Engineered Soluble Monomeric IgG1 CH3 Domain

**GENERATION, MECHANISMS OF FUNCTION, AND IMPLICATIONS FOR DESIGN OF BIOLOGICAL THERAPEUTICS**

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Tianlei Ying1, Weizao Chen†, Yang Feng†, Yanping Wang‡, Rui Gong‡, and Dimiter S. Dimitrov‡

From the †Protein Interactions Group, Cancer and Inflammation Program, Center for Cancer Research, NCI, National Institutes of Health, Frederick, Maryland 21702 and ‡SAIC-Frederick, Inc., Frederick, Maryland 21702

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**Background:** The CH3 domain of an antibody is a homodimer.

**Results:** Soluble monomeric IgG1 CH3 (mCH3) exhibits pH-dependent binding to FcRn.

**Conclusion:** The mCH3 can be used as a new scaffold for generation of binders with potentially enhanced half-life.

**Significance:** The mCH3 is a promising fusion partner for therapeutic proteins with increased therapeutic efficacy.

Most of the therapeutic antibodies approved for clinical use are full-size IgG1 molecules. The interaction of the IgG1 Fc with the neonatal Fc receptor (FcRn) plays a critical role in maintaining their long half-life. We have hypothesized that isolated Fc domains could be engineered to functionally mimic full-size IgG1 (nanoantibodies) but with decreased (10-fold) size. Here, we report for the first time the successful generation of a soluble, monomeric CH3 domain (mCH3). In contrast to the wild-type dimeric CH3, the mCH3 exhibited pH-dependent binding to FcRn similar to that of Fc. The binding free energy of mCH3 to FcRn was higher than that of isolated CH2 but lower than that of Fc. Therefore, CH3 may contribute a larger portion of the free energy of binding to FcRn than CH2. A fusion protein of mCH3 with an engineered antibody domain (m36.4) also bound to FcRn in a pH-dependent fashion and exhibited significantly higher neutralizing activity against HIV-1 than m36.4-Fc fusion proteins. The m36.4-mCH3 fusion protein was monomeric, stable, soluble, and expressed at a high level in *Escherichia coli*. We also found that engineering an additional disulfide bond in mCH3 remarkably increased its thermal stability, whereas the FcRn binding was not affected. These data suggest that mCH3 could not only help in the exploration of the dual mechanisms of the CH3 contribution to Fc functions (dimerization and FcRn interactions) but could also be used for the development of candidate therapeutics with optimized half-life, enhanced tissue penetration, access to sterically restricted binding sites, and increased therapeutic efficacy.

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The vast majority of the monoclonal antibodies (mAbs) approved for clinical use are full-size antibodies in IgG1 format (1–4). The IgG1 CH3 domain has two important functions: dimerization of the IgG1 Fc and interaction with the neonatal Fc receptor (FcRn).2 It is well established that the Fc interaction with the FcRn plays a critical role in maintaining the long half-life of IgG1 (5–7). The antibody interacts with FcRn by a pH-dependent mechanism that results in the binding of the IgG1 Fc to FcRn in the acidic environment of the endosomes, the recycling of the IgG1 to the cell surface, and the subsequent release of IgG1 back into the circulation at physiological pH. This process decreases IgG1 degradation, thereby extending its *in vivo* half-life. The Fc–FcRn interaction has been the focus of a number of engineering efforts seeking to modulate the antibody pharmacokinetics, and fusion to IgG1 Fc (molecular mass ~55 kDa) has been developed as an important strategy for extending the half-life of therapeutic proteins (8, 9). It is known that both the CH2 and the CH3 domains of the IgG1 Fc interact with FcRn. Identification of the involved residues has led to the development of Fc variants with increased pH-dependent FcRn binding and *in vivo* half-life (7, 10–12). However, the individual contribution of Fc domains to the pH-dependent mechanism of FcRn binding is not known. Identification of a domain that could best mimic Fc in terms of binding to FcRn is also important for the development of therapeutic proteins of both optimized half-life and small size for enhanced tissue penetration, access to sterically restricted binding sites, and lower production cost.

We have previously generated isolated single CH2 domains and monomeric Fc (mFc) and characterized their interactions with FcRn (13–15). Here, we report for the first time the successful generation of a soluble, monomeric CH3 domain (mCH3). We found that the engineering of CH3 by structure-based mutagenesis, which resulted in soluble mFcs (15), was not effective in the generation of soluble mCH3. This was likely due to the absence of the highly soluble CH2. In this current study, we found that a specific combination of four mutations is essential in generating soluble mCH3. In contrast to the wild-type dimeric CH3 (CH3), the mCH3 exhibited pH-dependent binding to a human single-chain soluble FcRn (sFcRn) (15, 16), which resembled that of bacterially expressed Fc but with lower affinity (KD = 940 nM) at pH 6. The free energy of mCH3 bind-
ing to sFcRn was higher than that of isolated CH2 and dimeric CH3 (which did not bind FcRn) but lower than that of mFc. These results indicate that CH3 in Fc may contribute a larger portion of the free energy of binding to sFcRn than CH2.

