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Production and characterization of anti-human interferon γ receptor antibody fragments that inhibit cytokine binding to the receptor

Angela Bridges, Fiona Stuart, Julia Späth, Stefan Lang, Christoph Henke, Ashley Birch and John A.Robinson[1]

Institute of Organic Chemistry, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

[1]To whom correspondence should be addressed

Three single-chain antibody fragments that recognize the extracellular human interferon γ receptor α-chain (IFNγR), and inhibit the binding of human IFNγ, have been produced in *Escherichia coli*. These fragments are derived from murine anti-receptor monoclonal antibodies, and comprise the variable heavy (V_H) domain linked to the variable light (V_L) chain through a 15 amino acid linker [$(GGGGS)_3$]. Using surface plasmon resonance technology (BIAcore), the soluble proteins were shown to retain a high affinity for recombinant IFNγR, and by radioimmunoassay to possess high inhibitory activity towards IFNγ-binding to human Raji cells. The antibody fragments most likely recognize epitopes that overlap the cytokine binding site on the receptor surface. Attempts to dissect further the antibodies to isolated V_H- and V_L-chains and to synthetic linear and cyclic peptides derived from the individual complementarity determining regions failed to afford fragments with significant IFNγR binding affinity. Nevertheless, these native-like variable region fragments and petidomimetics derived from them are of interest in the design of novel IFNγR antagonists.

Keywords: antagonist/epitope/Fv fragment/ligand design/mimetic/monoclonal antibody

Introduction

The physico-chemical basis of the binding of protein ligands at specific sites on the surface of receptor proteins is not well understood. The extracellular portion of the human interferon γ receptor α-chain (IFNγR) is an interesting target for studies of protein–protein recognition, since both the natural ligand and several surrogate ligands in the form of neutralizing anti-receptor monoclonal antibodies (mAbs) are available for investigation (Aguet and Merlin, 1987; Garotta *et al.*, 1990). Moreover, the crystal structure of the IFNγ–IFNγR was solved recently, which revealed in atomic detail the residues on the receptor that are buried upon complex formation with the cytokine (Walter *et al.*, 1995). It is then of interest to enquire how the structural epitopes bound by neutralizing anti-IFNγR mAbs are related to the ligand binding site on the receptor surface. The study of such neutralizing mAbs is also fuelled by the prospect that fragments derived from them may provide a useful starting point in the design of novel small molecule IFNγR antagonists.

In some cases much smaller fragments of an antibody may retain the ability to interact selectively with a protein antigen. This includes the Fv fragment (Raag and Whitlow, 1995), which contains an intact copy of the antigen binding site, as well as the individual H-chain or L-chain variable regions (Ward *et al.*, 1989; Hamers-Casterman *et al.*, 1993; Davies and Riechmann, 1995; Monfardini *et al.*, 1995), each with just three complementarity determining regions (CDRs). Even relatively small linear and cyclic peptides derived from the sequences of individual CDRs of some mAbs have been identified that retain a weak antigen-binding activity (Saragovi *et al.*, 1992; Doring *et al.*, 1994).

The diverse functions of IFNγ in host defence, inflammation and autoimmunity arise through its association with the IFNγR (Farrar and Schreiber, 1993). This ~90 kDa transmembrane glycoprotein binds IFNγ with high affinity ($K_D \approx 10^{-10}$ M), and consists of an extracellular portion of 229 amino acid residues folded into two Ig-like domains, a single transmembrane region of 22 residues and an intracellular region of 221 residues. The recombinant extracellular portion alone, as well as the intact membrane-bound receptor, binds IFNγ tightly in a 2:1 receptor:ligand stochiometry (Fountoulakis *et al.*, 1992; Greenlund *et al.*, 1993), as revealed recently in the X-ray crystal structure of the IFNγ–IFNγR complex (Walter *et al.*, 1995). IFNγ-induced signal transduction, however, requires a species-specific interaction of the α-chain–hormone complex with a separate transmembrane receptor β subunit (Hemmi *et al.*, 1994; Soh *et al.*, 1994; Marsters *et al.*, 1995), and additional receptor subunits may be required to mediate the anti-viral actions of IFNγ (Soh *et al.*, 1994). IFNγR antagonists are of potential value in the treatment of autoimmune diseases, chronic inflammations, allograft rejection and delayed-type hypersensitivity.

