Immunoglobulin Heavy Chain and Binding Protein Complexes Are Dissociated In Vivo by Light Chain Addition

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Abstract. Immunoglobulin heavy chain binding protein (BiP, GRP78) associates stably with the free, nonsecreted Ig heavy chains synthesized by Abelson virus transformed pre-B cell lines. In cells synthesizing both Ig heavy and light chains, the Ig subunits assemble rapidly and are secreted. Only incompletely assembled Ig molecules can be found bound to BiP in these cells. In addition to Ig heavy chains, a number of mutant and incompletely glycosylated transport-defective proteins are stably complexed with BiP. When normal proteins are examined for combination with BiP, only a small fraction of the intracellular pool of nascent, unfolded, or unassembled proteins can be found associated. It has been difficult to determine whether these BiP-associated molecules represent assembly intermediates which will be displaced from BiP and transported from the cell, or whether these are aberrant proteins that are ultimately degraded. In order for BiP to monitor and aid in normal protein transport, its association with these proteins must be reversible and the released proteins should be transport competent. In the studies described here, transient heterokaryons were formed between a myeloma line producing BiP-associated heavy chains and a myeloma line synthesizing the complementary light chain. Introduction of light chain synthesis resulted in assembly of prelabeled heavy chains with light chains, displacement of BiP from heavy chains, and secretion of Ig into the culture supernatant. These data demonstrate that BiP association can be reversible, with concordant release of transportable proteins. Thus, BiP can be considered a component of the exocytic secretory pathway, regulating the transport of both normal and abnormal proteins.

DATA from many laboratories have demonstrated that protein transport from the ER is highly specific and tightly regulated. Nascent polypeptides are usually assembled and folded in the ER and aberrant proteins that cannot fold or assemble properly are often blocked at this point in the exocytic transport pathway (reviewed Pfeffer and Rothman, 1987). The retention of at least some of these abnormal proteins appears to be due to their association with Ig heavy chain binding protein (BiP,[1] GRP78), a soluble resident ER protein (Haas and Wabl, 1983; Bole et al., 1986; Munro and Pelham, 1986). BiP associates stably with the free, nonsecreted heavy chains synthesized by Abelson virus transformed pre-B cell lines (Haas and Wabl, 1983) and transiently with the incompletely assembled Ig precursors in plasmacytoid lines (Bole et al., 1986). In addition to Ig heavy chains, a number of mutant and incompletely glycosylated transport-defective proteins have been found to be stably associated with BiP (Gething et al., 1986; Dorner et al., 1987; Kassenbrock et al., 1988). These BiP-associated proteins are not secreted and are eventually degraded.

Attempts to demonstrate the combination of normal secreted or membrane proteins with BiP have revealed that nascent, unfolded, or unassembled proteins are transiently complexed with BiP, but in most cases, these represent only a small fraction of the intracellular pool of such proteins (Bole et al., 1986; Gething et al., 1986; Dorner et al., 1987; Kassenbrock et al., 1988). Because of this, it has been difficult to demonstrate with any certainty that these proteins represent folding or assembly intermediates which will eventually be secreted and not aberrant dead-end products that are degraded. This has led to divergent hypotheses as to whether BiP functions primarily to bind defective proteins in the ER and target them for intracellular degradation or whether it may also be involved in retaining nascent proteins in the ER until their folding and assembly are complete, perhaps even facilitating these processes.

For BiP to regulate normal protein transport, its association with target proteins must be reversible. Although in vitro experiments have demonstrated that ATP can release BiP from heavy chains and peptides (Munro and Pelham, 1986; Flynn et al., 1989), there is no clear evidence that BiP binding in vivo is reversible. I now show that introduction of light chains into cell lines synthesizing only Ig heavy chains can result in the disruption of BiP-heavy chain complexes, leading to secretion of assembled Ig molecules. These data show that BiP-associated proteins are not inherently defective and can be released in a transport competent state. Further, they strongly support the idea that BiP is a

1. *Abbreviation used in this paper*: BiP, heavy chain binding protein.

component of the exocytic secretory pathway, and is able to regulate the transport of both normal and abnormal proteins.

