

Posttranslational Association of Immunoglobulin Heavy Chain Binding Protein with Nascent Heavy Chains in Nonsecreting and Secreting Hybridomas

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Abstract. A rat monoclonal antibody specific for immunoglobulin (Ig) heavy chain binding protein (BiP) has allowed the examination of the association of BiP with assembling Ig precursors in mouse B lymphocyte-derived cell lines. The anti-BiP monoclonal antibody immunoprecipitates BiP along with noncovalently associated Ig heavy chains. BiP is a component of the endoplasmic reticulum and binds free intracellular heavy chains in nonsecreting pre-B (μ^+ , L^-) cell lines or incompletely assembled Ig precursors in (H^+ , L^+) secreting hybridomas and myelomas. In the absence of light chain synthesis, heavy chains remain associated with BiP and are not secreted. The associa-

tion of BiP with assembling Ig molecules in secreting hybridomas is transient and is restricted to the incompletely assembled molecules which are found in the endoplasmic reticulum. BiP loses affinity and disassociates with Ig molecules when polymerization with light chain is complete. We propose that the association of BiP with Ig heavy chain precursors is a novel posttranslational processing event occurring in the endoplasmic reticulum. The Ig heavy chains associated with BiP are not efficiently transported from the endoplasmic reticulum to the Golgi apparatus. Therefore, BiP may prevent the premature escape and eventual secretion of incompletely assembled Ig molecules.

THE process by which B lymphocytes synthesize immunoglobulin (Ig)¹ involve the assembly of heavy and light chains, glycosylation, posttranslational modification of oligosaccharides, and transport between intracellular compartments before eventual secretion (41). Ig appears to be transported along the same intracellular pathway as other secretory glycoproteins.

Comparative examination of the intracellular transport of many secretory proteins indicates that transport from the rough endoplasmic reticulum (RER) to the Golgi apparatus is selective and can be the rate-limiting step in the secretory process (11, 24). This selectivity and the demonstration of different rates of transport between the RER and Golgi apparatus for different secretory proteins within a cell line has led to the hypothesis that secretory proteins express specific transport signals within their structure (11, 24) and that this transport may be receptor mediated (12, 24, 40).

Expression and selective intracellular transport of Ig in B-lymphocyte lineage cells is developmentally regulated. Pre-B cell lymphocytes differentiate from bone marrow multipotential stem cells and are first identified by the presence of cytoplasmic μ -heavy chains in the absence of light chain synthesis (8, 27). The μ -chains of bone marrow derived pre-

B cells are of the membrane type but are not expressed on the cell surface (42). The onset of light chain synthesis coincides with and appears to be necessary for the expression of surface and secretory Ig (26).

Mouse pre-B-derived hybridomas and Abelson virus transformed pre-B cell lines synthesize membrane and secretory μ -chains which remain intracellular and are not secreted or expressed on the cell surface (17, 32, 37). The analysis of intracellular μ -chains from pre-B derived cell lines as well as variant nonsecreting myelomas has identified a protein of 78,000 daltons, Ig heavy chain binding protein (BiP), that co-immunoprecipitates with Ig heavy chains (13, 28). Because BiP was readily observed in cell lines representative of the pre-B cell stage of development, others have speculated that BiP functions in the regulation of Ig synthesis or prevents heavy chain insolubility in pre-B cells (43).

The results of the presently described studies demonstrate that BiP binds free heavy chains as they occur in pre-B derived cell lines, and incompletely assembled Ig molecules in secreting cell lines. Our interpretation of these observations are that BiP plays a role in the posttranslational processing of nascent Ig heavy chains in the endoplasmic reticulum. Heavy chains associated with BiP are not efficiently transported from the RER to the Golgi apparatus. This suggests that the posttranslational interaction of BiP with Ig heavy chains interferes with the expression of transport signals inherent in the Ig molecule.

1. Abbreviations used in this paper: BiP, immunoglobulin heavy chain binding protein; Ig, immunoglobulin; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum.

We propose that association of BiP with incompletely assembled Ig molecules prevents their transport from the endoplasmic reticulum. Once assembly with light chain is complete, disassociation of BiP occurs, allowing transport of the assembled molecule to the Golgi apparatus and eventual secretion.

Materials and Methods

Reagents

Affinity-purified isotype specific antibodies used for immunoprecipitation were obtained from Southern Biotechnology Associates Inc., Birmingham, AL. [³⁵S]Methionine was purchased from Amersham Corp., Arlington Heights, IL. Sugar nucleotides were obtained from New England Nuclear, Boston, MA. Dolicol phosphate and protein A were purchased from Sigma Chemical Co., St. Louis, MO, and tunicamycin was obtained from Calbiochem-Behring Corp., San Diego, CA. Tissue culture media and fetal calf serum were purchased from GIBCO, Grand Island, NY. All other reagents were obtained from different sources and were of analytical grade.

Cell Culture

Cell lines were maintained in stationary culture in RPMI 1640 supplemented with L-glutamine (2 mM), penicillin-streptomycin, β -mercaptoethanol (50 μ M), fungizone 1:1,000, and 15% fetal calf serum at 37°C in 10% CO₂. Pre-B cell-derived hybridoma 15-58 was obtained by fusion of 19-d fetal liver with P3-X63-Ag8.653, as previously described (8). Ag8(8) was isolated in collaboration with A. Radbruch and K. Rajewsky (Department of Genetics, University of Cologne, FRG), by fluorescent-activated cell sorting as a spontaneous variant of P3X63-Ag8, and produces only cytoplasmic γ_1 heavy chains. RD3-2 and MM60 are conventional Ig-secreting mouse hybridomas with anti-idiotypic specificity (33, 38). 11-11 is a rat lymph node x Ag8.653 heterohybridoma which secretes rat IgG with anti-mouse IgD specificity.

Preparation of Monoclonal Anti-BiP

The immunogen containing mouse BiP: μ -chain complexes was obtained by immune precipitation of 5×10^7 , 15-58 pre-B hybridoma cells. The cells were lysed in 15 ml of 1% Nonidet P-40 lysis buffer and immunoprecipitated with 200 μ g of goat anti-mouse μ -chain antibody and *Staphylococcus aureus*. The *S. aureus* pellet was washed three times with 0.75 M guanidinium hydrochloride buffered in 10 mM Tris, pH 7.5. The immune complexes were eluted from the *S. aureus* with 2.5 M buffered guanidinium hydrochloride. Proteins in the eluate were precipitated with 9 volumes of ethanol at -20°C for 1 h and resuspended in PBS for injection. A Fisher rat was immunized five times, every third day, in one rear footpad with this antigen. The first injection of immunogen was emulsified in complete Freund's adjuvant the others administered in phosphate-buffered saline (PBS). 1 d after the last injection the draining popliteal lymph node was fused with mouse myeloma Ag8.653 (16). Hybridomas were initially screened by an enzyme-linked immunosorbent assay for reactivity against both BiP: μ -chain complex and purified IgM. The hybridomas secreting antibody specific only for BiP by the enzyme-linked immunosorbent assay were further screened for their ability to immunoprecipitate BiP from a labeled cell lysate as detected by SDS PAGE.

