Variable Region Domain Exchange Influences the Functional Properties of IgG

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Variable Region Domain Exchange Influences the Functional Properties of IgG

Sherie L. Morrison, Stephen B. Porter, K. Ryan Trinh, Letitia A. Wims, Jerrod Denham, and Vernon T. Oi

In the present study we have characterized a family of anti-dansyl Abs with the variable region of the heavy chain on human Cκ and the variable region of the light chain on different human γ constant regions (creating inside-out molecules). Although fully assembled molecules were secreted, this variable region exchange slowed the kinetics of Ab assembly. Although the variable region exchange does not lead to a detectable change in the microenvironment of the combining site, it did alter the kinetic parameters of binding to immobilized Ag, slowing both the on and off rates. When effector functions were evaluated, inside-out IgG1 and IgG3 were more effective in complement-mediated cytolysis than their wild-type counterparts. Variable region domain exchange may be one approach to obtaining Abs of identical specificity with altered binding characteristics.


Ig molecules of the γ class consist of two heavy (H) and two light (L) chains. The H and L chains fold into 12,000-Da globular β barrel domains with homologous sequences but different functions. There are four domains for the H chain and two for the L chain. H chain and L chain are held together by disulfide bonds and by extremely strong, noncovalent interactions (1). Ig domains other than CH2 interact strongly in a lateral fashion to form modules: Vγ1-VL, Cκ-Cγ1, and Cγ3-Cγ3. This lateral pairing buries hydrophobic residues that would be exposed in isolated domains. Longitudinal interactions along the H chain or L chain are weak or nonexistent. Within the IgG1 myeloma protein Kol, there are no nonbonded longitudinal contacts (2).

Although Ab molecules and their domains have been described as pearls on a string with weak longitudinal noncovalent interactions between the domains, but strong lateral interactions (except for Cγ2), the questions still remain as to whether longitudinal domain interaction exerts any influence and whether the context of the variable regions influences their functional properties. An earlier study (3) in which variable regions from anti-(4-hydroxy-3-nitrophenyl)acetyl (anti-NP) Abs were exchanged between H and L chain or L chain are weak or nonexistent. Within the IgG1 myeloma protein Kol, there are no nonbonded longitudinal contacts (2).

Although Ab molecules and their domains have been described as pearls on a string with weak longitudinal noncovalent interactions between the domains, but strong lateral interactions (except for Cγ2), the questions still remain as to whether longitudinal domain interaction exerts any influence and whether the context of the variable regions influences their functional properties. An earlier study (3) in which variable regions from anti-(4-hydroxy-3-nitrophenyl)acetyl (anti-NP) Abs were exchanged between H chains and L chains of the murine Cκ and the murine Vκ on different human γ constant regions (creating inside-out (io) molecules). We found that this variable region exchange alters the kinetics of assembly of the io protein compared with that of their isotype-matched wild-type (wt) protein. Although the variable region exchange does not lead to a detectable change in the microenvironment of the combining site, we found that it does change the kinetic parameters of binding to immobilized Ag, slowing both the on and off rate. Moreover, io IgG1 and IgG3 are more effective in complement-mediated cytolysis than their comparable wild types. These results indicate that for some variable regions, associated functions can be influenced by their constant region context and suggest that variable region exchange may be one strategy for changing the Ag binding characteristics of Abs while maintaining their specificity.

Materials and Methods

Construction of chimeric IgG molecules

The expressed Vκ and Vλ genes from the mouse anti-dansyl (anti-DNS) hybridoma 27–44 had previously been joined to human Cκ in the pSV184 Δhneo expression vector and to human IgG heavy chain in the pSV184 Δhgt vector, respectively (4). To produce the vectors encoding the io molecules, the EcoRI in the intervening sequence immediately 5′ of Cκ in pSV184 Δhneo was converted to a SalI site using oligonucleotide linkers (Fig. 1). As a result of this manipulation, Cκ and the four human IgG constant regions existed as SalI-BamHI cloning cassettes (5). Using these cassettes, the IgG constant regions were then joined to Vκ and Cκ was joined to Vκ.

