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Structure/function studies of murine interferon-α1 using site-directed mutagenesis followed by in vitro synthesis

May C. Lai, Manfred W. Beilharz, Anthony A. Scalzo, Kerryn L. Garrett, James F. Cannon, Stuart J. Boyer and Nalini Swaminathan

Department of Microbiology, The University of Western Australia, Nedlands, Australia

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Summary

Site-directed in vitro mutagenesis followed by in vitro transcription and translation has been used to study structure/function relationships for murine interferon-α1 (MuIFN-α1). The mature form of the MuIFN-α1 protein was expressed as well as analogue forms with amino acid substitutions at positions 33, 71, 72, 123 and 133. These positions were chosen on the basis of known human interferon-α structure/function relationships. Biological assays for antiviral activity on murine cells and natural killer cell activation have been performed for each of the proteins produced. The data obtained have been interpreted in the light of previous human and murine interferon-α structure/function work and the recently published three-dimensional structure of murine type I interferon.

Murine interferon-α1; Site-directed mutagenesis; Structure/function; Antiviral activity; Natural killer cell activation

Introduction

The murine alpha interferons (MuIFNs-α) comprise a family of at least 10 distinct functional genes analogous to the human alpha interferon (HuIFN-α) genes. The genes coding for MuIFNs-α and HuIFNs-α show a high degree of

Correspondence to: M.W. Beilharz, Department of Microbiology, Queen Elizabeth II Medical Centre, University of Western Australia, Nedlands, W.A. 6009, Australia.

similarity in their nucleotide and amino acid sequences with 50 amino acid residues being strictly conserved between 14 human and 6 mouse alpha interferons (IFNs-α). Comparison of the flexibility and hydrophobicity indices of the amino acid residues of HuIFN-α2 and MuIFN-α1 has shown them to be almost identical (Raj et al., 1988).

A previous approach to identifying functionally important regions of the MuIFN-α molecule has been the construction of HuIFN-α/MuIFN-α and MuIFN-α/MuIFN-α hybrids. The human/murine hybrid studies have attempted to define regions responsible for the species-specificity of the alpha interferons. The amino-terminal half of the HuIFNs-α seems to predominate in conferring activity on human cells, while the carboxy-terminal 104 amino acids of MuIFNs-α appears to be responsible for the activity on mouse cells (Weber et al., 1987; Raj et al., 1988). The MuIFN-α/MuIFN-α hybrid studies have indicated that the amino-terminal sequence determines high activity on hamster cells and that the carboxyl terminal determines activity on mouse cells (Van Heuvel et al., 1987; Trapman et al., 1988). A third region, amino acids 68–123, has been shown to be important for the low activity of MuIFN-α10 on mouse cells (Trapman et al., 1988). However, hybrid IFN studies cannot delineate individual amino acids responsible for differences in biological activities. Site-directed mutagenesis allows individual amino acids to be assessed for their role in determining biological activity, but few such studies have been performed with MuIFNs-α.

In direct contrast to the limited structure/function work done on MuIFNs-α, quite a lot of human studies have been performed. In recent years, much of the structure/function work on IFNs-α has focused on the human subtypes. The availability of cloned HuIFN-α genes has contributed greatly to the understanding of the molecular functions of these proteins. Structure/function studies on HuIFNs-α have included chemical modification of amino acids (Wetzel et al., 1982; Morehead et al., 1984), generation of fragments and synthetic peptides to test their biological and receptor binding activity (Arnheiter et al., 1981; Ackerman et al., 1984), IFN hybrid production (Weck et al., 1981; Rehberg et al., 1982; Weissmann et al., 1982; Horisberger and De Staritzky, 1987; Weber et al., 1987), cross-species IFN hybrid production (Raj et al., 1986, 1988), epitope mapping using monoclonal antibodies directed against IFN molecules or peptide fragments (Arnheiter et al., 1981, 1983; Lydon et al., 1985; Trown et al., 1985; Kontsek et al., 1989, 1990), truncations and alterations to the IFN sequence using total gene synthesis (Edge et al., 1986) and site-directed mutagenesis (Nisbet et al., 1985; Beilharz et al., 1988, 1991; Cheetham et al., 1991). Structure/function studies have aided in identifying and characterising the biologically important regions of the HuIFN-α molecule and the amino acid residues that are important for the in vitro activities. Fish et al. (1989) have implicated 3 major regions (amino acids 10–35, 78–107, 123–136) as being important in the active configuration of HuIFNs-α in vitro.

