Increasing the serum persistence of an IgG fragment by random mutagenesis

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The major histocompatibility complex (MHC) class I-related receptor FcRn is involved in regulating serum gammaglobulin (IgG) levels in mice. With the aim of increasing the serum half-life of a recombinant murine Fcγ1 fragment, the affinity for binding to FcRn at pH 6.0 has been increased by random mutagenesis of Thr252, Thr254, and Thr256 followed by selection using bacteriophage display. These residues were chosen as they are in proximity to the FcRn-IgG (Fc) interaction site. Two mutants with higher affinity (due to lower off-rates) than the wild-type Fc have been isolated and analyzed in pharmacokinetic studies in mice. The mutant with the highest affinity has a significantly longer serum half-life than the wild type fragment, despite its lower off-rate from FcRn at pH 7.4. The results provide support for the involvement of FcRn in the homeostasis of serum IgGs in mice. The indications that a homologous FcRn regulates IgG levels in humans suggest that this approach has implications for increasing the serum persistence of therapeutic antibodies.

Keywords: antibody engineering, pharmacokinetics, phage display, surface plasmon resonance

IgGs constitute the most prevalent immunoglobulin class in the serum of man and other mammals and are maintained at remarkably constant levels. The MHC-class I related receptor, FcRn (n, neonatal), is involved in the homeostasis of serum IgGs[1–3]. This receptor most likely acts as a salvage receptor, which would be consistent with its known ability to transcytose IgGs in intact form across the neonatal gut[4,5] and yolk sac[6,7] or placenta[8–10]. The interaction site of FcRn on mouse IgG1 (mIgG1) has been mapped using site-directed mutagenesis of recombinant Fc-hinge fragments, followed by analysis of these fragments both in vivo and in vitro[11–13]. I253 (EU numbering[14]), H310, H435 and to a lesser extent, H436, play a central role in this interaction. These amino acids are located at the CH2-CH3 domain interface[15], and the mapping of the functional site to these residues is consistent with the X-ray crystallographic structure of rat FcRn complexed with rat Fc[16].

The FcRn interaction site encompasses three spatially close loops comprised of sequences that are separated along the primary amino acid sequence. The central role of Fc histidines in building this site accounts for the marked pH dependence (binding at pH 6.0, release at pH 7.4) of the Fc-FcRn interaction[5,17,18], as the pKa of one of the imidazole protons lies in this pH range. I253, H310, H435 and to a lesser degree, H436, are highly conserved in both human and rodent IgGs[19]. This, taken together with the isolation of a human homolog of FcRn[20], indicate that the molecular mechanisms involved in IgG homeostasis are common to both mouse and man and this has implications for the modulation of the pharmacokinetics of IgGs for use in therapy.

Mutations of Fc-hinge fragments have been made to identify FcRn interaction sites that reduce the serum half-lives of the Fc-hinge fragment[13,21]. In total, six mutated Fc-hinge fragments have been analyzed and the correlation between serum half-life and binding affinity for FcRn is excellent[11,13,18]. This suggests that if the affinity of the FcRn-Fc interaction could be increased, while still retaining pH dependence, this would result in an Fc fragment with prolonged serum persistence. The demonstration that such a fragment can be produced has obvious consequences for the engineering of a new generation of therapeutic IgGs with improved pharmacokinetics such as increased persistence in the circulation, and this was the goal of the current study.

Results

Mutagenesis and selection. Three residues (T252, T254, and T256) that are in close proximity to I253, solvent exposed (Fig. 1) and not highly conserved in IgGs[19] were chosen for random, PCR-directed mutagenesis. The recombinant Fc-hinge fragments were expressed as a phage display library (approx. 20,000 clones) using the vector pHEN1[22]. Immunoblots of the recombinant phage using anti-mouse Fc-horseradish peroxidase conjugate showed 30 kDa and 90 kDa polypeptides, corresponding to the expected mobilities of Fc-hinge polypeptides and Fc-hinge:gene III coat protein fusions, respectively (data not shown). In addition, a protein of 70 kDa was seen, which is possibly a proteolytic product of the fusion protein. The expression products observed on immunoblots of recombinant phage are most likely due to the leakiness of readthrough of the amber codon resulting in a mixture of Fc-hinge:gene III coat protein and soluble Fc-hinge fragments being expressed and assembled as phage displayed Fc-hinge homodimers linked to the gene III coat protein. Two rounds of panning were carried out by incubating phage preparations with recombinant, soluble FcRn (polyhistidine tagged) in solution followed by capture of phage-FcRn complexes using Ni^{2+}-NTA-agarose beads. Bound phage were eluted at pH 7.4 to select for Fc-hinge fragments that retained pH dependence of binding characteristic of the wild-type (WT) Fc-hinge.

