

INDUCTION OF DNA BINDING COMPLEXES BY
TYPE I AND TYPE II INTERFERONS

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**INDUCTION OF DNA BINDING COMPLEXES BY TYPE I AND TYPE II
INTERFERONS**

By

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ABSTRACT

The Interferon (IFN) family is divided into two mainly classes which is type I IFN and type II IFN. Type I IFNs activate phosphorylation of STAT1 and STAT2 and induces their heterodimerization and association with IRF9 to form IFN-stimulated gene factor 3 (ISGF3) complex that binds to IFN-stimulated response elements (ISREs). Type II IFN induces STAT1:STAT1 homodimers (which is also known as GAF) that bind to IFN- γ activated site (GAS) element. Besides, type I IFNs form GAF that bind to GAS element and activate STAT3 phosphorylation. Activated STAT3 is able to form either homodimers with STAT3 or heterodimers with activated STAT1 and bind to GAS element as well. Both ISGF3 and GAF complexes has distinct biological function but the balance between ISGF3 and GAF in type I IFN signalling pathway is not well understood. This project was aimed to investigate IFN-mediated induction of ISGF3 and GAF complexes of different cell lines. Electrophoresis mobility shift assay (EMSA) and western blot analysis were used to study the formation of ISGF3 and GAF complexes of each cell lines.

My experiments demonstrated serial dilution of protein extracts and increased final concentration of DTT in extraction buffers did not affect the formation of STAT3 homodimers, STAT3:STAT1 homodimers and GAF. Several optimization of experiment protocols were done to reveal these three DNA binding complexes. 4 out of 8 cell lines did not induce STAT3 homodimers although activated STAT3 was detected in these cells. 2 of these cells showed the formation of STAT3:STAT1 heterodimers but the concentration was lower than GAF.

On the other hand, 4 out of 8 cell lines induced ISGF3 complex under an hour of type I IFN-stimulation. Pre-treatment of low concentration of IFN- β (priming) for 24 hours prior stimulation increased the DNA binding activity of ISGF3, GAF, STAT3 homodimers and STAT3:STAT1 heterodimers. However, priming affected differently on 8 cell lines.

In summary, there is cell type specific regarding the DNA binding complex such as ISGF3, STAT3 homodimers and STAT3:STAT1 heterodimers and IFN priming.

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Table of Contents

ABSTRACT.....	i
ACKNOWLEDGEMENT	ii
LIST OF ABBREVIATIONS	v
INTRODUCTION	1
1.1 Overview of Interferons and JAK-STAT pathway	1
1.2 Type I and type II IFNs	2
1.2.1 Type I and type II interferon signalling pathway	3
1.3 STATs: Signal Transducers and Activators of Transcription.....	6
1.3.1 Function and structure of STATs.....	6
1.3.2 Serine phosphorylation of STATs	6
1.4 IFNs priming	7
1.5 Research hypothesis and aims	8
MATERIALS AND METHODS	9
2.1 Materials.....	9
2.1.1 Buffer solutions	9
2.1.2 Chemicals.....	9
2.1.3 Enzymes	9
2.1.4 DNA primers and oligonucleotides	9
2.1.5 Cell lines and cell culture reagents.....	9
2.1.6 Antibodies.....	10
2.1.7 Common buffer recipes.....	11
2.1.8 Software.....	13
2.2 Methods	14
2.2.1 Cell Culture techniques	14
2.2.2 Cell extraction	15
2.2.3 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)	16
2.2.4 Immunoblotting / Western Blot analysis.....	16
2.2.5 Electrophoretic mobility shift assay (EMSA).....	18
RESULTS.....	20
3.1 IFN-β priming induced ISGF3-DNA binding complex in 2fTGH but not HeLa.....	20
3.2 Jurkat and RAMOS did not induce ISGF3-DNA binding complex.....	20
3.3 STAT2 mediate GAF DNA binding activity	21
3.4 DNA binding complex of STAT3 homodimers and STAT3:STAT1 heterodimers cannot be detected in HepG2 cells.	23

3.5 Triple bands formation in HeLa cells	23
3.6 Concentration of extracts does not affect the formation of three DNA binding complexes	27
3.7 DNA binding activities are reduced by increasing the concentration of DTT	29
3.8 Optimization of experiment protocol to reveal three DNA binding complexes that bind to GAS elements	29
3.9 IFN-induced DNA binding complexes formed in macrophages.....	30
3.10 IFN-induced DNA binding complexes in HeLa and 2fTGH.....	36
3.11 IFN-induced DNA binding complexes in HepG2 and MCF7	37
3.12 IFN-induced DNA binding complex in Jurkat and RAMOS	39
3.13 Chapter summary	41
DISCUSSION.....	42
IFN-induced DNA binding complexes in different cell lines.....	42
Serine phosphorylation of STAT1 and STAT3 play a role in formation of GAS binding complex	43
Formation of STAT3 homodimers and STAT3:STAT1 heterodimers is cell types specific	45
Role of STAT2 in phosphorylation of STAT1.....	47
Balance between ISGF3 and GAF complex induced by type I IFNs.....	47
CONCLUSION AND FUTURE WORK	49
REFERENCES	51

LIST OF ABBREVIATIONS

Ab	Antibody
APS	Ammonium persulfate
BSA	Bovine Serum Albumin
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulfoxide
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediaminetetracetic acid
EGTA	Ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid
EMSA	Electrophoretic mobility shift assay
FCS	Fetal Calf Serum
GAF	γ -activated factor
GAS	γ -activated sites
HCl	Hydrogen chloride
IFN	Interferon
IFNAR	Interferon alpha receptor
IFNGR	Interferon gamma receptor
IgG	Immunoglobulin G

IRF9	Interferon regulatory factor 9
ISG	Interferon stimulated gene
ISGF3	Interferon stimulated gene factor 3
ISRE	Interferon stimulated response element
JAK	Janus Kinase
KCl	Potassium chloride
MgCl ₂	Magnesium chloride
NaCl	Sodium chloride
Na ₃ VO ₃	Sodium orthovanadate
NaF	Sodium fluoride
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PMSF	Pheynylmethanesulphonylfluoride
pSTAT1	Tyrosine 701 phosphorylated STAT1
pSTAT2	Tyrosine 688 phosphorylated STAT2
pSTAT3	Tyrosine 705 phosphorylated STAT3
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH2	Src homology 2
STAT	Signal transducer and activator of transcription
TAD	Transactivation domain
TBE	Tris/Borate/EDTA

TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
TYK	Tyrosine kinase
Tyr	Tyrosine
WB	Western blot
WT	Wild type

INTRODUCTION

1.1 Overview of Interferons and JAK-STAT pathway

Interferons (IFNs) are cytokines that play a role in innate and acquired immunity against infectious pathogens and tumours (Nallar and Klvakolanu, 2014). They were first described by Isaacs and Lindenmann (1957) and named as Interferons due to their ability to interfere the viral replication. Later, their antiviral property, IFNs regulate apoptosis, inflammation and cell proliferation have been continuously investigated and revealed by other researchers (Borden *et al.*, 2007; Kalvakolanu, Choi and Borden, 2001; Sen and Sarkar, 2007; Vesely *et al.*, 2011).

To date, there are three classes of IFNs family have been discovered: type I IFN, type II IFN and type III IFN (Platanias, 2005). There are numerous type I IFNs which include IFN- α , IFN- β , IFN- δ , IFN- ε , IFN- κ , IFN- τ and IFN- ω . All of these type I IFNs have considerable structural homology. IFN- α , IFN- β , IFN- ε , IFN- κ and IFN- ω exist in humans, whereas IFN- ε and IFN- τ have been described only for pigs and cattle, respectively, and do not have human homologues (Platanias, 2005). In addition, IFN- α has 13 different subtypes, which are IFN- α 1, - α 2, - α 4, - α 5, - α 6, - α 7, - α 8, - α 10, - α 13, - α 14, - α 16, - α 17 and - α 21. In contrast, there is only one cytokine belongs to type II IFN family, IFN- γ . There are three IFNs are included in type III IFN family, IFN λ 1, IFN λ 2 and IFN λ 3 (Platanias, 2005).

All type I IFNs bind a common cell-surface receptor which is known as the type I IFN receptor whereas type II IFN binds a different cell-surface receptor which is known as the type II IFN receptor (Platanias, 2005). Type II IFN (IFN- γ) is a markedly different cytokine than the type I IFNs. Due to its ability to 'interfere' with viral infections, IFN- γ is classified in the IFN family (Platanias, 2005).

Each of these cell-surface receptor subunits interacts with a member of the Janus activated kinase (JAK) family (Platanias, 2005). The JAK family consists of four protein tyrosine kinases which are Janus Kinase 1 (JAK1), Janus Kinase 2 (JAK2), Janus Kinase 3 (JAK3) and tyrosine kinase 2 (TYK2) with a common domain structure (Decker, Müller, Stockinger, 2005). Type I and type

II IFN cell-surface receptors associate permanently with JAKs protein. JAKs will then tyrosine phosphorylate several members of signal transducer and activator of transcription (STAT) proteins and stimulate other signalling responses (Decker, Müller, Stockinger, 2005). Type I IFN receptor, known as IFN- α/β receptor (IFNAR), contains two subunits which are IFNAR1 and IFNAR2. IFNAR1 subunit is constitutively associated with TYK2 and IFNAR2 is associated with JAK1. On the other hand, type II IFN receptor, known as IFN- γ receptor (IFNGR) consists of IFNGR1 and IFNGR2. IFNGR1 subunit associates with JAK1, whereas IFNGR2 is constitutively associated with JAK2 (Platanias, 2005).

The first signalling pathway shown to be activated by IFNs was the JAK-STAT pathway. The original discovery of this pathway in 1990s (Schindler *et al.*, 1992; Fu *et al.*, 1992; Shuai *et al.*, 1992; Silvennoinen *et al.*, 1993) gave a simple model for type I and type II IFN mediated signalling pathway which involved rapid nuclear translocation and initiation of IFN induced gene transcription by STATs. JAK-STAT signalling pathway will be further discussed in section below.

1.2 Type I and type II IFNs

Most of the cells are capable in inducing IFN- α/β (Schneider, 2014). However, massive amount of IFN- α are produced by specialized immune cells known as plasmacytoid dendritic cells during the course of an infection.

IFN- β is a pleiotropic cytokine with potent antiproliferative, antiviral and host innate and adaptive immune regulatory functions (Fink and Grandvaux, 2013). The binding of IFN- β to IFNAR is well characterized to trigger a cascade of signalling events initiated by the activation of the TYK2 and JAK1 which subsequently phosphorylate the intracellular domain of IFNAR on tyrosine residues, providing the binding sites for the Src-homology-2 (SH2) domain of the STAT proteins. Tyrosine phosphorylation of STAT proteins induces their heterodimerization or homodimerization to form DNA binding complexes and then translocate to the nucleus whereby transcription of IFN stimulated genes (ISGs) begins (Fink and Grandvaux, 2013; Levy and Darnell, 2002).

Type I IFNs (α and β) are currently approved for treating variety of diseases including chronic hepatitis B and C virus infection, multiple sclerosis, and melanoma (Schneider, 2014).

There is only one cytokine belongs to type II IFN family, IFN- γ . It is secreted by B cells, neutral killer cells and professional antigen-presenting cells (APCs) (Schroder *et al.*, 2004). IFN- γ interacts with IFNGR, results in receptor activation. This leads to the induction of homodimers which will then enter the nucleus and bind to a specific promoter element to initiate or suppress the transcription of IFN- γ regulated genes (Schroder *et al.*, 2004).

1.2.1 Type I and type II interferon signalling pathway

Type I IFN signalling pathway initiates with the binding of IFN- α or other type I IFNs to the IFNAR1 and IFNAR2, resulting in the rapid autophosphorylation and activation of the receptor associated JAKs, TYK2 and JAK1 respectively (Platanias, 2005). Activated JAKs will then phosphorylate specific tyrosine residues on the receptor polypeptides, which provides docking sites for the STAT proteins through their SH2 domains (Nallar and Klvakolanu, 2014). This will lead to phosphorylation of STATs at a specific tyrosine residue (Y⁷⁰¹ on STAT1, Y⁶⁸⁵ on STAT2 and Y⁷⁰⁵ on STAT3) by JAKs. Phosphorylated STATs form homodimers or heterodimers that translocate to the nucleus and initiate transcription by binding specific sites in the promoters of ISGs (Platanias, 2005). Figure 1.1 showed a basic model of type I and type II IFNs signalling pathways.

Type I IFNs induce an important transcriptional complex which is the IFN stimulated gene factor 3 (ISGF3) complex. The ISGF3 complex consists of the phosphorylated (activated) forms of STAT1 and STAT2, together with IRF9 (also known as ISGF3 γ / p48). IRF9 belongs to the IFN gene regulatory factor family of transcription factors. Even though the IRF9 protein has a DNA binding domain, it weakly binds to DNA on its own and is incapable of activating IFN induced transcription without associating with the STAT1:STAT2 heterodimers (Nallar and Klvakolanu, 2014).

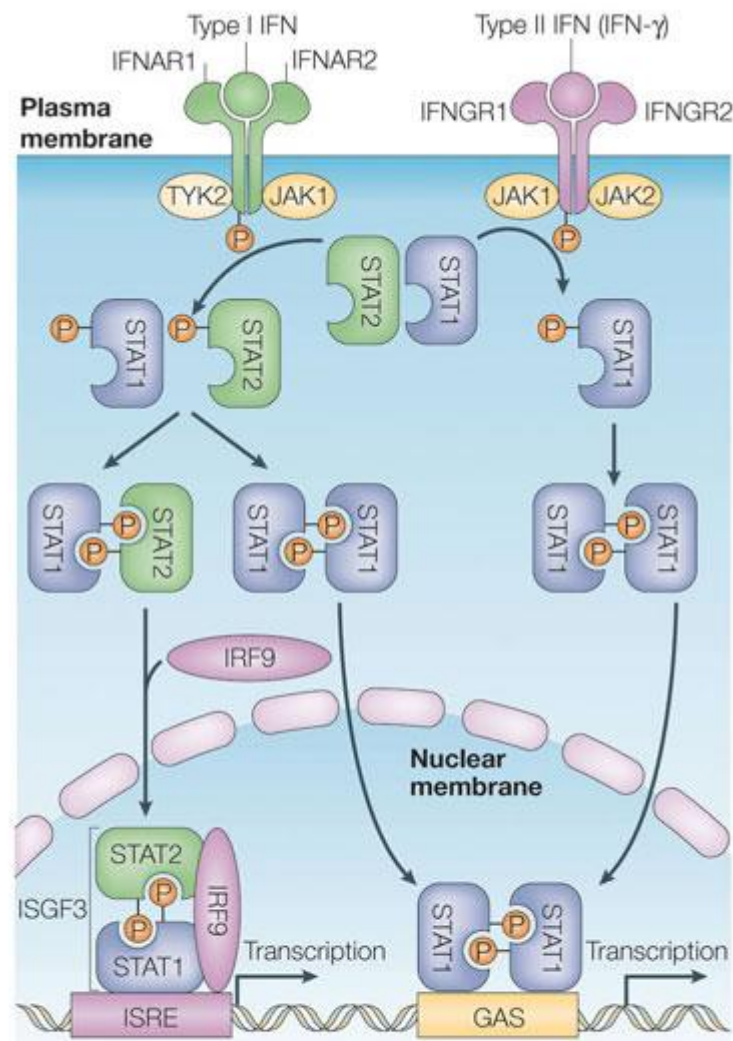
ISGF3 complex then translocate to the nucleus and binds to a specific elements known as IFN-stimulated response elements (ISREs; consensus sequence TTTCNNTTTC) that are present in the promoters of certain ISGs, thus initiating their transcription (Platanias, 2005). The ISRE consensus DNA sequence is found at the promoters of most direct type I or III ISGs (Au Yeung, Mandhana and Horvath, 2013). The interaction with the core of the ISRE consensus is mediated by IRF9, but DNA contacts of STAT1 and STAT2 are required to provide stability of the interaction (Fink and Grandvaux, 2013).