To increase the stability of isolated mCH3, we engineered an additional disulfide bond, which resulted in a remarkable increase in the melting temperature, \( T_{\text{m}} \), from 40.6 to 76.0 °C, and a 5-fold increase in protein expression with retained binding to FcRn. These data suggest that a stable, soluble mCH3 can be generated and used as a new scaffold for the generation of binders with potentially enhanced half-life.

We also demonstrated that a fusion protein of mCH3 with an antibody heavy-chain variable domain (VH), m36.4 (17), bound to FcRn in a pH-dependent fashion and exhibited significantly higher HIV-1-neutralizing activity than the large size VH-Fc fusion proteins. This provides direct evidence that the size of therapeutic proteins is important for targeting sterically restricted epitopes. In addition, we demonstrated that the VH-mCH3 fusion protein was monomeric, stable, and soluble expressed at a high level in Escherichia coli. Thus, the mCH3 is also a promising candidate for a therapeutic protein fusion partner with potentially better tissue penetration, reduced steric hindrance, and increased therapeutic efficacy.

**EXPERIMENTAL PROCEDURES**

**Cloning of mCH3**—The following primers were used: Omp-F, 5'-AAAGACACGTATGCCAGTGCAG-3'; gIIl-R, 5'-ATCACCGGAAACCAGGCCACCAC-3'; CH3-F, 5'-GTTGATGTAACGGCCCAGGCCGAGCCCCGAG-3'; dCH2-F, 5'-GCCAAAGACAAAACTCACACAGCAGCTGAACCTGGGGAGAC-3'; dCH2-R, 5'-CAGGAGTTCAAGGTGCTGTGATGGAGTTTTGCTTTGAGATGTTTTTCT3'- (where F and R indicate forward and reverse). The wild-type CH3 gene was amplified by PCR from an Fc-expressing plasmid constructed in the pComb3x vector (primer: CH3-F and gIII-R). Four residues were mutated in mCH3 as compared with wild-type CH3 (residues 351, 366, 368, and 395). The dimeric CH3 gene was generated by joining two CH2 genes together with a human IgG1 hinge (DKTHT) using an overlap-extension PCR (primer: Omp-F, dCH2-R; dCH2-F, gIII-R).

**Cloning of mCH3 Fusion Proteins**—The m36.4 gene was amplified from the m36.4-encoding plasmid pCom36.4, as described previously (17), and was joined to the mCH3 gene to construct the m36.4-mCH3 fusion protein. A (G4S)3 linker was inserted between m36.4 and mCH3 by overlap-extension PCR. The products were digested with SfiI and cloned into a pComb3x vector. The wild-type CH3 gene was amplified by PCR from the m36.4-encoding plasmid pCom36.4, as described previously (15). Protein purity was judged by SDS-PAGE, and protein concentration was measured spectrophotometrically (Nanovue, GE Healthcare).

**Size Exclusion Chromatography**—Purified antibody domains and fusion proteins were loaded onto a Superdex 75 10/300 GL column running on an FPLC ÄKTA BASIC pH/C system (GE Healthcare). PBS (pH 7.4) was used as the running buffer throughout (flow rate 0.5 ml/min), and eluting proteins were 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).

**Circular Dichroism (CD)**—The CD spectra were collected with an AVIV Model 202 spectropolarimeter (Aviv Biomedical). Purified antibody domains and mCH3 fusion proteins were dissolved in PBS, pH 7.4, at the final concentration of 0.25 mg/ml. For native structure measurement, spectra of mCH3 and wild-type CH3 were collected from 200 to 260 nm (0.1-cm path length) at 25 °C. For evaluation of thermal stability, CD signals at 225 nm were recorded for wild-type CH3, and signals at 216 nm were recorded for all other antibody domains and fusion proteins. The instrument was programmed to acquire spectra at 1 °C intervals over the range 25–90 °C.

**Surface Plasmon Resonance Binding Experiments**—Surface plasmon resonance measurements were performed using a BIacore X100 instrument (GE Healthcare). Purified human soluble single-chain FcRn was immobilized on a CMS biosensor chip using a primary amine coupling in 10 mM sodium acetate buffer (pH 5.0). To test binding at pH 7.4, the proteins were diluted in PBS plus 0.005% Tween 20. To test binding at pH 6.0, the same running buffer was adjusted to pH 6.0 with HCl. The running buffer was allowed to flow through the cells at a rate of 30 μl/min. The analytes consisted of serial dilution of proteins between 1 μM and 62.5 μM. The chip was regenerated with pH 8.0 buffer (100 mM Tris, 50 mM NaCl, pH 8.0) after 10 min of dissociation.

