MECHANISMS UNDERLYING SUPPRESSION OF INTERFERONα-INDUCED ANTI-VIRAL RESPONSES BY THE ACTIVATED Ras/Raf/MEK PATHWAY

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Mechanisms Underlying Suppression of Interferon α-Induced Anti-Viral Responses by the Activated Ras/Raf/MEK Pathway

By

Thaddeus William Collier

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Abstract

Interferon α (IFN) is important for anti-viral defense. IFN binds to cell surface receptors and stimulates the Janus kinase-signal transducer and activator of transcription (Jak/STAT) pathway which activates transcription of anti-viral effector genes 2',5'-oligoadenylate synthetase (OAS), myxovirus resistance gene 1 (Mx1), and double-stranded RNA-dependent protein kinase (PKR). Upon virus infection, viral double stranded RNA (dsRNA) activates the OAS/latent ribonuclease (RNase L) pathway which degrades viral RNA whereas the PKR/eIF2α pathway blocks translation of viral RNA. Mx1 has been shown to be involved in blockage of viral nucleocapsid transport or viral RNA synthesis. Previously, we demonstrated that activation of the Ras/Raf/MEK pathway inhibits IFN-induced anti-viral responses. The objective of this study was to determine how activated Ras/Raf/MEK pathway suppresses IFN-induced responses. First, we stimulated vector control NIH 3T3 cells (BABE) and Ras transformed NIH 3T3 cells (RasV12) with IFN and then examined mRNA expression levels of OAS, Mx1, and PKR mRNA at 6 and 12 hours after the stimulation. We found that OAS, Mx1, PKR mRNA expression were higher in BABE compared to RasV12. We were able to rescue Mx1 and OAS gene expression induced by IFN in RasV12 by treatment of U0126, a specific inhibitor of MEK. Similarly, western blot analysis revealed that OAS protein levels were restored in RasV12 stimulated with IFN using U0126 and RNA interference (RNAi) to Ras. To further determine how activated Ras/Raf/MEK pathway suppresses IFN-induced gene transcription, we investigated activation status of the Jak/STAT pathway in IFN stimulated BABE and RasV12 by Western blot analysis using antibodies

against phosphorylated STAT1, total STAT1, phosphorylated STAT2 and total STAT2. As a result, we observed higher phosphorylation and total levels of STAT1 and STAT2 in BABE compared to RasV12. When treated with U0126, phosphorylation of both STAT proteins was restored in RasV12. These results suggest that activated Ras/Raf/MEK pathway inhibits activation of the JAK/STAT pathway leading to suppression of IFN-induced gene transcription.

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Also, I extend my thanks to all my friends who during my masters supported me and provided much appreciated laughter.

Finally, I thank my parents Bill and Shirley, my brother Aaron and his family Jill, Robyn, and Liam who always inspired me to push further and attain my goals.

List of Abbreviations

2'5'-OAS – 2'5'-oligoadenylate synthetases

2-5A – 2'5'-oligoadenylates

ADAR – RNA-specific adenosine deaminase

BPB – Bromophenol blue

CARD – N-terminal caspase recruitment domain

CD – Cluster of differentiation

cDNA – Complementary DNA

CIS – Cytokine-inducible SH2 domain-containing protein

c-Myc – Myelocytomatosis viral oncogene homolog

CPE – Cytopathic effects

CpG – Cytosine-phosphate-guanine

CREB – cAMP response element-binding protein

CRM – Chromosome region maintenance

dsRNA – Double-stranded RNA

eIF2α – Eukaryotic translation initiation factor 2α

ELK – Ets-like protein

ERK – Extracellular signal-regulated kinase

ETS – E26 transformation-specific

FBS – Fetal bovine serum

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

GAS – Gamma activated site

GDP – Guanosine diphosphate

GEF – Guanine nucleotide exchange factor

GTP – Guanosine triphosphate

HSV – Herpes simplex virus

IFN – Interferon

IFNAR – Interferon alpha receptor

IFNGR – Interferon gamma receptor

IgG – Immunoglobulin G

IKK – IκB kinase

IL – Interleukin

iNOS – Inducible nitric oxide synthase

ISG – Interferon-stimulated gene

ISGF3 – Interferon stimulated gene factor 3

ISRE – Interferon stimulated response element

IRF – Interferon regulatory factor

Jak1 – Janus kinase 1

Jak-STAT – Janus kinase and signal transducer and activator of transcription

LRR – Leucine rich repeat

MAVS – Mitochondrial anti-viral signalling protein

MEF – Mouse embryonic fibroblast

MEK – Mitogen-activated protein/extracellular signal-regulated kinase kinase

MHC – Major histocompatibility complex

MyD88 – Myeloid differentiation primary response gene 88

Mx – Myxovirus resistance gene

NK – Natural killer

PBS – Phosphate buffered saline

PIAS – Protein inhibitors of activated STATs

PKC – Protein kinase C

PKR – Double stranded RNA-dependent protein kinase

PMSF – Phenylmethylsulfonyl fluoride

P-STAT – Phosphorylated-STAT

RasGAP – p21GTPase-activating protein

RIGI – Retinoic acid gene I

RIPA – Radioimmunoprecipitation assay

RNAi – RNA interference

RNase L – Latent ribonuclease

RT-PCR – Reverse transcription polymerase chain reaction

SDS-PAGE – Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SH2 – Src-homology 2

SHP – SH2-containing phosphatase

SOCS – Suppressors of cytokine signalling

ssRNA – Single strand RNA

STAT – Signal transducer and activator of transcription

TBS – Tris-buffered saline

T-ERK – Total extracellular signal-regulated kinase

TIR – Toll/IL-1 receptor

TKB – TANK-binding kinase

TLR – Toll-like receptor

TRIF – TIR-domain-containing adaptor-inducing IFN

T-STAT – Total STAT

Tyk2 – Tyrosine kinase

VSV – Vesicular stomatitis virus

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Chapter 1: Introduction

1.1 *Interferons*

The interferon (IFN) system is important for early anti-viral defense (Samuel, 2001). When infected by viruses, cells respond by releasing IFNs which bind to cellular receptors and activate an intracellular anti-viral state to protect the host from viral infection. Discovered in 1957, IFNs were given their name due to their ability to interfere with viral replication (Issacs and Lindenmann, 1957). Since their discovery, IFNs have been shown to be involved in several cellular functions other than anti-viral defense including proliferation and activation of immune cells. Classification of IFNs is based on the surface receptors which they interact with and on sequence homology (Malmgaard, 2004). Within the IFN superfamily, IFNs are classified into three different types, type I, II, or III.

1.1.1 *Type I IFNs*

Type I IFNs consist of a number of different members including IFNα, IFNβ, IFNτ, and IFNω. In humans, all type I IFN genes are located on chromosome 9 whereas in mice, these genes are clustered on chromosome 4 (Samuel, 2001). There are several different subtypes of IFNα and IFNτ whereas IFNβ and IFNω are both unique cytokines each encoded by a single gene. All cell types possess the ability to produce type I IFNs in response to viral infection. In virus infected cells, the ratio of IFNα, IFNβ, and IFNω production depends upon several factors such as host cell type, the species of organism, and type of virus (Pestka *et al*, 2004). All type I IFNs bind with the same surface

receptor, IFNα receptor (IFNAR), which is comprised of two subunits IFNAR1 and IFNAR2, to mediate a wide breadth of cellular functions (Samuel, 2001). The biological activities of type I IFNs include anti-viral defense (Van den Brock *et al*, 1995), inhibition of proliferation (Clemens, 2003), activation of natural killer cells and lymphocytes (Ortaldo *et al*, 1983), regulation of major histocompatibility complex (MHC) class I antigen expression (Dolei *et al*, 1983), and suppression of angiogenesis (Kerbel and Folkman, 2002).

1.1.2 *Type II IFN*

Type II IFN consists of only one member, IFNγ, which is a cytokine also important for mediation of anti-viral responses (Costa-Pereira *et al*, 2002). Production of IFNγ occurs during later stages of viral infection where it also has an important role in regulating adaptive immune responses (Pestka *et al*, 2004). IFNγ initiates cellular signalling events by binding with surface receptors interferon γ receptor 1 (IFNGR1) and 2 (IFNGR2). IFNγ is involved in upregulating the transcription of about 1300 genes whereas IFNα and β regulate transcriptional control of 100-300 genes (MacMicking, 2004). Only certain immune cells are able to synthesize IFNγ, including natural killer (NK) cells, CD8 and CD4 cells (Costa-Pereira *et al*, 2002). Effector functions of IFNγ include differentiation of CD4 cells, macrophage activation, and anti-viral defense (Pestka *et al*, 2004).

1.1.3 *Type III IFN*

The type III interferon (IFN) family consists of IFN-λ1, -λ2 and -λ3 which are also referred to as interleukin-29 (IL-29), IL-28A, and IL-28B respectively. Although type III IFNs bind to a unique receptor, they share many functional characteristics with type I IFNs. Both type I and type III IFNs are induced by viral double-stranded RNA, transduce signals to the nucleus via the Janus kinase-signal transducer and activator of transcription (Jak-STAT) pathway, activate interferon stimulated response element (ISRE) regulated gene expression and upregulate MHC class I antigen expression. In addition, cells treated with type III IFNs are resistant to viral infection (Kotenko *et al*, 2003; Sheppard *et al*, 2003). A recent study has demonstrated that type III IFNs also possess potent anti-viral and immunostimulatory activity *in vivo* (Bartlett *et al*, 2005).

1.2 *Induction and transcriptional control of type I IFNs*

The mechanism of induction of type I IFNs during viral infection is a complex molecular system involving both viral components and host cell proteins. Two major pathways which regulate induction of IFN include (1) retinoic acid gene I (RIGI)-dependant pathway and (2) Toll-like receptor (TLR)-dependent pathway.

1.2.1 *The RIGI-dependent pathway*

RIGI is an intracellular receptor capable of binding to viral double stranded (dsRNA) or synthetic dsRNA (poly (I:C)), and has recently been identified as an important inducer of IFNα and β (Figure 1) (Yoneyama *et al*, 2004). RIGI consists of a

C-terminal RNA helicase domain which is capable of binding with dsRNA and an N-terminal caspase recruitment domain (CARD). Interaction of the helicase domain with viral dsRNA induces a conformational change of RIGI that promotes interactions between RIGI and CARD and downstream CARD-containing adaptor proteins (Honda *et* al, 2005). Recent studies have identified mitochondrial anti-viral signalling protein (MAVS) as an important downstream adaptor protein in RIGI signalling (Seth *et al*, 2005). Upon activation by RIGI, MAVS phosphorylates TANK-binding kinase 1 (TBK1), and inducible IκB kinase (IKKε/*i)* which in turn phosphorylate interferon regulatory factor 3 (IRF3) and 7 (IRF7). Once activated, IRF3 and IRF7 translocate into the nucleus to activate transcription of IFNα and IFNβ (Takaoka and Yanai, 2006). The regulatory role of RIGI in the induction of IFN was confirmed in mouse knockout studies in which loss of RIGI resulted in inhibition of IRF3 activation (Kato *et al*, 2005).