Production of transfectoma proteins

P3X63Ag8.653, an Ig-nonproducing mouse myeloma cell line, was transfected with the H and L chain expression vectors (4), transfecants were selected, and surviving clones were screened for Ab production by ELISA using DNS/BSA-coated plates. DNS/BSA was prepared using dansyl chloride and BSA of RIA grade from Sigma Chemical Co. (St. Louis, MO). The amount of bound chimeric Ab was determined using alkaline phosphatase-conjugated polyclonal goat Ab (Sigma Chemical Co.) against human IgG constant regions. Clones producing large quantities of anti-DNS Ab were expanded and maintained in Iscove’s modified Dulbecco’s medium containing 5% calf serum. Chimeric Abs were purified by DNS-coupled affinity chromatography as described previously (6). The concentrations of purified Abs were determined by a bicinchoninic acid-based protein assay (BCA Protein Assay, Pierce, Rockford, IL). Concentration and purity were confirmed by SDS-PAGE analysis or by chromatography on a Superose 12 column (Pharmacia, Piscataway, NJ).
Biosynthetic labeling

Transfectomas were washed twice in methionine-free DMEM (Irvine Scientific (Irvine, CA)) supplemented with nonessential amino acids (Life Technologies, Grand Island, NY) and glutamine (29.2 μg/ml). Cells were labeled in 1 ml of DMEM with 15 μCi of [35S]methionine (Amersham, Arlington Heights, IL). Be-

 Biosensor analysis of Ag binding

DNS-BSA80 was immobilized to a commercially available IAsys cuvette precoated with a high m.w. carboxymethylated dextran (Fisons Applied Technology, Cambridge, U.K.), using the conditions recommended by the supplier. The covalently bound DNS-BSA80 increased the sensor-

 Fluorescence emission spectrum

To determine the fluorescence emission spectra of ε-dansyl-l-lysine bound to the proteins, protein at a concentration of 54 μg/ml was mixed with hapten at a concentration sufficient to saturate the binding sites. The exci-

 Biosynthesis of io Ab molecules

Clones expressing V_{L} joined to C_{K} along with V_{L} joined to the four human y isotypes were also isolated and characterized. The proteins produced by these transfectomas were designated io to
HL half-molecules are a consequence of the IgG3 also assembling using H2 and H2L as intermediates (Fig. 4). Transfectomas were labeled by overnight growth in the presence of [35S]methionine, and the Igs were precipitated from the culture supernatants. The immune precipitates were analyzed on 5% PO4 gels without addition of a reducing agent. Lanes 1 and 2, V_LC_k; lanes 3 and 4, V_HC_k; lanes 5 and 6, V_LC_m and V_HC_m.

distinguish them from their wt isotype-matched controls consisting of classical V_HC_m and V_LC_m heavy and light chains. To characterize the proteins produced, io and wt cell lines were grown overnight in medium containing [35S]methionine, and the secreted Igs were immunoprecipitated with DNS-BSA-Sepharose and analyzed by SDS-PAGE (Fig. 3). IgG1, IgG2, and IgG3 io Abs assemble and secrete H2 L2 heterodimers that bind Ag. Both HL half-molecules and fully assembled H2 L2 that bind Ag are produced by io and wt IgG4. About 10% of wt IgG4 anti-DNS and 30% of io IgG4 anti-DNS consist of HL molecules (Fig. 3A). The presence of the HL half-molecule is a consequence of the ∆4 hinge (7, 8). The nature of the noncovalent interactions between HL half-molecules remains unclear. Following reduction, the io heavy chains were similar in apparent m.w. to their wt counterparts (Fig. 3B); as shown in Figure 2, V_HC_k migrated with a larger apparent m.w. than V_LC_m.