Radiolabeling of Cell Lines

Before labeling with [³⁵S]methionine, cells were preincubated at a density of $1-5 \times 10^6$ /ml in methionine-free RPMI 1640 containing 15% dialyzed fetal calf serum for 30 min. When tunicamycin was used, it was included in the preincubation media at a final concentration of 2.5 μ g/ml from stock. Cell cultures were then labeled with 10 μ Ci of [³⁵S]methionine 1,400 Ci/mmol. In pulse-chase experiments, either complete medium or cold methionine was added at the time of chase to a concentration of 30 μ g/ml. Unless otherwise stated, cells were labeled 10-15 min before lysis or chase. Cells were harvested by centrifugation at 1,000 g for 5 min and lysed for 10 min at 0°C in 1 ml of lysis buffer containing 1% Nonidet P-40, 0.15 M NaCl, 50 mM Tris, pH 7.5, and 2 mM phenylmethylsulfonyl fluoride. In pulse-chase experiments with short chase periods, 2x lysis buffer was added directly to the cell culture. Nuclei and cellular debris were then removed by centrifugation in a Brinkmann centrifuge for 10 min. Supernatant was removed and immunoprecipitated by direct binding with monoclonal anti-BiP covalently linked to Sepharose 4B or indirectly with 50 μ l of anti-BiP culture supernate followed by addition of protein A-Sepharose. Ig heavy chains were immunoprecipitated with 10 μ g of

affinity-purified isotype-specific goat anti-mouse antibody and protein A-Sepharose. Protein A or anti-BiP antibody, 1.0 mg per ml Sepharose, was coupled to CNBr-activated Sepharose 4B (31). Lysates were allowed to incubate with precipitating antibody for 1 h at 4°C with gentle mixing. Sepharose-bound immune complexes were washed three times with lysis buffer containing 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 0.5 M NaCl, 50 mM Tris, pH 7.5, and once with PBS to remove detergents and salt. Precipitated proteins were then boiled in SDS sample buffer and subjected to SDS PAGE (21). Before autoradiography gels were fixed and then impregnated with EN³HANCE (New England Nuclear) as directed by the manufacturer. Kodak X-omat AR X-ray film was used to visualize labeled proteins.

Subcellular Fractionation

Subcellular fractionation was performed by a modification of an unpublished procedure (Gary Sahagian, Department of Physiology, Tufts University, Boston, MA, personal communication). 5×10^7 RD3-2 or Ag8(8) hybridoma cells were harvested by centrifugation at 1,000 g for 5 min at 4°C, and first washed twice with PBS and then twice with 0.25 M sucrose in 5 mM Hepes buffered to pH 6.8. The cells were then resuspended in 2 ml of isotonic sucrose and transferred to a nitrogen cavitation bomb (Kontes Co., Vineland, NJ). The cells were pressurized to 40 psi for 30 min at 0°C. After decompression the cells were dispersed with 10 gentle strokes in a tight-fitting dounce homogenizer. Nuclei and cellular debris were removed by centrifugation at 800 g for 10 min. The postnuclear supernate was then layered on top of a discontinuous sucrose gradient containing 1 ml/2.0 M, 3.4 ml/1.3 M, 3.4 ml/1.0 M, and 2.75 ml/0.6 M sucrose in 5 mM Hepes (pH 6.8) in a Beckman 14 x 89 mm Ultra-Clear tube. After 2 h of centrifugation at 40,000 rpm in a Beckman SW 41 rotor, ~19 fractions containing 10 drops each were collected from the bottom of the tubes. Each fraction was then assayed for endoplasmic reticulum and Golgi marker enzymes.

Marker Enzyme Assays

Mannosyltransferase was measured by methods as previously described (39). Membrane fractions were assayed in a total volume of 80 μ l containing 5×10^4 cpm GDP-mannose [mannose-3,4-³H(N)] 40-60 Ci/mmol, 0.05 mg dolicol phosphate, 1.25 mM MgCl₂, 0.015% Triton X-100, 20 mM Tris, pH 7.5, and 0.15 M NaCl. The assay mix was sonicated before addition of suitably diluted membrane fractions. Assays were incubated for 10 min at room temperature. Reactions were terminated by addition of 1.5 ml of chloroform/methanol (2:1), followed by 0.3 ml of saline. The tubes were vortexed, and chilled on ice for 10 min. Each tube was then spun for 5 min at 2,000 g and 0.6 ml of the chloroform was removed, dried in scintillation vials, and counted in scintillation cocktail.

Galactosyltransferase was measured by a modification of a procedure previously described (7). Membrane fractions were incubated in a final volume of 40 μ l containing 5×10^4 cpm UDP-Galactose [Galactose-¹⁴C(U)] 337.0 mCi/mmol, 0.28 mg ovalbumin (Sigma Chemical Co.; grade V), 50 mM Tris, pH 7.5, 20 mM MnCl₂, 0.5% Triton X-100. Reactions were incubated at 37°C for 60 min. 30 μ l of each assay was then spotted on Whatman 3-MM filter paper and dried at room temperature. The filter paper was then washed twice in 10% trichloroacetic acid, 2% phosphotungstic acid, and 1% pyrophosphate at 0°C for 15 min. The filter paper was then washed in methanol, dried, and counted.

Identification of Immunoglobulin and BiP in Subcellular Fractions

BiP and Ig were identified in subcellular fractions by immunoprecipitation with monoclonal anti-BiP or with goat anti-heavy chain specific antibody. 1×10^6 [³⁵S]methionine-labeled cells were fractionated by the above procedure. Before immunoprecipitation each fraction was diluted with an equal volume of 2x lysis buffer to solubilize membranes. Each fraction was then divided for precipitation with anti-BiP or anti-Ig heavy chain.

