School of Medicine

Oregon Health Sciences University

CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. thesis of

Tammy M. Martin

has been approved

[redacted]

Professor in charge of thesis

[redacted]

Associate Dean for Graduate Studies

Dedicated to the memory of
Viola (Tootie) Lewis Martin,
one of my first mentors.

TABLE OF CONTENTS

ACKNOWLEDGMENTS

I am indebted to Qing Chen, Susan Stevens, Christine Kowalczyk and Greg Wiens for the large contributions that they made to this project. I would like to thank the talented people that I have worked with in the Rittenberg lab whose guidance and support have been extremely helpful, especially McKay Brown and Greg Wiens. I am also most grateful to the Department of Molecular Microbiology and Immunology, and for expert advice and guidance provided by Dr. Mary Stenzel-Poore, Dr. Miles Wilkinson, Dr. Scott Landfear, Dr. Caroline Enns and Dr. James Rosenbaum.

In addition, I extend my appreciation to the outstanding individuals who have helped to make my graduate experience enjoyable and successful. Most notably my husband, Jeff Bringenberg, and my friends Anne Wertheimer, Mark Carter, Suzanne Lindsey, Janet Duncan and Elizabeth Whitcomb (to name only a few).

Lastly, I would like to acknowledge Dr. Marvin Rittenberg for the exceptional training that I have received not only as a scientist, but also as a problem-solver and writer. Without his insight, wit, and guidance I would have not attained this level of expertise during my graduate studies.

ABSTRACT

To understand antibody function, an *in vitro* random mutagenesis system has been employed to study the negative effects of somatic hypermutation. This approach has revealed that heavy (H) chain variable (V) region structure affects antibody secretion. Four mutant antibodies, expressed in stable transfectants were shown to be defective in secretion. They differ from the wild type (WT) T15 antibody by 2 to 4 amino acid replacements in the H chain complementarity determining region 2 (HCDR2). To explore the structural basis for this defect, various molecular characteristics of these mutants have been assessed. Normal intracellular levels of H chain are produced, and some assembly with L chain occurs based on anti-idiotype recognition and, for one mutant, antigen binding. To analyze the contributions of individual amino acid changes to the secretion defect, a panel of the representative single mutations was assayed for secretion. None of these single amino acid replacements disrupted secretion. One low secretor mutant has only two changes, and another double mutant (from a low secretor with three mutations) was shown to be secretion-impaired. Therefore, it appeared that this H chain tolerates a single amino acid replacement very well, and that the defective phenotype results from more that one change in HCDR2. When these HCDR2 mutations were placed in the context of a related antibody utilizing the same VH gene, but different diversity (D) and joining (J) gene segments and a different L chain, secretion was not impaired. The differences between these two antibodies (T15 and D16) were explored, and it was shown that secretion was defective upon expression of the mutant T15 H chains with the D16 L chain, or another

unrelated L chain. Thus, the T15 L chain was not uniquely responsible for the secretion defect. However, the HCDR3 may play a role, since a four-residue deletion in this region of T15 (to mimic the length of D16 HCDR3) was able to restore secretion competence of the T15 mutant H chains. Pulse-chase analyses revealed that the mutant T15 H chains accumulated in the endoplasmic reticulum (ER) for long chase times (up to 26 h), in a manner similar to T15 WT expressed without L chain. In addition, these mutant H chains were associated with ER-resident molecular chaperones, BiP and Grp94, at levels 7 to 20-fold greater than the WT H chain (expressed with L chain, and efficiently secreted). The assembly of T15 WT and mutant H chains progressed rapidly to an H_2 intermediate. However, only a portion of the mutant H chains assembled with L chain, and the H_2 intermediate appeared to be relatively long-lived for the mutants. Therefore, the mutant H chains interact inefficiently with L chain, suggesting that the HCDR2 mutations disrupt, either directly or indirectly, H-L association. This study demonstrates that mutations in an H chain hypervariable region can disrupt immunoglobulin secretion, in contrast to the paradigm that CDR loops do not make significant functional contributions other than antigen binding.

Abbreviations

α, γ, μ	heavy chains of IgA, IgG, and IgM, respectively
C	constant domain
CDR	complementarity determining region
D	diversity
ER	endoplasmic reticulum
Fab	antibody VH-VL + CH1-CL fragment
FRW	framework residues
Fv	antibody VH-VL fragment
H	heavy chain
H_2L_2, H_2L, HL, H_2 & L_2	covalent polymers of heavy and light chains
Hsp	heat shock protein
Ig	immunoglobulin
J	joining
L	light chain
MHC	major histocompatibility complex
PC	phosphocholine
PDI	protein disulfide isomerase
sFv	single chain Fv
V	variable region or gene
WT	wild type

Statement of problem

The development of the memory immune response involves successive changes in antibody molecules through the process of somatic hypermutation (1). Generally, mutations that increase affinity for antigen are selected positively, while detrimental changes are presumed to be selected against, although evidence for this latter point has been difficult to obtain. The immune response to PC-KLH (phosphocholine-keyhole limpet hemocyanin) has been utilized as an evolutionary model of antibody diversity and antigen-antibody interactions. A panel of antibodies generated by random saturation mutagenesis of the VH CDR2 region of a prototype anti-PC antibody (T15) has been characterized (2). The study allowed analysis of molecules with decreased affinity for antigen (i.e. antibodies that would likely be selected against and therefore absent *in vivo*). Interestingly, a few of the mutants that were generated exhibited a phenotype in which little or no antibody was secreted from the cells. The only difference in these "low secretors" from the T15 WT molecule resides in 2-4 amino acid replacements in HCDR2. The CDRs of antibodies are intricately involved in antigen binding and frequently exhibit mutations incurred during the somatic hypermutation process. For this reason, it was expected that deleterious mutations introduced into T15 HCDR2 would only affect antigen binding and the discovery that a secretion-deficient phenotype could result from HCDR2 perturbations was therefore surprising. The four low secretor mutants of T15 HCDR2 were novel, not only due to the primary functional role of CDRs in antigen interaction, but also because current models of immunoglobulin transport have emphasized a major role for

the L chain and not the H chain. This concept has been generated from observations that most L chains are secreted in the absence of H chain, but the secretion of H chains is normally possible only after assembly with L chain.

The overall goal of the studies described here was to investigate the structural basis for the failure of these proteins to be secreted. The secretion defective phenotype first had to be defined quantitatively so as to be able to compare subtle differences in secretion. Secondly, it was necessary to evaluate the intracellular production of non-secreted Ig proteins. The fate(s) of the four mutant H chains were to be examined by: (i) assessment of the time that nascent mutant proteins accumulate intracellularly, (ii) determining the subcellular site of accumulation, and (iii) determining to what extent they oligomerize with each other or with L chains. The possible contribution of the T15 L chain to the defect was examined by expressing other L chains with the mutant H chains in stable transfectants, and assessing secretion competence. Another avenue of analysis was to use site-directed mutagenesis to investigate the individual contributions of each amino acid replacement found in the four mutants which have multiple substitutions. This research addresses basic immunological concepts concerning the evolution of antibody diversity as well as the broader issue of protein trafficking.

General Features of Immunoglobulin Structure

The immunoglobulin domain is a general structural motif found in proteins, of which antibodies are the first-described and classical examples (3). The immunoglobulin fold is described as a "simple" Greek key β-barrel (4), and there are several variations of this theme grouped into subclasses of immunoglobulin domain-like structures (5). Other members of the immunoglobulin superfamily include cell surface receptors (CD2, CD4, CD8, and MHC class I and class II), matrix proteins (tenascin and fibronectin), regulatory proteins (PapD), and enzymes such as myosin light chain kinase (5). The Ig superfamily contains a continually expanding number of members, cell surface and secreted proteins, from outside, as well as within the immune system (6). When the vast number of related sequences is considered, the immunoglobulin-like domain is one of the most widespread protein modules found in nature (7).

Antibodies are found on the B cell surface as H_2L_2 tetramers, which is also the polymeric form of secreted IgG. Each H chain consists of one variable (V) domain at the amino-terminus and three or four constant (C) domains. L chains consist of one V and one C domain. Each of these antibody V and C domains exhibits the immunoglobulin fold common to antibodies, which consists of 7-9 hydrogen-bonded, anti-parallel β-strands that form 2 distinct, stacked β-pleated sheets linked by an intrachain disulfide bond (8, 9). There is a tight hydrophobic core that results from the packing of inward-facing hydrophobic amino acid residues that stabilize the domain structure (10). The homologous

interacting domains (i.e. VH and VL, or CH1 and CL) are very similar to one another in three-dimensional structure (8).

Two variable domains (VH and VL) associate non-covalently to form the antigen-combining site of an antibody. The resulting interface is composed of two tightly packed β-sheets which forms a barrel that lines the bottom and sides of the combining site (4, 11). There are large differences in the shape and size of antibody combining sites, and three classes have been described, these include cavity (haptens), groove (DNA, carbohydrate and peptide antigens), and planar (protein antigens) (12). These structural variations are predominantly attributed to sequence differences found between the hypervariable regions in the V domains (13). There are three hypervariable regions (or CDRs) in each V domain that form loop structures which link the strands of the β sheets (made up of highly conserved framework sequences that flank the CDRs) (14). The six CDR regions of an Ig dictate the specificity and affinity of antigen binding (13). Upon systematic examination of CDR sequences in the Ig database, it was noted that there were conserved residues within certain sets of CDRs, suggesting that this sequence conservation may be important structurally (15). A comparison of various Ig structures revealed that some CDRs were similar in main chain conformation despite differences in amino acid sequence (16, 17). Using the available Ig structures, Chothia and colleagues determined that for five of the six CDRs, a small set of canonical structures exists (18), and these predictions were tested by modeling canonical CDR structures prior to their experimental structural determination (19). Therefore, the CDRs are highly variable at positions important for antigen binding

differences, but are somewhat structurally constrained as directed by key, highly conserved residues.

T15 and the Anti-Phosphocholine Response

PC is a cell wall component of various microorganisms, and is considered to be an important and common environmental epitope (20). Myeloma proteins frequently are representative of antibodies which have been induced by environmental antigens. Many of the myeloma proteins that have been tested are precipitable with pneumococcal preparations, with an immunodominant reactivity to PC (21, 22). In the initial, or primary response to PC, the antibodies produced have been characterized as Group I anti-PC antibodies which recognize free PC, utilize a very small number of different V genes, and are highly restricted in idiotype expression (23-26). The myeloma protein, TEPC15 (T15), binds free PC and represents a prototypical Group I anti-PC antibody (23, 27, 28). The majority of anti-PC antibodies from BALB/c mice are T15 Id^+ (29, 30) and these T15 Id^+ antibodies are protective against *Streptococcus pneumoniae* challenge (31).

One of the early crystal structures of an antibody Fab (a monovalent antibody fragment with VH-VL + CH1-CL domains covalently linked) was that of McPC603, an anti-PC protein (21). It was revealed that the PC binding site was a wedge-shaped cleft lined by residues of the hypervariable regions, and that PC bound in the site asymmetrically with the choline moiety toward the interior (21, 32). These results were

confirmed in a refined structure of McPC603 to 2.7 Å, and here it was shown that 45% of the VH-VL interface contacts were between residues from hypervariable regions (33). In addition, other Fab structures have been shown to exhibit significant CDR-CDR interactions and to presumably contribute to VH-VL association (11). Thus, there was intriguing speculation that these hypervariable contacts may be able to dictate which H-L chain pairings were permissible (33). However, it was also possible that unfavorable VH-VL interactions could be overcome by the strong CH1-CL association.

The S107 myeloma cell line utilizes the same germ-line sequences for VH and VL as T15 (34, 35) and a model of this combining site was derived based primarily on the McPC603 structure (36). This was possible due to the high degree of homology between McPC603 and S107 VH sequences (they both use the VH1 gene product and the same DJ segments, but with different somatic mutations (37)), even though the VL segments are different. The current model of the T15 combining site (38) is a refinement of an earlier S107 model (36).

Protein Folding and Transport

Early studies of spontaneous protein refolding revealed that for some proteins, all of the necessary information required to attain the correct three-dimensional conformation was present in the amino acid sequence (39). Based on these self-assembly demonstrations, it had been concluded that protein folding *in vivo* was also a generally spontaneous

event. It is now know that the acquisition of native protein conformation in the cell is energy-dependent and assisted by various intracellular activities. Interestingly, this was predicted by Anfinsen who had studied the *in vitro* folding of ribonuclease and staphylococcal nuclease; he suggested that some other large protein might actually interact with the unfolded protein to influence the *in vivo* folding process, reviewed by R. J. Ellis (40).

There are numerous enzymatic activities that assist in proper protein folding. For instance, protein disulfide isomerase (PDI) may facilitate the retention of proteins until the appropriate disulfide bond pattern is achieved by catalyzing the redox reaction of cystine/cysteine residues and the "shuffling" of disulfide bonds (41-43). This protein acts on secretory and membrane proteins, as it is localized to the ER lumen (44). Peptidylprolyl cis-trans isomerases (PPIs), or immunophilins are two distinct families of proteins with members that reside in the ER and play a functional role in protein folding, where they catalyze rotation around the X-Pro peptide bond (where X is any amino acid residue) (42, 45, 46). Proline residues place unique restrictions on protein conformations as they are more constrained stereochemically than other amino acids and are commonly found at turns of the polypeptide backbone (4).

Another large group of proteins involved in assisting proper folding and assembly are the chaperones (47). The general model of how chaperones provide such assistance is that they interact specifically with non-native proteins to suppress the innate tendency of proteins to form and be trapped as aggregates (48, 49). Chaperones are fundamental cellular

tools that seem to be conserved evolutionarily and utilized by all organisms, including bacteria, yeast, plants, insects and mammals. Many of the chaperones were first described as proteins inducible by stress or heat shock, and are referred to as heat shock proteins (Hsps). However, most chaperones are constitutively expressed, abundant cellular proteins (43). There are three well-defined, conserved families of chaperones: chaperonin-60, stress-70, and stress-90 (43). In addition, there is a family of small Hsps, which includes alpha-crystallin, as well as other large proteins that do not fall into one of these Hsp classes, such as Hsp 104 (50). Other molecular chaperones have been described, most notably, calnexin (also known as p88 and IP90) which is an integral membrane protein associated with the ER translocation machinery (51), and has been shown to bind numerous polypeptides transiently, including MHC class I molecules and T cell receptor components (46, 52).

The chaperonin-60 family includes GroEL of bacteria, and mitochondrial Hsp60 proteins from yeast and mammals. These proteins provide subunits for very large oligomeric chaperone structures. The GroEL/GroES heterooligomer exerts its chaperone activity on partially folded proteins by binding them in a large interior cavity of the complex (53-55). The cavity provides a protected environment that prevents the interaction of the partially folded intermediate with other partially folded proteins, an interaction that could lead to aggregation (40). To date, there is no evidence that members of the chaperonin-60 family are found in the ER of eukaryotic cells (43, 56). Therefore, these chaperones

will not be discussed further since the focus of the research described here is concerned with proteins that are folded and assembled in the ER.

The stress-70 chaperone family is large compared to the chaperonins, with members found in bacteria (such as DnaK from *E. coli*), as well as proteins found in the cytosol, ER and mitochondria of eukaryotic cells. The most widely studied stress-70 protein is BiP which was first identified as Ig heavy chain binding protein (57-59), and then cloned and identified as the previously described protein Grp78 (60-62). The notion that BiP binds to and sequesters misfolded or improperly assembled proteins in the ER (63-65) is now generally accepted. BiP has a peptide-dependent ATPase activity (66), and the *in vitro* addition of ATP will dissociate BiP from misfolded proteins (64). In addition to Ig H chain, BiP interacts with many other proteins including influenza haemagglutinin (65) and von Willebrand factor (67). The analyses of haemagglutinin and BiP interaction have revealed that the accumulation of unassembled protein can cause upregulation of BiP mRNA (68), and that BiP recognizes the subunit interfaces of haemagglutinin stem domains which are important for homotrimer association (65, 69, 70). Interface residues are generally hydrophobic and become buried within the protein upon subunit assembly. Indeed, extensive analyses of peptides that are bound by BiP reveals a propensity for short, hydrophobic extended peptides (71-73). Although no crystal structure of an Hsp70 molecule is available, modeling studies have predicted similarities in BiP and other Hsp70s with the peptide-binding grooves present in MHC molecules (74, 75). These findings are consistent with the observations that stress-70 proteins bind to proteins during

translation and translocation (i.e. when short stretches of extended nascent polypeptide chains may be accessible) prior to folding to prevent misfolding and aggregation (76-79).

Members of the stress-90 chaperone family exhibit sequence conservation between prokaryotes and eukaryotes. In vertebrate cells, there are distinct stress-90 proteins in the cytosol and the ER. Cytosolic Hsp90 is thought to function as a molecular chaperone, assisting in the folding and multimeric association of proteins (80). The ER-resident member of this family, Grp94 (81), has also been identified as endoplasmin (82), Hsp108 (83), ERp99 (84) and gp96 (85). The association of Grp94 with unassembled Ig, thyroglobulin, MHC class II molecules, and a mutant viral protein that is retained in the ER, all indicate that Grp94 may also possess a molecular chaperone activity (86-90). Consistent with a role in chaperone activity, Grp94 expression is increased under stress conditions which cause an accumulation of malfolded proteins in the ER (68). To complicate matters, there is evidence for both an ER transmembrane and a luminal form of Grp94 (82, 84, 91, 92). The structural moiety recognized by Grp94 is unknown, however, binding to peptides has been reported (85). It is thought that Grp94 has a more limited substrate repertoire than BiP, and that Grp94 interacts with more mature folding intermediates of proteins (46, 88).

Secretory and membrane proteins pass from the ER, through the Golgi and to the plasma membrane via a vesicular transport process consisting of repeated cycles of vesicle budding and fusion. There are two general models concerning the control of protein transport through the cell.

One model, the bulk flow model, suggests that proteins inherently traffic through the vesicular transport pathway unless they are retained along the way be specific retention signals (93). This model is supported by the KDEL retention signal found on ER resident proteins (64), a population of transport vesicles that bud from the ER and the Golgi which are thought to serve as unselective bulk carriers (94, 95), and kinetic analyses that suggest bulk flow and protein secretion have similar rates (96). In contrast the selectivity model proposes that proteins destined to be secreted or reside on the cell surface are tagged with a positive transport signal. In a study where murine leukemia viral glycoproteins encoded by the *env* gene were analyzed, it was found that they were transported to the cell surface with random kinetics and were sequestered in the ER (97). This provides evidence for a selectivity model because the arrival of these proteins did not occur in a linear fashion or in the order in which they were synthesized (97).

Immunoglobulin Biosynthesis

The availability of radioactive amino acid compounds coupled with the ability to culture and clone plasmacytoma and myeloma tumor cell lines prompted a burst of research activity on the kinetics of Ig production and secretion during the 1970s. In primary cultures of normal lymphoid cells, antibody is secreted into the culture medium 40-80 min after translation (98). In the case of myeloma cells, the secretion kinetics are more variable with antibody appearing in the supernatant 20-150 min after polypeptide chain synthesis (98, 99). As it was known

that the H and L chains were independently synthesized from different genes, an early question in Ig biosynthesis was whether H and L chains were produced in equimolar amounts (99). It was shown that an intracellular pool of free L chains is maintained and constantly turned over in antibody-producing cells (100). Initially, it was reported that approximately equimolar amounts of intracellular H and L chains were produced from plasma cell tumor lines (101-103), suggesting that this was a general driving force in the assembly of H_2L_2 molecules (104). A later survey of lymph node cells from immunized mice, as well as several myeloma tumors and lines thus derived, demonstrated that an excess of L chains was synthesized in most cells tested, whereas some produced roughly equimolar ratios (105). From these studies it was suggested that a pool of excess L chain may be advantageous to drive the association of H chains with L chains, but was not a requirement (106). Consistent with this notion was the demonstration that in the MPC-11 myeloma, completed (not nascent) L chains were covalently assembled onto nascent H chains (107). It was also of interest that there were no known examples of production of excess H chain in any of the cells examined (106). Along these lines, myelomas that produce only L chains were readily available for study, however, L chain-loss variants which produced normal H chains were very rare (57).

Not only were the kinetics of Ig secretion analyzed, but the identification of assembly intermediates and role of glycosylation were two additional areas of great interest. Ig assembly proceeded in a discrete step-wise fashion, with a major and a minor pathway that was specific to the particular isotype of the antibody (104, 106). These pathways were

found to correlate with the formation of disulfide bonds, as well as the order of non-covalent interactions (104, 108). The major assembly intermediates for murine IgG1, murine IgG2a, and human IgG are H_2L and H_2, but no HL molecules are used (104, 108-111). In contrast, murine IgG2b exhibits all possible intermediates (H_2L, H_2, and HL), with HL being a predominant species (107, 108), whereas murine and human IgM molecules assemble solely through HL subunits (112, 113). The role of glycosylation was assessed in a cell line derived from the MPC-11 myeloma (M3.11) and it was found that even though as much as 50% of its H chains were not glycosylated, these nonglycosylated H chains were normally assembled and secreted (114). Tunicamycin treatment blocks N-linked oligosaccharide addition, and this was used in a set of classic experiments by Hickman and colleagues, to show that glycosylation was a prerequisite for murine IgM, IgA and IgE secretion, but was not needed for IgG secretion (115, 116). Additionally, murine IgA is degraded intracellularly with a >90% loss of secretion upon removal of α H chain glycosylation sites by site-directed mutagenesis (117).

A common strategy in the study of all biological processes, is to analyze mutants that are dysfunctional in order to gain additional insight into the mechanisms involved. In the case of antibody biosynthesis, an important advance was the availability of cell lines that have lost their ability to secrete Ig. The MPC-11 tumor, which produces IgG2b antibody, was widely used in all of these types of analyses and several hundred tissue culture variants have been made (57). This tumor is very interesting in that it secretes all possible assembly intermediates as well as the H_2L_2 Ig (118). There are differences in the relative proportions of

these intermediates between the original plasmacytoma and the tissue culture clones derived from it (104). Another unique feature of this tumor is that the HL intermediate is a dead-end species, due to the non-covalent interaction of HL with excess L chain, which blocks formation of H_2L_2 (119). The HL assembly intermediate has been shown to be secreted in other cells, however in these examples, this occurs in the absence of formation of complete H_2L_2 molecules and it is thought that these H chains are aberrant (104).

As alluded to previously, the H and L chains seem to play different roles in the assembly and transport processes. Evidence for this comes partially from the different fates of H and L chains that are not assembled with the other. In general, normal H chains are retained intracellularly (57, 120), while most L chains are secreted either as monomers or L_2 dimers (121, 122). Pulse-chase analysis of L chain-loss variants of the P3 (IgG1) cell line demonstrated that H chains labeled during a 3 min pulse were still detected intracellularly, and not secreted after 24 h chase (57). In contrast, excess L chains are often produced in plasma cells, and these L chains are degraded intracellularly if they are not secreted (123). When L chains that cannot be secreted are followed over time, they are degraded relatively quickly, with half-life values ranging from 40-100 min (124). These fundamental observations of Ig biosynthesis laid the foundation for two important concepts: 1) that the intracellular production of nonsecreted H chains in the absence of L chain frequently was toxic to the cell and is a model of heavy chain disease, and 2) that since L chains are generally secreted in the absence

of H chain, they probably confer secretion competence on the assembled Ig.

There are a number of secretion-defective L chains with point mutations that have been described. A derivative of the H chain-loss variant of MOPC 315 which produces, but does not secrete λ2 L chain, has a Gly→Arg mutation in VL at position 15 (125), and this same change in a λ1 L chain also prevents L chain secretion (121). The MOPC 21 L chain has a conditional secretory defect, in that it is not secreted unless provided with a H chain partner (123). In most L chains an aromatic residue is found at position 87 in VL, whereas the secretion defect of the MOPC 21 L chain can be attributed to the presence of a histidine at this position (126). This change is thought to affect the hydrophobic surface of the VH-VL interface (126), and the resultant intracellular L chains are associated with BiP, the expression of which is increased in these cells (127). Mutagenesis of the HOPC 2020 L chain revealed that a single amino acid replacement in the λ1 VL (Phe62→Ser) was sufficient to block secretion of L chain (121). This secretion defect cannot be rescued by co-expression with H chain, and the mutation is at a highly conserved residue in most L chains (121). All of these data affirm the importance of the VL domain on L chain (and in some instances Ig) transport competence. These observations have led Argon and colleagues to suggest that these highly conserved residues form a solvent exposed patch in the native VL conformation that is required for L chain and Ig secretion (121).

Native Ig domain conformations are achieved in the cell with the assistance of numerous enzymatic and chaperone activities. The domain architecture is dependent on the proper formation of disulfide bonds, the patterns of which are highly conserved and used for structural alignment in other members of the Ig superfamily (128). The dependence on appropriate disulfide bonds for the folding and stability of VH and VL domains is confirmed in an extensive biochemical analysis of McPC603 Fv (monovalent antibody VH-VL fragment), sFv (single chain Fv), and Fab fragments (129). PDI has been found to specifically associate with nascent Ig chains (130).

As mentioned above, the chaperone BiP was first described due to its ability to bind to Ig H chains (57, 58) and was subsequently found to associate also with Ig L chains, binding preferentially to VL domains (88, 127). Analyses of the interaction of BiP with Igs suggested that BiP plays an active role in retaining Ig proteins in the ER until they have achieved a native conformation (63, 131, 132). However, one cannot rule out that in the case of nonsecreted H chains, BiP association may be the result of retention in the ER, and not the driving force (133). As BiP recognizes short stretches of hydrophobic peptides, it is presumed that there are various sites on H chains where BiP may interact. However, it has been shown that BiP binds to the H chain CH1 domain, and that a deletion of this region abrogates BiP binding and allows secretion of free H chains (131, 134). In fact, most secreted H chains in heavy chain disease lack L chain and have large CH1 deletions (135). The CH1 domain is demonstrated to be a requirement for high affinity binding of BiP to H chains, and this region contributes to the interface that interacts with L

chain CL domains during assembly, providing further documentation that BiP mainly associates with interface regions (43, 131). Even though the CH1 domain is necessary for BiP binding, there is evidence that this binding may be influenced by residues in the VH domain (136). For instance, a secreted H chain disease protein has been described with an intact CH1, but a deletion in VH (137), and a human μ H chain containing a VH deletion was found to be transported without L chain to the cell membrane (138). Lastly, the replacement of VH in a chimeric CD4-γ1 protein with an intact CH1 was secreted without L chain (139).

Covalent assembly of H and L chains begins on nascent proteins before completion of translation (107) and proper oligomerization of Ig occurs in the ER lumen before transport to the cell surface, similar to other secretory multimeric proteins (140). BiP is released from H chains upon oligomerization with L chains (58, 63), and IgG H chains have been shown to remain bound with BiP and subsequently released upon association of L chain to form secreted IgG (141). More recently, this model has been further complicated by the findings that other molecular chaperones interact transiently with Igs. For instance, Grp94 binds to unassembled H and L chains, but not assembled Ig (87). Interestingly, evidence suggests that nascent Ig proteins interact with BiP and Grp94 in a sequential manner, with rounds of binding and release (87, 88). BiP binds preferentially to an early folding intermediate, whereas a more mature folding intermediate is thought to be bound by Grp94, consistent with the notion that BiP activity facilitates Grp94 interaction (46, 88). In addition to BiP and Grp94, calnexin has been reported to interact with membrane Ig chains (142, 143), and the stress

protein, Grp170, which is a putative molecular chaperone, has been shown to associate with Ig polypeptides (46, 144). Even though many of the chaperone interactions have been elucidated that occur prior to proper Ig assembly, it is still unclear as to the specific requirements, kinetics and order of these transient associations.

Defective Secretion of an Immunoglobulin Caused by Mutations in the Heavy Chain Complementarity Determining Region 2

Ching Chen, Tammy M. Martin, Susan Stevens and Marvin B. Rittenberg

J. Exp. Med. 180: 577 (1994)

Summary

We have investigated four secretion-deficient antibodies derived from a panel of 46 mutant T15 anti-phosphocholine Abs, all of which have point mutations in the heavy chain (H) complementarity determining region 2 (CDR2). The level of secretion for these four Abs was <10% of wild type when expressed together with the T15 light chain (L) in either SP2/0 or P3X63Ag8.653 myeloma cells although normal levels of H and L chain mRNA were produced. Moreover, abundant intracellular H and L chain proteins were detected. Three of the four mutants had little or no assembled H and L complexes intracellularly whereas one had a significant amount of intracellular immunoglobulin (Ig) which was shown to be capable of binding Ag. Thus, we demonstrate for the first time that point mutations confined to CDR2 of the H chain variable (V) region can impede Ab assembly and secretion. We then introduced the same CDR2 mutations into a related H chain which is encoded by the same T15 VH gene but different diversity (D) and joining (J) genes. When these H chains were expressed with a non-T15 L chain, the resulting Abs were secreted normally. The results thus suggest that the effects of the CDR2 mutations on Ab secretion are dependent on their interactions with L chain and/or H chain D-J sequences. These results also reveal a novel mechanism that could contribute to B cell wastage.

Introduction

The process of somatic mutation appears to operate randomly throughout Ab V regions, however, because of the selective force of Ag, specific mutations that confer a binding advantage to the B cell become dominant and thus provide a biased view of the structural consequences of somatic mutations. Mutations that fail to improve Ag binding are less well understood since such Abs are not easily recovered, however, this is the category of mutations that is likely to contribute to B cell wastage during an Ag-driven response. We have chosen to analyze the structural consequences of V region mutations that result in loss of Ab function. Because formation of a functional Ab molecule is a complex, multi-step process, a number of mechanisms could contribute to loss of function, including those affecting interactions with Ag, protein assembly and Ab secretion.

The pathways of Ig assembly were delineated in classic studies by Scharff and his colleagues (1, 2) who showed that the assembly of H and L chains of each isotype followed a major and a minor pathway. For IgG2b, the major pathway was $H+L \rightarrow HL \rightarrow H_2L_2$. The minor pathway consisted of $H+L \rightarrow HL \rightarrow HL+H \rightarrow H_2L+L \rightarrow H_2L_2$. The structure of the H chain isotype appeared to determine the pathway of assembly since most of the tumor cells studied utilized κ chains (1). Furthermore it has been suggested based on H + L chain reassociations in vitro, somatically mutated H chains may be more restricted in their ability to pair with heterologous L chains than unmutated H chains (3). Thus, mutations in either chain that affect assembly have the potential to prevent proper transport and secretion.

