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Complementarity-Determining Region 2
Results in a Severe Ig Secretion Defect

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Mutation of a Single Conserved Residue in $V_H$ Complementarity-Determining Region 2 Results in a Severe Ig Secretion Defect

Gregory D. Wiens, Annemarie Lekkerkerker, Imke Veltman, and Marvin B. Rittenberg

During an immune response, somatic mutations are introduced into the $V_H$ and $V_L$ regions of Ig chains. The consequences of somatic mutation in highly conserved residues are poorly understood. Ile$^{51}$ is present in 91% of murine $V_H$ complementarity-determining region 2 sequences, and we demonstrate that single Ile$^{51}$→Arg substitutions in the PCG1-1 Ab are sufficient to severely reduce Ig secretion (1–3% of wild-type (WT) levels). Mutant $H$ chains, expressed in the presence of excess $L$ chain, associate with Ig binding protein (BiP) and GRP94 and fail to form HL and H$_2$L$_2$ assembly intermediates efficiently. The mutations do not irreversibly alter the $V_H$ domain as the small amount of mutant H chain, which assembles with L chain as H$_2$L$_2$, is secreted. The secreted mutant Ab binds phosphocholine-protein with avidity identical with that of WT Ab, suggesting that the combining site adopts a WT conformation. A computer-generated model of the PCG1-1 variable region fragment of Ig (Fv) indicates that Ile 51 resides in the CDR2 or framework 2 (FRW2). The role of single mutations Ile$^{51}$→Arg or Ile$^{51}$→Lys mutations impair association with the PCG1-1 L chain via indirect interactions. These interactions are in part dependent on the nature of the L chain as the PCG1-1 $V_L$, single Ile$^{51}$→Arg or Ile$^{51}$→Lys mutants were partially rescued when expressed with the J558L λ1 L chain. These results represent the first demonstration that single somatic mutations in $V_H$ residues can impair Ig secretion and suggest one reason for the conservation of Ile$^{51}$ in so many Ig $V_H$.


The ability of somatic mutation to improve Ag-binding characteristics of Abs during immune responses is well documented (1–5); however, harmful effects of somatic mutation are less well characterized (6). The number of B cells directed to wastage pathways as a result of harmful somatic mutation probably far exceeds the number of cells with beneficial mutations (7, 8). Understanding the negative impact of somatic mutation on Ig structure is important as B cell survival appears to be dependent on the expression of functional Ig receptors (9). Failure to express functional Ig receptors due to harmful somatic mutations may also contribute to the genesis of pathological conditions such as Hodgkin’s disease (10, 11).

Previously, we created a collection of anti-phosphocholine (PC)$^-$ and anti-nitrophenylphosphocholine (NPPC)-specific Abs as a model system to understand the deleterious nature of somatic mutation. In a panel of 160 random mutants generated from 2 Abs, T15 and PCG1-1, 16 $V_H$ mutants had defects in Ig trafficking and secretion (8, 12). These mutants secreted $<10\%$ of wild-type (WT) levels of Ig but expressed levels of $V_H$ mRNA and intracellular H chain equivalent to or greater than those of WT cells. Of the 16 secretion-defective mutants, all contain 2–4 residue changes, some in highly conserved residues in either $V_H$ complementarity-determining region 2 (CDR2) or framework 2 (FRW2). The role of these conserved residues in proper Ig folding and assembly is not clear, nor is it known how the quality control system within the endoplasmic reticulum (ER) of B cells maintains transport of properly assembled Ig while targeting improperly assembled Ig for degradation.

Here we conduct a molecular analysis of the secretion defect by determining the contribution of individual substitutions to the low secretion phenotype. Generation and analysis of a subset of single-site PCG1-1 mutants indicate that in each case, a single substitution in the highly conserved $V_H$ CDR2 position 51 impaired secretion markedly and led to intracellular accumulation of partially assembled H chains. A small amount of fully assembled Ab (1–3% of WT) was secreted and bound Ag similar to WT Ab, indicating the formation of functional combining sites. A computer model of the PCG1-1 combining site indicates that mutation at Ile$^{51}$ indirectly impairs $H$ and $L$ chain pairing.

