Localization and Characterization of Antiviral Neutralizing Epitopes of HuIFN-gamma

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In Partial Fulfilment of the Requirement for
the Degree of Doctor of Philosophy

By

James Shanwei Zu

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BY

JAMES SHANWEI ZU

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

This work is dedicated to:

my parents: Mrs. Linxiong Chen and Mr. Yuqing Zu

my wife Nancy and son Michael

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I dedicated this thesis to my parents who taught me that one's abilities are limited only by one's fears and who give me financial and spiritual support during my education. Special thanks also go to Nancy, my loving wife for her continuous emotional and technical support and for sharing with me both the excitement and frustration during this peroid. Without her strong support, I would not have achieved today's accomplishments. I am also very grateful to my loving son Michael, for his cooperation and for being such a terrific child.

ABSTRACT

Human interferon-gamma (HuIFN-γ) has multiple biological activities which include antiviral, growth inhibitory and immunomodulatory activities. Its structure and functions have been studied in detail. However, the structure-functional relationship of HuIFN-γ was not completely understood. The objective of this study is to localize and characterize the functional epitopes of HuIFN-γ.

Two distinct functional epitopes, E_1 and E_2, were identified previously by analysing the cross-reactivity of 21 anti-HuIFN-γ monoclonal antibodies (mAbs) in their binding to HuIFN-γ. In the present study, functional epitope E_1 was mapped to amino acid residues #84-94 by using synthetic octapeptides. Its sequence was "Ser^{84}-Asn-Lys-Lys-Lys-Arg-Asp-Asp-Phe-Gln-Lys^{94}". A novel functional epitope E_3 was identified by mAb MIF3037. This functional epitope was first mapped to the C-terminal 21 amino acid residues of HuIFN-γ by the failure of MIF3037 to bind to MIF3037 to Del_{122}, a HuIFN-γ variant lacking the C-terminal 21 amino acids. Based on the specific reactivity of MIF3037 to the synthetic octapeptides, E_3 functional epitope was precisely mapped to residue #130-138. The sequence of E_3 epitope was "Lys^{130}-Arg-Ser-Gln-Met-Leu-Phe-Arg-Gly^{138}". It was found that although MIF3037 (recognize E_3 epitope) neutralized the antiviral activity of HuIFN-γ, the neutralization activity was incomplete. However,

MIF3037 (recognize E_3 epitope) and MIF3061 (recognize E_1 epitope) have a synergistic effect in neutralizing the antiviral activity of HuIFN-γ. These results, taking together, suggested that E_1 and E_3 epitopes may have similarly functional roles. In order to identify the critical amino acid residues that confer the antiviral function of HuIFN-γ, three variant clones, K86Q, K87E and K88Q, were constructed. The polypeptides expressed by the three variant clones were specifically recognized by anti-HuIFN-γ polyclonal antibody and were purified by immunoaffinity chromatography. The antiviral specific activity of Del_{122}, K86Q, K87E and K88Q were 2.9×10^7, 2.1×10^7, 2.9×10^7 and 7.0×10^6 (IU/mg), respectively. The antiviral specific activity of K86Q and K87E was not significantly different from that of Del_{122}, while the antiviral specific activity of K88Q was about 4 times lower than that of Del_{122}. It was suggested that Lys^{88} is probably more important than Lys^{86} or Lys^{87} in maintaining the antiviral function of HuIFN-γ.

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List of Abbreviations

3':	three prime
5':	five prime
aa:	amino acids
Asn:	asparagine
Arg:	arginine
Asp:	aspartic acid
bp:	base pair
BSA:	bovine serum albumin
Ci:	curie
CIAP:	calf intestinal alkaline phosphatase
CIP:	calf intestinal phosphatase
CPE:	cytopathic effect
cpm:	count per minute
CSF-1:	colony stimulating factor-1
Cys:	cystine
Del_{122}:	deletion variant of HuIFN-γ without 21 residue in the C-terminus
DMF:	dimethyformamide
DNA:	deoxyribonucleic acid
dsDNA:	double stranded deoxyribonucleic acids
E. coli:	Escherichia coli
E_1:	antiviral neutralizing epitope 1
E_2:	antiviral neutralizing epitope 2
E_3:	antiviral neutralizing epitope 3
EGF:	epithelial growth factor
EIA:	enzyme immunoassay
eIF-2:	initiation factor 2 for protein syntheses
ELISA:	enzyme linked immunosorbent assay
EMCV:	encephalomyocarditis virus
F-MOC:	fluoroennylme-thyloxycabonyl
Fig.:	figure
g	gram
GAS:	gamma-activated site
Gly:	glycine
Gln:	glutamine

Glu:	glutamic acid
HIV:	human immunodeficiency virus
HLA:	human leukocyte antigen
hr:	hour
HuIFN:	human interferon
IDO:	indolamine 2,3-deoxygenase
IFN:	interferon
IFN-β:	interferon-beta (β)
IFN-γ:	interferon-gamma(γ)
IFN-α:	interferon-alpha (α)
Ig:	immunoglobulin
IL-6:	interleukin-6
IRE:	interferon gene regulatory element
IRF-1:	interferon regulatory factor-1
IRF-2:	interferon regulatory factor-2
ISG:	interferon-a-stimulated gene
ISGF-3:	interferon-stimulated gene factor 3
ISGF-3α:	interferon-stimulated gene factor 3-α
ISGF-3γ	interferon-stimulated gene factor 3-γ
ISRE:	interferon-stimulated responsive element
Kb:	kilobase
KDa	kilodalton
Lys:	lysine
M:	molar
mAb:	monoclonal antibody
Met:	methionine
MHC:	major histocompatibility complex
MIF:	mAb of interferon
min:	minute
ml:	millilitre
mm:	millimetre
mM:	milimolar
MOI:	multiplicity of infection
mtr:	methoxytrimethyphenyl sulphonyl
NK:	natural killer cells
NLS	nuclear localization signal
nm:	nanomolar
°C:	degrees centigrade
OD:	optical density
PBS:	phosphate buffered saline

PDGF:	platelet-derivated growth factor
Phe:	phenylalanine
RBP9-27	ribonucleic acid binding protein 9-27
rHuIFN:	recombinant human interferon
RNA:	ribonucleic acids
RNase L:	ribonuclease L
RNase A:	ribonuclease A
SDS:	sodium dodecryl sulfate
SDS-PAGE:	sodium dodecryl sulfate polyacrylamide gel
sELISA:	sandwich ELISA
Ser:	serine
sRIA:	competition sandwich ELISA
ssRNA:	single stranded RNA
Stat	signal transducer and gene activator
Stat91	signal transducer and gene activator 91
TCA:	trichloroacetic acid
trp:	tryptophan
Tween 20:	polyoxyethylene-sorbitan monolaurate
ug:	microgram
um:	micrometer
UV:	ultraviolet
v/v:	volume per volume
V:	volt
w/v:	weight per volume
X-gal:	5-bromo-4-chloro-3-indoxyl-b-D-galactosidase

CHAPTER I

INTRODUCTION

Interferon (IFN) was first described by Issacs and Lindnaman (1957) as an agent that induced the resistance to viral infection in susceptible cells (Issacs et al., 1957). Since then, at least 3 distinct classes of interferon have been identified (Pestka et al., 1987). They are interferon-α (IFN-α), interferon-β (IFN-β) and interferon-γ (IFN-γ). It has been shown that IFNs have multiple biological effects, including antiviral, anti-proliferation and immunomodulatory activities. IFNs have been used in the treatment of certain diseases, such as hepatitis B virus (HBV) hepatitis, hairy cell leukaemia and chronic granulomatous diseases (Pestka et al., 1987).

IFNs are grouped into 2 types based on their physical and biological properties. Type I includes interferon-α which is secreted by leukocyte and interferon-β which is secreted by fibroblast. Type II interferon is referred to as interferon-γ, which is secreted by lymphocytes. Type I interferons are stable in pH 2.0 and bind to the same receptor on cell surface. Type II interferon (interferon-γ) is not stable in pH 2.0 and bind to an unique extracelluar receptor.

The physicochemical and biological characteristics of these 3 classes of interferon are compared in Table 1.

Table 1. Comparison of the properties of IFNs.

Property	IFN-α	IFN-β	IFN-γ
NOMENCLATURE	Type I	Type I	Type II
	Leukocyte	Fibroblast	Immune
MAJOR INDUCERS	Virus	Virus,LPS	Antigens
	dsRNA	dsRNA	Mitogens
PHYSICAL PROPERTIES			
Molecular Weight(kDa)	20	20	17
Amino Acids	165-166	166	143
N-Linked Glycosylation	some	+	2 sites
Subunit	Single	Single	Homodimer
pH Stability(2.0)	Stable	Stable	Labile
GENE STRUCTURE			
No. of Genes	26	1	1
Chromosome Location	9	9	12
Introns	None	None	3
CELLULAR SOURCE	T, B cells	Fibroblast	T cells
		Epithelial	Macrophages
			NK cells
RECEPTOR	Single, share	Single, share	Single,
	with IFN-β	with IFN-α	

1. Induction of IFN

1.1 The Inducers.

Normal cells do not produce IFN constitutively. A variety of chemical and biological agents can be used to induce the production of IFN. These inducers include viruses, synthetic double stranded RNA (dsRNA) and mitogens. Among all of these inducers, viruses and dsRNA were well characterized.

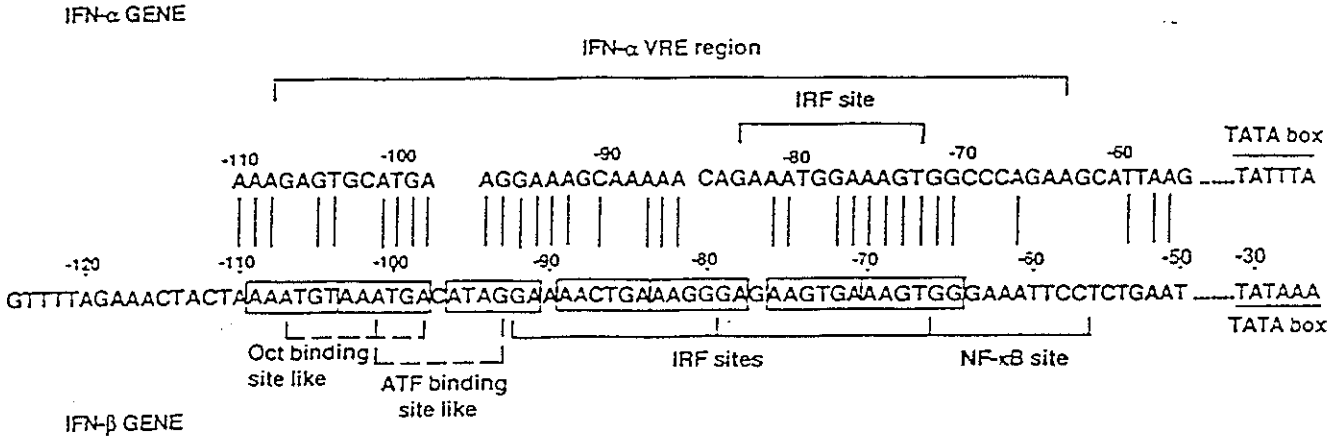
IFN was produced by cells infected with complete virions, either infectious or inactivated virus. It was reported that the synthesis of IFN began after the initiation of virus maturation and maintained at same rate for 20-50 hours (Smart et al., 1966). Viruses which replicated slower were more potent inducer for IFN production and the potency was related to the amount and half life of virus RNA inside the infected cells (Clavell et al., 1971). It was also found that the double stranded RNA (dsRNA) was the major chemical stimulus for IFN production and the capacity of inducing IFN production was mainly dependent on its high molecular weight and resistance to enzyme degradation (Lengyel, 1982). The synthetic polymer consisting of one chain of polyriboinoisinic and one chain of polyribocytidylic acid (poly I:C) is one of such dsRNA molecules. The poly I:C chain usually contained more than 100 residues and it was very resistant to the digestion of cellular ribonucleases. (Field et al., 1970). These properties made poly I:C one of the most potent synthetic inducer for IFN production.

1.2 Mechanism of IFN Induction

IFN production induced by virus infection or poly I:C is due to the transcriptional activation of IFN genes. The mechanism for induction of type I IFN in the transcriptional level was well-documented, while the mechanism for induction of type II IFN (IFN-γ) remained mainly unknown.

The promoter sequences in 5' upstream region of IFN-α and IFN-β genes shared some degree of homology (Figure 1). The *cis*-acting elements that are responsible for virus or poly I:C induction have been identified (Rylas et al., 1985). It was found that a region between -117 to -50 bp in human IFN-β gene promoter was required for IFN expression (Fujita et al., 1987 and 1989a). This region contained seven repeated hexanucleotide sequences [AA(A/G)(T/G)GA]. These hexanucleotide sequences were tested by placing 4 hexanucleotide repeats into an *in vitro* gene expression system with chloramphenicol acetyltransferase (CAT) gene as a reporter (Fujita et al., 1987, 1989 and Kuhl et al., 1987). It was shown that these repeats inhibited CAT gene expression when the host cells were not infected by virus. However, the repeats enhanced CAT gene expression when the host cells were infected by virus (Fujita et al., 1987, 1989 and Kuhl et al., 1987). It was suggested that the same DNA element could functions as either an "activator" and a "silencer" depending on virus infection of the host cells. These hexanucleotides repeats between -117 to -50 bp in human IFN-β gene promoter were

Figure 1. The regulatory DNA sequences and factor binding sites within IFN-alpha and IFN-beta genes. (modified from Tanaka et al., 1992) The TATA box and each transcriptional factor binding sites are indicated; the transcriptional factor binding site indicated with broken lines were not well characterized. The numbers refer to the distance in nucleotides from the transcriptional start site. The repetitive hexaemeric sequence are framed. Gaps are introduced to maximize the sequence homology between the two genes.


IFN-α GENE
IFN-α VRE region
IRF site
TATA box
-110
-100
-90
-80
-70
-60
AAAGAGTGCATGA
AGGAAAGCAAAAA
CAGAAATGGAAAGTGGCCCAGAAGCATTAAG
TATTTA
-120
-110
-100
-90
-80
-70
-60
-50
-30
GTTTTAGAAACTACTA
AAATGT
AAATGA
CATAGGAA
AACTGA
AAGGGA
AAGTGA
AAGTGG
GAAATTCCTCTGAAT
TATAAA
TATA box
Oct binding site like
ATF binding site like
IRF sites
NF-κB site
IFN-β GENE

hence considered as functional *cis*-acting DNA elements that regulated IFN-β gene transcription in response to the virus infection. The second *cis*-acting DNA element was found between -66 bp and -57 bp of IFN-β gene promoter. This DNA element (GGGAAATTCC) have consensus sequence for the binding of transcriptional factor NF-*k*B (Lenardo et al., 1989a). It was found that mutations in this region decreased IFN production, while increasing copies of the motif enhanced IFN production induced (Lenardo et al., 1989a and Fujita et al., 1989b). Thus, DNA element (GGGAAATTCC) between -66 bp and -57 bp of IFN-β gene promoter was also a functional *cis*-acting element that regulated IFN-β gene transcription in response to virus infection.

Two types of transcriptional factors, NF-*k*B and interferon regulatory factor (IRF) were shown to regulate gene expression of type I IFN. NF-*k*B was originally identified as B-cell specific nuclear protein that bound to *k*B *cis*-DNA regulatory element in immunoglobulin *k*-chain gene (Sen et al., 1986). It was shown that NF-*k*B bound DNA sequence GGGAAATTCC which was between -66 bp to -57 bp in human IFN-β gene promoter. NF-*k*B was shown to be translocated into cell nucleus after host cells were infected by virus or stimulated by poly I:C (Lenardo et al., 1989b). These observations suggest that NF-*k*B was a positive regulator for the induction of IFN-β gene expression. IRF was the second type of transcriptional factors that regulated IFN-β gene expression. It contained 2 proteins, IRF-1 (Miyamoto et al., 1988) and IRF-2 (Harada et al., 1989).

Both protein have been cloned and characterized (Miyamoto et al., 1988 and Harada et al., 1989). The polypeptide sequences of IRF-1 and IRF-2 were 62% identical in the amino terminal 154 residues. It was found that DNA binding activity of both IRF-1 and IRF-2 resided in the amino terminal 154 amino acid residues. Both IRF-1 and IRF-2 specifically bound to hexanucleotides repeats AA(A/G)(T/G), a *cis*-DNA element between -117 bp to -50 bp in human IFN-β gene promoter. It has been estimated that a single IRF binding site required at least 3 to 4 hexanucleotides repeats (Harada et al., 1989). It was demonstrated *in vitro* that co-transfection of IRF-1 cDNA and CAT gene linked with four repeats of AAGTGA into the host cells could induce CAT gene expression in the absence of virus infection (Fujita et al., 1989a). However, co-transfection of IRF-2 cDNA into the cells containing IRF-1 and CAT report gene inhibited IRF-1 dependent gene activation (Harada et al., 1989). Hence, IRF-1 functioned as an "activator", while IRF-2 functioned as a "repressor" in the transcriptional regulation of IFN-β gene. It was shown that IRF-1 and IRF-2 had similar affinity to the specific DNA binding sequence (Watanabe et al., 1991). In a variety of cells, both IRF-1 and IRF-2 were expressed at a basal level. However, the half life of IRF2 was far longer than IRF1 (8 hours versus 30 minutes) (Watanabe et al., 1991). Based on these observations, it was hypothesized that IRF binding element (*cis*-acting DNA element) was usually occupied by IRF-2 and resulted in repression of IFN-β gene expression. When the host cells were infected by virus or stimulated by poly I:C, more IRF-1 was produced, at least

transiently (Watanabe et al., 1991). IRF-1 in high concentration may displace IRF-2 in its binding to the *cis*-DNA element through competition and caused activation of IFN-β gene transcription (Watanabe et al., 1991). In conclusion, IFN-β gene expression induced by virus or poly I:C was regulated through the regulation of IRF-1 and IRF-2.

2. Interferon Genes

Interferon-α, interferon-β and interferon-γ are encoded by different genes. The gene for IFN-α has been shown to be polymophic. Twenty-three separated genes have been found (Owerbach et al., 1981 and Trent et al., 1982). All of the genes were located in chromosome 9 and did not contain intron (Pestka et al., 1987). Based on the amino acid sequence of the polypeptides, the IFN-α family was subdivided into two classes. The genes for the first class encoded polypeptides of 165 or 166 amino acids and the genes for the second class encoded polypeptide of 172 amino acids. Both classes exhibited potent antiviral activity and were expressed in response to viral infection (Capon et al., 1985).

A single gene located on chromosome 9 encoded human IFN-β polypeptide (166 amino acids) (Owerbach et al., 1981 and Trent et al., 1982). Human IFN-γ (HuIFN-γ) was encoded by a single 6 Kb gene in chromosome 12q24.1. This gene contained 4 exons and three introns (Naylor et al., 1983 and 1984). An enhancer element was found in a 220 bp sequence within the first intron of HuIFN-γ gene (Ciccarone et al., 1990). Positive and

negative *cis*-acting regulatory elements have been identified in a region of 700 bp upstream to the transcriptional start site (Ciccarone et al., 1990). HuIFN-γ gene has no significant sequence similarity to human IFN-α or IFN-β (Ciccarone et al., 1990).

3. Structure of IFN-γ.

The cDNAs for HuIFN-γ and murine IFN-γ were cloned in 1982 by Gray and Goeddel, respectively (Gray et al., 1982 and Goeddel et al., 1983). The sequence of HuIFN-γ was determined through sequencing of cDNA and polypeptide (Gray et al., 1983 and Rinderknecht et al., 1985). Activation of HuIFN-γ gene leaded to the transcription of a 1.2 Kb mRNA that encoded for an 166 amino acid polypeptide. The amino terminal 23 residues were shown to be a typical hydrophobic signal peptide, which was removed proteolytically in posttranslational process. The mature polypeptide contained 143 residues with a predicted molecular weight 17 kDa (Gray et al., 1982 and Rinderknecht et al., 1985). The natural protein of HuIFN-γ had two N-linked glycosylation sites at residues 25 and 97. It has been shown that these two sites were independently and differentially glycosylated, thereby giving rise to subunits of different molecular weight. The subunits with molecular masses of 17, 20 or 25 kDa were corresponding to the molecules with 0, 1 or 2 carbohydrate chains (Kelker et al., 1983 and 1984). It was found that glycosylation was not essential for the biological activities of

IFN-γ, but it increased the half life of IFN-γ *in vivo* (Kelker et al., 1983 and Rutenfranz et al., 1988).

The murine IFN-γ gene transcribed a 1.2 Kb mRNA that encoded a mature 134 amino acid polypeptide (Gray et al., 1983). Similar to its human counterpart, murine IFN-γ has two glycosylation sites (residue 25 and 97). Human and murine IFN-γ share only modest similarity at either cDNA or amino acid levels (60% and 40%). The IFN-γ polypeptide sequences of four species are compared in Figure 2.

The polypeptides of IFN-γ were self-assembled to form homodimers. No monomer was detected at physiological conditions. It has been suggested that homodimer was the functional form (Fountoulakis et al., 1989 and Dighe et al., 1993). Since IFN-γ polypeptide was devoid of cysteine, homodimer was held together by noncovalent forces. The x-ray crystallographic structure of HuIFN-γ was solved in 1992 by Ealick and Cook (Figure 3, Ealick et al., 1991). This study has confirmed the dimeric nature of natural HuIFN-γ. The individual subunit had a flattened elliptical shape, but overall structure of the dimer was compact and globular. The molecule was mainly α-helical (62%) and lacked β-sheet structure. Each subunit had six α-helices held together by short nonhelical regions. The dimer was formed by a unique intertwine of the helices across the subunits. This type of intimate subunit interaction was very rare among the proteins with known x-ray crystallographic structure. The model has shown that the subunits were associated in

Figure 2. The amino acid sequences of IFN-gamma in different species: human (Tanaka et al., 1983), bovine (Grey & Goeddel, 1987), sheep (McInnes et al., 1990), mouse (grey & Goeddel, 1983) and rat (Dijkema., 1985). (modified from Ealick et al., 1991).

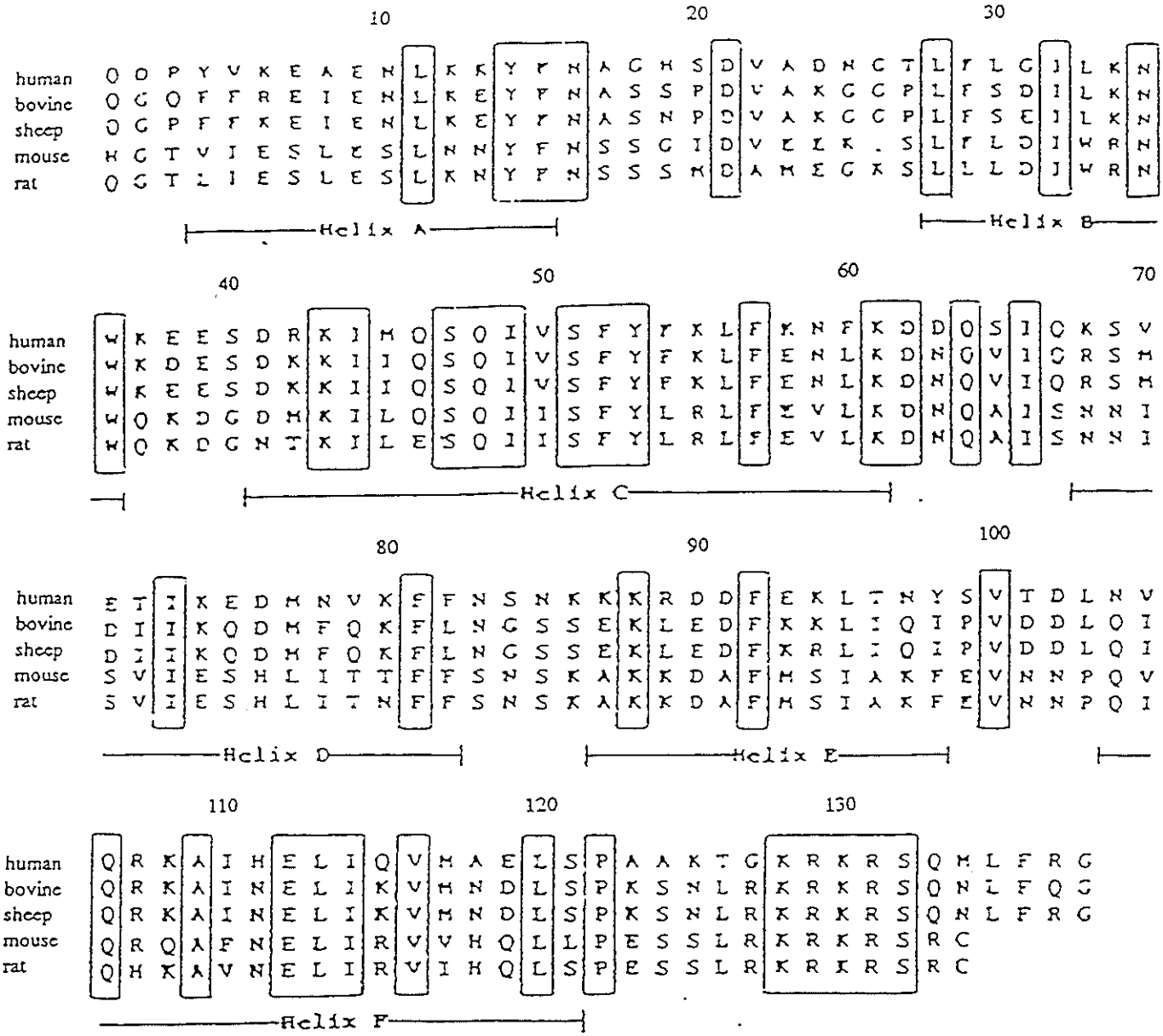
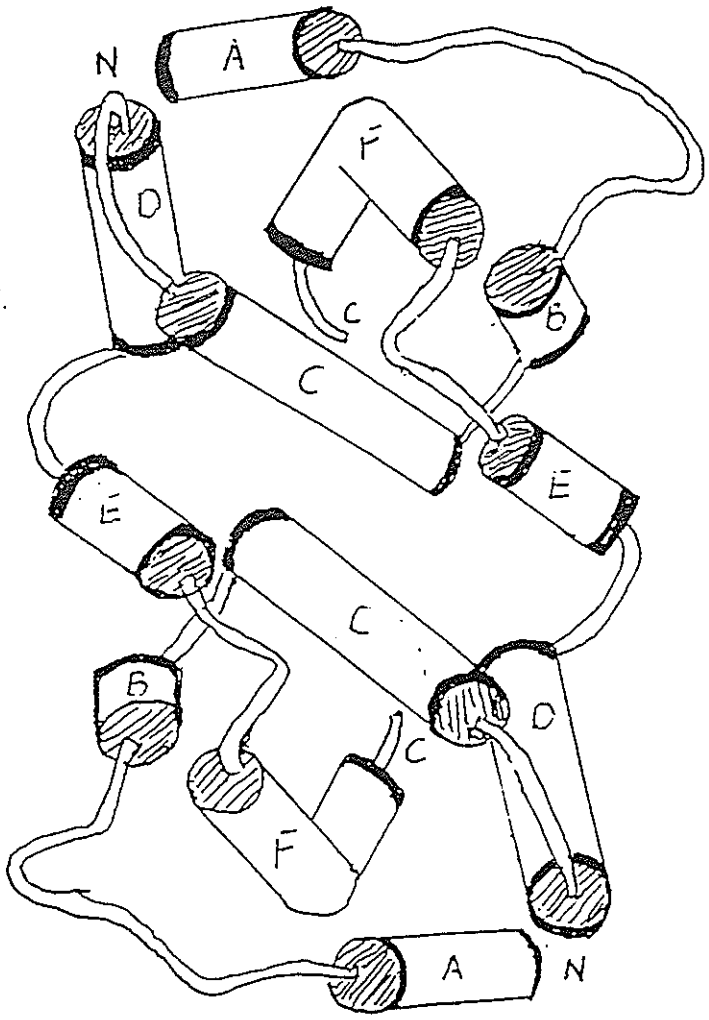

10
20
30
human
bovine
sheep
mouse
rat
Helix A
Helix B
40
50
60
70
Helix C
80
90
100
Helix D
Helix E
110
120
130
Helix F

Figure 3. The model of x-ray crystallographic structure of HuIFN-gamma (modified from Ealick et al., 1993). The functional form of HuIFN-gamma is a homodimer that has 6 alpha-helices (from A to F, represented by the cylinders) and loops between the helices. The two monomer are completely bound by the non-covalent forces.


N
A
D
F
C
B
C
E
E
C
B
C
D
F
A
N

antiparallel fashion, thereby having a juxtaposition of an amino and carboxyl terminal portion of the opposing polypeptide chains. The amino terminus contained a firm α-helix, while the carboxyl terminus did not have a rigid conformation in the crystallographic model, which was consistent with the structure of IFN-γ in solution with magnetic resonance approach (Lundell et al., 1991).

4. The Receptor of IFN-γ

IFN-γ exerts its pleotropic effects on cells through its interaction with a specific receptor on the cell surface. Based on immunochemical, radioligand binding and molecular genetic analyses, a single type of IFN-γ receptor was identified in various cells (Farrar et al., 1993; Finbloom et al., 1985; Littman et al., 1985; Celada et al., 1985). It has been shown that IFN-γ bound to its receptor with high affinity ($Ka=10^9$-10^{10} M^{-1}). The density of the receptor on most cells was between 200-25,000 per cell (Farrar et al., 1993). The levels of IFN-γ receptor on skin, nerve and syncytial trophoblasts of placenta were 10-100 fold higher than those on lymphocytes and haematopoietic cells (Farrar et al., 1993). The physiological significance for the different levels of expression in various cells was unknown.

