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Creating stable stem regions for loop elongation in Fcabs — Insights from combining yeast surface display, in silico loop reconstruction and molecular dynamics simulations

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ABSTRACT

Fcabs (Fc antigen binding) are crystallizable fragments of IgG where the C-terminal structural loops of the CH3 domain are engineered for antigen binding. For the design of libraries it is beneficial to know positions that will permit loop elongation to increase the potential interaction surface with antigen. However, the insertion of additional loop residues might impair the immunoglobulin fold. In the present work we have probed whether stabilizing mutations flanking the randomized and elongated loop region improve the quality of Fcab libraries. In detail, 13 libraries were constructed having the C-terminal part of the EF loop randomized and carrying additional residues (1, 2, 3, 5 or 10, respectively) in the absence and presence of two flanking mutations. The latter have been demonstrated to increase the thermal stability of the CH3 domain of the respective solubly expressed proteins. Assessment of the stability of the libraries expressed on the surface of yeast cells by flow cytometry demonstrated that loop elongation was considerably better tolerated in the stabilized libraries. By using in silico loop reconstruction and mimicking randomization together with MD simulations the underlying molecular dynamics were investigated. In the presence of stabilizing stem residues the backbone flexibility of the engineered EF loop as well as the fluctuation between its accessible conformations were decreased. In addition the CD loop (but not the AB loop) and most of the framework regions were rigidified. The obtained data are discussed with respect to the design of Fcabs and available data on the relation between flexibility and affinity of CDR loops in Ig-like molecules.

1. Introduction

The immunoglobulin-like fold (Ig-like) is one of the most common structural motifs, exhibiting a β-sandwich structure of two interacting antiparallel β-sheets with a Greek Key topology [1]. Immunoglobulin domains evolved an outstanding capacity to tolerate variability in the length of loops (that connect the β-strands), the amino acid sequence as well as loop conformation while maintaining their overall structure and function. This is most evident for the three CDR-loops (CDR1, CDR2 and CDR3) of variable domains of antibodies, but is generally observed in protein domains belonging to the immunoglobulin fold family [2]. Besides this natural loop variation, it could be demonstrated that artificially introduced sequences in loops can also be tolerated by Ig-like folds and used for the design of specific binders. This was shown for example with a fibronectin type III domain [3], the CH_2 domain of IgG1-Fc [4] and the CH3 domain in the context of the crystallizable fragment (Fc) of IgG1 [5,6]. The latter turned out to be a promising starting scaffold for the design of a novel antibody-based therapeutic format called Fcab, i.e. antigen binding Fc fragment. The Fc protein has – except for an antigen-binding site – all properties of a full-size IgG1, i.e. the ability to bind Fcγ-receptors, the complement activator C1q and the neonatal Fc receptor (FcRn). Upon engineering the C-

Abbreviations: IgG1, immunoglobulin G class 1; IgG1-Fc, crystallizable fragment of immunoglobulin G class 1; Fc-wt, recombinant wild-type human IgG1-Fc; Fab, antigen binding fragment; mAb, monoclonal antibody; scFv, single-chain variable fragment; FcγRI, Fcγ-receptor I (also termed CD64); ADCC, antibody dependent cell-mediated cytotoxicity; CDC, complement dependent cytotoxicity; aCH_2, antibody recognizing the intact fold of the CH_2-domain of human IgG1; FACS, fluorescence activated cell sorting; DSC, differential scanning calorimetry; MD, molecular dynamics; DSSP algorithm, Define Secondary Structure of Proteins algorithm

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terminal structural loops of the CH3 domains in IgG1-Fc, small 50 kDa HER2/neu-binding homodimeric Fcabs could be designed which elicited potent antibody-dependent cell-mediated cytotoxicity in vitro and which have a long half-life in vivo [6].

Successful introduction of novel binding sites in the C-terminal AB-, CD- and EF-loops of the IgG1-Fc CH3 domains needs detailed information about the correlation between primary and tertiary structure and stability. For the design of libraries that ideally contain a high percentage of well-folded clones to guarantee the efficient selection of binders, it is important to know those amino acids that can be randomized without significantly decreasing the conformational stability of resulting variants. Individual loop residues may exhibit important non-covalent interaction(s) with the β-strands or with other loops and thus should not be mutated. Additionally, those sites in loops have to be identified that allow the insertion of additional amino acids in order to increase loop length and thus the potential interaction surface with antigen. The optimization of Fcab libraries according to these criteria was reported recently [7]. However, no information on the conformation and dynamics of elongated structural loops of constant domains has been reported so far.

In the work described in this paper we constructed 13 yeast surface libraries in which the C-terminal part of the EF-loop of the CH3 domain of IgG1-Fc was randomized and additional residues were inserted. We evaluate how the insertion of stabilizing mutations in the EF loop may support the preservation of the overall stability of the respective libraries. Rapid stability assessment on a library scale together with novel in silico loop reconstruction and molecular dynamics simulations is demonstrated. This gives valuable information on the effect that the insertion of additional residues, in the absence and presence of stabilizing stem residues, has on the fluctuation between conformations of the EF-loop itself as well as on neighboring structural loops and the dynamics of the immunoglobulin framework. Results are discussed with respect to the impact that these findings will have on the selection of Fcabs.

