MOLECULAR CLONING, EXPRESSION AND MUTAGENESIS OF AN
ANTI-INSULIN SINGLE CHAIN Fv (scFv)

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Abstract—The immunoglobulin variable region genes of a murine anti-insulin IgG-producing
hybridoma were rescued and cloned into a bacterial expression vector. The variable regions of the
gamma heavy chain and the kappa light chain were expressed independently and together as a single
chain antibody (scFv). The variable heavy chain alone demonstrated the ability to bind to insulin.
The kappa light chain did not show any binding activity towards insulin. The scFv was constructed
by PCR assembly using a (Gly,Ser), linker between the carboxyl end of the variable heavy chain
and the amino terminus of the kappa light chain. The scFv bound insulin at an IC50 of 3.5 x 10-6 M
whereas the parent antibody bound insulin at 1.0 x 10-7 M. Mutagenesis of the variable heavy chain
complementarity determining regions (CDR) indicated that CDR1 and CDR3 were important for
binding to insulin. Position 99 in CDR3 of the heavy chain was found to be a critical position for
the ability of the scFv to bind to insulin.

Key words: single chain Fv, anti-insulin.

INTRODUCTION

Gene cloning technology has allowed many groups to
rescue and express immunoglobulin genes (Better et al.,
1988; Bird et al., 1988; Hoogenboom et al., 1991; Huston
et al., 1988; Larrick et al., 1989; Skerra and Pluckthun,
1988). Single chain Fvs (scFv) are antigen-binding pro-
teins, composed of immunoglobulin variable heavy (VH)
and variable light (VL) chains joined together by a
flexible peptide linker. ScFvs have been constructed from
both monoclonal antibody-producing hybridomas (Huston et al., 1988) and immunoglobulin cDNA
libraries (Barbas et al., 1991; Marks et al., 1992). Rescue
and molecular cloning of immunoglobulin variable region genes into expression vectors allows one to gain
access to immunoglobulin antigen-binding genes and
their gene products. Expression of immunoglobulin
variable-regions also provides a means to observe and
characterize the antigen binding activity of the antibody
fragments. The immunoglobulin variable region genes
can also be manipulated with the intent of improving
binding capability, humanizing the antibody (Riechmann et al., 1988) or identifying critical residues
involved in binding to antigen (Hasemann and Capra,
1991). Expression of the antibody genes in bacteria or on
the surface of bacteriophage, allows one to evaluate gene
products quickly and efficiently (Marks et al., 1992).

In this study our objective was to identify the specific
immunoglobulin variable regions, complementarity
determining regions, and residues involved in binding to
an antigen. To accomplish this objective, a murine
anti-insulin IgG 1-producing hybridoma (Schroer et al.,
1983) was obtained from the American Type Culture
Collection (ATCC). This antibody was designated
HB125 by the ATCC. This antibody recognizes an
assembled epitope on insulin; HB125 will not bind to
insulin unless both A and B chains form the intact
molecule. The gene usage of HB125 IgG has been
studied by Ewulonu et al. (1990). The structures of
HB125 VH and VL have also been modeled by Nell et al.
(1992) and Tanner et al. (1992) in two reports. However,
their predictions were not tested biologically. In this
study we have constructed a scFv and performed site-
directed mutagenesis to determine immunoglobulin
variable region residues involved in binding to insulin.

METHODS

Messenger RNA isolation and cDNA synthesis

Invitrogen's Fast Track kit was used to isolate mRNA
and synthesize cDNA according to the instructions
provided with the kit. A brief explanation is provided
below. Hybridoma cells were cultured in RPMI 1640
medium containing 10% fetal calf serum and 100 μg/ml
gentamycin sulfate in a 5% CO2 humidified chamber.
Cells were grown to a density of 1 x 106 cells/ml after
which 10 ml of cells were pelleted by centrifugation at
1000 g. The cells were washed once in phosphate
buffered saline and resuspended in lysis buffer in the
presence of protease K at 50°C for 30 min. The
chromosomal DNA was fragmented with a 20-gauge
needle and syringe so that the mRNA could be recovered
with oligo dT cellulose. The mRNA was incubated with the oligo dT cellulose and then eluted from the oligo dT cellulose in distilled water and reverse transcribed for 2 hr at 42°C using oligo dT as the reverse transcriptase primer to synthesize cDNA from the mRNA template. Reverse transcriptase was subsequently inactivated for 2 min at 99°C.

Polymerase chain reaction (PCR) of immunoglobulin variable regions

PCR amplification (Saiki et al., 1988) was performed for both immunoglobulin heavy and light chain variable region genes using cDNA as a template and oligonucleotide primers corresponding to the 5' and 3' sense and anti-sense sequences of the immunoglobulin variable regions, respectively. Table I shows the primer sequences used for the initial amplification from cDNA. PCR was performed according to standard protocols using Taq thermostable DNA polymerase. PCR products were visualized on a 1% agarose gel stained with ethidium bromide.

