Recovering Antibody Secretion Using a Hapten Ligand as a Chemical Chaperone*

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Engineered antibodies have come to the forefront as research reagents and clinical therapeutics. However, reduced stability or expression levels pose a major problem with many engineered antibodies. As a model for understanding functional consequences of variable region mutation, we have studied the assembly and trafficking of anti-phenylphosphocholine antibodies. Previously, we identified severe secretion defects because of mutations in the heavy chain second complementarity determining region, which is involved in antigen binding. Here we demonstrate that immunoglobulin secretion is increased up to 27-fold by incubating stably transfected PCG1–1 cells with cognate hapten p-nitrophosphocholine. Secretion was unaffected by nonbinding analogs. Radiotracer and metabolic labeling experiments demonstrated specific cellular uptake of p-nitrophosphocholine and increased intracellular heavy and light chain assembly. Brefeldin A inhibited hapten-mediated immunoglobulin secretion but not assembly, indicating that assembly occurs early within the biosynthetic pathway. Recovery of secretion correlated with antigen binding capacity, suggesting that the rescue mechanism involves stabilization of heavy and light chain variable domains. This model system provides the first demonstration that cognate ligands can increase intracellular assembly of functional anti-hapten antibody within mammalian cells and suggests that small molecules of appropriate specificity and affinity acting as chemical chaperones may find application for increasing or regulating immunoglobulin expression.

* This work was supported by a research award grant from the Oregon Chapter of the American Cancer Society (to G. D. W.) and by National Institutes of Health Grants AI-14985 and AI-26827 (to M. B. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: V-region, variable region; CDR, complementarity determining region; ER, endoplasmic reticulum; V10, variable region of Ig heavy chain; V11, variable region of Ig light chain; PC, phosphocholine; NPPC, p-nitrophosphocholine; APPC, p-amino-phenylphosphocholine; WT, wild type; H, heavy; L, light; ELISA, enzyme-linked immunosorbent assay; BFA, brefeldin A; PAGE, polyacrylamide gel electrophoresis.

Recently, it has been demonstrated that defective maturation of mutant proteins from the ER can be partially or fully corrected by incubation with low molecular weight compounds termed chemical chaperones (9, 10). Chemical chaperones can rescue both mutant secretory and transmembrane proteins that are otherwise destined for degradation (10–15). High concentrations of chemical chaperones, often in the millimolar range, are generally required to reverse trafficking defects. The mechanism(s) by which chemical chaperones function are not fully understood but are thought to include stabilization of improperly folded proteins (10), reduction of aggregation (13), and prevention of nonproductive interactions with ER proteins (11). Chemical chaperones such as glycerol and trimethylamine N-oxide appear to be nonspecific as they are able to rescue diverse types of proteins (16), whereas other chemical chaperones only function with a narrower set of proteins (13, 17). Until now chemical chaperones have not been shown to restore secretion of heteropolymers such as Ig.

Here we used anti-phenylphosphocholine antibodies as a model system to determine whether a defect in Ig secretion could be corrected by the use of the cognate hapten acting as a chemical chaperone. Incubation of stable transfectants with the cognate hapten, p-nitrophosphocholine (NPPC), led to rescue of secretion in five of six low secreting PCG1–1 mutants. NPPC was specifically taken up by cells and resulted in increased intracellular heavy and light chain assembly. In contrast, NPPC did not affect the amount of secretion of wild type (WT) Ig nor did it nonspecifically induce the release of incompletely assembled Ig chains, indicating that the interaction is highly specific. The capacity of trafficking-impaired Ig to bind antigen correlated with ligand-mediated rescue of secretion. These data indicate that the mechanism of rescue involves the intracellular stabilization of VH–VL pairing. Hapten ligands may find application for regulating or increasing assembly and secretion of anti-hapten Ig from hybridomas.