Materials and Methods

Plasmid constructs and site-directed mutagenesis

The subcloning of the PCG1-1 $V_H$ gene into pTZ18U has been described previously (8). Single mutations Ile$^{51}$→Lys and Ile$^{51}$→Arg were introduced into the PCG1-1 $V_H$ gene using the MutA-Gen Phagemid in vitro mutagenesis kit (Bio-Rad, Richmond, CA). Mutations were introduced using the primers Ile$^{51}$→Arg 5’-CCATCCCACCTGCTATCCAG-3’ and Ile$^{51}$→Lys 5’-CCATCAAGTCATCCAG-3’. Mutant and
WT V_H genes were subcloned into the pSV2gpt expression vector containing a murine y2b C region gene (13), and the entire V_H coding region was sequenced to ensure that no other mutations had been introduced. The forward primer (5'-TTAACGGCTATGGTGTAC-3') and the reverse primers (5'-TTGGCTCTGGGAGTTGTC-3' and 5'-CAGAATCCCCCACCAGTGC-3') were used.

Cell culture and stable transfection

The PCG1-1 heavy chain loss variant (PCG1-1 H- expressing the \(\text{V}_{\kappa}1\) gene) has been described previously (8). J558L is a V1-1-producing H chain loss variant of the IgA-secreting mouse myeloma J558 (14). Cell culture was done as described (8). Transfections were performed using the Lipofectin reagent (Life Technologies, Gaithersburg, MD), and mycoplasmfree, acid-resistant clones were selected as described (13). Stable transfectants with intracellular H and L chains similar to WT, as determined by ELISA, were stored for further analysis.

Ig secretion assay

Stable transfectants were plated in triplicate at \(1 \times 10^5\) cells in 1 ml of tissue culture medium in a 12-well plate. Culture supernatants and cell lysates were collected after 4 h as described (12).

Metabolic labeling, immunoprecipitation, and SDS-PAGE

Transfected cells were labeled with 150 \(\mu\)Ci \([^{35}\text{S}]\)Express Protein labeling mix (NEN Life Sciences, Boston, MA) for 20 min for assembly experiments or for 4 h as previously described for chaperone-binding experiments (15). For chaperone-binding experiments, 100 \(\mu\)g/ml of the thiocleavable, chemical cross-linker dithiobis(succinimidyl propionate) (DSP; Pierce, Rockford, IL) was added to the lysis buffer immediately before use (16). Immunoprecipitates were washed with buffers as described previously (15). Ig was immunoprecipitated from clarified cell lysates or supernatants by incubation with protein A-Sepharose CL-4B (Pharmacia, Piscataway, NJ). All labeling experiments were quantified using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA) and IP lab gel software (version 1.5; Analytics, Vienna, VA).

ELISA

The concentration of Ig proteins in supernatants and cell lysates was determined by sandwich ELISA as described previously (12). Briefly, to determine H or L + L in the supernatant or lysate, ELISA plates were coated with rabbit anti-mouse IgG2b (Zymed Laboratories, San Francisco, CA), and the amount of Ab in the lysate or supernatant was then determined by a secondary alkaline phosphatase-conjugated goat anti-mouse \(\kappa\) or rabbit anti-IgG2b Ab (Southern Biotechnology Associates, Birmingham, AL). All antisera were used at a 1/1000 dilution. Standard curves were generated using affinity-purified Ab from WT transfectant PCG1-1 (\(\kappa\)y2b, \(\kappa\)). Ag binding was determined by direct binding ELISA on plates coated with PC-histone (1 \(\mu\)g/ml) as described (13). Hapten NPPC was obtained from Sigma-Aldrich (St. Louis, MO).

Computer modeling of WT PCG1-1 combining site

ABGEN, a fully automated web-based molecular modeling program (17), was used to construct a model of the PCG1-1 fragment variable (Fv) region.