Cloning and sequencing of immunoglobulin variable regions

Since Taq was used to amplify the Ig variable regions, the PCR products were ligated into a sequencing vector (pT7 blue) with overhanging 5' thymidines (Marchuck et al., 1989) so that the 3' adenosines on the PCR products could base-pair with the vector. Ligations and subsequent transformations into Novablue cells (Novagen) were performed according to the protocols described in Ausubel et al. (1989). Transformants containing insert were identified by white colonies (plus insert) versus blue colonies (minus insert) and the ability to grow on LB plates containing 50 μg/ml carbenicillin. DNA was then digested with XbaI and Sac1 which flank an XhoI site were added to HB125 V, template along with the appropriate mixtures of dNTPs, PCR buffer and Taq DNA polymerase. For the V, PCR a 5' (Gly, Ser) leader sequence and a 3' anti-sense primer with an NcoI restriction endonuclease site were added to HB125 V, template along with the appropriate mixtures of dNTPs, PCR buffer and Taq DNA polymerase. The resultant scFv was cloned into pT7 blue, sequenced and then the scFv was inserted into the expression vector using the methods stated for V, and V, above.

Expression of immunoglobulin fragments in E. coli

PET21d or 22b plasmid DNA containing immunoglobulin variable regions was isolated from Novablue cells and purified on glass beads (Gene Clean, Bio 101). The plasmid DNA was then used to transform E. coli BL21(DE3) cells (Novagen). BL21(DE3) cells carry a chromosomal copy of the T7 RNA polymerase which is inducible by IPTG via a lambda phage lysogen (Novy, 1992). BL21(DE3) cells transformed with the expression plasmid containing either V, or scFv were grown at 37°C, 200 rpm to mid log after which IPTG was added for a final concentration of 1 mM and the cells further

Double stranded sequencing (Sanger et al., 1977) was performed using modified T7 DNA polymerase (United States Biochemical) and Sequetide (Dupont). DNA sequences obtained were compared to Genbank to ensure that they were anti-insulin immunoglobulin variable regions.

Both V, and V, were digested out of pT7 blue with NcoI and XhoI. V, and V, inserts were separated from the parent plasmids by agarose gel electrophoresis, purified on glass beads (Gene Clean, Bio 101) and ligated into an NcoI/XhoI digested expression vector (pET21d or pET22b, Novagen). Novablue cells were transformed with 50% of the ligation reaction and transformants were identified on carbenicillin plates.

Construction of scFv antibody fragments

HB125 V, and V, PCR products were linked together by three repeating units of Gly, Ser using PCR-based single overlap extension (Horton et al., 1989; Yon and Fried, 1989). For the V, PCR a (Gly, Ser) anti-sense 3' primer and a 5' sense primer with an NcoI restriction endonuclease site were added to HB125 V, template along with the appropriate mixtures of dNTPs, PCR buffer and Taq DNA polymerase. The resultant scFv was cloned into pT7 blue, sequenced and then the scFv was inserted into the expression vector using the methods stated for V, and V, above.

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Table 1. Oligonucleotide PCR primer sets for initial amplification of HR125 immunoglobulin variable regions from cDNA. Nomenclature: I = inosine, R = A or G, W = A or T, Y = C or T, D = A or G or T, S = C or G, V = A or C or G, M = A or C, B = C or G or T. Numbers in parentheses indicate codon numbering according to Kabat et al. (1991). Negative numbers correspond to leader sequences

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Sequence</th>
<th>Codon Number</th>
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<tbody>
<tr>
<td>5' murine V, leader (sense)</td>
<td>5'-ACTAGTGCACTGGCTTGGGTGGGAACTTGCTATTTCG-3'</td>
<td>-20 to -11</td>
</tr>
<tr>
<td>3' murine V, leader (anti-sense)</td>
<td>5'-GTTGCACTGGCTTGGGTGGGAACTTGCTATTTCG-3'</td>
<td>119-126</td>
</tr>
<tr>
<td>5' murine V, leader (sense)</td>
<td>5'-ACTAGTGCACTGGCTTGGGTGGGAACTTGCTATTTCG-3'</td>
<td>-22 to -13</td>
</tr>
<tr>
<td>3' murine V, leader (anti-sense)</td>
<td>5'-AGGTAGAAGGGTGGTAGGTCATTCGAACCC-3'</td>
<td>116-122</td>
</tr>
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</table>
incubated for 3–5 hr. Samples of the cultures were taken at various time points prior to and during induction with IPTG for SDS-PAGE analysis. Except where stated, all SDS-PAGE was performed under reducing conditions.