Type I IFNs induce other STAT complexes, for example STAT1:STAT1, STAT3:STAT3, STAT4:STAT4, STAT5:STAT5 and STAT6:STAT6 homodimers, as well as STAT1:STAT2, STAT1:STAT3, STAT1:STAT4, STAT1:STAT5, STAT2:STAT3 and STAT5:STAT6 heterodimers (Platanias, 2005). These STAT complexes do not recruit IRF9 and bind another type of element which is known as an IFN- γ activated site (GAS; consensus sequence TTCNNNGAA) element that is present in the promoter of ISGs (Platanias, 2005).

Type II IFN (IFN- γ) induces the activation of gene which is regulated by GAS elements (Schroder, *et al.*, 2004). STAT1 is the main IFN- γ -activated transcription factor for the regulation of these transcriptional responses. After binding of the type II IFN to IFNGRs, JAK1 and JAK2 are activated and ultimately induce the phosphorylation of STAT1 on the tyrosine residue at position 701 (Tyr701) (Schroder *et al.*, 2004). This leads to the formation of STAT1:STAT1 homodimers (which is also known as γ -activated factor; GAF), which then enter to the nucleus and bind GAS elements to initiate transcription. In contrast to type I IFNs, type II IFN does not induce the formation of ISGF3 complexes and thus it cannot induce the transcription of genes that have only ISREs in their promoter (Platanias, 2005).

Both type I and type II IFNs can induce STAT1:STAT1 homodimers and bind to the same GAS elements. Therefore, STAT1 plays an important role in both type I and II type IFNs signalling (Platanias, 2005). However, the specific reason for phosphorylated STAT1 to form either heterodimers with

phosphorylated STAT2 and IRF9 or homodimers with itself is yet to be investigate.



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Figure 1.1 Type I and type II IFNs signalling pathway. Binding of type I and type II IFNs to their respectively cell-surface receptors (IFNAR and IFNGR) activates the Janus Kinases (JAKs) which are permanently associate to the receptors (JAK1 and tyrosine kinase 2 (TYK2) for IFNAR; JAK1 and JAK for IFNGR). The activated JAKs phosphorylate tyrosine residues in the receptor, which will then provide a docking sites for signal transducer and activator of transcription 1 (STAT1) or 2 (STAT2). STATs are phosphorylated on tyrosine residues by JAKs. Tyrosine phosphorylated STATs induce STAT1 homodimers and STAT1:STAT2 heterodimers. These dimers move to nucleus and bind to specific promoter sequences. STAT1 homodimers bind to IFN-g activated site (GAS) promoter sequences

whereas STAT1:STAT2 heterodimers recruit IFN regulatory factor (IRF9) to form IFN-stimulated gene factor 3 (ISGF3) and bind to IFN-stimulated response element (ISRE) promoter sequence to initiate the transcription of IFN-stimulated genes. (Figure adopted from Decker, Müller and Stockinger, 2005).

1.3 STATs: Signal Transducers and Activators of Transcription

STAT is an abbreviation of signal transducers and activators of transcription. STATs were first described as latent cytoplasmic transcription factors (Shuai *et al.*, 1992). They are able to transduce signals from cell-surface receptors into nucleus and activate the gene transcription (Darnell, Kerr and Stark, 1994). To date, there are seven STAT family members identified in mammals, namely Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b and Stat6. All of them have diverse normal biological functions, which play a role in apoptosis, cell differentiation, proliferation, development, and inflammation (Turkson and Jove, 2000).

1.3.1 Function and structure of STATs

All seven STATs have the similar structural and functional domain organization. STAT proteins are within 700-850 amino acids long. STAT proteins consists of 6 well conserved functional domains which are amino terminal N-domain, coiled-coil domain, DNA binding domain, linker domain, Src homology 2 (SH2) domain and C-terminal transactivation domain (TAD) (Lim and Cao, 2006). N-domain take parts in STATs dimerization and tetramerization. Coiled-coil domain mediates protein-protein interaction such as IRF9 for STAT1 and STAT2. DNA binding domain is involved in nuclear translocation. Linker domain participates in transcriptional activation. SH2 domain is the most conserved domain among STATs and it is important for the receptor association and phosphor-dimer formation for example GAF, STAT1:STAT2 heterodimers and STAT1:STAT3 heterodimers. The TAD is least conserved among the STATs and involved in dimerization, nuclear exportation, transcriptional activity and protein-protein interaction (Lim and Cao, 2006).

1.3.2 Serine phosphorylation of STATs

The phosphorylation of tyrosine residues in STATs by activated JAKs is an essential step in Type I and type II IFN-mediated signalling pathway. However,

in order to optimize the IFN-regulated gene transcription, biochemical modification of STATs, or the interaction of STATs with transcriptional coactivators, are also required. Both type I and type II IFNs trigger activation of STAT1 and STAT3 on the serine residue at position 727 (Ser727) (Wen, Zhong and Darnell, 1995). Although phosphorylation at Ser727 is not required for translocation of homodimers, heterodimers to the nucleus or for their binding to the promoters of ISGs, it is understood that it functioned to enhance the transcriptional activation to maximum level (Wen, Zhong and Darnell, 1997).

1.4 IFNs priming

During inflammation, pre-exposure to certain cytokines greatly changes the cells' responses subsequently to type I IFNs. Mechanism to enhance the expression or specific transcription factors by pre-treatment of low concentration of cytokines is known as priming. For example, priming with IFN- γ increased the concentrations of STAT1, STAT2 and IRF9, resulting in increased concentration of ISGF3 complex to enhance responsiveness of IFN-resistant melanoma cells to type I IFNs (Wong et al., 1998).

Priming with very low concentrations of IFN- γ for two days in primary human monocytes also increased activated STAT1 in response to IFN- α (Hu *et al.*, 2002). Priming with IFN- γ of human Hep3B hepatoma cells for a day increased phosphorylation of STAT1 by IFN- α (Radaeva *et al.*, 2004). In contrast, priming with IFN- γ of Hep3B cells for three to six days reduced the concentration of phosphorylated STAT1 and totally abolishes the activation of STAT2 and STAT3 by IFN- α , despite increased expression of total STAT1 and STAT2 and normal expression of STAT3 (Radaeva *et al.*, 2004). It showed that priming might provide negative effect on cells. In summary, pre-treatment of cells with IFN- γ can either positively or negatively affects the quality or quantity of signal transduction of type I IFNs. Further investigation is required to identify the process of priming which could possibly contributes to negative effect on different cell types.

1.5 Research hypothesis and aims

As mentioned above, type I and type II IFNs are able to induce the formation of GAF and bind to GAS elements. Type I IFNs also trigger ISGF3 complex formation. Furthermore, the fact of cells to determine phosphorylated STAT1 will be transforming to either homodimerize or heterodimerize is yet to be understand. Thus, the main for this research project is to investigate the IFN-induced DNA binding complexes by type I and type II IFNs in certain cell types.

MATERIALS AND METHODS

2.1 Materials

2.1.1 Buffer solutions

All aqueous solutions were prepared in deionised water. pH value of the solutions were adjusted with pH-Meter 540 GLP (WTW, Weilheim).

2.1.2 Chemicals

Standard laboratory chemicals were purchased from Sigma-Aldrich, Merck, Carl Roth GmbH, Fluka, Qiagen, Amersham-Biosciences, Roche, Bio-rad, Melford and Fisher Scientific. Radiolabelled nucleotides α -[^{32}P] dNTPs (α -[^{32}P] dATP, α -[^{32}P] dCTP, α -[^{32}P] dGTP and α -[^{32}P] dTTP; 10 mCi/mL; 3000 Ci/mmol) were purchased from PerkinElmer.

2.1.3 Enzymes

Klenow fragment of DNA polymerase I was purchased from New England Biolabs (NEB). Complete protease inhibitors were purchased from Roche.

2.1.4 DNA primers and oligonucleotides

Oligonucleotides used for electrophoresis mobility shift assay were purchased from Sigma-Aldrich. The sequences of oligonucleotides are stated below. 1x GAS (M67) forward, 5'-CGACATTTCCCGTAAATCTG-3' and reverse, 5'-CAGATTTACGGGAAATGTCG-3'; 1x ISRE (ISG15) forward, 5'-GGGAAAGGGAAACCGAAACTGA-3' and reverse, 5'-TTCAGTTTCGGTTTCCCTTCCC. All the oligonucleotides has 5'-ACGT-3' overhangs at 5' ends were used.

2.1.5 Cell lines and cell culture reagents

Cell lines used in this project are shown in table 2.1

Table 2.1 Cell lines used in this project

Cell line	Species	Description	Supplier
HeLa	Human	Epitheloid cervix carcinoma	ATCC
2fTGH	Human	Fibrosarcoma	ECACC

HepG2 (HB-8065)	Human	Hepatocellular carcinoma	ATCC
MCF7 (HTB-22)	Human	Breast cancer cell	ATCC
RAMOS	Human	B lymphocyte	Dr. Ian Daniels (Nottingham University Hospitals Trust, NHS)
Jurkat	Human	T lymphocyte	ATCC
Macrophage S2 WT	Mouse	Immortalized	Drs. William
Macrophage S2 ^{-/-}	Mouse	bone marrow-derived macrophages	Alazawi & Ana Gamero (Bart's Health NHS Trust)

Cell culture reagents such as Dulbecco's Modified Eagle Medium (DMEM), Phosphate buffered saline (PBS), fetal calf serum (FCS), Penicillin Streptomycin (Pen/Strep) solution, Roswell Park Memorial Institute (RPMI) were purchased from Sigma. Culture dishes, flasks, plates were obtained from Corning (United States of America).

2.1.6 Antibodies

Primary antibodies used in this project are stated in the table 2.2

Table 2.2 Primary antibodies used in this project

Name of antibodies	Species	Supplier
anti-Tyr701-phosphorylated STAT1 (D4A7; #7649)	Rabbit, monoclonal	Cell Signalling Technology
anti-STAT1 (#610116)	Mouse, polyclonal	BD Transduction Laboratories
anti-Tyr688-phosphorylated STAT2	Rabbit, polyclonal	EMD Millipore

Anti-human STAT2 (C20; sc-476)	Rabbit, polyclonal	Santa Cruz Biotechnology
Anti-mouse STAT2 (L20; sc-950)	Rabbit, polyclonal	Santa Cruz Biotechnology
Anti-Tyr705-phosphorylated STAT3	Rabbit, monoclonal	Cell Signalling Technology
Anti-STAT3 (C20; sc-482)	Rabbit, polyclonal	Santa Cruz Biotechnology
Anti- β Actin (#A5441)	Mouse, monoclonal	Sigma-Aldrich
Anti-ISGF-3 γ p48 / anti-IRF9(C20; sc-496)	Rabbit, polyclonal	Santa Cruz Biotechnology

IgG secondary antibodies used in this project were IRdye800CW-conjugated anti-rabbit (#926-32213) and Alexa Fluor® 680 goat anti-mouse (#A-21057) which were purchased from Li-Cor Bioscience and Thermo Fisher Scientific respectively.

2.1.7 Common buffer recipes

The buffers mentioned in the table 2.3 were freshly prepared in the laboratory.

Table 2.3 Common buffers used in this project

Name of Buffer	Ingredients	Application
10x SDS running buffer	1.92 M Glycine, 250 mM Tris, 1% (w/v) SDS	SDS-PAGE
10x TBS	1.4 M sodium chloride, 100 mM Tris-HCl pH 7.3	WB
TBS-T	1x TBS, 0.05% (v/v) Tween-20	WB
5x TBE	0.7 M Tris-HCl pH 8.0, 0.44 M Boric Acid, 10 mM EDTA	EMSA

10x Blotting buffer	0.48 M Tris, 13 mM SDS, 0.39 M Glycine	WB
1x Blotting buffer	1x Blotting buffer, 20% (v/v) Methanol	WB
6x SDS sample buffer	7 mL Stacking gel buffer (4x), 3 mL glycerol, 1 g SDS, 0.93 g dithiothreitol (DTT), 1.2 mg bromphenol blue, top up to 10 mL final volume	SDS-PAGE
4x Resolving gel buffer	1.5 M Tris-HCl pH 8.8, 0.4% (w/v) SDS	SDS-PAGE
4x Stacking gel buffer	0.5 M Tris-HCl pH 6.8, 0.4% (w/v) SDS	SDS-PAGE
Whole cell extraction buffer	50 mM Tris-HCl pH 7.4, 2 mM EGTA, 0.2 mM EDTA, 1 mM Na ₃ VO ₃ , 50 mM NaF, 280 mM NaCl, 10% (v/v) Glycerol, 1 mM DTT, 0.5% (v/v) IGEPAL, 0.1 mM (phenylmethylsulfonyl fluoride) PMSF, Complete protease inhibitor	Whole Cell Extraction
Cytoplasmic extraction buffer	10 mM KCl, 1 mM EDTA, 20 mM HEPES-OH pH 7.4, 0.1 mM Na ₃ VO ₃ , 10% (v/v) Glycerol, 1 mM DTT, 0.2% (v/v) IGEPAL, 0.1	Cytoplasmic extraction

	mM PMSF, Complete protease inhibitor	
Nuclear extraction buffer	420 mM KCl, 1 mM EDTA, 20 mM HEPES-OH pH 7.4, 0.1mM Na ₃ VO ₃ , 10% (v/v) Glycerol, 1mM DTT, 0.1 mM PMSF, Complete protease inhibitor	Nuclear extraction
Western blot reprobinding buffer (Stripping buffer)	25 mM Glycine, 2% (w/v) SDS, adjust pH with HCl until pH 2.0	WB
Blocking solution	4% (w/v) Milk in 1x TBS	WB
5x EMSA binding buffer	100 mM HEPES, 20% (v/v) Ficoll, 5 mM MgCl ₂ , 200 mM KCl, 0.5 mM EGTA, 2.5 mM EDTA, adjust pH with HCl until pH 7.9	EMSA
Oligobuffer	20 mM Tris-HCl pH 7.5, 10 mM MgCl ₂ , 50 mM KCl	DNA methods

2.1.8 Software

Odyssey 2.1 (LI-COR Biosciences) was used to visualize nitrocellulose membranes from western blots with infrared dye-conjugated antibodies as well as for the quantification of band intensity. Raw data quantifications and normalization were recorded in Microsoft Excel spreadsheet (Microsoft). SPSS (Statistical analysis software package, IBM) and Prism4 (GraphPad Software) were used for statistical analysis of western blot, EMSA and

statistical tests. The Image J software was used to quantify band intensity of EMSA.

2.2 Methods

2.2.1 Cell Culture techniques

2.2.1.1 Subculturing cell line

Cell lines used in this project were listed in Table 2.1. All cell lines were kept at 37°C in humidified incubator with 5% CO₂ (Panasonic). Cells (except Jurkat and RAMOS) were grown in DMEM supplemented with 10% (v/v) heat deactivated FCS and 1% (v/v) penicillin/streptomycin. Jurkat and RAMOS cell line were cultivated in RPMI medium supplemented with 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin. Subculture took place in every 2 to 3 days when adherent cells were grown to 90% confluence whereas suspension cells were grown to not more than 1×10^6 viable cells/mL. In order to detach adherent cells, sterile Phosphate Buffered Saline (PBS, Sigma) was used to remove traces of DMEM and were incubated at room temperature (RT) in Trypsin/EDTA (Sigma) for 5 minutes. DMEM was added to stop trypsinization.

Freezing cells

Detachment of cells by trypsinization when cells confluence reached 80-90% under standard grown conditions. Cells were pelleted by centrifugation at 1000x g for 5 minutes at RT and resuspended in appropriated volume of freezing medium (FCS, 10% (v/v) dimethylsulfoxide (DMSO)). These cells were then transferred to cryo-vials (Greiner Bio-One, Germany) which were stored in a cryo freezing container (Nalgene, Neerijse Belgium) that filled with isopropanol. The cells were stored in liquid nitrogen after incubation overnight at -80°C.

