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Acknowledgements

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ABSTRACT   Humanized antibody plasmid DNA was modified to allow the distance between the Fc fragment and antigen binding sites of immunoglobulin G (IgG) antibodies to be studied. Specific variable heavy (VH) and variable light (VL) genes were inserted into heavy and light chain plasmids so that dye molecules can be easily attached to the expressed protein, and further inspection of antibody structure and function can be conducted via single molecule Förster resonance energy transfer (FRET). First, VH and VL genes were inserted into humanized antibody plasmids through the technique of ligation. The ligation product was then transformed into Escherichia coli cells, allowing the success of the ligation reaction to be determined by methods of agarose gel electrophoresis and sequencing of the plasmid DNA.

INTRODUCTION/BACKGROUND

Antibodies are composed of four chains: two identical light chains of DNA and two identical heavy chains of DNA. The Fc fragment is the lower lobe of the antibody, and is attached to the two upper lobes, where antigen binding occurs, by a hinge region, as shown in Figure 1. The binding of effector molecules to the Fc region leads to an immune response within the body. Sugars within the Fc fragment help give structure to this region, presumably allowing effector molecules to more readily bind to this region, resulting in an immune response. When these sugars are removed, the Fc fragment is only supported by the hinge region, allowing the lobe to move freely, thereby reducing the binding affinity of effector molecules, such as Fc receptors.[1]

The particular sugars present within the Fc region do not always maximize the binding ability of the Fc region. The precise nature of the sugar molecules present in the Fc region has been shown to impact the binding of effector molecules,[2,3] leading to a desire to develop therapeutic antibodies containing sugars that best promote effector molecule binding. Scientists have been tediously working to develop therapeutic antibodies, or antibodies used for treatment of diseases, containing sugars within the Fc region that increase the binding affinity of the antibodies for effector molecules.[2-4] This process, known as glycoengineering, is not only time consuming, but also very expensive, as proteins containing sugars must be produced in mammalian cells. By developing a way to keep the Fc region stable and receptive to effector molecules without the need for glycoengineering or the use of mammalian cells, the tedious process of glycoengineering could be avoided.

A cheaper alternative to protein synthesis is expressing antibodies in E. coli, however, antibodies synthesized in E. coli do not have the sugars thought to be necessary for maximal binding of effector molecules. By mutating the amino acids present at the bottom tip of the Fc fragment (see Figure 4), Jung et al. have been able to stabilize the Fc region of IgG and achieve binding to

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effector molecules at a level similar to that observed in naturally occurring IgG.4 It is believed that because the mutations are so far from the binding sites for the effector molecules, a conformational change is occurring within the Fc region, so that it takes on a conformation similar to that of the Fc region when it contains sugars (Figure 4).4 By synthesizing these sugar free antibodies in E. coli, it may be possible to develop a cheaper route to therapeutic antibodies. While it has been suggested that the tight binding of the mutated protein arises from a conformational change, this has not been confirmed with structural studies. The structures of these mutated antibodies with and without the sugars present must be further examined.

One technique that can be used to do this is Förster resonance energy transfer (FRET). FRET can be used to examine the structure of the Fc region with and without the mutations introduced and with and without the sugar molecules present. FRET is also capable of determining the flexibility of a protein, as well as determining the number of conformations of a protein present. The technique involves placing a donor and an acceptor dye molecule on a protein. The donor absorbs light and can transfer this energy to the acceptor. The closer the acceptor is to the donor, the more likely it is that energy transfer will occur. By examining the relative amounts of fluorescence from the donor and the acceptor, the distance between the two can be calculated. In addition, the distance between the antigen binding sites and the Fc region is of interest, as it has been suggested that the Fc region curls up toward the antigen binding site to promote the binding of effector molecules

In order to attach a dye molecule to the antigen binding site, the DNA for the antigen binding site of a humanized (containing both human and mouse parts) IgG antibody was modified. This modification process was the goal of this research project.

EXPERIMENTAL

VL and the VH genes from the plasmids for the humanized antibody (see Figures 5 and 6) were cut and removed using restriction enzymes (HindIII and BamHI) and replaced with the VL and VH regions from a mouse antibody through the process of ligation. (see Figure 7) The VL and VH regions from the mouse antibody are desirable because they will produce a new antigen binding site on the Fc region, containing a reactive lysine residue, which is necessary for dye attachment.

The ligation products were then introduced into E. coli cells. By introducing the plasmid into E. coli cells, the DNA can be replicated as the cells divide and grow overnight. The DNA in any colonies observed was then isolated and examined to see if the ligation reaction was successful.