Results

Ile\(^{51}\) is highly conserved in mouse and human V_H

Previously, we generated 16 multisite secretion-defective mutants from 2 parent Abs; these mutants secrete \(<10\%\) WT Ig levels but synthesize intracellular H and L chains at levels similar to WT transfecteds (8, 12). Mutants P28 and P35 were identified for further analysis as both contain a nonconservative substitution at position 51, replacing isoleucine with the positively charged residue arginine or lysine (Fig. 1). None of the other mutations in P28 and P35 is sufficient to cause secretion defects because replacement of Asp\(^{54}\) with alanine or glutamic acid occurs singly in secretion competent Abs P20 and P9 (Fig. 1A). In addition, replacement of Met\(^{50}\) with valine occurs in a secretion competent anti-PC hybridoma, W140 (data not shown). Therefore, we focused our analysis on the position 51 substitutions. Strikingly, a compilation of all mouse V_H sequences present in the Kabat database indicated that V_H CDR2 Ile\(^{51}\) was highly conserved, occurring in 91% of all tabulated murine sequences (Fig. 1B). In addition, Ile\(^{51}\) is highly conserved in 81% (1348 of 1659) of human V_H sequences. An explanation for the high degree of conservation of position 51 has not been reported. No V_H sequences with arginine at position 51 were present in the Kabat database of murine V_H sequences as of March 10, 1998 (27).

Mutation of Ile\(^{51}\) to arginine or lysine inhibits secretion and increases chaperone association

We tested whether a single substitution at position 51 was sufficient to impair secretion by introducing either an Ile\(^{51}\)→Arg or Ile\(^{51}\)→Lys into the V_H CDR2 of PCG1-1. At least 20 clones of each mutant from 3 separate transfections were screened by ELISA. Stable transfectants secreted 1–3% of WT amounts of Ig (Table I). The presence of the intended mutation and lack of any other mutations in the V region were confirmed by directly sequencing V_H RT-PCR products (data not shown). The secretion defect was not due to impaired H chain synthesis in that intracellular levels of the mutant H chains were 170% of WT as determined by H chain-specific ELISA (Table I). Similarly, the secretion defect was not due to decreased L chain synthesis as the amounts of intracellular and secreted L chain were similar between the WT and the mutants (Table I). To confirm the ELISA results,
stable transfectants were labeled for 4 h with $^{[35]}$S-Met/Cys. Ig were immunoprecipitated from equal amounts of supernatant using protein A-Sepharose, which binds the H chain. Labeled proteins corresponding to H chains and L chains were present in the supernatants of both WT and the secretion-competent mutant P20 (Fig. 2A). The H chain doublet is characteristic of asymmetrical O-glycosylation of murine IgG2b H chains (18). Mutants, P35, P28, and site-directed mutants Ile$^{51}$Arg and Ile$^{51}$Lys secreted <5% of WT amounts of Ig as determined by densitometry confirming the ELISA results. The presence of abundant intracellular H chain of the correct size indicates that the secretion defect is not due to lack of H chain production (Fig. 2B). Less L chain coimmunoprecipitated with P35, P28, and Ile$^{51}$Arg and Ile$^{51}$Lys H chains as compared with WT suggesting a defect in H-L pairing. The PCG1-1 H chain loss variant cell line, H$^-$, served as a control for nonspecific immunoprecipitation and demonstrates that the PCG1-1 L chain, in the absence of H chain, is not immunoprecipitated by protein A-Sepharose. In summary, both the ELISA and metabolic labeling experiments demonstrate that single substitutions of Ile$^{51}$ to arginine or lysine were sufficient to impair Ig secretion in stably transfected cells.

Previously, we demonstrated that CDR2 mutations in the T15 H chain led to increased association of ER-localized chaperones BiP and GRP94 (15). Thus, we investigated whether a similar increase in chaperone association occurred with the PCG1-1 secretion mutants. In the presence of the chemical cross-linker dithiobis(succinimidyl-propionate), secretion-impaired H chains exhibited a 7- to 13-fold increase in BiP association and a 10- to 16-fold increase in GRP94 association as compared with WT or the secretion-competent mutant P20 (Fig. 2B). Furthermore, intracellular H chains were sensitive to endoglycosidase H digestion, indicating that N-linked oligosaccharides on the majority of H chains had not undergone the normal enzymatic alterations in post-ER vesicles (data not shown). Taken together, these data suggest that the H chains of the multisite and single-site PCG1-1 low secretion mutants are retained within the ER as reported for other nonsecreted H and L chains (15, 19–22).

