REFERENCES


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Site-Specific Mutagenesis of Immunoglobulin Domains by Multiple-Fragment Homologous Recombination

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The polymerase chain reaction (PCR) is a widely used and versatile technique for the amplification and modification of immunoglobulin (Ig) genes. The rapidity and accuracy with which antibody genes can be modified in vitro has produced an assortment of novel antibodies. For example, PCR methods have been used for engineering IgGs with increased affinity to antigen, for “humanizing” antibodies and for modulating effector function (7–9).

As part of a more comprehensive study, we desired to introduce various site-specific mutations into the CH2 constant domain of human IgG1. Six specific amino acid residues distributed throughout the CH2 domain previously identified to play a role in immune effector function were marked as targets for mutagenesis (1,8,10). Five of the six residues were grouped into two clusters; one cluster consisted of two residues, two amino acids apart (Location 1 or L1), and the second cluster consisted of three residues spanning a sequence of five amino acids (L2). The remaining amino acid position (L3) brought the total to six residues. We were interested in constructing a panel of mutant CH2 domain IgGs consisting of each L mutation by itself as well as in combination with other L mutants (e.g., L1; L1 and L2; L1, L2 and L3 etc.).

Various in vitro methods have been described in which PCR is used to simultaneously introduce multiple distally located site-specific mutations within a gene sequence (2,3). Alternatively, an in vivo procedure termed recombination PCR has also successfully been used for rapidly and efficiently generating distally located site-specific mutations (4,5). Recombinant PCR uses *E. coli*’s recombination machinery to generate intact circular recombiant plasmids from a transfected mixture of linear PCR-generated product and

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linearized vector. In vivo recombination is mediated through the joining of nucleotide sequences designed into the 5’ ends of both PCR primers that are homologous to DNA sequences encoded by the vector. We describe an extension of the recombinant PCR procedure for simultaneously introducing complex combinations of mutations into an antibody CH2 domain.

Humanized BR96 variable (V) region heavy- and light-chain genes, previously cloned and coexpressed as an assembled active Fab fragment in an M13 phage expression vector, provided the starting material (9). The heavy- and light-chain V genes were amplified by PCR from a single-stranded M13 DNA template and subcloned by in vivo recombination (4) into vectors pD17-hG1a and pD16-hCκ to form pBR96-hG1a and pBR96-hCκ, respectively. pD17-hG1a and pD16-hCκ are eukaryotic Ig expression vectors.

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Figure 1. Outline of strategy for introducing multiple mutations by recombinant PCR. (A) Diagram of the 1.4-kbp IgG heavy-chain region showing the hinge, CH2 and CH3 domains as boxed regions. Site-specific mutations to be introduced into CH2 positions L1, L2 and L3 are encoded by complementary sets of mutant PCR primers (A1 and A2; B1 and B2; and C1 and C2). The asterisks (*) indicate the number of amino acid changes introduced at each L position. The two PCR primers, Rs (Recombination-sense) and Ra (Recombination-antisense), flank the Eco47III restriction sites and mediate homologous recombination with vector ends. The 3’ ends of the oligonucleotides are represented by arrowheads. (B) A three-way homologous recombination event between fragments RsA2, A1Ra and the linearized vector produces the L1 mutant IgG. Two distally located sets of mutations (L1 and L2) are simultaneously introduced by increasing the number of recombining PCR products as is shown in the four-way recombination of RsA2, A1B2 and B1Ra with vector to produce the L1 + L2 mutant IgG.
derived from pcDNA3 (Invitrogen, Carlsbad, CA, USA). pBR96-hG1a contains two \textit{Eco}47III restriction sites flanking the Ig hinge, CH2 and CH3 domains. The recipient vector was then prepared by digesting pBR96-hG1a with \textit{Eco}47III, isolating the vector backbone by agarose gel electrophoresis followed by extracting the vector DNA from the excised gel slice using the QIAquick™ Gel Extraction Kit (Qiagen, Chatsworth, CA, USA).