To see if the ligation reactions were successful, the polymerase chain reaction (PCR) was used to amplify the VL and VH regions that should be in the ligated plasmid DNA. In addition, restriction digests were used to cut out the VL and VH genes. The PCR products and restriction digest fragments were isolated, cleaned, and run on an agarose gel. An agarose gel separates substances by molecular weight. By running the different fragments and knowing how many base pairs each gene has, it is possible to tell if the reaction worked based on how far the bands are visualized down the gel. (see Figure 8). If the ligation was successful, bands should be visualized around 360bp. Single cut plasmid and uncut plasmid were also run on the gel for control samples.

The ligation and PCR reactions for the VL and the VH genes were performed separately. Gel results for the VH chain suggested that the ligation product was produced, while the gel results for the VL chain (data not shown) were inconclusive. A sample of the VH chain was then prepared and sent to Macrogen for sequencing. Because the desired DNA sequence is known, the sequencing results received from Macrogen could be compared to the desired sequence using CLC Sequence Viewer 6. Sequencing confirmed the PCR results, indicating that the ligation reaction was successful.

CONCLUSION

The VL and VH plasmids were ligated and analyzed several times. The VH ligation was determined to be a success by the agarose gel and sequencing results, whereas the VL ligation products were inconclusive from the agarose gel and therefore not sent out for sequencing. It is believed that because the VL plasmid contains a significantly larger number of base pairs than the VL insert, the ligase enzyme responsible for finding and connecting the vector and insert was unable to find the two vectors to combine them, and the ligation reaction did not proceed. Different techniques were incorporated into each new reaction, however, further troubleshooting throughout the entire ligation process proved unsuccessful. By successfully inserting the VH vector into the plasmid DNA of an IgG antibody, the dye molecule can be attached and FRET can be used to further understand the structure of the Fc region of antibodies for future therapeutic applications.
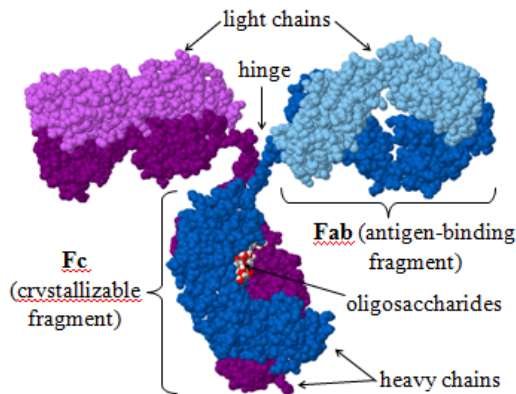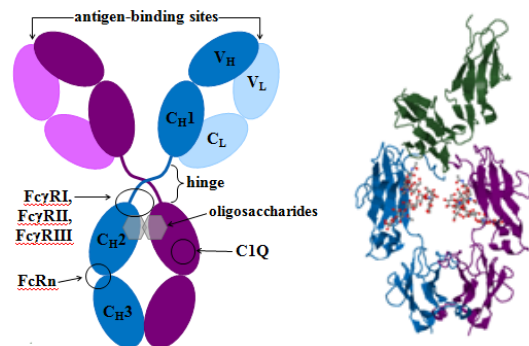

FIGURE 1

Crystal structure of an IgG antibody. The Fc region is attached to the two upper lobes by the hinge region. Antibody drawn from the PDB file 1IGT. Harris, L.J.; Larson, S.B.; Hasel, K.W.; McPherson, A. (1997) Biochemistry 36, 1581.

FIGURES 2, 3

2: Schematic of an IgG antibody showing the locations at which various effector molecules bind.

3: Crystal structure of human FcγRIII (green) bound to a human IgG Fc region (blue and purple). The binding occurs in the lower hinge and upper CH2 regions. Drawn from the PDB file 1E4K. 6Sondermann, P.; Huber, R.; Oosthuizen, V.; Jacob, U. (2000) *Nature* 406, 267.
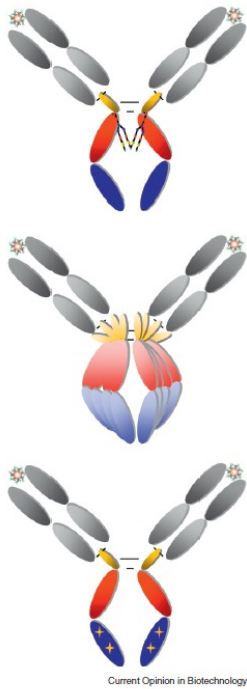
FIGURE 4