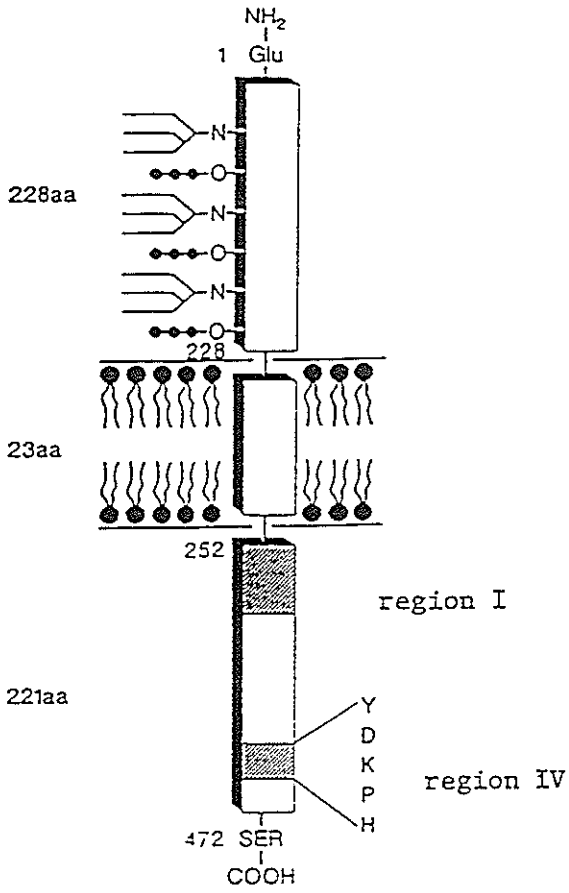
Human IFN-γ receptor was cloned by Aguet and colleagues in 1988 (Aguet et al., 1988). Subsequently, murine IFN-γ receptor was cloned (Gray et al., 1989). The genes for

HuIFN-γ and murine IFN-γ receptors were located on chromosomes 6 and 10, respectively. The genes for human and murine receptors were both 7 Kb in size, each consisting of 7 exons (Merlin et al., 1989). It was found that HuIFN-γ receptor (472 amino acids) and murine IFN-γ receptor (451 amino acids) were organized in a similar manner (Bazan, 1990). Based on the cDNA sequences, It was estimated that molecular weight for human and murine receptors was 52.5 and 48.5 kDa, respectively (Aguet et al., 1988 and Gray et al., 1989). Sequences of both human and murine receptors had the characteristics of typical transmembrane proteins. It was found that both human and murine receptors were symmetrically oriented around a single transmembrane domain of 23 amino acids. The intracellular domain contained 221 amino acids in human receptor and 200 amino acid residues in murine receptor (Bazan. 1990). Comparing to the extracelluar domain, intracellular domain was rich in serine and threonine residues (approximately 25% of all residues in intracellular domain). Five tyrosine residues were also found in this domain. The intracellular domains of human and murine IFN-γ receptors had no significant structure similarity to any other known receptor and it did not possess any identifiable kinases, phosphatases, SH2 or SH3 domains (Bazan, 1990). The extracelluar domain contained 228 amino acid residues in both human and murine receptors. Ten cystine residues and 5 potential N-linked glycosylation sites were identified in the extracelluar domain. Usually, all 5 glycosylation sites were occupied (Hershey et al., 1989

and Mao et al., 1989). The N-linked glycosylation was required for ligand binding (Fischer et al., 1990). The IFN-γ receptors had neither the common structures of type I cytokine receptor family (characterized by the position of 4 conserved cystine residues and a membrane proximal WSXWS motif) nor the common structure of immunoglobulin superfamily (Bazan, 1990). Based on the analyses of predictive algorithms, IFN-γ receptor belonged to a new family of cytokine receptors (termed type II cytokine receptor). The other members of this receptor family was IFN-α receptor and receptor for tissue factor (a protein-phospholipid complex that initiates blood coagulation through external pathway). All members in type II cytokine receptor family shared a similar structure of 210 amino acid ligand binding domain which contained conserved cysteine pairs at both amino and carboxyl termini (Bazan, 1990).

The extracelluar domain of HuIFN-γ receptor was responsible for ligand binding. Using proteolytic digestion, it was found that the amino terminal 6 residues were not essential for ligand binding. The smallest proteolytic fragment with ligand binding capacity encompassed residues 6-227, which was almost the entire extracelluar domain of the receptor (Fountoulakis et al., 1989). The functional sites in the intracellular domain were mapped to two regions, region I (residue 256-303, Figure 4) and region IV (residue 434-472, Figure 4) (Farrar et al., 1991). A mutant receptor molecule devoid of region I (residue 256-303) was able to bind to HuIFN-γ, but the host cell was unable to internalize

Figure 4. The structure of HuIFN-gamma receptor (modified from Farrar et al., 1991). The polypeptide of HuIFN-gamma receptor is symmetrically oriented around a single 23 amino acids transmembrane domain. The extracellular domain and intracellular domain are composed of 228 and 221 residues, respectively. The protein contains O-linked and N-linked glycosylation in some cells. The region I, from 256 to 303 (shaded area) is needed for both receptor induced ligand internalization and biological function. The region IV (shaded area) is only needed for biological function.


NH2
1 Glu
N
O
N
O
N
O
228aa
228
23aa
252
region I
221aa
Y
D
K
P
H
region IV
472 SER
COOH

receptor-ligand complex. Subsequently, the host cell could not have a functional response to the ligand binding. It was found that a leucine-isoleucine sequence in region I (residue 270-271) was critical in receptor mediated ligand internalization (Farrar et al., 1991). Region IV (residue 434-472) was essential for the induction of functional responses. Mutants that had residues 440 (tyrosine), 441 (aspartic acid) or 444 (histidine) changed to alanine produced a receptor that was able to bind to ligand but unable to confer functional responses (Farrar et al., 1991). The mechanism for region IV mediated functional responses was unknown.

The receptors expressed in host cells of different species has confirmed that receptors bound ligand in a species-specific manner. Although receptor-ligand internalization showed a similar kinetics as the receptors expressed on homologous cells, the biological responses in the transfected cells were not induced (Aguet et al., 1988 and Gray et al., 1989). These observations suggested that receptor-ligand binding was necessary, yet was insufficient for inducing a functional response. One or more additional species matched components were required to form a functionally active receptor. Pestka and colleagues have utilized the human:murine somatic cell hybrids to investigate the minimal requirements for the formation of a functional HuIFN-γ receptor in murine cells (Yeivin et al., 1992 and Jung et al., 1990b). The fused somatic cells contained whole genome of murine cell but had a random assortment of human chromosomes. It was found that hybrid cells containing human chromosome 6 could bind HuIFN-γ. However,

biological responses were only observed in hybrid cells that contained both human chromosome 6 and 21 (Jung et al., 1990a). In murine cells transfected with cDNA of HuIFN-γ receptor, only human chromosome 21 was required to confer the biological responses (Jung et al., 1990b). The obligate requirement of two chromosomes in the hybrid cells suggested that at least one additional species matched component from chromosome 21 was required to form a functional receptor. This component has been called accessory protein of HuIFN-γ receptor. The site for the interaction of accessory protein to HuIFN-γ receptor was localized to the extracelluar domain of HuIFN-γ receptor (Hibino et al., 1992). Recently, one type of the accessory proteins for HuIFN-γ and murine IFN-γ receptors was cloned. The polypeptide encoded by cDNA of human accessory protein contained 337 amino acid residues, including amino terminal 27 residues signal peptide (Soh et al., 1994). Based on cDNA sequence, it was predicted that the extracelluar domain contained 220 amino acid residues, while the intracellular domain consisted of 64 amino acid residues. The transmembrane domain contained 26 amino acid residues. The cDNA of murine accessory protein of IFN-γ receptor encoded a polypeptide containing 332 amino acids, including 18 amino acids signal peptide on the amino terminus (Hemmi et al., 1994). Similar to the human accessory protein, this protein also had a putative extracelluar domain containing 224 amino acid residues and an intracellular domain with 66 amino acid residues. Based on computer-assisted search of

sequence data banks, it has been shown that both human and murine accessory proteins had similar structure to receptors of human IFN-γ, human IFN-α, murine IFN-γ and murine IFN-α. These two accessary proteins shared a characteristic structure with all the members of class II cytokine receptor family. The human accessory protein cDNA could replace the human chromosome 21 in murine cells that contained human IFN-γ receptor and confer the induction of MHC class I molecule in response to HuIFN-γ, but failed to confer antiviral activity in response to HuIFN-γ under similar conditions (Soh et al., 1994). These observations suggested that a different accessory protein or different combinations of accessory proteins were required to form a functional human IFN-γ receptor for antiviral activity. The cloned accessory protein for mouse IFN-γ receptor was able to confer antiviral activity, anti-proliferation activity and induction of MHC class I molecule in a species-specific manner. This mouse protein has been termed as β-chain of murine IFN-γ receptor (Hemmi et al., 1994).

5. Mechanism of IFN Induced Gene Transcription

5.1 *Cis*-DNA Element

The biological activities result from IFN induced gene transcription and subsequent protein syntheses inside susceptible cells. It was observed that the effects of IFN required active cellular RNA and protein syntheses. Inhibition of *de novo* protein

syntheses abolished the effect of IFN (Willman et al., 1993). It was estimated that the numbers of genes induced by IFN were from 15 to 25 in most of the cells. These genes are listed in Table 2.

Based on the functional studies, genes induced by IFN were classified into 3 groups. The first group was induced by both type I and type II IFN, such as (2'-5')oligoadenylate synthetase (Benech et al., 1987) and 1-8 gene family (Friedman, 1979). The second group was mainly induced by type I interferon, such as Mx genes (Staeheli et al. 1986 and 1987). The third group was induced preferentially by IFN-γ. This group included genes for MHC class I (Eellous et al., 1982) and class II antigens (Basham et al., 1982) as well as the genes for receptor of IgG Fc fragment.

A highly homologous DNA element called IFN-stimulated response element (ISRE) has been located in the 5' flanking promoter-enhancer region of many type I IFN-induced genes. The consensus sequence of ISRE was ACTTTCAGTTTCAT. The functional roles of ISRE in gene expression were analyzed by *in vitro* gene transcriptional studies. By inserting ISRE sequence into a plasmid containing a report gene (such as CAT gene), function of ISRE could be measured by the expression of CAT activity. It was shown that ISRE sequence was required for the activation of type I IFN induced gene transcription (Lew et al., 1991). A similar *cis*-DNA element that responses to type II IFN (IFN-γ) was found in many IFN-γ induced genes, such as guanylate binding protein. The consensus sequence was AGTTTCATATTACTCTAAATC, termed as γ-activated

Table 2. Interferon induced genes/proteins.

Protein/Gene	Function	Interferon	Reference
2-5A Synthetase	synthesis of 2.5-A	α,β,γ	Baglioni, 1980
Class I Ag	MHC antigens	α,β,γ	Basham, 1982
B2-microglobulin	Small chain of class I Ag	α,β,γ	Fellous, 1982
RBP9-27	Inhibit HIV expression	α,β,γ	Contantou, 1993
IRF-1 and -2	gene transcription	α,β,γ	Harada, 1989
IDO	Indoleamine 2,3-dioxygenase	α,β,γ	Ozaki, 1988
6-16	unknown	α,β	Porter, 1988
ISG54	DNA binding	α,β	Levy, 1986
67 kDa GBP	GTP and GDP binding	α,β	Cheng, 1983
Mx protein	Inhibition of influenza virus	α,β	Horisberger,1983
67 kDa Kinase	Protein kinase	α,β	Lebleu, 1976
Metallothionein	binding of metal ions	α	Friedman, 1964
ISG15	DNA binding	α	Reich, 1988
56 kDa protein	unknown	β	Samnata, 1986
Class II Ag	MHC class II antigen	γ	Vilcek, 1985
Fc receptor	Ig receptor on lymphocyte	γ	Vilcek, 1985
IP-10	unknown	γ	Luster, 1988
Gamma. 1	unknown	γ	Fan, 1988

site (GAS). This *cis*-DNA element was required for the activation of IFN-γ induced gene transcription (Lew et al., 1991).

5.2 Transcriptional Factors in IFN Induced Gene Expression

DNA binding proteins that specifically recognized ISRE or GAS sequences have been identified. Two families of transcriptional factors (IRF family and ISGF3 family) were shown to be regulatory to IFN-induced gene expression. Interferon regulatory factor (IRF) family contained two transcriptional proteins, IRF1 and IRF2. Both proteins have been cloned and characterized (Harada et al., 1989 and Miyamoto et al., 1988). IRF1 and IRF2 were originally recognized as transcriptional factors in the induction of type I IFN by virus and poly I:C. The *cis*-DNA binding sequence of IRF1 and IRF2 was found in 5' regulatory sequences of many IFN-induced genes. The consensus hexanucleotide motif AA(A/G)(T/G)GA recognized by IRF1 and IRF2 was located within the ISRE sequence. Co-transfection of IRF1 cDNAthat has CAT gene linked with the ISRE sequence into host cell could activate the CAT gene expression, while co-transfection of IRF2 that has CAT gene n linked with the ISRE sequence inhibited the IRF1 dependent gene expression (Harada et al., 1989). In conclusion, the IRF1 was an activator in type I IFN induced gene expression, while IRF2 was a repressor in type I IFN induced gene expression (Harada et al., 1989).

ISGF3 was the second family of transcriptional factors involved in the IFN-

induced gene transcription. ISGF3 was a multicomponent cytoplasmic factor that assembled and transported into the cell nucleus in response to IFN-α stimulation (Larner et al., 1986; Levy et al., 1989; 1988 and 1990). ISGF3 proteins could be detected within 1 minute in the cytoplasmic fraction and within 3-4 minutes in the nuclear fraction after cells were treated with IFN-α. ISGF3 contained 2 dissociable protein subunits, ISGF3α and ISGF3γ. Both ISGF3α and ISGF3γ were expressed and located in the cytoplasm. ISGF3α consisted of 3 cytoplasmic polypeptides, termed Stat 84, Stat 91 and Stat 113 (Stat: signal transducer and activator of transcription) (Dale et al., 1989 and Levy et al., 1989). ISGF3γ was a 48 kDa DNA binding protein and recognized ISRE *cis*-DNA element which was located in the 5' flanking region of many IFN-α induced genes. Based on *in vitro* CAT gene transcriptional analyses, it was found that ISRF3γ by itself was a weak gene activator (Fu et al., 1990). However, when ISGF3γ formed a complex with ISGF3α, the capability for ISGF3γ to activate gene expression increased more than 10 times (Fu et al., 1990). In conclusion, ISGF3α and ISGF3γ were functionally associated protein complex that activated gene expression in response to HuIFN-α stimulation.

One subunit of ISGF3α complex, Stat 91 has been shown to bind GAS sequence specifically (Fu et al., 1992b). Stat 91 protein was usually in monomer form in cytoplasm. When the cells were stimulated by IFN-γ, it was found that Stat 91 formed

homodimer and translocated into cell nucleus in a few minutes. It has been demonstrated that Stat 91 protein was required and sufficient for the activation of gene transcription through specific binding to GAS DNA element (Shuai et al., 1993). Thus, it appeared that Stat 91 was a transcriptional activator in the IFN-γ induced gene expression.

6. Signal Transduction of IFN

IFN elicits its biological activities through specific binding to cell surface receptor and subsequent activation of gene expression. The initial studies on receptor-mediated signal transduction of IFN were focused on the studies of "second messengers". Yap et al. (1986) reported that intracellular diacylglycerol and inositol triphosphate were elevated in fibroblast and human B cell lymphoblastoid after exposure to IFN-γ (Yap et al., 1986). However, this intracellular event required 20,000 units/ml of IFN-γ which was much higher than the physiological concentration of IFN-γ. Another possible "second messenger" was protein kinase C (PKC) (Cernescu et al. 1989). It was found that γ.1 gene induction in macrophage was inhibited by PKC inhibitor H7 and sphingosine. Although these studies have provided some details for potential "second messengers" that mediate the biological responses induced by IFN-γ, none of these studies has clarified the signal transduction pathways for IFN.

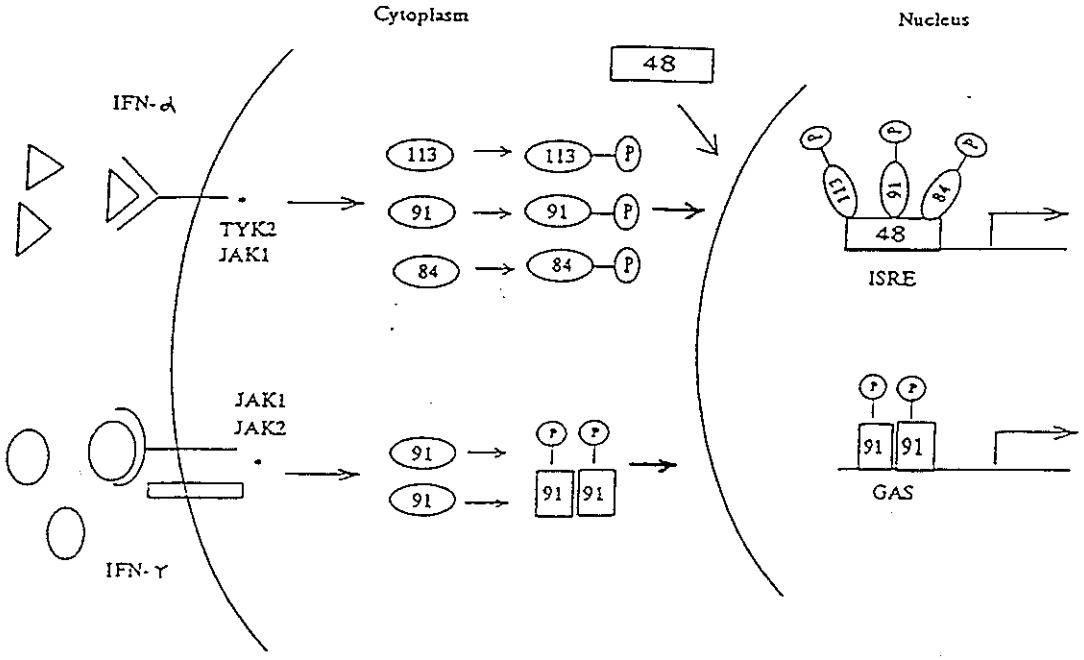
Fu first described that ISGF3α polypeptides (the cytoplasmic Stat 84, 91 and

113 kDa proteins) contained SH2 and SH3 Src homology domains (Fu, 1992a). The structure of these domains suggested that Stat proteins could be phosphorylated by tyrosine kinase. Stat proteins existed in the cell cytoplasm under normal conditions. Following the treatment of type I IFN, it was found that three Stat proteins (Stat 84, 91 and 113) were phosphorylated on tyrosine residues and then assembled into a protein complex with ISGF3γ (48 kDa DNA binding protein). This complex was translocated into the cell nucleus, where it bound to ISRE sequence in the 5' regulatory region of type I IFN induced genes (Veals et al., 1992 and Hunter, 1994). It was suggested that the initial "signal" from receptor-ligand binding of type I IFN was passed into the cytoplasm through phosphorylation of tyrosine residues of Stat proteins (Stat 84, 91 and 113). The subsequent phosphorylated Stat proteins transduced the "signal" into the cell nucleus by forming a complex with ISGF3γ protein (48 kDa), which bound to ISRE sequence (Shuai et al., 1992). Following the stimulation of IFN-γ, it was found that only the SH2 domain of Stat 91 was phosphorylated (Shuai et al., 1993). The phosphorylated Stat 91 protein was assembled into a homodimer and translocated into the cell nucleus. It was demonstrated that only the phosphorylated homodimer of Stat 91 could bind to GAS sequence, which was one of the *cis*-DNA regulatory element for activation of IFN-γ induced genes (Shuai et al., 1993).

The protein kinases that phosphorylated the Stat proteins have been identified

recently (Muller et al., 1994 and Baglioni et al., 1980). It was found that 2 tyrosine kinase, janus kinase (Jak1) and tyrosine kinase 2 (Tyk2) were activated by type I IFN receptor after ligand-receptor binding. The activated Jak1 and Tyk2 then phosphorylated Stat 84, 91 and Stat 113 proteins simultaneously and resulted in activation of gene transcription. It was found that the JAK1 and another protein kinase JAK2 were activated by IFN-γ receptor after receptor-ligand binding (Miller et al., 1994 and Baglioni et al., 1980). The activation of JAK1 and JAK2 simultaneously caused phosphorylation of Stat 91 but not Stat 84 and Stat 113. The phosphorylation and subsequent dimerization of Stat 91 protein caused activation of IFN-γ induced gene expression. In summary, type I IFN and type II IFN (IFN-γ) utilized overlapping but different signal transduction pathways in the activation of gene expression. Recent studies have shown that phosphorylated Stat 91 was also involved in the signal transduction of several other cytokines and growth factors, such as epithelial growth factor (EGF), colony stimulating factor 1 (CSF 1), platelet derived growth factor (PDGF) and interleukin 6 (IL 6). It was suggested that phosphorylation of Stat 91 protein was a common pathway for many growth factors and cytokines. However, other mechanisms might involved in order to produce a specific biological responses induced by IFNs (Hunter, 1994).

The signal transduction pathways for IFN-α and IFN-γ are schematically summarized in Figure 5.


Cytoplasm
Nucleus
48
IFN-α
TYK2
JAK1
113
91
84
P
ISRE
JAK1
JAK2
IFN-γ
GAS

7. Molecular Mechanisms of IFN Action

7.1 a. Antiviral effect of 2'-5'-Oligoadenylate Synthetase-Nuclease System

The 2'-5'-oligoadenylate (2,5-A_n) synthetase-nuclease system is one of the enzyme systems induced by IFN. This system was initially described by Brown et al. (1976). This system consists of three enzymes: 1) 2,5-A_n synthetase, 2) 2,5-A_n phosphodiesterase and 3) 2,5-A_n dependent RNase (RNase L).

The 2,5-A_n synthetase could be induced by both type I and type II IFN in a variety of cell types (Baglioni et al., 1980). Both human and mouse 2,5-A_n synthetases have been purified and characterized (Baglioni et al., 1980). A large (85-100 kDa) and a small form (20-30 kDa) of human enzyme have been described. It was found that the activity of both forms of 2,5-A_n synthetase was dependent on the presence of dsRNA. The 2,5-A_n synthetase catalyzed the synthesis of 2,5-A_n (n ranges from 1-15) in the presence of virus dsRNA. A series of 2,5-A_n were required for the activation of the RNase L, an cytoplasmic endoribonuclease that cleaved virus RNA (Schimtdt et al., 1979). The expression of RNase L was usually kept in low level, but it increased 10-20 fold in response to the stimulation of IFN (Pestka et al., 1987). It was shown that RNase L cleaved the single stranded RNA (ssRNA) of viruses, cellular ssRNA as well as synthetic ssRNA in the presence of 2,5-A_n (Silverman et al., 1983). The specific cleavage of the viral ssRNA *in vivo* was considered to be one of the direct mechanism for IFN-induced antiviral effect. The third enzyme in this system was 2'-5'A_n phosphodiesterase

which degraded the 2,5-A_n (Eppstein et al., 1979 and Minks et al., 1980). The specific 2'-5' phosphodiesterase hydrolysed the 2,5-A_n into AMP and ATP. Therefore, the activated RNase L returned to the latent state. In summary, a balance between 2'-5'-A_n synthetase and 2'-5'A_n phosphodiesterase determined the intracellular concentration of 2,5-A_n and hence, the activity of RNase L. In response to the exogenous IFN stimulation, more 2,5-A_n synthetase were synthesized, resulting in elevation of 2'-5'-A_n and activation of RNase L. Subsequently, cells become resistant to virus infection (Pestka et al., 1987).

7.1 b. P1/eIF-2α Protein Kinase and Phosphatase System

Phosphorylation of two intracellular proteins, P1 and eIF-2α (elongation factor 2α) was very sensitive to IFN treatment in many types of animal cells (Samuel et al., 1979 and Robert et al., 1976). Protein P1 was a ribosome-associated protein while eIF-2α was the smallest subunit of initiation factor eIF-2 that was required for protein syntheses. According to the studies of Samuel et al. (1985), both P1 and eIF-2α were phosphorylated in response to exogenous IFN stimulation. The protein kinase responsible for the phosphorylation of P1 and eIF-2α has been identified. It was found that P1 protein itself had kinase activity and specifically phosphorylated itself and eIF-2α protein in the presence of dsRNA (Samuel et al., 1984). The major phosphorylation sites were serine residues in both P1 and eIF-2α. It was found that phosphorylation of P1

and elF-2α resulted in inhibition of protein syntheses, and thus, virus replication (Krause et al. 1980). The inhibition of protein syntheses caused by the P1 and elF-2α phosphorylation was transient because a specific phosphoprotein phosphatase induced by IFN could subsequently remove the phosphate from P1 and elF-2α (Pestka et al., 1987).

7.1 c. Inhibition of Virus Gene Expression by RBP9-27

It was found that the replication of HIV-1 in cell lines could be inhibited by exogenous IFN (Poli et al., 1989). An IFN-induced cellular protein, named RBP9-27 could specifically bind to HIV RNA and inhibited the gene expression of HIV-1 (Contantoulakin and Campbell, 1993). The genome of HIV-1 contained three major genes (*gag*, *pol* and *env*) and several regulatory genes, such as *tat* and *rev* genes. The *rev* gene encoded a protein that recognized specifically a RNA sequence of HIV-1 termed *rev* response element (RRE, about 210 nucleotides). It was found that binding of *rev* protein to the RNA altered the splicing of HIV-1 primary transcript, which was required for the replication of HIV-1 (Haseltine, 1991). It was found that the cellular protein RBP9-27 induced by IFN-γ also specifically bound to RRE sequence of HIV-1 RNA (Contantoulakin & Campbell., 1993). The binding of RBP9-27 to viral RNA interfered the interaction of RRE sequence with *rev* protein, thus inhibited the gene expression of

HIV-1. These results suggested that IFN induced inhibition to HIV-1 replication was mediated by RBP9-27 protein through its interference with the function of HIV-1 *rev* protein. Further study on RBP9-27 protein may shed the light to the mechanism of IFN-γ induced antiviral state in HIV-1 infected cells.

7.2 Inhibition of Cell Proliferation

Both type I and type II IFN inhibited cell growth. Generally, IFN-γ was a more potent inhibitor than IFN-α and IFN-β. The anti-proliferative effects induced by IFN affected all phases of cell growth cycle (Pestka et al., 1987). Recently, HuIFN-α has been approved for the treatment of hairy cell leukemia because of its anti-proliferative effect (Quesada et al., 1984).

Ozaki et al. (1988). first showed that an enzyme called indolamine 2,3-dioxygenase (IDO) was involved in the anti-proliferative activity of IFN (Ozaki et al., 1988). IDO catalyzed the conversion of tryptophan into kynurenine in the metabolic pathway of tryptophan. It was found that IDO was induced by both type I and type II interferons. The intracellular level of IDO was directly related to the IFN dependent anti-proliferation activity (Ozaki et al., 1988). This observation suggested that the degradation of tryptophan by IDO caused cell starvation of this essential amino acid and hence caused inhibition of cell growth. This "amino acid starvation" hypothesis was mainly supported

by the following facts: 1) cell lines grown in medium containing relatively high level of tryptophan were more resistant to IFN-γ induced anti-proliferative effect; 2) anti-proliferative activity of IFN-γ can be reversed by addition of tryptophan in the medium of cell culture; 3) mutant cell line Me-180 which was defective in IDO induction was resistant to the anti-proliferation effect of IFN-γ (Taylor et al., 1991). In summary, these observations suggested that the induction of IDO by IFN and subsequently degradation of tryptophan was one of the mechanisms of IFN-mediated anti-proliferative effect.

8. Structure and Functional Relationship of HuIFN-γ

8.1 Neutralizing mAbs and Functional domains

Anti-HuIFN-γ monoclonal antibodies (mAb) bind to HuIFN-γ through specific recognition to the epitopes on the surface of HuIFN-γ. If the binding of a specific mAb to HuIFN-γ induces inactivation of the biological activities, this mAb is termed neutralizing mAb. In most circumstances, a neutralizing mAb inactivates HuIFN-γ through its specific binding to the epitope and causes a steric hindrance to the functional site, which is the epitope itself or a domain at close proximity to the epitope. Thus, the epitope recognized by the neutralizing mAb usually is the functional site or a domain adjacent to the functional site. In the very rare case, a mAb can inactivate HuIFN-γ by causing conformational change that affects a distant functional domain. In this exceptional case,

the functional site has no structural relationship with the epitope and can not be localized through the location of the neutralizing epitope. In general, the neutralizing mAbs approach is an effective method for studying the functional domains of HuIFN-γ.

8.1 a. Development of Anti-HuIFN-γ mAbs

The hybridoma technique developed by Kohler et al. (1975) is a common laboratory technique to produce monoclonal antibodies. The most important step in the production of mAb is the screening of hybridoma cells with an effective method so that the desired mAbs can be selected. In the production and screening of anti-HuIFN-γ monoclonal antibody, solid phase radioimmunoassay, enzyme immunoassay (EIA), radio-immunoprecipitation and direct enzyme linked immunoassay (ELISA) have been used (Le et al., 1984; Liang et al., 1985; Meager et al., 1984 and Farre et al., 1989). All these methods were based on the specific antibody-antigen interaction and using purified HuIFN-γ. The anti-HuIFN-γ mAbs produced with these screening methods contained both neutralizing and non-neutralizing mAbs. The functional assays (such as antiviral neutralizing assay and inhibition of the expression of HLA class II antigen) have also been used by some investigators as screening procedures. The anti-HuIFN-γ mAbs screened by these procedures were often the neutralizing mAbs only.