Materials and Methods

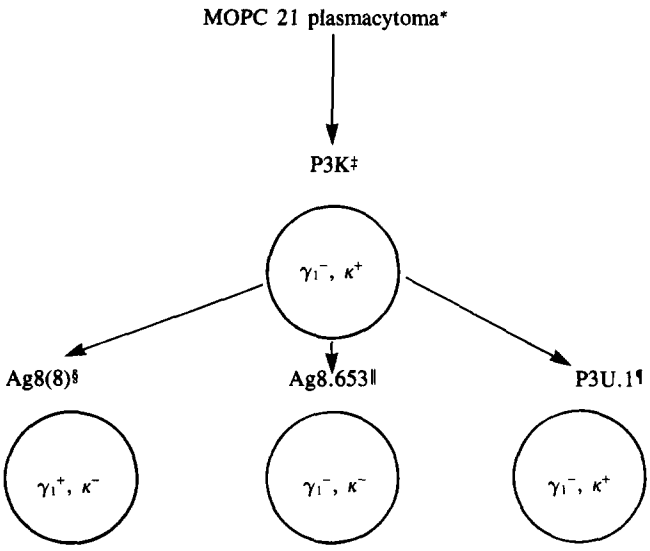
Cell Lines

All cell lines used in this study were derived from the P3K cell line (γ_1, κ), which is the tissue culture adaptant of the MOPC 21 plasmacytoma (Horibata and Harris, 1970) (Table I). The Ag8(8) cells express the parental γ heavy chains but no longer synthesize κ light chains (γ_1^+, κ^-) (Bole et al., 1986) and the P3X63 Ag8U.1(P3U.1) cells synthesize the parental κ chains but do not make γ heavy chains (γ_1^-, κ^+) (Yelton et al., 1978). The Ag8.653 cells have lost the ability to make either chain (γ_1^-, κ^-) (Kearney et al., 1979). The P3U.1 cells were maintained in DME with 4.5 g/liter glucose supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin. The Ag8(8) and Ag8.653 cells were maintained in RPMI-1640 medium with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin. All lines were screened for mycoplasma infection and found to be negative. Both Ag8(8) and P3U.1 cells were stained with fluorochrome-conjugated, Ig chain-specific antibodies and found to be >95% positive for their respective chain.

Transient Heterokaryon Formation

Transient heterokaryons were formed according to method of Baron and Maniatis (1986). A total of 6×10^7 cells were plated on poly-L-lysine-HBr-treated 100-mm tissue culture dishes and incubated with 50 μg/ml of phytohemagglutinin (PHA) for 1 h to allow the cells to form a "monolayer" on the bottom of the plate. The cell lines and their ratios varied with the individual experiments and are each described in Results. After incubation, the plates were carefully washed two times with serum-free medium and then fused with 2 ml of 50% PEG (Boehringer Mannheim Biochemicals, Indianapolis, IN). After 1 min, residual PEG was aspirated and the plates were washed three times with serum-free medium before replating them with complete RPMI-1640 medium. After incubation, culture supernatants and cell lysates were analyzed for Ig assembly and secretion. Mock-fused cultures were treated exactly the same as the fused cultures except that no PEG was added to the plates. In each experiment, a small aliquot of cells from the fused and mock-fused cultures was analyzed by staining ethanol/acetic acid (95:5) fixed cytocentrifuge preparations with FITC-conjugated anti-γ antibodies and TRITC-conjugated anti-κ antibodies (Southern Biotechnology Associates, Birmingham, AL).

Table I. Derivation of Cell Lines



* MOPC 21 plasmacytoma (Horibata and Harris, 1970).
‡ Tissue culture adaptation of MOPC 21 (Horibata and Harris, 1970).
§ Subclone of P3K synthesizing only γ heavy chains (Bole et al., 1986).
‖ Subclone of P3K that has lost the ability to make both heavy and light chains (Kearney et al., 1979).
¶Subclone of P3K synthesizing only κ light chains (Yelton et al., 1978).