Results

Immunoprecipitation of BiP and Immunoglobulin Heavy Chains from a Nonsecreting (γ_1^+ , κ^-) and a Secreting (γ_1^+ , κ^+) B-Cell Hybridoma

To compare the association of BiP with free and assembling heavy chains we have selected a nonsecreting variant of the myeloma P3X63-Ag8, designated Ag8(8) (γ_1^+ , κ^-), and a secreting hybridoma RD3-2 (γ_1^+ , κ^+). Like pre-B cell-derived

hybridomas, Ag8(8) produces only heavy chains which remain intracellular. Cells were labeled with [³⁵S]methionine for 15 min and lysed immediately with detergent or chased for 60 min in excess cold methionine before lysis. The lysates were divided and immunoprecipitated separately with monoclonal anti-BiP or anti- γ_1 heavy chain-specific antibody. When Ig-secreting RD3-2 cells were immunoprecipitated with anti- γ_1 antibody and the precipitated proteins separated by SDS PAGE under reducing conditions, heavy chains and light chains were detected (Fig. 1A, lane 1). When cell lysates from the 15-min pulse were immunoprecipitated with anti-BiP antibody, BiP with M_r 78,000 and a band migrating with the same mobility of γ_1 -heavy chain were detected (Fig. 1A, lane 2). No detectable band co-migrating with light chains is seen in the anti-BiP immunoprecipitate. After a 60-min chase, immunoprecipitation with anti-BiP yielded the same M_r 78,000 band, however, little protein with the molecular weight of γ_1 heavy chain is observed (Fig. 1A, lane 4). After the 60-min chase, labeled intracellular heavy and light chains are still present and are precipitated with anti- γ_1 antibody (Fig. 1, lane 3). These results suggest that heavy chains in RD3-2 associate transiently with BiP after their synthesis, and these heavy chains that are associated with BiP have not acquired light chains. When the same type of experiment was conducted with nonsecreting Ag8(8), BiP and γ_1 heavy chains were precipitated as a complex regardless of which precipitating antibody was used (Fig. 1B). In contrast to the results obtained with RD3-2, the BiP heavy chain complex is stable and does not dissociate after a 60-min chase (Fig. 1B, lanes 3 and 4).

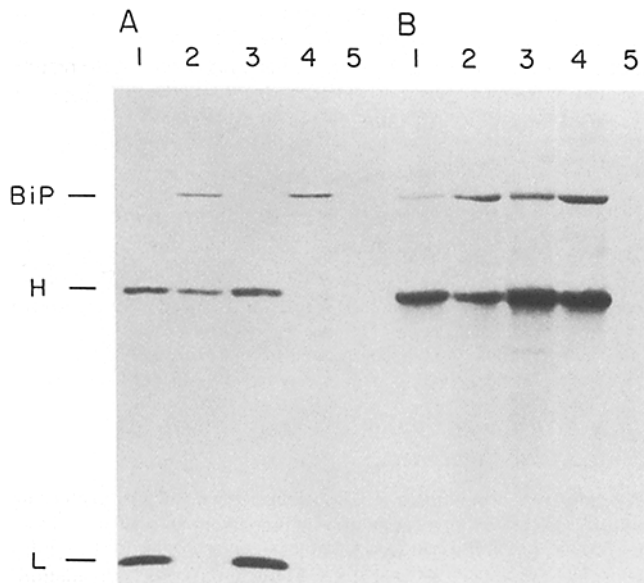


Figure 1. Association of BiP with γ_1 heavy chains in secreting RD3-2 and nonsecreting Ag8(8) hybridomas. Secreting RD3-2 cells (A) or nonsecreting Ag8(8) cells (B) were pulsed 15 min with [³⁵S]methionine, lysed in detergent, and immunoprecipitated with goat anti- γ_1 heavy chain antibody and protein A-Sepharose (lanes 1) or with monoclonal rat anti-BiP antibody coupled to Sepharose (lanes 2). Cells pulsed 15 min and chased 1 h in excess cold methionine before lysis were immunoprecipitated with goat anti- γ_1 heavy chain antibody (lanes 3) and anti-BiP Sepharose (lanes 4). Lanes 5 show immunoprecipitation of cell lysates after a 15-min label with a monoclonal rat anti-mouse IgD (11-11) as control. Immunoprecipitated proteins were reduced and resolved on 10% SDS PAGE.

A similar pattern of association between heavy chains and BiP was demonstrated in cells producing μ -chains. A nonsecreting pre-B-derived hybridoma, 15-58 which produces μ -chains in the absence of light chain synthesis and a conventional μ, λ secreting hybridoma MM60 were immunoprecipitated with anti- μ heavy chain-specific and anti-BiP antibody. The μ -chains from each of these cell lines migrate as two primary bands reflecting an apparent glycosylation heterogeneity that is observed among IgM oligosaccharides (1). After treatment with endoglycosidase H or when cells are labeled in the presence of tunicamycin a single band representing nonglycosylated μ -chain is resolved (Bole, D. G., L. M. Hendershot, J. F. Kearney, unpublished observations). After 15 min of labeling two bands with apparent mobility characteristic of μ -chains were immunoprecipitated from 15-58 with either anti-BiP or anti- μ chain antibodies (Fig. 2A, lanes 2 and 3). However, after a 1-h chase period, three bands are immunoprecipitated with both anti- μ and anti-BiP antibodies (Fig. 2A, lanes 4 and 5); the top band likely corresponds to BiP and the two bands below to μ -chains which now migrate with increased mobility. An increase in the mobility of the μ -chains could result from exoglycosidase trimming of the five oligosaccharide units of the μ -chains (15, 41). In contrast to 15-58, when lysates from MM60 were immunoprecipitated with anti-BiP antibody, very little co-precipitation of μ -chain is detected in the pulse and the chase (Fig. 2B, lanes 3 and 5). MM60 μ -chains immunoprecipitated with anti- μ antibody show an increase in mobility after the 60-min chase, similar to that observed in 15-58. Thus a prolonged association occurs between BiP and the μ -chains in nonsecreting 15-58 that is not observed in secreting MM60; similar to the results obtained with Ag8(8) and RD3-2.