Studies of faulty Ab assembly and secretion have revealed several abnormal Ig products: secreted L chain monomers and dimers (4), secreted mutant H chains (5, 6), and secreted partially folded Ig (7). In most of these mutants the non secreted Ab is retained in the endoplasmic reticulum (ER) (8). Free unmutated L chains can be secreted in the absence of H chains (9, 10), but free unmutated H chains are retained in the cell in the absence of L chain (11, 12). Mutant L chains that cannot be secreted have been found in the lumen of the ER (10, 13). Mutations at Gly 15 in Vλ2 (14) and Phe 62 in Vλ1 (15) have been shown to block secretion. Phe 62→Ser blocked transport of λ1 from ER to the Golgi and prevented secretion even in the presence of H chain. The mutation at residue 62 did not cause gross misfolding since the mutants contained intracellular IgM that could bind Ag. More recently, in another H chain loss variant, His 87 in the MOPC 21 κ chain caused the latter's retention in the ER (16). Most L chains contain Tyr or Phe at position 87, and changing His 87 to Phe in Vκ21 restored secretion.

The location of these L chain mutations led to the proposal that VL contains information essential for Ig secretion (15) in accord with earlier reports that transfected L chains can restore Ig secretion in cells expressing only H chains (17-19). It is interesting to note that defective secretion of the MOPC21 κ chain (containing His 87) can be "repaired" by coexpression with H chain (16) as can defective secretion of another L chain, CH12κ (20) Thus, some L chain secretion failures can be overridden by H chain. It was suggested that VH-VL assembly masks the negative influence of His 87, thereby allowing the protein to continue on the normal transport pathway. However, the molecular nature of the interaction is unclear, and it is important here not to rule out

the possibility of a positive transport signal resident on H chains, as has been suggested previously (7).

Analysis of the H chain contribution to Ig assembly and secretion has focused on two domains of the constant region: CH1 and the μs tailpiece. L chain-negative cell lines can secrete γ H chains in which CH1 is absent (7, 21, 22) and camels normally secrete IgG2 and IgG3 H chain dimers lacking CH1 and L chains (23). However in order for free μ chains to be secreted it appears that CH1 must be deleted and a site (Cys 575) in the the μs tailpiece must be modified (6). These studies suggest that retention sites in the H chain constant region may be masked by L chain association or by polymerization into IgM pentamers before Ig transport proceeds.

We show here for the first time that mutations in the VH CDR2 region can also result in loss of Ab secretion. We have created four mutants of the anti-phosphocholine Ab, T15, that only differ by two to four point mutations in the VH CDR2 yet their secretion is <10% of wild type (WT). This is the first evidence that the VH hypervariable region may be involved in the process of Ab secretion. In addition, these results reveal a novel mechanism through which somatic mutation could contribute to the process of B cell wastage.

Materials and Methods

Genomic Cloning of D16 VH Genes: Genomic DNA obtained from D16 hybridoma cells was partially digested with EcoRI and ligated to λ phage EMBL4 EcoRI arms (Stratagene, La Jolla, CA). Approximately 10^6 phage recombinants were screened with JH- and VH1-specific probes (24). A clone positive for both probes was isolated and characterized by restriction enzyme

analysis. The 7.1-kb EcoRI fragment containing the productive V_H1-$D_HSP2.2$-J_H2 rearrangement of D16 was subcloned into the pTZ18U phagemid (Bio-Rad, Richmond, CA) for sequencing and subsequent genetic manipulation.

Plasmid Constructs: The CDR2 of the germline S107 VH1 gene was randomly mutated as described previously (25) and the WT and mutated T15 VH fragments were cloned into the pSV2gpt vector provided by Dr. J. Sharon (Boston University, Boston, MA) which contains the γ2b C region gene (26). The pSV2neoVκ22Cκ plasmid, a gift from Dr. S. Morrison (University of California at LA, Los Angeles, CA), was described elsewhere (27). The germline CDR2 sequence of the D16 VH1 gene was replaced with mutated VH1 CDR2 sequences from the T15 mutants generated previously (25) using SmaI and XhoI digestion and subsequent ligation into pTZ18U. The CDR2-replaced D16 VH genes were then subcloned into the pSV2gpt γ2b plasmid. The VH1 sequences and the CDR2 mutations were verified by sequencing in both pTZ18U and pSV2gpt vectors using primers for VH1 framework 3 (25) and JH2 (24).

Cell Culture and Transfection. Cells were cultured in IMDM (Gibco BRL, Gaithersburg, MD) containing L-glutamine, nonessential amino acids, sodium pyruvate and 15% FCS. Mutant and WT T15 cell lines were described previously (25). The D16 hybridoma was produced and characterized in this laboratory by Dr. M. Stenzel-Poore. It was derived from an early secondary response to phosphocholine (PC)-KLH, in which a mouse received a primary injection of 100 μg PC-KLH in CFA and was boosted on day 14 with 100 μg PC-KLH in IFA. Spleen cells were fused with the myeloma cell line FO as described (28). D16 produces an IgM, Vκ1c, group II anti-PC-protein Ab.

Accordingly, it binds nitrophenylphosphocholine much better than PC, is T15 id^- and does not bind to PC-polysaccharide (29). D16 is encoded by the S107 VH1 germline sequence and thus is identical to T15 in this respect. It differs from T15 in DJ and in Vκ. An H chain loss variant of D16 was isolated by soft agar cloning (30) using a mouse embryonic fibroblast feeder layer ($C_3H/10T1/2$, kindly provided by Dr. M. Wilkinson, Oregon Health Sciences University, Portland, OR).

SP2/0 myeloma cells, P3X63Ag8.653 myeloma cells and H chain-loss D16 hybridoma cells were used as recipients for DNA transfection. Transfection was performed by the lipofectin method as described previously (25) except that 100 μl lipofectin and a 22-h incubation was used in each transfection in the case of D16 cells. In this study, the SP2/0 cells were first transfected with a pSV2neoVκ22Cκ plasmid and a stable Vκ22Cκ transfectant was then used as the recipient for subsequent transfection with WT or mutant VHS107-γ2b. In the case of P3X63Ag8.653 cells, cotransfection of Vκ22Cκ and VHS107-γ2b was performed.

ELISA. The basic procedures of ELISA were described previously (31). Ab concentration was determined by sandwich ELISA in which microtiter plates were coated with rabbit-anti-mouse γ2b (Zymed Laboratories Inc., South San Francisco, CA) or with goat-anti-mouse κ (Southern Biotechnology Associates, Birmingham, AL). Serial dilutions of culture supernatant or cell lysate were added to the plates and alkaline phosphatase-labeled rabbit-anti-mouse γ2b or goat-anti-mouse κ were used to detect the Ab. Purified Ab from hybridoma PCG2b-2 (γ2b, κ) was used to generate standard curves. For T15 idiotypic expression, three anti-idiotype reagents were used. The mAb B36-82

(32) and the goat-anti-T15 antiserum are specific for T15 VH and VL regions and were described previously (25). The mAb TC54 was a kind gift from Dr. M. Scharff (Albert Einstein College of Medicine, Bronx, NY) and is specific for S107 VH region (33). Purified anti-idiotypic Ab was coated on the plates and then overlaid with protein A-Sepharose-purified mutant Ab at 100 ng/ml. Binding was detected with alkaline phosphatase-labeled rabbit-anti-mouse γ2b.

Preparation of Cell Lysates. Cell lysates were prepared according to George et. al. (34). Briefly, cell pellets were lysed in ice cold PBS containing 25 mM iodoacetamide, 20 μg/ml soybean trypsin inhibitor, 50 μg/ml PMSF, and 0.25% NP-40 (all from Sigma Chemical Co., St. Louis, MO). Lysates were incubated for 30 min at 4°C before spinning out nuclei and insolubles. For ELISA, cells were plated in duplicate 4 ml cultures at 2 x 10^6 cells/ml, incubated for 4 hours at 37°C, washed twice in culture medium without FCS, counted and pelleted. The pellets were lysed in 100 μl lysing solution and assayed immediately. For Western blotting, confluent 25 cm^3 flasks of cells were lysed in a volume of lysing solution to give an equivalent of 2 x 10^8 cells/ml.

Western Blot Analysis. Cell lysates were run on a 12% SDS-PAGE gel under reducing conditions. The separated proteins were then electrophoretically transferred to an Immobilon P membrane (Millipore, Marlborough, MA). The blot was blocked overnight at 4°C in PBSA (PBS with 0.02% sodium azide) containing 3% nonfat dry milk (NFDM). The blot was incubated for 1.5 h at room temperature in rabbit anti-mouse IgG2b (Zymed) diluted to 1 μg/ml in PBSA with 1% NFDM. After three 15-min washes in PBSA with 0.05%

Tween-20, the blot was incubated in 0.5 μg/ml protein A-alkaline phosphatase conjugate (Sigma Chemical Co.) for 1 h. After washing as above, the blot was incubated 5 min in AMPPD chemiluminescent substrate from the Immuno-Lite Substrate Kit (BioRad), wrapped in Saran wrap and exposed to film.

Northern Analysis. Cytoplasmic RNA was isolated by a mini-prep method (35). Northern blot was performed as described (36). Briefly, 10 μg RNA was loaded on 0.7% agarose gel and was electrophoresed for 4-7 h. RNA was then transferred to Nytran membranes which were hybridized with probes for VHS107 (37), Vκ22 (38) and for the housekeeping gene CHO-A (39) which was kindly provided by Dr. M. Wilkinson. Autoradiographs were analyzed by densitometric scanning using a densitometer (model 620; Bio-Rad).

Results

Identification of Secretion-defective Abs. We previously established a panel of T15 Abs restricted to random point mutations in VH CDR2 (S107) to study the effects of V region mutation on Ab function (25). In the course of making transfectomas with the mutated H chain gene constructs, we found that five mutants yielded clones severely impaired in their ability to secrete Ab. As shown in Table 1, mutants M102, M153, M164, M166 and M241 had been transfected several times into an SP2/0 line containing the Vκ22 L chain gene. Of a total of 331 mycophenolic acid resistant clones, only 19 (6%) produced any level of detectable Ab, and, as described below, this level was extremely low. In contrast, transfectants containing WT or other H chains with different CDR2 mutations (M85, M171 and M154) yielded 51 out of 95 (54%) clones that secreted normal levels of Ab.

To determine if other mutations beyond the V region (we had sequenced the V regions of all mutants before transfection) were affecting Ig expression, up to 500 bp of the 5' flank were sequenced. Only M102 had an altered 5' flank, a deletion of 200 bp that is probably responsible for the non-secretor phenotype, and further studies on this mutant were not performed. In contrast, the only differences distinguishing the other four low secretor mutants from the WT and the normally secreting mutants are the particular CDR2 mutations shown in Fig. 1.

Since the same SP2/0 cell line and the same procedures were used for all transfections, the reproducibility of the low secretor phenotype suggested a defect intrinsic to the mutated H chains. Nevertheless, in order to rule out a possible contribution by the transfection recipient, SP2/0, we made additional transfectants of the four low secretor H chain genes (excluding M102 from further analysis) and the WT S107 H chain gene together with the Vκ22-Cκ gene using another myeloma line as recipient, P3X63Ag8.653. As shown in Table 1, the low secretor phenotype was also exhibited in this cell line as only 7 of 143 drug-resistant clones containing the low secretor H chains produced any detectable Ab.

Ig and mRNA Levels of Low Secretor Mutant Cells. Clones that exhibited Ig levels above background during the first screen were saved from each mutant (a few negative clones were also saved). The amount of Ab secreted was quantified. As shown in Table 2, after 4 d of culture, the low secretor clones had very low levels of Ig in the supernatants, ranging from undetectable to 6.8% of the WT transfectant. Further, in other experiments using biosynthetic labeling, Ig H_2L_2 complexes were detected by 6 h in WT

supernatants but not in low secretor supernatants (data not shown). Ig was detectable by ELISA in the supernatants of low secretor clones only after several days of culture and the amount was inversely proportional to cell viability. Thus it is possible that some or all of the mutant clones are true nonsecretors and that Ab is only detectable in the supernatants after cells begin dying. However since this point has not been formally established, we use the term low secretor to describe the phenotype.

To determine if the defect in Ig secretion is at the transcriptional level, the levels of H and L chain mRNA were examined. Northern analysis with the S107 VH probe revealed that the H chain mRNA levels of most low secretor clones were comparable to or higher than the WT clone (Table 2 and Fig. 2). Moreover, the sizes of the H chain transcripts in the WT and the mutant clones were the same, indicating that gross deletions or truncations were not present. Northern analysis using a Vκ22 probe also indicated that most transfectants had normal levels of L chain mRNA (Table 2). Clearly, transfection had been successful in most instances, suggesting the basis for failure to secrete Ig at normal levels is located at some point after transcription.

Binding Analysis of the Ab. Anti-idiotype reagents were used to determine whether gross conformational changes or misfolding might be present in the mutant Abs. The mutant clones were grown for several days to allow Ab to accumulate in the supernatant. Abs were purified from the supernatants of clones M153-1, M164-4, M166-1 and M241-2. Three different anti-idiotype reagents were used to test for conformational changes that might disrupt recognition by the anti-idiotype. TC54 is a mAb specific for the S107 VH gene

product present in T15 even in the absence of L chain (33). The mAb B36-82 and the polyclonal antiserum recognize T15 idiotopes requiring the presence of both T15 VH and Vκ22 (25). All four low secretor mutant Abs were recognized by B36-82 and by the anti-T15 antiserum, indicating that the epitope formed through the interaction of H and L chains was essentially intact (Table 3). M153 and M166 also bound to TC54 (although with reduced avidity) whereas binding to M164 was not detected. It appears that the epitope recognized by TC54 is vulnerable to alterations caused by CDR2 mutations since the normally secreting mutant, M85, has also lost this epitope. This is consistent with an earlier report that a mutant S107 molecule bearing six VH mutations one of which was in the CDR2 had also lost its ability to bind to TC54 (40) and with our own findings that other residues in this region may also affect the TC54 epitope (Chen, C. V. A. Roberts, S. Stevens, M. P. Stenzel-Poore and M. B. Rittenberg, manuscript in preparation).

The integrity of the Abs was also tested by their ability to bind to the Ag, PC-histone. Although, as shown previously, mutations in VH CDR2 of T15 frequently result in loss of Ag binding (25), the low secretor M153 Ab isolated from cell lysates was able to bind PC-protein. These data indicate that the mutated H chains are unlikely to be grossly misfolded, and in the case of M153, can combine with the L chain to form an intact Ab or an L/H dimer. However, the above experiments do not indicate whether the mutated H chains in the low secretors have a normal efficiency or pattern of pairing with the L chain.

Examination of Intracellular Ig Proteins. To determine if the mutant H chain proteins accumulate in the cells and if they assemble with the L chain

efficiently, we employed an ELISA to detect intracellular Ig in cell lysates. One representative clone of each low secretor mutant (M153-6, M164-3, M166-3 and M241-2) was analyzed and compared to WT. Cells were cultured for 4 h and Ig proteins in the lysates and supernatants were quantified by ELISA. As shown in Table 4, all four mutant clones had a large amount of H chain protein in the cells, ranging from 68 to 185% of the WT level. However, the intracellular H/L dimers (or tetramers) were greatly reduced in the mutants. Clones M164, M166 and M241 had only 1-8% of the WT level of assembled protein. The exception is M153, which contained intracellular H/L protein at 36% of WT level in keeping with our ability to detect Ag binding activity in this extract.

By 4 h of incubation, the WT clone already had secreted a significant amount of Ab into the supernatant (Table 4). However, at this time point, little or no Ab could be detected in the supernatants of the mutant clones, including M153. Thus, in addition to reduced assembly, a defect in transport/secretion may also account for the low secretor phenotype.

In addition to the ELISA analysis described above, intracellular H and L chains were visualzed by Western blot (Figure 3). The proteins in cell lysates were separated by SDS-PAGE under reducing conditions and H and L chains were detected with anti-γ2b Abs (which fortuitously cross-react with L chains). H and L proteins were seen in all four low secretor lysates, as well as WT lysates. These proteins run at apparent molecular mass of 54 (H chains) and 26 kDa (L chains), and both migrate very similarly to purified MOPC 195 proteins (γ2b, κ), indicating that no gross alterations of the protein have occurred. Together, these data suggest that the impaired Ab secretion in these

mutants is not due to defects in protein synthesis. Rather deficient H/L assembly or stability may be a major reason for the low secretor phenotype of these mutants. The exception is M153 which appears to assemble but fails to secrete the mutant molecules (consistent with higher levels of intracellular H and L proteins compared to WT in Figure 3).

Effect of Different L and H Chain D-J Sequences on Secretion. In an attempt to investigate whether the same set of T15 CDR2 mutations would have similar effects on Ab function when associated with a different Ab, the mutated CDR2s were placed in the VH of another mAb, D16, which is encoded by the same germline S107 VH gene as T15 but differs from T15 in D-J and VL (Fig. 4). Unexpectedly, all four D16 transfectants with the low secretor CDR2 mutations secreted high levels of Ab, ranging from 59 to 141% of the WT level (Table 5).

Thus, the contribution of the CDR2 mutations to low secretion is complex and may be modified through interaction with VL and/or D-J. This result also emphasizes that the CDR2 mutations are unlikely to have caused major structural alterations in VH.

Discussion

In this study we have shown that point mutations in the H chain CDR2 are the cause of defective secretion of an Ig. We drew this conclusion based on several observations. First, L chain-bearing cells transfected with the mutated H chain genes secreted little Ab whereas cells receiving the same H chain gene without mutations or with different mutations in CDR2 secreted normal amounts of Ab. Second, the low secretor phenomenon was observed in repeated transfections with the mutant H chains in two different cell lines and multiple transfectants, excluding the possibility that low secretion was caused by an error in transfection or a defect in the secretory apparatus of the cell itself. Third, no mutations other than those in VH CDR2 were present as determined by sequencing of the entire V gene. Fourth, transcription or stability of the H chain mRNA appears not to be affected by the mutations since the low secretor mutants express abundant H chain mRNA. Fifth, normal levels of H chain proteins were detected intracellularly; thus the defect appears to be posttranslational.

Somatic hypermutation in Ab V regions represents an important means of generating Ab diversity, in particular, improving Ab affinity after antigenic stimulation (41). Since mutations are introduced randomly into the V region, it is predicted that many mutations would result in nonfunctional Ab such as the low secretors described here. Based on the frequency of invariant residues in the frameworks it has been estimated that 50% of mutations in these regions would result in nonfunctional Ab (42). However, previous efforts to identify such mutations in the VH region have been limited. For example, substitution of the invariant VH residue Cys-92→Tyr or Trp-36→Ala did not show any apparent effect on Ab function (43, 44). Here we

show that four mutant Abs with two to four mutations in the VH CDR2 are secretion deficient. These results were unexpected because mutations in the CDRs are usually thought to be permissive due to the highly variable nature of CDR sequences and their importance for generating diversity. These four mutants came from a pool of 46 Abs all of which have point mutations in VH CDR2. Approximately 50% of these mutants had lost or displayed reduced binding to Ag (25). Of the 46 mutant Abs originally described (25), 2 of 9 with four mutations and 1 of 10 with three mutations are shown here to be low secretors. Thus the secretion defect does not reflect the number of mutations but rather their location. The high frequency (4 of 46) of low secretion mutants caused by random CDR2 mutations emphasizes that extensive B cell wastage may occur during the process of somatic mutation and that the creation of nonsecretor variants, in addition to loss of Ag binding, may be a significant factor leading to wastage.

There is increasing evidence that the correct folding and assembly of secreted proteins are necessary for their transport and secretion (45). To determine if the low secretor H chains were misfolded, we tested their binding to several anti-idiotypic Abs. Two of these reagents recognize epitopes provided by the correct combination of H and L chain V regions and the other recognizes structures provided by the H chain V region alone. All four low secretors appear to retain the T15 VH-VL epitope(s) while the TC54 VH epitope was lost in one of the three mutants tested. In addition, one of the low secretor Abs, M153, was able to bind Ag. Thus, the low secretor mutations in VH CDR2 do not appear to cause a gross misfolding of the H chain although local conformations such as those recognized by TC54 may be altered.

On the other hand, the levels of intracellular H-L dimer (or tetramer) in these low secretors were greatly reduced (three had <10% of the WT level) even though the amount of unassembled H chains was normal. The L chain expression in these cells should not be an affecting factor since the same SP2/0 cell line (which contains a previously transfected Vκ22 L chain gene) was used in all the transfections in this study and the L chain mRNA expression was normal in most clones of the low secretors. The finding that three mutants exhibit very little intracellular H-L protein could result from inefficient pairing of the mutant H chain with the Vκ22 L chain. Previous in vitro experiments have suggested that some H + L pairing may be favored over others (46-48) and, as indicated above, may be affected by somatic mutations (3). Our data may therefore suggest that local structural alterations caused by the CDR2 mutations interfere with H-L assembly and/or the stability of the assembled proteins, and may represent the major cause of the low secretor phenotype in three of the four mutants.

As shown above, mutant M153 makes a reasonable amount of Ab (36% of WT level), but it is not secreted. Thus, a defect in Ab transport/secretion appears likely. The prevailing view of Ab assembly and secretion is that unassembled H chains are retained in the ER via their interaction with the chaperone BiP. BiP is displaced when free L chains become available, and the assembled Ab is transported along the secretory pathway based on signals provided by the L chains (15, 49). Previous studies of λ chain secretion demonstrated that a single substitution at position 15 (Gly→Arg) of λ2, or position 62 (Phe→Ser) of λ1 L chains, was sufficient to block secretion (14, 15). Since both of these residues are located on the solvent-exposed surface and are close to each other as predicted by the McPC603 model, it has been

proposed that they are included in a surface patch that is required for intracellular Ig transport (15). Here we show for the first time that mutations in the H chain V region may also affect Ab transport. How a local alteration in VH CDR2 could be involved in this process is not clear. The alteration may destroy a transport signal or create a retention signal in VH. Or, it may block or alter the transport signal in the L chain through interactions at the L/H interface. This latter possibility is supported by our finding that the same mutant H chains were secreted when transfected into a cell line (D16) expressing a different Vκ gene. The relationship of the two light chains to the CDR2 mutations is not clear at present. Vκ22 alone is not able to be secreted (Scharff, M. D., personal communication as cited in reference 19 and confirmed in our transfectants). In contrast we have found that the D16 H chain loss variant secretes large amounts of Vκ1c L chain (data not shown). The role played by WT H chain in the secretion of T15 Ab and the role of Vκ1c in rescue of secretion defective mutant H chains remains to be determined. Further investigations on the intracellular transport of the mutant Abs in conjunction with their structural analysis should clarify these possibilities.

It is generally recognized that it is the framework or conserved V region residues that provide the structural basis for proper Ig folding and assembly (50). In the V domain, the CDRs loop out from the β-pleated sheets of the framework regions and form the surface that interacts with Ag. Thus it is surprising to see the low secretor phenotype associated with VH CDR2. Indeed, residues in VL regions known to affect secretion (10, 14-16), and V region positions important for VL-VH packing (51) have so far been localized to conserved sites. However, even though there is extensive sequence

diversity in the CDR residues, the many known Ab structures actually fit into a small number of main chain canonical structures in the hypervariable regions (50). This CDR structural constraint is believed to be important in forming an Ag binding site. Two of the low secretor mutations described here occur in canonical residues 51 and 52, and several other low secretor mutations are found in a region (56-63) where all known Ab structures exhibit essentially the same main-chain conformation (50) (Fig. 1). Thus it is intriguing to consider the possibility that canonical structures in the CDRs may also affect aspects of Igs not associated with Ag binding, such as assembly or intracellular transport. In addition, residues 59 to 65 form the second loop of VH CDR2 which is distant from the binding cavity but in proximity to the L chain framework 2 region in T15 (25). VL framework 2 is important for formation of the VL-VH interface in McPC603 (51), thus substitutions in VH CDR2 may change the conformation of this loop and interfere with or alter H-L assembly .

Collectively, these low secretors contain amino acid replacements in two general regions of VH CDR2, 51-52 and 58-63, which could potentially perturb several aspects of assembly or transport of the immunoglobulin. However other mutants we described previously also had mutations at these same canonical positions (but in different combinations) and yet were secreted normally (25). Further investigation to examine the effects of individual and combined mutations by site-directed mutagenesis and detailed computer modeling will help to clarify these issues and improve our understanding of the structural requirements for Ab assembly and secretion.

This work was supported by grants AI-14985, AI-26827 and EY-07123 from the National Institutes of Health. C. Chen and T. M. Martin were recipients of Tartar Foundation Research Awards and contributed equally to this work.

Acknowledgments: We thank Dr. Mary Stenzel-Poore for providing the D16 hybridoma. We also thank her and Arun Bhat for important contributions to the manuscript; and McKay Brown, Dr. Greg Wiens and Kurt Heldwein for critical review of the manuscript.

References

1. Baumal, R., M. Potter, and M.D. Scharff. 1971. Synthesis, assembly and secretion of gamma globulin by mouse myeloma cells. III. Assembly of the three subclasses of IgG. *J. Exp. Med.* 134:1316-1334.
2. Scharff, M.D. 1975. The synthesis, assembly, and secretion of immunoglobulin: A biochemical and genetic approach. *Harvey Lect.* 69:125-142.
3. Kranz, D.M., and E.W.J. Voss. 1981. Restricted reassociation of heavy and light chains from hapten-specific monoclonal antibodies. *Proc. Natl. Acad. Sci. USA* 78:5807-5811.
4. Knittler, M.R., and I.G. Haas. 1992. Interaction of BiP with newly synthesized immunoglobulin light chain molecules: cycles of sequential binding and release. *EMBO J.* 11:1573-1581.
5. Alberini, C.M., P. Bet, C. Milstein, and R. Sitia. 1990. Secretion of immunoglobulin M assembly intermediates in the presence of reducing agents. *Nature* 347:485-487.
6. Sitia, R., M. Neuberger, C. Alberini, P. Bet, A. Fra, C. Valetti, G. Williams, and C. Milstein. 1990. Developmental regulation of IgM secretion: the role of the carboxy-terminal cysteine. *Cell* 60:781-90.
7. Hendershot, L., B. Bole, G. Kohler, and J.F. Kearney. 1987. Assembly and secretion of heavy chains that do not associate posttranslationally with immunoglobulin heavy chain-binding protein. *J. Cell Biol.* 104:761-767.
8. Pfeffer, S., and J.E. Rothman. 1987. Biosynthetic protein transport and sorting by the endoplasmic reticulum and golgi. *Ann. Rev. Biochem.* 56:829-852.
9. Morrison, S.L., R. Baumal, B.K. Birshstein, M.W. Kuehl, J.L. Preud'homme, L. Frank, T. Jasek, and M.D. Scharff. 1974. The

identification of mouse myeloma cells which have undergone mutations in immunoglobulin production. *In* Cellular selection and regulation in the immune response. G.M. Edelman, editor. Raven Press, New York. 233-245.

10. Mosmann, T.R., and A.R. Williamson. 1980. Structural mutations in a mouse immunoglobulin light chain resulting in failure to be secreted. *Cell* 20:283-292.
11. Morrison, S.L., and M.D. Scharff. 1975. Heavy chain-producing variants of a mouse myeloma cell line. *J. Immunol.* 114:655-659.
12. Sonenshein, G.E., M. Siekevitz, G.R. Siebert, and M.L. Gefter. 1978. Control of immunoglobulin secretion in the murine plasmacytoma line MOPC 315. *J. Exp. Med.* 148:301-312.
13. Winberry, L., A. Marks, and R. Baumal. 1980. Immunoglobulin production and secretion by variant clones of the MOPC 315 mouse myeloma cell line. *J. Immunol.* 124:1174-1182.
14. Wu, G.E., N. Hozumi, and H. Murialdo. 1983. Secretion of a λ_2 immunoglobulin chain is prevented by a single amino acid substitution in its variable region. *Cell* 33:77-83.
15. Dul, J.L., and Y. Argon. 1990. A single amino acid substitution in the variable region of the light chain specifically blocks immunoglobulin secretion. *Proc. Natl. Acad. Sci. USA* 87:8135-8139.
16. Dul, J.L., O.R. Burrone, and Y. Argon. 1992. A conditional secretory mutant in an Ig L chain is caused by replacement of tyrosine/phenylalanine 87 with histidine. *J. Immunol.* 149:1927-1933.
17. Rice, D., and D. Baltimore. 1982. Regulated expression of an immunoglobulin κ gene introduced into a mouse lymphoid cell line. *Proc. Natl. Acad. Sci. USA* 79:7862-7865.

18. Pepe, V.H., G.E. Sonenshein, M.I. Yoshimura, and M.J. Shulman. 1986. Gene transfer of immunoglobulin light chain restores heavy chain secretion. *J. Immunol.* 137:2367-2372.

19. Oi, V.T., S.L. Morrison, L.A. Herzenberg, and P. Berg. 1983. Immuoglobulin gene expression in transformed lymphoid cells. *Proc. Natl. Acad. Sci. USA* 80:825-829.

20. Gardner, A.M., S. Aviel, and Y. Argon. 1993. Rapid Degradation of an Unassembled Immunoglobulin Light Chain Is Mediated by a Serine Protease and Occurs in a Pre- Golgi Compartment. *J Biol Chem* 268:25940-25947.

21. Morrison, S. 1978. Murine heavy chain disease. *Eur. J. Immunol.* 8:194-199.

22. Seligmann, M., E. Mihaesco, J.-L. Preud'Homme, F. Danon, and J.-C. Brouet. 1979. Heavy chain diseases: Current findings and concepts. *Immunol. Rev.* 48:145-167.

23. Hamers-Casterman, C., T. Atarhouch, S. Muyldermans, G. Robinson, C. Hamers, E. Bajyana Songa, N. Bendahman, and R. Hamers. 1993. Naturally occurring antibodies devoid of light chains. *Nature* 363:446-448.

24. Stenzel-Poore, M., and M. Rittenberg. 1989. Clonal diversity, somatic mutation, and immune memory to phosphocholine-keyhole limpet hemocyanin. *J. Immunol.* 143:4123-4133.