**ELISA**—Recombinant protein A (Sigma-Aldrich) and protein G (Invitrogen) were coated on ELISA plate wells at 50 ng/well in PBS overnight at 4 °C and blocked with protein-free blocking buffer (Thermo Scientific) at 37 °C for 2 h. 2-fold serially diluted protein was added and incubated at 37 °C for 2 h. The plates were washed with PBST (PBS plus 0.05% Tween 20), and horseradish peroxidase (HRP)-conjugated anti-FLAG tag antibody (Sigma-Aldrich) in PBS was incubated in the wells for 1 h at 37 °C. After extensive washes with PBST, the binding was detected by the addition of diammonium 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) substrate (Roche Applied Science) and monitored at 405 nm. For measurement of competition between mCH3 and IgG1, ELISA plates were coated with recombinant protein G and blocked as described above. mCH3 at a concentration of 500 nM was premixed with 2-fold serially diluted competitor human IgG1 antibodies. Mix-
Monomeric IgG1 CH3

atures were subsequently added to each ELISA well and incubated. Bound mCH3 was detected with HRP-conjugated anti-FLAG tag antibody, and the assay was developed as described above.

Pseudovirus Neutralization Assay—Viruses pseudotyped with HIV-1 Envs were prepared by co-transfection of 70–80% confluent 293T cells with pNL4-3.luc.E-R- and pSV7d constructs encoding HIV-1 Envs by using the PolyFect transfection reagent (Qiagen) according to the manufacturer’s instructions. Pseudotyped viruses were obtained after 48 h by centrifugation and filtration of cell culture through 0.45-μm filters. Neutralization assays were performed as follows. Viruses were mixed with different concentrations of antibodies for 1 h at 37 °C, and then the mixture was added to 1.5 × 10^4 HOS-CD4-CXCR4 cells grown in each well of 96-well plates. Luminescence was measured after 48 h by using the Bright-Glo luciferase assay system (Promega, Madison, WI) and a LumiCount microplate luminometer (Turner Designs). Mean relative light units for duplicate wells were determined. Relative infectivity (%) was calculated by the following formula: (average relative light units of antibody-containing wells/average relative light units of virus-only wells) × 100.

Simulation of Molecular Structures—The initial coordinates of mCH3 and mCH3cc were created based on the crystal structure of IgG1 Fc (Protein Data Bank (PDB) entry: 2WAH). The m36.4-mCH3 and m36.4h1Fc models were created with both the VMD program and the Swiss-Model homology-modeling server. The structures were further simulated with CHARMM force field by using the NAMD program. Water molecules were retained in the simulation. The structures were first minimized for 5000 steps with the conjugate gradient method and equilibrated for 10 ps with the time step of 1 fs, and then were further minimized for 100,000 steps for analysis with VMD program.

RESULTS

Generation of Soluble mCH3—We previously reported the generation of three mFc proteins using a novel multiple panning/screening procedure (15). A combination of six or seven specific mutations on the CH3 dimerization interface caused mFcs to be highly soluble and monomeric. We hypothesized that isolated single CH3 domains from these mFcs would also be soluble and monomeric. We therefore cloned the CH3 gene from the mFcs into the pComb3x vector and tested for soluble expression in E. coli. Unfortunately, the isolated CH3 domains were not expressed in soluble form in E. coli. To solve this problem, we performed structure-guided mutagenesis of seven contact residues at the CH3 dimerization interface. A specific combination of four mutations (residues 351, 366, 368, and 395) (Fig. 1) that is essential to generate mCH3 was identified. Mutation of any of these residues in mCH3 eliminated the soluble expression. The expression of mCH3 was relatively low (2 mg/liter of bacterial culture).

The isolation of the mCH3 provided an opportunity for comparative analysis of IgG1 Fc CH2 and CH3 domains in monomeric and dimeric formats (Fig. 2A). The CH2 domain was previously characterized and shown to have a structure similar to that of the intact glycosylated Fc (18). The dimeric CH2 (dCH2) was generated by linking two CH2 molecules with a five-residue linker (DKTHT) from the human IgG1 hinge region. The native dimeric CH3 was cloned from the Fc with the same primers that were used to generate mCH3. A monomeric Fc protein, mFc.67, and the wild-type Fc were also expressed. All domains and fragments were expressed and purified as described previously (15).

There were no detectable impurities as shown by SDS-PAGE (Fig. 2B). The mCH3 ran slightly slower than the wild-type CH3 likely due to the small difference in their molecular masses (14.4 kDa versus 14.3 kDa, respectively). The mCH3 was monomeric as demonstrated by size exclusion chromatography (Fig. 2C). The CH2 was also monomeric with a slightly larger size than the mCH3 (Fig. 2C). The CH3 was dimeric (molecular mass ~28 kDa) as expected, and the dCH2, which consists of two CH2 molecules, was of about the same size (molecular mass ~29 kDa).

Conformation and Stability of mCH3—The overall structure of CH2 and CH3 is a seven-stranded antiparallel β-barrel with a buried stabilizing disulfide bond (19). Despite the topological similarity, the CH3 was found to be much more stable than the CH2 (20, 21). Whether the high CH3 stability is due to the structure of the CH3 molecule per se or to the homodimerization was unknown. To investigate the influence of dimerization on the structure of CH3, we first compared the CD spectra of mCH3 and dimeric CH3. Interestingly, we found significant differences in their spectra. As shown in Fig. 2D, the spectrum of mCH3 exhibited a local minimum at 216 nm, which is very similar to that of an isolated CH22, indicating that isolated mCH3 is intact and well folded. In contrast, the spectrum of isolated wild-type (dimeric) CH3 exhibited a minimum at 225 nm. These results suggest that mCH3 has a conformational state different from that of an isolated CH3 dimer.