The successful establishment of the hybridoma cell lines secreting anti-HuFN-γ

mAb was first reported by Hochkeppel and Ley in 1982. Although these mAbs specifically recognized HuIFN-γ, the functional analyses of the mAbs were not reported. The first neutralizing anti-HuIFN-γ mAb was generated in 1983 (Rubin et al., 1983). This mAb was shown to neutralize the antiviral and anti-proliferation activities of natural HuIFN-γ. Wang et al. (1984) reported the generation of 5 other mAbs against natural HuIFN-γ. All of the 5 mAbs had high binding affinity to HuIFN-γ but exhibited different HuIFN-γ neutralizing activity (Wang et al., 1984).

Using radioimmunoassay to screen hybridoma cells, 2 mAbs (named B1 and B3) have been selected by Le et al. (1984). Both mAbs had neutralizing activity to natural and recombinant HuIFN-γ. B3 mAb was shown to be more potent than B1. Stafanos et al. (1985) produced 3 mAbs by using soluble-phase radioimmunoassay. All of the 3 mAbs recognized specifically the natural and recombinant HuIFN-γ. Two of the mAbs, S1-1 and S1-3 were able to neutralize the antiviral activity of HuIFN-γ. Soh et al. (1993) reported their production of 72 mAbs against HuIFN-γ by screening culture fluid of the hybridoma cells with solid phase immunoassay. Only 26 of these 72 mAbs showed high affinity binding to HuIFN-γ. Among them, 5 were neutralizing mAbs. Liang et al. (1985) selected 7 mAbs (BG1-7) by screening hybridoma cells with ELISA, four (BG1-4) of them were able to neutralize the antiviral activity of both natural and recombinant HuIFN-γ. With

similar screening procedures, Oda, et al. (1986) produced three anti-HuIFN-γ mAbs (KM45, KM48, KM61), only KM48 was able to inhibit the antiviral activity of rHuIFN-γ.

In addition to the reports mentioned above, other screening methods have been used. Radio-immunoprecipitation was used to screening anti-HuIFN-γ mAbs by Meager et al. (1984) and 5 mAbs were produced. Four of these 5 mAbs were able to inhibit the antiviral activity of HuIFN-γ. Two hybridoma cell lines were reported to secrete specific mAbs as detected by enzyme immunoassay (Van der Meide et al., 1985). Both mAbs (MD-1 and MD-2) neutralized the antiviral activity of natural and recombinant HuIFN-γ. Ziai et al. (1986) raised two anti-HuIFN-γ mAbs, M133.1 and M133.3 by screening the supernatant of hybridoma cells for its ability to block HLA class II enhancing effect of rHuIFN-γ. Both mAbs neutralized the antiviral, anti-proliferative and HLA class II enhancing activities of HuIFN-γ. By screening the hybridoma cells with the direct ELISA, immunoprecipitation, neutralizing antiviral assay and neutralizing HLA induction, Farre et al. (1989) developed five mAb against HuIFN-γ. It was reported that 3 mAbs (B22, B27 and B35) inhibited the antiviral activity of recombinant HuIFN-γ, while 2 other mAbs (B32 and B3-6) had no neutralizing effect.

In contrast to the small number of anti-HuIFN-γ mAbs produced by each group

described above, Alfa et al. (1987) has reported a large panel of anti-HuIFN-γ mAbs produced in our laboratory. A sandwich ELISA was designed for the screening of hybridoma cells. A polyclonal antibody which was absorbed with E. coli proteins was used to capture HuIFN-γ from the crude preparation of human recombinant IFN-γ. The captured HuIFN-γ was specifically recognized by the mAb tested. One hundred and thirty mAbs have been selected by this screening method (Alfa and Jay, unpublished data). All of these 130 mAbs have been characterized by antiviral neutralization assay and immunoblotting. Among them, 21 mAbs had neutralizing antiviral activity and high affinity binding to HuIFN-γ.

8.1 b. Characterization of Anti-HuIFN-γ mAbs

The large panel of neutralizing anti-HuIFN-γ mAbs produced in our laboratory allowed a comprehensive search for the antigenic epitopes of the recombinant HuIFN-γ. To determine the relatedness of the epitopes recognized by each individual mAb, a competition sandwich radioimmunoassay (sRIA) was developed. This assay was used to determine the efficiency of each mAb to compete with other mAbs (Alfa et al., 1988). The degree of the cross-reactivity would reveal the relatedness of the respective epitopes recognized by these mAbs. Each ^{125}I-labelled mAb was allowed to compete for binding to HuIFN-γ with various unlabelled mAbs. According to results of the cross-competition

analyses, 21 mAbs was classified into 3 reacting groups. The first group recognized E_1 epitope only and the second group recognized E_2 epitope only. The third group cross-reacted with some of the E_1 and E_2 mAbs and was termed group E_1/E_2 (Table 3).

The first group has 8 mAbs, which competitively recognized a single epitope E_1. Five of these mAbs reacted with sodium dodecyl sulfate (SDS) denatured HuIFN-γ, which suggested that E_1 was a linear epitope. The second reacting group also contained 8 mAbs which competitively recognized another distinct epitope E_2. All of the mAbs in E_2 group did not react with SDS denatured HuIFN-γ, suggesting that a tertiary structure-dependent epitope was recognized by this group of mAbs. The third reactivity group (E_1/E_2 group) contained 3 mAbs which had cross competition with the mAbs in both E_1 and E_2 reacting groups (Table 3). According to these results, two distinct epitopes were identified by the cross competition analyses. E_1 epitope was recognized by the mAbs in E_1 reacting group and E_2 epitope was recognized by the mAbs in E_2 reactivity group. Since these mAbs neutralize the biological functions of HuIFN-γ, E_1 and E_2 were considered as two distinct neutralizing epitopes (Alfa et al., 1988).

Each of the 21 neutralizing mAbs has been analyzed for its ability to block HuIFN-γ binding to the receptor on cell surface. None of the mAbs was able to block HuIFN-γ binding to the targeted cells (Alfa and Jay, unpublished data), suggesting that E_1 and E_2 epitopes were not involved in the binding to the receptor of HuIFN-γ. In

Table 3.anti-HuIFN-γ mAbs grouped according to the epitope specification.

E1	E1/E2	E2
MIF3061	MIF3102	MIF3070
MIF3015	MIF3094	MIF3098
MIF3009	MIF3059	MIF3055
MIF3081		MIF3045
MIF3075		MIF3041
MIF3169		MIF3069
MIF3074		MIF3054
MIF3152		MIF3052
MIF3091		MIF3125

The classifications of anti-HuIFN-gamma mAbs and the neutralizing epitopes were based on the studies of Alfa and Jay (1988).

summary, these analyses of anti-HuIFN-γ mAbs reported by Alfa and Jay (1988) can be concluded as: 1.) HuIFN-γ contained at least two functional epitopes, E_1 and E_2; 2.) these two functional epitopes were not involved in the binding to HuIFN-γ receptor.

Similar to the studies in our laboratory, three neutralizing anti-HuIFN-γ mAbs (BG1, BG3 and BG4) were analyzed by Liang and coworkers (Liang et al., 1995). The mAbs BG1 and BG4 recognized the same epitope, while mAb BG2 recognized another epitope that did not overlap with epitope recognized by BG1 and BG4. Chang et al. (1986) found that two neutralizing mAbs (B1 and B3) developed in their laboratory did not compete each other in binding to HuIFN-γ molecules. They concluded that two non-overlapped neutralizing epitopes were responsible for binding of mAbs B1 and B3. Stefanos et al. (1985) reported that two neutralizing mAbs, S1-1 and S1-2 recognized different epitopes based on their epitope blocking test. Van der Meide et al. (1985) studied the neutralizing mAbs MD1 and MD2 by immunodiffussion analysis. Two different mAb-HuIFN-γ diffusion zones were observed in this study. Although the method used in this study was not as accurate as mAb cross-competition assays, this observation also suggested that MD1 and MD2 recognized two separated neutralizing epitopes (Van der Meide et al., 1985).

Because of different mAbs and methods were used in characterize the functional epitopes of HuIFN-γ, it was not certain whether the epitopes identified in various studies

were comparable. In order to determine whether these anti-HuINF-γ mAbs were recognizing similar neutralizing epitopes, Yang et al.(1993) collected 13 independently isolated mAbs from 5 different laboratories and allowed these mAbs to compete against the mAbs developed in our laboratory. The mAb MIF3009, MIF3055 and MIF3125 were chosen to represent the mAbs in E_1, E_2 and E_1/E_2 reacting groups, respectively. Anti-HuIFN-γ mAbs developed in other laboratories were analyzed for their ability to compete with these mAbs (Table 4). The results suggested that these 13 mAbs could compete with the mAbs of either E_1, E_2 or E_1/E_2 groups (Table 4). Therefore, these 13 mAbs from 5 different laboratories were proven to bind the same two epitopes (E_1/E_2) (Yang, 1991). This study provided a comparison and classification of the mAbs developed across different laboratories. Therefore, it was concluded that E_1 and E_2 epitopes were the only 2 functional epitopes identified to date.

8.2 Mapping of the Functional Epitopes by Abs against HuIFN-γ Analogs

Although the analysis of mAbs against the entire HuIFN-γ molecule is one of the most common methods to study the structure-functional relationship of HuIFN-γ, some investigators preferred to approach this question by development and characterization of mAbs and polyclonal antibodies directed against HuIFN-γ peptide analogs. Johnson et al. immunized rabbits with a synthetic peptide corresponding to the N-terminal 20 amino

Table 4A. The mAbs compared in the coss-competition analyses for epitope assigmemt.

mAb name	Ig subtype	sample preparation	ref
mAbB1	IgG1	hybridoma supernatant	Le et al.
mAbB3	IgG1		
5J	unknown	Purified Ig	Meager et al.
45B3	unknown		
NIB42	unknown		
KM48	IgG1	Ascites fluid	Oda et al.
KM61	IgG1		
B133.1	IgG1	Ascites fluid	Ziai et al.
B133.3	IgG1		
69B.J	IgG1	Ascites fluid	Pestka et al.
220A12.J	IgG1		
73A.J	IgG1		
113B.J	IgG1		

Table 4B. Epitope assigment of 13 mAbs from 5 different laboratories.

MIF3009 (E_1)	MIF3094 (E_1/E_2)	MIF3055 (E_2)
69B.J	N1B42	B133.1
113B.J	B133.3	KM48
220A12.J	B1	5J
73A.J		B3
		4SB3
		KM61

The epitope assigment of the 13 mAbs were based on the cross-competition ananlyses of Yang (1991).

acids sequence. The antibodies developed recognized natural HuIFN-γ specifically and inhibited antiviral activity of natural HuIFN-γ (Johnson et al., 1982). The results suggested that the amino terminal 20 amino acid residues was important for antiviral activity of HuIFN-γ (Johnson et al., 1982). Leist et al. reported that the antibodies raised against the amino terminal peptide corresponding to residues 1-59 inhibited the antiviral activity of HuIFN-γ, while the antibodies against peptide from residues 24-59 did not inhibit the antiviral activity of HuIFN-γ (Leist et al., 1985). These results suggested that residues 1-20 were essential for the function, while residues 24-59 was not directly associated with biological function of HuIFN-γ. Ichimori et al. reported that a mAb against amino terminal peptide corresponding to 4-21 residues of HuIFN-γ was developed (Ichimori et al., 1987). This mAb inhibited antiviral activity of HuIFN-γ. It was suggested that biological activities of HuIFN-γ were determined by the amino terminal 4-21 residues (Ichimori et al., 1987).

The functional roles of HuIFN-γ C-terminus have also been studied by anti-peptide antibodies. Ichimori et al. reported that the mAb against C-terminal peptide corresponding to residue 131-146 did not inhibit antiviral activity of HuIFN-γ, although the mAb could bind to HuIFN-γ (Ichimori et al., 1987). Altrock et al. (1989) generated a mAb against the C-terminus peptide corresponding to residues 128-146 of HuIFN-γ. This

mAb was able to bind HuIFN-γ, but it did not inhibit antiviral activity of HuIFN-γ. Lord et al. (1989) also reported that a polyclonal rabbit serum against the C-terminal peptide corresponding to residues 121-130 was not able to inhibit the antiviral activity of HuIFN-γ, although the antibody specifically recognized HuIFN-γ. In summary, it was suggested that the C-terminal 121-146 residues of HuIFN-γ was not directly associated with the biological functions of HuIFN-γ (Figure 6).

8.3 Modification of the Primary Sequence of HuIFN-γ

A gene modification technique called site-directed mutagenesis has been widely used in constructing various mutant proteins. Specific amino acid residues can be specifically changed by site-directed mutagenesis. The structure-functional relationship of HuIFN-γ can be studied by comparing the activities of modified variants to the wild type of HuIFN-γ. The modification of HuIFN-γ molecule can also be obtained by protease cleavage in the specific peptide bond of HuIFN-γ. Only the truncated molecule of HuIFN-γ can be produced using this method. Therefore, this method is often used to study the functional roles of C-terminus or N-terminus of HuIFN-γ.

Hogrefe et al. (1989) reported a method to generate HuIFN-γ analogs lacking amino-terminal 8 or 10 residues by limited digestion with V8 protease, which cleaved "Ser-Leu" peptide bond. The truncated molecules containing fragments from

Figure 6. Antibodies to synthetic peptides corresponding to the sequence of HuIFN-gamma. The anti-HuIFN-gamma peptide antibodies were categorized into 2 types: antibodies that bound and neutralized antiviral activity of Human IFN-gamma (---); antibodies that bound to HuIFN-gamma but did not neutralize the antiviral activity (===).

```
          10        20        30        40        50
 QDPYVKEAENLKKYFNAGHSDVADNGTLFLGILKNWKEESDRKIMQSQIVSFYFKLFK
 1-----.----------17 (Johnson, 1982)
    4-------------18 (Ichimori, 1987)
                     21==================================55
                                                  (Leist,1986)
 1-----------------------------------------------------56
                                                   (Leist, 1985)
 60        70        80        90        100       110
 NFKDDQSIQKSVETIKEDMNVKFFNSNKKKRDDFEKLTNYSVTDLNVQRKAIHEILQ


  120       130       140
VMAELSPAAKTGKRKRSQMLFRGRRASD
118======127 (Lord, 1988)
       128==============143 (Ichimori, 1985)
     125================143 (Altrock, 1983)
```

residues 8-139 and 10-139 were subsequently purified. Both fragments did not show antiviral activity and failed to induce expression of MHC class II molecules in target cells. Examination of fragments 8-139 and 10-139 by far-UV circular dichroism revealed that cleavage of 8-10 residues at the amino terminus caused a dramatic change in the secondary structure of HuIFN-γ. Therefore, it was suggested that the N-terminal 10 amino acid residues were important for maintaining the tertiary structure of HuIFN-γ (Hogrefe et al., 1989).

The functional roles of HuIFN-γ C-terminus have been studied extensively with gene modification and other methods with controversial results. The C-terminus was highly hydrophillic with two pairs of Lys and Arg sequence from 128-132 (Figure 2). The x-ray crystallography has shown that the C-terminus was largely a random coil and move relatively free compared to other parts of the molecule. Pan et al. reported (1987) that natural HuIFN-γ purified from medium of peripheral blood leucocytes contained HuIFN-γ molecules with heterogeneity of C-terminus. The major form had 138 residues in length, 5 amino acid shorter than that predicted from the cDNA sequence of HuIFN-γ. Other forms had 127, 128, 129 and 131 amino acids in length. Protein sequencing results revealed that HuIFN-γ was trimmed at the C-terminal end at different sites. Limited proteolytic digestion of the recombinant full length HuIFN-γ with endoproteinase Lys-C and trypsin released the C-terminal 15 residues. The C-terminus 15 amino acids truncated

HuIFN-γ retained full antiviral activity (Pan et al., 1987). The presence of C-terminal heterogeneity suggested that the C-terminus 15 amino acids residues were not essential for the functions of HuIFN-γ molecule. Sakaguchi et al. (1985) constructed mutated cDNA clones that encoded HuIFN-γ variants lacking 19, 20, 21, and 22 C-terminal residues. The circular dichroism spectra of these C-terminus truncated mutants were very similar to that of wild type HuIFN-γ. All 4 variant molecules had approximately the same level of antiviral activity as the wild type. It was concluded from this study that the C-terminus 22 amino acid residues were not essential for the biological functions of HuIFN-γ. In contrast to the studies described above, Leinikki et al. (1987) generated a HuIFN-γ variant with C-terminal 11 amino acid residues removed by proteolytic enzyme digestion. This molecule displayed 1000-2000 fold lower binding capacity to surface receptor of HuIFN-γ and 10 fold reduction in its ability to induce Fc receptor on human cell lines. It was suggested that the C-terminus 11 residues contributed significantly to the formation of receptor binding site of HuIFN-γ (Leinikki et al., 1987). Using similar approach, Arakawa et al. (1989) generated two polypeptides lacking 13 and 14 residues from the C-terminus. These two polypeptides were reported to be identical to the intact HuIFN-γ in respect to the conformation and dimerization, as analyzed by circular dichroism and gel filtration. However, the antiviral activity was 1000 fold lower than that of the intact HuIFN-γ. Using protease digestion, Arakawa also reported the generation of another two

HuIFN-γ analogs, lacking C-terminal 4 and 15 residues, respectively (Arakawa et al., 1990). The HuIFN-γ analog without C-terminal 4 residues had activity indistinguishable from those of HuIFN-γ in antiviral and cell growth inhibition. The HuIFN-γ analog without 15 C-terminal residues not only had markedly decreased antiviral and growth inhibition activity, but also showed decreased binding to the cell surface receptors. These results suggested that the C-terminus was not involved in the protein conformation and self association, but it could alters antiviral activity (Arakawa et al., 1989 and 1990). de la Maza et al. (1987) reported that a HuIFN-γ variant lacking C-terminal 18 residues could inhibit virus replication, growth of Chlamydia trachomatous in various human cell lines. Comparing to the wild type HuIFN-γ, this variant was approximately 100-1000 fold less potent.

Luk et al. (1990) in our laboratory have demonstrated that removing C-terminal 21 amino acid residues resulted in 3-10 fold loss of anti-viral activity It was reported that although C-terminal 21 residues were not essential for antiviral activity of HuIFN-γ, it might have a substantial impact on *in vitro* re-naturing of HuIFN-γ (Luk et al., 1990). The variant clone Del_{122} (which lacks C-terminal 21 residues) had 5 fold lower antiviral activity and 10 fold lower anti-proliferation activity than that of wild type. Deletion of more than 21 residues from the C-terminus resulted in dramatic decrease of the biological function. These results indicated that the residues beyond the C-terminal 21 amino acid

were necessary to maintaine the structure of HuIFN-γ or were directly involved in biological functions. This study suggested that the C-terminal 21 amino acid residues were not essential for the biological function but were required for the full activity of HuIFN-γ. The amino acid residues 1-122 were more important in maintaining the functions of HuIFN-γ (Luk et al., 1990).

A HuIFN-γ mutant containing single amino acid substitution at residue Asn^{25} was constructed by Hsu et al. (1986). This mutant (Asn25Gln) had about 10 fold lower antiviral activity than that of wild type. No secondary structure change were found using circular dichroism and UV spectroscopy analyses. The mutant molecule was still in dimer form. Another HuIFN-γ mutant molecule created by Hsu et al. (1986) had two residues substituted, Asn^{25} by Gln and Asn^{78} by Lys. This mutant had 30 fold lower antiviral activity than that of wild type. The molecule was also in a dimer form and had no detectable secondary structure change. It was suggested that residue Asn^{25} and Asn^{78} were not essential for maintaining the structure and biological function of HuIFN-γ. Another HuIFN-γ mutant with Met^{45} substituted by Thr had 2.5×10^3 fold lower antiviral activity than the wild type HuIFN-γ (Hsu et al, 1986). However, there was an obvious change in the secondary structure since formation of molecular aggregation was detected. It was suggested that residue Met^{45} was required for maintaining the tertiary structure of HuIFN-γ. The decreased biological activity of Met45Thr variant was caused

by changes of tertiary structure (Hsu et al., 1986). A mutant with Phe^{92} substituted by Cys had 2.2 x 10^3 fold lower antiviral activity than wild type. Since residue Phe^{92} was in a very hydrophobic region and was folded inside the core of HuIFN-γ according to the crystal structure of HuIFN-γ, the reduced antiviral activity was probably caused by changes in the tertiary structure (Fish et al., 1988).

8.4 Receptor Binding Site and Effective Site

Neutralizing anti-HuIFN-γ mAbs developed in our laboratory have shown strong inhibitory effects to both antiviral and anti-proliferation activities of HuIFN-γ. However, none of these mAbs was able to block receptor binding of HuIFN-γ (Alfa and Jay, unpublished data). It was suggested that the receptor binding site of HuIFN-γ was separated from the effector site(s) (Alfa and Jay, 1989). The observation has also been confirmed by other investigators. Schreiber et al. (1985) demonstrated that mAbs H1 and H2 neutralized antiviral of mouse IFN-γ activity but did not interfere the binding of mouse IFN-γ to its. Zier et al. reported that two mAbs developed were not able to prevent HuIFN-γ from binding to its receptor, but neutralized antiviral, anti-proliferation and HLA induction activities of HuIFN-γ (Ziai et al., 1986).

Leinikki et al. (1987) reported that deletion of C-terminal 15 residues reduced the binding affinity of HuIFN-γ to its receptor about 10 fold . Substitution of residue His^{111}

by Asp eliminated the antiviral activity accompanied by markedly reduced receptor binding (Lunn et al., 1993). The studies with circular dichroism spectroscopy and NMR indicated that there were no major changes in the tertiary structure of this mutant comparing to the wild type of HuIFN-γ. It was suggested that the functional changes caused by His^{111} swubstitution was duo to the change in the delicate receptor binding domain. It was concluded that His^{111} was located within the receptor binding site of HuIFN-γ (Lunn et al., 1993). Jarpe et al. reported that a synthetic peptide corresponding to the residues 1-39 of murine IFN-γ was able to competitively bind to the cell surface receptor. It was suggested that the receptor binding domain or at least part of the receptor binding domain of murine IFN-γ was located in the N-terminal 1-39 amino acids (Jarpe et al., 1990). A mAb 5.102.12 raised against N-terminal 6-20 residues of murine IFN-γ was shown to neutralize the antiviral activity and block the binding of murine IFN-γ to its receptor. This blocking effect could be removed competitively by synthetic peptide corresponding to residue 6-20 of murine IFN-γ (Jarpe et al., 1990). These results suggested that N-terminal residues 6-20 of murine IFN-γ was directly involved in the binding of murine IFN-γ to its receptor.

Objectives

The understanding of how HuIFN-γ induces the various biological responses offers the opportunity to design new modelities to exploit the different biological

responses for disease intervention and prevention. To this end, the study of the signal transduction pathway was carried out extensively in the past 10-15 years. Although the intracellular signal transduction pathway for HuIFN-γ was quite well understood, the transduction process across the cell membrane as a result of HuIFN-γ binding to the cell surface structure is unknown (Shuai et al., 1993). Indeed, genetic studies has already shown that an additional species specific molecule coded by chromosome 21 is required in addition to the receptor to react with HuIFN-γ (Jung et al., 1990). Previous work in our laboratory has shown that the domain for effector function was separated from that for receptor binding (Alfa & Jay. 1987). Thus, the signal transduction across the cell member is not simply activated by binding of HuIFN-γ to its receptor.

Our aim, therefore, was to understand how HuIFN-γ interact with the cellular component in order to initiate the signal transduction process. The approach to this study is to identify the functional domains of HuIFN-γ and then identify the cellular component that interact specifically with these domains. To this end, we make use of the known observation that mAbs bind to a specific sites and the binding sites are relatively small (6-12 residues). Thus, the epitope of a neutralizing mAb must reside at or adjacent to a functional site in order to block the function of the domain. Our immediate objective, therefore, is to localize the neutralizing epitopes on HuIFN-γ and characterize their function.

CHAPTER II

MATERIALS AND METHODS

1. Extraction and Purification of HuIFN-γ and Its Mutants

The construction and cloning of HuIFN-γ gene (Jay et al., 1984a) and its efficient expressions by a plasmid vector (pJP_1R_3), in Escherichia coli (E. coli) have been previously described (Jay et al., 1984b). The deletion mutant Del_{122} was generated by Luk et al. (1990). The initiating methionine residue in the amino acid sequence of the recombinant HuIFN-γ (rHuIFN-γ) was not part of the mature natural HuIFN-γ and was considered as residue zero. The rHuIFN-γ used in this study was expressed by a modified plasmid vector $pJP_{14}R_9$. The respective recombinant proteins were extracted from transfected E. coli strain LE392 described as following. Cultures were grown from the frozen stock in 500 ml T-broth (1.2% tryptone, 2.4% yeast, 0.4% glycerol, 0.17 M KH_2PO_4, and 0.72 M K_2HPO_4) containing 20 μg/ml of tetracycline (Sigma) at 37°C on a rotary shaker overnight. Cells were harvested by centrifuging at 5000 rpm for 10 minutes at 4°C in a Sorvall GSA rotor and were washed twice with an equal volume of $T_{50}N_{30}$ (50 mM Tris.HCl, pH 8.0, 30 mM NaCl) at 4°C. The cell pellet was resuspended in 10 ml $T_{50}N_{30}$, frozen at -20°C and disrupted by passing through a Carver press (Fred S. Carver Inc., New Jersey) twice at -20°C. The supernatant containing the recombinant protein was separated from the cell debris by centrifuging at 10,000 rpm in a Sorvall HB4 rotor at 4°C for 20 minutes. The supernatant was further centrifuged at 40,000 rpm in Beckman Ti50 rotor for three hours and then was used for immunoaffinity purification.

The HuIFN-γ and its variant polypeptides were purified by immunoaffinity chromatography. The affinity column was prepared by coupling purified anti-HuIFN-γ

monoclonal antibody (MIF3052) to CNBr-activated sepharose (Pharmacia). Supernatant of E. coli lysate containing HuIFN-γ was adjusted to 50 mM Tris.HCl (pH 8.0), 500 mM NaCl and 1% nonidet P-40 (NP_{40}) before loading onto the affinity column. The column was washed with the same buffer at pH 8.0. The absorbed HuIFN-γ was then eluted with 50 mM diethylamide hydrochloride, pH 11.2. Fractions (1.0 ml) were immediately neutralized by collecting into tubes containing 0.1 ml 1 M Tris.HCl (pH 7.5). The purified HuIFN-γ proteins were stored at 0^0C. The protein concentration was estimated using an AmidoBlack (Sigma) adsorption micro-assay method (Schuffuer & Weissmann, 1973).

Purified proteins were also characterized by SDS-PAGE. Typically, protein in 100 µl of fractions was precipitated by addition of 11 µl of 100% (w/V) trichloroacetic acid at 4^0C for 10 minutes. The precipitate was collected by centrifuging at 12,000 x g for 15 minutes. The pellet was washed twice with five volumes of 80% ice cold acetone. The final pellet was air-dried and resuspended in 10 µl of SDS sample buffer(?). The samples were then electrophoresed on a 15% SDS polyacrylamide gel and were stained with Coomassie blue staining (0.04% Commassie brilliant blue, 20% methanol, 10% trichloroacetic acid and 7.5% acetic acid).

2. Estimate of Protein Concentration

Protein concentration of purified rHuIFN-γ and its variants was estimated by using the AmidoBlack (Sigma) adsorption assay (Schuffuer & Weissmann, 1973). The unknown sample

proteins were precipitated with 10% trichloroacetic acid (TCA, Fisher) in the presence of 1% sodium dodecyl sulfate (SDS). The precipitated proteins were collected on a nitrocellulose membrane (0.22 μM, Minipore) by filtration under vacuum suction. The proteins on the membrane were stained with 0.1% Amidoblack 10B in methanol/acetic acid/water (45:10:45, v/v) for 5 minutes. After rinsing the membrane with distilled water extensively, the protein-dye complexes were eluted by immersing the membrane in tubes containing 2 ml of the eluent solution (25 mM NaOH, 0.05 mM EDTA, 50% ethanol) at room temperature for 10 minutes with frequent vortexing. The eluted dye was measured at 630 nm wavelength on a spectrophotometer (Shimadzu, UV-160; Shimadzu Corp, Kyoto, Japan). A standard curve was made by analyzing 0, 0.3, 1.0, 2.0, 3.0 μg of bovine serum albumin (BSA) in parallel. The concentration of unknown proteins was calculated from the standard curve.

3. Anti-HuIFN-γ mAbs

The rabbit polyclonal anti-HuIFN-γ serum, PIF3003 was prepared previously (Alfa et al., 1987). The rabbit serum was prepared by immunizing rabbits with purified denatured HuIFN-γ. The polyclonal anti-serum was collected and purified by Protein-A Sepharose CL-4B affinity column according to the method of the manufacturer (Pharmacia). The purified immunoglobulin (Ig) was absorbed by $pJP_{14}R_9$ bacterial proteins immobilized on cyanogen bromide activated Sepharose 4B (Pharmacia). The reactivity to HuIFN-γ of the purified Ig was tested with enzyme linked immunosorbent assay (ELISA) and Western blot, and was stored at -

20°C.

The production and characterization of anti-HuIFN-γ mAbs have been previously described (Alfa et al., 1987). All mAbs used were IgG type, except MIF3037 which was a IgM type. The mAb ascetic fluid was used in the epitope mapping and neutralization studies. The production of mAb contained in the ascetic fluid was described previously (Alfa et al., 1987).

