Engineered Soluble Monomeric IgG1 CH3 – Generation, Mechanisms of Function and Implications for Design of Biological Therapeutics

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Running Title: Monomeric IgG1 CH3
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 E. coli. We also found that engineering an additional disulfide bond in mCH3 remarkably increased its thermal stability while 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.
INTRODUCTION

The vast majority of the monoclonal antibodies (mAbs) approved for clinical use are full-size antibodies in IgG1 format (1,2,3,4). The IgG1 CH3 domain has two important functions – dimerization of the IgG1 Fc and interaction with the neonatal Fc receptor (FcRn). It is well-established that the Fc interaction with the FcRn plays a critical role in maintaining the long half-life of IgG1 (5,6,7). 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 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 (7,10,11,12). 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 (13,14,15). 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 (K_D = 940 nM) at pH 6. The free energy of mCH3 binding 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_m, from 40.6°C 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 solubly expressed at a high level in E. 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'-AAGACAGCTATCGCGATTGCAG-3'; gIII-R, 5'-ATCACCGGAACCAGAGCCACCAC-3'; CH3-F, 5'-GTTGATGTAACGGCCCAGGCGGCCGGGCGCCCCGAGAAC-3'; dCH2-F, 5'-GCCAAAGACAAAACTCACACAGCACCTGACTCCTGGGGGGAC-3'; dCH2-R, 5'-CAGGAGTTCAGGTGCTGTGTGAGTTTTGTCTTTTGCTTTGGAGATGTTTTC-3'. 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 compared to wild-type CH3 (residues 351, 366, 368 and 395). The dimeric CH2 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. m36.4h1Fc, the fusion protein with a human IgG1 hinge region as a linker between m36.4 and Fc, was generated as described previously (17).

**Generation of mCH3 P343C/A431C mutant (mCH3cc)**– The following primers were used: P343C-F, 5'-GTTGATGTAACGGCCCAGGCGGCCGGGCA GTGCCGAGAACCACAGGTGTAC-3'; A431C-F, 5'-CTGCGTGTAGTGGTTGTGCAGACACTCATG CATCACGGAGC-3'; A431C-R, 5'-ATGCTCCGTGATGCATGAGTGTCTGCACAA CCACTACACGCAG-3'. The mCH3 P343C variant was generated by PCR using mCH3 as template (primer: P343C-F and gIII-R). The mCH3 P343C/A431C variant was generated by the QuikChange Site-Directed Mutagenesis Kit (Stratagene) using the primers A431C-F and A431C-R.

**Protein expression and purification**– mCH3, mCH3 fusion proteins, mCH3cc and other antibody domains were expressed in E. coli HB2151 by using a procedure similar to that described previously (15). Protein purity was judged by SDS-PAGE, and protein concentration was measured spectrophotometrically (NanoVue, 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 CM5 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 uM and 62.5 nM. 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, Carlsbad, CA) were coated on ELISA plate wells at 50 ng per well in PBS overnight at 4°C, and blocked with protein-free blocking buffer (Thermo Scientific) at 37°C for 2 hr. Two-fold serially diluted protein was added and incubated at 37°C for 2 h. The plates were washed with PBST, 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 ABTS substrate (Roche, Indianapolis, IN), 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 two-fold serially diluted competitor human IgG1 antibodies. Mixtures 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 hr 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 × 10⁴ HOS-CD4-CXCR4 cells grown in each well of 96-well plates. Luminescence was measured after 48 hr by using the Bright-Glo Luciferase Assay System (Promega, Madison, WI) and a LumiCount microplate luminometer (Turner Designs). Mean relative light units (RLU) for duplicate wells were determined. Relative infectivity (%) was calculated by the following formula: (average RLU of antibody-containing wells/average RLU of virus-only wells) × 100.

**RESULTS**

Generation of soluble monomeric CH3 (mCH3). We previously reported the generation of three monomeric Fc (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 be also 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/L 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 previously described (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 weights (MWs) (14.4 kDa vs 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 (MW ~28 kDa) as expected, and the dCH2 which consists of two CH2 molecules was of about the same size (MW ~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 homo-dimerization 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 difference 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 CH2, 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 \( T_m \) of mCH3 was 40.6 ± 0.3°C. This value is similar to that of an isolated CH2, 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 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 µM. We also noted that the CM5 chip could be better regenerated by the pH 8.0 buffer after the mCH3 binding measurement as compared to that after the CH2 binding measurement, although neither of the two proteins showed detectable binding at pH 7.4. This behavior is not surprising since 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 I253 and S254. 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 H433 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.

**FcRn binding.** We next explored whether mCH3 could bind FcRn in a pH-dependent manner. Binding to a human single-chain soluble FcRn (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. While 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. 4A-C). Since 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 more accessible to FcRn than in the isolated CH3 dimer, thereby conferring pH-dependent binding capability.

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 EC\textsubscript{50}. 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 G, although its binding affinities for both were decreased since 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 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.