An understanding of IFNγR recognition by mAbs will require high-resolution structural data on mAb fragment–receptor complexes, as well as the ability to produce mutants for structure–function studies. For both goals, an important step is the production in *Escherichia coli* of soluble scFv fragments. The variable region cDNAs encoding the H- and L-chains of the neutralizing anti-IFNγR mAbs A6, γR38 and γR99, studied here, have been described in previous work (Bridges *et al.*, 1995). The first two mAbs bind overlapping epitopes contained entirely within the N-terminal Ig-like domain of the IFNγR, between residues 1 and 108, whereas the last mAb binds an epitope within the membrane proximal Ig-like domain, between residues 105 and 229 (Ruegg *et al.*, 1995; Williams *et al.*, 1995). In all three cases, the mAbs recognize conformational (i.e. discontinuous) epitopes.

Materials and methods

Polymerase chain reaction

Amplifications were carried out in a reaction volume of 100 μl under mineral oil containing the following: dNTPs (0.2 mM of each), $MgCl_2$ (1.5 mM), sense and antisense primers (1 μM), plasmid or cDNA, and the recommended buffer (AmpliTaq or Vent). Filter tips were used throughout. The reactions were performed using a Perkin-Elmer 480 thermal cycler as follows: 5 min at 95°C, 1 min at 94°C, 2 min at 55°C and 2 min at 72°C.

DNA sequencing and analysis

The sequences of all the scFv and individual V_H and V_L genes cloned in pHEN1 were verified by dideoxy sequencing (Sanger *et al.*, 1977) of plasmid DNA isolated from *E.coli* TG1 using Sequenase (USB) and the following primers:

HENLEFT	5′-GCTATGACCATGATTACGCCAGCTT-3′
HENRIGHT	5′-CGATCTAAAGTTTTGTCGTCTTTCC-3′
LINKFORNEW	5′-TGGAGACTGAGTGAGCTCGATGTC-3′
LINK-BACKNEW	5′-GGCACCACGGTCACCGTGTGGTGA-3′

Variable region gene assembly

To assemble scFv genes, V_H and V_L cDNAs (Bridges *et al.*, 1995) were amplified by PCR using the Heavy and Light Primer Mixes in the Mouse scFv Module of the Pharmacia (Pharmacia Biotech, Uppsala, Sweden) Recombinant Phage Antibody System. The scFv gene was then assembled by PCR using the Linker–Primer Mix and RS–Primer Mix, according to the manufacturer's instructions (Pharmacia). The resulting product was rendered blunt-ended with T4 DNA polymerase and concatenated by ligation to improve subsequent digestion with *Not*I and *Sfi*I, before ligation into the vector pHEN1 (Hoogenboom *et al.*, 1991).

The individual V_H and V_L cDNAs were amplified by PCR, using the appropriate M13 clone (Bridges *et al.*, 1995) as template, and the following primers (Hoogenboom *et al.*, 1991) (*Not*I and *Sfi*I restriction sites underlined):

VH1BACKSFI15	5′-CATGCCATGACTCGCGGCCCAGCCGGCCATGG-CCSAGGTSMARCTGCAGSAGTCWGG-3′
VH1FOR2NOT1	5′-TTCTGCGGCCGCTGAGGAGACGGTGACCGTG-GTCCCTTGGCCCC-3′
VLPHENSFI	5′-ACGCGGCCCAGCCGGCCATGGCTGACGTCGT-GATGACMCARWCKCCA-3′
VLPHENNOTI	5′-TTCTGCGGCCGCCCGTTTCAGCTCCAGCTTGG-TCCC-3′

The PCR products were then digested with *Not*I and *Sfi*I and cloned in pHEN1, as above for the scFv genes.

Site-directed mutagenesis

Camelization of H-chains was carried out by site directed mutagenesis using the Altered Sites II *in vitro* Mutagenesis System (Promega, Madison, WI). V_H genes were amplified by PCR using the appropriate cDNA as template, the products were digested with *Xba*I and *Asp*718 and the products ligated into the vector pALTER-1. The following oligonucleotides were used for mutagenesis of A6, γR38 and γR99 H-chains, respectively:

A6VHCAMEL1	5′-CGTCAGTCTTCAGGGAAGGAACGCGAGGGGC-TGGCACACATTTGGTGGG -3′
R38VHCAMEL1	5′-CCGCCAGTCTCCAGAGAAGGAGCGCGAGGGG-GTTGCTGAAATTAGATTGA-3′
R99VHCAMEL1	5′-AAAACAGAGGCCTGGACAGGAACGCGAAGG-GATTGGATACATTAATCCTA-3′

Clones carrying the desired mutation were identified by DNA sequencing, and used as template for PCR using the primers VH1BACKSFI15 and VH1FOR2NOT1. The products were subsequently cloned into pHEN1 (see above).