IRF3 and IRF7 are constitutively expressed and in their inactive forms, are localized within the cytoplasm (Au *et al*, 1995). Upon phosphorylation, IRF3 dimerizes and translocates into the nucleus where it forms a complex with CRE-binding protein (CREB) and p300 to activate transcription of target genes (Juang *et al*, 1998). Dimerization of IRF7 also occurs upon its phosphorylation, followed by translocation into the nucleus and binding to promoter elements of the IFN genes. However, it has been shown that IRF3 activation is more important for transcription of IFNβ rather than IFNα genes, whereas IRF7 causes activation of both IFNα and IFNβ (Sato *et al*, 2000). IRF5 is also involved in both the induction and suppression of IFN expression. As with IRF3 and

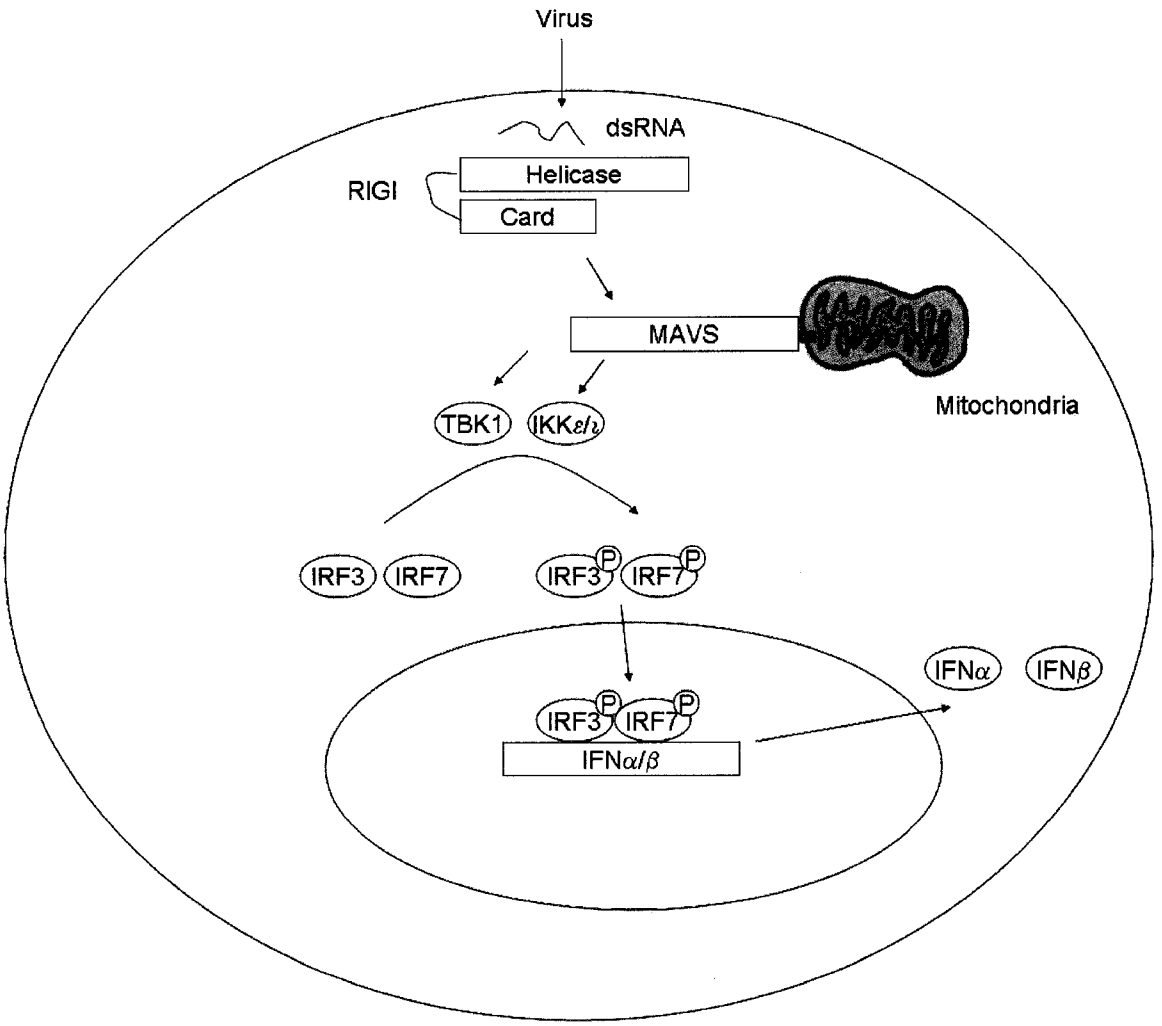


Figure 1. Induction of IFNα/β via the RIGI-dependent pathway. The C-terminal helicase domain of RIGI binds intracellular viral dsRNA to activate the N-terminal CARD domains, leading to the activation of downstream MAVS. Once activated, MAVS activates IRF kinases TBK1 and IKKε/ι which, in turn, phosphorylate IRF3 and IRF7. Phosphorylated IRF3 and IRF7 translocate into the nucleus to stimulate transcription of IFNα and IFNβ genes by binding to their promoters.

IRF7, IRF5 is activated via phosphorylation, after which the activated IRF5 translocates to the nucleus to activate transcription of the IFN genes (Malmgaard, 2004). Interestingly, IRF5 can also downregulate IFN gene transcription by binding to IRF7, thereby preventing IRF7 from binding to the IFN promoters (Barnes *et al*, 2003).

1.2.2 The TLR-dependent pathway

Another important discovery in recent IFN research is that activation of TLRs leads to the induction of IFNα and IFNβ. In mammals, there are 11 identified TLRs, of which TLR3, 7, 8, and 9 are involved in modulating anti-viral responses (Figure 2) (Akira and Takeda, 2005). The TLRs are capable of responding to various viral determinants at domains containing leucine rich repeats (LRR). TLR3 recognizes viral dsRNA whereas viral single stranded RNA (ssRNA) binds with TLR7 and TLR8, and TLR9 associates with unmethylated cytosine-phosphate-guanine (CpG) DNA from DNA viruses (Seth *et al*, 2006). TLRs contain a cytoplasmic toll/IL-1 receptor (TIR) domain which functions as the docking site for downstream adaptor proteins (Seth *et al*, 2006). With the exception of TLR3 (whose adaptor protein is TIR-domain-containing adaptor-inducing IFN (TRIF)), myeloid differentiation primary response gene 88 (MyD88) serves as an adaptor protein for all TLRs to activate downstream TBK1 and IKKβ/i (Takaoka and Yanai, 2006). Upon activation, these kinases phosphorylate IRF3 and IRF7 which translocate into the nucleus to stimulate IFN gene transcription.

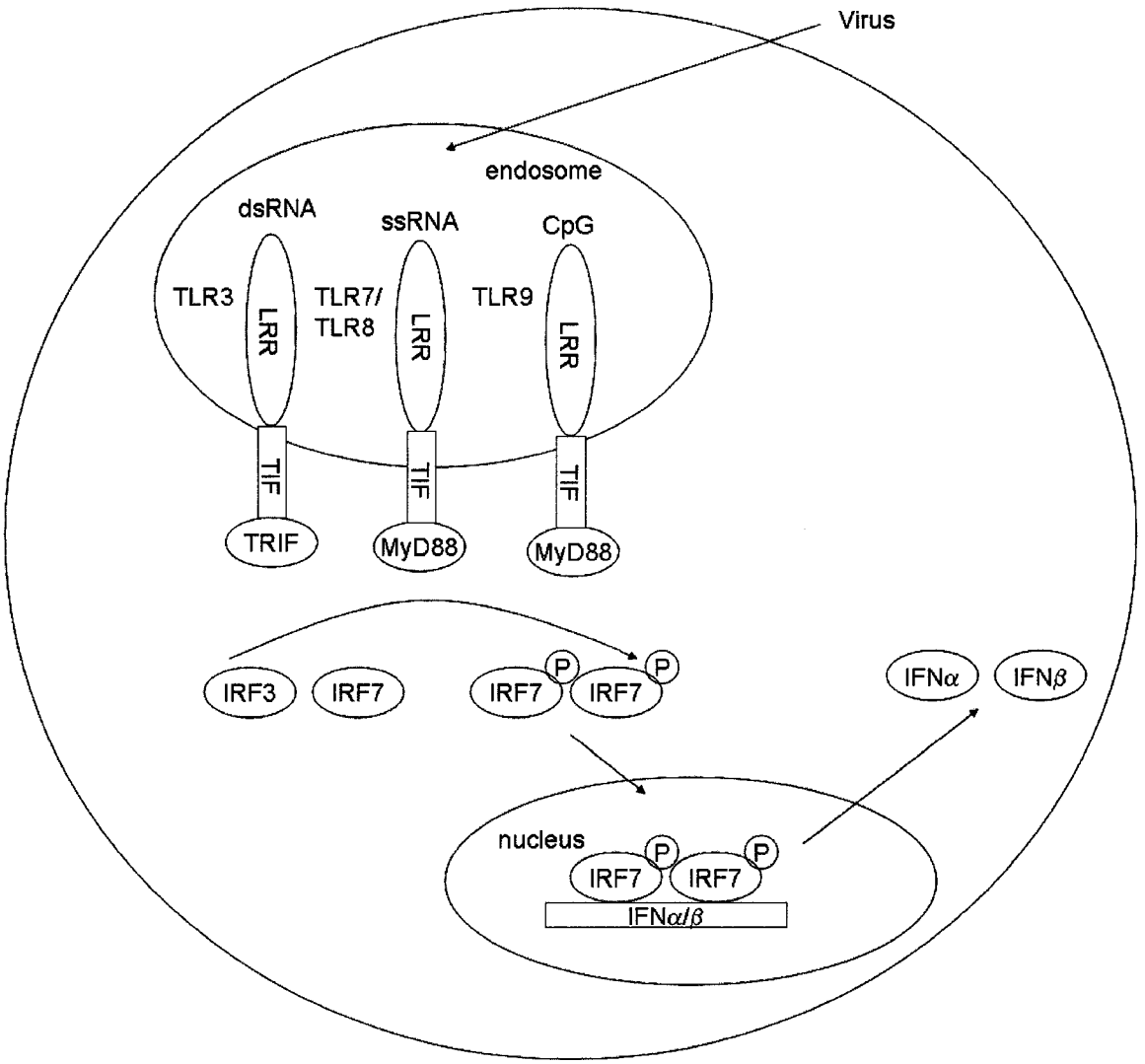


Figure 2. Induction of IFNα/β via the TLR-dependent pathway. Products generated during viral replication (dsRNA, ssRNA, and CpG) bind to LRR domains of TLRs. TLR3 recognizes viral dsRNA, TLR7 and TLR8 bind with ssRNA, and TLR9 interacts with CpG DNA. Upon recognition of dsRNA, TLR3 activates downstream adaptor protein TRIF whereas MyD88 is the downstream target of TLR7, TLR8, and TLR9. Once activated, these adaptor proteins activate TBK1 and IKKβ/i which phosphorylate IRF3 and IRF7 which then translocate into the nucleus and activate transcription of IFNα and IFNβ.

1.3 *Jak-STAT signalling pathway*

The Jak-STAT signalling pathway is a well characterized signalling cascade which is activated by IFNs and cytokines (Figure 3). IFNα binds to surface receptors IFNAR1 and IFNAR2 (Samuel, 2001). This binding leads to heterodimerization of the two receptors and subsequent apposition of two tyrosine kinases, Janus kinase 1 (Jak1) and tyrosine kinase 2 (Tyk2) which are associated with the intracellular domains of IFNAR (Kisselvela *et al*, 2002). After IFN binds with the receptor, Jak1 and Tyk2 phosphorylate each other to become activated (Aaronson and Horvath, 2002). Phosphorylated Jak1 and Tyk2 phosphorylate downstream transcriptional factors localized within the cytoplasm, signal transducer and activator of transcription 1 (STAT1) and 2 (STAT2) (Darnell *et al*, 1994). Phosphorylated STATs dimerize with one another to form a trimeric complex termed interferon stimulated gene factor 3 (ISGF3) along with a DNA binding protein termed IRF9. ISGF3 then translocates into the nucleus where it binds to specific promoter elements of IFN-inducible genes termed ISRE with the consensus sequence $AGTTT(N)_3TTTC$ (Aaronson and Horvath, 2002). Binding of ISGF3 to the ISRE sites activates gene transcription of the IFN-inducible genes. After gene transcription, the STATs become dephosphorylated and are exported back into the cytoplasm to be recycled for the Jak-STAT pathway (Kisseleva *et al*, 2002; McBride *et al*, 2000).

