Soluble Monomeric IgG1 Fc

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Running Title: Monomeric IgG1 Fc

Background: The Fc region of an antibody is a homodimer of two CH2-CH3 chains.
Results: Monomeric IgG1 Fcs (mFcs) were generated by using a novel panning/screening procedure.
Conclusion: The mFcs are highly soluble and retain binding to human FcRn comparable to that of Fc.
Significance: The mFcs are promising for the development of novel therapeutic antibodies of small size and long half-lives.

Antibody fragments are emerging as promising biopharmaceuticals because of their relatively small size and other unique properties. However, compared to full-size antibodies, these antibody fragments lack the ability to bind the neonatal Fc receptor (FcRn) and have reduced half-lives. Fc engineered to bind antigens but preserve interactions with FcRn and Fc fused with monomeric proteins are currently being developed as candidate therapeutics with prolonged half-lives; in these and other cases Fc is a dimer of two CH2-CH3 chains. To further reduce the size of Fc but preserve FcRn binding, we generated three human soluble monomeric IgG1 Fcs (mFcs) by using a combination of structure-based rational protein design combined with multiple screening strategies. These mFcs were highly soluble and retained binding to human FcRn comparable to that of Fc. These results provide direct experimental evidence that efficient binding to human FcRn does not require human Fc dimerization. The newly identified mFcs are promising for the development of mFc fusion proteins, and for novel types of mFc-based therapeutic antibodies of small size and long half-lives.

INTRODUCTION

Therapeutic monoclonal antibodies (mAbs) are being gradually improved during the last two decades (1,2,3,4,5,6,7,8,9,10). Most of the mAbs approved for clinical use are full-size (7,9,10). However, full-size antibodies exhibit poor penetration into tissues, especially solid tumors, and also poor or absent binding to regions of some antigens that are occluded and can only be accessed by molecules of smaller size (11,12,13). Engineering of a variety of antibody fragments of smaller size such as Fab, Fv, scFv, VH, VH, and other antibody fragment formats are under development (9,10,14,15,16,17,18). However, to date these antibody fragments have been of limited therapeutic applications, because they usually display greatly reduced half-lives compared to full-size IgG. One approach to increase half-lives is by fusion with Fc or by engineering binding sites to the neonatal Fc receptor (FcRn).

The Fc contributes to the long half-life of IgG through its unique pH-dependent association with the neonatal Fc receptor (FcRn) (19,20). The IgG Fc can bind to FcRn in the acidic environment of the endosome after internalization, then be recycled into the cell surface and released into circulation. This protects IgG from degradation and increases its serum half-life. A technology to produce Fc fusion proteins has been proven effective in extending half-lives of therapeutic molecules (21,22). A typical Fc fusion protein
contains two effector molecules because the Fc fragment of the IgG consists of a tightly packed homodimer, and each molecule is attached to one chain of the Fc dimer. Recently, so-called "monomeric Fc fusion proteins" were generated by fusing a single active protein to dimeric wild-type Fc (23,24,25). Such smaller molecules have been shown to possess extended half-lives compared with the dimeric version and are promising for therapeutic applications. Despite this advancement, the Fc domain in a fusion protein is still dimeric and of relatively large size (~50 kD).

It has been debated whether the dimeric Fc binds to two molecules FcRn or only one and whether the dimeric state of Fc is required for efficient binding to FcRn (20,26,27,28,29,30). Several experiments showed a 2:1 FcRn/Fc binding stoichiometry (26,28,29), while the 1:1 FcRn/Fc complex was also observed in some studies (27,29). A better understanding of these interactions could be helped by generating and using monomeric Fc (mFc).