**Impaired L chain assembly of mutant H chains**

To define the kinetics of secretion and H-L association, WT and Ile$^{51}$Arg cells were incubated for 20 min with medium containing $^{[35]}$S-Met/Cys followed by a chase period of 0, 2, or 10 h (Fig. 3). Immunoprecipitates were subjected to reducing SDS-PAGE to determine the Ig intracellular half-life and secretion kinetics (Fig. 3A), or subjected to nonreducing SDS-PAGE to follow H-L assembly (Fig. 3C). It is to be understood that H chains are secreted only as H-L complexes, but for simplicity we use the term H chain secretion. The WT H chains were secreted rapidly in comparison with H chains produced by the Ile$^{51}$Arg transfectant (Fig. 3A). The efficiency of H chain secretion was calculated by normalizing the amount of H chain to the amount of H chain present at time 0 (Fig. 3B). These analyses demonstrate that the majority of the labeled WT H chain was present in the supernatant by 2 h postchase. In contrast, only 1% of the labeled Ile$^{51}$Arg H chain was secreted into the supernatant by 2 h, and this percentage increased to only 5% by 10 h postchase. The WT H chain exhibited a shorter intracellular half-life ($t_{1/2}$ = 6 h) than the Ile$^{51}$Arg H chain ($t_{1/2}$ = 11 h). In summary, these data confirmed that H chains containing an arginine substitution for Ile$^{51}$ were not efficiently secreted but displayed a prolonged intracellular persistence, suggesting that they are only slowly targeted for intracellular disposal.

Interestingly, immunoprecipitation of intracellular mutant H chain from 0-, 2-, and 10-h chase time points coprecipitated a significant amount of L chain. We examined the stoichiometry of H chain to L chain to determine whether H-L chain association was altered in the Ile$^{51}$Arg mutant. The H:L ratio of secreted WT Ig was 1:1.1, and the H:L ratio in the small amount of secreted Ile$^{51}$Arg Ig was 0.9:1. Both ratios are within experimental error of the theoretical 1:1 ratio. However, the intracellular ratio of H to L in the Ile$^{51}$Arg mutant was higher than in WT. At the 2-h chase time point the ratio of H:L in the WT was 0.6:1, whereas in the mutant the ratio was 4.3:1. Thus, more L chain associates with WT H chain in WT cells than with H chain in the Ile$^{51}$Arg mutant cells, although the total amount of L chain present in the mutant cells is equal to that in WT cells (Table I).

### Table I. Single amino acid mutations at position 51 in the V$\kappa$ impair Ig assembly and secretion in PCG1-1 cells expressing V$\kappa$I

<table>
<thead>
<tr>
<th>Transfected H Chain</th>
<th>Supernatant</th>
<th>Intracellular</th>
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<tbody>
<tr>
<td></td>
<td>H + L (%)</td>
<td>H + L (%)</td>
</tr>
<tr>
<td>PCG1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>WT</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Ile$^{51}$Arg</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>PCG1-1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Ile$^{51}$Lys</td>
<td>100</td>
<td>100</td>
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</tbody>
</table>

* WT or mutant H chains were transfected into PCG1-1 H$^-$ cells. Triplicate cultures of stable transfectants were cultured for 4 h in fresh medium, and the concentrations of secreted and intracellular Ig were determined as described in Materials and Methods. Data are representative of at least three separate experiments.

