to Ed-MeV infection.

Ed-MeV RNA expression was subsequently analyzed in N2a-HSP-CD46 and N2a-V-CD46 cells (Figure 2.5). Viral N transcript signal above background was now detected at 2 h.p.i. Levels of transcript increased in a linear manner through 10 h.p.i., as observed in the parent N2a cells, although the quality of linear fit for the increase in transcript levels was improved ($R^2 = 0.86$ for both N2a-HSP-CD46 and N2a-V-CD46, compared to 0.69 and 0.65 for N2a-HSP and N2a-V cells, respectively). As before, there was no significant influence of hsp72 on primary transcript accumulation, as reflected by the comparable linear slopes and calculated polymerase elongation rates. The elongation
FIGURE 2.4. (A) Flow cytometric analysis of murine neuroblastoma cells stably transfected to express CD46. Transfected N2a-V and N2a-HSP expressed comparable levels of CD46 receptor (74.9% and 82.6%, respectively) relative to non-transfected control cells. (B) Nucleocapsid protein antigen expression in neuroblastoma cells that express CD46, with or without hsp72 overexpression (N2a-HSP-CD46 and N2a-V-CD46, respectively). Cells were infected at an MOI = 1.0 with Ed-MeV and fixed at 24 h.p.i. for indirect immunofluorescence of MeV N protein (Alex Fluor secondary antibody, Hoechst counterstain for nuclei). Confocal microscopic images show that the majority of cells express viral antigen at this time, illustrative of a more synchronous infection when cells express CD46.
FIGURE 2.5. MeV N transcript levels in CD46- and hsp72-expressing murine neuroblastoma cells (N2a-HSP-CD46) relative to CD46 expressing control neuroblastoma cells (N2a-V-CD46). Transcript levels were based upon RT-qPCR analysis of total cell RNA. Cells were infected at an MOI = 1.0 with Ed-MeV. The increase in transcript levels is exponential at ≥ 12 h.p.i. (A) and linear between 2-10 h.p.i. (B). Infections were performed in duplicate and RNA copy number is expressed as a mean ± SEM. The phases of viral polymerase activity are indicated at the top of each panel. High quality linear fitting of the change in transcript levels between 2 and 10 h.p.i. is consistent with primary transcript accumulation. Non-parametric repeated measures ANOVA of primary transcript levels did not reveal significant differences between N2a-V and N2a-HSP cells at 2-10 h.p.i.
rate was 0.69 nt/s for N2a-HSP-CD46 and 0.87 nt/s for N2a-V-CD46. The switch to an exponential increase in transcript accumulation, corresponding to phase II and III of RdRp activities of MeV infection, was identical in both cell lines. As with the parent non-CD46 expressing N2a cells, there was a decline in the rate with which transcripts were produced during the later stages of infection of N2a-V-CD46, in contrast to a continual rise in genome levels. This increase in replicase relative to transcriptase activity was expressed as a transcript to genome ratio (Figure 2.6) to illustrate the phase IV and V of RdRp activity during MeV infection. The onset of this change was comparable, albeit earlier, in N2a-V-CD46 relative to the N2a-V cells (i.e., 30 versus 42 h.p.i.). Infection of hsp72 overexpressing N2a-HSP-CD46 cells did not exhibit this decline in transcriptase activity so that the transcript to genome ratio remained constant through 48 h.p.i. The higher ratio reflected nucleocapsid transcriptional activity and not more nucleocapsid template, since significant differences in genome levels were not observed between N2a-HSP-CD46 and N2a-V-CD46 cells (not shown). Cell monolayers deteriorated after 48 h.p.i. due to advanced viral cytopathic effects. Results are representative of two separate experimental trials.

**Hsp72 enhancing effect on late transcription is also observed in primate cells**

Vero cells were used to show that the lack of effect of elevated heat shock protein expression on primary MeV transcription was not unique to cells of murine lineage. Transient heat shock is an effective method of inducing elevated hsp72 expression in Vero cells (Figure 2.7D), resulting in increased MeV transcription and genome replication\(^{186}\). Linear increases in transcript accumulation were documented through 6
FIGURE 2.6. Ratio of Ed-MeV N transcripts to full-length genomes in CD46 and hsp72 expressing murine neuroblastoma cells (N2a-HSP-CD46) relative to CD46 expressing control neuroblastoma cells (N2a-V-CD46). Cells were infected at an MOI = 1.0 with Ed-MeV, and viral genome and transcript levels determined by RT-qPCR of total cell RNA at 6-12 h intervals. Infections were performed in duplicate and results expressed as a mean transcript to genome ratio. The phases of viral polymerase activity are indicated at the top of the panel. The transcript:genome ratio declined in the later stages of N2a-V-CD46 infection, consistent with the greater replicase activity that has been described for stages IV and V of MeV RdRp activity during the infection cycle. In contrast, transcriptase activity levels are increased throughout the infection cycle for hsp72 overexpressing cells, even though genome levels were not significantly different between N2a-HSP-CD46 and N2a-V-CD46 cells (not shown), resulting in an increased transcript:genome ratio for infected N2a-HSP-CD46 cells. Differences in mean ratio between N2a-HSP-CD46 and N2a-V-CD46 cells are statistically significantly different at 36 and 48 h.p.i. (p < 0.05, two tailed t test).
h.p.i. in both heat shocked and non-shocked infected Vero cells (Figure 2.7A and B). Similar to neuroblastoma cells, primary transcription was unaffected by heat shock in Vero cells, this being reflected in polymerase elongation rates of 2.26 nt/s and 1.98 nt/s for infected heat shocked and non-shocked cells, respectively. The temporal switch to an exponential increase in transcriptase activity (corresponding to phase II RdRp activity) and transcriptase and replicase activity (corresponding to phase III RdRp activity) was identical for both shocked and non-shocked cells, the latter beginning at 12 h.p.i. for both cell populations and in perfect agreement with published results from MeV infected HeLa cells\textsuperscript{136}. Late transcript levels were greater for infected heat shocked Vero cells, as demonstrated by Northern blot analysis of N transcripts at 24 h.p.i. (Figure 2.7C). Phosphorimage analysis of signal intensities showed that transcript levels in heat shock Vero cells were increased by 1.80 ± 0.17 fold, a statistically significant difference (p = 0.01). Genome levels were also significantly increased by 3.02 ± 0.33 fold in heat shocked relative to non-shocked cells (p = 0.001). The magnitude of these changes is in good agreement with the previously reported 1.7 and 1.9- fold increases in Ed-MeV transcript and genome levels that are induced by heat shock in Vero cells\textsuperscript{186}.

**Hsp72 is part of nucleocapsids isolated from virions**

Hsp72 has been shown to associate with MeV cytoplasmic nucleocapsid with an affinity that allows for recovery of hsp72-nucleocapsid complexes following CsCl isopycnic density centrifugation\textsuperscript{175,186}. This suggests that virion nucleocapsid can contain hsp72, such that primary transcription can occur in the presence of hsp72 regardless of hsp72 levels in the target cell. To address this possibility, the Ed-MeV virions purified by
FIGURE 2.7. Measles virus transcript production in Vero cells that were heat shocked to elevate hsp72 expression (HS), compared to non-shocked controls (NS). Cells were infected at an MOI = 1.0 with Ed-MeV. (A) Real time RT-qPCR analysis of total RNA showed that exponential increases in viral N transcript levels occurred after 6 h.p.i., with 12 h.p.i. corresponding to the start of the previously described phase III of the RdRp activity of the MeV infection cycle – an exponential increase in transcript levels that reflect newly formed transcriptases acting upon newly formed templates. Differences in transcript levels and genome levels (not shown) emerge at 18 h.p.i., although these differences were not statistically significant. Infections were performed in duplicate and RNA copy number is expressed as a mean ± SEM. (B) The linear phase of primary transcript accumulation was observed between 2 and 6 h.p.i. Significant differences in primary transcript levels were not observed during this interval, resulting in a comparable calculated polymerase elongation rate for Ed-MeV in shocked and non-shocked control cells (i.e., 2.0-2.3 nt/s). (C) Northern blot analysis of total infected cell RNA at 24 h.p.i. illustrates the difference in secondary N transcript levels between shocked and non-shocked cells. GAPDH transcripts were probed as RNA loading controls, and total RNA from uninfected cells was included as a negative control. Infections were performed in triplicate. Phosphorimager signal intensities of viral genome and transcript levels, corrected for GAPDH, were statistically significantly greater for HS relative to NS cells (p < 0.5, two tailed t test). The fold change was 3.02 ± 0.33 for genome and 1.80 ± 0.17 for transcript. (D) Western Blot analysis of heat shocked and non-shocked infected cell protein lysates. Infections were performed in duplicate. Total cell protein was harvested at 0 and 6 h.p.i., resolved by 10% SDS-PAGE, transferred to nylon membranes, and probed with antibodies specific to hsp72 or β-actin (as a protein loading control). Hsp72 levels are elevated in HS cells over basal expression in NS cells.
Figure 2.7
sucrose gradient centrifugation for the kinetic analysis of viral RNA expression were analyzed by Western blot. Virions were derived from infected non-shocked Vero cells. Virion total protein contained hsp72 (Figure 2.8). The hsp72 signal was also retained in the nucleocapsids obtained after virus solubilization and sedimentation at 100,000 g (data not shown).

2.5 Discussion

The kinetic analysis of viral N transcript RNA levels showed no significant differences between hsp72-expressing and control murine neuroblastoma cells in the first 10 hours post infection. The increase in transcript levels was linear during this interval, supporting the conclusion that hsp72 in trans does not affect polymerase elongation rate during primary transcription. Expression of CD46 in neuroblastoma cells allowed us to better visualize primary transcription by increasing the synchronicity of infection. In the absence of CD46, a linear increase in transcript accumulation was not detected until 6 h.p.i. Increasing the efficiency of the infection through CD46 expression affected the slope of primary transcript accumulation and thus the calculated polymerase elongation rate, although no effect of hsp72 overexpression was detected. The apparent lower elongation rates in mouse cells not expressing CD46 is likely reflecting the very limited amount of genome that successfully penetrates the cell to initiate transcription relative to the amount of virions adsorbed to the cell surface. The inefficient entry is illustrated by the initial low percentage of cells expressing viral proteins. Purified total cell RNA would include these cell-surface virion genomes, and their inclusion would lower the apparent polymerase elongation rate, since that rate is calculated by dividing the number of
FIGURE 2.8. Western blot analysis of purified Ed-MeV virion total protein showed the presence of hsp72. Virions were isolated from infected Vero cell supernatant by sucrose gradient centrifugation. Uninfected control supernatants were processed in parallel as a negative control. Results illustrate isolates processed separately. Samples were stained separately with antibody specific for hsp72 (HSP) or MeV N protein (N).
polymerized nucleotides per time unit by the number of genomes within the total cell RNA (normalized for 18S RNA). Expression of CD46 supported an approximately 10-fold increase in the percentage of viral antigen positive cells at 24 h.p.i., and this was correlated to an approximately 10-fold increase in the apparent polymerase elongation rate. The calculated polymerase elongation rate in CD46 expressing neuroblastoma cells was 0.7-0.9 nt/s, which is still less than that reported for HeLa cells (2.8 nt/s). This suggests that there are differences in cellular co-factors available to support primary transcription in murine neuroblastoma cells relative to more highly permissive cell lines of primate origin. The effect of hsp72 was evaluated on Vero cells to determine if hsp72-dependent changes in primary transcription might emerge with a more permissive intracellular environment. Calculated polymerase elongation rates were increased to 2.0 – 2.3 nt/s in Vero cells, approximating rates calculated in HeLa, although there remained no effect of heat shock-induced hsp72 levels on primary transcription.