Association of BiP with Assembling Immunoglobulin Precursors in RD3-2

Fig. 1A demonstrated that nascent heavy chains in RD3-2 that were co-precipitated with BiP contained few if any light

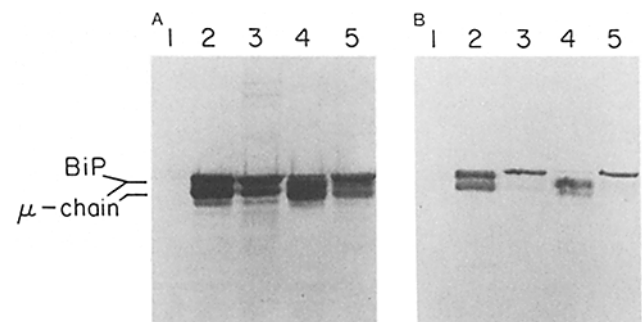


Figure 2. Association of BiP with μ -heavy chains in 15-58, a nonsecreting pre-B hybridoma and MM60, a conventional secreting hybridoma. Nonsecreting 15-58 cells (A) and secreting MM60 cells (B) were labeled 15 min with [³⁵S]methionine and immediately lysed in detergent and immunoprecipitated with goat anti- μ heavy chain antibody and protein A-Sepharose (lanes 2) or monoclonal anti-BiP antibody and protein A-Sepharose (lanes 3). Cells labeled for 15 min and then chased for 1 h were immunoprecipitated with goat anti- μ antibody and protein A-Sepharose (lanes 4) or monoclonal anti-BiP antibody and protein A (lanes 5). Cells labeled for 15 min and immunoprecipitated with protein A-Sepharose alone are shown in lanes 1. Immunoprecipitated proteins were reduced and resolved on 8% SDS PAGE.

chains. This observation suggested that the association of BiP with heavy chains in this secreting hybridoma was a transient posttranslational event occurring before light chain addition. To further investigate this association, we examined the assembly of Ig precursors in RD3-2 by immunoprecipitation with anti- γ_1 heavy chain-specific and anti-BiP antibody. Pulse-chase experiments were performed and immunoprecipitated proteins resolved on nonreducing gels. When heavy chains were immunoprecipitated with anti- γ_1 antibody free heavy chains (H), heavy chain dimers (H₂), dimers with one light chain (H₂L), and complete (H₂L₂) molecules were observed (Fig. 3A). A small amount of BiP was also detected when lysates were immunoprecipitated with anti-heavy chain antibody. The preferred sequence of assembly of mouse IgG₁ requires heavy chains to polymerize into heavy chain dimers before light chain addition occurs (5). With increasing chase time incompletely assembled Ig precursors polymerized with light chains into the mature completely assembled H₂L₂ molecules. When immunoprecipitation was performed with anti-BiP antibody, free heavy chains, heavy chain dimers, and dimers with one light chain were co-precipitated with BiP. However, no completely assembled H₂L₂ molecules were associated with BiP (Fig. 3B). Thus when Ig assembly is complete affinity of BiP for RD3-2 heavy chains is lost.

The Effect of Tunicamycin on Immunoglobulin Assembly and Association with BiP

Before polymerization with light chain, Ig heavy chains are glycosylated co-translationally (3). Inhibiting the glycosylation of Ig heavy chains with tunicamycin could affect the folding of the nascent molecule within the endoplasmic reticulum. We examined the assembly of heavy and light chains in J558 an α, λ myeloma and compared the association of BiP with J558 heavy chains in cells which had been cultured in the presence and absence of tunicamycin. Intracellular transport and secretion of IgA in myelomas is sensitive to but not completely blocked by tunicamycin treatment (14).

J558 cells were pulse labeled and chased for increasing periods of time in the presence or absence of tunicamycin before lysis. Immunoprecipitation of Ig from these cells with anti- α heavy chain-specific antibody precipitated heavy

chains with associated light chains (Fig. 4, A and C). At early time points after pulse labeling, very few light chains co-precipitated with heavy chains in lysates from the tunicamycin-treated cultures (Fig. 4C, lanes 1 and 2). In contrast, light chains were rapidly associated with heavy chains in control cultures (Fig. 4A, lanes 1 and 2). These results suggest that subunit assembly occurs less efficiently when glycosylation is inhibited or blocked. A lack of heavy chain oligosaccharide units has been shown to inhibit assembly of Ig in vivo (10) and in vitro (18). If displacement of BiP is a consequence of light chain assembly, it would be anticipated that BiP association would be enhanced in the presence of tunicamycin.

Fig. 4, B and D, shows that BiP and associated heavy chains are precipitated with anti-BiP antibody. The amount of heavy chains co-precipitating with anti-BiP antibody is much greater when tunicamycin is included in the culture (compare Fig. 4, B and D). The heavy chains precipitated with BiP from the control lysate contain multiple bands which may reflect glycosylation heterogeneity of the α -heavy chains. The relative enhancement of BiP heavy chain complex observed in the tunicamycin-treated cells was estimated by densitometer tracings of heavy chains co-precipitated with BiP (Fig. 4B, lane 1 [sum of three species] and Fig. 4D, lane 1 [one species]). Based on band intensity of the autoradiographs, densitometer analysis revealed that four times as many heavy chains coprecipitated with anti-BiP antibody from lysates of tunicamycin-treated cells compared to the control cell cultures without tunicamycin. The relative quantity of heavy chain co-precipitating with BiP was standardized for the densitometer value of BiP precipitated from each fraction. Thus, in J558, prevention of glycosylation by tunicamycin appears to delay the assembly of heavy and light chains and retards the displacement of BiP from nonglycosylated heavy chains. These results suggest strongly that there exists a direct correlation between assembly of Ig and BiP displacement.

Autoimmune Nature of Anti-BiP Antibody

The results of the above-described studies suggest that BiP is associated with heavy chains after their synthesis and before addition of light chain is complete. This requires that BiP, and partially and completely assembled functional Ig mole-