Here we describe the identification and characterization of highly soluble functional mFcs of half the Fc size and preserved binding to FcRn. Fc dimerization is mainly mediated by a large hydrophobic interface in its CH3 domain, which involves at least 16 residues in each polypeptide chain that make intermolecular interactions (31,32). Disruption of this large interaction interface would cause exposure of a hydrophobic surface resulting in poor solubility, instability and/or aggregation. To solve this problem, a large phage library was constructed in which more than 10^9 human IgG1 Fc individual molecules were displayed with extensive mutations in the CH3 dimer interface (L351, T366, L368, P395, F405, Y407, and K409). Amplified libraries with 10^12 phage-displayed Fc mutants were applied to a pre-equilibrated protein G column (Roche, Indianapolis, IN). The resins were washed extensively with PBS, and the bound phages were eluted by 0.1 M HCl-glycine (pH 2.2). The elution was then neutralized with 1 M Tris-base, mixed with TG1 cells for 1 h at 37˚C, and the phages capable of binding to protein G were amplified from the infected cells and used in the biopanning against FcRn. Human FcRn, containing both β and α chains in a 1:1 molar ratio, was expressed in mammalian cells and purified as a soluble protein as previously described (33). Libraries with 10^12 phages were mixed with PBS (pH 6.0) and incubated in ELISA wells coated with FcRn. Human FcRn, containing both β and α chains in a 1:1 molar ratio, was expressed in mammalian cells and purified as a soluble protein as previously described (33). Libraries with 10^12 phages were mixed with PBS (pH 6.0) and incubated in ELISA wells coated with FcRn for 2 h at 37˚C. After incubation, the wells were washed 10 times for the first round and 20 times for the later rounds with PBS (pH 6.0) containing 0.05% Tween 20. The bound phages were eluted with PBS (pH 7.4), amplified by infecting TG1 cells along with helper phage M13KO7 (Invitrogen, Carlsbad, CA). 80 clones were randomly picked from the fifth selection round, transferred into HB2151 cells, inoculated into 3 mL 2YT medium containing 100 µg/mL ampicillin, and incubated for 2 h at 37˚C with shaking at 250 rpm. After the addition of 1.5 µL isopropyl-1-thio-β-D-galactopyranoside, bacteria were grown for 3 additional hours, harvested by
centrifugation. The pellet was resuspended in PBS buffer containing 5 µU polymixin B (Sigma-Aldrich, St. Louis, MO), incubated at room temperature for 30 min, and centrifuged at 10,000 × g for 10 min. The supernatant was separated by non-reducing SDS-PAGE without boiling and then analyzed by Western blot. The clones that did not show evidence of dimeric Fc via Western blot (~54 kD bands) were selected for further characterization.

Expression and purification of monomeric Fc proteins– The selected clones (mFc.1, mFc.23 and mFc.67) were sequenced, and plasmids extracted from these clones were used for transformation of HB2151 cells. A single and freshly transformed colony was inoculated into 200 mL SB medium with 100 µg/mL ampicillin, and incubated at 37˚C with vigorous shaking at 250 rpm. When optical density of the culture at 600 nm reached around 0.6, expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM, and the culture was further incubated at 30˚C for 6 h. Cells were then harvested by centrifugation at 6,000 rpm for 15 min and resuspended in PBS buffer. Polymixin B (Sigma-Aldrich) (0.5 µu/mL) was added to the suspension (1:1000). After 30 min incubation with rotation at 50 rpm at room temperature, the culture was centrifuged at 12,000 rpm for 15 min at 4˚C. The supernatant was used for further purification by Ni-NTA resin (Qiagen, Valencia, CA) according to the manufacturer's protocols. Protein purity was estimated as >95% by SDS-PAGE and protein concentration was measured spectrophotometrically (NanoVue, GE Healthcare).

Size exclusion chromatography– The molecular composition of purified proteins was analyzed by size exclusion chromatography (SEC) using an FPLC AKTA BASIC pH/C system (GE Healthcare) with a Superdex 75 10/300 GL column (GE Healthcare). PBS (pH 7.4) was selected as the running buffer and a flow rate of 0.5 mL/min was used. Eluting protein was monitored at 280 nm. The molecular mass standards used were ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (44 kDa), bovine serum albumin (67 kDa) and aldolase (158 kDa).

Reverse mutation– The reverse mutation assay was conducted using a selected monomeric Fc clone, mFc.23, as template, in which the dependent residues were mutated back to their original counterparts in wild-type Fc. Six mFc.23 mutants, 351L, 366T, 368L, 366T/368L, 395P and 405F/407Y/409K were all generated by the overlap-extension PCR method. Each mutation was confirmed by automated DNA sequencing. The reverse mutations were expressed and purified using a similar procedure as monomeric Fc proteins described above.

