Full-length article

Antagonist peptides of human interferon- α 2b isolated from phage display library inhibit interferon induced antiviral activityWang TIAN¹, Gang BAI^{2,3}, Zheng-he LI², Wen-bo YANG²¹Department of Microbiology, College of Life Sciences, Nankai University, Tianjin 300071, China; ²College of Life Sciences, Nankai University, Tianjin 300071, China**Key words**interferon- α 2b; antagonist peptide; phage display; type I IFN receptor; cell selection³ Correspondence to Prof Gang BAI.
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Abstract

Aim: To screen human interferon (IFN)- α 2b antagonist peptides from a phage displayed heptapeptide library. **Methods:** WISH cells and polyclonal anti-IFN- α 2b antibodies were used to select IFN receptor-binding peptides from a phage displayed heptapeptide library. The specific binding of phage clones was examined by phage ELISA and immunohistochemistry. The specific binding activities of synthetic peptides to WISH cells were detected by competition assay. Effects of synthetic peptides to IFN-induced antiviral activity were analyzed by evaluating the cytopathic effect (CPE) using the MTT method. **Results:** Twenty-three positive clones were obtained after seven rounds of selection. Ten clones were randomly picked from the positive clones and were sequenced. The corresponding amino acid sequences suggested 3 groups homologous to the 3 domains of IFN- α 2b, defined by residues 24–41, 43–49, and 148–158 of IFN- α 2b. As they presented as corresponding to IFN receptor-binding domains, AB loop and E helix, clone No 26 and 35 were chosen for further characterization and shown to bind to WISH cells. Two peptides corresponding to clone No 26 and 35, designated SP-7(SLSPGLP) and FY-7(FSAPVRY) were shown to compete with GFP-IFN- α 2b for binding to its receptor and to inhibit the IFN- α 2b-induced antiviral activity. **Conclusion:** Both IFN- α 2b antagonist peptides, SP-7 and FY-7, were able to inhibit the IFN-induced antiviral activity, and could be helpful in laying the foundation for the molecular mechanism of the interaction between IFN and its receptor.

Introduction

Human type I interferons (IFN), including IFN- α , - β , - τ and - ω , are cytokines best known for their antiviral activities^[1]. IFN binding to its receptor triggers a cascade of events, activating a number of proteins that inhibit viral replication and cell growth and control apoptosis^[2]. IFN- α and - β bind to a common cell-surface receptor comprised of IFNAR1 and IFNAR2. IFNAR2 is the major ligand binding component of the receptor complex, exhibiting nanomolar affinity to both IFN- α and - β subtypes^[2]. The IFN- α / β signal is transduced by the induction of specific intracellular responses mediated by tyrosine phosphorylation of janus kinases (JAK). The

activation of these kinases induces phosphorylation of the cytoplasmic tail of the receptor itself, which lacks intrinsic kinase activity. The subsequent phosphorylation of signal transducer and activator of transcription (STAT) factors and activation of the transcriptional activator IFN- α -stimulated gene factor3 (ISGF3), resulting in the induction of an antiviral state^[1].

Suppression of any one of these steps would inhibit the IFN-induced antiviral activity. So far, several interferon antagonists have been identified including the Newcastle disease virus V protein^[3], which blocks the antiviral functions of IFN- α by targeting STAT1 for degradation. Another example is the sarcolectin^[4], an interferon antagonist extracted

from hamster sarcomas and normal muscles, which has no direct action on IFN molecules and does not compete with it for specific cell membrane receptors, and could act by triggering or enhancing the synthesis of an IFN regulatory protein, which restores virus sensitivity in the cells. The vasoactive intestinal peptide^[5] is another interferon antagonist, which inhibits Jak1-2 and STAT1 phosphorylation, resulting in the downregulation of IFN-induced gene expression. Not only do the antagonists block the IFN intracellular signaling but they also suppress IFN binding to its receptor, such as neutralizing antibodies. It has been reported that some patients treated with IFN have produced neutralizing antibodies inhibiting the antiviral activity of IFN^[6]. Previous studies have shown that IFN- α was able to induce the expression of MHC (major histocompatibility complex) class I antigens, and II antigens, both of which had been linked genetically and functionally to autoimmune diseases. There appears to be a correlation between these diseases and increased expression of IFN- α . Thus, IFN- α antagonists may be therapeutic candidates for autoimmune diseases^[7,8].