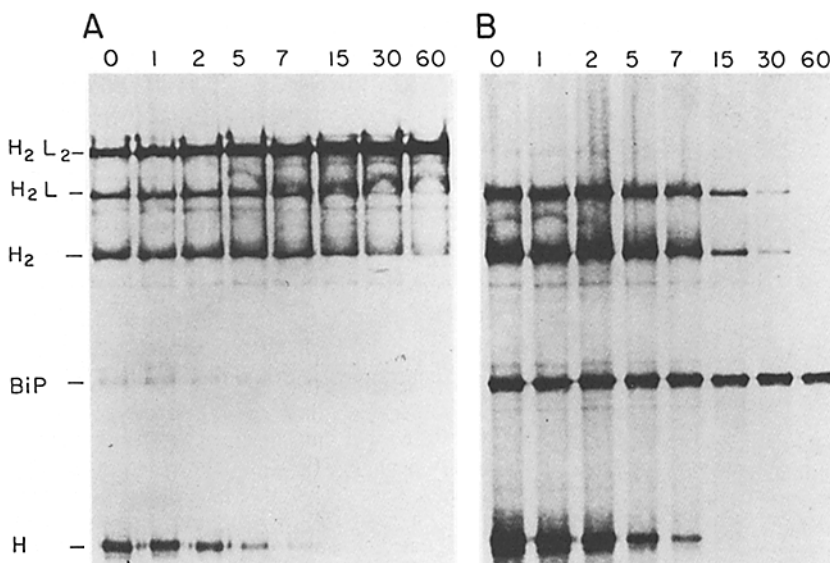


Figure 3. Association of BiP with assembling immunoglobulin precursors in RD3-2. RD3-2 cells were labeled for 10 min with [³⁵S]methionine and then chased with excess cold methionine for 0, 1, 2, 5, 7, 10, 20, and 60 min, at which time point an aliquot of the cell culture was lysed in detergent. Cell lysates from each time point were split and immunoprecipitated separately with either goat anti- γ_1 heavy chain antibody and protein A-Sepharose (A) or with monoclonal anti-BiP coupled to Sepharose (B). Assembling protein precursors were run on 8% SDS PAGE under nonreducing conditions. The fluoroenhanced gel represented in B was exposed to film approximately twice as long as the gel in A.

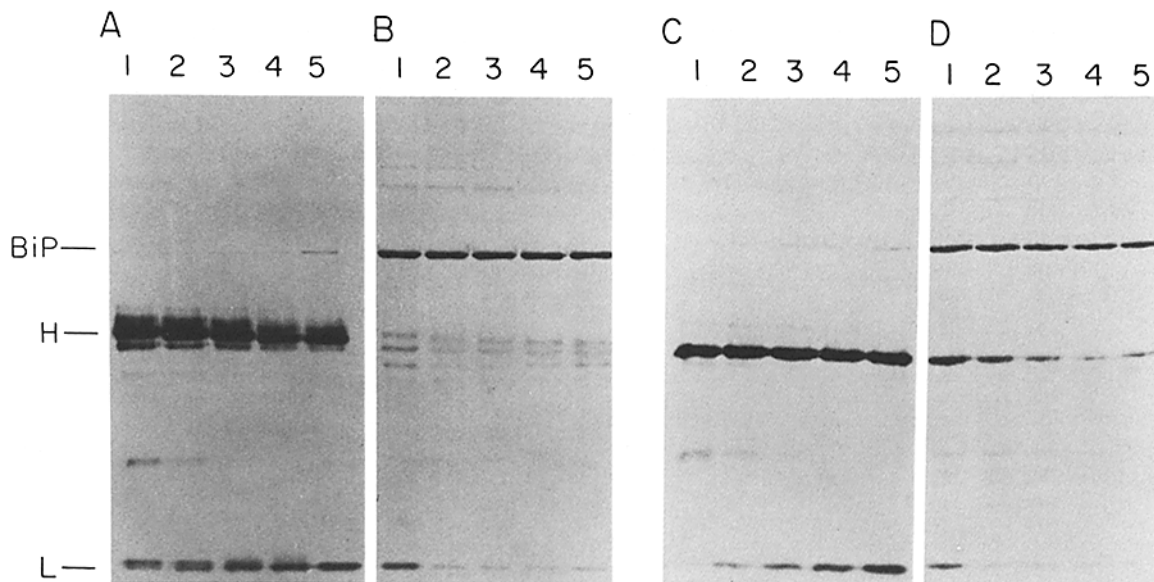


Figure 4. The effect of tunicamycin on immunoglobulin assembly and association with BiP in J558. J558 cells were cultured in the absence (*A* and *B*) or presence (*C* and *D*) of 2.5 µg/ml tunicamycin 30 min before labeling. Cells were labeled for 10 min with [³⁵S]methionine and then chased in excess cold methionine for 0, 10, 20, 30, and 60 min (lanes 1 to 5, each panel) before lysis in detergent. Lysates were immunoprecipitated with goat anti-α heavy chain antibody, and protein A-Sepharose (*A* and *C*) or with monoclonal rat anti-BiP and protein A-Sepharose (*B* and *D*). Immunoprecipitated proteins were reduced and resolved on 10% SDS PAGE. Fluoroenhanced gels containing anti-BiP immunoprecipitates (*B* and *D*) were exposed to film approximately twice as long as those containing anti-α immunoprecipitates (*A* and *C*).

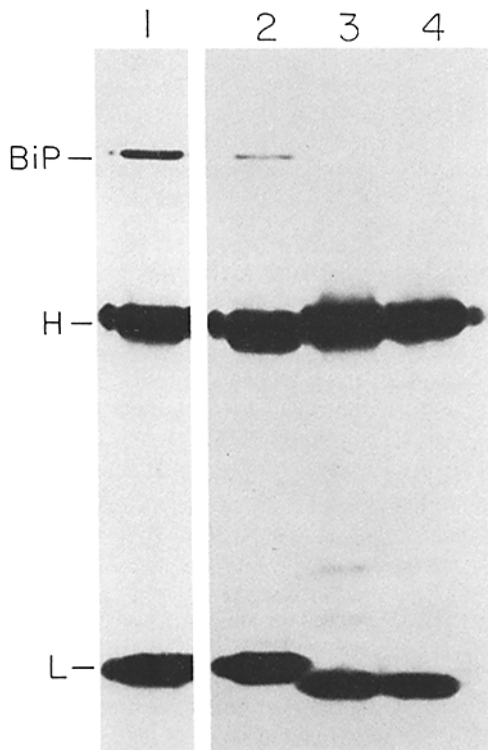


Figure 5. Autoimmune nature of anti-BiP antibody. Rat X Ag8.653 heterohybrid 2-1 cells secreting anti-BiP antibody were labeled for 15 min and then lysed in detergent and immunoprecipitated with protein A-Sepharose (lane 1). Cell-free culture supernatant from 2-1 cells labeled for 15 min and chased 4 h was immunoprecipitated with goat anti-rat IgG coupled to Sepharose 4B (lane 2). Cell-free culture supernatant from rat heterohybridoma 11-11 cells labeled for 15 min and chased for 4 h was immunoprecipitated with goat anti-rat IgG Sepharose (lane 3). Labeled cell free supernatant from 11-11 was

co-precipitated with secreted Ig from the 2-1 heterohybridoma (Fig. 5, lane 2). The co-precipitating protein comigrated on SDS PAGE with intracellular BiP immunoprecipitated from a cell lysate of 2-1 (Fig. 5, lane 1). We believe this to be BiP that has been secreted in an immune complex with a small fraction of the secreted antibody 2-1.