In contrast to the lack of effect on primary and secondary transcription, elevated cellular levels of hsp72 sustained late transcription in neuroblastoma and Vero cells. Hsp72 does not appear to enhance the level of viral protein that is expressed from primary transcripts. This conclusion is supported by the observation that the temporal change from primary to secondary transcription (i.e., the time where the increase in transcript levels no longer conforms to a linear regression) remained constant despite changes in hsp72 levels. Increased protein expression from primary transcripts would be expected to accelerate formation of new transcriptional complexes, and therefore to shorten the transition from primary to secondary transcription. Likewise, a shortening of
the lag period before the onset of the replication should also have been expected from an enhanced formation of N+P+L replicase complexes.

Hsp72-dependent increases in genome levels can only partly explain the increased levels of late transcripts by simply providing more templates for viral transcriptases, since the significant difference in the transcript to genome ratios in N2a-HSP-CD46 and N2a-V-CD46 cells was not accompanied by significant differences in genome levels. Heat shock did significantly increase genome levels by approximately 3-fold in Vero cells at 24 h.p.i., and more dramatic differences were observed between infected N2a-HSP and N2a-V cells. In these later scenarios, hsp72 could increase the genome levels by supporting nucleocapsid assembly, the latter being integral to genome replication.

Assembly of MeV nucleocapsid from N protein and genomic RNA requires a chaperone to maintain N protein monomers in a soluble and assembly competent state. The MeV P protein normally provides this function\textsuperscript{75}, but hsp72 has been found to substitute for P in cell free assays of nucleocapsid assembly, where RNase protection of minigenomic RNA is used as the indicator of encapsidation\textsuperscript{121}. A similar host chaperone function for 70-kDa HSPs has been documented in the intracellular viral protein assembly of polyomavirus\textsuperscript{42}. Here, cell free reactions supporting the oligomerization between the major and minor viral capsid proteins (VP1 and VP3) requires the presence of DnaK, the bacterial homologue of eukaryotic hsp72, in order to achieve nucleocapsid structures with the ordered symmetry of infectious intracellular virions. Furthermore, hsc70 and VP co-localize in the nucleus, which is the site of polyomavirus particle assembly\textsuperscript{44}. Variability in the genome response to elevated hsp72 levels in CD46 expressing cells of the current study may reflect the broader spectrum of induced HSP and co-chaperone expression that
follows heat shock, in contrast to the selective hsp72 overexpression of the N2a-HSP-CD46 cells. Furthermore, Ed-MeV induces hsp72 by 24 h.p.i. in N2a cells and perhaps earlier\textsuperscript{129}, so that some requirements for HSP in support of viral replication may be met in the absence of overexpression.

Elevated transcript:genome ratios at late PI intervals in the infected N2a-HSP-CD46 cells may reflect a role for hsp72 in maintaining nucleocapsid templates in a transcriptionally competent state. One mechanism may involve prevention of nucleocapsid aggregation as levels rise late in the infection cycle. Canine distemper virus (CDV) is a morbillivirus closely related to MeV that exhibits identical structural and functional interactions between hsp72 and N protein/nucleocapsid\textsuperscript{123,127}. Previous work shows that heat shock induction of hsp72 in persistently infected cells results in extensive co-localization between hsp72 and N protein within cytoplasmic inclusion bodies\textsuperscript{122}. The co-localization is associated with dispersal of these cytoplasmic aggregates, increased cell-free transcriptional activity of nucleocapsid that is isolated from the cytoplasm, and increased cellular accumulation of viral transcripts and thus viral protein and cytopathic effect\textsuperscript{122,127}. Similar enhancement of hsp72 on cell-free transcriptional activity of MeV nucleocapsid has been shown\textsuperscript{175,176}. CDV nucleocapsid bound to hsp72 also exhibits an altered morphology that enhances exposure of the genomic RNA and is associated with a reduced nucleocapsid buoyant density\textsuperscript{123,124}. This hsp72-dependent alteration of nucleocapsid buoyant density is also found in MeV, suggesting an additional (structural) basis by which hsp72 could maintain the nucleocapsid templates in a transcriptionally competent state\textsuperscript{175,176}. Hsp72 binds nucleocapsid with high affinity, being similar to that of P\textsuperscript{22,187}, which readily explains how hsp72 could accompany nucleocapsid assembly.
into the virion. Indeed, MeV assembly occurs within membrane rafts\textsuperscript{97,177} where large amount of hsp72 concomitantly accumulate\textsuperscript{63}. As such, virion nucleocapsid could be maintained in a transcriptionally competent state, allowing transcription to be initiated immediately following nucleocapsid penetration of the target cell.

The possibility that hsp72 could have a role in primary transcription was previously suggested by the fact that CDV nucleocapsid cell-free transcriptional activity requires hsp72\textsuperscript{128}. These reactions measure the elongation of pre-initiated transcripts from pre-formed transcriptional complexes, thereby modeling primary transcription. In this assay, pre-formed CDV nucleocapsid-hsp72 complexes exhibited enzymatic activity whereas nucleocapsid devoid of hsp72 did not. Addition of antibody specific for hsp72 suppressed enzymatic activity, and the addition of purified hsp72 stimulated CDV nucleocapsid cell-free transcription\textsuperscript{128}. In light of our current findings where exogenous hsp72 has no significant effect on MeV primary transcription, we propose that hsp72 carried on the incoming nucleocapsid may be sufficient to support primary transcription, potentially by maintaining nucleocapsid in an appropriate morphological state. Exogenous hsp72 may maintain nucleocapsid activity by preventing nucleocapsid aggregation, but this function may only be required at high nucleocapsid concentrations created in cell-free assays, not the low nucleocapsid concentrations that would normally be encountered in the early stages of cell infection.

Collectively, results of the kinetic analysis of hsp72-dependent MeV RNA production together with previous work allow us to construct a model for the role of hsp72 in MeV gene expression. In this model, elevated levels of hsp72 in the cell may stimulate late transcription by two mechanisms. First, hsp72 may enhance genome
replication which, in turn, enhances secondary transcription by virtue of creating additional templates for the viral RdRp. Second, hsp72 may maintain the nucleocapsid in a transcriptionally competent state. The relative contributions of genome replication (and thus nucleocapsid levels) and nucleocapsid functionality to late transcription may be determined by cell permissiveness. In a permissive cell where nucleocapsid levels rise rapidly, the greater impact of hsp72 may be on nucleocapsid functionality (e.g., solubility) whereas in a poorly permissive cell, the greater impact may be mediated by the stimulatory effect on genome replication. This model may be used to explain results with a mutant of Ed-MeV, in which an N\textsubscript{TAIL} N522D substitution selectively diminishes hsp72-dependent stimulation of transcription but not genome replication. In highly permissive Vero and HeLa cells, hsp72 increases the genome levels by only 1.7-fold on average and significant increase in transcript levels was observed only for the parent virus\textsuperscript{186}. In poorly permissive N2a cells, hsp72 stimulates genome replication by greater than 10-fold for both parent and N522D virus. It also stimulates virus transcription but to a lower extend for the latter virus\textsuperscript{35}. The increase in transcription in the latter instance may reflect the increase in genomic templates that would support transcription. The model therefore suggests that the role of the N\textsubscript{TAIL} motif containing the N522, which is a hallmark of all vaccine strains, is to mediate hsp72-dependent nucleocapsid functionality.

Ongoing studies should examine the relevance of this model to other paramyxoviruses such as respiratory syncytial virus (RSV), given the observation that hsp72-specific antibody suppresses the synthesis of nucleic acids by the RSV RdRp contained within lipid rafts of infected cells\textsuperscript{26}.
2.6 Acknowledgments

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CHAPTER 3

A TYROSINE RESIDUE WITHIN THE MEASLES VIRUS N PROTEIN C-TERMINUS CAN MODULATE VIRAL POLYMERASE ACTIVITY

3.1 Abstract

Measles virus (MeV) nucleocapsid (N) protein tyrosine phosphorylation has been associated with reduced viral gene expression and viral persistence in neurons, although a causal role of tyrosine phosphorylation in altering infection phenotype has not been established. The present work tested the hypothesis that one of two tyrosine residues (Y518) within the intrinsically disordered C-terminus of the N protein (N\textsubscript{TAIL}) is a determinant of viral gene expression and infection phenotype. Y518 resides within a conserved element of N\textsubscript{TAIL} (Box-3) that mediates binding to the viral polymerase co-factor protein (P) and the major inducible 70-kDa heat shock protein isoform (hsp72)-virus and host determinants of viral gene expression. The present work showed that Y phosphorylation in Box-3 peptides disrupted direct binding to hsp72, and this effect was mimicked by a Y to D substitution. Binding of Box-3 to the P protein X-domain (XD) is indirect and so was measured in context of N\textsubscript{TAIL}. Within N\textsubscript{TAIL}, the Y518D substitution reduced N\textsubscript{TAIL} binding for both XD and hsp72, whereas a Y451D substitution was without effect. Nucleocapsid protein containing the Y518D substitution supported
increased minireplicon reporter gene expression, which is a measure of transcription and/or genome replication. Infectious MeV encoding the Y518D substitution exhibited markedly attenuated RNA expression in Vero cells that was attributed to a high propensity for defective interfering particle formation. Collectively, results support the potential of the Box-3 tyrosine to modulate viral replicase activity, suggesting a mechanism by which host cell tyrosine kinase activity could promote viral persistence through enhanced production of DI particles.