Production and purification of scFvs

Escherichia coli HB2151 was transformed with phagemids pOCI455 (pHEN1 + A6 scFv), pOCI456 (pHEN1 + γR38 scFv) and pOCI461 (pHEN1 + γR99 scFv), and transformants were selected on 2×TY agar containing 1% glucose+100 μg/ml ampicillin at 30°C. Colonies were then grown in 2×TY medium, also with 1% glucose+100 μg/ml ampicillin at 30°C. Cells were pelleted after growth to OD_{550} = 0.5–0.8, washed once with 2TY medium and resuspended in TY medium with 100 μg/ml ampicillin and 0.01 mM IPTG. After growth for a further 16 h at 30°C, scFv was purified either from the culture supernatant (for A6 and γR99) or from cells (for γR38). For the former, PMSF (1 mM) benzamidine (1 mM) and EDTA (1 mM) were added to the culture, cells and debris were removed by centrifugation and the supernatant was applied to an immunoaffinity column prepared by cross-linking anti-c-myc tag mAb 9E10 (Evan *et al.*, 1985) to protein-G Sepharose (Pharmacia). The column was washed with Tris–HCl (50 mM, pH 7.5), EDTA (1 mM) and NaN_3 (0.04%), and bound protein was eluted with glycine–HCl (50 mM, pH 2.5), EDTA (1 mM) and NaN_3 (0.04%). This was neutralized immediately with sodium phosphate (4 M, pH 7). The protein was then applied to a second immunoaffinity column, prepared by immobilizing a thioredoxin–IFNγR$^{1-108}$ (for A6 scFv), or a thioredoxin–IFNγR$^{100-229}$ (for γR99 scFv) fusion protein (Ruegg *et al.*, 1995; Williams *et al.*, 1995) on CNBr-activated Sepharose. The bound scFv was eluted with glycine–HCl (50 mM, pH 2.5), EDTA (1 mM) and NaN_3 (0.04%) and neutralized immediately with sodium phosphate (4 M, pH 7). For the γR38 scFv, a cell lysate was prepared by resuspending *E.coli* cells in sucrose (20% v/v), Tris–HCl (pH 8.0), EDTA (1 mM) and Triton X-100 (0.1%) for 5 min, followed by centrifugation (27 000 *g*) to remove cellular debris. The γR38 scFv was then purified as for the A6 scFv. scFv concentrations were determined by quantitative amino acid analysis.

Gel filtration

Analytical gel filtration chromatography was performed on an Ultropac TSK G3000SW column (7.5×600 mm) equilibrated with MOPS (40 mM, pH 7.5) and $Ca(OAc)_2$ (100 mM) at a flow rate of 0.7 ml/min. The column was calibrated using BSA (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 29 000) and ribonuclease A (M_r 13 700).

Biosensor measurements

The intact extracellular IFNγR produced in insect cells (Gentz *et al.*, 1992) was immobilized on CM5 sensor chips by the random amine coupling method described in the BIAcore methods manual (Pharmacia). Binding curves, measured in duplicate, were recorded under the following conditions: A6 scFv—injection volume 40 μl, flow rate 10 μl/min, HBS buffer pH 8, HEPES (10 mM), NaCl (150 mM), EDTA (3.4 mM), surfactant P20 (0.005%), concentrations of scFv tested 38, 47, 66, 76, 85, 95, 104, 114 nM; γR38 scFv—injection volume 30 μl, flow rate 10 μl/min, HBS buffer pH 7.4, concentrations of scFv tested 20, 40, 60, 80, 100, 140, 180, 220 nM; and γR99 scFv—injection volume 30 μl, flow rate 20 μl/min, phosphate buffer (50 mM, pH 7.2), NaCl (150 mM), with P20 (0.005%) concentrations of scFv tested 6, 8, 10, 12, 14, 16, 18, 20 nM. After each measurement the surface was regenerated with glycine buffer (10 μl, 0.1 M, pH 2.0). The specificity of each interaction was tested using a control surface, and by preincubation of each scFv with the intact IFNγR or with thioredoxin (Trx)–IFNγR fragment fusion proteins (Ruegg *et al.*, 1995; Williams *et al.*, 1995). Preincubation of A6 and γR38 scFvs with the intact IFNγR or the Trx–IFNγR$^{1-108}$ fusion protein inhibited binding of scFv to the biosensor, whereas Trx–IFNγR$^{100-229}$ had no effect. For the γR99 scFv, the intact IFNγR and Trx–IFNγR$^{100-229}$ inhibited binding and Trx–IFNγR$^{1-108}$ had no inhibitory effect, as anticipated.

