A peptide mimetic of human interferon (IFN)- β

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Type I interferons (IFNs) are cytokines that are used clinically as antiviral and antitumour agents. The interaction of IFNs with their heterodimeric type I IFN receptor comprised of IFNAR1 and IFNAR2 is a first step to inducing biological actions. Here, we describe the successful mimicry of IFN- β by a peptide isolated by phage-display screening using a neutralizing anti-IFN- β monoclonal antibody. The 15-mer peptide, designated SYR6, was shown to compete with IFN- β for binding to type I IFN receptor in a concentration-dependent manner, and was shown to elicit antiviral activity on cultured cells. This antiviral activity

was not eliminated in the presence of neutralizing monoclonal antibodies to IFN- α , - β and - γ , and a low concentration of soluble type I IFN receptor, suggesting that it was not due to IFN contamination or the induction of endogenous IFNs by SYR6. This peptide might be a potent agonist to provide a mechanism of activating heterodimeric cytokine receptors.

Key words: cytokine, heterodimeric receptor, interferon, mimetic, neutralizing monoclonal antibody, phage display.

INTRODUCTION

Human type I interferons (IFNs), including IFN- α , - β , - τ and - ω , are cytokines best known for their antiviral activities [1]. Human IFN- α and - β have been used clinically as antiviral agents, especially against hepatitis B and C [2]. Mature IFN proteins consist of 166 amino acids, except for IFN- α 2 which consists of 165 amino acids [3]. Despite low amino acid identity ($\approx 25\%$), IFN- α and - β bind to a common cell-surface heterodimeric receptor comprised of IFNAR1 and IFNAR2 [4]. The IFN- α/β signal is transduced by the induction of specific intracellular responses mediated by tyrosine phosphorylation of Janus kinases ('JAKs'). The subsequent phosphorylation of STAT (signal transducers and activators of transcription) factors and activation of the transcriptional activator ISGF3 (IFN- α -stimulated gene factor 3) results in the induction of an antiviral state [5].

In general, proteins are not suitable drug candidates for a number of reasons, including antigenicity, susceptibility to proteolytic degeneration and high cost [6]. Clinically available protein-based drugs such as IFNs also have the drawback that they must be administered parenterally, e.g. by subcutaneous, intramuscular or intravenous injection. Thus there is a growing interest in the development of orally active mimetics for protein drugs. Recently, the discovery of small peptidic and non-peptidic agonists for cytokine receptors, for instance for erythropoietin ('EPO') [7], granulocyte colony-stimulating factor ('G-CSF') [8] and thrombopoietin ('TPO') [9–11], and receptor tyrosine kinases, such as fibroblast growth factor ('FGF') [12] and insulin [13], were reported. The mechanism by which these mimetics activate target receptors is believed to be through the induction of receptor homodimerization [14,15]. However, there have been no reports of the isolation of mimetics for heterodimeric cytokine receptors, and it has been questioned whether it is possible for small-molecule mimetics to activate heterodimeric cytokine receptors.

The phage-display approach has been successfully applied to the identification of peptide mimetic ligands in the absence of structural information on the interacting surfaces of the target molecule [16]. In order to obtain peptide mimetics for IFN, we screened a random phage display library with a soluble form of the human type I IFN receptor (sIFNR) and a neutralizing monoclonal antibody (mAb) against IFN- β . Whereas screening with sIFNR failed to identify any peptide ligands, the use of a neutralizing anti-IFN- β mAb as a target for phage-display screening resulted in the successful mimicry of IFN- β .

EXPERIMENTAL

Solid-phase ligand-binding assay

sIFNR was prepared as a chimaeric protein with the Fc region of human IgG₁. cDNAs encoding the extracellular domain (ECD) of IFNAR1 (amino acids 1–436) and IFNAR2 (amino acids 1–243) were cloned separately. sIFNR comprising the ECDs of both IFNAR1 and IFNAR2 (hIFNAR1AR2Fc) was expressed in COS-1 cells by transient co-transfection using DEAE-Dextran. To prepare sIFNR comprising the IFNAR2 ECD alone (hIFNAR2Fc), cDNA encoding the ECD of IFNAR2 was transfected into COS-1 cells and the chimaeric protein purified using a Protein A column, with bound sIFNR eluted with 0.1 M glycine/HCl buffer (pH 2.5). Microtitre plates (Maxisorp; Nunc) were coated with 100 μ l/well of 3 μ g/ml soluble hIFNAR1AR2Fc in PBS overnight at 4 °C and blocked with 1% BSA and 0.05% Tween 20 in PBS for 1 h at room temperature. IFN- β (Feron; Toray Industries) at the indicated concentrations was added to each well and incubated for 2 h at room temperature. After washing the wells four times with wash buffer (PBS containing 0.05% Tween 20), plates were incubated with 50 μ l/well horseradish peroxidase-labelled mouse anti-human IFN- β mAb (Fab'; YSB-1) [17] for 1 h at room temperature. Wells were then washed four times with wash buffer

