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Structural Basis for Antagonism of Innate Immunity by Poxvirus

A dissertation submitted to Trinity College, Dublin for degree
of Doctor of Philosophy

By

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Insulin Potentially by Foxvins

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*I dedicate this thesis to
my family, and my wife, Tomoko
for their constant support.*

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Declaration

This thesis is submitted by the undersigned to the University of Dublin for the examination of Doctorate in Philosophy. The work herein is entirely my own and has not been submitted as an exercise for a degree in any other university. The library at Trinity College, Dublin, has my full permission to lend or copy this thesis on request.

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Abstract

Vaccinia virus encodes many proteins that antagonize our innate immune system. It has been shown that some of them adopt a Bcl-2 like fold in spite of a lack of sequence identities to the Bcl-2 superfamily. Unlike viral Bcl-2 homologs that antagonize apoptosis by binding to cellular Bcl-2 targets, these viral proteins serve other functions. Despite the identification of numerous cellular targets for these proteins, there is currently little structural information regarding the protein-protein complexes formed during infection.

In previous studies, DDX3 was identified as one of the cellular targets of the *vaccinia virus* Bcl-2 fold protein K7. K7 antagonizes innate immune signaling by binding to DDX3. Previous NMR and biochemical studies of K7 revealed the solution structure of uncomplexed K7. However, a K7-DDX3 complex structure is needed for a more detailed analysis of the mechanism by which K7 antagonizes host immune signaling. Full-length K7 was co-crystallized with a DDX3 peptide that retained a majority of the binding affinity from the full-length protein. The crystal structure of K7 in complex with DDX3 peptide was solved at 1.6 Å resolution and revealed that the DDX3 peptide binds to a novel hydrophobic binding interface of K7 that is not utilized in other viral and cellular Bcl-2 proteins. The monomeric character and flexible $\alpha 6$ helix of K7 are crucial for binding to DDX3.

The *Vaccinia virus* A46 protein is reported to antagonize the innate immune system by binding to cellular TIR domain-containing adaptor proteins such as MyD88, Mal, TRAM, and TRIF. In previous modeling studies, it has been suggested that A46 adopts a Bcl-2 fold, but there is no biochemical or biophysical study of the protein yet published. Here we purified and characterized A46 and its interactions with cellular targets Mal and TRAM by biophysical methods. Although crystals of the isolated protein and its complex with targets remain elusive, some useful data for the design of future structural studies was generated. In particular, it was observed that an α -helical sub-domain of A46 is sufficient for binding to the TIR domain of Mal.

Overall, the structural studies described here provide some initial insight into the mechanistic basis for viral subversion of host immunity. The various tools and expertise developed during this work will be essential for future investigations into the pathogen-host relationship.

Abbreviations

AIM	absent in melanoma
ASC	apoptosis-associated speck-like protein containing a CARD
Bcl-2	B-cell lymphoma 2
BH	Bcl-2 Homology
BIR	baculovirus inhibitor repeat
Btk	Bruton's tyrosine kinase
CARD	caspase recruitment domain
CD	circular dichroism
CLRs	C-type lectin receptors
CMV	Cytomegalovirus
CTD	C-terminal domain
CTLD	C-type lectin domain
DAI	DNA-dependent activator of IRFs
DAMPs	damage-associated molecular patterns
DD	death domain
DDX3X	DEAD-box protein 3 X linked
DLS	dynamic light scattering
DTT	dithiothreitol
ER	endoplasmic reticulum
FADD	Fas-associated death domain protein
GAS	IFN- γ activated sequence
GTP	guanosine-5'-triphosphate
HA	hemagglutinin
HIN-200	hemopoietic IFN-inducible nuclear proteins with a 200-amino acid residue motif
IFI	IFN- γ inducible gene
IFNAR	IFN- α receptor
IFN	interferon
IKK	I κ B kinase
IL	interleukin
IPAF	Ice protease activating factor
IPS-1	IFN promoter stimulator-1
IRAK	interleukin-1 receptor-associated kinase
IRF	interferon regulatory factor

ISGF	IFN-stimulated gene factor
ISRE	IFN-stimulated response elements
JAK	Janus-family kinases
LGP	laboratory of genetics and physiology
LIC	ligation-independent cloning
LPS	lipopolysaccharides
LRR	leucine-rich repeat
Mal	MyD88 adapter like
MAPK	mitogen-activated protein kinase
MBP	maltose binding protein
MDA	melanoma differentiation-associated gene
MMTV	mouse mammary tumor virus
MyD88	myeloid differentiation factor 88
NACHT	NAIP, CIITA, HET-E and TP1
NBS	nucleotide-binding site
NEMO	NF- κ B essential modifier
NF- κ B	nuclear factor kappa-B
NK	natural killer
NLRP	NACHT, LRR and PYD domains-containing protein
NLRs	NOD-like receptors
NOD	nucleotide-binding oligomerization domain
ORF	open reading frame
PAMPs	pathogen-associated molecular patterns
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PIP2	phosphatidylinositol 4,5-bisphosphate
PRRs	pattern-recognition receptors
PYD	pyrin domain
RD	repressor domain
RIG	retinoic acid inducible gene
RIP	receptor-interacting protein
RLH	RIG-I-like helicase
RNF	RING finger protein
ROS	reactive oxygen species
RSV	respiratory syncytial virus
SAD	single-wavelength anomalous diffraction

SARM	sterile α - and armadillo-motif containing protein
SDS	sodium dodecyl sulfate
SLS	static light scattering
STAT	signal-transducing activators of transcription
STING	stimulator of interferon genes
TAB	TAK1-binding protein
TAK	TGF- β -activated kinase
TBK	TANK-binding kinase
TEV	<i>tobacco etch virus</i>
TIR	Toll/interleukin (IL)-1 receptor domain
TLRs	Toll-like receptors
TNF	Tumor Necrosis Factor
TNFR	TNF receptor
TRADD	TNF-receptor-associated death domain
TRAF	tumor necrosis factor receptor-associated factor
TRIF	TIR-domain-containing adaptor protein inducing IFN- β
TRIM	TRIF-related adaptor molecule
TRIM	tripartite motif
Tyk	tyrosine kinase
VACV	<i>Vaccinia virus</i>
VAR	<i>Variola virus</i>
VIPER	viral inhibitory peptide of TLR4

Chapter 1. Introduction

1.1 Innate Immune System

The mammalian immune system is comprised of the innate and adaptive immune response (1). We are highly dependent on both immune responses to defend our body from pathogens such as microbes, viruses, and fungi. The adaptive immune system has evolved as a specific defense mechanism for the efficient detection and clearance of pathogens. Moreover, it provides long-lasting protection against pathogens. While the adaptive immune system is crucial to eliminate or destroy pathogens, it takes at least a few days to be activated. As a consequence, we need an additional defense mechanism to provide a first line of protection. We encounter numerous pathogens in daily life of which we are unaware, because the innate immune system either prevents infection and/or alerts the adaptive immune system for effective clearance of pathogens (2).

The innate immune system is responsible for the initial immune response to the pathogens from a few hours to a few days until the adaptive immune system is activated (2). It also provides largely non-specific immunity, thus it does not eliminate pathogens as well as the adaptive immune system (1). If pathogens are not cleared, the innate immune system is essential to the development of an adaptive immune response, which eliminates pathogens with a more specific and targeted response (3).

The innate immune system consists of three defining defense mechanisms. The first line of defense is the physical and chemical barrier by which microorganisms are denied access to our organs, tissues and circulation. Our epithelium is a physical barrier to pathogens and the low gastric pH prevents penetration and the proliferation of infectious microorganisms (2). The second line comprises mainly of cell-intrinsic responses. Infected cells take measures to kill or cripple invaders. For example, the cells which have taken up a microorganism by phagocytosis will direct the phagosome to fuse with a lysosome to digest the microorganisms through lysosomal proteases (2). The third line depends on the specialized set of proteins that recognize characteristic signatures of pathogenic molecules such as dsRNA, lipopolysaccharides (LPS), and peptidoglycans, and other foreign cellular components. The signature molecules which are derived from bacteria, viruses, protozoa and fungi are known as pathogen-associated molecular patterns (PAMPs) and are recognized by pattern-recognition receptors

(PRRs) (4). The first example of PRRs reported in the literature, was the evolutionarily highly conserved receptor family, Toll-like receptors (TLRs) (5). Following activation, PRRs trigger the release of inflammatory cytokines and type I interferons (IFN) to orchestrate the host defense. In addition, stimulation of TLRs triggers dendritic cell maturation, which results in the induction of co-stimulatory molecules and increased capacity by antigen-presenting cells (5). Thus, this response is not only important for the host defense but also for the activation of the adaptive immune system by priming T cells (2).

1.2. Pattern Recognition Receptors (PRRs) and PRR signaling

To date, four types of PRRs have been well characterized; Toll-like receptors (TLRs), retinoic acid-inducible gene (RIG)-I-like helicase receptors (RLHs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and C-type lectin receptors (CLRs). The signals from these receptors result in the expression of a range of immune and pro-inflammatory genes. The architecture of these receptors involves seven distinct domains: the leucine-rich repeat (LRR) domain, the Toll/interleukin (IL)-1 receptor domain (TIR), the nucleotide-binding site (NBS), the caspase recruitment domain (CARD), the pyrin domain (PYD), the helicase domain and C-type lectin domain (CTLD) (6). PRRs are the central components of innate immune response and they also help to shape the adaptive immune response of vertebrates and the 'effector-triggered' immunity of plants. Invertebrates rely solely on PRRs as a universal and ancient defense mechanism for recognition of invading pathogens (6). Despite their diversity, PRR signals converge upon common mediators of the downstream cascade that alters the gene expression of immune modulators such as interferons and pro-inflammation cytokines.

1.2.1. TLR family proteins

TLRs were the first discovered and to date the best characterized family of PRRs. The TLRs are type I integral membrane glycoproteins (7). In mammals, 12 different members of TLRs are identified and they interact with lipid, protein, and nucleic acid PAMPs. TLRs are localized at the cell surface or intracellular vesicles such as the endosome and endoplasmic reticulum (ER) (1), and their ligands are distinct. The external domain (ectodomain) of the TLRs is made up of multiple leucine-rich repeats (LRRs) and this domain detects the PAMPs as homo/hetero dimers. TLR1, TLR2, TLR4, TLR5, and TLR6 are localized on the plasma membrane, whereas TLR3, TLR7, TLR8, TLR9, and TLR13 are localized to the intracellular vesicles including ER, endosomes, lysosomes, and endolysosomes (8). TLR11 is expressed on the cell surface and in the intracellular endosomal compartments (9). TLRs which are localized on the plasma membrane mainly detect microbial membrane components, whereas TLRs which are localized to the intracellular vesicles detect nucleic acid (8).

Viral PAMPs are detected by multiple TLRs. TLR3 detects double-stranded RNA, TLR7-TLR8 detects single-stranded RNA, and TLR9 detects DNA from viral pathogens (8). To date, it is known that TLR9 detects HSV-1, HSV-2, and MCMV (1,8,10,11). DNA recognition by TLR9 occurs independently of the nucleotide sequence. The sugar backbone 2'-deoxyribose of DNA is sufficient to confer signaling (12,13). Given that plasmacytoid dendritic cells (pDCs) produce vast amounts of type I interferon (IFN) in response to DNA virus infection, TLR9 expressed by pDCs may serve as a sensor for virus infection (14). On the other hand, TLRs on the cell-surface also detect viral pathogens. TLR4 detects both the fusion protein from respiratory syncytial virus (RSV) and the envelope protein from mouse mammary tumor virus (MMTV) (1). TLR2 detects hemagglutinin (HA) protein of measles virus, human CMV, and HSV-1. It has also been shown that TLR2 detects inactivated vaccinia virus in a cell specific manner, independently of nucleic acids (15).

The relationship between TLRs and their PAMPs including the PAMPs from bacteria and fungal pathogens is well characterized and summarized in Table 1.1 (3,14,16).

Table 1.1. TLRs and PAMPs

PRRs (domain organization)	Adaptor (domain)	PAMPs	Pathogens
TLR1-TLR2 (LRR-TIR)	MyD88 (TIR-DD), Mal (TIR)	Triacyl lipopeptide	Bacteria
TLR2-TLR6 (LRR-TIR)	MyD88, Mal (TIR)	Diacyl lipopeptide	Mycoplasma
		LTA	Bacteria
		Zymosan	Fungus
TLR2-TLR2 (LRR-TIR)	MyD88, Mal	PGN	Bacteria
		Lipoarabinomannan	Mycobacteria
		Porins	Bacteria (<i>Neisseria</i>)
		tGPI-mucin	Parasites (<i>Trypanosoma</i>)
		HA protein	Virus (Measles virus)
TLR3-TLR3 (LRR-TIR)	TRIF (TIR)	dsRNA	Virus
TLR4-TLR4 (LRR-TIR)	MyD88, Mal, TRIF, TRAM (TIR)	LPS	Bacteria
TLR5-TLR5 (LRR-TIR)	MyD88, TRIF	Envelope proteins	Virus (MMTV, RSV)
TLR7-TLR7 (LRR-TIR)	MyD88	Flagellin	Bacteria
TLR7-TLR7 (LRR-TIR)	MyD88	ssRNA	RNA virus
hTLR8-hTLR8 (LRR-TIR)	MyD88	ssRNA	RNA virus
TLR9-TLR9 (LRR-TIR)	MyD88	CpG DNA	Bacteria
		DNA	DNA virus
		Malaria homozoin	Parasite
mTLR11 (LRR-TIR)	MyD88	Not determined	Bacteria
		Profilin-like molecule	Parasite
mTLR13	MyD88	Not determined	Virus

This table has been adapted from Kawai and Akira, *Int Imuunol.* (2009) (14).

Ligand binding results in the dimerization of the ectodomain and it induces the dimerization of internal Toll/Interleukin-1 receptor (TIR) domain (17,18). The crystal structure of the TIR-domain-dimer of TLR10 suggested the physiological state of TIR when the ectodomain binds to the ligands (Fig. 1.1) (19). It showed that the BB-loop is crucial for the dimer formation. Moreover the inhibition of the signal transduction by the decoy peptides derived from the BB-loop of TLR1, TLR2, TLR4, TLR6, Mal, MyD88 supports the theory that TLR10-TIR represents the functional dimer of the TIR domain (20-24). However, the nature of the active complex remains obscure because the formation of the homotypic dimer in these TIR domains has not been observed in solution (19,25).

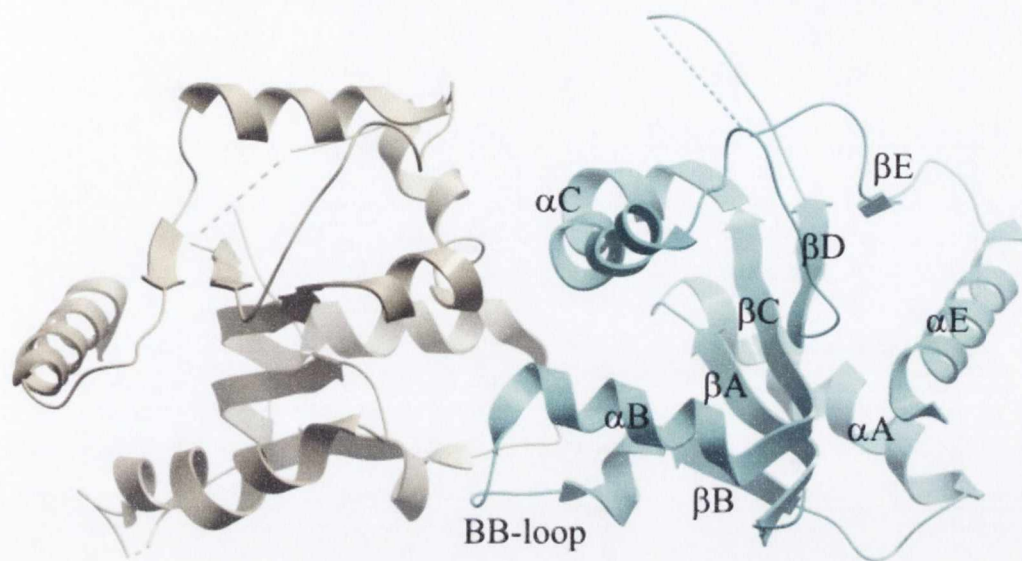


Figure. 1.1. Ribbon diagram of the TLR10 TIR dimer structure (PDB code: 2J67, Nyman, et al., *J.Biol.Chem.* (2008) (19)). TLR10 TIR domain forms a dimer in the crystal packing (each monomer is shown in different color). The dimer interface contains residues from the BB-loop and α C-helix, which have previously been identified as important structural motifs for signaling in homologous TLR receptors.

The crystal structures of the TLR ectodomains revealed how TLRs recognize their ligands (Fig. 1.2.) (26-28). TLRs belong to the typical leucine-rich-repeat (LRR)-containing protein family. Each LRR 24 amino acid repeat possess the conserved motif XLXXLXXLXXNXLXXLPXXXFX and displays the unique horse-shoe shape associated with LRR proteins (29). Each TLR structure in complex with its cognate ligand has the same structural arrangement despite having a diverse chemistry of ligands (30). TLRs require adapter proteins that have TIR domains to confer functionality. Dimerization of the ectodomains leads to the oligomerization of the TIR domain, located on the cytosolic side of membranes. It initiates the recruitment of TIR domain-containing adapter proteins to the TIR domain of TLRs. The cytoplasmic TIR-containing-adapter subgroups comprise of five members: myeloid differentiation factor 88 (MyD88), MyD88 adapter like (Mal, also known as TIRAP), TIR-domain-containing adaptor protein inducing IFN- β (TRIF), TRIF-related adaptor molecule (TRAM), and sterile α - and armadillo-motif containing protein (SARM) (7).

TLR1, TLR2, TLR4, and TLR6 recruit Mal and MyD88 (31). TLR4 also recruits TRAM and TRIF, while TLR5 recruits TRIF in addition to MyD88 (31,32). TLR7, TLR9 and TLR11 recruit MyD88, whereas TLR3 recruits TRIF (31). MyD88 is used by all of the TLRs except for TLR3. Only TLR4 has been shown to utilize four TIR domain-containing adaptor molecules (MyD88, Mal, TRIF, and TRAM) for signaling (31). Although other TIR-containing adaptor proteins have been assigned activating function, only SARM was identified as a TRIF-specific negative regulator protein (33).

A recent study of TLR4 signaling revealed that MyD88 and Mal have different roles in signaling pathways (34). MyD88 has a role as an essential “signaling adaptor” which transmits signals from ligand-activated TLRs to downstream factors to initiate a kinase-dependent signaling cascade (7). Mal serves as a ‘sorting adaptor’ which recruits MyD88 to the plasma membrane by binding to phosphatidylinositol 4,5-bisphosphate (PIP2) *via* its PIP2 binding motif to promote interaction between activated TLR4 and MyD88 (34,35). The solution structure of MyD88 was solved in 2009 (Fig. 1.3.), and this study revealed that MyD88 has a direct interaction with Mal-TIR *in vitro* (36). Moreover, they showed that Mal has a direct interaction with TLR4-TIR (36). In the TLR4-TRIF pathway, TRAM has been proposed to serve as a sorting adaptor and it recruits TRIF to a specific membrane compartment *via* its myristoylation site (37). The TIR domain is the common signaling domain, but clearly differences occur in the utilization of the different TIR-domain-containing adaptors. Other factors such as TRAF3 or IKK ϵ are shared with the other PRR signaling pathways.

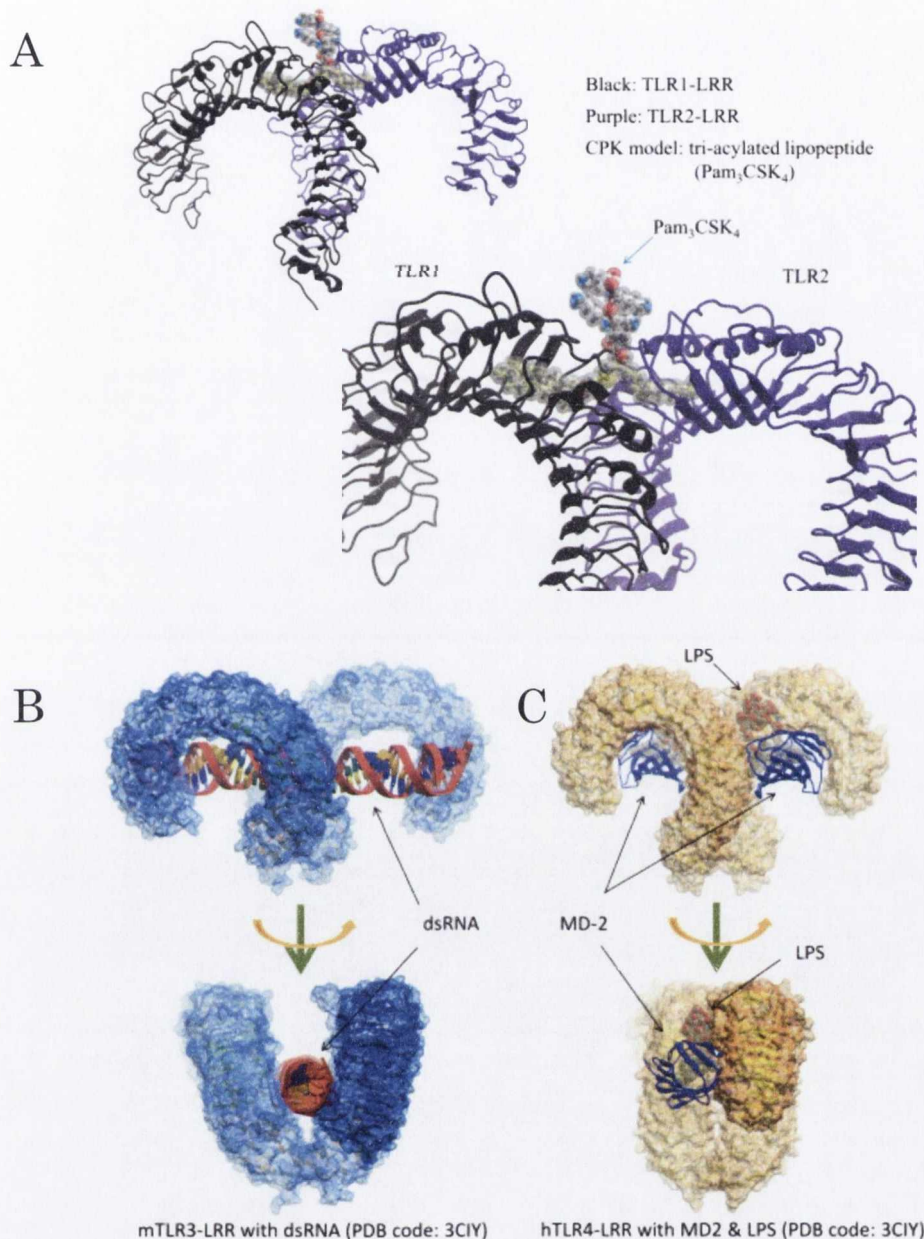


Fig. 1.2. Three dimensional model of TLR ectodomains A: TLR1-TLR2 heterodimer in complex with tri-acylated lipopeptide (Pam₃CSK₄), PDB code: 2Z7X, Jin et al., Cell (2007), B: Mouse TLR3 ectodomains (blue) form a dimer on interaction with dsRNA (red). PDB code: 3CIY, Liu et al., Science (2008) (26). C: Side view of the symmetrical dimer of the TLR4-MD-2-LPS complex. PDB code: 3FXI, Park et al., Nature (2009) (28). The primary interface between TLR4 and MD-2 is formed before binding LPS, and the dimerization interface is induced by binding LPS. In every case, TLR ectodomain forms M-shaped dimers despite the diverse chemistry of the ligands.

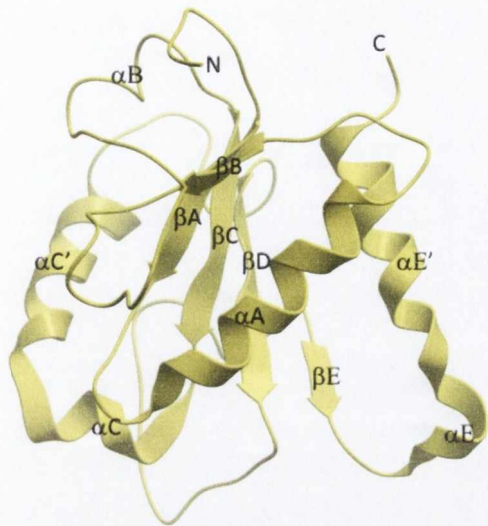


Figure 1.3. Solution structure of the TIR domain of human Myd88 (PDB code: 2Z5V, Ohnishi et al., PNAS (2009) (36)). A representative ribbon drawing of the NMR structure of MyD88. The helix, sheet and loop notation used in this figure is based on the TIR domains of TLR.

Signal transduction is initiated by the oligomerization of the intracellularly-situated TIR domains after binding of their cognate PAMPs, and TIR-domain-containing adaptor proteins are recruited to a TIR domain signaling platform. MyD88 is the critical adaptor for signal transduction of all TLR except for TLR3 (38). MyD88 contains a death domain (DD) at the N-terminus and it interacts with the downstream interleukin-1 receptor-associated kinase (IRAK) adaptor proteins, IRAK1, IRAK2, IRAK4, and IRAK-M *via* homotypic DD interactions (39). All of these IRAKs have an N-terminus DD-domain and C-terminus Ser/Thr kinase or kinase-like domain (1,7,40). Activation of the IRAKs involves auto- and cross-phosphorylation (41).

The crystal structure of the DD of MyD88, IRAK4, and IRAK2 complex was solved and it revealed how they interact (39) (Fig. 1.4). IRAK2 can functionally substitute for IRAK1 (42). The overall assembly consists of 6xMyD88, 4xIRAK4, and 4xIRAK2 DDs, and adopts a left-handed helical oligomer called the ‘Myddosome’. The formation of this structure is dictated by both molecular complementarity and surface electrostatics. Formation of the Myddosome leads to the apposition of IRAK1/2, IRAK4, and MyD88, and induces the phosphorylation of IRAK1 by IRAK4 (41).

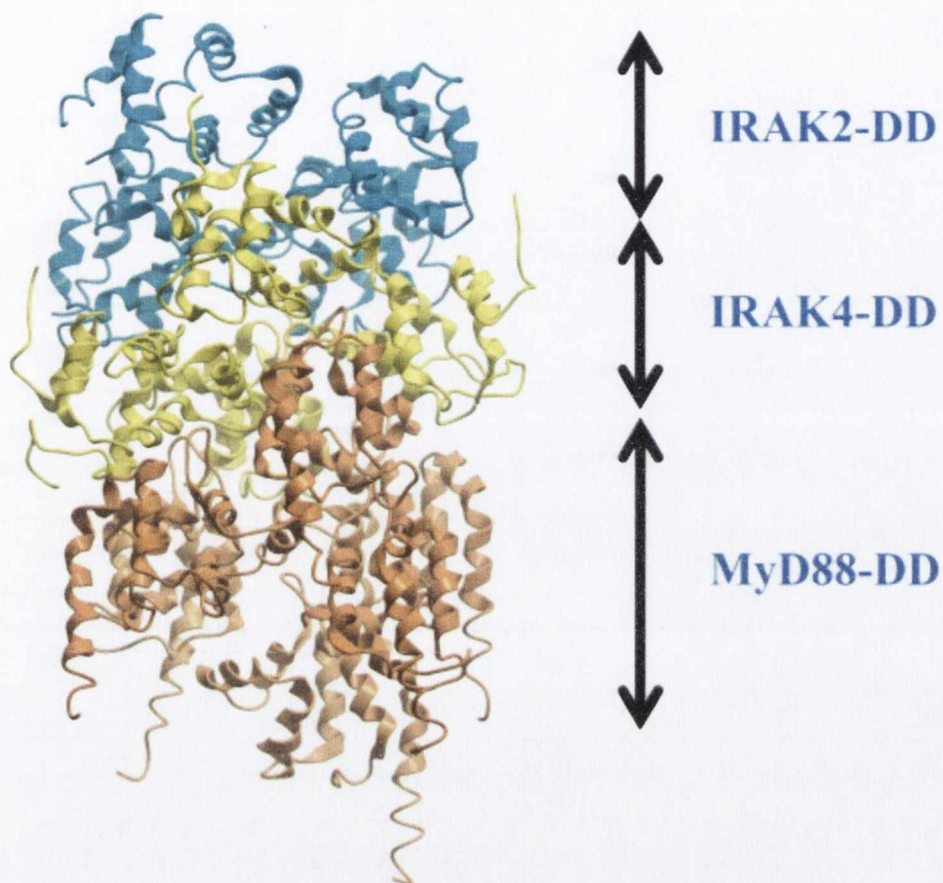


Figure 1.4. 3D structure model of the Myddosome. The Myddosome contains four IRAK2, four IRAK4, and six MyD88 death domains (DD) (PDB code: 3MOP, Lin et al., Nature (2010) (39)). The formation of this structure is dictated by both molecular complementarity and compensation of surface electrostatic interaction within each layer.

1.2.2. Downstream signaling cascade

The signaling pathways of TLRs consist of a complex assortment of kinases and adaptor proteins, and involve protein-protein interactions, ubiquitination, and phosphorylation. The signals from TLRs lead to the phosphorylation and activation of nuclear factor kappa-B (NF- κ B) and interferon regulatory factors (IRFs), which result in the release of type-I interferons and other pro-inflammatory cytokines.

Since signal transduction from TLR4 is currently the best characterized pathway, the TLR4-MyD88 pathway will be utilized as an example to discuss downstream signaling (Fig. 1.5). Dimerization of TLR-TIR induced by ligand-binding to the extracellular receptor domain recruits Mal and MyD88. MyD88 promotes association with the IL-1R-associated kinases, IRAK4 and IRAK1, which shares the function with IRAK2. IRAK4 is initially activated, and during the formation of this complex, IRAK1 or IRAK2 are phosphorylated by activated IRAK4 (42). This phosphorylation induces the interaction between tumor necrosis factor receptor-associated factor 6 (TRAF6) and the complex. TRAF6 is an E3 ubiquitin ligase, which forms a complex with E2 ubiquitin-conjugating enzymes such as UBC13 and UEV1A (ubiquitin-conjugating enzyme variant 1A). This complex polyubiquitinates TRAF6 itself and NEMO [NF- κ B essential modifier, also known as IKK γ (I κ B kinase γ)] through a Lys63 linkage (43,44). The IRAKs/TRAF6 complex interacts with another protein complex of the TGF- β -activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1), TAB2, and TAB3. The Lys63 polyubiquitinated TRAF6 activates the transforming growth factor β activated kinase-1 (TAK1) (16,43). TAK1 in complex with TABs subsequently activates two distinct pathways involving the I κ B kinase (IKK) complex or mitogen-activated protein kinase (MAPK) (14). Phosphorylation and activation of TAK1 leads to the activation of IKK complex or MAPK. Activated IKK complex, which is composed of the catalytic subunit IKK α , IKK β and a regulatory subunit NEMO, catalyzes the phosphorylation and subsequent Lys48-linked ubiquitination of I κ B leading to its degradation (1,14,45). Structural and biochemical studies of NEMO with both linear and Lys63-linked ubiquitin suggested the importance of linear ubiquitin for IKK activation (46,47) (Fig. 1.6). These studies suggested that structural changes of NEMO by binding to the ubiquitin chain induce the activation of IKK α/β . Degradation of I κ B allows the release of NF- κ B, which translocates to the nucleus to induce the transcription of pro-inflammatory cytokines. The NF- κ B family consists of five members that can exist as dimers and the heterodimer composed of

RelA and p50 is considered to be the most frequently activated during PRR signaling (14).

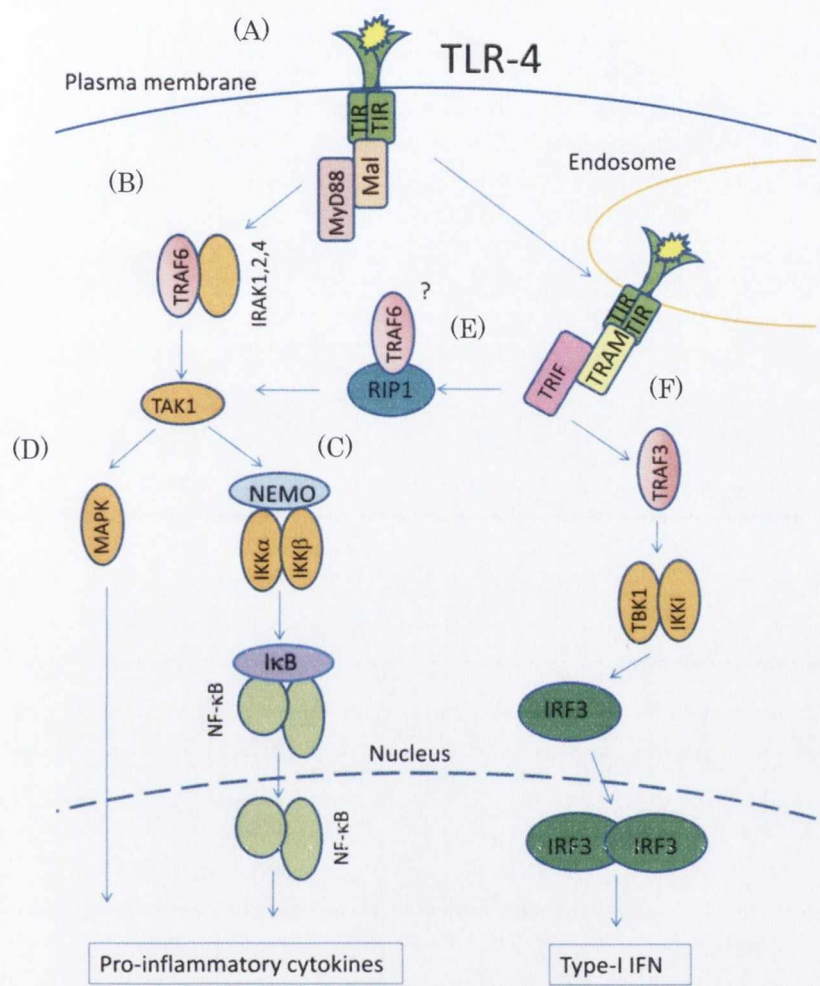


Fig. 1.5. Signaling pathway triggered by TLR4. (A) The TLR4-MD-2 complex engages with LPS on the cell surface and then recruits a TIR domain-containing adapter complex including Mal and MyD88. The TLR4-MD-2-LPS complex is subsequently trafficked to the endosome, where it recruits TRAM and TRIF adapters. (B) Mal-MyD88 recruits IRAK family members and TRAF6 to activate TAK1. (C) The TAK1 complex activates the IKK complex composed of IKK α , IKK β , and NEMO, which catalyze phosphorylation of I κ B proteins. Phosphorylated I κ B proteins are degraded, allowing NF- κ B to translocate to the nucleus. (D) TAK1 simultaneously activates the MAPK pathway. The activation of NF- κ B and MAPK results in the induction of inflammatory cytokine genes (MyD88 dependent pathway). (E) TRAM-TRIF recruits TRAF6 and RIP1 for the activation of TAK1. (F) TRAM-TRIF also recruits TRAF3 for activation of TBK1 and IKK ϵ which phosphorylates IRF3.

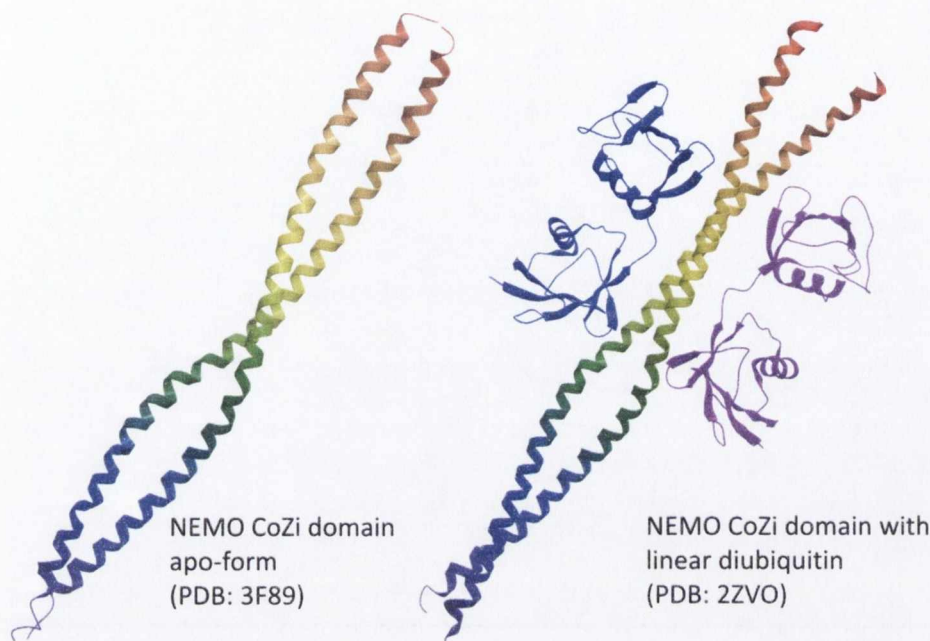


Figure 1.6. Crystal structure of CoZi (coiled zipper) domain of the NEMO ubiquitin-free form (left) and in complex with linear diubiquitin (right). CoZi domain which include UBAN (ubiquitin binding in ABIN and NEMO proteins) motif forms a coiled-coil structure and linear diubiquitin (colored blue and yellow) interacts with the coiled-coil. Rahighi et al., Cell (2009) (46).

A second key example of signaling is the TLR4-TRIF pathway (Fig. 1.5). This pathway is MyD88 independent and activates interferon regulatory factor-3 (IRF3) at the early endosome (34,48). The trafficking of TLR4 in complex with ligand is performed under the control of the small GTPase Rab11a (49), where the dimerized TIR domains of TLR4 recruit TRAM and TRIF. TRAM works as a bridging adaptor between the receptor's TIR domain and TRIF. TRIF associates with TRAF3, TRAF6 and a receptor-interacting protein-1 (RIP1) as downstream signaling molecules (50-56). RIP1 is inducibly ubiquitinated and facilitates interaction with TAK1. TRAF6 and RIP1 activate NF- κ B by sharing the signaling pathway with the TLR4-MyD88 pathway. On the other hand, TRAF3 activates both TANK binding kinase 1 (TBK1) and inducible I κ B kinase epsilon (IKK ϵ), both of which are involved in the phosphorylation and activation of IRF3 and/or IRF7 (57-60). Phosphorylated IRF3 or IRF7 form homodimers, which can subsequently translocate into the nucleus and induce type I IFNs (IFN- α is induced by the IRF7 dimer while IFN- β is induced by the IRF3 dimer) (61).