4. Peptide synthesis

Overlapping octapeptides were synthesized using a commercially available kit (Cambridge Research Biochemicals, Cambridge, UK). The detailed procedures for the peptide synthesis were described in the software and manual supplied by the manufacturer. The amino acid derivatives used in the peptide synthesis were pentafluorophenyl (opfp) active esters of fluoroennylme-thyloxycabonyl (Fmoc) protected L-amino acids. The t-butyl group was linked to the side chains of these amino acid derivatives, except arginine with a methoxytrimethyphenyl sulphonyl (mtr) side chain protecting group (Geysen et al., 1987). Peptides were synthesized onto the tip of solid polyethylene rods in which Fmoc-β-alanine was covalently coupled by the manufacturer. The rods were held in position by specially molded polyethylene holders in the format of 96-well microtiter plate.

A series of 136 overlapping octapeptide homologous of HuIFN-γ were synthesized, each with seven identical residues to its consecutive peptide (one residue's shifts in the sequence between successive peptides). Thus, peptide one had a sequence identical to residue one to eight

of HuIFN-γ; and peptide two, from residue two to nine; and so on. Fmoc-β-alanine that covalently coupled on the polyethylene pins was deprotected by incubation with 20% piperidine in dimethyformamide (DMF) for 30 minutes. The pins were washed in a DMF bath (1 x 5 min), and then methanol bath (4 x 2 min). The pins after deprotection were incubated with the appropriate Fmoc-L-amino acids (30 mM) in DMF for 18 hours. After the coupling reaction, the pins were again washed in a DMF bath (1 x 5 min) and the methanol bath (4 x 2 min). These deprotection-coupling-washing cycles were repeated for eight times. The coupling reactions were carried out in polypropylene microtiter trays designed for 96-well microtiter plate. The distal N-terminus of the synthesized peptide was then acetylated with acetic anhydride and diisopropylethylamine in DMF (5:1:50) for 90 minutes, and the pins were washed again as described above. The side chains were deprotected with trifluoroacetic acid, phenol and ethanedithiol (190:5:5) for four hours, followed by washing in baths of dichoromethanol (2 x 2 min), 5% diisopropylethylamine in distilled dichloromethane (2 x 5 min) and dichoromethane (1 x 5 min) again. Finally, the pins were washed in the methanol bath for 18 hours. The pins were kept dry in a vacuum over silica gel for another 18 hours. The pins were then ready for use in ELISA. Octapeptides with sequences of "T-L-N-P-T-I-A-G" (Peptide A) and "L-A-P-T-I-A-G-K" (Peptide B), which have no sequence homology with HuIFN-γ, were synthesized in parallel. Peptide A and Peptide B were synthesized as positive and negative controls for the peptide synthesis process and for the specificity of reactions in the subsequent mAb mapping by ELISA. Anti-peptide A mAb was kindly provided by Drs. G. Zhong and R. Brunham.

5. Peptide ELISA

The reactivity of anti-HuIFN-γ antibodies (both mAbs and polyclonal antibody) with synthetic octapeptides of HuIFN-γ homologus was detected by a modified ELISA. Each octapeptide was covalently coupled to the tip of a polypropylene rod (pin) which was arranged in the format of 96-well microtiter plate (Dynatech Laboratories, Inc.). The tips of the plastic pin fit exactly into the wells of the standard ELISA plate and were totally immersed when the solution in each well was above 150 µl. The ELISA plate and the plastic pins were pre-incubated with the carrier buffer (1% bovine serum albumin, 1% chicken egg albumin in phosphate buffered saline) at room temperature for two hours to reduce nonspecific absorption in the subsequent steps. Anti-HuIFN-γ antibodies at appropriate dilution, were incubated with the pins at 4^0C overnight. The pins then were washed with 0.05% Tween-20 in phosphate buffered saline (PBS: 136 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4) three times for 15 minutes. Antibodies bound to the synthetic peptides on the pins were detected by incubating with peroxidase-conjugated secondary antibodies for two hours (goat anti-mouse Ig-A, IgG, IgM for mAb and goat anti-rabbit IgG for polyclonal antibodies, supplied by Bethesda Research Laboratory). After washing with the 0.05% Tween-20 in PBS (3 x 15 min), the pins were incubated with peroxidase substrate, 2,2'-azino-di-[3-ethybenzthiazolinsulfonate] (Bio-Rad) at 0.35 mg/ml in citrate buffer (80 mM citric acid, 100 mM Na_2PO_4, pH 4.0). After 15 minutes incubation at 25^0C, the pins were removed and the absorbance was measured with an ELISA reader (BioTek Instrument) at

nm wavelength. Reactions of the pins that carried Peptide A "T-L-N-P-T-I-A-G" and Peptide B "L-A-P-T-I-A-G-K" with anti-Peptide A mAb served as an indicator for the specificity of the ELISA. The average absorbance of anti-Peptide A mAb with Peptide B was taken as background and was subtracted from the absorbance for each pin. Each peptide was numbered according to its N-terminal residues as found on the sequence of HuIFN-γ. The absorbance was plotted according to the peptide numbers that form a consecutive sequence on the HuIFN-γ molecule. The reaction is considered positive if the absorbance at 405 nm is more than 3 times of the mean of the background value since the variation of the nonspecifc reaction is nerver higher than 3 times of the mean of the background value. In addition, any reactivity peak is considered positive only upon repeated assays of not less than 3 times. After each assay, the pins were washed in a sonicator with 1% (w/v) SDS and 0.1% (v/v) 2-mercaptoethanol for 30 minutes at 60^0C to strip the proteins bound to the pins. After washing the pins with water (3 x 10 min), the pins were stored in vacuum and ready to be reused.

6. Direct ELISA

A soluble peptide that contained residue 128-139 of HuIFN-γ was synthesized. The peptide sequence was "Cys-Lys^{128}-Arg-Lys-Arg-Ser-Gln-Met-Leu-Phe-Arg-Gly-Arg^{139}", in which the cystine was not in the natural sequence of HuIFN-γ. A direct ELISA was used to test the reactivity of the anti-HuIFN-γ antibody (both polyclonal antibody and mAbs) to this

soluble peptide. The peptide was dissolved in the carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.0) at final concentration of 50 μg/ml. Fifty microliters of the dissolved peptide (50 μg/ml) were added into each well of a standard 96-well ELISA plate and were incubated at 4°C overnight to allow the attachment of the peptide to the solid phase of ELISA wells. Nonspecific protein binding to the solid surface was reduced by incubating with 3% BSA in PBS (150 μl/well) at 37°C for 3 hours. The anti-HuIFN-γ antibodies, at appropriate dilution in 1% BSA/PBS were serially diluted (1:2) in the ELISA plate and were allowed to react with peptide at 37°C for 2 hours. The ELISA wells were then washed with 0.1% Tween-20 in PBS (3 x 10 minutes). The antibodies bound to the peptide were detected by incubating with peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG for the polyclonal antibody and goat anti-mouse Ig-A, G, M for the mAbs) in 1% BSA/PBS at 37°C for 2 hours. After washing with 0.1% Tween-20 in PBS, the wells were incubated with the peroxide subtract, 2,2'-azino-di-[3-ethybenzthiazolinsulfonate] (Bio-Rad) at 0.35 mg/ml in citric buffer at room temperature for 5 minutes. The color development was stoped by adding 50 μl of 1% SDS into each wells. The absorbance was measured at 405 nm wavelength with an ELISA reader. The negative control was defined as the ELISA reaction without the anti-HuIFN-γ antibody. The average absorbance of three negative controls was taken as the background for all the reaction in the same plate and was subtracted from the absorbance of each well.

7. Western Blot

Proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were transferred electrophoretically onto nitrocellulose membrane (Schleicher Schull, BA83, 0.2 μM pore size) at 300 mA for two hours. Nonspecific binding sites on the nitrocellulose were blocked by incubating with 3% bovine serum albumin (BSA) in TTBS (20 mM Tris.HCl, 100 mM NaCl and 0.05% Tween20) at 4^0C overnight. Primary antibodies (both polyclonal antibody and mAbs) at appropriate dilution in 1% BSA/PBS were incubated with the nitrocellulose at 37^0C for two hours. The nitrocellulose membrane was then washed with TTBS (3 x 10 minutes) at room temperature. The antibodies bound to the nitrocellulose were detected by incubating with the peroxidase-conjugated secondary antibody (goat anti-rabbit for polyclonal serum, goat anti-mouse for mAbs) diluted in 1% BSA/TTBS. After incubation at room temperature for two hours, the nitrocellulose membrane was again washed with TTBS (3 x 10 minutes). The blot was then developed in 100 ml TBS containing 60 μl 33% hydrogen peroxide and 60 mg of 4-chloro-1-napthol (Bio-Rad) dissolved in 20 ml cold methanol. After the color development for 5-20 minutes, the membrane was washed with water and dried at room temperature.

8. *In Vitro* Antiviral Assay

The antiviral assay of HuIFN-γ was based on its ability to inhibit the cytopathic effect (CPE) induced by virus infection. The human lung carcinoma cell line A_{549} was used as target cell

in the antiviral activity assay of various HuIFN-γ preparations. Cells were seeded in 96-well tissue culture plate (Corning, New York) at a concentration of 4 x 10^4/well and were allowed to attach at 37^0C for 4 hours in 5% CO_2. HuIFN-γ preparations were then added in the first well and were twofold serially diluted. After 18 hours incubation, the cells were challenged with encephalomyocarditis virus (EMCV) at 2 multiplicity of infection (MOI) each well. After 24 hours incubation, the supernatant was removed and the cells were fixed and stained with staining solution (0.25% crystal violet, 20 mM Tris.HCl, pH 7.5; 0.9% NaCl, 20% methanol) for 10 minutes at room temperature. The excess staining solution was then washed out with tape water. After the plate was dried at room temperature, 100 µl of methanol was added into each well to dissolve the dye adsorbed by the cells. The absorbance at 590 nm was detected by using an ELISA reader Bio-Tek Instrument EL 308. Since the absorbance at 590 nm represented the amount of dye uptake by the cells, it was inversely proportional to the cytopathic effect (CPE) caused by EMCV infection. The average absorbance of the untreated cells infected with EMCV was used as calibration for 100% CPE. The endpoint was the reciprocal of the dilution that produced 50% CPE. A laboratory HuIFN-γ standard calibrated against the NIH standard was included in each assay for the conversion into international units.

9. Antiviral Activity Neutralization Assay

The neutralizing effect of anti-HuIFN-γ mAbs was analyzed by using antiviral activity neutralization assay. A constant amount of HuIFN-γ (usually 4 working units/ml) was usually

used in the assay. The concentration of 4 working units per ml of HuIFN-γ produced approximately >80% protection against EMCV challenge. The A_{549} cells (4×10^4 per well) were seeded at 37°C in 5% CO_2 for 4 hours to allow cell attachment. The mAbs were serially diluted (1:2) in another 96-well plate and a constant amount of HuIFN-γ was added. After incubation at 4°C for one hour, the solutions of mAb and HuIFN-γ were transferred into the corresponding wells of cell culture plate and incubated at 37°C in 5% CO_2 for 18 hours. The subsequent virus challenge, cell staining and the reading of the absorbance were similar as in the antiviral assay. The antiviral activity neutralization unit was calculated as the highest dilution of the mAb that produced 50% CPE and was normalized to one reference unit of HuIFN-γ per ml input in the neutralization assay.

10. DNA Extraction with Phenol/Chloroform and Precipitation with Ethanol

Distilled phenol was pre-saturated with 250 mM Tris.HCl (pH 8.0) once and was mixed with chloroform/isoamyl alcohol solution (24: 1) in a volume ratio of 1:1. The organic phase (lower phase) was then equilibrated with TE buffer (10 mM Tris.HCl, pH 8.0, 1 mM EDTA). When DNA was extracted from a liquid solution or suspension, an equal volume of phenol/chloroform/isoamyl solution was added into the sample tube and was immediately vortexed for one minute. The tube was then centrifuged at 12,000 x g for one minute and the organic phase was removed. The extraction with phenol/chloroform/isoamyl alcohol was repeated for another two times. The aqueous phase was then collected into a new tube and extracted 3

times with chloroform/isoamyl alcohol (24:1, V/V). In the last extraction, the aqueous phase was aspirated and transferred into another microcentrifuge tube. The solution was then adjusted with potassium acetate to a final concentration 0.3 M and mixed with 2.5 volumes of cold ethanol. After freezing the sample at -70^{0}C for 10 minutes, DNA was collected by centrifugation at 12,000 x g for 30 minutes. The DNA was washed once by 70% ethanol and collected by centrifugation. After removing the ethanol from the tube, the DNA was dried in vacuum and redissolved in TE buffer in appropriate volume.

11. Plasmid Isolation (Miniprep)

The construction and the cloning of the HuIFN-γ gene and its efficient expression with recombinant plasmid pJP_1R_3-IFNγ-171 in E. coli has been previously described (Jay et al., 1984a and 1984b). The deletion mutant Del_{122} has also been described previously (Luk et al., 1990). Plasmid DNA was isolated from both Del_{122} and $P_{14}R_9$ transfected E. coli strain LE392. A loop of bacteria from the frozen stock was inoculated into 3 ml Terrific Broth (TB) containing 20 μg/ml tetracycline and incubated at 37^{0}C overnight with vigorous shaking. The overnight culture was then collected by centrifugation at 12,000 x g for 2 minutes. The cell pellet was resuspended in 100 μl solution I (25 mM Tris.HCl, pH 8.0; 10 mM EDTA, 15% sucrose) after removing the medium by aspiration. The cell pellet was resuspended in the residual liquid by tapping. The cells were then lyzed by adding 200 μl of solution II (0.2 N NaOH, 1% SDS) followed by inverting the microcentrifuge tube five times to mix the suspension. This solution was neutralized

by adding 150 µl solution III (3 M potassium acetate, 11.5% acetic acid) and gently mixed by vortexing. After centrifugation of the suspension at 12,000 x g for five minutes, the supernatant containing the plasmid DNA was transferred into another tube. DNase-free RNase A (Boehringer Mannheim) was added into the supernatant at a final concentration of 50 µg/ml and incubated at room temperature for 30 minute. The supernatant was extracted with phenol/chloroform/isoamyl three times and chloroform/isoamyl alcohol three times. Then, 2.5 volume of cold ethanol was mixed with the aqueous phase. The sample was frozen at -70^{0}C for 10 minutes followed by centrifugation at 12,000 x g for 30 minutes. The precipitated plasmid DNA was washed once with 70% ethanol and was collected again by centrifugation. Finally, DNA pellet was dried in a spinning-vacuum system and redissolved in 20 µl of TE buffer.

12. Preparation of Single Stranded Phage DNA

The ssDNA was isolated from the recombinant phage clone M13mp/IFN. The coding sequence of HuIFN-γ was inserted into the Hind III site of M13mp8 phage vector (Luk, 1990b). The restriction map within the HuIFN-γ coding sequence was identical to that of the HuIFN-γ coding sequence in the recombinant plasimd $pJP_{14}R_9$/IFN. A single clone of M13mp8/IFN phage was inoculated into 2 ml cultures of logarithmic growing E. coli strain MV1193 (OD_{600} 0.9) and was allowed to grow at 37^{0}C for four hours. Cells were pelleted by centrifugation at 12,000 x g for 2 minutes and the supernatant was collected. The phage was precipitated by adding 200 µl of 20% Polyethylene glycol (PEG, type 8000) in 2.5 M NaCl solution into the supernatant and

incubated at 37^{0}C for 15 minutes. The phage was collected by centrifugation at 12,000 x g for 10 minutes and then redissolved in 100 µl TE. This suspension was extracted with phenol/chloroform/isoamyl alcohol three times and then chloroform/isoamyl extract three times. The final liquid phase containing single stranded DNA (ssDNA) was adjusted with 3 M potassium acetate to a final concentration of 0.3 M and was mixed with 2.5 volumes of ethanol. The mixture was frozen at -70^{0}C for 10 minutes and was centrifuged at 12,000 x g for 15 minutes. Finally, the pellet was washed with 70% ethanol and redissolved in 20 µl TE. The ssDNA was stored at -20^{0}C.

13. Estimation of DNA Concentration and Purity

The purity and concentration of DNA in the aqueous solution (both dsDNA and ssDNA) were estimated spectrophotometrically as described (Sambrook et al., 1989). DNA was diluted in TE buffer and its optical density (OD) was measured at 260 and 280 nm wavelength against the appropriate solvent blank. The OD ratio at 260/280 nm was used as an indicator for DNA purity. DNA samples with 260/280 nm ratio higher than 1.75 were considered relatively pure and were used in the subsequent experiments. DNA concentration was determined based on measuring its OD at 260 nm wavelength. For the dsDNA, one OD at 260 nm equals 50 µg/ml of DNA. For the ssDNA, one OD at 260 nm equals 40 µg/ml of DNA. When the ratio of 260/280 nm of the DNA sample was lower than 1.75, the DNA quantitation was not accurate.

14. Restriction Endonuclease Enzyme Digestion of DNA

Restriction enzymes were obtained from Boehringer Mannheim or Bethesda Research Laboratory (BRL). The 10 x reaction buffers were supplied with the enzyme by manufacturers. The reactions of restriction enzyme digestion of double stranded DNA (dsDNA) with a variety of enzymes were done following the manufacturer's specifications. Reaction was carried out in 1 x reaction buffer, dsDNA (from 0.1 to 10 μg) and restriction enzyme in a final volume of 10-30 μl. Usually, 1 unit of enzyme was described as the amount of enzyme required to completely digest 1 μg of specific DNA in an hour. The enzymes were stored at -20°C in buffer containing 50% glycerol. The volume of the restriction enzyme in the reaction was less than 10% of the total volume of the reaction to reduce the inhibitory effect of the glycerol. The reactions were carried out at 37°C for 1-4 hours.

15. Agarose Gel Electrophoresis

DNA was separated by electrophoresis in agarose gel in this study. The molecular weight of the DNA fragments was determined by comparing to the standard DNA molecular weight markers run in parallel. Electrophoresis was performed as described (Sambrook et al., 1989). TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) was made in 10 times concentrated (10X) stock solution and diluted to 1 x during electrophoresis. Agarose gel (0.7-1.8%) containing 1 μg/ml ethidium bromide was used. The DNA sample mixed with 0.1 volumes of 10 x loading

buffer (30% ficoll, 0.25% bromophenol blue, 0.25% xylene cyanole FF, and 0.2 M EDTA, pH 8.0; in 10 x TAE) were loading onto the gel. The electrophoresis was carried out in 1 x TAE buffer at room temperature under constant voltage of 75 volts for 1-3 hours. After electrophoresis, the DNA bands were visualized with UV light. Photographs were taken using Polaroid film type 57 and a Polaroid 545 land camera (Polaroid Corporation, Cambridge, Massachusetts).

16. Autoradiography of Agarose Gel

The radioisotope labeled DNA fragments were separated on agarose gel by electrophoresis as described in the previous section. The agarose gel was fixed in 10% acetic acid at room temperature for 10 minutes. The gel was washed in 5% acetic acid for 10 minutes followed by wash in 1% acetic acid for 10 minutes and in water for 10 minuts. The gel was left between two pieces of 3 mm filter paper (whatman). A flat5 pound object weighted was placed on top of the gel overnight to remove water from the gel. The dried agarose gel was exposed to Kodark XAR film in a cassette for 4-12 hours.

17. Library Construction

17.1 General Scheme of Library Construction

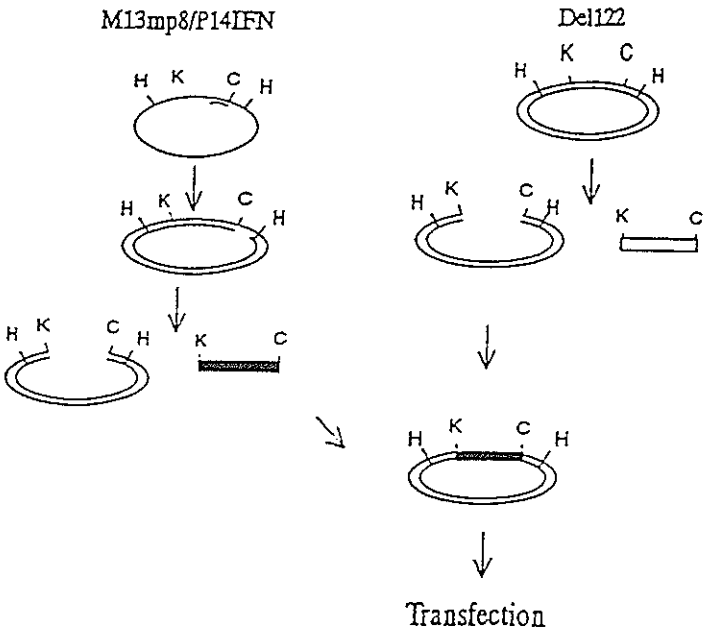
The sequence of HuIFN-γ was modified by site-directed mutagenesis. The parental molecule used in the site-directed mutagenesis was Del_{122} (Luk et al., 1990). The experiment was

designed to substitute each single amino acid from residues #86 to #91. The 3' end of the codon for residues #91 was immediately followed by a Csp45 I restriction enzyme site. The oligonucleotide primers (29 nucleotides, including a Csp45 I restriction site) containing mismatched nucleotides was synthesized according to DNA coding sequence for residues #86-91. The designed oligonucleotide primers had random substitutions (A, C, G, T) in position 1 of each codon for amino acid residues #86-91. Thus, this pool of oligonucleotide primers contained oligonucleotides that have the potential to create substitution for each single amino acid from residue #86 to #91.

M13mp8/IFN phage ssDNA was used as a template for the DNA polymerization (Figure 7). The primer extension reaction was catalyzed by Tag DNA polymerase at 70°C for a single cycle after the primers have annealed to ssDNA template. The dsDNA synthesized during primer extension was digested by restriction enzyme Kpn I and Csp45 I. The Kpn I/Csp45 I DNA fragment from HuIFN-γ gene (196 bp) was isolated. After dephosphorylation of the dsDNA at the 5' ends, it was ligated to the vector (Figure 7).

The Del_{122} plasmid DNA was also digested with restriction enzyme Kpn I and Csp45 I. Restriction fragments were separated by electrophoresis in agarose gel. The 196 bp Kpn I/Csp45 I fragment from the primer extension was ligated into the 4.6 Kb Kpn I/Csp45 I fragment from Del_{122} by T_4 ligase. The ligated DNA plasmid restored the DNA sequence of HuIFN-γ gene except the mutations introduced by the primer. After transforming the ligated DNA into E. coli strain HB101 host cells and grown in the presence of tetracycline, the surviving clones were

Figure 7. General scheme of site-directed mutagenesis in E1 domain. The dsDNA and ssDNA are represented by double or single lines, separately. The Csp45 I, Kpn I and Hind III restriction enzyme sites are labelled as C, K and H , respectively. The HuIFN-gamma gene which is between the 2 HinD III sites has identical restriction enzyme map in both M13mp8/P14IFN and Del122.


M13mp8/P14IFN
Del122
H K C H
K C
H K C H
K C
Transfection

collected and pooled. The plasmid DNA extracted from these pooled clones was transformed again into HB101 host cells in order to separate the plasmids derived from the primer and those from parental DNA. The clones from the second transformation were stored at -70°C as the mutagenesis library (Figure 7).

17.2 Oligonucleotide Primer

A degenerative pool of oligonucleotide primers was synthesized according to DNA coding sequence for amino acid residue #85-93. The sequences of the oligonucleotides and its relationship to the DNA and amino acid sequences of HuIFN-γ are showing as below.

Residue No.	85	86	87	88	89	90	91	92	93
Amino Acid	N	K	K	K	R	D	D	F	E
+ Strand	GC	AAC	AAA	AAG	AAA	CGT	GAT	GAC	TTC GAA
									Csp45 I
Oligo Z_1	CG	TTG	NTT	NTC	NTT	NCA	NTA	NTG	AAG CTT
Substitutio			---	---	---	Cys	Tyr	Tyr	
			Glu	Glu	Glu	Gly	His	His	
			Gln	Gln	Gln	Ser	Asn	Asn	

In the synthetic oligonucleotide primers, the nucleotide in the first position of each codon for residue #86-91 was substituted randomly by one of the four nucleotides (A, C, G, T) in equal proportion represented by "N" in these positions. The possible amino acids substitutions at

residue #86 to #91 were deduced from the putative nucleotide substitutions in each site. The "---" represent the nonsense mutation that would result in a premature chain termination. Based on this design, the primer would have the potential to substitute each of the single amino acids from residue 86-91.

17.3 Primer-Extension and Labelling

M13mp8/IFN ssDNA was used as a template. The synthetic oligonucleotide primer was allowed to mix with the template in a microcentrifuge tube. The molar ratio of oligonucleotide primer to the template was 50:1. In the presence of Taq DNA polymerase reaction buffer (30 mM Tris.HCL pH 9.0, 5 mM $MgCl_2$, 30 mM KCl), and substrate (dATP, dCTP, dGTP, dTTP, 0.2 mM each) and α-^{32}P-dATP (specific activity > 5,000 Ci/mmol, Amersham), the microcentrifuge tube was heated to 95^0C in a water bath for 5 minutes to disrupt the secondary structure of ssDNA. One unit of Taq DNA polymerase was then added into the reaction after the tube was gradually cooled down to 25^0C in one hour to allow the annealing of oligonucleotide primer to the template. DNA polymerization was carried out at 70^0C. After one hour, the reaction was adjusted to 100 µl with TE buffer and the sample was extracted with phenol/chloroform/isoamyl three times followed by extraction with chloroform/isoamyl three times. DNA in aqueous phase was recovered by ethanol precipitation after addition of potassium acetate to 0.3 M. The precipitated DNA was redissolved in 15 µl of TE buffer. The success of the primer extension reaction was assessed by agarose gel electophoresis and subsequent

autoradiography.

17.4 Restriction Digestion by Kpn I/Csp45 I

The dsDNA synthesized in the primer extension reaction was digested by restriction enzyme Kpn I (BRL) and Csp45 I (Boehringer Mannheim). The reaction buffer for the restriction enzyme digestion was tested prior to the experiment. The reaction buffer IV (1X=20 mM Tris.HCl, pH 7.4; 5 mM $MgCl_2$, 50 mM KCl) supplied by BRL was used for restriction enzyme digestion of Kpn I and Csp45 I. One unit of each enzyme was added into the reaction in the presence of 1 x reaction buffer IV to digest dsDNA. The restriction enzyme digestion was carried out at 37°C for 3 hours. After adjusting the volume to 100 µl, DNA was extracted with phenol/chloroform/isoamyl three times followed by chloroform/isoamyl three times. The extracted DNA was recovered by ethanol precipitation. After washing the DNA once with 70% ethanol, the pellet was redissolved in 20 µl TE. The success of the restriction enzyme digestion was assessed by agarose gel electrophoresis and subsequent autoradiography.

17.5 Recovery of the 196 bp Kpn I/Csp45 I DNA Fragment

DNA fragments after Kpn I/Csp45 I digestion was separated on agarose gel by electrophoresis. The molecular weight of DNA fragment was estimated by running parallel standard molecular weight markers (DNA ladder 123, BRL) on the same gel. The bands of DNA fragments were visualized with UV light. A small pieces of gel containing only the 196 bp DNA

fragment was excised out with a surgical blade. The DNA in the excised agarose piece was recovered by electroelution as described below.

Electroelution was used to recover DNA fragment (196 bp) from agarose gel. A diethylaminoethyl (DEAE) cellulose minicolumn was used as matrix for DNA recovery and cleaning. DEAE cellulose type DE-52 (Whatman) was suspended in 2 M NaCl. A minicolumn was made in a 1ml pipete. The column was washed with 200 µl TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). After the DNA band was excised from agarose, the agarose was crushed into pieces and allowed to settle on the top of DE-52 column. The column was placed in a vertical electrophoresis apparatus with TAE buffer. DNA in the gel was electroeluted into DE-52 column at constant 300 voltage for two hours. After washing the column with 200 µl $T_{10}E_1N_{50}$ buffer (10 mM Tris.HCl, 1mM EDTA, 50 mM NaCl, pH 8.0) 2 times, DNA was eluted with 300 µl buffer containing 1.0 M NaCl, 20% ethanol. The collection of DNA fractions was monitered by detecting radioactivity of the elution using a Ganger's counter (Measurement INC.). The collected DNA was further cleaned by phenol/chloroform/isoamyl extraction twice, followed by chloroform/isoamyl extraction twice. The DNA was precipitated with ethanol and then washed once with 70% ethanol. Finally, DNA was redissolved in 10 µl of TE. The success of the recovery was confirmed by electrophoresis of DNA sample on agarose gel and subsequent autoradiography.

17.6 Dephosphorylation of 196 Kpn I/Csp45 I DNA Fragment

The recovered DNA fragment (196 bp) with free 5'-phosphate was dephosphorylated with calf intestinal phosphatase (CIP) in order to prevent the formation of multiple insertion in subsequent ligation reaction. CIP was obtained from Bethesda Research Laboratory (BRL) and 5 x reaction buffer was supplied together with the enzyme. In the reaction of dephosphorylation, 0.25 unit of CIP per microgram of DNA were used. The volume for reaction was 10-20 µl with the reaction buffer adjusted to 1 x. The reaction was carried out in a microcentrifuge tube at 37°C for one hour. The CIP was then inactivated by heating at 75°C for 10 minutes after adjusting the EDTA concentration to 5 mM. The reaction was extracted by phenol/chloroform/isoamyl 2 times followed by chloroform/isoamyl two times. DNA was then precipitated by ethanol as described. Finally, the dephosphorylated DNA was redissolved in 10-20 µl of TE buffer.

17.7 The Restriction Enzyme Digestion of Del_{122} Plasmid

DNA from Del_{122} clone was digested by Kpn I and Csp45 I. Condition for restriction enzyme digestion were the same as the reaction in the digestion of dsDNA derived from primer extension. DNA fragments after restriction enzyme digestion were separated on agarose gel by electrophoresis. The molecular weight was assessed by comparing to the migration of molecular weight markers (lamda Hind III) run in parallel. A 4.6 Kb DNA fragment was excised from the agarose gel and was recovered by the method as described below.