Thawing cells

Frozen cells were thawed rapidly at 37°C in a water bath, followed by centrifugation at 1000x g for 5 minutes at RT. Cell pellets were resuspended in appropriate volume of DMEM containing 10% FCS and 1% penicillin/streptomycin. Culture medium was changed to remove traces of DMSO on the following day.

2.2.1.2 Cytokines treatment of cells

Human IFN- β (#11415-1; PBL Assay Science), IFN- γ (#407306; Calbiochem), mouse IFN- β (#12401-1; PBL Assay Science) and IFN- γ (#407320; Calbiochem) were used to stimulate the cells in table 2.1. The cells were incubated with 500 U/mL IFN- β or 50 U/mL IFN- γ for 1 hour in culture medium at 37°C. Priming of cells was performed by culturing them for 24 hours in culture medium with 10 U/mL IFN- β or 1 U/mL IFN- γ . After 24 hours of priming, cells were treated with standard IFN stimulation. Meanwhile, the unprimed cells were cultured in culture medium.

2.2.2 Cell extraction

Preparation of whole cell extracts

Adherent cells were allowed to grow up to 90% confluence. The cells were washed with PBS to remove traces of DMEM and 120 μ L of whole cell extraction buffer (WCE) was added into each well. The cells were then scraped off from the culture plate with a cell scraper (TPP, Switzerland). Cell lysates were transferred into a pre-cold Eppendorf tubes and incubated on ice for 30 minutes. Subsequently, cell lysates were centrifuged at 16,000x g for 15 minutes at 4°C. Supernatants were transferred into a fresh tube.

Suspension cells were pipetted gently into a conical tube and centrifuged at 250x g for 5 minutes at RT. Pellets were washed with PBS and transferred into Eppendorf tube. Then, the pellets were centrifuged at 16,000x g for 5 minutes at 4°C and 120 μ L of WCE buffer was added to lyse the pellets. After incubation on ice for 30 minutes, cell lysates were then centrifuged at 16,000x g for 15 minutes at 4°C. Supernatants were transferred into a fresh tube.

Concentration of supernatants of both adherent and suspension cells were determined by Bradford method (Bradford, 1976). The whole cell extracts were kept at -80°C for further analysis.

Preparation of cytoplasmic and nuclear extracts

Cytoplasmic and nuclear extracts were combined for DNA binding complexes analysis by EMSA. It was similar to the preparation of whole cell extraction except two extraction buffers were used. 100 μ L of cytoplasmic extraction

buffer (table 2.3) was used to lyse the cells. After 5 minutes of incubation on ice, centrifugation of 16,000x g for 30 seconds, 4°C was performed. Cell pellets was recognized as nuclei while the supernatants were transferred into fresh tube, centrifuged again at 16,000x g for 5 minutes, 4°C. The supernatants were used as cytoplasmic extracts. Equal volume of nuclear extraction buffer was added to dissolve the nuclei pellets. After 30 minutes of incubation on ice, cell lysates were centrifuged at 16,000x g for 15 minutes, 4°C and the supernatants were used as nuclear extracts. Both cytoplasmic and nuclear extracts were combined and the protein concentration of extracts were determined by Bradford method (Bradford, 1976). Cyto-nuclear extracts were stored at -80°C for further analysis.

2.2.3 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Protein extracts were separated on one dimensional, vertical, discontinuous SDS-polyacrylamide gel (Laemmli, 1970). The acrylamide concentration was adjusted to 10% in the resolving gel buffer using the 30% acrylamide/bisacrylamide 37.5:1 mixture (Sigma). After degassing the gel buffer mixture, 0.01% (w/v) ammonium persulfate (APS, Fluka) and 0.1% (v/v) N,N,N',N'-Tetramethylethylenediamine (TEMED, Sigma) were added. Stacking gels were then added on top of the layer of polymerized resolving gel. The composition of stacking gel was 3.3% (v/v) acrylamide in stacking gel buffer. 6x SDS sample buffer was added to the protein extracts (Method 2.2.2) and boiled at 95°C for 4 minutes. Electrophoresis was performed with SDS running buffer at 120V for 15 minutes followed by 160V for 1 hour or when bromphenol blue tracking dye has reached the bottom of resolving gel. Protean®II electrophoresis chambers (Biorad) and electrophoresis power supply EPS E835 (Consort; Turnhout, Belgium) was used to conduct the experiment. The pre-stained molecular weight marker SeeBlue® Plus2 (Invitrogen) was used as a molecular weight reference.

2.2.4 Immunoblotting / Western Blot analysis

Western blot analysis was used to investigate the immunodetection of proteins. Samples were first separated by SDS-PAGE and then transferred to a nitrocellulose membrane (Protran, Whatman) with semidry system. Blotting took place at 16V for 1 hour with the Trans-Blot®SD Semi-Dry Transfer Cell

System (Biorad) and electrophoresis power supply EPS E835 (Consort). Membranes were then incubated for 1 hour at RT in blocking solution. The primary antibody was added at a standard dilution of 1:1000 (anti-human STAT2 and anti- β actin which were 1:4000 and 1:1500 respectively) in TBS-T and incubated for 1 hour at RT or overnight at 4°C on rotary shaker. The membranes were then washed three times with TBS-T for 5 minutes at RT. Secondary antibody with respective species was added at standard dilution of 1:4000 in TBS-T for 40 minutes at RT on rotary shaker. Then, the membranes were washed three times for 5 minutes with TBS-T and once with TBS. They were then taken to be scanned with the Odyssey Infrared Imaging System (LI-COR Biosciences).

Stripping buffer was added to membranes with β -mercaptoethanol (β -MeOH) in order to strip off the primary and secondary antibodies. The membranes were incubated for 1 hour at 65°C in water bath with one buffer change after 30 minutes. The membranes were then washed three times with TBS and blocked for 1 hour in blocking solution prior adding the new primary and secondary antibodies.

Three different SDS- Polyacrylamide gels were combined to present most of the western blot results (otherwise stated) in the results section. Protein extract of cells from different treatments was loaded in equal amount for three gels. Electrophoresis and Western blotting were performed at the same time and condition for all the gels. After blocking with blocking solution, Membrane A was probed with anti-Tyr701-phosphorylated STAT1, anti-STAT1 and anti-Tyr688-phosphorylated STAT2; Membrane B was probed with anti-Tyr705-phosphorylated STAT3; and Membrane C was probed with anti-IRG9. After developed images using Odyssey Infrared Imaging System, the membranes were incubated in stripping buffer, followed by blocking solution. Membrane A was reprobed with anti-human STAT2 or anti-mouse STAT2 and anti- β Actin; Membrane B was reprobed with anti-STAT3 and anti- β Actin; and Membrane C was reprobed with anti- β Actin. All the images generated from membranes were then composed and combined into one figure. Since Membrane A, B and

C were probed with anti- β Actin as a loading control and the band intensities were similar, anti- β Actin image from Membrane A was used.

2.2.5 Electrophoretic mobility shift assay (EMSA)

EMSA was used to study the DNA binding activity of protein extracts (Fried and Crothers, 1981). The DNA probes used in this project were GAS site DNA probe and ISRE site DNA probe. The sequences of oligonucleotide were mentioned in material 2.1.4

Annealing of oligonucleotide into double stranded DNA probes

50 μ L of each complementary oligonucleotide (100 pmol/ μ L) diluted in oligobuffer (table 2.1.7) was combined in 1:1 ratio to a total volume of 100 μ L and denatured at 95°C in water bath for 5 minutes. Annealing was completed by cooling down the water bath to RT. The double stranded DNA probes were stored at -20°C.

Labelling of probes with radioactive nucleotides

1.0 μ L of double stranded DNA probes (1 pmol/ μ L) were labelled with 8.0 μ L of each radiolabelled nucleotides, 5.0 μ L 10x Klenow enzyme buffer (NEB) and 1.0 μ L Klenow-fragment of *E.coli* DNA polymerase I (NEB) to a total volume of 50 μ L. The mixture was then incubated for 25 minutes at RT. 1.0 μ L of non-radioactive dNTPs (dATP, dTTP, dCTP and GTP, 6.5 mM each) were then added to the mixture, followed by 5 minutes incubation at RT. Subsequently, 1.0 μ L 0.5M EDTA was added to stop the labelling reaction. MicroSpinTM G-25 columns (GE Healthcare) were used to separate the radiolabelled probes from free radioactive nucleotides according to the manufacturer's instructions. The radiolabelled probes were stored at 4°C.

EMSA with protein extracts

For the preparation of two 4.9% gels, 4.8 mL 5x TBE buffer (table 2.3), 12.0 mL 40% Acrylamide/bis-Acrylamide [29:1 (Sigma)] and 81.4 mL H₂O were mixed and degassed. After degassing the gel, 2.0 mL 10% (w/v) APS and 100 μ L TEMED were added to begin the polymerization of non-denaturing polyacrylamide gels. Prior loading DNA protein mixtures, non-denaturing

polyacrylamide gels were pre-equilibrated in 0.25x TBE buffer by running at 400V and 4°C for 3 hours and 30 minutes.

Cytoplasmic and nuclear extracts were prepared. 5 µL of the protein extracts were incubated in 1x EMSA binding buffer with 2 µg poly (dIdC) (Sigma), 8 mM DTT, 5 µL of radiolabelled probes in total volume of 12.5 µL for 15 minutes at RT to allow the DNA protein complex formation. DNA protein complexes were resolved at 400V and 4°C on 4.8% non-denaturing polyacrylamide gels by using 0.25x TBE buffer.

After the process of electrophoresis, the gels were transferred to Whatman paper (GE Healthcare) and covered with plastic wrap. Drying for 1 hour at 85°C on a vacuum gel drier (Biometra, Gottingen) was performed to eliminate diffusion of radiolabelled probes and improve the quality of the band. Dried gels were then exposed to a phosphoimager screen (Fujifilm) and incubated overnight at RT. Gel images were developed using phosphoimager (FLA-3000 Fujifilm) and analysed using Advanced Image Data Analyzer (AIDA; Amersham).

RESULTS

3.1 IFN- β priming induced ISGF3-DNA binding complex in 2fTGH but not HeLa

First, DNA binding activities in HeLa and 2fTGH cells were examined. As expected, an hour stimulation of IFN- β induce the formation of STAT1:STAT1 homodimers complex (GAF) in both cells (Figure 3.1A, lane 4 and 8). On the other hand, there was no ISGF3 DNA complex induced in HeLa under IFN- β stimulation (Figure 3.1B, lane 4). The formation of ISGF3 DNA complex was showed in 2fTGH cells, but the concentration of these complex was too low to be detected (Figure 3.1B, lane 8). IFN- β priming did not make any difference between the intensity of GAF in both primed and unprimed cells (Figure 3.1A, HeLa: compare lane 2 and 4; 2fTGH: compare lane 6 and 8) despite there was an increase in expression and activation of STATs in HeLa (Figure 3.1C). Although IFN- β priming showed to increase the concentration of IRF9 in HeLa and 2fTGH, no DNA binding activities of ISGF3 complex in HeLa had been noticed (Figure 3.1B, lane 2). However, there was ISGF3 complex detected in 2fTGH primed cell (Figure 3.1B, lane 6). The reason of the primed HeLa did not show ISGF3 DNA binding complex was suggested that the stability of DNA protein complex is weaker in HeLa than that in 2fTGH or HeLa required more activated (phosphorylated) STAT1s, STAT2s and IRF9 in order to form ISGF3 complex. Western blot analysis showed that IFN- β priming did increase the expression and activation of STAT1, STAT2 and STAT3 in HeLa but not 2fTGH cells. Therefore, these results conclude that increased level of ISGF3 components does not improve the stability of ISGF3 complex binds to DNA sequence.

3.2 Jurkat and RAMOS did not induce ISGF3-DNA binding complex

Cell type specific DNA binding activities was carried out. Jurkat and RAMOS cells were investigated. Similar to HeLa and 2fTGH, GAF DNA binding activity was observed in both Jurkat and RAMOS primed and unprimed cells after an hour of IFN- β stimulation (Figure 3.2A). It was surprising to found out that no ISGF3 DNA complex was detected in Jurkat and RAMOS cells, neither in primed nor unprimed state (Figure 3.2B). Referring to western blot result (Figure 3.2C), the activation of STAT2 was lower in Jurkat cells than in

RAMOS cells. In addition, RAMOS cells had more STAT2 expression than Jurkat cells. IFN- β priming increased both concentration of STAT1 and IRF9 in both cells. However, the level of phosphorylated STAT1 remained unchanged. The expression and activation of STAT3 in both cells were not affected by IFN- β priming. In summary, Jurkat and RAMOS do not induce ISGF3-DNA binding complex regardless of the concentration pSTAT1, pSTAT2 and IRF9 presented in cells.

3.3 STAT2 mediate GAF DNA binding activity

DNA binding activities of mouse macrophage STAT2 wild type (STAT2 WT) and STAT2 deficient cells (STAT2^{-/-}) was studied. Figure 3.3A showed that GAS DNA binding complex was formed among the cells, either in prime or unprimed IFN-stimulated cells. However, in STAT2^{-/-} cells, the intensity and concentration of GAS DNA complex was lower than STAT2 WT cells. On the other hand, a clear and sharp band was detected in EMSA gel containing ISRE probe in macrophage STAT2 WT cells (Figure 3.3B lane 2 and 4). As expected, there was no ISRE binding activity found in macrophage STAT2^{-/-} cells. Western blot result (Figure 3.3C) revealed that IFN- β priming only increased the expression of STAT1 in macrophage STAT2 WT. Increased expression of STAT1 did not followed by the increased phosphorylation of STAT. The expression and activation of STAT2 in macrophage STAT2 WT remained unchanged after IFN-priming. The expression of STAT1 in macrophage STAT2^{-/-} was lower than macrophage STAT2 WT. It was interesting to find out that IFN- β stimulation activated less STAT1 as compared to IFN- γ stimulation in macrophage STAT2^{-/-} (Compare lane 8 and 9; lane 11 and 12). The reduced concentration of pSTAT1 in IFN- β stimulation resulted in the decreased DNA binding activity of GAF in macrophage STAT2^{-/-} cells. Priming effect on macrophage STAT2^{-/-} was negligible. In brief, these results showed that STAT2 mediated the expression of STAT1 and had an impact on the GAF DNA binding activity.