The thermal stability of mCH3 and other Fc domains was then assessed by following changes in the CD spectra in response to increasing temperature (Fig. 3). The midpoint transition (melting) temperature (Tm) of mCH3 was 40.6 ± 0.3 °C. This value is similar to that of an isolated murine CH2 domain, which was reported to have a Tm of 41 °C. The isolated human CH2 domain (Tm = 54.5 ± 0.2 °C) and mFc.67 (Tm = 51.0 ± 0.5 °C) are more stable than mCH3. Notably, the isolated CH3 (Tm = 82.4 ± 0.6 °C) was found to be more stable than the wild-type Fc (Tm = 78.9 ± 0.5 °C). The dimeric CH2, in which two CH2 molecules were joined by a linker, did not show
enhanced stability ($T_m = 49.2 \pm 0.5 \degree C$). Taken together, these results indicate that strong intermolecular hydrophobic interactions are critical for stabilizing the CH3 homodimer, although the four mutations required for mCH3 generation could also have destabilizing effects.

**FcRn Binding**—We next explored whether mCH3 could bind FcRn in a pH-dependent manner. Binding to a human sFcRn immobilized on a BIAcore chip was measured as described previously (15). The measurements were carried out at pH 6.0 or pH 7.4, and the chips were regenerated after binding by injection of a pH 8.0 buffer. Although the isolated wild-type dimeric CH3 did not show any FcRn binding at either pH 6.0 or pH 7.4, significant binding was observed at pH 6.0 for mCH3, but not at pH 7.4 (Fig. 4, A–C). Because mCH3 has a different conformation than that of an isolated CH3, it is likely that the conformational changes resulted in a binding site on mCH3 that is more accessible to FcRn than in the isolated CH3 dimer, thereby conferring pH-dependent binding capability.

The calculated affinity ($K_d$) of mCH3 was 940 nM. As expected, it was not as high as that of the wild-type bacterially
expressed Fc, which was shown previously to bind with a $K_d$ of 126 nM (15). Both CH2 and CH3 contribute to the Fc interaction with FcRn. In an attempt to dissect their relative contribution, we also measured the affinity of an isolated CH2 to FcRn. We found that there was an unusual delay during the association phase, and the dissociation of CH2 from the chip was very slow (Fig. 4D). The calculated affinity was 4 nM. We also noted that the CM5 chip could be better regenerated by the pH 8.0 buffer after the mCH3 binding measurement as compared with that after the CH2 binding measurement, although neither of the two proteins showed detectable binding at pH 7.4. This behavior is not surprising because most of the interface residues of the Fc-FcRn complex in the CH2 domain are involved in hydrophobic interactions, which are thought to be inherently “sticky,” as exemplified by residues Ile-253 and Ser-254. In contrast, the FcRn binding residues in the CH3 domain may participate in the formation of titratable salt bridges, which are likely to confer most of the pH dependence to the Fc-FcRn interactions, as exemplified by residues His-433 and H435. These results indicate that the CH2 and CH3 domain may have distinct binding properties and function differently when participating in the pH-dependent interaction with FcRn.

Protein A/G Binding—It is well documented that the Fc can bind a diverse set of proteins (22, 23). Examples include proteins A and G, the bacterial cell wall proteins, which have been widely used to detect and purify immunoglobulins. Although they do not share homology in sequence or structure, their binding sites on the Fc were found to overlap at the inter-CH2/CH3 domain region, which is also used by the Fc to interact with FcRn (23). We further examined protein A and G binding to mCH3 and CH2 using ELISA. As shown in Fig. 5, the wild-type Fc binds to protein A or G with very low EC50. The monomeric Fc, mFc.67, also has a high binding capability to protein G but a weakened binding to protein A. mCH3 can bind both protein A and protein G, although its binding affinities for both were decreased because it only possesses part of the binding site of the wild-type Fc. The isolated CH2 domain can bind protein G, but its binding to protein A is very weak. Consistent with the FcRn binding results described above, the isolated dimeric CH3 did not bind to either protein A or protein G. Additionally, the isolated CH2 monomer can bind protein G, but no detectable binding was observed for the dimeric single-chain CH2 in which two CH2 molecules were connected by a hinge. These results suggest that the appropriate conformation of an antibody domain is a key requirement for its binding to protein A/G (Table 1).

To analyze the binding site of protein G on mCH3, we developed a competition ELISA assay. IgG1 was shown to bind protein G more potently than mCH3 and thus was used as a strong competitor in this experiment. As shown in Fig. 5C, mCH3 binds well to protein G and was competitively inhibited by IgG1, suggesting that mCH3 and IgG1 share the same binding site on protein G.