Radioimmunoassays

Raji cells (10^7 cells/ml) in Hanks BSS with 1% BSA and HEPES buffer (15 mM, pH 7.2) were aliquoted (100 µl ≈ 10^6 cells) into wells in a COSTAR 96U-well microtitre plate each containing [^{125}I]IFNγ (~2.5 ng, 1.5×10^4 c.p.m./ng) and scFv protein in the concentration range 0.1–10^{-6} mg/ml). After incubation at 4°C for 90 min, the plates were centrifuged (1500 r.p.m., 5 min) and the supernatant was discarded. The cells were washed twice with Hanks BSS with 0.1% BSA in HEPES buffer (15 mM, pH 7.2) and 0.01% Triton X100. The amount of [^{125}I]IFNγ bound to the cells was determined by liquid scintillation counting (Garotta *et al.*, 1990). The assays were performed in triplicate and the results given are the mean values (Table II).

Peptide synthesis

Linear peptides were assembled on an ABI430A synthesizer (Applied Biosystems, Foster City, CA), using Fmoc chemistry. Each HPLC-purified peptide gave a satisfactory amino acid analysis, a molecular ion (M + H or M + Na) on electrospray mass spectrometry and 600 MHz ^{1}H NMR spectra that were consistent with the expected connectivity. Cyclic peptides were assembled in the same way except that Fmoc-Cys(Trt)-COO-*p*-alkoxybenzyl alcohol resin (Bachem, Switzerland) was used to initiate the synthesis, the fully assembled peptides were bromoacetylated, the resin was then treated with TFA containing water (5% v/v), thioanisole (5% v/v), phenol (5% v/v) and triisopropylsilane (5% v/v), and cyclization was performed in acetonitrile–water (1:1, ~1 mg/ml) at pH 8 (NH_3), with stirring under N_2 for 14 h. The cyclic peptides were also purified by reversed-phase-HPLC and characterized by electrospray mass spectrometry and 600 MHz 1D and 2D NMR spectrometry.

Inhibition assays

Competitive ELISA was performed by minor variation of an established method (Ozmen *et al.*, 1992). Competitive binding assays were performed with the BIAcore apparatus. Intact antibody and free (linear or cyclic) peptide were co-injected over immobilized receptor. No inhibition of antibody binding to the biosensor in the presence of up to ~500 µM peptide concentration could be detected.

Results

Cloning and expression of scFv genes

Genes encoding scFv fragments derived from the mAbs A6, γR38 and γR99 were assembled by PCR (Marks *et al.*, 1991) from cDNAs encoding individual H- and L-chains (Bridges *et al.*, 1995). Each V_H and V_L was connected via a linker encoding the peptide $(GGGGS)_3$, in the order V_H–linker–V_L. The three scFv genes were each cloned as *Sfi*I–*Not*I fragments into the phagemid vector pHEN1, in-frame behind a *pel*B leader sequence, and before a linker encoding a peptide c-myc tag, such that the tag is attached to the C-terminus of V_L in the scFv (Hoogenboom *et al.*, 1991). The anti-myc tag mAb 9E10 (Evan *et al.*, 1985) was used to detect secretion of soluble scFv after growth in *E.coli* HB2151 and induction with IPTG at 30°C. No significant improvement in yield was observed by induction at lower temperatures. Production of the A6 and the γR99 scFv gave rise to extensive cell lysis, whereas cell division continued after induction of the γR38 scFv, which could subsequently be isolated from a periplasmic extract.

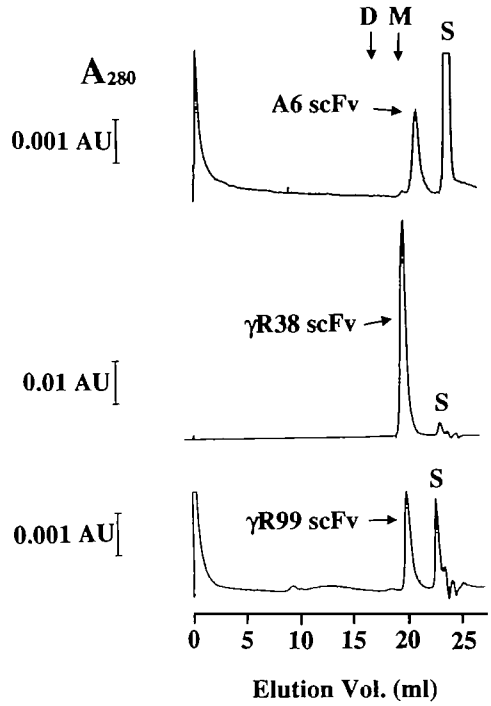

Fig. 1. Elution profile of each scFv from a gel filtration chromatographic column (see Results and Materials and methods sections). S = salt peak; M=elution volume expected for monomeric scFv; D=elution volume expected for dimeric scFv.

