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# A single amino acid substitution in the variable region of the light chain specifically blocks immunoglobulin secretion

**(protein transport/transient transfection/site-directed mutagenesis)**

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ABSTRACT **Although immunoglobulin light chains are usually secreted in association with heavy chains, free light chains can be secreted by lymphocytes. To identify the structural features of light chains that are essential for their secretion, we mutated a conserved sequence in the variable domain of a λI light chain. The effects of the mutations on secretion were assayed by transient expression in COS-1 cells. One mutant (AV60), which replaced Ala-60 with Val, was secreted as efficiently as wild-type λI by transfected COS-1 cells. This result was not surprising because secreted λII chains contain valine in this position. However, a second λI mutant (AV60FS62), which replaced Phe-62 with Ser as well as Ala-60 with Val, was not secreted. This mutant was arrested in the endoplasmic reticulum, as judged by immunofluorescence and by its association with a lumenal endoplasmic reticulum protein, immunoglobulin heavy chain binding protein (BiP). The defect in secretion was not due to gross misfolding of the λI chain, since cells cotransfected with AV60FS62 and an immunoglobulin heavy chain gene produced functional antigen-binding antibodies. These assembled IgM molecules were still not secreted. Hence, the replacement of Phe-62 with Ser specifically affects a determinant on the λI light chain that is necessary for the intracellular transport of this molecule.**

Two major requirements for secretion of proteins are proper folding and correct assembly of their subunits (1–5). Misfolded or unassembled polypeptides are usually retained intracellularly, often in the endoplasmic reticulum (ER) in association with a lumenal protein, immunoglobulin heavy chain binding protein (BiP) (2, 4, 6, 7). For oligomeric proteins like immunoglobulins, noncovalent chain association and formation of interchain disulfide bonds begin cotranslationally and are completed in the ER (8, 9). When the amount of light chain is limiting, immunoglobulin assembly and secretion are impeded (10). Interestingly, the secretory capabilities of free immunoglobulin heavy and light chains differ. Mutants that lack heavy chains secrete free light chains (1, 11), but free heavy chains are usually not secreted (12–14). Thus, free light chains are transport competent, but free heavy chains are not.

One possible explanation for this difference in transport competence is that light chains bear determinants that are required not only for their own secretion but also for the secretion of assembled immunoglobulin molecules. Several observations suggest, somewhat surprisingly, that portions of the variable domain of light chains ($V_L$) may contribute to one such site. Mutant κ chains lacking a $V_L$ domain, because of direct attachment of a signal sequence to the constant (C) domain, are translocated across the ER membrane but are not transported further (15). Moreover, a point mutation in residue 15 of the $V_L$ domain is sufficient to block secretion of the λII chain of the myeloma MOPC-315 (16). The interpretation of this mutation is limited, however, because it is not known how the mutation affects the folding of the protein. Since even minor changes in the amino acid sequence can have deleterious effects on protein folding (17, 18), structural analysis is required before the defect in secretion can be directly attributed to local changes in the polypeptide.

If there are structural features of immunoglobulin light chains that are crucial for secretion, they are likely to be conserved. The variable (V) domains contain a surprisingly large number of invariant or conserved residues (19). *In vitro* mutagenesis of these residues can be used to test their role in light chain secretion. We report here that substitution of one amino acid within a conserved region of the V domain of mouse λI blocked its secretion, even though the mutant chain maintained a normal conformation. Hence, the mutation exerted a direct effect on the secretory competence of this protein.