3.2 Introduction

Like all members of the Paramyxoviridae family, the measles virus (MeV) nucleocapsid is a helical ribonucleoprotein complex (RNP) consisting of a single negative–sense RNA genome encapsidated by repeating monomers of the virus–encoded nucleocapsid (N) protein. The viral RNA-dependent RNA polymerase (L) and polymerase co–factor (P) bind the RNP in support of both transcription and genome replication. Complex formation is mediated by the C-terminal 125 amino acids of the N protein (N\textsubscript{TAIL}), which is an intrinsically unstructured domain that is exposed on the surface of the viral RNP\textsuperscript{21}. Typical of unstructured protein domains, N\textsubscript{TAIL} exhibits a hypervariable amino acid sequence, with the exception of three separate conserved elements (Box-1, aa 401-420; Box-2, aa 489-506; and Box-3, aa 517-525). Of these, Box-2 and Box-3 mediate binding to the viral P protein, specifically the C-terminal X domain (XD, aa 459-507)\textsuperscript{22}. The P protein also binds L, thereby establishing the linkage between L and the RNP template for transcription and genome replication.
Box-2 is a component of the \( \text{N}_\text{TAIL} \) \( \alpha \)-Molecular Recognition Element (\( \alpha \)-MoRE, aa 488-499), so named because it undergoes induced \( \alpha \)-helical folding upon XD binding, aligning the hydrophobic residues within Box-2 with the binding groove of XD that is formed by an anti-parallel three helix bundle\(^\text{22,83}\). Heteronuclear NMR studies show that \( \text{N}_\text{TAIL} \) undergoes additional structural changes upon binding XD, causing the more C-terminal Box-3 to fold upon XD\(^\text{22}\). These secondary contacts between XD and Box-3 are necessary to maintain the high binding affinity that is observed between XD and \( \text{N}_\text{TAIL} \) (\( K_D = 80 \text{ nM} \))\(^\text{22}\). Movement of the polymerase along its RNP template during transcription and replication requires cycles of binding and release between the P and \( \text{N}_\text{TAIL} \), so that high affinity interactions between P and \( \text{N}_\text{TAIL} \) should constrain the rate of transcript and/or genome elongation. This is supported by the finding that deletion of Box-3 reduces \( \text{N}_\text{TAIL} \) binding affinity for XD\(^\text{22}\) and N protein lacking Box-3 supports enhanced minireplicon reporter gene expression relative to that mediated by parent N protein\(^\text{187}\). In addition, direct binding of the major inducible 70-kDa heat shock protein (hsp72) to Box-3 is also associated with enhanced minireplicon reporter gene expression and increased transcriptional activity for Edmonston MeV, consistent with a model whereby the contribution of Box-3 to stabilizing \( \text{N}_\text{TAIL} \) binding of P is neutralized\(^\text{186,187}\).

The N protein Box-3 contains a tyrosine residue at position 518, providing a target for phosphorylation that is readily accessible when incorporated in nucleocapsid due to its surface location. Phosphorylation of Y imparts an overall negative charge. A negative charge may discourage P binding to \( \text{N}_\text{TAIL} \), given the observation that negative charges diminish hsp72 binding\(^\text{59,187}\) and that hsp72 and the P protein XD exhibit similar binding preferences for \( \text{N}_\text{TAIL} \) hydrophobic motifs\(^\text{186}\). Tyrosine phosphorylation of MeV
N has been demonstrated and is associated with profound alteration of virus infection phenotype. N protein purified from murine neuroblastoma cells persistently infected with Edmonston MeV is phosphorylated on tyrosine residues, but not N protein isolated from acutely infected cells\textsuperscript{152}. The persistent infection is associated with both interferon production and elevated levels of protein tyrosine kinase activity, although the location of the tyrosine phosphorylation events has not been mapped\textsuperscript{142,152}.

The current work addresses the hypothesis that the negative charge associated with Y518 phosphorylation can induce changes in MeV polymerase activity by altering the interaction between P protein XD and N_{TAIL}. The functional and structural significance of the negative charge will be examined using a Y518D substitution mutant. A second highly conserved Y residue exists within N_{TAIL} (Y451), located in a region between Box-1 and -2 that is not known to influence P/N_{TAIL} interaction. The structural and functional significance of a negative charge at this location will also be examined as a control.

3.3 Materials and Methods:

**Binding reaction analysis**

Surface plasmon resonance (SPR) technology (BIAcore\textsuperscript{®}, GE Healthcare) was used to examine the influence of negative charge within MeV N_{TAIL} sequences on binding to P protein XD and hsp72. In this system, one protein partner (i.e., ligand) is covalently linked to CM5 sensor surfaces while the other flows over the sensor surface in solution (i.e., analyte). Binding events alter SPR, a specific property of light that is reflected off the opposing surface of the sensor chip. Changes in SPR are proportionate to
loss or gain of mass on the opposite surface and are plotted as response units (RU) in sensorgrams. The unit can thus monitor association and dissociation rates between molecules in real time. Experimental data are fitted to reference data in order to calculate association and dissociation rate constants, and equilibrium dissociation constants ($K_D$) that describe the binding reaction. The $K_D$ is the numerical measure of binding affinity.

SPR was used to separately analyze the binding of purified hsp72 or XD with three synthetic 15–amino acid peptides representing C–terminal NTAIL sequences that include Box-3 (Genemed Synthesis, Inc.). The tyrosine residue within this domain was either replaced by aspartic acid (Box-3 Y518D) or was phosphorylated (Box-3 Y518P). Mass spectroscopy confirmed the phosphorylation status of Y518. Each sensor surface contains four separate flow channels that can be conjugated to different ligands. One channel was activated and blocked in order to measure non-specific interactions between ligand and sensor. This background was subtracted from all sensorgrams. Purified hsp72 (Assay Designs, Inc./StressGen) was conjugated to a second flow channel at 8,500 RU and 500 RU of XD was conjugated to a third flow channel. High immobilization levels of ligand were used since the peptide analytes were small, contributing only minor changes in RU’s following a binding event. The level of ligand immobilization represented molar equivalents of hsp72 and XD. Individual peptide analytes were diluted in HBS-P running buffer (GE Healthcare) supplemented with 2.5 mM magnesium acetate and 2.5 mM adenosine triphosphate and used in separate binding reactions. Measurements were made on a range of peptide concentrations, and the resulting curves fit globally to a 1:1 binding model to calculate the equilibrium dissociation constant ($K_D$) for each variant. Chi$^2$
analysis and residual values were used to judge the quality of fit between experimental and reference data.

Additional binding studies were performed with synthetic $N_{TAIL}$ molecules as analytes. Expression and purification of $N_{TAIL}$ molecules, including the alteration of individual amino acids, has been described previously\textsuperscript{22}. Here, the two tyrosine residues present within the Ed-MeV $N_{TAIL}$ sequence (position 451 and 518) were separately replaced with aspartic acid, with the resulting molecules designated $N_{TAIL\ Y451D}$ and $N_{TAIL\ Y518D}$, respectively. Generation and use of an $N_{TAIL}$ construct lacking Box-3 (i.e., $N_{TAIL\ A3}$) has been previously described\textsuperscript{22}. Ligand immobilization levels were reduced since the mass of the $N_{TAIL}$ analytes were significantly greater than that of the peptides, reducing the amount of analyte binding required to elicit detectable responses on the sensorgrams. Immobilization levels were 1,000 RU for hsp72 and 165 RU for XD.

**XD-induced folding of $N_{TAIL}$**

Folding propensities of $N_{TAIL}$ proteins were measured as a function of changes in the initial CD spectrum upon addition of either increasing concentrations of TFE (Fluka), or a two-fold molar excess of XD or lysozyme (Sigma). TFE measures the intrinsic ability of a protein to fold, and lysozyme serves as a negative control for folding events that may be specifically attributed to XD binding. The CD spectra were recorded on a Jasco 810 dichrograph using 1-mm thick quartz cells in 10 mM sodium phosphate pH 7 at 20°C. CD spectra were measured between 185 and 260 nm, at 0.2 nm/min and were averaged from three independent acquisitions. Mean ellipticity values per residue ($[\Theta]$) were calculated as $[\Theta] = (3300 \Delta A)/(l \ c \ n)$, where $l$ (path length) = 0.1 cm, $n$ = number of residues, $m$ = molecular mass in daltons and $c$ = protein concentration expressed in
mg/ml. Number of residues (n) are 132 for NTAIL variants, 56 for XD, and 129 for lysozyme, while m values are approximately 14,676 Da for NTAIL variants, 6,690 Da for XD, and 14,300 Da for lysozyme. Protein concentrations of 0.1 mg/ml were used when recording spectra of both individual and protein mixtures. In the case of protein mixtures, mean ellipticity values per residue (\(\Theta\)) were calculated as 
\[
\Theta = 3300 \Delta \lambda / \left[ \left( \left( C_1 n_1 / m_1 \right) + \left( C_2 n_2 / m_2 \right) \right) \lambda \right],
\]
where \(\lambda\) (path length) = 0.1 cm, \(n_1\) or \(n_2\) = number of residues, \(m_1\) or \(m_2\) = molecular mass in daltons and \(c_1\) or \(c_2\) = protein concentration expressed in mg/ml for each of the two proteins in the mixture. The theoretical average ellipticity values per residue (\(\Theta_{\text{Ave}}\)), assuming that neither unstructured-to-structured transitions nor secondary structure rearrangements occur, were calculated as follows: 
\[
\Theta_{\text{Ave}} = \left( \left( \Theta_1 n_1 \right) + \left( \Theta_2 n_2 R \right) \right) / \left( n_1 + n_2 R \right),
\]
where \(\Theta_1\) and \(\Theta_2\) correspond to the measured mean ellipticity values per residue, \(n_1\) and \(n_2\) to the number of residues for each of the two proteins, and \(R\) to the excess molar ratio of protein 2. The \(\alpha\)-helical content was derived from the ellipticity at 220 nm as described by Myers and others\textsuperscript{117}.

**Analysis of N protein function using minireplicons**

The MeV minireplicon system is composed of separate plasmids encoding the Ed–MeV N, P, and L proteins and a plasmid encoding a negative–sense copy of a luciferase reporter gene flanked by the non–coding Ed–MeV genomic termini, all under the control of a T7 promoter. Simultaneous transfection of all plasmids into subconfluent Hep–2 cell monolayers and concurrent infection with a replication–deficient recombinant vaccinia virus as a source of T7 (i.e., MVA T7) results in assembly of a functional viral nucleocapsid complex. Luciferase activity in cell lysates is a measure of transcription and/or genome replication that is mediated by the viral polymerase (L) and polymerase...
cofactor (P). These results can thus be used to determine how changes in the template for the viral polymerase (in this case, N\textsubscript{TAIL}) influence viral transcription and genome replication.

For creation of the pT7MV–N–Y518D plasmid, electrocompetent \textit{Escherichia coli} RZ1032 were transformed with the parent pT7MV–N plasmid. Single–stranded DNA representing the 5’– 3’ strand of the plasmid was produced by the R408 helper phage and isolated. For oligonucleotide site–directed mutagenesis, the appropriate primer (Y518D = 5’-GAAGATTTCTGTCATTGT\textsubscript{CG}ACTATAGGGG-3’; Y451D = 5’-GCCCGGTTTCTCGATCGCTCTCCCTGGC-3’) was annealed to the single–stranded pT7MV–N, extended and ligated \textit{in vitro}. Aside from the alteration of N protein coding sequence, mutagenic primers also introduced a unique restriction enzyme site to differentiate the product from the parent sequence. The resultant double–stranded mutant plasmid was amplified in \textit{E. coli} DH5α. Restriction enzyme digestion was used to confirm the presence of the mutation and sequence analysis ruled out the presence of spurious second site mutations.