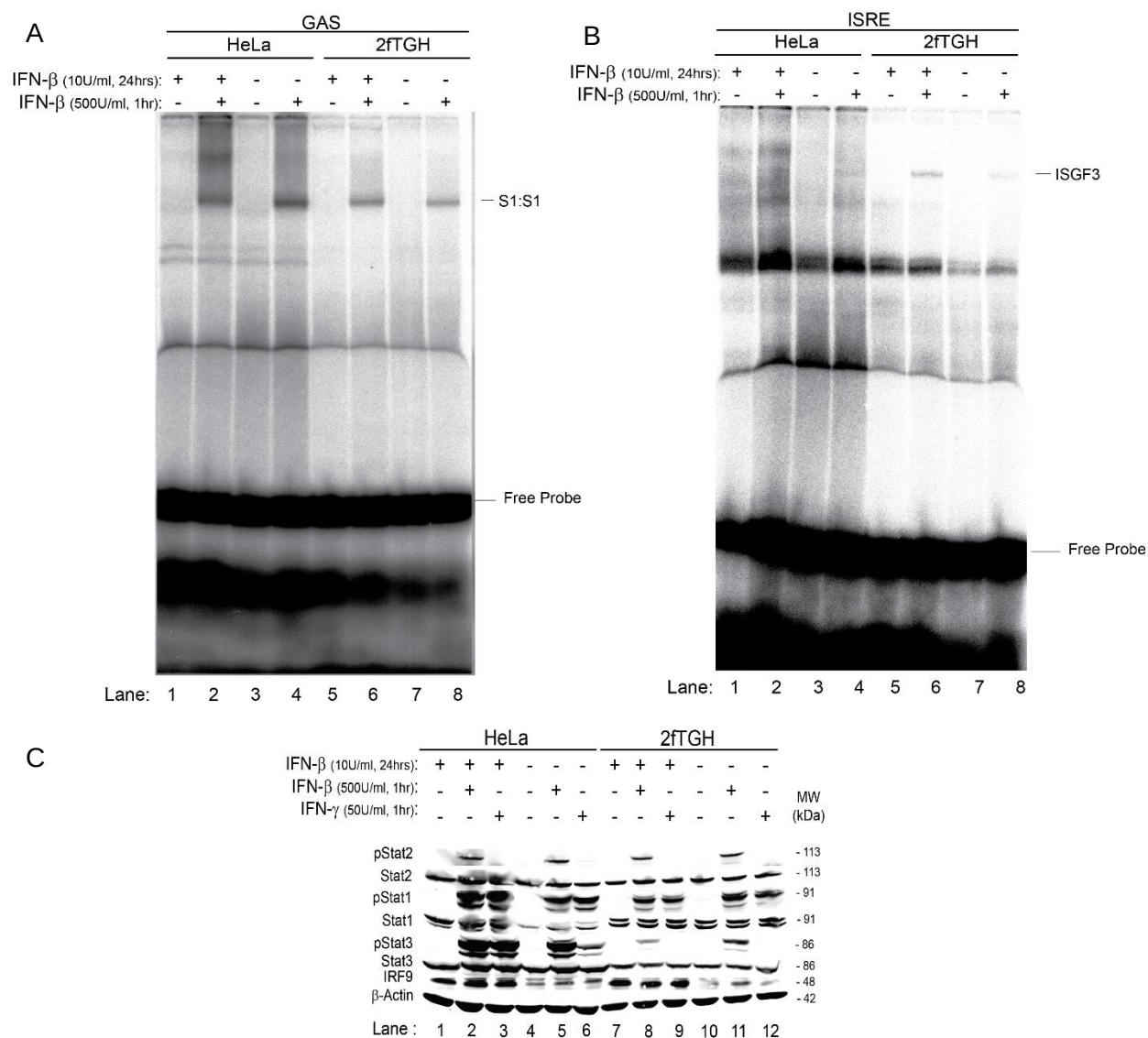


Figure 3.1 IFN- β priming induces ISGF3-DNA binding complex in 2fTGH but not 2fTGH. EMSA using probes containing GAS (A) or ISRE (B) with extracts from HeLa and 2fTGH primed or unprimed with IFN- β for 24hrs followed by treatment of IFN- β for 1hr or left untreated. The positions of GAF (S1:S1) and ISGF3 complex are labelled. (C) Western blot analysis of phosphorylated STAT1 at Tyr701 (pStat1), total STAT1 (Stat1), phosphorylated STAT2 at Tyr688 (pStat2), total STAT2 (Stat2), phosphorylated STAT3 at Tyr 705 (pStat3), total STAT3 (Stat3), IRF9 and β -Actin in HeLa and 2fTGH primed or unprimed with IFN- β for 24hrs followed by treatment of IFN- β or IFN- γ for 1hr or left untreated. β -Actin was used as loading control. MW, molecular weight. The western blot image in (C) were composed of three different polyacrylamide gels (refer Methods 2.2.4). Experiment was performed only once.

3.4 DNA binding complex of STAT3 homodimers and STAT3:STAT1 heterodimers cannot be detected in HepG2 cells.

HepG2 and MCF7 cells were the last pairs of cell to be tested in this project. GAF DNA complex and ISGF3 DNA complex were observed on EMSA gel containing GAS probe and ISRE probe respectively in both HepG2 and MCF7 cells after IFN- β priming for 24hrs prior an hour of IFN- β stimulation (Figure 3.4A). Concentration of GAF in primed MCF7 cells was two-fold higher than primed HepG2 cells (Figure 3.4A, lane 2 and 4). Referring to western blot result (Figure 3.4B), there was approximately three-fold of phosphorylated STAT1 was detected in MCF7 cells than HepG2 cells. These result correlates with the finding of concentration of GAF DNA binding complex. In addition, the concentration of phosphorylated STAT2, phosphorylated STAT1 and IRF9 was lower in HepG2 as compared with MCF7, resulted in band intensity of ISGF3 DNA complex. IFN- β priming did not increase the concentration of STATs protein and IRF9 in HepG2 cells. Moreover, priming did increase the expression and activation of STAT1 and concentration of IRF9 in MCF7 cells. Remarkably, no STAT3 homodimers and STAT3:STAT1 heterodimers were not detected in HepG2. In short, these results demonstrated that MCF7 cells are more capable of activating STATs protein and trigger the formation of IFN-induced DNA binding complex than HepG2 cells.

3.5 Triple bands formation in HeLa cells

By comparing Figure 3.1, 3.2, 3.3 and 3.4, a smear background was observed above the GAF DNA complex. It indicated that there were some DNA binding complexes migrate slower than GAF DNA complex. If the smear background is able to be resolved, new DNA binding complex(s) would possibly able to be detected. DNA binding activities experiment was studied again. HeLa cells were primed with either IFN- β or IFN- γ for 24hrs followed by 1hr stimulation of IFN- β or IFN- γ or left untreated. Figure 3.5 revealed that there were three DNA binding complexes detected in unprimed, IFN- β stimulated HeLa cells (lane 6). Zhong and his colleagues (1994) showed that the three DNA binding complexes were STAT3 homodimers, STAT3:STAT1 heterodimers and STAT1 homodimers (GAF). In contrast, lane 2 and lane 4 showed similar DNA binding pattern with Figure 3.1A, 3.2A, 3.3A and 3.4A. In addition, a GAF

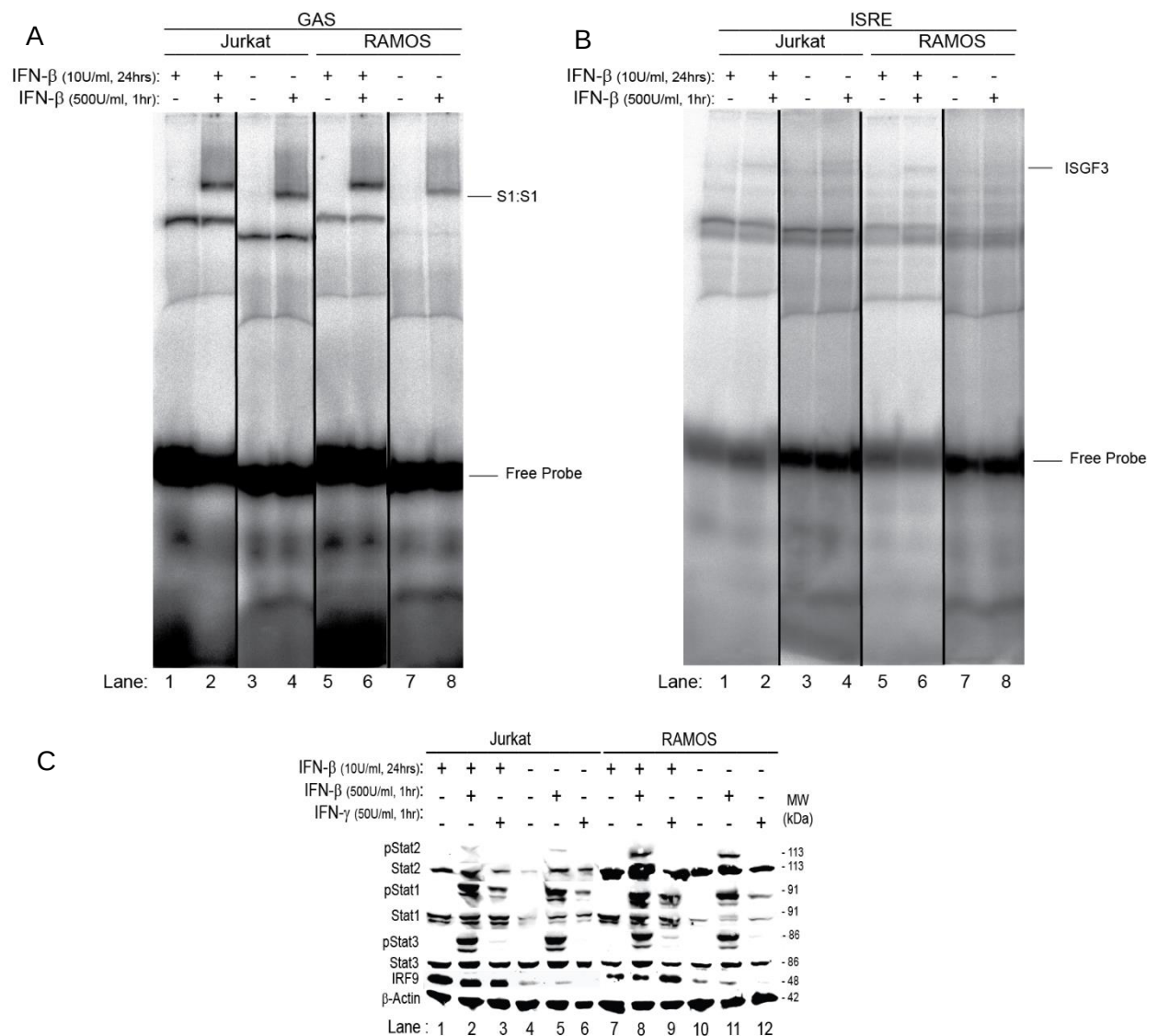


Figure 3.2 Jurkat and RAMOS do not form ISGF3-DNA binding complex. EMSA using probes containing GAS (A) or ISRE (B) with extracts from Jurkat and RAMOS primed or unprimed with IFN- β for 24hrs followed by treatment of IFN- β for 1hr or left untreated. (C) Western blot analysis of phosphorylated STAT1 at Tyr701 (pStat1), total STAT1 (Stat1), phosphorylated STAT2 at Tyr688 (pStat2), total STAT2 (Stat2), phosphorylated STAT3 at Tyr 705 (pStat3), total STAT3 (Stat3), IRF9 and β -Actin in Jurkat and RAMOS primed or unprimed with IFN- β for 24hrs followed by treatment of IFN- β or IFN- γ for 1hr or left untreated. The positions of GAS (S1:S1) and ISGF3 are indicated. EMSA image (A and B) were composed of two different EMSA gels, lane 1-2 and 5-6 were from one gel whereas lane 3-4 and 7-8 were from another gel. The experiment conditions for both EMSA gels were identical. β -Actin was used as loading control. MW, molecular weight. The western blot image in (C) were composed of three different polyacrylamide gels (refer Methods 2.2.4). Experiment was performed only once.

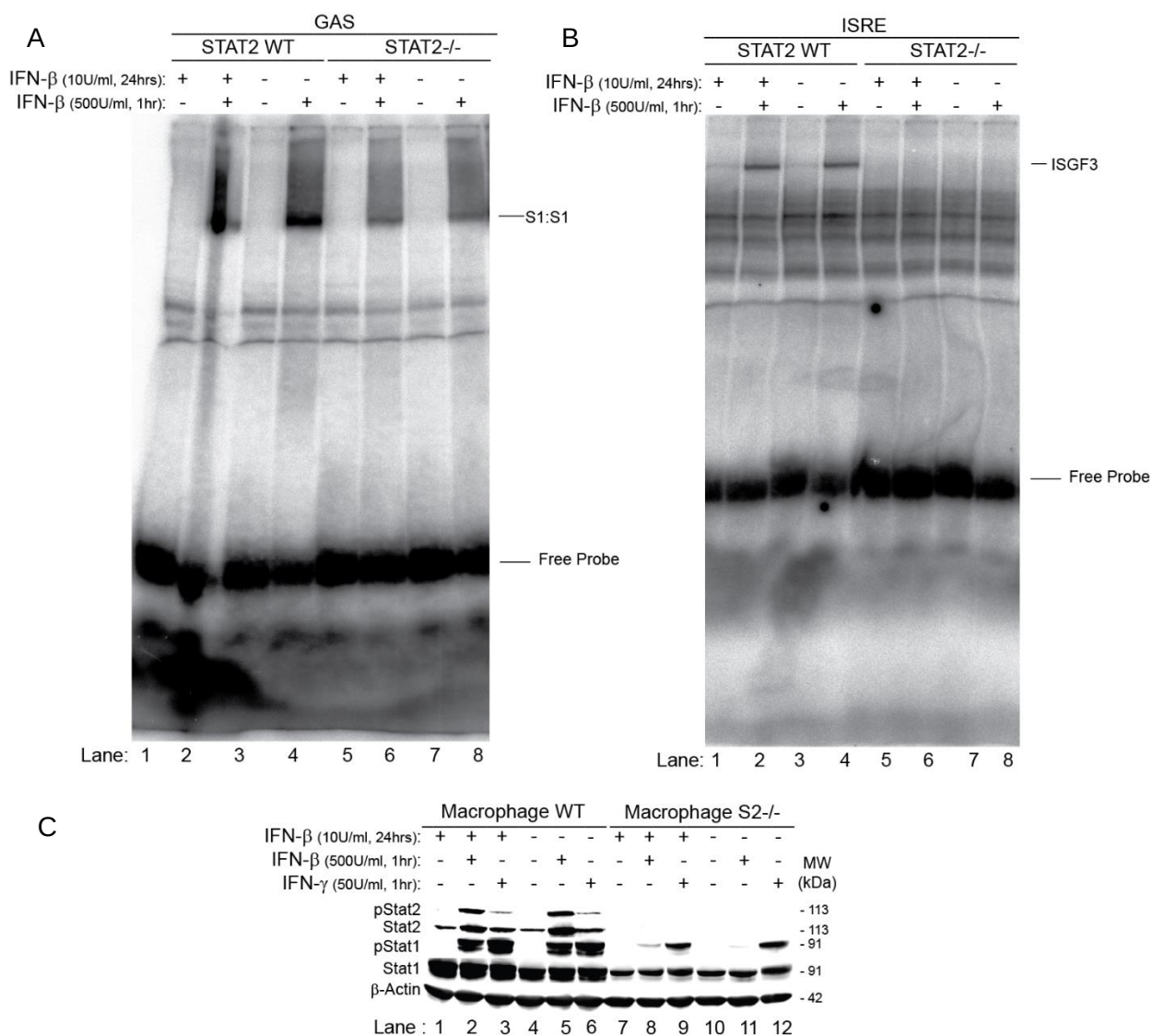


Figure 3.3 Macrophage STAT2 deficient cell (STAT2^{-/-}) reduces DNA binding activity of GAF. EMSA using probes containing GAS (A) or ISRE (B) with extracts from mouse macrophage STAT2 wild type (STAT2 WT) and STAT2 deficient cells (STAT2^{-/-}) which was primed or unprimed with IFN- β for 24hrs followed by treatment of IFN- β for 1hr or left untreated. The positions of GAF (S1:S1) and ISGF3 are indicated. (C) Western blot analysis of phosphorylated STAT1 at Tyr701 (pStat1), total STAT1 (Stat1), phosphorylated STAT2 at Tyr688 (pStat2), total STAT2 (Stat2) and β -Actin in mouse macrophage STAT2WT and STAT2^{-/-} primed or unprimed with IFN- β for 24hrs followed by treatment of IFN- β or IFN- γ for 1hr or left untreated. β -Actin was used as loading control. MW, molecular weight. Experiment was performed only once.