**Preparation of bacterial cell lysates for Ig fragment isolation**

Five hour cultures (50 or 100 ml) containing E. coli producing either VH or VL Ig fragments were subjected to centrifugation for 10 min in an SS-34 rotor at 8000 rpm. The cell pellet was washed twice in PBS and sonicated three times for 15 sec each to solubilize the Ig fragments. The lysate was then incubated at 4°C for 4 hr or overnight. Finally the urea solubilized cell pellet was subjected to centrifugation at 12,000 rpm in an SS-34 rotor for 10 min and the supernatant was removed for SDS-PAGE analysis or purification.

**Superose 12 column fractionation**

Bacterial cell lysates solubilized in 6 M urea were filtered through a 0.45 μm membrane and loaded onto a 32 cm Superose 12 molecular weight sieving column in a maximum volume of 1 ml. The elution solvent was 2 M urea in PBS. One or two milliliter fractions were collected and analysed by SDS-PAGE.

**Purification of immunoglobulin fragments on a nickel–agarose column**

A nickel–agarose column was used to purify the Ig fragments from the bacterial cell lysates. The nickel–agarose column was prepared by charging the metal chelating linker on the agarose with 50 mM NiSO$_4$·6H$_2$O (Porath and Olin, 1983; Smith et al., 1988). The bacterial cell lysates were filtered through a 0.45μm filter and loaded onto the nickel–agarose column which was pre-equilibrated with binding buffer (6 M urea, 50 mM NaCl, 5 mM imidazole, 20 mM Tris–HCl, pH 7.9). The purity of the fractions were then analysed by SDS-PAGE followed by Coomassie Blue staining. Fractions containing purified proteins were pooled, diluted to a concentration of less than 150 μg/ml and dialysed against 1 l of refolding buffer for a minimum of 4 hr with four buffer changes.

SDS-PAGE was performed, essentially, as described by Laemmli (1970). For SDS-PAGE under reducing conditions, 10% 2-mercaptoethanol was included in the sample buffer. Non-reduced SDS-PAGE sample buffer did not contain 2-mercaptoethanol.

**Variable heavy chain inhibition ELISA**

Fractions from the Superose 12 gel filtration column were dialysed against PBS and coated onto an ELISA plate for 1 hr and then the plate was blocked using PBSTw-0.1% gelatin. Unlabeled insulin (40 μg/ml) was added to one set of fractions while 0.1% gelatin was added as a control to the other. After a 1 hr incubation, the plate was washed once in PBSTw-0.1% gelatin and 4 μg/ml of biotinylated insulin was added in either 0.1% gelatin or in the presence of 40 μg/ml of unlabelled insulin. One hour later the plate was washed three times with PBSTw-0.1% gelatin and streptavidin coupled to alkaline phosphatase was added. The plate was incubated and washed as before after which 1 mg/ml p-nitrophenyl phosphate (pnp) substrate in carbonate buffer pH 9.6 was added and the optical densities at 405 nm were quantified on a Titertek multiskan ELISA reader.

**Biotinylation of immunoglobulin fragments**

Purified fractions containing immunoglobulin fragments (e.g. VH, VL or scFv) were dialysed against 2 l of 50 mM NaHCO$_3$, pH 8.0 for at least 6 hr. The protein content of the purified material was then quantified (Bradford). NHS-LC-Biotin was then solubilized in 50 mM NaHCO$_3$, pH 8.5 and added to the dialysed fractions such that the molar ratio between biotin and immunoglobulin fragment was approximately 14:1. Biotinylation was performed at room temperature for 2 hr after which Tris–HCl, pH 7.9 (15 mM final) was added to stop the biotinylation reaction. The immunoglobulin fragments were tested in ELISA against a control biotinylated molecule.
Insulin–agarose purification of HB125 scFv

Bacterial cell lysates solubilized in 6 M urea were dialysed overnight, stepwise against 41 of 2 M urea in PBS, 2 M urea, 0.1 mM glutathione (reduced), 5 mM dithiothreitol (DTT), and PBS alone. After dialysis, precipitated material was removed by centrifugation at 12,000 rpm for 10 min in an SS-34 rotor. Soluble material containing HB125 scFv was filtered through a 0.45 μm membrane and loaded onto a PBS-equilibrated insulin–agarose column. The column was washed with 20 bed volumes of PBS. HB125 scFv was eluted from the column with 5 ml of 100 mM glycine, pH 2.3 in fractions of 1 ml each. Fractions were neutralized to pH 7.0 with 1 M Tris-base. Ten microliters from each fraction were analysed by SDS-PAGE followed by Coomassie Blue staining.

