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Species-Specific Determinants in the IgG CH3 Domain Enable Fab-Arm Exchange by Affecting the Noncovalent CH3–CH3 Interaction Strength

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A distinctive feature of human IgG4 is its ability to recombine half molecules (H chain and attached L chain) through a dynamic process termed Fab-arm exchange, which results in bispecific Abs. It is becoming evident that the process of Fab-arm exchange is conserved in several mammalian species, and thereby represents a mechanism that impacts humoral immunity more generally than previously thought. In humans, Fab-arm exchange has been attributed to the IgG4 core-hinge sequence (226-CPSCP-230) in combination with unknown determinants in the third constant H chain domain (CH3). In this study, we investigated the role of the CH3 domain in the mechanism of Fab-arm exchange, and thus identified amino acid position 409 as the critical CH3 determinant in human IgG, with R409 resulting in exchange and K409 resulting in stable IgG. Interestingly, studies with IgG from various species showed that Fab-arm exchange could not be assigned to a common CH3 domain amino acid motif. Accordingly, in rhesus monkeys (Macaca mulatta), aa 405 was identified as the CH3 determinant responsible (in combination with 226-CPACP-230). Using native mass spectrometry, we demonstrated that the ability to exchange Fab-arms correlated with the CH3–CH3 dissociation constant. Species-specific adaptations in the CH3 domain thus enable Fab-arm exchange by affecting the inter-CH3 domain interaction strength. The redistribution of Ag-binding domains between molecules may constitute a general immunological and evolutionary advantage. The current insights impact our view of humoral immunity and should furthermore be considered in the design and evaluation of Ab-based studies and therapeutics. *The Journal of Immunology, 2011, 187: 3238–3246.

Immunoglobulin molecules are typically composed of two H chains and two L chains that are covalently connected via disulfide bonds: one disulfide bond connecting each L chain to a H chain and a number of disulfide bonds linking the two H chains (1). The cysteines involved in the inter-H chain disulfide bond formation are located in the hinge region and differ in number between IgG subclasses with two disulfide bonds present in both human IgG1 and IgG4. Unlike IgG1, however, the disulfide bonds in the IgG4 hinge can easily adopt an intra-H chain conformation, facilitated by the 226-CPSCP-230 core-hinge sequence of IgG4 (compared with 226-CPACP-230 in IgG1) (2). As a result, a fraction of IgG4 molecules exists as noncovalently associated half molecules, which becomes apparent when analyzed by non-reducing SDS-PAGE (2–4). A single S228P mutation enhances the disulfide linkage in the core-hinge of IgG4 and reduces the amount of half molecules (2–4).

Another characteristic of the human IgG4 molecule is its ability to exchange half molecules with other IgG4 molecules. This process, termed Fab-arm exchange, occurs naturally in vivo and can be mimicked in vitro by the addition of mild reducing agents such as reduced glutathione (GSH) (5, 6). As a consequence, blood-derived IgG4 is characterized by an inability to cross-link identical Ags (homologous cross-linking), which has been referred to as functional monovalency and, as a result, is nonprecipitating (7). Furthermore, through Fab-arm exchange, natural IgG4 Abs acquire the ability to cross-link two different Ags (heterologous cross-linking), making them bispecific (5). More recent studies suggest that IgG subclasses from other mammalian species share this ability (6, 8–10), making it a mechanism that could impact humoral immunity more generally.

Human IgG4 is classically considered nonactivating because of its limited ability to bind C1q and FcRs, resulting in a poor ability to induce complement and cell activation (11, 12). As such, IgG4 is commonly chosen for immunotherapeutic applications where recruitment of the immune system’s effector functions is undesired (13, 14). On infusion in patients, however, unmodified (bivalent) IgG4 molecules engage in Fab-arm exchange with the patient’s endogenous IgG4 pool (15). Thus, in time, bivalent IgG4 therapeutics convert into functionally monovalent IgG4 therapeutics, which could potentially affect both their initial binding strength and cross-linking behavior. This kind of unpredictability is an
undesired trait for a therapeutic Ab, and eliminating the molecular features enabling Fab-arm exchange should be considered when designing therapeutic IgG4 Abs.

The ability to exchange half molecules has been attributed to the IgG4 core-hinge sequence (226-CP5CP-230) in combination with determinants in the third constant H chain domain (CH3) (6). In accordance, mutating the core-hinge sequence (S228P) inhibited Fab-arm exchange to undetectable levels in vivo (8, 9, 15), whereas the molecular determinants in the CH3 enabling Fab-arm exchange remain to be elucidated. In this article, we identify R409 as the crucial CH3 residue governing Fab-arm exchange in human IgG4. We furthermore confirm that the ability to engage in Fab-arm exchange is conserved in a number of other species, but that this does not correspond to a particular CH3 consensus sequence. We demonstrate that in IgG4 from rhesus monkeys (Macaca mulatta) of Chinese origin, the 226-CP5CP-230 core-hinge sequence and the CH3 residue L405 are responsible. Moreover, we establish that the ability to exchange Fab-arms is largely determined by the strength of the noncovalent CH3–CH3 interaction.