Abbreviations used: ECD, extracellular domain; IFN, interferon; mAb, monoclonal antibody; sIFNR, soluble human IFN receptor; SV, sindbis virus; VSV, vesicular stomatitis virus.

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The nucleotide sequence of phage 6 has been deposited in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under accession no. AB058940.

Figure 1 Inhibitory effect of anti-IFN- β mAb, YSB-2, on the binding of IFN- β to immobilized sIFNR

(A) Solid-phase ligand-binding assay of IFN- β . IFN- β bound the immobilized sIFNR in a dose-dependent and saturable manner. Inset shows the specific binding of IFN- β , with no IFN- β binding to immobilized human IgG₁ observed. Signal was obtained when 0.5 nM IFN- β was added, but disappeared in the presence of 27 nM sIFNR. (B) Inhibitory effect of anti-IFN- β mAb, YSB-2, on IFN- β binding to immobilized sIFNR. Purified sIFNR (3 μ g/ml) coated each well of a microtitre plate. IFN- β (0.5 nM) was incubated with the immobilized sIFNR in the presence of YSB-2 mAb or mouse IgG₁ (negative control) at the indicated concentrations. Bound IFN- β was detected by the addition of horseradish peroxidase-labelled mAb YSB-1 (Fab'). All data represent means $\pm$ S.D. from triplicate wells; some error bars are small enough to be obscured by the circles denoting the means.

and bound enzyme detected by the addition of 3',3',5',5'-tetramethylbenzidine ('TMB') substrate. Absorbance readings were measured at 450 nm on an ELISA plate reader. For the competitive binding assay, synthetic peptides or antibodies at the indicated concentrations were added to each well during the IFN- β -binding step.

Phage selection

A phage-display library consisting of random sequences of 15 amino acids inserted into the minor coat protein pIII was constructed as described previously [18]. The library consisted of 2×10^8 unique phage clones. Microtitre plates were coated with 100 μ l/well of 20 μ g/ml mouse anti-human IFN- β mAb (YSB-2) in PBS overnight at 4 °C and blocked with 1% BSA and 0.05% Tween 20 in PBS (dilution buffer) for 1 h at room temperature. Aliquots of the 15-amino-acid random phage-display library (10^{12} virions) in dilution buffer were incubated in the YSB-2-coated wells for 1 h at room temperature. Unbound phage were removed by washing 10 times with washing buffer (PBS containing 0.05% Tween 20) and bound phage eluted with 0.1 M glycine/HCl (pH 2.5) containing 1 μ g/ml BSA. Eluted phage were neutralized and amplified in *Escherichia coli* K91 kan. Biopanning was repeated for three rounds. Individual phage clones were picked from agar plates and amplified. YSB-2-binding phage were identified using a phage ELISA as described previously [18,19]. When the library was screened with sIFNR, 3 μ g of sIFNR (hIFNAR1AR2Fc or hIFNAR2Fc) was coated

on each well of a microtitre plate. Further screening was the same as described above.

Antiviral assay

The antiviral activities of the SYR synthetic peptides were measured by inhibition of the virus-induced cytopathic effect on human FL cells using sindbis virus (SV) or vesicular stomatitis virus (VSV) as the viral challenge [20]. Briefly, peptides were dissolved to 18.4 mM in DMSO and diluted in Eagle's minimal essential medium containing 5% fetal calf serum. The maximum concentration of DMSO in the antiviral assay was 0.65% (DMSO at 2% had no effect on this assay). Serial 2-fold-diluted samples of SYR synthetic peptides were added to confluent monolayers of human FL cells in 96-flat-bottom-well plates and incubated for 24 h. Cells were challenged with virus and incubated for about 14 h, after which cells were fixed with paraformaldehyde and stained with Crystal Violet. Antiviral activity was calibrated against Japan NIH reference standard human IFN- β . For bioassays in the presence of low sIFNR (hIFNAR1AR2Fc) concentration or neutralizing IFN mAbs, 180 nM hIFNAR1AR2Fc or various neutralizing IFN mAbs at the indicated concentrations were added to the confluent monolayers of human FL cells, together with the SYR6 peptide. For bioassays with neutralizing IFN mAbs, each neutralizing IFN mAb was also added to the wells during the virus infection step.