Biosynthetic Labeling

Cells were metabolically labeled with [^{35}S]methionine (Amersham Corp., Arlington Heights, IL) in RPMI-1640 medium devoid of methionine. For prelabeling experiments, Ag8(8) cells were labeled for 2 h, washed twice with PBS, and plated with a fivefold excess of unlabeled P3U.1 or Ag8.653 cells for 1 h before fusion. After fusion, the cultures were reincubated in complete medium for 3 h. For postlabeling experiments, equal numbers of the two cell lines to be fused were plated, incubated, and fused as described above. After fusion, the cells were recultured in medium devoid of methionine and labeled for 3 h. In both cases, culture supernatants were saved and reacted with anti-mouse Ig antibodies to detect Ig secretion. Cell lysates were prepared and immunoprecipitated with antibodies specific for mouse Ig and BiP (Bole et al., 1986). Precipitated material was analyzed under both reducing and nonreducing conditions on SDS-polyacrylamide gels and proteins were visualized by fluorography.

Cycloheximide Treatment of Culture

Ag8(8) cells were prelabeled with [^{35}S]methionine, washed, and plated with excess P3U.1 cells in the presence of PHA for 1 h. 5 min before fusing the cells, 100 μM cycloheximide was added to the cultures to block protein synthesis. After fusion, cells were recultured in complete medium with cycloheximide and Ig assembly and secretion were analyzed as described above.

Endo H Treatment of γ Heavy Chain

Immunoprecipitated proteins were divided in half and each was resuspended in 45 μl of 0.1 M Na acetate buffer, pH 5.5. To one portion, 5 μl (5 mU) of Endo H (Boehringer Mannheim Biochemicals) was added. Both tubes were incubated at 37°C for 15 h.

Results

Quantitation of the Percentage of γ Heavy Chains Associated with BiP

To first confirm that all γ_1 heavy chains in the Ag8(8) cell line were complexed with BiP, the cells were metabolically labeled with [^{35}S]methionine, and cell lysates were prepared, divided in half, and immunoprecipitated with either anti-Ig or anti-BiP antibodies. After precipitation with anti-BiP, the remaining cell lysate was cleared once with protein A bearing *Staphylococcus aureus* and then reprecipitated with anti-Ig to detect any non-BiP-associated heavy chains. The anti-Ig antibody precipitated γ_1 heavy chains as well some of the BiP molecules (Fig. 1). The anti-BiP monoclonal antibody precipitated BiP as well as associated heavy chains. Reprecipitation of the residual lysate from the anti-BiP reaction with antibodies specific for immunoglobulin revealed that no free γ_1 heavy chains were present (Fig. 1). Pulse-chase experiments indicated that the BiP-heavy chain complexes were stable with a half-life of >4 h (data not shown).

Introduction of Light Chains into Cell Lines with Stable BiP/Heavy Chain Complexes

To determine whether BiP binding to heavy chains could be dissociated in vivo, a system was constructed to introduce free light chains into cells with stable BiP-heavy chain complexes. Transient heterokaryons were formed between a cell line producing BiP-associated heavy chains and one syn-

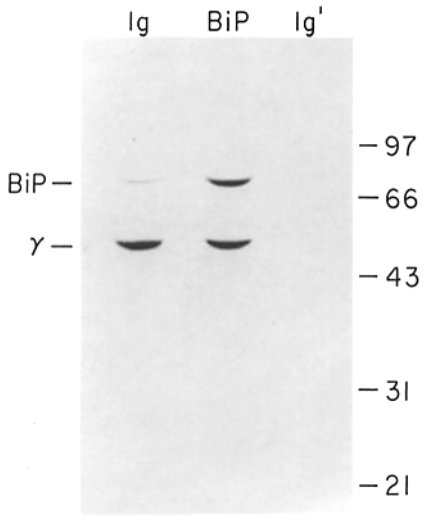


Figure 1. Quantitation of γ chain association with BiP. Ag8(8) cells (4×10^6) were labeled with 50 μCi [^{35}S]methionine for 2 h. A cell lysate was prepared and divided in half; one portion was immunoprecipitated with goat anti-mouse Ig antibodies and the other with the anti-BiP monoclonal antibody. The lysate remaining after anti-BiP precipitation was cleared once more with *S. aureus* to remove any residual complexes and then reprecipitated with anti-Ig antibodies (*Ig'*). All three immunoprecipitates were then analyzed on 10% SDS-polyacrylamide gels under reducing conditions.