17.8 Recovery of DNA Fragments by Glass Milk

The mechanism of recovery and purification of DNA with glass milk was based on different affinity of glass milk to DNA and other macromolecules (protein, carbohydrate and RNA). The reagents and detailed method for purifying DNA in this method were included in a kit "Geneclean" supplied by Bio 101 Inc (La Jolla, CA). The excised agarose gel containing 4.6 Kb Kpn I/Csp45 I DNA fragment was weighted and then mixed with 2.5 volumes of 6 N NaI in a microcentrifuge tube to dissolve agarose (a density of the 1 g/ml was used). The tube containing the gel and NaI was placed in a water bath at 50°C. The gel was dissolved completely in about five minutes in the solution. After adding 10 µl of glass milk suspension into the solution, the tube was placed in ice for 15 minutes to allow absorption of DNA to glass milk. The tube was vortexed every 2 minutes in order to keep glass milk in suspension. The glass milk was collected by centrifugation at 12,000 x g for one minute and the supernatant was transferred into another tube. The agarose/NaI solution was treated with glass milk 3 times, and the combined glass milk was collected into a tube and washed 3 times with cold washing solution (10 mM Tris.HCl, pH 8.0; 1 mM EDTA, pH 8.0; 100 mM NaCl, 50% ethanol). The DNA was eluted from the glass milk by incubating with 50 µl TE buffer at 50 °C for 3 minutes. After centrifugation at 12,000 x g, supernatant was collected by aspiration without disturbing glass milk pellet. The elution process was repeated for three times and all supernatant was pooled and extracted twice with phenol/chloroform/isoamyl and twice with chloroform/isoamyl. DNA was then precipitated by ethanol. After washing once with 70% ethanol, DNA was redissolved in 20 µl of TE buffer. The

success of DNA recovery was confirmed by electrophoresis of DNA fragment on agarose gel.

17.9 Ligation of DNA Fragement

The 196 bp Kpn I/Csp45 I fragment from the primer extension reaction was ligated into 4.6 Kb Kpn I/Csp 45 I large fragment from Del_{122}. DNA T4 ligase was obtained from Bethesda Research Laboratory (BRL) and a 5 x reaction buffer was supplied by the manufacturer. The molar ratio of DNA vector to insert in the ligation reaction was adjusted to 1:1. Before addition of T4 ligase, the reaction was heated at 95°C for five minutes and then cooled down to room temperature gradually in about one hour to allow annealing of the cohesive ends. The amount of T4 ligase used was one unit per microgram of vector DNA. The total volume of the reaction was 10-20 µl and reaction buffer was adjusted to 1 x concentration. The ligation reaction was incubated overnight at 12°C in a water bath. Since the DNA fragments had two different cohesive ends created by the restriction digestion of Kpn I and Csp45 I, the insert was ligated into the vector in only one orientation. The success of the ligation was confirmed by electrophoresis of the ligated DNA on agarose gel and subsequent autoradiography.

17.10 Transfection

The competent E. coli strain HB101 was obtained from Bethesda Research Laboratory (BRL). The competent cells were aliqotted into 40 µl per tube and stored at -70°C. The ligation reaction was diluted 1:5 in TE buffer. One µl of DNA was added into the tube containing HB101

competent cells and incubated in ice for 30 minutes to allow even mixture of competent cells and DNA molecules. Cells in the tube were shocked by heating at 42°C for 2 minutes and then transferred immediately into ice to cool the cells for 2 minutes. Two hundred microliters of warm SOC medium (2% bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added into the tube to mix with the cells. The cells were allowed to recover at 37^0C for one hour in SOC medium. Appropriate volume of cell suspension was spread onto the LB agar (1% bactotryptone, 0.5% yeast extract, 1.5% agar, 0.18 M NaCl, pH 7.0) plate containing 12 μg/ml tetracycline as selection reagent. The colonies were allowed to grow overnight at 37°C.

18. DNA Sequencing of Constructed Del_{122} Mutants

The constructed Del_{122} mutants were sequenced by using the dsDNA cycle sequencing kit (BRL). The principle of double stranded DNA (dsDNA) cycle sequencing system was based on the chain termination by dideoxy nucleotides. This sequencing kit used a thermal stable Taq DNA polymerase in chain extension instead of phage or bacterial DNA polymerase. In order for the DNA polymerase to synthesize a complementary DNA strand, DNA to be sequenced must be in single strand form, at least temporarily. Since the optional temperature for Taq DNA polymerase was 70^0C, the opening of dsDNA and chain extension occured simultaneously in the sequencing reaction of dsDNA cycle system. This characteristics of Taq DNA polymerase allowed direct sequencing of dsDNA. In addition, a repetitive series of temperature changes in the

sequencing reaction allowed chain extension to repeat many times and therefore, amplify the sequencing signal.

The main experimental procedures for sequecing Del_{122} and its mutants are described in the following sections.

18.1 End Labeling of the Oligonucleotide Primer

The oligonucleotide was chemically synthesized without phosphorylation of the 5' end (DNA Laboratory, University of Manitoba). The primer was complementary to the DNA coding sequence correspoding to amino acid residue 137 to 143, which was located after the termination coden in Del_{122} gene. The sequence of the synthetic primer and its relationship to the template is as following:

AA location		137	138	189	140	141	142	143	
(+)Strand	5'	CGT	GGT	CGT	CGT	GCA	TCC	CAG	3'
Primer	3'	GCA	CCA	GCA	GCA	CGT	AGG	GT	5'

The sequence primer was labeled on the 5' end by using γ-^{32}P-ATP (Amersham, specific activity > 5000 Ci/mmol). The molar ratio of γ-^{32}P-ATP to the primer was adjusted to 2:1 before the labelling reaction. The labelling reaction was catalysed by T4 kinase in T_4 kinase buffer (60 mM Tris.HCl, pH 7.8; 10 mM $MgCl_2$, 15 mM 2-mercaptoethanol, 0.41 μM ATP) at 37^{0}C for

one hour. Then, the reaction was heated at 55^{0}C for 10 minutes to inactivate the T4 kinase.

18.2 Sequencing Reaction

The amount of plasmid DNA used for sequencing reaction was 50 fmol, which equals approximately 0.2 μg Del_{122} plasmid DNA. The plasmid DNA was pre-mixed with the end labelled sequencing primer, Taq DNA polymerase reaction buffer and Taq DNA polymerase. The total volume was adjusted to 36 μl with double distilled water. A typical procedure of this reaction mixture was as following.

component	Volume
10 x Taq sequencing buffer	4.5 μl
Template DNA (50 fmol or 0.2 μg DeL_{122})	2.0 μl
Labelled Primer (1 pmol)	5.0 μl
Taq Polymerase (2.5 Units/μl)	1.0 μl
H_2O	12.5 μl

For each of the DNA sequencing sample, four tubes marked as tube A, C, G, T were prepared. Two microliters of the termination mixtures of either Mixure-A, Mixture-C, Mixture-G or Mixture-T were added into each tube as marked. The components of the termination mixtures were as following.

Four nucleotide stock mixes:

Mix-A (2 mM ddATP, 50 μM each dATP, dCTP, 7-deaza-dGTP, dTTP),

Mix-C (1 mM ddCTP, 50 μM each dATP, dCTP, 7-deaza-dGTP, dTTP),

Mix-G (0.2 mM ddGTP, 50 μM each dATP, dCTP, 7-deaza-dGTP, dTTP),

Mix-T (2 mM ddTTP, 50 μM each dATP, dCTP, 7-deaza-dGTP, dTTP).

Eight microliters of the reaction mixture were transferred into each of the 4 tubes that contained 2 μl of respective termination mixture. Ten microliters of mineral oil were overlaid in each tube and the tubes were centrifuged at 12,000 x g for a few second in the microcentrifuge. The components in each tube were summarized as following:

Component	Tube A	Tube C	Tube G	Tube T
Nucleotides Mixture				
Mixture-A	2 μl	-	-	-
Mixture-C	-	2μl	-	-
Mixture-G	-	-	2 μl	-
Mixture-T	-	-	-	2 μl
reaction Mixture	8 μl	8 μl	8 μl	8 μl
Mineral Oil	10 μl	10 μl	10 μl	10 μl

After centrifugation, the tubes were placed in a thermal cycler (Pharmacia) and incubated according to the following thermocycling schedules:

Temperature	Time
94°C	30 sec
55°C	30 sec
70°C	1 min
20 cycles	
94°C	30 sec
70°C	1 min
10 cycles	

When the thermal cycles were finished, the tubes were immediately transferred to an ice bucket. Five microliters of stop solution [95% (v/v) formamide, 10 mM EDTA, pH 8.0; 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol] were added into each tube to terminate the sequencing reaction. Four microliters of sample were loaded in each lane of a sequencing gel and electrophoresed on the same day.

18.3 Electrophoresis of Sequenceing Gel

The sequencing reaction was run on a 6% sequencing gel using a Bio-Rad sequencing unit in TBE buffer (90 mM Tris, 88 mM boric acid, 1 mM EDTA). The gel was pre-run for 15 minutes at constant voltage of 1750 V to heat the gel to approximately 50^0C. The sequencing reaction samples were heated at 95^0C for three minutes before loading. Four microliters of final sample solution which contained either termination mixture-A, -C, -G or -T were loaded on each

lane and were electrophoresed at constant voltage of 1750 V for 2 to 3 hours. The gel was fixed with a solution containing 10% methanol and 10% acetic acid for 10 minutes and dried on a heating plate under vacuum. The sequencing gel was exposed to Kodak XAR film for at least 12 hours.

CHAPTER III

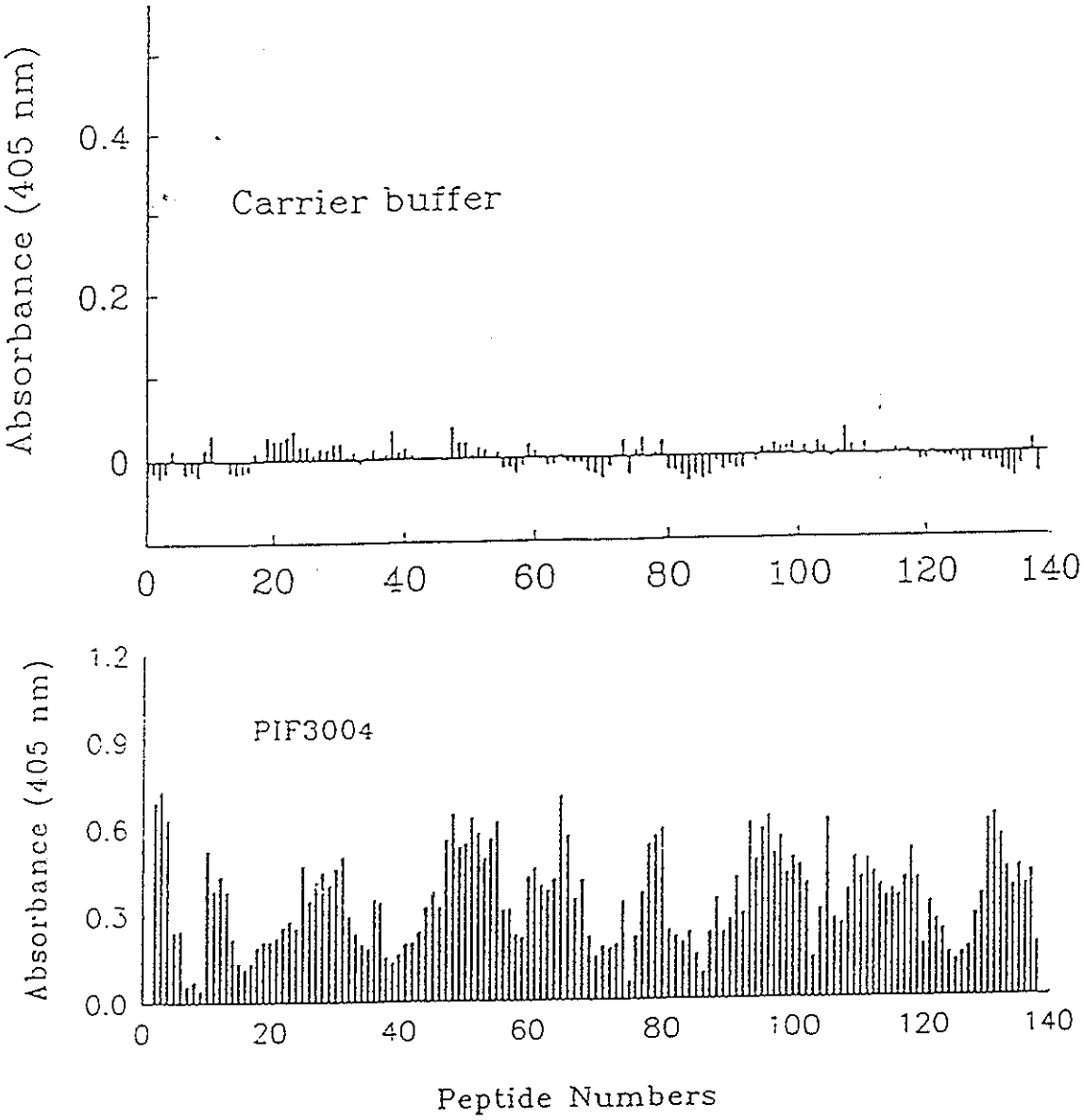
RESULTS

1. Epitopes Mapping of Polyconal Antibody PIFF3003

A series of 136 overlapping octapeptides of HuIFN-γ sequence were synthesized. Each octapeptides was covalently linked on the carboxyl terminus to the tip of a polypropylene rod for reaction with anti-HuIFN-γ antibodies in ELISA. The success of peptide syntheses was confirmed by parallel syntheses contol peptides and the specific recognition by mAbs. The synthesized octapeptides were first incubated with carrier buffer (1% BSA and 1% chicken egg albumin in PBS) to detect nonspecific reactions. The specificity of the interactions was confirmed by positive reaction of anti-peptide A mAb against Peptide A "T-L-N-P-T-I-A-G" (OD_{405} >0.8) and negative reaction with Peptide B "L-A-P-T-I-A-G-K" (OD_{405} <0.05) in ELISA. The absorbance at 405 nm was measured and plotted against the peptide numbers (Figure 8). Since the octapeptides were arranged in sequential order according to the linear sequence of HuIFN-γ, the plotted OD_{405} formed a specific pattern that allowed us to localize the amino acid sequences of HuIFN-γ. The reaction of carrier buffer with the octapeptides clearly demonstrated a pattern of nonreactivity which represented background of the ELISA (Figure 8).

PIF3003 was a polyclonal antibody (rabbit serum) against denatured HuIFN-γ. This polyclonal antibody was extensively adsorbed with E. coli proteins extract and

Figure 8. The reactivity of PIF3003 to synthetic octapeptide homologs of HuIFN-gamma. A series of 136 octapeptide homologs of HuIFN-g, each one having a 7-residue sequence homology with its consecutive peptide, was synthesized on plastic pins. The pins were arranged in an 8 x 12 format for ELISA. Antibody PIF3003 that specifically bound to the octapeptides was recognized by HRP-conjugated goat anti-rabbit Ig. The subsequent colour reaction was detected at 405 nm. The reactivity of carrier buffer (1% BSA and 1% chicken egg albumin in PBS) to the octapeptides was tested in order to detect any nonspecific interactions. PIF3004, which was purified IgG of rabbit serum against denatured HuIFN-g, was diluted 1:500 in the carrier buffer. The absorbency at 405 nm was plotted against the octapeptide numbers, which were arranged according to the sequence of HuIFN-gama.

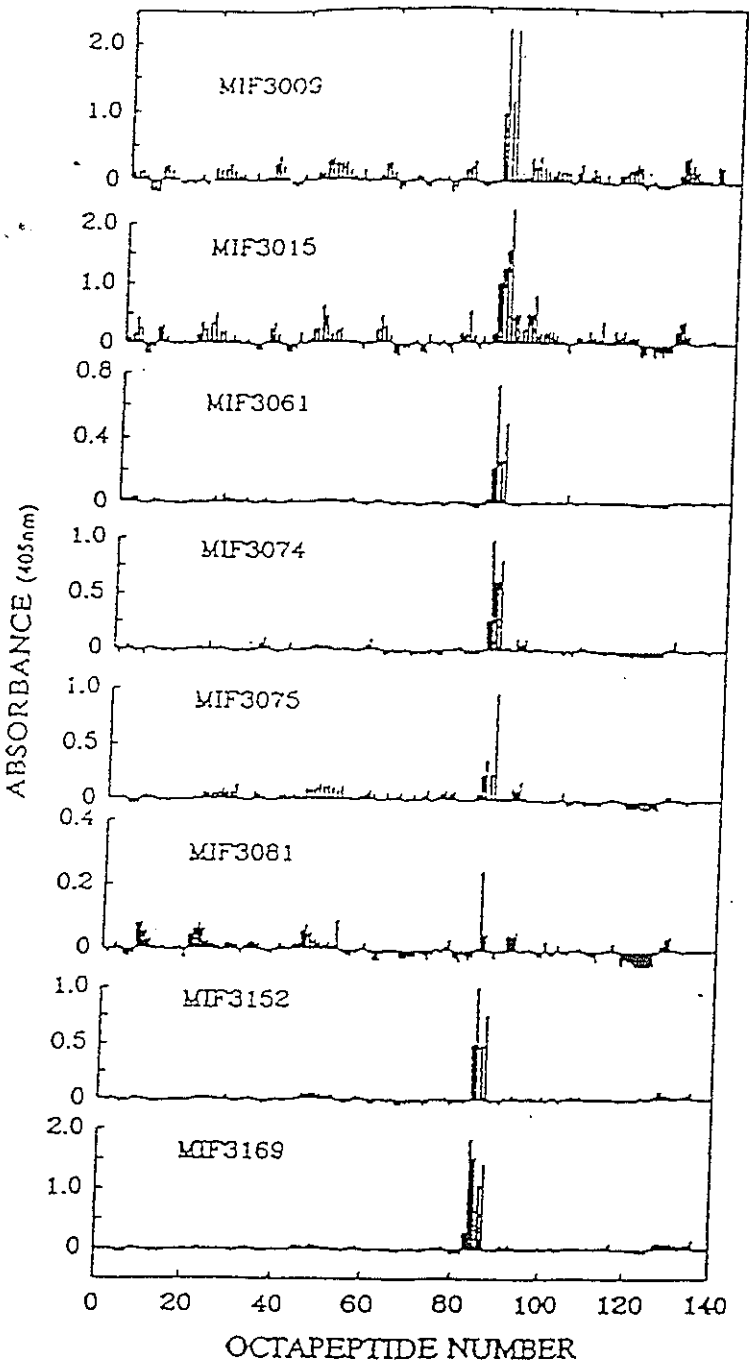

Absorbance (405 nm)
0.4
0.2
0
Carrier buffer
0
20
40
60
80
100
120
140
Absorbance (405 nm)
1.2
0.9
0.6
0.3
0.0
PIF3004
0
20
40
60
80
100
120
140
Peptide Numbers

purified by protein-A affinity column before interaction with the set of synthetic octapeptide homologs of HuIFN-γ. The specificity of antibody-octapeptide interaction was confirmed by positive reaction of anti-Peptide A mAb with Peptide A and negative reaction with Peptide B in ELISA. The polyclonal antibody against denatured HuIFN-γ recognized many octapeptides homologs of HuIFN-γ and the octapeptides recognized were clustered in 9 groups (Figure 8). In contrast to the background reaction of carrier buffer, the pattern of the reaction with PIF3003 represented a positive reaction to this set of octapeptides, which confirmed the successful syntheses of the octapeptides.

2. Epitope Mapping of mAbs in E_1 Reacting group

Eight independently isolated anti-HuIFN-γ neutralizing mAbs, MIF3009, 3015, 3061, 3074, 3075, 3081, 3152, and MIF3169 have been shown to recognize functional epitope E_1 by competitive binding analyses (Alfa & Jay, 1988). In order to map E_1 epitope, each mAb in the E_1 reacting group was tested for its reactivity to the synthetic octapeptide of HuIFN-γ in an ELISA and results were plotted as described. Six mAbs, MIF3009, 3015, 3061, 3074, 3075 and MIF3152 reacted with four consecutive octapeptides, #84-87 (Figure 9). The peaks in each reactivity pattern have a good signal-to-noise ratio (positive reaction was at least 4 times higher than background). Although background reaction of MIF3015 was relative high, the reactivity pattern was reproducible and essentially similar to the pattern of MIF3009, 3015, 3061 and

Figure 9. Epitope mapping of mAbs in E_1 reacting group with synthetic octapeptides. MIF3009, 3015, 3061, 3074, 3075, 3081, 3152, and MIF3169 are anti-HuIFN-gamma mAbs recognized in E_1 reacting group (Alfa & Jay, 1988). Each of these mAbs were diluted 1:500 in carrier buffer. The mAbs that specifically bound to synthetic octapeptides were recognized by HPR-conjugated goat anti-mouse (anti-IgA, -IgG and -IgM) IgG. The absorbance at 405 nm was plotted against octapeptide numbers, which were arranged according to the sequence of HuIFN-gamma.


MIF3009
MIF3015
MIF3061
MIF3074
MIF3075
MIF3081
MIF3152
MIF3169
ABSORBANCE (405nm)
OCTAPEPTIDE NUMBER
0
20
40
60
80
100
120
140

MIF3152. MIF3081 reacted with a single peptide #84. MIF3169 was the only mAb that reacted with five consecutive octapeptide, #84-88, which suggested that MIF3169 recognized the epitope with a wider region.

Based on these reactivity patterns, it was indicated that mAbs in the E_1 reacting group recognized an overlapping sequences of HuIFN-γ ranged from octapeptide #84 to #88. The amino acid sequence of this region was "Ser^{84}-Asn-Lys-Lys-Lys-Arg-Asp-Asp-Phe-Gln-Lys^{94} ", which was named E_1 epitope or E_1 domain. Within E_1 doamin, sequence "Asn^{85}-Lys-Lys-Lys-Arg-Asp-Asp-Phe^{92}" is a common motif recognized by all mAbs in E_1 reacting group. The E_1 domain contains 5 basic residues (4 Lys and 1 Arg) and two acidic residues (2 Asp) and was exposed on the surface of HuIFN-γ molecule according to x-ray crystal structure model of HuIFN-γ (Ealick et al., 1991). In summary, E_1 functional epitope was mapped to residue #84-94 of HuIFN-γ.

3. Epitope Mapping of mAbs in E2 Reacting Group

Based on cross competition studies (Alfa & Jay, 1988), 8 neutralizing mAbs, MIF3070, 3098, 3055, 3045, 3043, 3069, 3054 and MIF3052 competitively bound to the neutralizing epitope E_2. All mAbs in E_2 reacting group have been shown to recognize native HuIFN-γ in ELISA, but none recognized SDS-denatured HuINF-γ in Western blot analyses (Alfa & Jay, 1988).

In an attempt to map epitope E_2, two of the mAbs in E_2 group, MIF3055 and

MIF3070 were incubated with overlapping octapeptide homologs of HuIFN-γ. Antibody-octapeptide reaction was detected in an ELISA. The results were plotted as described. MIF3055 had no specific reaction to any of the octapeptide homologs (Figure 10). MIF3070 had slightly higher reactivity (OD_{405}) to some of the octapeptides than that of MIF3055. However, the reaction pattern demonstrated a higher background and none of the antibody-peptides reaction was significant higher (OD_{405} 4 times higher) than background. This result suggested that mAbs MIF3055 and MIF3070 did not specifically recognize any of the 136 synthetic octapeptide homologs of HuIFN-γ. Therefore, E_2 functional epitope was not mapped in this study.

4. Epitope Mapping of mAbs in E_1/E_2 Reacting Group

Three neutralizing mAb, MIF3102, MIF3094 and MIF3095 generated in our laboratory have been shown to cross-react with mAbs in E_1 or E_2 groups in their binding to HuIFN-γ (Alfa & Jay, 1988). This group of mAbs was classified as E_1/E_2 reacting group.

In an attempt to map E_1/E_2 epitope, one mAb, MIF3094 was tested for its reaction to synthetic octapeptide homologs of HuIFN-γ in ELISA and the result (OD_{405}) was plotted as described (Materials and Methods). No specific antibody-octapeptide reaction was detected (Figure 11). Thus, the binding site of mAb in E_1/E_2 reacting group was not mapped in this study.

Figure 10. Epitope mapping of mAbs in E_2 reacting group with synthetic octapeptide. MIF3055 and MIF3070 are anti-HuIFN-gamma mAbs in E_2 reacting group (Alfa & Jay, 1988). Each of the mAbs were diluted 1: 500 in carrier buffer. The mAbs that bound to octapeptides on the pins were recognized by HRP-conjugated goat anti-mouse (anti-IgA, -IgG and -IgM) IgG. The absorbance at 405 nm was plotted against the synthetic octapeptide numbers, which were arranged according to the sequence of HuIFN-gamma.

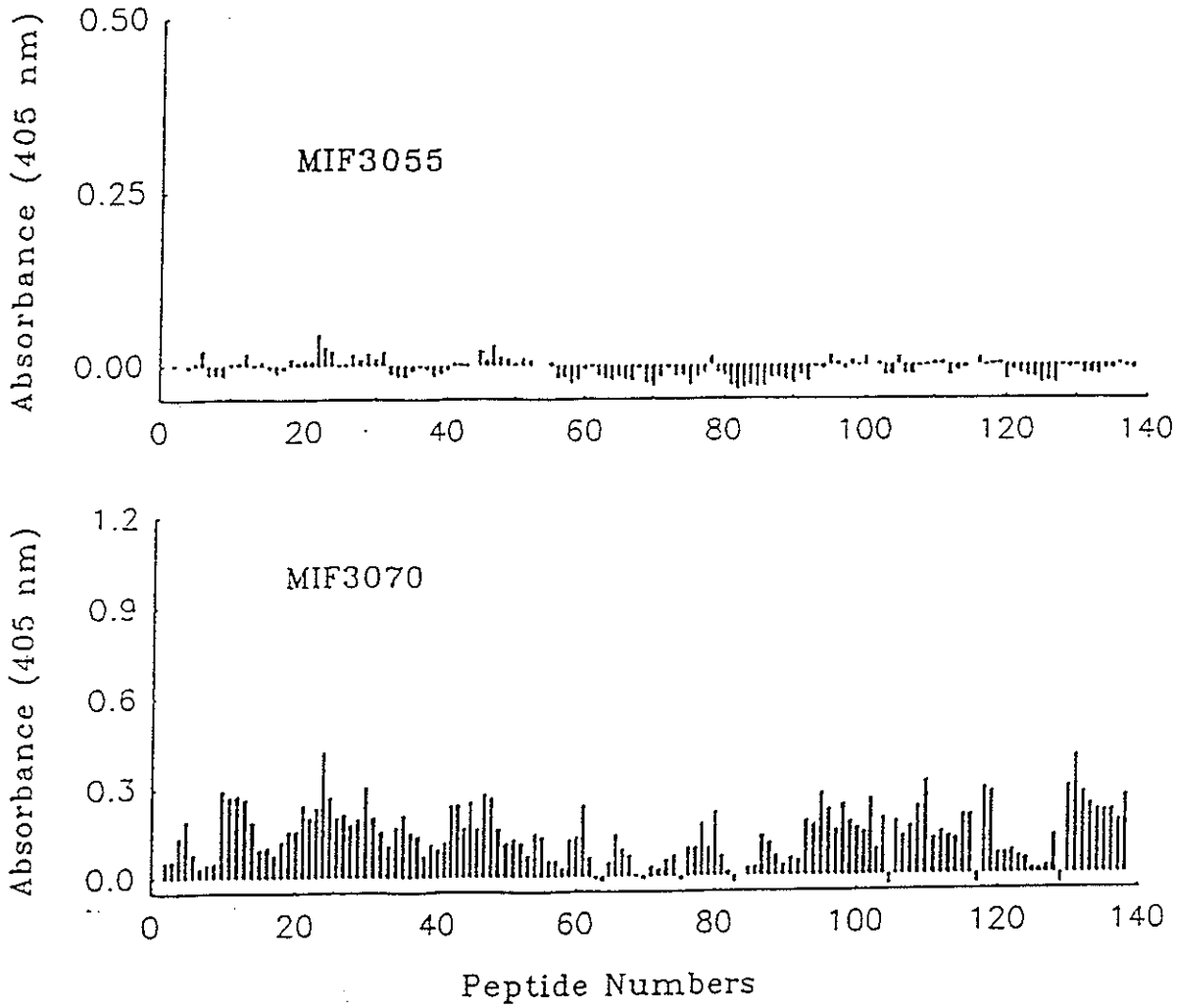
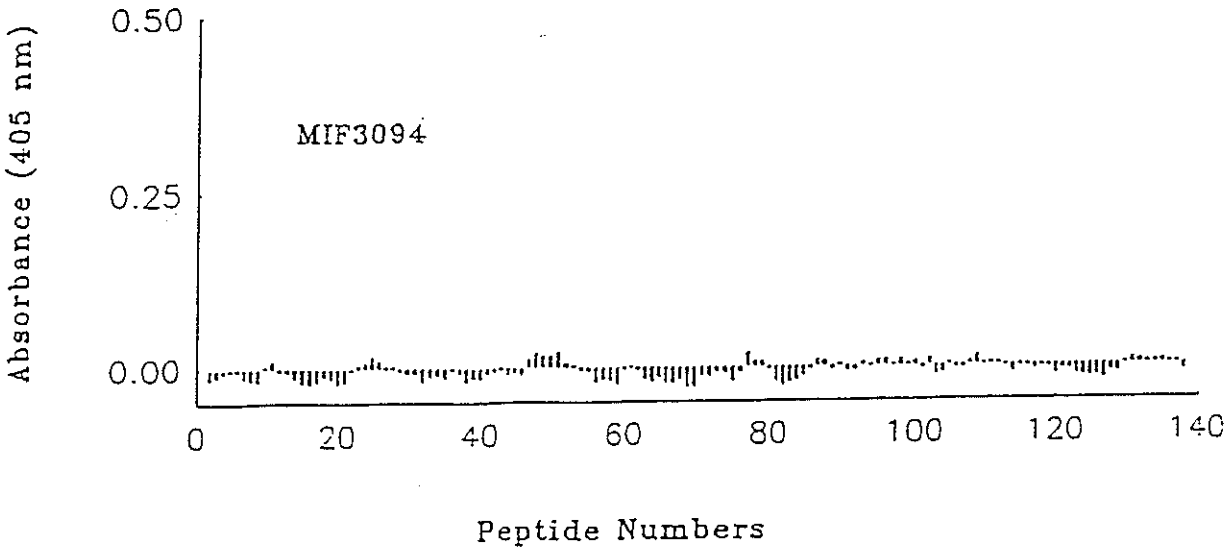

Absorbance (405 nm)
0.50
0.25
0.00
MIF3055
0
20
40
60
80
100
120
140
Absorbance (405 nm)
1.2
0.9
0.6
0.3
0.0
MIF3070
0
20
40
60
80
100
120
140
Peptide Numbers

Figure 11. Epitope mapping of mAb in E_1/E_2 reacting group with synthetic octapeptide. MIF3094 is a mAb in E_1/E_2 reacting group (Alfa & Jay, 1988). MIF3094 was diluted 1:500 in carrier buffer. The mAb bound to octapeptides on the pins was detected by HRP-conjugated goat anti-mouse (anti-IgA, -IgG, -IgM) IgG. The absorbency at 405 nm was plotted against the octapeptide numbers, which were arranged according to the sequence of HuIFN-gamma.