* PCG1 WT supernatant H + L = 154 ± 6 ng/10$^6$ cells; intracellular H + L = 64 ± 4 ng/10$^6$ cells; intracellular H = 105 ± 12 ng/10$^6$ cells; intracellular L = 1171 ± 145 ng/10$^6$ cells. SE were <15% of mean values.
To further investigate the kinetics of H-L assembly, samples were separated using nonreducing SDS-PAGE (Fig. 3C). In WT cells, H and L chain assembly occurred rapidly and was essentially complete in the 2-h lysate sample. In contrast, the majority of the Ile51→Arg H chain was blocked at the H2 assembly step. The amount of H2 in each sample was calculated using a PhosphorImager and normalized to the total H chain (Fig. 3D). At time 0, the WT H2 intermediate accounted for 20% of the total WT lysate H chain. This was reduced to 7% at 2 h and to <5% by 10 h. In contrast, the H2 intermediate in the Ile51→Arg mutant accounted for 55% of the total lysate H chain at 0 h, and this percentage remained stable at 2 h (59%) and 10 h (55%). During this time period, the total amount of H chain decreased by 54%. Furthermore, no discernable HL assembly intermediate was detected in the 0-h mutant lysate, whereas a strong band was present in the 0-h lysate of the WT (Fig. 3C). These data and the high ratio of H chain to L chain in the mutant suggest that impaired H-L assembly is responsible for the secretion defect. L chain that is coimmunoprecipitated with mutant H chain (Fig. 3A) is covalently associated because L chains were not dissociated under nonreducing SDS-PAGE conditions (Fig. 3C). Remarkably, the covalent attachment of L chain to small amounts of H chain occurs rapidly in that bands corresponding to H2L and H2L2 were present at the 0-h time point in the Ile51→Arg mutant. The failure to detect noncovalent L chains is not an artifact of insufficient denaturing conditions because BiP was displaced from the complex under the same conditions. Furthermore, it is unlikely that the covalent attachment is an artifact of the immunoprecipitation procedure because the lysis buffer contains 50 mM iodoacetamide to alkylate free sulfhydryl groups. A small amount of fully assembled H2L2 was present in the supernatant of the Ile51→Arg mutant, indicating that the block in secretion is not complete and is consistent with the small amount of Ig detected by the ELISA and steady state labeling experiments. In summary, these data indicate that the majority of H chain containing the Ile51→Arg mutation rapidly forms a covalent H2 assembly intermediate but that further assembly is blocked or aberrant.

**PCG1-1 WT and single mutants bind to PC-histone**

The presence of a small amount of fully assembled Ig in the supernatant led us to investigate whether this secreted Ig contained functional hapten-binding sites. We were able to purify a small amount of mutant Ig from culture supernatants using protein A chromatography followed by binding to PC-histone. Both the Ile51→Arg and Ile51→Lys single mutants showed binding curves similar to those of PCG1-1 WT transfectant Ab (Fig. 4). Binding specificity was demonstrated by inhibition with 0.01 M NPPC, a hapten that mimics the diazophenyl linkage between PC and carrier protein (23). These data indicate that a low level of functional Ab was released from the mutants and that the conformation of this Ab is indistinguishable from that of WT, at least with respect to Ag binding.

**Partial rescue of secretion by expression with the J558 L chain**

The capacity of mutant PCG1-1 H chains to form a small amount of functional Ab suggests that altering the fit between V\(\text{H}\) and V\(\text{L}\) may alter secretion. To test this possibility, we examined the secretion capacity of the PCG1-1 WT and mutant H chains with other L chains. Mutant H chains coexpressed with either the T15L
or the Vκ10 L chain were not secretion competent (data not shown). However, coexpression of mutant PCG1-1 H chains with the J558 L chain partially rescued secretion (Table II). Secretion of the Ile51→Arg mutant was 28% of WT amounts, whereas the Ile51→Lys mutant was 53% of WT amounts. Furthermore, abundant intracellular H-L complexes were present in both the WT and mutant cells (Table II). L chain amounts were similar between transfectants (Table II and data not shown). These results were confirmed by 4-h metabolic labeling experiments. Fully assembled Ig (H1L2) was released into the supernatant (Fig. 5A) containing H and L chains (Fig. 5B). Immunoprecipitation of intracellular H chain coprecipitated similar amounts of L chain (Fig. 5C). In contrast to WT H chain, mutant H chain coprecipitated with elevated amounts of BiP (4- to 6-fold) and GRP94 (6- to 8-fold). These data indicate that the PCG1-1 WT and mutant H chains fully assemble with J558 L chains, although the mutant H chains do so slightly less efficiently.