Circular dichroism (CD)– The circular dichroism spectra of CH2, Fc and mFc proteins were collected from 190 to 250 nm (0.1 cm path length), with an AVIV Model 202 spectropolarimeter (Aviv Biomedical). The protein samples were dissolved in PBS, pH 7.4 a at a final concentration of 0.4 mg/mL. Spectra were first recorded at 25˚C for native structure measurements. For evaluation of the refolding, the samples were heated slowly to 90˚C (1˚C/min), kept at 90˚C for 10 min, then rapidly cooled down to 25˚C (10˚C/min), and the spectra were recorded again. For evaluation of thermal stability, CD signals at 216 nm were recorded and the instrument was programmed to acquire spectra at 1˚C intervals over the range 25-90˚C.

Spectrofluorometry– Fluorescence spectra were measured using a Fluoromax-3 spectrofluorometer (HORIBA Jobin Yvon Inc., NJ). For urea denaturation tests, Fc and mFc proteins were dissolved in urea-containing buffers (50 mM Tris-Cl, 450 mM NaCl, pH 8.0, 0 to 8 M Urea), to give a final protein concentration of 10 µg/mL. The samples were kept overnight at 4˚C and fluorescence measurements were performed with the excitation wavelength at 280 nm. The emission spectra were recorded from 320 to 380 nm, and fluorescence intensity at 343 nm was used for quantitative evaluation of urea unfolding.

Serum stability assay– Normal human serum was collected from healthy human donors approved by the NCI-Frederick Research Donor Program (RDP). Wild-type human Fc (30 µg) and mFc proteins (15 µg) were incubated with normal human serum in PBS at 37˚C. An aliquot was taken out at each time point and immediately
stored at −80˚C. Western blot and ELISA assays were applied to check the serum stability. For Western blot, samples were electrophoresed through SDS-PAGE and transferred onto a 0.2 µm nitrocellulose membrane (Bio-Rad). The membrane was blocked with 3% milk in PBS for 1 h at room temperature, and then incubated with anti-His Tag monoclonal antibody (ABM, Vancouver, Canada) for 1 h. Washing with PBST was followed by incubation with anti-mouse IgG-alkaline phosphatase antibody (Sigma-Aldrich). The BCIP/NBT substrate solution (Sigma-Aldrich) was used for detection. For the ELISA test, wells were coated with 50 µL of anti-Fc Fab (Sigma-Aldrich) and then blocked in 100 µL protein-free blocking buffer (Thermo Scientific) for 1 h at 37˚C. After five washes with PBST, wells were incubated with samples for 2 h at 37˚C. Following six washes with PBST, 50 µL horseradish peroxidase (HRP)-conjugated anti-FLAG tag antibody (Sigma-Aldrich) was added and incubated for 1 h at 37˚C. The assay was developed with ABTS substrate and monitored at 405 nm.

Binding ELISA— Antigens were coated on ELISA plate wells at 50 ng per well in PBS overnight at 4˚C and blocked with protein-free blocking buffer for 1 h at 37˚C. Threefold serially diluted protein was added and incubated at 37˚C for 2 h. The plates were washed with PBST, and HRP-conjugated anti-FLAG tag antibody in PBS was incubated with wells for 1 h at 37˚C. After extensive washes with PBST, binding was detected with the addition of ABTS substrate, and signals were read at 405 nm.

Surface plasmon resonance (SPR) experiments— The interaction of samples with immobilized FcRn was monitored by SPR detection using a BIAcore X100 instrument (GE Healthcare). Purified human FcRn was diluted in 10 mM Na-acetate buffer, pH 5.0, and immobilized on a CM5 biosensor chip using an amine coupling kit. The running buffer was PBS with 0.005% Tween 20 for binding at pH 7.4 or PBS pH 6.0 with 0.005% Tween 20 for binding at pH 6.0. The proteins diluted with running buffer were allowed to flow through the cells, at concentrations ranging from 125 nM to 2000 nM. After 10 min of dissociation, the chip was regenerated with pH 8.0 buffer (100 mM Tris, pH 8.0, 50 mM NaCl). Another test with a protein concentration of 500 nM was repeated to monitor the regeneration efficiency. As a negative control for the FcRn binding assay, the Fc I253A/S254A/H435A/Y436A mutant was constructed using the overlap-extension method.