Absorbance (405 nm)
0.50
0.25
0.00
MIF3094
0
20
40
60
80
100
120
140
Peptide Numbers

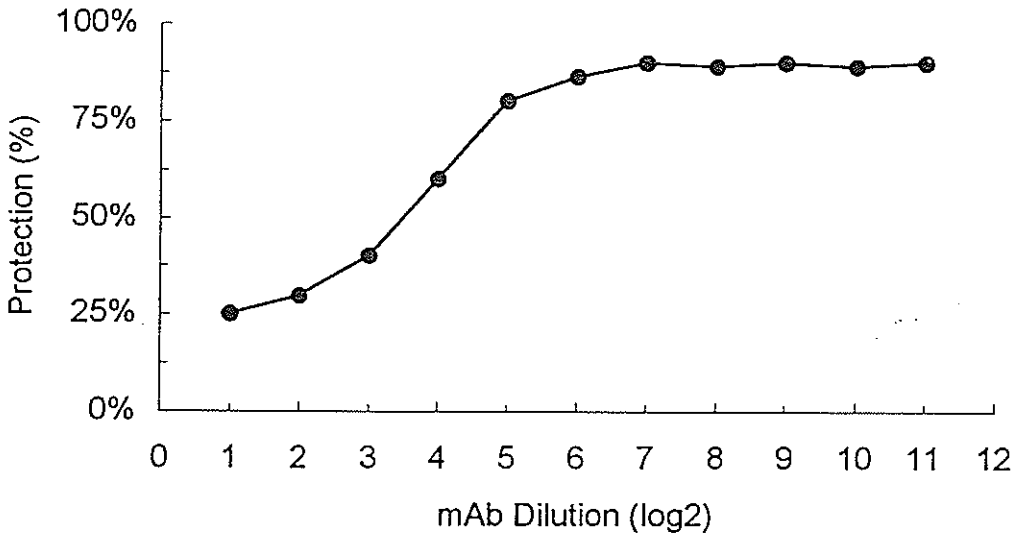
5. Identification of Functional Epitope E_3

MIF3037 was an IgM anti-HuIFN-γ mAb developed in our laboratory and its biological properties were not fully characterized. It was shown previously that MIF3037 did not belong to E_1, E_2 or E_1/E_2 reacting groups (Kwok et al.,1993). A neutralizing antiviral assay was carried out to determine whether MIF3037 could neutralize anti-viral activity of HuIFN-γ. MIF3037 in twofold serial dilution was incubated with a fixed concentration of HuIFN-γ (4 working unit/ml). The results of the antiviral assay were expressed as a percentage of protection against EMCV infection and were plotted against mAb dilution (Figure 12). The neutralization curve demonstrated a dose-dependent inhibition to the antiviral activity of HuIFN-γ. The pattern of the neutralization curve suggested that MIF3037 is a neutralizing mAb against HuIFN-γ. Thus, the epitope recognized by MIF3037 must be a functional epitope. Since MIF3037 had no cross reactions to any mAbs in either E_1 or E_2 reacting groups (Kwok et al., 1993), the epitope recognized by MIF3037 was a novel functional epitope named as E_3.

6. Kinetics of Neutralizing Effect of MIF3037

The neutralizing activities of anti-HuIFN-γ mAbs in E_1 and E_2 reacting groups were described previously (Alfa & Jay, 1988). MIF3037 was also shown to be a neutralizing mAb in the current study. In order to characterize the kinetics of neutralization effect, MIF3037 was twofold serially diluted and incubated with a constant

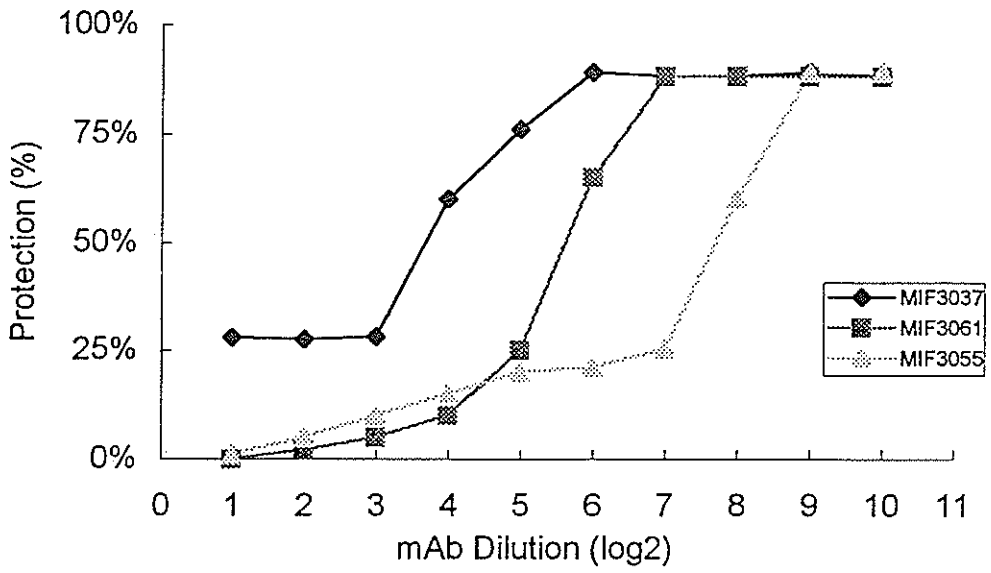
Figure 12. Neutralizing antiviral assay of MIF3037. MIF3037 was twofold serially diluted into the microtitre plate and HuIFN-gamma was added to a final concentration of 4 working units/ml. After incubation at 4°C for 1 hour, this mixture was transferred to cell culture plate containing 4 x 10^4 A_{549} cells per well. After incubation at 37°C for 18 hours, A_{549} cells were infected with EMCV for 24 hours. Viable cells were stained with crystal violet and the absorbency was detected at 590 nm spectrophotometrically. The average absorbency of culture with EMCV infection but without HuIFN-gamma treatment served as background in each assay. Results were expressed as percentage of protection comparing to that of HuIFN-g treated and EMCV infected culture in the absence of any mAb (100% protection).


100%
75%
50%
25%
0%
Protection (%)
0
1
2
3
4
5
6
7
8
9
10
11
12
mAb Dilution (log2)

concentration (4 working units per ml) of HuIFN-γ. MIF3061 (recognize E_1 epitope) and MIF3055 (recognize E_2 epitope) were also twofold serially diluted and incubated with HuIFN-γ (4 working units/ml). After incubation at 4^0C for 1 hour, each solution was transferred into the plate containing 4 x 10^4 A549 cells per well for antiviral assay. MIF3061 (E_1 mAb) or MIF3055 (E_2 mAb) neutralized the antiviral activity of HuIFN-γ in a dose dependent manner (Figure 13). The protection against EMCV challenge increased gradually as concentrations of MIF3061 or MIF3055 decreased.

MIF3037 (E_3 mAb) also neutralized antiviral activity of HuIFN-γ in a dose-dependent manner (Figure 13). About 80% cells were protected from EMCV challenge at the lowest concentration of MIF3037. However, there were about 25% cell protection at the highest concentration of MIF3037 (Figure 13). The neutralization curve of MIF3037 formed a plateau at 30% cell protection, suggesting that the binding of HuIFN-γ to MIF3037 was saturated at this concentration. Thus, 30% of HuIFN-γ activity was not neutralized by MIF3037 even at excess amount of mAb. Since neutralization of HuIFN-γ by MIF3037 was mediated through specific binding of MIF3037 to E_3 epitope, the residual activity suggested that the binding of MIF3037 to E_3 epitiope resulted in an incomplete inactivation of antiviral activity of HuIFN-γ.

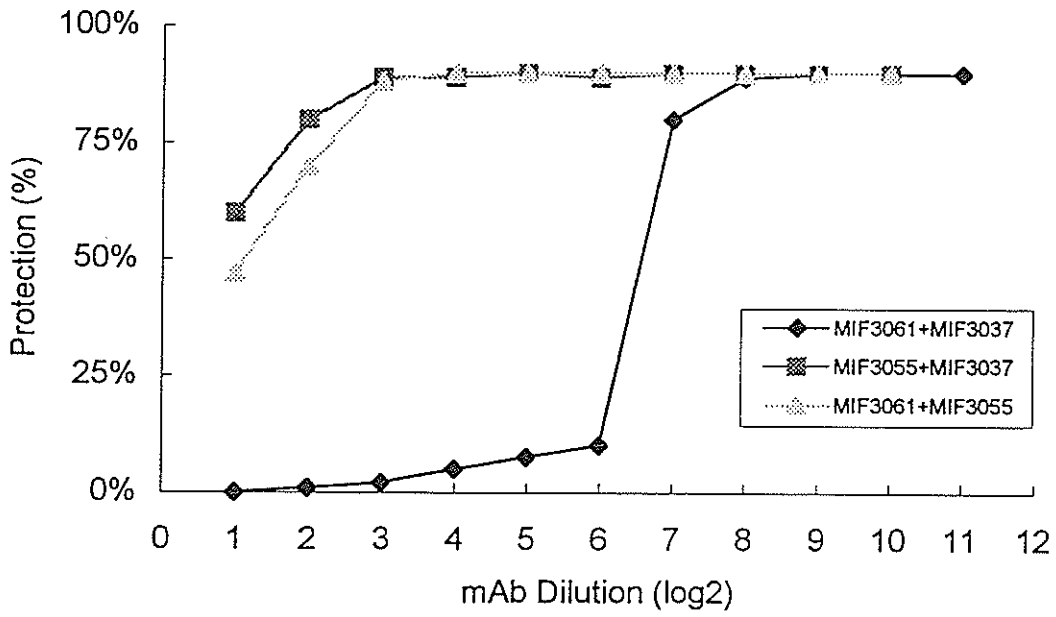
Figure 13. The neutralizing antiviral effect of MIF3061, 3055 and MIF3037. A constant amount of HuIFN-gamma (4 working units/ml) was incubated with twofold serially diluted mAbs at 4°C for 1 hour. This incubation mixture was transferred to cell culture plate containing A_{549} cells and processed as standard antiviral assay. The initial dilution for MIF3061, 3055 and MIF3037 were 1:2500, 1:1000 and 1:40, respectively. Results were expressed as percentage of protection comparing to that of HuIFN-gamma treated and EMCV challenged culture in the absence of any mAb (100% protection).


100%
75%
50%
25%
0%
Protection (%)
0
1
2
3
4
5
6
7
8
9
10
11
mAb Dilution (log2)
MIF3037
MIF3061
MIF3055

7. Neutralizing Antiviral Effect of mAbs in Combination

Previous studies have shown that neutralizing mAbs from different groups (E_1, E_2 or E_3 reacting groups) bound to HuIFN-γ independently (Kwok et al., 1993). Any 2 mAbs from different groups could bind to HuIFN-γ simultaneously without interfering with each other (Alfa & Jay, 1988 and Kwok et al., 1993). In order to examine functional relationships of epitopes E_1, E_2 and E_3, mAb combinations of MIF3061 (E_1 mAb), MIF3055 (E_2 mAb) and MIF3037 (E_3 mAb) were analyzed in the neutralizing antiviral assay (Figure 14). Each of the mAbs was titrated for its neutralizing antiviral units (NAU) before experiment. Twelve NAU of each mAb were mixed and twofold serially diluted before incubating with HuIFN-γ (12 working units/ml). As shown in Figure 14, combinations of MIF3061/MIF3055 (E_1/E_2 mAbs) and MIF3055/MIF3037 (E_2/E_3 mAbs) demonstrated similar neutralizing effect to each mAb alone because the NAU of the combination (either MIF3061/MIF3055 or MIF3055/MIF3037) was about the same amount of NAU added into the assay. However, the combination of MIF3061/MIF3037 (E_1/E_3 mAbs) generated much stronger neutralization effect compared to each mAbs alone (Figure 14). The neutralizing effect of the MIF3061/MIF3037 (E_1/E_3 mAbs) combination was about 1200 NAU, which was 100 time higher than that of either MIF3061 or MIF3037. In summary, this experiment demonstrated that combinations of MIF3061 (E_1 mAb) and MIF3055 (E_2

Figure 14. Neutralizing antiviral activities of mAbs in combinations. MIF3061 (recognizing E_1), MIF3055 (recognizing E_2) and MIF3037 (recognizing E_3) were titrated into concentration of 6 NAV/ml prior to the experiment. Combinations of MIF3061/MIF3055, MIF3055/MIF3037 and MIF3061/MIF3037 were made by 6 NAV/ml of each mAb in the combination and were twofold serially diluted separately. HuIFN-gamma (final concentration of 12 working units/ml) was incubated with each mAb combination at 4°C for 1 hour. The incubation mixture was then transferred into the cell culture plate and processed as standard antiviral assay. Results were expressed as percentage of protection comparing to that of HuIFN-gamma treated and EMCV challenged culture in the absence of any mAb (100% protection).

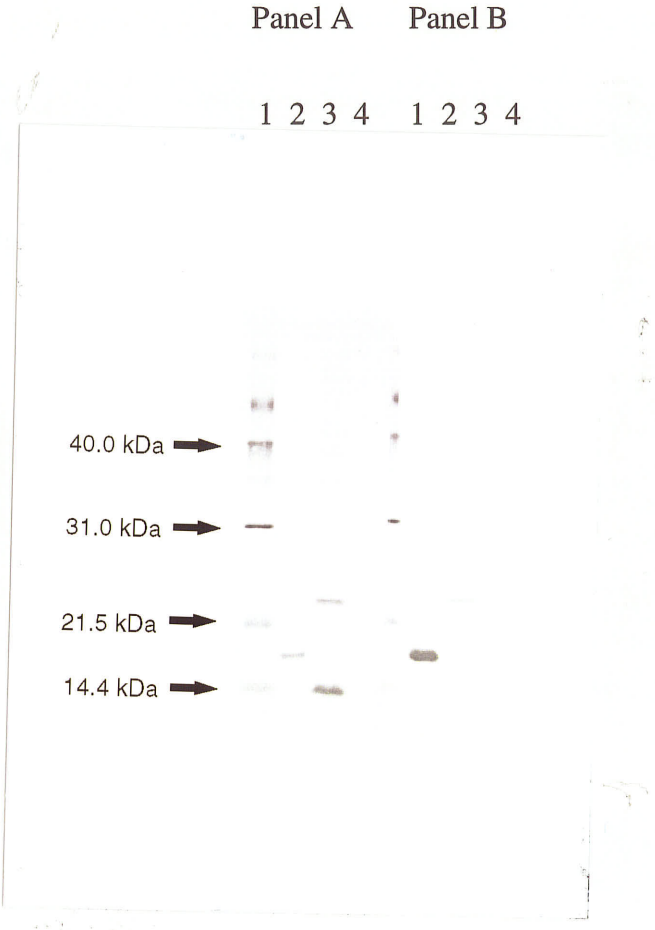

100%
75%
50%
25%
0%
Protection (%)
0
1
2
3
4
5
6
7
8
9
10
11
12
mAb Dilution (log2)
MIF3061+MIF3037
MIF3055+MIF3037
MIF3061+MIF3055

mAb) or MIF3055 (E_2 mAb) and MIF3037 (E_3 mAb) has only additive neutralization effect to antiviral activity of HuIFN-γ. However, MIF3061 (E_1 mAb) and MIF3037 (E_3 mAb) has synergistic effect in neutralizing the antiviral activity of HuIFN-γ. Because MIF3061 (E_1 mAb) and MIF3037 (E_3 mAb) could bind to HuIFN-γ without interfering with each other, epitope E_1 and E_3 should be functionally associated.

8. Mapping of E_3 Epitope with C-terminus Deleted HuIFN-γ Mutant Del_{122}

Del_{122} was a variant molecule of HuIFN-γ that lacks the C-terminus 21 residues (Luke & Jay, 1990). It has been shown that antiviral activity of Del_{122} was 2-10 fold lower than that of full-length molecule of HuIFN-γ (Luke & Jay, 1990). A Western blot analysis was carried out to test the binding of MIF3061 and MIF3037 to Del_{122} and wild type HuIFN-γ (Figure 15). In panel A, HuIFN-γ and Del_{122} were run on SDS-PAGE and transferred on nitrocellulose membrane, then incubated with MIF3061, a mAb recognizing E_1 epitope (residue 84-94). The result showed that MIF3061 recognized both HuIFN-γ (17 kDa) and Del_{122} (14 kDa) (lane 2 and 3 of panel A, Figure 15). Similarly, in panel B, HuIFN-γ and Del_{122} on nitrocellulose membrane were incubated with MIF3037 (mAb recognizing E_3 epitope). MIF3037 reacted with 17 kDa HuIFN-γ (lane 2 of panel B, Figure 15). However, 14 kDa Del_{122} polypeptide was not recognized by MIF3037 (lane 3 of panel B, Figure 15). A non-specific band at 24 kDa was also observed in both

Figure 15. Western blot analyses of the reactivity of MIF3037 and MIF3061 to HuIFN-g and Del_{122}, respectively. Proteins from cell lysate of clone $P_{14}R_9IF_2$ containing HuIFN-gamma gene (lane 2) and Del_{122} (lane 3) were separated on 15% SDS polyacrylamide gel and transferred electrophoretically onto 2 nitrocellulose membranes. MIF3061 (diluted with 1% BSA/PBS in 1:1000)(panel A) and MIF3037 (diluted with 1% BSA/PBS in 1:2500)(panel B) were incubated with each of the 2 nitrocellulose membranes at 37°C for 2 hours. The mAbs specifically bound to proteins on the nitrocellulose membranes were detected with HRP-conjugated goat anti-mouse (anti-IgA, -IgG, -IgM) IgG. The biotinylated molecular marker standards were specifically recognized by HRP-conjugated avidin.

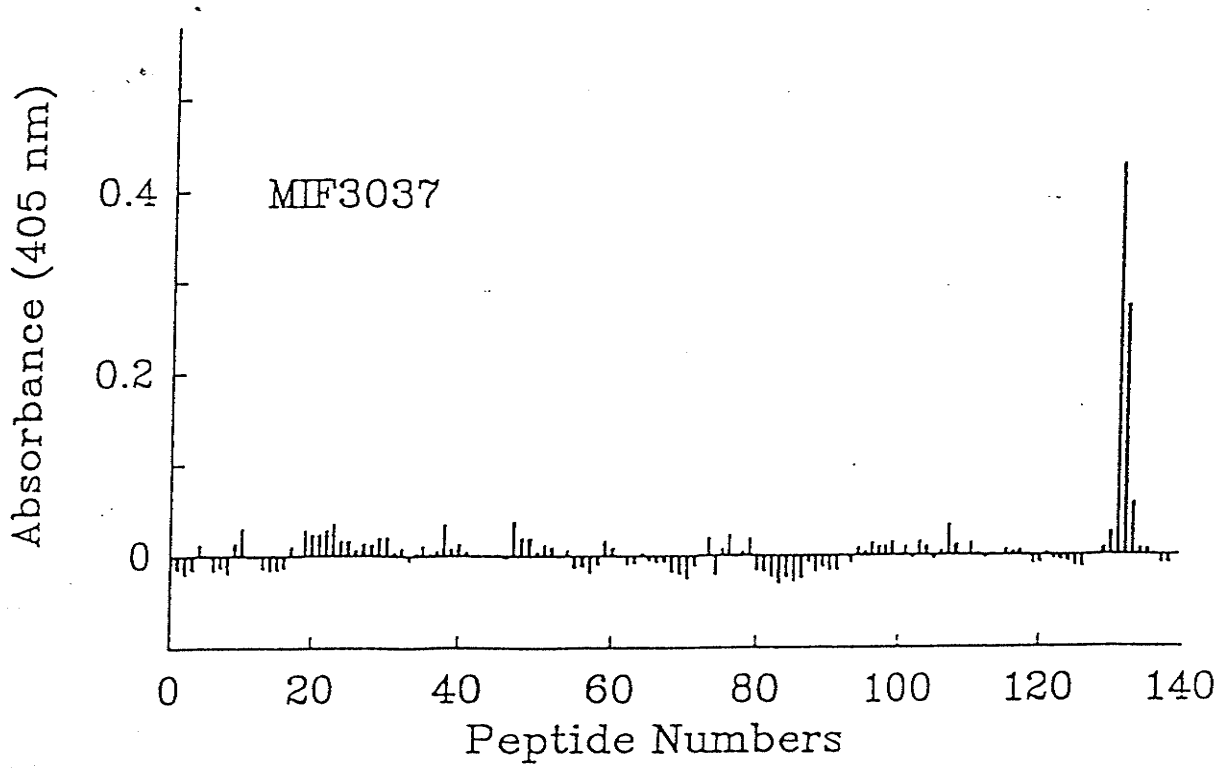

Panel A
Panel B
1 2 3 4 1 2 3 4
40.0 kDa
31.0 kDa
21.5 kDa
14.4 kDa

immunoblot with MIF3061 and MIF3037, respectively. The higher intensity of the 24 kDa band in Del_{122} than HuIFN-γ reflected the increased amount of Del_{122} cell extract loaded in the gel to compesant for the lower level of expression. The differential binding property of MIF3037 to HuIFN-γ and Del_{122} suggested that MIF3037 recognized an epitope (E_3) located within C-terminal 21 amino acid residues (from residues #123 to #143) of HuIFN-γ. In summary, functional epitope E_3 is located from residue #123 to #143 of HuIFN-γ.

9. Mapping of E_3 Epitope with Synthetic Octapeptide of HuIFN-γ

In order to map the precise location of E_3 epitope, MIF3037 was tested for its reaction to the set of octapeptide homologs of HuIFN-γ in ELISA. The specificity of the antibody-octapeptide reaction in ELISA was confirmed by positive reaction of anti-Peptide A mAb with octapeptide "T-L-N-P-T-I-A-G" (Peptide A) and negative reaction of the same mAb with octapeptide "L-A-P-T-I-A-G-K" (Peptide B). The results were plotted as described (Material and Methods). MIF3037 reacted with 2 consecutive peptides, #130 and #131 (Figure 16). The OD_{405} of the peaks was more than four times higher than background. These results were obtained at least in 3 contiunous experiments. These 2 consecutive peptides represent the E_3 epitope recognized by MIF3037. This epitope contained 9 amino acid residues ranged from #130 to #138. The sequence was "Lys^{130}-Arg-Ser-Gln-Met-Leu-Phe-Arg-Gly^{138}". This domain contained three charged

Figure 16. Epitope mapping of mAb recognizing E3 epitope with synthetic octapeptide. MIF3037 is a mAb (IgM type) that recognized E_3 epitope. This mAb was diluted 1:500 in carrier buffer. The specific reactivity of this mAb to octapeptide homologs of HuIFN-gamma was detected by HRP-conjugated goat anti-mouse (anti-IgA, -IgG and -IgM) IgG. The absorbance at 405 nm was plotted against the octapeptide numbers, which were arranged according to the sequence of HuIFN-gamma.


Absorbance (405 nm)
0.4
0.2
0
MIF3037
0
20
40
60
80
100
120
140
Peptide Numbers

basic amino acid residues (1 Lys and 2 Arg), which made this region highly hydrophilic. In summary, the functional epitope E_3 was mapped to amino acid residues #130-138.

According to the sequence of epitope E_3, an oligopeptide (Pepetide C) was synthesized. The sequence of Peptide C was "Cys-Lys^{128}-Arg-Lys-Arg-Ser-Gln-Met-Leu-Phe-Arg-Gly-Arg^{139}", in which the cystine was added for coupling purposes and was not part of the natural sequence of HuIFN-γ. The interaction of Peptide C to MIF3037 was detected with an indirect ELISA. The polyclonal anti-denatured HuIFN-γ serum (PIF3003) was used as a positive control. MIF3061 which was known to bind to E_1 epitope (residue 84-94), served as negative control. The interaction of Peptide C with MIF3061 produced a low absorbency (OD_{405} < 0.05) equivalent to background absorbency, which indicated a lack of specific peptide-antibody reaction (Figure 17). The anti-denatured HuIFN-γ polyclonal serum (PIF3003) reacted with Peptide C in a dose-dependent pattern, which suggested that a specific antibody-peptide reaction was detectable in ELISA. MIF3037 reacted with Peptide C in a similar dose-dependent pattern. The dose-dependent pattern of the reaction demonstrated by MIF3037 suggested that Peptide C (Cys-Lys^{128}-Arg-Lys-Arg-Ser-Gln-Met-Leu-Phe-Arg-Gly-Arg^{139}) was specifically recognized by MIF3037.

As described previously, MIF3037 was a neutralizing mAb against epitope E_3 of HuIFN-γ. An experiment was carried out to test if Peptide C containing E_3 sequence (residues 128-139) could block neutralizing activity of MIF3037 in antiviral

Figure 17. The binding activity of MIF3037 to Peptide C in ELISA. The Peptide C was absorbed on ELISA plate at 4°C overnight. MIF3061, MIF3037 and rabbit polyclonal antibody PIF3004 were twofold serially diluted into ELISA plate to react with the absorbed Peptide C. The mAbs and rabbit polyclonal antibody that specifically bound to the octapeptides were detected by HRP-conjugated goat anti-mouse (anti-IgA, -IgG, -IgM) IgG and HRP-conjugated goat anti-rabbit IgG, respectively. Subsequent colour reaction was detected at 405 nm. The A_{405} was plotted against the dilution of antibodies.