Neutralizing IFN mAbs used in this study were as follows: anti-IFN- α antibody MIF-1 (Hayashibara Biochemical Laboratories; final concentration, 895 neutralizing units/ml), anti-IFN- β antibody YSB-1 (final concentration, 133 neutralizing units/ml) and anti-IFN- γ antibody 1598-00 (Genzyme R&D systems; 1.0 μ g/well).

RESULTS

As no sIFNR-reactive phage clones were obtained, we used an alternative approach to screening the phage-display libraries with neutralizing mAbs that blocked IFN-receptor interactions. Antibody-binding sequences may mimic the spatial structures of the IFN molecule receptor-binding site. Prior to the selection of the inhibitory mAb, a solid-phase IFN- β receptor-binding assay was developed based on an ELISA. IFN- β was found to bind immobilized sIFNR in a dose-dependent and saturable manner (Figure 1A). Using this specific ligand-binding assay, neutralizing mAbs that showed an inhibitory effect on IFN- β receptor binding were selected. The neutralizing mAb YSB-2 [21] was found to inhibit the binding of IFN- β to immobilized sIFNR in a dose-dependent manner (Figure 1B) and was therefore considered to be a good candidate as the target molecule for phage-clone selection.

A 15-mer phage library [18,19] was screened with mAb YSB-2. After three rounds of screening, three clones (phage 1, 2 and 6) were identified as binding YSB-2. These three clones recognized mAb YSB-2 by ELISA, but did not recognize mAb YSB-1 [21], another neutralizing mAb to IFN- β , suggesting that the selected phage had binding specificity for mAb YSB-2 (Figure 2). The deduced amino acid sequences of the three phage clones (Table 1)

Figure 2 Specific binding of selected phage clones to mAb YSB-2

Three phage clones isolated from a phage-display library specifically bind to mAb YSB-2 in phage ELISA. Bound phage was detected by the addition of horseradish peroxidase-labelled anti-M13 IgG. Absorbances at 450 nm are shown. Binding was eliminated by addition of 40 nM IFN- β . No cross-reaction of the phage clones to mAb YSB-1 was observed. The control phage, fuse2, with no random insert, showed no reactivity. Data represent means $\pm$ S.D. from triplicate wells.

Table 1 YSB-2-binding sequences isolated from a phage-display library

Frequency indicates the number of times each sequence was independently isolated. The (L/V)Q(A/E)XWE motif is shown in bold.

Clone no.	Sequence	Frequency
1	RLQAW WE MWADSYRP	5/11
2	QKRPN W ISTLQEL WE	3/11
6	SVQAR WE AAFDLDLY	3/11

showed no similarity to the primary sequences of IFN- β and other known proteins, consistent with the fact that mAb YSB-2 recognizes a discontinuous epitope on IFN- β . Three sequences identified from the phage library contained the common epitope sequence (L/V)Q(A/E)XWE. Peptides (15-mers) corresponding to the identified amino acid sequences were synthesized chemically and used for further characterization, and were designated as SYR1, 2 and 6, corresponding to phage clones 1, 2 and 6, respectively.

To test whether the synthetic SYR peptides that bound neutralizing anti-IFN- β mAb were able to interact with the type I IFN receptor, competitive binding of IFN- β and SYR peptides to sIFNR, made up of the ECDs of IFNAR1 and IFNAR2 (hIFNAR1AR2Fc), was investigated using a solid-phase ligand-binding assay. Although SYR1 and 2 had no effect on IFN- β binding, SYR6 inhibited IFN- β binding to immobilized sIFNR in a concentration-dependent manner (Figure 3A), with the half-maximal inhibition concentration (IC_{50}) of SYR6 estimated to be ≈ 50 μ M. As IFN- α 2, a member of the type I IFN family, was reported to have binding affinity for the ECD of IFNAR2 only [22], we then tested whether SYR6 interacted with IFNAR2 alone. Like IFN- α 2, IFN- β was found to bind the ECD of IFNAR2 (hIFNAR2Fc; Figure 3B), which was inhibited by SYR6 in a concentration-dependent manner. Thus SYR6 interacted with IFNAR2 in a similar manner to IFN- β .