Generation of m36 fusion proteins— The following primers were used: Omp, 5’- AAGACAGCTATCGCGATTGCAG-3’; gIIIF, 5’- ATCACCGGAACCAGACGCCAACCAC-3’; m36R, 5’-TGAGGAGACGCGTAGCACGAGGTGCCTG-3’; mFcF, 5’- CTGTTACCGGCTCTTCTAGCACACTCCTGG-3’; mFcCH3F, 5’- CTGTTACCGGCTCTTCTACCCAGAACTCAGGGTGAC-3’; mFc.67 gene (primer: mFcF and gIIIF) and mFc.67 CH3 domain gene (primer: mFcCH3F and gIIIF) were amplified from mFc.67 plasmid, which was constructed in a pComb3x vector. The m36 fragment was joined to mFc.67 or the CH3 domain of mFc.67 by overlap-extension PCR, and the products were digested with SfiI enzyme and cloned into a pComb3x vector. The fusion proteins were expressed and purified using a similar procedure as for monomeric Fc proteins described above.

RESULTS

Identification of mFc from a library of Fc mutants. To identify mFcs we utilized a combination of structure-based rational protein design combined with multiple screening strategies of Fc mutant libraries. Fc dimerization is mainly mediated by a large (1000 Å buried surface), tightly packed interface between the two CH3 domains (Fig. 1A). This interface is composed of multiple regions containing at least 16 residues in each chain, most of which are hydrophobic (31,32). We identified four regions in the CH3 domain of human IgG1 contributing to the interchain interactions with the following critical contact residues: L351, T366 and T368, P395, F405, Y407 and K409 (Fig. 1A). This is in agreement with previous studies, e.g. significant destabilizing effects were found by mutation of the
above seven residues in human IgG1 CH3 (34). Two problems must be solved to generate the soluble Fc monomer: disruption of the strong interactions between these residues and prevention of protein aggregation due to exposure of the hydrophobic residues.

We hypothesized that functional and highly soluble Fc monomers could be produced by panning and screening of a large Fc library with extensive mutations in the hydrophobic interface for proper folding, FcRn binding and solubility. Thus a phage library was constructed by randomly mutating the above seven residues (L351, T366, L368, P395, F405, Y407, K409) in human IgG1 Fc. Initially, this library was panned directly against human single-chain soluble FcRn (33). Buffer with pH 6.0 was used for washing and buffer with pH 7.4 was used for elution to select pH-dependent binders. However, enrichment was not observed after two rounds of panning. It is likely that functional binders were masked by ubiquitous misfolded molecules in the library. Thus, the library was first panned against protein G resulting in a library enriched for phage-displayed soluble mFcs. This library was further panned against human FcRn for 5 rounds as described above. To select for highly expressed monomers, 80 clones from the final enriched library were expressed in E. coli and screened by non-reducing SDS-PAGE and Western blot. This panning/screening procedure is schematically depicted in Fig. 1B.

Using this procedure three mFc proteins, mFc.1, mFc.23 and mFc.67, were identified (Fig. 1C). The mFc.1 and mFc.23 contain seven mutations in the CH3 dimer interface while mFc.67 contains six. Although contain different mutations, all of them were expressed in E. coli with high efficiency. Purified mFcs were obtained with yields of 15-20 mg L\(^{-1}\) bacterial culture. All proteins were monomeric with molecular weights of approximately 27 kD as demonstrated by size exclusion chromatography (Fig. 2A).

Identification of mutations in mFc that are essential and sufficient to maintain a soluble monomer. To determine which residues/mutations are essential for generation of a soluble mFc we reversed certain mutations in mFc.23. Six mFc.23 mutants, S351L, R366T, H368L, R366T/H368L, K395P and E405F/K407Y/A409K were generated. They were expressed and purified using a similar procedure as used for the other mFc proteins, and then analyzed by size exclusion chromatography (Fig. 2B). Shoulder peaks appeared near the monomer peaks for the reverse mutants S351L, R366T and R366T/H368L, suggesting formation of a small proportion of dimers. Although only single peaks were observed for H368L and K395P, their shapes were broad and distorted. The E405F/K407Y/A409K mutant of mFc.23 appeared completely monomeric but its expression was relatively low (5 mg protein/L). Taken together, these results suggest that four specific mutations are essential to produce mFc and that the number of mutations needed for formation of soluble mFc can be reduced but results in lower expression.

Stability of mFc. To establish the thermal stability of the mFcs, their CD ellipticity at 216 nm was measured as a function of temperature (Fig. 3A). The stability of the wild-type dimeric Fc and the isolated monomeric CH2 domain were also examined. The midpoint transition temperatures (T\(_m\)) for Fc, mFc.1, mFc.23, mFc.67 and CH2 were 75.1 ± 0.5°C, 45.0 ± 0.6°C, 45.2 ± 0.6°C, 51.0 ± 0.5°C and 54.0 ± 0.8°C, respectively. The thermal refolding was reversible as indicated by the fact that CD spectra of the samples after cooling from 90°C were similar to the original measurements (Fig. 3B).

