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Structural consequences of aglycosylated IgG Fc variants evolved for FcγRI binding

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ABSTRACT

In contrast to the glycosylated IgG antibodies secreted by human plasma cells, the aglycosylated IgG antibodies produced by bacteria are unable to bind FcγRs expressed on the surface of immune effector cells and cannot trigger immune effector functions. To avoid glycan heterogeneity problems, elicit novel effector functions, and produce therapeutic antibodies with effector function using a simple bacterial expression system, FcγRI-specific Fc-engineered aglycosylated antibodies, Fc11 (E382V) and Fc (E382V/M428I), containing mutations in the CH3 region, were isolated in a previous study. To elucidate the relationship between FcγRI binding affinity and the structural dynamics of the upper CH2 region of Fc induced by the CH3 mutations, the conformational variation of Fc variants was observed by single-molecule Förster resonance energy transfer (FRET) analysis using alternating-laser excitation (ALEX). In sharp contrast to wild-type Fc, which exhibits a highly dynamic upper CH2 region, the mutations in the CH3 region significantly stabilized the upper CH2 region. The results indicate that conformational plasticity, as well as the openness of the upper CH2 region, is critical for FcγR binding and therapeutic effector functions of IgG antibodies.

1. Introduction

Abnormalities in immune surveillance mechanisms and imbalances in immune cell activation states result in serious acute or chronic immunological disorders including infection, tumors, or autoimmune diseases. To reinforce the human body's immune regulatory system and to effectively remove pathogens or defective cells, over 30 highly specific monoclonal IgG antibodies have been approved by the US Food and Drug Administration (US FDA) and the European Medicines Agency (EMA) and successfully commercialized for targeted immunotherapy (Elvin et al., 2013). Of the ten best-selling biopharmaceutical products of 2013, six were monoclonal antibody products, and the second best-selling biopharmaceutical product was an antibody Fc fusion protein (Walsh, 2014).

The human IgG antibody, a complex tetrameric molecule composed of two identical heavy chains and two identical light chains, can be divided into a Fab region, which binds to highly variable pathogenic antigens, and an Fc portion, which recruits and activates immune effector leukocytes such as macrophages, dendritic cells, and natural killer (NK) cells. Each chain of a homodimeric Fc is N-linked glycosylated at an Asn297 residue and the appended biantennary glycans play critical roles in the structure and function of IgG antibodies. The N-linked glycans are known to cause the CH2 domain to protrude from the internal hydrophobic region (Jefferis, 2005; Krapp et al., 2003), allowing the IgG antibody Fc region to associate with various activating FcγRs (FcγRI, IIa, and IIIa) and inhibitory FcγRIIb, which are expressed on the surface of various immune cells. The engagement of antibody Fc with the

Abbreviations: Fc, fragment crystallizable; IgG, immunoglobulin G; Fab, fragment antigen binding; FcγR, Fc gamma receptor; ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cell-mediated phagocytosis; CDC, complement-dependent cytotoxicity; FRET, Förster resonance energy transfer; smFRET, single-molecule FRET; ALEX, alternating-laser excitation; PBS, phosphate buffered saline; TCEP, tris-carboxyethylphosphine; ELISA, enzyme-linked immunosorbent assay; SAXS, small-angle X-ray scattering; PDB, Protein Data Bank.

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activating FcγRs promotes the recruitment of effector cells and triggers effector functions for the removal of defective cells, such as cancer cells or infected cells, by bridging humoral and cellular immune functions (Jefferis, 2009a,b; Schwab and Nimmerjahn, 2013).

Mammalian cells have been exhaustively exploited as primary hosts for the production of full-length IgG antibodies. However, production of glycosylated IgG using mammalian expression systems requires a long time for cell line development and a high capital investment in the facility. To express IgG antibody in bacteria, increase the ease of downstream processing, and bypass glycan heterogeneity issues, which can result in side effects and inconsistent efficacy, an aglycosylated IgG format, lacking the N-linked glycan, would possess many advantages over the conventional glycosylated format, from a bioprocessing standpoint. Aglycosylated IgGs display antigen binding, solubility, stability at physiological temperature, and serum persistence *in vivo* similar to those of glycosylated IgGs produced in mammalian cells (Hristodorov et al., 2013), therefore, several aglycosylated antibodies have been developed for clinical trials (Ju and Jung, 2014).

Despite their many favorable properties, aglycosylated antibodies show almost no binding to FcγRs, resulting in the loss of effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC) for the clearance of defective cells (Jefferis, 2005; Simmons et al., 2002). Recently, to overcome the limitations of wild-type aglycosylated IgG, comprehensive directed evolution of an aglycosylated Fc region has been used to generate aglycosylated IgG Fc variants with effector functions. Two mutations in the upper CH2 region of aglycosylated Fc, which is the FcγR-binding region of the Fc domain, restored the FcγRIIa binding affinity and the FcγRIIa-mediated effector functions shown by glycosylated IgG Fc (Sazinsky et al., 2008). Using bacterial display and more comprehensive aglycosylated Fc library screening by flow cytometry, an aglycosylated Fc mutant was isolated that displayed high selectivity for activating FcγRIIa over inhibitory FcγRIIb and greatly enhanced macrophage-mediated ADCP (Jung et al., 2013).