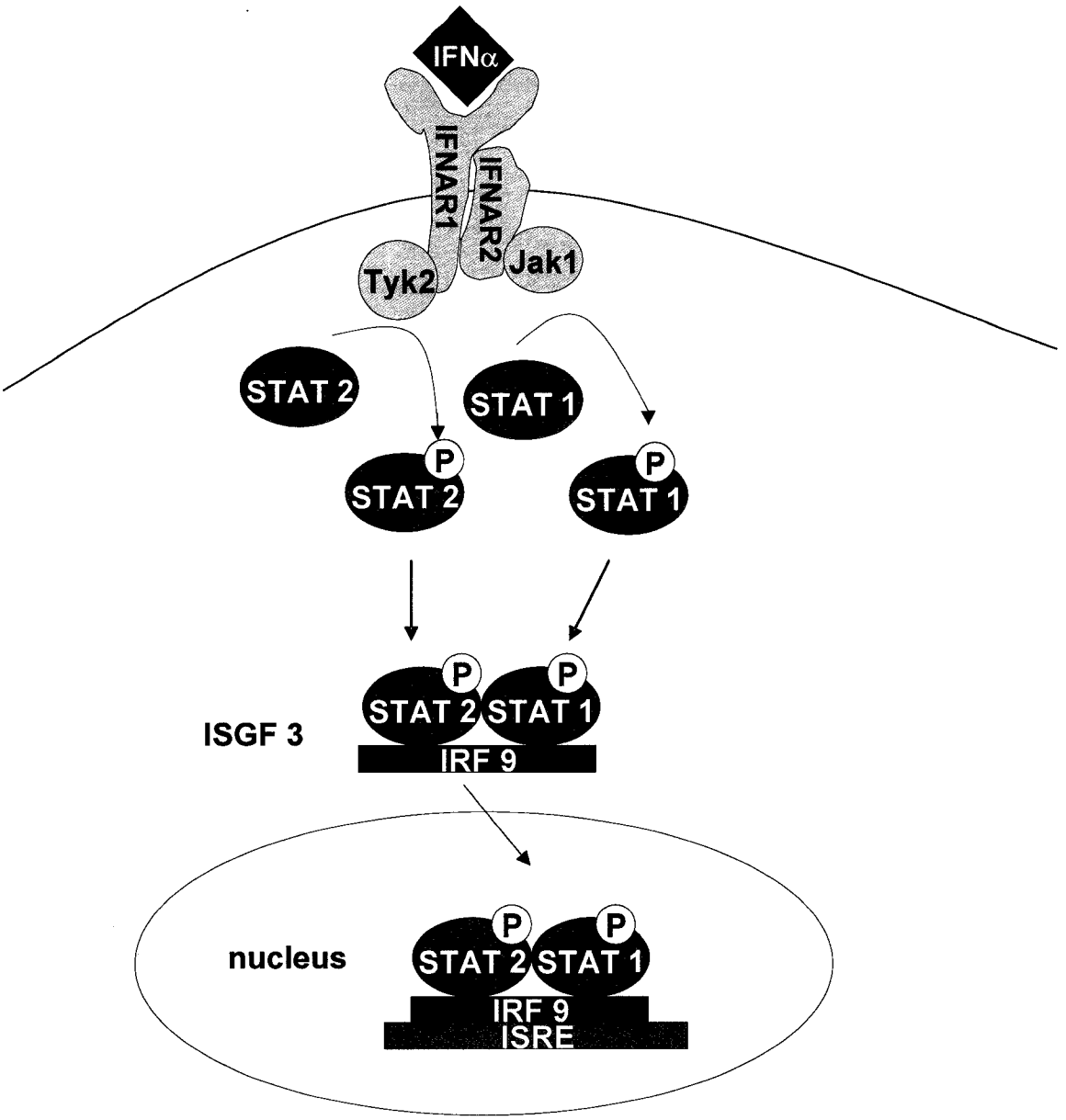


Figure 3. Jak-STAT pathway. IFNα binds to surface receptors IFNAR1 and IFNAR2 leading to the activation of receptor associated kinases, Tyk2 and Jak1. Upon activation, Jak1 and Tyk2 phosphorylate STAT2 and STAT1 which dimerize and form a trimeric complex with IRF9 termed ISGF3. ISGF3 then translocates into the nucleus and binds to ISRE sites to activate the transcription of IFN-inducible genes.

Unlike the type I IFN pathway, IFNγ binds with IFNγ receptor (IFNGR) which consists of two subunits IFNGR1 and IFNGR2 leading to the activation of Jak1 and Jak2. Activated Jak1 and Jak2 phosphorylate cytoplasmic STAT1 which dimerizes and translocates to the nucleus. After entering the nucleus, the STAT1 dimer binds at gamma activated sites (GAS) of DNA which possess the consensus sequence $TTC(N)_{3-4}GAA$ to promote transcription of IFN-inducible genes. (Aaronson and Horvath, 2002)

1.4 *IFN-inducible anti-viral genes*

Three anti-viral genes induced by IFN include 2'5' –oligoadenylate synthetase (OAS), dsRNA-activated protein kinase (PKR), and myxovirus resistance gene (Mx1) (Figure 4) (Samuel, 2001). Each of these genes contains an ISRE promoter element which can be recognized by ISGF3. Studies have shown that in mouse embryonic fibroblasts (MEF) with knockouts of latent ribonuclease (RNase L), PKR, and Mx1 are still able to resist viral infection in the presence of IFN, indicating that they are not the only proteins responsible for IFN-induced anti-viral defense (Samuel, 2001). Other IFN-inducible genes include RNA-specific adenosine deaminase (ADAR), inducible nitric oxide synthase (iNOS), and MHC proteins all of which inhibit different stages of the viral replicative cycle. ADAR inhibits viral infection by editing viral RNA transcripts (Samuel, 2001). Nitric oxide produced from iNOS mediates the killing of cells infected by a virus (Michel and Feron, 1997). MHC proteins contribute to anti-viral defense by presenting viral antigens to T cells (Biron, 1998).

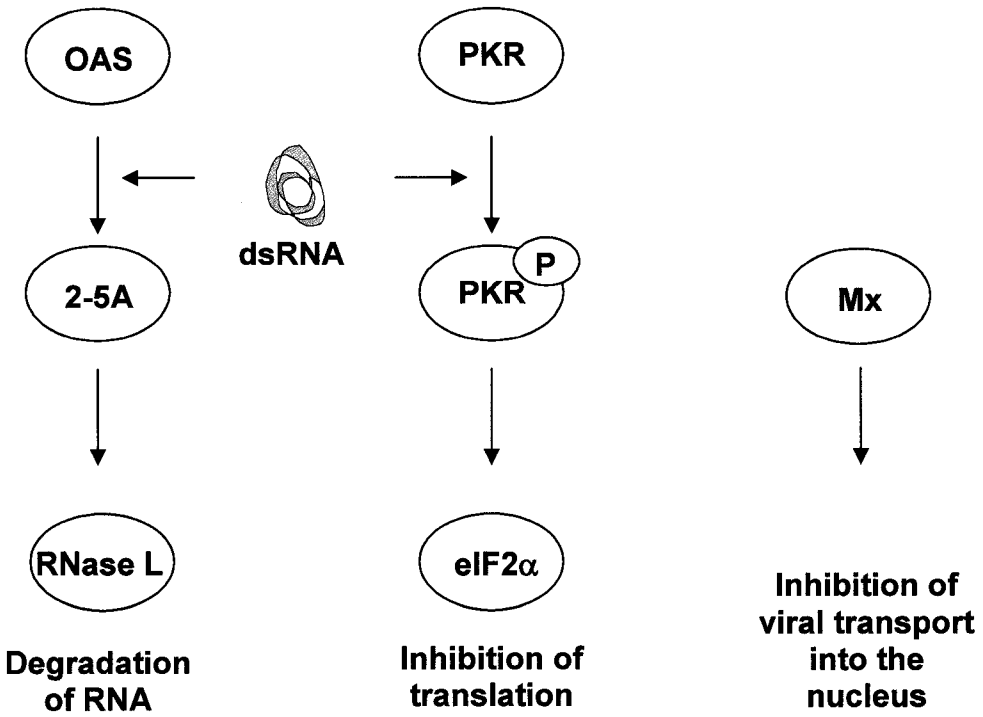


Figure 4. IFN-induced anti-viral proteins. Upon recognition of viral dsRNA in the cytoplasm, OAS synthesizes 2'-5'-oligoadenylates which activate the ribonuclease RNase L, leading to degradation of viral and host RNA. After binding with viral dsRNA, PKR becomes activated via autophosphorylation. Activated PKR phosphorylates eIF2α, resulting in inhibition of translation. Mx inhibits viral replication by blocking transport of viral nucleocapsid into the nucleus.

1.4.1 *OAS/RNase L*

OAS is a cytoplasmic enzyme which is activated by viral dsRNA (Samuel, 2001). dsRNA is a product of the replicative cycle of RNA viruses. dsRNA is also produced by the annealing of complementary RNA strands transcribed from some DNA viruses (Boone *et al*, 1979). Upon activation by dsRNA, OAS catalyzes the formation of 2-5A oligoadenylates (Kerr, 1987; Stark *et al*, 1998). The 2-5A oligoadenylate molecules then bind with the dormant ribonuclease RNase L. RNase L activity is dependent on the amount of 2-5A molecules produced (Li *et al*, 1998). Low levels of 2-5A results in selective degradation of viral mRNA whereas a higher level leads to cleavage of 18S and 28S ribosomal RNA (Li *et al*, 1998; Wreschner, 1981).

RNase L is an endonuclease which is constitutively expressed in most types of cells (Floyd-Smith and Denton, 1981). In its inactive form, RNase L is a monomer without endonuclease activity. Upon binding with 2-5A oligomers, RNase L dimerizes to become active. Activated RNase L cleaves both host and viral RNAs on the 3' side of the UpXp sequences (Floyd-Smith *et al,* 1981; Wreschner *et al,* 1981). The degradation of RNA results in suppression of both host and viral mRNA translation, leading to an inhibition of viral replication.

1.4.2 *PKR/eIF2α*

PKR is ubiquitously expressed and its expression can be further induced by IFN (Stark *et al*, 1998). Prior to IFN stimulation, PKR is predominantly localized to the cytoplasm

where it associates with ribosomes, with lower amounts localized within the nucleus (Thomis *et al*, 1992). After IFN stimulation, PKR levels in the cytoplasm increase whereas levels in the nucleus remain stable (Jeffery *et al*, 1995). During viral infection, PKR is activated by dsRNA generated from the process of viral replication (Kerr, 1987; Stark *et al*, 1998). PKR can associate with dsRNA duplexes ranging in length from 11 bp to an optimal size of 80 bp (Bevilacqua and Cech, 1996). Upon binding with dsRNA, PKR becomes activated through autophosphorylation (Samuel, 2001). Activated PKR catalyzes the addition of a phosphate group to the translation factor eukaryotic initiation factor 2α (eIF2α) (Samuel, 1979).

eIF2α is a subunit of larger complex eIF2. eIF2 contains three subunits α, β, and γ and functions in association with guanosine triphosphate (GTP) and methionyl-tRNA, forming the 43s preinitiation complex along with the 40s ribosomal subunit (Samuel, 2001). Once mRNA and the 60S subunits bind with the eIF2 complex, GTP is hydrolyzed to guanosine diphosphate (GDP). For translation to continue, GDP must be converted back to GTP by a reaction which is catalyzed by guanine nucleotide exchange factor (GEF) eIF2B. However, when the eIF2α subunit of eIF2 complex is phosphorylated, recycling of GDP to GTP by eIF2B is inhibited, which results in a translational block. Therefore, the phosphorylation of eIF2α by PKR induces translational blocks of both host and viral mRNA (Samuel, 2001).

1.4.3 *Mx*

The Mx proteins belong to a family of dynamin-like GTPases (Horisberger, 1992). The two different Mx proteins (Mx1 and Mx2) are localized to different regions of the cell with Mx1 residing in the nucleus and Mx2 within the cytosol (MacMicking, 2004). The anti-viral mechanisms of Mx are not clearly understood. There is evidence to suggest that Mx may be involved in inhibiting transport of viruses across the nuclear membrane (Haller *et al*, 1998; Pavlovic *et al*, 1993; Weber *et al*, 2000). Viruses which replicate within the nucleus, such as orthomyxoviruses and thogaviruses, are sensitive to Mx1 activity whereas cytosolic viruses such as bunyaviruses are susceptible to the effects of Mx2 (Haller and Koch, 2000).

1.5 *Therapeutic strategies for IFNα*

IFNα has been proven useful for the treatment of both viral diseases and different types of cancers. IFNα is widely used for treatment of hepatitis B and C virus (Mazzella *et al*, 1999; Pianko and Mchutchinson, 2000). IFNα has also been approved as a treatment for genital herpes caused by herpes simplex virus (HSV) (Ophir *et al*, 1995) and for rhinovirus (Gwaltney *et al*, 2002).

In addition to anti-viral activity, IFNα also exhibits potent anti-tumor activity (Gutterman, 1994). Cancers that are treated with IFNα include chronic myelogenous leukemia (Talpaz *et al*, 1987) and melanoma (Kirkwood et al, 2002).

Unfortunately, IFN therapy is not a completely effective form of therapy. For the treatment of chronic hepatitis C, patients given IFN treatment have a response rate of 54-

63% (Manns *et al*, 2001, Fried *et al*, 2002; Hadziyannis *et al*, 2004). Also, patients with high risk melanoma have also demonstrated resistance to IFN therapy (Fluck *et al*, 2005). In this study, the 2-year and 5-year relapse-free survival rates of patients treated with IFNα were 48% and 36% respectively. Identification of the mechanisms underlying IFN resistance is an important endeavor in developing more effective forms of therapy for patients.