To examine what possible effects such interaction may have on the cellular location of intracellularly immune-complexed BiP, the heterohybridoma, 2-1, and a control heterohybridoma, 11-11, were labeled with [³⁵S]methionine and the cell-free culture supernatants immunoprecipitated with goat anti-rat Ig. Heavy and light chains were secreted from both the 2-1 and 11-11 culture supernatants (Fig. 5, lanes 2 and 3). However, a small amount of an additional protein of *M_r* 78,000 co-precipitated with secreted Ig from the 2-1 heterohybridoma (Fig. 5, lane 2). The co-precipitating protein comigrated on SDS PAGE with intracellular BiP immunoprecipitated from a cell lysate of 2-1 (Fig. 5, lane 1). We believe this to be BiP that has been secreted in an immune complex with a small fraction of the secreted antibody 2-1.

If BiP is membrane-associated or is tightly associated with an intracellular structure, it is probable that antibody immune complexed to BiP would remain within intracellular sites. The co-precipitation of BiP from the culture supernatant of 2-1 suggests that BiP is a soluble protein which when immune complexed to specific antibody can be transported in unison along the secretory pathway and subsequently secreted together with Ig. This demonstrates in unbroken, viable cells

mixed with an equal volume of unlabeled 2-1 supernatant and immunoprecipitated with goat anti-rat Ig Sepharose (lane 4). Immunoprecipitated proteins were reduced and resolved on 10% SDS PAGE.

that BiP and Ig at some time must co-exist within the same intracellular compartment.

To rule out the possibility that a small amount of BiP leaked from 2-1 cells while in culture and that immune complex formation occurred after secretion of antibody into the culture fluid, we immunoprecipitated unlabeled supernate from 2-1 that was mixed with labeled supernate from 11-11 with goat anti-rat Ig. Precipitated proteins analyzed by SDS PAGE revealed only the precipitation of 11-11 heavy and

light chains; no band migrating with M_r 78,000 was detected (Fig. 5, lane 4). Therefore, the anti-BiP antibody in the unlabeled 2-1 supernatant was unable to immune complex any labeled BiP in the supernatant of 11-11. Immunoprecipitation of cell culture supernatants from other cell lines with anti-BiP antibody has failed to detect extracellular BiP. This suggests that the presence of BiP in the supernatant of 2-1 is due to specific co-transport with anti-BiP antibody and is not a result of nonspecific leakage.

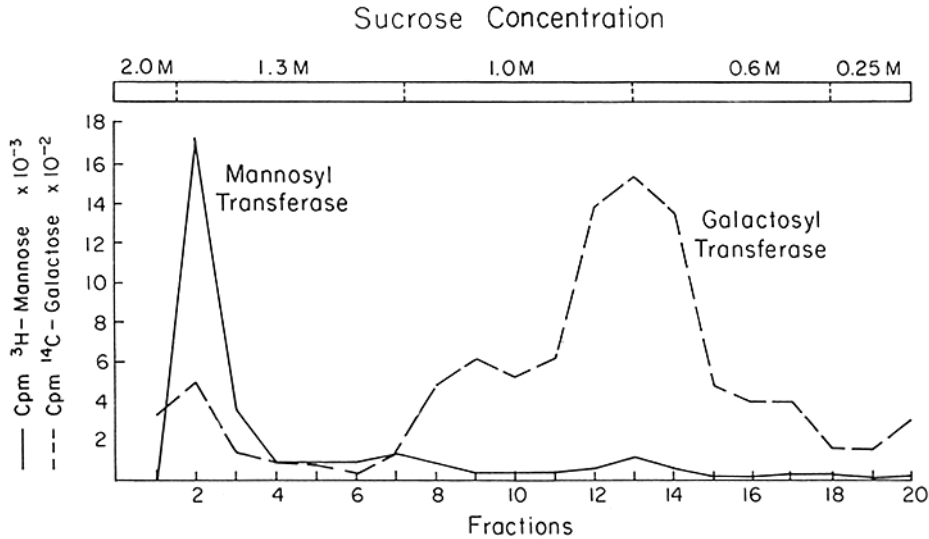
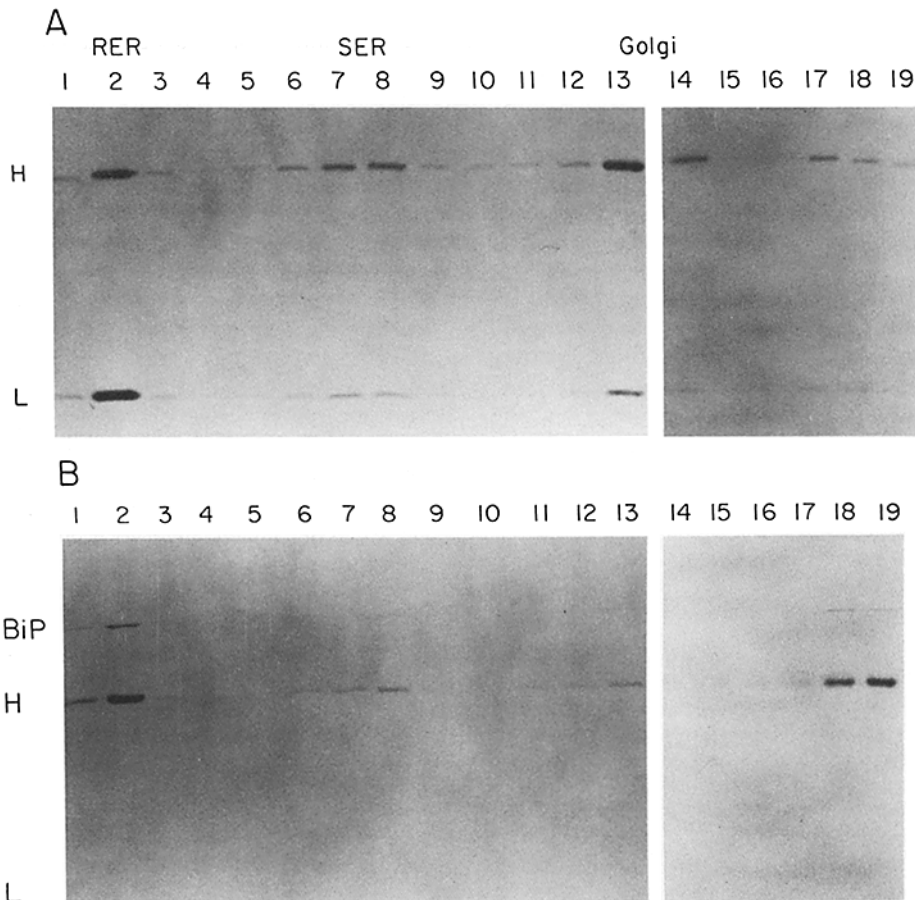