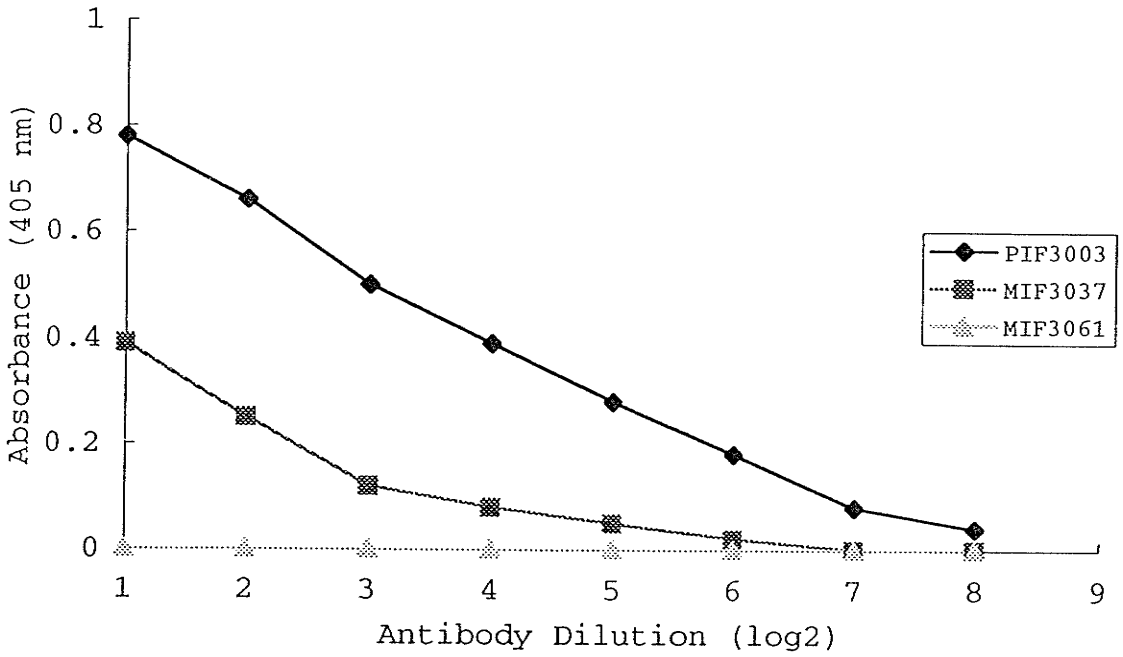
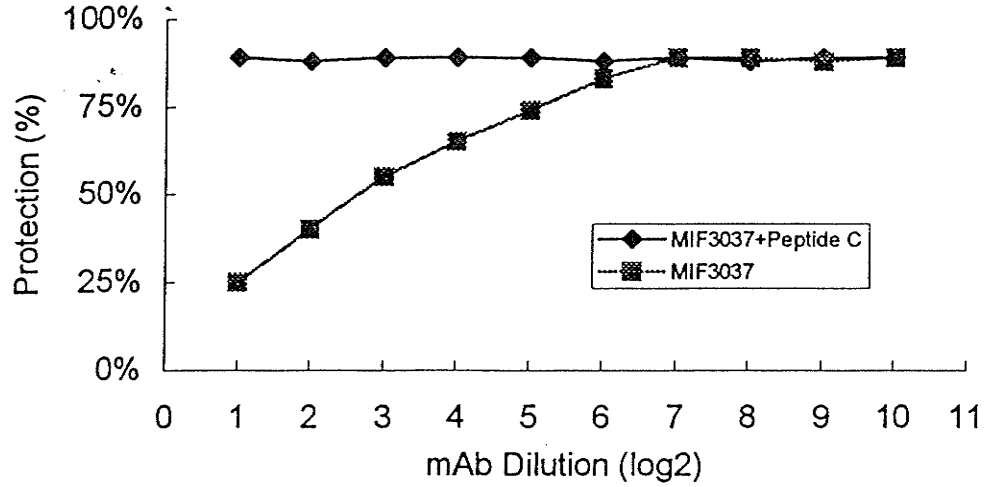
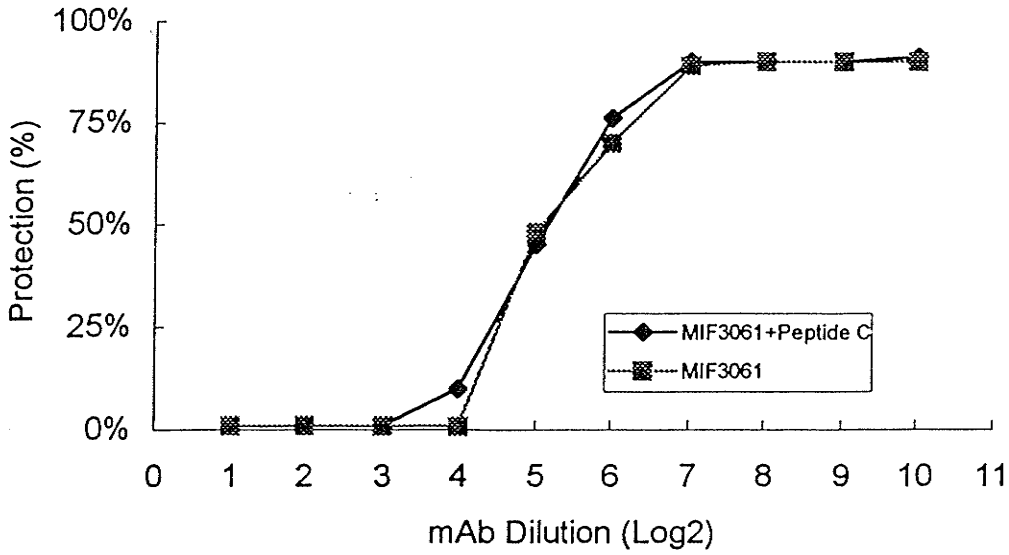

Absorbance (405 nm)
1
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
9
Antibody Dilution (log2)
PIF3003
MIF3037
MIF3061

Figure 18. Blocking effect of Peptide C to the neutralizing antiviral activity of MIF3037. The neutralizing antiviral activity of MIF3061 (bottom) and MIF3037 (top) were tested in the presence or absence of Peptide C. Twofold serially diluted MIF3061 (bottom) and MIF3037 (top) were incubated with constant amount of Peptide C (50 ug/ml) or PBS at 4°C for 1 hour followed by incubation with HuIFN-gamma at 4°C for another hour. The antigen-antibody mixture from this reaction was then transferred into the cell culture plate containing A_{549} cells and processed as standard antiviral assay. Results were expressed as percentage of protection comparing to that of HuIFN-gamma treated and EMCV infected culture in the absence of any mAb (100% protection).


100%
75%
50%
25%
0%
Protection (%)
0 1 2 3 4 5 6 7 8 9 10 11
mAb Dilution (log2)
MIF3037+Peptide C
MIF3037


100%
75%
50%
25%
0%
Protection (%)
0 1 2 3 4 5 6 7 8 9 10 11
mAb Dilution (Log2)
MIF3061+Peptide C
MIF3061

neutralization assay. A constant amount of Peptide C (5 ng per well) was pre-incubated with serially diluted MIF3037 for 1 hour prior to incubation with a constant amount of HuIFN-γ (4 working units, Figure 18). MIF3061 (which did not recognize Peptide C) was processed in the same way to serve as a control group. In this experiment, antiviral activity of HuIFN-γ was neutralized by MIF3061 and neutralizing effect was not affected by addition of Peptide C (Figure 18, bottom). However, neutralizing effect of MIF3037 was completely blocked by Peptide C under similar conditions (Figure 18, top). In conclusion, this experiment has demonstrated that neutralizing activity of MIF3037 was dependent on its specific binding to epitope E_3 which was sequence "^{130}Lys-Arg-Ser-Gln-Met-Leu-Phe-Arg-Gly^{138}" of HuIFN-γ.

10. Site-Directed Mutagenesis in E_1 Epitope

Epitope E_1 was mapped to residue 84-94 of HuIFN-γ polypeptide. The sequence of this domain was "Ser^{84}-Asn-Lys-Lys-Lys-Arg-Asp-Asp-Phe-Gln-Lys^{94}". In order to characterize the functional roles of the E_1 epitope, modifications in the E_1 epitope were made by site-directed mutagenesis. Because all of the 8 anti-HuIFN-γ mAbs in the E_1 reacting group recognized the common sequence of "Lys^{86}-Lys-Lys-Arg-Asp-Asp^{91}", experiments were designed to modify each amino acid from residue #86 to #91.

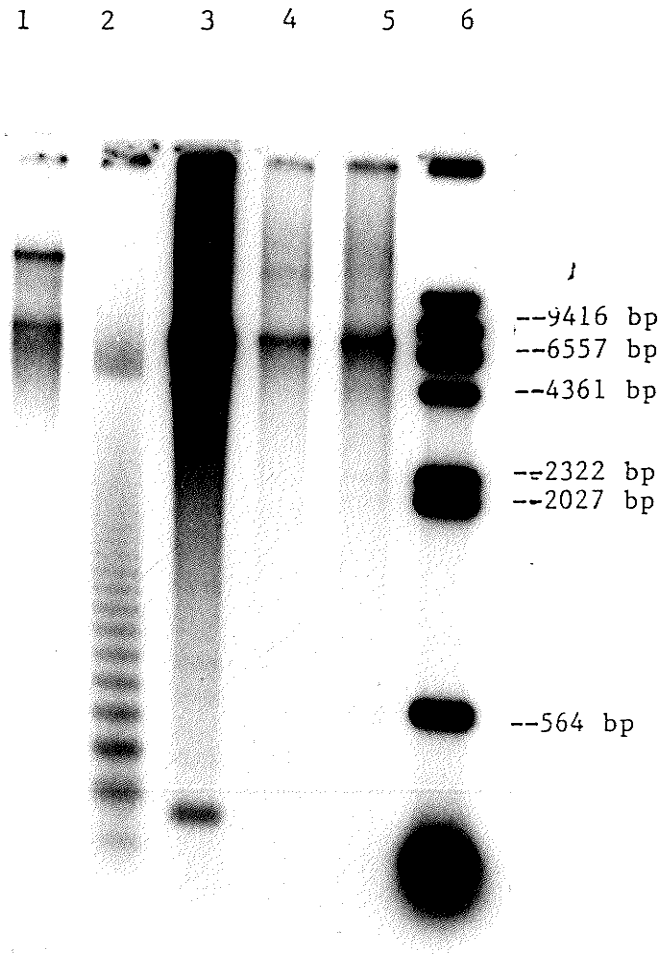
The E_1 epitope was located between two unique restriction enzyme sites (Kpn I and Csp45 I) in the recombinant HuIFN-γ cDNA and the cDNA of Del_{122}. Since E_3

epitope was functionally associated with E_1 epitope, Del_{122} was used as the parental molecule in order to exclude any possible effect from the E_3 epitope. Site-directed mutagenesis at the designed amino acids were made by using random nucleotide substitutions in the first nucleotide of each codon for amino acid residues #86-91. Thus, mutations were introduced by the oligonucleotide prime in the primer extension reaction using Del_{122} as a template (Figure 7).

10.1. Primer Extension and Restriction Digestion

Single stranded DNA (ssDNA) from recombinant phage clone of $M_{13}mp8/P_{14}IFN$ was extracted and the sequence of HuIFN-γ cDNA was confirmed by DNA sequencing. Single stranded DNA (ssDNA) of $M_{13}mp8/P_{14}IFN$ was used as a template and the oligonucleotide that carried the mutations in codons for residues #86-91 was used as primer in DNA primer extension reaction. The α-^{32}P-ATP was added into the reaction as a tracer for *in vitro* syntheses of dsDNA. Following the primer extension reaction, synthesized dsDNA was digested by specific restriction enzymes and then electrophoresed on agarose gel for analyses. DNA molecular weight markers (DNA 123 ladders in lane 2 and lamda Hind III in lane 6, Figure 19) which were end-labelled with γ-^{32}P-ATP were electrophoresed in parallel to serve as indicators for the migration of DNA fragments (Figure 19). It was shown that synthesized dsDNA after primer extension (lane 1, Figure 19) had two major fragments, about 14 Kb and 8 Kb. After restriction digestion

Figure 19. Agarose gel electrophoresis of DNA from primer extension. Radiolabelled DNA materials were visualized by autoradiography. DNA from primer extension (lane 1) was labelled by the incorporation of a-^{32}P-dATP during primer extension reaction. The DNA synthesized during primer extension was subsequently digested by Kpn I (lane 4), Csp45 I (lane 5) or Kpn I/Csp45 I (lane 3). Samples were loaded onto 1.8% agarose gel for electrophoresis. Radiolabelled DNA molecular weight markers (lane 2 for DNA ladder 123 and lane 6 for lamda Hind III fragments) were run in parallel to serve as indicator for the migration rate of DNA samples.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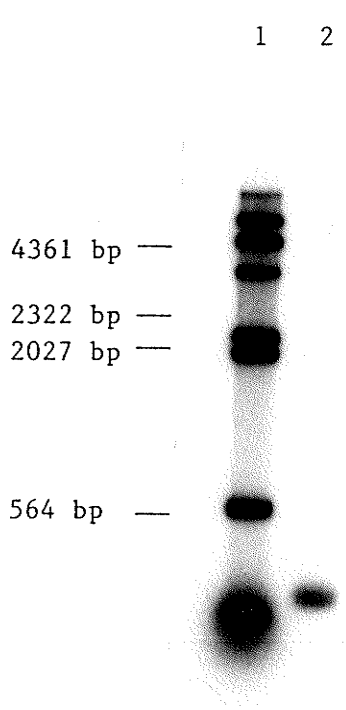
4
5
6
--9416 bp
--6557 bp
--4361 bp
--2322 bp
--2027 bp
--564 bp

with either Kpn I or Csp45 I, only one major band was shown (8 Kb in lane 4 and 5, Figure 19). It was approximately the same size as M_{13}mp8/P_{14}IFN dsDNA (8.4 kb). Since M_{13}mp8/P_{14}IFN DNA had only one restriction enzyme site for either Kpn I or Csp45 I, the 8 Kb fragment must be the linear DNA form after primer chain extension and 14 Kb DNA band (lane 1, Figure 19) must represent the circular form of synthesized dsDNA. After Kpn I and Csp45 I restriction digestion, another band appeared on the agarose gel (lane 3, Figure 19). The size of this fragment was about 196 bp, which was the Kpn I/Csp45 I fragment (196 bp) of HuIFN-γ gene that carried mutations as designed.

10.2. Recovery of the 196 bp Kpn I/Csp45 I Fragment

Following restriction enzyme digestion with Kpn I and Csp45 I and subsequent separation by agarose gel electrophoresis, the 196 bp Kpn I/CSp45 I fragment was recovered from agarose gel by electroelution with DEAE cellulose (type 52) column. After purifying DNA by phenol/chloroform/isoamyl extraction, DNA was dephosphorylated by calf intestinal alkaline phosphatase (CIAP). The recovered DNA was loaded again on agarose gel for electrophoresis and DNA bands were visualized by autoradiography. Molecular weight markers (lamda Hind III) labelled with γ-^{32}P-ATP were electrophoresed in parallel for estimation of DNA fragment size. The recovered DNA was a single band of 196 bp (lane 2, Figure 20). Therefore, the 196 bp Kpn I/CSp45 I fragment was recovered from agarose gel. This recovered Kpn I/Csp45 I fragment

Figure 20. Agarose gel electrophoresis of the 196 bp Kpn I/Csp45 I fragment. The 196 bp Kpn I/Csp45 I DNA fragment was labelled by incorporation of a-^{32}P-dATP during primer extension reaction and subsequently recovered from agarose gel. The recovered material from agarose gel was then loaded onto 1.8% agarose gel for electrophoresis and visualized by autoradiography. Radiolabelled DNA molecular weight marker of lamda Hind III fragment (lane 1) was run in parallel for the determination of DNA molecular weight.


1
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4361 bp
2322 bp
2027 bp
564 bp

(196 bp) which carried mutations was subsequently used as the insert for DNA ligation reaction (Figure 20).

10.3. Enzyme Digestion of Del_{122} and Recovery of Kpn I/Csp45 I Fragment

The Del_{122} dsDNA was extracted and digested with Kpn I and Csp45 I. DNA samples after restriction enzyme digestion were loaded onto agarose gel for electrophoresis. The molecular weight marker (lamda Hind III fragments) was run in parallel for estimation of DNA molecular weight. Restriction digestion of Del_{122} with Kpn I resulted in a single band of 4.6 kb, representing the linear form of Del_{122} plasmid (lane 1, Figure 21). When Del_{122} plasmid DNA was digested by Kpn I and Csp45 I, the DNA band was slightly smaller than the linear form of the Del_{122} plasmid (comparing lane 2 to lane 1, Figure 21), which indicated that a small Kpn I/Csp45 I fragment (196 bp) was removed from the plasmid. The large Kpn I/Csp45 I fragment of Del_{122} had molecular weight about 4.58 kb. This fragment contained the $pJ_{14}R_9$ vector and N- and C-termini of the Del_{122} coding sequence.

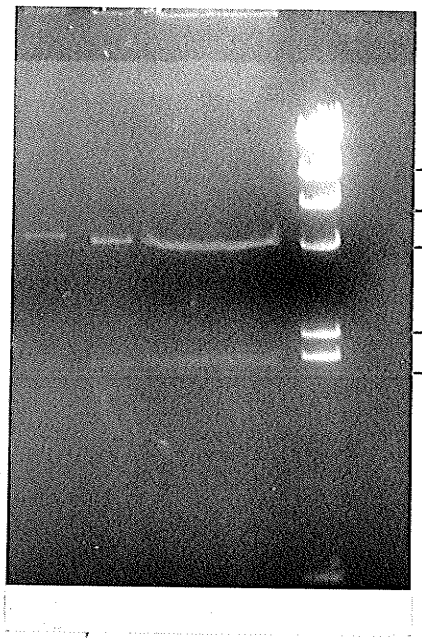
The 4.58 kb Kpn I/Csp45 I band (lane 3, Figure 21) was sliced out from agarose gel and DNA was recovered from agarose gel using "Geneclean" kit from BRL. The recovered 4.58 kb Kpn I/Csp 45 I fragment was used as vector for DNA ligation reaction.

10.4. Ligation of 196 bp Kpn I/Csp45 I Fragment into Del_{122} Vector

The 196 bp Kpn I/Csp45 I fragment from primer extension reaction was labelled

Figure 21. Agarose gel electrophoresis of Del_{122} DNA digested with Kpn I & Csp45 I. Del_{122} DNA was digested by Kpn I (lane 1) or Kpn I/Csp45 I (lane 2 and 3) and subsequently loaded onto 1% agarose gel for electrophoresis. Molecular weight marker (lamda Hind III fragments) was run in parallel for the estimation of DNA molecular weight.

1 2 3 4



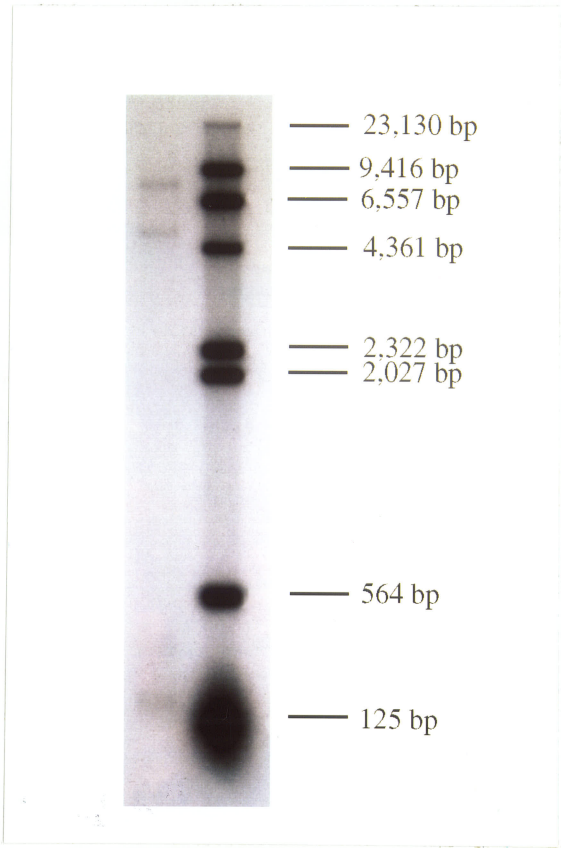
by the incorporation of α-^{32}P-dATP during DNA syntheses. This fragment which carried nucleotide substitutions was ligated to 4.58 kb Kpn I/Csp45 I vector fragment of Del_{122}. Since both fragments had Kpn I and Csp45 I cohesive ends, the 196 bp fragment should be ligated into vector (4.58 kb fragment) in only one orientation. The amount of DNA insert to vector was adjusted to achieve molar ratio of one in the ligation reaction and the reaction was catalyzed by T_4 phage ligase. After the ligation reaction, an aliquot of DNA (one tenth) was electrophoresed on agarose gel and the results were visualized by autoradiograghy (Figure 22). The molecular weight marker (lamda Hind III fragments) 5' end-labelled with γ-^{32}P-ATP and run on the gel as the marker in ligation reaction (Figure 22). Only the labelled DNA fragments could be visualized on the film. Two major bands appeared on the autoradiograghy in Figure 22. The first band had a molecular weight of 4.6 Kb (same molecular weight as linear molecule of Del_{122} plasmid). The second band had a migration rate between 13 to 15 Kb, which was the circular form of the ligated plasmid DNA .

10.5. Transfection of Ligated DNA into Host Cells

The ligated DNA was transfected into host E. coli strain HB101. The transfected E. coli was grown on LB plates containing tetracycline to select clones containing recombinant plasmid. About 500 clones were obtained in this transfection. In each transfected clone, there were 2 types of plasmid, a wild type derived from the template

Figure 22. Agarose gel electrophoresis of DNA after ligation reaction. The amount of 196 bp Kpn I/Csp45 I fragment from primer extension reaction and 4.6 kb Kpn I/Csp45 I fragment from Del_{122} were adjusted to equal molar. Only the 196 bp Kpn I/Csp45 I fragment was radiolabelled. Ligated DNA sample (lane 1) was loaded onto 1% agarose gel for electrophoresis. The labelled DNA molecular weight marker (lamda Hind III fragment) was run in parallel for the determination of DNA molecular weight. DNA bands were visualized by autoradiography.

1 2



and a variant type from the primer extension of the oligonucleotide primer. In order to separate these two types of plasmids, all clones were collected and pooled. DNA extracted from pooled E. coli was transfected again into E. coli strain HB101 and the transfected cells with recombinant plasmid were selected by growing on LB plate containing tetracycline. These clones were collected and stored. Subsequently, each clone was grown from this stock and DNA of the clones was sequenced to screen for mutations.

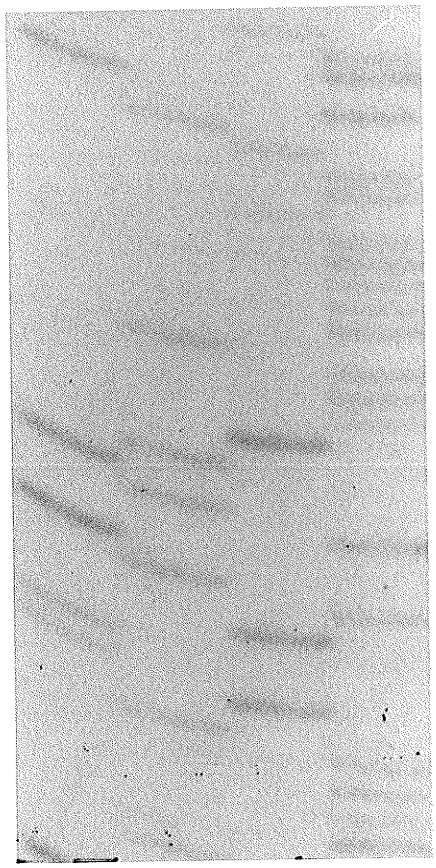
10.6. DNA Sequencing of Selected Clones

The clones were grown from the frozen stocks on LB plate medium. Each clone was picked up from the plate and grew in TB medium overnight. DNA was extracted and sequenced by using dsDNA cycle sequencing system (BRL).

Based on the sequencing results, three clones were found to have mutations at the designed sites. The sequences of the parental DNA and 3 variant clones were showed in Figure 23-26, respectively. The DNA sequence showing the specific nucleotide substitutions and the corresponding changes in the polypeptide sequence were diagrammatically shown in Figure 27. The 3 variant clones were named K86Q, K87E, K88Q according to sequence changes in the polypeptide. In K86Q, the first nucleotide A of codon AAA for Lys^{86} was substituted by C to form the new codon CAA for amino acid Gln^{86} (Figure 24 and 27). In clone K87E, the first nucleotide A of codon AAG for Lys^{87} was substituted by G to form the new codon for amino acid Glu^{87} (Figure 26 and

Figure 23 Nucleotide sequence of Del_{122}. DNA was sequenced with the dsDNA cycle sequencing system (BRL). The lane A, C, G, and T represent the chain termination of the sequencing reaction at base A, C, G and T, respectively. The DNA sequence encoding for amino acid residues #85-93 are shown in the figure.

A C G T



CGAAGTCATCACGTTTCTTTTTGTTG

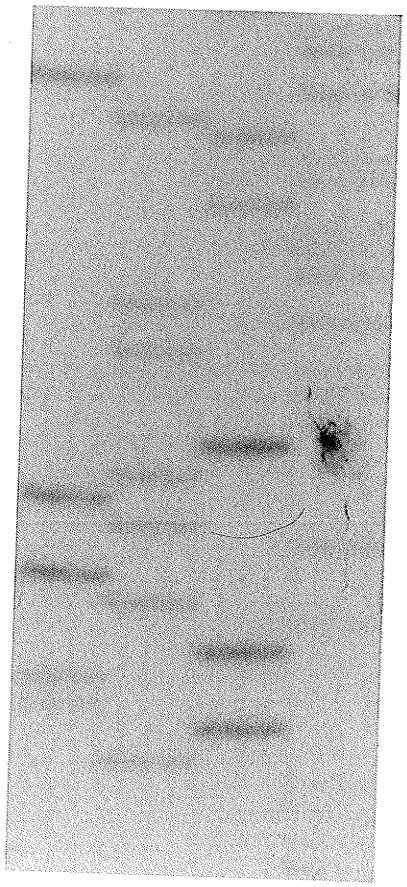
Figure 24. Nucleotide sequence of K86Q. DNA was sequenced with the dsDNA cycle sequencing system (BRL). The lane A, C, G, and T represent the chain termination of the sequencing reaction at base A, C, G and T, respectively. The DNA sequence encoding for amino acid residues #85-93 are shown in the figure. The substituted nucleotide was underlined in the sequence.

A C G T

CGAAGTCATCACGTTTCTTTTGGTTG

Figure 25. Nucleotide sequence of K87E. DNA was sequenced with the dsDNA cycle sequencing system (BRL). The lane A, C, G, and T represent the chain termination of the sequencing reaction at base A, C, G and T, respectively. The DNA sequence encoding for amino acid residues #85-93 are shown in the figure. The substituted nucleotide was underlined in the sequence.

A C G T



CGAAGTCATCACGTTTCTCTTTGTTG

Figure 26. Nucleotide sequence of K88Q. DNA was sequenced with the dsDNA cycle sequencing system (BRL). The lane A, C, G, and T represent the chain termination of the sequencing reaction at base A, C, G and T, respectively. The DNA sequence encoding for amino acid residues #85-93 are shown in the figure. The substituted nucleotide was underlined in the sequence.

A C G T

CGAAGTCATCACGTTGCTTTTTGTTG

Figure 27. Amino acid residue substitutions in clone K86Q, K87E and K88Q. The substitutions in the nucleotide and amino acid sequences of K86Q, K87E and K88Q are underlined.

Del_{122}

	84	85	86	87	88	89	90	91	92	93	94
AA sequence:	S	N	K	K	K	R	D	D	F	E	K
(+) strand: 5'-	AGC	AAC	AAA	AAG	AAA	CGT	GAT	GAC	TTC	GAA	AAG
										Csp45 I	

K86Q:

	S	N	Q	K	K	R	D	D	F	E	K
5'-	AGC	AAC	CAA	AAG	AAA	CGT	GAT	GAC	TTC	GAA	AAG

K87E:

	S	N	K	E	K	R	D	D	F	E	K
5'-	AGC	AAC	AAA	GAG	AAA	CGT	GAT	GAC	TTC	GAA	AAG

K88Q:

	S	N	K	K	Q	R	D	D	F	E	K
5'-	AGC	AAC	AAA	AAG	CAA	CGT	GAT	GAC	TTC	GAA	AAG

27). In clone K88Q, the first nucleotide of codon AAA for Lys^{88} was substituted by C to form the new codon CAA for Gln^{88} (Figure 25 and 27).

11. Analyses of Variant Clones with Western Blot

The expression of the variant HuIFN-γ polypeptides in K86Q, K87E, K88Q clones was examined by Western blot analyses. The expressed polypeptides were detected by immunoblotting with polyclonal antibodies PIF3003 against denatured HuIFN-γ. This antibody (PIF3003) was absorbed with E. coli host cell proteins prior to the reaction.

The specific reactions of these polypeptides with anti-HuIFN-γ antibody were shown in Figure 28. The protein extracts of $pJ_{14}R_9$ host cell which did not carry the HuIFN-γ cDNA were not recognized by PIF3003 (lane 5, Figure 28). A 14.4 kDa polypeptide (same molecular weight as predicted from the sequence of Del_{122}) was detected by anti-HuIFN-γ antibody PIF3003 in K86Q, K87E, K88Q as well as Del_{122}. In conclusion, this experiment has demonstrated that each of the variant clones (K86Q, K87E and K88Q) expressed a polypeptide similar in size as that of Del_{122}.

12. Specific Antiviral Activity of K86Q, K87E and K88Q.

Polypeptides of K86Q, K87E and K88Q were purified by immunoaffinity chromatography. Del_{122} polypeptide was also purified for comparison of the specific

Figure 28. Western blot analyses of HuIFN-g variants K86Q, K87E and K88Q. Whole cell lysate from Del_{122} (lane 1), K86Q (lane 2), K87E (lane 3), K88Q (lane 4) and the host cell without recombinant HuIFN-g gene (lane 5) were dissolved in 1 x sample buffer and loaded on 15% SDS polyacrylamide gel for electrophoresis. The proteins on the gel were transferred electrophoretically to the nitrocellulose membrane and blotted with polyclonal rabbit antibody PIF3003 (1:500 diluted). The specific antigen-antibody binding was detected by HRP-conjugated goat anti-rabbit IgG (1:1000 diluted).

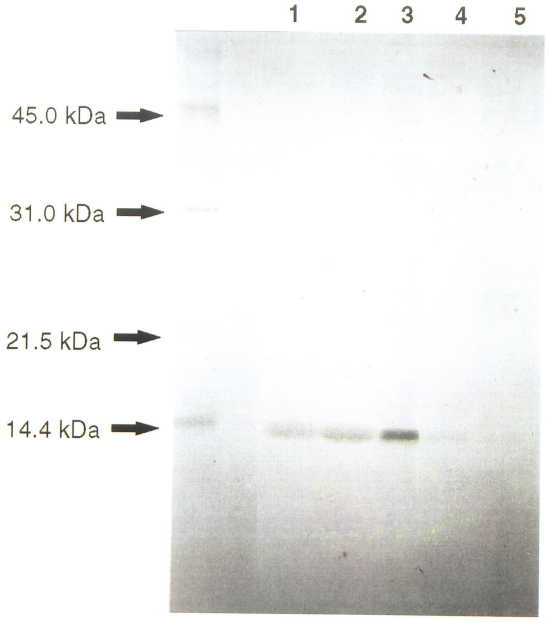
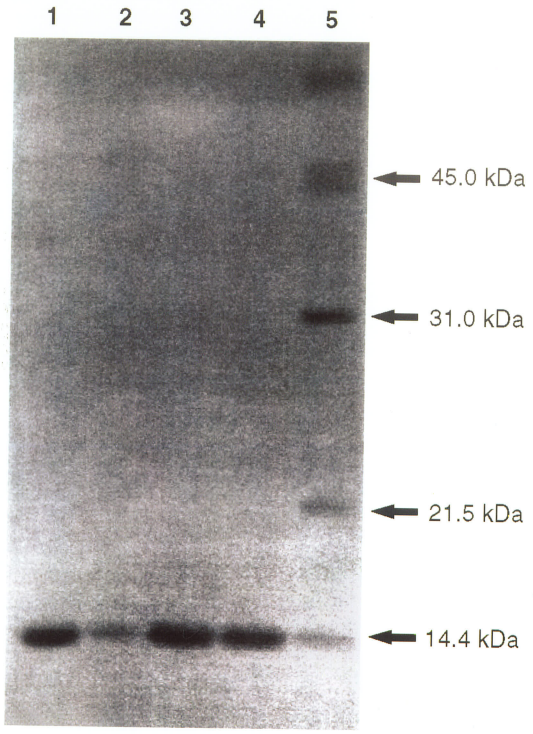

1
2
3
4
5
45.0 kDa
31.0 kDa
21.5 kDa
14.4 kDa

Figure 29. SDS-PAGE analyses of purified proteins from K86Q, K87E, K88Q and Del_{122} clones. Purified polypeptides of Del_{122} (lane 4), K86Q (lane 3), K87E (lane 2) and K88Q (lane 1) were precipitated by 10% TCA and run on 15 % SDA polyacrylamide gel. The molecular weight markers (lane 5) were run in parallel for the estimation of molecular weight of the purified proteins.