Recombinant Fc-hinge fragments from ten of the isolated clones were analyzed further as soluble fragments in osmotic shock fractions of *Escherichia coli* transfectants. Using surface plasmon resonance (SPR), five were observed to have a similar or higher affinity for binding to immobilized FcRn than that of the WT Fc-hinge fragment. The genes encoding these Fc-hinge fragments were recloned into a vector with an in-frame C-terminal polyhistidine tag to allow purification of soluble protein on Ni^{2+}-NTA-agarose. Following purification, three Fc-hinge fragments were found to have HPLC and SDS-PAGE profiles similar to the WT Fc-hinge (55 kDa, comprising a mixture of sulfydryl linked and noncovalently linked homodimers; data not shown) and these proteins were analyzed in subsequent studies.

The nucleotide sequences of the three mutants were deter-

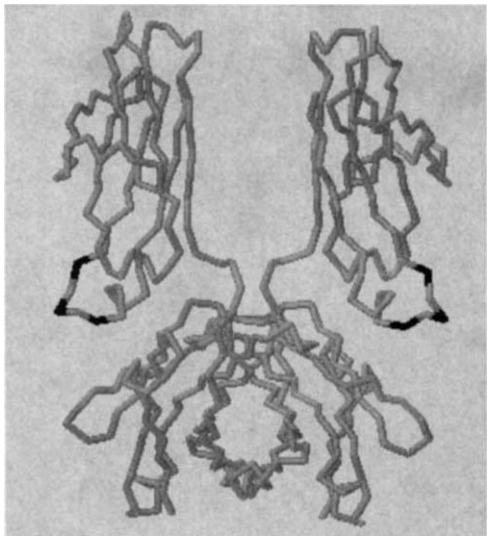

Figure 1. Location of residues targeted for mutagenesis on the three-dimensional structure of human Fcg1[16]. T252, T254, and T256 are indicated in black, and the α-carbon backbone of the remainder of the molecule in grey.

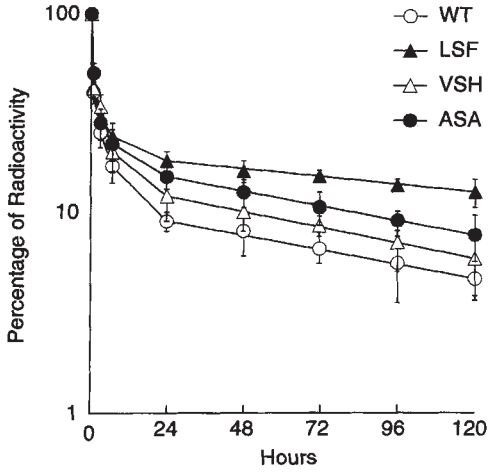

Figure 2. Clearance curves of Fc-hinge fragments in Swiss mice. Data points represent average values of percent radioactivity remaining (with standard deviations indicated by error bars) for each Fc-hinge fragment at the indicated times postinjection.

mined and these Fc-hinge fragments were named according to the encoded amino acids at positions 252, 254 and 256 (ASA, T252 to A252, T254 to S254 and T256 to A256; VSH, T252 to V252, T254 to S254 and T256 to H256; LSF, T252 to L252, T254 to S254 and T256 to F256). The sequences of the remainder of the corresponding genes were the same as that of the WT Fc-hinge fragment with the exception of mutant ASA, which had a G to A change at codon 272 resulting in a change of glutamic acid to lysine. This amino acid is located in proximity to the hinge region of IgG[15].

Pharmacokinetics of the Fc-hinge fragments. The Fc-hinge fragments were radioiodinated and their pharmacokinetics in Swiss mice analyzed (Table 1; Fig. 2). Of the three mutants, mutant LSF has a significantly longer β phase half-life than either the WT Fc-hinge fragment or mutants ASA or VSH. The half-lives of the latter three fragments are not significantly different from each other. Pharmacokinetic studies were also carried out in Balb/c mice, and again mutant LSF had a significantly longer β phase half-life than either the WT Fc-hinge or mutants ASA and VSH (Table 1). In Balb/c mice, as in Swiss mice, the half-lives of the latter three fragments were not significantly different. In addition, HPLC analyses of serum samples at 24 hours postinjection for the WT and mutated Fc-hinge fragments indicated that they persisted as intact 55 kDa proteins (data not shown).