Figure 6. Subcellular fractionation of RD3-2 and Ag8(8). RD3-2 cells were disrupted by nitrogen cavitation and a postnuclear supernatant layered over a discontinuous sucrose gradient. After centrifugation membrane containing fractions were collected from the bottom of the tube. Marker enzymes for RER, mannosyltransferase, and Golgi apparatus galactosyltransferase were assayed. The marker enzyme profiles were obtained from RD3-2. A similar distribution of marker enzyme activity was obtained from Ag8(8) (not shown). For immunoprecipitation of Ig heavy chains from fractionated membrane vesicles of RD3-2 or Ag8(8), cells were labeled for 15 min and then chased 30 min in the presence of excess cold methionine. Labeled cells were then subjected to nitrogen cavitation and membrane fractions were resolved over a discontinuous sucrose gradient. To each fraction was added an equal volume of $2\times$ lysis buffer. Labeled Ig was immunoprecipitated from membrane fractions of RD3-2 (A) or Ag8(8) (B) with goat anti- γ_1 antibody and protein A-Sepharose. Immunoprecipitates were reduced and proteins separated by 10% SDS PAGE.



Intracellular Location of BiP and Immunoglobulin Heavy Chains in Secretory RD3-2 and in Nonsecretory Ag8(8) Hybridomas

The analysis of the association of BiP with Ig heavy chains suggests this interaction occurs in the endoplasmic reticulum. Since heavy chains, in heavy chain-only cell lines, are not secreted and have a prolonged association with BiP it is possible that heavy chains associated with BiP remain within the RER. Resistance of oligosaccharides to endoglycosidase H cleavage has been used as a marker for measuring intracellular transport of many glycoproteins to the Golgi apparatus. However, endoglycosidase H-resistant oligosaccharides cannot be demonstrated among intracellular Ig of many mouse myelomas (15, 41). Thus terminal glycosylation is observed as a co-secretory event within mouse myeloma cells. For this reason we separated membranes of the RER, smooth endoplasmic reticulum (SER), and Golgi apparatus to examine the intracellular location of BiP and associated heavy chains in secreting RD3-2 and nonsecreting Ag8(8).

RD3-2 and Ag8(8) cells were disrupted by nitrogen cavitation and a postnuclear supernatant containing membrane vesicles was fractionated on a discontinuous sucrose gradient. Membranes enriched for RER, SER, and Golgi apparatus were recovered at the three interfaces of the sucrose layers after centrifugation. Fig. 6 shows the location of the marker enzyme mannosyl transferase for RER and galactosyl transferase for the *trans*-Golgi region. Membranes banding at the second interface between fractions 7 and 8 are believed to be enriched for SER. Mannosyltransferase activity can be shifted from fraction 2 to fraction 7 in the presence of EDTA (data not shown). The majority of galactosyltransferase activity is concentrated around fraction 13 (Golgi membrane); however, significant amounts of galactosyltransferase activity are also present in fractions designated RER and SER. The fractions designated RER and SER do not contain mannosyltransferase activity above background, and thus were considered free of RER-derived membranes.

Cells were labeled with [³⁵S]methionine for 15 min and given a 30-min chase before disruption. Membrane fractions from RD3-2 and Ag8(8) were analyzed for presence of Ig and BiP. Membrane vesicles in each fraction were solubilized with lysis buffer and then immunoprecipitated separately with anti- γ_1 heavy chain and anti-BiP antibody. Immunoprecipitated Ig from RD3-2 cells was present in greatest quantities in fractions enriched for RER, SER, and Golgi membranes (Fig. 6A). In addition, Ig was immunoprecipitated from fractions 17, 18, and 19 which represents Ig in the cell disruption buffer that does not sediment with membrane vesicles. This most likely is Ig that has leaked from membrane vesicles during the cell disruption procedure (36). Fractionated membranes from Ag8(8) show a different migrational profile for heavy chains within the sucrose gradient than those of RD3-2 (Fig. 6B). Heavy chains which are associated with BiP are present in greatest amounts in fraction 2 containing RER membranes; only a very small amount of heavy chain is seen in the SER, or Golgi fractions. Densitometer tracings were performed on the autoradiographs (Fig. 6, A and B) to quantitate the relative amounts of heavy chain within each fraction. The results obtained are shown in Table I. In RD3-2, 41% of the γ_1 heavy chains were found in the RER and 38% in the Golgi apparatus, whereas in Ag8(8) 73% of the heavy chains were within

Table I. Subcellular Localization of Intracellular Ig and BiP in RD3-2 and Ag8(8)

Cell type and protein precipitated	Subcellular fraction			
	RER	SER	Golgi apparatus	Cytosol
	%	%	%	%
Ag8(8), γ_1 30-min chase	73	6	3	18
RD3-2, γ_1 30-min chase	41	17	38	4
Ag8(8), BiP 30-min chase	61	2	<2	36
RD3-2, BiP 30-min chase	82	<1	<1	17
RD3-2, BiP 4-h chase	60	3	<2	33

Labeled Ig heavy chains or BiP was quantitated by densitometer tracings. Fractions 1, 2, and 3 were designated RER, fractions 7 and 8 SER, fractions 12, 13, and 14 Golgi apparatus, and fractions 17, 18, and 19 were designated cytosolic or nonmembrane vesicle-associated protein. The percent of total intracellular heavy chain or BiP within each subcellular fraction was determined by dividing the relative densitometer value of a subcellular fraction by the value of the total of all fractions.

the RER and only 3% in the Golgi apparatus. The subcellular fractions from Ag8(8) and RD3-2 were also examined for presence of BiP by immunoprecipitation. Table I shows that BiP is found largely in RER fractions from each cell type, and is not present in other membrane fractions in quantities larger than 3% of total. The presence of BiP in the cytosolic fractions supports the hypothesis that BiP is soluble and not a membrane-associated protein. BiP was also extracted into the aqueous phase during Triton X-114 detergent partitioning (not shown), a procedure which promotes separation of integral membrane proteins into a detergent phase and soluble proteins into the aqueous phase (6). Thus BiP is most likely located within the RER and can be demonstrated to behave as a freely soluble protein.