25. Chen, C., V.A. Roberts, and M.B. Rittenberg. 1992. Generation and analysis of random point mutations in an antibody CDR2 sequence: Many mutated antibodies lose their ability to bind antigen. *J. Exp. Med.* 176:855-866.

26. Sharon, J., M.J. Gefter, L.J. Wysocki, and M.N. Margolies. 1989. Recurrent somatic mutations in mouse antibodies to p-azophenylarsonate increase affinity for hapten. *J. Immunol.* 142:596.

27. Morrison, S.L., M.J. Johnson, L.A. Herzenberg, and V.T. Oi. 1984. Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. *Proc. Natl. Acad. Sci. USA* 81:6851.

28. de St.Groth, S.F., and D. Scheidegger. 1980. Production of monoclonal antibodies: strategy and tactics. *J. Immunol. Meth.* 35:1-21.

29. Stenzel-Poore, M., U. Bruderer, and M. Rittenberg. 1988. The adaptive potential of the memory response: clonal recruitment and epitope recognition. *Immunol. Rev.* 105:113-136.

30. Coffino, P., R. Baumal, R. Laskov, and M.D. Scharff. 1971. Cloning of mouse myeloma cells and detection of rare variants. *J. Cell. Physiol.* 79:429-440.

31. Chen, C., M.P. Stenzel-Poore, and M.B. Rittenberg. 1991. Natural auto and polyreactive antibodies differing from antigen-induced antibodies in the H chain CDR3. *J. Immunol.* 147:2359.

32. Cerny, J., R. Wallach, and G.J. Hammerling. 1982. Analysis of T15 idiotopes by monoclonal antibodies: variability of idiotypic expression on phosphorylcholine-specific lymphocytes from individual inbred mice. *J. Immunol.* 128:1885.

33. Desaymard, C., A.M. Giusti, and M.D. Scharff. 1984. Rat anti-T15 monoclonal antibodies with specificity for VH- and VH-VL epitopes. *Mol. Immunol.* 21:961-967.

34. George, A.J.T., R.C. Foreman, and F.K. Stevenson. 1991. Characterization of a light chain loss variant of the BCL1 lymphoma. *Mol. Immunol.* 28:789-799.

35. Wilkinson, M. 1988. RNA isolation: a mini-prep method. *Nucleic Acids Res.* 16:10933.

36. Buenafe, A., and M.B. Rittenberg. 1989. Molecular analysis and fine specificity of antibodies against an organophosphorus hapten. *J. Immunol.* 143:539.

37. Early, P., H. Huang, M. Davis, D. Livant, R. Wall, and L. Hood. 1980. An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: VH, D, and JH. *Cell* 19:981.

38. Kwan, S.P., S. Rudikoff, J.G. Seidman, P. Leder, and M.D. Scharff. 1981. Nucleic acid and protein sequences of phosphocholine-binding light chains. *J. Exp. Med.* 153:1366.

39. Harpold, M.M., R.M. Evans, M.S. Salditt-Georgieff, and J.E. Darnell. 1979. Production of mRNA in chinese hamster cells: relationship of the rate of synthesis to the cytoplasmic concentration of nine specific mRNA sequences. *Cell* 17:1025.

40. Chien, N.C., R.R. Pollock, C. Desaymard, and M.D. Scharff. 1988. Point mutations cause the somatic diversification of IgM and IgG2a antiphosphorylcholine antibodies. *J. Exp. Med.* 167:954.

41. French, D.L., R. Laskov, and M.D. Scharff. 1989. The role of somatic mutation in the generation of antibody diversity. *Science* 244:1152.

42. Shlomchik, M.J., A.H. Aucoin, D.S. Pisetsky, and M.G. Weigert. 1987. Structure and function of anti-DNA autoantibodies derived from a single autoimmune mouse. *Proc. Natl. Acad. Sci. USA* 84:9150.

43. Rudikoff, S., and J.G. Pumphrey. 1986. Functional antibody lacking a variable-region disulfide bridge. *Proc. Natl. Acad. Sci. USA* 83:7875-7878.

44. Sharon, J. 1988. The invariant tryptophan in an H chain V region is not essential to antibody binding. *J. Immunol* 140:2666.

45. Hurtley, S.M., and A. Helenius. 1989. Protein oligomerization in the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 5:277-307.

46. Grey, H.M., and M. Mannik. 1965. Specificity of recombination of H and L chains from human γG-myeloma proteins. *J. Exp. Med.* 122:619-632.

47. Hamel, P.A., M.H. Klein, and K.J. Dorrington. 1986. The role of the VL- and VH-segments in the preferential reassociation of immunoglobulin subunits. *Mol. Immunol.* 23:503-510.

48. Roholt, O.A., G. Radzimski, and D. Pressman. 1965. Preferential recombination of antibody chains to form effective binding sites. *J. Exp. Med.* 122:785-798.

49. Bole, D.G., L.M. Hendershot, and J.F. Kearney. 1986. Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol.* 102:1558-1566.

50. Chothia, C., A.M. Lesk, A. Tramontano, M. Levitt, S.J. Smith-Gill, G. Air, S. Seriff, E.A. Padlan, D. Davies, W.R. Tulip, P.M. Colman, S. Spinelli, P.M. Alzari, and R.J. Poljak. 1989. Conformations of immunoglobulin hypervariable regions. *Nature* 342:877-883.

51. Chothia, C., J. Novotny, R. Bruccoleri, and M. Karplus. 1985. Domain association in immunoglobulin molecules; The packing of variable domains. *J. Mol. Biol.* 186:651-663.

Table 1. Identification of S107 Low Secretor Mutant Cell Lines

	Expt. 1		Expt. 2		Expt. 3		P3X63Ag8.653	
	mpa^R	Ig^+	mpa^R	Ig^+	mpa^R	Ig^+	mpa^R	Ig^+
NORMAL SECRETORS								
WT	6*	2‡	23	8	ND	ND	33	8
M85	5	3	8	5	ND	ND	ND	ND
M171	5	3	12	6	ND	ND	ND	ND
M154	22	17	14	7	ND	ND	ND	ND
A61T	ND	ND	ND	ND	ND	ND	29	18
TOTAL	**38**	**25**	**57**	**26**			**62**	**26**
LOW SECRETORS								
M102	13	-	47	-	ND	ND		
M153	22	1, 2§	5	-	32	2, 2§	45	1§
M164	13	2§	ND		40	2§	55	4§
M166	18	2§	33	3§	17	-	24	2§
M241	27	1§	ND	ND	64	2§	19	-
TOTAL	**93**	**8**	**85**	**3**	**153**	**8**	**143**	**7**

WT and VHCDR2 mutated T15 transfectants were generated as described in Materials and Methods. Each experiment represents a separate transfection. SP2/0-Vκ22 cells were the recipient cells in experiments 1 - 3 whereas in the last experiment P3X63Ag8.653 cells were transfected with VHS107 together with Vκ22.

* mpa^R is the number of mycophenolic acid resistant clones (which indicates the presence of transfected H chain DNA) compared to the number of normal antibody secreting clones (Ig^+) obtained in each transfection.

‡ Ig^+ antibody secretion was determined by direct-binding ELISA as indicated in Materials and Methods.

§ Transfectants had an OD_{405} reading <0.3 at a time point when WT readings were ≥ 1.0. (-) Undetectable.

Table 2. Quantitation of Antibody and Ig mRNA Levels in S107 Low Secretor Mutants

	Antibody Secreted		Ig mRNA	
mutants	ng/ml	Percent WT	VHS107	VK22
WT SP2/0*	736‡	100	1.0§	1.0
WT P3X63Ag‖	296	100	1.0	1.0
M153-1	2.8	0.4	8.54	11.8
M153-5	-		2.88	0
M153-6	2.6	0.4	2.92	6.97
M153-7	2.7	0.4	0.40	ND
M153 P3X63Ag‖	-		0.51	0.16
M164-1	-		0.28	0
M164-2	-		1.57	0.58
M164-3	2.0	0.3	1.42	0.11
M164-4	12.7	1.7	0.26	2.03
M164 P3X63Ag‖	7.5	2.8	2.29	3.05
M166-1	15.6	2.1	3.08	2.85
M166-2	28.0	3.8	6.58	2.75
M166-3	8.3	1.1	2.18	3.41
M166-4	50.0	6.8	5.70	2.79
M166-5	4.8	0.7	2.08	3.48
M166-6	-		1.25	0.79
M166 P3X63Ag‖	7.2	2.7	14.02	5.65
M241-1	-		3.72	0.72
M241-2	2.6	0.4	1.29	0.81
M241-3	-		2.13	1.07
M241-4	-		0	0.57
M241 P3X63Ag‖	-		0.36	0.93

* Each clone of the mutants was independently isolated (represents an independent transfection event of the designated mutant H chain).

‡ The same number of cells (10^5) was plated for each clone and the supernatants were collected on day 4. Antibody was quantitated on anti-IgG-coated plates using purified isotype-matched antibody as standard. (-) < 0.1 ng/ml.

§ mRNA was determined by Northern analysis using VH S107 or Vκ22 specific probes. The relative amount of mRNA was estimated by densitometry scanning and normalized to the mRNA signal from the CHO-A house-keeping gene. ND indicates not done.

‖ Clones are from P3X63Ag8.653 transfectants.

Table 3. Binding Features of S107 Low Secretor Mutant Ig as an Indication of Conformational Changes

	anti-T15 id			
	TC54	B36-82	antiserum	PC-his
WT	+++	+++	+++	+++
M85	-	+++	+++	-
M171	+	+++	+++	-
M154	++	+++	+++	-
M153	++	+++	+++	++
M164	-	+++	+++	-
M166	+	+++	+++	-
M241	ND	+++	+++	-

Binding to various anti-T15 antibodies and to PC-histone was determined by direct-binding ELISA as described in Materials and Methods. Affinity purified antibody was used in anti-idiotype analysis (the low secretor clones M153, M164, M166, and M241 were cultured for extended period of time in order to accumulate sufficient Ig in the supernatant for testing). Either lysates (M153, M164, M166, and M241) or affinity-purified antibody (M85, M171, and M154) were used in PC-histone binding assays. WT antibody had high OD.readings in the PC-histone binding assay regardless of whether it was affinity purified or from lysate. Antibodies were added to the anti-T15 or PC-histone coated plates at a concentration of 100 ng/ml. The OD readings of triplicate wells were averaged and are expressed in a simplified scale: +++ (> 0.8); ++ (0.2 - 0.8); + (0.01 - 0.2); - (< 0.01). ND: not done. The mutant clones M85, M171, and M154 secrete Ig which is not able to bind antigen as described previously (25).

Table 4. Quantitation by ELISA of Intracellular Ig Proteins in WT and Mutant Clones

	lysate				supernatant	
	H		H+L		H+L	
	ng	%	ng	%	ng	%
WT	16.31	100	9.24	100	31.02	100
M153-6	30.17	185	3.34	36	0.03	0.1
M241-2	16.71	102	0.51	5	-	
WT	16.67	100	15.70	100	35.35	100
M164-3	12.40	74	1.29	8	-	
M166-3	11.35	68	0.13	1	-	

Cells were cultured for 4 h before collection. Cell lysates were prepared as described in Materials and Methods. Intracellular protein was detected by ELISA using either anti-γ2b (H) or anti-κ (H+L) coated plates, all of which were developed by an anti-IgG2b-alkaline phosphatase conjugate. Total ng of Ig protein was determined using isotype-matched, purified antibody as a standard. The number of cells collected for lysis was used to calculate the amount of protein per million cells. Mutants M153, M241 and mutants M164, M166 were tested on separate days, each with a WT control. (-) < 0.01 ng per million cells.

Table 5. Effects of L and H Chain D-JH Replacement on Antibody Secretion

Ig combination			antibody secreted	
VH	D-JH	VL	ng/ml	% of WT
WT S107	T15	Vκ22	736.0	100.0
M153	T15	Vκ22	2.8	0.4
M164	T15	Vκ22	12.7	1.7
M166	T15	Vκ22	50.0	6.8
M241	T15	Vκ22	2.6	0.4
WT S107	D16	Vκ1-C	642.0	100.0
M153	D16	Vκ1-C	376.0	58.6
M164	D16	Vκ1-C	904.0	140.8
M166	D16	Vκ1-C	483.0	75.2
M241	D16	Vκ1-C	381.0	59.3

Quantitation of secreted antibody was done as in Table 2.

NORMAL SECRETORS

	50	51	52	a	b	c	53	54	55	56	57	58	59	60	61	62	63	64	65	Ig
WT	A	S	R	N	K	A	N	D	Y	T	T	E	Y	S	A	S	V	K	G	+
M85	-	-	I	-	-	-	-	-	H	-	-	-	-	-	-	-	-	-	-	+
M107	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	+
M154	-	-	-	K	-	-	-	-	-	I	-	-	-	-	-	-	-	-	-	+
M171	-	-	G	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
A61T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	+

LOW SECRETORS

M102*	-	-	-	-	-	-	-	Y	-	A	P	Q	-	-	-	T	-	-	-	-
M153	-	-	-	-	-	-	-	-	-	-	-	-	-	T	V	-	M	T	-	±
M164	-	-	I	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	±
M166	-	-	-	K	-	-	-	-	-	-	-	G	-	-	-	Y	M	-	-	±
M241	-	R	-	-	-	-	-	E	-	-	-	-	S	-	-	-	-	-	-	±

Fig. 1. Amino acid sequences of VHS107 CDR2 WT and mutant H chains of normal secretor and low secretor transfectant clones. The remainder of the H chain and L chain sequences are identical to WT T15. (Ig $^{+}$) normal secretion; (Ig $^{\pm}$) secretes <10% of wild type; (Ig $^{-}$) no Ig detected. Status defined quantitatively in Table 2. M102 was unable to produce any Ig presumably because of a 200-bp deletion in the 5' flanking region as discussed in the text. These sequence data are available from EMBL/Gen-Bank/DDBJ under accession number X79818.

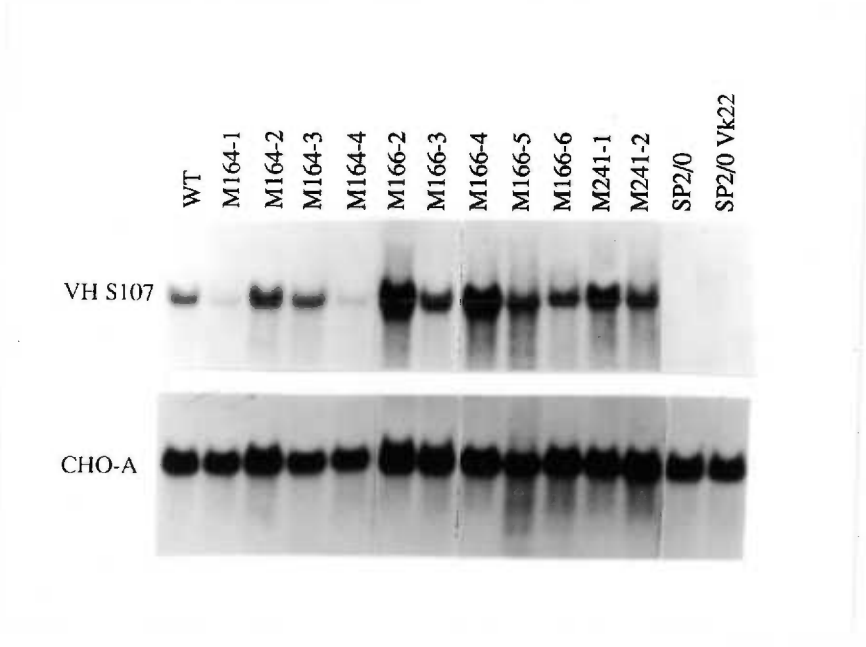


Fig. 2. H chain mRNA levels of low secretor clones. Autoradiograms of Northern blots for WT and some of the low secretor clones are shown. RNA samples were first probed for VHS107 (top) and the membranes stripped and reprobed for CHO-A (bottom) as described in Materials and Methods. Clone names are indicated above each lane.

	VH	DH	JH	VL
T15	S107	DYYGSS	YWYFEVWGAGTTVTVSS	Vκ22
D16	S107	GHYD	YFEYWGQGTTLTVSS	Vκ1-C

Fig. 4. Comparison of T15 and D16 D-JH sequences, their VH sequences are identical. Amino acid sequences are shown as single letter code.

Deletion in HCDR3 Rescues T15 Antibody Mutants from a Secretion Defect Caused by Mutations in HCDR2

Tammy M. Martin, Christine Kowalczyk, Susan Stevens, Gregory D. Wiens, Mary P. Stenzel-Poore, and Marvin B. Rittenberg

Abstract

We recently described mutants of the murine anti-phosphocholine antibody, T15, with changes in heavy chain complementarity determining region 2 (HCDR2) that caused loss of secretion. Surprisingly, the T15 HCDR2 mutations did not alter secretion when placed into a related anti-phosphocholine antibody, D16, which differs from T15 only in HCDR3 and light (L) chain. Here, we exploit the differences between these two antibodies to assess the basis of the secretion defect. The T15 L chain is not secreted in the absence of heavy (H) chain, while the D16 L chain in contrast, is secreted in the absence of H chain, as are most L chains. We co-expressed the T15 wild type (WT) and mutant H chains with the D16 L chain, as well as with another secreted L chain, J558L. The mutant H chains were not secreted when expressed with either heterologous L chain. These results establish that the T15 L chain is not uniquely associated with the defect. The T15 and D16 antibodies also differ in HCDR3 length in that D16 lacks four amino acid residues (Ser99, Ser100, Tyr100a, Trp100b) present in T15. We deleted these four residues from T15 WT and mutant H chains. Secretion of T15 WT was unaffected by the deletion but shortening HCDR3 restored secretion in the HCDR2 mutants regardless of L chain association. Together these data demonstrate that both the HCDR2 and HCDR3 domains contain structural information that may affect the secretion competence of antibodies.

Introduction

Native and mutant immunoglobulins have been an excellent model for studying the folding and trafficking of oligomeric proteins. Generally, oligomeric proteins first attain a correctly folded and assembled state in the endoplasmic reticulum (ER) before being transported to the plasma membrane or out of the cell (1). The overall assembly patterns of heavy (H) and light (L) chain IgG intermediates have been known for more than 20 years (2). In one current model of antibody secretion L chains are viewed as conferring transport competence on the assembled H_2L_2 complex (3-5). While H chains accumulate intracellularly in the absence of L chain and are secreted only when complexed with L chain (6-8), most L chains can exit the ER and be secreted in the absence of H chains (4, 5, 9). In addition, highly conserved variable region L chain residues have been identified by mutagenesis to be required for antibody transport (3, 4, 10). Other observations have suggested that the role of L chain in antibody transport is not always required. For example, free H chains can be secreted after removal of the CH1 domain, a region known to contain a binding site for the molecular chaperone BiP (11-13). Replacement of the μ_S tailpiece Cys575 of IgM increases transport kinetics, and when coupled with deletion of CH1 allows secretion of monomeric μ_S chains (14). Moreover, a substantial portion of serum antibodies in camels are functional H chain dimers (i.e. not assembled with L chains), where the H chains lack the CH1 domain (15).

The body of knowledge of antibody secretion has been greatly expanded since Scharff et. al. (2) described the isotype-specific assembly pathways. However, many steps in folding and transport remain to be elucidated. It might have been expected that residues critical to assembly and secretion would lie in highly conserved regions of the H and L chain sequences. Therefore, it was surprising

when we identified mutations in the H chain complementarity determining region 2 (HCDR2) of a murine anti-phosphocholine (PC) antibody, T15, which reduced secretion to less than 10% of wild type levels (16). The secretion defect in these mutants appears to result from inefficient H-L assembly, although the precise mechanism is not yet clear. Interestingly, when the same HCDR2 mutations were placed in a closely related antibody, D16, with an identical VH segment but different HCDR3 and L chain, antibody secretion was not affected. In this report we explore the basis for the different effects of these mutations on antibody secretion in T15 and D16 by testing the influence of L chain association and HCDR3 length, which are the main distinctions between the two antibodies. We demonstrate that replacement of the L chain does not restore antibody secretion in the T15 HCDR2 mutants. However, shortening the length of T15 HCDR3 to equal that of D16, rescues antibody secretion from the defect caused by mutation in HCDR2 in all four T15 mutant antibodies. Thus, mutations in HCDR2 of T15 antibodies that lead to defective secretion can be successfully complemented by altering the length of HCDR3. These results suggest that the sequence integrity of both HCDR2 and HCDR3 may be critical to Ig secretion competence.

Materials and Methods

Plasmid constructs: To create the deletion mutants, a synthetic mutant oligonucleotide was designed to eliminate the SSYW amino acid sequence at positions 99 - 100b (99-100i according to Kabat (17)) of the H chain DJ region of T15 wild type (WT) and T15 low secretor mutants, see Figure 1. Site-directed mutagenesis was performed with the mutagenic oligo, 5'-ccagacatcgaagtaaccgtagtaatctct-3', using the Muta-gene kit (Bio-Rad Laboratories, Richmond, CA) as described previously (18). The mutant VDJ genes were subcloned into pSV2gptγ2b and re-sequenced as described previously (18) to ensure that the deletion was the only change present in the T15 WT and each of the four HCDR2 mutants.

Cell culture and stable transfection: SP2/0 cells were previously transfected with a murine Vκ22Cκ L chain gene under neomycin selection to produce the SP2/0 Vκ22 transfectant (18). D16H$^-$ is a D16 heavy chain loss variant produced in this laboratory that secretes endogenous Vκ1c L chain (16). J558L (a gift from Dr. S. Morrison, UCLA) is a Vλ1-producing heavy chain loss variant of the IgA-secreting mouse myeloma J558 (19). Transfection was performed by the lipofectin (Gibco BRL, Grand Island, NY) method described previously (20). SP2/0 Vκ22 and D16H$^-$ cells were grown in Iscove's DMEM (Gibco BRL, Grand Island, NY) plus 20% FCS to 50-80% confluence in six-well tissue culture plates and transfected with 10 μg DNA plus 30 μl lipofectin (SP2/0 Vκ22) or 30 μg DNA plus 100 μl lipofectin (D16H$^-$), or 10 μg DNA plus 50 μl lipofectin (J558L). After 24-48 h., the cells were transferred to 96-well plates in the presence of gpt selection drugs (6 μg/ml mycophenolic acid, 15 μg/ml hypoxanthine, and 250 μg/ml xanthine). Transfectants that survived selection were screened for antibody in the supernatants by sandwich ELISA. If mycophenolic acid-resistant

colonies grew yet did not secrete immunoglobulin, then Northern analysis was performed to verify adequate levels of Ig mRNA. Antibody secreting and non-secreting transfectants were expanded and frozen in liquid N_2. For transfections into J558L, the initial ELISA screening process included cellular lysates to verify adequate levels of Ig production.

Northern blot analyses: Northern analysis was done as previously described (21) to determine that H and L chain mRNA was present in transfectants of SP2/0 and D16H$^-$ that exhibited a secretion defect. Cytoplasmic RNA (5 μg) isolated by a miniprep method (22), was electrophoresed on a 1% agarose gel for 5 hours and then transferred to Nytran membranes (Schleicher & Schuell, Keene, NH). Blots were hybridized with probes for VHS107 (23), Vκ22 (24), Vκ1 (25), and the housekeeping gene CHO-A (26). Northern analyses indicated that low secretor clones contained H chain and L chain mRNA at levels comparable to or above those of T15 WT transfectants (data not shown).

ELISA: A sandwich ELISA method described previously (27) was used to screen for and quantify immunoglobulin proteins from the stable transfectants. Plates were coated at 1 μg/ml with goat anti-mouse IgG, or goat anti-mouse λ, or goat anti-mouse κ (Southern Biotechnology Associates, Inc., Birmingham, AL), or rabbit anti-mouse IgG2b (Zymed, South San Francisco, CA). Captured immunoglobulin proteins were then detected with alkaline phosphatase-labeled rabbit anti-mouse IgG2b (Zymed Laboratories, South San Francisco, CA), or goat anti-mouse κ or goat anti-mouse λ (Southern Biotechnology Associates, Inc., Birmingham, AL). Purified antibody from the T15 WT stable transfectant (γ2b, κ) or the hybridoma M3C65 (γ2b, λ) (20) was used to generate standard curves.

Antigen binding was determined by direct binding ELISA on plates coated with PC-histone (1 μg/ml) as described previously (27). Supernatants from 80-90% confluent cultures (which were initially plated at the same cell density) were tested by ELISA. Detection of antibody was as above. Immunoglobulins were assessed as either binding or non-binding based on an O.D. >0.1 under conditions where WT supernatants gave an OD of >0.8 (D16) or >1.5 (T15).

Preparation of four-hour titer samples: To determine the amount of antibody in supernatants and lysates, assays were performed based on a previously described method (16). $1x10^6$ or $2x10^6$ cells were cultured for 4 h. before supernatants and lysates were collected. Cells were lysed in the same volume in which they were cultured using ice-cold PBS containing 25 mM iodoacetamide, 20 μg/ml soybean trypsin inhibitor, 50 μg/ml PMSF and 0.25% NP-40 (Sigma Chemical Co., St. Louis, MO). Lysates were incubated on ice for 1 h. and nuclei and debris were then removed by centrifugation. All samples were kept ice-cold and assayed within 24 h. of preparation. Samples were titered in triplicate wells by sandwich ELISA and quantified relative to standard curves of isotype-matched purified antibodies (described above).

Statistical analysis: Results from quantitative ELISA titers for Figure 2 represent three or more determinations and are expressed as mean + SEM. For titers that were undetectable, the detection limit (0.4 ng/ml) of the assay was used. For statistical analysis, one-way analysis of variance was performed using Multistat software (Biosoft, Cambridge, UK). Statistical significance was set at $p < 0.05$.

Computer modeling: The model of T15 Fv was constructed previously (28). The positions of amino acid side chains were examined with computer graphics program Insight II, Biosym Technologies, San Diego, CA.

Results

Defective secretion of mutant T15 H chains paired with other L chains: Previous work showed that four HCDR2 mutants of the anti-PC antibody T15 (M153, M164, M166, and M241) are defective in their ability to secrete antibody (16). Each of the HCDR2 mutants has from two to four amino acid changes in this region, spanning residues 50-65 (Figure 1). When the same HCDR2 mutations were placed in a related antibody, D16, normal antibody secretion was observed (16). These two antibodies, T15 and D16, share identical unmutated VH gene segments and both bind to PC-protein. One major difference between them that could potentially influence secretion of antibodies containing the mutated H chains is the use of different L chains. T15 utilizes the Vκ22 L chain, while D16 uses Vκ1c. The T15 L chain is one of only a small number of L chains that are not secreted in the absence of H chain (5, 16, 29). In contrast the D16 L chain is readily secreted in the absence of H chain. Failure of the T15 L chain to be secreted on its own might account for the differences between the T15 and D16 mutants, particularly if secretion with such L chains was more dependent on the H chain than for L chains that can be secreted with or without H chain.

To examine the possible contribution of the L chain to defective secretion, T15 WT or T15 mutant H chains were transfected into a D16 H chain loss variant ($D16H^-$), resulting in antibodies comprised of a T15 H chain (WT or mutant) with the D16 L chain. As measured by ELISA, none of the mutant H chains were secreted with D16 L chain even though cells co-expressing the T15 WT H

chain/D16 L chain produced 100-fold greater levels of supernatant Ig than T15 WT itself (Table I). In addition to D16 L chain, the J558L L chain, which is also secreted in the absence of H chain, was tested for its ability to influence secretion when paired with T15 WT or mutant H chains. This was accomplished by stable transfection of the T15 H chains into J558L, an H chain loss variant of the murine myeloma J558 which expresses Vλ1. As shown in Table I, the T15 WT H chain/J558L L chain complex was readily secreted (see supernatant column), while <1% of WT levels was detected in supernatants of the corresponding T15 mutant H chain/Vλ1 transfectants.

The amounts of intracellular H chain, L chain, and H + L complexes in these transfectants were also quantified (Table I). All transfectants contained abundant intracellular H and L chain protein, and for the T15 WT H chains, essentially all of the intracellular H chain appeared to be complexed with L chain (compare the "Intracellular H" and "Intracellular H + L" columns of Table I). In contrast, little of the mutant T15 H chain protein appeared complexed with L chain. Thus neither D16 L chain nor J558L L chain was better at pairing with the mutant H chains than the original T15 L chain. In comparison to the original T15 mutants, the D16 and J558L L chains were expressed at much higher intracellular levels, possibly because the latter are endogenous rather than transfected. D16 and J558L L chains were secreted in the absence of H chains and the level of intracellular and supernatant L chain protein in untransfected $D16H^-$ and J558L cells was comparable to that of the transfectants (data not shown). These results indicate that mutations in T15 HCDR2 which result in defective assembly and secretion do not depend on the inability of the L chain itself to be secreted and thus are not due exclusively to particular interactions with the Vκ22 L chain.

Secretion analysis of T15 H chains with a shortened HCDR3: Although the T15 and D16 WT H chains have identical VH segments, they differ in the DJ region (of which HCDR3 is a component). While there are differences at residues 95, 96, 98, and 101 the most striking feature is that T15 is longer than D16 by four residues at the DJ junction (Figure 1). Since differences in L chain association could not account for the inability of the low secretor mutations to prevent secretion of the D16 antibody, we investigated the effect of DJ length. Four amino acid residues, SSYW (99 - 100b), were deleted from the DJ junctions of T15 WT and mutant H chains to mimic the length of DJ in D16 (Figures 1 and 3). The shortened H chains were expressed with either T15 or D16 L chains. Secretion of antibodies containing the four residue deletion was determined by quantitative ELISA. Placing the deletion in the T15 WT H chain did not affect its secretion when paired with either T15 L chain (Figure 2A) or D16 L chain (Figure 2B). In marked contrast, deleting the four residues in DJ of the mutant H chains had a profound effect: antibody secretion was restored to 50-80% of WT with T15 L chain (Figure 2A) and 30-75% of WT with D16 L chain (Figure 2B). Purified supernatant antibody of mutant H chains with shortened HCDR3s co-expressed with either the T15 or D16 L chain were run on non-reducing SDS-PAGE and migrated appropriately for H_2L_2, as did the T15 WT with a shortened H chain expressed with T15 L chain (data not shown). It is clear from these data that shortening T15 HCDR3 restores secretion of defective antibodies containing HCDR2 mutations.