1.2.3. RIG (retinoic acid-inducible gene)-I like helicase (RLH) family proteins

Once viruses enter the cytoplasm, they generate ssRNA or dsRNA in the cytoplasm to establish infection (62). RLHs are cytosolic PRRs, so they act as intracellular sensors for detection of RNA derived from RNA viruses and replicating DNA viruses leading to the activation of intrinsic anti-viral signaling pathways (Fig. 1.9). These interactions occur in the cytoplasm of both immune and non-immune cells and are independent of the TLRs that can detect the RNA species present within endosomes (14,63). RLHs, which are DExD/H-box RNA helicases, consist of retinoic acid inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). The recognition of RNA by intracellular sensors subsequently activates innate antiviral responses through activation of IRF3/7 and NF- κ B in most cell types (16,64). RIG-I and MDA5 contain two tandem repeats of the caspase recruitment domain (CARD) at their N-terminus, which are important for downstream signaling (Fig. 1.7). The intermediate region of these proteins contains the helicase domain. Although the helicase domain of RIG-I contains an ATP-binding region which is essential for RIG-I function, MDA5 does not have an ATP-binding region. In addition, RIG-I and MDA5 contain a repressor domain (RD) at the C-terminus, which represses the activity of RIG-I (65,66). On the other hand, LGP2 does not contain a CARD. The helicase domain and RD are significant for the RNA recognition, but the CARDs are essential for triggering intracellular signaling cascades (67,68). The 5'-triphosphate moiety of RNA is essential for RIG-I recognition (69) and it is not related to the strand properties (single or double) of RNA (70). Although the 5'-triphosphate moiety of RNA from the pathogen is detected by the C-terminal domain (CTD) of RIG-I (66) (Fig. 1.8), host-cell RNA is not recognized by RIG-I because, during synthesis of cellular RNA, the 5'-ends are either modified by the addition of a 7-methylguanosine cap or the 5'-triphosphate is removed before transportation to the cytoplasm (16). Thus RIG-I can discriminate host-cell RNA from viral RNA. RIG-I can bind to 25 base pair (bp) dsRNA for efficient induction of type I IFN (66). It was shown that RIG-I recognizes small dsRNA species ranging from 21 to 27 nucleotides (71), and RIG-I and MDA5 distinguish dsRNA by size; RIG-I binds relatively short dsRNA (approx. 1.2-1.4 kbp), whereas MDA5 binds relatively long dsRNA (longer than 3.4 kbp) (72) (Fig. 1.7). RNA terminal properties (blunt end, 5'-overhang and 3'-overhang) and its nucleotide sequences are not critical for binding to RIG-I (62). LGP2 was suggested to serve as a negative regulator of RNA virus-induced responses, because the LGP2 RD binds to that of RIG-I and suppresses signaling by interfering with the

self-association (62). However, a recent study showed that LGP2 facilitates viral RNA recognition by RIG-I and MDA5 through its ATPase domain (73,74).

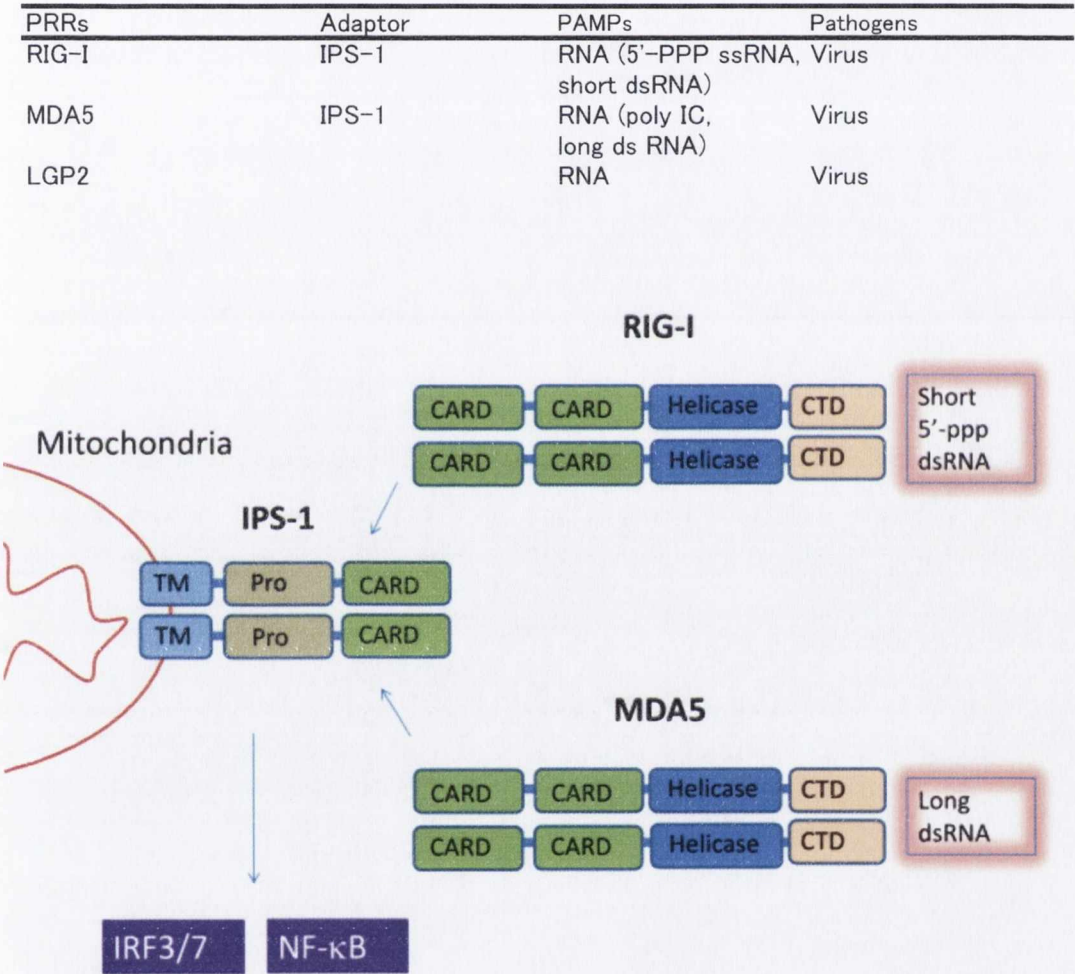


Figure 1.7. Signaling from RIG-I like helicase. RIG-I and MDA5 recognize short 5'-pp dsRNA and long dsRNA respectively. RLHs interacts with interferon beta promoter stimulator protein 1 (IPS-1) via homotypic CARD-CARD interactions after the detection of PAMPs. The signal from RLHs leads the activation of IRF3/7 and NF-κB. Pro: proline-rich domain, TM: transmembrane domain.

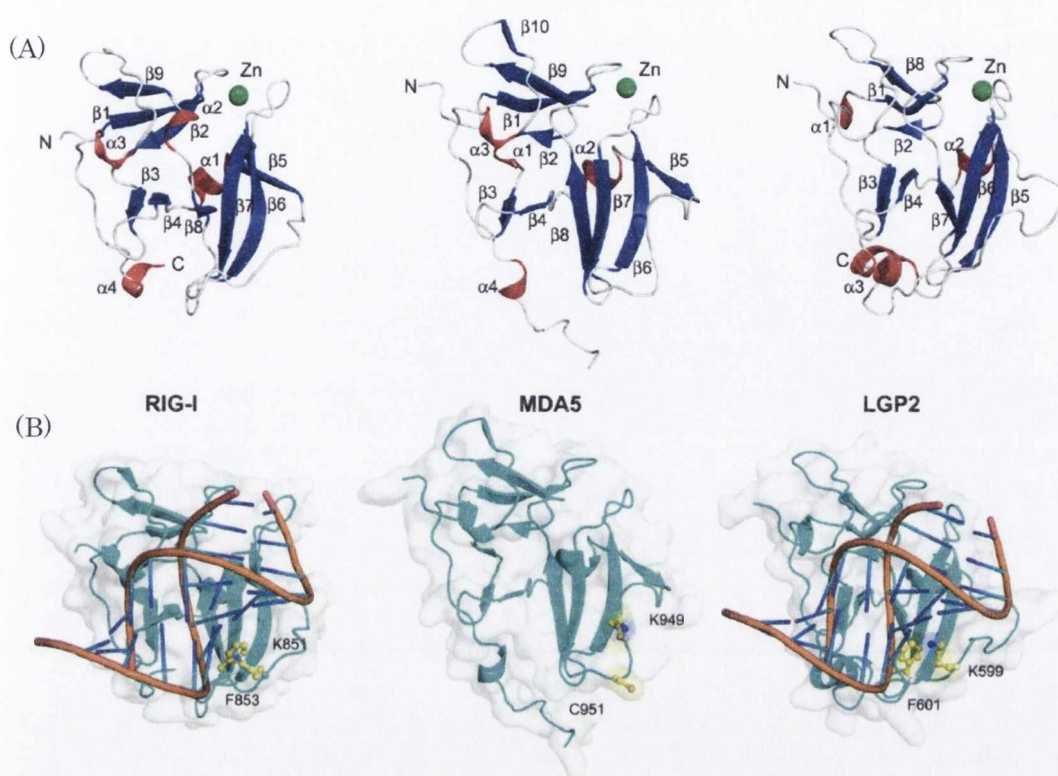


Figure. 1.8. Structural comparisons of RLH CTDs. (A) Ribbon diagrams of the NMR structure of RIG-I CTD, MDA5 CTD, and LGP2 CTD (left to right). (B) dsRNA-bound model of RIG-I CTD (left) and LGP2 CTD (right). The CTD domains have a similar fold and a similar basic surface (not shown) but there is the distinct structural feature of a RNA binding loop. The CTD is responsible for the binding affinity of RLRs to viral RNAs. Figures taken from Takahashi et al., J. Biol. Chem. (2009).

In resting cells, RIG-I is inactive as a monomer, but on virus infection and RNA binding, conformational changes are triggered to facilitate self-association, which promotes CARD interaction with downstream signaling molecules (14). The CARD of RIG-I and Mda5 interacts with the CARD-containing adaptor protein known as IPS-1 [IFN promoter stimulator-1, also known as MAVS (mitochondrial antiviral signaling), Cardif (CARD adaptor inducing IFN- β) and VISA (virus-induced signaling adaptor)], which is localized in the outer membrane of mitochondria (75) (Fig. 1.7). IPS-1 is the sole adaptor for RIG-I/Mda5 and has an essential role in host defense against various RNA viruses (63,76). Signaling through RIG-I is also regulated by ubiquitination. TRIM25 which is an E3 ubiquitin ligase interacts with the CARD of RIG-I and leads to

Lys63-linked ubiquitination (77). By contrast, RNF125 which induces ubiquitination and proteasomal degradation of RIG-I inhibits RIG-I (78). These studies suggest that the ubiquitination is an additional regulatory mechanism for the RIG-I-mediated signaling pathway (78). IPS-1 recruits TNFR-I (TNF receptor-I) signaling adaptor TRADD (TNF-receptor-associated death domain), which in turn assembles Fas-associated death domain protein (FADD), RIP1, TRAF3, TRAF6, and TANK (14,79,80), leading to the activation of IRF3 and NF- κ B (Fig. 1.9). TRAF3 is recruited by IPS-1 (81,82), and TRAF3 also interacts with TRADD which is essential for the activation of NF- κ B and IRF3/7 (80). IPS-1 also recruits TANK which, by definition, interacts with TBK1 (TANK-binding kinase). TBK1 forms a kinase complex with IKK ϵ and initiates the activation of IRF3/7 as described in the previous section (52,53,82). Michallet *et al.* also demonstrated that FADD recruitment to IPS-1 requires the presence of RIP1 and TRADD (80). They interact with each other through homotypic interactions of death domains. This means that RIP1 and TRADD work as adaptors for FADD to form the complex with IPS-1. Tkahashi *et al.* reported that FADD interacts with caspase-8 and caspase-10 which are essential for the RIG-I mediated activation of NF- κ B (83). TRAF3 is also required for the ubiquitination of RIP1 (80). Ubiquitinated RIP1 recruits NEMO and activates the IKK kinase complex to activate NF- κ B. On the other hand, TRAF6 constitutes the protein complex with IPS-1 (81). Thus, TRAF6 is expected to contribute to the activation of NF- κ B and MAPK *via* TAK1.

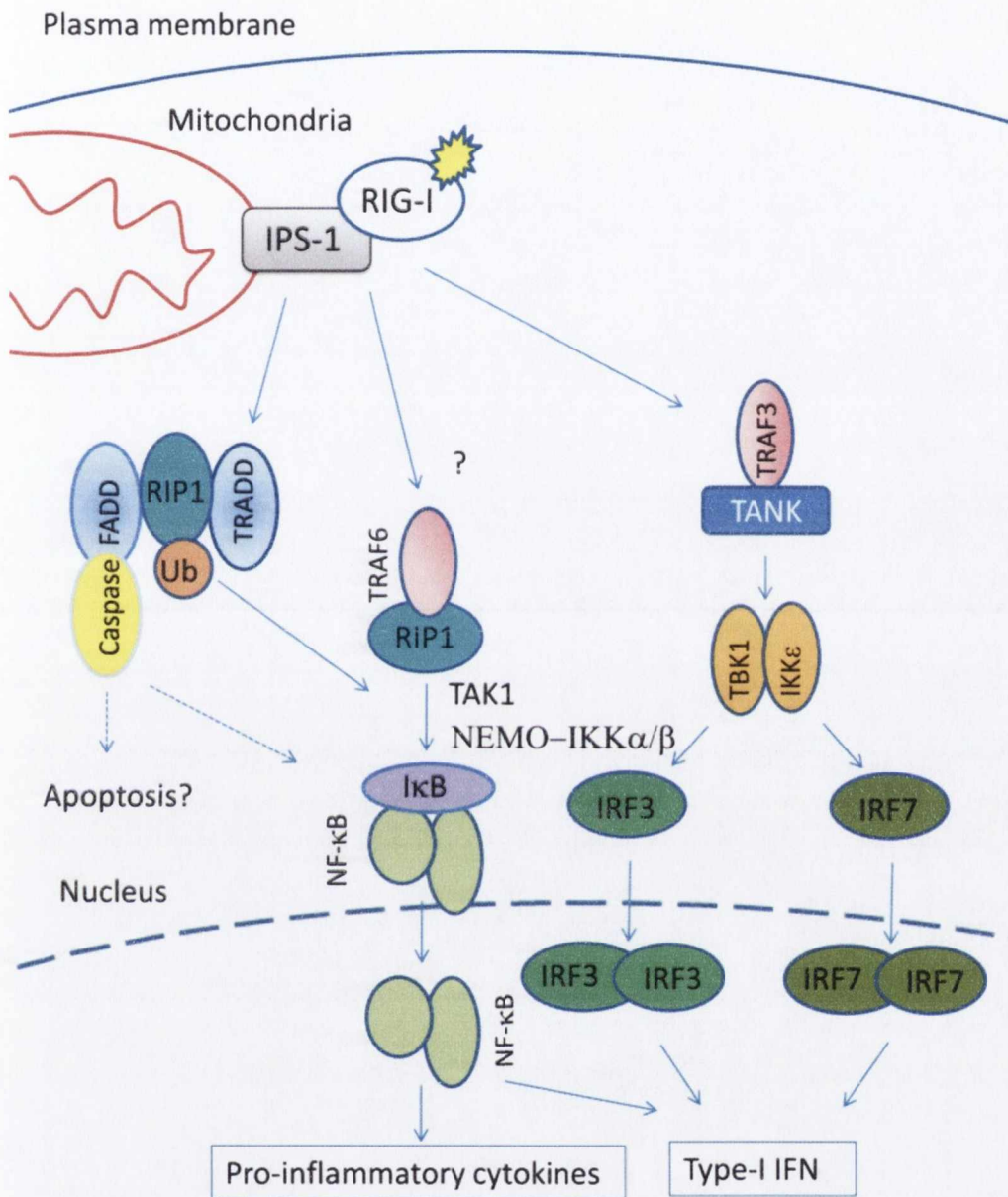


Fig. 1.9. Recognition of viral nucleic acids by RLHs. In conventional dendritic cells (cDCs), macrophage and fibroblast cells, viral RNA species are recognized by RLRs. RIG-I and MDA5 recruit the adapter IPS-1 via CARDs. IPS-1 is localized on mitochondrial membrane, and recruits TRADD, which then forms a complex with FADD, caspase-8 and caspase-10 to activate NF- κ B. TRADD also recruits TRAF3 to activate the TBK2-IKK ϵ -IRF3/7 pathway. TRAF6 interacts with IPS-1 but the role of TRAF6 in this signaling pathway is unclear.

Both signals from TLRs and RLHs activate the transcription factors IRF3/IRF7 and NF- κ B. The signal from TLRs activates IRFs and NF- κ B *via* TIR-domain-containing adaptors, but the signal from RLHs activates IRFs and NF- κ B *via* IPS-1. Activated IRFs and NF- κ B translocate to the nucleus and induce type I interferon production and/or other inflammatory cytokines. Phosphorylation, which is catalyzed by TBK1 and/or IKK ϵ , is a key signal for translocation of IRF and NF- κ B. Recently, TBK1/IKK ϵ have also been observed to interact with an RNA helicase, DEAD-box protein 3 X linked (DDX3X) (84,85). The role of DDX3 in innate immunity will be discussed in section 1.4.2.5.

1.2.4.NOD-like receptor (NLR) family proteins

NLRs are a large receptor family that have a conserved nucleotide oligomerization domain (NOD) motif (86,87). NLRs have a trimodular structure and consist of three domains: (i) the C-terminal LRR domain which is essential for recognition of the microbial components; (ii) a centrally located nucleotide-binding and oligomerization NACHT (NAIP, CIITA, HET-E and TP1) domain which is essential for self oligomerization and formation of a complex for the activation and recruitment of downstream signaling proteins; (iii) the variable N-terminal domains (16) (Fig. 1.10). The N-terminal domain is effector binding region that consists of protein-protein interaction domains, such as the caspase-recruitment domain (CARD), pyrin domain (PYD) or baculovirus inhibitor repeat (BIR) domain (88). Database searches have revealed that there are 22 NLR genes in the human genome and at least 34 NLR genes in the mouse genome (86,87). LRRs are proposed to function in ligand sensing (89). Binding of ligand triggers a conformational change of the protein molecule and oligomerization through the NACHT domain in an ATP-dependent manner (88). In turn, N-terminal effector domains (CARD or PYD domains) exert homotypic protein-protein interactions for downstream signaling (88). Three distinct NACHT domains within the NLR family are identified by phylogenetic analysis: the NODs (NOD1-2, NOD3/NLRC3, NOD4/NLRC5, NOD5/NLRX2, CIITA), NLRPs (NLRP1-14, also called NALPs) and the IPAF subfamily (IPAF/NLRC4 and NAIP) (90). This phylogenetic classification reflects somewhat the domain organization of these proteins (Fig. 1.10). If NLR family proteins are classified based on the domain organization, the NLR family proteins consist of at least five subfamilies distinguished by an N-terminal

protein interaction domain; NLRA/CIITA (which contain an acidic trans-activation domain), NLRB (which contains a baculovirus inhibitor of apoptosis protein repeat), NLRC (which contains a CARD), NLRP (which contains a Pyrin domain), and NLRX (which contains an uncharacterized domain) (91). There are a vast variety of proposed NLR family type proteins which remain uncharacterized.

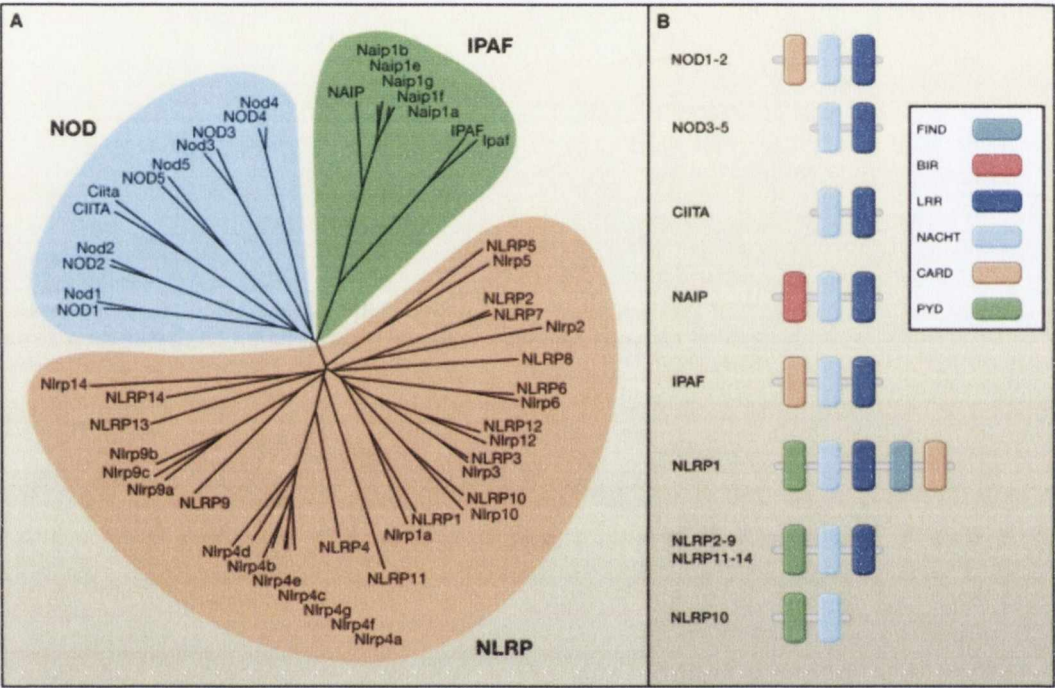


Figure 1.10. Phylogenetic tree and domain organization of NOD
 Figure taken from Schroder and Tschopp, Cell (2010)

1.2.4.1. NLR as a component of inflammasome

Inflammasomes are a high-molecular-mass (~700 kDa) IL-1 β -processing complex (90,92,93). Inflammasomes are assembled by spontaneous oligomerization of proteins that form a “scaffold”. Several PRRs, including NLRP3 [NLR (NOD-like receptor)-related protein 3], NLRP1, IPAF (IL-1 β -covering enzyme protease-activating factor), RIG-I, and AIM2 (absent in melanoma 2), have been identified as components of particular inflammasomes (88,94). Caspase 1, the adaptor protein ASC [apoptosis-associated speck-like protein containing a CARD (caspase–recruitment

domain)] and the CARD-containing protein Cardinal, were also identified as components of these complexes. Inflammasomes are formed in response to the stimuli of PAMPs and endogenous molecules in the cytosol (14,89,95-99). The complex is formed by the protein-protein interaction of homotypic-CARD and/or Pyrin domain interactions (90) (Fig. 1.11). Caspase-1 is a cysteine protease that is synthesized as an inactive zymogen. The catalytic activity of Caspase-1 is tightly regulated by signal-dependent auto-activation within inflammasomes (90,93). The inflammasome in effect provides a scaffold for proximity-induced auto-activation of capase-1 which mediates the cleavage of immature IL-1 β and IL-18 for maturation (74,88,90,100,101). IL-1 β and IL-18 are secreted following cleavage of their cytosolic precursors by caspase-1 (102). IL-1 β is mainly produced by blood monocytes and mediates a wide range of inflammatory reactions including fever, hypotension, the release of adrenocorticotrophic hormone, and the production of other cytokines, such as IL-6 (103). These activities induce leukocytosis, thrombocytosis, and the synthesis of acute-phase proteins required to sustain an inflammatory response (104). The main function of IL-18 seems to be the induction of IFN γ production by activated T cells and natural killer (NK) cells, thereby contributing to T helper 1 (T_H1) cell polarization (102,105). A vast range of viruses and virus components activate caspase-1 and subsequent activation of IL-1 β and Il-18 (88).

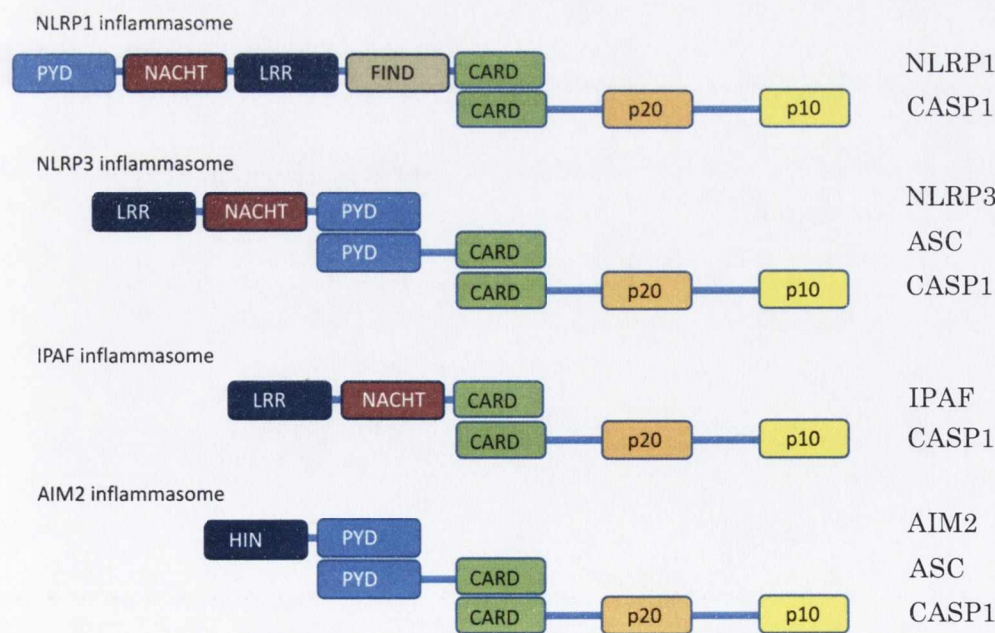


Figure 1.11. Domain arrangement in activated inflammasome complex. Inflammasome formation depends on either homotypic CARD-CARD or PYD-PYD interactions.

The NLRP3 inflammasome is one of the most well characterized inflammasomes. This inflammasome consists of the NLRP3 scaffold, the ASC adaptor, and caspase-1 (106). Since a vast range of stimuli including PAMPs and DAMPs (damage-associated molecular patterns) activates the NLRP3 inflammasome, the hypothesis that a second messenger helps to mediate the activation has been proposed (94). The exact mechanism by which structurally distinct molecules activate NLRP3 is still not clear, but it has been proposed that reactive oxygen species (ROS) may play some part (107). Recent reports indicate that the source of ROS is most likely mitochondria, and this could explain the frequent association of mitochondrial dysfunction with inflammatory disease (94,108). Such a mechanism centering on stress detection, is somewhat different from the other inflammasome types mediated by the IPAF and AIM2 inflammasomes (108). On detection of the stress, ASC and caspase-1 are recruited to NLRP3, and as a result, caspase-1 is auto-activated for IL-1 β and IL-18 maturation (88) (Fig. 1.12).

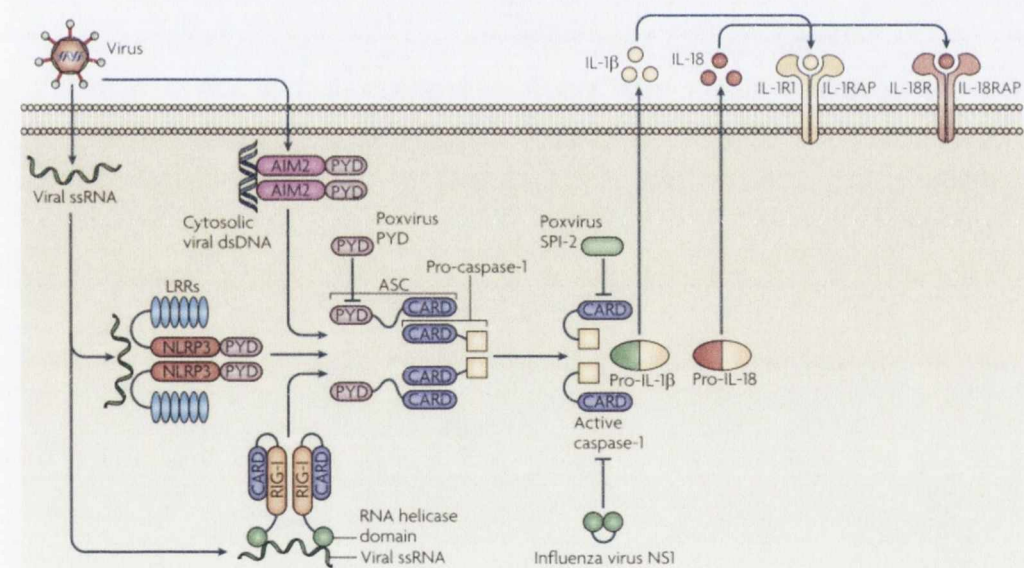


Figure 1.12. Schematic diagram of inflammasome mediated activities. Formation of various inflammasomes is induced by PAMPs. As a result, pro-caspase-1 which mediates the activation of pro-IL-1 β and pro-IL-18 is activated leading to the production of cytokines and other inflammatory inducing signals. (Figure taken from Kanneganti, Nat. Rev. Immunol. 2010 (88))

1.2.4.2. The Function of non-inflammasome NLRs

There are members of the NLR family other than NLRP1, NLRP3, and IPAF, which have roles in the innate immune system beyond that of inflammasome signaling (88,109). It has been shown that non-inflammasomal NLR proteins function include the regulation of canonical and non-canonical NF- κ B activation, mitogen-activated protein kinase activation, cytokine and chemokine production, antimicrobial reactive oxygen species production, type I interferon production, and ribonuclease L activity (110). The NLR proteins, NLRX1 and NOD2, function to regulate the IRF3 activation through the multimeric protein complex called the “mito-signalosome” which involves the mitochondrial IPS-1 protein which acts in response to viral stimuli as previously mentioned in section 1.2.3 (110). NLRX1 prevents the interaction of RIG-I to IPS-1 (MAVS) by binding to IPS-1 (111) (Fig. 1.13). Thus, NLRX1 negatively regulates the induction of type I interferons (111) by sequestering IPS-1 and it amplifies the NF- κ B and JNK pathways by inducing ROS production simultaneously (112). IPS-1 is released from NLRX1 on stimulation by PAMPs. On the other hand, NOD2 interacts with IPS-1 and activates both IRF3 and NF- κ B, which induces the production of both type I interferons and other pro-inflammatory cytokines, in response to both synthetic and viral ssRNA (113). In the studies of NOD1 and NOD2, LRR domains were indispensable to confer responsiveness to the respective stimuli. Thus, they are thought to interact with PAMPs such as peptidoglycan and ssRNA but neither NOD1 nor NOD2 have been shown to interact with their PAMPs in a manner consistent with the other pattern recognition receptors (110).

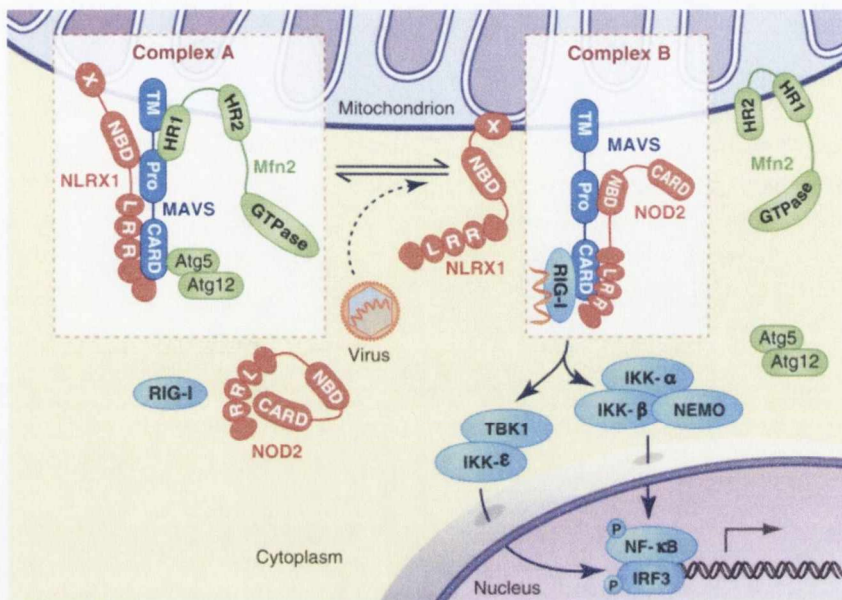


Figure 1.13. In complex A, MAVS/IPS-1 is released from NLRX1 on stimulation of PAMPs. In complex B, NOD2 and RLHs interact with MAVS/IPS-1 and activate the IRF3 and NF-κB inducing the production of type-I INF. Figure taken from Jenny P.Y. Ting, *et al.* Science (2010) (110).

1.2.5. HIN-200 family receptors

Hemopoietic IFN-inducible nuclear proteins with a 200-amino acid residue motif (HIN-200) was identified in both mouse and human (114). Four human (IFI16, MND A, AIM2, IFIX) and 13 mouse members of HIN-200 family proteins have been identified (115,116). Most of HIN-200 family proteins possess C-terminal conserved HIN domain and N-terminal conserved PYRIN domain (117-122) (Fig. 1.14). HIN domains have been observed in interactions with DNA and other proteins such as transcription factors (119-122). PYRIN domains belong to the death domain-containing protein superfamily involved in apoptosis, inflammation, and immune responses. HIN-200 family proteins are important not only for immune regulation but also for other fundamental processes such as cellular differentiation (121). Recently, several groups reported that the HIN-200 family protein members AIM2 and IFI16, function as cytosolic DNA sensors (123-127).

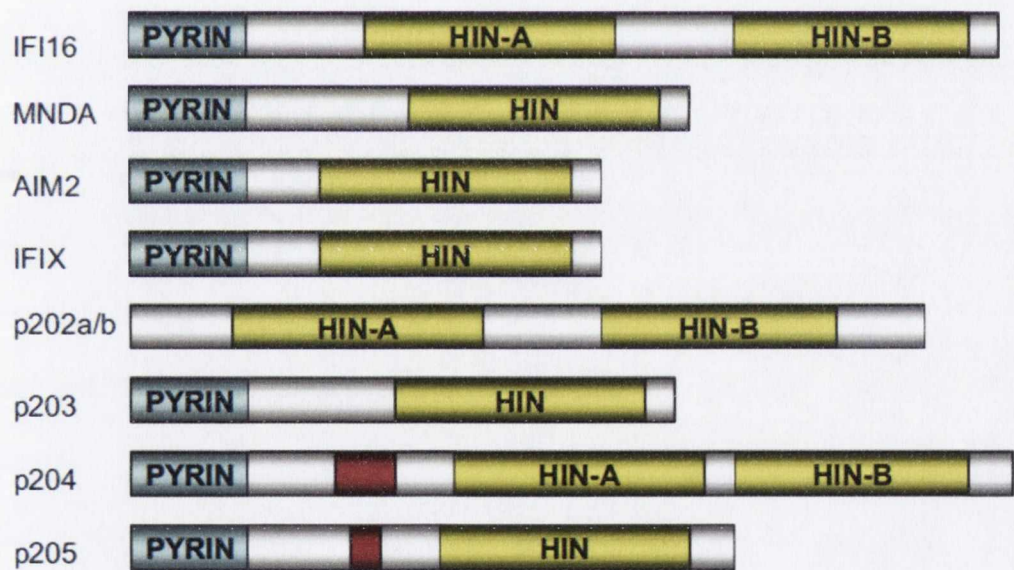


Figure 1.14. Domain organization of HIN-200 family receptors. Most of HIN-200 family receptors possess a C-terminal conserved HIN domain and N-terminal conserved PYRIN domain. Figure taken from Liao *et al.*, Structure (2011) (115).

1.2.5.1. Absent In Melanoma 2 (AIM2)

Several groups have identified AIM2 as an IFN- β inducible DNA sensor (124-127). AIM2 detects dsDNA and AT-rich dsDNA in the cytosol (124-127). AIM-2 consists of one HIN domain and one Pyrin domain. However, AIM2 is not a primary mediator of DNA-induced IFN- β production. Instead, on binding to its ligand, AIM2 recruits ASC (apoptosis-associated speck-like protein containing a CARD) and interacts with it through homotypic interactions of the Pyrin domains (128). Caspase-1 interacts with ASC through its CARD domain (90). Formation of the inflammasome leads to activation of both NF- κ B and caspase-1, and then caspase-1 catalyzes the maturation of IL-1 β and IL-18 (129). Subsequently, pro-inflammatory genes are induced by secreted IL-1 β and IL-18. Although AIM2 is a potential sensor of cytosolic DNA, the exact function of AIM2 in innate immune response against DNA viruses is yet to be elucidated.

1.2.5.2. Interferon- γ inducible gene 16 (IFI16)

IFI16 is one of the HIN-200 family proteins. It consists of one N-terminal Pyrin domain and two C-terminal tandem HIN domains. It has been implicated that it binds to the transcription factors including pRB, E2F1, p53, and BRCA1 and modulates the transcription of the target genes (120,130). Recent studies showed that the IFI16 protein directly associates with an IFN- β -inducing viral DNA motif and induces IFN- β production through the STING-TBK1-IRF3 pathway and not *via* the inflammasome (123) (Fig. 1.16). In contrast to IFI16, AIM2 is not involved in the signaling pathway *via* STING (stimulator of interferon genes) (131). STING was identified as an essential adapter for the induction of type I IFN by intracellular DNA (132,133). STING predominantly localizes in the endoplasmic reticulum (ER) (131). Overexpression of STING induces the activation of both NF- κ B and IRF3 to stimulate type-I IFN production (132,134,135). The structure of IFI16 tandem HIN domains was recently solved (115) (Fig. 1.15). This revealed that both of the tandem HIN domains of IFI16 has high structural homology to the oligonucleotide/oligosaccharide binding (OB) fold (115). Since interaction with nucleotides and downstream adapter proteins is still unclear, understanding the signal transduction mechanism of IFI16 remains to be elucidated.

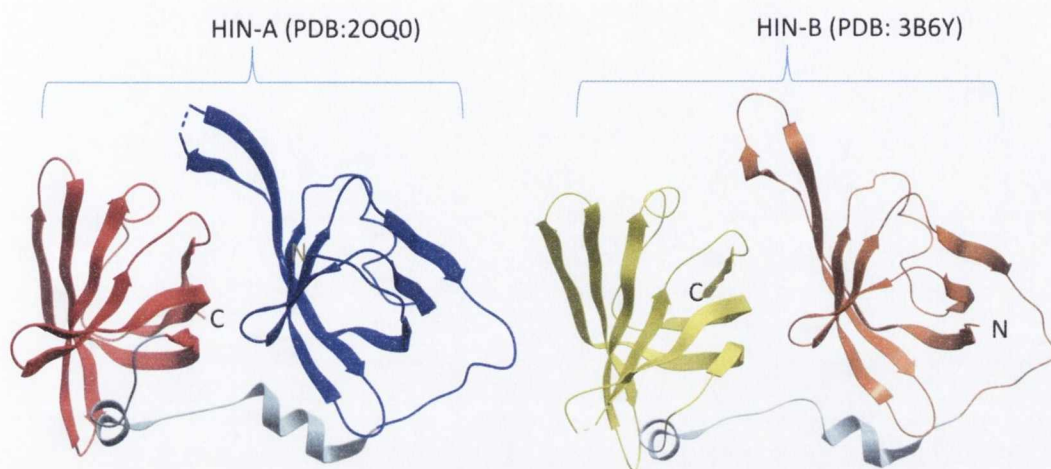


Figure 1.15. Crystal structures of HIN-A and HIN-B domain of IFI16. Each HIN domain of IFI16 consists of two OB-fold sub-domains (shown in red, blue, yellow, and orange) connected by a linker (grey). Liao *et al.*, Structure (2011) (115).