The stability was further tested by urea denaturation experiments monitored by fluorescence spectroscopy (data not shown). The 50% unfolding of Fc occurred at higher urea concentrations (5.8 M) than that of mFc.1 (4.1 M), mFc.23 (4.1 M), mFc.67 (4.3 M) and CH2 (4.2 M).

We next evaluated the serum stability of the mFc proteins. Samples were incubated with human serum at 37°C, and an aliquot was taken out at each time point and stored at −80°C before Western blot (Fig. 4A) or ELISA (Fig. 4B) analyses. Fig. 4A shows that the band for mFc.1 disappeared after 3 days incubation, while the bands for Fc, mFc.23 and mFc.67 were not evidently diminished even after an 11-day incubation. We further assessed protein degradation by ELISA. Anti-Fc Fab was coated on ELISA plates to capture Fc and mFcs, and anti-FLAG-HRP conjugate were used for detection. From Fig. 4B, it is evident that Fc, mFc.23 and
mFc.67 were degraded more slowly than mFc.1, in agreement with the Western blot results. These data suggest that Fc, mFc.23 and mFc.67 have high serum stability. It is possible that some mutations in mFc.1 make it more accessible to proteases in human serum.

*mFc* bind to FcRn. To test whether these mFcs are functional and behave in a similar manner as wild-type Fc, we first checked if they could still bind protein A and protein G. A monomeric Fc, mFc.1, was applied to a pre-equilibrated protein A and a protein G columns, respectively. The flow-through and eluted factions from the columns were collected and analyzed by SDS-PAGE. As shown in Fig. 5, all proteins were bound to the columns, indicating mFc could bind strongly to protein A and protein G.

We next examined whether monomeric Fc could functionally bind FcRn. Surface plasmon resonance (SPR) experiments were used to validate the pH-dependent FcRn binding and to obtain reliable binding constants. Recombinant human FcRn was immobilized on a CM5 biosensor chip followed by analysis of the interaction using a BIAcore X100 as described in Experimental Procedures. The binding was performed under pH 6.0 and pH 7.4, respectively, and the chip was regenerated with pH 8.0 buffer. As shown in Fig. 6, the mFcs displayed a similar behavior to that of the wild-type Fc. At pH 6.0, the calculated binding affinities (K_D) of wild-type Fc, mFc.1, mFc.23 and mFc.67 to human FcRn were 126, 204, 59 and 111 nM, respectively (Fig. 6A-D). The binding of an Fc mutant (Fc 253A/254A/435A/436A mutant), in which four residues in the FcRn binding interface of Fc were mutated and served as a negative control in this analysis, was too weak to quantify (Fig. 6E). At pH 7.4 neither wild-type Fc nor the mFcs showed detectable binding to FcRn (Fig. 6F). These results demonstrate that the mFcs we generated maintained characteristic pH dependent FcRn binding.

**Fusion proteins with mFcs.** In a final series of experiments, we explored whether our monomeric Fc can be used to produce fusion proteins. m36, an engineered human antibody heavy chain variable domain (VH) targeting the HIV-1 envelope glycoprotein (Env) (13), was used to construct fusion proteins with a monomeric Fc, mFc.67. Two versions of fusion proteins were generated. As shown in Fig. 7A, one is m36 fused with mFc.67 (MW ~39 kD) and the other is m36 fused with only the CH3 domain of mFc.67 (MW ~27 kD). We found that both of them can be efficiently expressed in *E. coli*, with yields of ~20 mg L^{-1} for m36-CH3mFc.67 fusion protein and 5-10 mg L^{-1} for m36-mFc.67 fusion protein. Size exclusion chromatography results indicated that they are both in pure monomeric forms, with molecular weight of ~27 kD for m36-CH3mFc.67 and ~39 kD for m36-mFc.67 (Fig. 7B).