## MATERIALS AND METHODS

**Plasmid Constructions and Mutagenesis.** The double-primed method of Carter *et al.* (20) was used to generate mutations in the λI gene from the murine myeloma HOPC 2020 (21). The V and C regions of this gene were cloned separately into the mutagenesis vector M13K19 as follows: For the V region construct, a *Sal* I site was first added to the vector polylinker by linker ligation into the *Sma* I site. A 0.9-kilobase (kb) *Xba* I fragment containing the rearranged V region of λI was treated with the Klenow fragment of DNA polymerase I (Klenow polymerase) and cloned into the *Bam*HI site (also treated with the Klenow polymerase) of this vector. In the resulting construct the λI insert was flanked by unique *Sal* I and *Xba* I sites from the polylinker of the vector. Similarly, a 3.5-kb *Xba* I fragment containing the C region of λI was treated with Klenow polymerase and cloned into the *Sma* I site of M13K19. In this construct, the insert is flanked by unique *Xba* I and *Eco*RI sites.

λI was expressed in COS-1 cells by using the vector pJDE, which provides high levels of expression of cloned genes under the control of the simian virus 40 late promoter. This vector was derived from pJC119 (22) to permit directional cloning of inserts. This was done by destroying the internal *Sal* I and *Eco*RI sites and adding *Sal* I and *Eco*RI sites to the unique *Xho* I cloning site. To clone the wild-type λI gene in pJDE, a three-point ligation was done with λ fragments isolated from the above M13K19 constructs. The 0.9-kb V region fragment was isolated from a *Sal* I–*Xba* I digest, the 3.5-kb C region fragment was isolated from an *Xba* I–*Eco*RI digest, and they were ligated into pJDE, which had been cut with *Sal* I and *Eco*RI. Since pJDE had no *Xba* I sites,

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Abbreviations: V, variable; $V_L$, V domain of light chains; C, constant; BiP, immunoglobulin heavy chain binding protein; NIP, (4-hydroxy-5-iodo-3-nitrophenyl)acetyl; ER, endoplasmic reticulum.
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mutations created in the M13K19 clones were then introduced into pJDEλ by cassette cloning of the V region fragment. Mutations were confirmed by sequence analysis using the dideoxynucleotide chain-termination method (23).

To express μ heavy chains in COS-1 cells, a rearranged genomic μ gene, isolated from the plasmid pμΔ3 (24) by digestion with *Sal* I and *Xho* I, was cloned into the *Xho* I site of pJC119.

**Cell Culture and Transfection.** COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 2 mM L-glutamine. Subconfluent monolayers of cells were transfected by the DEAE-dextran method (25), which included a 3-hr incubation with 100 μM chloroquine. After incubating 4–12 hr in medium alone, to allow recovery from the chloroquine shock, the cells were treated for 12 hr with 10 mM sodium butyrate (26). Cells were analyzed 2 days after transfection.

**Immunofluorescence.** Two days after transfection, COS-1 cells were permeabilized and stained with a tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse λ antibody (Southern Biotechnology Associates, Birmingham, AL) as described (27).

**Immunoprecipitation.** Metabolic labeling and immunoprecipitation were performed as described (28). Briefly, transfected COS-1 cells at $5 \times 10^6$ cells per ml were labeled with [$^{35}$S]methionine (Amersham) at 100–250 μCi/ml (1 Ci = 37 GBq) for the indicated times. Cell lysates and supernatants from equal numbers of cells were immunoprecipitated with rabbit anti-λ antiserum (Organon Teknika, Durham, NC) or monoclonal antibody to BiP (generous gift of D. Bole, Yale University) that was followed by protein A-Sepharose and were analyzed by electrophoresis in 10% polyacrylamide gels containing $NaDodSO_4$. Functional immunoglobulin molecules were isolated from transfected cells by immunoadsorption on NIP-Sepharose [NIP is (4-hydroxy-5-iodo-3-nitrophenyl)acetyl] (28) and analyzed by one- or two-dimensional gel electrophoresis.

## RESULTS

To define structural features of light chains that may be required for secretion, we examined the $V_L$ sequences of murine light chains. We found that residues 57–65 (nomenclature of Kabat *et al.* (19)] are highly conserved, not only among murine λ and κ chains but also across species lines. Inspection of the crystal structure of mouse and human κ chains shows that these residues, which follow the second hypervariable loop, form part of the external surface of the molecule (Fig. 1). This surface is not involved in antigen binding and does not form contacts with heavy chain (31, 32). We hypothesize that this conserved sequence is involved in the intracellular transport of light chains.