Single chain Fv insulin binding ELISA

Purified scFv wild type and scFv mutants were serially diluted and incubated in a 96-well microtiter ELISA plate coated with 1 μg/ml insulin. The plates were blocked with PBSTw-gel at 37°C for 1 hr. The plates were then washed twice with PBSTw-gel followed by the addition of streptavidin-horseradish peroxidase (HRP). The plates were further incubated for 1 hr at room temperature and washed three times as before. Finally, TMBZ substrate was added to the plates and the optical density of each well was quantified on an ELISA reader.

Single chain Fv inhibition assays

To determine 50% inhibitory concentrations (IC₅₀) for the scFv by insulin, ELISA plates were coated with

![Fig. 2.](image-url)

(A) Nucleotide and amino acid sequence of HB125 heavy chain variable region. Frameworks are designated by arrows and CDRs are boxed. (B) Nucleotide and amino acid sequence of HB125 kappa light chain variable region. Frameworks are designated by arrows and CDRs are boxed.
Anti-insulin scFv expression and mutagenesis

Fig. 3. (A) SDS-PAGE analysis of HB125 V₅₇-producing cultures in pET22b. Lane 1, molecular weight markers; lane 2, pET22b vector alone cell lysate, + IPTG; lane 3, HB125 V₅₇ cell lysate, + IPTG; lanes 4-6, HB125 V₅₇ cell lysates, 1, 2 and 4 hr post induction; lane 7, HB125 V₅₇ 4 hr supernatant; lane 8, HB125 V₅₇ 4 hr cell lysate (cell lysate in the presence of lysozyme); lane 9, HB125 V₅₇ 4 hr cell lysate; lane 10, HB125 V₅₇ 4 hr culture supernatant (cell lysate in the presence of lysozyme); lane 11, lysozyme treatment of 4 hr HB125 VH cell lysate; lane 12, urea solublized HB125 V₅₇ 4 hr cell lysate. (B) SDS-PAGE analysis of PET 21d bacterial expression of HB125 V₅₇, variable kappa chain and scFv. Lane 1, molecular weight markers; lane 2, pET21d transformed bacterial cell lysate, no insert; lane 3, HB125 V₅₇ expressed from pET 21d transformed cells; lane 4, HB125 V_kappa expressed from pET 21d transformed cells; lane 5, HB125 scFv expressed from pET 21d transformed cells.

The plate was washed, streptavidin-HRP was added and developed using TMBZ substrate as stated above. Optical densities were quantified at 450 nm. Percent inhibition by insulin was calculated as follows: 1 - (O.D. antibody with inhibitor)/(antibody without inhibitor) × 100%. Percent inhibition was plotted against each concentration of insulin and IC₅₀s were determined from the graph.

PCR-based mutagenesis

Multiple site-directed mutagenesis of the immunoglobulin CDRs was performed using PCR single overlap extension (PCR soeing). Overlapping sense and antisense primers were designed with one or more mutations which changed amino acids in the CDRs. An example is shown in Fig. 1.

RESULTS

Cloning and expression of HB125 variable gamma chain, variable kappa chain and scFv

Messenger RNA was isolated from anti-insulin producing hybridoma cells, cDNA was synthesized and PCR was performed using immunoglobulin V-region specific 5' sense and 3' antisense oligonucleotide primers. After PCR amplification of the heavy and light chain variable region genes, the PCR products were ligated into a sequencing vector (pT7 blue). The sequences of the antibody fragments were determined to ensure that immunoglobulin was specifically amplified and cloned. The nucleotide and amino sequences of the variable heavy and variable light chains are shown in Fig. 2. The heavy chain contains the entire V-region plus 16 amino acids from constant heavy 1 (C₅₇₁). The light chain contains the entire variable region plus two amino
acids from constant kappa 1 (C\textsubscript{k}1). The sequence of both V\textsubscript{H} and V\textsubscript{k} have been reported previously except that CDR3 of HB125 V\textsubscript{H} was not included in the report (Ewulonu et al., 1990). HB125 V\textsubscript{H} CDR3 was found to be very important in this study.

The immunoglobulin V-regions were cloned into the pET expression vector and BL21 (DE3) E. coli cells were transformed with the variable heavy, variable kappa and scFv containing vectors. Induction with IPTG resulted in over-production of the immunoglobulin fragments by the bacteria. Although the production rate could not be measured with specific anti-sera, the purified protein was quantitated. Variable heavy-producing cultures produced between 5 and 10 \mu g/ml.