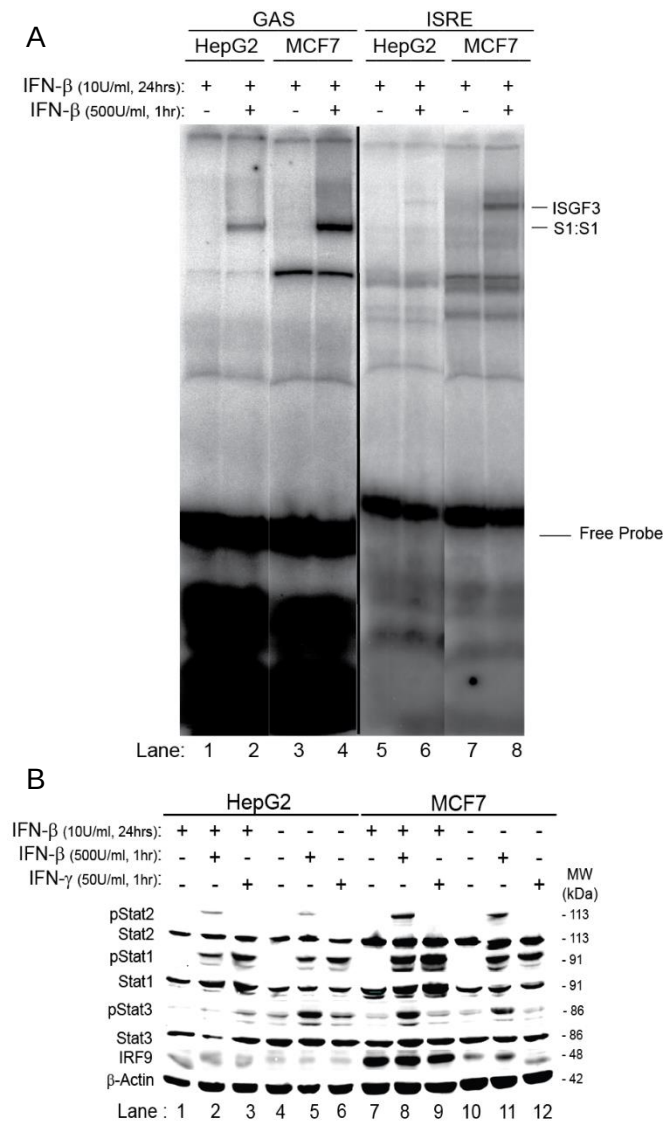


Figure 3.4 HepG2 does not induce STAT3 homodimers or STAT3:STAT1 heterodimers. (A) EMSA using probes containing GAS or ISRE with extract from HepG2 and MCF7 cells which was primed with IFN- β for 24hrs followed by treatment of IFN- β for 1hr or left untreated. The positions of GAF (S1:S1) and ISGF3 are indicated. The black vertical line shown in (A) indicated that EMSA image was composed of two different EMSA gels. One gel used GAS probe while another gel used ISRE probe. The experiment conditions for both gels were identical. (B) Western blot analysis of phosphorylated STAT1 at Tyr701 (pStat1), total STAT1 (Stat1), phosphorylated STAT2 at Tyr688 (pStat2), total STAT2 (Stat2), phosphorylated STAT3 (pStat3), total STAT3 (Stat3), IRF9 and β -Actin in HepG2 and MCF7. β -Actin was used as loading control. MW, molecular weight. The western blot image in (B) were composed of three different polyacrylamide gels (refer Methods 2.2.4). Experiment was performed only once.

DNA binding complex was detected in IFN- γ primed unstimulated cell. On the other hand, DNA binding activities with ISRE probe was examined (Figure 3.5B). As expected, IFN- γ priming increased concentration of IRF9 and activation of STAT1 and STAT2 tremendously. This lead to a formation of clear and sharp band which is ISGF3 DNA complex (Figure 3.5B, lane 2). In contrast, the concentration of ISGF3 DNA complexes in IFN- β primed and unprimed cells was lower than IFN- γ primed cells, approximately three-fold and ten-fold respectively (Figure 3.5B, compare lane 4 and 6 to lane 2). Western blot result (Figure 3.5C) further revealed the behaviour of HeLa to type I and type II IFN priming. IFN- γ priming elevated the expression of STAT1, STAT2 and IRF9 as well as the activation of STATs. The activation of STAT3 was detected in IFN-unstimulated and stimulated cell. Furthermore, activated STAT1 was also detected in IFN- γ primed unstimulated cell and this link to the formation of GAF DNA binding complex. In Figure 3.1B (lane 4) and Figure 3.5A (lane 6) showed that although the cytokine treatment of cells were similar, lane 4 showed that only GAF DNA complex had the presence of smearing background whereas in lane 6 showed the presence of three DNA binding complexes. However, in lane 2, lane 4 and lane 6 (Figure 3.5A) showed that in lane 2 and lane 4 smear background had been noticed although the experiment handling of three different treatments of HeLa cell was identical. Experimental protocol was manipulated to examine whether there are DNA binding complexes in the smearing area that migrate slower than GAF DNA complex.

3.6 Concentration of extracts does not affect the formation of three DNA binding complexes

Concentration of protein extract is modified to examine whether it contributes to the formation of DNA binding complexes. HeLa cells from Results 3.5 were used. The IFN-stimulated protein extract was diluted with IFN-unstimulated protein extract. Serial dilution of protein extract was loaded onto the ESMA gel. Figure 3.6 showed that concentration of protein extract did not affect the activity of DNA binding. More diluted reaction mixture gave a clearer background image as compare to higher concentration sample. Thus, result

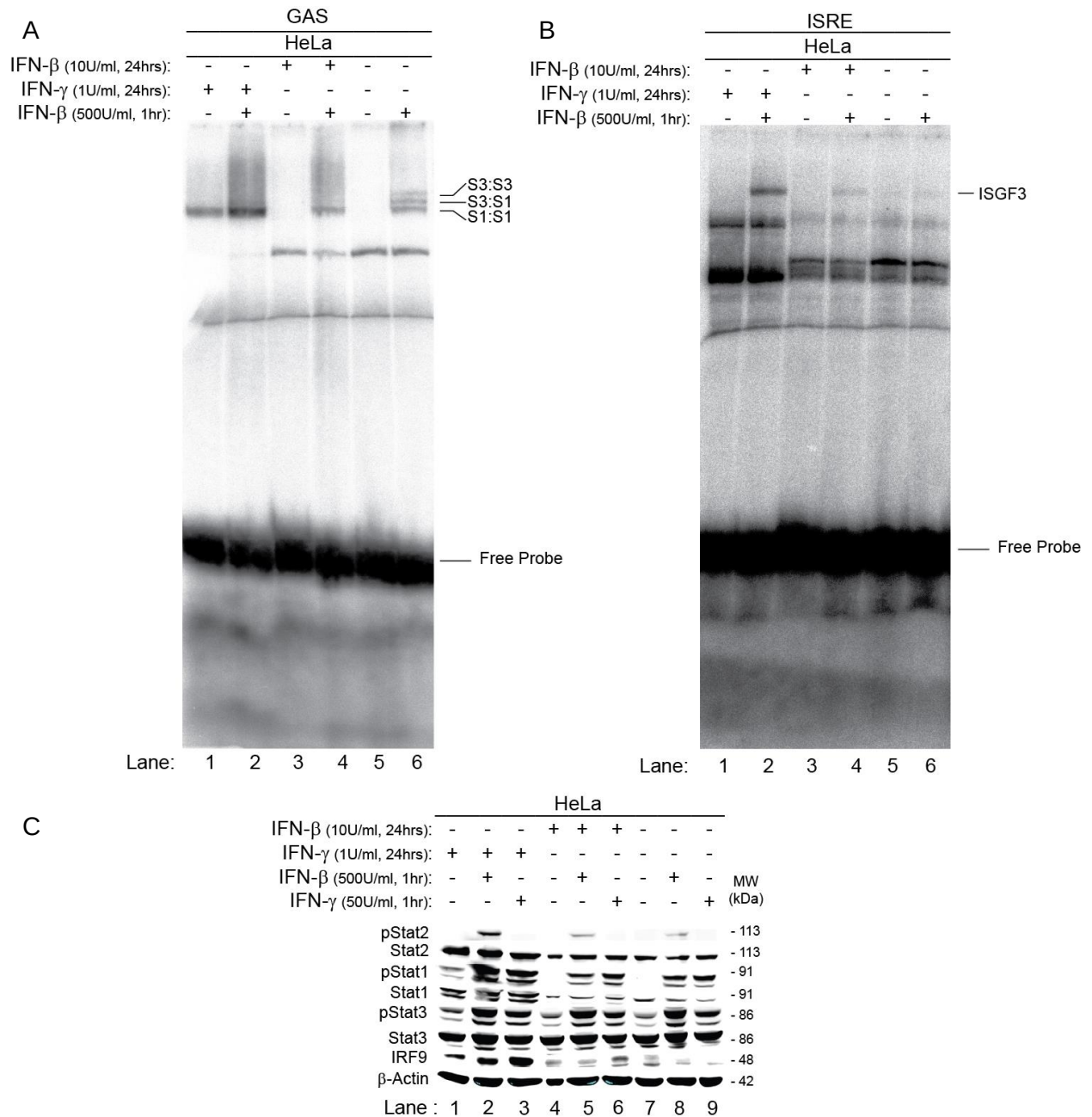


Figure 3.5. Triple bands formation was found in HeLa cells. EMSA using probes containing GAS (A) or ISRE (B) with extracts from HeLa primed or unprimed with IFN- β or IFN- γ for 24hrs followed by treatment of IFN- β for 1hr or left untreated. The positions of GAF (S1:S1), STAT3 homodimers (S3:S3), STAT3:STAT1 heterodimers (S3:S1) and ISGF3 complex are indicated. (C) Western blot analysis of phosphorylated STAT1 at Tyr701 (pStat1), total STAT1 (Stat1), phosphorylated STAT2 at Tyr688 (pStat2), total STAT2 (Stat2), phosphorylated STAT3 at Tyr 705 (pStat3), total STAT3 (Stat3), IRF9 and β -Actin in HeLa primed or unprimed with IFN- β or IFN- γ for 24hrs followed by treatment of IFN- β or IFN- γ for 1hr or left untreated. β -Actin was used as loading control. MW, molecular weight. The western blot image in (C) were composed of three different polyacrylamide gels (refer Methods 2.2.4). Experiment was performed only once.

showed that by diluting the protein extract does not induce the formation of three DNA binding complexes.

3.7 DNA binding activities are reduced by increasing the concentration of DTT

Concentration of DTT was manipulated to investigate its effect on DNA binding activities. HeLa cells were primed or unprimed with IFN- β or IFN- γ for 24hrs. Cells were then treated with or without IFN- β for an hour. In Figure 3.1-3.6, the final concentration of DTT used in both cytoplasmic and nuclear extraction buffer was 1mM. Increased final concentration of DTT in both cytoplasmic and nuclear extraction buffer was tested to study the DNA binding activities. Protein extraction with 1mM and 5mM final concentration of DTT were compared in this experiment. In Figure 3.7, only GAF DNA complex was detected in 5mM of DTT (lane 3, 6 and 9) whereas two or three DNA binding complexes were detected in the extracts that contained 1mM of DTT (lane 2, 5 and 8). In addition, increased DTT eventually decrease the DNA binding activities of the cells. Comparing both GAF DNA complexes from protein extract with different final concentration of DTT, there was less GAF DNA complexes when 5mM of DTT was used. This result clearly illustrated that increased concentration of DTT in both extraction buffers would negatively impact to the DNA binding activities of cells.

3.8 Optimization of experiment protocol to reveal three DNA binding complexes that bind to GAS elements

In Figure 3.7, there was no smear background detected in IFN-stimulated protein extract (Figure 3.7, lane 2, 3, 5, 6, 8 and 9). The difference between this experiment and the previous experiments (Figure 3.1-3.6) was protocol to prepare the protein extract (cytoplasmic and nuclear extracts). In previous experiments, the extraction buffers were prepared 30 minutes before washing the cells with PBS to remove the trace of culture medium. However, in this experiment, extraction buffers were prepared after the washing step of cells. Therefore, these results demonstrated that optimized protocol is able to robustly reveal IFN-induced DNA binding complexes.

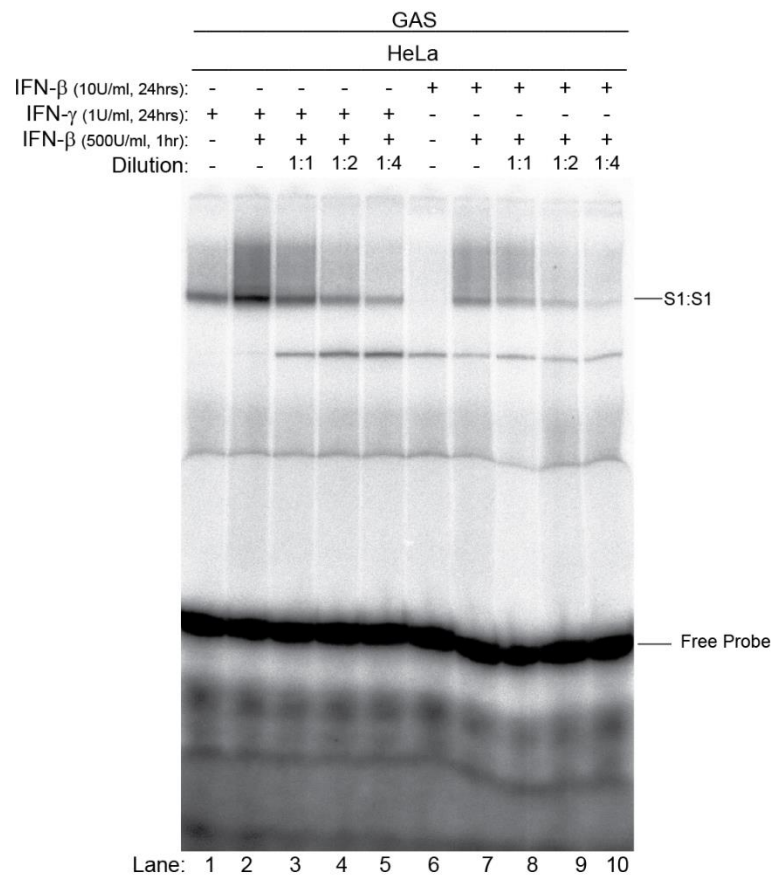


Figure 3.6. Concentration of protein extracts does not affect the formation of three DNA binding complexes. EMSA using GAS probe with extract from HeLa cells which were primed with IFN- β or IFN- γ for 24hrs followed by treatment of IFN- β for 1hr or left untreated. The position of GAF (S1:S1) DNA complex is indicated. IFN stimulated extract was diluted with untreated extract. The dilution factor was shown in the figure. Experiment was performed only once.