Ig assembly pathways

A pulse-chase experiment was performed to determine whether switching the variable regions to different constant regions had any effect on either the kinetics or the pathway of assembly of Ab. In each case the io Ab-producing cell lines were compared with an isotype-matched wt Ab-producing cell line. Two to four independent clones of each io isotype were examined, and within an isotype similar assembly patterns were observed. Results from a representative clone of each isotype are shown in Figure 4. Several things are noteworthy. The wt and io IgG1 and IgG2 assemble through an H2 intermediate. For wt IgG1 and IgG2, the H2 and the H2L intermediates are rapidly converted to H2 L2, with 80% of the Ig fully assembled by 30 min. In contrast, assembly of io IgG1 and IgG2 into H2 L2 takes longer and ∼20% of the Ig is assembled into H2 L2 by 30 min (Fig. 4, A–D, and data not shown). The wt and io IgG3 also assemble using H2 and H2L as intermediates (Fig. 4, E and F, and data not shown); however, the difference in assembly kinetics between wt and io IgG3 is less marked, and both have similar amounts of H2 L2 by 60 min. The wt and io IgG4 assemble H2 and H2L but also accumulate HL half-molecules that are found in the secretions (Figs. 3 and 4, G and H). HL was an even more prominent assembly intermediate in an io IgG4 clone in which the presence of the noncovalent interactions between HL half-molecules remains unclear. Following reduction, the io heavy chains were similar in apparent m.w. to their wt counterparts (Fig. 3B); as shown in Figure 2, V_HC_k migrated with a larger apparent m.w. than V_LC_m.

Ag reactivity

All the proteins containing both V_HDNS and V_LDNS could be isolated using Ag. Ag binding was characterized further by measuring the fluorescence emission spectra of bound DNS and by measuring Ab binding kinetics. Similar fluorescence emission spectra were obtained for DNS bound by both io and wt proteins (Fig. 5), indicating that the microenvironment of the hapten in the combining site was similar in all the proteins.

The kinetics of DNS binding by the engineered Abs were measured using an IAAsys biosensor. The a and kd in PBST and PBST plus DNS-lysine were calculated as described above (Table I). The Abs were also directly compared by adding equal quantities of the anti-DNS Abs (5 μg) to the DNS-BSA-coated cuvette (Fig. 6). The wt and io Abs exhibited different binding kinetics. The wt Abs showed a more rapid ka than the io Abs. The wt IgG1, IgG2, and IgG3 approached equilibrium by 300 s; the wt IgG4 required longer, reflecting its smaller initial ka (Table I). In contrast, the io Abs did not reach equilibrium during the 1000 s of the experiments. However, when the cuvette was washed with PBST, the io Abs dissociated more slowly than the wt (Fig. 6), as reflected in a smaller kd (Table I). Under the conditions of the experiment, rebinding occurred, and the off-rate represented the competition between dissociation and rebinding. Addition of DNS-lysine as a soluble competitor to prevent rebinding increased the kd (Table I), but again, the io Abs showed a smaller kd than their wt counterparts.
Ab-mediated complement activation

To evaluate the ability of the io Abs to activate the complement cascade, we performed a direct lysis assay using DNS80-BSA-coated SRBCs. Neither io nor wt IgG2 and IgG4 were able to effect lysis (data not shown), and as we had observed previously, wt IgG3 was more effective than wt IgG1 in causing lysis (9, 10). In addition, we found that io IgG1 and IgG3 were significantly more effective than wt IgG1 and IgG3 in activating complement-mediated cytolysis, with io IgG1 as effective as wt IgG3, and io IgG3 causing maximum lysis at a concentration less that one-tenth of that required for wt IgG3 (Fig. 7).

Discussion

These studies confirm that it is possible to create Ab molecules in which the variable regions of the H and L chains are exchanged. Molecules consisting of VH Ck and with VL joined to

Table I.  Kinetic constants for the wt and io Absa

<table>
<thead>
<tr>
<th>Ab</th>
<th>kₚ (M⁻¹·s⁻¹)</th>
<th>kₛ (M⁻¹·s⁻¹)</th>
<th>k₂ (s⁻¹)</th>
<th>k₃ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt IgG1</td>
<td>57,725</td>
<td>39,777</td>
<td>0.007</td>
<td>0.0182</td>
</tr>
<tr>
<td>wt IgG2</td>
<td>60,868</td>
<td>42,849</td>
<td>0.005</td>
<td>0.0169</td>
</tr>
<tr>
<td>wt IgG3</td>
<td>57,070</td>
<td>36,556</td>
<td>0.005</td>
<td>0.0328</td>
</tr>
<tr>
<td>wt IgG4</td>
<td>13,520</td>
<td>37,518</td>
<td>0.004</td>
<td>0.0211</td>
</tr>
<tr>
<td>io IgG1</td>
<td>22,290</td>
<td>4,697</td>
<td>0.0015</td>
<td>0.0135</td>
</tr>
<tr>
<td>io IgG2</td>
<td>22,731</td>
<td>4,808</td>
<td>0.0014</td>
<td>0.0089</td>
</tr>
<tr>
<td>io IgG3</td>
<td>36,269</td>
<td>5,337</td>
<td>0.0016</td>
<td>0.0119</td>
</tr>
<tr>
<td>io IgG4</td>
<td>22,859</td>
<td>16,478</td>
<td>0.0015</td>
<td>0.0109</td>
</tr>
</tbody>
</table>

