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Technical note

A method for rapid, ligation-independent reformatting of recombinant monoclonal antibodies

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ABSTRACT

Recombinant monoclonal antibodies currently dominate the protein biologics marketplace. The path from target antigen discovery and screening, to a recombinant therapeutic antibody can be time-consuming and laborious. We describe a set of expression vectors, termed mAbXpress, that enable rapid and sequence-independent insertion of antibody variable regions into human constant region backbones. This method takes advantage of the In Fusion™ cloning system from Clontech, which allows ligation-free, high-efficiency insertion of the variable region cassette without the addition of extraneous amino acids. These modular vectors simplify the antibody reformatting process during the preliminary evaluation of therapeutic or diagnostic candidates. The resulting constructs can be used directly for transient or amplifiable, stable expression in mammalian cells. The effectiveness of this method was demonstrated by the creation of a functional, fully human anti-human CD83 monoclonal antibody.

1. Introduction

Over the past 10–15 years there has been a surge of interest in the use of recombinant monoclonal antibodies (mAbs) as therapeutic agents. In 2007, mAb sales in the USA alone exceeded $14 billion, with a year on year growth rate of 22% (Aggarwal, 2008). With the number of approved mAbs approaching 30 and hundreds of new candidates in the pipeline, this trend shows no signs of slowing. Most therapeutic recombinant mAbs are members of the IgG family and owing to their large size and complex glycosylation patterns, these molecules are currently produced in mammalian cells, with the vast majority utilizing Chinese Hamster Ovary (CHO) cells as the production host (Wurm, 2004).

The path from discovery to the clinic for a therapeutic, recombinant mAb can be a long and tedious process, often taking several years. The first step of this process involves identification of a high-affinity binder to a target molecule, such as a surface antigen over-expressed during tumourigenesis. Considerable effort has been dedicated to elucidating methods that facilitate isolation of binding moieties to an antigen of interest. The first mAbs were produced utilizing hybridoma technology, however the resultant murine antibodies are not

Abbreviations: mAb, monoclonal antibody;CHO, Chinese hamster ovary; IgG, immunoglobulin G;V, variable region;scFv, single chain variable fragment;Fab, fragment antigen binding;ADCC, antibody dependent cellular cytotoxicity;SEC, size exclusion chromatography; LAK, lymphokine-activated killer.

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suitable for therapeutic applications (Berger et al., 2002). Subsequently, methods such as CDR grafting, phage, yeast and ribosome display were developed (for review see: Hoogenboom (2005)). Phage display is the most commonly used method. This technique identifies single chain variable fragment (scFv) or fragment antigen binding (Fab) elements, that bind to the target molecule isolated from libraries of high-complexity, emulating the naïve immune repertoire. This library may contain murine or human sequences and more recently, completely synthetic libraries have been created. Crucially, since these fragments contain antibody variable regions, they require "reformatting" into an expression vector containing both the requisite constant region sequences and the elements for high-level expression in mammalian cells. This reformatting step can be a protracted and complicated process since the sequences of the isolated fragments are by nature variable. This makes traditional PCR and/or restriction endonuclease cloning problematic. For example an anti-TNF antibody isolated from a naïve Fab immunoglobulin gene library was rebuilt as a complete antibody by a tripartite ligation; a fragment containing the leader sequence and the amino terminus of the V (variable) domain, a second fragment containing the remainder of the V domain and Cλ constant region, and the expression vector. The reformatting required PCR using fragment specific primers and appendage of compatible restriction sites (Mahler et al., 1997). Existing antibody reformatting vectors exhibit limited flexibility and the codons formed by restriction endonuclease recognition sequences result in the addition of several "foreign" amino acids into the primary sequence (Coloma et al., 1992; Persic et al., 1997; Jostock et al., 2004). These legacy vectors are also no longer commercially available and are difficult to source. In this paper we describe a set of novel vectors that will facilitate a high-throughput-compatible and sequence-independent method for rapid antibody reformatting. We demonstrate the effectiveness of this system by creating a functional, fully human anti-human CD83 mAb.