3.9 IFN-induced DNA binding complexes formed in macrophages

As an optimized protocol was established, the experiment results generated in each cell types (Figure 3.1, 3.2, 3.3 and 3.4) were considered as negative results. The experiments performed on the four pairs of cell was repeated with newly established protocol. First, the IFN-induced DNA binding complexes in macrophages STAT2 wild type (STAT2 WT) and STAT2 deficient cells (STAT2 $^{-/-}$) were examined. Figure 3.8A showed there were three DNA binding complexes clearly developed on the EMSA gel. As mentioned before, the DNA binding complexes that migrated the fastest was GAF, followed by STAT3:STAT1 heterodimers. The slowest migrating DNA binding complex

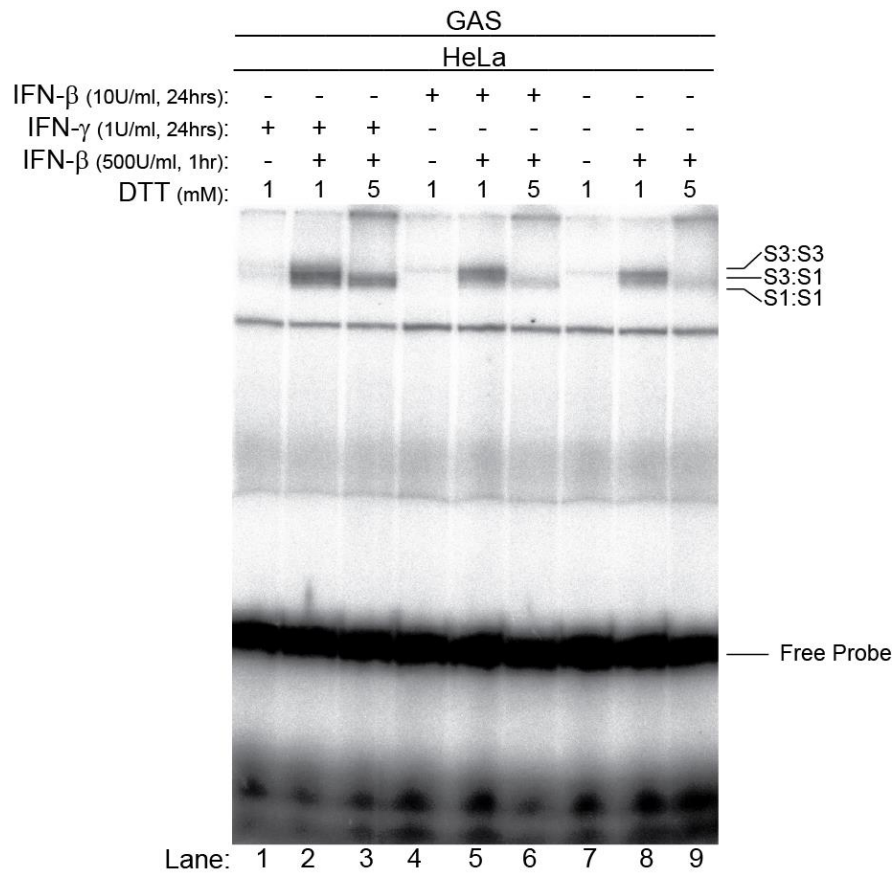


Figure 3.7 Increased final concentration of DTT reduces the DNA binding complexes of STAT3 homodimers and STAT3:STAT1 heterodimers. EMSA using probes containing GAS with extract from HeLa which was primed with IFN- β or IFN- γ for 24hrs prior an hour of IFN- β stimulation or left untreated. Final concentration of DTT used in extraction buffers and the positions of GAF (S1:S1), STAT3 homodimers (S3:S3) and STAT3:STAT1 heterodimers are indicated. Experiment was performed only once.

was STAT3 homodimers. Similar to Figure 3.3, the concentration of DNA binding complexes in wild type was more than that in STAT2^{-/-}, about 2 fold. In STAT2^{-/-} cells, the three DNA binding complexes formed equally (Figure 3.8A, lane 6 and 8). In contrast, GAF was more predominantly over STAT3:STAT1 heterodimers and STAT3 homodimers in STAT2 WT (Figure 3.8A, lane 2 and 4).

In Figure 3.8A, a DNA binding complex was detected that migrated slower than STAT3 homodimers. It was believed to be the tetramerization of STATs protein. Since Figure 3.8A was DNA binding activities induced by type I IFN (IFN- β), DNA binding activities induced by type II IFN (IFN- γ) was examined.

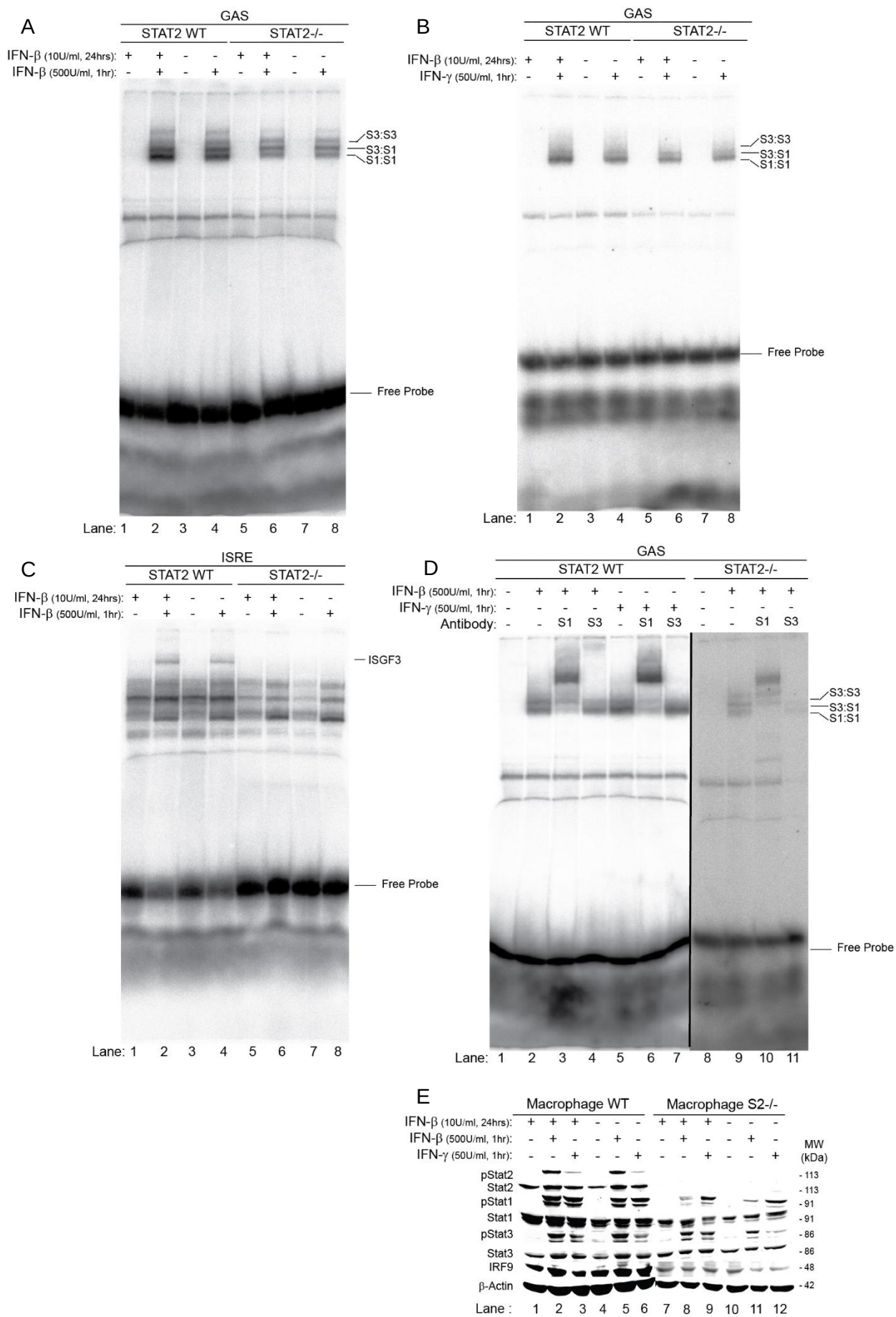


Figure 3.8. IFN-induced DNA binding complexes in macrophages. (A, B and C) EMSA using GAS or ISRE probe with extracts from mouse macrophage STAT2 wild type (STAT2 WT) and STAT2 deficient cell (STAT2^{-/-}) which were primed with IFN- β for 24hrs followed by IFN-stimulation of IFN- β or IFN- γ for 1hr or left untreated. (D) Supershift assay using GAS probe was performed on unprimed STAT2 WT and STAT2^{-/-} cells which were stimulated by IFN- β or IFN- γ for 1hr. Anti-STAT1 (S1) and anti-STAT3 (S3) antibodies were used to 'supershift' the DNA binding complexes. The ESMA image (D) were composed of two separate gels but the experimental conditions were performed identically. The positions of GAF (S1:S1), STAT3 homodimers (S3:S3), STAT3:STAT1 heterodimers (S3:S1) and ISGF3 complexes were labelled. (E) Western blot analysis of phosphorylated STAT1 at Tyr701 (pStat1), total STAT1 (Stat1), phosphorylated STAT2 at Tyr688 (pStat2), total STAT2 (Stat2), phosphorylated STAT3 at Tyr 705 (pStat3), total STAT3 (Stat3), IRF9 and β -Actin in mouse macrophage STAT2 wild type (Macrophage WT) and STAT2 deficient cells (Macrophage S2^{-/-}) which were primed with IFN- β for 24hrs followed by treatment of IFN- β or IFN- γ for 1hr or left untreated. β -Actin was used as loading control. MW, molecular weight. The western blot image in (E) were composed of three different polyacrylamide gels (refer Methods 2.2.4). Experiment was performed only once.

Figure 3.8B showed the three DNA binding complexes although the expression of the DNA binding complexes were not as obvious as Figure 3.8A. GAF was found to be more dominant than STAT3 homodimers and STAT3:STAT1 heterodimers when the cells were stimulated by IFN- γ (either primed or unprimed condition). In order to further clarify the three DNA binding complexes that bind to GAS elements were comprised of STAT3 and STAT1. Antibodies supershift assay was used. Anti-STAT1 and anti-STAT3 antibodies were added into the binding reactions which contained extract from unprimed IFN- β or IFN- γ stimulated STAT2 WT and STAT2^{-/-} cells. As shown in Figure 3.8D, the three DNA binding complexes reacted with anti-STAT1 and anti-STAT3. Assume the three DNA binding complexes to be DBC1 (the slowest migrating complex), DBC2 (the intermediate migrating complex) and DBC3 (the fastest migrating complex). DBC2 and DBC3 were reacted with anti-STAT1 whereas DBC1 and DBC2 were reacted with anti-STAT3. Anti-STAT1 totally blocked the formation of DBC3 (lane 3, 6 and 10), creating a slower DNA binding complex than DBC1. Therefore, DBC3 was determined as dimer of STAT1s. Anti-STAT1 reduced the formation of DBC2 which indicated that

DBC2 contained partial of STAT1 with another protein. Anti-STAT3 completely abrogated the formation of DBC1 (lane 4, 7 and 11), revealed this complex was consisted of STAT3 homodimers. Anti-STAT3 also reduced the formation of DBC2. Thus, these results concluded that DBC2 could be a heterodimeric complex that contained STAT3 and STAT1.

The DNA binding activity pattern using ISRE probe was identical to Figure 3.3B. It was obvious that no ISGF3 DNA complex was detected in STAT2^{-/-} cells as ISGF3 consisted of activated STAT1 and STAT2, associated with IRF9. Priming effect on macrophages was same with previous experiment. IFN- β priming only boosted the level of STAT1. The expression of STAT2, STAT3 and the activation of STATs were remained unchanged with or without IFN priming. There was no effect of priming on STAT2^{-/-} cells. It was interesting to found out that STAT2 WT cell was having a higher basal level of IRF9 than STAT2^{-/-} cell. Priming did not increase the concentration of IRF9. The high basal level of IRF9 might lead to the formation of ISGF3 DNA complex.

In summary, the three DNA binding complexes that bind to GAS element were STAT3 homodimers, STAT3:STAT1 heterodimers and GAF. In macrophage cells, GAF was able to form a more stable DNA binding complex than STAT3 homodimers and STAT3:STAT1 heterodimers because the intensity of the band formation of GAF was more intense than other two bands. Without IFN-priming for a longer period of time, macrophage WT was able to induce the ISGF3 DNA complex.

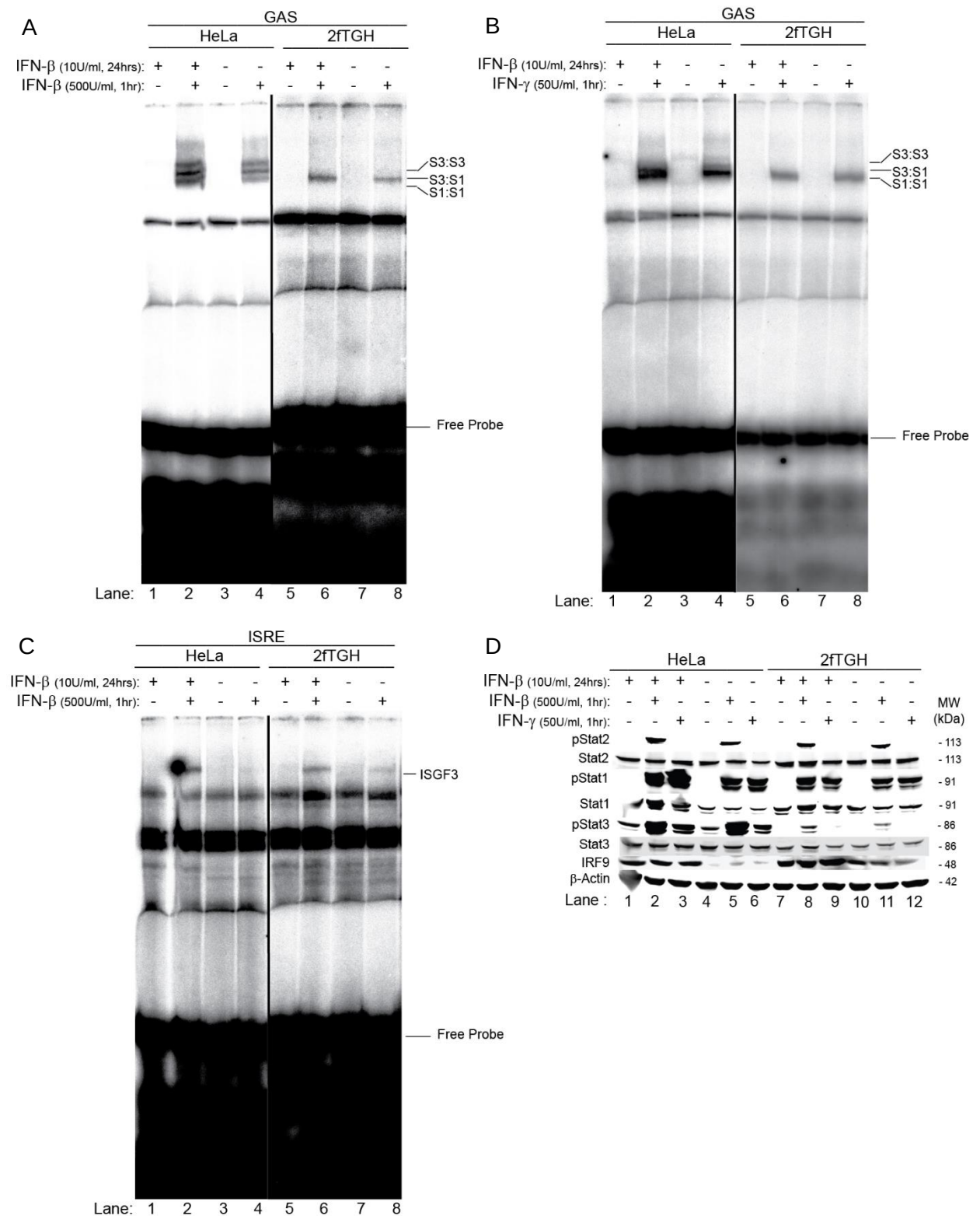


Figure 3.9 IFN-induced DNA binding complexes in HeLa and 2fTGH cells. (A, B and C) EMSA using GAS or ISRE probe with extracts from HeLa and 2fTGH cells which were primed with IFN- β for 24hrs followed by IFN-stimulation of IFN- β or IFN- γ for 1hr or left untreated. EMSA images (A, B and C) were composed of two different gels but the experimental conditions were identical. The positions of GAF (S1:S1), STAT3 homodimers (S3:S3), STAT3:STAT1

heterodimers (S3:S1) and ISGF3 complexes were labelled. (D) Western blot analysis of phosphorylated STAT1 at Tyr701 (pStat1), total STAT1 (Stat1), phosphorylated STAT2 at Tyr688 (pStat2), total STAT2 (Stat2), phosphorylated STAT3 at Tyr 705 (pStat3), total STAT3 (Stat3), IRF9 and β -Actin in HeLa and 2fTGH cells which were primed with IFN- β for 24hrs followed by treatment of IFN- β or IFN- γ for 1hr or left untreated. β -Actin was used as loading control. MW, molecular weight. The western blot image in (D) were composed of three different polyacrylamide gels (refer Methods 2.2.4). Experiment was performed only once.