The present study has used the technique of site-directed mutagenesis to

create specific amino acid substitutions within MuIFN-α1. The extensive HuIFNs-α structure/function data were used as a guide to identifying the biologically important amino acid residues and regions in MuIFN-α1.

Materials and Methods

In vitro mutagenesis of IFN genes

The MuIFN-α1 genomic clone was a gift from Prof. Pitha-Rowe. Site-directed mutagenesis of the MuIFN-α1 gene and the expression of the mature protein has been described previously (Beilharz et al., 1991). The sequences of the 5 synthetic oligonucleotides used to direct specific amino acid substitutions in MuIFN-α1 are shown in Fig. 1. These oligonucleotides, prepared using an Applied Biosystems Model 380A DNA Synthesizer, were purified by electrophoresis on a 15% polyacrylamide gel prior to phosphorylation as described by Zoller and Smith (1983).

Site-directed mutagenesis and selection of mutated DNA by differential plaque hybridisation (Nisbet and Beilharz, 1985) was performed. The sequences of the modified clones were determined by dideoxy sequencing (Sequenase, Version II, U.S.B.).

OLIGONUCLEOTIDES DIRECTING ALTERATIONS IN MuIFN-α1

Numbered amino acids of MuIFN-α1 with corresponding codons shown base paired to oligonucleotides directing amino acid substitutions	Nomenclature for modified form
29 30 31 32 33 34 35 36 37 5' - TGC CTG AAG GAC AGG AAG GAC TTT GGA - 3' * 3' - CG GAC TTC CTG TAC TTC CTG AAA CC - 5'	Arg33Met
83 84 85 86 87 88 89 90 5' - GAC TCA TTC TGC AAT GAC CTC CAC - 3' * 3' - CTG AGT AAG AGG TTA CTG GAG G - 5'	Cys86Ser
120 121 122 123 124 125 126 5' - GTG AGG AAA TAC TTC CAC AGG - 3' * 3' - CAC TCC TTT AGG AAG GTG TCC - 5'	Tyr123Ser
67 68 69 70 71 72 73 74 75 76 5' - CTC TTC ACA TCA AAG GAC TCA TCT GCT GCT - 3' * * * * 3' - G AAG TGT AGT GAC GAG AGT AGA CGA CG - 5'	Lys71Leu,Asp72Leu
129 130 131 132 133 134 135 136 5' - GTG TAC CTG AGA GAG AAG AAA CAC - 3' * * * 3' - C ATG GAC TCT AAG TTC TTT GTG - 5'	Glu133Phe

Fig. 1. Partial nucleotide sequence of MuIFN-α1 (upper nucleotide sequence 5′ to 3′ of each panel) shown in codon triplets with the corresponding amino acid number indicated. The amino acids are numbered using the first cysteine of the mature protein as number 1. Aligned (base-paired) nucleotide sequences of the synthetic oligonucleotides are shown 3′ to 5′ for each sequence. The underlined bases direct the desired amino acid substitutions and asterisks show the number of mismatched bases for each annealing.

In vitro expression of MuIFN-α1 and its analogues

The 634-bp gene fragment of the wild-type MuIFN-α1 and its analogue Cys86Ser were subcloned into the *Bam*HI site of the expression vector pBM1 (Cheetham et al., 1991). All the other analogues of MuIFN-α1 were subcloned into the *Bam*HI site of the expression vector pGem3Zf(+) (Promega, U.S.A.) as described previously (Beilharz et al., 1991). DNA templates purified on cesium chloride gradients were linearized by restriction enzyme digestion downstream of the site of subcloning and subsequently transcribed using SP6 or T7 RNA polymerase as appropriate. The transcripts synthesized in vitro were translated in rabbit reticulocyte lysate (RRL), from Promega, U.S.A.; in the presence of ^{35}S-labelled methionine (specific activity 1000 Ci/mmol, NEN, Dupont, U.S.A.) as detailed in Tymms and McInnes (1988).