Table 1. Pharmacokinetics of the WT and mutant Fc-hinge fragments in Swiss and Balb/c mice.

Fc-hinge fragment	Swiss mice Number of mice	Swiss mice β phase half-life (h)*	Balb/c mice Number of mice	Balb/c mice β phase half-life (h)*
WT	9	123.5±13.3	4	92.8±12.9
ASA	4	116.0±19.9	9	104.6±10.4
VSH	4	98.2±6.5	9	107.1±10.8
LSF	9	152.3±16.0	4	152.8±12.0

*By student t test the values for WT are not significantly different from those of the ASA and VSH mutants, whereas the values for the LSF mutant are significantly different from those of WT ($p<0.003$ and $p<0.001$ for Swiss and Balb/c mice, respectively).

Table 2. SPR analyses of the kinetics of binding of Fc-hinge fragments to FcRn at pH 6.0.

Fc-hinge	k_a ($M^{-1}s^{-1}$)/10^5	k_d (s^{-1})/10^{-3}	K_D (k_d/k_a) (nM)
Wild-type	6.20±0.19	4.61±0.10	7.44±0.19
ASA	5.01±0.17	2.07±0.03	4.13±0.10
VSH	5.38±0.14	4.48±0.40	8.33±0.68
LSF	4.72±0.20	1.02±0.20	2.16±0.36

To demonstrate that the half-life differences observed in mice that express FcRn are solely due to altered interaction with FcRn rather than some other factor, the β phase half-lives of the WT and mutant LSF Fc-hinge fragments were also determined in C57BL/6 mice that do not express FcRn due to homozygous knockout of the β2-microglobulin gene[23]. The β phase half-lives in these mice are 14.7 ± 0.3 hours (WT; four mice analyzed) and 14.8 ± 0.3 hours (LSF mutant; four mice analyzed) and are not significantly different.

Surface plasmon resonance analyses. The interaction kinetics of each of the three mutants with recombinant, immobilized mouse FcRn were analyzed at pH 6.0 using SPR (Table 2). All mutants have similar on-rates (k_as) to that of the WT Fc-hinge fragment. The affinity (K_D) differences for mutants ASA (4.13 nM) and LSF (2.16 nM) compared with WT (7.44 nM) are primarily due to significant differences in off-rates, with ASA and LSF having approximately two and fourfold lower k_ds, respectively (Table 2; Fig. 3). The relative affinities of these two mutants were also analyzed using inhibition binding studies in which the ability of each Fc-hinge fragment to inhibit the binding of radiolabeled, soluble FcRn to murine IgG1 coupled to Sepharose was quantitated. Consistent with the SPR analyses, the amount of Fc-hinge fragment needed for 50% inhibition of binding decreased in the order WT>ASA>LSF (data not shown).

Importantly, the mutations have little effect on the characteristic pH dependence of the Fc-FcRn interaction that is believed to be essential for the correct functioning of this Fc receptor. The off-rates at pH 7.4 for WT, ASA, and VSH Fc-hinge fragments are immeasurably fast by SPR. However, mutant LSF has a lower off-rate at pH 7.4 than the WT, ASA or VSH fragments (Fig. 3), although the off-rate of approximately 0.027 s^{-1} is still about 25-fold faster than at pH 6.0 (Table 2). The retention of the pH-dependent binding is consistent with the suggested involvement of H310, H435, and H436, located in loops adjacent to the mutated residues, in mediating this property[13,17].