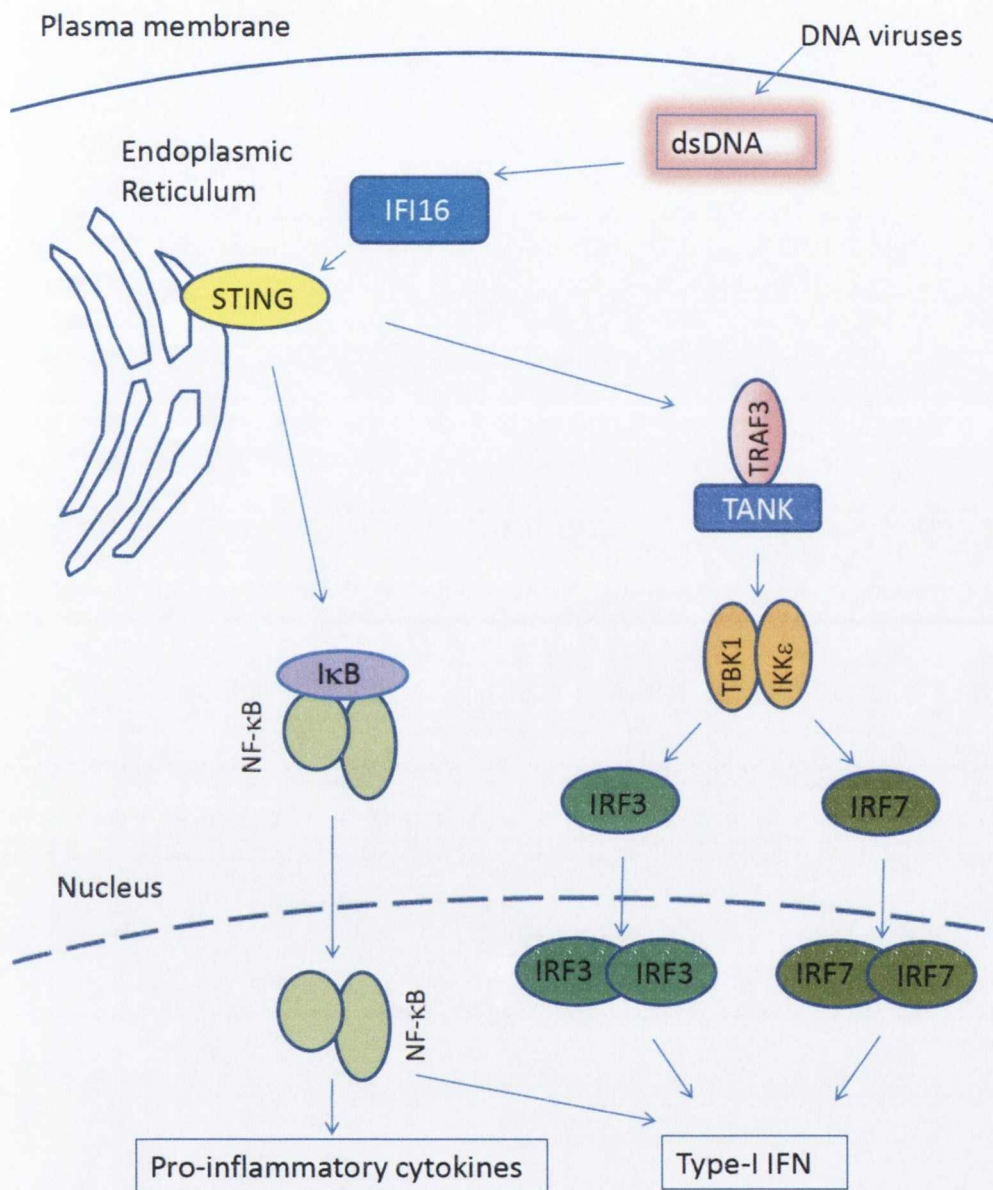


Figure 1.16. Schematic diagram of STING-dependent signaling. dsDNA is detected by IFI16 and then IRFs and NF-κB are activated via STING to induce the production of type-I IFN and other pro-inflammatory cytokines.

1.2.6. Other DNA sensors

Although not all of them are mentioned in this section, several DNA sensor proteins have been discovered recently including DAI, RNA polymerase III, LRRFIP1, DHX9, DHX36, and Ku70 (116).

1.2.6.1. DNA-dependent activator of IRFs (DAI)

DNA-dependent activator of IRF (DAI) has been isolated as a DNA sensor with DNA-binding and TBK1-activating properties (136). Double-stranded DNA induces anti-viral and inflammatory responses after infection with DNA viruses or certain bacteria through a TLR9- and RLR-independent mechanism, *via* DAI (14,137,138). In response to right-handed B-form dsDNA, DAI induces both IRF3 and NF- κ B activation in the cytoplasm of many cell types including immune and non-immune cells (14,139,140). DAI binds directly to the dsDNA and recruits TBK1 and IRF3 (128). IRF3 is then activated, undergoes nuclear translocation and stimulates type-I interferon production (128). This is in contrast to endosome-localized TLR9, which functions in both DCs and B-cells and triggers type-I IFN without the need for TBK1 (141). DAI also induces NF- κ B activation *via* the protein kinases, RIP1 and RIP3 (142). However, responses against dsDNA were unaffected by DAI-deficient mice, suggesting a redundant or non-essential role for DAI (143).

1.2.6.2. RNA polymerase III

DAI-deficient mice still produce interferon in response to B-form DNA and retain the innate and adaptive immune responses of wild-type mice (143). Thus, another mechanism of dsDNA sensing was expected and a cytosolic DNA-dependent RNA-polymerase III dependent pathway was found (144). Cytosolic poly DNA is converted to 5'-ppp RNA by this enzyme and type-I interferon is induced through the RIG-I pathway.

1.3. Interferons (IFNs) and the Jak/STAT signaling pathway

Interferons (IFNs) are known as one of the cytokines which are induced in response to the infection by pathogens and they exert anti-pathogenic cellular actions through the regulation of many downstream effector genes (145).

IFNs are categorized as, Type I to III IFNs (146). Type I IFNs make several contributions in fighting viral infection. IFN- α and IFN- β are the best characterized and most broadly expressed among the members of the type I IFN family of class II α -helical cytokines in human and mice (147). Type I IFN gene transcription is regulated by the activation of IRF-3, IRF-7, AP-1 [AP-1 is heterodimer of activating transcription factor 2 (ATF2) and c-Jun], and NF- κ B, which bind to the specific site on type I interferon promoter (148-153). IRFs are activated through signal transduction from the endosomal TLRs, RIG-I-like receptors, and other cytoplasmic receptors in response to cytosolic viral invasion (95,148). Type I IFNs, which are produced and secreted in specialized immune cells such as DCs and macrophages in response to extracellular stimuli of viral or bacterial origin, also exert the regulatory function of adaptive immunity by leading the maturation of DCs, the differentiation of cytosolic T cells, and activation of natural killer (NK) cells (137).

The type I interferons produced in infected cells are secreted and bind to the heterodimer receptor known as the IFN- α receptor (IFNAR) complex that is composed of IFNAR1 and IFNAR2 which are associated with Janus-family kinases (JAK) by their cytoplasmic domain (147) (Fig. 1.17). IFNAR1 and IFNAR2 associate with tyrosine kinase Tyk2 and Jak1 respectively. There are 4 mammalian JAKs including Jak1, Jak2, Jak3, and Tyk2. Type I IFN binding to the receptor complex brings Tyk2 and Jak1 into close proximity and allows their cross-phosphorylation and activation (154-157). The activated JAKs phosphorylate the receptor cytoplasmic domains, which create docking sites for SH2-containing signaling proteins, including signal-transducing activators of transcription (STAT) (158). Thus, STAT1 is recruited to cytoplasmic domain of the receptor and then JAKs phosphorylate STAT (158). Subsequently, the STAT1 forms a heterodimer with receptor-bound STAT2. STAT1-STAT2 heterodimers dissociate from the receptors and migrate into the nucleus (147,159). In the nucleus, the STAT1-STAT2 heterodimer associates with IRF9 to form IFN-stimulated gene factor 3 (ISGF3) (160). ISGF3 translocates to the nucleus to induce several hundred of IFN-stimulated genes (ISGs) (161) from IFN-stimulated response elements (ISREs).

Type II IFN, IFN- γ , which is a distinct structure from type I IFNs, is expressed in T-cells and natural killer cells (153,162). In the case of type II interferon, IFN- γ , the activation of Jak/STAT signaling is somewhat different from the case of type I IFNs. IFN- γ binds to a heterodimeric receptor formed by the two chains IFNGR1 and IFNGR2. These two subunits are isolated in the absence of stimulation (163-165) and become assembled (166,167). By binding of IFN- γ to these receptors, Jak family kinases Jak1 and Jak2 are activated (164). Activated kinases phosphorylate and activate STAT1 to form a homodimer of STAT1 (168). This homodimer translocates to the nucleus and induces IFN-stimulated genes (ISGs) by binding to the IFN- γ activated sequence (GAS) (147) (Fig. 1.17).

Only a fraction of ISGs encoding host proteins are well characterized, and little is known of the remainder (169). ISGs can be interferon-type-specific or common to both type I and type II interferons (161). For instance, antiviral genes that are activated by IFNs include three enzymes that bind double-stranded RNA; the dsRNA-activated protein kinase R (PKR) inhibits translation; the family of 2'-5'-oligoadenylate synthetases mediates RNA degradation through activation of RNAase L; and adenosine deaminase inactivates viral RNAs by RNA editing (147). One family of GTPases - Myxovirus resistance (Mx) family of proteins - is mainly activated by IFN- α and/or IFN- β . These proteins affect the intracellular transport of viral particles and inhibit viral replication, but these proteins do not explain the antiviral response completely (170). Thus, the other effector proteins require further characterization (170).

IFN- γ target genes included the gene that encodes inducible nitric oxide synthase (iNOS, also called NOS2). This enzyme generates NO radicals which have been shown to have an antibacterial effect (171). The other target genes of IFN- γ are subunits of the phagocyte NADPH oxidase, which generates O_2^- radicals, and indoleamine-2,3-dioxygenase which suppresses the proliferation of bacteria and virus by catalyzing degradation of tryptophan (172). IFN- γ also targets the genes of members of the GTPase superfamily that alter the trafficking and/or maturation of phagosome to counteract pathogen strategies that are based on survival in intracellular compartments (169).

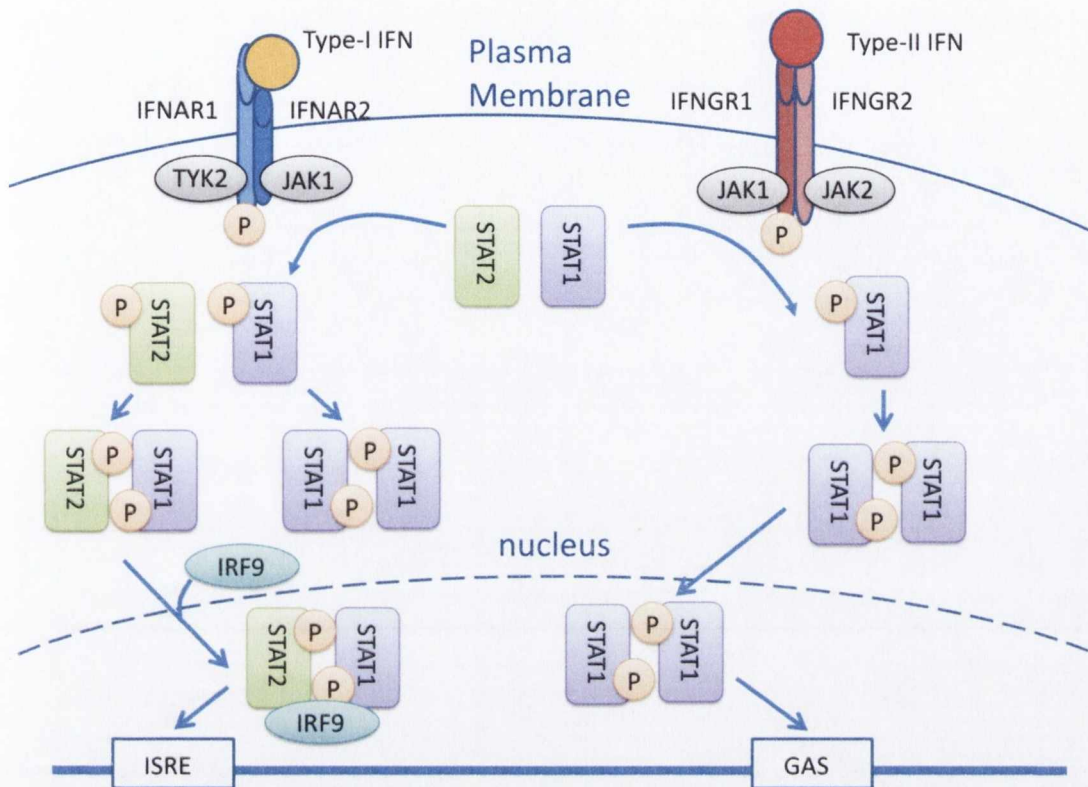


Figure 1.17. Schematic diagram of the Jak/STAT signaling pathway. Secreted type I and type II IFNs are recognized by corresponding IFN receptors (IFNAR for type I and IFNGR for type II IFNs) and associate with Janus kinases (JAKs). JAKs associated with IFNRs are activated by cross-phosphorylation which subsequently phosphorylate and activate the signal transducer and activator of transcription (STAT) 1 or 2. Tyrosine phosphorylated STATs form STAT1 homodimers on stimulation by both IFNGR and IFNAR, and STAT1-STAT2 heterodimer on stimulation by IFNAR. STAT dimers then move to the nucleus. STAT1-STAT2 heterodimers forms IFN-stimulated gene factor 3 (ISGF3) with IFN regulatory factor 9 (IRF9) and ISGF3 binds to the IFN-stimulated response element (ISRE) promoter sequence to stimulate IFN-regulated gene transcription. The STAT1-STAT1 homodimer binds to the IFN- γ -activated site (GAS) promoter sequences and induce the gene transcription.

Viruses that infect our body have evolved strategies to antagonize our immune system. Some proteins encoded by virus genomes interact with proteins which mediate the PRR signaling pathways, thereby inhibiting subsequent signal transduction. Viruses have developed these evasion strategies throughout their evolution and many viral proteins are critical for virulence.

1.4. Vaccinia Virus (VACV)

Viruses are obligate parasites that depend on host cells for survival and, throughout evolution, they have developed strategies to evade or subvert key aspects of the host antiviral response (173). Poxviruses represent a family of large double-stranded DNA viruses that replicate in the cytoplasm. *Variola virus* (VAR), the cause of smallpox, is the most notorious poxvirus and was eradicated by vaccination with *Vaccinia virus* (VACV) (174). The poxvirus family is subdivided into the entomopoxvirus (EnPV) and chordopoxvirus (ChPV) subfamilies which infect insects and chordates, respectively. The ChPVs are further divided into eight genera (*Avipoxvirus*, *Molluscipoxvirus*, *Orthopoxvirus*, *Capripoxvirus*, *Suipoxvirus*, *Leoporipoxvirus*, *Yatapoxvirus*, and *Parapoxvirus*), whereas the EnPVs are divided into three genera (*A*, *B*, and *C*) (174).

VACV encodes about 200 open reading frames (ORFs) (175). In addition to viral RNA/DNA polymerase (176,177) and virion assembly proteins, poxviruses encode numerous proteins that antagonize host immunity (178,179). The central portion of orthopoxvirus genomes (~100 kb) is highly conserved and contains genes essential for virus replication (174). Because VACV encodes its own DNA polymerase to replicate the viral dsDNA genome, it replicates in the cytoplasm and so does not rely on the host nuclear replication system (180). They have evolved numerous mechanisms for evasion of innate immune signaling pathways to favour their replication.

Many proteins are expressed during infection by VACV and each protein is utilized in distinct ways, for example; in (i) antagonizing interferon production (A46, A52, K7, N1, B14, K1, M2, B19, B8, H1, E3, K3, and C7); (ii) capturing chemokines (C23); and (iii) intercepting cytokines (A53, B13, B16, C12) (155). These proteins antagonize the immune response of the host cell and may help enhance viral propagation. The targets of some of those proteins are critical components of PRR

signaling pathway such as TRIF, TRAF6, IRF7, IRAK2, and DDX3 (155) (Fig. 1.18. and Fig. 1.19.).

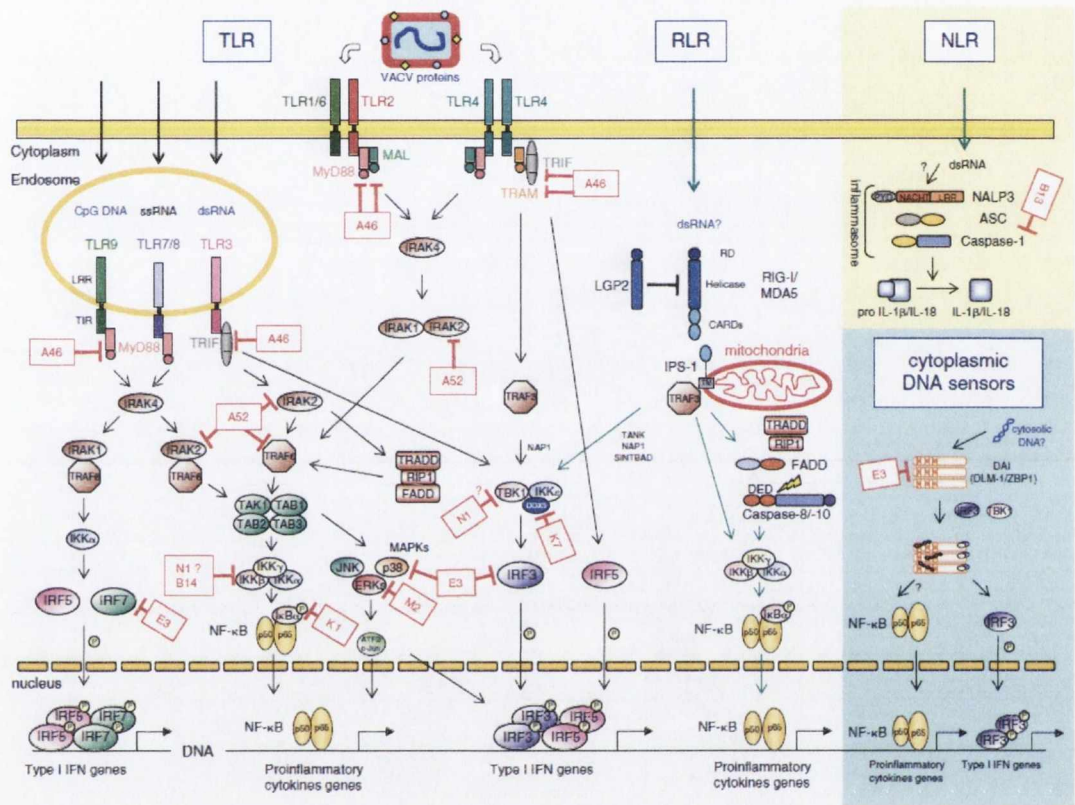


Figure 1.18. Relationship between PRRs, their signaling pathways, and VACV proteins. Four host PRRs (TLRs, RLRs, NLRs, and DAI) are involved in the sensing of VACV-specific components (highlighted by blue boxes). VACV encodes several immunomodulatory proteins which interfere with these signaling pathways (shown in the red boxes). Figure taken from Perdiguero, B. & Esteban, M. (2009) (155).

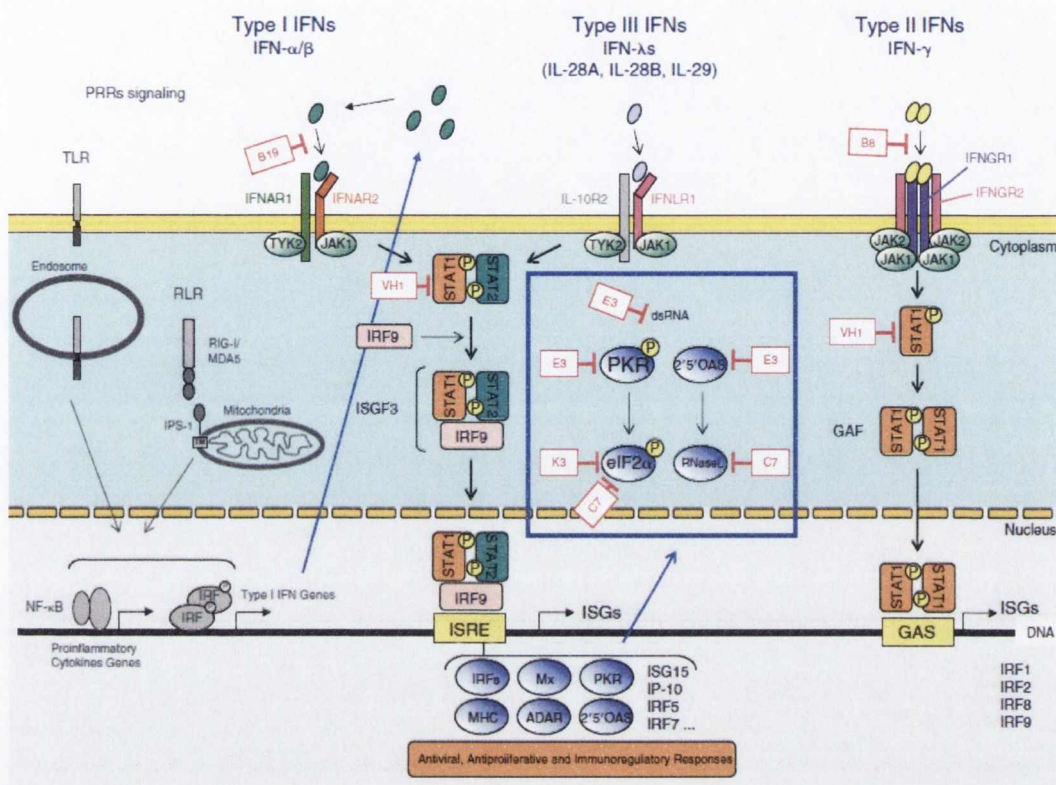


Figure 1.19. Relationship between IFN signaling pathways and VACV proteins. VACV also encodes proteins interfering with the components of IFN signaling pathways (shown in the red boxes). Figure taken from Perdiguero, B. & Esteban, M. (2009) (155).

1.4.1. The Vaccinia virus A46 protein and its cellular targets

1.4.1.1. The Vaccinia Virus A46 protein

The VACV protein A46 was discovered during database searches for TIR domain-containing proteins and was characterized as an antagonist of IL-1 and TLR4 signaling (181). A46 suppresses the MAP kinase and TLR3-TRIF mediated IRF3 activation, but not TLR3 mediated NF- κ B activation (179).

A46 is a 240 amino acid residue protein that binds directly to the TIR domain-containing adaptors, MyD88, Mal, TRIF, and TRAM, which are mentioned below, interfering with the downstream activation of MAPKs, NF- κ B, and IRFs triggered by multiple TLR pathways (Fig. 1.18) (179,181). It has been speculated that A46 has a TIR domain, which would make it the only viral member of the IL-1R/TLR family identified to date (179,181). Considering A46 interacts with a range of TIR-domain containing adaptor proteins, it was speculated that A46 interacts with TIR domain-containing adaptors *via* its TIR domain, and sequesters the adaptor proteins from signaling. In direct contradiction to this model, another group predicted that A46 adopts a Bcl-2 like fold based on bioinformatics analysis (182). Database searches revealed that A46 belongs to the Orthopox_N1 family of the Pfam database (182). Secondary structure prediction showed that A46 consists of a C-terminal α -helical sub-domain (residue 90 to 220) and N-terminal region (1-89) which consists largely of β -strand (Fig. 1.20). Furthermore, multiple sequence alignment showed that the N-terminal α -helical sub-domain of A46 revealed significant sequence similarity with other Orthopox_N1 family of proteins, which include other poxvirus proteins, such as N1, A52, and K7 (182). Seven alpha helices which are observed in canonical Bcl-2 fold protein were predicted on the N-terminal alpha-helical sub-domain of A46. Although three conserved TIR domain sequences were identified along the A46 sequence (179) (named box 1, box 2, box 3, located on the N-terminus, α -1 helix, and α -7 helix of predicted Bcl-2 fold sub-domain, respectively), there is no significant sequence similarity of A46 with TIR domains, in which the central five-stranded parallel beta-sheet is surrounded by alpha-helices as seen in the TLR10 or MyD88 TIR domains (Fig. 1.1 and 1.3), using tools for remote sequence search and fold recognition (182).

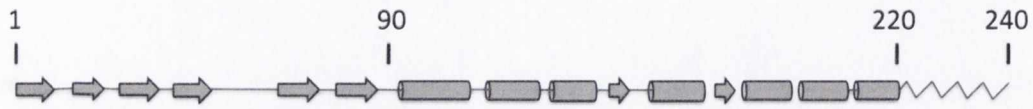


Figure 1.20. Schematic diagram of secondary structure prediction of A46. β -strands are represented by arrows, and α -helices are cylinders. The C-terminal 20 residues (220-240) are predicted to be disordered (zigzag line). Two short β -strands are predicted in the region from 90 to 220, but otherwise the region is uniformly α -helical. Only those regions of the sequence that are consistent with both PsiPred and PredictProtein servers are shown.

An 11-aa-long A46 peptide called VIPER (viral inhibitor peptide of TLR4), which encompasses residues KYSFKLILAEY and corresponds to residues 88 to 98 of the VACV A46 protein, and has been identified as an antagonist of TLR signaling (183). It was demonstrated that this peptide interacts only with Mal and TRAM, but not with MyD88 and TRIF, which have direct interaction with the A46 protein (183). VIPER inhibits the signal transduction from TLR4 specifically, and not through other TLR signaling pathways (183). This peptide was derived from the predicted alpha 1 helix, which is located on the positively charged molecular surface of A46. The positively charged character matches the predicted negatively charged character of Mal and Tram (183). It was shown that leucine 6 of VIPER, which corresponds to leucine 93 of A46, is critical for interaction of VIPER with Mal and TRAM (183). This peptide may, at least partly, explain the binding of the TIR domain by A46, but the detailed interactions and mode of inhibition remains unclear.

1.4.1.2. Toll/IL1 receptor (TIR) domain-containing adaptor proteins

TIR domains constitute the cytoplasmic portion of TLRs, the IL-1 receptor family, and TIR domain-containing adaptor proteins. As it is mentioned earlier, signaling by TLRs is initiated by the recruitment of the TIR domain-containing adaptor proteins to the activated proximal TIR domain portion of TLRs. As of now, five TIR domain-containing proteins have been identified: i) myeloid differentiation factor 88 (MyD88) (184,185), ii) MyD88 adaptor-like [Mal (186), also called TIR domain-containing adaptor protein (TIRAP) (187,188)], iii) TIR domain-containing

adaptor-inducing IFN- β [(TRIF) (189-191), also called TIR domain-containing adaptor molecule 1 (TICAM-1) (192)], iv) TRIF-related adaptor molecule (TRAM, also called TICAM-2 (193-195)), and v) SARM (33) are known. Each TLR uses a different set of adaptors and the recruitment of adaptors determines the direction of signaling pathway of TLRs. Vaccinia virus (VACV) protein A46 (as mentioned in section 1.4) interacts with these TIR domain-containing adaptors (MyD88, Mal, TRAM, and TRIF) and inhibits signal transduction (179). In this study, the interaction of A46 and some TIR domain-containing adaptor proteins was examined. Therefore, it is necessary to discuss in some detail of the functions of Mal and TRAM.

1.4.1.3. MyD88 adaptor like protein (Mal)

Mal is one of the TIR domain-containing adaptor proteins. Mal is a 235 amino acid residue protein which has a phosphatidylinositol-4,5-bisphosphate (PIP2)-recognition domain on its N-terminus and it functions to localize Mal to the plasma membrane, where it interacts with TLR4 (34). Mal shuttles between the plasma membrane and the endosome by an ADP ribosylation factor 6 (ARF6) dependent process (34). Mal shares 24% sequence identity with MyD88 whose TIR domain structure was solved by NMR (36) (Fig. 1.3). This protein is only used in the signal transduction from TLR2 and TLR4 (196) and there is a different interaction mechanism for each receptor (the DD-loop is used for TLR2 and BB-loop is used for TLR4 interactions) (197-199). The signal from TLR2 and TLR4 activates NF- κ B and pro-inflammatory cytokine production (187,189,194). Mal bridges between the TIR domains of MyD88 and TLR *via* heterotypic TIR domain interactions (7) (Fig. 1.1 and 1.21). Electrostatic surface complementarity between the TIR domain of TLR 4, 3 and MyD88 allows interaction and the functioning of Mal as a bridging adaptor (197). The cleavage of the C-terminal fragment of Mal by Caspase-1 and its phosphorylation on several sites are necessary for activation (200).

The cleavage of Mal by caspase-1 is performed at Asp198 (201). It was shown that the inhibition of caspase-1 resulted in a failure of NF- κ B activation in response to LPS and phosphorylation of p38 MAPK in response to Pam₃Cys (201). Therefore, it was thought that the cleavage at the C-terminus of Mal was crucial for Mal activation and signal transduction from TLR through Mal. Furthermore, it was expected that the

activation of the inflammasome potentiates signaling by TLR2 and TLR4 *via* the cleavage of Mal by caspase-1 (202). However, recent studies show that Mal cleavage by caspase-1 is not required for Mal interaction with TLR4 or MyD88, or for Mal induced NF- κ B activation (200). Instead, it was shown that the negatively charged character of the caspase-1 cleavage site is crucial for the interaction and function of Mal (200). This matter requires further study and clarification.

Phosphorylation of Mal at several sites is also crucial for Mal activity. Brutons tyrosine kinase (Btk) mediates the phosphorylation of Mal on tyrosine 86, 106, and 159 (202). Two separate studies have reported that phosphorylation is induced by LPS on these sites (203,204). Mutations in Mal at these phosphorylation sites result in a decreased ability to phosphorylate p38, degrade I κ B and activate NF- κ B (204). The mutations prevent the interaction of Mal with Btk and enhance the ability of Mal to bind the TIR domain of TLR4. As a result, the mutations lead to a dominant negative effect of the Mal mutant over wild type (204).

1.4.1.4. TRIF-related adaptor molecule (TRAM)

TRAM is the other TIR domain-containing adaptor protein. It is required for MyD88-independent signal transduction pathway of TLR4. TRAM bridges TIR domains of TLR4 and TRIF (205). The signal through TRAM and TRIF induces IRF3-dependent type-I interferon production (57,189,191-194) .

TRAM is a 235 amino acid residue protein containing C-terminal TIR domain (residue: 73-235) and N-terminal bipartite localization motif (residue: 1-20). TRAM interacts with TLR4 and TRIF *via* homotypic interaction of TIR domains (198). The signal from TLR4 induces the signal transduction in two distinct ways, Mal-MyD88 and TRAM-TRIF dependent signaling pathways. TLR4 induces the Mal-MyD88 dependent signaling pathway from the plasma membrane at an early stage. After the endocytosis of TLR4 induced by LPS, the TRAM-TRIF dependent signaling pathway is induced from the early endosome (205). TRAM localizes on the plasma membrane or Golgi apparatus *via* the bipartite localization motif at the N-terminus, which consists of a myristoylation site and subsequent polybasic residues (205). Myristoylation facilitates membrane binding *via* its insertion into the hydrophobic interior of the lipid bilayer (37) and the polybasic sequence helps TRAM localization at the membrane by interacting with

acidic phospholipids (205). TRAM co-localizes and translocates with TLR4 but is independent of the interaction with the receptor itself (205). Phosphorylation of both Ser6 and Ser16, which are located in the myristoylation motifs and polybasic motifs respectively, catalyzed by protein kinase C ϵ (PKC ϵ), induces the translocation of TRAM from plasma membrane to early endosome (206).

1.4.2. Cellular and Viral B-cell lymphoma-2 (Bcl-2) fold proteins

Programmed cell death (apoptosis) plays a vital role in normal development, tissue homeostasis, and the removal of damaged and infected cells in higher organisms (207). The Bcl-2 family proteins act as gatekeepers for mitochondria-mediated apoptosis (208). The three-dimensional structures of Bcl-2 family members consist of two central, predominantly hydrophobic α -helices surrounded by six or seven amphipathic α -helices of varying lengths (207) (Fig. 1.22). Although they have the same fold, they diverge in amino acid sequence and function. One group has pro-apoptotic, and another group has anti-apoptotic functions. In mammals, at least 12 core Bcl-2 family proteins are currently known. Structural and functional studies have identified the importance of conserved Bcl-2 homology motifs (BH1, BH2, BH3, and BH4) in many family members (207,209-212). Bcl-2 family members have been grouped into three classes that are characterized by the presence of Bcl Homology (BH) motifs (213-215). One class including Bcl-2, Bcl-xL, Bcl-W, Mcl1, Bcl-B, and A1, inhibits apoptosis (anti-apoptotic proteins) by protecting the integrity of the mitochondrial outer membrane (216). This class possesses three or four of the BH motifs. The second group includes Bax, Bak, and Bok which are directly responsible for disrupting the mitochondrial membrane, and are pro-apoptotic proteins. This group of proteins contains multiple BH domains (BH1, BH2, and BH3) and are required downstream of BH3-only proteins to induce apoptosis (217). The third group including Bad, Bik, Bid, Hrk, Bim, Bmf, NoxA, and PUMA are pro-apoptotic BH3-only proteins. This latter group become activated in response to various forms of cellular stress (210) and promote apoptosis by inhibiting anti-apoptotic proteins as well as activating pro-apoptotic proteins (216). Anti-apoptotic proteins inhibit apoptosis by forming dimers with pro-apoptotic proteins (218,219). During apoptosis, BH3-only proteins interfere with the binding of these complexes releasing pro-apoptotic proteins, which are then able to form oligomers. The oligomers form a pore in the mitochondrial outer membrane leading to the loss of membrane potential and the release of apoptotic agents

from the mitochondria (220).

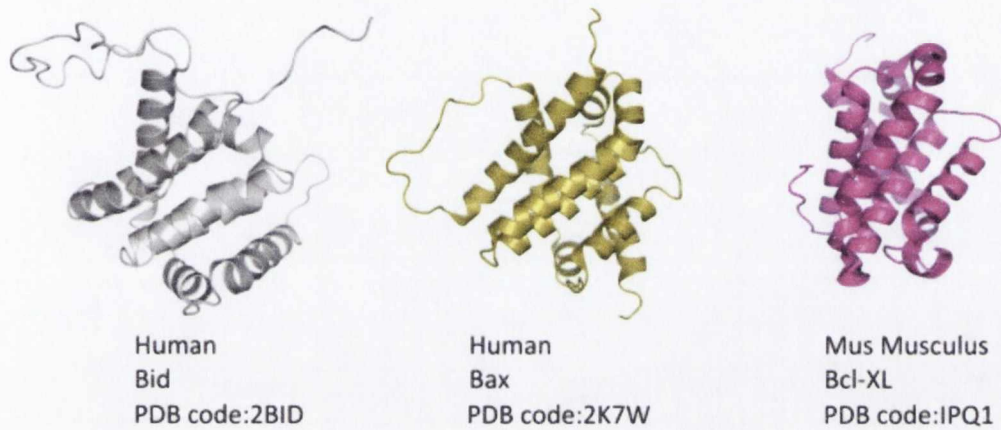


Figure 1.22. Three dimensional structure of cellular Bcl-2 fold proteins.

Cellular Bcl-2 fold proteins are known to be regulators of apoptosis. Human Bid (PDB code: 2BID. Chou et al., Cell (1999)), Human Bax (PDB code: 2K7W. Gavathiotis et al., Nature (2008)), and Mus Musculus Bcl-XL (PDB code: 1PQ1. Liu et al., Immunity (2003)).

The viral Bcl-2 like proteins are required for successful viral propagation and/or persistence. Some viruses express anti-apoptotic Bcl-2 homologs which can act either by binding and sequestering the BH3-only proteins or by directly binding to pro-apoptotic proteins, forming complexes that are resistant to BH3-only protein interference (216,221,222). The viral Bcl-2s are able to accomplish this because of their structural similarity to anti-apoptotic proteins. Myxoma virus M11 (223), cytomegalovirus vMIA (224) and vaccinia virus F1 (225) are such proteins. The structural studies of these proteins revealed that anti-apoptotic Bcl-2 proteins inhibit apoptosis by binding the BH3 peptide of pro-apoptotic Bcl-2 and BH3-only proteins, in a hydrophobic groove on the surface of the anti-apoptotic protein (226).

In recent years, several Bcl-2 fold viral proteins have been identified which do not appear to play any role in apoptosis (227,228). However, poxvirus proteins such as A52, A46, and K7 appear to have roles in antagonizing immune signaling pathways (85,181). Structural, sequence and evolutionary analysis suggest that these proteins

have evolved from an ancestral mammalian Bcl-2 protein and have acquired novel roles in immune signaling to favour viral replication (182,227). The crystal structure of A52, B14, and N1, revealed that they adopt Bcl-2 fold (227,229,230). The NMR structure of K7, determined by a Leeds group in collaboration with the Khan lab in Dublin, also revealed a Bcl-2 fold (228).

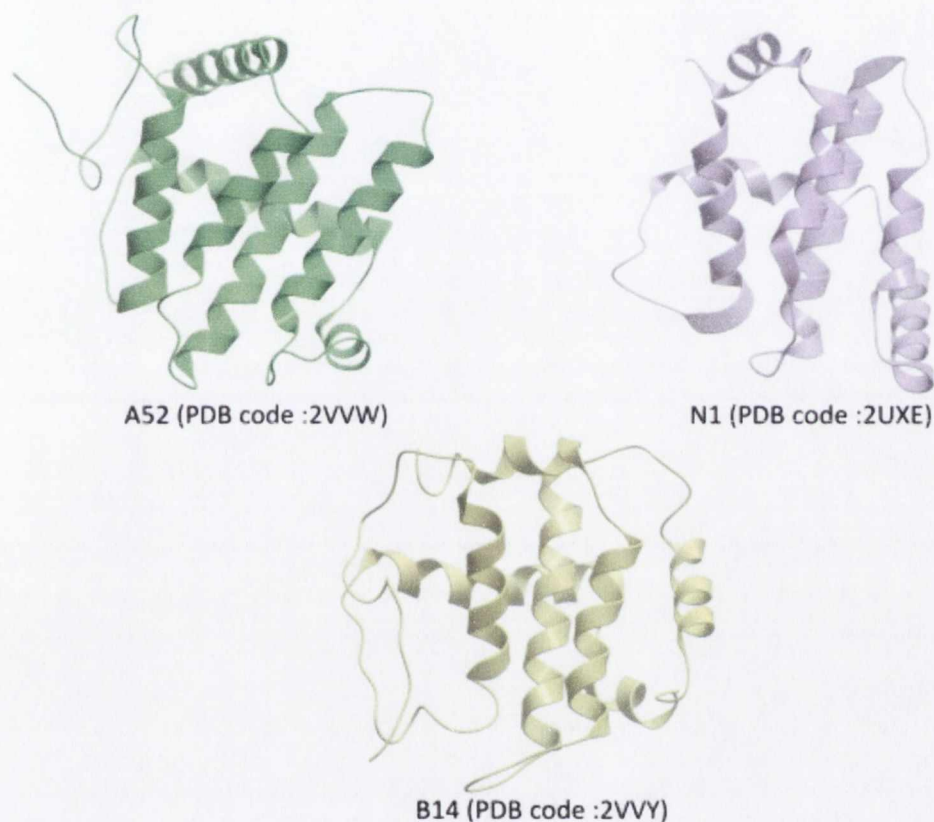


Figure 1.23. Three-dimensional structures of vaccinia virus Bcl-2 fold proteins. Viral Bcl-2 fold proteins adopt an α -helical Bcl-2 fold despite sharing low sequence homology with cellular Bcl-2 fold proteins and they have different cellular targets. A52 (PDB code: 2V VW. Graham et al., Plos Pathog. (2008)). N1 (PDB code: 2UXE. Cooray et al., J.Gen. Virol (2007) (230)). B14 (PDB code: 2VVY. Graham et al., Plos Pathog. (2008) (227)).

1.4.2.1. The VACV protein A52

A52 is a 23 kDa intracellular viral protein with no obvious similarity to host proteins. It inhibits the activation of NF- κ B by interacting with the downstream

TLR-signaling molecule IRAK2 (231). A52 also interacts with TRAF6 directly and enhances MAPK activation (181,232). Activation of p38 MAP kinase is enhanced by the direct binding of A52 to TRAF6 and drives the p38-dependent production of IL-10, which is a cytokine that inhibits inflammatory and cell-mediated immune responses (155). The crystal structure of A52 (Fig. 1.23) was solved but both the mechanism by which it binds cellular targets and its regulation mechanism of PPR signaling by A52 are still ambiguous (227) (Fig. 1.23).

P38 MAP kinase activation was enhanced by the direct interaction of A52 with the TRAF domain (Fig. 1.24) of TRAF6 while truncation of the C-terminal 46 residues abolishes this interaction and activation of the p38 MAP kinase (178,232). A52 forms a stable dimer in solution and adopts an α -helical Bcl-2 like fold (Fig. 1.23). The dimer interface comprises the N-terminal region, α -1, and α -6, which is conserved among the dimeric Bcl-2 superfamily.