Gel electrophoresis of products of in vitro translation

^{35}S-labelled MuIFN-α1 and analogue samples of 5 μl each from the in vitro translation reactions were electrophoresed on a 12% SDS-PAGE gel. ^{35}S-labelled IFNs were visualised by soaking the gel in fluorography enhancer (Amplify; Amersham, U.K.) and then drying and exposing it to X-ray film at −80°C (Shoeman and Schweiger, 1982).

Biological assays

Antiviral (AV) assay

In vitro expressed IFNs were assessed for their AV activity using the cytopathic effect reduction assay (Jilbert et al., 1986) in which murine L929 cells were challenged with murine encephalomyocarditis virus (EMCV). The activities of the MuIFN-α1 and analogue preparations were titrated against a MuIFN-α reference standard at 4.5×10^5 IU/ml (Lee Biomolecular, U.S.A.). In order to check for the effect of variable ^{35}S incorporation into the IFN proteins (see Fig. 2), the bioassays were performed in two ways: (a) equal ^{35}S cpm from each preparation were used and the volume variations compensated for by the addition of RNA-free RRL; (b) equal volumes of each preparation were used and the activities were subsequently normalized on input ^{35}S cpm. In both cases the number of methionines in each protein was taken into account and results were the same with both approaches. The AV activity of each analogue was then expressed as a percentage of MuIFN-α1 activity, based on a minimum of 3 independent determinations.

Natural killer (NK) cell activation assays

Single cell suspensions from the spleens of 8-week-old C3H/HeJ mice, housed under minimal disease conditions, were prepared as previously described (Allan et al., 1982) and resuspended at 2×10^7 cells/ml in culture medium [RPMI-1640 (Gibco Laboratories, Grand Island, NY, U.S.A.)

supplemented with 40 mg/ml gentamicin, 100 U/ml penicillin, 2 mM L-glutamine, 50 μM 2-mercaptoethanol and 10% FCS]. One-ml volumes of cells were added to the wells of 24-well tissue culture trays (Costar, Cambridge, MA, U.S.A.) and an equal volume of IFN diluted in culture medium was added. After incubating for 4 h at 37°C in 10% CO_2 in air, the cells were recovered, washed three times with RPMI-1640 containing 20 mM Hepes and 2% FCS, and finally resuspended in culture medium. Viable cell counts were performed by trypan blue exclusion and the cell concentrations were adjusted to 1×10^7 cells/ml. NK cell activity of these IFN-activated spleen cells (effectors) was then assayed in a standard 4-h ^{51}Cr-release assay against YAC-1 cells (target cells), using 1×10^4 target cells and $0.125–1.0 \times 10^6$ effectors per well, each sample in triplicate (Dawkins and Shellam, 1979). Controls of media only and RRL only were included to indicate basal levels. Data are presented as % specific cytotoxicity, calculated from 4 independent assays as follows:

$$\% \text{ specific cytotoxicity} = \frac{\text{test cpm} - \text{spontaneous release cpm}}{\text{maximum release cpm} - \text{spontaneous release cpm}} \times 100$$

Maximum release cpm represents the radioactivity released from target cells incubated with 1% Triton X-100. Spontaneous release cpm is the background release of ^{51}Cr from target cells incubated in the absence of effectors. Test cpm represents ^{51}Cr release into wells containing effector and target cell populations. As was the case with the AV assays, appropriate normalisations based on both input cpm and equal RRL volumes were undertaken.

Results

IFN analogue generation

The MuIFN-α1 gene, subcloned into M13mp8, was used as a template for site-directed mutagenesis. A *Bam*HI site and an ATG start codon were created immediately 5′ to the first cysteine of the mature protein coding region, for the expression of mature IFN protein in vitro (Beilharz et al., 1991). The new *Bam*HI site facilitated the excision of the entire coding region since a 3′ *Bam*HI site was present as part of the M13mp8 polylinker region. This modified MuIFN-α1 wild type gene was then used as a template for directing amino acid substitutions at various points along the IFN molecule. The sequences of the oligonucleotides used to alter specific amino acids, and the sites of the substitutions on the MuIFN-α1 template are shown in Fig. 1. Successful mutagenesis and the fidelity of the entire IFN coding sequence was confirmed by dideoxy sequencing (data not shown).