Discussion

These studies demonstrate that it is possible to use phage display and selection to isolate a recombinant, mutated Fc-hinge fragment that has longer serum persistence than the WT fragment from which it was derived. The longer β phase half-life of the LSF mutant relative to WT would, for example, result in approximately fourfold (Balb/c mice) and twofold (Swiss mice) more LSF in the serum 20 days postinjection. The

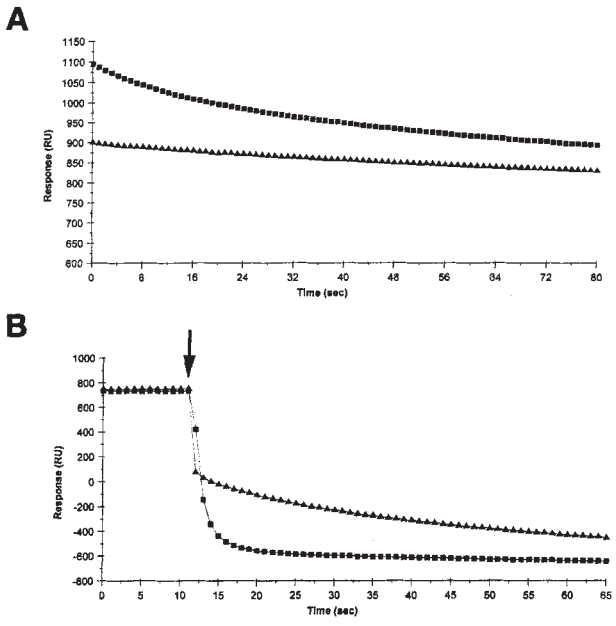

Figure 3. Regions of SPR sensorgrams showing dissociation of WT (filled squares) and LSF mutant (filled triangles) Fc-hinge fragments at (A) pH 6.0 and (B) pH 7.4. The arrow indicates the point at which the buffer was changed from pH 6.0 to 7.4. Plots are drawn using BIAevaluation 2.1 software. The bulk shift downwards due to the pH 7.4 buffer relative to the pH 6.0 buffer (latter used as baseline) results in the negative RU values.

SPR data indicate that the longer half-life of mutant LSF relative to WT can be accounted for by an approximately 3.5-fold higher affinity for interaction with FcRn, and this is primarily due to a decrease in off-rate. This is consistent with the concept that FcRn is saturable[24], and that higher affinity Fc fragments have a competitive advantage with endogenous IgGs for being salvaged and recycled rather than degraded.

However, the ASA mutant has a 1.8-fold higher affinity for FcRn than WT and yet does not show a significantly longer in vivo half-life. This suggests that for significant effects on serum persistence to be observed, substantial increases in affinity for FcRn binding are required. Two alternative explanations are possible. Firstly, the mutations in the ASA mutant may result in an Fc-hinge fragment that has lower stability in the serum, due to enhanced susceptibility to attack by serum proteases or to denaturation. Secondly, based on a 6.5 Å resolution structure of rat FcRn dimers complexed with rat Fc[16] (2:1 stoichiometry), it has been suggested that Fc may have two distinct regions of contact with the FcRn dimer in a 'lying down' complex[16]. The first region, located at the CH2-CH3 domain interface, interacts with the α1/α2 domains of one FcRn molecule. A second contact region encompassing His285 and Glu272 may interact with the α3 domain of the second FcRn molecule in the FcRn dimer[16]. Although we have excluded a role for His285 of the murine IgG1 molecule in regulating serum persistence and other FcRn-related functions[12,13], mutations of Glu272 have not to our knowledge been analyzed. Therefore, it cannot currently be excluded that the mutation of Glu272 to Lys272 (by PCR error) in the ASA mutant may play a minor role in counteracting the higher affinity FcRn-ASA mutant interaction in vivo by affecting a step downstream of the initial FcRn-Fc interaction. This second interaction may play a role in the efficient trafficking of bound IgGs/Fcs by FcRn. Such a step could be the recruitment of a second FcRn molecule to the initial FcRn-Fc complex in the model proposed by Bjorkman and colleagues[16].

Analysis of the sequences of the three Fc-hinge mutants indicates that a number of different residues can be tolerated at positions 252, 254, and 256 without being detrimental to FcRn binding (mutant VSH) and for mutants ASA and LSF, the affinity is even improved. In this respect, the data suggest that for positions 252 and 256, the WT residues may not make favorable contacts with FcRn as replacement of both T252 and T256 with alanine in the ASA mutant results in an affinity increase. This contrasts with the apparently essential role of I253 in mediating a high-affinity Fc-FcRn interaction, as substitution with alanine at this location results in a substantial loss (3,000-fold) in affinity[18]. It cannot, however, be excluded that other residues could substitute for I253 without alteration in FcRn binding ability, but this is made unlikely by the conservation of I253 across all mammalian IgGs[19].