We then assessed whether the effector molecules in these fusion proteins are still active. m36 exhibits potent broadly neutralizing activity against HIV-1 by targeting a highly conserved CD4 binding-induced structure on the Env gp120 (13). As shown in Fig. 7C, m36 and the two fusion proteins, m36-mFc.67 and m36-CH3mFc.67, exhibited comparable binding to gp120_{Bal}-CD4, a single-chain fusion protein of gp120_{Bal} with soluble two-domain human CD4 (35), as measured by ELISA. Wild-type Fc and mFc.67 monomer did not show any binding ability. Taken together, these results confirmed that monomeric Fc can be used to develop functional monomeric fusion proteins.

**DISCUSSION**

In the present study, several mFcs were generated using a combination of rational design and multiple panning/screening methods. In nature many proteins are in oligomeric states, and some are "compulsory complexes" in which free monomers are not available (36,37). Although much effort has been put into disrupting such oligomers, most of the engineered monomers were insoluble or poorly structured. To address this challenge large libraries could be constructed and desired monomers could be selected by directed evolution. Besides, a multiple screening strategy is very valuable because it provides multiple evolutionary pressures. Support for this view is provided by the fact that the screening of our library directly against FcRn did not produce mFcs but an additional round against protein G before FcRn screening successfully produced some highly soluble and functional monomers. We expect that our strategy could be extended to other
cases of monomer development, and expand the arsenal of protein engineering.

A significant application of monomeric Fc is for the so-called "monomeric Fc fusion" technology where a monovalency of the active protein is presented but currently it is fused to dimeric wild-type Fc (23,24,25). This "monomeric" technology is an upgraded version of the traditional dimeric Fc fusion molecules which contain two effector molecules. It has been shown to be more robust due to better tissue penetration offered by the smaller size, and a reduced steric hindrance which can make effector protein and/or Fc part more effective. For instance, it has been found that "monomeric" factor IX (FIX)-Fc fusion protein not only has an extended half-life compared to the FIXFc dimer, but also greatly enhanced pharmacokinetics, with a 10-fold increase in $C_{\text{max}}$ and more than a 12-fold increase in AUC (25). These advantages are valuable because they provide cheaper therapeutics and enhance the delivery of therapeutic proteins by non-invasive routes. In this study we have shown that mFc, which is only half the size of the Fc dimer but retains FcRn binding ability, can replace dimeric Fc and generate mFc fusion proteins. Such constructs consist of only one effector molecule and one Fc monomer, thus the size is largely reduced compared to the current "monomeric Fc fusion", and may lead to a new type of promising therapeutics.

Moreover, mFc represents a novel antibody format in the field of therapeutic antibodies. Currently, monoclonal antibodies have enjoyed widespread therapeutic applications, and represent the largest class of biological drugs. However, antibody therapeutics have demonstrated difficulty in penetrating tissues due to their large size. A variety of small antibody formats, such as Fab, Fv, scFv, VH and VHH, have been developed but at the expense of their in vivo half-lives (16,17). Thus, it is emerging that the generation of small engineered antibodies with appropriately long half-lives may lead to a new therapeutic revolution. Our work establishes the development of functional fusion proteins of antibody fragments with monomeric Fc that have molecular weights of ~30 and 40 kD, only one-fourth the size of IgG.

We also hypothesize that mFcs themselves could serve as novel antibody formats and be used as scaffolds for construction of libraries containing diverse binders to various antigens. They are relatively stable, can bind FcRn in a pH-dependent manner, and can be produced in large quantities in bacteria. Importantly, compared to wild-type Fc a large surface area is exposed in the Fc monomers due to the disruption of the CH3 dimerization interface, providing more access for protein engineering by designed point mutations and CDR-grafting onto a mFc framework. It is anticipated that binders generated from such a design would have molecular weights of approximately 27 kD, similar to that of scFv but possess much longer in vivo half-lives.