The screening of phage-displayed libraries is a powerful technique for identifying peptides with desirable biological or physical properties, particularly when it is combined with iterative cycles of phage selection and amplification^[9]. New agonists and antagonists of cell membrane receptors have been successfully identified using this process^[10], and examples include the RGD(Arg-Gly-Asp)-containing peptides that bind to specific cell surface integrins and inhibit integrin-mediated cell adhesion^[11,12]. Peptide display libraries have also been used to derive a peptide-(ATWLPPR), which specifically inhibited human endothelial cell proliferation *in vitro* and totally abolished VEGF(vascular endothelial growth factor) induced angiogenesis *in vivo*^[13]. Thus, phage-displayed technology has been shown to be effective to the identification of novel peptides that may inhibit cell adhesion.

In this study, we attempted to identify IFN- α 2b antagonist peptides that might block the binding of IFN- α 2b to its receptor and be helpful in laying the foundation for the molecular mechanism of the interaction between IFN and its receptor.

Materials and methods

Cell lines WISH cells and VSV(vesicular stomatitis virus) viruses were kindly provided by Hualida Co (Tianjin, China). The WISH cells, naturally carrying the type I IFN receptors on the cell surface, were cultured in RPMI-1640 (Roswell Park Memorial Institute) medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 1×10^5 U/L of penicillin

and 0.1 g/L of streptomycin.

Phage peptide library and bacterial strain The heptapeptide library [1.5×10^{16} plaque forming units (pfu)/L] and the host bacterial strain *E coli* ER2738 were purchased from New England Biolabs (Beverly, MA, USA).

Interferon, antibodies and other reagents Standard IFN- α 2b was kindly provided by Hualida and had a specific activity of 1.0×10^{11} U/g protein. Anti-IFN- α 2b antibodies were prepared as outlined by Tian *et al*^[14]. Wild-type M13 phage and horseradish peroxidase (HRP)-conjugated anti-M13 phage antibody were purchased from Pharmacia Biotech (Uppsala, Sweden). Vector pET32a(+) was purchased from Novagen Co LTD (Darmstadt, Germany). Vector pEGFPCII containing cDNA coding green fluorescence protein (GFP) was a kind gift from Prof Xiao-dong ZHANG (College of Life Sciences, Nankai University, Tianjin, China). O-Phenylenediamine (OPD) and diaminobenzidine (DAB) were purchased from Sigma (St Louis, MO, USA). Other chemicals used in this study were of analytical grade and commercially available.

Phage selection with WISH cells and polyclonal anti-IFN- α 2b antibodies Selection procedure was based on the ph.D-7 kit standard procedure (New England Biolabs, Beverly, MA) with some modifications. The exponentially growing WISH cells were fixed on 96-well culture plates ($\sim 2.0 \times 10^5$ cells/well; Nunc, Roskilde, Denmark) with glutaraldehyde (0.25% final concentration). Phages of approximately 2.0×10^{10} pfu were preincubated for 1 h with blocking buffer [5 g/L bovine serum albumin (BSA), 0.1 mol/L NaHCO₃] and then were transferred to the 96-well culture plate and incubated for 2 h at room temperature. Unbound phage particles were removed by washing with 0.1% TBST (0.05 mol/L Tris-HCl, pH 7.5, 0.15 mol/L NaCl, 0.1% Tween 20). Cell-bound phages were eluted for 10 min with 0.001 g/L IFN- α 2b. The eluted phages were replicated by infecting *E coli* ER2738 cells. The amplified phage particles were purified using polyethyleneglycol (PEG), and then used for the subsequent round of selection with WISH cells.