Materials and Methods

Cells

FreeStyle 293F (human embryonic kidney [HEK]-293F) and FreeStyle Chinese hamster ovary (CHO)-S cells were cultured in FreeStyle 293 expression medium and FreeStyle CHO expression medium, respectively (Invitrogen, Carlsbad, CA).

Commercial Abs

Purified human, pig, horse, bovine, sheep, rabbit, rat, goat, and guinea pig IgG were obtained from Jackson ImmunoResearch (West Grove, PA). Purified cynomolgus monkey and rhesus monkey IgG were purchased from Sera Laboratories International (Haywards Heath, U.K.). Commercial Abs (Invitrogen, Carlsbad, CA).

Cloning and production of Abs and His-second constant H chain domain-CH3 constructs

Expression vectors for IgG1-epidermal growth factor receptor (EGFR), IgG4-EGFR, IgG4-S228P-EGFR, IgG1-CD20, and IgG4-CD20 have been described previously (6). For the expression of His-second constant H chain domain (CH2)-CH3 (nchCH3), His-CH2-CH3 (H1), and His-CH2-CH3 (H2), constructs were designed that contained N-terminal poly-histidine (6xHis) tags followed by aa 230–447 (EU numbering conventions) of rhesus IgG, human IgG1, and human IgG4, respectively. All His-CH2-CH3 constructs were codon optimized, synthesized de novo by Geneart (Regensburg, Germany), and cloned into pETE12.4 (Lonzà, Slough, U.K.). A Quikchange site-directed mutagenesis kit (Strategene, La Jolla, CA) was used to introduce the Q355R, R409K, E419Q, L445P, K370E, R409M, Q409L, L536D, R409A, L351K, Y349D, F405A, F405L, R409W, E357T, R409F, and T411V mutations in IgG4 using pTomG4-2FS, pTomG4-7FD, and pEE12.4His-CH2-CH3-G4 as templates. In the same way, the R355Q, K409R, Q419E, P445L, T530L, K370F, F405L, and T505K370T/F405L mutations were introduced in IgG1 using pConG1f2F8, pConG1f7D8, pG1f2F8CPSC, pG1f7D8CPSC, and pEE12.4His-CH2-CH3-G1 as templates. Rhesus IgG4 constructs were based on the sequences described by Scincicariello et al. (16) and synthesized de novo by Geneart AG.

All Abs were produced under serum-free conditions (FreeStyle medium) by cotransferring relevant H and L chain expression vectors in HEK-293F cells, using 293fectin (Invitrogen), or CHO-S cells, using FreeStyle MAX Reagent (Invitrogen), both according to the manufacturer’s instructions. All His-CH2-CH3 constructs were produced by transfecting relevant expression vectors in HEK-293F cells as described earlier.

Abs were purified by protein A affinity chromatography (Protein A FF; GE Healthcare, Uppsala, Sweden), diazoyl overnight to PBS, and filtered-stereilized over 0.2-μm dead-end filters. The concentration of purified IgGs was determined by absorbance at 280 nm. The quality of the purified proteins was analyzed by SDS-PAGE.

GSH-mediated Fab-arm exchange in vitro

As described previously (6), combinations of Abs were mixed and incubated with GSH (Sigma, St. Louis, MO) at a final concentration of 4 μg/ml per Ab. The final concentration of GSH was 0.5 mM. The mixtures were incubated at 37˚C for 24 h, and samples were drawn in PBS-TB (PBS/0.05% Tween 20% BSA), in which (b)specific IgG concentrations were measured. For inhibition experiments, Ab mixtures were supplemented with 160 μg (200-fold excess) of purified animal IgG either during or after the 24-h incubation at 37˚C. All purified animal IgG were first diazoyl against PBS to remove additivities and preservatives.

Fab-arm exchange in vivo

Female SCID mice (6- to 8-wk-old) were obtained from Charles River Laboratories (Maastricht, The Netherlands) and housed in a barrier unit of the Central Laboratory Animal Facility (Utrecht, The Netherlands). The mice were kept in filter-top cages with water and food provided ad libitum. All experiments were approved by the Utrecht University animal ethics committee. Mixtures of Abs (600 μg in total per mouse) were administered to mice (n = 4) and blood samples were drawn from the saphenous vein at 3, 24, 48, and 72 h after administration. Blood was collected in heparin-containing vials, which were kept on ice, and centrifuged (5 min at 10,000 × g) to separate the plasma from cells. Plasma was transferred to a new vial and stored at −20˚C for determination of bispecific Ab levels.