Discussion

Normal peripheral B cell survival is predicated on maintenance of Ig expression (9). During affinity maturation, maintenance of functional receptors is complicated by the extensive somatic hypermutation of the Ig VH and VL domains. Here, we report that a single V region mutation at a highly conserved residue in H chain CDR2 impairs secretion. Mutant H chains are sensitive to endoglycosidase H and coimmunoprecipitate with BiP and GRP94, consistent with retention in the ER (19–22, 24). Furthermore, intracellular mutant H chains fail to associate covalently with L chain efficiently. These findings highlight the potential of somatic mutation in VH to alter the capacity of an H chain to assemble with its original VL counterpart (8, 25).

Sequence conservation of Ile51

Sequence analysis of 5000 Ig sequences has identified 47 positions conserved within the VH and VL of Igs (26). Most conserved residues are located in the FRW of the Ig domain and form contacts with residues in the same or adjacent β sheets (26). Highly conserved residues are also present in CDRs; position 51 is an isoleucine in 91% of all murine and 81% of all human VH sequences compiled from the Kabat database (27). In addition, it has been noted that Ile occurs at position 51 in V\textsubscript{H} from the South American alligator Caiman, suggesting a conservation during vertebrate evolution (28). Although a systematic mutational analysis of conserved residues has not been performed, single mutations in other invariant residues Trp56 or Cys92 in V\textsubscript{H} do not alter Ig assembly or secretion (29, 30). Conservation of Ile51 in combination with our data indicating a functional role in assembly suggest that this position may be important to forming secretion competent Ig. It is also possible that the conservation of Ile51 in the Kabat database reflects a protection from the hypermutation mechanism. Ile51 in the V\textsubscript{H} CDR2 of PCG1-1 is encoded by the nucleotide triplet ATA and only single nucleotide substitutions are required to change Ile51 (ATA) to Arg (AGA) or Lys (AAA). Both AT and TA have a statistically higher dinucleotide mutation frequency than indicated by random chance (31). In addition, the T in the ATA triplet is more likely to be mutated than would be expected by chance as determined by analysis of murine intronic DNA and nonproductively rearranged human VH genes (31). This would argue against the proposition that there is an intrinsic bias against Ile51 mutations in the M141 germline gene of BALB/c mice, although it is formally possible that other factors may serve to shield this region from somatic mutation. Thus, the high degree of amino acid conservation and our functional data more likely suggest that Ile51 is conserved for its role in assembly and secretion.

Mechanism of the secretion defect

Ig that differ by a single amino acid are the simplest system for dissecting the structural requirements necessary for Ig assembly and secretion. To investigate the molecular environment surrounding Ile51, we constructed a computer generated model of the PCG1-1 WT combining site using the program ABGEN. This program constructs a molecular model of the Fv using an automated homology-based scaffolding technique (17). In the model of the PCG1-1 WT Fv (Fig. 6A), Ile51 does not contact L chain and is buried between V\textsubscript{H} CDR2 and FRW3. This is in agreement with the buried position of Ile51 in other Ig crystal structures (32, 33). Thus, if the Ile51 to arginine or lysine substitutions alter the interface of the H chain that contacts L chain, the mutations must do so.

Table II. Secretion of PCG1-1 VH mutant H chains Ile51→Arg and Lys by the J558L cell line expressing X1

<table>
<thead>
<tr>
<th>Transfected H Chain</th>
<th>Supernatant H (%)</th>
<th>Intracellular H (%)</th>
<th>Intracellular L (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCG1-1 WT</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>PCG1-1 Ile51→Arg</td>
<td>28</td>
<td>56</td>
<td>146</td>
</tr>
<tr>
<td>PCG1-1 Ile51→Lys</td>
<td>53</td>
<td>130</td>
<td>159</td>
</tr>
</tbody>
</table>

* WT or mutant H chains were transfected into J558 L cells. Triplicate cultures of stable transfectants were cultured for 4 h, and the concentrations of secreted and intracellular Ig were determined as described in Materials and Methods. Data are representative of one of at least three separate experiments. SE were <15% of mean values.