For the selection with antibodies, all the steps were the same as described for the selection with WISH cells except for the fact that the 96-well plates were coated with polyclonal anti-IFN- α 2b antibodies (0.01 g/L in 0.1 mol/L NaHCO₃, pH 8.6).

Phage ELISA WISH cells naturally carrying type I IFN receptor on the cell surfaces were used to select IFN receptor-binding peptides from a phage displayed library that was described as phage ELISAA method. The exponentially growing WISH cells were fixed on 96-well culture plates ($\sim 2.0 \times 10^5$ cells/well) with glutaraldehyde (0.25% final concentration).

Approximately 2×10^{11} pfu amplified phages (or mixed with 0.01 g/L IFN- $\alpha 2b$) were added to each well, and then incubated with the cells for 2 h at room temperature. The wells were treated with five 3-min washes with 0.1% TBST and the amount of bound phages was detected with horseradish peroxidase (HRP)-conjugated anti-M13 phage antibody. The development was performed by the addition of OPD, and read at 490 nm in an ELISA Reader (Bio Rad). Original phage peptide library without selection was used as the negative control.

For detecting the binding reactivity of phage clones to antibodies with phage ELISA B method, all the steps were the same as the phage ELISA A method except that the 96-well plates were coated with polyclonal anti-IFN- $\alpha 2b$ antibodies (0.01 g/L in 0.1 mol/L NaHCO_3 , pH 8.6).

Immunohistochemistry Exponentially growing WISH cells ($\sim 2.0 \times 10^5$ cells/well) were fixed on 96-well culture plates using glutaraldehyde and incubated with detective phage clones (about 1.0×10^{10} pfu) for 2 h at room temperature. The cells were washed five times with PBS and incubated with HRP-conjugated anti-M13 phage antibody at 37 °C for 1 h. After washing five times with PBS, the wells were developed with DAB until brown. The DAB excess was washed away with water and photographed with an inverted microscope and digital camera.

Peptides sequencing and synthesis The single-stranded DNA (ssDNA) was prepared from identified phage clones as described in the pH D-7 kit guidelines and sequenced by the Shanghai Sangon Company. The primer used for sequencing was -96pIII: 5'-CCCTCATAGTTAGCGTAACG-3'. Corresponding amino acid sequences were deduced from DNA sequences, and a multiple sequence alignment was done using BLAST software package (obtained from <http://ncbi.nlm.nih.gov/BLAST>) to determine the groups of related peptides. Two peptides corresponding to positive clones No 26 and 35 were synthesized chemically by the GL Biochem (Shanghai, China), designated as SP-7 (SLSPGLP) and FY-7 (FSAPVRY), and then used for characterization analyses.

Cloning, expression and purification of GFP-IFN- $\alpha 2b$ fusion protein This procedure has been described in a previous studies^[15]. Briefly, the cDNA coding human IFN- $\alpha 2b$ was obtained from peripheral blood mononuclear cells (PBMC) by reverse transcription-PCR and was then cloned into expression vector pET32a(+) to construct pET32a(+)/IFN- $\alpha 2b$. The cDNA coding GFP was digested from pEGFPCII and was cloned into pET32a(+)/IFN- $\alpha 2b$. Thus, the prokaryotic expression vector pET32a (+)/GFP-IFN- $\alpha 2b$ was constructed and then transformed to *E coli* BL21. The expressed

fusion protein after induction with isopropyl-beta-D-thiogalactopyranoside (IPTG) was purified by nickel chelation chromatography.

Competition assay of synthesized peptides Exponentially growing WISH cells were fixed approximately 2.0×10^5 cells per well on 96-well culture plates using glutaraldehyde. After blocking with 0.06 mol/L phosphate buffer (pH 7.4, containing 1% BSA and 0.1% NaN_3) for 1 h at room temperature, 1 μg GFP-IFN- $\alpha 2b$ was added to each well and incubated at 37 °C for 2 h. After washing five times with 0.1% TBST, synthetic peptides or IFN- $\alpha 2b$ were added and incubated at 37 °C for 1 h with gentle shaking. The wells were then washed with 0.1% TBST again and observed with an inverted fluorescence microscope. Irrelative peptide 50 μg , (Lysine)₇, was used as a negative control.