Binding assay for the detection of CD20/EGFR bispecific Abs

The presence of CD20/EGFR bispecific Abs was determined using a sandwich ELISA as described previously (6). In short, ELISA plates (Greiner bio-one, Frickenhausen, Germany) were coated overnight with 2 μg/ml recombinant EGFR (extracellular domain) in PBS at 4˚C. The plates were washed and incubated with serial diluted plasma samples or purified Ab mixtures (in PBS-TB) for 90 min at 20˚C under shaking conditions (300 rpm). Next, the plates were washed and incubated with 2 μg/ml mouse anti-idiotypic mAb 2F2 SAB1.1 (directed against human mAb CD20; Genmab) diluted in PBS-TB for 75 min at 20˚C. Bound bispecific Abs were detected with HRP-labeled goat anti-mouse IgG (Jackson ImmunoResearch) and ABTS substrate (Roche Diagnostics). The color development reaction was stopped by addition of an equal volume of oxalic acid (Riedel de Haen, Buchs, Germany), and absorbance was measured at 405 nm. Bispecific Abs in plasma samples were quantified by nonlinear regression curve fitting (GraphPad Software, San Diego, CA) using an in vitro exchanged Ab mixture as reference (with the assumption that the maximal expected concentration of bispecific IgG4 was 50% of total IgG4 concentration).

Total human IgG ELISA

The total amount of human Abs in the mice was determined by sandwich ELISA. In short, ELISA plates were coated overnight with 2 μg/ml mouse anti-human IgG (MH16-1; Sanquin) in PBS at 4˚C. The plates were subsequently washed and blocked with PBS-C (PBS/2% normal chicken serum; Life Technologies) for 1 h at 37˚C. Next, the plates were washed and incubated with diluted plasma samples in PBS/0.05% Tween 20% normal chicken serum for 120 min at 20˚C under shaking conditions (300 rpm). Bound Abs were detected by HRP-labeled goat anti-human IgG (Jackson ImmunoResearch) and ABTS substrate (Roche Diagnostics). The color development reaction was stopped by addition of an equal volume of oxalic acid, and absorbance was measured at 405 nm. IgG was quantified by nonlinear regression curve fitting (GraphPad Software) using the injection mixtures as reference.

Determination of dissociation constant by native mass spectrometry

Apparent dissociation constant (Kd) values were determined by nano-electrospray ionization mass spectrometry, as described elsewhere (17). In brief, purified proteins (at 50 μM stock concentrations) were buffer exchanged into 100 mM NH4Ac (pH 6.9), serial diluted to concentrations ranging from 25 nM to 20 μM (monomer equivalent), and analyzed by nano-electrospray ionization mass spectrometry. Effect of time between analysis and sample dilution was investigated and did not significantly influence the results. Signals corresponding to the monomeric (M1) and dimeric (D2) states were integrated, and the relative proportion of each state at each concentration of total protein in monomer equivalents (M1) was determined using the following equations: [M1]e = [M1][M2] + [D2][M1]e.
(concentration monomer at equilibrium), and $[D]_{eq} = ([M]_0 - [M]_\infty)/2$ (concentration dimer at equilibrium). The apparent $K_D$ was subsequently extracted by plotting the $[D]_{eq}$ against $[M]_\infty$ values of each concentration and determining the gradient by least squares linear regression (Microsoft Office Excel 2007 software; Microsoft, Redmond, WA).

**Modeling in silico**

Crystal structures were studied using the Brugel modeling package (18). To propose mutations that would lead to a desired stabilization (or destabilization) of IgG4, we used a quantitative structure-based scoring methodology (19). In brief, each position in the CH3-CH3 dimer interface was subjected to in silico mutagenesis to all natural amino acids, except cysteine and proline. Subsequent to in silico mutagenesis, exploration of the conformational space was obtained by interdependent optimization of the side chains of all residues located in a sphere of 12 Å of the mutated residue, using the FASTER algorithm (20), whereby the side chain under investigation was kept fixed at a macrorotameric state. Subsequently, on each macrorotameric state thus obtained, 300 steps of steepest descent minimization was carried out, and finally a scoring function for the side chain under investigation was evaluated, as described previously (19).

Finally, per position in the CH3-CH3 dimer interface, the highest scores for each mutation were compared, and visual inspection of the resulting conformation was carried out in selected cases.

**Statistical analysis**

Data analysis was performed using GraphPad Prism for Windows, version 4.03 (GraphPad). Data sets were compared by using one-tailed paired Student $t$ tests. Statistical significance was accepted when $p < 0.05$.