1.6 *Molecular negative regulation of IFN-induced responses*

Prolonged activation of IFN-induced responses can have potent cytotoxic effects, leading to the development of asthma, cancer, and immunodeficiency (Schindler, 2002). Inhibition of IFN-induced responses involves three major families of negative regulators which are either constitutively expressed or are induced. These include src-homology 2 (SH2)-containing phosphatases (SHP), suppressors of cytokine signalling (SOCS), and protein inhibitor of activated STAT (PIAS) (Wormald and Hilton, 2004). Ras/Raf/MEK signalling pathway, which has been studied in our laboratory, is also involved in negative regulation of IFN-induced responses.

1.6.1 *SHP*

Two tyrosine phosphatases which have been implicated in the inhibition of IFN-induced responses include SHP1 and SHP2. Unlike SHP2 which is ubiquitously expressed, SHP1 is mainly expressed within hematopoietic cells but has also been detected in epithelial or smooth muscle cells (Banville *et al*, 1995; Valentino and Pierre, 2005). Both

phosphatases are constitutively expressed and predominately localized within the cytoplasm (Valentino and Pierre, 2005). Both SHP1 and SHP2 contain two N-terminal SH2 domains and a C-terminal protein-tyrosine phosphatase domain (Jones *et al*, 2004). The SH2 domains recognize phospho-tyrosine residues present on activated receptor associated kinases such as the Jaks. Deactivation of the Jaks leads to a loss of phosphorylation of STAT proteins, which in turn leads to decreased formation of ISGF3 and reduced transcription of IFN-inducible genes (Shuai and Liu, 2003).

SHP1 has been shown to negatively regulate the IFNα stimulated Jak/STAT pathway *in vivo* by selectively inactivating Jak1 (David *et al*, 1995). Another study demonstrated that IFNα-induced activation of Jak1, but not Tyk2, was enhanced in $SHP1^{-/-}$ macrophages underlying the importance of SHP1 in attenuating Jak activity (Costa-Pereira *et al*, 2002).

SHP2 serves diverse physiological roles by promoting mitogenic signals while also functioning as a negative regulator of IFN-induced growth-inhibitory and apoptotic pathways (Wormald and Hilton, 2003). SHP2 has also been implicated as a regulator of the Jak-STAT pathway. In $SHP2^{-/-}$ mouse embryonic fibroblasts (MEF), phosphorylation levels of STAT1 were increased in response to IFNα treatment when compared to wild-type cells suggesting that activation of STAT1 is dependent on SHP2 activity (You *et al*, 1999). A more recent study has shown that protein kinase C (PKC) and SHP2-dependent pathway inhibits IFNα-induced Jak-STAT signalling (Du *et al*, 2005).

1.6.2 *SOCS*

The SOCS family consists of 8 members: the cytokine-inducible SH2 domain-containing protein (CIS) and SOCS1 through SOCS7 (Shuai and Liu, 2003). Unlike the SHP and PIAS families which are constitutively expressed, expression of SOCS is induced by IFN Valentino and Pierre, 2005). SOCS family members possess a central SH2 domain that is flanked by a variable length amino-terminal domain and a novel conserved carboxyl-terminal motif termed the SOCS box (Starr and Hilton, 1998). Within the SOCS family, CIS, SOCS1, SOCS2, and SOCS3 have been shown to attenuate Jak-STAT pathway signalling (Wormald and Hilton, 2004). SOCS members inhibit Jak-STAT signalling by either: (1) binding to and inhibiting the activity of Jaks, (2) competing with STATs for phosphorylated binding sites on receptors, or (3) targeting bound signalling proteins for proteasomal degradation (Kamizono *et al*, 2001; Kile *et al*, 2002; Krebs and Hilton, 2000). Over expression of SOCS1 and SOCS3 have been shown to inhibit induction of OAS and Mx by both IFNα and type III IFNs (Vlotides *et al*, 2004; Brand *et al*, 2005).

1.6.3 *PIAS*

The mammalian PIAS family contains four members: PIAS1, PIAS3, PIASX, and PIASY (Shuai, 2000). Each of the PIAS members is capable of disrupting functionality of STAT proteins. PIAS1 and PIASY have been shown to inhibit STAT1-mediated gene activation by disrupting its ability to bind with DNA (Liu *et al*, 1998; Liu *et al*, 2001). PIASX and PIASY disrupt STAT1-dependent transcription without inhibiting the DNA-binding capability of STAT1 (Arora *et al,* 2003). The direct interaction between PIAS-STAT

occurs only after cytokine stimulation as PIAS proteins are unable to bind monomeric forms of the STAT proteins (Shaui and Liu, 2003).

1.6.4 *Activated Ras signaling pathways*

Ras is a membrane-bound guanosine triphosphate (GTP)-binding protein which is important for the regulation of several biological processes including proliferation, transformation, and differentiation (Campbell *et al*, 1998). Three isoforms of Ras have been identified including H-Ras, Ki-Ras, and N-Ras (Yan *et al*, 1998). Activation of Ras leads to the stimulation of a number of cellular signaling pathways. One of the downstream targets activated by Ras in a GTP-dependant manner is the serine/threonine protein kinase Raf which in turn leads to the phosphorylation of mitogen-activated protein/extracellular signal-regulated kinase kinase 1 (MEK1) and 2 (MEK2) (Campbell *et al*, 1998). When phosphorylated, MEK1 and MEK2 activate extracellular signal-regulated kinase 1 (ERK1) and ERK2, which regulate several transcriptional factors including E26 transformation-specific (Ets), Ets-like protein (Elk), and myelocytomatosis viral oncogene homolog (c-Myc) (Lewis *et al*, 1998).

Activated Ras is a negative regulator of the IFN-induced anti-viral state. When BALB/c-3T3 cells were transfected with viral oncogene (v-Ras), induction of MHC I by IFN was inhibited (Offermann and Faller, 1990). Inhibition of PKR by activated Ras was first reported by Mundschau and Faller (Mundschau and Faller, 1992). Activation of K-Ras suppressed GAS-mediated transcription of IFNγ stimulated genes by inhibiting

expression of STAT1/2 (Klampfer *et al*, 2003). However, the mechanism by which Ras suppresses the IFN pathway remains unknown.

1.7 *Mechanisms underlying abrogation of IFN-induced responses by activated Ras*

Studies conducted in this laboratory have demonstrated that activation of the Ras/Raf/MEK pathway suppresses IFN-induced anti-viral responses (Battcock *et al*, 2006). In this study, it was found that cells with activated Ras were not protected from viral infection by IFNα whereas the control cells containing normal Ras were protected by IFNα (Figure 6). IFN sensitivity of Ras activated cells was restored upon knockdown of downstream MEK with RNA interference (RNAi). Expression of PKR in IFN-stimulated control cells and Ras activated cells was similar suggesting that PKR levels are unaffected by activation of Ras/Raf/MEK. It is unknown which elements of the IFNα induced anti-viral pathway are targeted by the Ras/Raf/MEK pathway. In this study, we hypothesized that activated Ras/Raf/MEK pathway inhibits a component of the IFN pathway between activation of Jak-STAT pathway and induction of anti-viral proteins. Decreased levels of anti-viral proteins could decrease the ability of cells to resist viral infection. To identify the precise mechanism whereby activated Ras/Raf/MEK inhibits the IFN-induced anti-viral response, a systematic analysis of the IFN system including activation of IFN-inducible genes and of upstream Jak-STAT pathway was conducted in the present study.

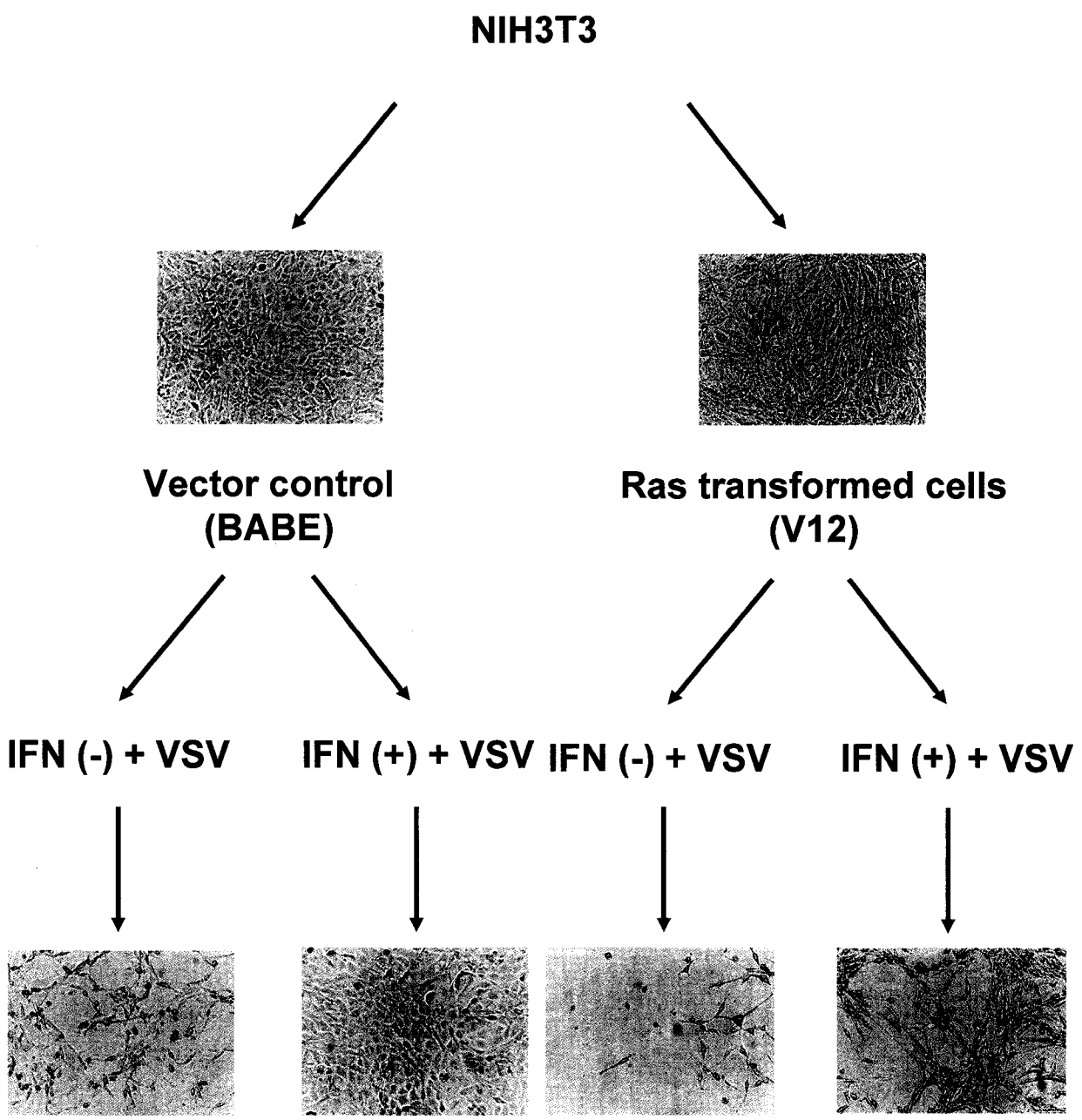


Figure 5. Activation of Ras suppresses IFN-induced anti-viral response. NIH 3T3 cells were transfected with either an empty vector control (BABE) or an vector encoding an activated Ras (V12). When challenged with VSV - Vesicular stomatitis virus (VSV) in the absence of IFN, both BABE and V12 were susceptible to infection as high levels of cytopathic effects (CPE) were observed. When treated with IFN (500UI/ml) prior to viral challenge, BABE was protected from infection as low levels of CPE were observed. Importantly, V12 was not protected from viral infection as high levels of CPE were observed after viral challenge.

1.8 *Objective*

The objective of this thesis was to determine which element of the IFN pathway is targeted by activated Ras/Raf/MEK

1.9 *Aims*

1) To determine whether activated Ras/Raf/MEK pathway suppresses the induction of PKR, OAS, and Mx by IFN
2) To determine whether activated Ras/Raf/MEK suppresses activation of the Jak-STAT pathway by IFN

Chapter 2: Materials and methods

2.1 *Reagents and antibodies*

The following reagents and antibodies were used in this study: IFNα (Sigma, St. Louis, MO, cat. no. I8782). U0126 (Calibiochem, La Jolla, CA, cat. no. 662005), anti-phospho-STAT1, anti-phospho-STAT2, anti-total-STAT1 and anti-total-STAT2 (Upstate Cell Signaling Solutions, Lake Placid, NY cat. no. 07-714, 07-224, 06-501, 06-502 respectively), anti-β-actin (Sigma, cat. no. A2228), anti-ERK, anti-phospho-ERK1/2 (Calibiochem, cat. no. 442704, 442706), and anti-OAS (provided by Dr. Yoshihiro Sokawa, Kyoto Institute of Technology, Kyoto, Japan).