Purification and characterization of scFvs

An immunoaffinity column of 9E10 mAb coupled to Sepharose was used to isolate scFv fused to the peptide tag from the culture supernatant or a periplasmic extract. Functionally active scFv protein was further purified by affinity chromatography using recombinant IFNγR fragments immobilized on Sepharose. The native-like N-terminal IFNγR domain (Williams *et al.*, 1995) fused to thioredoxin (Trx–IFNγR$^{1-108}$) was used for the A6 and γR38 scFvs, and the membrane proximal IFNγR domain as a thioredoxin fusion protein (Ruegg *et al.*, 1995) (Trx–IFNγR$^{100-229}$) for γR99 scFv. This affinity purification step afforded in each case >95% homogeneous scFv, with the correct relative mass by SDS–PAGE, and the expected N-terminal amino acid sequence. The yields were γR99 scFv (<0.2 mg/l), A6 scFv (~0.3 mg/l) and γR38 scFv (~1–2 mg/l).

Upon gel filtration chromatography, >95% of each protein eluted close to the volume expected for monomeric scFv (Figure 1). The A6 scFv eluted slightly slower than expected, but this property was also shared by A6 Fab fragment. The γR99 scFv and to a lesser extent the A6 scFv precipitated irreversibly from even dilute solutions (<1 mg/ml) upon storage at 4°C. The γR38 scFv was stable over several weeks at 4°C and remained soluble in more concentrated solutions (up to several mg/ml).

Isolated V_H- and V_L-chains

Each V_H and V_L gene from A6, γR38 and γR99 was amplified separately by PCR and cloned as a *Sfi*I–*Not*I fragment into the phagemid vector pHEN1 (Hoogenboom *et al.*, 1991), as described above for the scFvs. Production of tag-positive material after growth in *E.coli* HB2151 and induction with IPTG was detected with the 9E10 mAb. Although in several cases soluble tag-positive proteins were found, none was able

Table I. Sequences of linear and cyclic peptides derived from individual CDRs of mAbs A6, γR38 and γR99

Antibody A6	
CDRL1	Ac-LysAlaSerGluAspIleTyrAsnArgLeuAla-OH
CDRL2	Ac-LeuIleSerGlyAlaThrSerLeuGluThrGluValProSer-OH
CDRL3	Ac-GlnGlnTyrTrpSerThrTrpThrPheGly-OH
CDRH1	Ac-SerGlyPheSerLeuThrThrTyrGlyMetGlyValGlyTrp-OH
CDRH2	Ac-AlaHisIleTrpTrpAspAspAspLysTyrTyr-OH
CDRH3	Ac-AlaArgArgAlaProPheTyrGlyAsnHisAlaMetAspTyr-OH
CYCLL1	Cyclo(S.Ac-AlaLysAlaSerGluAspIleTyrAsnArgLeuAlaCys-OH)
CYCLL2	Cyclo(S.Ac-LeuIleSerGlyAlaThrSerLeuGluCys-OH)
CYCLL3	Cyclo(S.Ac-AlaGlnGlnTyrTrpSerThrTrpThrPheCys-OH)
CYCLH1	Cyclo(S.Ac-SerPheSerGlyPheSerLeuThrThrTyrGlyMetGlyValGlyCys-OH)
CYCLH2	Cyclo(S.Ac-HisIleTrpTrpAspAspAspLysTyrCys-OH)
CYCLH3	Cyclo(S.Ac-ArgAlaProPheTyrGlyAsnHisAlaMetCys-OH)
Antibody γR38	
CDRL3	Ac-GlnGlnTyrTrpSerThrProTyrThrPheGly-OH
CDRH1	Ac-SerGlyPheThrPheSerAsnTyrTrpMetAsn-OH
CDRH2	Ac-AlaGluIleArgLeuLysSerAsnAsnTyrAlaThrHisTyrAla-OH
CDRH3	Ac-ThrTrpTyrAspGlySerTyr-OH
CYCLL3	Cyclo(S.Ac-GlnGlnTyrTrpSerThrProTyrThrPheCys-OH)
CYCLH1	Cyclo(S.Ac-ValAlaSerGlyPheThrPheSerAsnTyrTrpMetAsnCys-OH)
CYCLH2	Cyclo(S.Ac-ArgLeuLysSerAsnAsnTyrAlaCys-OH)
CYCLH3	Cyclo(S.Ac-ThrTrpTyrAspGlySerTyrTrpCys-OH)
Antibody γR99	
CDRL1	Ac-ArgSerSerGlnSerLeuAlaAsnSerTyrGlyAsnThrTyrLeuSer-OH
CDRL2	Ac-LeuIleTyrGlyIleSerAsnArgPheSer-OH
CDRL3	Ac-LeuGlnGlyThrHisGlnProTrpThrPheGly-OH
CDRH1	Ac-LysAlaSerGlyTyrThrPheThrThrTyrThrMetHisTrp-OH
CDRH2	Ac-IleGlyTyrIleAsnProSerSerGlyTyrThrGluTyrAsnGln-OH
CDRH3	Ac-Cys(Acm)ValArgSerArgArgValProAspTyrTrpGly-OH
CYCLL1	Cyclo(S.Ac-ArgSerSerGlnSerLeuAlaAsnSerTyrGlyAsnThrTyrLeuSerCys-OH)
CYCLL2	Cyclo(S.Ac-LeuIleTyrGlyIleSerAsnArgCys-OH)
CYCLL3	Cyclo(S.Ac-GlnGlyThrHisGlnProTrpThrCys-OH)
CYCLH1	Cyclo(S.Ac-GlyLysAlaSerGlyTyrThrPheThrThrTyrThrMetCys-OH)
CYCLH2	Cyclo(S.Ac-TyrIleAsnProSerSerGlyTyrThrGluCys-OH)
CYCLH3	Cyclo(S.Ac-ArgSerArgArgValProAspTyrCys-OH)