Discussion

We previously identified mutations in the HCDR2 region of the T15 anti-PC antibody which resulted in greatly reduced antibody secretion (16). Each of four T15 mutants, M153, M164, M166, and M241, contains two to four amino acid substitutions (Figure 1), involving at least one highly conserved residue

important in determining the canonical HCDR2 structure (30) and/or assignment to H chain subgroup III(A) (17). Other HCDR2 mutants with similar amino acid changes, but in different combinations than those in the secretion defective mutants are capable of secretion (18). The defect is characterized by failure to secrete H+L complexes, despite abundant intracellular mutant H chain (16). Anti-idiotope binding analysis of the original secretion defective mutants indicated that, in the small fraction which contains assembled antibody, H and L chains are assembled accurately enough to be recognized by antibodies to idiotopes formed by the H-L complex, although an H chain-specific idiotope recognized by the monoclonal antibody TC54 was decreased or lost in at least three of the mutants (16) . Furthermore, associated H+L from the lysate of one mutant, M153, binds antigen similarly to T15 WT lysate preparations (16). Thus, even though the mutations in HCDR2 apparently affect H-L pairing, whatever conformational abnormalities may result, the idiotype is still expressed upon H-L assembly.

The finding that these same HCDR2 amino acid changes did not prevent secretion when placed into the H chain of a highly related antibody, D16, provided an opportunity to examine specific differences in structure that might influence antibody secretion. The role of L chain was of particular interest since unlike most L chains, the T15 L chain is not secreted on its own and thus, may be more sensitive to pairing defects with mutated H chains. The intracellular level of T15 L chain is lower in the four secretion mutants than in the T15 WT (Table I). The amount of T15 L chain in the lysate of SP2/0 transfectants expressing only this L chain is similar to quantities found in the secretion mutants (data not shown). This suggests that in the absence of functional association with an H chain, intracellular levels of T15 L chain are low, presumably due to degradation.

The T15 WT H chain was efficiently secreted when paired with either D16 or J558L L chain indicating that each of these heterologous H-L combinations are functional with respect to proper assembly and transport, although they did not bind PC-protein (data not shown). In contrast, pairing of the mutant T15 H chains with either D16 or J558L L chain did not restore secretion (Table I). These data demonstrate that the inability of the T15 L chain to be secreted in the absence of H chain is not directly involved in the low secretor defect since neither D16 nor J558L L chain could overcome the secretion defect, although they are secreted abundantly in the absence of H chain.

The mechanism by which mutations in HCDR2 might impair efficient assembly with L chain is not yet understood. Possibly mutant H chains are less stable, unable to fold properly, or have an increased tendency to aggregate. There is evidence that mutations in the second loop of HCDR2 can specifically affect folding of VH in *E. coli.* Upon introduction of Ser60 → Ala and Ala61 → Asp into McPC603 HCDR2 (numering according to Kabat (17)), aggregation was reduced and expression improved; interestingly these two changes were independent of effects on antigen binding or thermodynamic stability (31). Since McPC603 uses a VH sequence nearly identical to T15 (17), it is possible that the CDR2 mutants also have altered H chain folding. In addition, modified folding could alter interactions with molecular chaperones, which assist in protein folding and oligomerization. We have evidence that the HCDR2 mutations result in increased association with the ER-resident molecular chaperone, BiP (T. Martin, G. Wiens, and M. Rittenberg, manuscript in preparation).

The HCDR2 mutations may also perturb either the efficiency of VH-VL pairing or the stability of the complex once pairing has occured. One possibility is that these mutations directly disrupt contact with L chain. It is known that VH-VL interactions include framework (FRW) with FRW, FRW with CDR, and CDR with CDR and in general, CDRs contribute 26-57% of all atomic interactions between VH and VL (32). In some antibody structures, such as the anti-lysozyme HyHEL-10, HCDR2 makes a number of direct contacts with LCDR3 (32). However, it is not known how significantly HCDR2 and LCDR3 interactions contribute to H-L association in T15.

Besides direct contact with L chain, HCDR2 may have long range effects influencing other residues in the VH-VL interface. Recent crystallographic evidence demonstrated that replacement of the second loop of HCDR2 of the humanized anti-CD18 antibody, huH52, with murine residues 59-65 caused a 1.5Å change in the orientation of the side chain of H chain Trp47 (33), a residue with a large buried surface area in VH-VL interface (34). In addition the change in HCDR2 was associated with a 5.2Å shift in the rotation angle of the VH with respect to VL, although the explanation is not clear as the crystal packing structures were different and one was an Fv and the other an Fab (33). Nevertheless, these results suggest the possibility that mutation in T15 HCDR2 may have long range effects that influence the VH-VL interface.

The HCDR2 of T15 is depicted in the model of T15 by a solid black ribbon (Figure 3) and consists of two loop regions, amino acid residues 50-58 and 59-65 (28). Interestingly, all four of the low secretor mutants have at least one change in the second loop of HCDR2, a region generally considered to be outside the combining site and distant from the other hypervariable regions (28, 35-37),

suggesting that the HCDR2 mutations may indirectly affect H chain contacts with L chain. One residue in the second loop region, Tyr59, is highly conserved among all H chain sequences (17), and in the model of T15 (Figure 3), the Tyr59 side chain is oriented toward the FRW3 region of VH, i.e. away from the binding pocket and away from the L chain. Two of the four secretion mutants have a serine substituted here in addition to an amino acid change in the first loop at position 51 (M241) or 52 (M164) (Figure 1). It is not clear how this residue might contribute to secretion, and the Tyr59→Ser mutation alone is not capable of causing the defect as this single mutant secretes normally (T.M.M., unpublished results). The Tyr59→Ser change coupled with a mutation in the first loop of HCDR2 (residues 50-58) appears to be sufficient to create a secretion defect, as evidenced by M164 which has only these two changes. In addition, the mutation in M241 at position 54 probably does not contribute significantly to the defect since a double mutant containing only the two other changes in M241 (Ser51→Arg and Tyr59→Ser) also fails to secrete (T.M.M. unpublished data). Mutants M153 and M166 have a change at either position 58 (Glu→Gly) or 60 (Ser→Thr), respectively (i.e. flanking Tyr59), coupled with two other amino acid substitutions in this second loop. Taken together, these data suggest that particular, multiple amino acid changes perturb the structure of the HCDR2 sufficiently to prevent secretion, possibly by altering interaction with L chain.

In addition to the use of another L chain, the D16 antibody also differs from T15 in DJ, which is highly diverse among antibodies (17). Mutations in HCDR3 which involve amino acid substitutions as well as changes in length may affect antigen binding specificity, affinity or idiotype expression (38-42). The HCDR3s of T15 and D16 are comprised of residues 95-102 (Figure 1), where they differ at four positions and in length. Homology sequence alignment reveals that T15

HCDR3 contains four amino acid residues between 98 and 100c (98 and 100j in Kabat (17)) which are absent from D16 HCDR3. We tested the contribution of HCDR3 length to the secretion defect by removing these four amino acid residues from T15 H chains: Ser99, Ser100, Tyr100a, and Trp100b. The shortened HCDR3 had a dramatic effect on the T15 mutants; secretion capability was restored regardless of whether the mutant H chains were associated with T15 or D16 L chains (Figure 2). In the current model of T15 (28), these residues are located on the apex and side of the HCDR3 region (Figure 3) and residues 99, 100a, and 100b make a number of contacts with L chain CDR and FRW residues. These results suggest that shortening HCDR3 may correct or compensate for the disruption caused by the mutations in HCDR2. Our findings underscore the importance of HCDR3 length in determining the secretion phenotype of HCDR2 mutants. Detailed analysis of the contribution of the individual positions in HCDR3 to restoring the secretion defect is currently underway using site-directed mutagenesis.

We have shown that perturbing the HCDR2 region can have profound effects on immunoglobulin functions in addition to antigen binding (16). The secretion defect was unexpected in view of the hypervariable nature of CDRs. Here we demonstrate that the low secretor phenotype of the H chain is independent of the L chain partner, since two heterologous L chains efficiently assemble and are secreted with T15 WT, but not with the mutant H chains. In addition, we provide evidence that the HCDR2 and HCDR3 regions can both influence immunoglobulin secretion. Although the mechanism of the secretion defect is unknown, it is characterized by a failure to assemble efficiently with L chain. Most importantly, however, is the implication that the flexible and variable nature of the CDR loops that are so important for antigen binding, may as a

result of mutations disastrously alter other functions of antibodies, leading to their disappearance from the immune response.

Acknowledgments

We are grateful to Dr. Victoria A. Roberts for critical discussions and advice. Thanks to McKay Brown, Dr. Thomas O'Hare, and Dr. Elizabeth A. Whitcomb for critical reading of the manuscript. We also thank Jeremy C. Morse for superb technical assistance, and Janet E. Duncan for statistical advice.

References

1. Hurtley, S. M., and A. Helenius. 1989. Protein oligomerization in the endoplasmic reticulum. *Ann. Rev. Cell Biol. 5:277.*
2. Scharff, M. D. 1975. The synthesis, assembly, and secretion of immunoglobulin: A biochemical and genetic approach. *Harvey Lect. 69:125.*
3. Dul, J. L., and Y. Argon. 1990. A single amino acid substitution in the variable region of the light chain specifically blocks immunoglobulin secretion. *Proc. Natl. Acad. Sci. USA 87:8135.*
4. Dul, J. L., O. R. Burrone, and Y. Argon. 1992. A conditional secretory mutant in an Ig L chain is caused by replacement of tyrosine/phenylalanine 87 with histidine. *J. Immunol. 149:1927.*
5. Pillai, S. 1991. Immunoglobulin transport in B cell development. *Int'l. Rev. Cyt. 130:1.*
6. McCune, J. M., and S. M. Fu. 1981. Ig biosynthesis in a human pre-B cell line. *J. Immunol. 127:2609.*
7. Pepe, V. H., G. E. Sonenshein, M. I. Yoshimura, and M. J. Shulman. 1986. Gene transfer of immunoglobulin light chain restores heavy chain secretion. *J. Immunol. 137:2367.*
8. Morrison, S. L., and M. D. Scharff. 1975. Heavy chain-producing variants of a mouse myeloma cell line. *J. Immunol. 114:655.*
9. Knittler, M. R., and I. G. Haas. 1992. Interaction of BiP with newly synthesized immunoglobulin light chain molecules: cycles of sequential binding and release. *EMBO J. 11:1573.*
10. Wu, G. E., N. Hozumi, and H. Murialdo. 1983. Secretion of a λ2 immunoglobulin chain is prevented by a single amino acid substitution in its variable region. *Cell 33:77.*

11. Kaloff, C. R., and I. G. Haas. 1995. Coordination of immunoglobulin chain folding and immunoglobulin chain assembly is essential for the formation of functional IgG. *Immunity 2:629.*

12. Hendershot, L., D. Bole, G. Köhler, and J. F. Kearney. 1987. Assembly and secretion of heavy chains that do not associate posttranslationally with immunoglobulin heavy chain-binding protein. *J. Cell Biol. 104:761.*

13. Bole, D. G., L. M. Hendershot, and J. F. Kearny. 1986. Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol. 102:1558.*

14. Sitia, R., M. Neuberger, C. Alberini, P. Bet, A. Fra, C. Valetti, G. Williams, and C. Milstein. 1990. Developmental regulation of IgM secretion: the role of the carboxy-terminal cysteine. *Cell 60:781.*

15. Hamers-Casterman, C., T. Atarhouch, S. Muyldermans, G. Robinson, C. Hamers, E. B. Songa, N. Bendahman, and R. Hamers. 1993. Naturally occurring antibodies devoid of light chains. *Nature 363:446.*

16. Chen, C., T. M. Martin, S. Stevens, and M. B. Rittenberg. 1994. Defective secretion of an immunoglobulin caused by mutations in the heavy chain complementary determining region 2. *J. Exp. Med. 180:577.*

17. Kabat, E. A., T. T. Wu, H. M. Perry, K. S. Gottesman, and C. Foeller. 1991. *Sequences of Proteins of Immunological Interest*. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Washington, D. C.

18. Chen, C., V. A. Roberts, and M. B. Rittenberg. 1992. Generation and analysis of random point mutations in an antibody CDR2 sequence: Many mutated antibodies lose their ability to bind antigen. *J. Exp. Med. 176:855.*

19. Weigert, M. G., I. M. Cesari, S. J. Yonkovich, and M. Cohn. 1970. Variability in the lambda light chain sequences of mouse antibody. *Nature 228:1045.*

20. Brown, M., M. P. Stenzel-Poore, S. Stevens, S. K. Kondoleon, J. Ng, H. P. Bachinger, and M. B. Rittenberg. 1992. Immunologic memory to phosphocholine keyhole limpet hemocyanin. Recurrent mutations in the lambda 1 light chain increase affinity for antigen. *J. Immunol. 148:339.*

21. Buenafe, A., and M. B. Rittenberg. 1989. Molecular analysis and fine specificity of antibodies against an organophosphorus hapten. *J. Immunol. 143:539.*

22. Wilkinson, M. 1988. RNA isolation: a mini-prep method. *Nucleic Acids Res. 16:10933.*

23. Early, P., H. Huang, M. Davis, K. Calame, and L. Hood. 1980. An immunoglobulin heavy chain variable region is generated from three segments of DNA: VH, D and JH. *Cell 19:981.*

24. Kwan, S. P., S. Rudikoff, J. G. Seidman, P. Leder, and M. D. Scharff. 1981. Nucleic acid and protein sequences of phosphocholine-binding light chains. *J. Exp. Med. 153:1366.*

25. Stenzel-Poore, M. P., and M. B. Rittenberg. 1989. Clonal diversity, somatic mutation, and immune memory to phosphocholine-keyhole limpet homocyanin. *J. Immunol. 143:4123.*

26. Harpold, M. M., R. M. Evans, M. S. Salditt-Georgieff, and J. E. Darnell. 1979. Production of mRNA in Chinese hamster cells: relationship of the rate of synthesis to the cytoplasmic concentration of nine specific mRNA sequences. *Cell 17:1025.*

27. Chang, S. P., M. Brown, and M. B. Rittenberg. 1982. Immunologic memory to phosphorylcholine. II. PC-KLH induces two antibody populations that dominate different isotypes. *J. Immunol. 128:702.*

28. Brown, M., M. B. Rittenberg, C. Chen, and V. A. Roberts. 1996. Tolerance to single, but not multiple, amino acid replacements in antibody VH CDR2. A

means of minimizing B cell wastage from somatic hypermutation? *J. Immunol. 156:3285.*

29. Oi, V. T., S. L. Morrison, L. A. Herzenberg, and P. Berg. 1983. Immunoglobulin gene expression in transformed lymphoid cells. *Proc. Natl. Acad. Sci. USA 80:825.*
30. Chothia, C., A. M. Lesk, A. Tramontano, M. Levitt, S. J. Smith-Gill, G. Air, S. Sheriff, E. A. Padlan, D. Davies, W. R. Tulip, P. M. Colman, S. Spinelli, P. M. Alzari, and R. J. Poljak. 1989. Conformations of immunoglobulin hypervariable regions. *Nature 342:877.*
31. Knappik, A., and A. Plückthun. 1995. Engineered turns of a recombinant antibody improve its in vivo folding. *Protein Engineering 8:81.*
32. Padlan, E. A. 1994. Anatomy of the antibody molecule. *Mol. Immunol. 31:169.*
33. Eigenbrot, C., T. Gonzalez, J. Mayeda, P. Carter, W. Werther, T. Hotaling, J. Fox, and J. Kessler. 1994. X-Ray structures of fragments from binding and nonbinding versions of a humanized anti-CD18 antibody: Structural indications of the key role of V_H residues 59 to 65. *Prot. Struct. Funct. Genet. 18:49.*
34. Chothia, C., J. Novotny, R. Bruccoleri, and M. Karplus. 1985. Domain association in immunoglobulin molecules: the packing of variable domains. *J. Mol. Biol. 186:651.*
35. Davies, D. R., E. A. Padlan, and S. Sheriff. 1990. Antibody-antigen complexes. *Annu. Rev. Biochem. 59:439.*
36. Chen, C., V. A. Roberts, S. Stevens, M. Brown, M. P. Stenzel-Poore, and M. B. Rittenberg. 1995. Enhancement and destruction of antibody function by somatic mutation: unequal occurrence is controlled by V gene combinatorial associations. *EMBO 14:2784.*

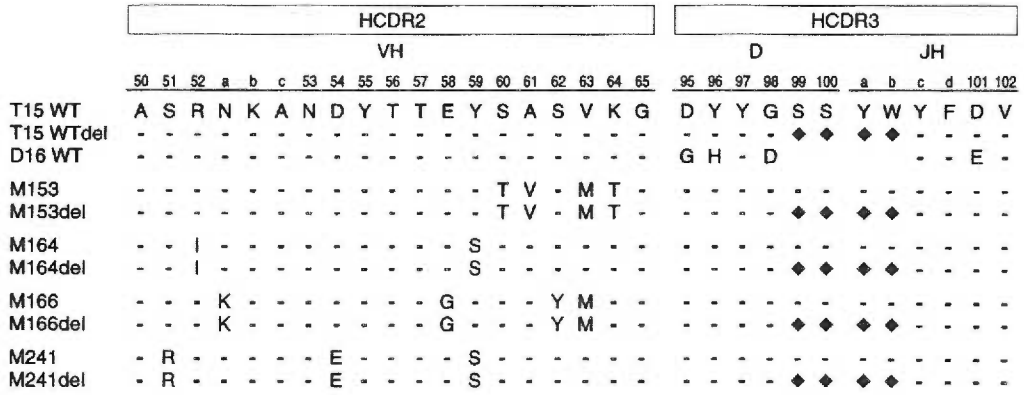


Figure 1. Relevant amino acid sequences of T15 WT and related H chains. The HCDR2 region is 19 residues in length (residues 50-65), and the changes in this region found in the secretion mutants of T15 are shown (i.e. M153, M164, M166, M241). The HCDR3 region is encoded by the D and part of JH (residues 95-102). The D16 WT DJ region is homology-aligned to that of T15 WT and does not contain residues 99-100b, corresponding to positions 99-100i, as defined in Reference 17. Three sequence differences between T15 WT and D16 WT occur in D at positions 95, 96, and 98 as shown. The JH region continues to amino acid residue 113, and there are three differences in JH between these two antibodies, one at position 101 (shown) and two at positions 105 and 109 (not shown). A dash denotes sequence identity with T15 WT, and a diamond indicates residues that were deleted via site-directed mutagenesis (see methods). All numbering is based on Kabat (17) except for residues 100a-100d (28).

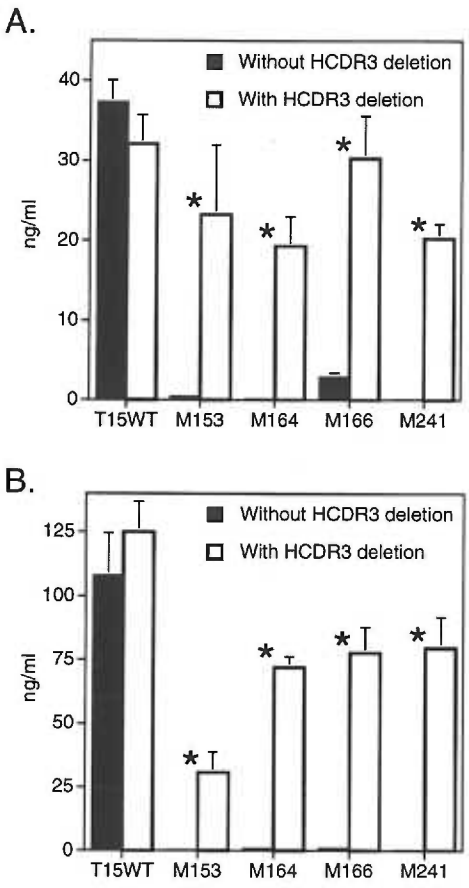


Figure 2. Effect of the four amino acid deletion in HCDR3 on antibody secretion. Antibody (ng/ml) secreted into the supernatant from $2x10^6$ cells cultured in 4 ml medium for 4 h. was quantified by sandwich ELISA (H+L) with triplicate wells per point. Data shown represent the mean (+ SEM) of three or more separate determinations done in triplicate per transfectant. *(A).* T15 WT and mutant H chains are expressed as stable transfectants with the T15 L chain in SP2/0 cells. *(B).* The same H chains are expressed as stable transfectants in $D16H^-$ cells which endogenously produce the D16 L chain. For sets A and B separately, paired secretion values for H chains with and without the HCDR3 deletion were tested for significance by analysis of variance. An asterisk denotes $p < 0.05$ for the difference between each pair. The values for the T15 WT pairs were not significantly different.

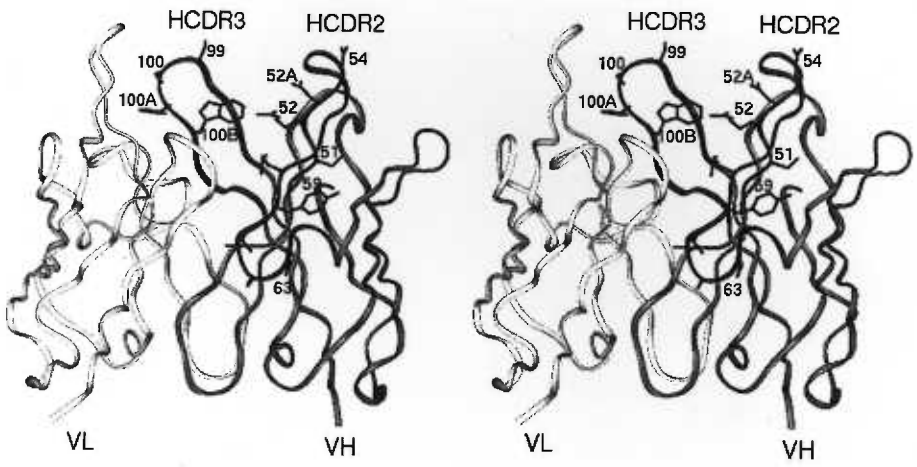


Figure 3. Stereo model of the T15 Fv (side view) with the VL on the left in light gray and VH on the right in dark gray. HCDR2 (residues 50-65) and HCDR3 (residues 95-102) of VH are depicted as black ribbons. The side chains shown in HCDR3 indicate the 4 amino acid residues (99, 100, 100a, and 100b) which were deleted from this loop region. Selected side chains are shown in HCDR2 to indicate the orientation of this loop region, and to depict certain amino acid residues which are mutated in one or more of the secretion-defective mutants. This model was constructed by V. A. Roberts based on the McPC603 crystal structure and is described elsewhere (28).

Table I. *Immunoglobulin protein levels of WT and mutant H chains expressed with different L chains.*

		SUPERNATANT				INTRACELLULAR					
		H + L		L		H		H + L		L	
H	L	%WT [a]	ng	%WT	ng	%WT	ng	%WT	ng	%WT	ng
T15 WT	T15	100 [b]	(33)	ND [c]	ND	100 [b]	(17)	100 [b]	(13)	100	(17)
M153	T15	<1		ND		185		36		18	
M164	T15	<1		ND		74		8		4	
M166	T15	<1		ND		68		1		17	
M241	T15	<1		ND		102		5		6	
T15 WT	D16	100	(3314)	100	(5491)	100	(3517)	100	(3311)	100	(4980)
M153	D16	<1		83		25		2		72	
M164	D16	<1		72		46		1		47	
M166	D16	<1		89		27		2		91	
M241	D16	<1		64		17		1		47	
T15 WT	J558	100	(769)	100	(6763)	100	(517)	100	(704)	100	(6580)
M153	J558	<1		69		21		1		63	
M164	J558	<1		65		157		3		104	
M166	J558	<1		81		99		1		102	
M241	J558	<1		103		65		2		115	

[a] Values are % of WT levels within each experimental group. Numbers in parentheses are ng protein/million cells of the WT. Data shown are the results from one representative of two experiments which gave similar results. The data represent average of samples from two separate cell cultures each assayed in triplicate ELISA wells.

[b] Data for the T15 WT and mutant H chains expressed with the T15 L chain were previously published (16).

[c] Not done because Vk22 is not secreted in the absence of H chain (16, 29)

Secretion-Defective Antibodies with Mutations in a Heavy Chain Hypervariable Region: Intracellular Accumulation and Inefficient Assembly

Tammy M. Martin, Gregory D. Wiens, and Marvin B. Rittenberg

Abstract

We previously described secretion defects in four mutants of the murine anti-phosphocholine antibody, T15. The mutant heavy chains had amino acid replacements in the VH complementarity determining region 2 and were expressed at normal intracellular levels. Here, the mechanism of the secretion defect was investigated by exploring the fate of the antibody proteins in the secretion-defective mutants. Pulse-chase/immunoprecipitation analyses revealed that the T15 wild-type antibody was secreted efficiently, within 4 hour chase, while the mutant heavy chains accumulated with intracellular half-life values of 10 to >26 hours. Mutant heavy chains associated with the molecular chaperone, BiP, at 7 to 17-fold greater levels than wild-type heavy chains and remained endoglycosidase H sensitive at 6 hour chase. The T15 wild-type antibody exhibited an $H_2 \rightarrow H_2L \rightarrow H_2L_2$ assembly pathway. The mutant heavy chains failed to associate efficiently with L chain, exhibiting a relatively stable H_2 subpopulation. Therefore, the secretion defect in these mutants is consistent with impaired or inefficient assembly with L chain and accumulation of mutant heavy chains in the ER. This study strengthens the emerging view that hypervariable regions make structural contributions to antibody functions other than antigen binding.

Introduction

Somatic hypermutation in rearranged variable regions (V) introduces additional diversity into an ongoing antibody response (1). Thus, antibodies provide a unique system in which to study the effects of mutation not only on antigen binding, but also on fundamental processes common to most proteins such as folding, assembly, transport and secretion.

Generally, oligomeric proteins first attain a correctly folded and assembled state in the endoplasmic reticulum (ER) before being transported through the Golgi and to the plasma membrane or out of the cell (2). An array of enzymatic processes contribute to the proper folding of a protein, including protein disulfide isomerization, addition of oligosaccharides, modifications of amino acid side chains, and interaction with molecular chaperones (2). The immunoglobulin (Ig) folding process is marked by the transient and sequential interaction of nascent heavy (H) chains and light (L) chains with BiP and Grp94, two ER-resident molecular chaperones (3). Abnormal Igs resulting from mutations in H chains or L chains have led to misfolding, altered chaperone binding, destabilization or aggregation (4-8). The aforementioned defects in protein processing may ultimately contribute to a loss of secretion.

We previously generated a library of mutants of the T15 antibody with the mutations confined to heavy chain complementarity determining region 2 (HCDR2) (9). We identified four secretion-defective variants with two to four amino acid replacements, which provided the first evidence that a heavy chain variable region could contribute to secretion competence of an Ig molecule (10). The HCDR2, like the other CDRs of antibody H and L chains,

frequently displays mutations acquired during the process of affinity maturation. Thus the secretion defect of these T15 HCDR2 mutants was surprising since this region would be expected to be highly tolerant of mutation, consistent with its functional role in antigen binding.

The basis for the secretion defect in these mutants is unclear. From the previous study, it was apparent that there was little association between the mutant H chains with L chain based on detection of complexes by sandwich ELISA (10). The HCDR2 of T15 is 19 amino acid residues in length (50-65, numbering based on Kabat (11)) and forms two loop structures, the first loop (50-58) which is highly solvent exposed and contains residues that provide important antigen contacts, and a second loop (59-65) which is oriented away from the combining site and is not known to contact antigen (12); the second loop is in close proximity to VH framework residues (13). Mutant M153 has four changes that are all confined to the second loop of HCDR2: S60→T, A61→V, V63→M, and K64→T. The other three mutants have at least one change in the first loop coupled with one or more replacements in the second loop, M164 (R52→I and Y59→S), M166 (N52a→K, E58→G, S62→Y, and V63→M), and M241 (S51→R, D54→E, and Y59→S). When these same mutations were placed in HCDR2 of another antibody with the identical VH gene sequence, but differing in HCDR3 sequence and L chain association, secretion was not impaired. Thus it was clear that the mutations do not completely destroy this domain of the protein (10). Furthermore, deleting four residues in HCDR3 restored secretion in the T15 mutants[4], again supporting the notion that the mutations in HCDR2 do not irreversibly destroy domain function.

In order to gain further insight into the nature of the defect, we have analyzed the fate of the T15 mutant H chains compared to the efficient assembly and transport of the T15 wild type (WT) antibody. In the present study, we show that the mutant H chains accumulate intracellularly in the ER with long half-lives, and are associated with high levels of the molecular chaperone BiP, relative to T15 WT. We also demonstrate that assembly with L chain is inefficient, with a substantial portion of mutant H chains arrested at an H_2 assembly intermediate stage.Somatic hypermutation in rearranged variable (V) regions introduces additional diversity into an ongoing antibody response (1). Thus, antibodies provide a unique system in which to study the effects of mutation not only on antigen binding, but also on fundamental processes common to most proteins such as folding, assembly, transport and secretion.

Generally, oligomeric proteins first attain a correctly folded and assembled state in the endoplasmic reticulum (ER) before being transported through the Golgi, and to the plasma membrane or out of the cell (2). An array of enzymatic processes contribute to the proper folding of a protein, including protein disulfide isomerization, addition of oligosaccharides, modifications of amino acid side chains, and interaction with molecular chaperones (2). The immunoglobulin (Ig) folding process is marked by the transient and sequential interaction of nascent heavy (H) chains and light (L) chains with BiP and Grp94, two ER-resident molecular chaperones (3). Abnormal Igs resulting from mutations in H chains or L chains have led to misfolding, altered chaperone binding, destabilization or aggregation (4-8). The aforementioned defects in protein processing may ultimately contribute to a loss of secretion.

We previously generated a library of mutants of the T15 antibody with the mutations confined to heavy chain complementarity determining region 2 (HCDR2) (9). We identified four secretion-defective variants with two to four amino acid replacements, which provided the first evidence that a heavy chain variable region could contribute to secretion competence of an Ig molecule (10). The HCDR2, like the other CDRs of antibody H and L chains, frequently displays mutations acquired during the process of affinity maturation. Thus the secretion defect of these T15 HCDR2 mutants was surprising since this region would be expected to be highly tolerant of mutation, consistent with its functional role in antigen binding.