For minireplicon assays, subconfluent Hep2 cell monolayers in six-well plates were transfected with plasmids encoding MeV N (0.8 µg of parent, Y451D or Y518D mutant per well), P (0.6 µg) and L (0.2 µg) transcripts and minigenome RNA containing the luciferase coding sequence (0.2 µg), with six replicates for each transfection group. The parent and Y518D reaction groups were further supplemented with 0 or 2.5 µg of an hsp72 expression plasmid. Firefly luciferase activity in 20 µL of cell lysate was measured with a luciferase assay kit (Promega) and a Perkin–Elmer LS–5B luminescence spectrometer. Western blot analysis was performed on total transfected cell protein to
measure the following: MeV N protein (mouse mAb 2D7, Abcam Inc., diluted 1:500) to control for potential variation in N protein expression between the different constructs; hsp72 (mouse mAb C92, Assay Designs, diluted 1:2000) in instances where hsp72 supplementation was performed; β-actin (rabbit mAb 13E5, Cell Signaling Technology, Inc., diluted 1:400) as a protein loading control.

**Analysis of N protein function in infectious virus**

A plasmid-based reverse genetics system was used to produce recombinant MeV incorporating either the Y518D or the Y451D substitution in the N protein as previously described\(^\text{186}\). The approach is similar to that of the minireplicon, except that plasmid encoding the minigenome is replaced by the p(+)MV plasmid. This construct contains the full-length viral genomic cDNA, with the N coding sequence altered by site-directed oligonucleotide mutagenesis to express the desired N protein amino acid substitution. Mutagenesis, using the same primers described above, was performed on a 2310 bp MeV cDNA genomic subclone that included the complete N gene and 239 bp of the 5’ P gene coding region\(^\text{186}\). Restriction digest and sequence analysis confirmed the presence of the desired mutation. The subgenomic cDNA was released by SfiI and SacII, and ligated into p(+)MV that was similarly linearized with SfiI and SacII. Plasmids were amplified in *E. coli* DH5α. Hep-2 cells were infected with MVA T7 and transfected with plasmids to express N, P and L proteins, and plus strand genomic RNA of either parent or mutant virus. Hep-2 cells were subsequently harvested by scraping and co-cultured with Vero cell monolayers. After allowing three hours for Hep-2 cell adhesion, the culture media was replaced with fresh media containing 0.75% methylcellulose. Individual plaques were harvested and passed two times on Vero cells in order to generate working viral
stocks. Total RNA was isolated from aliquots of these stocks, the 3’ end of the N gene amplified by RT-PCR, and restriction analysis of the amplicon used to confirm the presence of the desired mutation.

For the measurement of infectious viral progeny release, subconfluent Vero cells monolayers were infected at an MOI = 0.01 with either Ed-MeV (designated Ed N for these studies), Ed N-Y518D or Ed N-Y451D to establish multistep viral growth curves. Infections were performed in triplicate. Cell-free viral progeny was sampled at 24, 36, 48, 60, 72 and 84 hours post-infection (h.p.i.) and titrated on Vero cells, calculated as the 50% tissue culture infective dose (TCID$_{50}$)/mL.

For the analysis of viral RNA expression, subconfluent Vero cells monolayers were infected at an MOI = 1.0 in triplicate, and total RNA was isolated at 36 h.p.i. (Qiagen RNeasy Kit). Northern blot analysis of viral transcript and genomic RNA levels was performed as previously described$^{175,176}$. Virus strand and gene specific [$^{32}$P]UTP-labeled riboprobes were prepared from Ed-MeV N and H cDNAs$^{145}$. A 359-base riboprobe derived from the mouse GAPDH coding sequence (pTRI-GAPDH-Mouse, Ambion) defined the cellular GAPDH transcript levels, which served as an RNA loading control. Image J software was used to quantify phosphorimager signal intensities of the respective RNA bands.

For the biological interference assay, subconfluent Vero cell monolayers were infected with Ed N at an MOI = 0.01 and co-infected with either Ed N, Ed N-Y451D or Ed N-Y518D at an MOI = 0.001. Infections were performed in triplicate. Cell monolayers were harvested at 24 h.p.i. Cell associated virus was titrated on Vero cells, calculated as the 50% tissue culture infective dose (TCID$_{50}$)/mL.
3.4 Results

The Y518D substitution reduced XD binding affinity for N\text{TAIL}.

A 15 amino acid peptide representing the C-terminus of the Ed-MeV N protein and containing the Box-3 sequence bound hsp72 with a low but physiologically relevant affinity based upon SPR analysis. The $K_D$ describing the binding reaction ($9 \ \mu M$, Table 3.1) was in good agreement with previously published reports of hsp72 binding to the same 15 amino peptide ($K_D = 1.0 \ \mu M$)\textsuperscript{187} and or a 21 amino acid peptide that also contained Box-3 ($K_D = 3.6 \ \mu M$)\textsuperscript{186}. Direct binding of XD to Box-3 peptide was weak and physiologically irrelevant, having a $K_D$ of 42 µM. This low affinity binding is consistent with XD/Box-3 interactions that are secondary to XD-induced conformational changes in N\text{TAIL} mediated by Box-2\textsuperscript{22}. Phosphorylation of the tyrosine residue within the Box-3 peptide reduced the XD binding affinity by 2.9-fold and the hsp72 binding affinity by 333-fold, so that neither XD nor hsp72 bound Box-3 peptide with a physiologically relevant affinity. These changes were mirrored by loss of XD and hsp72 binding affinity for peptides that contained the Y518D substitution. Changes were 2-fold and 1398-fold for XD and hsp72, respectively. Aspartic acid mimics the additional negative charge that results from tyrosine phosphorylation.

Binding studies that utilize the N\text{TAIL} molecule more closely reflect the effect that amino acid alterations have on binding between the nucleocapsid protein and its partners\textsuperscript{22,186}. The synthetic N\text{TAIL} molecule maintains the intrinsic properties of this domain that are expressed when it is incorporated into the full-length N protein, in particular the ability to bind to the C-terminus of the MeV P protein (PCT) that includes...
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Table 3.1. Calculated equilibrium dissociation constants ($K_D$) for the binding of three Box-3 peptide variants with both XD and hsp72. High quality fitting of experimental to reference data for a 1:1 Langmuir binding model are reflected in residuals of ± 2 and $\text{Chi}^2$ values < 1.0.
XD. In addition, hsp72 binding reactions are similar for MeV nucleocapsid fragments and N_{TAIL}. In the current work, binding affinity of both XD and hsp72 for N_{TAIL} was in the nM range (31.1 and 34.6 nM, respectively – Table 3.2), consistent with results of previous studies. The N_{TAIL} Y518D exhibited a dramatic reduction in both XD and hsp72 binding affinity, whereas substitution of aspartic acid for tyrosine at position 451 of the N protein (i.e., N_{TAIL} Y451D) had no significant affect. Substitution of aspartic acid for tyrosine in Box-3 mimics the XD and hsp72 binding of N_{TAIL} that lacks Box-3 (i.e., N_{TAILΔ3}), where the K_D’s are 12.0 and 14.1 μM, respectively. Results support the critical role of Box-3 in maintaining high affinity complex formation between XD and N_{TAIL}, and are consistent with the previous observation that N protein mutants lacking Box-3 support a lower level of co-immunoprecipitation with hsp72 relative to parent N protein.

The Y518D substitution does not alter the ability of N_{TAIL} to undergo induced folding, only the folding that is induced by XD

Circular dichroism (CD) was used to determine if tyrosine to aspartic acid substitutions could affect the overall secondary structure content of the N_{TAIL} variants. Notably, secondary structure predictions provided by PSI-PRED pointed out no differences amongst the N_{TAIL} variants, with an α-helix spanning residues 488-502 being predicted as the sole secondary structure element in all cases (data not shown). In order to directly assess the possible impact of the acidic substitutions on the N_{TAIL} structure, the far-UV CD spectra of N_{TAIL} variants was recorded at neutral pH. The CD spectra of mutated N_{TAIL} proteins are very similar to that of parent N_{TAIL}, and are all typical of
Table 3.2. Calculated equilibrium dissociation constants ($K_D$) for the binding of four $N_{TAIL}$ protein variants with both XD and hsp72. High quality fitting of experimental to reference data for a 1:1 Langmuir binding model are reflected in residuals of ± 2 and Chi$^2$ values < 1.0.

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<td>$1.41 \times 10^{-5} = 14.1$ μM</td>
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unstructured proteins, as seen by their large negative ellipticity at 200 nm and moderate ellipticity at 185 nm (Fig. 3.1A-C). However, ellipticity values at 200 and 222 nm indicate that both mutated and parent N_{TAIL} possess a certain residual compactness typical of the premolten globule state\textsuperscript{94,174}. The α-helical content of the N_{TAIL} proteins was derived from the ellipticity at 222 nm (Fig. 3.1D), and showed that the N_{TAIL} variants have an α-helical content similar to parent N_{TAIL}.

In order to further investigate the structural impact brought by the acidic substitutions, the ability of the N_{TAIL} variants to undergo induced folding in the presence of 2,2,2-trifluoroethanol (TFE) was analyzed. The solvent TFE mimics the hydrophobic environment experienced by proteins in protein-protein interactions and is therefore widely used as a probe to unveil disordered regions having a propensity to undergo an induced folding\textsuperscript{72}. Previous reports note that the addition of increasing amounts of TFE to N_{TAIL} triggers a gain of α-helicity, and that Box-2/α-MoRE plays a major role in this α-helical transition\textsuperscript{21,22,94}. In the present work, CD spectra of mutated N_{TAIL} proteins were recorded in the presence of increasing concentrations of TFE, and all proteins showed a gain of α-helicity (Fig. 3.1). This was indicated by the characteristic maximum at 190 nm and minima at 208 and 222 nm. Furthermore, the alpha helical content was comparable between the mutant and parent N_{TAIL} constructs under different TFE concentrations, indicating that the acidic substitutions do not impair the ability of N_{TAIL} to undergo α-helical folding.

The next series of experiments determined whether the mutated N_{TAIL} proteins retain the ability to undergo induced folding in the presence of XD. Far-UV CD spectra
Figure 3.1. Far-UV CD spectra and analysis of the α-helical propensities of NTAIL proteins. Far-UV CD spectra of wt NTAIL (A), NTAIL Y451D (B) and NTAIL Y518D (C) at 0.1 mg/ml in 10 mM Sodium Phosphate pH 7 in the presence of increasing concentrations of TFE (0, 10, 20, and 30%) recorded at 20°C. Each spectrum is the mean of three independent acquisitions. (D) α-helical content of NTAIL proteins in the presence of increasing TFE concentrations. The α-helical content was derived from the ellipticity value at 222 nm as described by Myers et al. The error bar (10% of the value) corresponds to the experimentally determined standard deviation from three independent experiments.
were recorded in the presence of a two-fold molar excess of XD, a condition where XD induces the most dramatic structural transition within parent NTAIL (Fig. 3.2A)\textsuperscript{77}. As a negative control, the CD spectrum of parent NTAIL was recorded in the presence of lysozyme (data not shown). The far-UV CD spectrum of XD (Fig. 3.2, grey line) is typical of a structured protein with a predominant $\alpha$-helical content, as indicated by the positive ellipticity between 185 and 200 nm, and by the two minima at 208 and 222 nm. After mixing parent NTAIL with a two-fold molar excess of XD, the observed CD spectrum differs from the corresponding theoretical average curve calculated from the two individual spectra (Fig. 3.2A). Since the theoretical average curve corresponds to the spectrum that would be expected if no structural variations occur, deviations from this curve indicate structural transitions. The observed deviations are consistent with an XD-induced $\alpha$-helical transition of parent NTAIL, as judged by the much more pronounced minima at 208 and 222 nm, and by the higher ellipticity at 190 nm of the experimentally observed spectrum compared to the corresponding theoretical average curve (Fig. 3.2A).