1.4.2.2. TNF receptor-associated factor 6 (TRAF6)

TRAF6 has a crucial role in innate immune signal transduction. Most TRAFs consist of an N-terminal RING (really interesting new gene) domain, zinc finger domains, and a C-terminal TRAF domain that comprises a coiled-coil and a conserved TRAF-C domain (Fig. 1.24). The C-terminal domain of TRAFs forms a trimer *via* the coiled-coil domain. The TRAF-C domain functions as an interaction motif with receptors and adaptor proteins (233-235). On the other hand, the N-terminal RING domain and zinc finger domains form dimers in the known crystal structures, and in solution (236). Because of presence of both of trimeric and dimeric assemblies, the existence of an extended structure of TRAF6 was proposed (236).

TRAF6 is a RING-domain-containing E3 ubiquitin ligase which does not seem to catalyze ubiquitination but provides a bridge between the E2 ubiquitin ligase complex, Ubc13/Uev1A, and substrate (54) (Fig. 1.24). The crystal structure of TRAF6 in complex Ubc13 revealed how they interact (Fig. 1.25) but the ubiquitination mechanism of TRAF6 is still ambiguous. TRAF6 mediates self Lys-63-linked poly-ubiquitination. This Lys63-linked ubiquitination is non-degradative and functions as a signaling moiety in DNA-damage repair processes and innate immunity pathways, unlike Lys48 linked ubiquitination. It is reported that this Lys63 linked ubiquitin recruits TAB2 and TAB3. TAB2 and TAB3 bring TAK1 to TRAF6, and the IKK

complex is also recruited. IκB is phosphorylated, and it then undergoes Lys48-linked ubiquitination, releasing NF-κB to the nucleus.

It was reported that the Lys63-linked polyubiquitin chain, which is not anchored to any target proteins, activates TAK1 by binding to TAB2 *in vitro* (237). The same group also reported that unanchored polyubiquitin chains synthesized by TRAF6 and E2 ubiquitin ligase UBCH5C activated the IKK complex. Therefore, it was suggested that Lys63-linked ubiquitin chain provides the scaffold for a kinase complex which leads to the oligomerization and autophosphorylation of kinases (237).

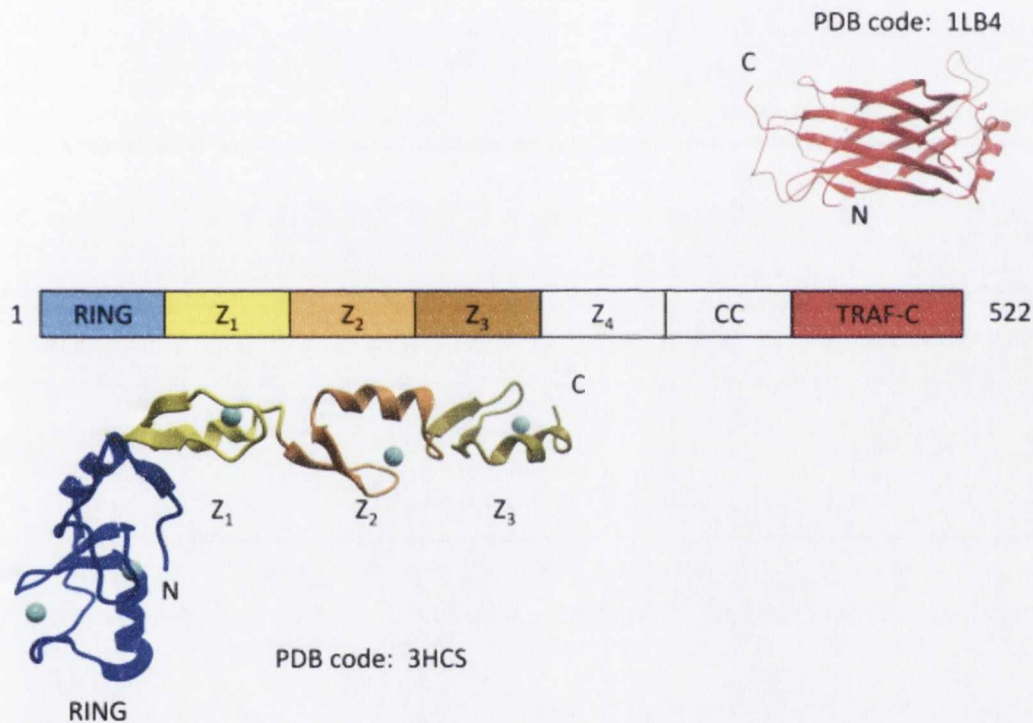


Figure 1.24. Domain organization and three-dimensional structure of TRAF6.

TRAF6 contains RING, four zinc finger (Z₁ to Z₄), coiled-coil (CC), and TRAF-C, domains. RING (blue) and Zinc finger (yellow and orange) structure and TRAF-C domain (red) structure was solved separately. Zinc atoms are represented in ball models. TRAF-C domain of TRAF6 (PDB code: 1LB4. Ye et al., Nature (2002) (235)). N-terminal domain of TRAF6 (PDB code: 3HCS. Yin et al., Nat. Struct. Mol. Biol. (2009) (236)).

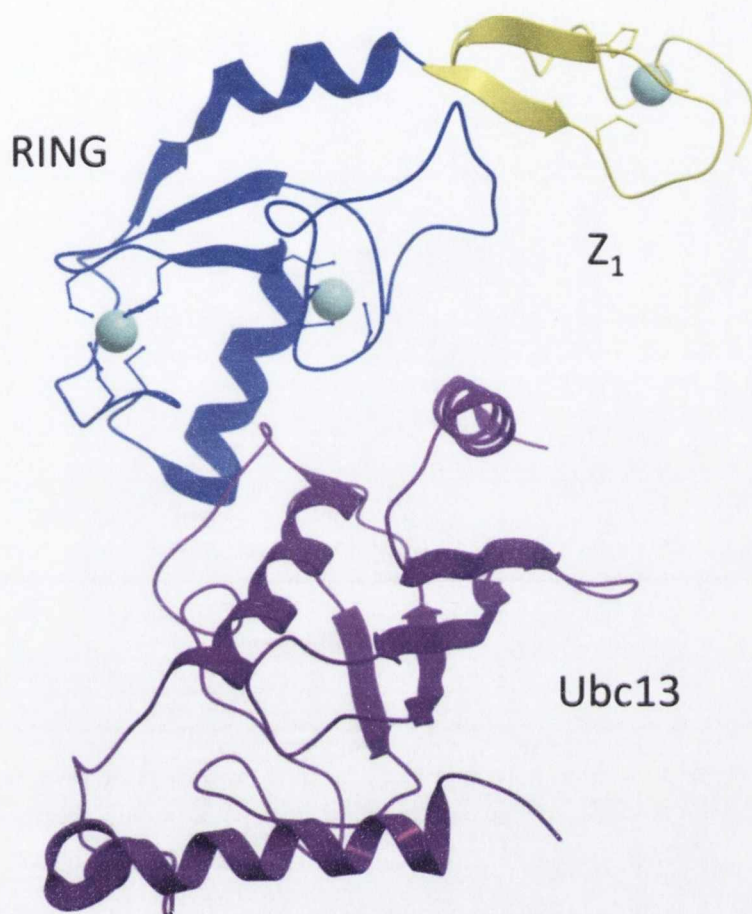


Figure 1.25. Three dimensional structure of TRAF6 RING-Z1 in complex with the E2 ubiquitin conjugating enzyme Ubc13 (PDB code: 3HCT, Yin et al., *Nat. Struct. Mol. Biol.* (2009) (236)). TRAF6 interacts with Ubc13 via the RING domain. Ubc13 is colored purple and the RING-domain and zinc-finger motif of TRAF6 are colored blue and yellow respectively. Zinc atoms are represented in ball model.

1.4.2.3. Protein K7 of VACV

K7 is a 149 amino acid protein and is very highly conserved within the poxvirus family, suggesting that it plays an important role (85). The NMR structure of K7 revealed that K7 adopts a Bcl-2 fold although it is devoid of sequence similarities to other Bcl-2 family proteins (228). As mentioned earlier, viral and cellular Bcl-2 fold proteins regulate cell death by forming hetero-oligomers *via* a surface groove (BH3 groove) that binds to α -helical BH3 motifs of partner Bcl-2 proteins. The BH3 groove is formed by helices $\alpha 2$ - $\alpha 5$ and is observed in various conformational states, from “open” to “closed” in the absence of BH3 ligands (217,238-242). The BH3 groove is “open” in the ligand-bound state of conventional Bcl-2 proteins that regulate apoptosis, thus enabling intimate hydrophobic contacts with amphipathic BH3 α helices (207,217,240,241). Although the N1 protein from vaccinia virus, which interacts with pro-apoptotic cellular proteins, has an open BH3 groove, the NMR structure of K7 revealed that the BH3 groove in K7 is closed and it is consistent with the function of K7 not being linked to the regulation of apoptosis (228) (Fig.1.26).

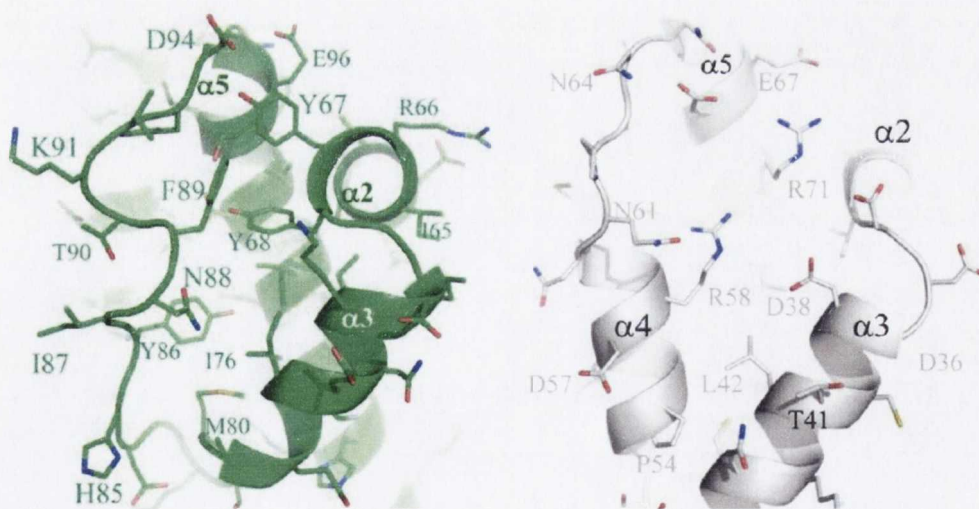


Figure 1.26. The BH3 groove of K7 and N1.

Side chains are represented as stick models. The BH3 groove of K7 (green) (PDB code: 2K36. Kalverda et al., J. Mol. Biol. (2009) (228)) is filled with hydrophobic side chains (Y67, Y68, and F89). The N1 surface is relatively open (grey) (PDB code: 2UXE. Cooray et al., J. Gen. Virol. (2007) (230)).

K7 and A52 have a Bcl-2 type fold and they antagonize the innate immune system of the host cell by interacting with the proteins in the innate immune signaling pathway. K7 shares 20% overall sequence identity with A52. The crystal structure of A52 was determined by the group of Prof. David Stuart (227). K7 and A52 have similar three-dimensional structures, although A52 is a dimer in solution, while K7 is monomeric. Both K7 and A52 interact with TRAF6 and IRAK2 and inhibit TLR-induced NF- κ B activation (85,232). However, unlike A52, K7 binds to human DDX3 and inhibits TLR-dependent and -independent IRF3/7 activation and induction of the IFN- β promoter. The investigation of this K7-mediated viral evasion strategy led to the discovery of a novel role for DDX3 in innate immunity (85). DDX3 binds to, and is phosphorylated by IKK ϵ and/or TBK1 (84,85), two kinases which are essential for IRF3/7 activation. Following PRR stimulation, DDX3 enhances IRF3 activation and IFN- β induction as mentioned earlier (84,85,243). K7 binding to DDX3 interferes with the activation of IRF3/7 and subsequent induction of the IFN- β promoter. Previous biochemical studies in our laboratory showed that K7 recognizes a segment of DDX3 (residues 61-90) that is upstream of the conserved tandem RecA-like helicase domains (228). This region correlates with a functional role for DDX3 (1-139) which is necessary to enhance IRF3 activation (85). This domain is enriched with 27 serine residues, 20 glycine residues, 25 positively charged amino acids (Lys, Arg, and His), and 22 negatively charged amino acids (Asp and Glu). Thus, this segment of DDX3 is presumably unstructured and is not conserved in other RNA helicases or known proteins in the database.

Despite the identification of numerous cellular targets of VACV proteins, there is currently little structural information regarding the protein-protein complexes formed during infection. The only structure of a viral Bcl-2 protein with its target involves a BH3 peptide from Bak, which binds to the canonical BH3 groove of myxoma M11 protein (240). Intriguingly, K7 and A52 share similar three-dimensional structures and common cellular targets, but A52 is unable to bind DDX3 (85).

1.4.2.4. Human DEAD-box RNA helicase 3 (DDX3)

The human DEAD-box protein 3 (DDX3) belongs to the superfamily 2 (SF2) family of RNA helicases. DEAD/H-box helicases constitute a large superfamily of proteins that comprises at least 38 members in humans (244). These enzymes are characterized by the presence of two conserved RecA-like domains (Fig. 1.27). Within these domains, nine conserved sequence motifs are found, including the eponymous D-E-A-D/H sequence that interacts with ATP (84). Other motifs are typically linked to RNA-binding, ATP-binding, or both, such as the Walker sequence that is observed in all NTP-binding domains. The classic ‘nucleic acid RNA unwinding’ activity of these enzymes is facilitated by a complex structural bridge between the RNA-binding site (which spans both RecA-like domains), and the ATP-binding site, which resides mostly in the N-terminus (245,246). Although DEAD-box helicases are usually classified as RNA helicases, it has been suggested that some, including DDX3, can also bind to DNA (247). DEAD-box helicases are involved in a large variety of cellular processes involving RNA, such as splicing, mRNA export, transcriptional and translational regulation, RNA decay and ribosome biogenesis (248). Recent studies of DEAD/H-box helicases highlighted the function as RNA sensors of innate immune signaling (249). DDX3X is ubiquitously expressed in a wide range of tissues (250). In contrast, expression of DDX3Y, the Y chromosome-linked gene, is expressed in a narrow range of tissue (e.g., testis) (251) and will not be discussed further. The role of DDX3 includes both transcriptional and post-transcriptional regulation of gene expression (252-255), cell cycle regulation and innate immune signaling (243). It was also shown that DDX3 plays a role in enhancing the signal from RLHs at the early phase of virus infection, by interacting with both IPS-1 and RLHs, and subsequently enhancing the activation of IRF3 (256). DDX3 is a soluble 73 kDa protein, which consists of an N-terminus unstructured region (1-166), a tandem Rec-A like domain (residue 167-576), and a C-terminal region (577-662). The crystal structure of DDX3 (residue 168-582) was solved as shown in Fig. 1.25 (246). It consists of two RecA-like domains (246), as well as conserved regions that include the nine consensus sequence motifs. However, the N- and C- termini of DEAD-box helicases, including DDX3, are much more divergent and thought to confer functional specificity to individual DEAD-box helicases (248).

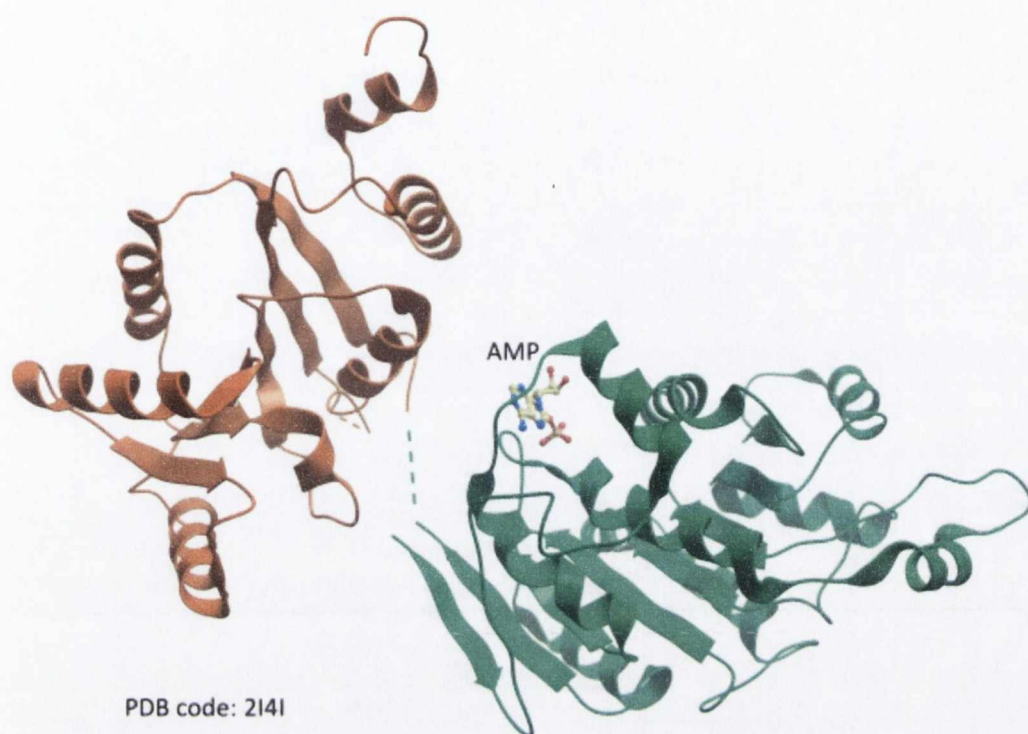


Figure 1.27. Overall structure of tandem RecA-like domain of DDX3 (PDB code: 2I4I. Hogbom et al., J. Mol. Biol (2007) (246)). The disordered part of the sequence connecting the two domains is indicated by broken line.

DDX3 appears to be a prime target for viral manipulation by at least four different viruses, namely Hepatitis C virus (HCV) (252,257-259), Hepatitis B virus (HBV) (260), Human Immunodeficiency Virus (HIV) (261), and poxviruses (85). They encode proteins that interact with DDX3 and modulate its function (243), but each virus has a distinct strategy to co-opt the various functions of DDX3.

In the case of HIV, the chromosome region maintenance 1 (CRM1)-DDX3 interaction is exploited by HIV. DDX3 interacts with CRM1 which mediates the export of RNA through the nuclear pore complex, exporting both ribosomal RNAs and small nuclear RNAs (snRNAs) (262). HIV's incompletely spliced RNAs are recruited to CRM-DDX3 complex and exported out of the nucleus. This is mediated *via* an interaction between the viral RNA-binding protein Rev and DDX3(261). This function of DDX3 was shown to be dependent on DDX3 helicase activity (261).

HCV core protein was the first viral protein to be described as a DDX3-interacting protein (243). Three independent publications described the interaction between DDX3 and the HCV core protein (252,257,258). Another study recently revealed that DDX3 is required for HCV RNA replication (259) and this function is unrelated to DDX3's interaction with the viral core protein (263). Moreover, the other study revealed that DDX3 enhanced IPS-1 signaling, is interfered by the core protein of HCV (264). Thus, on the one hand, HCV requires DDX3 for HCV replication, but on the other hand, HCV can suppress the innate immune signaling *via* DDX3. The precise mechanism involved however needs clarification.

It was recently shown that DDX3 binds to HBV polymerase, in an interaction that is not mediated by RNA (260). HBV polymerase is essential for replication of HBV. Interestingly, although DDX3 was incorporated into nucleocapsids together with HBV polymerase and inhibited the initial step of reverse transcription, in a manner that seemed to depend on the ATPase activity of DDX3 (260), HBV polymerase traps DDX3 and antagonizes activation of IRF3 *via* TBK1/IKK ϵ by hampering the interaction of them (265,266). These two functions seem to balance out but exact mechanism of inhibition remains unclear. Overall, the links between HBV infections, and DDX3 function appears to be complicated, and requires further investigation.

DDX3 was recently shown to be a part of the innate immune signaling pathway, and found to contribute to the induction of anti-viral mediators (243). This recently identified role of DDX3 appears to be its clearest and most well-characterized biological function. DDX3 belongs to the same family of helicases as RIG-I and MDA5 and yet, its mode of action in innate immunity is distinct. Unlike RIG-I and MDA5, DDX3 does not exert an effect as a PRR, but is situated downstream in the signaling cascade that controls IFN- β production (84). As mentioned earlier, the signal from PRRs funnels into the TBK1/IKK ϵ -IRF3 pathway to activate the IFN- β gene. Recently, two groups simultaneously discovered that DDX3 has an essential role in the type-I interferon production thorough a PRR signaling pathway (84,85). Soulat et al. demonstrated that DDX3 interacts with TANK-binding kinase 1 (TBK1) which is thought to phosphorylate and activate IRFs (84). Based on their data, DDX3 is a critical effector of TBK1. They suggested that DDX3 is phosphorylated by activated TBK1 and DDX3 is recruited to the IFN promoter region. Their experiments imply that DDX3 is required for TBK1/IRF3-mediated IFN- β production as a transcriptional co-factor, but the mechanism of this IFN stimulation is still unclear (84). On the other hand, Schröder

et al. showed that DDX3 interacts with IKK ϵ and activates IRF3, resulting in the IFN- β production (85). Their study also showed that the DDX3 N-terminal region is required for *ifnb* promoter induction and the vaccinia virus protein, K7, inhibits the IFN- β production by binding to this functionally important N-terminus (85). In both studies, they share the opinion that DDX3 induces the IFN- β production *via* IRF activation but does not activate NF- κ B. They also showed that the helicase activity of DDX3 is not necessary for this function.

The method by which K7 antagonizes DDX3 and inhibits its innate immune signaling pathway, may be similar to the signal inhibition mechanism of HBV, but it is clearly different in comparison to the subverting mechanism of HIV and HCV (267). K7 binds to the DDX3 N-terminus to disrupt the function of DDX3 which enhances IRF3 activation (85). Instead of inhibiting DDX3, HIV and HCV co-opt DDX3 for replication or translocation, and sequester DDX3 away from its innate immune effector function in the IRF pathway (85).

1.5. The innate immune system, viral antagonists and the project aims

The signals from pattern recognition receptors, including TLR, RLH, NLR, CLR, and other receptors, recognize PAMPs and DAMPs and induce the expression of immune and pro-inflammatory genes, including type I IFN. PRRs also trigger innate cell maturation. Despite their diversity, PRR signals converge upon common mediators of the downstream cascade, which ultimately lead to altered gene expression. The signaling pathways of PRRs consist of a complex assortment of kinases and adaptor proteins, and involve protein-protein interactions, ubiquitination, and phosphorylation.

Viruses have evolved strategies to hijack these pathways to antagonize our immune system. Some viral proteins interact with proteins which mediate the PRRs signaling pathways, thereby inhibiting subsequent signal transduction. Viruses have developed and sophisticated these evasion strategies throughout their evolution, and encoded viral proteins are critical for virulence.

Many proteins are expressed during infection by VACV and each protein is utilized in distinct ways. Especially, Bcl-2 fold proteins, A52, K7, N1, B14, and probably A46, have same fold despite their low sequence similarity and have distinct cellular targets. Cellular Bcl-2 fold proteins function as a regulator of apoptosis. In recent years, several Bcl-2 fold viral proteins have been identified have roles in antagonizing immune signaling pathways.

A46 antagonizes TLR signaling by binding to the TIR-domain containing adaptor proteins, MyD88, Mal, TRIF, and TRAM. Although A46 interacts with a range of TIR-domain containing adaptor proteins, database searches revealed that A46 belongs to the Orthopoxvirus N1 family on the Pfam database. It has been inferred that A46 has a Bcl-2 like fold at its C-terminus, because some of the N1 family protein structures, such as A52, B14, N1, and K7 have been determined and they adopt Bcl-2 fold.

Both A52 and K7, which adopt Bcl-2 fold, they share only 25% sequence identity. They interact with IRAK2 and TRAF6, to evade detection. Additionally, K7 interacts with DDX3 and inhibit the type I IFN production via IKK ϵ /TBK1 kinase complex.

Interactions of viral Bcl-2 fold proteins with innate immune signaling adaptors have not been well characterized. In this study, the major focus was on the characterization from a structural and biochemical point of view, how the Bcl-2 like fold viral proteins K7, A46, and A52 interact with components of the innate immune system thereby facilitating virulence.

Project Aims

1. Structural determination of K7-DDX3

Protein K7 of VACV binds to human DEAD-box RNA helicase DDX3 and inhibits TLR-dependent and -independent IRF3/7 activation and induction of *ifnb* promoter. Since K7-DDX3 interaction has not been well characterized, antagonizing properties of K7 were unclear. Thus, the aim of this project is to identify the binding region of K7 to DDX3 and reveal the binding mechanism by x-ray crystallography and biophysical analysis.

2. Biophysical analysis of A46 and TIR domain-containing proteins interactions, and crystallization trial of A46, Mal, and TRAM

Vaccinia virus protein A46 binds to TIR domain-containing adaptor proteins including Mal, TRAM, TIRF, MyD88, resulting in antagonism of NF- κ B, IRF3 and MAP kinase signaling pathways. No experimental biophysical and structural information of this protein-protein interaction have been reported and as a result it is difficult to elucidate the antagonizing properties of A46. The aim of this project is to advance our understanding of the interaction of A46 with TIR domain-containing proteins from a biophysical point of view.

3. Analysis of the effect of the F154A mutation of A52 for dimer formation by biophysical techniques

Vaccinia virus protein A52 protein interacts with both IRAK2 and TRAF6. Activation of p38 MAP kinase is enhanced by the direct binding of A52 to TRAF6 and drives the p38-dependent production of IL-10, which is a cytokine that inhibits inflammatory and cell mediated immune responses. The Phe154 residue sits at the edge of the A52 dimer interface, and the F154A mutant may therefore disrupt dimer formation. The aim of this project is to advance our understanding of the structural implications of Phe154 and the consequence of a F154A mutation on the dimerization of A52.

Chapter 2. Materials and Methods

2.1. Expression constructs

For K7 expression, a synthetic gene encoding the full-length K7 protein (Geneart AG) was sub-cloned to pET15b plasmid. A52, A46, Mal, and TRAM expression constructs were established following the protocol below. Inserts were cloned into both the pNIC28-Bsa4 and pNIC-CH vectors (kindly provided by O. Gileadi, SGC Oxford). The pNIC28-Bsa4 vector produces a cleavable N-terminal His-tagged fusion protein once the desired PCR products have been ligated in-frame. The His tag is removed by cleavage with rTEV protease. This removes the 2466 Da tag (mhhhhhssgvdlgtenlyfq*sM – The rTEV cleavage site is denoted with an asterisk while the open reading frame start codon is represented by the uppercase M). Vectors pNIC28Bsa4 and pNIC-CH rely on the lac-regulated T7 promoter to drive expression, and the plasmids confer kanamycin resistance on transformed cells. DNA was inserted into these vectors through a ligation-independent cloning (LIC) process. Full-length Mal (1-235), TRAM (1-235), A46 (1-230; A46-230), and A52 (1-190), and an N-terminally truncated TRAM (62-235) and A46 construct (81-230; A46ΔN-230) were amplified by PCR primers and inserted into the pNIC28-Bsa4 plasmid. To prepare the pNIC28-Bsa4 vector for insertion of amplified products, the vector (5 µg) was initially linearized by BsaI (New England Biolabs) in a final volume of 100 µL using 30 units of the restriction enzyme at 323 K for 4 hours. The digested vector was then purified using a High Pure PCR Clean Up Kit (Roche). Purified PCR amplified products (5 µL) were treated with 1.25 units T4 DNA polymerase Novagen (LIC qualified standard) in the presence 2.5 mM dCTP. In tandem, the purified *BsaI* linearized pNIC28-Bsa4 vector was treated with 12.5 units of T4 DNA polymerase in the presence of 2.5 mM dGTP and 5 mM DTT (final concentrations) in the Novagen supplied T4 polymerase buffer (1x). The T4 treatment of vector and inserts was allowed to proceed for 30 minutes at 295 K followed by 20 minutes at 343 K. The T4 DNA polymerase treated vector (1µl) and insert (3µl) which have the T4 generated complementary overhangs are allowed to anneal by incubating at 295 K for 15 minutes. This DNA mix was then transformed into competent *E.coli* *XL1-Blue* cells using heat shock treatment.

2.1.2. Site-directed mutagenesis

Site-directed mutagenesis, A52 phenylalanine 154 to alanine mutant (F154A), was performed by using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Plasmid encoding wild-type A52 was used as the template.

2.2. Protein expression

Escherichia coli BL21(DE3) cells were transformed with the pET15 plasmid (harboring synthetic gene encoding the full-length K7 protein), pNIC28Bsa4 plasmids [harboring synthetic genes encoding A46 (1-230; A46-230) and an N-terminally truncated A46 (81-230; A46 Δ N-230), full-length Mal (1-235), full-length TRAM (1-235), and N-terminally truncated TRAM (62-235)], and pNIC-CH plasmid [(harboring synthetic gene encoding the full-length A52 (1-190))]. Protein expression construct of MBP-tagged *human* TRAF6 (residue: 1-508) using *E.coli* rosetta-gami was provided by the lab of Prof. Andrew Bowie. Liter volume cultures of cells were inoculated with an overnight culture and grown in 2xYT medium (Formedium, UK). Cells were grown to mid-log phase (OD₆₀₀ = 0.6) at 310 K, and the culture was cooled in iced water for a few minutes. Protein expression was subsequently induced by addition of 0.3 mM IPTG (final concentration) with vigorous shaking at 291 K. Following induction for 20 hr, cells were harvested by centrifugation and stored frozen if necessary at 250 K.

The expression of selenomethionine-derivatized K7 followed a similar protocol, except that a minimal media (Molecular Dimensions, UK) was used in place of 2xYT. In addition, immediately prior to induction at mid-log phase, the media was supplemented with amino acids Lys, Thr, and Phe (100 mg each); Leu, Ile, and Val (50 mg each); and 50 mg of L(+)-selenomethionine.

2.3. Protein purification

2.3.1. Protein purification of K7

All subsequent purification procedures were performed at 277 K. The cells were suspended in lysis buffer (20 mM Tris-HCl, pH 8.0) supplemented with 300 mM NaCl, 10 mM imidazole, and 10 mM β -mercaptoethanol) with a protease inhibitor cocktail (complete mini cocktail, Roche) and were lysed using a Branson sonicator. Following centrifugation at 20,000 *g*, the soluble fraction was loaded onto a Ni²⁺ ChroMatrix resin (Jena Bioscience) equilibrated with lysis buffer. After successive steps of washing with lysis buffer supplemented with up to 30 mM imidazole, K7 was eluted with a step gradient of 200 mM imidazole. The eluted protein was mixed with 10 units thrombin (GE Healthcare)/mg K7 to cleave the His-tag, and dialyzed against 20 mM Tris-HCl (pH 8.0) supplemented with 150 mM NaCl, 10 mM imidazole, and 10 mM β -mercaptoethanol. The dialyzed protein was applied to the Ni²⁺ ChroMatrix resin a second time, and the flow-through fraction contained the cleaved K7 protein.

Further purification involved dialysis into a low-salt buffer (20 mM Tris-HCl [pH 8.0] supplemented with 20 mM NaCl, and 1 mM dithiothreitol), followed by anion-exchange chromatography with a linear salt gradient to 1 M NaCl. To prepare the K7-DDX3 peptide complex, a two-fold molar excess of peptide was added to the affinity-purified K7 protein, just prior to ion-exchange chromatography. Purified peptides for all studies (WT and mutant) were obtained from EMC Microcollections GmbH. Fractions containing the K7-DDX3 complex were pooled and applied to a gel filtration column (Superdex-75, GE Healthcare) equilibrated with 20 mM Tris-HCl (pH 8.0) supplemented with 150 mM NaCl, and 1 mM DTT. The final purified complex was concentrated and subjected to crystallization procedures. All purification procedures were performed on ice, or at 277 K.

2.3.2. Protein purification of Mal

Mal was initially purified by Ni²⁺-affinity chromatography. Following the Ni²⁺ affinity capture and elution steps as described in section 2.3.1, Mal containing fractions as determined by SDS-PAGE analysis were dialyzed in low salt buffer (20 mM Tris-HCl pH 7.4, supplemented with 20 mM NaCl, 1 mM DTT) for 3 hours and applied to a Mono Q column (GL 5/50; GE Healthcare). Mal was eluted with a linear gradient

from 20 to 300 mM NaCl in the same buffer system. Recombinant TEV protease was added to the eluted Mal sample to cleave the 6xHis-tag while dialyzing against 10 mM Tris pH 8.0, supplemented with 150 mM NaCl, 10 mM β -mercaptoethanol. The cleavage was performed for 20 hours and the sample was subsequently applied to a 2 mL bed volume Nickel ChroMatrix resin a second time. The flow-through fraction containing the cleaved and purified protein was concentrated by a spin concentrator (Pierce Inc.) and loaded onto a HiLoad 16/60 Superdex 75 gel-filtration column (GE Healthcare) equilibrated with 150 mM NaCl, 1 mM DTT in 20 mM Tris-HCl, pH 7.4. The peak containing Mal was collected and used for biophysical experiments and crystallography trials.

2.3.3. Protein purification of TRAM

TRAM was initially purified by Ni^{2+} -affinity chromatography as described in section 2.3.1. 6xHis-tag was cleaved by adding recombinant TEV protease while dialyzing against 10 mM Tris pH 8.0 supplemented with 150 mM NaCl, 10 mM β -mercaptoethanol. The cleavage performed for 20 hours and the sample was subsequently applied to an approximately 2 ml of Nickel ChroMatrix resin a second time. The flow-through fraction containing the cleaved and purified protein was dialyzed against low salt buffer for about 3 hours. Sample was loaded onto a Mono Q column (GL 5/50; GE Healthcare) and was eluted with linear gradient from 20 to 300 mM NaCl in the same buffer system. The peak fractions containing TRAM was collected and used for biophysical experiments and crystallography trial.

2.3.4. Protein purification of A46

The cell pellets were suspended in lysis buffer (300 mM NaCl, 20 mM imidazole, and 10 mM β -mercaptoethanol in 50 mM potassium phosphate pH 8.0) with a complete mini-protease inhibitor cocktail (Roche) and were lysed by sonication (Branson). Following centrifugation at 20,000 g for 1 hour, the supernatant was loaded onto a 6% CL Nickel-ChroMatrix resin (Jena Bioscience) equilibrated with lysis buffer. After the resin was washed with more than 10 column volume of lysis buffer, A46 was eluted with a step gradient of 200 mM imidazole. The eluted protein sample was dialyzed in ammonium sulfate buffer (0.5 M ammonium sulfate, 10 mM

β -mercaptoethanol in 20 mM potassium phosphate pH 8.0) for 20 h. The protein pellet was collected by centrifugation at 4000 g for 20 min and washed twice with ammonium sulfate buffer. The pellet was then re-suspended into the 2nd Ni^{2+} -agarose buffer (150 mM NaCl, 20 mM imidazole, 10 mM β -mercaptoethanol, 20 mM potassium phosphate pH 8.0). Recombinant TEV protease was added to the protein solution to cleave the 6xHis tag while dialysing at 277 K for 20 hours. The protein solution was applied to the 6% CL Nickel-ChroMatrix resin a second time, and the flow-through fractions containing the cleaved A46 protein. A46 protein solution was equilibrated to hydrophobic column buffer (300 mM ammonium sulphate, 1 mM DTT in 20 mM Tris-HCl pH 7.4) and was applied to the phenyl sepharose column (HiTrap Phenyl HP 1ml; GE Healthcare). A46 was eluted with a linear gradient from 300 to 0 mM ammonium sulphate. The peak fraction containing A46 was collected and used for subsequent biophysical experiments and crystallography trials.

2.3.5. Protein purification of A52

The cell pellets were suspended in lysis buffer (300 mM NaCl, 20 mM imidazole, and 10 mM β -mercaptoethanol in 50 mM potassium phosphate pH 8.0) with a complete mini protease inhibitor cocktail (Roche) and were lysed by sonication (Branson). Following centrifugation at 20,000 g for 1 hour, the supernatant was loaded onto a 6% CL Nickel-ChroMatrix resin equilibrated with lysis buffer. After the resin was washed with more than 10 column volume of lysis buffer, A52 was eluted with a step gradient of 200 mM imidazole. The eluted protein sample was concentrated and applied to a gel filtration column (Superdex-75, GE Healthcare) equilibrated with 150 mM NaCl, and 1 mM DTT in 20 mM Tris-HCl pH 8.0. The fractions containing A52 wild-type and A52 (F154A) were pooled and used for biophysical experiments.

2.3.6. Protein purification of *human* TRAF6

The cell pellets were suspended in lysis buffer (300 mM NaCl, 1% Triton-X 100, and 10 mM β -mercaptoethanol in 50 mM potassium phosphate pH 8.0) with a complete mini protease inhibitor cocktail (Roche) and were lysed by sonication (Branson). Following centrifugation at 20,000 g for 1 hour, the supernatant was loaded onto the amylose resin (New England Biolabs). After the resin was washed with more

than 10 column volume of lysis buffer, MBP-tagged TRAF6 was eluted with a step gradient of 10 mM maltose. The eluted protein sample was concentrated and applied to a gel filtration column (Superdex 200, GE Healthcare) equilibrated with 150 mM NaCl, and 1 mM DTT in 20 mM Tris-HCl pH 8.0. The fractions containing MBP-TRAF6 (1-508) was pooled and used for biophysical experiments.

2.4. SDS- and Native- polyacrylamide gel electrophoresis (PAGE)

Proteins were analyzed by SDS-PAGE using Tris-Glycine gels on a mini-slab size electrophoresis system (ATTO corporation). Protein solutions were mixed with sample buffer including SDS and boiled at 343 K for 30 min and then cooled at 277 K for 5 min. Sample solution was spun down by flash centrifugation and loaded to the SDS-PAGE gel. About 5-10 μ l of proteins were loaded into the wells (12%-15% gels), and run at constant 100-200V. The running buffers and stacking/separating gel compositions were standard (268). Protein bands were visualized using either the Coomassie stain (1%) or Instant Blue (Molecular Dimensions Inc.).

Proteins were characterized by Native-PAGE using Tris-Glycine gels on a mini-slab size electrophoresis system (ATTO corporation). 10-20 μ l of proteins were mixed with Native-PAGE sample buffer and loaded into the wells (8%-10% gels), and run at constant 100-200V. The running buffers and stacking/separating gel compositions were standard. Protein bands were visualized using either the Coomassie stain (1%) or Instant Blue (Molecular Dimensions Inc.).

2.5. *In vitro* pull-down experiments

Purified 6xHis-tagged bait proteins were incubated with 40 μ l of 50% suspended Ni^{2+} ChroMatrix resin for 30 min. Ligand protein solution was added to the mixture of bait proteins and Ni^{2+} agarose. The solution was incubated at 277 K with gentle rotation for 2 hours. The Ni^{2+} agarose resin was washed three times with 1 ml extraction buffer solution of Ni^{2+} purification. 40 μ l of SDS sample buffer was added to the Ni^{2+} agarose solution. This solution was boiled at 369 K for 5 min and then cooled at 277 K for 5 min. Ni^{2+} ChroMatrix resin was removed by flash centrifugation and

supernatant solution was loaded to the SDS-PAGE gel as outlined in section 2.3.

2.6. Circular Dichroism (CD) spectroscopy

The His-tagged fragment 5-167 of DDX3 was purified by Ni²⁺-agarose chromatography, and dialyzed into 50 mM phosphate buffer pH 7.0. The His-tag and associated thrombin cleavage site (20 residues total) was not removed prior to CD analysis. The spectrum was recorded at 15 µg/ml concentration in a 1 cm path length cell using Jasco-815 spectropolarimeter (Jasco Inc.). Secondary structure content was estimated using K2d algorithm (269) as provided on the DICHROWEB server (270). An identical protocol was employed for the Mal, A46, and A52 protein.

CD spectroscopy of Mal, A46, and A52 protein, was performed using a Jasco-815 spectropolarimeter (Jasco Inc.). Typical protein and peptide concentrations ranged from 0.01-0.5 mg/mL in 50 mM phosphate buffer at pH 7, using either a 1 mm or 1 cm cell path length. Data was again submitted to the DICHROWEB server (270) and analyzed using the CDSSTR algorithm (271) against the SP175 set of reference spectra (272).