The basis for the secretion defect in these mutants is unclear. From the previous study, it was apparent that there was little association between the mutant H chains with L chain based on detection of complexes by sandwich ELISA (10). The HCDR2 of T15 is 19 amino acid residues in length (50-65, numbering based on Kabat (11)) and forms two loop structures, the first loop (50-58) which is highly solvent exposed and contains residues that provide important antigen contacts, and a second loop (59-65) which is oriented away from the combining site and is not known to contact antigen (12); the second loop is in close proximity to VH framework residues (13). Mutant M153 has four changes that are all confined to the second loop of HCDR2: S60→T, A61→V, V63→M, and K64→T. The other three mutants have at least one change in the first loop coupled with one or more replacements in the second loop, M164 (R52→I and Y59→S), M166 (N52a→K, E58→G, S62→Y, and V63→M), and M241 (S51→R, D54→E, and Y59→S). When these same mutations were placed in HCDR2 of another antibody with the identical VH

gene sequence, but differing in HCDR3 sequence and L chain association, secretion was not impaired. Thus it was clear that the mutations do not completely destroy this domain of the protein (10). Furthermore, deleting four residues in HCDR3 restored secretion in the T15 mutants (T. M. Martin, C. Kowalczyk, S. Stevens, G. D. Wiens, M. P. Stenzel-Poore, and M. B. Rittenberg, submitted for publication), again supporting the notion that the mutations in HCDR2 do not irreversibly destroy domain function.

In order to gain further insight into the nature of the defect, we have analyzed the fate of the T15 mutant H chains compared to the efficient assembly and transport of the T15 wild type (WT) antibody. In the present study, we show that the mutant H chains accumulate intracellularly in the ER with long half-lives, and are associated with high levels of the molecular chaperone BiP, relative to T15 WT. We also demonstrate that assembly with L chain is inefficient, with a substantial portion of mutant H chains arrested at an H_2 assembly intermediate stage.

Materials & Methods

Cell Culture and Transfection: SP2/0 cells and stable transfectants of these cells were cultured in Iscove's modified Dulbecco's medium (IMDM, Gibco BRL, Grand Island, NY) containing 20% FCS as described previously (10). The transfectants expressing T15 WT H chains with T15 L chains, T15 mutant H chains with T15 L chains, and T15 L chains alone were previously described (9, 10). Transfections of T15 WT and mutant H chains into SP2/0 (without L chain) were performed by the lipofectin (Gibco BRL, Grand Island, NY) method (14) . Briefly, cells grown to sub-confluence in 6-well plates were

transfected with 15 μg of the plasmid construct pSV2gptS107γ2b (9), containing T15 WT or mutant H chains mixed with 30 μl lipofectin. After 1-2 days, cells were plated with selection reagents (6 μg/ml mycophenolic acid, 15 μg/ml hypoxanthine, and 250 μg/ml xanthine) in 96-well plates. Approximately 2-3 weeks post transfection, drug-resistant colonies were screened for production of intracellular H chain. Cells to be screened were suspended in lysis buffer (PBS containing 25 mM iodoacetamide, 20 μg/ml soybean trypsin inhibitor, 50 μg/ml PMSF, and 0.25% NP-40; all from Sigma Chemical Co., St. Louis, MO) and lysates were analyzed by ELISA on plates coated with goat anti-mouse IgG (Southern Biotechnology Associates, Inc., Birmingham, AL) or rabbit anti-mouse IgG2b (Zymed Laboratories, South San Francisco, CA). Bound protein was detected using alkaline phosphatase (AP)-labeled rabbit anti-mouse IgG2b (Zymed Laboratories, South San Francisco, CA). Stable transfectants producing intracellular H chain were expanded and frozen in liquid N_2.

Metabolic Labeling, Immunoprecipitation and SDS-Polyacrylamide Gel Electrophoresis: Washed monolayers of SP2/0 transfectants from sub-confluent 35 mm culture wells were depleted of intracellular stores of methionine (met) and cysteine (cys) by a 20 min incubation in DMEM without met, cys (Sigma Chemical Co., St. Louis, MO). Cells were then metabolically labeled in 0.4 ml labeling medium (DMEM without met, cys + 1% FCS + 0.4 mCi/ml [^{35}S] Express Protein Labeling Mix (Dupont NEN, Wilmington, DE)) for a 15' pulse, except BiP assays which were labeled for 4.5 h with no subsequent chase. Labeling medium was removed and cells were washed in ice-cold PBS (except in BiP binding experiments where 130 mM NaCl, 20 mM Bicine, pH 8.0 was used), then incubated in IMDM + 20% FCS for the duration of the chase. At the end of each chase, supernatants were

collected and cells were washed again. The monolayers were lysed on the plate in lysis buffer for 3 min, lysates were collected, iced 60 min, and then centrifuged to spin out the nuclei and debris. The lysis buffer used depended on the particular experiment - for the accumulation/degradation analyses: 0.025 M iodoacetamide, 20 μg/ml soybean trypsin inhibitor, 0.25% NP-40, 50 μg/ml PMSF, 1% sodium deoxycholate, and 0.1% SDS in PBS; for assembly studies: 0.005 M iodoacetamide, 20 μg/ml soybean trypsin inhibitor, 0.5% triton X-100, 50 μg/ml PMSF, 1% sodium deoxycholate, and 0.1% SDS in a TSA buffer (0.01 M Tris·Cl pH 8.0, 0.14 M NaCl, 0.025% NaN_3); and for BiP binding experiments: 50 mM Bicine, 40 mM NaCl, 5 mM $CaCl_2$, 5 mM KCl, 10 mM $Na_2MoO_4 \cdot 2H_2O$, 1% NP-40, pH 8.0 as in (15).

Immunoprecipitations were performed by incubating samples with rabbit anti-mouse κ (Cappel Organon Teknika, Durham, NC) on ice for 60 min, followed by Protein A-Sepharose (Pharmacia, Piscataway, NJ) overnight at 4°C to precipitate the anti-κ antibodies as well as the T15 H chains. The anti-κ incubation was not included for all experiments (see figure legends). Immunoprecipitates were washed twice in the appropriate lysis buffer, once in TSA buffer and then once in 0.05 M Tris·Cl, pH 6.8. Proteins were eluted from the Sepharose pellet by the addition of 1X SDS-PAGE loading buffer (2% SDS, 10% glycerol, 60 mM Tris pH 6.8, and 0.005% bromophenol blue) ± 0.1 M dithiothreitol (DTT), except for the assembly intermediates, where proteins were first eluted with 0.2 M glycine·HCl, pH 2.5 followed by the same volume of 2X SDS-PAGE loading buffer. Proteins were heated 5 min at 100°C, electrophoresed on SDS/polyacrylamide gels, subjected to fluorography with Enhance (Dupont NEN, Wilmington, DE), and visualized by

autoradiography. Densitometric analyses were done on images scanned with a Hewlett/Packard ScanJet IIC using NIH Image software.

Endoglycosidase H Digestion: To test the T15 H chains for sensitivity to endoglycosidase H (endo H), cells were labeled with [^{35}S]-met, cys and proteins were immunoprecipitated as above through the last wash step. Pellets were resuspended in 25 µl 0.05 M sodium citrate, 0.1% SDS, pH 6.0 ± 100 mU/ml endo H (Boehringer Mannheim, Indianapolis, IN), and incubated at 37°C overnight. Proteins were eluted with 25 µl 2X SDS-PAGE loading buffer + 0.2 M DTT.

Western Blot Analysis: Lysates from unlabeled cell cultures were subjected to immunoprecipitations as above. Proteins were separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA). To assess bands containing H chains, blots were probed with rabbit anti-mouse IgG2b (Zymed Laboratories, South San Francisco, CA), followed by AP-conjugated Protein A (Sigma Chemical Co., St. Louis, MO). For identification of BiP (also known as Grp78), blots were probed with rabbit anti-Grp78 (StressGen, Victoria, BC, Canada), then detected with sheep $(Fab)'_2$ anti-rabbit IgG-AP conjugate (Sigma Chemical Co., St. Louis, MO). The same blots were stripped and reprobed with anti-H chain reagent to confirm the location of Grp78 in relation to H chain. All reactions were visualized using the AMPPD chemiluminescent substrate (Bio-Rad, Richmond, CA).

Results

Secretion of T15 WT Ig: Our previous study indicated that the T15 WT and mutant H chains were present at similar intracellular levels, but that Ig secretion was impaired in the mutants relative to the WT (10). These data suggested that the T15 WT Ig was secreted efficiently, while the mutant H chains in the absence of secretion were accumulating intracellularly. To assess the fate of the Ig proteins more directly, cells were metabolically pulse-labeled and chased for various times. Ig H chains and L chains were subsequently immunoprecipitated from cellular lysates and supernatants. As can be seen in Fig. 1a, the T15 WT antibody is secreted, with H chain and L chain visible in the supernatant by 2 h chase, and at a peak by 4 h. The T15 WT antibody bands in Fig. 1a were quantified by densitometry and depicted in Fig. 1b as the % of maximum intracellular label for each Ig chain. The concurrent disappearance of H chain and L chain from the lysate, and subsequent appearance of these two proteins in the supernatant indicated efficient oligomerization and antibody secretion which was essentially complete by 4 h chase (Fig. 1b).

Pulse-Chase Examination of T15 WT and Mutant Heavy Chains: In contrast to the T15 WT antibody which was secreted within 4 h, cells expressing T15 mutant H chains (M153, M164, M166, and M241) retained these labeled mutant H chains for long periods of time, up to 26 h chase (Fig. 2), indicating that they were not targeted for rapid degradation. The mutant H chains were not present in appreciable quantities in the supernatants (with the exception of M153, Fig. 2, discussed below), consistent with the previously described secretion defect in these mutants. The faint bands that were visible in M164, M166, and M241 supernatants were possibly caused by cell death during the course of the experiment. This explanation is supported by the lack of

reproducibility of these supernatant bands, and a correlation between the amount of Ig and the cytosolic enzyme lactose dehydrogenase (LDH) in 4 h culture supernatants as detected by ELISA (data not shown). LDH activity is effectively used as a marker of cell lysis (16, 17).

In order to assess the intracellular fate of T15 H chains expressed in the absence of L chain, the H chains were stably transfected into SP2/0, the same cell line used to produce all of the other transfectants (9). Pulse-chase/immunoprecipitation analyses demonstrates that the T15 WT H chain is not secreted in the absence of L chain (Fig. 2, T15 WT H), but is retained intracellularly for at least 26 h. The mutant H chains also accumulated intracellularly when expressed alone, suggesting that they did not depend on the presence of L chain to be long-lived within the cell (data not shown).

The rate of degradation (or secretion) was examined for the T15 WT and mutant H chains by densitometrically quantifying the bands from 2-3 pulse-chase experiments. These results are shown in Fig. 3, where the % label remaining intracellularly was plotted for the various chase times. H chains from all 4 mutants were degraded slowly (in the presence of L chain) with ~15-60% of H chain protein remaining even at 26 h chase (Fig. 3, M153-M241). When the intracellular T15 WT H chain was analyzed in the absence of L chain, degradation was slow and very similar to the mutants (Fig. 3, T15 WT H). It was interesting to note that individual HCDR2 mutations may differentially affect the degradation of these proteins, as evidenced by a faster decay of the M166 H chain compared to the other 3 mutant H chains (Fig. 3, M166).

In order to compare the degradation of T15 H chains quantitatively, the intracellular half-life ($t^{1}/_{2}$) of each H chain was calculated from the curve fit lines of the plots in Fig. 3, and of similar plots for transfectants expressing mutant H chains alone (data not shown). As shown in Table I, the T15 WT H chain is secreted in the presence of L chain with a $t^{1}/_{2}$ of 3 h, whereas in the absence of L chain it is not secreted and has a $t^{1}/_{2}$ of 15 h, again demonstrating that the T15 WT H chain has the capacity to be long-lived when denied an assembly partner. From the plots in Fig. 3 it is evident that the mutant H chains in the presence of L chains are also long-lived proteins. However, upon examination of $t^{1}/_{2}$ values for the mutant H chains with L chains, it was apparent that three out of four mutants were degraded slower ($t^{1}/_{2}$ >26, 25 and 23 h) than the WT without L chain ($t^{1}/_{2}$ 15 h), and one mutant, M166, decayed more quickly ($t^{1}/_{2}$ 10 h), Table I. For two of the mutant H chains, M153 and M166, the half-life in the presence of L chain was similar to that in the absence of L chain. In contrast, co-expression with L chain resulted in a longer $t^{1}/_{2}$ for M164 (25 h vs. 4 h) and M241 (23 h vs. 12 h). It is possible that these two mutant H chains are retained longer due to an interaction with L chain, even though it may not be an interaction that leads to efficient secretion.

Degradation of T15 Light Chains: The T15 L chain, unlike most L chains, is not secreted in the absence of H chain (10, 18, 19). When T15 L chains expressed in the absence of H chain were metabolically labeled and immunoprecipitated, L chain was easily detected in the lysates, but no L chain appeared in the supernatant over the course of the experiment, 26 h (Fig. 2, T15 WT L). These data were consistent with the inability of T15 L chain to be secreted. The levels of intracellular L chain decreased dramatically during the

chase (Figs. 2 and 3, T15 WT L) demonstrating that without an assembly partner the T15 L chain was efficiently targeted for degradation, with an intracellular $t^1/_2$ of 1.2 h (Table I). In the presence of T15 mutant H chains, the majority of labeled L chains disappeared from the lysates, in contrast to the H chains (Fig. 2, M153-M241). This was also evident when the L chains were plotted as % label vs. time (Fig. 3, M153-M241). However, unlike the degradation of L chain in the absence of H chain, in the presence of mutant H chains the L chain decay plots were biphasic. There was an initial rapid decay of L chains expressed with mutant H chains which was similar to the decay observed for L chain expressed alone (Fig. 3, T15 WT L). The $t^1/_2$ values of this population of L chains from the low secretors were also similar to that of L chain alone, indicating that a major portion of the nascent L chains were targeted for efficient degradation when expressed with the mutant H chains (Table I, Light Chain, phase 1 column). The second subpopulation of intracellular L chains (~5-20 %) was more slowly degraded, with L chains still detectable at late time points (up to 26 h, Fig. 3, M153-M241). Interestingly, this minor population decayed in a manner that paralleled the mutant H chains, with $t^1/_2$ values that were similar to the mutant H chains with which they were expressed (Table I, Light Chain, phase 2 column). These data provided evidence that the mutant H chains affected the fate of at least some of the T15 L chains. Thus even though the mutant H chains did not readily form secretable Ig, they reduced the efficiency of L chain degradation supporting the notion that assembly interactions occurred, but were not appropriate for export.

Association of T15 Heavy Chains with BiP: It is known that the ER-resident molecular chaperone BiP binds to Ig chains and this interaction may be

intricately involved in coordinating antibody assembly (15, 20-23). In addition, mutant proteins that accumulate intracellularly have been found to be associated with BiP for prolonged times compared to their unmutated, secreted counterparts (24-26). We therefore hypothesized that the mutant H chains may have an increased association with BiP. To test this hypothesis, H chains from metabolically labeled cells were immunoprecipitated with Protein A-Sepharose under conditions that allowed non-covalently associated proteins to be recovered. As evident in Fig. 4, the H and L chains were readily immunoprecipitated from the T15 WT and mutant H chain transfectants. It was noted that lysates from the low secretors generally had more background proteins that the control cell lines or T15 WT transfectants. There are several bands that appear more intense, including proteins that migrate with the dye front at the bottom of the gel. There are two additional bands that are easily discernible, one at ~32 kDa apparent MW which remains unidentified, and one that migrates slower than H chain near the 66 kDa marker which we have identified as BiP by Western blotting (data not shown). It is possible that the unidentified band is a H chain degradation product, as many H chains have been found to undergo acid hydrolysis under conditions typically used for SDS-PAGE (27). However this has not been formally tested and we cannot exclude the possibility that this band represents another protein associated with H chain. The BiP band was much stronger in the samples from the low secretor transfectants than from cells expressing the T15 WT, Fig. 4. The H chain, L chain, and BiP bands were quantified by densitometry, and the relative intensities of the L chain and BiP bands were normalized to those of the H chain for each lysate from two independent experiments. The levels of L chain were 3-10 times lower in the mutants than in the T15 WT. Conversely, there were from 7-17 times higher levels of BiP associated with

the mutant H chains compared to the T15 WT H chain. These data demonstrate that the mutant H chains have a decreased association with L chains and an increased association with BiP relative to T15 WT H chain.

Endo H Analysis of T15 WT and Mutant Heavy Chains: The increased association of the mutant H chains with BiP suggested that the mutant H chains were retained in the ER, since BiP is a resident ER protein. To gain additional evidence that the ER was the intracellular site of mutant H chain accumulation, we digested H chains immunoprecipitated from lysates of metabolically labeled cells with endo H. Sensitivity to endo H is conferred by the high mannose form of N-linked oligosaccharides, indicative of proteins that are in the ER and have not yet been modified in the Golgi (28). The murine γ2b H chain possesses a conserved N-linked carbohydrate addition site at position 297 in the CH2 domain (11, 29, 30), and cleavage of this oligosaccharide should cause a discernible increase in band mobility under SDS-PAGE conditions. A pulse-chase/immunoprecipitation analysis with time points up to 6 h was performed on lysates from cells expressing T15 WT and mutant Igs (Fig. 5). At all time points tested, the intracellular H chains remained endo H sensitive, indicating that these H chains had not trafficked to the medial Golgi apparatus. Importantly, by 6 h chase the T15 WT H chains from supernatants had gained endo H resistance, demonstrating that the oligosaccharide on T15 H chain is normally altered by the enzymatic processes of the post-ER vesicles (Fig. 5). Additionally, the H chain doublet that was present in the T15 WT supernatant (SN) sample was indicative of the characteristic asymmetrical O-glycosylation of murine IgG2b H chains, a process which occurs primarily in the Golgi (31). The failure of the mutant H

chains to gain endo H resistance by 6 h chase time suggests that these proteins are retained in the ER for long time periods, possibly until they are degraded.

Secretion of Ig from mutant M153: As mentioned above, M153 exhibited Ig H chain and L chain bands in culture supernatants at late chase times (Fig. 2), a result that was reproducible in subsequent experiments. H and L chain supernatant bands from 3 pulse-chase experiments including the one shown in Fig. 2 were quantified by densitometry and the percent of labeled H and L chains detected in the supernatant of M153 cells reached ~ 15% by 18 h chase, and seemed to increase slightly between 18 and 26 h (data not shown). Therefore, it appeared that a portion of mutant M153 H chains were secreted as Ig, although this level of secretion was much lower, and had slower kinetics than that seen with T15 WT. Even though this observation was reproducible, it was possible that the supernatant proteins were the direct result of cell lysis. To determine if the supernatant proteins were actually secreted, we subjected the M153 H chains from late chase times to endo H digestion. As shown in Fig. 6, the lysate H chains remained endo H sensitive, consistent with the data for this mutant from Fig. 5. However, the M153 H chains immunoprecipitated from the supernatants had gained endo H resistance (Fig. 6, last 4 lanes), confirming that these mutant Igs had trafficked through the Golgi, consistent with the secretion pathway. These samples had been precipitated only with Protein A-Sepharose and the presence of L chains was noted (data not shown), indicating that H-L complexes were formed at these late time points, which is also consistent with secretion.

Assembly Intermediates of T15 WT and Mutant Immunoglobulins: To determine if the defect in the low secretors is at the assembly level, the

assembly intermediates of the T15 WT and mutant Igs were compared. Assembly intermediates immunoprecipitated from lysates of metabolically labeled cells expressing the T15 WT H and L chains were analyzed under non-reducing SDS-PAGE. These data are shown in the first six lanes of Fig. 7a. At 0 min chase, all four bands (H, H_2, H_2L, and H_2L_2) were visible although the H_2 and H_2L bands were relatively more intense than the H and H_2L_2 bands, which were barely detectable. At 30 min chase the H_2 band was decreased in intensity, and stronger H_2L and H_2L_2 bands had emerged while the H chain monomer had essentially disappeared (Fig. 7a). By 1 h chase an intense H_2L_2 band was observed and the H_2 species was barely visible. The presence of H chains in these bands was confirmed by Western blot (data not shown). In addition, proteins from cells expressing only T15 WT H chains rapidly assembled to an H_2 intermediate (by 30 min chase), but the two higher molecular weight bands were not present, consistent with the interpretation that L chain is a component of these slower migrating bands in WT (Fig. 7a, lanes 7-12). Together, these data demonstrate that the T15 WT Ig follows an $H_2 \rightarrow H_2L \rightarrow H_2L_2$ assembly pathway.

The assembly intermediates of the secretion impaired mutants were examined at 1 h and 6 h chase as shown in Fig. 7b, which also included T15 WT assembly intermediates for direct comparison. An H_2 band was readily visible, and a less intense H_2L band was seen at both time points for all four mutants. Mature Ig was not properly assembled by 6 h chase as indicated by a lack of discernible H_2L_2 bands in the mutants (Fig. 7b). The assembly intermediates of the mutant H chains expressed in the absence of L chains indicated efficient H_2 assembly, and lacked bands that migrated at positions representing the H_2L and H_2L_2 species (data not shown). From these results,

it is evident that the mutant H chains efficiently formed dimers and a portion of these labeled H_2 dimers assembled with L chain, although this interaction progressed only to the H_2L intermediate by 6 h chase.

As was evident in the chaperone binding experiments (Fig. 4), the low secretor lysates have additional bands of unknown identity. The band that migrates near the 66 kDa marker can be seen in T15 WT and mutant H chain assembly without L chain, and is likely to be BiP, although this has not been confirmed (Fig. 7 and data not shown). Another band is seen that migrates slightly faster than H chain, near the 97 kDa marker in T15 WT (without L chain) and in the low secretors, but not T15 WT with L chain, Fig. 7. This band is also seen on Western blots of low secretor lysates probed for H chain and on assembly intermediates of mutant H chains without co-expression of L chain (data not shown). A less discrete band(s) was visible in the mutants that migrated slower than the H_2L_2 band, near the 220 kDa marker (Fig. 7b). The possibility exists that these bands may represent an aberrant H_2L_2 species in the mutants. However when T15 H chains were examined in the absence of L chain, a similar band was seen (i.e. one that migrated slower than H_2L_2), but it may or may not be the same species (Fig. 7a).

Discussion

It has become increasingly apparent that CDR domains of antibodies may play very important structural roles other than shaping the antigen combining site. Therefore, the secretion defective mutants analyzed here provide a model with which the deleterious structural effects of mutation on CDR regions can be explored. We have previously demonstrated that two to four amino acid replacements in HCDR2 cause a secretion defect of the T15

antibody (10). Normal amounts of mutant H chain are produced in stable transfectants at levels comparable to T15 WT transfectants. However the amount of intracellular H chain associated with L chain is low as measured by ELISA, and only extremely low levels of intact antibody are found in the culture supernatants. Nonetheless, these HCDR2 mutations do not completely destroy the VH domain: mutant antibodies from culture supernatants exhibit anti-idiotype binding, and when these mutations are placed in a different but related antibody with the same VH gene product, there is no secretion impairment (10). These observations suggest that the mechanism(s) of the secretion defect lies within the assembly and/or transport pathways. Here we further characterize the secretion impairment of the T15 mutants in an effort to explore these possibilities.

The secretion of T15 WT was characterized by examining the fate of the H and L chains in SP2/0 stable transfectants. Antibody was efficiently secreted from these cells so that within 2-4 h, H and L chains appeared in the supernatant and concomitantly disappeared from the cell lysates. This finding is within the range of secretion kinetics observed for other antibody-producing cell lines where antibody appears in the supernatant from 20-150 min after protein synthesis (32, 33). Unlike the secretion profile of intact T15 WT antibody (i.e. H and L chain), the T15 WT H chain accumulated intracellularly in cells that were devoid of L chain. This demonstrated that the T15 WT H chain is intrinsically retained inside the cell unless it is paired with an L chain with which it can be secreted. These findings are consistent with studies done by others suggesting that Ig H chains are not normally secreted in the absence of L chains (34-36), and that H chain secretion can be "rescued" by assembly with L chains (37).

The relative abundance of mutant H chains detected by ELISA in cell lysates led us to examine the intracellular accumulation of these mutant H chains more closely. We found that the mutant H chains were retained intracellularly (independent of L chain co-expression), with a significant fraction of labeled H chain remaining up to 26 h chase. The T15 WT H chain was also retained at late chase times when expressed alone. Thus, the fate of the mutant H chains (which was largely independent of the presence of L chain) appeared to be consistent with that of T15 WT H chain by itself. This suggests that the mutant H chains are defective in their ability to be "rescued" by L chain, which further implicates a defect in proper assembly with L chain.

The possibility that the mutant H chains are defective in proper interaction with L chain suggested that the site of accumulation of these proteins would likely be the ER, i.e. the site of oligomerization. Other analyses of non-secreted Ig proteins have implicated the ER as the site of accumulation and/or degradation of impaired H and L chains, and most of these studies have demonstrated a prolonged interaction with BiP, an ER-resident molecular chaperone (5, 7, 24, 25, 38, 39). Although BiP binds transiently to native chains of many proteins, its binding is enhanced with misfolded, mutant polypeptides, leading to extended retention time in the ER and eventual degradation of the mutant proteins (15, 18, 40, 41). It was recently reported that Ig L chains stably associated with BiP mutants were not secreted and unable to reach their native conformation, including proper disulfide bond formation, suggesting that Igs may not complete essential folding steps until after release from BiP (42). The finding that H chains from the low secretor mutants were associated with 7-17 fold more BiP than the T15 WT is

consistent with accumulation in the ER. We have also found that the T15 WT H chain has an increased association with BiP when expressed without L chain, compared to T15 WT co-expressed with L chain (data not shown). Furthermore the continued sensitivity of the mutant H chains to endo H digestion confirms the ER as the site of accumulation of these non-secreted proteins, at least through 6 h chase after pulse labeling of nascent H chains.

Upon examination of the assembly intermediates we found that the T15 WT antibody oligomerizes efficiently following an $H_2 \rightarrow H_2L \rightarrow H_2L_2$ assembly pathway. It appeared that the H chains form homodimers very quickly, as the H_2 band was more intense than the H chain monomer immediately after the pulse label (Fig. 7a). This observation also held true for the mutant H chains regardless of the presence of L chain, where a strong H_2 band was seen at all time points (Fig. 7b), including 0 min chase (data not shown) suggesting that a large fraction of the labeled H chains did not progress past this point in assembly. This finding was reminiscent of an early report showing that full length, mutant H chains dimerize rapidly, but persist in the cytoplasm as stable proteins (43).

Some assembly to the H_2L intermediate did occur in the low secretor mutants, however the mutants did not form discrete H_2L_2 oligomers (Fig. 7b). When the rates of disappearance of intracellular L chains were compared, it was noted that the curves were biphasic (Fig. 3). The first phase accounted for the rapid degradation of ≥80% of the L chains, with $t^1/_2$ values of 1-2 h consistent with other nonsecreted L chains (38). A more long-lived minor portion of L chain was present at late chase times in the cells expressing mutant H chains (Fig. 3, M153-M241). This second fraction of labeled L chain

decayed similarly to the H chains from the same transfectants (Fig. 3) and had essentially the same $t^1/_2$ values as the mutant H chains (Table I, Light Chain, phase 2). These observations are consistent with the detection of H_2L bands in the assembly intermediates (Fig. 7b). Thus, it is likely that the L chains present in H_2L oligomers were those detected in the second phase, at late chase times in the aforementioned immunoprecipitations. The H_2L complexes are formed within 1 h chase, and are still detected at 6 h chase (Fig. 7b), indicating that they may be relatively stable over time. These data suggest that one L chain may be added to an H_2 dimer with relative ease, compared to the subsequent addition of a second L chain. It is intriguing to speculate that the H_2L species (in the case of mutant H chains) is at this time exhibiting a conformation that prevents interaction with another L chain (or inability to dissociate from chaperones). In addition, a non-covalent assembly of $H_2L + L \rightarrow H_2L_2$ may be formed, a possibility that cannot be excluded from the data presented here.

The assembly patterns of antibodies were elucidated more than 20 yrs ago and follow discrete isotype-specific steps (33, 44, 45). The analysis described here was performed on the T15 WT and mutant VH domains linked to the murine γ2b constant region expressed in stable transfectants of SP2/0 (9). According to published reports on IgG2b assembly, this murine isotype typically assembles an HL intermediate in the major assembly pathway, with H_2 usually present in the minority of assembly intermediates (46, 47). Our data demonstrated that the combination of T15 WT VH/γ2b + Vκ22/Cκ proteins assembled via an H_2 homodimer intermediate, and no discernible HL band was detected on the gels. In the mutants, but not the T15 WT, there is an unidentified band that migrates slightly faster than the H_2 intermediate,

at an apparent MW similar to (but slower than) that expected for an HL species near the 97 kDa marker, Fig. 7b. This band probably contains H chain as evidenced by Western blot analysis (data not shown), however, it is unlikely that it represents an HL intermediate because a band of similar size is also present in immunoprecipitations of T15 WT and mutant H chains from lysates of cells devoid of L chain (data not shown). Moreover, H chain dimerization occurs extremely efficiently, as it is seen at the earliest time points, while the monomeric H chain is only faintly detectable. Therefore if a minor assembly pathway utilizing HL intermediates exists in these antibodies it must be extremely transitory as it was not detected under the experimental conditions used.

To our knowledge this is the only known example of an IgG2b that does not appear to assemble HL heterodimers into H_2L_2 oligomers, with the exception of the aberrant H chains present in the IgG2b-producing MPC 11 tumor (48). It is interesting to note that HL heterodimers do form in the MPC 11 tumor, but they are non-covalently associated, do not participate in the formation of H_2L_2, and actually get secreted from the cell (48). The inconsistency between the findings presented here and other reports of IgG2b assembly, i.e. lack of HL heterodimers, is suggestive of an unfavorable interaction of nascent H and L chains. It is intriguing to speculate that this unique combination of VH and L chain may somehow disfavor HL formation in IgG2b oligomerization. It was shown previously that the T15 L chain does not compete as well for association with T15 H chain *in vitro* as L chains from other anti-PC antibodies (those of MOPC167 and McPC603, which use Vκ24 and Vκ8, respectively) or by heterologous Vκ21 L chains (49). This is consistent with our data suggesting that the T15 H chains may favor H_2 formation over HL.