Comparison of expression vectors containing or lacking pel B signal peptide

Initially the variable heavy chain and scFv were cloned into the expression vector, pET22b (plus signal peptide). After the cultures were induced with IPTG, samples of the cultures were taken at various time points. SDS–PAGE analysis of the cell lysates and the culture supernatants indicated that no variable heavy chain or scFv was secreted into the supernatant (Fig. 3A). All of the protein of interest remained associated with the cell. As shown in Fig. 3A, V\textsubscript{H}-producing cell lysates are represented as plus and minus signal peptide. Even after lysozyme treatment of the cell pellet (lanes 10 and 11), the immunoglobulin fragments were not liberated from the cell lysate, suggesting that they were not soluble and were located in inclusion bodies inside the cells as reported by others (Huston et al., 1991). HB125 scFv was expressed in pET 22b with the same result; the protein was not soluble and the pel B signal peptide was not cleaved (data not shown).

Since the immunoglobulin fragments of this particular antibody were not secreted from E. coli via pel B signal peptide, the genes were cloned into pET21d (Novagen), an expression vector identical to pET22b, but lacking the pel B signal peptide. As shown in Fig. 3B, expression of all antibody variable region genes from pET21d resulted in antibody fragments without pel B signal peptide.

Purification of anti-insulin V\textsubscript{H}

IPTG-induced liquid cultures were pelleted by centrifugation and sonicated in 6 M urea as described in Materials and Methods. The supernatant was obtained and run through a Superose 12 gel filtration column. Fractions were collected and analysed by SDS–PAGE (data not shown).

Antigen-binding activity of anti-insulin V\textsubscript{H}

HB125 V\textsubscript{H} inhibition ELISA was performed according to the procedures in Materials and Methods. Fractions 47–53 from the Superose 12 column were dialysed in PBS and directly coated onto an ELISA plate. Using unlabeled insulin as the inhibitor, it was observed that HB125 V\textsubscript{H} alone, coated onto the ELISA plate, could bind to biotinylated insulin and was inhibited by insulin itself. The results of this inhibition are shown in Fig. 4. The binding of HB125 V\textsubscript{H} was weak compared to HB125 parent antibody. The difference in insulin binding capacity by each fraction reflects the different amounts of protein in each fraction. For example, fractions 47–51 contained more protein than fractions 52 and 53. Fraction 49 was inhibited by 82% from binding to labeled insulin.

HB125 V\textsubscript{k} expression and purification

The HB125 kappa chain variable region was expressed in BL21(DE3) bacteria using the same procedures as HB125 V\textsubscript{H}. Interestingly, V\textsubscript{k} expression in 100 ml of E. coli culture occurred at a high level. Over 2.7 mg was purified from the cell lysate of a 100 ml HB125
Expression and purification of HB125 scFv

The assembled scFv PCR product was cloned into the pET 22b vector via the 5' NcoI and the 3' XhoI sites at the ends of the V<sub>H</sub> and V<sub>K</sub>, respectively. Then the vector was used to transform BL21(DE3) host cells for expression. Figure 5 shows expression of HB125 scFv from pET 22b in BL21(DE3) cells solubilized in urea in...
Table 2. HB125 scFv CDR3 mutagenesis. Mutations were made in CDR3 in the heavy chain of HB125 scFv. Binding was characterized by an insulin inhibition ELISA. Mutant amino acids are underlined.

<table>
<thead>
<tr>
<th>HB125 scFv mutants CDR3 mutations</th>
<th>Binding to insulin</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDR3 wild type</td>
<td>GYGKGYFDV</td>
<td>35 nM</td>
</tr>
<tr>
<td>CDR3 mutant no. 1</td>
<td>GFGEJYFDV</td>
<td>Loss of binding</td>
</tr>
<tr>
<td>CDR3 mutant no. 2</td>
<td>GFGEGYFDV</td>
<td>20 nM</td>
</tr>
<tr>
<td>CDR3 mutant no. 3</td>
<td>GFGMYFDV</td>
<td>10 nM</td>
</tr>
<tr>
<td>CDR3 mutant no. 4</td>
<td>GFGETYFDV</td>
<td>Loss of binding</td>
</tr>
</tbody>
</table>

Lane 2. Urea solubilized cell lysates were dialysed in PBS (lane 3) and loaded onto an insulin-agarose column for purification of the scFv. Running the scFv-containing cell lysates through the insulin-agarose column permitted both purification of the scFv and a check to ensure retention of anti-insulin binding activity. It was observed that the majority of HB125 scFv was eluted in the second fraction with very little in the third fraction, and none in fractions 4–6. The SDS–PAGE in Fig. 5 also shows a doublet at approximately 34 kD and 32 kD which corresponds to HB125 scFv plus signal peptide and scFv minus signal peptide indicating that the pelB signal peptide did not interfere with the capacity to bind to insulin. However, the decision was made to express the scFv from the vector without the pelB signal peptide.