Inhibition assay of antiviral activity The antiviral activity of IFN was determined *in vitro* by protection of human amnion WISH cells against VSV-induced cytopathic effects as described by following the traditional method^[16]. In brief, 2.0×10^4 cells were seeded into each well of 96 well plates and incubated with two-fold serial dilutions of synthetic peptides samples [or (Lysine)₇ as a negative control] and IFN- $\alpha 2b$ giving 50% protection of WISH cells for 18 h at 37 °C. After incubation, the cells were challenged with VSV and the plates were incubated at 37 °C for 24 h. Virus-induced cytopathic effects were assayed by the MTT method^[17].

Results

Specific enrichment of positive phages In order to enrich IFNAR-binding phages from the phage display library, four rounds of selection with WISH cells were performed. The enrichment was determined by the use of the output/input ratio of phages after each round of selection. The ratio increased approximately 78-fold (from 4.5×10^{-7} to 3.5×10^{-5}) after the second round of selection. After the third and the fourth rounds of selection, the output/input ratio of phages increased approximately 710-fold and 3700-fold respectively. When the library was screened with polyclonal antibodies, the output/input ratio of phages increased about 5-fold and 70-fold after the sixth and seventh rounds of selection, which indicates an obvious enrichment for the specific binding of phages to IFNAR-expressing cells and polyclonal antibodies against IFN- $\alpha 2b$ (Table 1).

Identification of the positive phages Ninety six clones were picked out from the sample after the fourth round of selection with whole cells and the specificity was examined by phage ELISA A. Thirty-four of the 96 clones (35.4%) showed a binding ability to WISH cells, while the positive rate increased to 40/96 (41.6%) after the seventh round of

Table 1. Enrichment of phages for each round of selection from phage displayed heptapeptide library

Rounds		Selected phages (pfu) (input)	Eluted phages (pfu) (output)	Ratio (output/input)
Cell selection	1	3.0×10^{10}	1.4×10^4	4.5×10^{-7}
	2	1.6×10^{10}	5.6×10^5	3.5×10^{-5}
	3	2.0×10^{10}	6.4×10^6	3.2×10^{-4}
	4	2.3×10^{10}	4.4×10^7	3.7×10^{-3}
Antibody selection	5	2.0×10^{10}	6.8×10^4	3.4×10^{-6}
	6	2.5×10^{10}	4.5×10^5	1.8×10^{-5}
	7	2.2×10^{10}	5.3×10^6	2.4×10^{-4}

pfu, plaque forming units.

selection with antibodies. In contrast, the original phage library cannot bind to WISH cells. Further testing demonstrated that 23 out of the 40 positive clones could be specifically competed by 1 μ g IFN- α 2b in the binding to WISH cells and anti-IFN antibodies using phage ELISA A and B method (Figure 1). These results indicated the enrichment for the specific binding of phages to IFNAR-carrying cells and polyclonal antibodies against IFN- α 2b.

Analyses of exogenous sequences of positive phage clones The ssDNA was prepared from positive phage clones and sequenced, and the amino acid sequences of the mimetic peptides were then deduced from DNA sequences. Homologous analysis was performed to find an optimal alignment between the selected motifs and the primary sequence of IFN- α 2b. The homologous analysis of amino acid se-

quences with IFN- α 2b showed that 3 groups were obtained, which corresponds to three domains defined by residues (group I, 24–41; group II, 43–49; and group III, 148–158) of IFN- α 2b (Table 2). It had been proposed that the AB loop (residues 26–35) and E helix (residues 144–153) are implicated in the interaction with receptors^[18]. By comparing the homology with functional domain amino acid sequences of IFN- α 2b and the absorbance at 490 nm, two clones No 26 in group I and No 35 in group III were selected for further testing.