We must also note that the original T15 antibody uses an α H chain, whereas we have expressed it as a γ2b. However, in the reassociation experiments *in vitro*, the competitive advantage was determined primarily by the Vκ gene segment (49), and other studies in different antibody systems concluded that preferential reassociation was not influenced by differences in CL (50, 51), consistent with the notion that VH-VL interactions are important in H-L association. Another possible explanation for the lack of HL intermediates lies in the stoichiometry of H and L chains produced in these stable transfectants. Antibody-producing cells commonly synthesize an excess of L chain (44) which may drive HL interaction. We do not see an excess of L chain produced from transfected genes compared to H chain. Therefore, it would be interesting to examine the assembly pathway of these H chains with endogenously expressed L chains, which would likely be in excess of the transfected H chains.

Even though all of the mutants have changes in the HCDR2 domain, they each have a unique combination of mutations and were originally produced as part of a larger panel of T15 HCDR2 mutants where the others were secreted normally (10). Despite their common secretion defect, some phenotypic differences have been observed among these four mutants. Most notably, the M153 mutant does not appear to be as impaired as the other three mutant Igs. It has a significant amount of intracellular H-L as detected by sandwich ELISA, which is probably assembled at least to the H_2L form (Fig. 7b) as this lysate Ig could also bind antigen (10). In this study we demonstrated that by 18 h, ~ 15% of labeled H and L chain from M153 is in the supernatant. This appears to be bona-fide secretion, as evidenced by the acquisition of endo H-resistant H chains (Fig. 6), but with much slower kinetics and lower

efficiency than the WT antibody. As noted earlier, this mutant also differs from the other low secretors in that all four amino acid changes are in the second loop of HCDR2, fueling the notion that a more disruptive change results when both loops of HCDR2 contain mutations.

Other examples of variation among the mutants were evident upon examination of the intracellular H chain half-life values, Table I. The M166 H chain had a short half-life compared to the other mutant H chains (10 h), and this characteristic remained essentially unchanged in the absence of L chain. In contrast, the other three mutants had $t^1/_2$ values ≥23 h. Interestingly, for M153 the $t^1/_2$ was >26 h regardless of L chain co-expression, whereas M164 and M241 had shortened half-life values in the absence of L chain, suggesting that the nature of interaction with L chain may vary among the mutants. The $t^1/_2$ values of mutant H chains (without L chain), compared to the intrinsic T15 WT $t^1/_2$ of 15 h, are particularly interesting. Three of four low secretor H chains disappear (presumably degraded) from the cells more quickly than T15 WT H chains. Especially for M164 ($t^1/_2$ 4 h) and M166 ($t^1/_2$ 9 h), it is possible that the HCDR2 mutations introduce or reveal a degradation signal, or otherwise decrease the stability of the protein.

Even though CDR regions are targets of somatic hypermutation, the process of positive selection drives the expansion of rare mutants that retain or increase ability to bind antigen, whereas those mutants that lose antigen binding are presumed to be lost *in vivo*. Examination of the database of hybridoma sequences reveals that within the hypervariable CDR regions, there are frequent examples of amino acid residues that are highly conserved among antibodies (11). It is also apparent from the growing body of structural

data that CDRs are more than solvent-exposed flexible loops that contact antigen; they can also interact directly with other CDRs and with framework regions from both VH and VL (52). However, the VH CDR2 domain of T15 is not an integral part of the interface with VL according to the most recent model of this antibody (13). It is possible that conformational changes in the HCDR2 may have long-range effects on other domains of the molecule such as those directly involved in L chain association. In support of this hypothesis, we have demonstrated that a four amino acid deletion in the HCDR3 region of these T15 mutants actually restores secretion competence[4]. The notion that CDR regions may contribute important structural information to an antibody is supported by our findings that the secretion impairment of T15 Igs with HCDR2 mutations is characterized by altered $t_{1/2}$ values of intracellular H chains that accumulate in the ER, and an inability to assemble properly with L chain.

Acknowledgements

We are grateful to Dr. Caroline Enns for critical discussions and advice. We thank McKay Brown, Dr. Elizabeth A. Whitcomb, Dr. Thomas O'Hare, Dr. Mary Stenzel-Poore, Kurt A. Heldwein, Jennifer K. Hill, and Kristie Absalon for critical review of the manuscript.

References

1. French, D. L., R. Laskov, and M. D. Scharff. 1989. The role of somatic hypermutation in the generation of antibody diversity. *Science 244:1152.*
2. Hurtley, S. M., and A. Helenius. 1989. Protein oligomerization in the endoplasmic reticulum. *Ann. Rev. Cell Biol. 5:277.*
3. Melnick, J., and Y. Argon. 1995. Molecular chaperones and the biosynthesis of antigen receptors. *Immunol. Today 16:243.*
4. Helms, L. R., and R. Wetzel. 1995. Destabilizing loop swaps in the CDRs of an immunoglobulin V_L domain. *Prot. Sci. 4:2073.*
5. Knittler, M. R., S. Dirks, and I. G. Haas. 1995. Molecular chaperones involved in protein degradation in the endoplasmic reticulum - quantitative interaction of the heat shock cognate protein BiP with partially folded immunoglobulin light chains that are degraded in the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA 92:1764.*
6. Hurle, M. R., L. R. Helms, L. Li, W. Chan, and R. Wetzel. 1994. A role for destabilizing amino acid replacements in light-chain amyloidosis. *Proc. Natl. Acad. Sci. USA 91:5446.*
7. Dul, J. L., and Y. Argon. 1990. A single amino acid substitution in the variable region of the light chain specifically blocks immunoglobulin secretion. *Proc. Natl. Acad. Sci. USA 87:8135.*
8. Wu, G. E., N. Hozumi, and H. Murialdo. 1983. Secretion of a λ2 immunoglobulin chain is prevented by a single amino acid substitution in its variable region. *Cell 33:77.*
9. Chen, C., V. A. Roberts, and M. B. Rittenberg. 1992. Generation and analysis of random point mutations in an antibody CDR2 sequence: Many mutated antibodies lose their ability to bind antigen. *J. Exp. Med. 176:855.*

10. Chen, C., T. M. Martin, S. Stevens, and M. B. Rittenberg. 1994. Defective secretion of an immunoglobulin caused by mutations in the heavy chain complementary determining region 2. *J. Exp. Med. 180:577.*

11. Kabat, E. A., T. T. Wu, H. M. Perry, K. S. Gottesman, and C. Foeller. 1991. *Sequences of Proteins of Immunological Interest.* U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Washington, D. C.

12. Davies, D. R., E. A. Padlan, and S. Sheriff. 1990. Antibody-antigen complexes. *Annu. Rev. Biochem. 59:439.*

13. Brown, M., M. B. Rittenberg, C. Chen, and V. A. Roberts. 1996. Tolerance to single, but not multiple, amino acid replacements in antibody VH CDR2. A means of minimizing B cell wastage from somatic hypermutation? *J. Immunol. 156:3285.*

14. Brown, M., M. P. Stenzel-Poore, S. Stevens, S. K. Kondoleon, J. Ng, H. P. Bachinger, and M. B. Rittenberg. 1992. Immunologic memory to phosphocholine keyhole limpet hemocyanin. Recurrent mutations in the lambda 1 light chain increase affinity for antigen. *J. Immunol. 148:339.*

15. Melnick, J., J. L. Dul, and Y. Argon. 1994. Sequential interaction of the chaperones BiP and GRP94 with immunoglobulin chains in the endoplasmic reticulum. *Nature 370:373.*

16. 1988. Cytotoxicity assays that may be run on molecular devices' kinetic microplate readers. Lactate dehydrogenase release. In *Applications Bulletin, vol. February 11, 1988.* Molecular Devices Corporation, Palo Alto, CA.

17. Korzeniewski, C., and D. M. Callewaert. 1983. An enzyme-release assay for natural cytotoxicity. *J. Immunol. Methods 64:313.*

18. Pillai, S. 1991. Immunoglobulin transport in B cell development. *Int'l. Rev. Cyt. 130:1.*

19. Oi, V. T., S. L. Morrison, L. A. Herzenberg, and P. Berg. 1983. Immunoglobulin gene expression in transformed lymphoid cells. *Proc. Natl. Acad. Sci. USA 80:825.*

20. Knittler, M. R., and I. G. Haas. 1992. Interaction of BiP with newly synthesized immunoglobulin light chain molecules: cycles of sequential binding and release. *EMBO J. 11:1573.*

21. Kaloff, C. R., and I. G. Haas. 1995. Coordination of immunoglobulin chain folding and immunoglobulin chain assembly is essential for the formation of functional IgG. *Immunity 2:629.*

22. Hendershot, L. M., and J. F. Kearney. 1988. A role for human heavy chain binding protein in the developmental regulation of immunoglobin transport. *Mol. Immunol. 25:585.*

23. Haas, I. G., and M. Wabl. 1983. Immunoglobulin heavy chain binding protein. *Nature 306:387.*

24. Bole, D. G., L. M. Hendershot, and J. F. Kearny. 1986. Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol. 102:1558.*

25. Dul, J. L., O. R. Burrone, and Y. Argon. 1992. A conditional secretory mutant in an Ig L chain is caused by replacement of tyrosine/phenylalanine 87 with histidine. *J. Immunol. 149:1927.*

26. Gething, M.-J., K. McCammon, and J. Sambrook. 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: The role of folding in intracellular transport. *Cell 46:939.*

27. Davagnino, J., C. Wong, L. Shelton, and S. Mankarious. 1995. Acid hydrolysis of monoclonal antibodies. *J. Immunol. Methods 185:177.*

28. Tarentino, A. L., T. H. Plummer, Jr., and F. Maley. 1974. The release of intact oligosaccharides from specific glycoproteins by endo-beta-N-acetylglucosaminidase H. *J. Biol. Chem. 249:818.*

29. Lund, J., N. Takahashi, H. Nakagawa, M. Goodall, T. Bentley, S. A. Hindley, R. Tyler, and R. Jefferis. 1993. Control of IgG/Fc glycosylation: a comparison of oligosaccharides from chimeric human/mouse and mouse subclass immunoglobulin Gs. *Mol. Immunol. 30:741.*

30. Nose, M., and H. Wigzell. 1983. Biological significance of carbohydrate chains on monoclonal antibodies. *Proc. Natl. Acad. Sci. USA 80:6632.*

31. Kim, H., Y. Yamaguchi, K. Masuda, C. Matsunaga, K. Yamamoto, T. Irimura, N. Takahashi, K. Kato, and Y. Arata. 1994. O-glycosylation in hinge region of mouse immunoglobulin G2b. *J. Biol. Chem. 269:12345.*

32. Baumal, R., and M. D. Scharff. 1973. Synthesis, assembly and secretion of mouse immunoglobulin. *Transplant. Rev. 14:163.*

33. Scharff, M. D., and R. Laskov. 1970. Synthesis and assembly of immunoglobulin polypeptide chains. *Progr. Allergy 14:37.*

34. Wall, R., and M. Kuehl. 1983. Biosynthesis and regulation of immunoglobulins. *Ann. Rev. Immunol. 1:393.*

35. Wilde, C. D., and C. Milstein. 1980. Analysis of immunoglobulin chain secretion using hybrid myelomas. *Eur. J. Immunol. 10:462.*

36. Morrison, S. L., and M. D. Scharff. 1981. Mutational events in mouse myeloma cells. *Crit. Rev. Immunol. 3:1.*

37. Pepe, V. H., G. E. Sonenshein, M. I. Yoshimura, and M. J. Shulman. 1986. Gene transfer of immunoglobulin light chain restores heavy chain secretion. *J. Immunol. 137:2367.*

38. Gardner, A. M., S. Aviel, and Y. Argon. 1993. Rapid degradation of an unassembled immunoglobulin light chain is mediated by a serine protease and occurs in a pre-Golgi compartment. *J. Biol. Chem. 268:25940.*

39. Lenny, N., and M. Green. 1991. Regulation of endoplasmic reticulum stress proteins in COS cells transfected with immunoglobulin μ heavy chain cDNA. *J. Biol. Chem. 266:20532.*

40. Kim, P. S., and P. Arvan. 1995. Calnexin and BiP act as sequential molecular chaperones during thyroglobulin folding in the endoplasmic reticulum. *J. Cell Biol. 128:29.*

41. Klausner, R. D., and R. Sitia. 1990. Protein degradation in the endoplasmic reticulum. *Cell 62:611.*

42. Hendershot, L., J. Wei, J. Gaut, J. Melnick, S. Aviel, and Y. Argon. 1996. Inhibition of immunoglobulin folding and secretion by dominant negative BiP ATPase mutants. *Proc. Natl. Acad. Sci. USA 93:5269.*

43. Morrison, S. L., and M. D. Scharff. 1975. Heavy chain-producing variants of a mouse myeloma cell line. *J. Immunol. 114:655.*

44. Scharff, M. D. 1975. The synthesis, assembly, and secretion of immunoglobulin: A biochemical and genetic approach. *Harvey Lect. 69:125.*

45. Bevan, M. J., R. M. Parkhouse, A. R. Williamson, and B. A. Askonas. 1972. Biosynthesis of immunoglobulins. *Progr. Bioph. Mol. Biol. 25:133.*

46. Baumal, R., M. Potter, and M. D. Scharff. 1971. Synthesis, assembly and secretion of gamma globulin by mouse myeloma cells. III. Assembly of the three subclasses of IgG. *J. Exp. Med. 134:1316.*

47. McKeever, P. E., M. E. Neiders, G. B. Nero, and R. Asofsky. 1980. Murine plasma cells secreting more than one class of immunoglobulin. VII. Analysis of the IgG2B and IgA precursors within the cytoplasm of

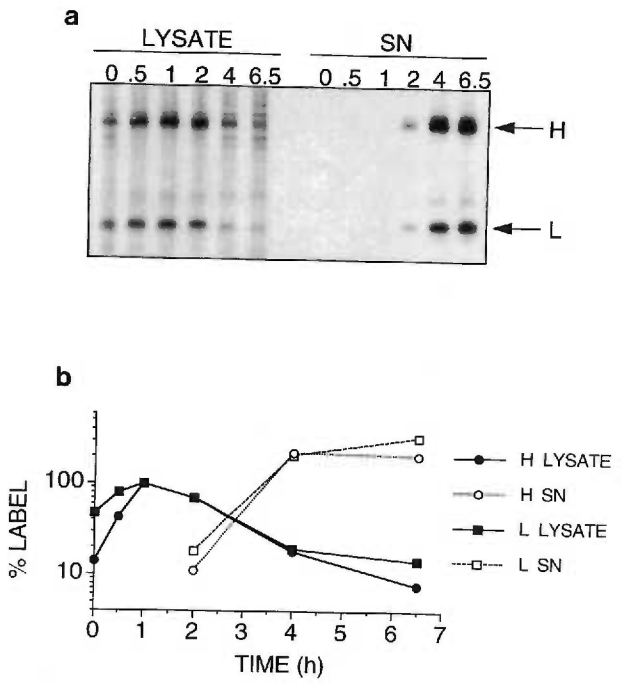


Fig. 1. Secretion profile of T15 WT antibody. *a*. Stable transfectants expressing immunoglobulin proteins were pulsed for 15 min and chased for the indicated times (h). Lysate and supernatant (SN) samples were immunoprecipitated with rabbit anti-mouse κ followed by Protein A-sepharose. Immunoprecipitated proteins were loaded on 12% SDS-polyacrylamide gels under reducing conditions. Note blank lane between lysate and SN. *b*. Densitometric analysis of data from the pulse-chase experiment shown in Fig. 1a. The % label from immunoprecipitated H chains and L chains are plotted vs. chase time points. The maximum incorporation of label into precipitable lysate proteins was reached at 1 h chase and denoted as 100%. All other samples were quantified relative to this value individually for H chain and L chain bands.

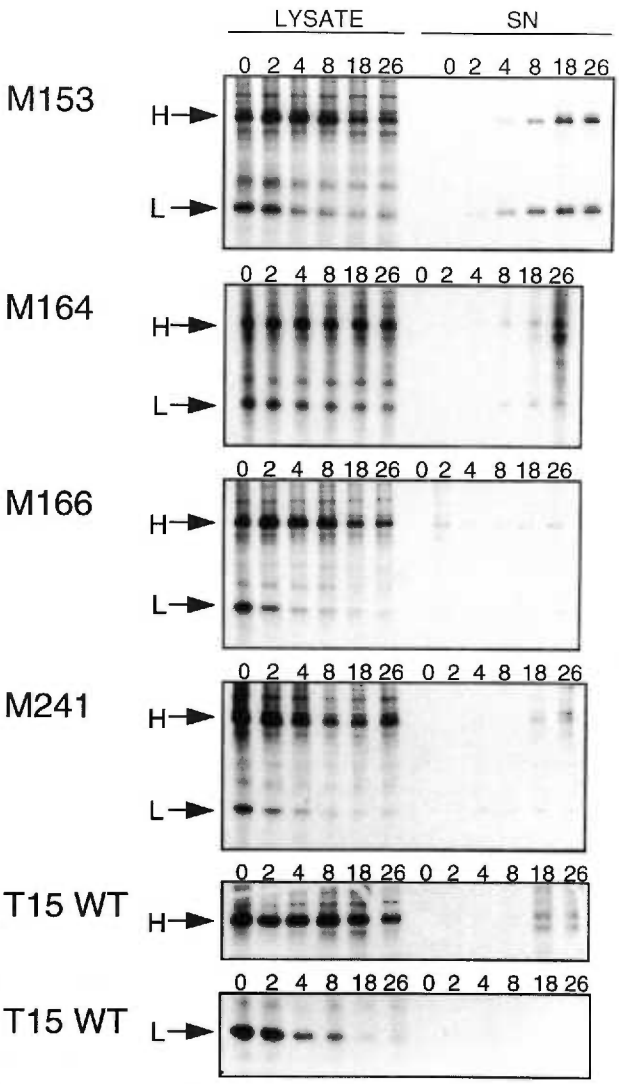


Fig. 2. Pulse-chase immunoprecipitation of [^{35}S]-labeled T15 heavy and light chains from lysates and supernatants. Immunoglobulin proteins were labeled, immunoprecipitated and analyzed by SDS-PAGE as in Fig. 1a except that a longer time course was used. *T15 WT H* (no L chain), the anti-κ incubation was omitted. *T15 WT L* (no H chain).

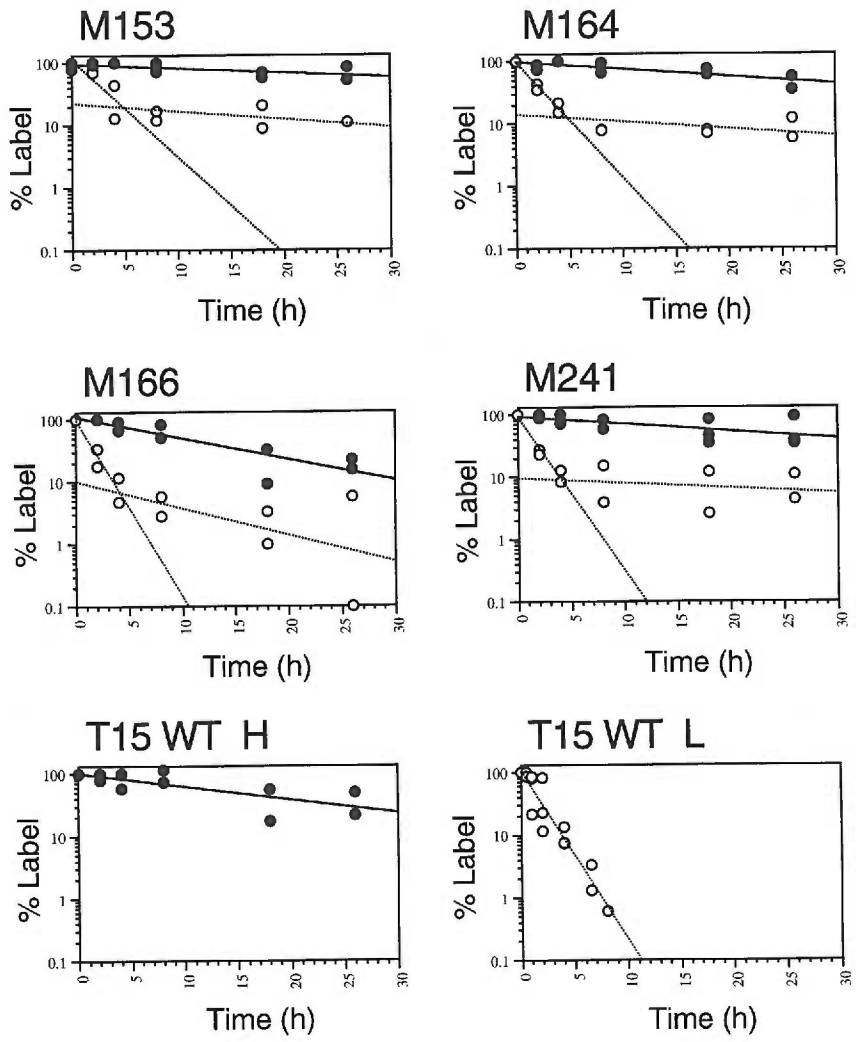


Fig. 3. Kinetics of intracellular degradation of T15 heavy chains and light chains. Densitometric analyses of pulse-chase immunoprecipitation data. Each graph represents 2 to 3 experiments such as those shown in Fig. 2, where the H chains and L chains were plotted for various chase times as a percentage of the total labeled H chain (●) and L chain (○). *T15 WT H* (no L chain). *T15 WT L* (no H chain). Curve fit lines were generated with Cricket Graph software. For L chains co-expressed with T15 mutant H chains (M153-M241) the degradation was biphasic, thus two curve fit lines were generated. The curve fit for the initial, more rapidly decreasing, L chain population was for 0 to 4 h chase, whereas the second phase was fitted for data collected ≥4 h chase.

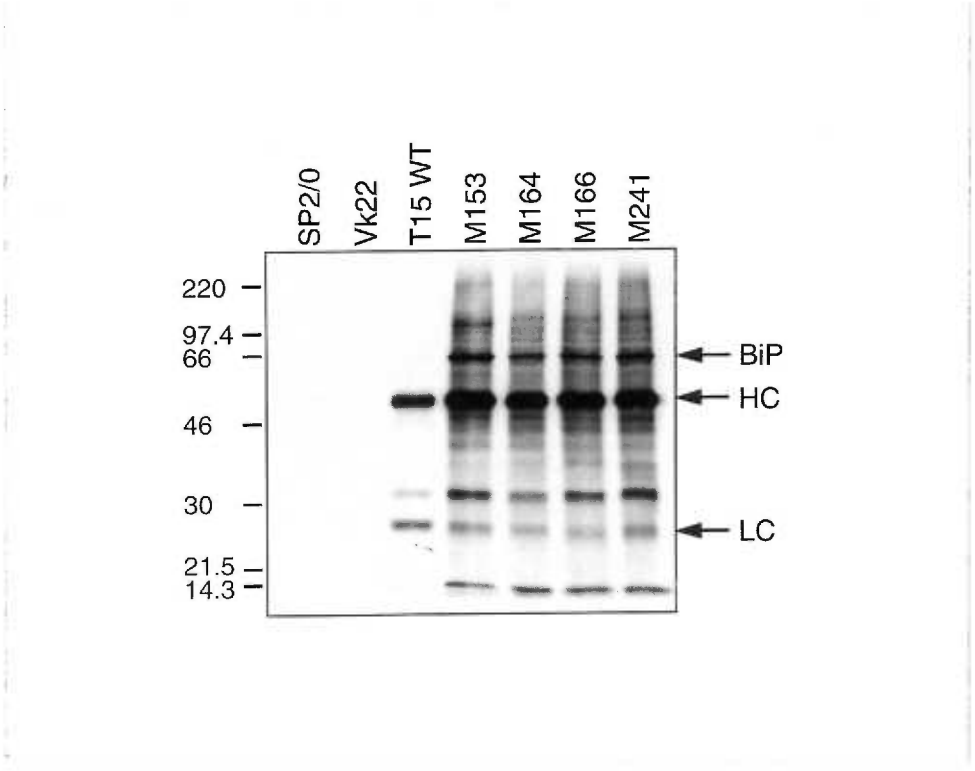


Fig. 4. Association of BiP with T15 heavy chains. Protein A immunoprecipitation of [^{35}S]-labeled cell lysates from SP2/0 transfectants run on a 12% SDS-polyacrylamide gel under reducing conditions. Untransfected cells (SP2/0) and transfectants expressing T15 L chain (Vκ22) were negative controls that do not produce heavy chain. Numbers on the left represent the migration of molecular weight markers. Arrows on the right indicate proteins identified as BiP, H chain (HC), and L chain (LC).

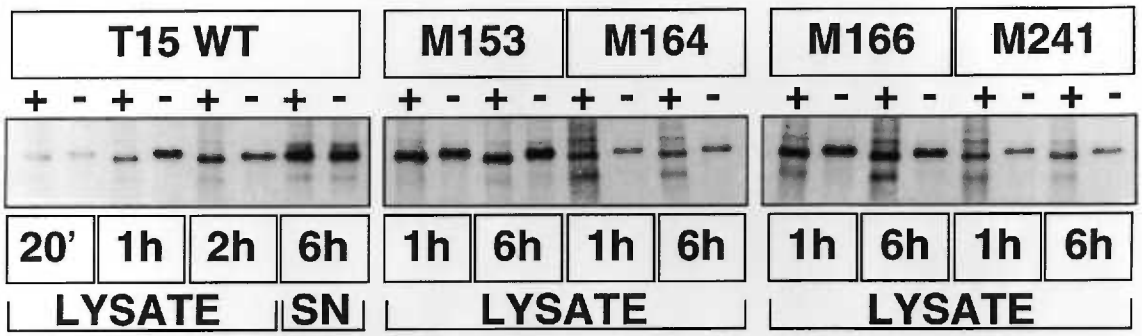


Fig. 5. Endo H digestion of T15 WT and mutant heavy chains. T15 WT and mutant H chains were isolated by Protein A immunoprecipitation after a 15 min [^{35}S]-pulse and indicated chase times. Proteins from lysates or supernatants (SN) were subsequently digested with (+) or without (–) endo H at 37° overnight, and analyzed on an 8% SDS-polyacrylamide gel under reducing conditions.

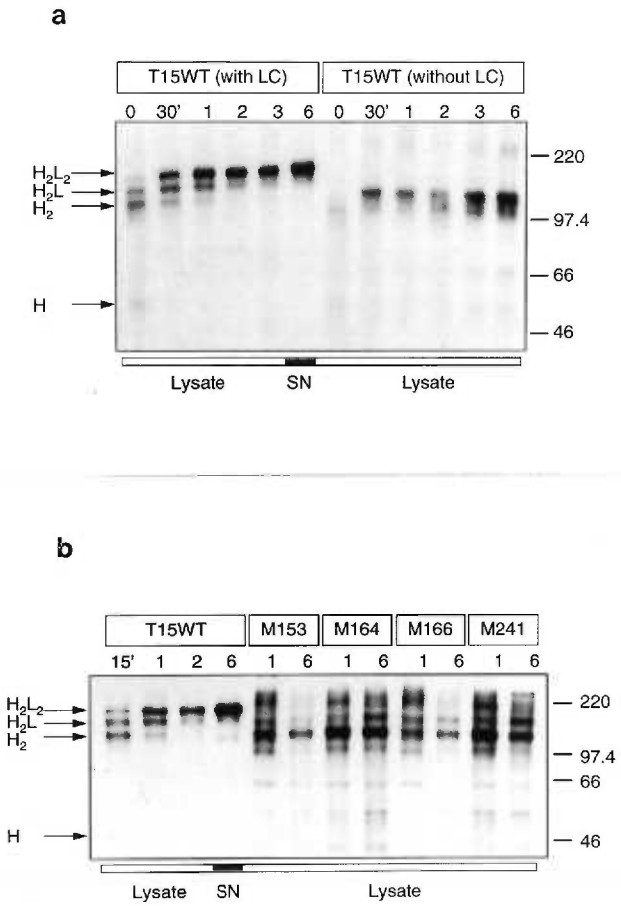


Fig. 7. Assembly intermediates of T15 WT and mutant immunoglobulins. Pulse-chase/immunoprecipitation of immunoglobulin proteins from T15 WT and mutants. Samples were analyzed on an 8% (*a*.) or 10% (*b*.) SDS-polyacrylamide gel under non-reducing conditions. *a*. Various chase times for T15 WT with and without L chain (LC), including 0 h. *b*. Comparison of T15 WT and mutants when H chain and L chain are co-expressed. For both analyses of T15 WT (with LC) in *a* and *b*, the 6 h time point was performed on antibody from supernatant (SN). The assignment of intermediate oligomeric species is shown on the left, and the migration of molecular weight markers is indicated on the right.

Table I

Calculated half-lives of intracellular Heavy and Light Chains [a]

	Heavy Chain (LC present)	Heavy Chain (LC absent)	Light Chain (phase 1) [b]	Light Chain (phase 2)
T15 WT	3	15	2.2	n.a. [c]
M153	>26	>26	2.3	>26
M164	25	4	1.6	>26
M166	10	9	1.0	11
M241	23	12	1.1	>26
Light Chain	n.a.	n.a.	1.2	n.a.

[a] Based on curve fit lines of semi-log plots such as those shown in Figure 3. Data from 2-3 experiments were graphed for each plot. Values are the chase time (h) where 50% of the labeled intracellular protein is present.

[b] For the L chains co-expressed with mutant H chains, there were two curve fit lines due to the biphasic nature of the graphs (see Figure 3). Therefore, the "phase 1" column above refers to the more rapidly degraded population, and "phase 2" represents the more slowly degraded population line. Since the second phase of L chain degradation uses time points begining at 4 h (Figure 3, legend), the $t^{1}/_{2}$ was calculated using half of the % label value at 4 h derived from the second phase line.

[c] "n.a." means not applicable.