EXPERIMENTAL PROCEDURES

Cell Culture—The PCG1–1 heavy chain loss variant (PCG1–1 H–), and cell culture conditions were described previously (7). For antibody purification, cells were cultured in HyQ-CCM1 serum-free medium (HyClone Laboratories, Logan, UT) for 4 days in the presence of 5 μM...
p-nitrophenolphosphocholine (NPPC). Antibody was purified from sterile filtered supernatants using protein A-Sepharose.

**Ig Secretion Assay**—Stable transfectants were plated in triplicate at 1 × 10⁶ cells ml⁻¹ of tissue culture medium in a 12-well plate. After designated incubation periods, culture supernatants and cell lysates were collected as described (6).

**Hapten—Hapten**—Stable transfectants NPPC and p-aminophosphocholine (APP) were obtained from Sigma-Aldrich and dissolved in tissue culture medium prior to use. Phosphocholine (PC, Sigma-Aldrich) was dissolved in 0.1 M EDTA (pH 8.0) and diluted 1:10 in 0.1 M sodium phosphate buffer (pH 8.0). The solution was adjusted to pH 8.0 with 1 M KOH, precipitated salt was removed by centrifugation, and supernatant stored at −20 °C.

**[3H]**NPPC Synthesis—All reagents were from Sigma-Aldrich except [methyl-²H]choline chloride (specific activity = 75.00 Ci mmol⁻¹), which was from PerkinElmer Life Sciences. The procedure for the synthesis of [³H]NPPC was based on the method of the Cheng et al. (18) for the synthesis of p-nitrophenyl-3,3,N,N-trimethylpropylammonium phosphate, as described by Moulton (19). Ethanol solutions of unlabeled choline chloride (0.32 mmol) and [methyl-²H]choline chloride (800 μCi) were combined and evaporated to dryness (final specific activity = 2.5 mCi mmol⁻¹). In a separate tube, p-nitrophenolphosphorochloridate (0.35 mmol) and quinoline (101 μl; 0.85 mmol) were dissolved in dry CH₃CN (372 μl) and mixed with radiolabeled choline on ice. The tube containing the reaction mixture was rotated end-over-end at 4 °C for 8 h. Pyridine (300 μl) and H₂O (50 μl) were then added, and the solution was agitated at 25 °C for 30 min. Solvent was removed, and the resulting oil was dissolved in H₂O (1.0 ml), then passed through a TMD-8 mixed bed ion exchange column (2 cm × 12 cm) with H₂O as the elution solvent. The first 45 ml of flow-through was lyophilized, yielding [³H]NPPC as a white solid (0.11 mmol; 2.5 mCi mmol⁻¹; 34% isolated yield). The product was analyzed by cellulose thin layer chromatography (Merk silica gel, Kieselgel 60 F₂₅₄; Rf: 0.26 for NPPC). Product obtained from a side-by-side synthesis in which the radiolabeled choline chloride was replaced by the unlabeled choline was examined by ¹H NMR, ¹³C NMR, and ³¹P NMR. Each NMR spectrum was identical to the corresponding spectrum of authentic NPPC. The final [³H]NPPC was tested alongside authentic NPPC in an inhibition ELISA and 24-h secretion assay and found to be indistinguishable from the unlabeled hapten.

**Metabolic Labeling, Immunoprecipitation, and SDS-PAGE**—Transfected cells were cultured and labeled with 150 μCi of [³⁵S]Express protein labeling mix for 20 min as described (20) either in the presence or absence of NPPC. Unincorporated label was removed by washing cells two times and resuspending in complete tissue culture medium containing Met/Cys for the indicated chase time periods. Ig was immunoprecipitated from clarified cell lysates or supernatants by incubation with protein A-Sepharose CL-4B (Amersham Pharmacia Biotech). For assembly experiments the immunoprecipitates were washed with buffers as described previously (20). All labeling experiments were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and IP lab gel software (version 1.5; Analytix, Vienna, VA). In some experiments, cells were cultured in 5 μg ml⁻¹ brefeldin A (BFA; Sigma) to block secretion. Cells were treated for 1 h with brefeldin A prior to pulse labeling and during chase time points.