**Results**

**Identification of the CH3 residue mediating Fab-arm exchange in humans**

IgG4 Fab-arm exchange was previously shown to depend on the CPSCP sequence in the core-hinge combined with the IgG4 CH3 domain (6). To identify the specific amino acid residues enabling Fab-arm exchange, we aligned the IgG4 CH3 sequence with that of IgG1 (which does not support Fab-arm exchange) and identified sequence differences (Fig. 1A). The whole CH3 domain of IgG1 or individual IgG1-specific CH3 residues (excluding those resulting from allelic variation) were introduced into IgG4 by mutagenesis. Vice versa, the CH3 domain of IgG4 or individual IgG4-specific CH3 residues were introduced into IgG1 (in combination with the P228S mutation to mimic the IgG4 core-hinge). The mutations were introduced into fully human mAbs 2F8 (21), directed against EGFR, and 7D8 (22), directed against the CD20 Ag. The resulting proteins (listed in Fig. 1A), were compared with wild-type IgG4 in their ability to support Fab-arm exchange (Fig. 1B, 1C). Swapping CH3 domains between IgG1 and IgG4 activated Fab-arm exchange for IgG1-P228S and abrogated the activity for IgG4. Introduction of individual subclass-specific point mutations showed that residues at positions 355, 419, and 445 in the CH3 domains of both IgG1 and IgG4 had no influence on Fab-arm exchange activity. In contrast, mutations in the 409 position abrogated or enabled Fab-arm exchange activity in IgG4 and IgG1-P228S, respectively. Thus, residue R409 in the CH3 domain is crucial for Fab-arm exchange in vitro.

To investigate whether the effects observed in vitro also apply to in vivo conditions, we injected mixtures of IgG4-CD20 with IgG1-EGFR, IgG4-EGFR, IgG4-S228P-EGFR, IgG4-R409K-EGFR, or IgG1-P228S-K409R-EGFR (in a 5:1 molar ratio) into mice. Blood samples were drawn at 3, 24, 48, and 72 h after injection, and bispecific Abs were quantified using in vitro exchanged 5:1 mixtures (IgG4-CD20/IgG4-EGFR) as reference standard (Fig. 1D).

![FIGURE 1](http://www.jimmunol.org/)
Bispecific Abs appeared in the blood of mice injected with IgG4-EGFR or IgG1-P228S-K409R-EGFR, whereas samples containing IgG1-EGFR, IgG4-S228P-EGFR, or IgG4-R409K-EGFR tested negative. These results confirm the importance of residues S228 and R409 for Fab-arm exchange.

In silico model for IgG4 CH3–CH3 interaction

In human IgG1, the noncovalent interaction between the CH3 domains involves 16 residues located on 4 antiparallel β-strands that make intermolecular contacts (23). Alanine scanning mutagenesis showed that stabilization of the IgG1 CH3–CH3 interaction was largely mediated by five of these residues, including K409 (24). To get a better understanding of the role of K409 in the IgG1 CH3–CH3 interaction, we studied the 1.65-Å resolution crystal structure of IgG1 (Protein Data Bank [PDB] code 1L6X) (25) in more detail. In this structure (Fig. 2A), the volume of the K409 side chain is accommodated by a cavity that is bordered by L368′, K370′, D399′, F405′, and Y407′ in the opposite CH3 domain. K409 forms hydrogen (H-) bonds with D399′ and a buried water molecule, which prevents an electrostatic clash between R409 and K370′. The buried water molecule also forms H-bonds with S364 and T411. Because the 3.15-Å resolution of the only available IgG4 structure (PDB code 1ADQ) (26) is too low for direct comparison, the K409R substitution was modeled in the 1L6X structure by optimizing side-chain conformations of the arginine residue and its surrounding residues, using the FASTER algorithm (20). The resulting model (Fig. 2B), confirmed the R409 and K370′ side-chain conformations observed in structure 1ADQ (26) and suggested that the R409 guanidinium group maintains a favorable electrostatic interaction with the D399′ carboxylate side chain, as is also the case for the K409 ammonium group in IgG1. Contrary to the latter, however, the R409 guanidinium group in IgG4 takes up the space of the water molecule observed in high-resolution IgG1 structures, thereby negating the H-bonds with S364, T411, and K370′, and causing an electrostatic clash between R409 and K370′.