2.7. Static light scattering

Purified proteins (typically 0.1-0.5 mg total) were loaded onto a Superdex 200 10/300 gel filtration column (GE Healthcare), equilibrated with buffer (10 mM Tris-HCl pH 7.5 supplemented with 100 mM NaCl, 1 mM DTT). Following elution from the column, the FPLC system was connected in-line with the miniDAWN multiangle light-scattering system, followed by an OPTILAB refractometer (Wyatt Technologies). Data processing and absolute molecular mass calculations were performed using ASTRA software (Wyatt Technologies).

2.8. Dynamic light scattering

Suitable sample buffer conditions for protein used in crystallization screens were assessed by dynamic light scattering (DLS; DynaPro NanoStar, Wyatt Technology Corporation). The protocol is largely based on the Proteau et al. in *Current Protocols in Protein Science* (2010). A wide range of buffer (from pH 4.0 to 10.0) and additive conditions were screened. 2 μ l of protein solutions were mixed with 2 μ l of mother liquor, which contained different pH buffers and various additives, and incubated on 24-well hanging drop plate for 20 hours. Protein samples which were not aggregated during the equilibration were measured by DLS at 298 K using a 2 μ l quartz cuvette. Data analyses were performed using DYNAMICS software version 7.0.1.12.

2.9. Crystallization

Protein sample of K7 purified with peptide was concentrated and used for crystallization. Initial crystals of K7 in complex with DDX3 peptide were grown using the sitting drop vapor diffusion method at 291 K by mixing 100 nl of protein sample at $\sim$ 10 mg/ml and 100 nl of reservoir solution using a Mosquito robot (TTP Labtech) in 96-well sitting drop plates. Crystals appeared after a few days in 100 mM citrate pH 5.5 and 20% PEG3350. Final optimized crystals were grown in Linbro 24-well plates using hanging drop vapor diffusion methods with a reservoir solution containing 100 mM sodium citrate pH 5.25 and 15.0% (w/v) PEG3350.

The buffer system of Mal and TRAM was exchanged with the buffer which maximized the monodispersity as found in the DLS screening. Initial screening was set up following the procedure mentioned above using Mosquito robot (TTP Labtech).

2.10. Diffraction data collection and structure determination of K7

For cryoprotection, the crystals were plunged directly into mother liquor supplemented with 20% xylitol. Diffraction data at the selenium peak (0.979 Å) were collected at beamline BM14 at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. Data were integrated and scaled with DENZO/SCALEPACK package (273). The structure of full-length K7 in complex with the DDX3 peptide

(71-90) was solved by the single-wavelength anomalous diffraction method. Five of six expected selenium sites were found using ShelxD, and density modification was performed using ShelxE (274). The resulting map was readily interpretable, and continuous backbone density of K7 between Tyr7 to Asn149 could be traced with COOT (275), model building was initiated with ARP/wARP 7.0.1 (276), which resulted in automated placement of 272 of 300 residues of K7 (dimer in the asymmetric unit). The remaining K7 residues and the DDX3 peptide were manually built by alternating cycles of model building with COOT and refinement using Refman5 (277). Subsequent iterative model improvement and refinement were performed with COOT and Refmac5. There are two K7-DDX3 complexes in the asymmetric unit, and residues Ser82-Arg88 of DDX3 are well-defined and identical in conformation for both complexes.

The root-mean-square (rms) deviation for all atoms in the two complexes is 0.93 Å. The rms.deviation for K7 atoms is 0.94 Å, whereas the rms deviation for equivalent DDX3 peptide atoms is 0.10 Å. However, terminal residues Lys81 and Gly89 of DDX3 are poorly defined and disordered. The sixth SeMet residue was situated in a flexible loop (Met80) in molecule A, thus explaining its poor contribution to the anomalous signal, and was built into the model in later refinement cycles. A Ramachandran plot maps 97.9% of residues in the preferred regions, and only one residue (Ser118) lies slightly outside the generous α -helical conformational space. The data and refinement statistics are summarized in Table 3.1.1.

2.11. Isothermal titration calorimetry

Calorimetric measurements were performed using the ITC-200 instrument (MicroCal, Inc.). Purified K7 (alone) was dialyzed against 10 mM Tris-HCl pH 7.5 supplemented with 100 mM NaCl, and 1 mM DTT. Following dialysis and concentration of the protein to approximately 60 μ M, the flow-through buffer from the concentrator (iCON, Pierce Inc.) was used to directly solubilize the pure peptide (lyophilized powder; EMC Microcollections GmbH) to give a final concentration 600 μ M. This strategy minimized the buffer mismatch in the subsequent titrations. The same buffer was used to dilute K7 and the DDX3 peptides (WT and mutant) to obtain ideal concentrations for binding isotherms suitable for subsequent data analyses. Titrations were performed at 293 K with K7 in the reference cell and peptide in the injection cell. The concentrations varied from 250 to 400 μ M for the peptide and from 20 to 50 μ M for

K7 protein. For the titration involving the nearly complete fragment of DDX3 (5-580), K7 was loaded into the injection syringe, and DDX3 was placed in the cell. Data analysis by peak integration and curve fitting were performed using the Origin software 7.0, and curves were fit to a single-site binding model to give the binding constant (K_d), enthalpy (ΔH), and entropy (ΔS) associated with complex formation.

2.12. Surface plasmon resonance studies

A Biacore X-100 instrument (GE healthcare) was used for collection of surface plasmon resonance (SPR) data. Ligand proteins were coupled to a CM5 chip as per manufacturers coupling instructions and various analytes were injected in Biacore running buffer (100 mM NaCl, 1 mM DTT, 0.005% P20 in 10 mM HEPES pH 7.4). Prior to experiments, proteins used as analyte were dialyzed in the running buffer to minimize buffer mis-match during injections. The purified (>98%) VIPER peptide was obtained from Genscript, and was solubilized to a concentration of 1.5 mM in 10 mM Tris-Cl pH 7.5 supplemented with 100 mM NaCl, 1 mM DTT. This stock solution was further diluted with Biacore running buffer to concentrations ranging from 0.1 μ M to 50 μ M before being injected over the Mal-coupled CM5 chip. SPR data were analyzed using Biacore Evaluation software version 2.0.

2.13. Mutagenesis and cellular studies

These studies were performed in the lab of our collaborator, Dr. Martina Schroder (NUI Maynooth). Briefly, the F84A and F85A mutations were simultaneously introduced into full-length DDX3 by PCR using the previously described pCMV-myc-DDX3 construct (85) as a template for Pfu fusion DNA polymerase (New England Biolabs). Co-immunoprecipitations were performed as described elsewhere (179). In brief, Ha-tagged K7 and myc-tagged DDX3 or FFAA were co-transfected into HEK293T cells, cells were harvested 48 hr after transfection, and cell lysates were prepared. Antibodies were pre-coupled to Protein G-Sepharose beads (Sigma) and then were used to immunoprecipitated proteins from cell lysates. Immunocomplexes were analyzed by SDS-PAGE and Western blotting with anti-Ha (Covance) and anti-myc antibodies (Sigma). Promoter induction and transcription factor activation were measured using HEK293 cells seeded onto 96-well plates and were

transfected 24 hr later with expression vectors and luciferase reporter genes (85,178,179). IRF3 activation was measured using a fusing protein between the DNA-binding domain of the yeast transcription factor GAL4 and IRF3 in conjunction with GAL4-responsive reporter gene construct. Data are expressed as the mean fold induction $\pm$ SD relative to control levels, for an individual experiment performed in triplicate.

Chapter 3. Structural determination of K7-DDX3

3.1. Introduction

The innate immune system is critical for the detection of invading pathogens and generation of the early inflammatory response. Pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and RIG-like helicases (RLHs), recognize distinct molecular patterns on viruses and bacteria (278,279). TLR and RLH engagement results in complex downstream signaling cascades involving adaptor proteins and kinases, ultimately leading to activation of the transcription factors NF- κ B and interferon regulatory factors (IRFs) 3 and 7. This activation subsequently leads to the release of proinflammatory cytokines and type I interferons (147). Stimulation of TLR3, TLR4, and RLH leads to downstream activation of the kinases TBK1 and IKK3, which is essential for activation of IRF3/IRF7 and subsequent interferon- β (*ifnb*) promoter induction.

Poxviruses are large double-stranded DNA viruses that have evolved numerous mechanisms for evasion of innate immune signaling pathways. Vaccinia virus (VACV), the prototypical member of the Poxviridae family that is used for the smallpox vaccine, replicates within the cytosol of infected cells and encodes over 200 open reading frames (175). During poxvirus infection, several viral proteins are expressed in the cytosol that antagonize the inflammatory response and favor viral replication (280). In particular, VACV proteins A46 and A52 were shown to specifically antagonize TLR signaling (178,179,181,232). The discovery of this viral evasion strategy provided early evidence for a role of TLRs in the antiviral immune response. A46 contributes to virulence by targeting multiple TLR adaptor proteins, resulting in membrane-proximal inhibition of TLR signaling and suppression of NF- κ B and IRF3 activation (179,280). A52 acts further downstream and inhibits NF- κ B activation by targeting IRAK2 (178,231,232). In addition, binding of A52 to TRAF6 mediates p38 MAP kinase activation and enhances TLR-mediated activation of the anti-inflammatory cytokine IL-10 (178). VACV protein K7 has 20% overall sequence identities to A52.

Both K7 and A52 interact with TRAF6 and IRAK2 and inhibit TLR-induced NF- κ B activation (85). However, unlike A52, K7 binds to human DEAD-box RNA helicase DDX3 and inhibits TLR-dependent and -independent IRF3/7 activation and induction of the *ifnb* promoter. The investigation of this K7-mediated viral evasion

strategy led to the discovery of a novel role for DDX3 in innate immunity (85). DDX3 binds to and is phosphorylated by IKK ϵ and/or TBK1 (84,85), the two kinases that are essential for IRF3/IRF7 activation. Following PRR stimulation of innate immune signaling, DDX3 enhances IRF3 activation and *ifnb* induction (84,85). K7 binding to DDX3 interferes with the activation of IRF3/IRF7 and subsequent induction of the *ifnb* promoter. DDX3 appears to have multiple cellular functions and has previously been implicated in transcriptional and translational control of gene expression, RNA splicing and export, and cell cycle control (281). Hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) also express proteins that interact with DDX3 during their replication cycle (259,260,282). The Y chromosome-encoded DDX3Y isoform is highly conserved throughout its amino acid sequence with DDX3X, the subject of this study. However, DDX3Y gene expression is restricted to male reproductive organs, where it is essential for spermatogenesis (283). Recent structural work has provided some insight into the molecular basis for poxvirus antagonism of innate immunity. The crystal structures of VACV proteins A52, B14, and N1 revealed that they adopt an α -helical Bcl-2 fold. However, these proteins are devoid of sequence similarities to each other and to viral and cellular members of this superfamily (227,230). Similarly, the NMR structure of VACV protein K7 also revealed a Bcl-2 fold, but in contrast to the other VACV proteins, K7 was found to be a monomer in solution (228). Viral and cellular Bcl-2 proteins regulate cell death by forming hetero-oligomers *via* a surface groove that binds to α -helical BH3 motifs of partner Bcl-2 proteins. The groove is formed by helices α 2- α 5 and is observed in various conformational states, ranging from “open” to “closed” in the absence of BH3 ligands (217,238-242). The groove is “open” in the ligand-bound state of conventional Bcl-2 proteins that regulate apoptosis, thus enabling intimate hydrophobic contacts with amphipathic BH3 α helices (207,217,240,241). The structures of K7, A52, and B14 revealed a closed BH3 groove that is apparently incompatible with binding to α -helical BH3 motifs, because these three viral proteins do not regulate apoptosis (85,227). Our previous biochemical studies showed that K7 recognizes a segment of DDX3 (residues 61–90) that is upstream of the conserved tandem RecA-like helicase domains (residues 168–576) (228). This region correlates with a functional role for the DDX3 N terminus (residues 1–139) in the induction of the *ifnb* promoter (85), suggesting that K7 binds to the region of DDX3 that mediates this effect. The N terminus of DDX3X (1–139), the X chromosome isoform of DDX3, is enriched in 27 serine residues, 20 glycine, and charged amino acids (25 Lys+Arg+His; 22 Asp+Glu). This segment of DDX3 is presumably unstructured and is not conserved in other RNA helicases or known

proteins in sequence databases. This N-terminal segment was removed in the published crystal structure of DDX3, which was determined using the fragment 168–582 (246). Despite the identification of numerous cellular targets of VACV proteins, there is currently little structural information regarding the protein-protein complexes formed during infection. The only structure of a poxvirus Bcl-2 protein with its target involves a BH3 peptide from Bak, which binds to the canonical BH3 groove of myxoma M11 protein (240). Intriguingly, K7 and A52 share similar three-dimensional structures and common cellular targets, but A52 is unable to bind DDX3 (85). Here, we have identified a peptide motif in the N-terminal region of DDX3, ahead of the core RNA helicase domains, that largely retains the binding affinity to K7. The crystal structure of full-length 149-residue K7 in complex with a 20-residue DDX3 peptide (residues 71–90) has been determined by single-wavelength anomalous diffraction (SAD) methods. The complex reveals the molecular basis for K7-DDX3 specificity *via* a binding mode that is novel within the Bcl-2 superfamily. We identify key residues that mediate the interaction between DDX3 and K7 and demonstrate that a tandem phenylalanine motif in DDX3 that is sequestered by K7 is also required for IRF3 activation and *ifnb* promoter induction.

3.2. Results

3.2.1. K7-interacting region of DDX3

The N-terminal region of DDX3 (1-167) was presumed to be unstructured, and a circular dichroism (CD) analysis supports this prediction (Fig. 3.1). Previous truncations of DDX3 had minimized the K7-binding region to 30 residues (61-90) (228). The interacting region was further narrowed using a series of overlapping DDX3 peptide (Fig. 3.2). All peptides were synthesized with a hexahistidine-tag at the N-terminus to facilitate a pull-down assay with purified, untagged K7. The results indicate that DDX3 segment 81-90 is necessary for binding to K7. Isothermal titration calorimetry experiments confirmed that DDX3 peptides encompassing the segment 81-90 largely retained the affinity of a nearly complete fragment of DDX3 (5-580) that included the N terminus and the two RecA-like domains. Consequently, peptides 61-90, 71-90, and 81-100 lacking the hexahistidine tag were synthesized, because these were deemed suitable for co-crystallization with K7. The best crystals were obtained with peptide 71-90, and, following selenomethionine derivatization of K7, the crystal structure of K7-DDX3 was solved by single-wavelength anomalous diffraction (SAD) methods.

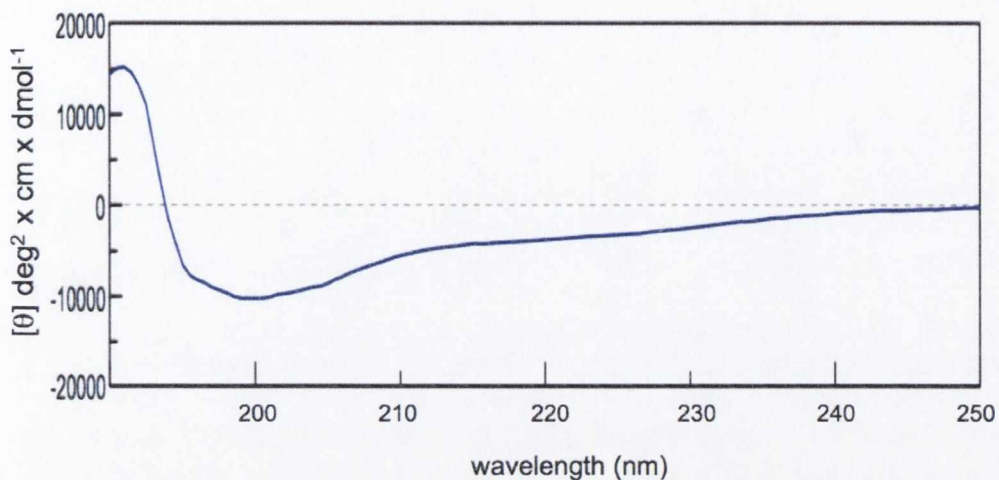


Figure 3.1. Circular dichroism (CD) spectroscopy of the N terminus DDX3

The His-tagged fragment 5-167 of DDX3 was purified by Ni^{2+} -agarose chromatography, and dialyzed into 50 mM phosphate buffer (pH 7). The His-tag and associated thrombin cleavage site (20 residues total) was not removed prior to CD analysis. The spectrum was recorded at 15 $\mu\text{g/ml}$ concentration in a 1 cm pathlength cell using Jasco-815 spectropolarimeter (Jasco Inc.). Secondary structure content was estimated using the K2d algorithm (Andrade et al., 1993) as implemented on the DICHROWEB server (Whitmore and Wallace, 2004). The content of secondary structure was calculated as 10% α -helix, 30% β -sheet, and 60% coil (unstructured).

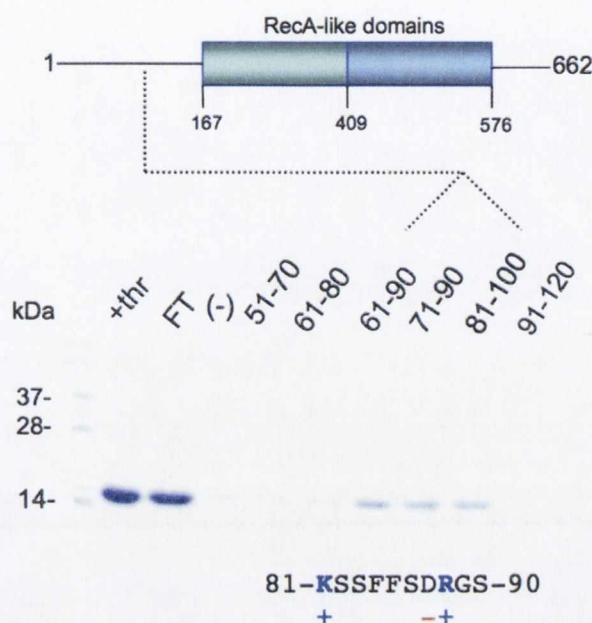


Figure 3.2 . Biochemical Mapping of the Interaction Between K7 and DDX3

Previous work had mapped the K7-interacting region to residue 61-90 of DDX3. Smaller overlapping His-tagged peptides were used to pull down untagged K7 and visualize the protein by Coomassie blue stain. The lane marked “+thr” denotes K7 treated with thrombin, which migrates at 16 kDa. This protein was subjected to further purification by second Ni^{2+} -agarose column (lane FT). In the negative control lane (-), Ni^{2+} , but not peptide, was added to K7. Only those His-tagged peptides spanning the region 81-90 of DDX3 were able to pull down K7. The sequence of this region of DDX3 is shown for convenience. The peptides are approximately 2-3 kDa in size and are too small to be visualized in this SDS-PAGE gel. This experiment was performed by Amir Khan.

3.2.2. Crystallization of K7 in complex with DDX3 peptide

The crystallization conditions for the recombinant K7 in complex with DDX3 peptide were obtained by the sparse-matrix method and optimized to produce a single crystal suitable for X-ray analysis. Only selenomethionine derivatives of K7 in complex with 20-residue DDX3 peptide (residue 71-90) generate good diffracting crystals (Fig. 3.3).

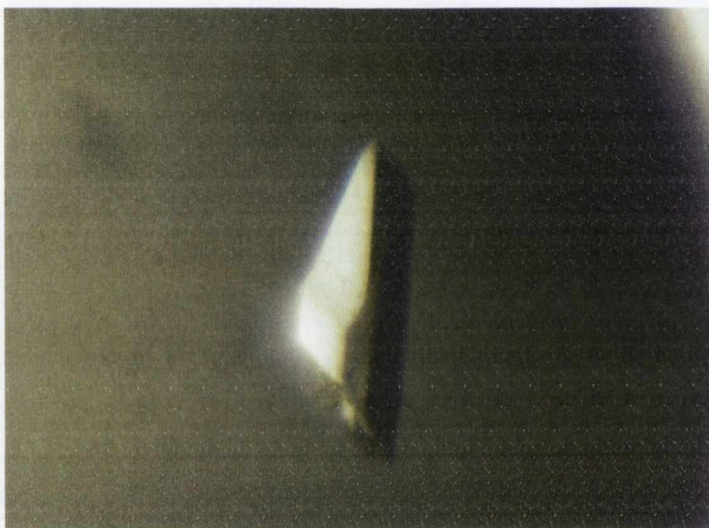


Figure 3.3. The image of the crystal of K7 in complex with 20-residue DDX3 peptide

3.2.3. X-ray diffraction data collection and refinement

The crystals diffracted X-rays to 1.60 Å resolution on beamline BM14 at ESRF. The crystals belong to the monoclinic space group *C2*, with unit-cell parameters $a=90.89$, $b=69.53$, $c=65.63$, $\alpha=90.00$, $\beta=122.17$, $\gamma=90.00$. A summary of the data collection statistics is given in Table 3.1. One K7 dimer is in the asymmetric unit.

Table 3.1

SeMet (Peak Data)

Data collection	
Space group	C2
Cell dimensions	
a, b, c (Å)	90.89, 69.53, 65.63
α, β, γ (°)	90.00, 122.17, 90.00
Resolution (Å)	30–1.60 (1.66–1.60) ^a
R_{sym}	0.081 (0.373)
$I / \sigma I$	35.4 (3.39)
Completeness (%)	97.3 (84.9)
Redundancy	7.0 (4.6)
Shelx D/E phasing	
SeMet sites	5
CC all/weak	47.46/34.15
PATFOM	44.09
Pseudo-free CC	77.92%
Connectivity	0.937
Refinement	
Resolution (Å)	30.0–1.60
No. of reflections	45370 (2267)
$R_{\text{work}} / R_{\text{free}}$ (%)	14.1/18.9
No. atoms	
K7	2316
DDX3 peptide	160
Water	251
<i>B</i> -factors	
K7 all atoms	17.2
K7 main chain	15.0
K7 side chain	19.4
DDX3 all atoms	19.6
DDX3 main chain	17.8
DDX3 side chain	21.4
Water	28.4
Rmsds	
Bond lengths (Å)	0.0146
Bond angles (°)	1.497
^a Values in parentheses are for highest-resolution shell.	

3.2.4. Overall structure of K7

The final model of K7-DDX3 was refined using data to 1.6 Å and includes residues 6-149 of K7, 82-88 of DDX3, and 261 water molecules. Consistent with the uncomplexed NMR structure, K7 adopts an α -helical topology belonging to the Bcl-2 family that consists of a core of seven α helices (Fig. 3.4). However, K7 is a non-canonical member of the Bcl-2 family in several respects. First, K7 is a monomer, whereas the other VACV Bcl-2 fold proteins A52, B14, and N1 –are dimers. As a consequence, an additional surface is exposed in K7 that is critical for DDX3 binding. Second, two of the seven canonical α helices (α 4 and α 6) are non-helical in K7. The atypical conformation of these two segments of K7 is necessary to form a deep hydrophobic pocket to accommodate the DDX3 peptide. Third, the canonical groove that binds to BH3 domains of cellular pro-apoptotic Bcl-2 proteins is closed in K7. Unlike conventional Bcl-2 homologs, K7 does not antagonize innate cell death pathways. In this latter respect, K7 resembles VACV proteins A52 and B14, whose crystal structures were recently determined. K7 and A52 share 27% sequence identities (54% homology) within their core Bcl-2 domain, and both proteins have a closed BH3 groove. Upon superposition of K7 and A52 secondary structures, the root-mean-square deviation (rmsd) of 108 aligned C α atoms is 1.89 Å. Apart from A52, the closest structural relative of K7 is VACV protein N1 (PDB code 2I39; 2.0 Å rmsd over 96 residues). N1 is a conventional anti-apoptotic protein with an open BH3 groove that interacts with cellular Bcl-2 proteins Bad, Bax, and Bid. K7 and A52 are devoid of significant sequence identities to N1, B14, and other viral and cellular Bcl-2 proteins. Nevertheless, K7, A52, and B14 share a Bcl-2 scaffold with a closed BH3 groove and antagonize distinct immune signaling pathways.

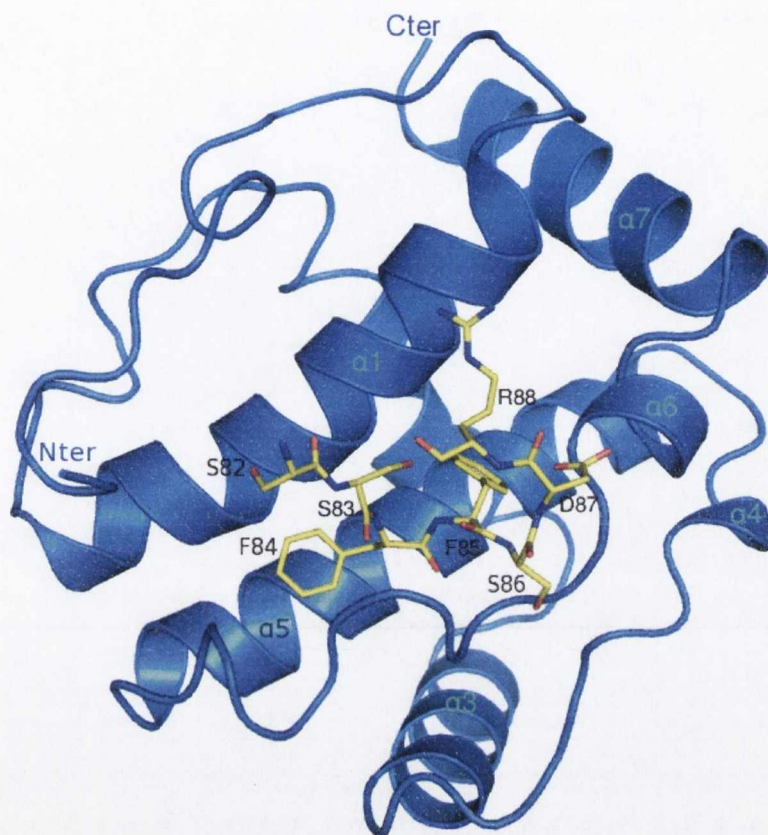


Figure 3.4. Overall structure of K7 in complex with DDX3 peptide
 K7 is shown in blue ribbons, and the peptide 82-88 is a yellow stick model. Canonical Bcl-2 family nomenclature is used for α helices, although almost K7 lacks $\alpha 4$ and $\alpha 6$ helices.

3.2.5. Structure of the K7-DDX3 complex

The DDX3 peptide binds to a negatively charged face of K7 and protrudes into a deep hydrophobic pocket (Fig. 3.7). Residues 82-88 of DDX3 are ordered in both molecules of the asymmetric unit and have an identical conformation. The bound DDX3 peptide segment is entirely consistent with our in vitro pull-down assays and thermodynamic studies. The hydrophobic binding pocket in K7 is bordered by the N terminus (Tyr7), $\alpha 1$, and the region 118-127, which is predominantly non-helical but is labeled “ $\alpha 6$ ” to remain consistent with the canonical Bcl-2 fold. Despite lacking secondary structure, the backbone in this segment is well ordered in electron density

maps. A52, which is the closest relative of K7, is α -helical ($\alpha 6$) in this corresponding segment (residues 154-168; Fig. 3.6). Strikingly, the dimeric interface of A52 comprises $\alpha 1$, $\alpha 6$, and the N terminus, which maps directly onto the DDX3-binding site of K7 (Fig. 3.5). All of the known VACV Bcl-2-family proteins are homodimers, and the dimeric interface is broadly conserved in the superfamily (227). In contrast, K7 is a monomer, and this face is therefore free to interact with DDX3. Furthermore, this face of K7 has a predominantly negatively charged character that is complementary to positively charged DDX3. The overall pI of K7 is 4.8, whereas the N-terminal segment of DDX3 (1-143) preceding the α -helical “flanking region” (residues 144-167) and core helicase domains has a pI of 7.8.

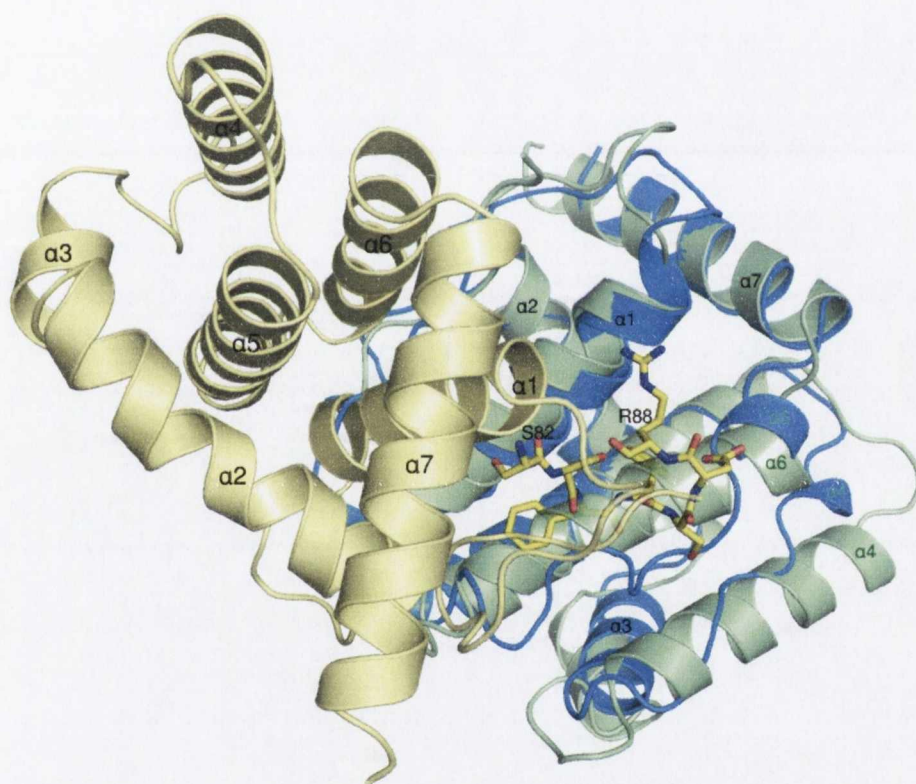


Figure 3.5. Dimer interface of A52

A52 dimerization is mediated by its N terminus, $\alpha 1$ and $\alpha 6$, which maps onto the DDX3 binding site of K7.

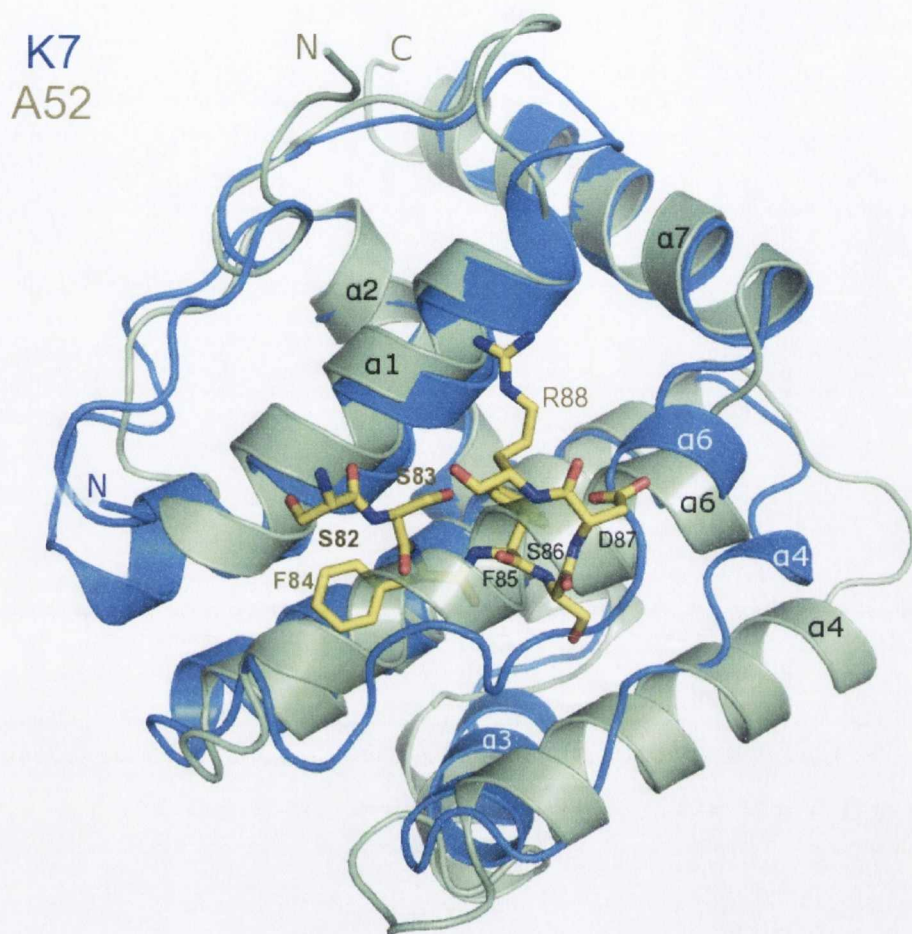


Figure 3.6. Superposition of K7 and A52

The DDX3 peptide (stick) occupies the position of $\alpha6$ in the Bcl-2 family.

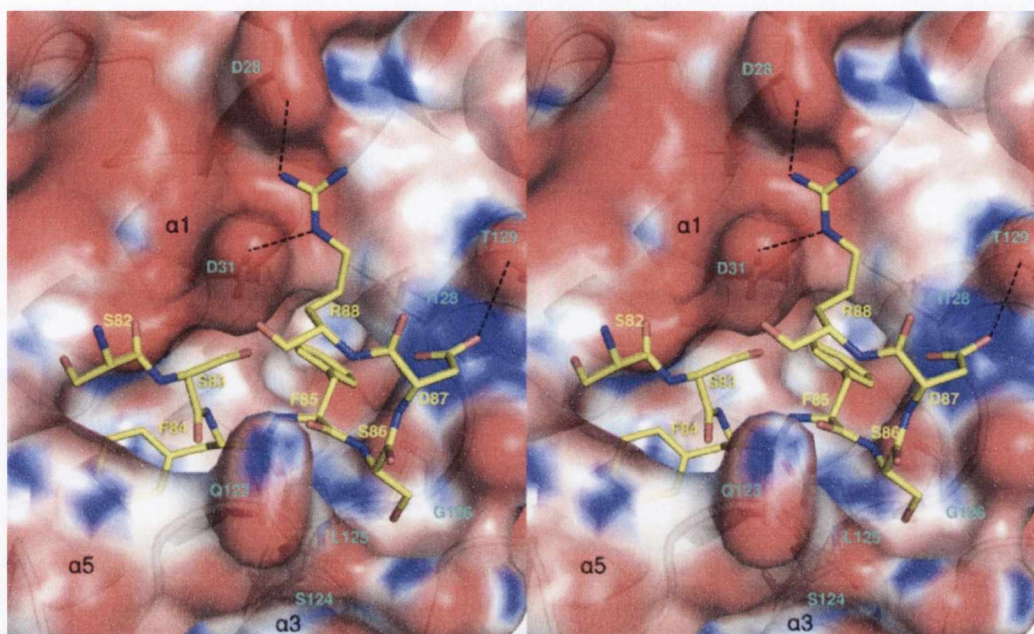


Figure 3.7. Electrostatic Surface View of K7 in Divergent Stereo

The peptide is represented as a stick model. Electrostatic surface was calculated by APBS and depicted by PyMOL. Stereo rendering reveals the depot of penetration by Phe84 and Phe85 into the K7 pocket.

The conformation of the bound peptide begins with an extended segment (Ser82-Ser83), followed by a loop that is anchored at the tip by two phenylalanine side chains, Phe84 and Phe85. Strikingly, the insertion of these aromatic residues into a deep hydrophobic cleft effectively means that the DDX3 peptide occupies the position of $\alpha 6$ in the canonical Bcl-2 fold. The intimate association of K7-DDX3 is reflected in hydrogen bonds between the side chain of Asp87 and the backbone amides of Ile128 and Thr129, which nucleate a single turn α helix ($\alpha 6$). The peptide conformation is stabilized by two internal hydrogen bonds. The first involves Ser83 ($O\gamma$) and the backbone amide of Phe85. The second internal hydrogen bond (3.4 Å) comprises the backbone of Phe85 (CO) and the backbone amide of Arg88 that is reminiscent of an α -helical 3_{10} hydrogen bond. The N and C termini of the peptide approach close in space such that the α -carbon atoms of Ser82 and Arg88 are within 6 Å. Thus, the overall peptide adopts a thumb-like projection into a hydrophobic pocket of K7. The peptide is likely disordered in solution, as evidenced by CD spectroscopy of the fragment 5-167 of DDX3 (Fig. 3.1). Furthermore, a second K7-binding site for the

peptide reveals a completely different conformation for the diphenyl motif. This second site is situated on the surface of molecule B in the asymmetric unit and mediates crystal contacts along the layer aligned with the AC face of the monoclinic lattice (Fig. 3.8). Although this second site is not biologically relevant, the variable conformation of the peptide adopted during crystallization suggests significant flexibility in solution, prior to complex formation with K7.

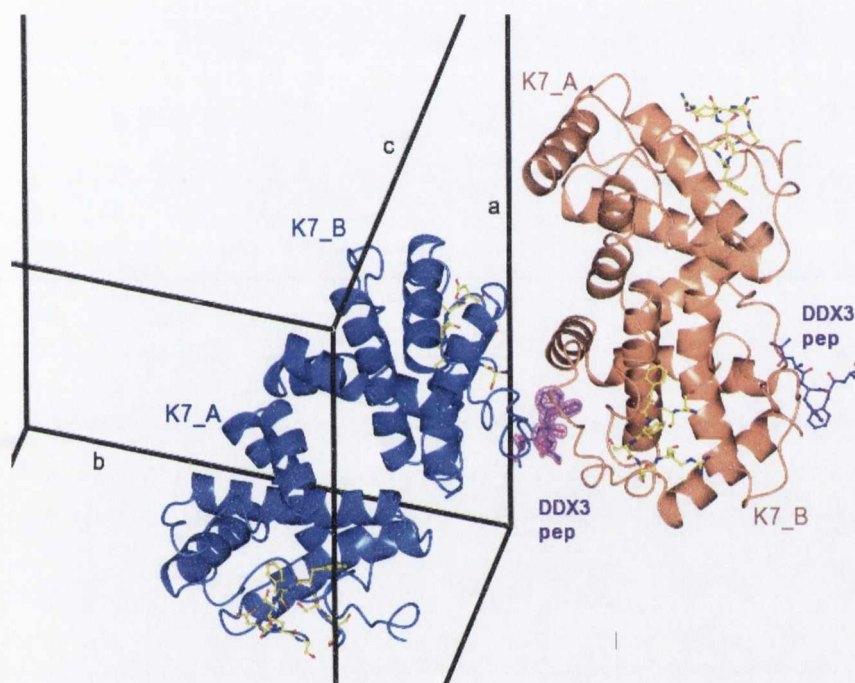


Figure 3.8. Crystal Packing of the K7-DDX3 Complex

The asymmetric unit is shown in blue and orange ribbons (K7), yellow and magenta stick (DDX3). In addition to the biologically relevant DDX3 binding site (yellow; 1:1 complex with K7), the peptide (depicted in magenta) also binds molecule B and propagates the crystal layer aligned to the AC face of the monoclinic lattice. The central DDX3 peptide is shown with electron density ($2f_o - f_c$), contoured at 1.5σ . This location is surface exposed and links the N-terminus of K7 to the $\alpha 3$ - $\alpha 5$ loop, which is generally $\alpha 4$ in Bcl-2 proteins. Residues Ser82-Ser86 of the peptide are ordered in electron density maps, which is relevant only for crystal growth. There is one peptide in this location per asymmetric unit. A crystallographically-related DDX3 peptide (magenta) is shown to the right, in order to emphasize its role in propagating lattice growth

Interaction between K7 and the DDX3 peptide involve hydrophobic contacts, hydrogen bonds, and electrostatic interactions. The packing of the diphenyl motif of DDX3 completes the central piece of continuous stretch of K7 aromatic residues comprising Phe43, Tyr7, Phe122, and Phe89. The key determinant of electrostatic complementarity is Arg88 of DDX3, which forms salt bridges to both Asp28 and Asp31 (O δ 2) (Fig. 3.9). Asp31 (O δ 1) is also hydrogen bonded to DDX3 residue Ser83 (O γ). A total of 545 Å² of K7 is buried in the complex, whereas 126 Å² (30% of total surface area) of the DDX3 peptide becomes buried after complex formation. Superposition of the uncomplexed NMR structure of K7 with the bound complex reveals significant flexibility around the DDX3 pocket (Fig. 3.11). The N terminus of K7 is disordered and points away from the DDX3 pocket (α 5/ α 6) in the NMR models. However, the N terminus shifts toward the DDX3 pocket in the complex, as evidenced by van der Waals contacts between Tyr7 (K7), Phe84 (DDX3), and Phe122 (K7). Overall, the monomeric state of K7 and its conformational flexibility enables the binding of DDX3 into a deep pocket. A cartoon depiction of DDX3 with K7 is shown in (Fig. 3.9 and Fig. 3.10.).