Specificity of positive phage clones to IFNAR The inter-

Table 2. Exogenous amino acid sequences of the heptapeptides in pIII coat proteins of positive phage clones selected from the peptide library

Group	Clone	Sequence	Homologous sequences set
I	41	ASYPPRL	24–30
	25	ISYHDLR	24–30
	26	SLSPGLP	25–31
	29	STSFTPH	28–34
	36	MADRIGT	31–37
	38	FAGVPSW	35–41
	IFN	ISLFSCLKDRHDFGFPQE	24–41
II	33	LNNQFWY	43–49
	22	TPGQYWA	43–49
	IFN	FGNQFQK	43–49
III	39	YTRPLPT	149–154
	35	FSAPVRY	152–158
	IFN	MRSFSLSTNLQ	148–158

Like this: IFN MRSFSLSTNLQ

Figure 1. Specific binding of 23 selected phage clones to WISH cells and anti-IFN antibodies. Phage clones binding to WISH cells and antibodies were detected by horseradish peroxidase-conjugated anti-M13 phage antibody in the presence or absence of IFN- α 2b. The original phage peptide library without selection, represented as No 0, was used as a negative control. Average values from three independent experiments are shown.

action of the positive phages and WISH cells was detected by immunohistochemical staining. As a result, dark brown staining on the cell surfaces indicated that positive phage clones (No 26 and 35) could bind to WISH cells (Figure 2B, 2E). In contrast, fewer positive staining was observed in the presence of 1 μ g IFN- α 2b (Figure 2C, 2F). Primary phage peptide library without selection and irrelevant phage clone were used as the negative control, showing no binding to WISH cells (Figure 2A, 2D).

Inhibition reactivity of synthetic peptides to IFNAR The ability of the synthetic peptides, SP-7 and FY-7 to inhibit the

Figure 2. Immunohistochemical staining of WISH cells bound with the positive phage clone 26 and 35. Cell-bound phages were detected using horseradish peroxidase-conjugated anti-M13 phage. The positive phages (No 26 and 35) showed binding to WISH cells (B,E), fewer positive staining was observed in the presence of IFN- α 2b (C, F). The primary phage peptide library without selection and irrelevant phage clone showed no binding to WISH cells (A,D) ($\times 100$).

binding of GFP-IFN- α 2b to IFNAR was determined by competitive assay. The fluorescence reduction was almost not observed when 1 μ g synthetic peptides (Figure 3E, 3G) added, while the fluorescence was obviously reduced or obsolescent in the conditions of 10 μ g synthetic peptides (Figure 3F, 3H), equaling approximately 1 μ g IFN- α 2b (Figure 3C). This result revealed that the binding of IFN- α 2b to WISH cells was inhibited by the two synthetic peptides.

Inhibition of IFN-induced antiviral activity by synthesis peptides It is well known that the binding of IFN to its receptor mediates the activation of different signal transduction pathways. Because peptides SP-7 and FY-7 have the ability to block the interaction between IFN and its receptor, we decided to test whether they might also suppress the antiviral activity mediated by IFN. As a result, when the added amount was from 6.25 μ g to 100 μ g, both mimetic peptides could inhibit the IFN-induced antiviral activity in a dose-dependent manner. The IC_{50} values of both peptides were approximately 25 μ g (Figure 4).

Discussion

There is a growing interest in the development of active mimetic or synthetic peptides for IFN. For instance, a synthetic peptide (WLDPRH) was recognized by a neutralizing antibody, in the presence of which, the protective effects of suboptimal dose of IFN- α were increased^[19]. Another example is the peptide mimetic of IFN- β isolated by phage-display screening also using a neutralizing monoclonal antibody (mAb), which elicits antiviral activity on cultured cells^[20]. In contrast, there are no reports of the isolation of synthetic

Figure 3. Competitive binding inhibition between GFP-IFN- α 2b and WISH cells by synthetic peptides. WISH cells were fixed on 96-well culture plates using glutaraldehyde. The cell-bound GFP-IFN- α 2b was detected by fluorescence microscope (B). The fluorescence reduction was not obvious when 1 μ g SP-7 (E) or FY-7 (G) were added, while the fluorescence was reduced or obsolescent when 1 μ g IFN (C), 10 μ g SP-7 (F) or FY-7 (H) were added. The strong fluorescence was observed in the conditions of irrelevant peptide (D). No fluorescence was detected for the normal WISH cells with GFP protein (A) ($\times 200$).