Fig. 3.2B and C shows the results obtained with the mutant NTAIL proteins in the presence of a two-fold molar excess of XD. Folding of NTAIL\textsubscript{Y451D} was induced by XD to an extent similar to parent NTAIL. In contrast, the Y518D substitution fully abrogated XD-induced folding of NTAIL, as judged by the superimposition of the experimental and average spectra. Under these experimental conditions, the XD concentration is approximately 7 $\mu$M, a concentration well above the reported $K_D$ for wt NTAIL (80 nM)\textsuperscript{22} but approximating the $K_D$ value determined by surface plasmon resonance analysis of NTAIL\textsubscript{Y518D}. The TFE data showed that NTAIL\textsubscript{Y518D} is capable of folding similar to parent NTAIL, so that results are consistent with the reduced binding affinity of XD for NTAIL.
Figure 3.2. Induced folding of NTAIL proteins in the presence of XD. Far-UV CD spectra of NTAIL (A), NTAIL Y451D (B) and NTAIL Y518D (C) in the presence of a two-fold molar excess of XD (full circles). Under these conditions, the concentration of NTAIL proteins and of XD is approximately 3.5 and 7 μM, respectively. The CD spectra of NTAIL proteins alone (black line) or XD alone (grey line), as well as the theoretical average curves calculated by assuming that no structural variations occur (see Materials and Methods) are also shown (diamonds). Data are representative of three experimental trials.
Figure 3.2
In other words, while $N_{\text{TAIL}} Y_{518D}$ is unable to undergo XD-induced folding under conditions where the parent protein is capable of such folding, it remains possible that induced folding would be possible in the presence of much larger amounts of XD.

An N protein Y518D substitution mutant enhances MeV minireplicon reporter gene expression

The Y518D substitution within Box-3 markedly reduces the binding affinity of $N_{\text{TAIL}}$ for the MeV P protein X domain. Such a change would alter polymerase interactions with the template by eliminating the constraint imposed on the cycles of binding and release required for elongation of transcripts or nascent genomic RNA, either of which would be reflected as enhanced minireplicon reporter gene expression. Consistent with this structure-function relationship, the N protein containing a Y518D substitution supported a significantly greater level of minireplicon reporter gene expression than that supported by parent N protein (Fig. 3.3A). The difference was 3.5-fold. A statistically significant increase was also observed for N protein Y451D, although the difference was only 1.7-fold. Differences do not reflect variation in the level of expression of the different N protein constructs, based upon Western blot analysis of transfected cell total protein.

Result are consistent with previously published data in which deletion of Box-3 significantly enhances the level of reporter gene expression that is supported by N protein$^{187}$. In that work, hsp72 failed to enhance reporter gene expression when N protein lacking Box-3 was used, in contrast to the stimulation of reporter gene expression with parent N. The current work reproduced this observation with N protein
Figure 3.3. Luciferase reporter gene expression from MeV minireplicons using either parent N protein or N protein Y to D substitution mutants as template. (A) Basal reporter gene expression supported by parent N, N-Y518D and N-Y451D. Significantly increases levels of reporter gene expression relative to that supported by N are indicated (*). Western blot analyses of representative transfected cell lysates show that differences in reporter gene expression do not reflect variations in the level of MeV N protein (α–N). β-actin protein levels are indicated as loading controls. (B) Hsp72 responsiveness of reporter gene expression supported by N or N-Y518D. A plasmid (2.5 µg) encoding a VSV-G tagged hsp72 was used to supplement hsp72 levels within the transfected cells. Hsp72 supplementation significantly enhanced reporter gene expression for minireplicons encapsidated by N but not N-Y518D. Western blot analysis of transfected cell total protein shows the levels of N and hsp72 in representative samples.
Y518D. The level of reporter gene expression supported by N-Y518D was not significantly affected by hsp72 supplementation (Fig. 3.3B).

In context of an infectious virus, N-Y518D is associated with a marked attenuation of viral gene expression that is attributed to increased DI particle formation

Changes in minireplicon reporter gene expression can be due to either changes in transcription or genome replication, restricting the approach to evaluating polymerase activity but not the nature of that activity (i.e., transcriptase versus replicase). For this reason, recombinant infectious virus encoding N-Y518D and N-Y451D was rescued.

The increased polymerase activity supported by N-Y518D in the minireplicons was associated with an attenuation of viral replication (Figure 3.4). Multistep growth curves showed parent Ed-MeV (i.e., Ed N) and Ed-MeV expressing N-Y451D to be comparable in terms of cell-free infectious viral progeny release. Peak viral titers were observed at 48-60 h.p.i., corresponding to 100% syncytial coverage of Vero cell monolayers. In contrast, Ed-MeV expressing N-Y518D yielded cell-free infectious viral progeny at titers that were two orders of magnitude lower than Ed N or Ed N-Y451D for the first 48 h.p.i. The attenuated progeny release for N-Y518D was associated with a slower progression of syncytial development, where cytopathic effect and peak progeny release was delayed until 72 h.p.i.

Northern blot analysis of total infected cell RNA revealed that the basis for attenuation of Ed N-Y518D replication was reduced viral transcript and full length genome expression within infected cells (Fig. 3.5). When Vero cells are infected at an
Figure 3.4. Cell free infectious viral progeny release by Ed N, Ed N-Y451D, and Ed N-Y518D mutant following infection of Vero cells (MOI = 0.01). Cells were infected in triplicate and results expressed as the average titer ± the standard deviation of the mean. Progeny release was statistically significantly suppressed for Ed N-Y518D for the first 48 h.p.i. relative to both Ed N and Ed N-Y451D.
Figure 3.5. (A) Northern blot analysis of total RNA derived from Vero cells infected at an MOI = 1.0 with Ed N, Ed N-Y451D or Ed N-Y518D. Samples were harvested at 36 h.p.i. and promoter proximal (N) and distal (H) transcripts and genome levels detected with strand and gene-specific riboprobes. (B) Transcript levels were not significantly different between Ed N and Ed N-Y451D, shown here based upon comparison of N transcript signals, but were markedly reduced for Ed N-Y518D (p<0.005, paired t-test). Findings were the same H transcripts (not shown). Genome levels for Ed N-Y518D were below the limits of detection, but were comparable between Ed N and Ed N-Y451D. GAPDH RNA expression was included as an RNA loading control.
MOI = 1.0 with Ed-MeV and RNA expression is examined at 36 h.p.i., both secondary transcription and genome replication have commenced\textsuperscript{136}. The Y518D mutation was associated with decreased cellular levels of both promoter proximal and promoter distal transcripts (i.e., N and H gene transcripts, respectively) and with decreased levels of viral genomic RNA. GAPDH-corrected N transcript signals were quantified for all constructs and were decreased 17-fold for Ed N-Y518D compared to parent Ed N (p < 0.005, paired \textit{t}-test). Genome levels were below the threshold of detection for Ed N-Y518D, precluding a quantitative comparison of genome levels by this approach. Transcript and genome levels were not significantly different between Ed N and Ed N-Y451D.

Attempts to amplify the original stocks of Ed N-Y518D virus were met with a progressive decline in titer, despite using low MOI’s (i.e., 0.01) for the infections. Titers were $1 \times 10^5$ TCID$_{50}$/mL for the first passage after rescue and had declined to $3.95 \times 10^2$ by the fourth passage (Fig. 3.6A). Such changes are consistent with DI particle activity that is otherwise observed with serial, high MOI passage of Ed-MeV in Vero cells\textsuperscript{180}. To determine if Ed N-Y518D exhibits an interference phenotype, co-infection experiments were performed. Vero cells were infected with Ed N at an MOI = 0.01 and co-infected with either Ed N-Y518D or Ed N-Y451D at an MOI = 0.001. Cell-free infectious viral progeny release was measured at 24 h.p.i. (Fig. 3.6B). Progeny release from cells infected with Ed N at an MOI of 0.01 or 0.011 (a negative control for the co-infections), or cells co-infected with Ed N and Ed N-Y451D were equivalent. In contrast, cells co-infected with Ed N and Ed N-Y518D exhibited a 15-fold reduction in titer that was statistically significant. Results are consistent with enhanced DI particle activity in Ed N-
Figure 3.6. (A) Serial low MOI (0.01) passage of Ed N-Y518D on Vero cells resulted in a progressive decrease in cell-free infectious viral progeny release, causing a greater than 250-fold loss in titer in just three passes. (B) In a viral interference assay, the titer that results from Ed N infection of Vero cell monolayers (MOI = 0.01) is significantly reduced by approximately 15-fold when co-infected with Ed N-Y518D (MOI = 0.001) (p-value<0.05, paired t-test). There is no effect when either Ed N or Ed N-Y451D are used in the co-infection at an MOI = 0.001. Cell-free progeny were titrated at 24 h.p.i.
Y518D relative to Ed N or Ed N-Y451D, and provide a basis for the attenuation of viral gene expression and infectious viral progeny release observed for Ed N-Y518D.

### 3.5 Discussion

The results of these studies show good correlation between XD binding affinity for and induced folding of $N_{TAIL}$. Peptide studies support the ability of an aspartic acid substitution to mimic tyrosine phosphorylation in terms of disrupting binding reactions for two molecules with similar binding preferences (i.e., hsp72 and XD). When incorporated into the full-length $N_{TAIL}$ molecule, the Y to D substitution within Box-3 results in a loss of both XD and hsp72 binding that is equivalent to deletion of the entire Box-3 domain. Comparison of the XD binding affinity between Box-2 and Box-3 shows that Box-2 binds much stronger, having a $K_D$ approximating that which is observed for XD binding to $N_{TAIL}$ (M. Oglesbee, unpublished observation). This suggests that Box-2 drives XD/$N_{TAIL}$ interaction, although it is clear that Box-2 is not sufficient to maintain such high affinity binding in the context of $N_{TAIL}$ because of the destabilizing effects of Box-3 deletions\(^{22}\). The same Y to D substitution outside of Box-2 and -3 (i.e., Y451D) has no effect on XD binding to $N_{TAIL}$. This tyrosine is well conserved amongst MeV strains even though it resides within a more highly variable region of $N_{TAIL}$ (i.e., outside of Box-1, -2, and -3)\(^{85}\). Results with $N_{TAIL}$ Y451D thus illustrate the ability of $N_{TAIL}$ to tolerate changes in sequence (a defining feature of disorder domains\(^{24}\)) without altering interaction with key binding partners, in this instance XD. Disorder domains are recognized for their ability to support interaction with a much greater diversity of molecules than more highly ordered structures\(^{51,52,86}\), and it is likely that Y451D does
influence the interaction with an undefined substrate, helping to explain the conservation of this amino acid. However, that substrate does not appear to be relevant to the replication of infectious virus in Vero cells, based upon similarity in growth curves and RNA expression between parent and Ed N-Y451D.