**ELISA**—The concentration of Ig proteins in supernatants and cell lysates was determined by sandwich ELISA as described previously (7). Briefly, to determine H+L in the supernatant or lysate, ELISA plates were coated with rabbit anti-mouse IgG (Zymed Laboratories Inc., San Francisco, CA), and the amount of bound Ig from the lysate or supernatant was determined by a secondary alkaline phosphatase-conjugated, goat anti-mouse κ antibody (Southern Biotechnology Associates, Birmingham, AL). All antisera were used at a 1:1000 dilution. Standard curves were generated using affinity-purified antibody from WT transfectant PCG1–1 (γ2b, κ) or hybridoma PCG2b-2 (γ2b, κ). Antibody binding to PC-histone was determined by a direct ELISA as described previously (7).

**[³H]**NPPC Uptake Assay—Trypsinized cells were washed twice and resuspended in tissue culture medium containing 20% fetal bovine serum, then allowed to adhere to 12-well tissue culture plates. For dose-response experiments, 1.0 × 10⁶ cells were plated, whereas 0.8 × 10⁶ cells were used for the time-course and inhibition experiments. In all experiments, after 12 h of incubation, nonadherent cells were removed with four washes of 1 ml of serum-free CCM-1 medium (HyClone) and dilutions of [³H]NPPC were added to cells in 0.5 ml of CCM-1 medium. As a negative control, cells were fixed with 4% paraformaldehyde solution in Dulbeco’s phosphate-buffered saline (pH 7.4) for 15 min prior to the addition of labeled hapten. For the dose-response and inhibition experiments, cells were incubated for an additional 14 h at 37 °C prior to harvest. At harvest, free [³H]NPPC was removed by washing cells four times with 1 ml of phosphate-buffered saline, and cells were solubilized in 0.5 ml of 0.5% SDS in 0.2 M NaOH solution as described (21). Lysates were transferred to vials, diluted in 10 ml of ScintiVerse (Fisher Scientific, Fair Lawn, NJ), and the radioactivity associated with the cells was quantified by liquid scintillation counting (Beckman model LS 3801).

**RESULTS**

NPPC Rescues Ig Secretion from Ile⁵¹ → Arg Stable Low Secretion Transfectants—Previously we proposed that V₅ mutations may impair Ig assembly and/or destabilize the H-L complex (20). A single mutation at position 51 appears sufficient to prevent stable H-L complex formation; we predicted that compounds stabilizing H-L interaction should increase secretion of this antibody. Because ligand binding can stabilize recombinant V₁-V₅ pairing in vitro (22, 23), we tested whether addition of the hapten NPPC (5 μm) to cultured cells would increase the secretion of Ig from the surrounding medium. Incubation of PCG1–1 Ile⁵¹ → Arg transfectant cells in NPPC significantly increased the amount of Ig in culture supernatant as determined by ELISA (15-fold ≥ 3 (mean ± S.E.); n = 7 independent experiments, Table I). Similar results were obtained with three independently created PCG1–1 Ile⁵¹ → Arg transfectants, indicating this is not a transfectant-specific effect (Data not shown). The addition of NPPC also augmented secretion of another Ile⁵¹ single-site secretion mutant, PCG1–1 Ile⁵¹ → Lys (9-fold ≥ 3; n = 4 independent experiments). The increase of Ig in the supernatant was dose-dependent, and the effect was specific to NPPC (Fig. 1A). Addition of the structurally related hapten PC, which does not bind to PCG1–1 WT antibody, did not increase the amount of Ig in culture supernatants. Similarly, the addition of 5 mm choline or 0.05 μm p-nitrophenol had no effect on secretion (data not shown). The
addition of 5 mM APPC resulted in a modest, 2.4-fold increase in secretion. APPC binds with -12-fold lower affinity as compared with NPPC (24), suggesting that binding affinity may correlate with efficiency of rescue. Increased supernatant Ig could not be attributed to toxicity as cell viability was greater than 95% in medium alone or medium with NPPC (Fig. 1B). To examine whether the hapten nonspecifically increased Ig chain secretion, the secretion of PCG1–1 L chains was measured as a control as it is produced in excess over transfected H chains (7). NPPC addition did not increase free PCG1–1 L chain secretion (Fig. 1C). Furthermore, NPPC did not increase secretion of H/L from WT PCG1–1 or T15 cells (data not shown).