Purification of recombinant immunoglobulin V-regions on a nickel-agarose column

Immunoglobulin fragment-producing bacterial cultures were processed as described in Materials and Methods. Various commercially available anti-sera were characterized for the ability to bind to HB125 V<sub>H</sub>, V<sub>K</sub> and scFv for affinity purification and detection purposes. No anti-sera were found which had specificity for the variable regions, so it was necessary to obtain an alternative method for purifying the HB125 variable regions. Since the immunoglobulin fragments produced from the pET 21d transformed cells contained a carboxyl (His)₆, it was possible to purify them on a nickel-agarose column using immobilized metal affinity chromatography (IMAC) (Smith et al., 1988). Figures 6A and 6B show SDS–PAGE analysis of the purification of HB125 V<sub>H</sub> and scFv, respectively. In Fig. 6A, lanes 2–5, nearly all of HB125 V<sub>H</sub> was purified from the cell lysate. Lanes 6–9 represent fractions 1–4 from the nickel-agarose column elution profile. A few faint contaminating bands can be seen in lane 6 of Fig. 6A, but the main V<sub>H</sub> band is clearly prominent at approximately 17 kD. As shown in the gel, all of the V<sub>H</sub> immunoglobulin fragment was eluted from the column in the first 1 ml fraction. A faint signal eluted at approximately 31 kD in lane 6. Nearly all of the scFv was purified (lane 6) from the cell lysate (lane 2), however, a small amount of the scFv band can be seen in lane 5 which represents the 60 mM imidazole column wash. In addition, another band can be seen in lane 6 just below the main band, at approximately 79 kD. This band was not characterized, but it is most likely a degradation product from the scFv. It is probably not a bacterial contamination because it cannot be seen in the V<sub>K</sub> and V<sub>H</sub> nickel-agarose column purifications. HB125 V<sub>K</sub> could also be purified using the same methodology.
Anti-insulin scFv expression and mutagenesis

2

1.6

1.6

0.6

0.4

0.2

0 /

10

6 2.5 1.25 0.625 0.3125

Antibody concentration (µg/ml)

Fig. 9. Insulin binding capacities of HB125 scFv heavy chain CDR1 and CDR2 mutants. Biotinylated mutants were incubated with insulin-coated ELISA plates. The mutants were detected with streptavidin-alkaline phosphatase. IC50 of CDR2 mutant is shown in Table 3.

Because scFv dimers or multimers may affect affinity determinations, the scFvs were subjected to both reducing and non-reducing SDS-PAGE as shown in Figs 6C and D, respectively. There was not evidence of scFv dimer or multimer formation in the non-reduced gel compared to the reduced gel.

Site-directed mutagenesis of HB125 scFv

Mutational analysis of complementarity determining region 3 (CDR3) of HB125 scFv heavy chain variable region. As diagrammed in Materials and Methods, PCR single overlap extension (Horton et al., 1989; Yon and Fried, 1989) was employed in CDR mutagenesis studies. Initially, three amino acid mutations were made in the heavy chain of CDR3 in HB125 scFv: Y99 to F96, K98 to E95, and G99 to V99 (CDR3 mutant no. 1, Table 2). After cloning and expression of the scFv CDR3 mutant, it was tested for the ability to bind to insulin. Based upon initial analysis, the mutations made in the VH of CDR3 of HB125 scFv resulted in a complete loss of the ability to bind to insulin as shown in Fig. 7. However, when V99 was restored to G99 through the PCR mutagenesis process, while the two other mutations, F96 and E95, were preserved (mutant no. 2) complete binding activity to insulin was restored. In addition, there was no significant difference in the IC50 of insulin between wild type scFv and the CDR3 mutants (Fig. 8) and mutant no. 2, V99 to G99 restoration. At this point, questions about the nature of position 99 in CDR3 if the heavy chain of HB125 scFv arose. It was decided to generate two more HB125 scFv mutants and substitute alanine and threonine separately at position 99 in CDR3. Alanine was chosen because it is close in size to valine, but has a relatively hydrophobic character like glycine. A summary of the results of HB125 scFv variable heavy CDR3 mutagenesis is shown in Table 3. It was observed that the A99 mutant (no. 3) was able to bind to insulin, but the T99 mutant (no. 4) was not able to bind to insulin. This suggests that a small amino acid is necessary in position 99 in CDR3 of the heavy chain of HB125 for retention of insulin binding. However, more extensive mutagenesis needs to be performed to test the effects of charged residues at position 99.