Figure 4. Inhibition of IFN-induced antiviral activity by synthetic peptides. WISH cells prepared in a 96-well plate were incubated with 2.5×10^3 IU/L standard IFN- $\alpha 2b$ and a series of 2-fold dilutions of each peptide for 24 h. The cells were exposed to VSV and incubated for 24 h. MTT 100 μ L (5 g/L) was then added to the cultures and incubated for 4 h. Both SP-7 and FY-7 showed the capability of inhibiting the IFN-mediated antiviral activity in a dose-dependent manner. $n=5$. Mean $\pm$ SD. (Lysine)₇ was used as a negative control.

peptides for IFN that inhibit the antiviral activity as antagonists. We report here the identification of two IFN antagonist peptides by screening a phage-displayed peptide library.

To obtain peptides blocking the binding of IFN to its receptor, screening of a phage-display library with IFN receptor should be a straightforward strategy. However, there are some difficulties for membrane receptors to be purified and maintain the natural conformation. Previous studies have shown that screening with a soluble form of type I IFN receptor failed to identify any peptide ligands, while the use of a neutralizing anti-IFN- β mAb as a target resulted in the successful mimicry of IFN- β ^[20]. It is better to use whole native cells or target receptor gene transfected cells to select the ligands^[13,21]. Whole cells usually make receptors maintain the native conformation with normal post-translational modification, so the ligands of the receptor can be selected even without information about the receptor^[22].

In this study, we performed four rounds of biopanning against WISH cells carrying IFN receptors on the cell membranes. This resulted in the enrichment of IFN-receptor-specific binding clones from the phage library. The second step was then subjected for three rounds of selection with polyclonal anti-IFN antibodies, which resulted in the enrichment of phage clones binding both to IFN receptors and anti-IFN antibodies. Compared with screening only using monoclonal antibody, this strategy has the advantage of allowing us to obtain different epitopes of IFN simultaneously. The phage ELISA and immunohistochemistry staining showed that positive clones could bind specifically

both to WISH cells and anti-IFN antibodies.

Ten out of the 23 positive clones were randomly picked out and sequenced. The results revealed that these sequences were divided into 3 groups, which correspond to three domains defined by residues of IFN- $\alpha 2b$ (group I, 24–41; group II, 43–49; and group III, 148–158) (Table 2). It has been recently determined by X-ray crystallography that the IFN- $\alpha 2b$ molecule is composed of five α -helices (A–E) linked by one long connection (AB loop) and three short segments (BC, CD and DE loops)^[23]. Mutational studies have revealed the mutual binding sites on IFN- $\alpha 2$. On IFN- $\alpha 2$, IFNAR2 binds to the A helix (residues 12–15), the AB loop (residues 26–35), and the E helix (residues 144–153)^[19,24], which induced antiviral activity. Therefore, it could be possible that peptides of group I are a structural mimic of discontinuous or conformational epitopes of the IFN AB loop, and those of group III might be E helix.

Clone No 26 of group I and clone No 35 of group III were selected for further analysis. They were synthesized chemically, and designated as SP-7 and FY-7. The two peptides were not only capable of specific binding to IFN receptor (Figure 3) but also inhibited the IFN-induced antiviral activity in a dose-dependent manner (Figure 4). However, no additive effect on inhibition of IFN-induced antiviral activity was observed when both peptides were added simultaneously.

Research has shown that the increased expression of IFN- α correlated with several autoimmune diseases such as insulin-dependent diabetes mellitus (IDDM) and systemic lupus erythematosus (SLE), but IFN antagonists could be candidates for treatment of IFN-induced diseases^[25]. In this paper, we demonstrated that two IFN antagonist peptides, SP-7 and FY-7, which can compete with IFN for binding to its receptors, inhibit IFN-induced antiviral activity. We hope they might also partly reduce IFN-induced autoimmune diseases or side effects.

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