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A large library based on a novel (CH2) scaffold: Identification of HIV-1 inhibitors

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ABSTRACT

Isolated immunoglobulin CH2 domains were proposed as scaffolds for selection of binders with potential effector functions. We tested the feasibility of this approach by constructing a large (size 5×10^{10}) library where all amino acids in two loops (BC and FG) were mutated to four residues (Y, A, D, or S). Three binders were selected from this library by panning against a gp120–CD4 complex. The strongest binder, m1a1, recognized specifically a highly conserved CD4i epitope and inhibited to various extents seven out of nine HIV-1 isolates from different clades. The loop BC and the conformational state of the scaffold are critical for its binding. These results provide a proof of concept for the potential of CH2 as a scaffold for construction of libraries containing potentially useful binders. The newly identified HIV-1 inhibitors could be further improved to candidate therapeutics and/or used as research reagents for exploration of conserved gp120 structures.

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Introduction

Monoclonal antibodies are well-established therapeutics and invaluable tools for research. Most of these antibodies are full-size immunoglobulins (Igs) but antibody fragments of smaller size including Fabs (~60 kDa), single chain Fv fragments (scFvs) (20–30 kDa) and domain antibodies (dAbs) (12–15 kDa) are being increasingly used [1]. These antibody fragments can be selected and produced with relative ease. They penetrate solid tissues including solid tumors better and can bind to structures that are sterically constrained. A large amount of work has been aimed at developing novel human or non-human scaffolds of small size and high stability for use as therapeutics and diagnostics [2,3].

Recently, isolated immunoglobulin constant CH2 (CH3 for IgE and IgM) domains were proposed as scaffolds for construction of libraries containing diverse binders that could confer some effector functions [4]. Isolated human CH2 is independently folded and can be expressed and purified from bacterial expression systems as a soluble, monomeric protein at a high level [4,5]. It exhibits thermostability that is comparable to a dAb and can be further improved [6]. Importantly, as a fragment in all IgGs, which are at high concentrations in blood, γ1 CH2-based therapeutics are likely to be well tolerated in concentrations needed for achieving therapeutic efficacies. Furthermore, CH2 binders can be engineered so as to retain some of the effector functions that are possessed only by IgGs and not by other scaffolds [7]. Based on its similarities to a variable domain, one could hypothesize that the CH2 framework could sustain diversification of the loops for the development of libraries containing diverse binders. Here we provide evidence supporting this hypothesis by demonstrating that antigen specific binders based on the CH2 scaffold with synthetic loops can be obtained and that these binders are functional, i.e. capable of neutralizing HIV-1 isolates from different clades.

Materials and methods

Primers, peptide, and proteins. All the primers used in this study were purchased from Invitrogen (Carlsbad, CA). The biotin-labeled peptide was from Sigma (St. Louis, MO). Bal gp120–CD4 was provided by Tim Fouts (University of Maryland, Baltimore, MD) and other recombinant proteins (gp120s and gp140s) were provided by Christopher Broder (USUHS, Bethesda, MD). SCD4 was obtained through AIDS research and reagent program.

Library construction. Overlapping PCR was used to introduce mutations to the loops BC and FG to generate the library (Fig. 1). Degenerate primers containing codon KMT were utilized to introduce desired combinations of four residues to each location indicated (Fig. 1). PCR fragments were subjected to SfiI digestion and ligated to the pCOM3X (provided by Dennis Burton, Scripps Institute, La Jolla, CAL). The ligated product was desalted and transformed to the electrocompetent TG1 cells using an electroporator (Bio-Rad, Hercules, CA).

Similar overlapping PCR method was employed to graft the loop BC sequence of m1a1 to N terminus, loop DE, and loop FG of wild-type CH2, respectively.

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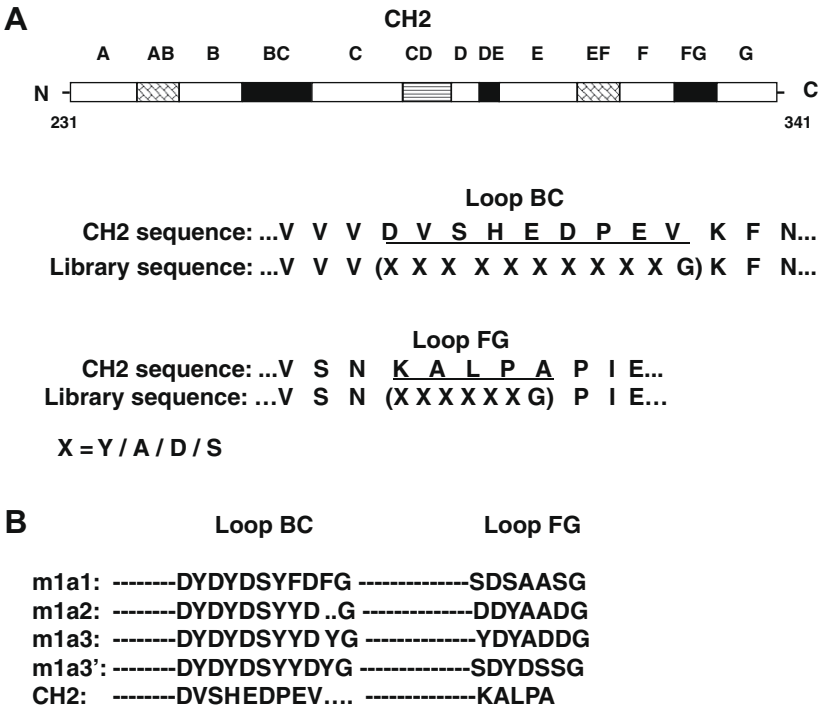