a Binding constants were calculated using the FASTfit program as described in Materials and Methods.
C regions of different human \( \gamma \) isotypes were secreted, with covalent assembly occurring between the H and L chains (Fig. 3). The L chain heterodimers of \( \text{V}_H \text{C}_\kappa - \text{V}_L \text{C}_\kappa \), although mostly lacking covalent bonds, also were secreted and bound Ag (Fig. 2). Presumably the \( \text{V}_H \text{C}_\kappa - \text{V}_L \text{C}_\kappa \) form noncovalent heterodimers capable of binding Ag and being secreted. However, L chains consisting of \( \text{V}_H \text{C}_\kappa \) were unable to bind Ag or be secreted. The precise structure of the \( \text{V}_H \text{C}_\kappa \) appears to determine whether it is secreted from cells. Another \( \text{V}_H \text{C}_\kappa \) with the anti-NP variable region was secreted in the absence of an associated L chain (3), while a \( \text{V}_H \text{C}_\kappa \) with a human variable region was not secreted even with the endogenous J558L L chains (11). In several cases failure to secrete L chains can be traced to amino acid sequences present in the variable region (12–14).

The change in domain position results in a slowed kinetics of covalent intracellular assembly. In contrast with an earlier study of domain exchanged anti-NP Abs, where L, H, HL, H\(_2\)L, and H\(_2\)L\(_2\) were present in the secretions (3), only fully assembled H\(_2\)L\(_2\) and free L chain are seen in the secretions of io IgG1, io IgG2, and io IgG3. Like wt IgG4, io IgG4 also secretes HL half-molecules; the formation of half-molecules has been shown to be a consequence of the structure of the \( \gamma_4 \) hinge (7, 8).

During in vivo assembly of the molecule, noncovalent association of the subunits must precede interchain disulfide bond formation, since sulfhydryl groups must be brought close together before oxidation. Covalent assembly of H chains can begin while they are still nascent chains, and disulfide-linked nascent H chains are present in the murine cell line MOPC-21 that assembles its Ig through an H\(_2\) intermediate (15). Similarly, H-H disulfide bond formation occurs early in the assembly of the io molecules. Strong noncovalent interactions in the Fc also serve to bring H chains into close proximity, and they appear to be dimers at concentrations as low as \( 10^{-10} \) M, which would facilitate their covalent assembly (16). The L chain is released from the ribosome before the terminal Cys involved in disulfide bond formation has transited the membrane of the endoplasmic reticulum and is available. Therefore, covalent attachment of L chain must take place after its translation has been completed. Excess L chain has been observed to facilitate Ig assembly and secretion (17), and a variant of the murine cell line MPC-11 which synthesizes less L chain has a delay in the secretion of its Ig, with the predominant intermediate in assembly now H\(_2\) instead of the normally occurring HL. However, for the io Abs, the L chain appears to be present in excess, so the quantity of L chain present should not delay their assembly. In fact, additional transfectants making a large excess of L chain showed similar H chain assembly kinetics (data not shown).
During in vitro reassembly, H-L interaction is accompanied by conformational changes in both subunits (18, 19), and previous studies (20) have suggested that the binding of one \( \kappa \)-chain domain, either \( \eta \) or \( \zeta \), to Fd induces a conformational change in the adjacent domain in Fd, modifying its reactivity toward the complementary \( \kappa \)-chain domain. In the \( \theta \) molecules, Fd now consists of \( \nu \) and \( \epsilon \) and the \( \lambda \) chain consists of \( \eta \) and \( \zeta \). In this altered orientation, the interaction of \( \epsilon \) and \( \zeta \) and that of \( \nu \) and \( \eta \) may fail to induce the appropriate conformational changes, thereby slowing the covalent assembly process.