The CD analyses show that the ability of N_{TAIL}Y518D to undergo induced folding in the presence of TFE is comparable to parent N_{TAIL}. This suggests that the ability of XD to induce N_{TAIL} folding is intact, but that the secondary contacts that normally occur between Box-3 and XD are disrupted, destabilizing the XD/N_{TAIL} complex, accounting for the reduced XD binding affinity for N_{TAIL} and failure to document stable XD-induced conformational changes in N_{TAIL}. Functional interactions remain, based upon the ability of N-Y518D to support minireplicon reporter gene expression, and this may reflect the fact that P is functional as an oligomer^{45}, so that multiple linked XD domains may have a greater potential for weak albeit functional interactions with N_{TAIL}. The enhanced reporter gene expression that is observed with N-Y518D supports enhanced cycles of release and binding that a reduced XD/N_{TAIL} binding affinity would predict and that would result in enhanced polymerase elongation on the template. The enhanced elongation rate could influence transcription as well as genome replication, a distinction that is not readily made with minireplicons. Minireplicon reporter gene expression that is supported by N-Y518D is also unresponsive to hsp72 supplementation. Elevated basal reporter gene activity and lack of response to hsp72 supplementation is also seen with N_{TAIL}\Delta^{187}. Collectively, these findings provide support for a model whereby Box-3 stabilizes XD/N_{TAIL} complexes, imposing constraint on polymerase elongation by slowing cycles of N_{TAIL} binding and release, and where that Box-3 stabilizing influence
can be removed by Y phosphorylation or neutralization through direct binding to hsp72. Although N-Y451D supports a small but statistically significantly greater level of reporter gene expression than parent N protein, analysis of infectious virus encoding N-Y451D suggests that this difference is not biologically significant (at least in vitro) based upon similarities to parent virus in terms of RNA expression and progeny release.

Infectious virus encoding the N-Y518D substitution was rescued in order to determine how template mediated changes in polymerase activity influenced transcription and/or genome replication. However, expression of both viral transcripts and full length genomes by Ed N-Y518D were significantly attenuated compared to Ed N (parent) and Ed N-Y451D following high MOI infections, providing a basis for the reduced cell-free infectious viral progeny release that was observed following low MOI infections. Low MOI passage of Ed N-Y518D was performed in order to amplify the low passage viral stocks, but this resulted in a progressive reduction in total viral yield, consistent with high-level defective interfering (DI) particle production. The interference capability of Ed N-Y518D was demonstrated when used in co-infections with Ed N, where the ratio of Ed N to Ed N-Y518D was 10:1, and the total MOI was only 0.011. More recently, primer pairs specific for 5’ copy-back DI genomes were used in the RT-PCR analysis of total infected cell RNA, revealing the presence of two DI species in Ed N-Y518D infected cells under conditions where none are detected in Ed N infected cells (Oglesbee et al., unpublished observation). The DI genomes were 480 and 420 nucleotides in length, their identity being confirmed by sequence analysis of cloned amplicons. Such particles are produced when the 5’ end of the negative strand genome is being produced from plus strand genomic replicative intermediates, and the polymerase switches templates from the
plus strand template to that formed by the nascent negative strand genome\textsuperscript{30}. The strong encapsidation signals present on these copy-back particles, combined with their short lengths, assures their efficient production at the expense of parent virus, consuming N, P, and L proteins produced by that parent virus in order to support DI particle replication\textsuperscript{30}. Reduced binding affinity of P (i.e., XD) for the N\textsubscript{TAIL} template thus appears to facilitate template switching of the viral polymerase during replication. While it may enhance minireplicon reporter gene expression, the result is to attenuate replication of infectious virus.

Defective interfering particles are linked to the establishment of viral persistence\textsuperscript{158}, and their production in the Ed N-Y518D mutant is consistent with published data linking N protein tyrosine phosphorylation to persistence in neurons\textsuperscript{152}. The latter finding may be DI-mediated, both by interference with replication of wild-type virus and through the induction of the Type I Interferon (IFN-αβ) response\textsuperscript{157}. The persistent state of infection was maintained by high level IFN-αβ secretion\textsuperscript{142}, and the cells are characterized by elevated activity of the IFN-αβ-responsive proteins 2,5-oligoadenylate cyclase and p68, a double-stranded RNA-activated protein kinase\textsuperscript{152}. We propose that a positive feedback loop may be active in persistently infected cells, where IFN-αβ production at the time of infection induces tyrosine phosphorylation, which promotes DI formation that in turn stimulates greater IFN-αβ expression in infected cells. Activation of one such pathway studied in persistently infected neuroblastoma cells results in the increased expression and phosphorylating activity of members of the PKC family of kinases, specifically the -α, -ε and -ζ isoenzymes\textsuperscript{10,155}. PKCα activity has been linked to downstream activation of the tyrosine kinase c-Src, leading to increased
expression of intracellular adhesion molecule-1 (ICAM-1) and cyclooxygenase-2 (COX-2) in a TNF-α-dependent pathway. The Scansite website (http://scansite.mit.edu/index.html) scans a protein of interest for similarities to known domains of well-characterized proteins, including sites that may support phosphorylation. When analyzed in this database, many sites on the MeV N protein had positive hits, in particular a portion of the molecule including the tyrosine at position 518 that showed a similarity to the Src-dependent phosphorylation site in inositol polyphosphate-5-phosphatase. This sequence scored in the top 1.8% of sites analyzed and compared to the reference sequence. The Y451D residue was not identified as a potential phosphorylation site. Finally, casein kinase II, which is known to mediate phosphorylation of the MeV P protein, can exhibit both serine/threonine and tyrosine kinase functions, and can be linked to general MeV N protein phosphorylation (Banerjee et al., unpublished observation). Future studies will focus upon identification of candidate N protein kinases and signal transduction pathways that may lead to their activation.

3.6 Acknowledgments

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CHAPTER 4

HSP40 CO-CHAPERONES ARE REQUIRED FOR HIGH AFFINITY BINDING BETWEEN HSP72 AND THE MEASLES VIRUS N PROTEIN UNSTRUCTURED DOMAIN

4.1 Abstract

Members of the 70-kDa heat shock protein family, including the major inducible isoform (hsp72), engage in high affinity binding to protein substrates in an ATP-dependent manner. Hydrolysis of ATP to form ADP allows hsp72 to form high affinity complexes with client proteins. The 40-kDa family of heat shock proteins (HSP40) can enhance the inherently low ATPase activity of hsp72, thereby acting as a co-chaperone for hsp72 function. Hsp72 enhances measles virus (MeV) gene expression by binding to the nucleocapsid protein (N), but the contribution of hsp72 co-chaperones to this functional interaction has yet to be determined. In this study, we examined the hypothesis that co-chaperone activity is required for the high affinity binding between MeV N and hsp72. We identified a prokaryotic HSP40 homologue (DnaJ) as a contaminant of commercial hsp72 preparations used previously to establish the high affinity nature of the binding between hsp72 and NTAIL, the unstructured C-terminus of the MeV N protein. High affinity binding was not observed in more highly purified hsp72 preparations lacking co-chaperones. Addition of purified recombinant mammalian HSP40 (i.e., Hdj1)
to purified hsp72 in substoichiometric amounts reconstituted the high affinity binding of hsp72 to NTAIL. These results demonstrate that the presence of a co-chaperone is necessary for high affinity binding of hsp72 to NTAIL, and that this effect may be mediated by enhancement of hsp72 ATPase activity.

4.2 Introduction

The members of the 70-kDa HSP family (HSP70) possess two main functional domains: the C-terminal 25-kDa contains the substrate binding domain (SBD) while the 44-kDa N-terminus comprises the nucleotide binding domain (NBD). The chaperone function of HSP70 is dependent upon transient associations with the target, and the reversible nature of these associations is mediated by the cyclic binding, hydrolysis and release of ATP. The phosphorylation state of the nucleotide bound to the NBD (either ATP or ADP) determines the conformation of the SBD reviewed in 27,28. With ATP bound, the SBD is open, able to rapidly associate with and dissociate from exposed hydrophobic domains on target peptide sequences. In this state, SBD has a low affinity for target substrates. Hydrolysis of bound ATP to ADP causes a conformational change in the SBD that locks it to the substrate, stabilizing the association between HSP70 and the target. ATP hydrolysis to ADP is thus required for the enhanced binding affinity between HSP70 and its substrate target that is critical to its function as a protein chaperone. Only upon ADP exchange for ATP is the SBD opened and the peptide released, allowing the binding cycle to repeat. The intrinsic ATPase activity of HSP70 is low, so co-chaperones are required for the high-affinity binding cycle to take place. HSP70 co-chaperones have been widely demonstrated in prokaryotes and eukaryotes, and
all share the J domain motif, a 70 amino acid sequence that mediates HSP70-NBD
binding\textsuperscript{67,172}. In mammalian cells, this role is filled by the members of the 40-kDa HSP
family (HSP40) which bind to the NBD and accelerate the ATP hydrolysis to ADP\textsuperscript{108}.

The measles virus N protein (MeV N) is a binding partner of hsp72, the highly
inducible HSP70 family member. Overexpression of hsp72 enhances measles virus
(MeV) gene expression and cytopathic effect both \textit{in vitro} and \textit{in vivo} as a result of this
interaction\textsuperscript{34,186}. The hsp72-binding domains of MeV N are found on the unstructured C-
terminal 125 amino acids ($\text{N}_{\text{TAIL}}$)\textsuperscript{186,187}. Hsp72 binds $\text{N}_{\text{TAIL}}$ with high affinity
characterized by rapid association and slow dissociation\textsuperscript{186}, consistent with the presence
of a co-chaperone. Purified recombinant $\text{N}_{\text{TAIL}}$ mimics MeV nucleocapsid fragments in
terms of hsp72 binding\textsuperscript{186,187}, and the ATP-dependence of these binding reactions have
been shown\textsuperscript{175,188}. For both $\text{N}_{\text{TAIL}}$ and nucleocapsid, ATP supplementation enhances the
dissociation of hsp72. Thus, the hypothesis examined in this study is that the high affinity
hsp72/$\text{N}_{\text{TAIL}}$ interaction requires co-chaperone activity. Analysis of recombinant human
hsp72 preparations subjected to varying degrees of purification revealed differences in
co-chaperone content. Highly purified hsp72 did not exhibit the binding affinity typical
of those that also contain the appropriate co-chaperones, where equilibrium dissociation
constants ($K_D$) are in the nM range. Finally, co-chaperone supplementation of highly
purified hsp72 reconstituted the high affinity for $\text{N}_{\text{TAIL}}$ that conforms to the expected
binding model.
4.3 Materials and Methods

Proteins

The commercially prepared product (Assay Designs, StressGen) is a recombinant form of human hsp72 (i.e., hsp70 A gene product) expressed in *Escherichia coli*. It is reported by the manufacturer to be >90% pure based on densitometric analysis of protein samples separated by SDS-PAGE and stained with Coomassie blue. Highly purified hsp72 was provided by Dr. Sonia Longhi (University of Marseille I et II and the CNRS), where His-tagged proteins are purified by both Ni\(^{2+}\) affinity and gel filtration chromatography (see below for production and purification). The same process was used by Dr. Longhi for the production of Hdj1, one of the most widely expressed and well-studied forms of mammalian HSP40. Like most HSP40 family members, Hdj1 shares homology with the prototype J domain protein, the bacterial DnaJ, and the latter may substitute for the former in providing co-chaperone functions for hsp72\(^{108}\). The purification process for the proteins produced by Dr. Longhi is summarized in Fig. 4.1. Production of purified recombinant MeV N\(_{TAIL}\) has been previously described\(^{22}\).