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 purified protein per liter culture). Size exclusion chromatography analysis confirmed that the fusion protein was monomeric, with a molecular size of approximately 26 kDa (Fig. 6A). It was also found to have a relatively high T\textsubscript{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 those of mAbs, suggesting that the FcRn binding region of the Fc fusion proteins may exhibit some differences compared to native mAbs (24). We investigated the FcRn binding of the VH-mCH3 fusion protein. As shown in Fig. 6C, the K\textsubscript{D} value calculated from this experiment was 685 nM at pH 6.0, which is comparable to 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 (MW ~60 kDa) and Fc fusion (MW ~110 kDa) did not show any measurable neutralizing activity against these two isolates. By contrast, as shown in Fig. 7, m36.4 exhibited particularly strong neutralization, with an IC\textsubscript{50} (antibody concentration resulting in 50% inhibition of virus infection) of 8 nM (Bal) and 25 nM (JRFL). The IC\textsubscript{50} 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 compared to m36.4 is not surprising, since 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 IC\textsubscript{50} of 270 nM against Bal and JRFL (17,25). The C34 exhibits HIV-entry inhibitory activity comparable to or higher than that of the 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...
Increasing the mCH3 stability by an engineered disulfide bond. The thermal stability of mCH3 was relatively low ($T_m = 40.6^\circ C$). 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 mCH3 P343C/A431C mutant (mCH3cc) which has an additional disulfide bond between the Cys343 and Cys431 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/L bacterial culture) in E. coli than mCH3 and is significantly more stable than mCH3. The $T_m$ 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 ($K_D$) of mCH3cc to sFcRn was calculated to be 1.1 uM 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 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 report 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, while the dimeric CH2 did not show enhanced stability compared to 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, since 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, since mutation of any of these residues results in greatly reduced binding affinity (27,28,29). In this study, we found that the absolute value of the free energy of binding ($\Delta G$) 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 FcγR 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 while 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 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 (32,33,34,35,36). Their small size enables better tissue penetration compared to 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 compared to 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 while 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, 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 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 approximately 85 Å after CD4 attachment (37), making the CD4-induced gp120 epitopes accessible to small-size molecules (eg., 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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FOOTNOTES

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The abbreviations used are: mAbs, monoclonal antibodies; FcRn, neonatal Fc receptor; mCH3, monomeric CH3; SEC, size exclusion chromatography; CD, circular dichroism; SPR, surface plasmon resonance.

Table 1. Summary of FcRn, protein A and protein G binding capabilities of antibody domains characterized in our study.

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<tr>
<th>Antibody domain</th>
<th>FcRn</th>
<th>Protein A</th>
<th>Protein G</th>
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<tr>
<td>Fc</td>
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FIGURE LEGENDS

Figure 1 Schematic representation of the generation of monomeric CH3 from IgG1 (Protein Data Bank code 1HZH). The mutated residues located at the CH3 dimerization interface (residues 351, 366, 368 and 395) in monomeric CH3 were colored red.

Figure 2 A. Schematic representations of different IgG1 Fc domains. B. SDS-PAGE analysis of purified IgG1 Fc domains. C. Size exclusion chromatography of IgG1 Fc domains, the insets show a standard curve for the gel filtration standards. D. CD spectra of mCH3 and CH3.

Figure 3 Plots of the change in fraction folded for mCH3, mFc.67, CH2, Fc and CH3.

Figure 4 FcRn binding to mCH3 (A), CH3 (C), CH2 (D) at pH 6.0, and mCH3 at pH 7.4 (B) measured by BIAcore.

Figure 5 Protein A (A) and protein G (B) binding of IgG1 Fc domains measured by ELISA. C. Competition of mCH3 (500 nM) with IgG (0-1 mM, two-fold serially diluted) for binding to protein G (blue, dot). Protein G binding of mCH3 (0-1 mM, two-fold serially diluted) is also shown for comparison (pink, triangle).
Figure 6  A. Size exclusion chromatography of m36.4-mCH3, the insets show a standard curve for the gel filtration standards. B. Plots of the change in fraction folded for m36.4-mCH3. C. sFcRn binding of m36.4-mCH3 measured at pH 6.0 by BIAcore.

Figure 7  Neutralization of viruses pseudotyped with Envs of HIV-1 isolates Bal (A) and JRFL (B). C. Schematic representations of the HIV-1 envelope glycoprotein gp120 interacts with the CD4 receptor at the cell membrane. The predicted distance between the membrane-proximal portion of gp120 and the host cell membrane is up to 85 Å. This sterically restricted epitope could be reached by the small-size m36.4 and m36.4-mCH3 molecules but could be blocked by the targeting of large-size m36.4h1Fc. Due to their linker flexibilities, only the minimum hydrodynamic dimensions of mCH3-m36.4 and m36.4h1Fc were shown.

Figure 8  A. Schematic representation of the mCH3 P343C/A431C variant (mCH3cc). Its native disulfide bond was colored yellow. The introduced disulfide bond between Cys343 and Cys431 was colored red. B. Plots of the change in fraction folded for mCH3cc. C. Size exclusion chromatography of mCH3cc. D. Amino acid sequence alignment of wide-type CH3, mCH3 and mCH3cc.
Figure 2
Figure 4
Figure 5

A

B

C

Concentration (nM)

Concentration (nM)

Concentration (nM)

OD405

OD405

OD405

0 200 400 600 800 1000

0 200 400 600 800 1000

0 200 400 600 800 1000

1.0 1.5 2.0 2.5

1.0 1.5 2.0 2.5

0.2 0.4 0.6

Fc, mFc, α7, CH2, αCH2, mCH3, CH3

Fc, mFc, α7, α, mCH3, nCH3, O-Q

mCH3, mCH3 + IgG (competitor)
Figure 6

A

B

C

Absorbance (mAU)

Elution volume (mL)

Fraction folded

Temperature (°C)

Response

Time (s)

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Figure 7

(A) 

(B) 

(C)
Figure 8

A. Cys343 and Cys431

B. Fraction folded vs. Temperature

C. Elution volume (mL) vs. Log MW

D. Sequence comparison:

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