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indirectly. We have recently reported the structure of an anti-PC single-chain variable fragment, M3C65 at 2.35 Å, complexed with the hapten NPPC (34). M3C65 uses the same germline V<sub>H</sub> gene as PCG1-1, and both Abs bind NPPC. In the computer-generated PCG1-1 model and in the M3C65 crystal structure, Ile<sup>51</sup> is surrounded by five residues, Gly<sup>55</sup>, Thr<sup>57</sup>, Ile<sup>69</sup>, Ser<sup>70</sup>, and Lys<sup>71</sup> (Fig. 6B). Modeling the replacement of Ile<sup>51</sup> with arginine in the PCG1-1 and M3C65 combining sites suggests that the side chain of arginine is accommodated within the folded Fv and that the side chain may extend into the solvent in between H chain CDR2 and FRW3 (data not shown). This accommodation would be consistent with the complete assembly of a small amount of mutant Ab that binds Ag indistinguishably from WT. One possibility is that the mutations at Ile<sup>51</sup> have unfavorable interactions with the surrounding residues to create an energetic barrier to folding of CDR2. Examination of the mutant side chain placement suggests potentially unfavorable electrostatic interactions with Lys<sup>71</sup>. Residue 71 of the heavy chain has been proposed to fix the relative dispositions of CDR1 and CDR2 depending on whether it is a bulky side chain (lysine or arginine) or a smaller side chain (alanine or valine) (35). Position 71 is a lysine or arginine in 38% (1125 of 2961 sequences) and an alanine or a valine in 56% (1661 of 2961) of sequences obtained from the Kabat database (27). Thus, we constructed a double mutant containing both Ile<sup>51</sup>→Arg and Lys<sup>71</sup>→Ala. This double mutant remained secretion impaired (data not shown), suggesting that an unfavorable interaction between the side chains of Arg<sup>51</sup> and Lys<sup>71</sup> is not the molecular explanation for the secretion defect. Furthermore, an unfavorable charge interaction would not explain the paucity of other substitutions at position 51 such as glycine, alanine, cysteine, tyrosine, phenylalanine, histidine, aspartic acid, glutamic acid, glutamine, and proline (Fig. 1B). Future experiments are required to determine the nature of interactions between Ile<sup>51</sup> and surrounding residues, and the effects of substitutions at position 51 on the proper folding of the V<sub>H</sub> domain.

Besides influencing folding, it is possible that substitution of Ile<sup>51</sup> may alter H chain interactions with molecular chaperones such as BiP. In our experiments, BiP coimmunoprecipitated with mutant H chain, whereas very little BiP was associated with WT H chain. Continuous binding of BiP to H chains in the absence of L chain results in H chain intracellular retention (19, 36) and that nonreleasing BiP ATPase mutants prevent secretion of L chains that are otherwise secretion competent (37). Furthermore, dissociation of BiP from the H chain is temporally linked to L chain arrival (38). Experiments using a truncated, two-domain heavy chain (V<sub>H</sub>-CH1) indicate that the disulfide bond within the CH1 domain is not formed until BiP dissociates (39). This suggests that the CH1 domain is not fully folded in the ER until it assembles with an L chain. One possibility is that the PCG1-1 L chain is unable to release BiP from the CH1 domain of the mutant H chains (39). It is also possible that mutation of Ile<sup>51</sup> to arginine or lysine may directly enhance BiP binding or prevent its release from the V domain, thus impeding H chain maturation. Although BiP-binding sites on V<sub>H</sub> have not been precisely localized, a computer program (40) has predicted peptides from several V<sub>H</sub> that stimulate BiP ATPase activity in vitro (41). Interestingly, Knarr et al. (41) identified a potential BiP-binding site encompassing Ile<sup>51</sup> in the V<sub>H</sub> of the 3D6 Ab. A peptide corresponding to V<sub>H</sub> residues 47–52a of the 3D6 Ab had a score of +7 (scores of +6 to +10 have a 3:1 chance of binding BiP) and stimulated BiP ATPase activity in vitro. We have used the same algorithm (40) to evaluate whether the single mutations affected the predicted BiP score in the same set of residues (47–53) from the V<sub>H</sub> of PCG1-1. The predicted BiP-binding score was +7 for WT but was reduced to −3 by the Ile<sup>51</sup>→Lys substitution or to +6 by the Ile<sup>51</sup>→Arg mutation. Thus, these results provide no evidence for the creation of a better BiP site, but rather predict reduced or no change in BiP binding to this putative site. Although the predicted BiP score does not suggest increased BiP binding, this result should be interpreted with caution given that Hellman et al. (42) demonstrated that there was no correlation between the presence of predicted BiP binding sequences and actual BiP binding to murine Ab variable and constant domains. Further analysis is required to determine whether Ile<sup>51</sup> is part of a BiP-binding site used in vivo and whether the single mutations affect this interaction.