Construction of hsp72 expression plasmids

The hsp72\(_{HN}\) gene construct, encoding the hsp72 full-length protein with a hexahistidine tag fused to its N-terminus, was obtained by recursive PCR, using pT7 HSP2 VSV1 as template\(^{187}\) and *Pfx* polymerase (Invitrogen). Forward primer (5’-gatagaaccatgCATCATCATCATCATCATgccaaagccgcggcagtcggcatcgac -3’) was designed to introduce a hexahistidine tag encoding sequence (upper case) at the N-terminus of hsp72, while reverse primer (5’-ggggaccacctttgtacaagaaagctgggtcttaatctacctctcaatgtggggcc -3’) was designed to
Figure 4.1. Purification of recombinant hsp72 (A) and Hdj1 (B) from bacteria. Coomassie blue staining of a 12% SDS-PAGE. TF: Bacterial Lysate (Total Fraction); SN: Clarified Supernatant (Soluble Fraction); FI-T: Flow-Through (Unretained Fraction). E_{Ni}: Eluent from Immobilized Metal Affinity Chromatography; E_{GF}: Eluent from Gel Filtration.
introduce an *AttB2* site (bold). The PCR amplification product was further used as template in a second PCR step, using forward primer (5’-*ggggacaagtgtgatcataacaagaacgct*tcgaaggagatagaaccatcatacatcatcata -3’), designed to introduce an *AttB1* site (bold), and reverse primer as above. After *Dpn*I digestion, to remove methylated parental DNA, and purification (Montage PCR, Genomics, Millipore), the PCR product was cloned into the pDest14 vector (Invitrogen) using the Gateway recombination system (Invitrogen). The final construct is referred to as pDest14/hsp72<sub>HN</sub>. The sequences of the coding region of all the Hsp72 constructs were confirmed by sequence analysis (GenomeExpress).

**Construction of Hdj1 expression plasmids**

The Hdj1<sub>HN</sub> gene construct, encoding the Hdj1 protein with a hexahistidine tag fused to its N-terminus, was obtained by PCR as described above. Forward primer (5’-*gatagaacctgCATCATCATCATCATCATAgtagaagactacagacgttg* -3’) was designed to introduce a hexahistidine tag encoding sequence (upper case) at the N-terminus of Hdj1, while reverse primer (5’-*ggggaccactttgatcataacaagaacgctggt*tcgtatatttgaagaacctcataagtactcata -3’) was designed to introduce an *AttB2* site (bold). The PCR amplification product was further used as template in a second PCR step, using forward primer (5’-*ggggacaagtgtgatcataacaagaacgct*tcgaaggagatagaaccatcatacatcatcata -3’), designed to introduce an *AttB1* site (bold), and reverse primer as above. After *Dpn*I digestion, to remove methylated parental DNA, and purification (Montage PCR, Genomics, Millipore), the PCR product was cloned into the pDest14 vector (Invitrogen) using the Gateway recombination system (Invitrogen). The final construct is referred to as
pDest14/Hdj1_{HN}. The sequences of the coding region of all the Hdj1 constructs were confirmed by sequence analysis (GenomeExpress).

**Expression of recombinant proteins**

The *E. coli* strain Rosetta [DE3] pLysS (Novagen) was used for the expression of all the constructs. Since both Hdj1 and hsp72 genes contain several rare codons that are used with a very low frequency in *E. coli*, co-expressions of Hdj1 or hsp72 constructs with the plasmid pLysS (Novagen) were carried out. This plasmid, which supplies six rare tRNAs, carries also the lysozyme gene, thus allowing a tight regulation of the expression of the recombinant gene, as well as a facilitated lysis. Cultures were grown overnight to saturation in LB medium containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. An aliquot of the overnight culture was diluted 1/25 in LB medium and grown at 37°C. At OD_{600} of 0.6, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM, and the cells were grown at 37°C for 3.5 hours. The induced cells were harvested, washed and collected by centrifugation. The resulting pellets were frozen at –20°C.

Isotopically substituted (\(^{15}\)N) N_{TAIL} and Hdj1 were prepared by growing bacteria transformed by the pDest14/N_{TAILHN} and pDest14/Hdj1_{HN} constructs, respectively, in minimal M9 medium supplemented with \(^{15}\)NH_{4}Cl (0.8 g/l) as already described\(^{22}\).

**Purification of recombinant proteins**

Purification of all recombinant proteins was carried out by re-suspending the cellular pellets of recombinant bacteria in 5 volumes (v/w) of lysis buffer, consisting of buffer A (50 mM Sodium Phosphate pH 8, 300 mM NaCl, 10 mM Imidazole, 1 mM phenyl-methyl-sulfonyl-fluoride (PMSF)) supplemented with lysozyme 0.1 mg/ml,
DNase I 10 µg/ml, and protease inhibitor cocktail (Complete® Roche) (one tablet per 50 ml of lysis buffer). After a 30 min incubation with gentle agitation, the cells were disrupted by sonication (using a 750 W sonicator and 4 cycles of 45 s each at 50% power output), and the lysate was clarified by centrifugation at 30,000 g for 30 min.

For bacteria transformed with the pDest14/hsp72HN construct, the clarified supernatant obtained from a 500 ml culture was incubated for 1 h at 4°C with 2 ml of chelating Sepharose Fast Flow Resin preloaded with Ni²⁺ ions (GE Healthcare), previously equilibrated in buffer A. The resin was washed with buffer A, and the protein was eluted in buffer A containing 250 mM Imidazole. The eluate was analyzed by SDS-PAGE for the presence of the desired product, and the fractions containing the recombinant product were pooled and further purified by gel filtration using a Sepharose 200 16/60 column (GE Healthcare). The protein was eluted with 10 mM Sodium Phosphate buffer pH 7. Following elution, the fractions containing Hsp72 were collected and concentrated using Amicon® ultra 10-kDa (Millipore).

For bacteria transformed with the pDest14/Hdj1HN expression plasmid the clarified supernatant obtained from a 500 ml culture was incubated for 1 h at 4°C with 2 ml chelating Sepharose Fast Flow Resin preloaded with Ni²⁺ ions previously equilibrated in buffer A. The resin was washed with buffer A, and elution was carried out in buffer A containing 250 mM Imidazole. The eluate was analyzed by SDS-PAGE for the presence of the desired product. The eluted fractions containing the recombinant product were pooled, loaded onto a Sepharose 200 16/60 column (GE Healthcare) and eluted with 10 mM Sodium Phosphate pH 7, NaCl 300 mM. Following elution, the fractions containing
Hdj1 were collected and concentrated as described above. All the purified proteins were stored at –20°C.

**Determination of protein concentration**

Protein concentrations were calculated using OD$_{280}$ measurements and the theoretical absorption coefficients $\varepsilon$ (mg/ml.cm) at 280 nm as obtained using the program ProtParam at the EXPASY server (http://www.expasy.ch/tools).

**Circular dichroism**

The folding state of hsp72 proteins and Hdj1 was checked by far-UV circular dichroism. The CD spectra were recorded on a Jasco 810 dichrograph using 1-mm thick quartz cells in 10 mM Sodium Phosphate pH 7 at 20°C. They were measured between 185 and 260 nm, at 0.2 nm/min and were averaged from three independent acquisitions. The small contribution of buffer was subtracted from experimental spectra. Spectra were smoothed using the "means-movement" smoothing procedure implemented in the SpectraManager package. Mean ellipticity values per residue ([\(\Theta\)]) were calculated as $[\Theta] = 3300 \Delta A/(l \cdot c \cdot n)$, where $l$ (path length) = 0.1 cm, $n$ = number of residues, $m$ = molecular mass in daltons and $c$ = protein concentration expressed in mg/ml. Number of residues ($n$) is 346 for Hdj1 and 647 for hsp72. The molecular mass ($m$) is 38,867 Da for Hdj1 and 70,861 for hsp72. Protein concentrations of 0.1 mg/ml were used when recording spectra. The $\alpha$-helical content was derived from the ellipticity at 222 nm as described in Myers et al$^{117}$.

**Mass Spectrometry (MALDI-TOF)**

The identity of purified proteins was confirmed by mass analysis of tryptic fragments obtained after digesting 1 µg of purified protein obtained after separation onto
SDS-PAGE with 0.25 µg trypsin. Search for possible traces of contaminating bacterial GroEL (AAS75782), GroES (NP_290775), DnaJ (NP_414556) and human Hdj1 (NP_006136) proteins in both recombinant or commercial (StressGen) hsp72 protein samples was carried out by mass analysis of tryptic fragments obtained upon incubation of the proteins (2 µg each) in 40 µl of a 12.5 ng/µl modified trypsin solution (Promega) in 50 mM ammonium bicarbonate pH 8.6 overnight at 37°C.

The tryptic peptides were analyzed in the Autoflex matrix-assisted laser desorption ionization/time of flight (Bruker Daltonics, Bremen, Germany). Peptide fingerprints were obtained and compared with in-silico protein digest (Biotools, Bruker Daltonics, Germany). The mass standards were either autolytic tryptic peptides or peptide standards (Bruker Daltonics).

Analysis of commercial and highly-purified hsp72 samples

Protein content of commercial versus highly purified hsp72 was assessed by Western blot analysis. Total protein concentration of hsp72 preparations was determined with the Pierce Micro BCA protein assay kit. For Western blot analysis, 10 µg of total protein was loaded onto pre-cast 10% Bis-Tris polyacrylamide gels (Invitrogen) for electrophoresis, then transferred to nitrocellulose membranes. Membranes were blocked with 5% bovine serum albumin (BSA) in 1x PBS with 0.01% Tween-20. Membranes were then incubated with either an anti-DnaJ rabbit pAb (Assay Designs) diluted 1:5000 or an anti-Hsp40 rabbit pAb (Assay Designs) diluted 1:5000 in 1x PBS with 1% BSA and 0.01% Tween-20 for 1 hour, followed by incubation with horseradish peroxidase-labeled goat anti-rabbit polyclonal antibody (pAb) (Zymed) diluted 1:2000 in 1x PBS with 1% BSA and 0.01% Tween-20. Signal was detected by chemiluminescence (Superwest pico
chemiluminescence detection kit, Pierce, Inc.). For Coomassie blue staining, hsp72 preparations separated by SDS-PAGE were incubated in fixing solution (25% v/v isopropanol, 10% v/v acetic acid, 65% H₂O) for one hour, stained (10% v/v acetic acid, 90% H₂O, 0.006% v/v Coomassie blue) for two hours, then de-stained (10% v/v acetic acid, 90% H₂O) for two hours. Stained gels were transilluminated and photographed using an Alpha Innotech Alphaimager.