Fab-arm exchange is conserved in other species but cannot be explained by the 409 position

Homologs of the human IgG4 subclass have been identified in other species, including several nonhuman primates often used to model human disease (16, 27, 28). Furthermore, previous studies suggest that Fab-arm exchange occurs in rhesus monkeys (Macaca mulatta) (6), cynomolgus monkeys (Macaca fascicularis) (8, 9), and rabbits (Oryctolagus cuniculus) (10). In addition, non-precipitating Abs have been reported in guinea pig (Cavia porcellus), rat (Rattus norvegicus), horse (Equus caballus), sheep (Ovis aries), cow (Bos taurus), donkey (Equus asinus), mouse (Mus musculus), goat (Capra hircus), and pig (Sus scrofa) (29, 30). To investigate whether other species contain IgG subclasses that engage in Fab-arm exchange, purified IgG preparations from different species were assessed for their ability to interfere with in vitro exchange of an equimolar mixture of human IgG4-EGFR and IgG4-CD20 (Fig. 3). We found that purified monkey, pig, horse, rabbit, and rat IgG was able to significantly inhibit the formation of bispecific Abs to varying degrees, suggesting the ability to undergo Fab-arm exchange. Of all tested species, cynomolgus and rhesus monkey IgG preparations were most efficient, inhibiting the formation of bispecific Abs by >50% when added in 200-fold excess. Mouse IgG4 was not included in these experiments because it interfered with detection of bispecific Abs, for which an anti-mouse conjugate was used as secondary Ab. In a different experimental setup, however, we did confirm that mouse IgG3 engages in Fab-arm exchange with human IgG4 (data not shown), as previously described by Lewis et al. (8).

In an attempt to correlate the ability to engage in Fab-arm exchange to specific CH3 residues, available sequences of IgG subclasses from multiple species were aligned (Supplemental Table I). Examination of the 96 CH3 sequences, however, revealed no obvious consensus sequence relating to Fab-arm exchange within the 16 aa implicated in the CH3–CH3 interface. R409 was observed only in the CH3 of human IgG4 (and one human IgG3 allotype) and sheep IgG1 and IgG2. The combination of S228 in the core-hinge and R409 in the CH3 was specific for human IgG4. Thus, the ability to engage in Fab-arm exchange appears to be conserved in several species, but presumably regulated by different molecular determinants.

Identification of the molecular determinants mediating Fab-arm exchange in rhesus monkeys (Macaca mulatta)

To directly investigate Fab-arm exchange in rhesus monkeys, the V regions of both 2F8 and 7D8 were cloned into rhesus IgG4 backbones corresponding to polymorphisms found in rhesus monkeys of Chinese (rhCh) and Indian (rhIn) origin (16). Because the available sequences (Fig. 4) were incomplete, we supplemented the H chain with the appropriate C-terminal amino acid residues derived from the rhesus macaque genome sequence (31). Furthermore, because the rhesus IgG4 H chain unexpectedly did not appear to contain a cysteine for L chain pairing, variants

**FIGURE 2.** In silico model for the IgG4 CH3–CH3 interface. A, The IgG1 CH3–CH3 interface centered around residue K409 as resolved in the 1.65-Å resolution crystal structure (PDB code 1L6X) (25) and (B) an in silico model of the IgG4 CH3–CH3 interface centered around residue R409, based on the same structure. Only polar H atoms (white) of relevant side chains and relevant H-bonds (black dashed lines) are depicted. The H2O molecule depicted is also the case for the K409 ammonium group in IgG1. Contrary to the latter, however, the R409 guanidinium group in IgG4 takes up the space of the water molecule observed in high-resolution IgG1 structures, thereby negating the H-bonds with S364, T411, and K370′, and causing an electrostatic clash between R409 and K370′.

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containing a cysteine (at the position equivalent to human IgG4) were constructed to address potential H–L chain mispairing (Fig. 4). The resulting proteins, rhChIgG4, rhChIgG4-S131C, and rhIgG4-S131C, were expressed and analyzed by nonreducing SDS-PAGE (Fig. 5A). The rhChIgG4 molecules existed as a mixture with major species of ∼150, ∼90, and ∼44 kDa as a result of heterogeneous disulfide linkage between H and L chains (Fig. 5A). The S131C mutation promoted the disulfides linking the H chains to the L chains, as demonstrated by one major protein species of ∼150 kDa. As expected (15), the rhIgG4-S131C Abs, containing the CPACP core-hinge sequence, contained stabilized inter-H chain disulfides as demonstrated by the low abundance of half molecules compared with the rhChIgG4-S131C Abs (containing a CPACP core-hinge sequence). Furthermore, the rhCh I gG4 molecules were compared with human IgG4 in their ability to support Fab-arm exchange (Fig. 5B). Both IgG4 molecules based on rhCh enabled Fab-arm exchange, whereas IgG4 based on rhIn did not. Furthermore, rhChIgG4 did engage in Fab-arm exchange, despite the presence of protein species containing an atypical disulfide linkage between the two half molecules including an inter-L chain disulfide bond next to two inter-H chain bonds.