Although NPPC did not affect the amount of secretion of WT Ig, the presence of this compound might nonspecifically induce the release of intracellularly retained, incompletely assembled Ig chains. This possibility was tested in two ways. First, we incubated NPPC with stable transfected cell lines that only express either the T15 H chain or the T15 L chain (V22 gene). We have previously demonstrated that both the T15 H chain and the T15 L chain, when expressed alone, are retained in the ER and that the T15 L chain is degraded via a proteasome-dependent pathway (25). The addition of 5 mM NPPC did not increase the amount of either T15 H chain or T15 L chain in supernatants, as measured by ELISA (Table I). These data indicate that NPPC does not nonspecifically disrupt the retention of unassembled Ig H or L chains in transfected cells. The presence of NPPC also did not alter the steady state balance between synthesis and degradation of the nonsecreted T15 H and T15 L chain (data not shown) or the intracellular amount of the mutant PCG1–1 H chain (Table I). Second, we examined the binding activity of Ig secreted in the presence of hapten (Fig. 1D). If NPPC increased secretion of partially assembled or nonfunctional antibody, then antigen binding activity would be reduced. However, protein A-purified WT and mutant antibody exhibited similar binding activity for PC protein, indicating that functional antibody was secreted into supernatant. Thus, these data indicate that NPPC increases the amount of mutant Ig in the supernatant and this secreted Ig is stable with respect to antigen binding.

[\[^3H\]NPPC Is Specifically Taken up by Cells—Because NPPC would not be expected to enter cells passively, we tested its capacity to be taken up by cells in culture. Specific uptake of [\[^3H\]NPPC was determined by incubating [\[^3H\]NPPC with paraformaldehyde-fixed cells. B, uptake of [\[^3H\]NPPC is inhibited by the addition of nonradiolabeled NPPC. A nonlinear, one-site binding curve is plotted. C, light chain secretion as determined by ELISA. D, secreted WT and mutant Ig display similar binding activity to PC-histone. Cells were incubated with NPPC for 4 days and antibody purified using protein A-Sepharose. Purified antibody was extensively dialyzed in phosphate-buffered saline to remove any bound hapten, and purified using protein A-Sepharose.

Addition of NPPC resulted in a 10-fold increase in secretion. NPPC binds with -12-fold lower affinity as compared with NPPC (24), suggesting that binding affinity may correlate with efficiency of rescue. Increased supernatant Ig could not be attributed to toxicity as cell viability was greater than 95% in medium alone or medium with NPPC (Fig. 1B). To examine whether the hapten nonspecifically increased Ig chain secretion, the secretion of PCG1–1 L chains was measured as a control as it is produced in excess over transfected H chains (7). NPPC addition did not increase free PCG1–1 L chain secretion (Fig. 1C). Furthermore, NPPC did not increase secretion of H/L from WT PCG1–1 or T15 cells (data not shown).

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delay, increased secretion parallels the uptake of labeled NPPC.