Immunogenicity is a potential problem for any protein-based therapeutics. Many factors could affect immune responses elicited by therapeutic proteins, such as molecular size, structural features, storage conditions, T-cell epitopes and others (5). Although currently immunogenicity cannot be predicted and only human clinical trials can definitely show whether these new proteins are immunogenic, it is likely that their immunogenicity is relatively low compared to similar foreign proteins for several reasons. First, mFcs are fully human proteins and the probability that several additional mutations will dramatically increase immunogenicity is relatively low. Second, they are smaller in molecular size than the wild-type Fc and perhaps more importantly they are monomeric and possible avidity effects may not exist. Third, mFcs seems to retain the structural characteristics of the wild-type Fc, as suggested by their similar CD spectra (Fig. 3B). Fourth, some of the mFcs have slightly less aromatic amino acids (AAA) (which may contribute more to immunogenicity than non-aromatic) than wild-type Fc (20 AAA in Fc and mFc.1, 18 in mFc.23, 19 in mFc.67). Further engineering of these proteins to reduce the number of mutations compared to wild-type Fc could also contribute to a potential decrease in immunogenicity if successful. Finally, our results provide direct evidence that mFc can bind FcRn comparably to Fc and therefore dimerization is not required for effective binding to FcRn. The stoichiometry of the interaction between FcRn and its ligand has been a matter of debate (20,26,27,28,29). In the cocrystals, a long, repeating oligomeric ribbon with a 2n:n stoichiometry was observed, in which FcRn...
dimers are bridged by Fc molecules so that every Fc dimer interacts with two receptors (38). Studies also suggested that the 2:1 FcRn/Fc complex is formed in solution, although FcRn is not capable of dimerization in these conditions (28,29). However, in some studies 1:1 FcRn/Fc stoichiometry was also observed under non-equilibrium conditions (27,29). It is not clear which of the complexes are physiologically relevant, but clearly the stoichiometry of the FcRn-Fc interaction is essential to further our understanding of the mechanism by which FcRn transports IgG in vivo, and also important to facilitate antibody engineering to modify the IgG-FcRn interactions in vitro. Our mFcs presented a unique opportunity to access the Fc-FcRn interactions; the fact that an Fc monomer is capable of binding FcRn offers direct evidence for the 2:1 FcRn/Fc stoichiometry. It is noteworthy to point out that some discrepancies among different assay systems have been reported in the FcRn binding studies (20); however, our data definitely show that mFc binds one molecule FcRn and doesn’t require avidity effects. It is also interesting to note that mFcs have an affinity ($K_D$) similar to that of the dimeric Fc for FcRn binding (mFc.1 126 nM, mFc.23 204 nM, mFc.67 59 nm and wild-type Fc 111 nM). Two distinct FcRn-Fc binding mechanisms could account for this similarity. In one, the two FcRn molecules bind symmetrically to two Fc monomers in a dimeric Fc, and in the other, the two FcRn bind asymmetrically: the first FcRn molecule binds to an Fc monomer in a similar manner to the FcRn-mFc binding, and the binding of the second FcRn to Fc must have a low affinity, to give an overall comparable $K_D$ to the one-step FcRn-mFc binding. The latter mechanism seems more likely, since a bent, asymmetrical Fc structure was found in the 1:1 FcRn/IdFc (heterodimeric Fc that can only binds one FcRn) cocrystal (38). The low affinity of the second FcRn binding is probably due to the conformational change in Fc caused by the binding of the first FcRn. The low affinity also explains the observation of the 1:1 FcRn/Fc stoichiometry under non-equilibrium conditions.

In addition to binding FcRn to protect antibodies from degradation the Fc also mediates effector functions, such as antibody-dependent cellular cytotoxicity (ADCC) by binding Fcγ receptors, and complement-dependent cytotoxicity (CDC) by binding the complement factor C1q. The N-linked glycosylation on Asn297 of the Fc CH2 domain plays an important role in these functions. The mFcs described in this study are expressed in E. coli and are not glycosylated. In addition, they are monomeric and therefore their binding to Fcγ receptors and C1q if any is expected to be significantly weaker compared to glycosylated wild-type dimeric Fc. In many cases, e.g., when developing binders against soluble monomeric ligands, effector functions may not be needed. In cases when such functions are needed they could be engineered and the proteins could be expressed in mammalian cells. It is reasonable to expect that mFc would have similar glycosylation profile as wild-type Fc, because the mutations in mFc are in the CH3 domain while the CH2 domain is the same. In conclusion, we have generated several mFcs using a novel panning/screening strategy. The monomers could be expressed in E. coli with high efficiency, are relatively stable, and retain the ability to bind FcRn in a pH dependent manner. These proteins are promising as tools to explore mechanisms of Fc interactions with FcRn and for the development of mFc fusion proteins; they could also represent robust building blocks for mFc based antibody engineering which could produce a new type of therapeutic antibodies with small size, long half-lives, and antigen-binding capabilities.

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**FOOTNOTES**

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The abbreviations used are: mAbs, monoclonal antibodies; FcRn, neonatal Fc receptor; mFc, monomeric Fc; SEC, size exclusion chromatography; CD, circular dichroism; SPR, surface plasmon resonance.