Mutant ϒ- and μ-chains, with reduced BiP binding due to a lack of the CH1 domain, partially assemble with L chain to form insoluble aggregates in the ER resembling Russell bodies (43, 44). However, our data do not support insoluble aggregation as the mechanism of secretion impairment, because soluble mutant PCG1-1 H chains were isolated at the 10-h chase time point, and we have failed to detect structures resembling Russell bodies.

**FIGURE 6.** A, Computer-generated model showing a side view of the PCG1-1 WT Fv. Ile<sup>51</sup> is buried between CDR2 and FRW3 and does directly contact L chain. H chain colors: yellow = CDR2; green = CDR3; red = CDR1; turquoise = FRW3. L chain is on the left. B, Close-up side view of Ile<sup>51</sup> and the surrounding residues Thr<sup>57</sup>, Gly<sup>55</sup>, and Lys<sup>71</sup>. Atoms are displayed as balls and sticks. Colors: red, oxygen; blue, nitrogen. The C<α> backbone of Ile<sup>69</sup> and Ser<sup>70</sup> (side chains not shown) also encloses Ile<sup>51</sup>. Van der Waal’s radii surrounding each atom are represented as dots. The model was generated using ABGEN, and graphics were rendered using RasMol.
within cells expressing mutant H chain (data not shown). The failure to form Russell bodies may be due to the isotype or specificity of the mutants (45). It is also possible that the increased association of BiP and GRP94 with the mutant H chains may prevent aggregation and thus formation of Russell bodies. Our results do not exclude the possibility that a small amount of H chain forms insoluble aggregates or that aggregation might be detected at a later time point.

Although sequence analysis indicates that Ile51 is highly conserved and the presence of arginine or lysine mutations at this position rare, we have found that an arginine substitution for isoleucine does not necessarily disrupt H-L pairing in all Lgs. We have introduced this substitution into the M3C65 H chain where it is paired with the J558 A1 L chain. In this setting, the mutation had no effect on Ig secretion. However, the rate of intracellular assembly was modestly reduced (data not shown). Expression of the PCG1-1 Ile51 → Arg H chain mutation in J558L A1 L cells resulted in a partial rescue of the secretion defect, thus indicating that at least some L chains can compensate for a substitution at this residue. These results suggest that different combining sites may be differentially affected by changes at position 51. This is in accord with our previous findings that identical CDR2 mutations can have different effects on Ag binding and secretion depending on whether they are placed within the T15 or D16 Abs which use the same VH sequences (12, 46). Taken together, these results highlight the possibility that diversity in Ig sequence not only increases the repertoire for Ag but also reduces the potential for a particular mutation to harm Ig expression.

In summary, we demonstrate that even a single VH mutation may have dramatic consequences on Ig assembly and secretion. The PCG1-1 single-mutant H chains showed impaired association with L chains, although a small amount of covalently associated, functional H1L1 was secreted. These data and our data from multisite mutants (8) clearly demonstrate that mutations in H chain CDR2 and FRW can disrupt the production of functional Ig. Light chain somatic mutations in conserved residues can disrupt L chain secretion and also lead to L chain pathologies (47). Therefore, it is likely that somatic hypermutation in conserved residues in H and L chains may frequently generate B cells with impaired Ig formation with potentially pathological consequences. Single mutants provide a useful system for further elucidation of the molecular mechanisms governing B cell homeostasis and ER-mediated Ig quality control.

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References