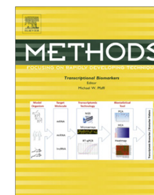
Methods 65 (2014) 44–56

Contents lists available at SciVerse ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth



Mammalian cell display for the discovery and optimization of antibody therapeutics

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ARTICLE INFO

Article history:
Available online 20 June 2013

Keywords:
Antibody
Somatic hypermutation
Mammalian cell display
AID

ABSTRACT

Recent advances are described for the isolation and affinity maturation of antibodies that couple *in vitro* somatic hypermutation (SHM) with mammalian cell display, replicating key aspects of the adaptive immune system. SHM is dependent on the action of the B cell specific enzyme, activation-induced cytidine deaminase (AID). AID-directed SHM *in vitro* in non-B cells, combined with mammalian display of a library of human antibodies, initially naïve to SHM, can be used to isolate and affinity mature antibodies via iterative cycles of fluorescence-activated cell sorting (FACS) under increasingly stringent sort conditions. SHM observed *in vitro* closely resembles SHM observed in human antibodies *in vivo* in both mutation type and positioning in the antibody variable region. In addition, existing antibodies originating from mouse immunization, *in vivo* based libraries, or alternative display technologies such as phage can also be affinity matured in a similar manner. The display system has been developed to enable simultaneous high-level cell surface expression and secretion of the same protein through alternate splicing, where the displayed protein phenotype remains linked to genotype, allowing soluble secreted antibody to be simultaneously characterized in biophysical and cell-based functional assays. This approach overcomes many of the previous limitations of mammalian cell display, enabling direct selection and maturation of antibodies as full-length, glycosylated IgGs.

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1. Introduction

In vitro display systems offer a number of advantages for antibody discovery and optimization [1]. They have the potential to overcome issues associated with *in vivo* immunization-based approaches for generation of antibodies such as tolerance to conserved antigens, poor immunogenicity, toxicity, and immunodominant epitopes. In addition, *in vitro* techniques have the potential to be significantly faster and can be carried out in high throughput mode. Phage display was the first *in vitro* display technology developed for antibody discovery, and continues to be widely used due to its simplicity and the benefits of the large libraries which can be presented [2,3]. Yeast display has also been widely used and, although not able to be used with libraries as large as those that can be screened with phage display, it has the advantage of a eukaryotic secretory pathway which has the potential for the selection of antibodies with improved folding characteristics [4,5]. However, phage, yeast and other microbial expression-based systems have a number of features which are less than ideal for displaying and selecting antibodies as potential therapeutic agents. These include the inability to work with native glycosylated IgG, lack of post-translational modification, the inability to select directly for high-level expression in mammalian cells, and most importantly, the inability to carry out direct functional screening. Mammalian cell display systems can overcome these limitations. As large, complex proteins that possess several functional domains each of which plays a significant role in the overall functionality of the molecule, antibodies are efficiently expressed and assembled into functional form during their path though the mammalian expression and secretory system. The ability to identify and affinity mature antibodies directly from mammalian cells therefore enables selection of antibodies with desirable properties from an early stage.

Mammalian cell display systems have been explored by a number of groups, and can be broadly categorized as those taking advantage of transient expression, in which DNA introduced into the cells persists for a few days resulting in rapid but relatively inefficient protein expression, and those that utilize stable cell lines in which heterologous DNA is retained through selection with appropriate marker genes [6–10].

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1046-2023/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ymeth.2013.06.010

Transient expression systems are suitable for a single round of antigen selection before recovery of the antibody genes and are therefore most useful for the selection of antibodies from smaller libraries. For example, transient expression in COS cells has been used to isolate antibodies to hepatitis B surface antigen from a library of approximately 1×10^6 antibodies recovered from an immunized human donor [11]. Stable expression systems are ideal for multiple rounds of selection. However, most stable expression systems rely on integration of DNA into the host cell genome, with selection through the simultaneous introduction of an antibiotic resistance or other selectable marker. Stable integration is a relatively rare event, and only a minority of transfected cells outgrows the antibiotic treatment, while most are killed. Although such systems are viable for single antibodies or small libraries, selection from large libraries using integrated gene expression systems is extremely inefficient. In addition, gene integration often results in the incorporation of multiple gene copies per cell which renders libraries difficult, or impossible, to resolve.

Stable episomal vectors offer an attractive alternative. Episomal vectors can be transfected at high efficiency and stably maintained at low copy number [12], permitting multiple rounds of selection, and the resolution of more complex antibody libraries. In early experiments, an EBV-derived episomal vector was successfully used to display IgG on the surface of HEK293 cells and isolate antibodies to IL-12 from a library isolated from the splenocytes of immunized chickens [13]. Similar systems have now been more extensively developed and extended to permit the screening and isolation of human antibodies from larger naïve libraries [14].