VH-mCH3 Fusion Protein Is Monomeric and Stable—We next examined whether mCH3 can be used for generating monomeric and stable fusion proteins. In this proof-of-concept study, m36.4, a cross-reactive HIV-1 neutralizer (17), was used to generate the VH-mCH3 fusion protein. m36.4 was joined to mCH3, resulting in a high expression efficiency in E. coli (more than 10 mg of purified protein per liter of culture). Size exclusion chromatography analysis confirmed that the fusion protein was monomeric, with a molecular size of ~26 kDa (Fig. 6A). It was also found to have a relatively high $T_m$ of 62.9 ± 0.3 °C (Fig. 6B).

Binding of the VH-mCH3 Fusion Protein to FcRn—Fc fusion proteins have been found to possess, in many cases, lower FcRn binding affinities than wild-type Fc. The isolated CH2 domain can bind protein G, but its binding to protein A is very weak. Consistent with the FcRn binding results described above, the isolated dimeric CH3 did not bind to either protein A or protein G. Additionally, the isolated CH2 monomer can bind protein G, but no detectable binding was observed for the dimeric single-chain CH2 in which two CH2 molecules were connected by a hinge. These results suggest that the appropriate conformation of an antibody domain is a key requirement for its binding to protein A/G (Table 1).

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binding affinities than those of mAbs, suggesting that the FcRn binding region of the Fc fusion proteins may exhibit some differences as compared with native mAbs (24). We investigated the FcRn binding of the VH-mCH3 fusion protein. As shown in Fig. 6C, the $K_D$ value calculated from this experiment was 685 nM at pH 6.0, which is comparable with that of the isolated mCH3 (940 nM). No binding was observed at pH 7.4, indicating that the fusion protein binds to sFcRn in a strictly pH-dependent manner. These results confirmed that mCH3 could be used as a fusion partner to confer pH-dependent FcRn binding capability.

Potent Neutralization of Pseudotyped HIV-1 Isolates by m36.4-mCH3—The engineered antibody domain m36.4 targets highly conserved CD4-induced structures on the HIV-1 envelope glycoprotein (17). We have previously shown that this epitope is sterically obstructed and fully accessible only by relatively small-size molecules during virus entry (17). To determine the potency of HIV-1 neutralization by m36.4-mCH3, viruses pseudotyped with Envs from the HIV-1 isolates Bal and JRFL were used. The dimeric CH3 fusion (molecular mass ~60 kDa) and Fc fusion (molecular mass ~110 kDa) did not show any measurable neutralizing activity against these two isolates. By contrast, as shown in Fig. 7, m36.4 exhibited particularly

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**TABLE 1**

Summary of FcRn, protein A, and protein G binding capabilities of antibody domains characterized in our study

| Antibody domain | FcRn | Protein A | Protein G |
|-----------------|------|-----------|-----------|
| Fc              | +++  | +++       | +++       |
| mFc             | +++  | ++        | +         |
| dCH2            | --   | --        | --        |
| CH2             | +    | +         | +         |
| CH3             | --   | --        | --        |
| mCH3            | +    | ++        | +         |

**FIGURE 5.** Protein A and protein G binding of IgG1 Fc domain. A and B, protein A (A) and protein G (B) binding of IgG1 Fc domains measured by ELISA. OD, optical density. C, competition of mCH3 (500 nM) with IgG (0–1 mM, 2-fold serially diluted) for binding to protein G (blue dot). Protein G binding of mCH3 (0–1 mM, 2-fold serially diluted) is also shown for comparison (pink triangle).
strong neutralization, with an IC₅₀ (antibody concentration resulting in 50% inhibition of virus infection) of 8 nM (Bal) and 25 nM (JRFL). The IC₅₀ of m36.4-mCH₃ was slightly higher: 38 and 30 nM for Bal and JRFL, respectively. The slightly lower neutralizing activity of m36.4-mCH₃ as compared with m36.4 is not surprising because its size (28 kDa) is twice that of m36.4 (14 kDa). Although side-by-side comparison was not done, it is noteworthy to point out that m36.4-mCH₃ appears to be more potent than the gp41-derived peptide C34, which has an IC₅₀ of 270 nM against Bal and JRFL (17, 25). The C34 exhibits HIV entry inhibitory activity comparable with or higher than that of the Food and Drug Administration (FDA)-approved peptide entry inhibitor T20 (brand name Fuzeon), which is in clinical use. These results suggest that m36.4-mCH₃ could have potential as an HIV entry inhibitor for therapeutic/prophylactic applications.