Expression of MuIFN-α1 and analogues in vitro

Each of the correctly mutated M13mp8 clones was individually subcloned into pBM1 (MuIFN-α1 and Cys86Ser) or pGem3Zf(+) for all other clones as a 634-bp *Bam*HI fragment. Both pBM1 and pGem3Zf(+) are plasmid expression vectors for in vitro transcription which contain the promoters for SP6 and/or T7 RNA polymerase. The subclones, linearized at a site downstream to the *Bam*HI insertion site, were used as templates for in vitro transcription. Radiochemically pure IFN was produced by adding the in vitro synthesized transcripts to a cell-free protein synthesizing system comprising methionine-depleted RRL and ^{35}S-methionine. The interferons produced using this system were radiolabelled and the ^{35}S-methionine incorporated was measured as trichloroacetic acid-insoluble material. The integrity and radiochemical purity of the radiolabelled IFNs was assessed by polyacrylamide gel electrophoresis (Fig. 2). A single radioactive band in the region of 17–20 kDa was seen for each of the proteins (Fig. 2, lanes 1, 2, 5–8), whilst no bands were

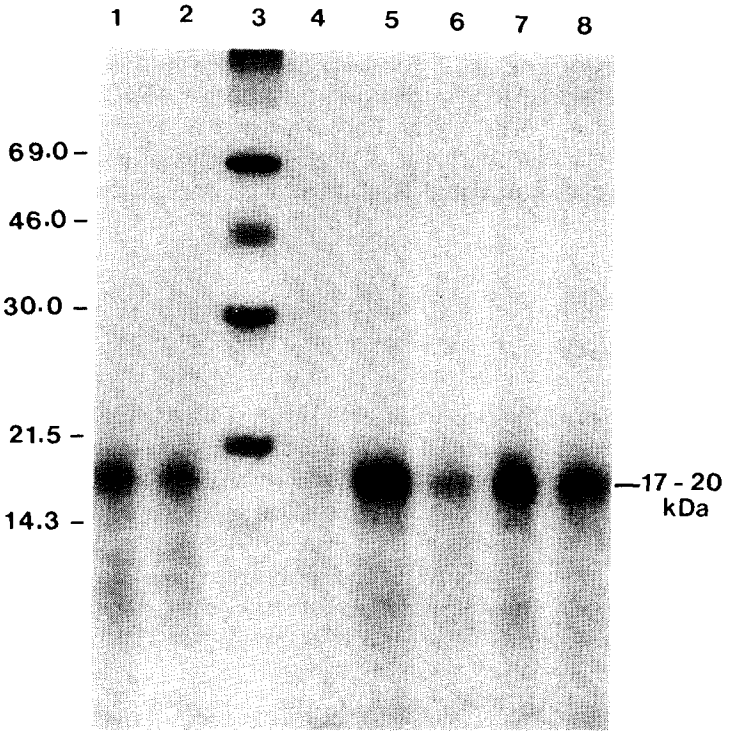

Fig. 2. Fluorograph of a 12% SDS-PAGE gel showing the in vitro expressed proteins of MuIFN-α1 (lane 5) and the analogues Cys86Ser (lane 1), Tyr123Ser (lane 2), Arg33Met (lane 6), Lys71Leu,Asp72Leu (lane 7) and Glu133Phe (lane 8). 5 μl of each respective translation mix was loaded into each lane. A radioactively labelled size standard (lane 3) and a 5-μl RRL/no RNA control (lane 4) were also included. All proteins are ^{35}S-labelled and fluorographically detected.