2.2 *Cell culture*

Murine fibroblast cell line, NIH3T3, was obtained from the American Type Culture Collection. Bosc23 cells were generously provided by Dr. Patrick Lee (Dalhousie University, Halifax, Nova Scotia, Canada). All cell lines utilized in this study were maintained in high-glucose Dulbecco's modified medium (DMEM) (Invitrogen, Burlington, Ontario, Canada) containing 10% fetal bovine serum (FBS) (Cansera, Etobicoke, Ontario, Canada). pBABE retroviral vectors containing activated Ras were generously provided by Dr. Patrick Lee (Norman *et al*, 2004). To generate Ras transformed cell lines, the retroviral vectors were transfected into Bosc23 packaging cells (provided by Dr. Patrick Lee) using Superfect (Qiagen, Valencia, CA) according to the manufacturer's instructions. Following a transfection period of 48 hours, supernatants

containing retroviruses were harvested, filtered, and stored at -70 °C. NIH3T3 cells infected with retroviruses were selected with 2 μg of puromycin/ml for 2 weeks.

2.3 *RNAi*

Two oligonucleotides corresponding to nucleotide sequences of H-Ras were synthesized by Invitrogen: 5'-ccacuauagaggauuccuaccggaa-3' [positions 289 to 314] and 5'-ccugugugguuugccauaacaac-3' [positions 422 to 446]. Negative control RNAi comprised of a random nucleotide sequence was used as a control for non-specific effects due to transfection of duplex RNA. Cells were grown to 40% to 50% confluence in 24-well plates, washed twice with phosphate buffered saline (PBS), and then incubated with a transfection mixture containing DMEM without antibiotics, 10% FBS, RNAi Lipofectamine (20 μg/ml), and oligonucleotides (final concentration range, 10 to 50 pM). Transfection was repeated after 24 hours for greater suppression of the Ras gene. To confirm the efficacy of the Ras RNAi on silencing Ras, phosphorylation of downstream ERK was assessed by western blot analysis.

2.4 *Northern blot analysis*

2.4.1 *Preparation of RNA samples*

Cells were plated in 100 mm X 20 mm tissue culture dishes (4 x 10^6 cells/dish) and were incubated at 37 °C until cells were 80% confluent. Following IFN stimulation (500 U/ml), RNA was extracted from cells with TRIzol (Invitrogen) according to the manufacturer's directions. RNA concentrations were determined according to the

Warburg-Christian method using DU Series 500 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). Experiments and subsequent analyses were repeated (n =2) to improve accuracy. Collected RNA samples were used for preparation of complementary DNA (cDNA) probes or for northern blot analysis. Samples for northern blot analysis were diluted in Northern Max Formaldehyde Load Dye (Ambion), heated at 60 °C for 10 min, and then stored at -45 °C until needed.

2.4.2 *Preparation of probes*

To construct cDNA probes, the following primers were designed: PKR 5'-gttaaagagcccgccgaa-3' (forward) and 5'- ttctcatccattgctccaaa-3' (reverse); OAS 5'-attacctccttcccgacacc-3' (forward) and 5'-gcatcaggaggtggagtttg-3' (reverse); Mx 5'-aaacctgatccgacttcacttcc-3' (forward) and 5'- tcttcttctctctggtgtcactc-3' (reverse); glyceraldehyde-3-phosphatase dehydrogenase (GAPDH), 5'-gggtggagccaaacgggtca-3' (forward) and 5'-tgcgacttcaacagcaactcc-3' (reverse).

One microgram of RNA isolated from IFN-stimulated BABE was subjected to reverse transcription (RT) using random hexanucleotide primers and reverse transcriptase according to the First-Strand cDNA Synthesis Kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The cDNAs generated by RT reaction were then subjected to polymerase chain reaction (PCR) using specific primers for OAS, Mx, PKR, and GADPH in a Mastercycler gradient (Eppendorf, Hamburg, Germany). PCR for each target gene were carried out as follows: denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min with 28 cycles used for OAS and PKR and 30 cycles used for Mx and GADPH. Final extension was performed at 72 °C for 10 min for each of

the target sequences. PCR amplification products were cloned using the TOPO TA Cloning Kit (Invitrogen). The cloning reaction was carried out as specified by the manufacturers' instructions. Clones were digested using *Hind* III and *Xho* restriction enzymes (Invitrogen) and subjected to electrophoresis on a 1% agarose gel (Invitrogen) containing ethidium bromide (0.4 mg/ml). The PCR product of interest was first visualized by 254 nm shortwave UV (Spectroline, Universal X-ray Company of Canada) and was then excised from the gel using a gel extraction kit (Qiagen) and then amplified using a kit according to the manufacturer's instructions (Qiagen). The prepared probes were stored at -20 °C until needed.

2.4.3 *Gel electrophoresis and transfer*

Ten micrograms of each RNA sample were denatured at 60 °C for 10 minutes and then fractionated in a 1% agarose gel containing 2.2 M formaldehyde gel and 1X 3-(N-morpholina) propansulfonic acid (MOPS) buffer at 100V in 1X MOPS/0.22M formaldehyde running buffer. RNA was transferred overnight to a nylon membrane (Hybond-XL; Amersham Biosciences, Little Chalfont, Buckinghamshire, England) by upward capillary action using 2X saline sodium citrate (SSC) buffer. RNA was cross-linked to the membrane using a UVC-508 ultraviolet cross-linker (Ultra-Lum., Paramount, CA). Membranes were stored at –20 °C until needed.

2.4.4 *Hybridization*

Membranes were prehybridized in hybridization buffer (Ambion) for 1 hour at 42 °C in a hybridization oven (Robbins Scientific, Sunnyvale, CA). cDNA probes were

labelled with ^{32}P- dCTP (Amersham Biosciences) using a Megaprime DNA Labeling kit (Amersham Biosciences) according to the manufacturers instructions. Unincorporated nucleotides were removed using a Nick column containing G50 Sephadex (Amersham Biosciences). Hybridizations were performed overnight at 42 °C. After the hybridization, membranes were washed twice for 5 minutes each at 42 °C with 2X SSC and 0.1% SDS followed by washing twice at 42 °C with 0.2X SSC and 0.1% SDS for 15 minutes each. The membranes were exposed to Kodak BioMax XAR scientific imaging film which was then developed using a Konica SRX-101A Medical Film Processor. The membranes with weak signals were exposed to Cyclone Storage Phosphor Screens (Perkin Elmer Life Sciences, Boston, MA) and analyzed using a Cyclone Storage Phosphor System (Packard) and using an Epson Perfection 3170 Photo Scanner (Seiko Epson Corporation, Japan). Quantifications of band intensities were performed using Kodak Molecular Imaging Software (Eastern Kodak Company, Rochester, NY).

2.5 *Western blot analysis*

2.5.1 *Preparation of protein samples*

Cells were plated in 6 or 24 well plates with 4 x 10^6 cells/well or 4 x 10^4 cells/well respectively. Following IFN stimulation (500 U/ml), the cells were lysed in radioimmunoprecipitation assay (RIPA) buffer, PBS containing 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS with 10 μg/ml aprotinin, 100 μg/ml phenylmethylsulponyafluoride (PMSF) and 1% phosphatase inhibitor cocktail (Sigma). After 3 minutes cooling on ice, protein samples were cleared of debris by centrifugation

at 12,000x *g* for 3 minutes. Protein concentrations in samples were determined by the bicinchoninic acid (BCA) method (BCA Protein Assay Kit, Pierce Biotechnology, Inc., Rockford, IL) or the Bradford method (Bio-Rad) using a Beckman DU Series 500 spectrophotometer (Beckman Instruments, Inc.). Experiments and subsequent analyses were repeated (n = 2) in order to improve accuracy. Following protein determination, the protein samples were boiled at 100 °C for 5 min in 3X bromophenol blue (3X BPB). The samples were stored at -45 °C until needed.

2.5.2 *Gel electrophoresis and transfer*

Protein samples were subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100V for 2 hours and transferred to nitrocellulose membrane at 100v for 1 hour (Bio-Rad, Mississauga, Ontario, Canada). A protein ladder containing bands ranging from 10-200 kDa (Benchmark Prestained Protein Ladder) was loaded to assess the molecular weight of the target proteins.

2.5.3 *Immunoblotting*

Membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) (20 mM Tris and 137 mM NaCl [pH 7.3]) containing 0.1% Tween 20 for 20 minutes prior to incubation with primary antibodies. Dilutions for primary antibodies were prepared as recommended by provider (Table 1). All primary antibody incubations were performed overnight at 4°C with shaking. After washing, the membrane was incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin (IgG) or anti-mouse IgG (1:5000 to 1:10000) (Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature, and

Table 1 Primary antibody dilutions for Western blot analysis

Antibody	**Dilution**
Anti-phospho-STAT1	1:2000
Anti-phospho-STAT2	1:2000
Anti-STAT1	1:2000
Anti-STAT2	1:2000
Anti-phospho-ERK	1:5000
Anti-ERK	1:5000
Anti-actin	1:10000
Anti-OAS	1:50

specific bands were detected with the SuperSignal West Pico Chemiluminescent detection kit (Pierce Biotechnology, Inc., Rockford, IL). Bands were visualized on Kodak BioMax XAR scientific imaging film (Eastman Kodak Company, Rochester). The film was developed using a Konica SRX-101A Medical Film Processor (Konica Medical and Graphic Corporation, Japan), and then scanned onto a computer using an Epson Perfection 3170 Photo Scanner (Seiko Epson Corporation, Japan).

Chapter 3: Results

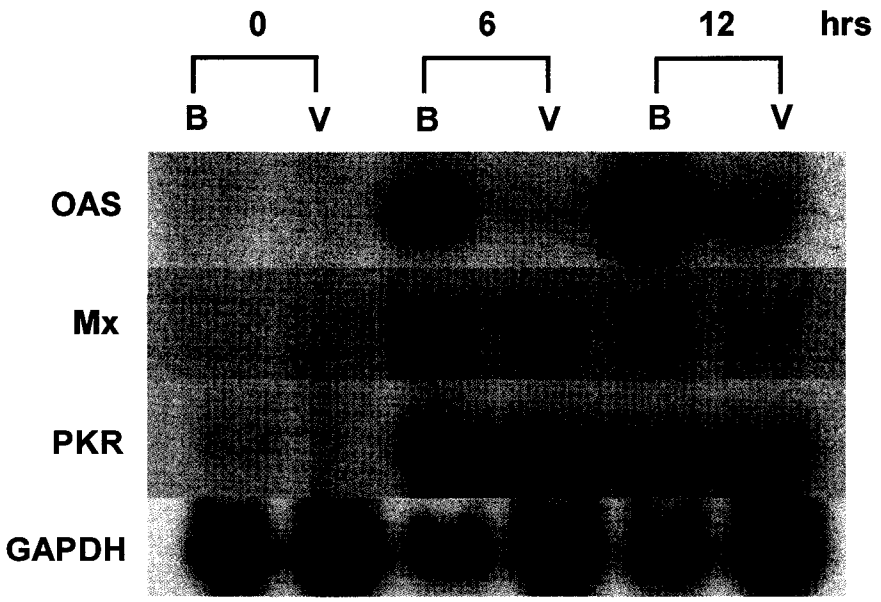
3.1 *Activation of Ras/Raf/MEK suppresses transcription of IFN-inducible genes*

To determine the effect of activated Ras on anti-viral genes induced by IFN, Ras activated NIH 3T3 cells (RasV12) and vector control NIH 3T3 cells (BABE) were grown to 80% confluency and then treated with IFNα (500 U/ml) for 6 and 12 hours or left untreated (time 0). At each time point, the cells were lysed and RNA was extracted for Northern blot analysis to identify expression of IFN-induced anti-viral genes, OAS, Mx, and PKR, and a housekeeping gene GAPDH (Figure 6A). In the absence of IFN stimulation, there was no detectable expression of any of these anti-viral genes. At 6 and 12 hours following IFN treatment, induction of OAS, Mx, and PKR mRNA was observed in both BABE and RasV12. Importantly, expression levels of these genes were higher in BABE at both 6 and 12 hours after IFN treatment when compared to RasV12. This was confirmed using densitometry analysis (Figure 6B). Thus, Northern blot analysis revealed that activation of Ras/Raf/MEK suppresses induction of anti-viral genes by IFN.