NPPC Increases Intracellular H-L Assembly—Because NPPC is taken up by PCG1–1 cells, we investigated whether rescue of secretion occurred via increased intracellular assembly and whether the secreted Ig consisted of fully assembled H₂L₂ heterodimers. The kinetics and pathway of assembly were examined by subjecting cells to [35S]Met/Cys-labeled pulse-chase analysis either in the presence or absence of 5 mM NPPC. Heavy chains were immunoprecipitated with protein A-Sepharose and separated by nonreducing SDS-PAGE. Ig assembly was followed at 0-, 2-, and 10-h time points after a 20-min pulse. In the absence of NPPC, the majority of the single Ile⁵¹ → Arg H chain was arrested at the H₂ step in Ig assembly (Fig. 3A). As expected, secretion-impaired mutant H chains co-immunoprecipitated with BiP (29, 30). A small amount of intracellular H₂L₂ was detected at 0 and 2 h, and then levels decreased by 10 h. In contrast, assembly of H₂L₂ in WT cells was essentially complete by 2 h and co-immunoprecipitated BiP was not detectable (Fig. 3C). In agreement with ELISA data (Table I), a small amount of the mutant Ig was visible in the supernatant at the 2- and 10-h chase time points in the absence of NPPC (Fig. 3A). The small amount of secreted mutant Ig was fully assembled H₂L₂, as judged by two-dimensional electrophoresis (data not shown). The addition of NPPC markedly increased the amount of H₂L₂ in the mutant supernatant. By 2 h there was a 2.2-fold increase in fully assembled Ig, and by 10 h there was a 6.4-fold increase in supernatant Ig (Fig. 3B). Importantly, NPPC stimulated an increase in the amount of intracellular H₂L₂ in the Ile⁵¹ → Arg transfectant, indicating an intracellular mechanism of rescue. The relative amount of H₂L₂ assembly intermediate was increased by 1.7-fold at 2 h and 2.9-fold at 10 h in comparison to control cultures. In agreement with ELISA data, the effect was specific to the mutated antibody, as NPPC did not increase secretion of WT antibody nor was there an increase in WT assembly intermediates (Fig. 3D). To independently confirm that NPPC augments intracellular assembly, we inhibited secretion by preincubation with brefeldin A (Fig. 4A). In the presence of BFA, NPPC caused a modest increase in intracellular H₂L₂ (2.0-fold) and a substantial increase (4.1-fold) in H₂L₂ by 10 h (Fig. 4C). The formation of H₂ was not altered by the presence of the hapten and BFA, indicating that increased assembly is targeted to H and L chain assembly. These data indicate that assembly occurs early within the biosynthetic pathway. No H₂L₂ was detected in the supernatant, indicating that secretion was completely blocked by BFA treatment (Fig. 4A). This was independently confirmed by a subsequent immunoprecipitation of free supernatant L chain (2-h chase time point) from either BFA-treated or vehicle (methanol)-treated PCG1–1 Ile⁵¹ → Arg culture supernatants (Fig. 4B). The addition of BFA completely blocked L chain secretion at both the 2-h (Fig. 4B) and 10-h chase time points (data not shown). PCG1–1 L chains are secreted as both apparent monomers and covalent dimers similar to other murine L chains (31, 32). As observed for the WT antibody, NPPC had no effect on L chain secretion or on the assembly of L chain into covalent dimers (Fig. 4B, data not shown). Taken together, these data indicate that NPPC specif-
shown are representative of three experiments.  

Kinetics of Rescue—We investigated whether the presence of NPPC was required early in biosynthesis (Fig. 5). A 27-fold increase in Ig secretion was achieved by addition of NPPC 12 h prior to and during the labeling (−12 h, Fig. 5A). A 10-h pulse of NPPC ending 2 h prior to labeling increased secretion 14-fold, indicating that hapten taken up by cells is necessary for rescue. Addition of NPPC at 2- and 4-h chase time points augmented secretion of labeled Ig only 3- and 2-fold, respectively. These data indicate that the presence of NPPC within the cell is required during the early steps of H chain and L chain biosynthesis for maximal restoration of secretion.