**FIGURE LEGENDS**

**Figure 1** A. Structure of human IgG1 Fc CH3 domain, showing key residues in its dimerization interface (PDB entry 2WAH). B. Schematic of the multiple panning/screening strategies. C. Amino acid sequence alignment of human IgG1 Fc, mFc.1, mFc.23 and mFc.67. Here the numbering begins with the IgG1 Fc, thus residues 121, 136, 138, 165, 175, 177, 179 correspond to residues 351, 366, 368, 395, 405, 407 and 409 in IgG1 numbering system, respectively.

**Figure 2** Size exclusion chromatography of (A), wild-type IgG1 Fc, mFc.1, mFc.23, mFc.67 and (B), the 351L, 366T, 368L, 366T/368L, 395P, and 405F/407Y/409K mutants of mFc.23. In both panels, the insets show a standard curve for the gel filtration standards.

**Figure 3** A. Plots of the change in fraction folded (calculated from CD molar ellipticity at 216 nm) for Fc, CH2, mFc.1, mFc.23 and mFc.67. B. CD spectra of Fc, mFc.1, mFc.23, and mFc.67 at 25°C (—) and refolding samples at 25°C after heating to 90°C (····).

**Figure 4** Serum stability of Fc, mFc.1, mFc.23 and mFc.67 measured by Western blot (A) and ELISA (B). For ELISA, plates were coated with anti-Fc F(ab')2 and anti-FLAG HRP conjugate was used for detection.

**Figure 5** The flow-through and elution of mFc.1 from protein A and protein G columns were analyzed by SDS-PAGE.
Figure 6 FcRn binding of mFc.1 (A), mFc.23 (B) mFc.67 (C), Fc (D), the Fc 253A/254A/435A/436A mutant (E) at pH 6.0, and mFc.1 at pH 7.4 (F) measured by BIAcore.

Figure 7 A. Schematic of different antibody fragments and monomeric Fc fusion proteins. B. Size exclusion chromatography of m36-CH₃mFc.67 and m36-mFc.67 fusion proteins. C. Binding of m36 and fusion proteins to gp120Bal-CD4 measured by ELISA.
Figure 3

A

B

[Graphs and data plots are shown here, depicting fraction folded vs. temperature and molar extinction coefficient vs. wavelength for different samples.]
Figure 4

A

|   | Fc   | mFc.1 | mFc.23 | mFc.67 |
|---|------|-------|--------|--------|
| 1 | ![Image 1](image1.png) | ![Image 2](image2.png) | ![Image 3](image3.png) | ![Image 4](image4.png) |
| 3 | ![Image 5](image5.png) | ![Image 6](image6.png) | ![Image 7](image7.png) | ![Image 8](image8.png) |
| 6 | ![Image 9](image9.png) | ![Image 10](image10.png) | ![Image 11](image11.png) | ![Image 12](image12.png) |
| 9 | ![Image 13](image13.png) | ![Image 14](image14.png) | ![Image 15](image15.png) | ![Image 16](image16.png) |
| 11| ![Image 17](image17.png) | ![Image 18](image18.png) | ![Image 19](image19.png) | ![Image 20](image20.png) |

Incubation time (day)

B

![Graph](graph.png)

- mFc.23
- mFc.67
- Fc
- mFc.1

Incubation time (day)

OD405
Figure 5

1: Marker; 2: mFc.1 before applying ProteinA/G; 3: Protein A flow-through; 4: Protein A elution; 5: Protein G flow-through; 6: Protein G elution.
Figure 6

A

mFc.1, pH 6.0

B

mFc.23, pH 6.0

C

mFc.67, pH 6.0

D

Fc, pH 6.0

E

Fc 253A/254A/435A/436A, pH 6.0

F

mFc.1, pH 7.4
Figure 7

A. IgG ~ 150 kDa
   Fab ~ 50 kDa
   scFv ~ 28 kDa
   VH ~ 15 kDa
   monomeric Fc ~ 27 kDa
   Fusion of VH and CH3 of mFc ~ 27 kDa
   Fusion of VH and mFc ~ 39 kDa

B. Emission volume (mL)
   Absorbance (AU)

C. Antibody concentration (nM)
   OD450
   m36-mFc.67
   m36
   m36-CH3-mFc.67
   mFc.67
   Fc