To test this hypothesis, we used site-directed mutagenesis of a cloned λI gene and generated mutations in this region of the protein. In one mutant, the codon for Ala-60 was changed to a codon for valine (AV60). This substitution reconstituted the local sequence of wild-type λII in a λI chain and as such was not expected to alter the phenotype of the protein. Indeed, when expressed in COS-1 cells, AV60 was synthesized and secreted as efficiently as wild-type λI (Fig. 2*a*, lanes 1, 2, 5, and 6). Pulse-labeled AV60 chains could be chased into the medium, as shown in Fig. 2*b*. This demonstrated that the extracellular appearance of light chains was not due to cell death but was the result of bona fide secretion by the cells.

A second mutant (AV60FS62) containing two substitutions was constructed. As in AV60, Ala-60 was changed to Val and, in addition, Phe-62 was changed to Ser. We chose to alter Phe-62 because its side chain seems to pack closely

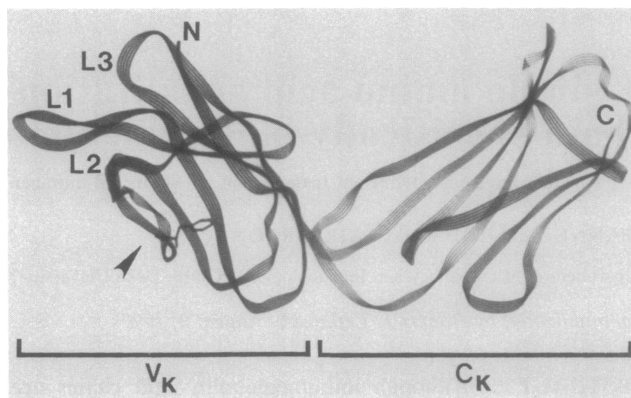


FIG. 1. Ribbon diagram of the McPC 603 κ chain (29). $V_κ$, variable domain; $C_κ$, constant domain; N, amino terminus; C, carboxyl terminus; L1, L2, and L3, first, second, and third hypervariable loops of the antigen binding site, respectively. The contacts with the heavy chain are on the back side and across the top of the molecule as drawn. The arrow indicates the loop formed by residues 57–65. The single side chain shown is that of Phe-62. This model was generated using file 2MCP from the Brookhaven data bank (30).

between the conserved residues Pro-59 and Tyr-86. Alteration of this residue would thus be expected to change the local conformation of the polypeptide. This double mutant AV60FS62 was expressed efficiently in COS-1 cells (Fig. 2*a*, lane 3) but was not secreted (Fig. 2*a*, lane 7). In a pulse-chase regime, the light chain did not appear extracellularly in any detectable amounts (Fig. 2*b*). The amount of intracellular AV60FS62 decreased slightly during the chase period (although not by nearly as much as did AV60), indicating the