HB125 scFv CDR1 and CDR2 mutagenesis in HB125 scFv

As controls for the CDR3 mutants, CDR1 and CDR2 were mutagenized. Three positions out of the five total residues in CDR1 of the heavy chain were changed. Aspartic acid at position 31 (D31) was changed to a lysine (K31), serine 33 (S33) was changed to glycine (G33) and histidine 35 (H35) was changed to an asparagine (N35). Since the amino acid sequence of CDR2 aligned very closely with many other antibody sequences in the Kabat database, only a glutamic acid at position 53 was changed to an asparagine, making CDR2 identical to CDR2 in the heavy chain of NC-41, an anti-neuraminidase antibody which has been crystallized. The mutagenesis was performed in the same manner as before. Both HB125 scFv CDR1 and CDR2 mutants bound to insulin as shown in Fig. 9, but the binding of the CDR1 mutant was weak. Figure 9 shows the results of HB125 scFv CDR1 mutant binding to insulin. Again, binding was weak. The degree of biotinylation of the CDR1 mutant was tested, but it showed that the biotinylation was equal to that of HB125 scFv wild type (data not shown). Because of this weak binding to insulin, inhibition curves could not be generated for the CDR1 mutant. However, the CDR2 V99 mutant showed nearly identical inhibition curves to the scFv wild type. Table 3 shows the IC50 values for the CDR1 and CDR2 HB125 VH mutants.

CONCLUSIONS

The two step cloning of immunoglobulin PCR produced worked very well. By first ligating the immunoglobulin PCR products into the T-vector, cloning efficiencies were 10- to 100-fold higher than digesting purified PCR products with the chosen restriction enzymes.

Table 3. IC50 values of CDR1 and CDR2 HB125 VH mutations. Mutant amino acids are underlined.

<table>
<thead>
<tr>
<th>Amino acid sequence</th>
<th>Affinity for insulin</th>
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<tbody>
<tr>
<td>HB125 scFv wild type</td>
<td>DYSMH WINTETGVPTYADDFKG</td>
</tr>
<tr>
<td>scFv CDR1 mutant</td>
<td>KYGMIN WINTETGVPTYADDFKG</td>
</tr>
<tr>
<td>scFv CDR2 mutant</td>
<td>DYSMH WINTNTGVPTYADDFKG</td>
</tr>
</tbody>
</table>
endonucleases and ligating into a pre-digested expression vector.

PCR assembly was used to construct HB125 scFv. This technique was surprisingly successful and simple to perform. Since HB125 V\textsubscript{H} contains 16 amino acids from CH\textsubscript{1}, it was uncertain as to whether the scFv would retain the ability to bind to insulin with the (Gly\textsubscript{9}, Ser\textsubscript{9}) linker. As reported (Huston \textit{et al.}, 1991), there is only a minimum length of 10 or 12 residues for a scFv linker. From analysis of crystal structures (Satow \textit{et al.}, 1986), a proposed linker must span at least 35 Å between the carboxyl and amino ends of the variable regions in order not to distort native Fv conformations. The glycine and serine residues in (Gly\textsubscript{9}, Ser\textsubscript{9}) would confer maximum flexibility and hydrophilicity so as not to constrain the scFv from binding to its antigen in an induced fit type of interaction. After PCR assembly of HB125 scFv, a band at the predicted molecular weight was observed, strongly suggesting that the new gene was assembled such that the (Gly\textsubscript{9}, Ser\textsubscript{9}) linker was flanked on both sides by V\textsubscript{H} and V\textsubscript{L}. Subsequent cloning, sequence analysis and protein expression proved that HB125 scFv was assembled correctly. Binding studies of the scFv demonstrated that the scFv retained its insulin-binding activity.

Solubility was a problem with the scFv. Upon dialysis of the urea-solubilized cell lysate into refolding buffers, there was considerable precipitation of the scFv. Even after purification of the scFv on the insulin agarose column (Fig. 2), the pure scFv fractions precipitated in the collection tube. Solubility problems for scFvs have been reported by other groups, as well (Cheadle \textit{et al.}, 1992; Huston \textit{et al.}, 1991). Several different conditions were used in attempts to renature the scFv from the urea-solubilized material. It was found that two rounds of 6 M urea solubilization and dialysis into refolding buffer at protein concentrations of less than 150 µg/ml gave the best yields of soluble, active scFv.

Monoclonal antibodies directed against the variable regions of heavy and light chains would be extremely useful reagents for detecting and purifying scFvs and antibody fragments. Anti-immunoglobulin variable region monoclonal antibodies would eliminate the need to label the antibody fragments prior to detection. Other groups have used a c-myc carboxyl terminal tag for which monoclonal antibodies exist for detection and purification (Marks \textit{et al.}, 1989). Attempts were made to couple horse radish peroxidase to nickel so that the antibody fragments containing a poly-histidine tag could be detected, but they were unsuccessful.