thesizing the complementary light chain. For these experiments, two single-chain mutant cell lines isolated from P3 × 63Ag8 cells (γ_1, κ) were used: Ag8(8) (γ_1^+, κ^-) and P3U.1 (γ_1^-, κ^+).

Ag8(8) cells (3×10^6) were mixed with fivefold excess P3U.1 (1.5×10^7) cells and plated in 60-mm dishes. Cells were fused or mock fused as described in Materials and Methods and recultured for 1 or 3 h. P3U.1 cells were plated in excess of Ag8(8) cells to increase the chance that heavy chain–containing cells would fuse with light chain–producing cells instead of with other heavy chain–positive cells. Cells were then carefully scraped from the plates, washed once with PBS containing 10% serum, and cytocentrifuge preparations were made and stained with FITC-conjugated anti-γ antibodies and TRITC-conjugated anti-κ antibodies. About 3–5% of the γ^+ cells costained with antibodies to κ after fusion (Table II). There was no significant difference in the percentage of double staining cells present at 1 or 3 h after fusion. There was no evidence of double-staining cells in the mock-fused cultures.

Stability of BiP/Heavy Chain Complexes after Incubation on Poly-L-Lysine Plates and PEG Treatment

To ensure that prolonged incubation on poly-L-lysine plates or PEG treatment did not disrupt BiP/heavy chain complexes, biosynthetically labeled Ag8(8) cells (γ_1^+, κ^-) were distributed on four 35-mm poly-L-lysine–treated plates and incubated for 1 h with PHA. The cells from two plates were fused with PEG as described, and the other two plates were washed and served as mock-fused controls. Immediately after fusing, the cells from one fused and one mock-fused culture were lysed. Cell lysates were divided in half and precipitated with either anti-γ antibodies or with the anti-BiP

Table II. Detection of Double Positive Cells After Cell Fusion

Cells	γ^+	κ^+	γ^+/κ^+
	%	%	%
Ag8(8)	>95*	<0.5*	<0.5*
P3U.1	<0.5*	>98*	<0.5*
Ag8.653	<0.5*	<0.5*	<0.5*
Ag8(8)–P3U.1 fused (1:5)	20‡	80‡	3–5§
Ag8(8)–P3U.1 mock (1:5)	20‡	80‡	<0.2§

* 200 cells were counted and scored as positive for γ, positive for κ, and positive for both.
‡ In each of three fusions and mock fusions, 500 cells were counted and scored as either positive for γ or positive for κ.
§ In each of three fusions and mock fusions, 500 γ^+ cells were scored for costaining with anti-κ antibodies. All double staining cells were binucleated.

monoclonal antibody. The resulting supernatant from the anti-BiP precipitate was reprecipitated with anti-γ as in Fig. 1. After 2 h, the second set of plates were lysed and immunoprecipitated in the same way. Both immediately after fusion and 2 h after fusion, there was no evidence for any disruption of heavy chain/BiP complexes in either the fused or mock-fused cultures. In all cases, all of the heavy chains were associated with BiP (Fig. 2).