The linear peptides are labelled according to H-chain (H1–3) or L-chain (L1–3) CDR, and CYCL indicates the corresponding cyclic peptides. Note that the L-chains of A6 and γR38 have identical L1 and L2 CDR sequences since both mAbs use the same Vk34C germline gene (Bridges *et al.*, 1995).

to bind the IFNγR fragments immobilized on Sepharose. Triple G44E, L45R and W47G mutations were introduced into each V_H-chain by site-directed mutagenesis. All three mutated V_H-chains could be produced in a soluble form in *E.coli* HB2151 following induction with IPTG. However, none of these proteins retained significant IFNγR binding activity.

CDR peptides

Sequence comparisons of each Fv with the structural database (Chothia *et al.*, 1989; Bridges *et al.*, 1995) were used to determine which amino acid residues are likely to be located in CDR loops. These, together with one or more residues from the flanking framework regions, were incorporated into synthetic linear peptides, as shown in Table I. Cyclic derivatives were made separately by starting the synthesis with cysteine at the C-terminus, addition of a bromoacetyl group at the N-terminus and subsequent cyclization by displacement of bromine with the S atom of cysteine, to afford a thioether.

The ability of each linear and cyclic peptide to compete with intact mAb for binding recombinant IFNγR was analysed on a BIAcore instrument and by ELISA. In both assays, no inhibitory activity (up to ~0.5 mM peptide) was found amongst the peptides investigated.

Biosensor measurements and radioimmunoassay

The receptor affinity and inhibitory activity of each scFv protein was analysed on a BIAcore instrument and by radioimmunoassay using native receptor on human Raji cells. For BIAcore measurements, the amount of recombinant insect cell-derived (Gentz *et al.*, 1992) IFNγR immobilized on the biosensor chip was selected to give responses that could be fitted accurately to a monoexponential function (Karlsson *et al.*, 1991). The dissociation rate constants were determined by non-linear fitting of the dissociation phase to an integrated form of the first-order rate equation. Two methods of fitting the association phases were then used. The first makes use of the k_{off} value to determine k_{on} by direct non-linear fitting of the instrument response to an integrated form of the rate equation, using protocols supplied by the manufacturer (Pharmacia). In the second method (Karlsson *et al.*, 1991), the response is used to calculate k_s, where $k_s = k_{on}C + k_{off}$, and a linear regression of a secondary plot of k_s against C yields k_{on} and k_{off}. Within experimental error, both methods gave the same values for the interaction constants. Binding curves from duplicate assays at eight analyte concentrations were analysed for each scFv (see Figure 2). Owing to the high k_{on} observed for the γR99 scFv [seen also for the intact mAb in earlier