3.2 *Restoration of induction of IFN-inducible genes in Ras activated cells*

From the previous experiment, activated Ras suppressed transcription of IFN-inducible genes. To determine whether induction of IFN-inducible genes by IFN was restored by suppression of Ras/Raf/MEK, Northern blot analysis was conducted on RasV12 pretreated with an MEK inhibitor (U0126) prior to IFN stimulation. RasV12 were pretreated with U0126 for 16 hours prior to IFN treatment. At time 0, BABE, RasV12,

A)



B)

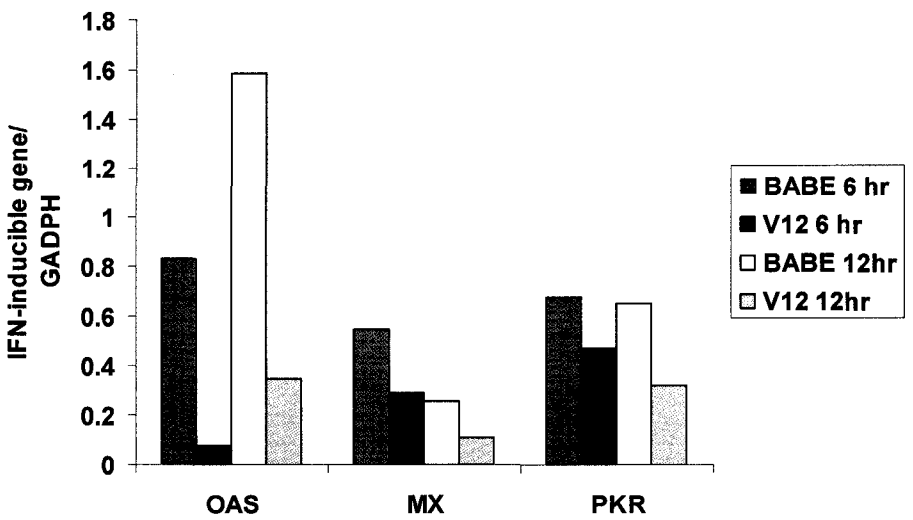


Figure 6. Activation of Ras suppresses induction of IFN-inducible genes. (A) Vector control cells (B) and RasV12 cells (V) were incubated with or without IFNα (500 U/ml) for 6 and 12 hours. RNA was extracted using TRIzol reagent followed by Northern blot analysis for OAS, Mx, PKR, and GAPDH transcription. (B) Densitometry analysis. Values were reported as the ratio of OAS/GADPH, Mx/GADPH, and PKR/GADPH.

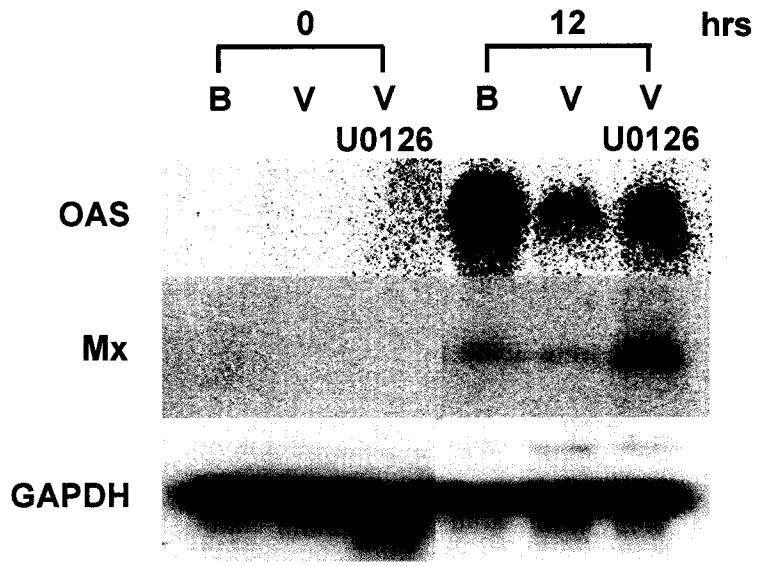
and U0126 treated RasV12 cells were stimulated with IFNα (500 U/ml). RNA samples were obtained at 0 and 12 hours following the IFN treatment and analyzed by Northern blotting (Figure 7A). As observed in Figure 7A, OAS and Mx genes were not transcribed in the absence of the IFN treatment in BABE or RasV12 cells. As well, there was no apparent induction of the IFN-inducible genes in U0126 treated RasV12 before the IFN stimulation. After 12 hours of IFN stimulation, induction of OAS and Mx mRNA were observed in BABE, RasV12, and U0126 pretreated RasV12. Induction of the IFN-inducible genes by IFN was higher in BABE than in RasV12. Importantly, when RasV12 were pretreated with U0126 prior to IFN treatment, induction of the IFN-inducible genes by IFN was restored. This was further confirmed using densitometry analysis (Figure 7B).

In summary, suppression of IFN-inducible genes by activated Ras can be reversed when the cells are treated with a MEK inhibitor prior to IFN stimulation. These results suggest that activation of MEK inhibits induction of anti-viral genes by IFN.

3.3 Inhibition of activated Ras/Raf/MEK pathway restores induction of OAS protein levels

To further confirm that activation of Ras/Raf/MEK inhibits transcription of IFN-inducible genes, it was investigated whether different temporal expression patterns of OAS protein occurred between BABE and RasV12 following IFN stimulation. BABE and RasV12 were treated with IFNα (500 U/ml) for 24 hours followed by Western blot analysis using antibodies against OAS, phosphorylated ERK (p-ERK), and total ERK (t-ERK) (Figure 8).

A)



B)

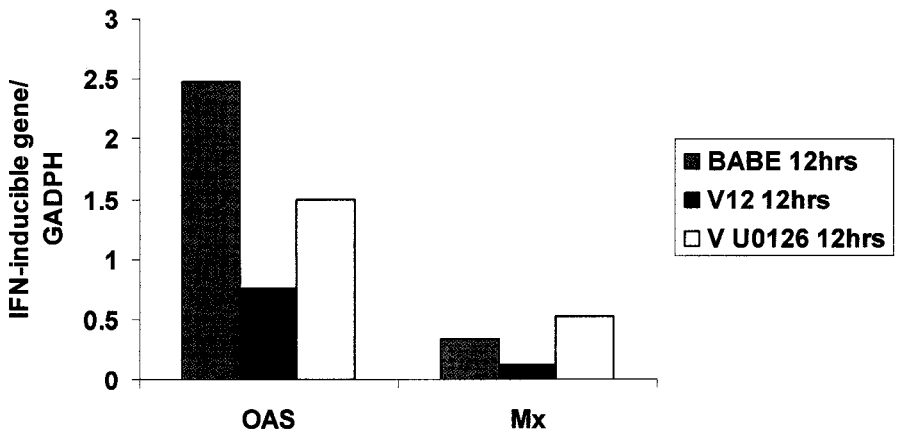


Figure 7. Inhibition of activated Ras/Raf/MEK pathways restores induction of IFN-inducible genes in RasV12. RasV12 were incubated with or without U0126 16 hr prior to IFNα treatment. Vector control cells (B), RasV12 (V), and U0126 pretreated RasV12 (V U0126) were stimulated with or without IFNα (500 U/ml) for 12 hours. RNA samples were obtained using TRIzol reagent followed by Northern blot analysis for OAS, Mx, and GAPDH. (B) Densitometry analysis. Values are reported as ratio of OAS/GAPDH and Mx/GAPDH.

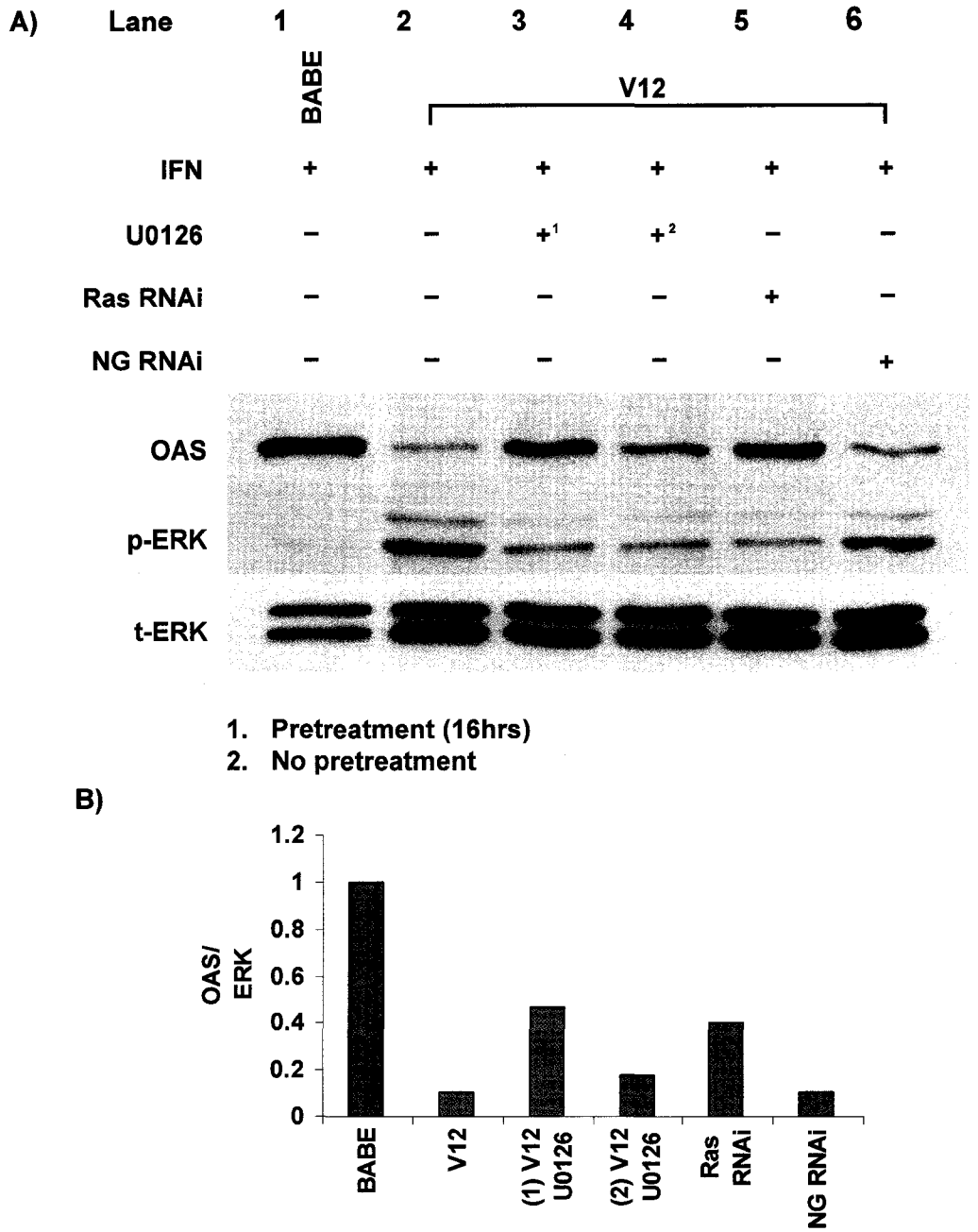


Figure 8. Inhibition of activated Ras/Raf/MEK pathway restores induction of OAS protein. (A) BABE (lane 1) and RasV12 (lane 2). RasV12 pretreated with U0126 (lane 3) or without U0126 (lane 4) 16hours prior to IFNα treatment. Ras-specific oligonucleotide sequence (RasRNAi) (lane 5) or random sequence (NGRNAi) were transfected to RasV12 (lane 6) twice during a 24 hour interval. All cells were incubated with IFNα (500 U/ml) for 24 hours followed by cell lysis and Western blot analysis with antibodies against OAS, phosphorylated ERK, and total ERK. (B) Densitometry analysis. Values are reported as ratio of OAS/ERK.