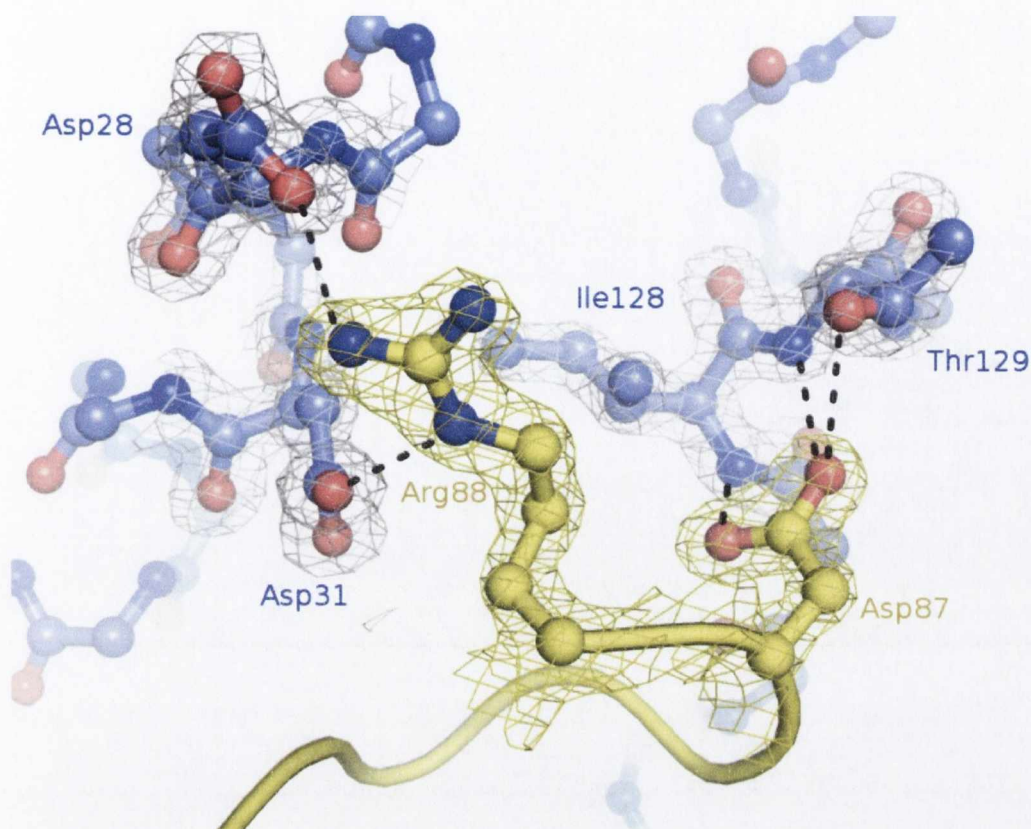


Figure. 3.9. Electrostatic interaction between K7 (Blue) and DDX3 peptide (Yellow)

2Fo-Fc map contoured at 1.5σ was displayed around the residues which are related to their interactions. Residue Arg88 and Asp87 of DDX3 seem to partially determine the binding position by making the hydrogen bonds to K7 residues.

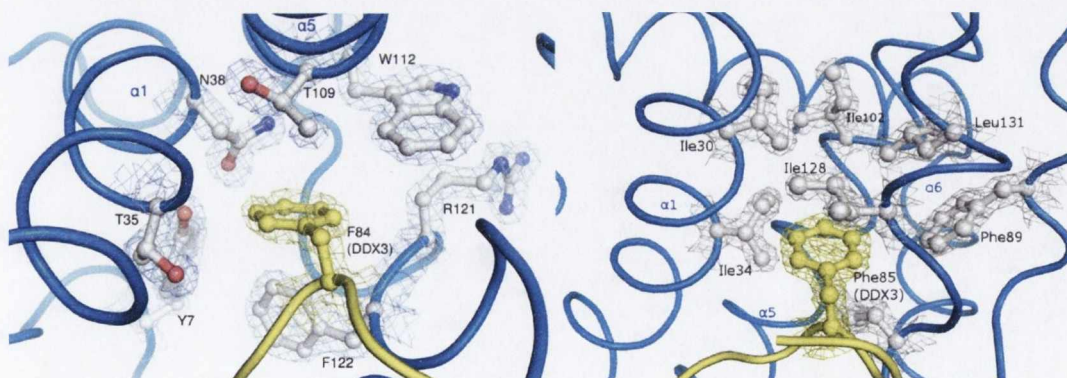


Figure. 3.10. Hydrophobic Environment for Phe84 and Phe85
The pocket for Phe84 and Phe85 is composed of mainly aromatic and hydrophobic residues.

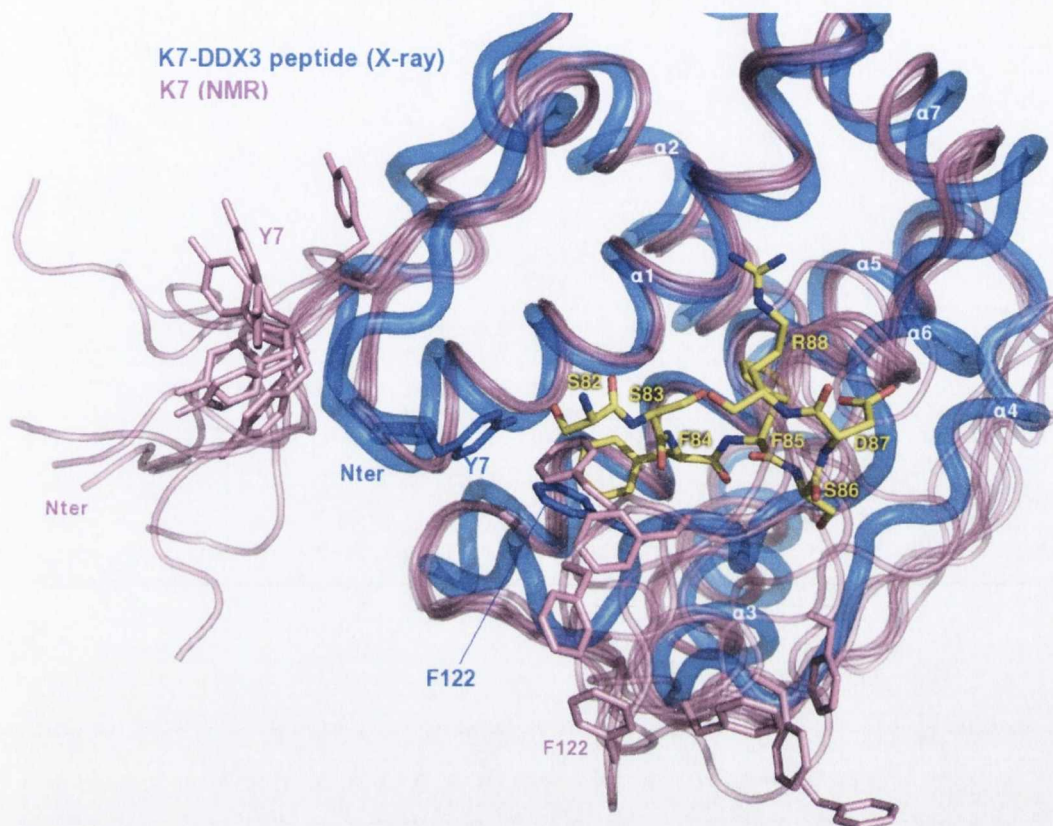


Figure. 3.11. Superposition of K7-DDX3 with the NMR ensembles of (peptide-free) K7.

The NMR models are shown as tubes of magenta. Despite its flexibility, the N-terminus generally points away from the DDX3 pocket in peptide-free K7, as evidenced by the position of Tyr7. Similarly, Phe122 in $\alpha 6$ is flexible, and both regions clamp the peptide in the complex (blue)

The structure of the K7-DDX3 complex implied that the diphenylalanine motif of DDX3, Phe84, and Phe85 is critical for the interaction with K7. In the lab of our collaborator, Dr. Martina Schroder (NUI Maynooth), these two phenylalanines were mutated to alanine to test whether this would disrupt the interaction between DDX3 and K7. Full-length myc-tagged DDX3 (662 residues) harboring two mutations, F84A +F85A (FFAA mutant), was co-transfected with Ha-tagged K7 into HEK293 cells, and immunoprecipitation experiments were performed. K7 failed to co-immunoprecipitate with the FFAA mutant, while in the same experiment it clearly co-immunoprecipitated

with wild-type DDX3 (Fig. 3.12). These cellular assays are consistent with in vitro thermodynamic studies in which the FFAA mutant peptide (71-90) of DDX3 failed to demonstrate measurable affinity with K7 by isothermal titration calorimetry (Table 3.2).

After having identified residues Phe84 and Phe85 as being essential for K7-DDX3 recognition, the functional relevance of the diphenylalanine motif of DDX3 in the antiviral response was investigated by immune signaling studies by the group of Dr. Martina Schroder (NUI maynooth). In reporter-based assays, the FFAA mutant failed to enhance IRF3 activated and IFN- β promoter induction, whereas wild-type DDX3 enhanced IKK ϵ -induced signals, as described elsewhere (Fig. 3.12). Altogether, our structural and functional work suggests a critical role for the diphenylalanine motif of DDX3 in IRF3 activation and type I interferon induction. In addition, these studies suggest that K7 binding to a short DDX3 segment encompassing the diphenylalanine motif is sufficient to prevent DDX3 from executing its role in innate immune signaling.

Table Thermodynamics of K7 Interactions with DDX3 Fragments

DDX3 Fragment	K _d (μM)	ΔH (kcal/mol)	ΔS (cal/mol/deg)
5–167	0.51	–22	–46
5–580	0.46	–30	–72
71–90 ^a	0.93	–26	–61
81–90	1.1	–22	–48
81–100	1.2	–15	–25
71–90 (FF → AA)	No binding		

^a Successfully crystallized in complex with full-length K7.

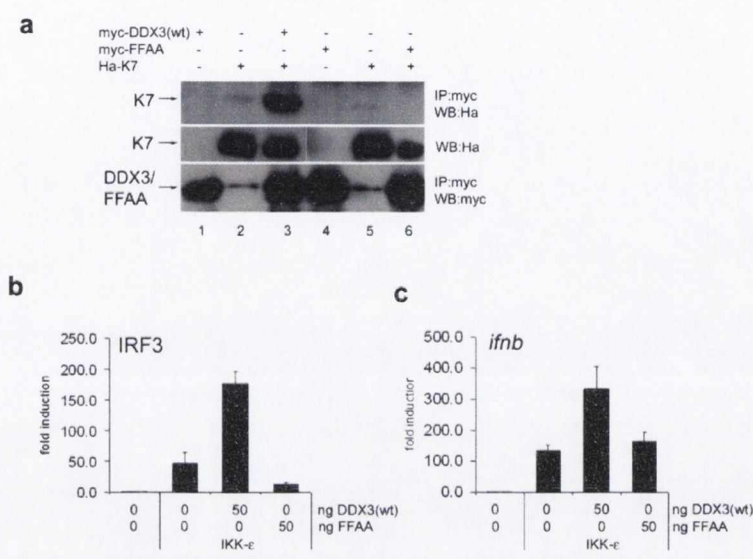


Figure 3. 12. Phe84 and Phe85 Are Required for the Effect of DDX3 on the IRF Pathway

(a) HEK293T cells were transfected with Myc-DDX3 (wild-type) or myc-FFAA mutant together with Ha-K7 for 48 hr, and the cell lysates were subjected to immunoprecipitation (IP) with the indicated antibodies. The precipitated complexes were analyzed by SDS-PAGE and Western blotting (WB) with the indicated antibodies. One representative experiment out of two is shown.

(b and c) HEK293 cells were transfected with the indicated amount (nanograms) of pCMV-DDX3 or pCMV-FFAA, 25 ng of IKKε expression plasmid, and either the IRF3-GAL4 fusion protein in conjunction with GAL4-dependent promoter or the IFNβ promoter reporter gene. A constitutively active *Renilla* luciferase construct was cotransfected to control for transfection efficiency. Luciferase activity was measured 36 hr after transfection. Data are expressed as the mean fold induction $\pm$ SD relative to control levels, for an individual experiment performed in triplicate. The first lane in (b) and (c) is a transfection with an empty vector (no IKKε expression) that is normalized to control (basal) levels of transcription. A representative of at least three independent experiments is shown. Data are shown as the mean fold induction $\pm$ SD relative to control levels, for an individual experiment performed in triplicate. **These experiments were performed by Dr. Martina Schroder (NUI Maynooth, Ireland).**

The K7-DDX3 complex represents a unique binding mode within the superfamily of Bcl-2 proteins. In effect, DDX3 occupies the position of the canonical helix $\alpha 6$ and interacts with the hydrophobic interior of K7. Hydrophobic contacts (3.6 Å) between Phe85 (DDX3) and Trp112 (K7 and $\alpha 5$) are indicative of the depth of penetration by the diphenylalanine motif into the globular core of K7. The NMR structure of K7 revealed considerable flexibility in the region 118-127, which is an α helix ($\alpha 6$) in all other known viral and cellular Bcl-2 proteins. Furthermore, the DDX3-binding face of K7 overlaps with the dimerization interface of A52, its closest structural and functional relative. These data support a model in which monomeric K7 has evolved as a conformational trap for DDX3, guided by electrostatic complementarity and anchored by the diphenylalanine motif in its hydrophobic core. The high density of negative charges on this face likely serves as the dual function of DDX3 binding, as well as contributing to the monomeric state of K7 via electrostatic repulsions. Several Asp and Glu residues on this face of K7 (Asp28, Glu119, Asp130, and Asp134) are not conserved in A52. The structure therefore explains the specific binding of K7, but not A52, to DDX3. We would also predict that none of the other described dimeric Bcl-2-like VACV proteins interacts with DDX3. Despite clearly possessing Bcl-2 topology, it is not evident why $\alpha 4$ and $\alpha 6$ of K7 are largely devoid of α -helical content. It is particularly striking that 8 of 15 residues in $\alpha 4$ are identical in A52 and K7, with conservative changes in an additional four residues. Given that K7 and A52 share some cellular binding partners, such as TRAF6 and IRAK2, these proteins presumably have common epitopes elsewhere. Apart from a study showing that an A52 peptide derived from the $\alpha 4$ - $\alpha 5$ loop inhibits NF- κ B in isolation (284), there is a lack of structural and biochemical information regarding the IRAK2 and TRAF6 binding sites.

The recent determination of structures of three viral proteins (K7, A52, and B14) that apparently fail to bind BH3 domains allows for their comparison to canonical Bcl-2 family members. The common structural element appears to be closure of the groove on the C-terminal side of the BH3 helix binding site (Fig. 3.13). This is due to the orientation of $\alpha 2$, which is closer to $\alpha 5$ in these three “non-BH3-binding” proteins. The beginning of $\alpha 5$ contains the “NWGR” signature (BH1) motif of cellular Bcl-2 proteins, which is lacking in the viral homologs. VACV protein N1 –which binds to the BH3 domains of Bid, Bad, and Bax–adopts an $\alpha 2$ orientation similar to that of K7, A52, and B14, but it is one helical turn shorter (Fig. 3.13), thus leaving this end of the BH3

groove open. The pocket that accommodates the N-terminal side of BH3 α helices displays conformational heterogeneity in the absence of ligand among the viral and cellular proteins. This side of the pocket is formed by $\alpha 3$ and $\alpha 4$, relative to Bcl-2 (207,242). However, even uncomplexed Bcl-xL appears to be more “open” at the $\alpha 2/\alpha 5$ end of the pocket.

Figure 3.13. Cartoon models of the BH3 groove of viral and cellular Bcl-2 proteins

In addition to its recently described role in innate immunity, the biological functions ascribed to DDX3 include RNA splicing, export, and translational regulation (243). In contrast to the antagonistic effect of VACV K7, other viruses, including HCV and HIV, have been shown to co-opt DDX3 during infection. The function of DDX3 that is targeted or exploited by viral proteins presumably correlates with their binding sites. The interaction between the nuclear export shuttle protein CRM-1 and DDX3, which is co-opted by the HIV Rev protein, has been roughly mapped to residues 260-517 of DDX3 (261). HCV core protein binding maps to the C-terminal region of DDX3 (553-611) (252). In contrast, K7 binds to the N terminus of DDX3, and the effect of DDX3 on innate immune signaling appears to be independent of its RNA helicase function. A DDX3 mutant lacking ATPase activity (K230E) retained its ability to enhance IFN- β induction by TBK1/IKK ϵ (84,85). Furthermore, a DDX3 truncation lacking residues 1-139 was unable to enhance IFN- β promoter induction, suggesting that this function of DDX3 is contained within its N terminus and correlates with the region that is targeted by the viral protein K7 (85).

The structural and functional data presented in this study support a model in which K7 masks a key epitope of DDX3 that is required for the IRF activation pathway *via* TBK1/IKK ϵ . One possibility is that the diphenylalanine motif interacts directly with IKK ϵ or another effector molecule linking it to the IRF activation pathway. A second hypothesis is that serine/threonine phosphorylation of DDX3 plays a role in downstream activation and that K7 interferes with this process by masking the substrate-binding site. The N terminus of DDX3 is enriched in serine/threonine residues (eight within the segment 71-90) that may be candidates for phosphorylation by IKK ϵ or other protein kinases. These various models can now be tested in future mutagenesis and signaling studies. Uncovering the molecular details of interaction between viral immune evasion proteins and their host targets may provide vital information for the design of antiviral and immunosuppressive drugs that mimic the strategy of the virus.

Chapter 4. Biophysical analysis of A46 and TIR domain-containing proteins interactions, and crystallization trial of A46, Mal, and TRAM

4.1. Introduction

Vaccinia A46 is a 240-residue protein that binds to several TIR-domain containing adaptors including MyD88, Mal, TRAM and TRIF, resulting in antagonism of NF- κ B, IRF3 and MAP kinase signaling pathways. Due to its interactions with TIR domain-containing proteins, it was initially believed to adopt a TIR domain fold, despite the lack of sequence identity with TIR domain-containing proteins over the majority of its primary sequence (179,181). In direct contradiction to this model, another group predicted that A46 adopts a Bcl-2 fold based on bioinformatics analysis (182). Pfam database searches revealed that A46 belongs to the Orthopox_N1 family. Secondary structure prediction provides some evidence that A46 consists of a C-terminal α -helical region (residue 90 to 220) and on N-terminal region (1 to 89) which consist largely of strand. Multiple sequence alignments showed that the predicted N-terminal α -helical region revealed significant sequence similarity with other Orthopox_N1 family of proteins, which include other poxvirus Bcl-2 fold proteins, such as N1, A52, and K7 (182).

An eleven amino acid peptide derived from A46, called VIPER (viral inhibitor peptide of TLR4), which encompasses the residues KYSFKLILAEY, has been identified as an antagonist of TLR signaling (183). This peptide corresponds to residues 88 to 98 of the VACV A46 protein (183). It was demonstrated that this peptide interacts only with Mal and TRAM, but not with MyD88 and TRIF, which interact directly with the A46 protein (183). This peptide corresponds to one-half of the N-terminal helix of a predicted Bcl-2 globular fold and the helix surface is a conserved dimerization interface of the other Bcl-2 fold viral proteins.

No experimental biophysical and structural information of this protein-protein interaction have been reported and as a result it is difficult to elucidate the antagonizing properties of A46. Thus, the studies outlined in this chapter intend to advance our understanding of the interaction of A46 with TIR-domain containing proteins from a biophysical point of view. Initially, A46, Mal, and TRAM proteins were expressed recombinantly and purified followed by biophysical characterization using CD spectroscopy, static light scattering, and surface plasmon resonance, and the results of which will be interpreted with respect to A46/TIR-domain interaction.

4.2. Results

4.2.1. Purification and biophysical analyses of A46

Poxvirus A46 protein and human Mal/TIRAP (Mal) were purified and their secondary structure examined by circular dichroism spectroscopy. Full-length A46 (1-240) was highly insoluble, but it was found that a modest truncation at the C-terminus (1-230) rendered the protein much more soluble (henceforth referred to as A46-230), thereby facilitating biophysical analyses. Secondary structure analyses and fold predictions suggest that A46 adopts a Bcl-2 structure (183). Bearing in mind that the most likely region comprising the all α -helical/Bcl-2 like fold lies between residues 81-200, as shown in Fig. 1.20, a further truncation to generate the fragment 81-230 (A46 Δ N-230) was prepared and the two constructs were solubly expressed in *E.coli* and subsequently purified to homogeneity in milligram amounts (Fig 4.2). The workflow of purification is summarized in Table 4.1. The C-terminal region of A46 (200-240) is predicted to be highly flexible, and therefore the last 10 residues are unlikely to be an integral part of the A46 globular structure. Both 81-230 and 1-230 constructs of A46 have very hydrophobic character. Thus, ammonium sulphate precipitation and hydrophobic column were added as purification steps (Fig 4.1). Ammonia precipitation was added to the protocol because His-tag was uncleavable after Ni²⁺ affinity purification. In all subsequent studies, full-length Mal (1-235) was used, as the protein was found to be soluble and could be purified to homogeneity (Fig 4.2 and Table 4.1). Full-length Mal protein had a tendency to aggregate after Ni²⁺ affinity purification. Thus, anion exchange chromatography was performed after 3 hours incubation against low-salt buffer for anion exchange. Addition of an immediate purification step seemed to stabilize the nature of the protein fraction possibly through separation. Both full-length and truncated fragment 62-235 constructs of TRAM were solubly expressed in *E.coli* and subsequently purified (Fig 4.2 and table 4.1). The full procedures for protein expression and purifications are outlined in Materials & Methods sections 2.3.2 (Mal), 2.3.3 (TRAM), and 2.2.4 (A46).

Table 4.1. Purification of A46, Mal, and TRAM

A46	Mal	TRAM
Ni affinity	Ni affinity	Ni affinity
Ammonium Sulfate precipitation	Anion Exchange	His tag cut
His tag cut	His tag cut	Ni through
Ni through	Ni through	Anion Exchange
Hydrophobic Interaction	Cation exchange	Size exclusion
Size exclusion	Size exclusion	

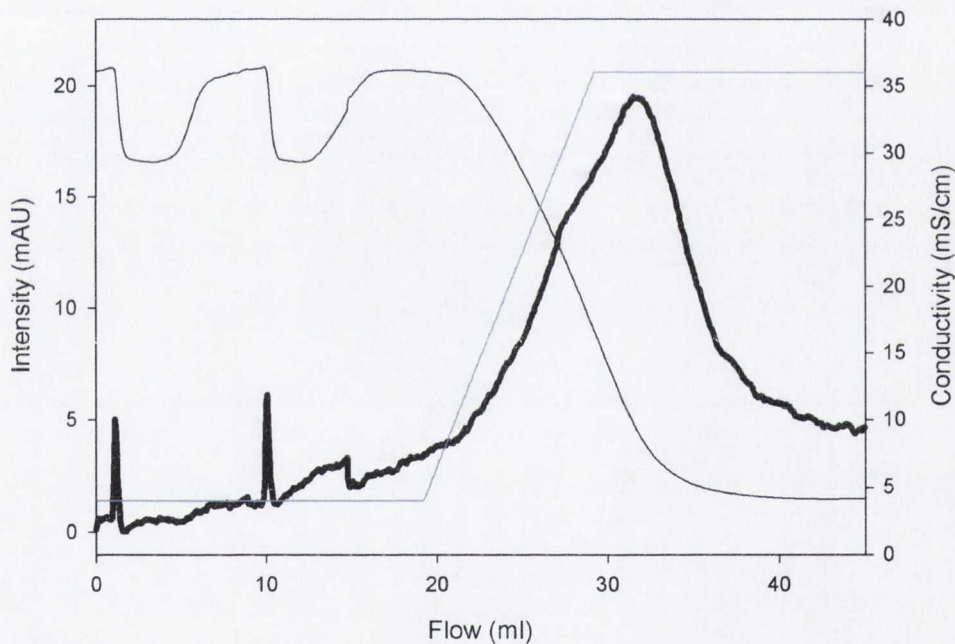


Figure 4.1. Chromatogram of A46 using a hydrophobic interaction column.

Approx. 1 mg protein was applied to the phenyl sepharose column (GE health care) equilibrated with 50 mM potassium phosphate pH 8.0 supplemented with 500 mM sodium sulphate and 1 mM DTT. Bold line, thin line, and grey line represent $A_{280\text{ nm}}$ absorbance of A46, conductivity of the buffer solution, and buffer ratio, respectively. His-tag cleaved A46 came to the peak and A46 protein containing uncleaved tag protein came as a sholder of the peak.

Upon analyses by circular dichroism (CD) spectroscopy, A46-230 was predicted to be a mixture of α -helix and β -sheet (Fig 4.3 and Table 4.2). However, A46 Δ N-230 revealed a largely α -helical structure by empirical analyses of the spectra (Fig 3.3 and Table 3.1). These properties of A46 Δ N-230 are consistent with a Bcl-2 fold, although it is essential to point out that TIR domains can also adopt significant α -helical type structure. Similar analyses of TIR domain-containing Mal in solution revealed a 47% α -helical content (Fig 4.3 and Table 4.2). The CD spectrum of an 11-residue fragment of A46 (residues 88-98) termed VIPER revealed a mixture of secondary structures, dominated by an extended conformation, and is discussed in more detail below. In addition to secondary structure analyses, the aggregation state of proteins was investigated by static light scattering coupled to gel filtration. Analyses of A46 Δ N-230 were consistent with a dimer in solution (37 kDa), with a small amount of tetramer (70 kDa) (Fig 4.4). Although the secondary structure and aggregation state of A46 Δ N-230 displays some consistency with a Bcl-2 fold, the data remains inconclusive. Many TIR domain-containing proteins contain significant α -helical content (Table 4.2) and their aggregation state may also be dimeric in some cases. Upon analysis of Mal by SLS coupled to gel filtration, the full-length protein revealed a monomeric state (31 kDa) (Fig. 4.4) which is inconsistent with previous *in vitro* studies (197). Upon analysis of TRAM by SLS coupled to gel filtration both full-length and truncated fragment revealed a monomeric state (27.1 and 16.8 kDa, respectively) (Fig 4.4) which is inconsistent with previous *in vivo* studies (193).

SDS-PAGE

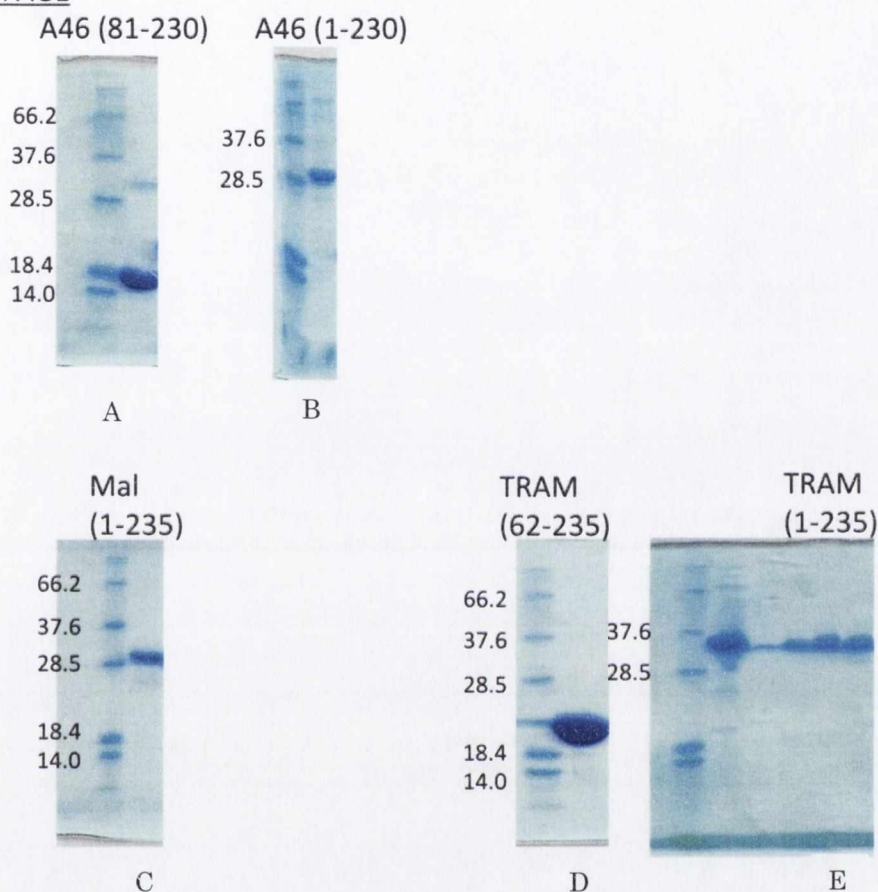
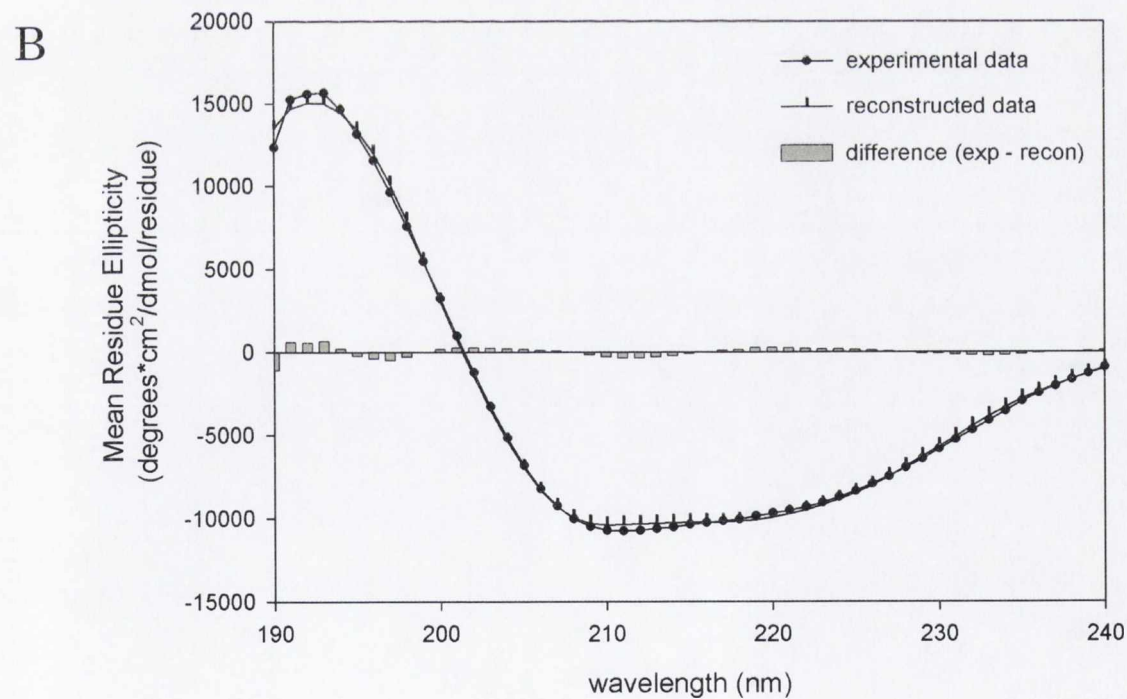
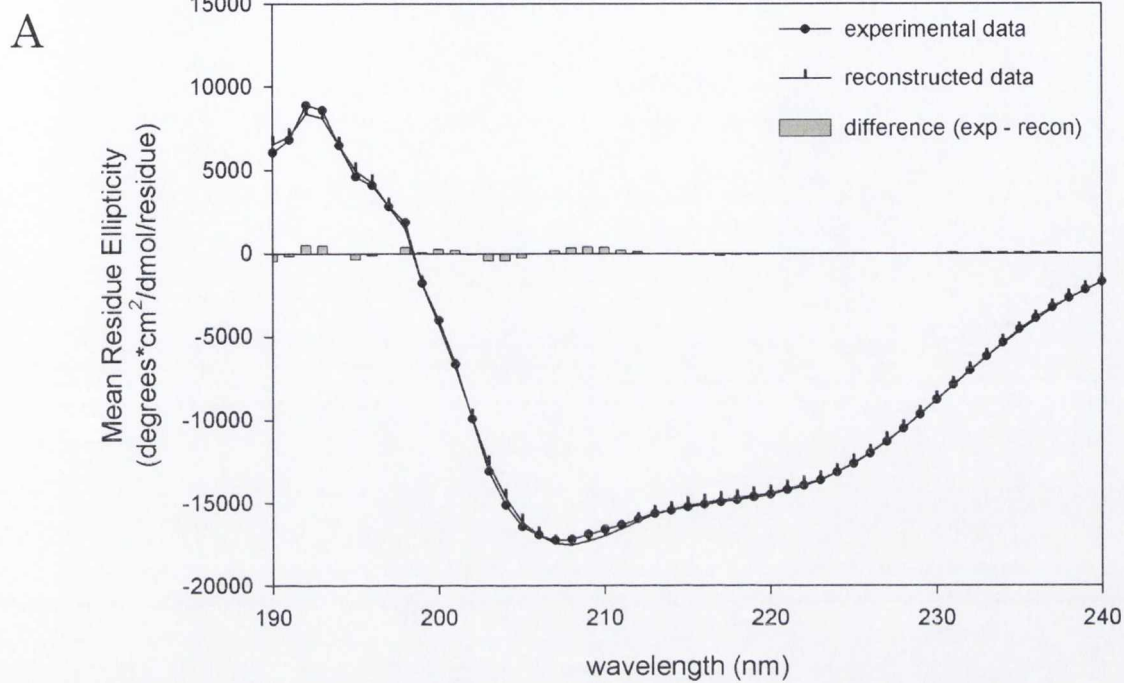


Figure 4.2. SDS-PAGE analyses of expressed and purified A46, Mal and TRAM proteins in this section.

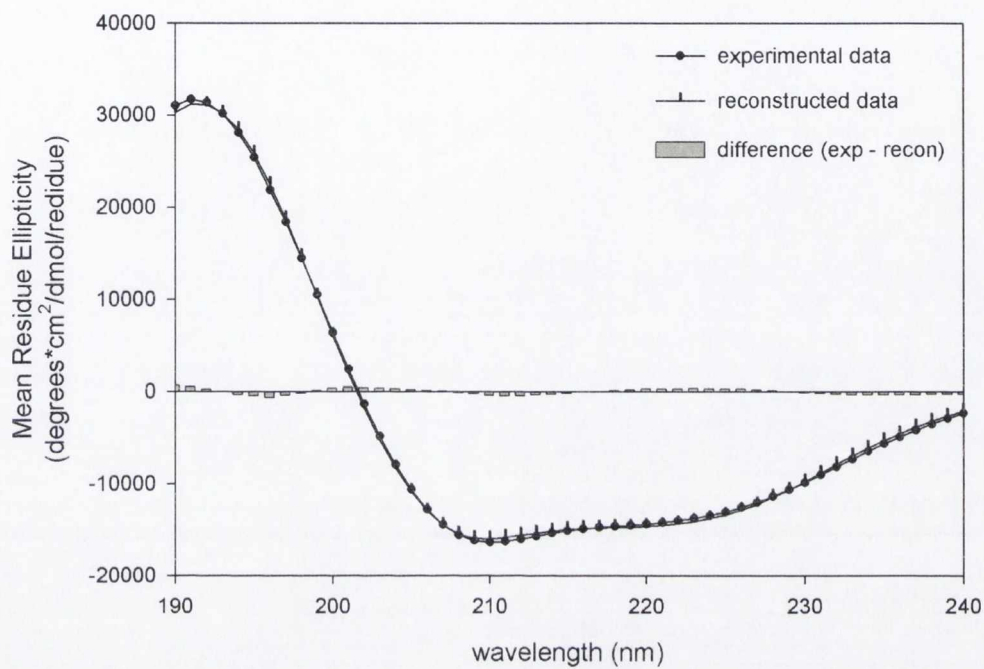
Proteins were purified as described in Materials and Methods (section 2.3). About 5 to 20 μg of each protein were generally loaded onto the gel. The lane on the left side of each gel is molecular weight markers (Jena Bioscience). Gels were stained using Instant Blue (Molecular Dimensions Inc.). Gel A: A46 (81-230); B: A46 (1-230); C: Mal (1-235); D: TRAM 62-235); E: TRAM (1-235).

Protein	Helix (%)	Strand (%)	Turns (%)	Disordered (%)
Mal (1-235)	47	7	15	32
A46 (1-230)	27	25	12	35
A46 (81-230)	44	18	12	28
VIPER	21	44	9	27
A52*	72	7	10	10

Table 4.2. Empirical determinations of secondary structure by CD spectroscopy. The content of α -helix, β -strand, turns and disordered regions is shown for each protein, as well the peptide VIPER. The three dimensional structure of A52 is known, and was used as a control to estimate the degree of reliability of the empirical methods. Calculations were performed using the DICHROWEB server, as described in Materials and Methods section 2.3.