Differential Rescue of Secretion of PCG1–I Mutants—To examine whether the ability of NPPC to enhance secretion extended to other nonsecreted Ig mutants specific for phenylphosphocholine binding, we tested the original panel of four low secretion PCG1–1 mutants (Fig. 6). Secretion-impaired mutants P35, P34, P32, and P28, as well as secretion-competent mutant P20, were incubated with or without NPPC, and the amount of supernatant Ig was determined by ELISA (Fig. 6A). The addition of NPPC significantly increased the amount of Ig present in culture supernatants of three of the four multisite mutants; P35 (7.3 ± 0.6-fold), P34 (5.0 ± 0.4-fold), and P28 (3.9 ± 0.2-fold). As with the PCG1–1 WT transfectant, NPPC did not increase the amount of Ig present in culture supernatant from secretion-competent mutant P20. Secretion of mutant P32 (1.6 ± 0.3-fold) was not improved by inclusion of NPPC. Mutant P32 is of particular interest because antigen binding is undetectable, presumably because of the substitution of Cys at Trp52, a residue critical in PC protein binding (Fig. 5B) (7). Mutants P34 and P35 have reduced binding avidity for PC protein as compared with PCG1–1 WT, whereas the binding activity of P28 has not been determined. The secretion rescue of mutant P34 demonstrates that NPPC can increase secretion of Ig from low secreting mutants that have V-region mutations at positions other than Ile51. These data also indicate that the mutations in P28 and P35 other than the mutation at Ile51 do not block the ability of NPPC to rescue secretion. Taken together, these data indicate that the capacity of secretion-impaired Ig to bind antigen correlates with ligand-mediated rescue of secretion.

**DISCUSSION**

A severe Ig secretion defect was specifically reversed in a panel of single and multisite mutant antibodies by incubation of cells with the cognate hapten ligand. NPPC increases intracellular assembly of fully functional Ig and does not induce nonspecific release of ER-retained Ig H or L chains. Secretion rescue was ligand-specific, as evidenced by the inability of related haptens such as choline, phosphocholine, or $p$-nitrophenol to rescue secretion. Failure to rescue secretion, at least for choline, cannot be attributed to lack of uptake. Rather, the rescue of Ig secretion correlated with antigen binding capability. NPPC did not significantly rescue secretion of a multisite mutant (P34) that does not exhibit detectable antigen binding (7). Finally, hapten increased intracellular assembly of H2L2 even when Ig secretion was blocked by brefeldin A. Taken together, these data support the notion that NPPC functions as a specific chemical chaperone to rescue assembly of Ig H and L chains within the ER, thus leading to increased secretion of mutant Ig. To our knowledge, this is the first report of a hapten affecting the secretion of its cognate Ig. Furthermore, other chemical chaperones ($1\%$ Me2SO or $1\%$ glycerol) that appear to affect the secretion of its cognate Ig. Although many steps in Ig assembly are known (reviewed in Refs. 33 and 34), the molecular events involved in H-L pairing are not clear. Molecular chaperones BiP and GRP94 are associated with Ig H chain prior to pairing (35, 36) and dissociation of BiP from H chain is temporally linked to L chain arrival (37). Experiments using a truncated, two domain heavy chain ($VH$) indicate that the disulfide bond within the CH1 domain is not formed until BiP dissociates and L chain associates with H chain (38). Furthermore, a mature L chain is required to displace BiP from H chain (38). A possible mechanism of NPPC action may be the stabilization of correctly paired mutant $VH$s with $Vc$ within the ER, thereby facilitating the dissociation of BiP and the folding of the CH1 and Cc domains. The requirement for antigen binding capacity supports this mechanism. Both
hapten and protein antigens have been shown to facilitate the pairing and folding in vitro of several V_{H} and V_{L} (22, 23, 39). Similarly, peptide has been shown to directly facilitate class I heavy chain association with β_{2}-microglobulin and folding in cell lysates in vitro (40, 41). In addition, the thermal denaturation of peptide-filled, purified K_{b} class I molecules results in simultaneous loss of peptide and dissociation of the heavy chain and β_{2}-microglobulin (42). The structural consequences of ligand binding have been investigated by comparison of crystal structures of ligand-bound and unbound antibodies (43). These computational comparisons have identified that small antigens or haptenas compact the V_{H}-V_{L} interface in the MepC603, 2B4, N19G, and DB3 antibodies. The increase in compactness has been estimated to contribute 1.47 kcal mol^{-1} to stability, representing a 10-fold improvement in binding affinity between the V_{H} and V_{L} domains (43). An improvement in affinity of this magnitude, occurring between the H and L chains in the mutant PCG1–1 antibodies, may be sufficient to offset the potentially destabilizing effect of the CDR2 mutation(s). WT Ig assembly and secretion was not affected, presumably because of the rapid and proper folding of the Ig H chain. We do not exclude the possibility that NPPC may directly facilitate mutant H chain folding prior to L chain pairing. In many antibody-antigen structures, H chain contributes a greater percentage of contacts than L chain (reviewed in Ref. 44). We recently reported the crystal structure of a single chain F_{v}, M3C65, complexed with NPPC (45). The M3C65 antibody uses the same V_{H} M141 gene as PCG1–1 but pairs with a V1 L chain instead of a Vx1 L chain used by PCG1–1 (46). In the M3C65-NPPC complex, residues in H chain CDR2 and CDR3 contact hapten, although mutations in the CDR2 of the V1 L chains confer high affinity binding (47). Thus, if the PCG1–1 combining site binds NPPC in a similar orientation, it is likely that residues from both the V_{H} and V_{L} contribute to binding. Further analysis will be required to determine the molecular contribution of hapten binding to mutant PCG1–1 Ig assembly.