3.10 IFN-induced DNA binding complexes in HeLa and 2fTGH

IFN-induced DNA binding activities in HeLa and 2fTGH were investigated again. The three DNA binding complexes that bind to GAS element were able to be resolved (Figure 3.9A and Figure 3.1A). Similar to previous experiment (Figure 3.1A), 2fTGH did not induce STAT3 homodimers and STAT3:STAT1 heterodimers. Western blot analysis revealed that low amount of phosphorylated STAT3 was able to be detected in 2fTGH (Figure 3.9D). HeLa cell contained more STAT3:STAT1 heterodimers DNA complex as compare to GAF and STAT3 homodimers when the cell was stimulated with IFN- β (Figure 3.9A). This pattern was same even though HeLa was primed with IFN- β for 24hrs. The case was totally different when it came to IFN- γ stimulation. Figure 3.9B showed that GAF DNA complex was more dominant than other two DNA protein complexes. Activated STAT3 was detected in HeLa cells which stimulated by IFN- γ or unstimulated in western blot result (Figure 3.9D). However, neither STAT3 homodimers nor STAT3:STAT1 heterodimers was able to be detected on EMSA gel. This could be explained as the stability of these two complexes were less than GAF DNA complex.

On the other hand, the DNA binding activities using ISRE probe in HeLa and 2fTGH cells were identical to previous result (Figure 3.9C and Figure 3.1B). Western blot result showed that HeLa had lower basal level of IRF9 than 2fTGH cells. This is the reason that ISGF3 DNA complex was detected in unprimed IFN- β stimulated 2fTGH cells. As expected, IFN- β priming rose the level of IRF9, resulted in formation of ISGF3 DNA complex. IFN- β priming acted different in HeLa and 2fTGH cells. Priming did increase the expression and activation of STAT1 in HeLa cells. There was no different in expression of

STAT2 and STAT3 in HeLa cells before and after priming. Similar to previous finding (Figure 3.1C), priming did not have any positive or negative effect on 2fTGH cells.

Thus, these results conclude that 2fTGH did not induce STAT3 homodimers DNA complex. Low amount of activated STAT3 might not be able to stabilize the DNA binding complex.

3.11 IFN-induced DNA binding complexes in HepG2 and MCF7

In Figure 3.4A (lane 2) only GAF DNA complex was present. The experiment was repeated. Then, in Figure 3.10A, three DNA binding complexes were detected in IFN-stimulated HepG2 cells. Moreover, STAT3 homodimers DNA complex was observed in unstimulated HepG2 cells (Figure 3.10A, lane 1 and 3). However there was no STAT3 homodimers DNA complex was found in MCF7 cells. MCF7 cells expressed more GAF DNA complex as compare to HepG2 cells. Priming did not have any effect on HepG2 but it induced one to two-fold more GAF DNA complexes on MCF7 cells. Similar result generated when comes to GAS binding complexes induced by IFN- γ (Figure 3.10B). The only difference between Figure 3.10 (A and B) regarding to DNA binding activities was a reduction in concentration of DNA binding complexes which contained STAT3 as IFN- γ does not activate STAT3 strongly. A DNA binding complex was detected and migrated slower than STAT3 homodimers could be tetramerization of STATs, as mentioned in Result 3.8.

Figure 3.10C revealed the formation of ISGF3 DNA complex in HepG2 and MCF7 cells. The ISGF3 DNA complex was barely detected in both unprimed cells. Western blot analysis (Figure 3.10D) was used as a reference to support the findings of EMSA. HepG2 activated more STAT2 than that in MCF7. MCF7 had slightly more phosphorylated STAT1 than HepG2. Furthermore, MCF7 activated more STAT3 than HepG2. Although there was large amount of phosphorylated STAT3 detected in MCF7, no STAT3 homodimers DNA complex was found. Besides, IFN priming did increase the expression of STAT1 in both cells about 0.5-fold. The concentration of IRF9 was increased

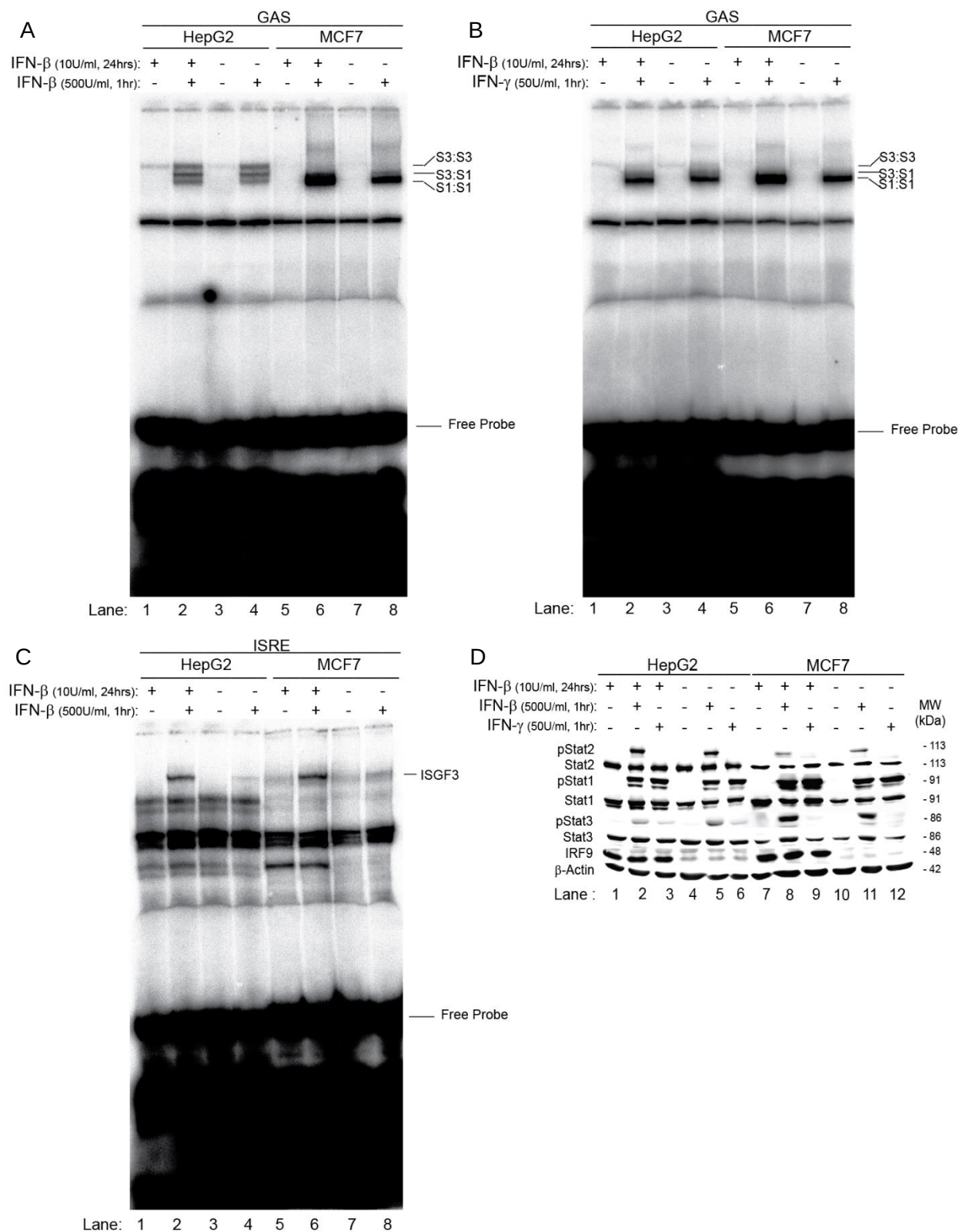


Figure 3.10 IFN-induced DNA binding complexes in HepG2 and MCF7 cells. (A, B and C) EMSA using GAS or ISRE probe with extracts from HepG2 and MCF7 cells which were primed with IFN- β for 24hrs followed by IFN-stimulation of IFN- β or IFN- γ for 1hr or left untreated. The positions of GAF (S1:S1), STAT3 homodimers (S3:S3), STAT3:STAT1 heterodimers (S3:S1) and ISGF3 complexes were labelled. (D) Western blot analysis of phosphorylated

STAT1 at Tyr701 (pStat1), total STAT1 (Stat1), phosphorylated STAT2 at Tyr688 (pStat2), total STAT2 (Stat2), phosphorylated STAT3 at Tyr 705 (pStat3), total STAT3 (Stat3), IRF9 and β -Actin in HepG2 and MCF7 cells which were primed with IFN- β for 24hrs followed by treatment of IFN- β or IFN- γ for 1hr or left untreated. β -Actin was used as loading control. MW, molecular weight. The western blot image in (D) were composed of three different polyacrylamide gels (refer Methods 2.2.4). Experiment was performed only once.

after pre-treatment of IFN- β for longer period of time. This phenomenon correlates to the formation of ISGF3 DNA complex (Figure 3.10 C).

In a nutshell, STAT3 is constitutively activated in HepG2 cell although western blot result did not contribute a strong evidence. MCF7 induce more GAF DNA complexes as compare to HeLa, 2fTGH, HepG2 and macrophages.

3.12 IFN-induced DNA binding complex in Jurkat and RAMOS

The last pair of cells to be examined was Jurkat and RAMOS. There was no three GAS binding complexes induced in both Jurkat and RAMOS cells (Figure 3.11A, lane 2, 4, 6 and 8). The STAT3:STAT1 heterodimers complex was barely detected as well. On the other hand, there was only GAF complex to be detected when the cells were stimulated with IFN- γ (Figure 3.11B). These findings were similar to 2fTGH (Figure 3.9) and MCF7 (Figure 3.10). Furthermore, western blot result (Figure 3.11C) showed that both Jurkat and RAMOS were able to activate STAT3 phosphorylation when stimulated by IFN- β .

Figure 3.11C demonstrated the DNA binding activities specific to ISRE element in Jurkat and RAMOS. Contrast to previous result (Figure 3.2B), ISGF3 DNA complex was barely detected in both cells even though they were primed with IFN- β for 24hrs IFN- β priming showed positive impact on Jurkat and Ramos cells. They expressed more STAT1 and STAT3 as well as the activation of STATs although there was only 10% more than unprimed cells. The positive effect was still able to be observed in EMSA (Figure 3.11A).

In summary, Jurkat and RAMOS induce more stable GAF DNA complex than STAT3:STAT1 heterodimers complex. Less ISGF3 DNA complex were detected in both cells indicate that the stability of the complex was low.

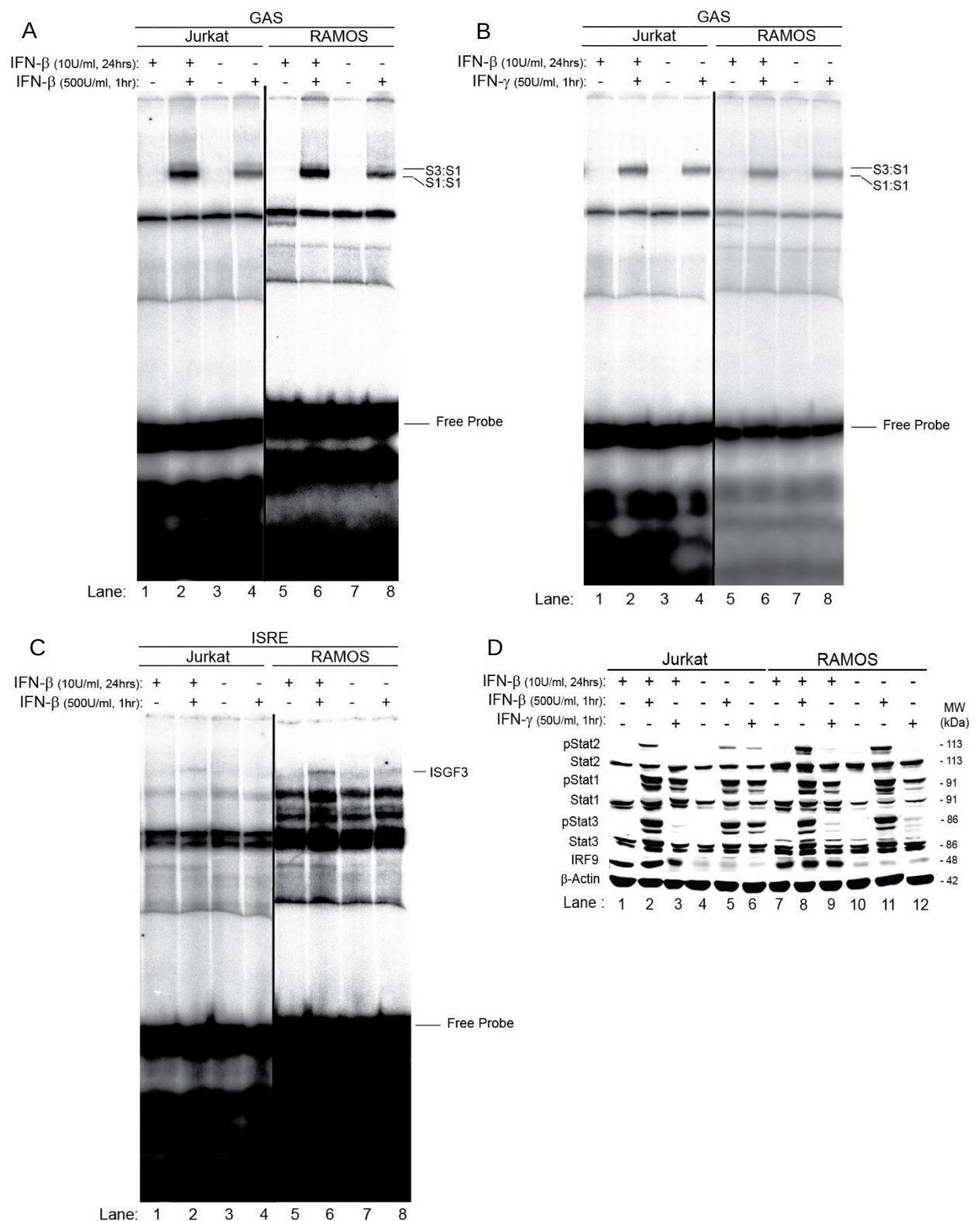


Figure 3.11 IFN-induced DNA binding complexes in Jurkat and RAMOS cells. (A, B and C) EMSA using GAS or ISRE probe with extracts from Jurkat and RAMOS cells which were primed with IFN- β for 24hrs followed by IFN-stimulation of IFN- β or IFN- γ for 1hr or left untreated. EMSA images (A, B and C) were composed of two different gels, the experimental conditions of both gels were identical. The positions of GAF (S1:S1), STAT3 homodimers

(S3:S3), STAT3:STAT1 heterodimers (S3:S1) and ISGF3 complexes were labelled. (D) Western blot analysis of phosphorylated STAT1 at Tyr701 (pStat1), total STAT1 (Stat1), phosphorylated STAT2 at Tyr688 (pStat2), total STAT2 (Stat2), phosphorylated STAT3 at Tyr 705 (pStat3), total STAT3 (Stat3), IRF9 and β -Actin in Jurkat and RAMOS cells which were primed with IFN- β for 24hrs followed by treatment of IFN- β or IFN- γ for 1hr or left untreated. β -Actin was used as loading control. MW, molecular weight. The western blot image in (D) were composed of three different polyacrylamide gels (refer Methods 2.2.4). Experiment was performed only once.

3.13 Chapter summary

Induction of DNA binding complexes by type I and type II IFNs on different cell types was investigated. Experimental protocol was optimized to robustly reveal the IFN-induced DNA binding complexes especially the GAS binding complexes (STAT3 homodimers, STAT3:STAT1 heterodimers and GAF). Diluting the concentration of protein extract and increasing the final concentration of DTT in extraction buffers did not affect the formation of three DNA binding complexes that specific to GAS element.

Once the protocol was optimized, cell specific DNA binding activities was carried out. Five out of eight cell lines showed there were formation of STAT3 homodimers, STAT3:STAT1 heterodimers and GAF that bind to GAS element. Four out of eight cell lines was able to induce ISGF3 DNA complex even the cells were unprimed. In order to induce the formation of ISGF3 DNA complex in the remaining cell lines, IFN-priming was used to elevate concentration of IRF9. However, IFN-priming affected cell differently. Three out of eight cell lines, the IFN-induced DNA binding activities remained unchanged before and after IFN-priming. Furthermore, the optimized protocol did not shown to have noticeable impact on the result of EMSA using ISRE probe and western blot. Both results were similar before optimized protocol was established.