(a) Glycosylated IgG1 antibody showing a fixed confirmation
(b) Aglycosylated IgG1 antibody showing high flexible conformations.
(c) Engineered aglycosylated IgG1 antibody showing a fixed confirmations.
Jung ST, Kang TH, Kelton W, Georgiou G. *Curr. Opin. Che, Biol.* 2011, 22, 858–867.
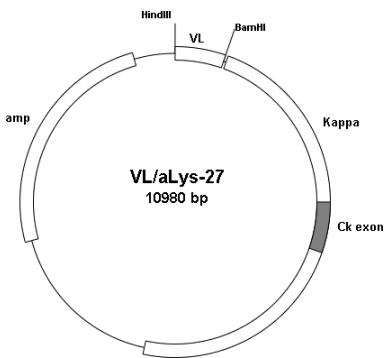

FIGURE 5

Plasmid map for the heavy chain of the humanized antibody showing the locations of the HindIII and BamHI restriction enzyme cut sites at either end of the VH gene. The locations of other genes for the heavy chain (CH1, hinge, CH2, and CH3) and the ampicillin resistance gene are also shown.
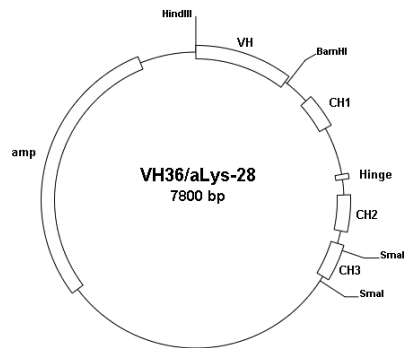

FIGURE 6

Plasmid map for the light chain of the humanized antibody showing the locations of the HindIII and BamHI restriction enzyme cut sites at either end of the VL gene. The Ck and ampicillin resistance genes are also shown.
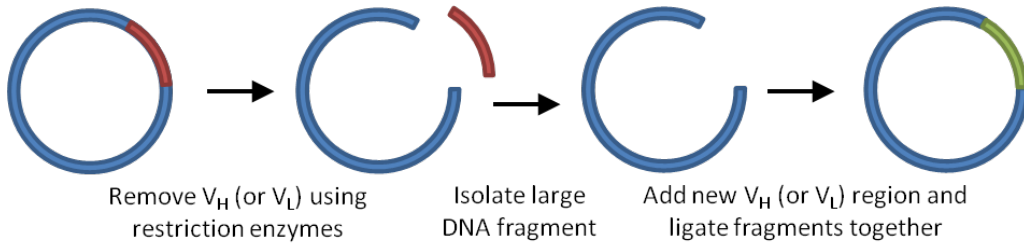
Remove V$_H$ (or V$_L$) using restriction enzymes

Isolate large DNA fragment

Add new V$_H$ (or V$_L$) region and ligate fragments together

FIGURE 7

Schematic showing the process used to alter the VH (or VL) gene of an antibody.
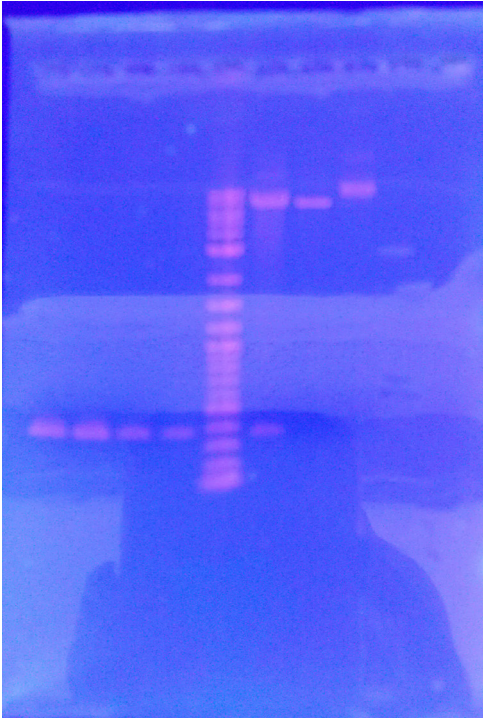

FIGURE 8

Stained agarose gel of the variable heavy chain from the VH36/aLys-28 plasmid. Lanes 1–4 represent the double cut variable chain region. Lane 6 represents the double digest of the VH fragment from the plasmid. Lane 7 is the single cut plasmid, and Lane 8 represents the uncut plasmid.

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Jung, ST.; Kang, TH.; Kelton, W.; Georgiou, G. *Curr. Opin. Che, Biol.* 2011, 22, 858-867.