Increasing the mCH₃ Stability by an Engineered Disulfide Bond—The thermal stability of mCH₃ was relatively low (Tₘ = 40.6 °C). We and others have reported that the introduction of additional disulfide bonds can enhance the stability of CH2 and Fc (13, 26). We used structure-based design to choose specific residues for introduction of additional cysteines and generated the mCH₃cc mutant, which has an additional disulfide bond between the Cys-343 and Cys-431, which joins the N-terminal A strand and the C-terminal G strand of the mCH₃ (Fig. 8A). We found that the mCH₃cc can be more efficiently produced (yield of more than 10 mg/liter of bacterial culture) in E. coli than mCH₃ and is significantly more stable than mCH₃. The Tₘ of the mCH₃cc was 76.0 ± 0.6 °C, 35.4 °C higher than that of the mCH₃ (Fig. 8B). The engineered CH₃ monomer was also >99% monomeric as indicated by size exclusion chromatography (Fig. 8C). The affinity (Kᵣ) of mCH₃cc to sFcRn was calculated to be 1.1 µM at pH 6.0, similar to that of the mCH₃ (940 nM). No binding was detected at pH 7.4, indicating that the strictly pH-dependent FcRn binding capacity was retained.

DISCUSSION

The biological function of CH3 is two-fold; it provides bivalency through dimerization, and it increases half-life in the circulation by contributing to the Fc interaction with FcRn. To understand the mechanisms of these functions, we generated...
an isolated IgG1 CH3 monomer and expressed it in *E. coli*. This is, to the best of our knowledge, the first study describing the development of a soluble monomeric antibody CH3 domain. We found that the isolated dimeric CH3 was more stable than the mCH3 and the wild-type dimeric Fc, whereas the dimeric CH2 did not show enhanced stability as compared with an isolated CH2 monomer. This result indicates that the strong intermolecular hydrophobic interactions in the CH3 dimer are important for stabilizing the Fc and the IgG1 homodimer. However, these results should be taken with a note of caution because we do not yet know to what extent the four mutations required for generation of the mCH3 contribute to its low thermal stability.

The critical role of FcRn in maintaining the long serum half-life of IgG1 is well established, and the engineering of the FcRn-Fc interaction holds promise for modulating the pharmacokinetics of therapeutic antibodies (7, 10, 12). The FcRn binds to a region at the CH2-CH3 domain interface, and both domains contribute to the interactions. For example, positions 253 and 254 of the CH2 domain and positions 435 and 436 of the CH3 domain were found to be critical for FcRn binding because mutation of any of these residues results in greatly reduced binding affinity (27–29). In this study, we found that the absolute value of the free energy of binding (ΔG) of mCH3 to sFcRn (−34.6 kJ/mol) was higher than that of isolated CH2 (−31 kJ/mol) and that of dimeric CH3 (which did not bind FcRn) but lower than that of mFc (−38.4 kJ/mol). These results indicate that CH3 in Fc may contribute a larger portion of the free energy of binding to FcRn than CH2. It also appears that in relation to binding to FcRn, the native CH3 conformation as part of Fc could be more similar to that of isolated mCH3 than to that of isolated dimeric CH3.

It was previously suggested that local conformational changes in the relative orientation of two CH2 domains play a pivotal role in the FcyR binding activity of IgG (30). It was also noted that the CD spectra of mCH3 and CH2 resemble the spectrum of the reduced CH3, which was shown to undergo a conformational change to the oxidized CH3, but the structure of the reduced CH3 remained intact and dimeric (31). We also found that the mCH3 exhibited pH-dependent binding to FcRn, whereas the isolated dimeric CH3 did not bind, suggesting that the loop containing residues 434–436 was more accessible to FcRn in mCH3 or that mCH3 (but not CH3) underwent other structural rearrangements resulting in enhanced interactions with FcRn. These results taken together suggest that structural mobility could be an inherent property of CH2 and CH3 domains and is likely to play a role in mediating interactions of Fc with other biomolecules. We expect that these
FIGURE 8. Increasing mCH3 stability by an engineered disulfide bond. A, schematic representation of the mCH3cc variant. Its native disulfide bond was colored yellow. The introduced disulfide bond between Cys-343 and Cys-431 was colored red. B, plots of the change in fraction folded for mCH3cc. C, size exclusion chromatography of mCH3cc. MW, molecular weight. D, amino acid sequence alignment of wide-type CH3, mCH3, and mCH3cc.
results could provide a better understanding of the mechanism of action of the Fc and help in producing antibodies with favorable pharmacokinetics and/or effector functions.

Antibody fragments with binding activity of full-size mAbs and engineered to possess other unique and superior properties may constitute the next wave of clinically useful antibody-based therapeutics (32–36). Their small size enables better tissue penetration as compared with full-size molecules, especially for penetration into solid tumors. Moreover, the small size of antibody domains and fragments provides better access to sterically restricted epitopes. Antibody fragments usually display greatly reduced half-life as compared with full-size mAbs; this has been a major issue for clinical therapies. Finding optimized size and half-life of antibody fragments is therefore of paramount value. Importantly, the IgG1 mCH3 as described in this study represents a very small structural unit that possesses strictly pH-dependent FcRn binding. We also proved that mCH3 can be engineered to be more stable and more efficient in soluble expression, whereas the FcRn binding remained unaffected.