2. Materials and methods

2.1. Differential scanning calorimetry

Differential scanning calorimetry (DSC) was used to analyze the thermal stability of wild-type IgG1-Fc and the variants Q418L and S424T. After adjusting the protein concentration to 5 μM, samples were degassed and measured on a VP–DSC Capillary Cell MicroCalorimeter (MicroCal, Northampton, MA) at a temperature range of 20 °C to 110 °C and a heating rate of 1 °C/min. The baseline was recorded by performing a rescan under the same conditions. After the subtraction of the baseline, data were normalized for protein concentration and fitted according to a non-2-state thermal unfolding model using the software MicroCal addin for OriginLab (Origin Lab Corporation, Northampton, MA).

2.2. Cloning and library construction

The gene encoding the human IgG1-Fc fragment (comprising hinge-region, CH2- and CH3-domains) was codon-optimized for the expression in yeast and cloned into the vector pYD1 (Invitrogen, Carlsbad, CA, USA) for expression as a fusion protein with Aga2p on the surface of *Saccharomyces cerevisiae* using BamHI and NotI [8]. A stop codon was introduced at the 3′ end of the region coding for the CH3 domain to exclude any C-terminal tags present on pYD1. To construct yeast cell surface display libraries, two novel *Bsm*BI restriction sites were introduced upstream of the region coding for the CD-loop of the CH3 domain and downstream of the EF loop (QuikChange Lightning Site-Directed Mutagenesis Kit, Agilent Technologies, Santa Clara, CA, USA). In accordance with that, a stuffer fragment (non-coding) was amplified containing two *Bsm*BI restriction sites. Vector as well as stuffer fragment were digested with *Bsm*BI and the fragment was ligated into the CIAP treated linearized vector using the T4 DNA ligase. The utilization of the resulting vector pYD1-2BN would therefore not lead to surface expression of wild-type IgG1-Fc as a consequence of incomplete *Bsm*BI-digest or religation.

A multi-step polymerase chain reaction (PCR) was performed to randomize loop sequences by saturated mutagenesis using NNK-oligonucleotides (N codes for a mixture of all four nucleotides, whereas K represents a mixture of G and T; ordered from Sigma, St. Louis, MO), resulting in fragments comprising regions of homology to the linearized pYD1 backbone flanking the regions coding for the EF-loop for homologous recombination in yeast. The stabilizing mutations Q418L and/or S424T (see Results Section) were introduced in the respective libraries by the modification of the oligonucleotide sequences (Supplementary Table 1).

S. cerevisiae EBY100 (Invitrogen, Carlsbad, CA, USA) were transformed with purified library inserts and BsmBI-digested pYD1-2BN using the lithium-acetate method [9]. Gap repair driven homologous recombination in *S. cerevisiae* due to the presence of homologous regions on inserts and BsmBI-digested pYD1-2BN resulted in reconstitution of the plasmids. The *S. cerevisiae*-libraries were cultured in SD-CAA medium [20 g/L glucose, 0.1 M KH_2PO_4/K_2HPO_4, pH 6, 10 g/L $(NH_4)_2SO_4$, 0.1 g/L L-leucine (all Sigma, St. Louis, MO), 3.4 g/L yeast nitrogen base, 10 g/L bacto casamino acids (all Difco, BD, Franklin Lakes, NJ)] at 28 °C for 48 h while shaking at 180 rpm. The isolation of pYD1 vector DNA for *Escherichia coli* transformation and ensuing sequencing was done using the Zymoprep Yeast Plasmid Miniprep Kit II (Zymo Research, Orange, CA). In total, 13 libraries were constructed as described in Table 1: Library stem-(0) consists of IgG1-Fc variants without stabilizing mutations or additional inserts, but with parts of the EF loop randomized (419–422). Library stem(0) is constructed similarly, but with two additional stabilizing mutations flanking the randomized region. In libraries stem-(1), stem-(2), stem-(5) and stem-(10), 1,2,3,5 or 10 additional residues are inserted into the randomized EF loop, without stabilizing mutations, while the EF loops in the corresponding libraries

Table 1
Library design, library identity (ID) and experimentally determined temperatures of half-maximal irreversible denaturation. Black lowercase letters in column 'EF loop design' represent amino acids that were kept constant in the design, blue uppercase letters represent sites of stabilizing mutations (Q418L, S424T), red upper case letters represent residues that have been randomized in the respective design and **bold red subscript numbers** represent the number of inserted random amino acids at the respective position.

EF loop design	Library ID	$\Delta T_{1/2}$ [°C]
dksrwqQGNVfsc	Stem–(0)	−4.6 ± 0.1
dksrwLQGNVfsc	Q418L	−3.9 ± 0.3
dksrwLQGNVfTc	Stem(0)	−2.9 ± 0.2
dksrwqQGNVX_1fsc	Stem–(1)	−5.8 ± 0.2
dksrwLQGNVX_1fTc	Stem(1)	−3.0 ± 0.1
dksrwqQGNVX_2fsc	Stem–(2)	−6.3 ± 0.1
dksrwLQGNVX_2fTc	Stem(2)	−4.8 ± 0.1
dksrwqQGNVX_3fsc	Stem–(3)	−6.7 ± 0.0
dksrwLQGNVX_3fTc	Stem(3)	−5.0 ± 0.1
dksrwqQGNVX_5fsc	Stem–(5)	−7.3 ± 0.2
dksrwlQGNVX_5fTc	Stem(5)	−5.5 ± 0.1
dksrwqQGNVX_{10}fsc	Stem–(10)	−7.3 ± 0.1
dksrwLQGNVX_{10}fTc	Stem(10)	−6.7 ± 0.2

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