Displacement of BiP from Heavy Chains by Light Chains

To test the reversibility of the BiP/heavy chain complex, the Ag8(8) cells (γ_1^+, κ^-) were labeled with [^{35}S]methionine, washed, mixed with a fivefold excess of unlabeled P3U.1 cells, (γ_1^-, κ^+), and incubated on culture dishes for 1 h. In addition to allowing the monolayer to form, the 1-h incubation also provided time to exhaust intracellular pools of [^{35}S]methionine in the Ag8(8) cells. The cells were then fused and incubated for another 3 h, after which Ig and BiP were immunoprecipitated from the cell lysates and medium. All immunoprecipitates were split in half and analyzed electrophoretically under both reducing and nonreducing conditions on denaturing polyacrylamide gels. Parallel control fusions and immunoprecipitations were performed between labeled Ag8(8) cells and unlabeled Ag8.653 cells that produce no Ig chains (Kearney et al., 1979). Examination of the culture supernatant from the Ag8(8) × P3U.1 fusion revealed the secretion of labeled IgG (Figure 3 *A*). In contrast, Ig was not detected in the medium of Ag8(8) cells fused to the Ig^- line (Ag8.653), nor was it secreted from mixed or mock-fused cells. While the molecular weight of the secreted Ig was consistent with it being an H_2L_2 heterodimer, the presence of both heavy and light chains in the secreted material was determined by immunoprecipitating the culture supernatants with either anti-γ, anti-κ, or Cowen's I formalin-fixed *S. aureus* alone. The secreted immunoglobulin could be precipitated with antibodies to either γ heavy chains or κ light chains demonstrating the presence of both and confirming the identity of the secreted protein as heterodimeric IgG (H_2L_2) (Fig. 4).

Reduction of immunoprecipitates from the cell lysates showed that only BiP and Ig heavy chains were radiolabeled (Fig. 3 *B*). The failure to detect any labeled light chains in the cell lysate suggested that proteins synthesized after fusion were not labeled. Most important, examination of the secreted Ig molecules under reducing conditions revealed

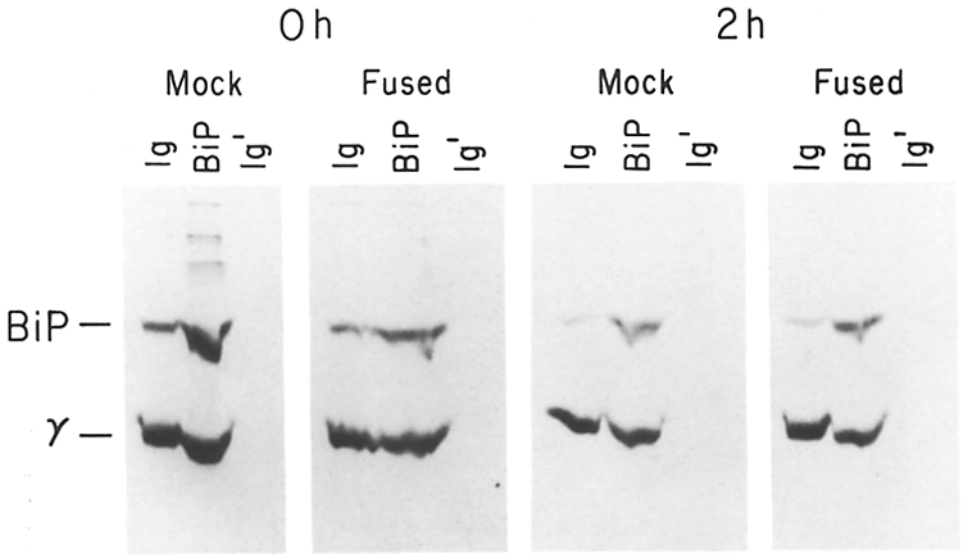


Figure 2. Stability of BiP/heavy chain association during incubation and PEG treatment. 24,000,000 Ag8(8) cells (γ^+, κ^-) were biosynthetically labeled with 100 μCi of [^{35}S]methionine for 3 h, washed, and plated on four 35-mm dishes for 1 h with PHA. After that, two were fused and two were only washed and served as controls. Immediately after fusion, the cells from one fused and one mock-fused culture were lysed (*0 h*). The two remaining cultures were reincubated for 2 h before being lysed (*2 h*). The lysates from all four cultures were split in two and precipitated with anti-Ig or anti-BiP antibodies and formalin-fixed *S. aureus*. The supernatant from the anti-BiP precipitate was reprecipitated with anti-Ig antibodies (*Ig'*) as in Fig. 1. All precipitates were analyzed on 10% SDS-polyacrylamide gels under reducing conditions. The 0-h precipitates were exposed to film for twice as long as the 2-h precipitates.