Our strategy for introducing multiple mutations within the Ig CH2 gene (Figure 1) relies on the in vivo homologous recombination of independently amplified PCR products with each other as well as with the pBR96-hG1a vector DNA. For introducing mutations at a single location, two PCR products are synthesized (Figure 1B). Introducing two distally located sets of mutations would require three PCR products (Figure 1B). One end of each PCR product is for recombining with a homologous end of the linear vector, and the other end, encoding the mutation(s) of interest, is for recombining with the neighboring PCR product. It might be possible to increase the number of distally located sets of mutations by increasing the number of PCR products proportionately. The recombination of neighboring PCR products occurs across the regions containing the desired mutations; therefore, the oligonucleotide primers encoding these ends (e.g., A1 and A2) contain complementary mutant residues. The mutagenic PCR primers contain at least 15 nucleotides of wild-type sequence flanking each side of the mutant residues for either priming the polymerization reaction or mediating recombination. Two 49-nucleotide PCR sense and antisense primers (Rs and Ra) contain sequences for recombining with the end regions of the \textit{Eco}47III-digested pBR96-hG1a vector.

Each L mutation was amplified in a separate PCR. The reaction conditions were 250 ng intact pBR96-hG1a DNA template, 10 µL of 10× \textit{Pfu} buffer (Stratagene, La Jolla, CA, USA), 10 nmol dNTPs, 200 ng each of the appropriate PCR primers, 10% dimethyl sulfoxide (ATCC, Rockville, MD, USA) and 2.5 U cloned \textit{Pfu} DNA Polymerase (Stratagene) in a 100-µL reaction volume. Samples were first denatured at 95°C for 5 min, annealed at 45°C for 5 min and extended at 72°C for 1 min followed by 25 cycles of denaturation at 94°C for 45 s, annealing at 45°C for 45 s, extension at 72°C for 1 min/kb, followed by a final extension at 72°C for 7 min in a DNA thermal cycler (Perkin-Elmer, Norwalk, CT, USA). The amplified products were purified from a 1% agarose gel, extracted with the QIAquick Gel Extraction Kit and quantitated.

<table>
<thead>
<tr>
<th>Mutant IgGs Constructed</th>
<th>PCR Fragments in Reaction</th>
<th>HR(^a) Events</th>
<th>HR(^a) Colonies Analyzed</th>
<th>Cloning Efficiency(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2</td>
<td>triple</td>
<td>24</td>
<td>45%</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>quadruple</td>
<td>24</td>
<td>29%</td>
</tr>
</tbody>
</table>

\(^a\) Homologous recombination
\(^b\) Number of clones containing 1.4-kbp insert/total number of colonies

Figure 2. \textit{Eco}47III restriction endonuclease analysis of DNAs prepared from colonies generated by multiple-fragment recombinant PCR. Lane M: 1-kb ladder DNA marker (Life Technologies). Lanes 1–12: twelve randomly selected colonies resulting from quadruple homologous recombination events were used to prepare plasmid and digested with \textit{Eco}47III. Clones 1, 2, 6 and 9 contain the fully assembled 1.4-kbp insert.
binding activity of the CH2 mutant IgGs, recombinant antibodies were transiently expressed in COS-7 cells (6). Miniprep DNAs from 6 clones derived from the triple recombination reaction and 6 clones derived from the quadruple recombination reaction (including the L1, L2 and L3 recombinant) exhibiting the diagnostic EcoRI restriction patterns were isolated, mixed with pBR96-hCκ DNA and used to co-transfect COS-7 cells. Supernatants (48 h spent) from 3-mL cultures secreted approximately 2–3 µg/mL Leα-reactive IgG (data not shown). The spectrum of Leα-binding activities was similar to that of native humanized BR96 IgG (data not shown), indicating that the homologously recombined antibodies did not acquire any gross mutations that affected antigen binding. To confirm that the desired CH2 mutations had been incorporated and to evaluate the recombined genes for misincorporated nucleotides, four of the clones producing functional antibody were sequenced using the Sequenase® Ver. 2 DNA Sequencing Kit (Amersham, Arlington Heights, IL, USA). Only one clone was found to contain any sequence errors, and this consisted of a single nucleotide change within the forward PCR primer used for mediating recombination with vector DNA. We are uncertain whether this error occurred during chemical synthesis of the oligonucleotide primer or during recombination in vivo, or was a result of misincorporation during the PCR, despite the fact that we used a thermostable polymerase with proof-reading activity.

We describe a recombinant PCR procedure for homologously recombining up to three separate PCR-generated mutated antibody sequence products into a eukaryotic expression vector for the rapid construction of engineered IgG molecules. The advantage of this approach is the ability to simultaneously introduce multiple distally located mutations with PCR products synthesized by a single round of PCR. Recombinant DNA molecules are produced with a reasonably high cloning efficiency and fidelity of correct nucleotide sequences. The ability to efficiently rejoin several distinct PCR products should permit combinatorial strategies for constructing complex mutated protein domains as well as broadening the number and location of desired mutations.

REFERENCES


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