We have also shown that the m36.4-mCH3 fusion protein was highly expressed, monomeric, soluble, and stable. Furthermore, it binds to FcRn in a strictly pH-dependent manner and possesses potent neutralization activity against HIV pseudoviruses. Thus, VH-mCH3 represents a novel antibody fragment format. This kind of antibody fragment is of fully human origin with only four mutations in the wild-type CH3, so the immunogenicity should be low if any. It is a bispecific format, which can target antigens using its VH domain and bind to FcRn using the mCH3 domain. The addition of mCH3 does not affect the activity and stability of VH; therefore, a number of engineered antibody domains targeting various epitopes, as well as other therapeutic proteins (e.g. interferons and interleukins), can be fused to mCH3 to acquire the additional pH-dependent FcRn binding activity. Moreover, the size of the VH-mCH3 is only 28 kDa, which is similar to that of a single-chain variable fragment (scFv). The small size offers many advantages. For instance, size is the determining factor for efficiently targeting sterically restricted epitopes. As shown in Fig. 7C, the distance between the membrane-proximal portion of gp120 and the host cell membrane is ~85 Å after CD4 attachment (37), making the CD4-induced gp120 epitopes accessible to small-size molecules (e.g. m36.4 and m36.4-mCH3) but not to molecules with relatively large size (Fc fusion protein). Other advantages include good tissue penetration and higher molar quantities per gram of products, leading to a significant increase in potency per dose and a reduction in overall manufacturing cost.

In conclusion, we report here the first successful development of a soluble monomeric IgG1 CH3 that could be a valuable tool for understanding the mechanisms of CH3 dimerization and FcRn binding as well as for development of novel types of small biological therapeutics.

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REFERENCES

1. Carter, P. J. (2006) Potent antibody therapeutics by design. Nat. Rev. Immunol. 6, 343–357
2. Dimitrov, D. S., and Marks, J. D. (2009) Therapeutic antibodies: current state and future trends—is a paradigm change coming soon? Methods Mol. Biol. 525, 1–27, xiii
3. Reichert, J. M. (2011) Antibody-based therapeutics to watch in 2011. MAbs 3, 76–99
4. Dimitrov, D. S. (2012) Therapeutic proteins. Methods Mol. Biol. 899, 1–26
5. Simister, N. E., and Mostov, K. E. (1989) An Fc receptor structurally related to MHc class 1 antigens. Nature 337, 184–187
6. Ober, R. J., Martinez, C., Vaccaro, C., Zhou, J., and Ward, E. S. (2004) Visualizing the site and dynamics of IgG salvage by the MHc class I-related receptor, FcRn. J. Immunol. 172, 2021–2029
7. Roopenian, D. C., and Akiles, S. (2007) FcRn: the neonatal Fc receptor comes of age. Nat. Rev. Immunol. 7, 715–725
8. Flanagan, M. L., Arias, R. S., Hu, P., Khawli, L. A., and Epstein, A. L. (2007) Soluble Fc fusion proteins for biomedical research. Methods Mol. Biol. 378, 33–52
9. Iazayeri, J. A., and Carroll, G. J. (2008) Fc-based cytokines: prospects for engineering superior therapeutics. BioDrugs 22, 11–26
10. Zalesky, J., Chamberlain, A. K., Horton, H. M., Karki, S., Leung, I. W., Sproule, T. J., Lazar, G. A., Roopenian, D. C., and Desjarlais, J. R. (2010) Enhanced antibody half-life improves in vivo activity. Nat. Biotechnol. 28, 157–159
11. Hinton, P. R., Jolif, M. G., Xiong, J. M., Hanestad, K., Ong, K. C., Bullock, C., Keller, S., Tang, M. T., Tso, J. Y., Vásquez, M., and Tsurshita, N. (2004) Engineered human IgG antibodies with longer serum half-lives in primates. J. Biol. Chem. 279, 6213–6216
12. Vaccaro, C., Zhou, J., Ober, R. J., and Ward, E. S. (2005) Engineering the Fc region of immunoglobulin G to modulate in vivo antibody levels. Nat. Biotechnol. 23, 1283–1288
13. Gong, R., Vu, B. K., Feng, Y., Prieto, A. D., Dyba, M. A., Walsh, J. D., Prabakaran, P., Veenstra, T. D., Tarasov, S. G., Ishima, R., and Dimitrov, D. S. (2009) Engineered human antibody constant domains with increased stability. J. Biol. Chem. 284, 14203–14210
14. Gong, R., Wang, Y., Feng, Y., Zhao, Q., and Dimitrov, D. S. (2011) Shortened engineered human antibody CH2 domains: increased stability and binding to the human neonatal Fc receptor. J. Biol. Chem. 286, 27288–27293
15. Ying, T., Chen, W., Gong, R., Feng, Y., and Dimitrov, D. S. (2012) Soluble monomeric IgG1 Fc. J. Biol. Chem. 287, 19399–19408
16. Feng, Y., Gong, R., and Dimitrov, D. S. (2011) Design, expression, and characterization of a soluble single-chain functional human neonatal Fc receptor. Protein Expr. Purif. 79, 66–71
17. Chen, W., Zhu, Z., Feng, Y., and Dimitrov, D. S. (2008) Human domain antibodies to conserved sterically restricted regions on gp120 as exceptionally potent cross-reactive HIV-1 neutralizers. Proc. Natl. Acad. Sci. U.S.A. 105, 17121–17126
18. Prabakaran, P., Vu, B. K., Gan, J., Feng, Y., Dimitrov, D. S., and Ji, X. (2008) Structure of an isolated unglycosylated antibody Cε2 domain. Acta Crystallogr. D Biol. Crystallogr. 64, 1062–1067
19. Bork, P., Holm, L., and Sande, C. (1994) The immunoglobulin fold. Structural classification, sequence patterns, and common core. J. Mol. Biol. 242, 309–320
20. Feige, M. J., Walter, S., and Buchner, J. (2004) Folding mechanism of the CH2 antibody domain. J. Mol. Biol. 344, 107–118
21. Thies, M. J., Mayer, J., Augustine, I. G., Frederick, C. A., Lilie, H., and Buchner, J. (1999) Folding and association of the antibody domain CH3: prolyl isomerization precedes dimerization. J. Mol. Biol. 293, 67–79
22. Jefferis, R., Lund, J., and Pound, J. D. (1998) IgG-Fc-mediated effector functions: molecular definition of interaction sites for effector ligands and the role of glycosylation. Immunol. Rev. 163, 59–76
23. DeLano, W. L., Ultsch, M. H., de Vos, A. M., and Wells, J. A. (2000) Convergent solutions to binding at a protein-protein interface. Science 287, 1279–1283
24. Suzuki, T., Ishii-Watabe, A., Tada, M., Kobayashi, T., Kanayasu-Toyoda, J.
T. Kawanishi, T., and Yamaguchi, T. (2010) Importance of neonatal FcR in regulating the serum half-life of therapeutic proteins containing the Fc domain of human IgG1: a comparative study of the affinity of monoclonal antibodies and Fc-fusion proteins to human neonatal FcR. J. Immunol. 184, 1968–1976