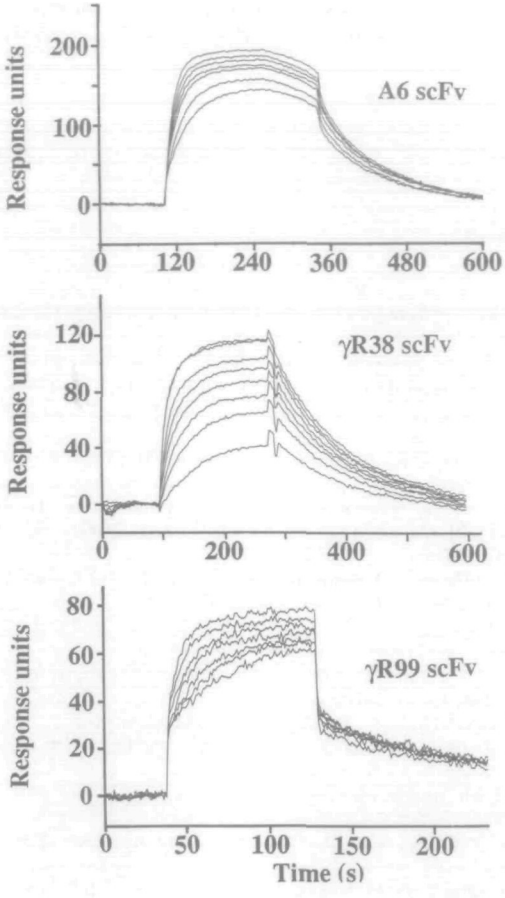

Fig. 2. Sensorgrams from a Pharmacia BIAcore instrument for scFv binding to immobilized recombinant IFNγR. The amount of IFNγR immobilized on the biosensor was varied by adjusting the exposure time of biosensor surface to activation reagent and of protein to the activated surface. Activation of the sensor surface was with NHS (50 mM) and EDC (0.2 M), and coupling to IFNγR was performed in sodium citrate buffer (10 mM, pH 3.95).

Table II. Kinetic and thermodynamic parameters for binding of scFvs derived from mAbs A6, γR38 and γR99 to the recombinant human IFNγR by BIAcore measurements and IC_{50}s for inhibition of IFNγ binding to the native receptor on human Raji cells

scFv	k_{off} ($\times 10^{-3}$ s^{-1})	k_{on} ($\times 10^{5}$ M^{-1} s^{-1})	K_D (nM)	IC_{50} (nM)
A6	10.4 ± 0.1	5.3 ± 0.2	20.0	10.3
γR38	9.2 ± 0.3	3.3 ± 0.4	27.8	25.8
γR99	6.9 ± 0.5	63.8 ± 0.5	1.1	8.5

work (Williams *et al.*, 1995)], the level of immobilized receptor had to be reduced to a low level to avoid mass transport limitation. Binding reactions run at different flow rates, and use of the BIAsimulation software indicated that none of the data used for estimation of K_Ds was influenced significantly by mass transport limitation. The interaction constants determined in this work are given in Table II.

The ability of each scFv to inhibit the binding of ^{125}I-labelled IFNγ to human Raji cells (Aguet and Merlin, 1987), which overexpress the IFNγR, was also tested by radioimmunoassay (Figure 3). The concentration of scFv needed to reduce the amount of bound [^{125}I]IFNγ by 50% (IC_{50}) is also given in Table II.

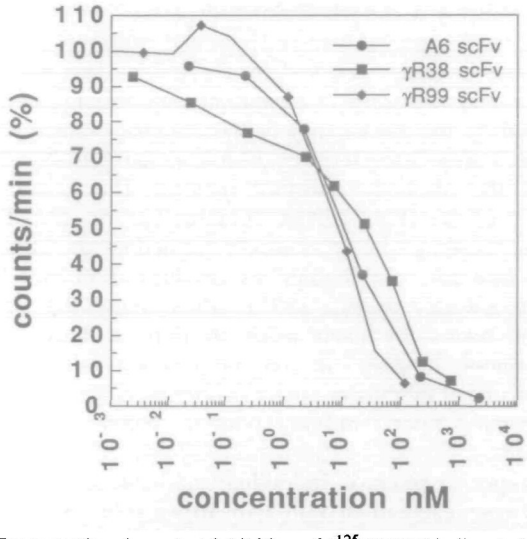

Fig. 3. Concentration-dependent inhibition of [^{125}I]IFNγ binding to human Raji cells by the A6, γR38 and γR99 scFvs using radioimmunoassay (see Materials and methods).

Discussion

The Fv region, comprising the entire variable region of the H- and L-chains, is the smallest fragment of an antibody that contains an intact copy of the antigen binding site. As such, it can be expected to maintain consistently the antigen-binding specificity and affinity of the whole antibody (Raag and Whitlow, 1995). Further dissection of the Fv fragment may lead to isolated V_H- or V_L-chains, which in some cases have been observed to retain a high antigen-binding affinity (Ward *et al.*, 1989; Monfardini *et al.*, 1995). However, the production of individual V_H-chains can be problematic owing to low expression and poor solubility. Attempts have been made to engineer the exposed interface on V_H for the light chain (Davies and Riechmann, 1994) to increase hydrophilicity, as seen in camelid antibodies which occur naturally as heavy chains without a light chain partner (Hamers-Casterman *et al.*, 1993).