At present the mechanism of uptake of NPPC into hybridoma cells is not clear. Interestingly, oligopeptides can be delivered directly to the ER of viable cells via a vesicular pathway resembling pinocytosis that conveys small extracellular substances to the ER without traversing the Golgi complex or the cytosol (48). This pathway is insensitive to brefeldin A, a compound that disconnects the proximal and distal portions of the Golgi, thus indicating that retrograde delivery of endocytosed molecules to the ER is not involved (48, 49). In PCG1–1 cells, assembly of mutant Ig occurs in the presence of brefeldin A, indicating that assembly is also not dependent on the retrograde delivery of endocytosed NPPC. Pinocytosis of NPPC by PCG1–1 cells would be consistent with the slow, low affinity uptake of NPPC and insensitivity to brefeldin A inhibition (48). However, a pinocytotic mechanism is inconsistent with the inhibition of [3H]NPPC uptake by both NPPC (Fig. 2B) and unlabeled choline (data not shown). Rather, the specific inhibition of [3H]NPPC uptake implicates the involvement of a transport-mediated process. Choline is known to be taken up by lymphocytes (26), and we also have evidence for choline uptake by PCG1–1 cells (data not shown). Recently a choline transporter has been identified, although its expression is restricted to cholinergic neurons (27, 28). Further experiments are required to differentiate between these potential mechanisms. The access of oligopeptides to the ER by a vesicular pathway (48) and our data indicating that hapten can increase intracellular assembly, even in the presence of brefeldin A, indicate that exogenous compounds may find entry by incompletely understood mechanism(s), and thus potentially stabilize subunit assembly of antigen receptor molecules.

The ability of a CDR mutation to disrupt assembly and, of hapten to repair this process suggest that V_{H}-V_{L} interactions play an important role in quality control of normal Ig secretion. Quality control processes are likely to be critical within the bone marrow during formation of new antigen receptors as well as in secondary lymphoid structures such as germinal centers in which high numbers of somatic mutations are introduced into the variable regions of the H and L chains. The requirement of V_{H} to pair well with V_{L} may prevent poorly paired H and L chains from being expressed, thus avoiding generation of potentially unstable molecules that could lead to heavy chain or light chain deposition diseases (50–53). These mutants and the ability to modulate secretion provide a useful system for further elucidation of the molecular mechanisms governing B cell homeostasis and ER-mediated Ig quality control.

We envision that cognate ligands may find application for increasing assembly and secretion of Ig from poorly secreting hybridomas. A drawback of NPPC, and most chemical chaperones, is that millimolar concentrations and long preincubation periods are required to rescue assembly and secretion. In addition, it is unclear whether assembly and secretion of antibodies that recognize naturally occurring peptides, carbohydrates, and nucleic acids can be enhanced in a similar manner as the ER access of larger molecules may be limiting. Future studies with more penetrable ligands of higher affinity as well as molecules that mimic larger and more complex antigen structures should clarify the feasibility of this approach. Such compounds could ultimately be useful in regulating Ig subunit assembly in cell lines and in vivo (54). With over 200 Igs in current clinical trials (55), understanding and maximizing Ig folding and secretion is of increasing importance.