Such improved technologies permit the display and resolution of libraries of approximately 1×10^9 members, sufficient to isolate antibodies to targets of interest, but likely insufficient to isolate antibodies with very high affinity to the target. This issue is similar to that faced by the human immune system on encountering a foreign antigen. A limited number of B cells are available, presenting unique antibodies derived from the rearrangement of a small number of V(D)J genes. To overcome this problem the immune system selects low affinity antigen-specific B cells for activation and initiates somatic hypermutation of their antibodies with the end result being survival of B cells producing the highest affinity antibodies. Thus B cells evolve weak germline-sequence antibodies to highly specific and potent molecules [15]. A similar process can be utilized *in vitro* in a mammalian cell display system to affinity mature antibodies from a library of modest size, producing highly specific and potent antibodies suitable for use as therapeutic agents [14].

As a system for both antibody display and *in vitro* evolution of antibodies, coupling *in vitro* somatic hypermutation (SHM) with mammalian cell display offers a powerful methodology for both antibody discovery and maturation. For example, a single antibody can be displayed and subjected to evolution via *in vitro* SHM to improve sub-optimal properties such as affinity, specificity, mammalian cell expression level, or stability [16]. Alternatively, diversity can be sampled from *in vivo* sources, such as antigen-biased libraries derived from immunized animals [17], or in naïve libraries [14]. Fully human libraries, designed to mimic primary rearranged immunoglobulin sequences, naïve to SHM, are particularly useful to isolate antibodies that can then be matured using *in vitro* SHM to reach stringent design goals.

2. Construction of a fully human ABELmAb library

The ABELmAb IgG library (AnaptysBio evolving library of monoclonal antibodies) is based on germline sequence V-gene segments joined to rearranged (D)J regions isolated from a panel of human donors. Human V-gene segments were chosen based on two criteria: frequent usage of the V-gene in humans and the ability of the selected ensemble of ABELmAb V-gene to represent the sequence diversity of the heavy chain (HC) and light chain (LC) repertoire. To identify the most commonly used V-gene in human, and to identify where AID-mediated mutations typically occur, germline allelic variants were assembled and compared with a database of *in vivo* HC and LC sequences. The NCBI archive of antibody sequences was downloaded from the PDB and NCBI databases, and mined for sequences annotated as human IgG or IgM in origin. Germline human IGHV, IGKV, and IGLV sequences, and their allelic forms were assembled from three online antibody sequence sources, including IMGT, NCBI Entrez and VBASE yielding a total of 232 IGHV, 56 IGKV and 66 IGLV germline alleles. The single germline sequence that provided the best unique alignment to each of the matured antibody sequences was identified using an ungapped BLAST alignment with an expectation score of $<1.0 \times 10^{-50}$ and a minimum 93% sequence identity over the entire length of the antibody variable region. Analysis of SHM events yielded a total of 106,909 IGHV, 24,378 IGKV and 24,965 IGLV mutations in 12,956, 4165 and 3811 alignments to germline sequences, respectively.

Comparison of V-gene usage from these two sources was contrasted with V-gene sequence similarity, shown in Fig. 1A. HC and LC V-gene usage was comparable with that identified elsewhere [19,20], with V-genes such as IGHV3-23 and IGKV1D-39 significantly over-represented. Based on these data, a total of 9 HC and 9 LC (5κ and 4λ) human germline V-gene segments were selected for the library. Notably, despite the high frequency of IGHV1-46 in our analysis of the PDB database, IGHV1-2 was more prevalent in our analysis of the larger NCBI dataset. These two sequences have strong sequence similarity and therefore, IGHV1-2 was selected for use in the ABEL library and not IGHV1-46. V-regions were chemically synthesized and fused to (D)J region sequences (encoding CDR3 and FR4 diversity) isolated by PCR from pooled peripheral blood mononuclear cells (PBMCs) of normal donors. PBMC mRNA, isolated from 68 healthy human donors, was reverse transcribed using a mix of oligo d(T) plus random hexamers primers (Superscript III, Life Technologies, Carlsbad, CA), followed by PCR using specific sets of oligo primers designed to amplify the HC and LC (D)J regions (Table 1). CDR3 diversity was inserted into synthesized germline V-regions (DNA2.0, Menlo Park, CA) selected based on the criteria above, and combined with the corresponding constant domains for the IgHC-γ1, IgKC and IgLC3 chains. Type I membrane protein transmembrane and cytoplasmic domains were added to the C-terminal end of the HC to enable presentation on the cell surface of HEK293 cells using vectors designed for simultaneous antibody cell surface display and secretion as described below. The assembled HC and LC chains were assembled in episomal vectors (ABELmAb library), and transfected as separate IGHV sub-libraries with the IGKV and IGLV libraries in HEK293 cells as described below (Fig. 1B). A sampling of the library by next generation sequencing providing a lower estimate of 6×10^5 HC and 5×10^5 LC for a total theoretical diversity of 3×10^{12} combinatorially-expressed antibody sequences (Fig. 1C). The library was designed to provide initial candidates with germline V-gene segments for further maturation by activation-induced cytidine deaminase (AID).

3. Simultaneous cell surface display and secretion of antibodies

A robust method has been developed that enables the display of IgG on the surface of mammalian cells and the simultaneous secretion of significant quantities of soluble IgG into the culture medium. The commonly used HEK293 cell line was chosen based on its ease of transfection, good growth characteristics, robustness and compatability with maintainence of episomal vectors. Epi-

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