In this work, three scFv fragments, derived from mAbs A6, γR38 and γR99, have been produced in *E.coli*, and isolated as soluble monomeric proteins that retain a high affinity for the recombinant human IFNγR (Table II). The relative affinities of the intact mAbs for the IFNγR, measured in earlier work using a laboratory-built SPR biosensor (Williams *et al.*, 1995), are similar within a factor of 2–3 to those measured in this work for the scFvs on a Pharmacia BIAcore instrument. The A6 and γR38 scFvs, which bind the N-terminal Ig-like domain of the IFNγR, have dissociation constants (K_Ds) of 20 and 28 nM, respectively. The γR99 scFv binds an epitope in the membrane proximal domain of the receptor, which includes residues in the interdomain linker region (Ruegg *et al.*, 1995), with a K_D of ~1 nM. Moreover, as found in earlier work with intact mAbs, the higher relative affinity for the immobilized receptor shown by the γR99 scFv, under these conditions, arises largely from an increased k_{on} rather than a lower off-rate.

The soluble scFvs also retain the ability of the whole mAbs to inhibit the binding of IFNγ to the native receptor on human Raji cells (Table II). The inhibitory activities expressed as IC_{50} values lie between 9 and 26 nM, which is approximately an order of magnitude weaker than the IC_{50}s seen for the intact mAbs (Bridges *et al.*, 1995). This is most likely due to an

avidity effect with the whole antibody, since the Fab fragments derived from each mAb have IC_{50}s that, within a factor of 2, are similar to those observed for the scFvs (data not shown). These results also show that the inhibitory effect of each mAb is caused by the association of the variable domain with the receptor, rather than steric exclusion of the hormone binding site by the antibody constant regions. The X-ray crystal structure of the IFNγ–IFNγR complex has revealed that the hormone binding site is centrally located in the rod-shaped IFNγR molecule and comprises residues from both Ig-like domains (Walter *et al.*, 1995). Most likely the structural epitopes bound by each mAb overlap, at least partially, the hormone binding site on the receptor, although other mechanisms of inhibition, such as allosteric changes in receptor conformation upon antibody binding, cannot be ruled out at present.

Attempts to produce individual, native-like V_H- and V_L-chains from each mAb failed to afford soluble protein that bound to the IFNγR. Of the V_H proteins, only those from antibodies A6 and γR38 could be isolated directly in a soluble form, following immunoaffinity chromatography using an anti-C-terminal peptide tag mAb. The γR99 V_H gave no soluble tag-positive protein in the expression system used here. To overcome potential problems arising from low solubility and non-specific interactions through the V_L interface, triple G44E, L45R and W47G mutations were introduced into each V_H-chain by site-directed mutagenesis, in analogy with camelid antibodies (Davies and Riechmann, 1994). All three 'camelized' V_H-chains could be produced in a soluble form in *E.coli* HB2151 following induction with IPTG. However, none of these proteins retained significant IFNγR binding activity. No IFNγR binding activity was observed with V_L-chains produced in this way, or with linear and cyclic peptides derived from the individual CDR sequences of each mAb (Table I).

The inability of the linear and cyclic peptides derived from individual CDR sequences (see Table I) to bind the IFNγR is most likely due to the absence in each of a sufficient number of the key residues that contribute significantly to the energetics of receptor binding, and/or to the fact that these are not displayed in the correct conformation. This point may be addressed quantitatively in future work by detailed structural and thermodynamic studies on native and mutant Fv–receptor complexes. This should now be feasible with the ability to produce both the mAb Fv fragments and native-like IFNγR fragments in *E.coli*, combined with the application of NMR and crystallographic techniques for structure determination.

The *de novo* design of new small-molecule ligands for the IFNγR, that block the cytokine binding site and act as antagonists, remains a considerable challenge. Conceivably, such molecules may be derived by design of small-molecule peptide mimetics of the key CDR loops derived from the mAbs described here. However, detailed mutagenesis studies on two different Fv fragment–protein antigen complexes (Hawkins *et al.*, 1993; Kelley and O'Connell, 1993) have shown already that the antibody residues which appear to be most important for antigen binding are grouped near the centre of the interface, and are located in more than one CDR loop. If these findings prove to be common, it seems likely that a general approach to mimetic design must take into account the necessity of retaining groups located in several CDRs to achieve high antigen-binding affinity. Possible solutions to this design problem may be found by grafting multiple loops on to alternate protein frameworks, for example a designer minibody (Martin *et al.*, 1994), or possibly by their incorporation into template-assembled synthetic proteins (Mutter and Vuilleumier, 1989).

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