Earlier pulse-chase analysis of cells from a patient with a \( \gamma_1 \) \( \lambda \) paraprotein had indicated HL half-molecules as an intermediate in the assembly of human IgG1 (21). In contrast, with all the chimeric proteins, both wt and \( \theta \) \( \Lambda \) is the predominant assembly intermediate; only wt and \( \theta \) IgG4 show significant quantities of HL molecules, which probably do not represent assembly intermediates but, instead, a final product. This difference from the previous studies may reflect the use of a \( \kappa \) instead of a \( \lambda \) constant region on the L chain. Alternatively, there may be alloype differences between the \( \gamma_1 \) H chain used in these experiments and the \( \gamma_1 \) H chain present in the patient from which the cells were obtained for the earlier pulse-chase experiments.

Ag reactivity of \( \theta \) anti-DNS molecules

Previous studies using anti-NP Abs in which the variable regions had been exchanged between the heavy and light chains had shown no alteration in the functional properties of these Abs and had suggested autonomy of the Ag binding domains (3). Similarly, the \( \theta \) anti-DNS Abs retain the local environment of the hapten binding site as measured by fluorescence enhancement, but, unlike the anti-NP Abs, they have been shown to exhibit altered binding kinetics. The wt 27.44 DNS combining site is believed to bind the dapsyl moiety in a lateral position near the rim of the binding pocket (22). In the \( \theta \) molecules, the combining sites may be positioned differently, and this difference in position could alter the structure of the array of Ab molecules on the Ag. This altered positioning could then affect the on and off rates by influencing the ease both of association with immobilized Ags and of rebinding. The constant region of murine Abs has been shown to influence Ag binding (23). At high epitope density, murine IgG3 was seen to have a significantly greater apparent functional affinity than murine IgG1, IgG2b, or F(ab\(^\prime\))\(_2\); these results are consistent with a model for cooperative binding by IgG3 that depends on noncovalent interactions between \( \gamma_3 \) Fc regions of Ab bound to epitopes in close proximity. However, in this case IgG3 was found to have advantages in both association and dissociation rate constants.

Changes in complement activation

Human IgG isotypes differ in their ability to fix complement. IgG1 and IgG3 are most effective; IgG2 is much less effective, functioning under only some assay conditions (6); and IgG4 is unable to bind C1q and activate the complement cascade. The \( \epsilon \)2 domain is the binding site for C1q with the exposed \( \beta \) strand of Glu\(^{319}\), Lys\(^{320}\), and Lys\(^{322}\) important (24). Amino acid changes within C\(_{1q}\) result in the inability of IgG4 to activate complement (9, 10) and contribute to the differential abilities of the other iso- types to activate complement (6, 7, 25–27). The \( \rho \) and IgG3 molecules were significantly more effective than their wt counterparts in their ability to effect complement-mediated cytolysis. Both \( \theta \) and wt IgG2 and IgG4 remained nonfunctional in this assay. Several aspects of the changed structure in the \( \theta \) Abs could contribute to these differences. The geometric presentation of hapten or antigenic epitopes recognized by Abs can determine their effectiveness in activating complement (28), probably due to the nature of the lattice formed by the Ag-antibody complexes. Switching the domains between the H and L chains in the anti-DNS Abs could alter the position of the binding site relative to the remainder of the molecule. This is possible given the reported position of the combining site at the edge of the variable region (22) (discussed above). A different array of \( \rho \) IgG1 and IgG3 more effective in complement activation could form on the surface of Ag coated-SRBC. It is also possible that the slower off-rate of the \( \theta \) molecules leads to a more stable Ab-C1 complex.

These studies demonstrate that it is possible to alter some of the functional properties of Ab molecules by changing the disposition of the different domains. While maintaining identical amino sequences in the variable region, it was possible to change the kinetics of Ag binding by exchanging the variable regions of the H and L chains. The resulting Abs had altered functional properties, including increased efficacy in complement mediated cytolysis. Domain exchange may provide an additional approach to producing Abs with the appropriate con- stellation of functional properties.

References