Fig. 1. (A) Experiment design. The filled rectangles represent the loops (BC, DE, and FG) which are on the same side of CH2. The shaded rectangles represent the two helices (AB and EF) and the loop (CD) which are on the opposite side of CH2. The empty rectangles labeled with letters A through G represent the seven β-strands. The numbers 231 and 341 denote the starting and ending residues corresponding to the CH2 in the context of the IgG1. Fragments containing loops BC and FG are shown below the CH2 representation where their sequences are underlined. The mutations introduced in the loops are shown in parentheses. (B) Sequence comparison among the isolated binders and WT CH2. M1a3' has the same loop BC as m1a3 but different loop FG.

Panning. Bal gp120–CD4 was coated directly to Maxisorp plates (Nunc, Denmark) in PBS buffer at 4 °C, o/n as the panning antigen. Monoclonal ELISA was then used to select for positive clones after five rounds of panning. Only clones displaying an ELISA signal that was at least 10 folds above the background signal were selected for further analysis.

CH2 expression and refolding. Escherichia coli strain HB2151 was used for protein expression. Fresh transformant was inoculated into 2YT with 100 units of amp and incubated at 37 °C with shaking. When the OD600 reached 0.5, IPTG was added to 1 mM and the culture was continued for another 3–5 h. Cells were collected, lysed with polymyxin B (Sigma, St. Louis) in PBS, and the supernatant was subjected to the Ni–NTA agarose bead (Qiagen, Hilden, Germany) purification. The pellet was re-suspended in a buffer containing 25 mM Tris–HCl, pH 8.0, 6 M Urea, 0.5 M NaCl, and subjected to a brief sonication. The supernatant was collected by centrifugation and subjected to the Ni–NTA agarose bead purification. CH2 obtained from the pellet was dialyzed against PBS and filtered through a 0.2 μm low protein binding filter (Pal, Ann Arbor, MI).

ELISA. Protein antigens were diluted in the PBS buffer in concentrations ranging from 1 to 4 μg/ml and coated to the 96-well plate at 4 °C for overnight. The mouse-anti-His-HRP (Qiagen) was used to detect the His tag at the C terminus of each of the CH2 clones binding to the antigens in most of the ELISA unless indicated otherwise. ABTS (Roche, Germany) was then added to each well and OD 405 was taken 5–10 min afterward.

Pseudovirus neutralization assay. HIV Env pseudotyped virus preparation and neutralization was performed essentially as previously described [8].

Structure modeling. For m1a1 and germline X5 modeling, coordinates were generated by replacing and/or inserting side chain residues based on CH2 crystal structure (PDB 3DJ9) and the X5 (PDB 1RHH), respectively. Changes were made only for residues that are in the primary binding sites, loop BC and HCDR3, respectively. Swiss-model was used to generate the coordinate [9]. VMD software was used to calculate structural alignment between the CH2 and m1a1 and to present the graphics [10].

Results

Construction of a large human CH2-based library and selection of specific binders

We mutated loops BC and FG because they are the longest loops on the same side of the molecule (see Fig. 1A). We selected four residues, A, Y, D, and S, which frequently occur in CDRs, to randomly replace all BC and FG residues. Another residue, G, was added to the C-terminal end of each loop to increase flexibility (Fig. 1A). It has been previously observed that these four residues are sufficient to form a specific binding surface on different frameworks [11,12]. The calculated diversity of this library is $4^{16} = 4.3 \times 10^9$. The number of clones from the final electroporation was 5×10^{10}, which would also include possible PCR mutants. More than 80% of randomly selected clones were with correct reading frames.

To test the library and select potentially useful binders we used an HIV-1 envelope glycoprotein, gp120, from the Bal isolate fused with a two-domain CD4 ($gp120_{Bal}$–CD4) as a panning antigen. After five rounds of panning 200 clones were screened by phage ELISA and 15 clones that exhibited the highest level of binding to the screening antigen were isolated for further characterization. Three clones, m1a1, m1a2, and m1a3, dominated; they were represented by 7, 5, and 2 (out of 15) sequences, respectively, suggesting a specific enrichment. They have similar loop BC sequences but different loop FG (Fig. 1B). The dominant clones, m1a1 and m1a2, have res-