Discussion

The studies described in this paper focus on the association of the microsomal protein BiP with intracellular Ig heavy chains from secreting (H^+ , L^+) and nonsecreting (H^+ , L^-) mouse B lymphocyte cell lines. A direct correlation exists between light chain addition, BiP displacement and the ability of assembled Ig molecules to be transported to the Golgi apparatus.

The detection of BiP heavy chain complexes in (H^+ , L^+) cell lines would be anticipated to be dependent on both the molar ratio of heavy and light chains and the mode and rate of assembly of the chains within a given cell line. Analysis of a variety of myelomas has shown that heavy and light chains are synthesized in variable ratios, however, the majority have a molar excess of light over heavy chains (2). Thus the detectable amount of free heavy chain associated with BiP may be very small when heavy chain concentration limits assembly. Our ability to readily detect BiP heavy chain complexes in RD3-2 and not in MM60 may reflect a difference in H:L chain ratios as well as the mode of light chain addition. RD3-2 assembles heavy chain dimers (H_2) before light chain addition, whereas in IgM-producing cell lines such as MM60, light chains are added directly to individual heavy chains (5, 30). Consequently, if BiP displacement is mediated solely by light chain addition, heavy chains available for BiP association would exist within RD3-2 until the last light chain is added forming the H_2L_2 tetramer, while in MM60 BiP would be

displaced immediately after the first light chain is added forming the HL dimer.

In RD3-2 and J558, co-precipitation of BiP with assembling Ig precursors was readily observed when anti-BiP antibody was used as the precipitating agent. However, when assembling heavy chains were precipitated with anti-heavy chain antibody, little or no co-precipitating BiP was observed. It is not known why the BiP heavy chain complex is more readily detectable from the anti-BiP immunoprecipitates. This observation may explain why BiP has not previously been identified to co-precipitate with assembling Ig heavy chains when analyzed with polyclonal anti-heavy chain antibody reagents.

Experiments with J558 revealed that when glycosylation of heavy chains was inhibited with tunicamycin, an increased level of BiP heavy chain complex was observed in pulse-chase experiments. Inhibition of heavy chain glycosylation has been reported to relieve a translational block in heavy chain synthesis. Relief of this block resulted in a higher H/L ratio (4). If tunicamycin relieves a translational block of heavy chain synthesis in J558, the increase in BiP heavy chain complexes observed in the presence of tunicamycin could result from an increase in the intracellular pool of heavy chain. Alternatively, inhibition of glycosylation could prevent normal protein folding resulting in a heavy chain conformation that associates less efficiently with light chain (10, 18).

Subcellular fractionation of Ag8(8) demonstrated that transport of free heavy chains to the Golgi compartment is either blocked or is inefficient when compared to the transport of completely assembled molecules in RD3-2. This finding is in agreement with the differential transport of membrane and secretory μ -chains within the human B-cell line, Daudi. Daudi cells produce both secretory and membrane μ -chains, however only the membrane μ -chains are externalized (10). The secretory heavy chains do not assemble with light chain and remain endoglycosidase H sensitive and thus are blocked from transport to, at least, the medial Golgi region (Hendershot, L., manuscript in preparation). BiP was only immunoprecipitated in significant quantities from the RER membrane-enriched vesicles. This finding demonstrates that BiP is a native component of the RER and maintains its subcellular location despite a continual synthesis and export of newly synthesized secretory protein. Thus, the transport of Ig from RER to Golgi apparatus is a very selective process restricting transport of native RER luminal components. A similar restriction of subcellular location has also been shown for three membrane-associated RER-specific proteins within a mouse myeloma (23).

Intracellular transport of secretory proteins from the RER to the Golgi apparatus has been hypothesized to be mediated by receptors within the endoplasmic reticulum (12, 24, 40). Receptor-mediated intracellular transport would ensure selective transport of only those proteins expressing determinants recognized by such receptors. Our data demonstrating selective transport of assembled Ig directly supports such a hypothesis. In the absence of light chain assembly, signals necessary for transport are not expressed on heavy chains associated with BiP. Transport signals within the heavy chain may be sterically masked by BiP, or BiP may influence the folding of the heavy chain such that proper conformation necessary for transport is not achieved. Light chain addition with disas-

sociation of BiP appears to be necessary for initiating transport. This suggests that BiP may play a regulatory role in the intracellular transport of Ig molecules by preventing the premature transport and possible expression of Ig heavy chains before their assembly with light chain.

Acquisition of proper conformation has been demonstrated to be necessary for the transport of other secretory proteins. In this regard, the transport of retinol-binding protein requires exogenous ligand for exit from the RER (34). One and two amino acid substitutions within secretory proteins can prevent their secretion presumably by altering conformation (44, 45). In addition, the prevention of glycosylation or its processing, which may influence protein folding, have been shown to affect efficiency of intracellular transport of Ig as well as other secretory proteins (22, 25). The signals necessary for directing transport and secretion of Ig molecules as well as other secretory proteins are enigmatic. Secretion of assembled Ig can occur in cells producing mutant heavy chains with deletions of any one of the constant region domains (20). Thus no one domain is necessary for secretion. In contrast to heavy chains, some but not all light chains can be secreted unassembled when light chain synthesis is in excess of heavy chain synthesis or when cells have lost the ability to produce heavy chains (19). These observations may suggest that neither the heavy chain nor light chain in itself is sufficient for signaling transport, but that it is the product of the two chains within the assembled molecule that signals transport. Heavy chains, however, can possess signals sufficient for secretion. In contrast to μ -chains in pre-B cells, heavy chains can be secreted in the absence of light chain synthesis in lymphoproliferative heavy chain disease (35) and in some mutant myelomas (9, 29). Examination of many heavy chain disease proteins has shown that they contain deletions that include portions of the first constant region (CH_1) and/or variable region domains where light chains associate (35). In preliminary experiments, we have been unable to demonstrate association of BiP with heavy chains containing deletions within the CH_1 domain. We speculate that association of nascent heavy chains with BiP requires an intact CH_1 domain.

We have described a novel posttranslational processing event involving the association of unassembled intracellular Ig heavy chains with the microsomal protein BiP. This association occurs early in the maturational process of immunoglobulin assembly within the endoplasmic reticulum. We propose that the association of BiP with unassembled heavy chains may prevent their transport along the secretory pathway by masking expression of transport recognition signals within the heavy chain molecule. We have found BiP to be ubiquitously distributed among cells of different lineages and from the results of preliminary experiments speculate that BiP may function in posttranslational processing and transport of other secretory proteins as well.

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