2. Methods and materials

2.1. Expression vector design

mAbXpress vectors were assembled using publically available human constant region heavy (IgG1 and IgG4 subtypes) and light chain (κ) sequences. Required DNA was synthesized and codon-optimized for mammalian expression by Geneart AG (Germany). These cassettes were then placed into mammalian expression vectors containing sequences for expression, selection and amplification in mammalian cells (Acyte Biotech, Australia) (Fig. 1). A single SacI site was included in the expression vector to facilitate linearization and In Fusion™ cloning of the variable region (see Section 3 for details). We plan to make these vectors available to academic research groups under a standard MTA and are available under license to commercial organizations.

2.2. Phage display panning against CD83 and Ligation-Independent, In Fusion™ cloning of scFvs

The extracellular domain of human CD83 was expressed in CHO cells and purified by immobilized metal affinity chromatography. This preparation was used to isolate binders from the human scFv phage display library of Sheets et al. (1998), kindly provided by Dr James D. Marks (University of California, San Francisco). Several unique binders to recombinant CD83 were isolated and clone 3C12 was selected for cloning and expression.

Variable regions for both the heavy and kappa light chains were PCR amplified from the phagemid vectors using primers against the 5′ and 3′ conserved regions of each chain. An additional 15 bp was included on each primer corresponding to upstream and downstream bases of the destination vector to enable ligation-independent In Fusion™ cloning (Clontech). Example primers for the heavy chain were: 3C12_VhFor 5′-CAGGTGTCCACTCCGAGGTGCAGCTGCAGGAG-3′ and 3C12_VhRev 5′-GCGGAGGACACGGTGAGCGTGGTCCCTTGGCCC-3′, and for the kappa chain the primers were: 3C12_VkFor 5′-CCGGCGTGCACTCCGAGATCGTGATGACCCAG-3′ and 3C12_VkRev 5′-GCCACGGTCCGCTTGAGTTCCAGCTTGGTCCC-3′. Underlined regions represent the scFv-specific sequence, which varies from clone to clone. The unpurified PCR products were inserted into the mAbXpress IgG1 heavy and κ light chain vectors using the In Fusion™ system (Clontech), as per the manufacturer's instructions.

2.3. Mammalian cell expression and purification

For antibody expression we used suspension-adapted Chinese Hamster Ovary (CHO) cells maintained in CD-CHO (Invitrogen). Heavy and light chain plasmids were co-transfected using PEI-Max (prepared in water) (Polysciences Inc). The transfection complex is prepared at a ratio of PEI:DNA of 3.5:1. For transient transfection a 0.75:0.25 v/v ratio of cells:transfection complex is used, therefore each 750 μL of cells (at 1.5×10^6 cells/mL) in CD-CHO is transfected with 1.6 μg DNA and 5.6 μg PEI in 250 μL of OptiPro SFM medium (Invitrogen). The complex was incubated for 15 min at room temperature without disruption before addition to the cell suspension. At 4 h post transfection the cells were diluted by doubling the total volume with CD-CHO and IGF-1 was added at 0.1 mg/L before transferring the cultures to humidified incubators at 32 °C and 7.5% CO_2 for 7–14 days with shaking (160–250 rpm, depending on the vessel and the shaker throw ratio). Expression studies were typically performed at small (2 mL), medium (30 mL) or large (400 mL) scale.

Cellular debris was removed by centrifugation and secreted antibody was purified from the culture media using Protein-A chromatography. A 1 mL Protein-A HiTrap™ column from GE Healthcare was used. After loading, the column was washed with 20 mL of Phosphate buffered saline (PBS) and protein was eluted using 0.1 M glycine pH 2.7 and neutralized with 1 M Tris pH 9. Purified antibody (3C12) was then analyzed by SDS-PAGE (NuPAGE system, Invitrogen) and analytical size exclusion chromatography (SEC). For SEC we used a TSK-GEL G3000SWxl , 30 cm×7.8 mm column (Tosoh Bioscience) on an Agilent 1200 series LC. The mobile phase was 100 mM Phosphate pH 6.7, 200 mM Sodium Chloride, filtered through a 0.22 μm filter. Flow rate was 0.8 mL/min. Calibration was done using gel filtration standards (Bio-Rad). Typical yields from transient transfection experiments using this system ranged from 20 to 60 mg/L.