TABLE 1

The relative antiviral and natural killer-cell activities by MuIFN-α1 and its analogues

MuIFN-α1 and analogues	Relative antiviral activities on murine cells (L929) (%)	Relative natural killer cell activation at effector: target cell ratio of 25:1 (%)
MuIFN-α1	100	100
Glu133Phe	31 ± 8.7	66 ± 14
Cys86Ser	15 ± 6	40.8 ± 2
Lys71Leu,Asp72Leu	7.9 ± 4.3	26.7 ± 5.6
Tyr123Ser	0.9 ± 0.5	0
Arg33Met	0	0

Results shown are the means of the percentages of the activity of MuIFN-α1 ± standard deviation of the mean, from 4 determinations. The activity of MuIFN-α1 has been set at 100%.

observed for the control incubation in the absence of added RNA (Fig. 2, lane 4).

Antiviral activity of MuIFN-α1 and analogues

RRL containing the IFNs synthesized in vitro was added directly to mouse L929 cell monolayers and the IFNs were tested for their ability to protect the cells against an EMCV challenge. The AV activity of MuIFN-α1 and the 5 analogues on mouse cells is presented in Table 1. All the amino acid substitutions resulted in IFNs with lowered AV activity ranging from 0-30% of MuIFN-α1.

NK cell activation of MuIFN-α1 and analogues

The NK cell activity of the IFN-activated effectors against ^{51}Cr-labelled target cells at various effector:target cell ratios was assessed. The NK-cell activities of the IFNs at effector:target cell ratio of 25:1, expressed as a percentage of MuIFN-α1 activity, are presented in Table 1. All the analogues had lowered abilities to activate NK-cells, with the Tyr123Ser and Arg33Met analogues being indistinguishable from the background control of RRL.

Discussion

Based on previous HuIFN-α studies, selected residues along the MuIFN-α1 molecule were altered to assess their role in MuIFN-α activity. The results are in good agreement with previous structure/function studies summarized in Table 2 and correlate well with the known three-dimensional structure of MuIFN-β (Senda et al., 1990). Three regions corresponding to those noted for HuIFNs-α1 are implicated in determining the biological activities of MuIFN-α1. The first is the region around position 33 which is within the domain (between amino acids 10 and 33) defined by Fish et al. (1989) for HuIFNs-α.

TABLE 2

The relative antiviral activities of IFN-α analogues

Amino acid substitution in analogue	Parental IFN-α species	Parental antiviral activity of analogue (%)	Reference
Arg33Ala	HuIFN-α2	0.04	Camble et al. (1986)
Arg33Lys	HuIFN-α2	0.15	Camble et al. (1986)
Arg33Met	HuIFN-α2	0.001	Camble et al. (1986)
Arg33Glu	HuIFN-α4	<0.1	Tymms et al. (1989)
Arg33Lys	HuIFN-α4	<0.1	Tymms et al. (1989)
Arg33Glu	MuIFN-α1	<0.03	Kerry et al. (1988)
Tyr123Trp	HuIFN-α1	40.0	McInnes et al. (1989)
Tyr123Phe	HuIFN-α1	2.0	McInnes et al. (1989)
Tyr123Lys	HuIFN-α1	3.0	McInnes et al. (1989)
Tyr123Asp	HuIFN-α1	4.0	McInnes et al. (1989)
Tyr123Ser	HuIFN-α1	15.0	McInnes et al. (1989)
Tyr123Ala	HuIFN-α1	9.0	McInnes et al. (1989)
Tyr123Gly	HuIFN-α1	<1.8	McInnes et al. (1989)
Tyr123Phe	MuIFN-α1	8.0	Kerry et al. (1988)
Tyr123Ser	MuIFN-α1	0.3	Kerry et al. (1988)
Glu133Lys	HuIFN-α4	143.0	Cheetham et al. (1991)
Cys86Ser	HuIFN-α1	39.0	Beilharz et al. (1988)
Ser86Cys	HuIFN-α4	91.0	Cheetham et al. (1991)
Cys86Ser	MuIFN-α1	23.0	Kerry et al. (1988)

The arginine residue at position 33 is a highly conserved residue and has previously been considered to be an absolute requirement for HuIFNs-α activity. The previous amino acid substitutions performed at this residue and their AV effects are summarized in Table 2. This residue is of equal functional importance in maintaining MuIFN-α1 activity, since the Met substitution at position 33 reported in the present study also abrogates AV activity. The same strong effect was previously reported for a Glu substitution at position 33 of MuIFN-α1 (Kerry et al., 1988).