In a previous study, aglycosylated Fc variants exhibiting selective binding to FcγRI and enhanced dendritic cell-mediated killing of tumor cells were isolated (Jung et al., 2010). Crystal structures of Fc–FcγR complexes (Radaev et al., 2001; Ramsland et al., 2011; Sondermann et al., 2000) have revealed that FcγRs directly contact the upper CH2 region of the Fc portion. However, the beneficial mutations conferring specific FcγRI binding were found to be located in the CH3 region, which is far from the FcγR-binding region, and it remains unclear how the two mutations induce structural changes and alter conformational dynamics to induce highly specific FcγRI binding.

X-ray crystallographic studies provide high-resolution, three-dimensional structural information. However, these results show only a snapshot of a single conformer among the numerous possible conformers that an aglycosylated Fc can adopt. Other biochemical techniques, such as circular dichroism, differential scanning calorimetry (DSC), fluorescence spectroscopic analysis, and isothermal titration calorimetry (ITC), have also been used for structural analysis of large proteins, such as antibody Fc. However, these techniques show only an ensemble average, representing the overall bulk behavior of the molecules, and do not provide data on the complete range of conformational variability.

Recently, single-molecule Förster resonance energy transfer (smFRET) between a single donor and a single acceptor fluorophore has been widely used to study the structure and dynamics of biological molecules (Deniz et al., 2000; Ha et al., 1999; Jeong et al., 2011). The smFRET assay enables high-throughput measurement of conformational changes because it acts as a spectroscopic "ruler" for measuring the distance between the two fluorophore probes (Stryer, 1978). Thus, it can provide powerful insight into the dynamic conformational changes of proteins, and reveal subpopulations in a heterogeneous mixture (Deniz et al., 2000; Kelliher et al., 2014; Kim et al., 2012a,b). The flexibility of a given protein conformer can be inferred from the width and position of the peaks observed in the histogram of a subpopulation. Further, combination of an alternating-laser excitation (ALEX) assay with smFRET has been shown to be useful for studying subpopulations in solution, without surface immobilization (Kim et al., 2012a,b).

Here, we analyzed the impact of isolated mutations on the conformational dynamics of aglycosylated antibody Fc variants at the single-molecule level. By observing the conformational variability of the aglycosylated Fc domain, we monitored the structural changes induced during a directed evolution process for highly selective FcγRI binding.

2. Materials and methods

2.1. Chemicals, reagents, and other materials

Restriction enzymes and Vent polymerase were purchased from New England BioLabs (Ipswich, MA). Oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA). Ampicillin and 1-Step™ Ultra TMB-ELISA substrate solution were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA). Protein A agarose resin and Terrific Broth (TB) medium were obtained from Genscript (Piscataway, NJ) and Becton Dickinson Diagnostic Systems (Sparks, MD), respectively. Recombinant human FcγRI purified from mouse myeloma NS0 cell line was purchased from R&D Systems (Minneapolis, MN). Cy3-maleimide and Cy5-maleimide were from GE Healthcare Life Sciences (Piscataway, NJ), and the horseradish peroxidase (HRP) conjugate of mouse anti-polyHistidine antibody and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Construction of plasmids for S267C Fc variants

All of the plasmids and primers used in this study are described in Tables 1 and 2. The pDsbA-*Nhe*I plasmid was constructed using a QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) using two primers (STJ#664 and STJ#665)

Table 1
Plasmids used in this study.

Plasmids	Relevant characteristics	Reference or source
pTrc99A	Ap_r, Trc promoter, $lacI_q$	Amersham Biosci.
pTrc99A-DsbA	DsbA signal sequence gene in pTrc99A	Jung et al. (2010)
pTrc99A-DsbA-Fc	Fc gene in pTrc99A-DsbA	Jung et al. (2010)
pTrc99A-DsbA-Fc5	Fc5 gene in pTrc99A-DsbA	Jung et al. (2010)
pTrc99A-DsbA-Fc11	Fc11 gene in pTrc99A-DsbA	Jung et al. (2010)
pDsbA-*Nhe*I	*Nhe*I restriction enzyme site in pTrc99A-DsbA	This study
pDsbA-Fc-S267C	Fc-S267C mutant gene in pDsbA	This study
pDsbA-Fc5-S267C	Fc5-S267C mutant gene in pDsbA	This study
pDsbA-Fc11-S267C	Fc11-S267C mutant gene in pDsbA	This study

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