Acknowledgments—We thank Drs. S. Landfear, M. Sanchez, J. Van Slyke, and K. Neve for helpful advice on uptake assays. We also thank M. Brown and Drs. Q. Chen, M. Stenzel-Poore, B. Wiens, and E. Whitcomb for critical comments. We especially thank Dr. C. Enns for helpful suggestions and commentary.

REFERENCES
1. Glennie, M. J., and Johnson, P. W. (2000) Immunol. Today 21, 403–410
2. Verma, R., Boleti, E., and George, A. J. (1998) J. Immunol. Methods 216, 165–181
3. Horwitz, A. H., Nadell, R., Reuguschat, F., and Better, M. (1994) Mol. Immunol. 31, 683–692
4. Somparum, S. R., Den, W., and Sharon, J. (1999) J. Immunol. 165, 1071–1081
5. Jost, C. R., Kurucz, I., Jacobus, C. M., Titus, J. A., George, A. J., and Segal, D. M. (1994) J. Biol. Chem. 269, 28267–28273
6. Chen, C., Martin, T. M., Stevens, S., and Rittenberg, M. B. (1994) J. Exp. Med. 179, 577–586
7. Wiens, G. D., Heldwein, K. A., Stenzel-Poore, M. P., and Rittenberg, M. B. (1997) J. Immunol. 159, 1293–1302
8. Wiens, G. D., Lekkerkerker, A., Veitman, I., and Rittenberg, M. B. (2001) J. Immunol. 167, 2179–2186
9. Tatzelt, J., Prusiner, S. B., and Welch, W. J. (1996) EMBO J. 15, 6363–6373
10. Sato, S., Ward, C. L., Krouse, M. E., Wine, J. J., and Kopito, R. R. (1996) J. Biol. Chem. 271, 653–658
11. Wang, J., and White, A. L. (1999) J. Biol. Chem. 274, 26263–26267
12. Burrows, J. A., Willis, L. K., and Perlmutter, D. H. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 1796–1801
13. Loo, T. W., and Clarke, D. M. (1997) J. Biol. Chem. 272, 709–712
14. Tamarappo, B. K., and Verkman, A. S. (1998) J. Biol. Chem. 273, 2257–2267
15. Tamarappo, B. K., Yang, B., and Verkman, A. S. (1999) J. Biol. Chem. 274, 34825–34831
16. Brown, C. R., Hong-Brown, L. Q., and Welch, W. J. (1997) J. Clin. Invest. 99, 1432–1444
17. Fan, J. Q., Ishih, S., Asano, N., and Suzuki, Y. (1999) Nat. Med. 5, 112–115
18. Cueno, E., and Metzger, H. (1972) Biochemistry 11, 766–771
19. Moult, H. M. (1996) Environmental Sciences and Resources: Chemistry, Ph.D. thesis, Portland State University, Portland, OR
20. Martin, T. M., Wiens, G. D., and Rittenberg, M. B. (1998) J. Immunol. 160, 5963–5970
21. Rajan, D. P., Huang, W., Kekuda, R., George, R. L., Wang, J., Conway, S. J., Devoe, L. D., Leibach, F. H., Prasad, P. D., and Ganapathy, V. (2000) J. Biol. Chem. 275, 14351–14355
22. Ueda, H., Tsunoto, K., Kubo, S., Suzuki, N., Nishimura, H., Schueler, P. A., Winter, G., Kumagai, I., and Mohoney, W. C. (1996) Nat. Biotechnol. 14, 1714–1718
23. Jager, M., and Pluckthun, A. (1999) J. Mol. Biol. 285, 2005–2019