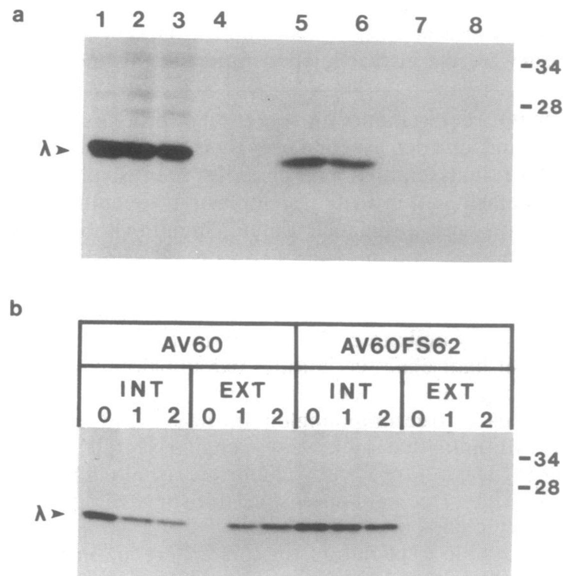


FIG. 2. Synthesis and secretion of λ chains by transfected COS-1 cells. (*a*) COS-1 cells were transfected with wild-type (lanes 1 and 5), AV60 (lanes 2 and 6), or AV60FS62 (lanes 3 and 7) DNA or were mock-transfected (lanes 4 and 8). Two days later the cells were labeled with [$^{35}$S]methionine for 2 hr. Cell lysates (lanes 1–4) and supernatants (lanes 5–8) from equal numbers of cells were immunoprecipitated with rabbit anti-λ antiserum and protein A-Sepharose and analyzed by $NaDodSO_4$/ PAGE on 10% gels. (*b*) COS-1 cells transfected with AV60 or AV60FS62 DNA were pulse-labeled with [$^{35}$S]methionine for 30 min and chased for 0, 1, or 2 hr, as indicated by lane labels. Cell lysates (INT) and supernatants (EXT) were immunoprecipitated as above. Apparent molecular masses are shown on the right in kDa, and the position of the λ light chain is indicated on the left.

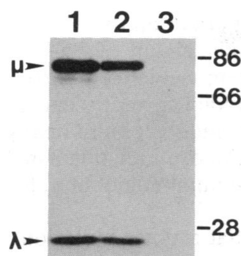


FIG. 3. Assembly of mutant λ chains with μ heavy chains to form functional antibodies. COS-1 cells were cotransfected with equal quantities of μ and AV60 DNA (lane 1) or of μ and AV60FS62 DNA (lane 2) or were transfected with μ DNA alone (lane 3). Two days later the cells were labeled with [$^{35}$S]methionine for 3 hr. Equivalent cell lysates were immunoadsorbed on NIP-Sepharose and analyzed by $NaDodSO_4$/PAGE on 10% gels. Apparent molecular masses are shown on right in kDa, and the positions of the μ heavy and λ light chains are indicated on the left.

nonsecreted chains are being degraded within the cell. Hence, the single amino acid change of Phe-62 to Ser was sufficient to arrest the transport of this protein.

This effect on the secretory capability of AV60FS62 could have been indirect if the Phe-62 to Ser mutation merely caused improper folding of the protein. Two lines of evidence show that this is not the case. (*i*) The nonsecreted polypeptide was recognized as efficiently as the secreted one by several polyclonal and monoclonal anti-λ reagents (data not shown), suggesting that it was not grossly misfolded. (*ii*) As a more direct assay for the conformation of the mutants, we determined whether they were capable of assembling with heavy chains to form functional antibodies. A μ heavy chain that binds the hapten NIP, when combined with λ light chains (33), was coexpressed in COS-1 cells with each of the mutants. [$^{35}$S]Methionine-labeled immunoglobulin was isolated from cell lysates by adsorption to NIP-Sepharose beads. Heavy chains alone or light chains alone do not bind NIP (ref. 33 and Fig. 3, lane 3). Therefore, only those heavy and light chains that were properly assembled into functional immunoglobulin molecules would be isolated in this way. As Fig. 3 shows, both the secreted and the nonsecreted λ mutants assembled with heavy chains to produce NIP-binding antibodies.

This ability to assemble into functional immunoglobulin is not limited to only a small portion of the AV60FS62 λ chains. To exclude this possibility, we used sequential immunoprecipitation protocols, where lysates of transfected cells were treated first with NIP-Sepharose, to isolate quantitatively the assembled chains, and then with anti-λ, to isolate the rest of the molecules. In two experiments, 61% and 68% of wild-type λ assembled with wild-type μ. Considering that we measure transient coexpression of gene products, this efficiency of assembly is quite good. In parallel experiments, the fraction of AV60FS62 that formed functional immunoglobulin was 28%. Thus, while this mutant assembled more poorly than wild-type λ, a large portion of the molecules were capable of assembly. The level of μ chain expression was not limiting, since we could still isolate free heavy chains after all the light chains were precipitated. To assess the relative affinity of the secreted and the nonsecreted mutants for NIP, we allowed binding of transfected cell lysates to NIP-Sepharose and performed sequential desorption with increasing concentrations of free NIP. The concentration of NIP required to elute half the IgM was ≈0.3 mM, whether the light chain was AV60 or AV60FS62. This concentration is similar to that required to elute half the NIP-binding IgD made by hybridoma B1-8.δ.1 (J.L.D., S. Aviel, and Y.A., unpublished data). Thus, at least by this assay the affinity of the assembled IgM for NIP is not affected by the use of the nonsecreted light chain. We consider the ability to form functional IgM as good evidence that the substitution of Ser for Phe in AV60FS62 affected secretion of λI directly without grossly altering its structure.