C



D

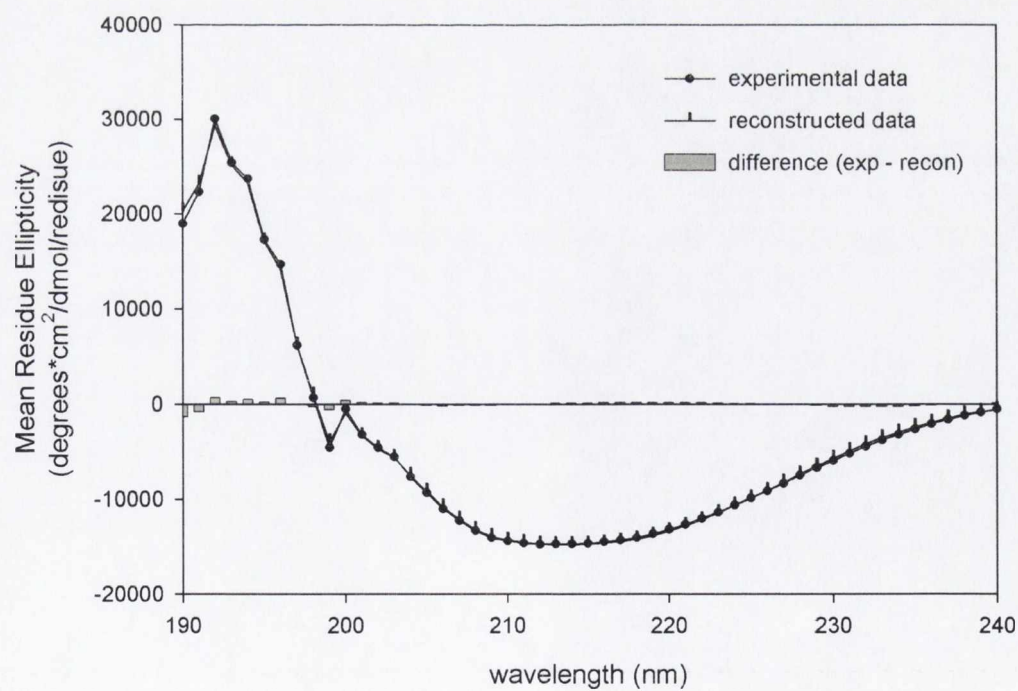
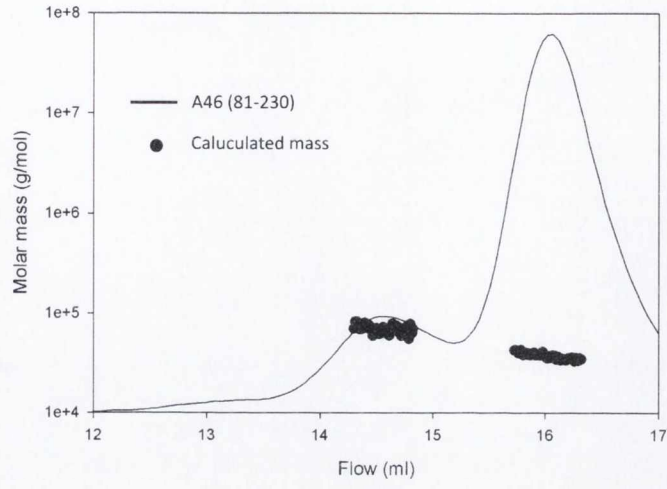


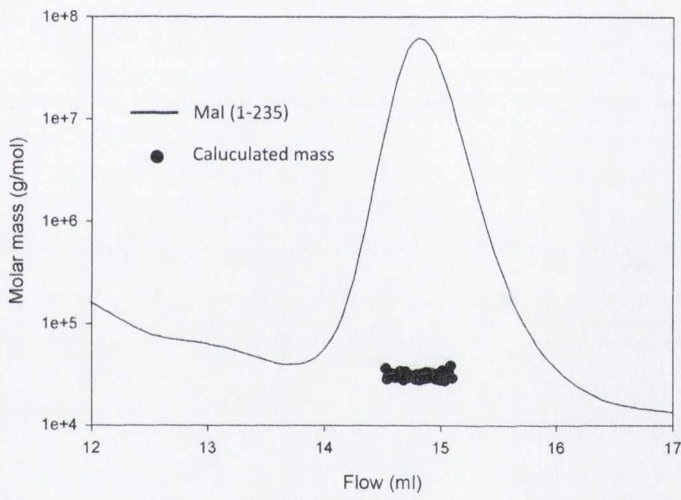
Figure 4.3. Circular dichroism spectroscopy analyses of A46 and Mal.

The proteins were analyzed as described in Material and Methods section 2.3. Purified protein solution was dialyzed into 50 mM phosphate buffer pH 7.4. The spectrum was recorded at 150 $\mu\text{g/ml}$ concentration in a 1 mm pathlength quartz cuvette. Data was submitted to the DICHROWEB server (Whitmore & Wallace, 2008) and analyzed using the CDSSTR algorithm (Sreerama & Woody, 2000) against the SP175 set of reference spectra (Lees & Wallace, 2006). (A) Mal (1-235); (B) A46 (1-230); (C) A46 (81-230); (D) VIPER peptide. Experimental data is shown as a line with a circle, and reconstructed data is shown as a line with a vertical mark. Difference between experimental data and reconstructed data is shown as bars along the zero mean residue ellipticity line over the wavelength range

A



B



C

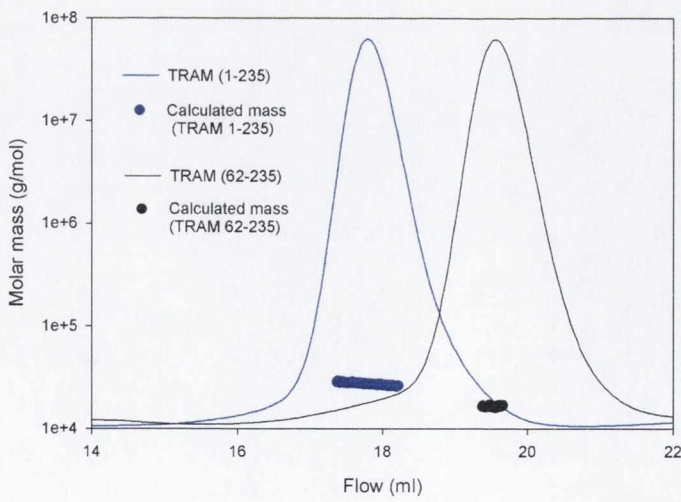


Figure 4.4. Determination of the molecular mass of purified A46, Mal, and TRAM by static light scattering. Purified proteins (about 0.5 mg total) were loaded onto a Supedex 200 10/200 gel filtration column (GE Healthcare), equilibrated with buffer (100 mM NaCl, 1 mM DTT in 10 mM Tris/HCl pH 7.5). (A) Elution of A46 Δ N-230; (B) Elution of full-length Mal; (C) Elution of full-length (1-235, blue line) and truncated TRAM (62-235, black line). Data was analyzed by ASTRA version 4.90.08. The absolute molecular weight distribution is shown (dotted line) along the elution profile ($A_{280\text{ nm}}$, solid line). A46 forms dimer (37 kDa) with a slight fraction of tetramer (70 kDa). Mal (31 kDa) and TRAM (62-235: 16.8 kDa; 1-235: 27.1 kDa) are monomeric in solution.

4.2.2. The interaction of Mal with A46 and the VIPER peptide

Full-length Mal was coupled to a CM5 chip and purified A46 proteins and the VIPER peptide were assayed for binding using a Biacore X100 system. SPR studies revealed that A46-230 and A46 Δ N-230 bind to Mal with similar affinities (Table 4.3). The affinity for the fragment 81-230 ($K_d = 1.5 \mu\text{M}$) is similar to A46-230 ($1.7 \mu\text{M}$), suggesting that the C-terminal α -helical domain retains the ability to interact with Mal (Fig 4.5). In contrast, the 11-residue VIPER peptide fragment of A46, which has been reported to retain the biological activity of full-length A46 (183), does not interact significantly with Mal by SPR (Fig 4.5). The molecular weight of the peptide (1,374 Da) is well above the threshold recommended for sensitivity by the Biacore X100 instrument ($>150 \text{ Da}$). Furthermore, full-length Mal was coupled to the CM5 chip at high density ($\text{RU} > 2,000$) to enable detection of weakly bound and low molecular weight analytes. Isothermal titration calorimetry experiments similarly failed to detect any significant affinity between VIPER and Mal (data not shown). These findings were corroborated further by co-purification experiments involving Mal and the VIPER peptide. Ion-exchange chromatography revealed that milligram amounts of full-length Mal incubated with at least 5-fold molar excess VIPER did not alter its migration (Fig 4.6). In contrast, incubation of a DDX3 peptide with poxvirus protein K7 resulted in the appearance of additional peaks corresponding to a stable peptide/protein complex (Fig 4.6). A modest shift in both uncomplexed proteins (K7 and Mal) is observed, and this can be attributed to the general effects of large amounts of peptide on the conductivity and pH of the mobile phase during ion exchange chromatography.

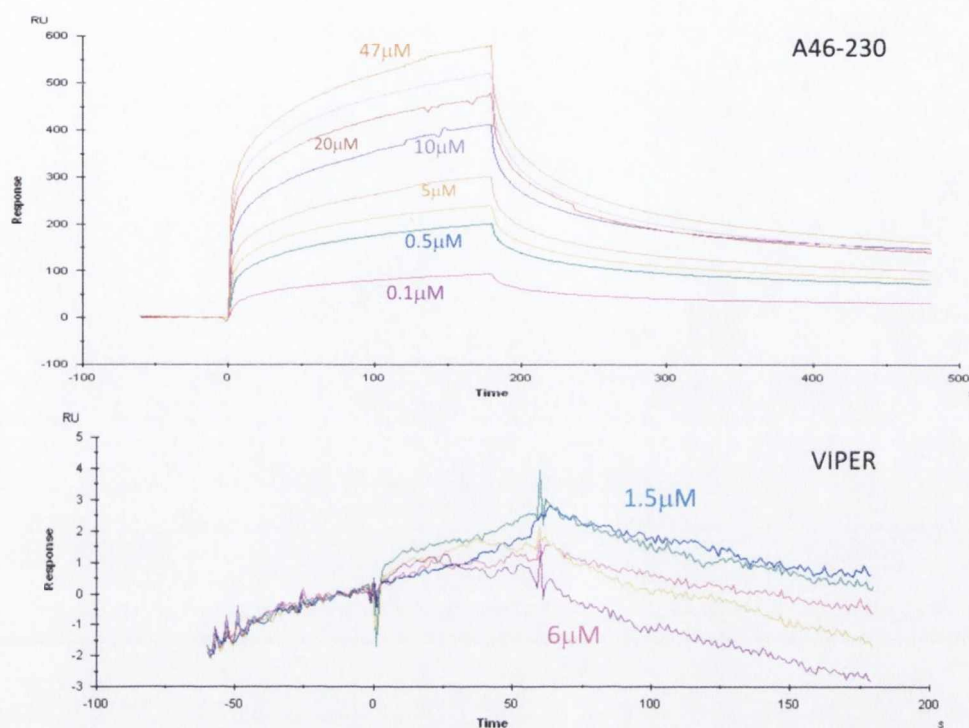


Figure 4.5. Sensorgram from surface Plasmon resonance analysis of A46 binding to Mal.

(A) Injection of various concentration of A46-230 onto a Mal-coupled CM5 chip. (B) VIPER peptide injected onto Mal/CM5 chip. Spectra have been corrected for non-specific binding to the reference flow cell. At higher concentrations, the peptide interacts non-specifically with the reference cell (Fc1), thus resulting in 'inverted' spectra. However, binding to the Mal-coupled flow cell (Fc2) is negligible [maximum Response Units (RU) = 2], and it can be concluded that there is no significant affinity of VIPER for Mal.

Protein	K_D (μ M) equilibrium	Response Units (RU _{max})
Mal_CM5		
A46-230	1.7	208
A46 Δ N-230	1.5	436
VIPER	No binding	~2

Table 4.3. Summary of SPR analysis.

Experiments were performed for A46 proteins and VIPER peptide with CM5-coupled Mal, as described in Materials and Methods. The maximum binding is indicated, and sensorgrams were fit to an equilibrium dissociation constant using Biacore Evaluation software, version 2.0 as discussed in 2.11.

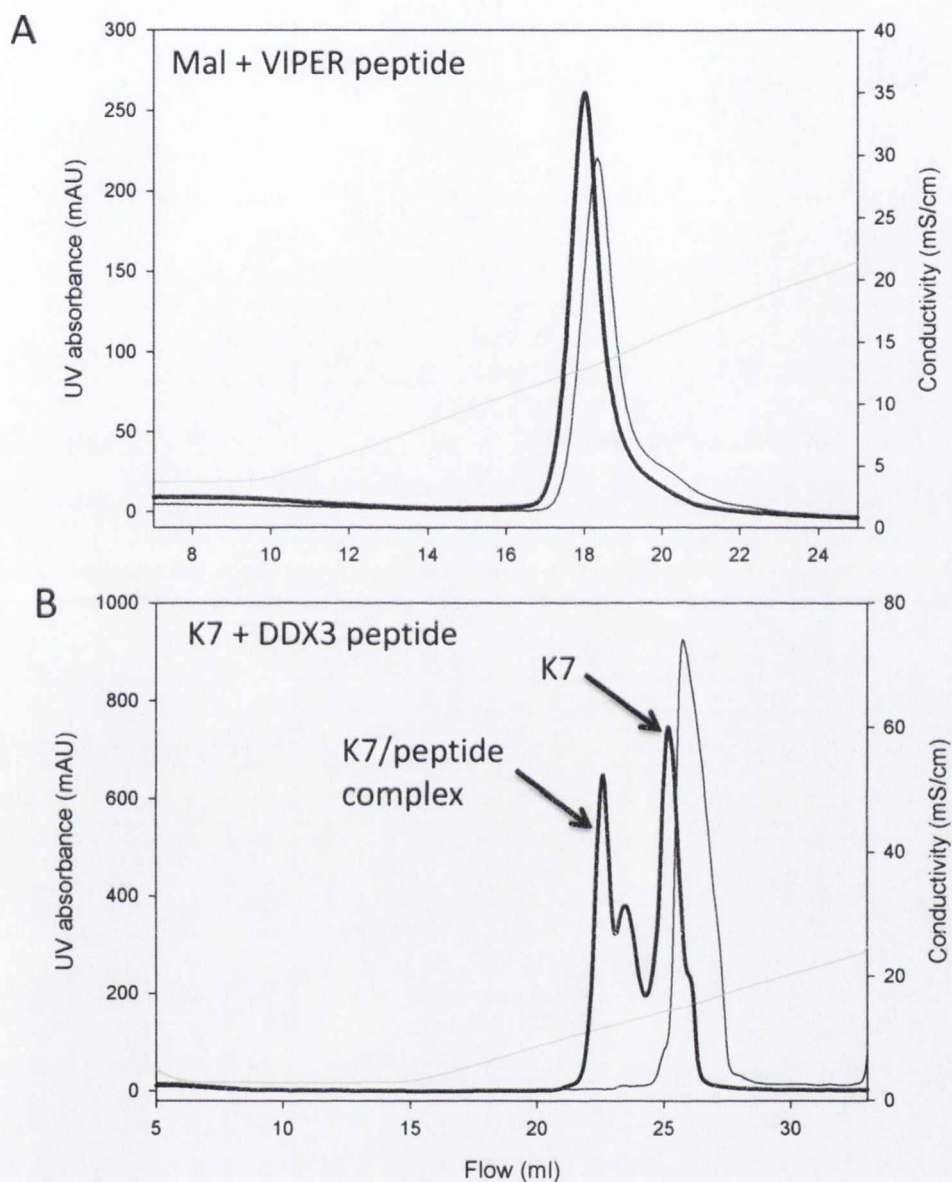


Figure 4.6. Ion-exchange chromatography analysis of proteins incubated with the VIPER peptide.

Thin/light lines represent the uncomplexed protein, while thick/dark lines represent the pre-incubation with peptide. A; full-length Mal incubated with VIPER. B; full-length K7 incubated with DDX3 peptide. The grey linear lines along the elution profile represent the gradient of salt used to elute protein.

4.2.3. Sample condition screening for crystallization

Truncated A46 and TRAM and the full-length Mal, and TRAM proteins were evaluated by Native-PAGE (Fig 4.7). The truncated A46 protein ran with a concentrated profile. While both full-length and truncated TRAM samples ran with a slightly broader profile, while full-length Mal fragment sample showed highly broad profile.

Both full-length Mal and TRAM are further characterized by dynamic light scattering for crystallization (Fig. 4.8). In both cases, a unimodal profile was achieved at pH 9.5 for Mal and pH 10.0 for TRAM. Moreover, 100 mM Arg was needed with TRAM to stabilize the unimodal profile of the protein sample. Protein crystals of these samples have not been obtained yet, but work is ongoing.

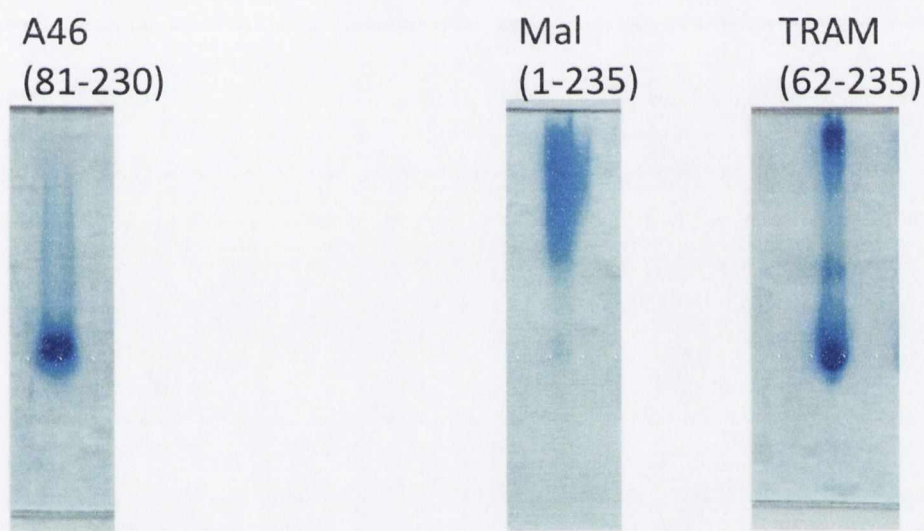


Figure 4.7. Native-PAGE analyses of A46 (81-230), full-length Mal, and TRAM (62-235).

Purified protein samples (about 20 μ g total) were mixed with Native-gel sample buffer and loaded onto the Native-PAGE gel as described in section 2.3. A46 sample gave a single band. The Mal sample gave a broad dispersed band. The TRAM sample gave one major band and several minor bands.

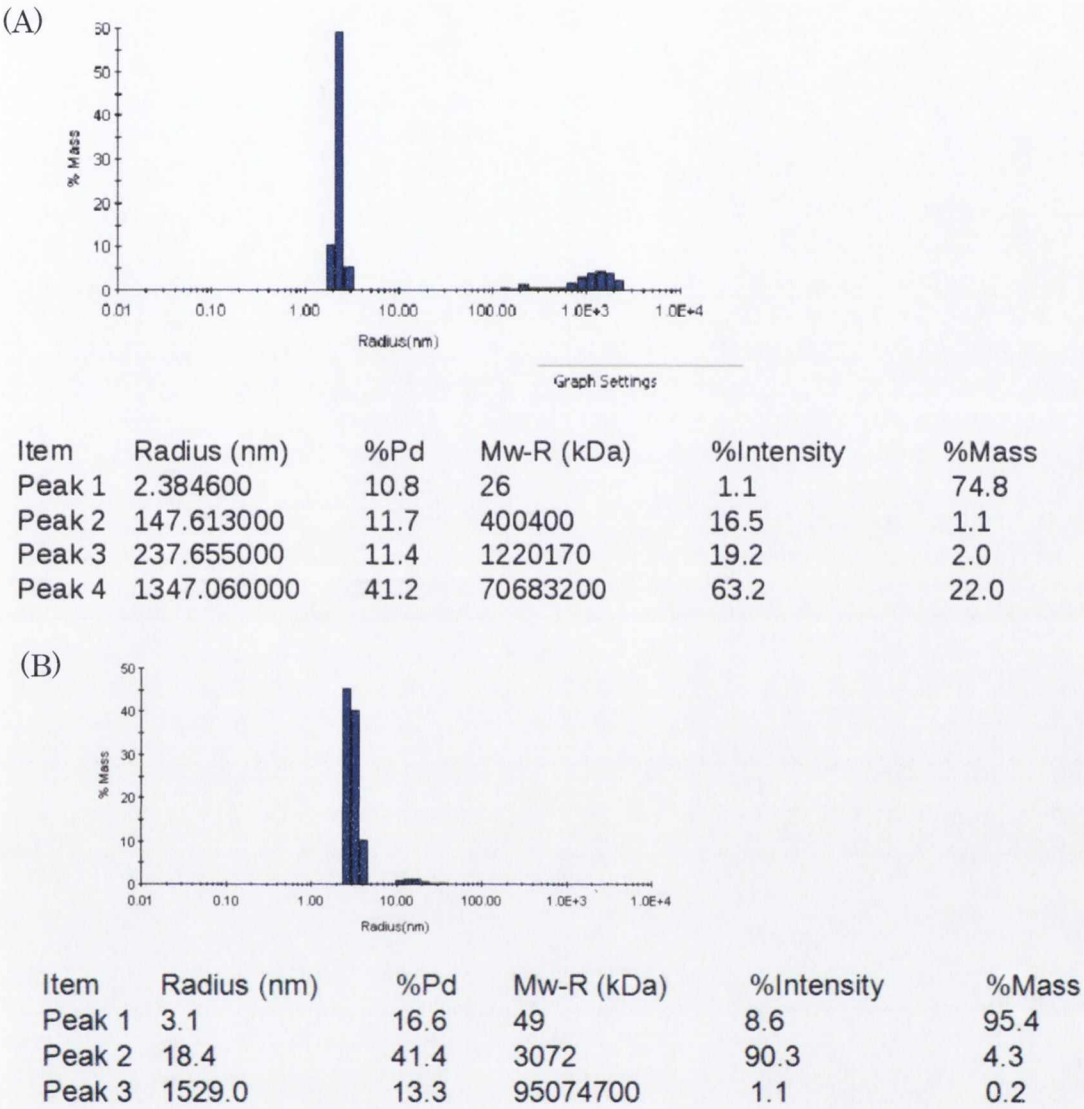


Figure 4.8. Dynamic light scattering analysis of full-length Mal (panel A) and TRAM (62-235) (panel B).

(A) Purified 2 mg/ml concentration Mal sample was prepared in 10 mM bicine pH 9.0 supplemented with 150 mM NaCl. (B) Purified 6 mg/ml concentration TRAM sample was prepared in 10 mM CAPS pH 10.0 supplemented with 150 mM NaCl, and 150 mM L-Arginine. Sample was loaded onto the 2 μ L volume quartz cuvette and measured by DynaPro NanoStar system (Wyatt Technology Corp). The data was analyzed by DYNAMICS (version 7.0.1.12). Percent polydispersity (%Pd), which represents the dispersity of the protein in the solution, is a good indicator of the crystallizability of the sample.

4.3. Discussion

Recently, an 11-residue peptide fragment of A46 (residues 88-98) has been observed to mimic some of the properties of the complete protein (183). In a hypothetical three-dimensional model of A46 (183), this peptide corresponds to one-half of the N-terminal helix of a Bcl-2 globular fold, part of which would be buried against the other α -helices in the intact protein. It is unlikely that the conformation of the isolated peptide is identical to a predicted three-dimensional epitope formed by this segment in intact A46, and our CD studies confirm that the peptide is not α -helical in solution. Although we observed affinity in the low micromolar range (1.5 μ M) between A46 Δ N-230 and Mal, we have found that there is no affinity of the VIPER A46 peptide (88-98) with Mal using SPR analyses, co-purification studies, and isothermal titration calorimetry. In contrast, a peptide from human RNA helicase DDX3, which we have previously crystallized in complex with poxvirus protein K7 (285), reveals positive interactions in all three assays under similar experimental conditions. The binding data from the VIPER *in vivo* experiments would perhaps be consistent with our observations if the affinity were extremely low. The TIR domain of Mal has a pI of 4.7, while the peptide 88-98 of A46 has a net positive charge with a predicted pI of 8.4, so that a component of the weak affinity may be electrostatic in nature. Indeed, the pulldowns reported in the VIPER paper required immunoprecipitation to visualize the binding partners (183), whereas the binding of K7 to His-tagged peptides of DDX3 was visualized by Coomassie stain, implying a more stable interaction (228,285). It is noteworthy that the affinity of K7 for a 10-residue DDX3 peptide (1.1 μ M) is on par with the strength of the interaction between full-length A46 and Mal (1-2 μ M). The clear difference in the nature of the peptide fragments is that the DDX3 peptide originates from a disordered segment of the protein, whereas the A46 peptide resides in a folded globular segment of the protein, as suggested by all secondary structure and fold predictions (182,183). These caveats must be considered when rationalizing the effects of the VIPER peptide on intracellular signaling pathways. Given the widespread effects of A46 on numerous signaling pathways, it is also conceivable that the antagonistic effects of VIPER on innate immune signaling are independent of Mal binding *in vivo*. Our biochemical and biophysical studies are consistent with the targeting of the TIR domain-containing Mal by a dimeric α -helical sub-domain of A46, situated within the segment 81-230. This study is the first to provide biophysical evidence for an interaction between a poxvirus protein and a monomeric TIR domain. The C-terminal region of A46 shares 30% sequence identity with residues 89-203 of the

M136R protein of myxoma virus, and shares 20% sequence identity with A52 between residues 94-201. The ensemble of evidence points to a Bcl-2 fold in the C-terminus of the protein that mediates binding to the TIR domain-containing Mal. However, it is important to remain cautious, especially taking into consideration that the C-terminal sub-domain of A46 may contain 18% β -strand or extended conformation, as evidenced by CD spectroscopy. This value is far higher than the Bcl-2 protein A52, whose overall assignments of secondary structures are correctly predicted by our CD experiments when comparing to the crystal structure (Tables 4.2). More detailed structural descriptions of A46 and the mechanism of antagonism of TIR adaptors will require X-Ray or NMR studies of the isolated proteins and complexes.

As yet, we have not obtained crystals of these proteins discussed in this section. The Native-PAGE profile of truncated A46 indicates the homogeneity of this sample, but it was difficult to concentrate it to over 2 mg/ml in neutral pH buffer. However, it was possible to concentrate it up to 10 mg/ml in alkaline pH buffer. These results suggest that charged residues on the surface decrease the solubility of the protein and possibly prevent crystallization. DLS data of both Mal and TRAM proteins showed a monodisperse profile only in alkaline pH. Thus, it is probable that some charged residues affect the dispersity of these samples as in the case of A46. It is conceivable that greater efforts to control the surface charge could result in obtaining crystals of these proteins, and this will now be an ongoing focus within the laboratory. The expression, purification, and an analysis of the behavior of these proteins *in vitro* presented in this study will greatly enhance the goal of obtaining crystals of an A46/TIR-domain complex.

Chapter 5. Analysis of the effect of the F154A mutation of A52 for dimer formation by biophysical techniques

5.1. Introduction

A52 is a 23 kDa intracellular viral protein with no obvious similarity to host proteins. It inhibits the activation of NF- κ B by interacting with the downstream TLR-signaling molecules IRAK2 (231). A52 also interacts with TRAF6 directly and enhances MAPK activation (181,232). Activation of p38 MAP kinase is enhanced by the direct binding of A52 to TRAF6 and drives the p38-dependent production of IL-10, which is a cytokine that inhibits inflammatory and cell-mediated immune responses (155). The crystal structure of A52 (Fig. 5.2) was solved but both the mechanism by which it binds cellular targets and its regulation of PPR signaling are still unknown (227).

P38 MAP kinase activation was enhanced by the direct interaction of A52 with the TRAF domain (Fig. 1.24) of TRAF6 and truncation of the C-terminal 46 residues abolishes the interaction with TRAF6 and activation of the p38 MAP kinase (178,232). A52 forms a stable homodimer in solution and adopts an α -helical Bcl-2 like fold (Fig. 1.23) (227). The dimer interface comprises the N-terminal region, α -1, and α -6, which is conserved among the dimeric Bcl-2 superfamily. Salt-bridges, hydrogen-bonds, and hydrophobic interactions mediate the dimer of A52 (Fig. 5.1).

Our collaborator, Prof. Andrew Bowie and his co-workers, revealed that A52 F154A mutant failed to activate the MAPK, but retained the NF- κ B inhibitory function by *in vivo* studies (Table 5.1). The Phe154 residue sits at the edge of the A52 dimer interface, and the F154A mutant may therefore disrupt dimer formation (Fig. 5.2).

The objective of the studies in this section was to advance our understanding of the structural implications of Phe154 and the consequences of a F154A mutation on the dimerisation of A52. Comparisons between wildtype A52 and the mutant were achieved through biophysical techniques specifically SPR and static light scattering.

A52 construct	TRAF6 interaction	NF-κB inhibition	MAPK activation
FL (1-190)	++++	+++	+++
ΔA52 (1-144)	-	++++	-
A52 (F154A)	++	++++	-

Table 5.1. Full-length A52 interacts with TRAF6. A52 (1-144) completely failed to interact and activate MAPK. The F154A mutant has a weaker interaction with TRAF6, but failed to activate MAPK. Both ΔA52 (1-144) and A52 (F154A) retained the ability to inhibit NF-κB function. **These experiments were performed by Prof. Andrew Bowie's group.**

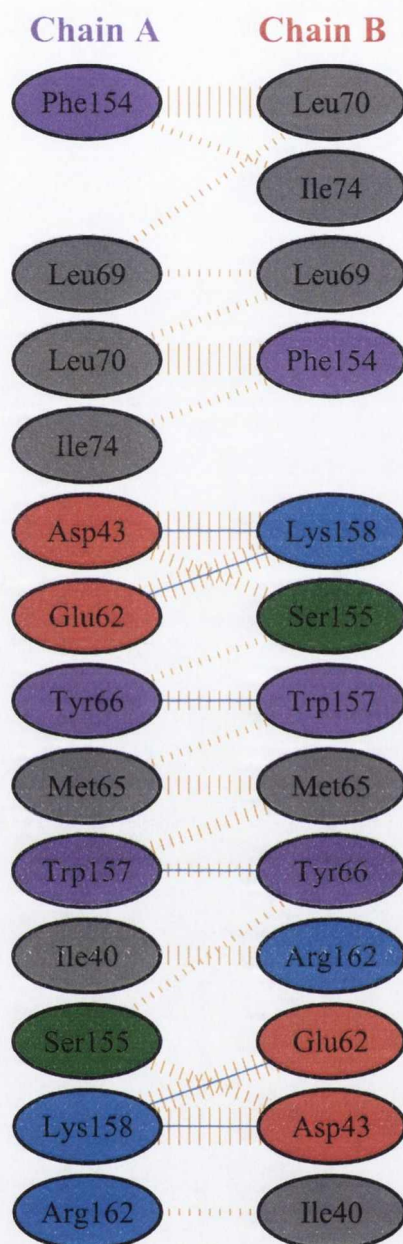


Figure 5.1. Schematic diagram of residue interactions across the A52 dimer interface.

The figure was generated by PDBsum (Laskowski R A (2009)). Light blue lines represent hydrogen bonds. Orange ladder represents non-bonded contacts. For non-bonded contacts, the width of the striped line is proportional to the number of atomic contacts. Overall the A52 dimer comprises mainly of hydrogen bonds and hydrophobic contacts.

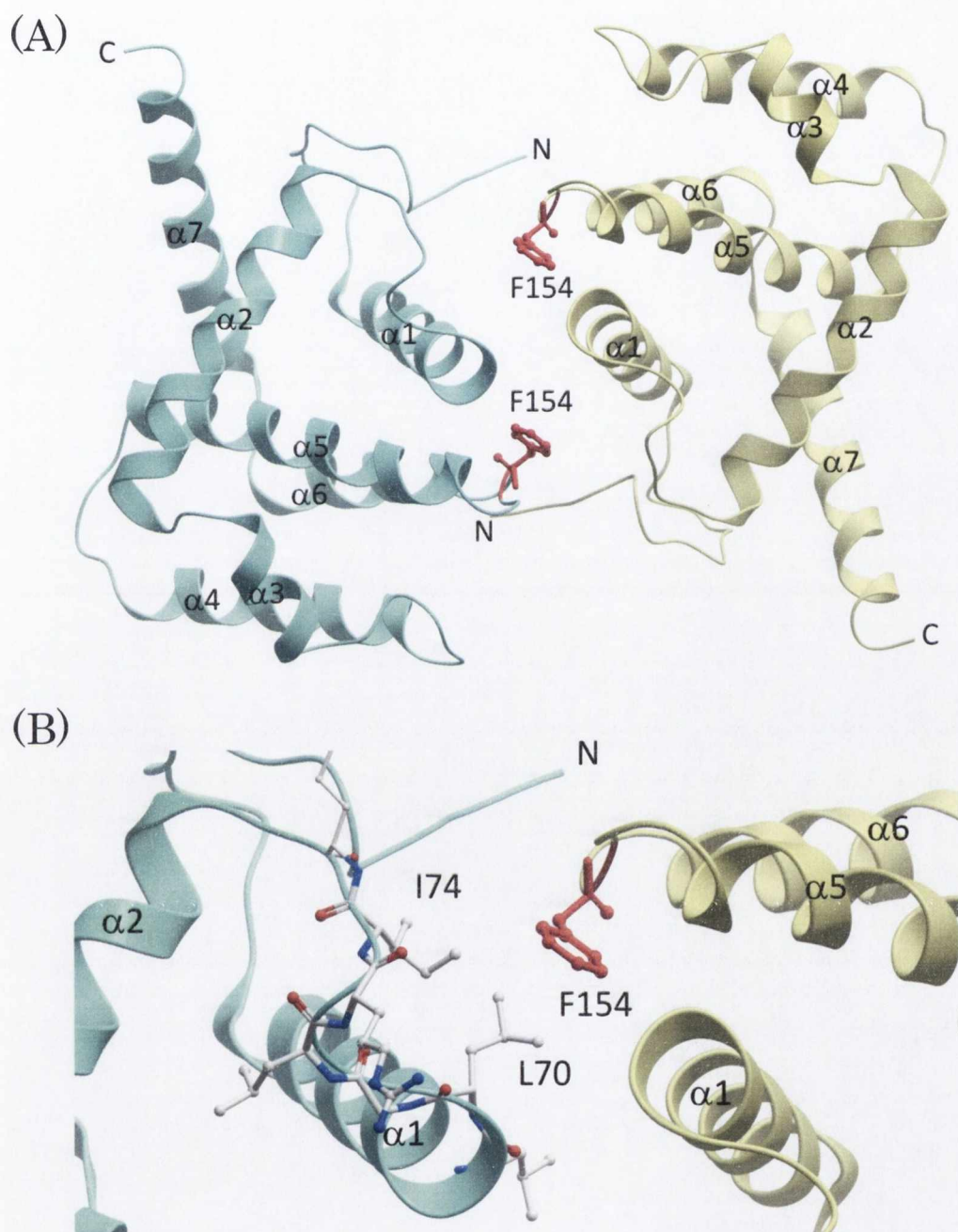


Figure 5.2. Dimer interface of A52 homodimer.

(A) Cartoon model of the A52 homodimer (PDB code: 2VVW). The Phe154 residues are represented as red stick models. The dimer interface comprises of the N-terminus, $\alpha 1$, and $\alpha 6$ of the Bcl-2 fold domain. (B) The local environment of Phe154 which resides on the loop between helices $\alpha 5$ and $\alpha 6$. It makes hydrophobic contacts with residues Leu70 and Ile74 at from the edge of $\alpha 1$ on the second molecule.

5.2. Results

5.2.1. Purification of MBP-TRAF6

MBP-tagged TRAF6 protein was cloned, expressed, and purified following the procedure outlined in Section 2.2.6. The molecular weight of MBP-tagged TRAF6 is 99 kDa and it forms oligomer in the solution (236). As a result much of the protein was observed in the void volume after size exclusion chromatography using superdex 200 column (GE healthcare) (Fig. 5.3).

5.2.2. Purification and biophysical analysis of A52

Proteins were cloned, expressed, and purified to homogeneity as detailed in Section 2.2. Results of the purification are presented in Figure 5.4. Elution peak of the F154A mutant shifted to higher retention volume than wild-type A52 protein (Fig. 5.4, panel A and B). The empirical analysis of wild-type A52 by CD spectrum showed the typical secondary structure composition of a Bcl-2 fold protein as shown in the crystal structure (Fig. 5.2 and Table 5.2) (227). The measurements of CD spectroscopy also revealed that the A52 (F154A) mutant remains folded but the helix content was decreased and unordered structure content increased as a consequence of the F154A mutation (Fig. 5.5 and Table 5.2). To investigate the aggregation state of the proteins, static light scattering was performed (Fig. 5.6). Analyses of A52 wild-type was consistent with that of a dimer in solution (47.9 kDa), but analysis of A52 (F154A) revealed a molecular mass of 37.3 kDa, which is an intermediate value between that of a dimeric and monomeric state.

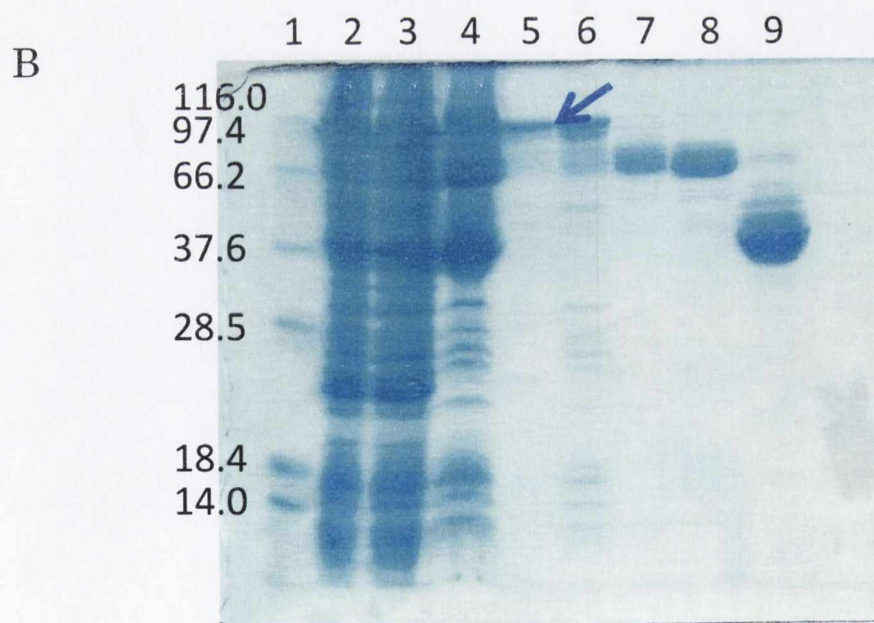
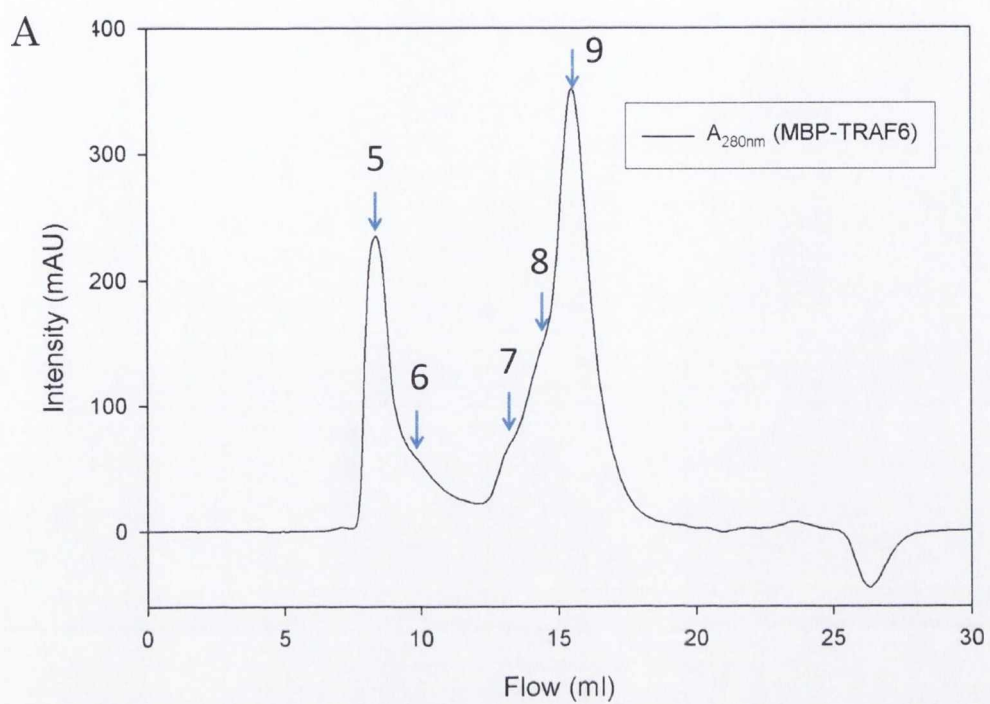
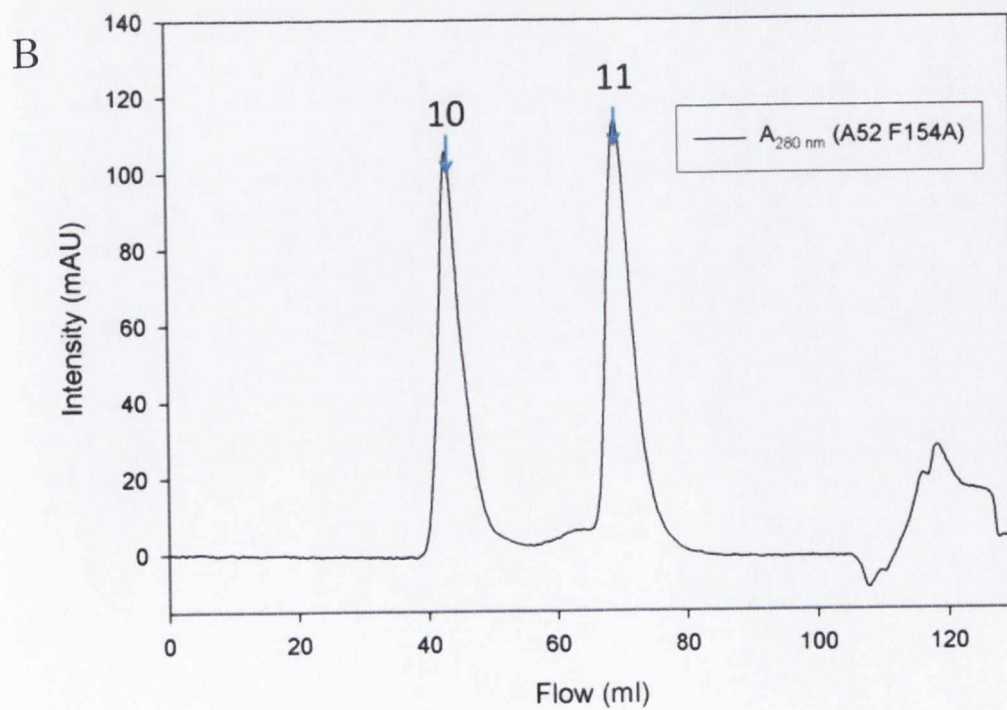
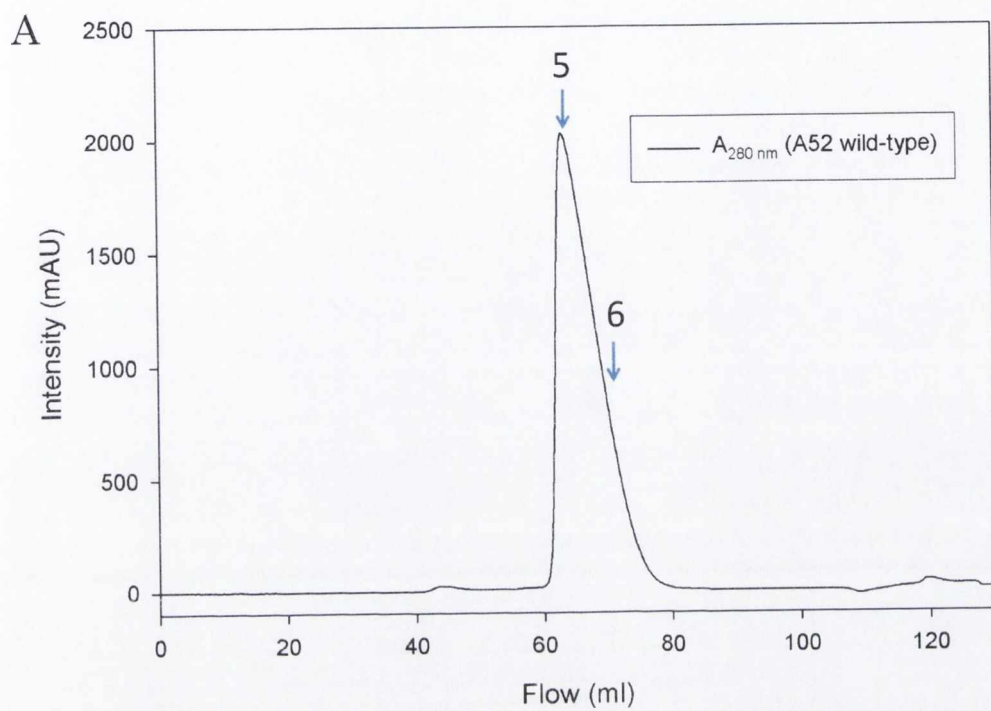


Figure 5.3. Chromatogram of size exclusion chromatography and SDS-PAGE analysis of MBP-tagged TRAF6.