The IC\textsubscript{50} of the scFv was measured to be 35 nM, while the IC\textsubscript{50} for the hybridoma-derived HB125 IgG was measured to be 10 nM which is 3.5-fold stronger than the scFv. This was not surprising for two reasons. First because the hybridoma-derived antibody is bivalent, and second because the scFv is an artificial molecule created in the laboratory which may not have the same conformation as an intact IgG or even a Fab. Several attempts were made to cleave HB125 IgG with papain for analysis of a Fab, but they were unsuccessful. On the other hand, the scFv is monovalent while HB125 IgG is bivalent. The binding affinities of each are similar suggesting that the binding affinity of the paratope remained intact in the scFv. SDS–PAGE under non-reducing conditions indicated that scFv dimers or multimers were not present in the material used to determine IC\textsubscript{50}.

Three of the five positions in the heavy chain of HB125 CDR1 were mutated: aspartic acid at position 31 (D31) was changed to a lysine (K31), serine 33 (S33) was changed to glycine (G33) and histidine 35 (H35) was changed to an asparagine (N35). Binding studies with the CDR1 mutant showed that it bound much less strongly to insulin than the wild type scFv, but did not lose binding altogether. However, it lost enough activity so that IC\textsubscript{50}s could not be determined. The CDR2 mutant with one residue change (E35 to N35), however, bound to insulin with an IC\textsubscript{50} value of 27 nM.

Since CDR3 has been reported to be the most important region involved in binding to antigen (Kabat and Wu, 1991; Sheriff \textit{et al.}, 1987) and since the results had already implicated HB125 V\textsubscript{H} in binding to insulin, CDR3 of the heavy chain from HB125 scFv was chosen for mutagenesis. Residues Y99, K33 and G99 were changed to F99, E99 and W99, respectively. The remainder of CDR3, Y100, F100A, D101 and W102 was remarkably similar to several other antibodies in the Kabat database and was left unchanged. Since the insulin-binding ability of CDR3 mutant no. 1 (Table 2) was abrogated, it was decided to restore the mutated amino acids one by one to the original (wild type) starting with V99 until binding to insulin was regained. Restoration of V99 to W99 completely restored binding by HB125 scFv to insulin without having to change the other two mutated amino acids to the wild type sequence. This result suggests that a glycine at position 99 in CDR3 of the heavy chain is important for binding to insulin. The other two mutations, Y99 to W99 and K99 to E99 did not appear to affect binding to insulin because the peptide IC\textsubscript{50}s for the CDR3 V99 to G99 mutants were restored to those of the wild type scFv.

There is a precedent for one amino acid mutation causing loss of binding activity to an antigen (Hasemann and Capra, 1991). Interestingly, in the study by Hasemann and Capra, mutation of position 99 in CDR3 of the heavy chain caused a loss of binding by an anti-arsenate antibody. In addition, they found that mutation of position 96 in the light chain also abrogated binding to arsename.

By mutagenizing position 99 in CDR3, heavy chain of HB125 scFv to alanine (A) and threonine (T) the effects of amino acid hydrophobicity and size could be investigated in binding assays for position 99. The HB125 scFv A99 mutant demonstrated the ability to bind to insulin while the T99 mutant was not able to bind. This suggests that residue size, not hydrophobicity, is an important factor for HB125 scFv binding. The molecular weights of glycine and alanine are 75 and 89 daltons, respectively, versus valine and threonine which are 117 and 119 daltons, respectively. It cannot be concluded that size is the most important determinant for position 99 because
V<sub>99</sub> may also have caused a collapse of the CDR loop. However, it is unlikely that V<sub>99</sub> would have been buried back into the flanking frameworks because position 99 is exactly in the center of a large and otherwise hydrophilic CDR3 loop according to Kabat et al. (1991). More extensive mutagenesis of position 99 using different sized, charged residues as well as computer modeling of the mutants should be performed. Tanner et al. (1992) and Nell et al. (1992) have modeled the antigen combining site of HB125 and suggested that four polar residues in the light chain (S<sub>57</sub>, S<sub>66</sub>, Y<sub>91</sub> and S<sub>94</sub>) and two polar residues in the heavy chain (Y<sub>12</sub> and Y<sub>96</sub>) were important for binding to insulin. This report does not confirm these suggestions, but may support their conclusions that perturbations in CDR3 of the heavy chain may affect binding. Although there are no canonical models for the structure of heavy chain CDR3, position 99 may be a necessary structural position for this particular antibody. Alternatively, any residue larger than alanine at position 99 may cause steric hindrance resulting in loss of binding.

Investigation of specific antigen--antibody interactions at the molecular level will further a basic understanding of how other proteins fit together and cause a biological effect. If one can accurately predict specific interactions between two molecules based upon known interactions and primary sequence, agents could be designed and tested for specific therapeutic function. These agents may bind specifically to the paraotope of a lymphoma immunoglobulin or to a cell-surface receptor.

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REFERENCES