Immunofluorescence of transfected COS-1 cells was used to localize the intracellular site of arrest of AV60FS62. Cells expressing either wild-type λ or mutant AV60 lacked any surface staining, displayed a reticular pattern typical of ER staining, and showed intense perinuclear staining indicative of the presence of the light chain in the Golgi complex (Fig. 4*a*). This overall pattern is consistent with that of a secreted protein. In cells transfected with nonsecreted AV60FS62, however, the prominent perinuclear staining was completely absent (Fig. 4*b*). Rather, the staining of the nuclear envelope and the ER was more pronounced, indicating that these mutant λ chains had accumulated in the ER.

Mutant heavy chains have been shown to be retained in the ER by association with the lumenal ER protein BiP (2, 6). Nakaki *et al.* (34) have also shown that BiP binds a nonsecreted κ chain. We found that immunoprecipitation of the nonsecreted λ mutant with anti-λ antiserum coprecipitated a second protein of 78 kDa (Fig. 5, lane 3). No similar 78-kDa band is enriched in immunoprecipitations of transfected wild-type λ or the secreted mutant AV60 (Fig. 5, lanes 1–4). This additional protein has the same molecular weight as BiP (2, 6). Reciprocal immunoprecipitation demonstrated that this protein was indeed BiP, since monoclonal anti-BiP antibodies coprecipitated BiP and λ (Fig 5, lanes 5 and 6). Since BiP is restricted to the lumen of the ER (35), the AV60FS62 chains associated with it must also be in the ER. Thus with the immunofluorescence data, these experiments

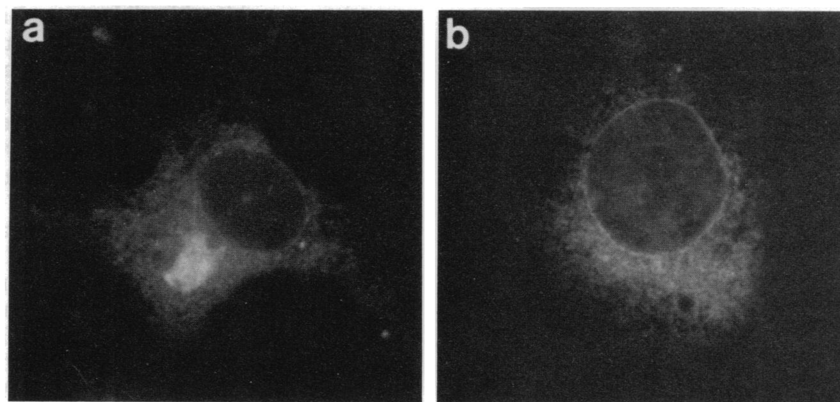


FIG. 4. Intracellular distribution of secreted and nonsecreted λ chains. Immunofluorescence of COS-1 cells transfected with wild-type (*a*) or AV60FS62 mutant (*b*) λ. Two days after transfection cells were permeabilized and stained with a tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse λ antibody.