In summary, these results were not strong enough to conclude the valid reason of IFN- β -induced phosphorylated STAT1 transformed to heterodimerize or homodimerize and bind to ISRE and GAS elements respectively.

DISCUSSION

This project was first aimed to study the behaviour of activated (or phosphorylated) STAT1 (pSTAT1) when cells are stimulated by type I IFNs (IFN- β). This is because pSTAT1 is able to form heterodimers with activated STAT2 (pSTAT2) and recruit IRF9 to form a heterotrimeric transcriptional complex which is called ISGF3 complex (Fu *et al.*, 1990). In addition, pSTAT1 is able to homodimerize with pSTAT to form GAF complex under type I IFNs stimulation and binds to the same GAS element from type II IFN (IFN- γ) signalling pathway (Decker, Lew and Darnell, 1991).

IFN-induced DNA binding complexes in different cell lines

The first experiment was to examine the IFN-induced DNA binding activities of 8 different cell lines. All the cell lines were able to induce GAF DNA complex when the cells were treated with IFN- β for an hour. Unlike GAF, ISRE binding complex showed to have different behaviour. Only IFN- β stimulated 2fTGH and macrophage STAT2 WT cells were able to show the ISGF3 DNA complex. Additionally, macrophage STAT2 WT showed a clear and sharp band on EMSA gel (Figure 3.3B) due to its high basal level of IRF9 that detected on western blot membrane (Figure 3.3C). In order to overcome this issue, cells were pre-treated with low amount of IFN- β prior IFN-stimulation. The results showed that the concentration of IRF9 has elevated and thus ISGF3 DNA complex was detected on EMSA gel. This process known as the IFN-priming. This technique was introduced in 1998 by Wong and his colleagues. They pre-treated IFN-resistant melanoma cell lines with IFN- γ before stimulation of type I IFN resulted in increased levels of ISGF3 components and hence enhanced DNA binding activation of ISGF3. In this project, IFN- β was used to prime the cells to investigate the effect of type I IFNs priming on cells. Based on the results in Figure 3.1 – 3.4, IFN- β priming elevated the level of IRF9 about two-fold among the cells. IFN- β priming also increased the expression of STAT1 in all the cells about one-and-half-fold. The fold expression of STAT1 in HeLa after IFN- β priming was the highest, indicated effect of IFN- β priming on the expression of STATs protein was cell type specific. There was a difference between IFN- β priming and IFN- γ priming (Figure 3.5C). Levy and his

colleagues (1990) demonstrated by pre-treatment of HeLa with IFN- γ prior IFN- α stimulation, the transcription of IFN- α stimulated genes was increased. They proposed that pre-treatment of IFN- γ increased the level of IRF9 which was consistent with the results in this project. In contrast, IFN- γ priming in HeLa cells also increase the expression and activation of STAT1 and STAT2 which are the crucial components of ISGF3 (Figure 3.5). Effect of IFN- β priming is weaker than IFN- γ and the reason remained unclear. Since the IFN priming is cell types specific, it makes the investigation more challenging.

Al Zaid Siddiquee and Turkson (2008) mentioned that STAT3 was constitutively phosphorylated in hepatocellular carcinoma (HepG2). On the other hand, Li *et al.* (1996) claimed that heterodimers and homodimers of STAT3 and STAT1 are able to bind to GAS sequence element to initiate gene transcription. However, the results in Figure 3.4A showed that STAT3 homodimers or STAT3:STAT1 heterodimers DNA binding activities were not present. There are three possible reasons to explain these observations. First, the heterodimers and homodimers of STAT3 and STAT1 did not form a stable DNA binding complex. They were disassociated from GAS element before they were detected by phosphorimaging. Second, there was a smearing background that migrated slower than the GAF DNA complex (Figure 3.4A). This phenomena was also been observed in other cell lines (Figure 3.1A, 3.2A and 3.3A). One possible explanation is homodimers and heterodimers of STAT3 and STAT1 was able to bind to GAS element and form a GAS binding complex but the complexes were degraded, thus resulted in a smearing background. Third, the homodimers or heterodimers of STAT3 and STAT1 did not bind to the GAS element. Hence, modification of experimental protocol was performed to reveal these DNA binding complexes.

Serine phosphorylation of STAT1 and STAT3 play a role in formation of GAS binding complex

Concentration of protein extracts in binding reaction was altered to examine its DNA binding activities. Figure 3.5A (lane 6) revealed a three DNA binding complexes. Another two conditions in lane 2 and lane 4 exhibited the band obviously as compare to the former. Serial dilution of protein extract was made

and subjected to gel shift assay. Although the intensity of band was diluted as the protein extracts were getting diluted, there were no trace of another two DNA binding complexes even though, pSTAT3 was able to be detected on western blot membrane. The overall results in this section determined that there might possibly mistakes presence in the protein extract.

DTT is a reducing agent and known as Cleland's reagent (Cleland, 1964). DTT was reported to enhance the DNA binding activity of nuclear factor κ B (NF κ B) (Hayashi, Ueno and Okamoto, 1993) and p53 (Rainwater *et al.*, 1995). Increased final concentration of DTT in both cytoplasmic and nuclear buffer from 1 mM to 5 mM surprisingly reduced the DNA binding activities of the cells (Figure 3.7). The mechanism by which DTT decrease the DNA binding activities in HeLa cells to GAS element is not known. According to Rainwater's research (1995), they hypothesized DTT would probably get involved in shifting protein from an oxidized state to a reduced state which resulted in a better DNA binding. They observed that specific cysteine residues in wild type p53 were involved in the binding process because cysteine are important for DNA binding of transcriptional regulatory proteins (Rainwater *et al.*, 1995). On the other hand, Buettner and his laboratory colleagues (2011) found out that there was a unique surface cysteine, Cys468 in STAT3 structure. This cysteine residue located in the DNA binding region of STAT3. Combining both findings, a hypothesis was generated that increased concentration of DTT in extraction buffers bring negative impact on DNA binding activities of STAT3 as DTT might reduce the cysteine residue that located in DNA binding region of STAT3. This could explain why STAT3 homodimers DNA complex was abolished. Another possible reason was that DTT caused the conformation change in structure of STAT3 and alter the binding of the STAT protein (Lin, Gether and Kobliska, 1996). Similar explanation could be used to explain the decreased of DNA binding activity of STAT3:STAT1 heterodimers. The modification of protocol did not apply in ISRE binding activity experiments because STAT3 is not a component of the ISGF3. In summary, these results suggested that STAT3 provides stability of the interaction between heterodimers or homodimers and DNA. More future research work should be conduct regarding on this issue.

Both cytoplasmic and nuclear extraction buffer contain PMSF, a serine protease inhibitor. At room temperature the half-life of PMSF in aqueous solutions are 110 min at pH 7.0, 55 min at pH 7.5 and 35 min at pH 8.0 (James, 1978). PMSF was added fresh into both extraction buffer (pH 7.4) at least 20 min before the cells were ready to be extracted. Since there was a short incubation time before adding of nuclear extraction buffer into Eppendorf tube contained nuclei pellet (refer Methods 2.2.2), there would be a possibility that PMSF had degraded in both extraction buffers although the extraction buffers were put on ice after preparation. Serine phosphorylation of STAT1 and STAT3 was reported for maximal activation of transcription (Wen, Zhong and Darnell, 1995). Without the inhibitory effect of PMSF, STAT1 and STAT3 would lost the serine phosphorylation and hence reduced the transcription activity. This could be the reason on the presence of smearing background above GAS DNA complex. Therefore, PMSF was added into cytoplasmic extraction buffer after the cells were washed by PBS. Then PMSF was added into nuclear extraction buffer after centrifugation when cells were incubated in cytoplasmic extraction buffer for 5 min. These two steps are crucial to ensure the activity of PMSF is sufficient to inhibit as much as serine proteases.

Comparing both negative (Figure 3.1 – 3.4) and positive result (Figure 3.8 – 3.11), this modified experimental protocol only affected the GAS binding activities of the cells. It did not show any significant difference in the ISRE binding activities and western blot analysis. Thus, it illustrated that serine phosphorylation plays a role on the formation of DNA complex from homodimers and heterodimers of STAT1 and STAT3.

Formation of STAT3 homodimers and STAT3:STAT1 heterodimers is cell types specific

Since a better experimental protocol to reveal the three GAS binding complexes was established, cell type specific DNA binding activities were carried out. STAT3 is predominantly activated by Interleukin-6 (IL-6) (Zhong, Wen and Darnell, 1994). Nevertheless, type I IFNs also trigger phosphorylation of STAT3 and form homodimers and heterodimers with STAT1 and STAT3 which are able to bind to GAS element (Zhong, Wen and Darnell, 1994). Activation of STAT3 varies among different cell lines. 2fTGH, MCF7,

Jurkat and RAMOS cell lines exhibited weak activation of STAT3 on western blot membrane. No STAT3 homodimers DNA binding activity was observed on these cells under IFN- β stimulation indicated that either STAT3 homodimers in these four cell lines did not bind to GAS element or the stability of DNA binding complex was weak.

STAT3:STAT1 heterodimers were reported induced with either STAT1 or STAT3 homodimers or both (Ho and Ivashkiv, 2006). These heterodimers DNA complex was barely observed in 2fTGH, MCF7, Jurkat and RAMOS cell lines. IFN- α strongly phosphorylates STAT3 and it has the ability to suppress phosphorylation of STAT1 (Ho and Ivashkiv, 2006). However, it does not correlate to the results in this project. Western blot results in this project revealed that the concentration of activated of STAT1 was equal to or more than activated of STAT3. Ho and Ivashkiv (2006) also claimed that IFN- α activated STAT3 suppressed the GAS dependent gene by sequestering STAT1 into STAT3:STAT1 heterodimers. Results in this project showed that concentration of GAS binding complexes consist of homodimers and heterodimers of STAT1 and STAT3 were similar. This result could not conclude that activated STAT3 inhibited the transcription activity of GAS induced by STAT1. More research such as real time polymerase chain reaction (RT-PCR) could be perform to investigate whether IFN- β activated STAT3 exhibit inhibitory effect on STAT1.

Interestingly, IFN- γ is also a weak activator of STAT3 as shown by additional slower migrating GAS binding complexes containing STAT3 homodimers and STAT3:STAT1 heterodimers (Qing and Stark, 2004). Referring to the western blot results, HeLa and macrophages cell exhibited high amount of phosphorylated STAT3 concentration with IFN- γ stimulation. Thus, it showed the activation of STAT3 by type II IFN is cell type specific. The evidence of type II IFN could trigger the activation of STAT3 is still remain unknown. Qing and Stark (2004) suggested STAT3 and STAT1 compete for the same binding site on IFNGR1, as activation of STAT3 was induced stronger in STAT1-deficient cells. Additionally, pSTAT3 was detected on unstimulated HeLa cells (Figure 3.5C and 3.9 C). This result showed contrast to previous result in

Figure 3.1C. There was a scientific report stated that heterodimers of STAT1 with STAT2 or STAT3 have been observed in unstimulated HeLa cells although the interactions was weak (Stancato *et al.*, 1996). It is unclear whether this preassociated complex consists of phosphorylated STATs protein as anti-STAT1 and anti-STAT2 antibodies were used to detect the complex on western blot membrane (Stancato *et al.*, 1996). There could be a possibility that the preassociated complex compose of inactivated or activated STAT. Another explanation would be STAT3 is constitutively active in several of cancer call lines and HeLa is cervical carcinoma (Garcia and Jove, 1998).

Furthermore, macrophages, HeLa and HepG2 cell lines presented a multimeric GAS binding complex when stimulated by IFN- β . This complex did not exhibit a very strong interaction with GAS element and the signal was weaker as compared to other three GAS binding complexes. The behaviour of this complex to be homomultimeric or heteromultimeric, or STAT1 and STAT3 is remain unknown. More studies are required to prove this, as it could not be concluded in this project (Figure 3.8 D). Even though weak signal of pSTAT3 was detected on western blot membrane, HepG2 was able to induce the three GAS binding complexes when stimulated with IFN- β or IFN- γ due to its constitutive activation of pSTAT3. Moreover, STAT3 homodimers binding complex could be found in unstimulated HepG2 cells as well. A possible explanation could be abnormal activation of STAT3 in hepatoma cancer cells.

Role of STAT2 in phosphorylation of STAT1

Figure 3.3 and 3.8 revealed a reduction of IFN- β stimulated pSTAT1 and basal level of STAT1 in STAT2 deficient mouse macrophage. Park and his colleagues noted the similar effect on STAT2 deficient mouse fibroblasts. They also found out that the IFN- α induced expression and activation of STAT1 in mouse macrophage was independent of STAT2. This finding is in striking contrast to the result in this project, suggesting that the effect was tissue specific.

Balance between ISGF3 and GAF complex induced by type I IFNs

In short, these results could not determine the balance between ISGF3 complex and GAF complex induce by type I IFN. Based on the observations,

cells did induce GAF complex favourably. Increased formation of ISGF3 binding complex through IFN- β priming did not really influence the induction of GAF binding complex. This is because IFN- β priming also increase the expression and activation of STAT1. Therefore, the DNA binding activities of GAF did not be affected.

In summary, this project established an optimized protocol to reveal the three GAS binding complexes that showed most of the cells induce homodimers and heterodimers of STAT1 and STAT3. Besides, the IFN-induced DNA binding complexes and how does cell react with IFN- β priming are cell type specific.

CONCLUSION AND FUTURE WORK

The work presented in this thesis were served to understanding how pSTAT1 choose its downstream signalling pathway when induced by IFN- β . In order to conduct this experiment, IFN-induced DNA binding complexes in different cell lines were examined by EMSA approach.

All cell lines induced the formation of GAF DNA complex when stimulated by IFN- β . 4 out of 8 cell lines were able to induce ISGF3 DNA complex. IFN- β priming did elevate the concentration of IRF9 and thus promoted the ISGF3 DNA complex formation for all the cell lines. One question arose when HepG2 did not exhibit STAT3 homodimers and STAT3:STAT1 heterodimers DNA complex because STAT3 was constitutively activated in HepG2 cells.

Several alteration of experimental protocol was performed. Diluting concentration of protein extract in EMSA binding reaction and increasing final concentration of DTT in extraction buffers did not show positive result. Soon later, it was found out that serine phosphorylation of STAT1 and STAT3 influenced the GAS binding complex.

Results showed that 4 out of 8 cell lines presented there DNA binding complexes that bind to GAS element. Using supershift assay, these three DNA binding complexes were homodimers and heterodimers of STAT3 an STAT1. STAT3 were reported to have inhibitory effect on GAF complex formation. However, the concentration of these three DNA binding complexes were similar, indicating that STAT3 did not suppress the transcription activity of GAF complex. Real time PCR should be used to further examine whether the transcriptional activity of GAF was affected by STAT3.

IFN-b priming increased the level of IRF9 in all the cell lines. It also promote the expression of STAT1 in every cell lines except 2fTGH. Effect of IFN- β priming was weaker than IFN-g because IFN- γ increased the expression level of IRF9, STAT1 and STAT2 in ten-fold induction.

Due to the 1 year of time constraint, more research to be conducted to investigate the behaviour of pSTAT to induce either homodimers or heterodimers. Real time PCR would be a tool to study the transcriptional

activities by the DNA binding complexes. Whether IFN- β induced pSTAT3 suppress the GAF dependent expression of gene is remained to be investigate.

In conclusion, a better experimental protocol was established to robustly reveal the IFN-induced DNA binding complexes especially GAS binding complex. Besides, the DNA binding activities and effect of IFN- β effecting showed cell type specific. However this work did not able to explain the fate of pSTAT1 to undergo heterodimerization or homodimerization. More researches is going to conduct for further exploration.

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