DISCUSSION AND CONCLUSIONS

This study provides the first demonstration that mutations in an H chain hypervariable region have a deleterious effect on Ig secretion. These data contribute to the evolving paradigm that even though the hypervariable regions directly contribute to functional antigen binding and confer antigen specificity on an antibody, they may also provide essential structural information necessary for proper folding, oligomerization and transport of the Ig.

The murine anti-PC antibody, T15, was subjected to a random *in vitro* mutagenesis of the HCDR2 region in order to "mimic" the somatic hypermutation process and obtain mutated antibodies for study that would normally be lost in an *in vivo* immune response (2). The rationale behind this approach was to learn more about the consequences and requirements of affinity maturation. By analyzing a mutant pool of antibodies with a random set of mutations, one might get a unique glimpse of the proteins available at the onset of positive selection during an *in vivo* response. This would provide quantitative insight into the extent of deleterious mutations that may normally occur. Indeed, from the panel of 46 HCDR2 mutant antibodies, the majority had either lost or decreased ability to bind antigen, whereas none had a significant binding advantage over the WT, suggesting that the somatic hypermutation toll is extensive wastage of B cells, where higher affinity antigen binding is an extremely rare event (2).

It was also found that among these 46 T15 HCDR2 mutants, four were deficient in the amount of antibody present in the culture supernatants. There were 2-4 amino acid replacements in the HCDR2 region of each mutant H chain, and upon analysis of the entire VH, no other sequence changes had occurred in the DNA during the mutagenesis and subcloning procedures (Paper 1, Fig. 1, p. 52). As these antibodies were expressed in stable transfectants of the SP2/0 cell line (already expressing a stably transfected T15 L chain), the transfections of the four H chains were repeated several times to ensure that the low production of antibody was not due to poor transfection events (Paper 1, Table 1, p. 45). In addition, the low secretor transfectants were not impaired in their ability to proliferate in culture (Appendix, Fig. 1). The T15 WT and mutant H chains were co-transfected (with the T15 L chain) into another myeloma cell line, P3XAg8.653, to make sure that the observed phenotype was a general phenomenon, and not unique to the SP2/0 cell line. Furthermore, RNA from the T15 WT transfectant and several clones of each low secretor mutant was probed by Northern blot analysis for H and L chain mRNA (as well as a housekeeping gene, ChoA). These data demonstrated that mRNA from the mutants was present at similar levels as the WT, with no detectable differences in gel migration, suggesting that the secretion defect was not due to inefficient or aberrant transcription of the transfected genes (Paper 1, Table 2, p. 47 and Fig. 2, p. 53).

Having established that the secretion defect occurred post-transcriptionally, a quantitative assessment of the low secretor phenotype and an analysis of the intracellular proteins was performed.

To this end cells were washed and plated at known densities, and the amount of antibody secreted into the culture supernatant during a 4 h incubation was quantified by ELISA. This short incubation time minimized the antibody that may be released due to cell death, and allowed a rigorous assessment of the difference between the WT and mutant antibodies. The intracellular levels of H and L chain were also quantified by ELISA using cell lysates that were prepared at the end of the 4 h incubation. These data demonstrated that all of the mutants produced relatively similar amounts of H and L chain as the WT, but that only the WT secreted appreciable quantities into the supernatant (Paper 1, Table 4, p.50). It was also noted that very little associated H+L protein was present intracellularly in the mutants, whereas this was easily detectable in the WT by sandwich ELISA. The initial characterization of intracellular proteins revealed that one mutant, M153, may be less affected by the perturbations of HCDR2 since it exhibited significantly more intracellular H+L than the other mutants. The finding that lysate proteins from M153 can bind antigen similar to T15 WT lysates was consistent with the observation that some assembly occurs for M153. These data therefore suggested that the M153 mutant may be more defective in transport than assembly.

The mutant H chains were not grossly aberrant in that when supernatants from overgrown cultures were collected, the antibody present was recognized by anti-T15 Id reagents (Paper 1, Table 3, p.49). Furthermore, the mutant H chains exhibited the same migration in SDS-PAGE as T15 WT H chains from cellular lysates (Paper 1, Figure 3, p. 54). The T15 WT and mutant H chains were found to be glycosylated, as

blocking N-linked carbohydrate addition with tunicamycin caused the same shift in gel mobility in the mutants as it did in the WT (data not shown). In fact, the tunicamycin treatment did not prevent secretion of T15 WT antibody, suggesting that N-linked glycosylation is not a requirement for assembly and transport of this antibody (data not shown). The mutations introduced into these H chains do not cause an unambiguous destruction of the Ig H chain as evidenced by the placement of these exact same HCDR2 mutations into another, very similar combining site, that of D16 (Paper 1, Table 5, p. 51).

D16 is an anti-PC antibody that uses the same VH, but differs in DJ and L chain (Paper 1, Fig. 4, p.55). Therefore, it was of interest to determine what difference(s) between these two antibodies could account for the opposite phenotypes upon introduction of the HCDR2 amino acid replacements. The largest difference between these two antibodies is the L chain usage. The T15 L chain (Vκ22) is uncommon in that it is not secreted by itself, whereas the D16 L chain (Vκ1c) is readily secreted in the absence of H chain. In order to test the contribution of the L chain to the secretion defect, the T15 WT and mutant H chains were co-expressed with other heterologous L chains, including that of D16. As described in Paper 2, the T15 WT H chain was readily secreted with the D16 L chain (Vκ1c) as well as the J558 L (λ1) chain. Thus the secretion defect could not be attributed to a unique property of the T15 L chain.

The other distinction between the T15 and D16 antibodies is in the HCDR3 region which is encoded by DJ sequences (Paper 2, Fig. 1, p. 77). There are four amino acid differences between the two HCDR3 regions,

and the T15 sequence is longer than D16 by four residues. To test the contribution of HCDR3 length to the secretion defect, site-directed mutagenesis was used to delete four residues (Ser99, Ser100, Tyr100a, Trp100b) from T15. This HCDR3 deletion had no effect on T15 WT secretion. However, the short HCDR3 was able to correct or compensate for the deficiency caused by the HCDR2 mutations in the low secretors (Paper 2, Fig. 2, p. 78).

In order to assess the contributions made by individual amino acid changes, site-directed mutagenesis was employed to produce H chains with the representative single mutations found in the original low secretor mutants. All of the single mutants were analyzed for secretion, and no defect was found in any of them (Appendix Table I). Therefore, the phenotype must be the result of at least two amino acid replacements in HCDR2. This is indeed the case for one of the low secretor mutants, M164, which only has two changes. Another example has been identified by the double mutant (S51R, Y59S) which was produced by mutagenesis. This double mutant contains two of the three changes present in M241, suggesting that the mutation D54E does not contribute significantly to the defect.

Pulse-chase metabolic labeling of T15 WT and low secretor mutants, followed by immunoprecipitation and SDS-PAGE analyses revealed that the fate of the mutant H chains differs appreciably from the T15 WT proteins, which are secreted efficiently in 2-4 h (Paper 3, Fig. 1, p. 114). The mutant H chains accumulated intracellularly throughout the chase, up to 26 h (Paper 3, Fig. 2, p. 115). In order to assess the differences

between the mutant H chains quantitatively, and to compare the accumulation of T15 WT and mutant H chains in the absence of L chain, the SDS-PAGE data were densitometrically quantified (Paper 3, Fig. 3, p. 116) and the half-life ($t^1/_2$) of each protein was calculated (Paper 3, Table I, p. 121). This analysis demonstrated that the T15 WT H chain is not degraded efficiently in the absence of L chain ($t^1/_2$ 15 h), and in general, this is also true for the mutant H chains, regardless of co-expression of L chain. However, there are some important differences between the mutants. M153 has a $t^1/_2$ of >26 h with or without L chain. L chain expression also did not effect the accumulation of M166 H chain, however, this mutant H chain is degraded significantly more rapidly than the other three low secretors in the presence of L chain. The other two mutants, M164 and M241, were more efficiently degraded in the absence of L chain, suggesting that a possible interaction with L chain may prolong the accumulation of these two mutant H chains.

It is apparent from several lines of evidence that the T15 mutant H chains accumulated in the ER. The initial ELISA analysis led to the hypothesis that the H chains might not traffic out of the ER, since very little H+L chain was detected from lysates, and it is known that for multimeric secretory proteins in general, oligomerization (an ER event) is a prerequisite for transport (140). Metabolic labeling and immunoprecipitation data demonstrate that the mutant H chains are retained in the ER. At 6 h chase, the intracellular mutant H chains are still endo H sensitive, indicating that they have not trafficked to the Golgi by this time, in contrast to the secreted WT H chain which did acquire endo H resistance (Paper 3, Fig. 5, p. 118). In addition, the

mutant H chains were shown to have an increased association with two ER-resident molecular chaperones, BiP (Paper 3, Fig. 4, p. 117) and Grp94 (Appendix, Fig. 2). This association is 7-20 fold higher with the mutant H chains than with the secreted WT H chain (Appendix, Table II), suggesting that the mutant H chains may be inappropriately folded and/or assembled.

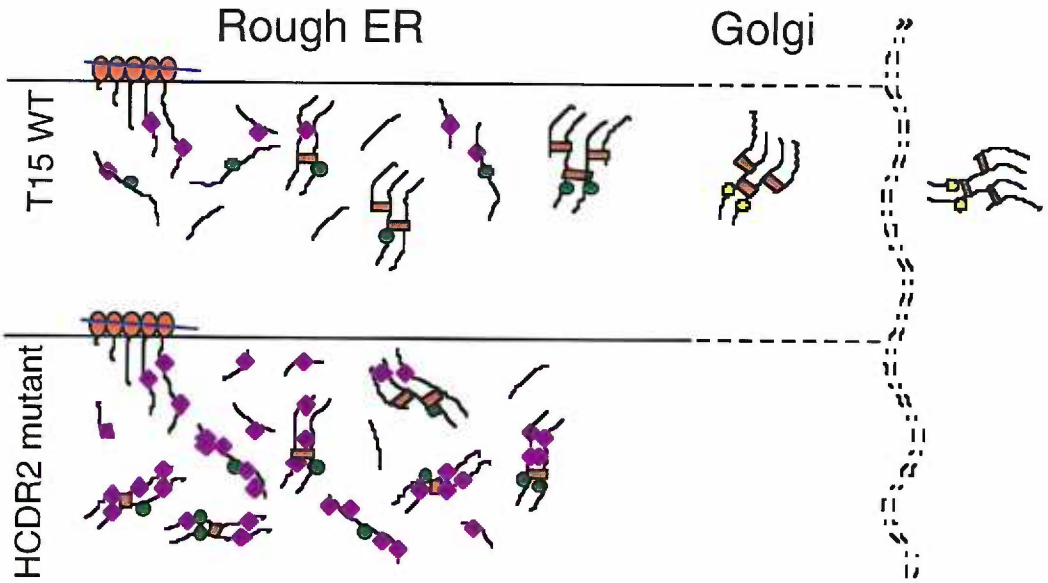
Since Igs follow a step-wise pattern of H and L chain assembly (106), it was of interest to determine what assembly intermediates, if any, that the mutant H chains were able to form. Pulse-chase, immunoprecipitation and non-reducing SDS-PAGE analysis was used to demonstrate that the T15 WT antibody (expressed as an IgG2b) produced assembly intermediates that corresponded to an $H_2 \rightarrow H_2L \rightarrow H_2L_2$ pattern of assembly (Paper 3, Fig. 7, p. 120). The formation of H_2 occurs very rapidly, and is produced irrespective of the presence of L chains. This population of H chain dimers is abundant in the low secretors and appears to be stable over time. There is no evidence for the formation of covalently-linked HL molecules, unlike other murine IgG2b antibodies (107, 108), suggesting that the nascent T15 H chains (WT and mutant) interact more favorably with each other than with L chains. This is consistent with an *in vitro* study in which T15 H chains more readily associated with other L chains than with T15 L chains (145) The mutant H chains in the low secretors did form an H_2L species (which was not as abundant as the H_2 intermediate), however, there was no evidence for discrete H_2L_2 bands in the low secretor lysates (Paper 3, Fig. 7, p. 120). The presence of a subpopulation of H chains that are in an H_2L configuration is supported by the findings that a minority of L

chains in the low secretors are as long-lived as the concomitant H chains (Paper 3, Table I, p. 121). These data expose the secretion defect as an inability to efficiently and properly assemble H and L chains into H_2L_2 Ig.

The following model of T15 assembly details the various interactions and assembly intermediates of the T15 WT antibody (top) and of the HCDR2 secretion mutants (bottom). As secretory proteins, the H and L chains are translated on polyribosomes and translocated into the ER lumen. Nascent H and L chains are bound by molecular chaperones, sometimes before translation/translocation is complete. The model depicted here indicates that the T15 WT antibody assembles in an $H_2 \rightarrow H_2L \rightarrow H_2L_2$ pattern, with fully assembled antibody trafficking via the Golgi and out through the plasma membrane. The N-linked carbohydrate present on the WT H chain is modified in the Golgi to an endo H-resistant, complex oligosaccharide moiety. In contrast, most of the HCDR2 mutant H chains are present as dimers with some H_2L molecules also present, but there is no evidence that fully assembled antibody is produced or secreted, consistent with the failure to find evidence of endo H resistant mutant H chains (with the exception of M153 discussed in Paper 3). The mutant H chains have a 7- to 20-fold greater association with the molecular chaperones BiP and Grp94, and this is depicted in the model as more chaperones per mutant H chain compared to WT H chains. However, it is not known whether this increase is due to more chaperone molecules bound per H chain, or if more H chains have chaperones associated with them, or both. It is also unknown if BiP or Grp94 have preferential associations with particular assembly intermediates or if

ternary complexes containing BiP, Grp94 and H chain exist. Given the multi-domain nature of Ig polypeptides it is intriguing to speculate that the mutations in VH CDR2 cause aberrant conformations of the VH domain. Thus, it is possible that an altered VH domain structure results in a more permanent interaction of mutant VH with molecular chaperones, which may prevent the mutant VH from achieving a native conformation or one that allows efficient VH-VL associations to be made. Taken together, the data suggest that these mutant complexes are retained in the ER, which would be most consistent with the bulk flow model of antibody secretion since the mutant H chains apparently cannot be freed of retentive chaperones.

MODEL OF T15 ASSEMBLY:



Legend:

- polyribosome with mRNA
- heavy and light chains
- intrachain disulfide bonds
- N-linked oligosaccharide (high mannose, endo H-sensitive)
- N-linked oligosaccharide (complex, endo H-resistant)
- molecular chaperones

FUTURE DIRECTIONS

The testing of all of the single mutations found in the low secretors revealed that the defect cannot be attributed to one change, but must result from two or more amino acid replacements (at least in this unique set of T15 HCDR2 mutations). Since in two of the four low secretors the defect can be observed with only two mutations (M164 and M241: S51R,Y59S), it will be of interest to dissect the roles of individual mutations in the other two low secretors, each of which have four amino acid changes. To this end, all representative double mutants will be tested for secretion competence. This information is expected to provide the minimum necessary changes needed to observe the defect, and hopefully will simplify future modeling endeavors. It is also possible that none of these double mutants will be as defective as the original low secretor mutants, and it is recognized that triple mutations may be required. The availability of a complete set of representative changes for each of the low secretors will also enable a more rigorous quantitative assessment of the defect. For instance, the single mutant secretion levels compared to WT ranged from ~25% to >100% (data not shown), however, the production of intracellular H chain will vary between transfectants, so it is not yet known if any of the single mutants have an intermediate secretion capability. It is possible that one may be able to identify a scheme within these low secretor mutants where, as mutations are added one at a time, secretion may become more impaired with each step.

Nascent H and L chains interact with BiP as they are translocated, and this interaction is thought to affect the protein conformation so as to

allow interaction with Grp94 (88). BiP is thought to associate and dissociate rapidly from nascent proteins, and to bind to immature folding intermediates. Grp94 is thought to interact with more mature conformations, however, a ternary complex containing BiP, L chain, and Grp94 has been identified (88). A current model for chaperone-assisted folding of Ig chains suggests that the molecules bound by Grp94 (with or without BiP) are oxidized (intrachain), but have not yet undergone subtle alterations that lead to a fully native conformation (46). This model implies that BiP may be displaced earlier in the folding process than Grp94, but that assembly of H and L chains requires a native conformation which also allows dissociation of Grp94. In another model of BiP interaction with H chains, the CH domains may be in a conformation that allows dimerization, while at the same time the VH domains might interact with BiP (146). The important aspect of all of these findings is that individual Ig domains undergo sequential (and sometimes simultaneous) associations with BiP and Grp94 prior to covalent assembly with other Ig domains (46, 50), and that changes in sequence which may cause only subtle structural changes in a protein, could result in a more stable chaperone interaction (46).

In the secretion-defective HCDR2 mutants described here, it appears that covalent H_2 assembly proceeds normally. This implies that the hinge, CH2 and CH3 domains are not aberrantly affected by the changes in VH. However, since association with L chain is inefficient and does not fully proceed to covalently-linked H_2L_2, it is possible that the CH1-CL association may be influenced by the VH alterations, or that the CH1-CL interaction is initiated normally, but is not by itself strong enough to

overcome a putative improper VH-VL interaction. Another possibility is that the addition of one L chain to an H_2 molecule has a long-range effect on the oligomer that disfavors the addition of the second L chain. The reason that only one L chain associates with H_2 might simply be that the kinetics and equilibrium of the system make this an infrequent event, rendering the assembly of a second L chain onto an H_2L species extremely rare, but otherwise no less favorable than the first L chain addition.

It may be possible to discriminate between some of the above mechanisms using differential pulse-chase/immunoprecipitation assays. For instance, with 2-dimensional gel electrophoresis, one could immunoprecipitate the H chains and proteins associated with them, and run the gels unreduced in the first dimension, then reduced. Thus, it might be possible to identify ternary BiP-H-Grp94 complexes, or H_2L-BiP complexes. The cross-linking reagent that was used to identify Grp94 association with mutant H chains (Appendix, Fig. 2) would probably need to be employed in these experimental strategies. With the aid of cross-linker, non-covalent associations of H and L chains might be demonstrated if they exist. Another approach to detection of heteromeric, non-covalent complexes would be sequential immunoprecipitations, utilizing antibodies to the chaperones in addition to reagents that bind Ig chains. If any of these complexes existed, they might provide intriguing insight into the mechanism of the secretion defect.

Another avenue of examination that could provide important data as to the conformational state of the mutant H chains is to assess the oxidation states of the various species of assembly intermediates or chaperone complexes. It may be that the mutant H chains do not achieve a fully oxidized state, but that this does not hamper their ability to dimerize.

Lastly, the HCDR2 mutations may destabilize the VH domain. A more rigorous biochemical analysis of mutant VH stability, as well as VH-VL association would be possible if these domains were expressed in E. coli. either separately or as sFv. Mutations that destabilize L chains have been found in the VL domain of human L chains, and these analyses provide an important model for L chain amyloidosis (147-149). These L chain studies are also relevant to the question as to why the T15 L chain is not secreted on its own, as are most other L chains. One prediction is that the T15 L chain has amino acid sequences that cause it to be destabilized compared to other L chains, but that interaction with H chain has a stabilizing effect. One of the destabilizing mutations that have been described is the replacement of a conserved Ala at position 84 with a Thr residue in L chains (149). Most L chains have an Ala or Gly at this position, but the T15 L chain contains Thr at position 84. Site-directed mutagenesis to change this Thr back to the conserved Ala does have a partial effect in that when expressed alone, this L chain mutant secretes a small proportion of L chains (compared to none detectable for the WT L chain, E. Whitcomb, unpublished results). Additional mutagenesis of other candidate residues of this L chain that may allow it to be secreted should prove insightful.

These data support the notion that CDR regions may make important contributions to the structural integrity of the antibody. The studies described here present a unique system whereby the mechanisms of defective assembly and secretion of Ig can be further elucidated.

REFERENCES

1. Tonegawa, S. 1983. Somatic generation of antibody diversity. *Nature 302:575.*
2. Chen, C., V. A. Roberts, and M. B. Rittenberg. 1992. Generation and analysis of random point mutations in an antibody CDR2 sequence: Many mutated antibodies lose their ability to bind antigen. *J. Exp. Med. 176:855.*
3. Poljak, R. J., L. M. Amzel, H. P. Avey, B. L. Chen, R. P. Phizackerley, and F. Saul. 1973. Three-dimensional structure of the Fab' fragment of a human immunoglobulin at 2,8-A resolution. *Proc. Natl Acad. Sci. USA 70:3305.*
4. Richardson, J. S. 1981. The anatomy and taxonomy of protein structure. *Adv. Prot. Chem. 34:167.*
5. Bork, P., L. Holm, and C. Sander. 1994. The immunoglobulin fold. Structural classification, sequence patterns and common core. *J. Mol. Biol. 242:309.*
6. 1993. Fundamental Immunology. W. E. Paul, ed. Raven Press, New York, p. 306.
7. Doolittle, R. F., and P. Bork. 1993. Evolutionarily mobile modules in proteins. *Sci. Am. 269:50.*
8. Padlan, E. A. 1994. Anatomy of the antibody molecule. *Mol. Immunol. 31:169.*
9. Lesk, A. M., and C. Chothia. 1982. Evolution of proteins formed by β-sheets. II. The core of the immunoglobulin domains. *J. Mol. Biol. 160:325.*

10. Chothia, C., J. Novotny, R. Bruccoleri, and M. Karplus. 1985. Domain association in immunoglobulin molecules: the packing of variable domains. *J. Mol. Biol. 186:651.*

11. Novotny, J., and E. Haber. 1985. Structural invariants of antigen binding: comparison of immunoglobulin VL-VH and VL-VL domain dimers. *Proc. Natl Acad. Sci. USA 82:4592.*

12. Webster, D. M., A. H. Henry, and A. R. Rees. 1994. Antibody-antigen interactions. *Curr. Biol. 4:123.*

13. Wu, T. T., and E. A. Kabat. 1970. An analysis of the sequences of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. *J. Exp. Med. 132:211.*

14. Davies, D. R., and H. Metzger. 1983. Structural basis of antibody function. *Annu. Rev. Immunol. 1:87.*

15. Kabat, E. A., T. T. Wu, and H. Bilofsky. 1977. Unusual distributions of amino acids in complementarity-determining (hypervariable) segments of heavy and light chains immunoglobulins and their possible roles in specificity of antibody-combining sites. *J. Biol. Chem. 252:6609.*

16. Padlan, E. A., and D. R. Davies. 1975. Variability of three-dimensional structure in immunoglobulins. *Proc. Natl Acad. of Sci. USA 72:819.*

17. de la Paz, P., B. J. Sutton, M. J. Darsley, and A. R. Rees. 1986. Modelling of the combining sites of three anti-lysozyme monoclonal antibodies and of the complex between one of the antibodies and its epitope. *EMBO J. 5:415.*

18. Chothia, C., and A. M. Lesk. 1987. Canonical structures for the hypervariable regions of immunoglobulins. *J. Mol. Biol. 196:901.*

19. Chothia, C., A. M. Lesk, A. Tramontano, M. Levitt, S. J. Smith-Gill, G. Air, S. Sheriff, E. A. Padlan, D. Davies, W. R. Tulip, P. M. Colman, S. Spinelli, P. M. Alzari, and R. J. Poljak. 1989. Conformations of immunoglobulin hypervariable regions. *Nature 342:877.*

20. Potter, M. 1971. Antigen-binding myeloma proteins in mice. *Ann. NY Acad. Sci. 190:306.*

21. Segal, D. M., E. A. Padlan, G. H. Cohen, S. Rudikoff, M. Potter, and D. R. Davies. 1974. The three-dimensional structure of a phosphorylcholine-binding mouse immunoglobulin Fab and the nature of the antigen binding site. *Proc. Natl. Acad. Sci. USA. 71:4298.*

22. Leon, M. A., and N. M. Young. 1970. Six mouse IgA myeloma proteins with phosphorylcholine specificity. *Fed. Proc. 29:437Abs.*

23. Chang, S. P., M. Brown, and M. B. Rittenberg. 1982. Immunologic memory to phosphorylcholine. II. PC-KLH induces two antibody populations that dominate different isotypes. *J. Immunol. 128:702.*

24. Claflin, J. L., and J. M. Davie. 1974. Clonal nature of the immune response to phosphorylcholine. IV. Idiotype uniformity of binding site associated antigenic determinants among mouse anti-phosphorylcholine-binding antibodies. *J. Exp. Med. 140:673.*

25. Perlmutter, R. M. 1984. The molecular genetics of phosphocholine-binding antibodies. In *The biology of idiotypes.* Plenum Press, New York, p. 59.

26. Perlmutter, R. M., S. T. Crews, R. Douglas, G. Sorenson, N. Johnson, N. Nevera, P. J. Gearheart, and L. Hood. 1984. The generation of diversity in phosphorylcholine-binding antibodies. *Adv. Immunol. 35:1.*

27. Claflin, J. L., S. Hudak, and A. Maddalena. 1981. Anti-phosphocholine hybridoma antibodies. I. Direct evidence for three distinct families of antibodies in the murine response. *J. Exp. Med. 153:352.*

28. Potter, M., and M. A. Leon. 1968. Three IgA myeloma immunoglobulins from the BALB/ mouse: precipitation with pneumococcal C polysaccharide. *Science 162:369.*

29. Sher, A., and M. Cohn. 1972. Inheritance of an idiotype associated with the immune response of inbred mice to phosphorylcholine. *Eur. J. Immunol. 2:319.*

30. Cosenza, K., and H. Köhler. 1972. Specific inhibition of plaque formation to phosphorylcholine by antibody. *Science 176:1027.*

31. Briles, D. E., C. Forman, S. Hudak, and J. L. Claflin. 1982. Anti-phosphorylcholine antibodies of the T15 idiotype are optimally protective against *Streptococcus pneumoniae. J. Exp. Med. 156:1171.*

32. Padlan, E. A., D. R. Davies, S. Rudikoff, and M. Potter. 1976. Structural basis for the specificity of phosphorylcholine-binding immunoglobulins. *Immunochemistry 13:945.*

33. Satow, Y., G. H. Cohen, E. A. Padlan, and D. R. Davies. 1986. Phosphocholine binding immunoglobulin Fab McPC603. An X-ray diffraction study at 2.7Å. *J. Mol. Biol. 190:593.*

34. Kwan, S. P., E. E. Max, J. G. Seidman, P. Leder, and M. D. Scharff. 1981. Two kappa immunoglobulin genes are expressed in the myeloma S107. *Cell 26:57.*

35. Crews, S., J. Griffin, H. Huang, K. Calame, and L. Hood. 1981. A single VH gene segment encodes the immune response to phosphocholine: somatic mutation is correlated with the class of the antibody. *Cell 25:59.*

36. Chien, N. C., V. A. Roberts, A. M. Giusti, M. D. Scharff, and E. D. Getzoff. 1989. Significant structural and functional change of an antigen-binding site by a distant amino acid substitution: proposal of a structural mechanism. *Proc. Natl. Acad. Sci. USA 86:5532.*

37. Kabat, E. A., T. T. Wu, H. M. Perry, K. S. Gottesman, and C. Foeller. 1991. *Sequences of Proteins of Immunological Interest.* U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Washington, D. C.