There appears to be a selection for amino acids with a hydrophobic side chain at position 252, and sequencing of a further mutant with FcRn binding activity similar to that of the WT Fc-hinge revealed Phe at this position (not shown). Interestingly, all three mutants have serine at position 254 which is also present in the majority of naturally occurring IgGs of mouse, rat, and man[19]. The apparent selection of mutants with serine at this position suggests that for retention of FcRn binding activity, this is the most favorable residue. However, the WT Fc-hinge (IgG1) has threonine at this position and still shows a high affinity Fc-FcRn interaction. Direct analysis of the effect of a threonine to serine change at residue 254, in the context of a WT IgG1-derived Fc-hinge fragment, requires the analysis of additional mutants.

The sequences of the mutants indicate that FcRn binding activity is retained with a diverse set of amino acids (Ala, His, and Phe) at position 256, suggesting that this region may not be in close proximity to the Fc-FcRn interaction. Residues at this position are, however, highly conserved in rodent and human IgGs (serine or threonine in 94% of IgGs analyzed[19]) indicating that this amino acid might have an as yet unidentified function. It is also possible that the hydrophobic side chain of F256 of the LSF mutant makes favorable interactions with FcRn that cannot be made by the less hydrophobic alanine or histidine residues in the ASA and VSH mutants, respectively. This would be the most likely explanation for the increased affinity of the LSF mutant relative to VSH, as these two mutants only differ markedly from each other at this position. However, elucidation of the molecular mechanisms by which the affinity for binding to FcRn of the LSF mutant is improved requires more detailed analysis such as X-ray crystallography of the corresponding Fc-FcRn complex.

Mutant LSF has a lower off-rate from FcRn than the WT Fc-hinge or mutants ASA and VSH at pH 7.4, although this off-rate is significantly faster than that at pH 6.0. This has implications concerning the mechanism by which FcRn binds, protects from degradation and recirculates IgGs back into the serum, indicating that the half-life of FcRn on the cell surface (pH 7.4) is sufficiently long to allow significant amounts of the LSF mutant to dissociate into the serum. In this respect the half-life of FcRn-LSF mutant complexes at pH 7.4 is about 26 seconds and this may partially offset the advantage of higher affinity binding at pH 6.0. It would be of interest to generate a mutant with similar affinity to that of LSF but more marked pH dependence of binding to determine whether the slower off-rate at pH 7.4 of mutant LSF counteracts, in part, the advantage of the higher affinity pH 6.0 interaction.

The data extend the correlation between affinity of an Fc-hinge fragment for FcRn and β phase half-life and further support the hypothesis[1–3] that FcRn is directly involved in IgG homeostasis. Further affinity improvements may be possible by targeting other regions of the mIgG1 molecule, such as those in the surface loop containing H310 in the CH2 domain. The observation that aglycosylated Fc-hinge fragments have the same half-life as complete glycosylated IgG1[21] indicates that these studies have direct relevance to prolonging the serum persistence of intact IgGs. Furthermore, the site that has been mutated is distal to the interaction sites of FcγRI, FcγRII, FcγRIII[25,26] and complement C1q[27], suggesting that the mutations resulting in longer serum persistence will not affect either ADCC or complement fixation. Finally, the identification of a human homolog of mouse FcRn[20] suggests that these studies are of relevance to the optimization of the pharmacokinetics of therapeutic antibodies for the treatment of diseases such as cancer and autoimmunity.

Experimental protocol

Construction of library of Fc-hinge mutants. The WT Fc-hinge gene[21] was used as a template in splicing by overlap extension[28] with the following oligonucleotides: HingebakNco[21], 5' ATC ACC ATG GCC GTG CCC AGG GAT TGT GGT TG 3' (NcoI site indicated by underlining); 252for, 5' CAA CAC ACG TGA CCT TAG GSN NCA GSN NAA TSN NGA GC 3' (N = T,C,G or A and S = A,G or C; S was inserted in complement at the wobble position to minimize the generation of stop codons); 252bak 5' GTC ACG TGT GTT G 3'; Xmafor, 5' GCT CCT CCC GGG GTT GCG T 3' (XmaI site indicated by underlining). The PCR product was digested with XmaI and NcoI and used to replace the corresponding segment of the WT Fc-hinge gene (NcoI site in pelB leader and XmaI at position 211 of Fc-hinge) in pHEN1[22]. A library size of 20,000 clones was generated by electroporation of *E. coli* TG1 as described[29].