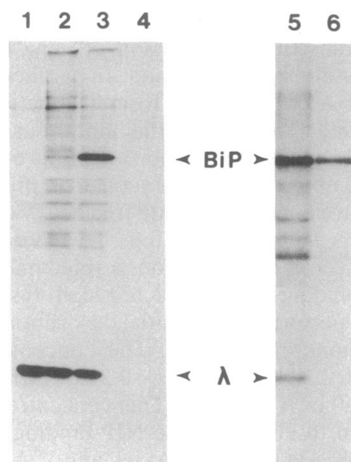


FIG. 5. Coprecipitation of nonsecreted λ with BiP. COS-1 cells were transfected with wild-type (lane 1), AV60 (lane 2), or AV60FS62 (lanes 3 and 5) DNA or were mock-transfected (lanes 4 and 6). Two days later they were labeled with [$^{35}$S]methionine for 2 hr. Equivalent cell lysates were immunoprecipitated with rabbit anti-mouse λ antiserum (lanes 1–4) or monoclonal anti-BiP antibody (lanes 5 and 6) and analyzed by NaDodSO$_4$/PAGE on 10% gels.

show that the Phe → Ser mutation in AV60FS62 blocks secretion of the protein by preventing its exit from the ER.

Since a large fraction of AV60FS62 chains assembled with $\mu$ in cotransfected cells, we next asked if this IgM is secreted. Pulse–chase analysis of the NIP-binding IgM (Fig. 6) shows that whereas the IgM formed with AV60 is secreted by COS cells, the IgM formed with AV60FS62 is not secreted. The oligosaccharides borne by these intracellular $\mu$ chains were sensitive to digestion with endoglycosidase H (data not shown), indicating that the IgM was likely to be arrested in the ER. Thus, the presence of heavy chains cannot rescue secretion of the mutant light chain. Rather, the presence of serine at position 62 blocks not only the secretion of free λ chains but also the secretion of functional IgM containing these light chains.

## DISCUSSION

The experiments described here show that substitution of a single amino acid within a conserved sequence of the V domain has a direct effect on the competence of light chains to be secreted. Replacement of Phe-62 with Ser created a nonsecreted λ mutant, which was arrested in the ER. The mutant chain was folded correctly inasmuch as it was able to assemble with an immunoglobulin heavy chain and form an antigen-binding antibody, indistinguishable from a secreted IgM. The functional immunoglobulin, however, was still not secreted.

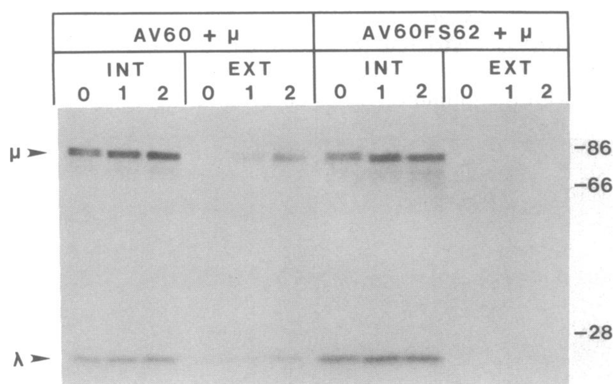


FIG. 6. Pulse–chase analysis of assembled IgM. COS-1 cells were cotransfected with equal quantities of AV60 and $\mu$ DNA or of AV60FS62 and $\mu$ DNA. Two days later, the cells were pulse-labeled with [$^{35}$S]methionine for 30 min and chased for 0, 1, or 2 hr, as indicated by lane labels. Cell lysates (INT) and supernatants (EXT) were immunoadsorbed on NIP-Sepharose and analyzed by NaDodSO$_4$/PAGE on 10% gels. Apparent molecular masses are shown on the right in kDa and the positions of the heavy and light chains are indicated on the left.