To identify the specific CH3 aa residues enabling Fab-arm exchange, we aligned the amino acid residues involved in CH3–CH3 interface of human and rhesus IgG (Fig. 5C). The three rhChIgG4-specific amino acids in the CH3 domain were introduced into human IgG1-P228S-EGFR and IgG1-P228S-CD20, as individual mutations or in combination, and assessed for their ability to support Fab-arm exchange (Fig. 5D). Whereas the T350I and K370T substitutions had no effect, the F405L substitution alone or the combination of all three mutations did enable Fab-arm exchange in an IgG1-P228S background.

Together, these results show that the CH3 of rhesus IgG4 enables Fab-arm exchange (in the context of A228 in the core-hinge), and that residue L405 mediates this ability (despite the presence of K409).

Probing and quantifying the CH3–CH3 interaction

Because the mechanism of Fab-arm exchange involves the dissociation and reassociation of CH3 domains, we hypothesized that the CH3–CH3 interaction strength would correlate with the ability to exchange half molecules. To investigate this correlation, we designed his-tagged constructs, lacking the hinge region to prevent covalent inter-H chain disulfide bonds, based on the Fc domains of rhChIgG4, human IgG1, and human IgG4 (termed His-CH2-CH3 [rhChG4], His-CH2-CH3[G1], and His-CH2-CH3[G4], respectively). Subsequently, variants of these constructs containing the mutations in the CH3–CH3 interface described earlier were generated by site-directed mutagenesis. Furthermore, additional mutations were designed based on the K409R model (Fig. 2B, Supplemental Fig. 1) and introduced into the CH3 domains of IgG4-EGFR, IgG4-CD20, and the His-CH2-CH3[G4] construct. The IgG4 variants were mixed pairwise and compared with wild-type IgG4 in their ability to support Fab-arm exchange (Fig. 6A, 6B). The resulting His-CH2-CH3 proteins listed in Table I were used to measure the strength of the noncovalent CH3–CH3 interactions by determining the apparent Kd by native mass spectrometry as described elsewhere (17) and ranked according to interaction strength (Table I).

CH3 mutations affected interdomain Kd and Fab-arm exchange differentially and could be divided into four groups. First, the R409A and S364D mutations only minimally affected Fab-arm exchange (in the context of A228 in the core-hinge), and that residue L405 mediated this ability (despite the presence of K409).

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CH3 mutations affected interdomain Kd and Fab-arm exchange differentially and could be divided into four groups. First, the R409A and S364D mutations only minimally affected Fab-arm exchange (in the context of A228 in the core-hinge), and that residue L405 mediated this ability (despite the presence of K409).

Probing and quantifying the CH3–CH3 interaction

Because the mechanism of Fab-arm exchange involves the dissociation and reassociation of CH3 domains, we hypothesized that the CH3–CH3 interaction strength would correlate with the ability to exchange half molecules. To investigate this correlation, we designed his-tagged constructs, lacking the hinge region to prevent covalent inter-H chain disulfide bonds, based on the Fc domains of rhChIgG4, human IgG1, and human IgG4 (termed His-CH2-CH3 [rhChG4], His-CH2-CH3[G1], and His-CH2-CH3[G4], respectively). Subsequently, variants of these constructs containing the mutations in the CH3–CH3 interface described earlier were generated by site-directed mutagenesis. Furthermore, additional mutations were designed based on the K409R model (Fig. 2B, Supplemental Fig. 1) and introduced into the CH3 domains of IgG4-EGFR, IgG4-CD20, and the His-CH2-CH3[G4] construct. The IgG4 variants were mixed pairwise and compared with wild-type IgG4 in their ability to support Fab-arm exchange (Fig. 6A, 6B). The resulting His-CH2-CH3 proteins listed in Table I were used to measure the strength of the noncovalent CH3–CH3 interactions by determining the apparent Kd by native mass spectrometry as described elsewhere (17) and ranked according to interaction strength (Table I).
mutations decreased the apparent $K_D$ of the CH3–CH3 interaction (3.2- to 24-fold in the His-CH2-CH3[G4] constructs) and blocked Fab-arm exchange to undetectable levels. The third group of mutations, consisting of L351K, E357T, Y394D, F405A, F405L, R409T, and R409W, all increased the apparent $K_D$ (16.7–833.3-fold) and influenced both maximal exchange levels and exchange kinetics. Thus, the maximal observed exchange was already reached after 6 h, followed by a reduction in the levels of bispecific reactivity. The fourth group consists of IgG1-based molecules, where all mutations, except T350I, increased the apparent $K_D$.