1
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45.0 kDa
31.0 kDa
21.5 kDa
14.4 kDa

activity. The purity of the proteins was estimated by SDS-PAGE and subsequent staining of proteins. In Figure 29, each samples from K86Q, K87E and K88Q clones (lane 3, 4 and 5, Figure 29) showed a 14.4 kDa band with the same molecular weight as thepolypeptide of Del_{122} (comparing lane 2 to lane 3, 4, 5) on the gel. Although there were some other proteins shown on the SDS-PAGE, the 14.4 kDa band represented at least 90% of the total proteins.

The concentration of the purified proteins was estimated by AmidoBlak adsorption micro-assay (Schaffuer & Weissmann, 1973). The antiviral activities were determined by standard antiviral assay as described. The specific antiviral activity was then calculated based on the amount of antiviral activity yielded by one mg of the purified proteins (Table 5).

In summary, three HuIFN-γ variants, K86Q, K87E and K88Q were cloned and the expression of these variant polypeptides were confirmed by Western blot. All three mutant molecules had antiviral activity. Since the variation of antiviral assay was usually 2 times of the detected activity, the specific activity of K86Q (2.1×10^7) and K87E (2.9×10^7) were not significantly different than that of Del_{122} (2.9×10^7). The specific antiviral activity of K88Q was about 4 time lower than that of Del_{122}.

Table 5. Comparsion of antiviral specific activities of Del_{122}, K86Q, K87E and K88Q.

clones	Del_{122}	K86Q	K87E	K88Q
specific activity (IU/mg)	2.9×10^7	2.1×10^7	2.9×10^7	7.0×10^6

The respective HuIFN-γ variant molecules were purified by immunoaffinity chromatography and the antiviral specific activity were expressed as NIH reference unit/mg protein.

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CHAPTER IV

DISCUSSION

HuIFN-γ is secreted by lymphocytes when they are stimulated by antigens or mitogens. It has multiple biological functions such as antiviral, cell growth inhibitory and immunomodulatory activities (Pestka, et al., 1987). The functional form of HuIFN-γ is a homodimer under physiological conditions. Each monomer is a polypeptide containing 143 amino acid residues. The activities of HuIFN-γ are elicited through the specific binding of HuIFN-γ to cell surface receptors (Farrar et al., 1993). Although the structure and the functions of HuIFN-γ have been extensively studied, the structure-functional relationships of HuIFN-γ have not been fully characterized and the functional domains of HuIFN-γ have not yet been identified. The purpose of the present study is to localize and characterize the functional site(s) of HuIFN-γ. This study may have the following biological and medical implications: 1.) it may help to understand the biological process of HuIFN-γ mediated effects and 2.) it may offer valuable information for the modification of HuIFN-γ which is a potential therapeutic agent for viral infection and cancer.

The functional sites of a protein are defined as parts of the molecule that are involved directly in the biological functions. The biological functions of a protein are usually dependent on its tertiary structure. Generally, hydrophobic portions of a polypeptide are folded inside the molecule to form the core of a protein, while hydrophilic portions of the polypeptide are exposed on the surface. The amino acid residues in the core of a protein are often critical in maintaining the tertiary structure,

while the residues on the surface are important for interaction with other molecules (Geysen et al., 1987). Therefore, functional sites are usually located on protein surface. Certain types of functional sites or functional domains, such as "zinc fingers" for DNA binding (Pabo & Sauer, 1984), "SH2, SH3 " phosphorylation domains for tyrosine kinase (Waksman et al, 1992) and "Arg-Gly-Asp" domain for the binding of cell matrix adhesion proteins (Pierschbacher, 1987) are shared by many proteins. These structural motifs are easily recognized because they exist in many proteins with similar functions. The structure-functional relationships of these functional domains have been well characterized. However, many proteins (such as HuIFN-γ) do not have an identifiable functional domain in the polypeptide sequences. In such a case, searching for functional sites requires an experimental approach.

In our laboratory, 21 neutralizing anti-HuIFN-γ mAbs were previously developed and characterized (Alfa & Jay, 1988). All of these mAbs could neutralize the antiviral activity of HuINF-γ. According to the degree of cross-reactivities in their binding to HuIFN-γ, these mAbs were classified into three reacting groups, E_1, E_2 and E_1/E_2 reacting groups. Eight mAbs recognized the E_1 epitope specifically, while another 8 mAbs recognized the E_2 epitope (Alfa et al., 1988). Thus, HuINF-γ had at least two functional epitopes (E_1 and E_2). Because mAbs in either E_1 or E_2 reacting group were able to neutralize the antiviral activity of HuIFN-γ, E_1 and E_2 epitopes must be functional epitopes. All of the 21 neutralizing mAbs developed in our laboratory have also been

tested for their capacity to block the binding of HuIFN-γ to cell surface receptor. None was found to be able to block the binding of HuIFN-γ to its receptor (Alfa & Jay, 1988). Therefore, E_1 and E_2 functional epitopes were not involved in receptor binding.

Although E_1 and E_2 epitopes were identified, the location and sequence of E_1 and E_2 epitopes were not localized and characterized. In order to map the functional epitopes of HuIFN-γ, a series of overlapping octapeptides were chemically synthesized to represent the polypeptide sequence of HuIFN-γ. Each of the octapeptides contained 8 amino acid residues with one amino acid shifting for each consecutive octapeptides. HuIFN-γ polypeptide (143 amino acid residues) was represented by a set of 136 overlapping octapeptides. Any specific interaction between octapeptides and anti-HuIFN-γ mAbs would represent the special interaction between corresponding sequences of HuIFN-γ with the mAbs. Thus, epitopes recognized by mAbs could be mapped wiwth an accuracy of a single amino acid.

The E_1 functional epitope was mapped by measuring the reactivity of 8 mAbs in E_1 reacting group to the octapeptide homologs of HuIFN-γ. The mAb-octapeptide interaction was quantitated by a modified ELISA as described in Materials and Methods. Among 8 mAbs in the E_1 reacting group, MIF3009, 3015, 3061, 3074, 3075 and MIF3152 all recognized four consecutive octapeptides #84-88 (Figure 9). MIF3081 reacted with one octapeptide #84 and MIF3169 recognized five consecutive octapeptides #84-89 (Figure 9). The sequence deduced from the peptides #84-89 represented E_1

epitope. Therefore, E_1 functional epitope was mapped to amino acid residues #84-94 of HuIFN-γ (Figure 9).

It was previously shown that mAbs 69B.J, 113B.J and 220A12.J developed by Kelde et al. (1986) could neutralize antiviral activities of HuIFN-γ. The cross competitive binding analyses of 69B.J, 113B.J and 220A12.J with MIF3009 (E_1 mAb) from our laboratory revealed that these mAbs recognized the same epitope as MIF3009 (Kwok et al., 1993). Thus, mAbs 69B.J, 113B.J and 220A12.J also belong to the E_1 reacting group of mAbs (Table 4). The sequences recognized by 69B.J, 113B.J and 220A12.J must be within E_1 domain or at least, overlapped with E_1 domain (residue 84-94) of HuIFN-γ. The sequence of the E_1 epitope was "Ser^{84}-Asn-Lys-Lys-Lys-Arg-Asp-Asp-Phe-Glu-Lys^{94}". This domain contained a basic tetrapeptide element "Lys^{86}-Lys-Lys-Arg^{89}" in the middle of the sequence followed by two acidic amino acid "Asp^{90}-Asp^{91}" and a basic residue "Lys^{94}" at the end. Other residues Ser^{84}, Asn^{85} and Glu^{93} have noncharged polar side chains. These amino acid residues with charged and noncharged polar side chains make the E_1 domain very hydrophillic. Thus, E_1 epitope should be exposed on the surface of HuIFN-γ. According to the x-ray crystallographic structure, HuIFN-γ consisted of 6 α-helices (from A to F) and 5 loops between these helices (Figure 3). The first three amino acids "Ser^{84}-Asn-Lys^{86}" in the E_1 domain was part of the loop between helices D and E. Other residues in E_1 domain "Lys^{87}-Lys-Arg-Asp-Asp-Phe-Glu-Lys^{94}" were within α-helix E (Figure 3). It was shown that E_1 domain was surface exposed in the x-ray

crystallographic structure model of HuIFN-γ (Figure 3). None of the residues in E_1 domain was shown to be involved in the dimerization of HuIFN-γ (Ealick et al., 1991).

According to previous studies in our laboratory, the E_2 functional epitope was a tertiary structure dependent epitope of HuIFN-γ because all of the mAbs in the E_2 reacting group were unable to react with the SDS denatured HuIFN-γ (Alfa & Jay, 1988). Two mAbs (MIF3055 and MIF3070) in the E_2 reacting group were chosen to represent this group of mAbs in the reaction to octapeptide of HuIFN-γ. No specific antibody-peptide binding was detected when MIF3055 or MIF3070 was incubated with the peptides homologs of HuIFN-γ. Therefore, our result confirmed that the E_2 functional epitope was a tertiary structure-dependent domain and could not be detected with the epitope mapping method used in this study. Similarly, the epitope recognized by the mAbs of E_1/E_2 reacting groups was also shown to be tertiary-structure dependent because all of the mAbs in E_1/E_2 reacting group were unable to react with SDS denatured HuIFN-γ (Alfa & Jay, 1988). Our experiment showed that MIF3094 (a mAb in E_1/E_2 reacting group) did not have any specific reactions with the synthetic octapeptides, which also confirmed that epitope recognized by mAbs in E_1/E_2 reacting group was tertiary-structure dependent (Figure 11). Thus, the epitope recognized by the E_1/E_2 reacting group was not localized with this epitope mapping method.

MIF3037 is an anti-HuIFN-γ mAb of IgM type. It has been previously shown that MIF3037 did not have any cross-reactivity with mAbs which recognized the E_1 and

E_2 epitopes (Kowk et al., 1993). Hence, the epitope recognized by MIF3037 must be different from E_1 and E_2 epitopes (Kowk et al., 1993). The present study demonstrated that MIF3037 neutralized the antiviral activity of HuIFN-γ in a dose dependent manner (Figure 12). Therefore, another functional epitope recognized by MIF3037 was identified and was named E_3 epitiope. MIF3037 was shown to react with SDS denatured HuIFN-γ in Western blot, but did not react with Del_{122} which was a HuIFN-γ mutant without the C-terminal 21 amino acid residues (Figure 15). Thus, the Western blot analyses suggested that E_3 epitope recognized by MIF3037 was a linear epitope located in C-terminal 21 amino acid residues of HuIFN-γ. Based on its reactivity with synthetic octapeptides, MIF3037 specifically recognized two consecutive octapeptides #130 and #131, which represented residues #130-138 of HuIFN-γ (Figure 16). It was found that MIF3037 recognized the soluble peptide (residue 128-139) which contained the E_3 sequence (Figure 17). This soluble peptide (residue 128-139) was shown to inhibit the neutralizing activity of MIF3037 in an antiviral assay (Figure 18). Thus, the E_3 epitope was mapped to residue #130 to #138 of HuIFN-γ. The sequence of E_3 epitope was "Lys^{130}-Arg-Ser-Gln-Met-Leu-Phe-Arg-Gly^{138}". According to x-ray crystallographic structure of HuIFN-γ, E_3 epitope was a random coiled structure following α-helix F and was exposed on the surface of HuIFN-γ (Figure 3). None of the amino acid residues in E_3 epitope was involved in maintaining the tertiary structure of HuIFN-γ or formation of the homodimer of HuIFN-γ

(Elick et al., 1991).

Based on the fact that MIF3037 was a neutralizing mAb, we concluded that the E_3 epitope (residue #130-138) was a functional epitope. However, Del_{122} (a HuIFN-γ mutant lacking C-terminal 21 amino acids) was functional, although its antiviral activity was 3-10 times lower than that of wild type HuIFN-γ. Thus, E_3 epitope was not essential but yet required for the full antiviral activity. This unique characteristic of being a functional but nonessential epitope suggested that E_3 epitope was a redundant functional domain. The redundancy of E_3 epitope was also supported by the kinetic studies of the neutralization activity of MIF3037. MIF3037 (E_3 mAb) neutralized antiviral activity of HuIFN-γ in a dose dependent manner similar to other mAbs, such as MIF3061 (E_1 mAb) and MIF3055 (E_2 mAb) (Figure 13). However, MIF3037 was not able to completely neutralize the antiviral activity of HuIFN-γ even at the vast excess of mAbs. Using 4 woking units of HuIFN-γ, about 25-35% residual activity of HuIFN-γ was detected when the binding of MIF3037 to HuIFN-γ was saturated (Figure 13). However, as the concentration of HuIFN-γ increased, the residual antiviarl activity at excess amount of MIF3037 increased proportionally. This was not observed with other mAbs in E_1 and E_2 groups. This observations is consistant with the fact that E_3 epitope is a redundant signal.

Because the E_3 functional epitope was redundant, there may be another domain in the sequence of HuIFN-γ which could replace or compensate the function of the E_3 epitope. We found that both E_1 and E_3 epitopes contained several basic amino acid

residues in their sequences. There was a basic tetrapeptide element "Lys^{86}-Lys-Lys-Arg^{89}" within E_1 domain. The first two amino acid residues in E_3 domain were "Lys^{130}-Arg^{131}". Together with two basic amino residues "Lys^{128}-Arg^{129}" immediately upstream from E_3 domain, it also formed a basic tetrapeptide element "Lys^{128}-Arg-Lys-Arg^{131}". Based on sequence similarity and redundancy of the E_3 epitope, we hypothesized that E_1 and E_3 epitopes had similar effects on the function of HuIFN-γ.

In order to test this hypothesis, neutralizing activities of MIF3061 (E_1 mAb), MIF3055 (E_2 mAb) and MIF3037 (E_3 mAb) were analyzed in various combinations. It was shown that the E_1 and E_2 mAbs combination (MIF3061 and MIF3055) or the E_2 and E_3 mAbs combination (MIF3055 and MIF3037) had additive neutralizing activity (Figure 14). However, combination of the E_1 and E_3 mAbs (MIF3061 and MIF3037) demonstrated an 100 fold increase in the neutralizing antiviral activity when compared to the that of each mAb alone (MIF3061 or MIF3037) (Figure 14). The synergistic effect of E_1 and E_3 mAb suggested a functional association of E_1 and E_3 epitopes and supported our speculation that the E_1 and E_3 epitopes had similar functions.

In our search for similar sequences in other proteins, we found that the sequence "Lys^{86}-Lys-Lys-Arg^{89}" and "Lys^{128}-Arg-Lys-Arg^{131}" resembled 2 types of functional domains. The first type was "Lys/Arg-X-Lys/Arg-Arg/Lys" (X represents any amino acid other than Lys and Arg), which was a consensus cleavage site for the subtilisin protease family (Rawlings and Barrett, 1994). The subtilisin protease family is a group of serine endopeptidases that exist in both eucaryotic and prokaryotic cells (Rawlings and

Barrett, 1994). In mammalian cells, subtilisin proteases (PC1, PC2 and furin convertases) were shown to function as the pro-protein and prohormone convertases which cleaved pro-protein or pre-hormone at peptide bond after two basic amino acid to release the functional protein or hormone. Seidah and Chretien (1994) showed that PC1 and PC2 proteases could cleave pro-renin, pro-enkephalin and pro-insulin to release the hormones. The cleavage site in all cases was found to be the peptide bond after a pair of basic amino acids (Lys and Arg). The recognition site of mammalian convertases was "Lys/Arg-X-Lys/Arg-Arg/Lys" (consensus sequence of subtilisin proteases) for furin convertase or just a pair of basic amino acids, such as "Lys-Lys", "Lys-Arg", "Arg-Lys" and "Arg-Arg" for PC1 or PC2 (Seidah and Chretien, 1994). The basic elements in E_1 and E_3 epitopes of HuIFN-γ is also resembled the consensus cleavage sequences of subtilisin protease. It has been demonstrated that\ mouse IFN-γ was cleaved by proteases intracellularly after internalization of the ligand-receptor complex. However, the nature of the specific proteases and the cleavage site on HuIFN-γ were not identified (Farrar et al., 1991). It is not clear the cleavage of HuIFN-γ was also a necessary step for the dissociation of HuIFN-γ from its receptor as in the case for the mouse system (Farrar et al., 1991). Thus, sequences "Lys^{86}-Lys-Lys-Arg^{89}" and "Lys^{128}-Arg-Lys-Arg^{131}" could serve as the protease cleavage sites of intracellular digestion and activation of HuIFN-γ. Based on this hypothesis, the E_1 and E_3 mAb mediated neutralizing activity could be related to the interference with protease binding to the cleavage sites on HuIFN-γ which could prevent

the putative activation of HuIFN-γ.

Nuclear localization signal (NLS) was the second type of functional domain similar to the basic elements of E_1 and E_3 epitopes. A Nuclear localization signal is usually a short stretch of basic amino acids that directs transportation of protein from cytoplasm into the cell nucleus. A comparison of the NLS from different proteins is shown in Table 6. A typical NLS consists of a series of basic amino acid residues (Lys and Arg). The NLS of SV40 T antigen " Pro-Lys-Lys-Lys-Arg-Val" have been extensively studied (Kalderon et al., 1984a). It was found that the NLS could function autonomously (tertiary structure independent) because it directed nuclear localization when it was translocated to other parts of SV_{40} T antigen or cloned into cytoplasmic proteins (pyruvate kinase and bacterial β-galactosidase) (Kalderon et al., 1984b). Substitution of the amino acids in NLS of SV40 T antigen suggested that all residues except lysine in the second position could be substituted independently without losing the nuclear localization function (Kalderon et al., 1984a). Some proteins, such as steroid hormone receptors had 2 or more nuclear localization signals (Hollenberg et al., 1985). It was shown that one NLS was essential for nuclear localization, but two or more NLS usually had higher efficiency (Guiochon et al., 1989). Based onthe known NLS sequences, a consensus sequence for the NLS was deduced (Table 6). The NLS usually has two consecutive basic residues at position 1 and 2 (second was always lysine). It was followed by at least 1 and up to 3 basic residues in position 3-5 with no more than one

Table 6. The comparison of amino acid sequences of NLS in various proteins and the putative NLS in IFN-gamma of different species.

PROTEIN	SPECIES	LOCATION	SEQUENCE	REFENCE
progesteron receptors	Human	637-642	R K F K K	Misrahi, 1987
	Rabbit	638-643	R K F K K	Guiochon, 1989
	Chicken	159-164	R K L K K	Conneely, 1987
Androgen receptor	Human	256-261	R K D R R	Chang, 1988
	Rat	261-266	R K D R R	Chang, 1988
Glucocorticoid receptor 1985	Human	491-499	R K T K K K I K	Hollenberg, 1985
	Rat	498-506	R K T K K K I K	Picard, 1987
	Mouse	510-519	R K T K K K I K	Danielsen, 1986
Mineralocorticoid receptor	Human	673-678	R K S K K	Arriza, 1987
Thyroid Hormone receptor	Human	130-133	K R K	Benbrook, 1987
Vitamin D receptor	Human	102-105	K R K	Baker, 1988
SV-40 T-antigen	Virus	127-132	K K K R K	Kalderon, 1984
Polymerase basic protein 2	Virus	736-740	K R K R	Mukaigawat, 1991
CONSENSUS SEQUENCE			K/R K K/R K/R K/R	
IFN-gamma	Human	86-89	K K K R	
		128-131	G K R K R	
	Bovine	127-131	R K R K R	
	Rat	127-131	R K R K R	
	Mouse	127-131	R K R K R	

single letter amino acid codes are used, lysine and arginine are underlined.

nonbasic residue separating them from the first two basic residues (Table 6). Based on the consensus sequence for NLS, the sequence "Lys^{86}-Lys-Lys-Arg^{89}" in E_1 epitope and "Lys^{128}-Arg-Lys-Arg^{131}" in E_3 epitope meet the criteria as nuclear localization signals.

It was not clear whether the sequences "Lys^{86}-Lys-Lys-Arg^{89}" and "Lys^{128}-Arg-Lys-Arg^{131}" of HuIFN-γ were protease cleavage sites or NLS sequences. In order to test these 2 hypotheses and to determine the critical residues for HuIFN-γ function, we substituted some amino acid residues in the E_1 domain by using site-directed mutagenesis. The sequence modifications were made in Del_{122} molecule to exclude any posssible effect from E_3 domain. In the E_1 epitope, the sequence "Lys^{86}-Lys-Lys-Arg-Asp^{90}" was a common binding site for all of the eight mAbs in the E_1 reacting group and contained basic amino acid residue "Lys^{86}-Lys-Lys-Arg^{89}". Our Experiment was designed to substitute amino acids from residue #86 to #90. Three clones (K86Q, K87E and K88Q), each containing single amino acid substitution in E_1 sequence were made (Figure 28). The amino acid residue Lys^{86}, Lys^{87} and Lys^{88} were substituted by Gln, Glu and Gln in the respective clones. The side chains of glutamate and glutamine were both hydrophillic, although the glutamine did not carry a negative charge. In each mutant clone (K86Q, K87E or K88Q), a positive charged side chain of Lys was changed into a neutral or negative charged side chain (Figure 24-26 and Figure 30). According to the model of x-ray crystallographic structure, the residue Lys^{86}, Lys^{87} and Lys^{88} were part of β-loop

structure exposed on the surface of HuIFN-γ (Ealick et al., 1992). The substitutions in Lys^{86}, Lys^{87} and Lys^{88} were unlikely to cause a significant change in the tertiary structure of HuIFN-γ because these three residues were not involved in maintaining the tertiary structure. All of the mutant clones (K86Q, K87E and K88Q) were shown to express the specific polypeptides detected by anti-HuIFN-γ antibodies in Western blot (Figure 28). The polypeptides expressed by the three mutant clones were subsequently purified by immunoaffinity column coupled with MIF3052 (E_2 mAb). Since MIF3052 recognized a tertiary structure-dependent epitope (E_2), its ability to bind the mutant proteins suggest that the 3 mutant proteins (K86Q, K87E and K88Q) did not have major changes in the tertiary structure. All of the 3 variant clones, K86Q, K87E and K88Q had antiviral activity (Table 5), indicated that Lys^{86}, Lys^{87} and Lys^{88} when replaced singly were insufficient to abolish the antiviral activity of HuIFN-γ.

According to the previous studies of NLS, Lys in the second position of NLS sequence was essential for the function of NLS. In the clone K87E, Lys^{87} was in the second position of the putative NLS "Lys^{86}-Lys-Lys-Arg^{89}" in E_1 epitope. However, antiviral activity of the K87E clone was not different from that of the parental Del_{122} molecule, suggesting that function of HuIFN-γ was not disrupted in mutant clone K87E. Thus, the characterization of K87E did not support the "NLS" hypothesis of E_1 functional epitope.

As mentioned previously, sequences "Lys^{86}-Lys-Lys-Arg^{89}" of the E_1 epitope

and "Lys^{128}-Arg-Lys-Arg^{131}" in E_3 epitope fit into the consensus cleavage sequence of protease of the subtilisin family. It has also been shown that protease cleavage of HuIFN-γ intracellularly was required for the dissociation of HuIFN-γ from its receptor and subsequent intracellular recycling of receptor (Farrar et al., 1991). Although the specific cleavage site(s) have not been identified, sequences "Lys^{86}-Lys-Lys-Arg^{89}" in the E_1 epitope and "Lys^{128}-Arg-Lys-Arg^{131}" in the E_3 epitope were potential sites for intracellular protease cleavage of HuIFN-γ. The recognition site for subtilisin proteases was not disrupted in mutant molecules of K86Q, K87E and K88Q. The sequence "Lys^{86}-Lys-Lys-Arg^{89}" after substitution in Lys^{86}, Lys^{87} and Lys^{88} still have a paired basic amino acid residues. These paired basic amino acids could be recognized by PC1, PC2 or other memebers of convertase in mammalian cells. Substitutions of Lys^{86}, Lys^{87} and Lys^{88} in this project were not sufficiently different from the consensus sequence to determine if the sequence "Lys^{86}-Lys-Lys-Arg^{89}" in E_1 functional epitope may function as a protease cleavage site for the intracellular digestion of HuIFN-γ.

In summary, a study in the structure-functional relationship of HuIFN-γ was carefully designed and carried out. The E_1 functional epitope recognized by 8 anti-HuIFN-γ mAbs was mapped to sequence "Ser^{84}-Asn-Lys-Lys-Lys-Arg-Asp-Asp-Phe-Glu-Lys^{94}" of HuIFN-γ. A novel functional epitope E_3 was identified by MIF3037. The E_3 epitope was also mapped to sequence "Lys^{130}-Arg-Ser-Gln-Met-Leu-Phe-Arg-Gly^{138}". The sequences of functional epitope E_1 and E_3 were both highly hydrophilic and were

exposed on the surface of HuIFN-γ, according to the x-ray crystal structure. Comparison of the kinetics of the neutralizing effect mediated by different mAbs indicated that the E_1 mAb (MIF3061) induced a complete inactivation to the antiviral activity of HuIFN-γ, while E_3 mAb (MIF3037) induced an incomplete inactivation. Since Del_{122} which did not have the sequence of E_3 epitope had 3-10 fold lower antiviral activity than that of wild type HuIFN-γ, E_3 epitope appears to be a functional but nonessential domain. Analyses of the functional relationship of the E_1, E_2 and E_3 epitopes indicate that the combinations of E_1/E_3 had synergistic neutralization activity, while E_1/E_2 mAbs or E_2/E_3 mAbs had only additive neutralizing activity. This synergistic effect indicated that there was functional association between E_1 and E_3 epitopes. A basic element "Lys^{86}-Lys-Lys-Arg^{89}" was found in E_1 epitope. A similar element "Lys^{128}-Arg-Lys-Arg^{131}" was also present on the N-terminal junction of E_3 epitope. Based on the the sequence comparsion to other well defined functional in other proteins, it is hypothesized that E_1 and E_3 epitopes may functions as nuclear localization signals. In an attempt to test these hypotheses, amino acid residues Lys^{86}, Lys^{87} and Lys^{88} (within E_1 epitope) were substituted by amino acids Gln, Glu and Gln, respectively. All three variant clones K86Q, K87E and K88Q, respectively, expressed the specific polypeptides and had antiviral activity. The specific antiviral activity of K86Q and K87E were not significantly different (within the limit of the sensitivity of the assay) from that of parental molecule Del_{122}. The specific antiviral activity of K88Q was about 4 times lower than that of Del_{122}. Thus, each individual

residue of Lys^{86}, Lys^{87} and Lys^{88} was not critical for the antiviral activity of HuIFN-γ. Baed on the studies of Kalderon et al. (1984) on the functional contribution of ecah of the basic residue within the SV_{40} T antigen NLS, the second basic residue of the NLS sequence is essential for the nuclear translocation function. In our study, the substitution in Lys^{87} (second position of the putattive NLS in E_1 epitope) with Gln did not affect the specific antiviral activity. This result suggested that E_1 epitope is unlikely to function as NLS. In examining the second hypotheses that the E_1 functioned as protease cleavage site, we found that the sequence of "Lys^{86}-Lys-Lys-Arg^{86}" after substitution in either Lys^{86}, Lys^{87}, or Lys^{88} still had a paired basic amino acid (Lys-Lys or Lys-Arg) which could be recognized by intracellular proteases such as PC1, and PC2 convertases or other similar proteases. Since intracellular cleavage of HuIFN-γ was a required process for eliciting the activity of HuIFN-γ, sequences "Lys^{86}-Lys-Lys-Arg^{86}" in E_1 epitope and "Lys^{128}-Arg-Lys-Arg^{131}" in E_3 epitope may serve as cleavage sites for the intracellular digestion of HuIFN-γ.

Based on our investigation, further studies for the structure-functional relationship of HuIFN-γ should be concentrated on the following aspects. The first is to search and map the receptor binding site of HuIFN-γ, which is essential for the activity of HuIFN-γ. The second is to identify the critical residues for HuIFN-γ function. Our study showed that substitutions in residues Lys^{86}, Lys^{87} and Lys^{88} did not result in the inactivation of HuIFN-γ. Modifications of other amino acids in E_1 epitope may be necessary to

determine the critical residues for HuIFN-γ function. Alternatively, substitutions of 2 or 3 amino acids simultaneously in E_1 epitope might be required. The third is to test whether the functional epitope E_1 and E_3 serve as protease cleavage sites for the intracellular digestion of HuIFN-γ. This could be achieved by modifying the putative cleavage sequence in E_1 or E_3 epitopes and by studying the function of the modified HuIFN-γ molecules in the presence or absence of specific proteases inhibitors.

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