**Binding reaction analysis**

Real time analysis of binding between the different hsp72 preparations and the NTAIL construct used BIAcore 3000 instrumentation (BIAcore, GE Healthcare) as previously described by our group. Hsp72 (5.0 μg/ml in acetate buffer pH 5.5) was covalently bound to activated carboxyl groups of CM5 sensor chips using amine-coupling chemistry (BIAcore, GE Healthcare). The immobilization level for the commercially prepared hsp72 was 640 RU, and for the purified hsp72 was 555 RU, where 1,000 RU equals a change in mass of 1 ng/mm² on the sensor surface. Reactions were performed at 25°C. Remaining flow channels on the sensor chip included a control for non-specific interactions between analyte and the sensor surface (i.e., activated/blocked flow channel). NTAIL was passed over the prepared surfaces in HBS-P buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 0.005% surfactant P-20) containing 2.5 mM magnesium acetate and 2.5 mM ATP. Sensorgrams plotted changes in surface plasmon resonance (measured in RU) as a function of time.

Multiple sensorgrams representing various analyte concentrations were analyzed by using BIAevaluation 4.1 software. Fitting of experimental data to well-characterized binding reactions was used to define reaction rate and equilibrium constants. Signal
changes on the activated/blocked control channel were subtracted from the hsp72 and \( N_{\text{TAIL}} \) protein interactions using in-line reference and the subtracted sensorgrams were analyzed. Kinetic and equilibrium constants were calculated from global analysis of reactions with multiple analyte concentrations (100, 50, 25, 10, 5, 2, 1, 0.5, 0.1, 0.05, 0 \( \mu \text{M} \) \( N_{\text{TAIL}} \) proteins). Curves generated with serial analyte concentrations were applied to the 1:1 binding with drifting baseline model.

The same procedure was followed for analysis of binding between \( N_{\text{TAIL}} \) and either purified hsp72 alone, purified Hdj1 alone or hsp72 combined with Hdj1. For the combined channel, the molar ratio of the hsp72/Hdj1 mixture was 10:1, approximating the 90% purification level reported for the commercially prepared hsp72. Ligand immobilization levels for the hsp72 alone or hsp72 + Hdj1 flow channels were 698 and 720 RU, respectively. Hdj1 binding level was 55 RU, approximating the expected immobilization level on the hsp72 + Hdj1 channel. Conditions for ligand immobilization, analyte flow and analysis of binding data were the same as described above.

### 4.4 Results

**Co-chaperones are only present in commercially prepared hsp72**

To examine the differences in protein content between commercial and purified preparations of hsp72, samples of each were subjected to SDS-PAGE and analyzed by Coomassie blue staining and Western Blot analysis for DnaJ and Hdj1 (Fig. 4.2). Coomassie staining confirmed the presence of additional protein components within the commercially prepared product, in particular a narrow band migrating with the 41-kDa band of a protein ladder. Western Blot analysis showed that the commercially prepared
Figure 4.2. Protein expression in two preparations of human recombinant hsp72. Hsp72 purified by gel filtration (1) and commercially prepared hsp72 (2) were resolved by SDS-PAGE and stained with Coomassie blue for 2 hours. The commercially prepared sample contains additional protein bands, including one at approximately 41-kDa. B. Western blot analysis of gel purified and commercially-prepared hsp72 for DnaJ and Hdj1. DnaJ, the bacterial analogue of human HSP40 is present only the commercially available product, while neither sample contained detectable levels of Hdj1. Heat-shocked A549 protein lysates were used as a control for Hdj1 expression (C).
sample contains DnaJ, the bacterial homologue of mammalian Hsp40, but no measurable levels of Hdj1, consistent with a product grown in and isolated from *Escherichia coli*, as reported by the manufacturer. More sensitive mass spectroscopic analysis confirmed that human Hdj1 as well as DnaJ sequences may be demonstrated in the commercial hsp72 preparation, but neither is detectable in the purified hsp72 (data not shown).

**Highly-purified hsp72 binds N\textsubscript{TAIL} with low affinity**

The commercial hsp72 preparation has been used in the past to demonstrate binding with MeV nucleocapsid\textsuperscript{187}, short polypeptides mimicking the C terminus of the MeV N protein\textsuperscript{187} and with N\textsubscript{TAIL}\textsuperscript{186}, the unstructured portion of the MeV N protein responsible for mediating the interactions between nucleocapsid and its binding partners. For the latter, the hsp72 binding affinity (i.e., the $K_D$) was found to be 11 nM\textsuperscript{186}. Here, N\textsubscript{TAIL} binding to the commercially prepared hsp72 was compared to that of the highly purified hsp72, also using surface plasmon resonance technology (BIAcore) (Table 4.1). The commercially prepared sample was able to achieve a high binding affinity with N\textsubscript{TAIL}, with a $K_D = 84$ nM, in good agreement with previously reported values, while the purified sample only achieved a $K_D = 64$ µM. Similarly, the association and dissociation rate constants ($k_a = 1.14 \times 10^2$/Ms, $k_d = 9.39 \times 10^{-6}$/s) for the commercially prepared hsp72 were in line with those reported previously, and consistent with a binding cycle characterized by a rapid association and slower dissociation of the interacting partners. Purified hsp72 exhibits a relatively unchanged association rate constant ($4.15 \times 10^2$/Ms), but a much more rapid dissociation ($k_d = 2.67 \times 10^{-2}$/s). Such a pattern suggests that
<table>
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<th>Ligand</th>
<th>Quality of Fit</th>
<th>$k_a(1/\text{Ms})$</th>
<th>$k_d(1/\text{s})$</th>
<th>$K_D$</th>
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<tr>
<td></td>
<td>Residuals</td>
<td>Chi$^2$</td>
<td></td>
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<tr>
<td>commercial hsp72</td>
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<td>1.14 x 10$^2$</td>
<td>9.39 x 10$^{-6}$</td>
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<td>purified hsp72</td>
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<td>0.157</td>
<td>4.15 x 10$^2$</td>
<td>2.67 x 10$^{-2}$</td>
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</table>

Table 4.1. Calculated association and dissociation constants ($k_a$, $k_d$) and equilibrium dissociation constants ($K_D$) for the binding of $N_{\text{TAIL}}$ protein with commercial and gel purified hsp72.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Quality of Fit</th>
<th>$k_a(1/\text{Ms})$</th>
<th>$k_d(1/\text{s})$</th>
<th>$K_D$</th>
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<td></td>
<td>Residuals</td>
<td>Chi$^2$</td>
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<td></td>
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<td>0.180</td>
<td>1.10 x 10$^2$</td>
<td>9.99 x 10$^{-6}$</td>
</tr>
</tbody>
</table>

Table 4.2. Calculated association and dissociation constants ($k_a$, $k_d$) and equilibrium dissociation constants ($K_D$) for the binding of $N_{\text{TAIL}}$ protein with hsp72 alone, Hdj1 alone, or a combination of hsp72 and Hdj1.
hsp72, without a co-chaperone to catalyze the hydrolysis of ATP, cannot efficiently engage the PBD clamp before rapid dissociation of the bound protein.

**Highly-purified hsp72 supplemented with Hdj1 binds N_{TAIL} with high affinity**

Separate binding studies confirmed that co-chaperones (in this case Hdj1, a widely expressed form of mammalian HSP40) are able to reconstitute the high affinity N_{TAIL} binding characteristic of commercially prepared hsp72 (Table 4.2). The approach used a 10:1 ratio of hsp72 to Hdj1 to mimic the conditions used in previous binding experiments with commercially available hsp72 (i.e., a sub-stoichiometric amount of co-chaperone relative to hsp72). The combined hsp72 + Hdj1 surface exhibited strong binding affinity for N_{TAIL} (K_D = 90.8 nM) with rapid association (k_a = 1.10 x 10^5) and slow dissociation (k_d = 9.99 x 10^{-6}). This represents a greater than 750-fold increase in binding affinity compared to hsp72 alone (K_D = 68.5 µM), closely matching the differences in N_{TAIL} binding between commercially available and purified hsp72. Again, the major differences in rate constants are in the rate of dissociation. These results support a role for Hdj1 in enhancing the affinity of hsp72 for N_{TAIL}. This effect is observed at sub-stoichiometric amounts of Hdj1, and appears to reflect an influence of Hdj1 on the ability of hsp72 to hydrolyze ATP.

**4.5 Discussion**

Hsp72 requires co-chaperone activity to achieve the high affinity for N_{TAIL} that has previously characterized their interactions\textsuperscript{186,187}. Critical to this link to past experimental observations is the fact that DnaJ, the bacterial homologue of the
mammalian HSP40 family of proteins, can effectively substitute for HSP40 and promote the high affinity binding between HSP70 and its substrates. We were able to identify DnaJ in a commercial hsp72 product used to define high affinity for both NTAIL and MeV ribonucleoprotein complexes. Use of a highly-purified hsp72 without contaminating co-chaperones drops NTAIL binding affinity by over three orders of magnitude. The dominant change when co-chaperones are omitted from binding experiments is a markedly increased rate of dissociation. This mirrors other studies which found that the critical step in achieving stable binding with target substrates is the rapid hydrolysis of bound ATP to ADP, and that co-chaperone activity is required to improve the otherwise slow inherent ATPase activity of HSP70 family members. Without co-chaperones, ATP hydrolysis proceeds too slowly to induce rapid closure of the HSP70 binding pocket around its target, allowing for rapid dissociation of the chaperone-substrate complex.

Binding studies utilized a sub-stoichiometric ratio of one molecule of Hdj1 to ten molecules of hsp72 to focus on the ability of Hdj1 to catalyze the ATPase activity of hsp72. J domain proteins also bind nascent, unstructured proteins and are proposed to direct them to HSP70’s, facilitating their association by bringing them into close apposition with one another. Like HSP70 molecules, they bind peptide sequences enriched in aromatic and hydrophobic residues, but with a lower affinity (i.e., in the micromolar range) for their targets. Similarly, at the low sensor-bound Hdj1 levels used to examine the interaction with NTAIL, there was only a low level affinity measured between the two partners (K_D = 254 µM). This suggests that, at least at low Hdj1 concentrations, there is no direct contribution to the affinity measured between
hsp72/Hdj1 and N\textsubscript{TAIL}, although such an effect cannot be excluded within a living cell based upon these results alone.

Future studies will confirm that the purified hsp72 used in these studies, without the benefit of co-chaperones, exhibit the low basal ATPase activity described previously, and that supplementation with purified Hdj1 is responsible for return of ATPase function necessary for high affinity binding with target substrates. Also to be established is the relevance of Hdj1, which is constitutively expressed\textsuperscript{43,65} and may also be augmented by heat shock\textsuperscript{65}, in cell systems used to analyze hsp72-MeV interactions.

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112


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129