The biological activity of SYR6 was investigated using an antiviral assay using human FL cells infected with SV or VSV. Since we obtained two major fractions of purified SYR6 (designated SYR6A and 6B), which were probably due to the conformational change in the solvent during the reversed-phase HPLC step, we examined antiviral activities of both peptides. As shown in Figure 4(A), SYR6A and 6B showed antiviral activity against both viruses in the same concentration-dependent manner, whereas SYR1 and 2 showed no antiviral activity. The fact that SYR6 showed antiviral activity against both SV and VSV indicated that the SYR6 peptide did not mediate its antiviral activity via the inhibition of specific viral attachment to cells. SYR6 also showed certain toxic or growth-inhibitory activity, as shown by the lower cell density at the higher peptide concentrations.

To rule out the possibility of IFN contamination or the induction of endogenous IFNs by SYR6, bioassays in the presence of excess amounts of various anti-IFN antibodies or a low concentration (180 nM) of sIFNR (hIFNAR1AR2Fc) were carried out. sIFNR (18 nM) showed complete inhibition of antiviral activity of 25 i.u./ml IFN- β (Figure 4C, upper panel). Neutralizing mAbs to IFN- α , - β and - γ , and low sIFNR concentration (180 nM), which is a 1.4×10^4 -fold higher concentration than that of 25 i.u./ml IFN- β , had no effect on the antiviral activity of SYR6 (Figures 4B and 4C). Furthermore, SYR6 did not show any antiviral actions on mouse LY cells infected with VSV (results not shown), indicating that SYR6 acted neither as an inhibitor of viral replication nor as an inducer

Figure 3 Competition assay for binding of IFN- β to immobilized sIFNR (hIFNAR1AR2Fc and hIFNAR2Fc)

(A) Synthetic peptide SYR6 inhibits IFN- β binding to immobilized hIFNAR1AR2Fc. Purified sIFNR (hIFNAR1AR2Fc; 3 μ g/ml) coated each well of a microtitre plate, and IFN- β (0.5 nM) plus various concentrations of each peptide added. Bound IFN- β was detected by the addition of horseradish peroxidase-labelled mAb YSB-1 (Fab'). (B) Synthetic peptide SYR6 inhibits IFN- β binding to immobilized sIFNR (hIFNAR2Fc). Assay was performed as in (A) except that soluble hIFNAR2Fc was used instead of hIFNAR1AR2Fc. All data represent means $\pm$ S.D. from triplicate wells.

of endogenous IFNs, and that the antiviral action of the peptide required the human IFN receptor. Taken together, we concluded that the antiviral action of SYR6 was exogenous, IFN-independent, and that SYR6 acted as type I IFN receptor agonist. Although cytotoxic effects due to high concentrations of SYR6 were observed, 1 mg of SYR6 had an antiviral effect comparable with 50 i.u. of IFN- β .

DISCUSSION

A molecular diversity approach with phage-displayed random peptide libraries has been utilized for discovering biologically active peptides. To obtain the peptides mimicking IFN activity, screening of a phage-display library with IFN receptor should be a straightforward strategy. Thus, first of all, a linear 15-mer and a cyclic 7-mer random peptide library were screened with sIFNR. This screening, however, did not lead to the discovery of IFN receptor-binding phage (results not shown). Another possible approach is to screen a phage-display library using neutralizing mAbs that block IFN-receptor interactions. Despite similar screening strategies successfully identifying peptide antagonists rather than agonists [23–25], the success of these methods encouraged us to attempt the screening of a peptide library with an inhibitory anti-IFN- β mAb. Although screening by affinity for an inhibitory anti-IFN- β mAb (YSB-2) is not a direct way to identify an IFN peptide mimetic, this strategy led to the successful mimicry of IFN- β (peptide SYR6). It is of interest to consider why an inhibitory anti-IFN- β mAb produced a peptide mimetic but sIFNR did not. Considering the fact that a phage clone displaying the sequence of SYR6 (phage 6) failed to bind sIFNR in phage ELISA due to the low binding affinity (results not shown), the use of an inhibitory anti-IFN- β mAb led to the identification of the phage clone that had too low an affinity

for sIFNR to be detected. Only one of three sequences isolated as binding an inhibitory anti-IFN- β mAb was active as the free peptide. Since three sequences showed the consensus sequence (L/V)Q(A/E)XWE, the amino acid residues in the non-motif positions may affect the conformation of the peptides that interact with IFN receptor.