38. Brown, M., M. B. Rittenberg, C. Chen, and V. A. Roberts. 1996. Tolerance to single, but not multiple, amino acid replacements in antibody VH CDR2. A means of minimizing B cell wastage from somatic hypermutation? *J. Immunol. 156:3285.*

39. Anfinsen, C. B. 1973. Principles that govern the folding of protein chains. *Science 181:223.*

40. Ellis, R. J. 1994. Roles of Molecular Chaperones in Protein Folding. *Curr. Opin. Struct. Biol. 4:117.*

41. Freedman, R. B., N. J. Bulleid, H. C. Hawkins, and J. L. Paver. 1989. Role of protein disulphide-isomerase in the expression of native proteins. *Biochem. Soc. Symp. 55:167.*

42. Jaenicke, R. 1993. Role of accessory proteins in protein folding. *Curr. Opin. Struct. Biol. 3:104.*

43. Gething, M. J., and J. Sambrook. 1992. Protein folding in the cell. *Nature 355:33.*

44. Noiva, R., and W. J. Lennarz. 1992. Protein disulfide isomerase. A multifunctional protein resident in the lumen of the endoplasmic reticulum. *J. Biol. Chem. 267:3553.*

45. Bose, S., and R. B. Freedman. 1992. The characterisation of peptidyl prolyl cis-trans isomerase (PPI) activity associated with the endoplasmic reticulum. *Biochem. Soc. Trans. 20:256S.*

46. Melnick, J., and Y. Argon. 1995. Molecular chaperones and the biosynthesis of antigen receptors. *Immunol. Today 16:243.*

47. Ellis, J. 1987. Proteins as molecular chaperones. *Nature 328:378.*

48. Seckler, R., and R. Jaenicke. 1992. Protein folding and protein refolding. *FASEB J. 6:2545.*

49. Buchner, J., M. Schmidt, M. Fuchs, R. Jaenicke, R. Rudolph, F. X. Schmid, and T. Kiefhaber. 1991. GroE facilitates refolding of citrate synthase by suppressing aggregation. *Biochemistry 30:1586.*

50. Buchner, J. 1996. Supervising the fold: functional principles of molecular chaperones. *Faseb 10:10.*

51. Wada, I., D. Rindress, P. H. Cameron, W. J. Ou, J. J. d. Doherty, D. Louvard, A. W. Bell, D. Dignard, D. Y. Thomas, and J. J. Bergeron. 1991. SSR alpha and associated calnexin are major calcium binding proteins of the endoplasmic reticulum membrane. *J. Biol. Chem. 266:19599.*

52. Ou, W. J., P. H. Cameron, D. Y. Thomas, and J. J. M. Bergeron. 1993. Association of folding intermediates of glycoproteins with calnexin during protein maturation. *Nature 364:771.*

53. Langer, T., G. Pfeifer, J. Martin, W. Baumeister, and F. U. Hartl. 1992. Chaperonin-mediated protein folding: GroES binds to one end of the GroEL cylinder, which accommodates the protein substrate within its central cavity. *EMBO J. 11:4757.*

54. Braig, K., M. Simon, F. Furuya, J. F. Hainfeld, and A. L. Horwich. 1993. A polypeptide bound by the chaperonin groEL is localized within a central cavity. *Proc. Natl Acad. Sci. USA 90:3978.*

55. Saibil, H. R., D. Aheng, A. M. Roseman, A. S. Hunter, G. M. F. Watson, S. Chen, A. auf der Mauer, B. P. O-Hara, S. P. Wood, N. H. Mann, L. K. Barnett, and R. J. Ellis. 1993. ATP induces large quaternary rearrangements in a cage-like chaperonin structure. *Curr. Biol. 3:265.*

56. Hendrick, J. P., and F. U. Hartl. 1993. Molecular chaperone functions of heat-shock proteins. *Annu. Rev. Biochem. 62:349.*

57. Morrison, S. L., and M. D. Scharff. 1975. Heavy chain-producing variants of a mouse myeloma cell line. *J. Immunol. 114:655.*

58. Haas, I. G., and M. Wabl. 1983. Immunoglobulin heavy chain binding protein. *Nature 306:387.*

59. Burrows, P. D., G. B. Beck, and M. R. Wabl. 1981. Expression of mu and gamma immunoglobulin heavy chains in different cells of a cloned mouse lymphoid line. *Proc. Natl Acad. Sci. USA 78:564.*

60. Munro, S., and H. R. Pelham. 1986. An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell 46:291.*

61. Haas, I. G., and T. Meo. 1988. cDNA cloning of the immunoglobulin heavy chain binding protein. *Proc. Natl Acad. Sci. USA 85:2250.*

62. Hendershot, L. M., J. Ting, and A. S. Lee. 1988. Identity of the immunoglobulin heavy-chain-binding protein with the 78,000-dalton glucose-regulated protein and the role of posttranslational modifications in its binding function. *Mol. Cell. Biol. 8:4250.*

63. Bole, D. G., L. M. Hendershot, and J. F. Kearny. 1986. Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol. 102:1558.*

64. Munro, S., and H. R. B. Pelham. 1987. A C-terminal signal prevents secretion of luminal ER proteins. *Cell 48:899.*

65. Gething, M.-J., K. McCammon, and J. Sambrook. 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: The role of folding in intracellular transport. *Cell 46:939.*

66. Flynn, G. C., T. G. Chappell, and J. E. Rothman. 1989. Peptide binding and release by proteins implicated as catalysts of protein assembly. *Science 245:385.*

67. Dorner, A. J., D. G. Bole, and R. J. Kaufman. 1987. The relationship of N-linked glycosylation and heavy chain-binding protein association with the secretion of glycoproteins. *J. Cell Biol. 105:2665.*

68. Kozutsumi, Y., M. Segal, K. Normington, M.-J. Gething, and J. Sambrook. 1988. The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. *Nature 332:462.*

69. Segal, M. S., J. M. Bye, J. F. Sambrook, and M.-J. Gething. 1992. Disulfide bond formation during the folding of influenza virus hemagglutinin. *J. Cell Biol. 118:227.*

70. Wilson, I. A., J. J. Skehel, and D. C. Wiley. 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature 289:366.*

71. Flynn, G. C., J. Pohl, M. T. Flocco, and J. E. Rothman. 1991. Peptide-binding specificity of the molecular chaperone BiP. *Nature 353:726.*

72. Blond-Elguindi, S., S. E. Cwirla, W. J. Dower, R. J. Lipshutz, S. R. Sprang, J. F. Sambrook, and M.-J. Gething. 1993. Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. *Cell 75:717.*

73. Landry, S. J., R. Jordan, R. McMacken, and L. M. Gierasch. 1992. Different conformations for the same polypeptide bound to chaperones DnaK and GroEL. *Nature 355:455.*

74. Flajnik, M. F., C. Canel, J. Kramer, and M. Kasahara. 1991. Which came first, MHC class I or class II? *Immunogenetics 33:295.*

75. Rippmann, F., W. R. Taylor, J. B. Rothbard, and N. M. Green. 1991. A hypothetical model for the peptide binding domain of hsp70 based on the peptide binding domain of HLA. *EMBO J. 10:1053.*

76. Pelham, H. 1988. Heat-shock proteins. Coming in from the cold. *Nature 332:776.*

77. Rothman, J. E. 1989. Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. *Cell 59:591.*

78. Hartl, F. U., R. Hlodan, and T. Langer. 1994. Molecular Chaperones in Protein Folding - The Art of Avoiding Sticky Situations. *Trends Biochem. Sci. 19:20.*

79. Stuart, R. A., D. M. Cyr, E. A. Craig, and W. Neupert. 1994. Mitochondrial Molecular Chaperones - Their Role in Protein Translocation. *Trends Biochem. Sci. 19:87.*

80. Wiech, H., J. Buchner, R. Zimmermann, and U. Jakob. 1992. Hsp90 chaperones protein folding in vitro. *Nature 358:169.*

81. Lee, A. S., J. Bell, and J. Ting. 1984. Biochemical characterization of the 94- and 78-kilodalton glucose-regulated proteins in hamster fibroblasts. *J. Biol. Chem. 259:4616.*

82. Koch, G., M. Smith, D. Macer, P. Webster, and R. Mortara. 1986. Endoplasmic reticulum contains a common, abundant calcium-binding glycoprotein, endoplasmin. *J. Cell Sci. 86:217.*

83. Sargan, D. R., M. J. Tsai, and B. W. O'Malley. 1986. hsp108, a novel heat shock inducible protein of chicken. *Biochemistry 25:6252.*

84. Mazzarella, R. A., and M. Green. 1987. ERp99, an abundant, conserved glycoprotein of the endoplasmic reticulum, is homologous to the 90-kDa heat shock protein (hsp90) and the 94-kDa glucose regulated protein (GRP94). *J. Biol. Chem. 262:8875.*

85. Li, Z. H., and P. K. Srivastava. 1993. Tumor Rejection Antigen gp96/grp94 Is an ATPase - Implications for Protein Folding and Antigen Presentation. *EMBO J. 12:3143.*

86. Kuznetsov, G., L. B. Chen, and S. K. Nigam. 1994. Several endoplasmic reticulum stress proteins, including ERp72, interact with thyroglobulin during its maturation. *J. Biol. Chem. 269:22990.*

87. Melnick, J., S. Aviel, and Y. Argon. 1992. The endoplasmic reticulum stress protein GRP94, in addition to BiP, associates with unassembled immunoglobulin chains. *J. Biol. Chem. 267:21303.*

88. Melnick, J., J. L. Dul, and Y. Argon. 1994. Sequential interaction of the chaperones BiP and GRP94 with immunoglobulin chains in the endoplasmic reticulum. *Nature 370:373.*

89. Navarro, D., I. Qadri, and L. Pereira. 1991. A mutation in the ectodomain of herpes simplex virus 1 glycoprotein B causes defective processing and retention in the endoplasmic reticulum. *Virology 184:253.*

90. Schaiff, W. T., K. A. Hruska, Jr., D. W. McCourt, M. Green, and B. D. Schwartz. 1992. HLA-DR associates with specific stress proteins and is retained in the endoplasmic reticulum in invariant chain negative cells. *J. Exp. Med. 176:657.*

91. Lewis, M. J., S. J. Turco, and M. Green. 1985. Structure and assembly of the endoplasmic reticulum. Biosynthetic sorting of endoplasmic reticulum proteins. *J. Biol. Chem. 260:6926.*

92. Kang, H. S., and W. J. Welch. 1991. Characterization and purification of the 94-kDa glucose-regulated protein. *J. Biol. Chem. 266:5643.*

93. Rothman, J. E. 1987. Protein sorting by selective retention in the endoplasmic reticulum and golgi stack. *Cell 50:521.*

94. Lodish, H. F., N. Kong, S. Hirani, and J. Rasmussen. 1987. A vesicular intermediate in the transport of hepatoma secretory proteins from the rough endoplasmic reticulum to the Golgi complex. *J. Cell Biol. 104:221.*

95. Orci, L., B. S. Glick, and J. E. Rothman. 1986. A new type of coated vesicular carrier that appears not to contain clathrin: its possible role in protein transport within the Golgi stack. *Cell 46:171.*

96. Wieland, F. T., M. L. Gleason, T. A. Serafini, and J. E. Rothman. 1987. The rate of bulk flow from the endoplasmic reticulum to the cell surface. *Cell 50:289.*

97. Fitting, T., and D. Kabat. 1982. Evidence for a glycoprotein "signal" involved in transport between subcellular organelles. Two membrane glycoproteins encoded by murine leukemia virus reach the cell surface at different rates. *J. Biol. Chem. 257:14011.*

98. Baumal, R., and M. D. Scharff. 1973. Synthesis, assembly and secretion of mouse immunoglobulin. *Transplant. Rev. 14:163.*

99. Scharff, M. D., and R. Laskov. 1970. Synthesis and assembly of immunoglobulin polypeptide chains. *Progr. Allergy 14:37.*

100. Williamson, A. R., and B. A. Askonas. 1968. Time course of labeling of light and heavy chains of immunoglobulin G and the turnover of the free light-chain intermediates. *Archs. Biochem. Biophys. 125:401.*

101. Bevan, M. J. 1971. Interchain disulfide bond formation studied in two mouse myelomas which secrete immunoglobulin A. *Eur. J. Immunol. 1:133.*

102. Askonas, B. A., and A. R. Williamson. 1967. Balanced synthesis of light and heavy chains of immunoglobulin G. *Nature 216:264.*

103. Schenkein, I., and J. W. Uhr. 1970. Immunoglobulin synthesis and secretion. I. Biosynthetic studies of the addition of the carbohydrate moieties. *J. Cell Biol. 46:42.*

104. Bevan, M. J., R. M. Parkhouse, A. R. Williamson, and B. A. Askonas. 1972. Biosynthesis of immunoglobulins. *Progr. Bioph. Mol. Biol. 25:133.*

105. Baumal, R., and M. D. Scharff. 1973. Synthesis, assembly and secretion of gamma-globulin by mouse myeloma cells. V. Balanced and unbalanced synthesis of heavy and light chains by IgG-producing tumors and cell lines. *J. Immunol. 111:448.*

106. Scharff, M. D. 1975. The synthesis, assembly, and secretion of immunoglobulin: A biochemical and genetic approach. *Harvey Lect. 69:125.*

107. Bergman, L. W., and W. M. Kuehl. 1979. Formation of intermolecular disulfide bonds on nascent immunoglobulin polypeptides. *J. Biol. Chem. 254:5690.*

108. Baumal, R., M. Potter, and M. D. Scharff. 1971. Synthesis, assembly and secretion of gamma globulin by mouse myeloma cells. III. Assembly of the three subclasses of IgG. *J. Exp. Med. 134:1316.*

109. Baumal, R., B. Bloom, and M. D. Scharff. 1971. Induction of long term lymphocyte lines from delayed hypersensitive human donors using specific antigen plus Epstein-Barr virus. *Nature New Biol. 230:20.*

110. Askonas, B. A., and A. R. Williamson. 1968. Interchain disulphide-bond formation in the assembly of immunoglobulin G. Heavy-chain dimer as an intermediate. *Biochem. J. 109:637.*

111. Sutherland, E. W. d., D. H. Zimmerman, and M. Kern. 1970. Synthesis and secretion of gamma-globulin by lymph node cells, VIII. Order of synthesis of the interchain disulfide linkages of immunoglobulins. *Proc. Natl Acad. Sci. USA 66:987.*

112. Parkhouse, R. M. 1971. Immunoglobulin M biosynthesis. Production of intermediates and excess of light-chain in mouse myeloma MOPC 104E. *Biochem. J. 123:635.*

113. Buxbaum, J., S. Zolla, M. D. Scharff, and E. C. Franklin. 1971. Synthesis and assembly of immunoglobulins by malignant human plasmacytes and lymphocytes. II. Heterogeneity of assembly in cells producing IgM proteins. *J. Exp. Med. 133:1118.*

114. Weitzman, S., and M. D. Scharff. 1976. Mouse myeloma mutants blocked in the assembly, glycosylation and secretion of immunoglobulin. *J. Mol. Biol. 102:237.*

115. Hickman, S., and S. Kornfeld. 1978. Effect of tunicamycin on IgM, IgA, and IgG secretion by mouse plasmacytoma cells. *J. Immunol. 121:990.*

116. Hickman, S., J. Kulczycki, A., R. Lynch, and S. Kornfeld. 1977. Studies of the mechanism of tunicamycin inhibition of IgA and IgE secretion by plasma cells. *J. Biol. Chem. 252:4402.*

117. Taylor, A. K., and R. Wall. 1988. Selective removal of α heavy-chain glycosylation sites causes immunoglobulin A degradation and reduced secretion. *Mol. Cell. Biol. 8:4197.*

118. Laskov, R., and M. D. Scharff. 1970. Synthesis, assembly, and secretion of gamma globulin by mouse myeloma cells. I. Adaptation of the Merwin plasma cell tumor-11 to culture, cloning, and characterization of gamma globulin subunits. *J. Exp. Med. 131:515.*

119. Laskov, R., R. Lanzerotti, and M. D. Scharff. 1971. Synthesis, assembly and secretion of gamma globulin by mouse myeloma

cells. II. Assembly of IgG2b immunoglobulin by MPC 11 tumor and culture cells. *J. Mol. Biol. 56:327.*

120. Sonenshein, G. E., M. Siekevitz, G. R. Siebert, and M. L. Gefter. 1978. Control of immunoglobulin secretion in the murine plasmacytoma line MOPC 315. *J. Exp. Med. 148:301.*

121. Dul, J. L., and Y. Argon. 1990. A single amino acid substitution in the variable region of the light chain specifically blocks immunoglobulin secretion. *Proc. Natl. Acad. Sci. USA 87:8135.*

122. Mosmann, T. R., and A. R. Williamson. 1980. Structural mutations in a mouse immunoglobulin light chain resulting in failure to be secreted. *Cell 20:283.*

123. Cowan, N. J., D. S. Secher, and C. Milstein. 1974. Intracellular immunoglobulin chain synthesis in non-secreting variants of a mouse myeloma: detection of inactive light-chain messenger RNA. *J. Mol. Biol. 90:691.*

124. Gardner, A. M., S. Aviel, and Y. Argon. 1993. Rapid degradation of an unassembled immunoglobulin light chain is mediated by a serine protease and occurs in a pre-Golgi compartment. *J. Biol. Chem. 268:25940.*

125. Wu, G. E., N. Hozumi, and H. Murialdo. 1983. Secretion of a λ2 immunoglobulin chain is prevented by a single amino acid substitution in its variable region. *Cell 33:77.*

126. Dul, J. L., O. R. Burrone, and Y. Argon. 1992. A conditional secretory mutant in an Ig L chain is caused by replacement of tyrosine/phenylalanine 87 with histidine. *J. Immunol. 149:1927.*

127. Nakaki, T., R. J. Deans, and A. S. Lee. 1989. Enhanced transcription of the 78,000-dalton glucose-regulated protein (grp78) gene and

association of grp78 with immunoglobulin light chains in a nonsecreting B-cell myeloma line (NS-1). *Mol. Cell. Biol. 9:2233.*

128. Williams, A. F., and A. N. Barclay. 1988. The immunoglobulin superfamily - domains for cell surface recognition. *Annu. Rev. Immunol. 6:381.*

129. Glockshuber, R., T. Schmidt, and A. Pluckthun. 1992. The disulfide bonds in antibody variable domains: effects on stability, folding in vitro, and functional expression in Escherichia coli. *Biochemistry 31:1270.*

130. Roth, R. A., and S. B. Pierce. 1987. In vivo cross-linking of protein disulfide isomerase to immunoglobulins. *Biochemistry 26:4179.*

131. Hendershot, L., D. Bole, G. Köhler, and J. F. Kearney. 1987. Assembly and secretion of heavy chains that do not associate posttranslationally with immunoglobulin heavy chain-binding protein. *J. Cell Biol. 104:761.*

132. Hendershot, L. M. 1990. Immunoglobulin heavy chain and binding protein complexes are dissociated in vivo by light chain addition. *J. Cell Biol. 111:829.*

133. Pillai, S. 1991. Immunoglobulin transport in B cell development. *Int'l. Rev. Cyt. 130:1.*

134. Morrison, S. 1978. Murine heavy chain disease. *Eur. J. Immunol. 8:194.*

135. Seligmann, M., E. Mihaesco, J. L. Preud'homme, F. Danon, and J. C. Brouet. 1979. Heavy chain diseases: current findings and concepts. *Immunol. Rev. 48:145.*

136. Haas, I. G. 1991. BiP - a heat shock protein involved in immunoglobulin chain assembly. *Curr. Topics Microbiol. Immunol. 167:71.*

137. Franklin, E. C., B. Frangione, and F. Prelli. 1976. The defect in mu heavy chain disease protein GLI. *J. Immunol. 116:1194.*

138. Pollok, B. A., R. Anker, P. Eldridge, L. Hendershot, and D. Levitt. 1987. Molecular basis of the cell-surface expression of immunoglobulin mu chain without light chain in human B lymphocytes. *Proc. Natl Acad. Sci. USA 84:9199.*

139. Capon, D. J., S. M. Chamow, J. Mordenti, S. A. Marsters, T. Gregory, H. Mitsuya, R. A. Byrn, C. Lucas, F. M. Wurm, J. E. Groopman, S. Broder, and D. H. Smith. 1989. Designing CD4 immunoadhesins for AIDS therapy. *Nature 337:525.*

140. Kreis, T. E., and H. F. Lodish. 1986. Oligomerization is essential for transport of vesicular stomatitis viral glycoprotein to the cell surface. *Cell 46:929.*

141. Bachhawat, A. K., and S. Pillai. 1991. Distinct intracellular fates of membrane and secretory immunoglobulin heavy chains in a pre-B cell line. *J. Cell Biol. 115:619.*

142. Hochstenbach, F., V. David, S. Watkins, and M. B. Brenner. 1992. Endoplasmic reticulum resident protein of 90 kilodaltons associates with the T- and B-cell antigen receptors and major histocompatibility complex antigens during their assembly. *Proc. Natl Acad. Sci. USA 89:4734.*

143. Galvin, K., S. Krishna, F. Ponchel, M. Frohlich, D. E. Cummings, R. Carlson, J. R. Wands, K. J. Isselbacher, S. Pillai, and M. Ozturk. 1992. The major histocompatibility complex class I antigen-

binding protein p88 is the product of the calnexin gene. *Proc. Natl Acad. Sci. USA 89:8452.*

144. Lin, H. Y., P. Massowelch, Y. P. Di, J. W. Cai, J. W. Shen, and J. R. Subjeck. 1993. The 170-kDa Glucose-Regulated Stress Protein Is an Endoplasmic Reticulum Protein That Binds Immunoglobulin. *Mol. Biol. Cell 4:1109.*

145. Hamel, P. A., M. H. Klein, and K. J. Dorrington. 1986. The role of the VL- and VH- segments in the preferential reassociation of immunoglobulin subunits. *Mol. Immunol. 23:503.*

146. Knarr, G., M.-J. Gething, S. Modrow, and J. Buchner. 1995. BiP binding sequences in antibodies. *J. Biol. Chem. 270:27589.*

147. Steipe, B., B. Schiller, A. Pluckthun, and S. Steinbacher. 1994. Sequence statistics reliably predict stabilizing mutations in a protein domain. *J Mol Biol 240:188.*

148. Helms, L. R., and R. Wetzel. 1995. Destabilizing loop swaps in the CDRs of an immunoglobulin V_L domain. *Prot. Sci. 4:2073.*

149. Hurle, M. R., L. R. Helms, L. Li, W. Chan, and R. Wetzel. 1994. A role for destabilizing amino acid replacements in light-chain amyloidosis. *Proc. Natl. Acad. Sci. USA 91:5446.*

APPENDIX

Cell Growth Comparisons

Even though there were no problems with routine cell culture and passage of the low secretor transfectants, it was possible that non-secreted H chains may be toxic to cells when accumulated intracellularly. Therefore we compared, by trypan blue exclusion, the expansion of a known number of T15 WT and mutant transfectants over time. Cells were cultured at 10^6 cells/well, and at 24 and 48 h cells were harvested from wells and counted. At all times, 0, 24, and 48 h, cell viability was greater than 90%, and the numbers of viable cells were recorded. Figure 1 illustrates a representative experiment of growth comparison. It is apparent from these data that the expression of mutant T15 H chains has no detrimental effect on cell proliferation, in fact, at 48 h the mutants consistently exhibited slightly better growth than the WT transfectant.

Single Mutant Analysis

Since the T15 low secretors have multiple HCDR2 mutations, it was of interest to determine the contribution of each individual mutation to the secretion phenotype. Single mutants with the representative changes present in all four low secretors were produced by site-directed mutagenesis and analyzed for secretion after stable transfection into SP2/0 Vκ22 (i.e. transfectant expressing the T15 L chain). In some cases, single mutants with different changes at these positions were available in the lab or convenient to make from a degenerate mutagenic oligo. The methods employed for mutagenesis, subcloning and transfection have

been described (38). Table I illustrates the secretion profile of these single mutants. It is evident from this analysis that none of the single mutants has the low secretor phenotype, indicating that this H chain is tolerant to one amino acid change, at least in the HCDR2 region. A similar finding was reported with this same region of T15 with respect to antigen binding (38). In two cases that have been identified, two mutations of this region can confer the low secretor phenotype: the original mutant M164 (R52I, Y59S), and a double mutant produced by site-directed mutagenesis S51R, Y59S. This latter double mutant has 2 of the 3 changes present in the original mutant M241, suggesting that the third replacement in M241 (D54E) does not significantly contribute to the secretion defect.

Molecular Chaperone Association

Molecular chaperones interact transiently with nascent proteins, and are believed to participate in the proper folding and assembly of proteins. Two such chaperones, BiP and Grp94, have been shown to interact with Ig proteins. Lysates from metabolically labeled cells were immunoprecipitated with Protein A-Sepharose, as was described in the methods of Paper 3 of this thesis. The experiment shown here (Figure 2) differs in that the cross-linker dithiobis(succinimidyl propionate) (DSP, Pierce, Rockford, IL) was added to the lysis buffer at 100 µg/ml, and after lysis, 10 mM glycine was added to block excess DSP. The Western blot detection of BiP was as described in Paper 3, and the Grp94 band was identified with rat anti-chicken Grp94 (StressGen, Victoria B.C.), followed by goat anti-rat IgG-AP (Sigma, St. Louis, MO).

The data from two independent experiments where proteins associated with H chains were immunoprecipitated in the presence of DSP are summarized in Table II. Densitometry was performed as described in Paper 3, and band intensities are normalized to the amount of H chain for each mutant and expressed relative to the levels of each protein associated with the T15 WT H chain. The increased association of Grp94 and BiP with the mutant H chains is similar, ranging from 7- to 13-fold, and 9- to 20-fold, respectively. The association with Grp94 is detectable in the presence of cross-linker, consistent with data reported from other labs (86, 87). It is also notable that more L chain is associated with the T15 WT H chain than with mutant H chains.

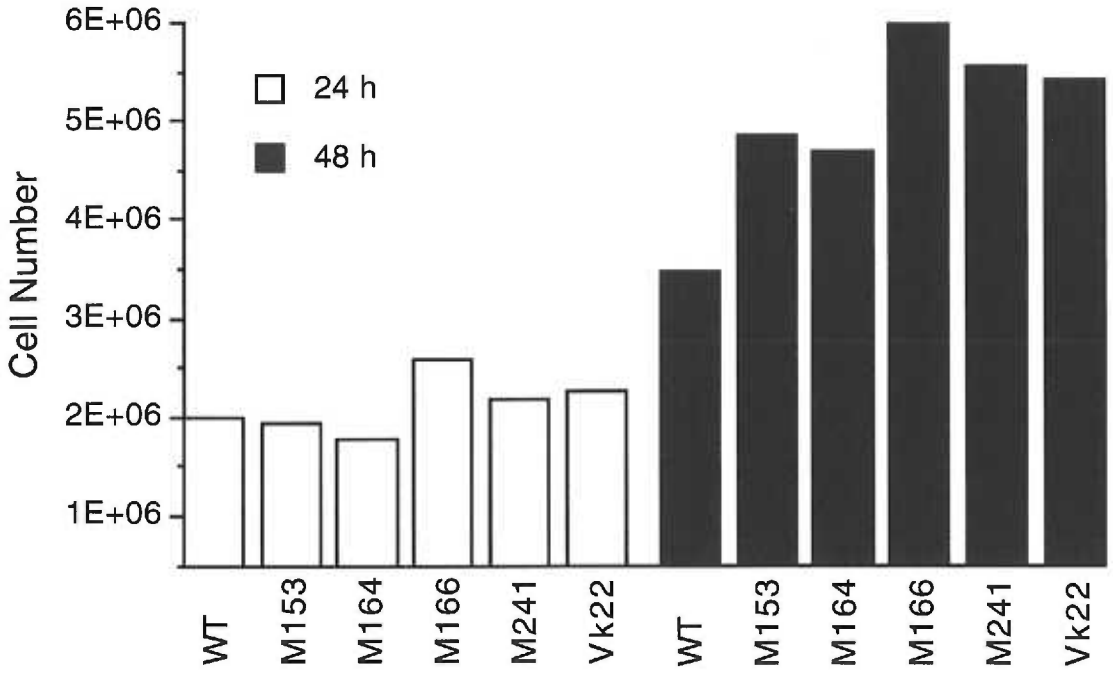


Figure 1. Comparison of cell growth in culture after 24 and 48 h. Stable transfectants were assayed for cell number after plating of 10^6 cells/well. WT indicates the T15 WT H chain expressed with the T15 L chain. M153, M164, M166, and M241 are mutant T15 H chains expressed with the T15 L chain. Vk22 is a transfectant expressing the T15 L chain alone, and is the cell line into which the mutant H chains were introduced.

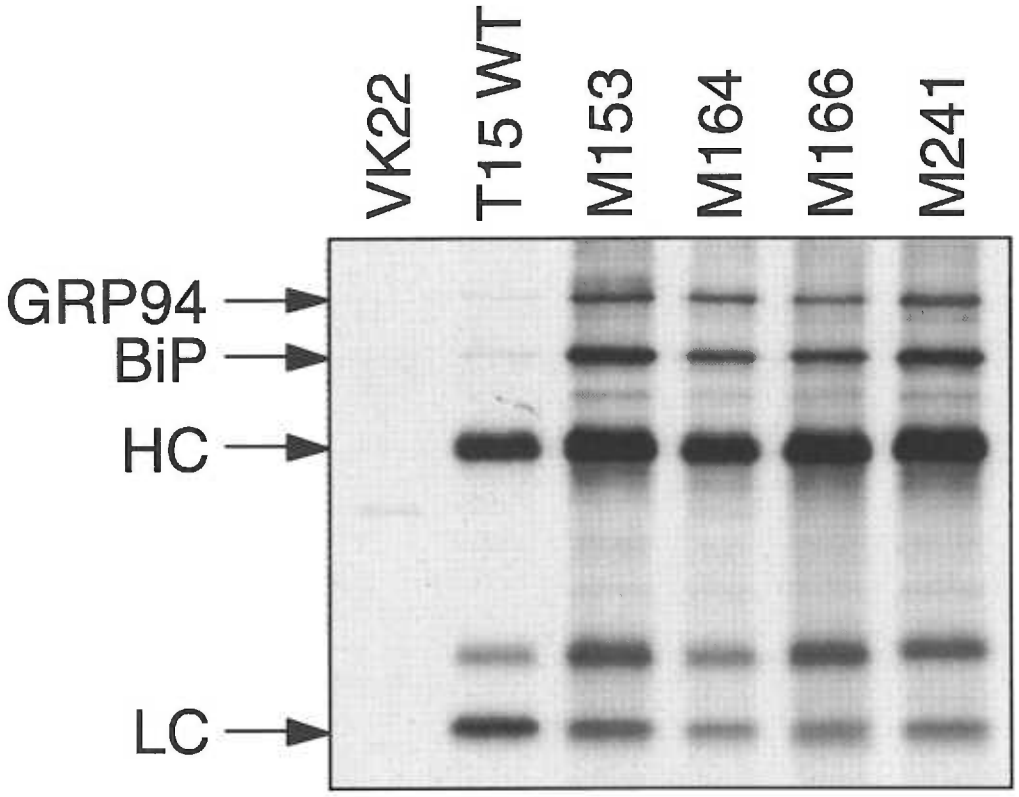


Figure 2. Immunoprecipitation of proteins associated with T15 WT and mutant H chains. Cells were metabolically labeled for 4.5 h and lysed in the presence of cross-linker (DSP). Lysates were immunoprecipitated with Protein A-Sepharose and analyzed by reducing SDS-PAGE. The identities of BiP and Grp94 were identified by Western Blot analysis (data not shown).

Table I. Secretion competence of T15 HCDR2 mutants.

	50	51	52	52 a	52 b	52 c	53	54	55	56	57	58	59	60	61	62	63	64	65	SN Ig
T15 WT	A	S	R	N	K	A	N	D	Y	T	T	E	Y	S	A	S	V	K	G	+
M153	-	-	-	-	-	-	-	-	-	-	-	-	-	T	V	-	M	T	-	-
M164	-	-	I	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-
M166	-	-	-	K	-	-	-	-	-	-	-	G	-	-	-	Y	M	-	-	-
M241	-	R	-	-	-	-	-	E	-	-	-	-	S	-	-	-	-	-	-	-
51R,59S	-	R	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-
S51R	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
S51C	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
S51G	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
R52I	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
R52G	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
R52K	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
R52S	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
R52T	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
R52aK	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
R52aT	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
D54E	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	+
E58G	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	+
E58A	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	+
Y59S	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	+
S60T	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	+
S60I	-	-	-	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-	-	+
A61V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	+
A61E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	+
S62Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-	-	+
S62C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	+
V63M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M	-	-	+
V63L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	+
V63C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	+
K64T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	+

Supernatant (SN) Ig was quantitated either in 4 h cultures or 2 day cultures and each assay was compared to the levels of T15 WT secreted. (-) <10% of T15 WT level; (+) >10% of T15 WT level.

Table II. Densitometric quantitation of association of Grp94, BiP and L chain with T15 WT and mutant H chains.

	Grp94	BiP	L chain
T15 WT	1	1	1.0
M153	13	20	0.4
M164	13	14	0.2
M166	7	9	0.2
M241	9	15	0.2

Relative association = $\frac{\text{(O.D. chaperone with mutant/ O.D. chaperone with WT)}}{\text{(O.D. mutant H chain/ O.D. WT H chain)}}$