Our data show that a point mutation in the light chain is sufficient to block secretion of the free light chain as well as of the assembled immunoglobulin. This extends a similar conclusion reached by Wu *et al.* (16), who found that a spontaneous mutation in a λII chain, whose Gly-15 was replaced by Arg, blocked the secretion of that light chain. When Gly-15 is replaced with Arg in λI, this mutant is also not secreted when expressed in COS-1 cells (S. Aviel, personal communication). It is interesting to note that both Phe-62 and Gly-15 are in the V domain. Thus, mutations in the V domain as well as in the C domain (11) can block immunoglobulin secretion. The altered residues in both mutants are highly conserved among more than 260 light chain sequences. Phe-62 is part of a conserved sequence, consisting of residues 57–65. Inspection of crystal structures of several mouse and human immunoglobulins reveals that this sequence forms a loop, which lies in striking proximity to the $\beta$-turn containing Gly-15. The results with both mutants, therefore, support the idea that these conserved noncontiguous elements contribute to a surface patch that is necessary for the transfer of light chains from the ER to Golgi complex. The effect of substituting Ser for Phe-62 suggests that the packing of Phe-62 against Tyr-86 and Pro-59 is important in maintaining the shape of this surface.

The intracellular stage where secretion is blocked is transport from the ER. This conclusion is based on microscopy and on association of the arrested λ chains with the resident ER protein BiP. It is interesting to note that exit from the ER is also the stage affected in several other nonsecreted immunoglobulin mutants (36). Even though our AV60FS62 was generated without any obvious phenotypic selection, it is arrested in the same organelle. This highlights the importance of ER-to-Golgi transport in the immunoglobulin secretory pathway.

The association with BiP demonstrates that BiP is not merely a heavy chain binding protein in B-lymphoid cells (2, 6). We show here that BiP is capable of binding λ light chains, and its binding to $\kappa$ chains has also been reported (34). By using a collection of λ chains that either do or do not complex with BiP, it should be possible to map the binding site.

We do not think that the binding to BiP is the mechanism responsible for the retention of the mutant in the ER. Preliminary data indicate that when AV60FS62 and other λ mutants assemble with wild-type $\mu$, BiP is not associated with the complex (S. Aviel and Y.A., unpublished data), as if it is displaced during immunoglobulin chain assembly. Since these functional IgM molecules are still arrested in the ER of COS cells, retention mechanisms other than association with BiP are likely to be operative. Given that BiP is known to bind to many misfolded proteins, this association could be interpreted as evidence for misfolding of AV60FS62. However, we show that this mutant light chain can assemble with heavy chain to form functional antigen binding sites, a finding that argues that the polypeptides fold normally. Moreover, BiP has been shown to associate transiently with wild-type proteins that are in the process of normal intracellular transport (4, 37), possibly assisting in their assembly (38). We therefore interpret the persistent binding of AV60FS62 to BiP as a consequence, rather than the cause, of its arrested transport.

It should be emphasized that our assembly assay for folding of the light chains only measures the mutant chains in the presence of heavy chains. We have not measured the

folding of free light chains, other than by serology, and it is conceivable that their conformation is slightly different from that of assembled light chains. However, from the known crystal structures of light chain dimers and immunoglobulin fragments, there is no evidence that chain association changes the folding of the various immunoglobulin domains significantly (39). We should also note that the nonsecreted $\lambda$ chains do not seem to aggregate or to form disulfide-linked dimers. In this respect they are again indistinguishable from the secreted $\lambda$ chains, which are secreted as monomers (S. Aviel and Y.A., unpublished data). Clearly, more direct biochemical studies are needed to resolve the structure of free nonsecreted $\lambda$ chains. Nevertheless, the mutation prevents the secretion of the whole functional IgM as well as the secretion of free $\lambda$ chains. We consider this strong evidence that it affects a determinant that is needed for proper intracellular transport of both free light chains and assembled immunoglobulin.

It is attractive to hypothesize that the patch on the external surface of light chains is recognized by the cellular transport machinery so as to allow the passage of the light chains to the Golgi complex. The data presented in this paper, however, do not answer the question of how this surface is involved in the transport of light chains. It cannot yet be determined whether the Phe $\rightarrow$ Ser mutation has disrupted a site that constitutes a positive signal for transport or alternatively has created a site that constitutes a retention signal, incompatible with further transport.

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