Panning of the library. The library stock was used to generate phage as described[29]. Phage (100 µl of 2×10^{12} pfu/ml) were panned using a solution panning approach as follows; phage were resuspended in 2% milk powder, 20 mM MES (MM buffer) pH 6.0 and mixed with 350 ng recombinant soluble FcRn[18] for 1 h at room temperature with agitation. Thirty microliters of a 50% suspension of Ni^{2+}-NTA-agarose (Qiagen, Chatsworth, CA) in MM buffer, pH 6.0 were then added for 10 min and pelleted by centrifugation. Beads were washed 20 times with 0.5 ml MM buffer pH 6.0 and phage eluted by incubation in 100 µl PBS pH 7.4 for 20 min at room temperature. Phage were used to reinfect exponentially growing *E. coli* TG1 as described[29,30]. Two rounds of panning were carried out.

Generation of osmotic shock fractions from selected clones. Colonies from plates resulting from panning were grown up as 1 ml cultures and induced for Fc-hinge expression as described[21]. Osmotic shock fractions were made by resuspension of cell pellets in 15 µl chloroform[31] and diluted tenfold in 50 mM MES, pH 6.0, 0.01% Tween, 150 mM NaCl. Cell debris was pelleted by centrifugation and the supernatants used in SPR experiments. To estimate the amount of each Fc-hinge fragment in osmotic shock fractions, these fractions were analyzed by immunoblotting using detection with the anti-c-myc antibody as described previously[32].

Expression and purification of soluble Fc-hinge fragments. The genes encoding mutated Fc-hinge fragments were recloned as NcoI-NotI fragments into a modified form of VβpelBhis[33] with an inframe NotI site inserted immediately 5' to the codons encoding the polyhistidine tag. Recombinant clones were grown, induced for expression and Fc-hinge fragments purified as described previously[21]. Prior to use in SPR studies, Fc-hinge fragments were further purified by size exclusion on a Superdex-75 (Pharmacia, Uppsala, Sweden) column if analytical analysis of the preparation on the same column showed more than one peak at a migration corresponding to 55 kDa.

Pharmacokinetic analyses. Proteins were radiolabeled with $Na^{125}I$ using Iodo-Gen (Amersham, Arlington Heights, IL) and pharmacokinetic analyses carried out in Swiss (Taconic, Germantown, NY), Balb/c (Harlan, Indianapolis, IN) and β2-microglobulin deficient (C57BL/6, Jackson Laboratories, West Grove, PA) mice as described previously[21]. The β phase half-lives were calculated using data obtained between 24 and 120 h postinjection of the radiolabeled proteins. The size of the radiolabeled protein in serum samples from mice at 24 h following injection were analyzed using HPLC and SEC-250 columns as described[21].

Surface plasmon resonance measurements. These were carried out using a BIAcore 2000 as described for analyzing Fc-FcRn interactions[18]. Binding activities of Fc-hinge fragments in osmotic shock fractions were semi-quantitatively analyzed using FcRn coupled to the BIAcore sensor chip (research grade CM5 chip), and clones producing fragments with apparently higher affinities (particularly those with lower off-rates at pH 6.0) than WT Fc-hinge were studied further as purified proteins. The binding of purified Fc-hinge fragments at concentrations ranging from 100–300 nM to immobilized FcRn was analyzed at pH 6.0 using a flow-rate of 40 µl/min. Fifty millimolar HEPES buffer, pH 7.4 was used to recover the chip as described[18], as the Fc-hinge rapidly dissociates from FcRn at this pH. Fc-hinge fragments were also flowed over an uncoated CM5 chip, and the sensorgrams from these analyses subtracted from those obtained with FcRn-coupled chips using BIAevaluation 2.1 software. For on- and off-rate calculations, the same software was used to fit data to the equations $R = R_{eq}(1-e^{-[(kaCn+kd)(t-to)]})$ and $R = R_o e^{-kd(t-to)}$, respectively. To minimize effects on k_ds due to rebinding, early parts (first 10–15 sec) of dissociation plots were used for analyses starting at about 10 sec after the buffer change. For the WT Fc-hinge, this resulted in a higher off-rate than that calculated previously by us[18], and this is the primary cause of the approximate fourfold lower affinity observed in this study than that described earlier. For each Fc-hinge fragment, values of k_a and k_d were extracted from 3–4 sensorgrams and the average value calculated. K_Ds (k_d/k_a) for each sensorgram were also calculated and the average value determined.

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