The second functionally important region identified is the region around Tyr123 and Glu133; this again corresponds to a HuIFN-α domain (between residues 123 and 136) noted by Fish et al. (1989). Previous amino acid substitutions at these positions are also summarized in Table 2. The HuIFN-α studies indicated the involvement of this Tyr residue and the region around this residue in maintaining the structure of HuIFNs-α in a form suitable for binding to the receptors. The inference also holds true for MuIFN-α1 as the Ser substitution at 123 results in significantly reduced AV activity. Interestingly, the conservative Phe substitution (see Table 2) in MuIFN-α1 at position 123 abrogates over 90% of the AV activity indicating subtle differences between MuIFNs-α and HuIFNs-α with respect to this residue's function. The importance of this region for MuIFNs-α is further supported by the studies of Weber et al. (1987) where the residues 121, 125 and 132 were implicated in the binding of HuIFN-α2 to the murine receptor.

Kontsek et al. (1989, 1990) used synthetic peptides to indicate that the region

spanning the residues 132–137 was involved with the binding of HuIFN-α2 and HuIFN-β to their common receptor. The present study has shown that substituting Glu133 with Phe133 reduces the AV activity of MuIFN-α1. The extent of the reduction is much less than that observed for substitution of Tyr123. Glu133 and the residues immediately around it in MuIFN-α1 may not be directly involved with binding to its receptor but may influence the affinity of binding by a distal conformational effect. Altered affinity for the receptor may also account for the slightly enhanced AV activity of HuIFN-α4 on mouse cells following a Glu133Lys substitution (Table 2).

The observations concerning the regions around amino acids 33 and 123 are also in agreement with the recently reported three-dimensional structure of MuIFN-β, a type I IFN related to the MuIFNs-α (Senda et al., 1990). In this study, high resolution X-ray diffraction data showed the regions around Arg33 and Tyr123 to be spatially juxtaposed on a protruding edge of the crystal structure (Y. Mitsui, pers. comm.). Specifically, the AB loop (amino acids 30–40) forms this structure at an extremity of the molecule together with part of the loop DE (amino acids 120–140). Taken together with the murine structure/function data summarized in the present report, it would appear highly likely that these two juxtaposed regions form the receptor binding domain.

A third region of functional significance in MuIFN-α1, corresponding to the 78–107 amino acid domain of HuIFNs-α (Fish et al., 1989), is apparent in the present study. Substitutions at residues 71/72 and 86 resulted in significant drops in the AV activity of MuIFN-α1. Comparable substitutions in the HuIFNs-α have been performed only for position 86 of HuIFN-α1 and HuIFN-α4 as indicated in Table 2. In both human cases, a relatively small decrease in AV activity was reported. The functional importance of this region was also observed in a MuIFN-α hybrid study by Trapman et al. (1988). It is more difficult, however, to interpret the role of this region in terms of its spatial relationship to the probable receptor binding site discussed above. Residues 71/72 and 86 are quite distal to the juxtaposed and protruding 33 and 123 regions. It is possible that conformational changes induced by the initial receptor binding event may bring this distal region into contact with functional epitopes on the receptor, but a structural effect on the whole IFN-α molecule cannot be ruled out.

The ability of natural and recombinant IFN-α species to augment NK cell activity has previously been demonstrated (Gidlund et al., 1979; Ortaldo et al., 1980, 1983). Data presented here demonstrate that MuIFN-α1 has the ability to augment NK cell activity and that substitution of residues along the MuIFN-α1 molecule has resulted in a lowering of NK cell activity from 66% in Glu133Phe to a total loss of measurable NK cell activity in Arg33Met and Tyr123Ser. The trend in the lowering of NK cell activity by the various amino acid substitutions is similar to that seen in AV activity (Table 1). As both NK cell and AV activities are thought to be mediated through the same receptor, the parallel trends in these activities suggest that receptor binding is being effected in the analogues studied.

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