FIGURE 6. The ability to engage in Fab-arm exchange is correlated to the CH3–CH3 interaction strength. A, Rhesus monkey IgG4 can mediate Fab-arm exchange. A, Rhesus monkey IgG4 formats of mAbs directed to EGFR and CD20 (rhChlgG4, rhChlgG4-S131C, and rhInIgG4-S131C) analyzed on nonreducing SDS-polyacrylamide gel. The molecular sizes of intact Abs (H2L2), H chain dimers (H2), half molecules (HL), L chain dimers (L2), and H and L chains are indicated. Possible conformations of disulfide linkages (red lines) between H chains (black lines) and L chains (gray lines) are depicted as cartoons. One representative experiment out of two is shown. B, Equimolar mixtures of identical rhesus monkey-derived IgG4-CD20 and IgG4-EGFR molecules, or (D) equimolar mixtures of IgG1-P228S-CD20 and IgG1-P228S-EGFR mutants, incubated in the presence of 0.5 mM GSH at 37°C. The generation of bispecific Abs was followed over time by ELISA and expressed as percentage of control (human IgG4 at 24 h). Data represent mean ± SEM of at least three separate experiments. C, Sequence alignment of relevant amino acid residues of human and rhesus monkey IgG subclasses.
Table 1. CH3–CH3 apparent $K_D$ of His-CH2-CH3 constructs based on rhesus IgG4 and human IgG1 and IgG4

<table>
<thead>
<tr>
<th>His-CH2-CH3 Construct</th>
<th>$K_D$ (M)</th>
<th>$R^2$</th>
<th>Fold Difference$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1-T350I</td>
<td>$1.0 \times 10^{-9}$</td>
<td>0.812</td>
<td>3</td>
</tr>
<tr>
<td>G4-K370E</td>
<td>$2.0 \times 10^{-9}$</td>
<td>0.8058</td>
<td>24</td>
</tr>
<tr>
<td>G4-R409M</td>
<td>$3.0 \times 10^{-9}$</td>
<td>0.7968</td>
<td>16</td>
</tr>
<tr>
<td>G1</td>
<td>$3.0 \times 10^{-9}$</td>
<td>0.992</td>
<td>1</td>
</tr>
<tr>
<td>G1-K370T</td>
<td>$5.0 \times 10^{-9}$</td>
<td>0.88</td>
<td>0.6</td>
</tr>
<tr>
<td>G4-R409K</td>
<td>$8.0 \times 10^{-9}$</td>
<td>0.9669</td>
<td>6</td>
</tr>
<tr>
<td>G4-K370O</td>
<td>$1.1 \times 10^{-8}$</td>
<td>0.994</td>
<td>4.3</td>
</tr>
<tr>
<td>G4-R409L</td>
<td>$1.5 \times 10^{-8}$</td>
<td>0.9513</td>
<td>3.2</td>
</tr>
<tr>
<td>G4-S364D</td>
<td>$4.7 \times 10^{-8}$</td>
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</tr>
<tr>
<td>G4</td>
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<td>0.9755</td>
<td>1</td>
</tr>
<tr>
<td>rHChG4</td>
<td>$1.1 \times 10^{-7}$</td>
<td>0.978</td>
<td>NA</td>
</tr>
<tr>
<td>G1-K409R</td>
<td>$1.1 \times 10^{-7}$</td>
<td>0.974</td>
<td>0.03 NA</td>
</tr>
<tr>
<td>G4-R409A</td>
<td>$1.6 \times 10^{-7}$</td>
<td>0.977</td>
<td>0.3</td>
</tr>
<tr>
<td>G4-R409T</td>
<td>$5.8 \times 10^{-7}$</td>
<td>0.896</td>
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<td>G4-L351K</td>
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<td>0.9171</td>
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<td>G1-F405L</td>
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<td>0.0035</td>
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<tr>
<td>G1-ITL</td>
<td>$1.2 \times 10^{-6}$</td>
<td>0.973</td>
<td>0.0025</td>
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<tr>
<td>G4-Y349D</td>
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<tr>
<td>G4-F405A</td>
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<tr>
<td>G4-E357T</td>
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<td>0.9773</td>
<td>0.0012</td>
</tr>
</tbody>
</table>

$^a$Compared with wild-type CH2-CH3(G1) or CH2-CH3(G4) molecules.

$K_D$, in which increases of >15-fold (K409R, F405L, and ITL) enabled Fab-arm exchange. Together, these data suggest that efficient Fab-arm exchange can occur only if the CH3–CH3 interaction is sufficiently weak. Increasingly weaker interactions, however, also make the generation of bispecifics less efficient (Figs. 1C, 5D, 6C, 6D).