Phosphorylation levels of ERK were analyzed to assess activity of the Ras/Raf/MEK pathway whereas total levels of ERK were used as the protein loading control. At 24 hours after IFN stimulation, induction of OAS protein was observed in BABE (Figure 8, lane 1) whereas induction of OAS was lower in RasV12 (Figure 8, lane 2). Western blot analysis revealed that RasV12 cells displayed higher levels of phosphorylated ERK than BABE cells, indicating greater levels of Ras/Raf/MEK activity in RasV12 cells. This was confirmed using densitometry analysis (Figure 8B)

In an attempt to rescue induction of OAS protein by IFN, RasV12 were pretreated with U0126 for 16 hours prior to IFN stimulation (Figure 8, lane 3) or were treated with U0126 at the same time as IFN stimulation (Figure 8, lane 4). In the pretreatment group, the induction of OAS was restored to levels comparable with that of BABE (Figure 8, lane 1) whereas, in the non-pretreatment group (Figure 8, lane 4), expression levels of OAS protein were increased but not as efficiently as observed in the pretreatment group. Levels of phosphorylated ERK were reduced by U0126 treatment suggesting that upstream MEK was inhibited.

To further demonstrate the restoration of OAS proteins levels by suppression of activated Ras, RasV12 were pretreated with Ras-specific oligonucleotide sequence (RasRNAi) or random sequence RNAi (NGRNAi) prior to IFN treatment (Figure 8, lane 5 and 6). Efficacy of the RasRNAi inhibition was confirmed since low levels of phosphorylated ERK were detected when compared to RasV12. RasRNAi treatment restored induction of OAS protein by IFN in RasV12 cells to levels comparable with that in BABE cells. However, NGRNAi treatment did not affect levels of phosphorylated ERK and failed to restore the induction of OAS. This experiment clearly indicated that

induction of OAS protein by IFN can be rescued in cells with activated Ras by disruption of Ras/Raf/MEK signaling.

3.4 *Activation of Ras/Raf/MEK suppresses activation of the Jak-STAT pathway*

The suppression of IFN-inducible genes by the Ras/Raf/MEK pathway could be the result of down-regulation of upstream Jak-STAT signaling. Therefore, activation status of the Jak-STAT pathway in BABE and RasV12 cells stimulated with IFN was determined (Figure 9). After 0.5, 1, and 2 hours following IFN treatment, proteins samples were prepared from BABE and RasV12 for Western blotting and analyzed using antibodies against phosphorylated STAT1, phosphorylated STAT2, total STAT1, total STAT2 and total ERK. In the absence of IFN treatment (time 0), there was no observable phosphorylation of STAT1 and STAT2 in BABE and RasV12. At 0.5, 1, and 2 hours of IFN treatment, phosphorylation levels of STAT1 and STAT2 were higher in BABE than in RasV12. Before and after IFN treatment, lower amounts of total STAT1 and STAT2 were detected in RasV12 than in BABE.

These results suggested that there were two possible mechanisms of how activated Ras suppresses the Jak-STAT pathway. First, activated Ras might inhibit cytoplasmic levels of STAT1 and STAT2 leading to reduced activation of the STAT proteins. Second, regardless of lower cytoplasmic availability, Ras activation may suppress the Jak-STAT pathway by inhibiting activity of kinases upstream of STAT1 and STAT2 including Jak1 and Tyk2 and/or reducing levels of IFNAR. Nonetheless, it was

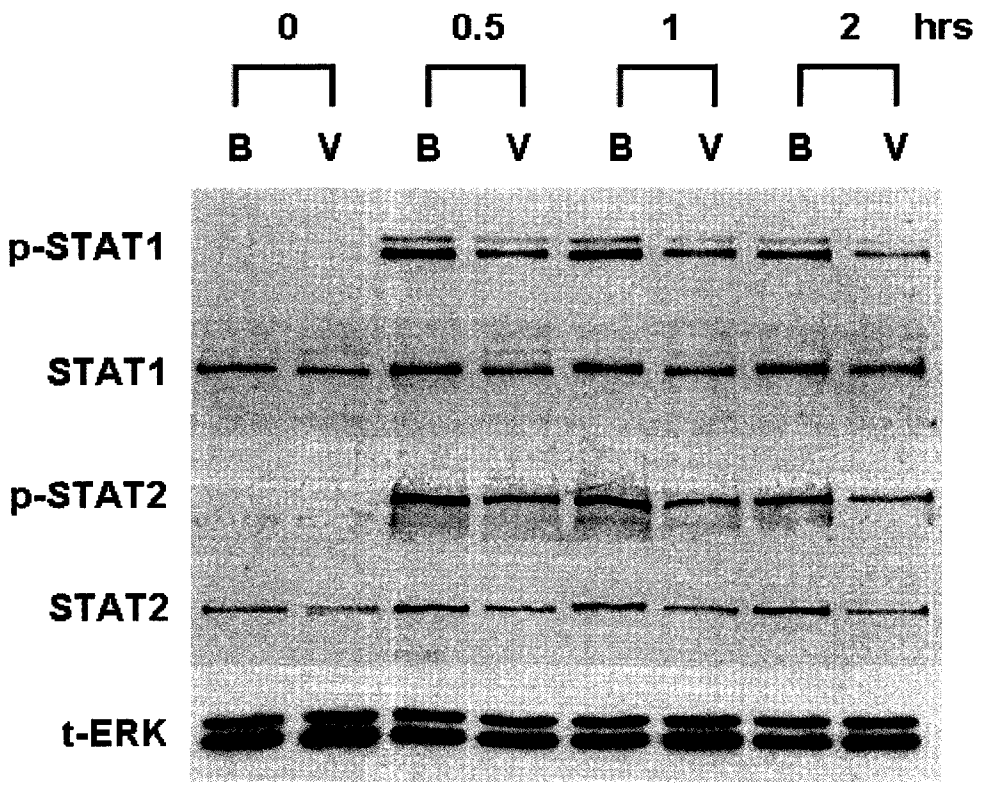


Figure 9. Activated Ras/Raf/MEK pathway inhibits activation of the JAK-STAT pathway. BABE (B) and RasV12 (V) were incubated with or without IFNα (500 U/ml) for 0, 0.5, 1 and 2 hours. Cellular proteins were harvested with RIPA buffer followed by Western blot analysis using antibodies against phosphorylated STAT1 (p-STAT1), total STAT1 (t-STAT1), phosphorylated STAT2 (p-STAT2), total STAT (t-STAT2) and total ERK (t-ERK).

concluded that inhibition of transcription of IFN-inducible genes is due to suppression of the Jak-STAT pathway by activated Ras.

3.5 *Inhibition of activated Ras/Raf/MEK pathway restores activation of the Jak-STAT pathway*

Upon observation that activated Ras pathway inhibited total and phosphorylated STAT1 and STAT2 levels, it was decided to determine whether the inhibition of the Ras/Raf/MEK pathway could rescue activation of the Jak-STAT pathway. RasV12 were pretreated with U0126 for 16 hours prior to IFN stimulation. At time 0, BABE, RasV12, and RasV12 with U0126 pretreatment were treated with IFNα for 0.5 hours (Figure 10, time 0). Western blot analysis was conducted using antibodies against phosphorylated STAT1, phosphorylated STAT2, total STAT1, total STAT2 and β-actin. In the absence of IFN, there was no phosphorylation of STAT1 or STAT2 in BABE in all cells tested (Figure 10, time 0). The total levels of STAT1 and STAT2 were reduced in RasV12 compared to those in BABE. However, treatment of RasV12 with U0126 restored levels of both total STAT1 and total STAT2. Treatment with IFN for 0.5 hours led to greater phosphorylation levels of STAT1 and STAT2 in BABE compared to RasV12 while phosphorylation levels of STAT1 and STAT2 in RasV12 pretreated with U0126 were restored.

In summary, activation of the Jak-STAT pathway was restored when activated MEK was inhibited. This indicated that MEK and its downstream elements were responsible for interruption of the Jak-STAT pathway.

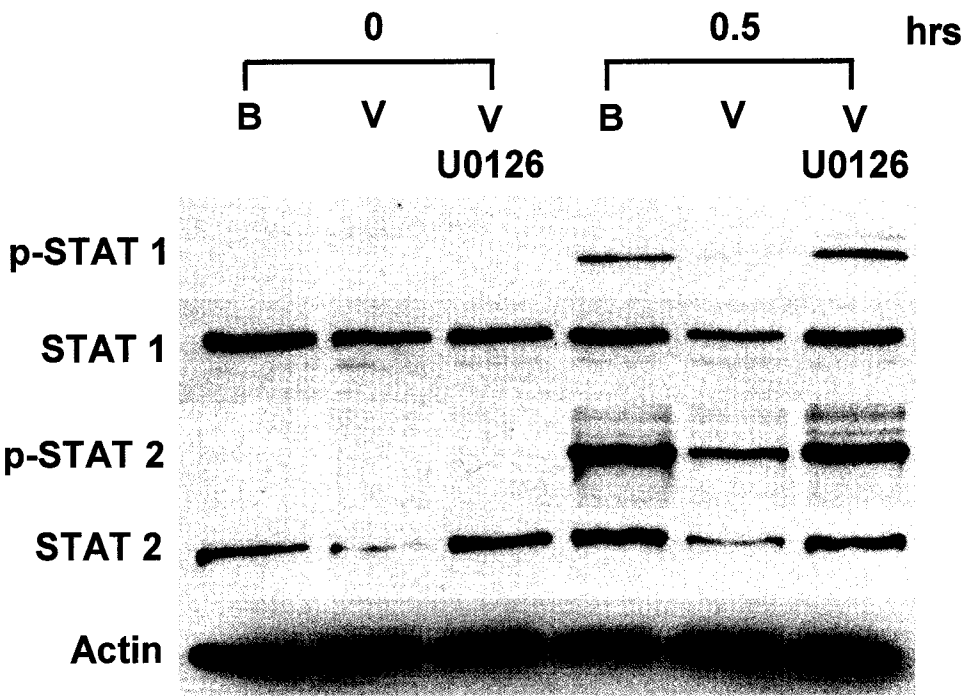


Figure 10. Inhibition of the Ras/Raf/MEK pathway restores activation of the Jak-STAT pathway in response to IFN. RasV12 (V) were incubated with or without U0126 16 hours prior to IFNα treatment. BABE (B), RasV12 (V), and U0126 pretreated RasV12 (V U0126) were incubated with or without IFNα (500 U/ml) for 0.5 hours. Proteins were harvested using RIPA buffer followed by Western blot analysis using antibodies against phospho phosphorylated STAT1 (p-STAT1), total STAT1 (t-STAT1), phosphorylated STAT2 (p-STAT2), total STAT (t-STAT2) and β-actin.

Chapter 4: Discussion

4.1 *Suppression of the IFN-induced anti-viral response*

The IFN system is an important first line of defense restricting viral replication. Intact Jak-STAT signalling is vital for maintaining robust induction of IFN-induced anti-viral genes. Several studies have demonstrated that disruption of Jak-STAT signalling leads to resistance to IFN (Landolfo, 2000; Wong, 1997). Some viruses containing genes that encode anti-IFN proteins can evade the IFN system by interrupting the Jak-STAT pathway (Young *et al*, 2002; Dideock *et al*, 1999). However IFN-sensitive viruses such as VSV, may selectively replicate within cells that are resistant to IFN due to high activities of negative regulators against the IFN-induced anti-viral response (Obuchi *et al*, 2003). Previous work from this laboratory demonstrated that activated Ras/Raf/MEK suppresses the IFN-induced anti-viral response (Battcock *et al*, 2006). As activation of the Ras/Raf/MEK pathway defines cellular sensitivity to IFN, the objective this masters project was to determine the molecular mechanisms underlying suppression of the IFN-induced response by the Ras/Raf/MEK pathway. To do this, a step-by-step analysis was performed to compare activation of the IFN pathway between Ras activated cells and vector control cells.

4.2 *Summary of key findings*

1) Activation of Ras/Raf/MEK downregulated expression of IFN-inducible genes.
2) Lower levels of cytoplasmic STAT1 and STAT2 occurred in cells with activated Ras/Raf/MEK.

3) Diminished induction of STAT1 and STAT2 phosphorylation by IFN occurred in cells with activated Ras/Raf/MEK.

4.2.1 *Inhibition of IFN-inducible genes by activated Ras*

Activation of Ras reduced IFN-induced transcription of OAS, Mx, and PKR genes (Figure 6). However, the degree of the inhibition observed was not equal among the three genes. OAS mRNA expression was clearly suppressed to a greater extent than the other genes. A possible explanation for the differential influence of activated Ras is the variation of promoter regions regulating transcription of these genes. IFN-inducible genes are transcribed upon binding of ISGF3 to the ISRE promoter site (Samuel, 2001). Since ISRE for OAS, Mx, and PKR genes varies in sequence and number within the promoters, they may compete for association with ISGF3 under insufficient activation of the STATs in RasV12 cells (Geiss *et al*, 2003; Kuhen and Samuel, 1997; Darnell *et al*, 1994). Although the mechanisms behind ISRE selectivity are not fully understood, the ISRE within the OAS promoter may have the weakest affinity for ISGF3 based on features of its sequence. Therefore, the Mx and PKR ISREs may out-compete the OAS ISRE promoter for binding with ISGF3, thereby leading to greater inhibition of transcription of the OAS gene.