A; Chromatogram of MBP-TRAF6. Approximately 1 mg protein was loaded to the superdex 200 10/300 column (GE Healthcare). The numbers across the peak correspond to the lane number on the SDS-PAGE gel. **B;** SDS-PAGE was performed following the protocol described in Section 2.3. 1; protein molecular weight marker. 2; Lysate. 3; Amylose resin flow through fraction. 4; Eluted samples from the amylose resin. 5-9; Fractions of size exclusion chromatography. Numbers correspond to the number on the chromatogram. Blue arrow indicates the band corresponding to MBP-tagged TRAF6.



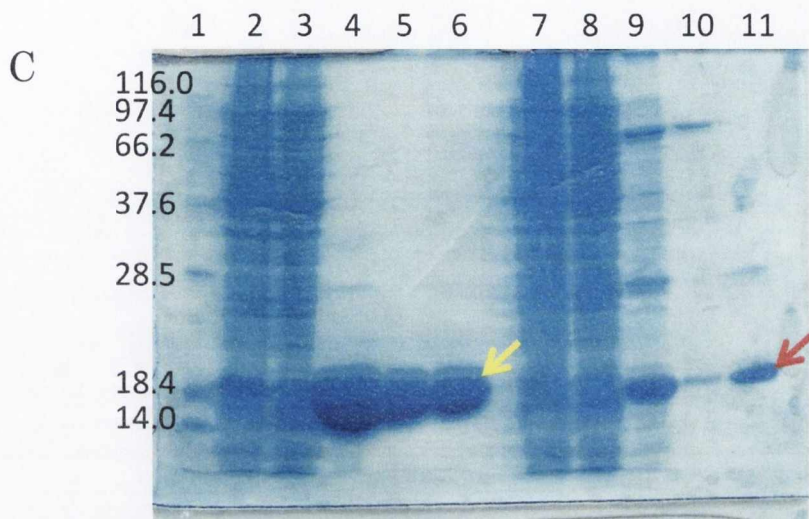


Figure 5.4. Protein purification of wild-type and the the F154A mutant of A52.

A; Chromatogram of wild-type of A52 from size exclusion chromatography;
 B; Chromatogram of F154A mutant of A52 from size exclusion chromatography. Numbers on the chromatogram correspond to the number on the SDS-PAGE gel. C; SDS-PAGE gel of wild-type and F154A mutant of A52. (1. Protein molecular weight marker, 2. Lysate, 3. Ni^{2+} resin flow through fraction, 4. Eluate from Ni^{2+} resin, 5-6. Size exclusion chromatography fraction of A52 wild-type; 7. Lysate, 8. Ni^{2+} resin Flow through fraction, 9. Eluate from Ni^{2+} resin, 10-11. Size exclusion chromatography fraction of A52 F154A mutant.) Yellow and red arrows indicate the induced wild-type and F154A mutant of A52, respectively.

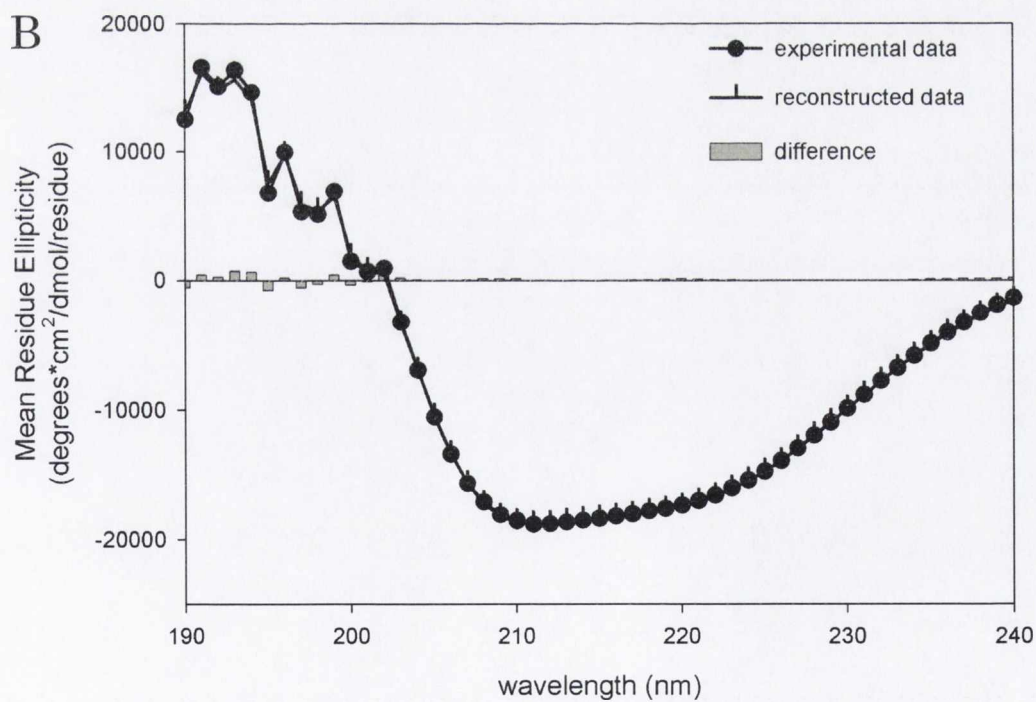
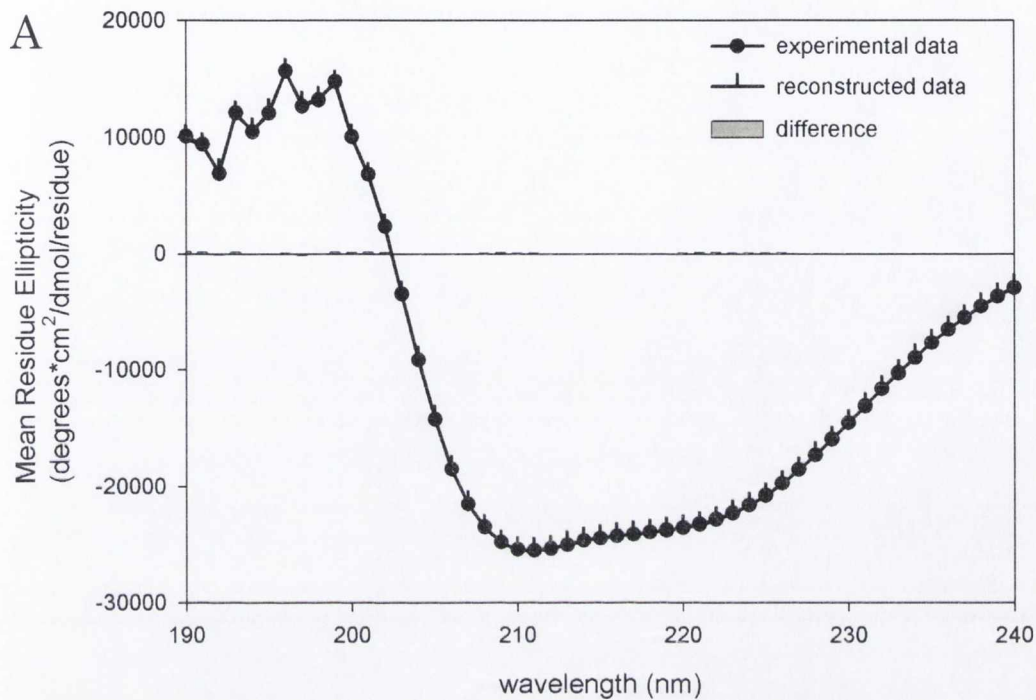


Figure 5.5. CD spectrum of A52 wild-type and the F154A mutant.

(A) A52 wild type. (B) A52 F154A mutant. The proteins were analyzed as described in Material and Methods Section 2.5. Purified protein solution was dialyzed into 50 mM phosphate buffer pH 7.4. The spectrum was recorded at 15 µg/ml concentration in a 1 cm lightpath quartz cuvette. Data was submitted to the DICHROWEB server (Whitmore & Wallace, 2008) and analyzed using the CDSSTR algorithm (Sreerama & Woody, 2000) against the SP175 set of reference spectra (Lees & Wallace. 2006).

	helix	strand	turns	unordered
A52 wild-type	0.71	0.08	0.10	0.11
A52 F154A	0.47	0.14	0.12	0.27

Table 5.2. Secondary structure content of wild-type and F154A mutant of A52.

Helix content decreased and unordered structure content increased as a consequence of the F154A mutation.

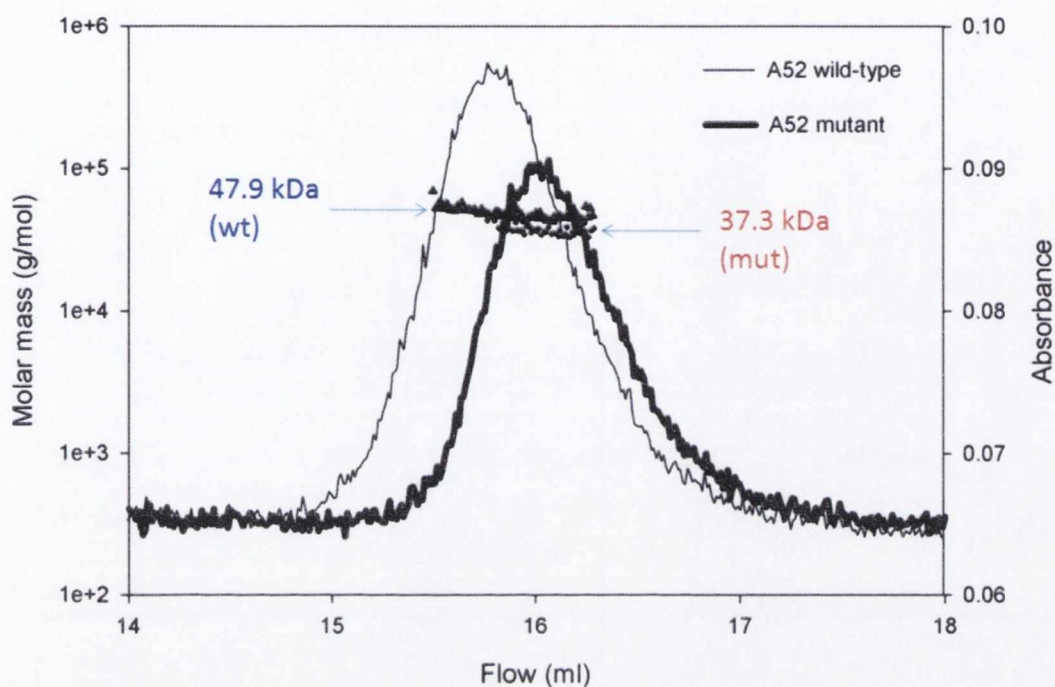


Figure 5.6. Static light scattering of A52 wild-type and F154A mutant.

Purified proteins (about 0.2 mg total) were loaded onto a Supedex 200 10/200 gel filtration column (GE Healthcare), equilibrated with buffer (100 mM NaCl, 1 mM DTT in 10 mM Tris/HCl pH 7.5). Thin line represents a chromatogram of A52 wild-type and bold line represents F154A mutant. Triangle and circle dots represent calculated molar mass of A52 wild-type and F154A mutant, respectively. Data were analyzed by ASTRA (version 4.90.08). The absolute molecular weight distribution is shown (dotted line) along the elution profile ($A_{280\text{ nm}}$, solid

5.2.3. Surface plasmon resonance

Kinetic analysis revealed that wild-type A52 has a slightly lower equilibrium K_d value than A52 (F154A) for the interaction with MBP-TRAF6 [0.13 μM (A52 wild-type) and 0.46 μM (A52 F154A)]. The first-order association constants (k_a) and dissociation constants (k_d) were distinct in each case (Table 5.3). In particular, despite a moderately slower on-rate, the wild-type complex dissociated much more slowly than the mutant, implying a more stable complex.

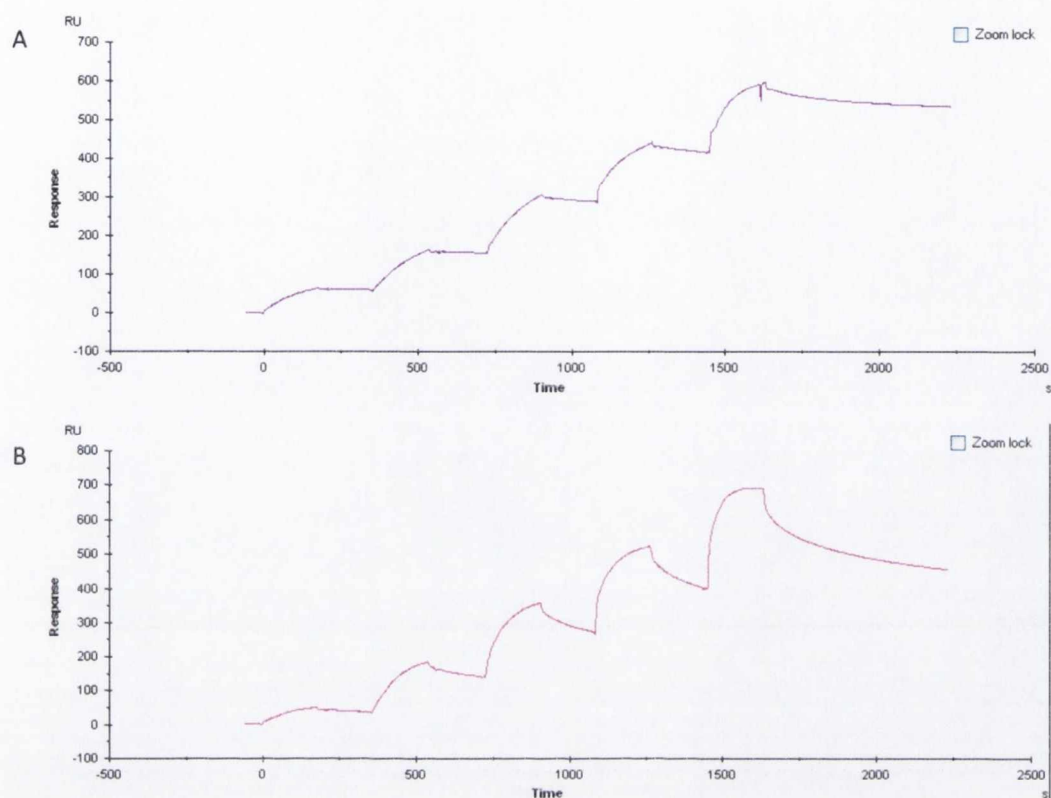


Table 5.3

	k_a ($M^{-1}s^{-1}$)	K_d (s^{-1})	KD (μM)
A52 wild-type/TRAF6	21,400	2.8×10^{-3}	0.13
A52 F154A/TRAF6	86,270	39.8×10^{-3}	0.46

Figure 5.7. and Table 5.3. SPR sensorgram of analysis of A52 binding to TRAF6

(A) A52 wild-type (analyte) was injected onto a MBP-tagged TRAF6-coupled CM5 chip. (B) A52 F154A mutant was injected onto TRAF6/CM5 chip described in Section 2.11. Spectra have been corrected for non-specific binding to the reference flow cell. Kinetic fit was performed using Biacore Evaluation software version 2.0. Six different analyte concentrations were used (0-22 μM) in each cycle. Biacore buffer, containing 10 mM HEPES, pH 7.4 supplemented with 150 mM NaCl, and 0.005% surfactant P20, was used for whole experiments.

5.3. Discussion

VACV protein A52 inhibits NF- κ B activation and enhances MAPK activation simultaneously. Both functions are independent of each other and MAPK activation is enhanced *via* binding of A52 to TRAF6. Our collaborator, Prof. Andrew Bowie's lab showed that although A52 (F154A) partially retained the interaction with TRAF6, but it failed to enhance MAPK activation (Table 5.1). The dimer interface of A52 comprises hydrogen bonds and hydrophobic contacts (Fig. 5.1). Phe154 resides on the loop between α 5 and α 6 on the dimer interface of A52 (Fig. 5.2). Phe154 has hydrophobic contacts with Leu70 and Ile74, which reside on the loop (or the edge of the α 1 helix) between α 1 and α 2, of opposing monomer units. It appears that the Phe154 residue enforces the correct folding of the helix. Considering the position of Phe154 and its interaction, CD data showed that the helix composition decreased on introduction with the F154A mutation. This could possibly indicate that the helices around Phe154 partially became unfolded by the mutation. SLS data indicated that the dimer of F154A become slightly unstable during migration through the size exclusion column. Since the elution is performed at low flow rates, the dissociation is unlikely to originate from shearing forces. Consistent with loss of α -helicity, the dissociation likely occurs from the lack of a tightly packed dimer in the mutant.

SPR data demonstrated that K_d of A52 wild-type is slightly lower than the F154A mutant, and moreover association and dissociation constants are obviously changed. This data indicated that the interaction of A52 with TRAF6 became slightly weakened by F154A mutation.

These studies indicated that the F154A mutation has a significant effect on α -helicity and dimer formation of A52. The instability of the dimer likely affects the interaction of A52 with TRAF6. It is also important to consider that protein K7 is a monomer in solution and also interacts with TRAF6. In the case of the F154A mutant, the structural defect may be propagated to the TRAF6 binding site, and although binding still occurs, it appears to be unstable – consistent with qualitative cellular pulldown assays.

Further investigation of the mechanism will be required, and in particular, a mutation that results in fully monomeric A52 without affecting TRAF6 binding/signaling would be useful to differentiate dimer formation from signaling. It is

possible that the TRAF6 binding site is very close the dimeric interface, and therefore the F154A mutation may have complicated effects on the structure and signaling. X-ray and/or NMR studies of K7/TRAF6 and A52/TRAF6 will be necessary to determine whether a common site on the Bcl-2 proteins is exploited for complex formation.

Chapter 6. Final discussion

Viruses have acquired an arsenal to facilitate entry into the host and the modulation of host proteins has been optimized through evolution to attack critical points in our immune system. Therefore, the study of interactions between viral proteins and cellular targets provides us with a clear insight into both the immune system, and mechanisms of viral subversion.

Since the innate immune system provides us with the first line of protection, we remain unaware of the majority of pathogens that we encounter in daily life. Pattern recognition receptors, which detect pathogen-associated molecular patterns, are one of the main components of the innate immune system and the signals from PRRs induce the production of IFNs, pro-inflammatory cytokines, and chemokines to prevent the infection or proliferation of pathogens. To date, four types of PRRs including TLRs, RLHs, NLRs, and CLRs, have been characterized. Each PRR detects distinct PAMPs and form the defense line against particular pathogens. Despite their diversity, PRR signals converge upon common mediators of the downstream cascade which alter the gene expression of immune modulators.

Since PRRs are a dependable sentinel within cells, they remain critical obstacles to be overcome for invading pathogens. Viruses are armed with different types of proteins which prevent the activation of the anti-viral responses with a variety of strategies antagonizing different stages of signal transduction pathways. For example, VP35 of the Ebola virus caps the viral RNA which sequesters it from the detection by RIG-I (286) and also vOTU of the Crimean Congo hemorrhagic fever virus functions as a deubiquitinase preventing the signal transduction via ubiquitination (287).

Many proteins are expressed during infection by VACV and each protein is utilized in distinct ways. Bcl-2 fold proteins, A52, K7, N1, B14, and probably A46, have the same fold despite their low sequence similarity and each have distinct cellular targets. Cellular Bcl-2 fold proteins function as regulators of apoptosis, but several Bcl-2 fold viral proteins have roles in antagonizing immune signaling pathways. Phylogenetic analysis (Fig. 6.1) reveals that viral and cellular Bcl-2 family proteins may share the same origin. Vaccinia virus protein N1, which has an anti-apoptotic function, is more related to the cellular Bcl-2 family proteins. Alignment of viral Bcl-2 fold domains show that $\alpha 1$, $\alpha 2$, and $\alpha 5$ helix sequences are relatively well conserved (Fig.

6.2). Especially, conserved is the hydrophobic region of $\alpha 5$ in the core for the Bcl-2 fold. Vaccinia virus N1 protein interacts with pro-apoptotic cellular proteins *via* the BH-3 groove consisting of helices $\alpha 2$, $\alpha 3$, $\alpha 4$, and $\alpha 5$. The 'open' conformation of this groove is significant for binding to the BH3 helix (207). Several Bcl-2 fold proteins such as K7, A52, and B15 has non-apoptotic function because the BH3 groove is 'closed' for binding to the BH3 helix (227,228). Moreover, sequence alignments show that characteristic charged residues of N1 (Asp38, Arg58, Lys70, and Arg72), which surround the hydrophobic BH3 groove, contribute to the interaction with the BH3 helix (230), and these residues are poorly conserved in non-apoptotic proteins. Therefore, non-apoptotic Bcl-2 fold proteins are expected to interact with their cellular targets *via* a different mechanism.

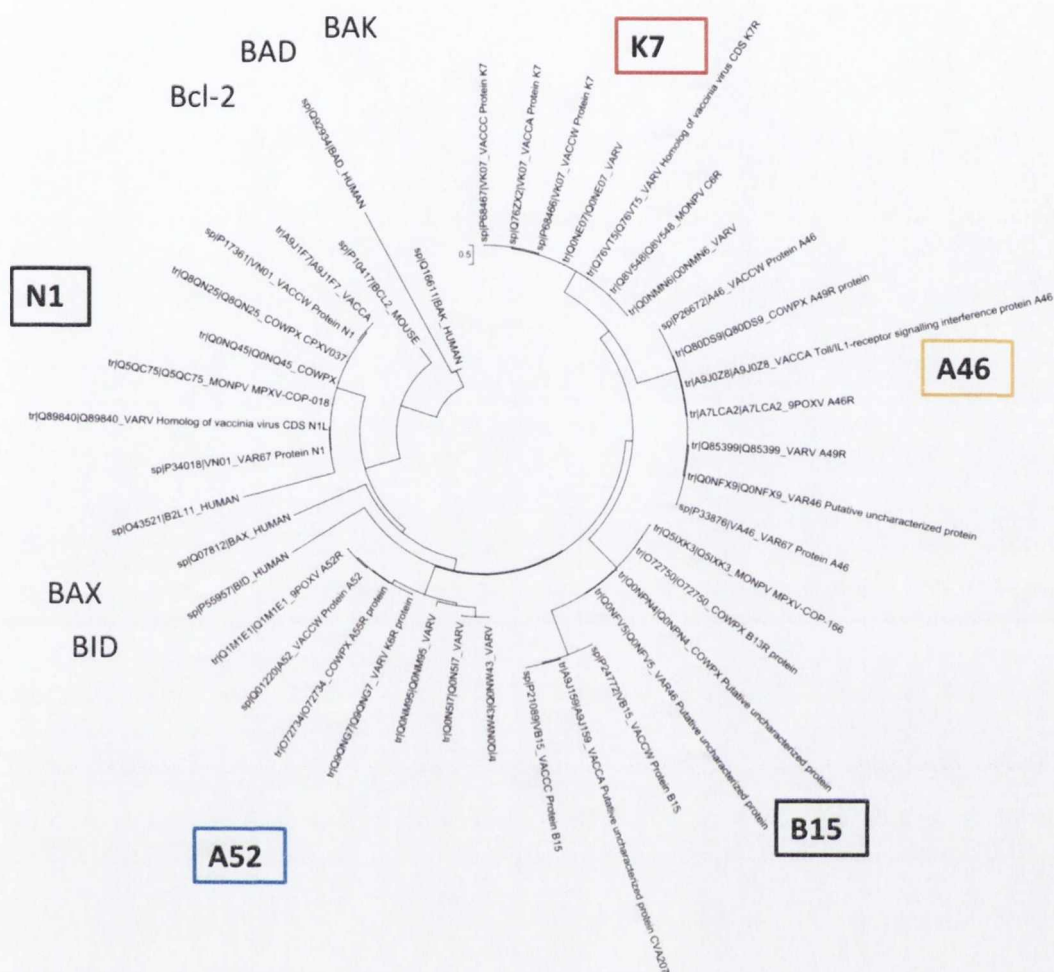


Figure 6.1. Phylogenetic tree of cellular and viral Bcl-2 fold proteins. Sequences of BAK, BAD, Bcl-2, BAX, BID, N1, A52, B15, K7, and A46 were aligned by Muscle (Edgar et al., 2004), and phylogenetic tree was generated by MEGA using maximum likelihood algorithm (Tamura et al., 2010). Bcl-2 family proteins share the origin and N1 is more related to the cellular Bcl-2 fold proteins than other viral Bcl-2 fold proteins. Accession numbers for each protein are presented in the branch identifiers.

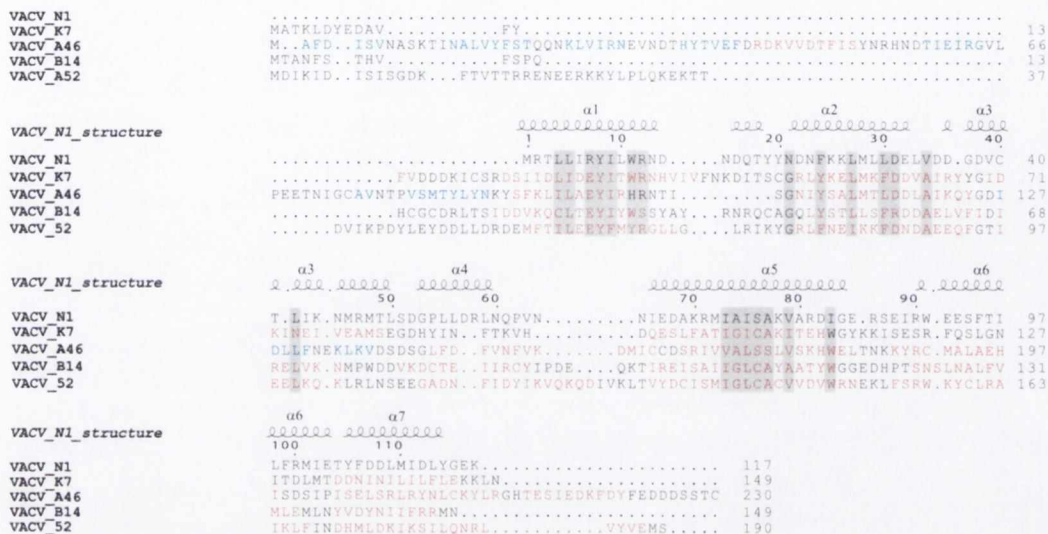


Figure 6.2. Sequence alignment of viral Bcl-2 fold proteins.

Amino acid sequences were aligned by Promals (Pei et al., 2007) and the alignment diagram was generated by ESPrpt (Gouet et al., 2003). Secondary structure elements aligned just above the sequence is based on the crystal structure of N1 (PDBcode: 2UXE). Texts colored red and blue correspond the helices and sheets, respectively, in the crystal structure except for A46. Only A46 is based on the secondary structure prediction.



Figure 6.3. Sequence alignment of TIR domains.

Amino acid sequence alignment of TIR domain of TLRs and TIR domain containing adaptors are aligned by Promals (Pei et al. 2007) and the alignment diagram was generated by ESPrnt (Gouet et al., 2003). Secondary structure model depicted just above the sequence correspond to the helices and strands of the TIR domain of TLR2 (PDB code: 1O77). Texts colored red and blue correspond the helices and sheets, respectively, in the crystal structure except for Mal and TRAM which colors are based on the secondary structure prediction.

In this study, antagonization mechanisms of the proteins K7, A46, and A52 were characterized by studying protein-protein interactions between K7-DDX3, A46-Mal, and A52-TRAF6 from a structural and biophysical point of view.

Both K7 and A52 share cellular targets, IRAK2 and TRAF6 but only K7 interacts with DDX3 (85). The precise molecular mechanism of K7's ability to antagonize innate immune signaling was unclear. Previous studies showed that K7 adopts a Bcl-2 fold and it interacts with the DDX3 N-terminal region (residues 61 to 90) (228). These structural studies of K7 and DDX3 revealed that K7 interacts with DDX3's N-terminal unstructured region from residues 81 to 90. The crystal structure of K7 in complex with DDX3 peptide (residue 71 to 90) revealed that the key determinant of this interaction is a tandem repeat phenylalanine (residue Phe84 and Phe85). These two residues reside in the hydrophobic cleft of K7 which is created by the flexible $\alpha 6$ region. This helix adopts an α -helical structure in a small number of the NMR

ensembles. Structure superposition of NMR ensembles highlights the flexibility of this $\alpha 6$ region (Fig. 3.11). Gly126, which is positioned in the middle of the $\alpha 6$ helix region, is not conserved in other viral Bcl-2 family proteins, and may be one of the main reasons for the $\alpha 6$ flexibility in K7. Furthermore, the $\alpha 4$ helix region of K7 does not adopt an α -helical structure in the NMR and X-ray structures. In the other viral Bcl-2 protein $\alpha 4$ and $\alpha 6$ are held in place by each other through antiparallel interactions. In K7, the $\alpha 4$ helix resembles more a twisted loop because residues in this K7 $\alpha 4$ region are fewer than the other Bcl-2 fold viral proteins. Thus, the non-helical structure of $\alpha 4$ is one of another reason for the instability of $\alpha 6$ in K7. K7 is a monomer in solution but the other Bcl-2 fold proteins form dimer and dimer interface of them consists of the N-terminus, $\alpha 1$, and $\alpha 6$, which is conserved. The binding pocket for DDX3 is stretched over the canonical dimer interface of the other Bcl-2 fold proteins. Interestingly, residues making up the hydrophobic cleft of K7 are Ile30, Ile34, Phe89, Ile102, Thr109, Trp112, Phe122, Ile128, and Leu131 are largely conserved. Thus, both the non-helical tendency of the $\alpha 6$ region and monomeric character of the K7 protein are important in ensuring the successful interaction of K7 with DDX3. Mutation of the two DDX3 phenylalanine results in the complete loss of its binding ability to K7 *in vitro* and *in vivo*, and failure of IRF3 activation and *ifnb* gene induction *in vivo*. Since a phosphorylation site is predicted around this tandem phenylalanine motif, TBK1 and/or IKK ϵ might interact with DDX3 around this motif. K7 may inhibit DDX3 function by binding to DDX3 and thus preventing phosphorylation.

The C-terminal domain of A46 aligned well with other Bcl-2 fold domains (Fig. 6.2). Since residues in $\alpha 1$, $\alpha 2$ and $\alpha 5$ which are well conserved in the other Bcl-2 fold viral proteins are conserved in the A46 sequence, the previous studies, which propose that A46 has a Bcl-2 fold, sound reasonable (Fig. 6.2) (182). In this study, experiments on A46 and Mal revealed that the putative Bcl-2 fold domain of A46 interacts with Mal. A46 forms a dimer, but Mal and TRAM form monomers in solution. Since previous studies showed that TRAM form homo-dimers, its dimerization may be buffer or condition dependent (193). Although homotypic TIR domain dimer interaction of TIR domain of any TLRs were not observed in solution, the BB-loop which connects the βB β -sheet and αB α -helix is involved in the dimer interface in the crystal structure of TLR-10 (19). A proline residue, corresponding to Pro680 in I077 sequence in Fig 6.3, on the BB-loop is conserved among the most of the TIR domain. Mutation of this conserved proline residue, or Ile679 at the BB-loop of TIR-domain of TLR2 impairs the signal transduction (288,289). Thus, the BB-loop is thought to be essential for

homotypic interactions of TIR domains of TLRs. The DD-loop which connects the β D sheet and the α D helix has also been implicated and shown to be important in homotypic interactions by mutation experiments and simulation studies (289). In the case of the TIR-domain containing adaptor protein, Mal, mutation studies of Pro125 which corresponds to the conserved proline, located at the BB-loop, failed to co-immunoprecipitate with TLR4 (186,188). Several natural variants of Mal were reported and D96N and S180L affect the function of Mal (290). Thus, the BB-loop of Mal is most likely important for binding to the TIR-domain of TLR4. The other natural variant of Mal, D96N, loses function by loss of interaction with the downstream adaptor protein, MyD88 (290). Three dimensional structure prediction studies reveal that Asp96 of Mal resides on the negatively charged surface which is the predicted MyD88/Mal interface (290). In the case of TRAM, mutation studies showed that the BB-loop of TRAM is also important for signal transduction (193,195). TRAM contains a cysteine residue following the conserved proline at the BB-loop in comparison to other TIR-domains (Fig. 6.3). A cysteine to histidine mutation impairs the signal transduction *via* TRAM (195,264). Cys to His mutant retains its binding ability to TLR4 but not to TRIF. Therefore, the BB-loop of TRAM may not to be used for binding to TLR4. Oshiumi et al., also showed that TRAM forms a homodimer and the Cys to His mutant fails to form a homodimer (193). In our *in vitro* studies, both Mal and TRAM behaved as monomers in solution. Biophysical experiments showed that A46 forms a homodimer in solution and binds to Mal *in vitro*. A46 probably inhibits the signal transduction *via* Mal and TRAM by binding to the TIR domain interface of TLR or TIR domain of downstream signaling adaptors. The VIPER peptide which is derived from α 1 of the putative Bcl-2 fold domain of A46 functioned as an antagonist of TLR4 signaling in preceding studies (183). However, the VIPER peptide has no observable interaction with Mal in our *in vitro* studies. This contradiction may indicate that the nature of the interaction of A46 and Mal is possibly too weak *in vitro*. In a hypothetical three-dimensional model of A46, this peptide corresponds to one-half of the N-terminal helix of a Bcl-2 globular fold (residue 88-98). Our CD studies confirmed that the peptide was not α -helical in solution. Since the corresponding VIPER region of A46 protein is folded correctly, interaction between A46 protein and Mal can be observed in this study. Thus, presumably, the peptide may not be folded correctly and so cannot exert its full antagonistic potential. Additionally A46 forms a dimer, but the dimer interface remains uncharacterized. However, the canonical dimer interface of viral Bcl-2 fold protein would partially cover the VIPER peptide region. Thus, possible interactions of A46 with TIR-domain containing proteins are still mysterious and

intriguing. An A46 structure in complex with Mal and TRAM would be necessary to define the interaction. Some difficulties remain, but the control of solubility of sample by changing the surface charge would be the key for crystallization and solving the structure.

Previous studies showed that A52 binds to TRAF6 and IRAK2 (178). A52 binds to the TRAF domain of TRAF6 (178). Deletion mutants of A52 containing residues 1-144 failed to interact with TRAF6 but not IRAK2 (178). Structural studies of A52 showed that it adopts the Bcl-2 fold (227). The elaborate binding mechanism of A52 to IRAK2 and TRAF6 was not understood. The crystal structure presented herein reveals that the C-terminal region is important for dimerization of A52 (Fig. 5.1, 5.2). Therefore, it can be expected that A52 (1-144) loses affinity for TRAF6 because A52 fails to dimerize, and the A52/TRAF6 binding interface encompasses the boundary of the A52 dimer. Cellular studies of our collaborator showed that F154A mutant of A52 partially lost the binding ability to TRAF6 and lost the MAPK activation function. The study of A52 and TRAF6 revealed that the F154A mutation affects the helical fold and consequently dimer formation of A52. Stability of dimer may affect the interaction of A52 with TRAF6. However, considering protein K7, which has 25% homology with A52 and is a monomer in solution, also interacts with TRAF6, disruption of the helical structure might affect the interaction.

Viral Bcl-2 proteins adopt a Bcl-2 fold despite having low homology and they have distinct cellular targets. Sequence alignment of viral Bcl-2 fold proteins showed that residues in $\alpha 1$, $\alpha 2$, and $\alpha 5$ helix region are relatively conserved in each proteins. It may be worth noting at this point that although these viral proteins have a Bcl-2 fold, specific host targets may be governed by residues outside of the fold. Since structures of Bcl-2 fold vaccinia virus proteins in complex with their cellular targets have not been solved with the exception of K7-DDX3, it is still difficult to depict a universal picture for their inhibition of innate immune signaling. Therefore, further investigations of protein-protein complexes of viral Bcl-2 fold proteins and their cellular target would be beneficial.

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