Discussion

The ability of human IgG4 to engage in Fab-arm exchange was shown to depend on the IgG4 core-hinge sequence, 226-CPACP-230, in combination with determinants in the CH3 domain (6). In this study, we identified residue R409 as the crucial CH3 determinant enabling Fab-arm exchange in human IgG4 (compared with K409 in the other human IgG subclasses). This can be rationalized because R409 is the only IgG4-specific residue directly involved in the noncovalent CH3–CH3 interaction. It is, however, striking that what appears to be a rather conservative K to R mutation has such a profound impact, although large effects of K to R substitutions on protein–protein interactions are not without precedence (32, 33). To explain the dramatic effect of this relatively conservative substitution, we used in silico modeling. The model suggests that because of the larger size of the R409 side chain, a buried water molecule is displaced that in IgG1 is involved in the stabilization of the CH3–CH3 interaction by forming four short H-bonds with S364, K409, T411, and K370', and preventing electrostatic clashes between K409 and K370’ (Fig. 3).

Previous studies suggested that IgG from rhesus monkeys (Macaca mulatta) (6), cynomolgus monkeys (Macaca fascicularis) (8, 9), and IgG3 from mice (Mus musculus) (8) could engage in Fab-arm exchange with human IgG4. In accordance, we now demonstrate that a variety of other species share this ability. The observed variation in efficiency (Fig. 2) could be explained by differences in the following factors: 1) interspecies compatibility, 2) relative concentrations of permissive subclasses, and 3) stability of the hinge disulfide bonds. A more detailed analysis with recombinant rhesus monkey IgG4 constructs identified the 226-CPACP-230 core-hinge sequence in combination with residue L405 in the CH3 domain as the molecular determinants enabling Fab-arm exchange in this species. Thus, these data, together with sequence analysis of available CH3 sequences of different species, suggest that ability to engage in Fab-arm exchange is regulated by species-specific molecular determinants.

The redistribution of Ag-binding domains by Fab-arm exchange results in bispecific IgG4 molecules that, in the polyclonal setting, are functionally monovalent. We have previously proposed that the resulting inability of IgG4 to cross-link homologous Abs contributes to the anti-inflammatory nature attributed to human IgG4 by interfering with immune complex formation (6). In this way, the functional monovalency of IgG4, typically induced on chronic Ag stimulation, may act as a natural buffer and dampen inflammatory reactions induced by other Ab subclasses. Because Fab-arm exchange is also observed in other species and because it is mediated by species-specific molecular determinants, one might speculate that this anti-inflammatory mechanism represents an evolutionary advantage. The fact that polymorphisms that block Fab-arm exchange exist in both humans (K/R409) and rhesus monkeys (P/A228), however, also suggests that there has been selective pressure favoring the preservation of bivalent IgG4 molecules and raises the possibility that such polymorphisms might also exist in other species, including those used to model human diseases.

Although evaluation of available CH3 sequences did not reveal specific common residues enabling Fab-arm exchange, all amino acids implicated are located at the CH3–CH3 interface. In accordance, we established a relation between the noncovalent CH3–CH3 interaction strength and the ability to exchange Fab-arms, consistent with a model in which strong interactions, such as found between IgG1 CH3 domains, block Fab-arm exchange by preventing the dissociation of half molecules. Weaker interactions, such as found between IgG4 CH3 domains, then allow the efficient exchange of Fab-arms. This interpretation is supported by a mechanistic study that showed dissociation of the CH3 domains to be the rate-determining step of IgG4 Fab-arm exchange (34). Increasingly weaker interactions, however, make the generation of bispecifics less efficient by precluding effective (re)association. The ~10-fold difference between the apparent $K_D$s of IgG4 and IgG4-R409K/IgG1 CH3 domains reported in this study (and thus the difference between allowing and inhibiting Fab-arm exchange) may be an underestimation, because the same mass spectrometric technique using IgG4hinge constructs reported similar IgG4 apparent $K_D$s, but ~100-fold stronger apparent $K_D$s for IgG4-R409K (17), which is consistent with the previously reported IgG1 CH3 interaction strength of $<10^{-10}$ M as determined by size-exclusion chromatography (35).

Consistent with a previously reported alanine scan of the IgG1 CH3 interface (24), our results show that mutating residues K409 and F405 considerably affect the $K_D$ of the CH3–CH3 interaction, ranging from $3.0 \times 10^{-9}$ to $3.4 \times 10^{-8}$ M (Table I). Substitution of K370 with alanine had a significant destabilizing effect on CH3 stability in IgG1 (24), but conversely, the same mutation increased the CH3–CH3 interaction in IgG4 (17), consistent with our proposed model (Fig. 2B) where a K370A substitution would relieve the electrostatic strain with R409’ in the partner CH3 domain. Likewise, the K370E and K370Q substitutions described in this article stabilize the CH3–CH3 interaction in IgG4 and are thought to act in a similar way (see Supplemental Fig. 1 for a detailed discussion of all mutations).

The ability of therapeutic IgG4 Abs to recombine with endogenous IgG4 in vivo may affect their pharmacokinetic (PK) and pharmacodynamic properties. Although PK modeling suggests that under normal conditions this will unlikely lead to biological effects,
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Disclosures


References