Previously in this laboratory, Western blot analysis using anti-PKR antibody revealed that activation of Ras/Raf/MEK did not affect induction of IFN-induced PKR protein (Battcock *et al*, 2006). Contrary to this finding, Northern blot analysis in the present study showed that activated Ras inhibited induction of PKR mRNA by IFN (Figure 6). The inconsistency between the two studies is most likely due to the difference

in the stability of the targets (i.e., PKR mRNA and PKR protein), detected by Western blot and Northern blot analysis. For example, in the previous study western blot analysis detected PKR protein in the absence of IFN treatment, while PKR mRNA was not detected in IFN untreated controls in the current study. Overall, Northern blot analysis appears to give a more accurate measure of expression of PKR because it detects newly synthesized mRNA.

4.2.2 *Inhibition of the Jak-STAT pathway by activated Ras*

In this study, we found that the STAT pathway was inhibited by activated Ras. We propose two possible mechanisms of how the downregulation of the STAT pathway may occur: (1) Disruption of upstream signaling impairing phosphorylation of STAT1 and STAT2 regardless of cytoplasmic STAT availability and (2) Insufficient availability of cytoplasmic STAT proteins leading to decreased phosphorylation of both STAT1 and STAT2. In either case, reduced levels of phosphorylated STAT1 and STAT2 would lead to decreased formation of ISGF3, thereby resulting in reduced transcription of anti-viral genes (Figure 11). However, it still remains to be explained precisely how Ras disrupts the functionality of the STAT pathway.

4.2.3 *Disruption of upstream signaling by activated Ras/Raf/MEK pathway*

Numbers of IFN receptors displayed on the cell surface can limit activation of the Jak-STAT pathway (Muller *et al*, 1994). A possibility is that activated Ras decreases levels of IFNAR, thereby impairing downstream signaling. However, based on a review of the literature, there is no clear evidence that activated Ras reduces cell surface levels of

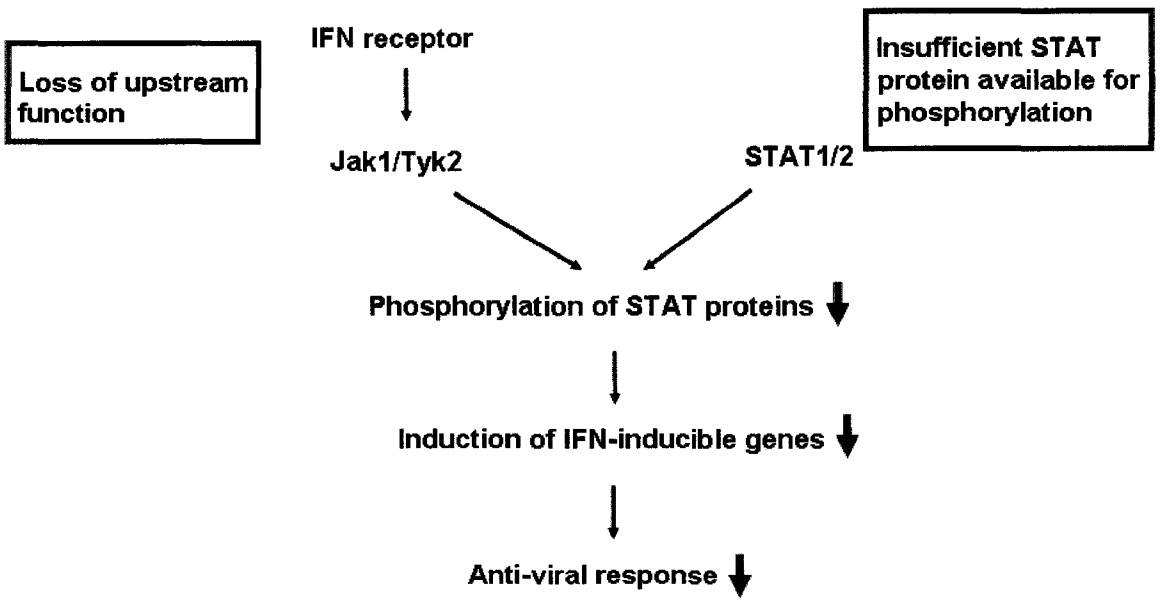


Figure 11. Possible mechanisms underlying suppression of IFN-inducible genes by activated Ras/Raf/MEK pathway. Activation of Ras/Raf/MEK inhibits Jak-STAT pathway signaling by inhibiting phosphorylation of the STAT proteins. Decreased phosphorylated-STAT levels could be the result of impaired upstream function or decreased cytoplasmic availability of STATs. This in turn results in decreased levels of ISGF3 and inhibition of IFN-inducible genes. A decreased level of anti-viral proteins leads to a weakened anti-viral response.

IFNAR. In a related study, activation of Ki-Ras did not affect levels of IFNγ receptor, suggesting that Ras is unable to target the IFN response at the receptor level (Klampfer *et al*, 2003).

Decreased phosphorylation of STAT1 and STAT2 could result from suppression of upstream kinases Jak1 and Tyk2 which are targeted by negative regulators within the cell. Negative regulators of Jak1 and Tyk2 include members of the SHP and SOCS protein families (Starr and Hilton, 1998). Jak1 has been shown to be dephosphorylated by SHP1 (David *et al*, 1995). It is also known that SHP1 can promote degradation of both Jak1 and Tyk2 (Wu *et al*, 2003). SOCS1 and SOCS3 can disrupt normal activity of the Jak kinases (Wormald and Hilton, 2004). Interestingly, research conducted in this laboratory has found that activation of Ras promotes transcription of the SHP1 and SOCS3 gene upon IFN stimulation (Battcock unpublished, 2006). Therefore, it is also possible that the inhibition of the Jak-STAT pathway in cells with activated Ras is due to increased expression of negative regulators, such as SHP1 and SOCS3, which leads to impaired function of Jak1 and Tyk2.

4.2.4 *Inhibition of STAT levels by activation of Ras/Raf/MEK*

Insufficient availability of cytoplasmic STAT1 and STAT2 may also attribute to their decreased phosphorylation. To transmit signals from upstream to downstream components of the Jak-STAT pathway, a certain amount of STAT proteins must be present within the cytoplasm (Darnell *et al*, 1994). The amount of cytoplasmic STAT

proteins is regulated by the following mechanisms: (1) transcription, (2) degradation, and (3) translocation.

The major inducer of STAT gene transcription is IFN (Samuel, 2001). Basal level expression of STAT1 and STAT2 genes is regulated by circulation of endogenous IFN. IFN binds to cognate surface receptors that activate the Jak-STAT pathway which in turn stimulates ISRE-mediated gene transcription of the STAT genes (Darnell, 1994). It is possible that downregulation of interferon regulatory factors (IRFs) by activated Ras would lead to insufficient production of endogenous IFN for maintaining the basal levels of STAT proteins within cytoplasm.

Increased degradation of the STAT proteins is another possible mechanism underlying decreased cytoplasmic availability of STAT1 and STAT2. Proteosome-dependent degradation is an important process in terms of reduction of the STATs (Hendry and John, 2004). Indeed, some viruses such as mumps virus or paramyxovirus promote proteosome-mediated destruction of STAT1 and STAT2 as a means of evading the IFN anti-viral system (Young *et al*, 2002; Dideock *et al*, 1999). As well, there is some evidence to suggest that the SOCS1 is involved in ubiquitination of STAT proteins (Wormald and Hilton, 2004). Promotion of proteosome-dependent degradation activity by activated Ras could result in reduced levels of STAT1 and STAT2 in the cytoplasm.

Dysfunction of mechanisms controlling translocation may also result in low levels of cytoplasmic STATs. Upon phosphorylation by upstream kinases Jak1 and Tyk2, STAT1 and STAT2 dimerize and subsequently bind with IRF9 to form ISGF3. This trimeric complex translocates into the nucleus and binds with ISRE sequences to activate IFN-inducible gene transcription (see Figure 3). Following this event, STATs are

dephosphorylated by nuclear phosphatases and are exported out into the cytoplasm where they are recycled into the Jak-STAT pathway (Haspel and Darnell, 1999). Transport of STAT proteins out of the nucleus is a tightly regulated process involving multiple cellular components (O'Shea *et al*, 2002). Accumulation of STAT1 and STAT2 in the nucleus is modulated by nuclear export via a Ras-related protein termed Ran (McBride *et al*, 2000). Ran-dependent interaction with chromosome region maintenance (CRM)1/exportin 1 regulates transport of STATs across the nucleus (McBride *et al*, 2000). Import of STATs into the nucleus is controlled by a nuclear import receptor, importinα5 and Ran (O'Shea *et al*, 2002). Although a review of the literature did not suggest any direct connection between Ras and exportin activity, it may be possible that the Ras/Raf/MEK pathway has a yet to be identified role in the modulation of exportin activity. Inhibition of exportin by Ras would result in an accumulation of STAT1 and STAT2 in the nucleus and thus explain the reduced cytoplasmic levels of these proteins in Ras activated cells.

4.3 *Clinical implications - cancer*

Defining the mechanism of cell resistance to IFN is critical for improving IFN-based cancer therapies. Therapeutic effects of IFN become limited when tumor cells display resistance to IFN treatment (Talpaz *et al*, 1991). Several studies have implicated attenuated Jak-STAT signaling as a possible cause of cancer cell resistance to IFN (Landolfo *et al* 2000; Pansky *et al*, 2000; Wong *et al*, 1997). Tumor cells are often resistant to the anti-proliferative effects of IFN due to the high activity of negative regulators targeting the IFN pathway. Ras/Raf/MEK pathway has been shown to be

constitutively active in a number of cancer cells (Scholl *et al*, 2005). If the Ras/Raf/MEK pathway underlies the resistance to IFN treatment in human cancer cells, a new treatment for cancer patients may be a combined therapy with IFN and chemical inhibitors against the Ras/Raf/MEK pathway. In future, in vitro experiments will be required to test the effectiveness of those inhibitors on suppression of tumor cell growth by IFN. As well, it will be necessary to conduct *in vivo* experiments to determine the efficacy of the combined therapy.

4.4 *Clinical implications – virus infection*

Resistance of cells infected with viruses to IFN is also an issue when treating virally infected patients. Viruses have evolved a wide plethora of strategies to evade the IFN system, such as releasing proteins that directly disrupt the IFN pathway (Grandvaux *et al*, 2002; Levy *et al*, 2001). Other mechanisms include manipulation of cellular signaling pathways that suppress the IFN-induced anti-viral response. They may also selectively replicate within cells insensitive to IFN due to activated negative regulators of IFN (Basler and Garcia-Sastra, 2002). However, it is still not clear that why the IFN therapy becomes ineffective in certain virally infected patients. Therefore, it is important to study the basis of cellular resistance to IFN in order to improve the effectiveness of IFN therapy for virus infection. Numerous studies have demonstrated the upregulation of Ras by viruses during infection. The Raf/MEK/ERK pathway is activated by Coxsackie virus during infection by inducing cleavage of p21GTPase-activating protein (RasGAP) (Huber *et al*, 1999). Borna disease virus activates the Raf/MEK/ERK pathway which is necessary for spreading to neighbouring cells (Planz *et al*, 2001). In the current study, it

was determined that activated Ras/Raf/MEK impairs normal Jak-STAT signaling which abrogates IFN-induced anti-viral responses. With this new insight, novel drugs could be designed to enhance the IFN's anti-viral ability by suppressing negative regulators of IFN within cells.

4.5 *Conclusion*

Previously, this laboratory has shown that activation of Ras suppresses IFN-induced anti-viral responses. However, the mechanism underlying this suppression was not clear. In this project, the objective was to determine the molecular basis of how Ras disrupted the IFN pathway. Based on evidence presented in this thesis, it is now better understood that activated Ras suppresses STAT signalling which leads to impaired transcription of IFN-inducible genes. This loss of IFN-inducible gene expression ultimately results in an abrogated anti-viral response. Identification of the precise mechanisms underlying suppression of IFN-induced responses by activated Ras/Raf/MEK pathway has important clinical applications in terms of improving IFN therapy for cancer and virally infected patients.

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