25. Chan, D. C., Fass, D., Berger, J. M., and Kim, P. S. (1997) Core structure of gp41 from the HIV envelope glycoprotein. Cell 89, 263–273

26. Wozniak-Knopp, G., Stadlmann, J., and Rüker, F. (2012) Stabilisation of the Fc fragment of human IgG1 by engineered intradomain disulfide bonds. PLoS One 7, e30083

27. Vaughn, D. E., Milburn, C. M., Penny, D. M., Martin, W. L., Johnson, J. L., and Bjorkman, P. J. (1997) Identification of critical IgG binding epitopes on the neonatal Fc receptor. J. Mol. Biol. 274, 597–607

28. Shields, R. L., Namenuk, A. K., Hong, K., Meng, Y. G., Rae, J., Briggs, J., Xie, D., Lai, J., Stadlen, A., Li, B., Fox, J. A., and Presta, L. G. (2001) High resolution mapping of the binding site on human IgG1 for FcγRI, FcγRII, FcγRIII, and FcRn and design of IgG1 variants with improved binding to the FcγR. J. Biol. Chem. 276, 6591–6604

29. Martin, W. L., West, A. P., Jr., Gan, L., and Bjorkman, P. J. (2001) Crystal structure at 2.8 Å of an FcRn/heterodimeric Fc complex: mechanism of pH-dependent binding. Mol. Cell 7, 867–877

30. Sondermann, P., Huber, R., Oosthuizen, V., and Jacob, U. (2000) The 3.2-Å crystal structure of the human IgG1 Fc fragment-FcγRIII complex.

31. Thies, M. J., Talamo, F., Mayer, M., Bell, S., Ruoppolo, M., Marino, G., and Buchner, J. (2002) Folding and oxidation of the antibody domain C1,3. J. Mol. Biol. 319, 1267–1277

32. Holt, L. J., Herring, C., Jespers, L. S., Woolven, B. P., and Tomlinson, I. M. (2003) Domain antibodies: proteins for therapy. Trends Biotechnol. 21, 484–490

33. Holliger, P., and Hudson, P. J. (2005) Engineered antibody fragments and the rise of single domains. Nat. Biotechnol. 23, 1126–1136

34. Saerens, D., Ghassabeh, G. H., and Muyldermans, S. (2008) Single-domain antibodies as building blocks for novel therapeutics. Curr. Opin. Pharmacol. 8, 600–608

35. Dimitrov, D. S. (2009) Engineered CH2 domains (nanobodies). MAbs 1, 26–28

36. Nelson, A. L., and Reichert, J. M. (2009) Development trends for therapeutic antibody fragments. Nat. Biotechnol. 27, 331–337

37. Labrijn, A. F., Poignard, P., Raja, A., Zwick, M. B., Delgado, K., Franti, M., Binley, J., Vizона, V., Grundner, C., Huang, C. C., Venturi, M., Petropoulos, C. J., Wrin, T., Dimitrov, D. S., Robinson, J., Kwong, P. D., Wyatt, R. T., Sodroski, J., and Burton, D. R. (2003) Access of antibody molecules to the conserved coreceptor binding site on glycoprotein gp120 is sterically restricted on primary human immunodeficiency virus type 1. J. Virol. 77, 10557–10565