Successful screening of conformationally constrained peptide libraries has been reported (for review, see [16]). To further examine the usefulness of screening a disulphide-containing peptide library, we screened a cyclic 7-mer peptide library with mAb YSB-2. Unfortunately, however, none of the mAb-binding sequences isolated from this library competed with IFN- β for binding to IFN receptor (results not shown). Phage 6 isolated from a linear 15-mer peptide library did not contain cysteine residues at any position. Thus introduction of the disulphide bonds into displayed peptides was not effective in our screening.

The 15-mer peptide SYR6 was shown to compete with IFN- β for binding to type I IFN receptor, and was shown to elicit antiviral activity. The observation that, like IFN- β , SYR6 interacted with IFNAR2 alone is in accordance with a previous report that human IFN- β bound to mouse L-929 cells stably expressing only the IFNAR-2c (a long form of IFNAR2) subunit, and induced antiviral activity [26].

The low biological activity of SYR6 compared with that of IFN- β can be explained by the low affinity of the 15-mer peptide for the type I IFN receptor. Attempts to examine the binding affinity of SYR6 for sIFNR by surface plasmon resonance (BIAcore) were not successful due to the low binding affinity. Likewise, detection of induced tyrosine phosphorylation of Janus kinases by 500 μ M SYR6 also failed.

Recently, the therapeutic effect of oral-use low-dose type I IFNs was reported (see the special issue *Oral Use of Interferons and Cytokines* [27]). Satoh et al. [28] reported oral administration

Figure 4 Evidence of an antiviral effect of SYR6

(A) FL cell cultures prepared in a 96-well culture plate were incubated overnight with a series of 2-fold dilutions of each peptide and human IFN- β . The next day, the cultures were exposed to SV (left-hand panel) or VSV (right-hand panel) and incubated for 14 h. Cultures were then fixed and stained with Crystal Violet. Both SYR6A and SYR6B, pooled from different fractions of a reversed-phase HPLC step, showed antiviral activities in the same concentration-dependent manner. (B and C) Neither endogenous IFN molecules nor contaminating IFN are involved in the antiviral activity of SYR6. (B) Antiviral effect of SYR6 in the presence of neutralizing IFN mAbs. The assay was performed as in (A) except that the culture steps were accompanied by excess neutralizing anti-IFN- α , - β and - γ mAbs. (C) Antiviral effect of SYR6 in the presence of a low concentration of hIFNAR1AR2Fc. The assay was performed as in (A) except that the culture steps were accompanied by 180 nM soluble hIFNAR1AR2Fc. sIFNR at concentrations of 18 nM showed complete inhibition of activity of 25 i.u./ml IFN- β (upper panel). sIFNR at 180 nM was expected to have very little influence on the binding of SYR6 to cell-surface IFN receptors.

of IFN- β in the guinea pig model of asthma and found that low-dose oral administration of IFN- β induced 2',5'-oligoadenylate synthetase activity, although the mechanism was uncertain. The small-peptide IFN mimetic we identified might be applied to oral administration despite its low activity.

It has been reported that small-peptidic or non-peptidic agonists can bind to and activate homodimeric cytokine receptors. Ligand binding is thought to induce receptor homodimeriz-

ation (see Introduction). In contrast, little is known of the mechanism by which heterodimeric cytokine receptors are activated. The relatively small peptide we obtained may be a potent agonist to provide the mechanism of activating heterodimeric cytokine receptors, because it is a ligand epitope responsible for binding to a cognate receptor [29].

In conclusion, an IFN peptide mimetic was successfully identified through the screening of a peptide phage display

library with a neutralizing mAb that blocked IFN-IFN-receptor interaction. This approach can be applied to other cytokine molecules. Although the biological activity of the 15-mer peptide was not high, the isolation of SYR6 is the first proof that a small peptide can mimic the biological activity of type I IFN molecule. This peptide might provide a tool to help explain how heterodimeric cytokine receptors are activated.

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