that only the γ_1 heavy chains were labeled (Fig. 3 *B*). These heavy chains must therefore have been labeled before fusion when they were complexed to BiP (Fig. 1). The lack of label in the κ chains was not due to their inability to be labeled with [^{35}S]methionine nor was it due to their synthesis at a much lower rate than the γ_1 heavy chains. Incorporation of [^{35}S]methionine into these light chains was shown to be at least equivalent to that in the γ_1 heavy chains when the Ag8(8) and P3U.1 cells were labeled after fusion (Fig. 5). Thus, preexisting BiP-heavy chain complexes can be dissociated by the introduction of light chains into the cell, resulting in the assembly and secretion of Ig.

Analysis of Ig Secretion after Cell Fusion

Secretion of Ig did not appear to be an artifact of fusion, because only assembled Ig molecules were secreted, and no heavy chains were secreted from the Ag8(8) cells that were fused to Ag8.653 (Ig^-) cells. To more closely examine secretion of Ig, Ag8(8) cells were prelabeled, fused, and then recultured for varying amounts of time. Increasing amounts of labeled immunoglobulin were detected in the culture supernatants with prolonged incubation (Fig. 6). This was not due to cell lysis at later time points, because no Ig was detected in the mock-fused cultures, and the secreted Ig was completely assembled. Very little Ig was detected in the 1-h supernatant showing that there was not an artifactual burst of "secretion" at the time of fusion. Instead, the data demonstrate that time was required for subunit assembly and passage of the Ig molecule through intracellular organelles.

To determine whether in fact the secreted IgG was being processed normally along the exocytic pathway, the secreted H_2L_2 molecules were examined for Endo H sensitivity. Glycoproteins that pass through the Golgi apparatus have their N-linked sugars modified and are no longer sensitive to Endo H digestion. The H_2L_2 molecules formed in the cell lysates from mixed, mock-fused, and fused cultures were also examined. Biosynthetically labeled Ag8(8) cells were cultured and set up in the same manner as in Fig. 3. After preparation of cell lysates, proteins were reacted with goat anti-mouse Ig antisera, immunoprecipitates were divided, and half of each sample was treated with Endo H. The proteins were separated on a SDS-gel run under nonreducing conditions and a 2-cm horizontal strip containing the H_2L_2 molecules was cut from the gel. This strip was equilibrated in SDS sample buffer containing 2-β-mercaptoethanol and then run on a second SDS gel under reducing conditions. In each case, the IgG from the cell lysates was Endo H sensitive as determined by a decrease in their molecular weight after treatment with the endoglycosidase (Fig. 7). This decrease is very slight because there is only a single glycosylation site on γ heavy chains. The secreted H_2L_2 molecules however were found to be Endo H resistant, demonstrating that they had been processed in the Golgi apparatus (Fig. 7). Because the shift in molecular weight is so slight, in a separate experiment the H_2L_2 molecules from the cell lysate and medium were mixed and analyzed as above. In this experiment, two bands are clearly present in the Endo H-treated sample demonstrating the presence of both Endo H-sensitive and -resistant heavy chains (Fig. 7). Thus, the IgG secreted after fusion appears to be transported and processed along the normal exocytic pathway as determined by both the kinetics of transport (Fig. 6) and the processing of N-linked sugars (Fig. 7). The H_2L_2 molecules found in the cell lysates of fused, mock-fused, and mixed cultures are not Endo H resistant and thus have not passed through the Golgi which is consistent with their being formed at the time of cell lysis.

Requirement of Protein Synthesis for Ig Assembly and Secretion

This method of heterokaryon production results in the fusion of the plasma membranes of cells in contact with each other but does not cause efficient fusion of intracellular membranes (Blau et al., 1983). Without adequate fusion of ER

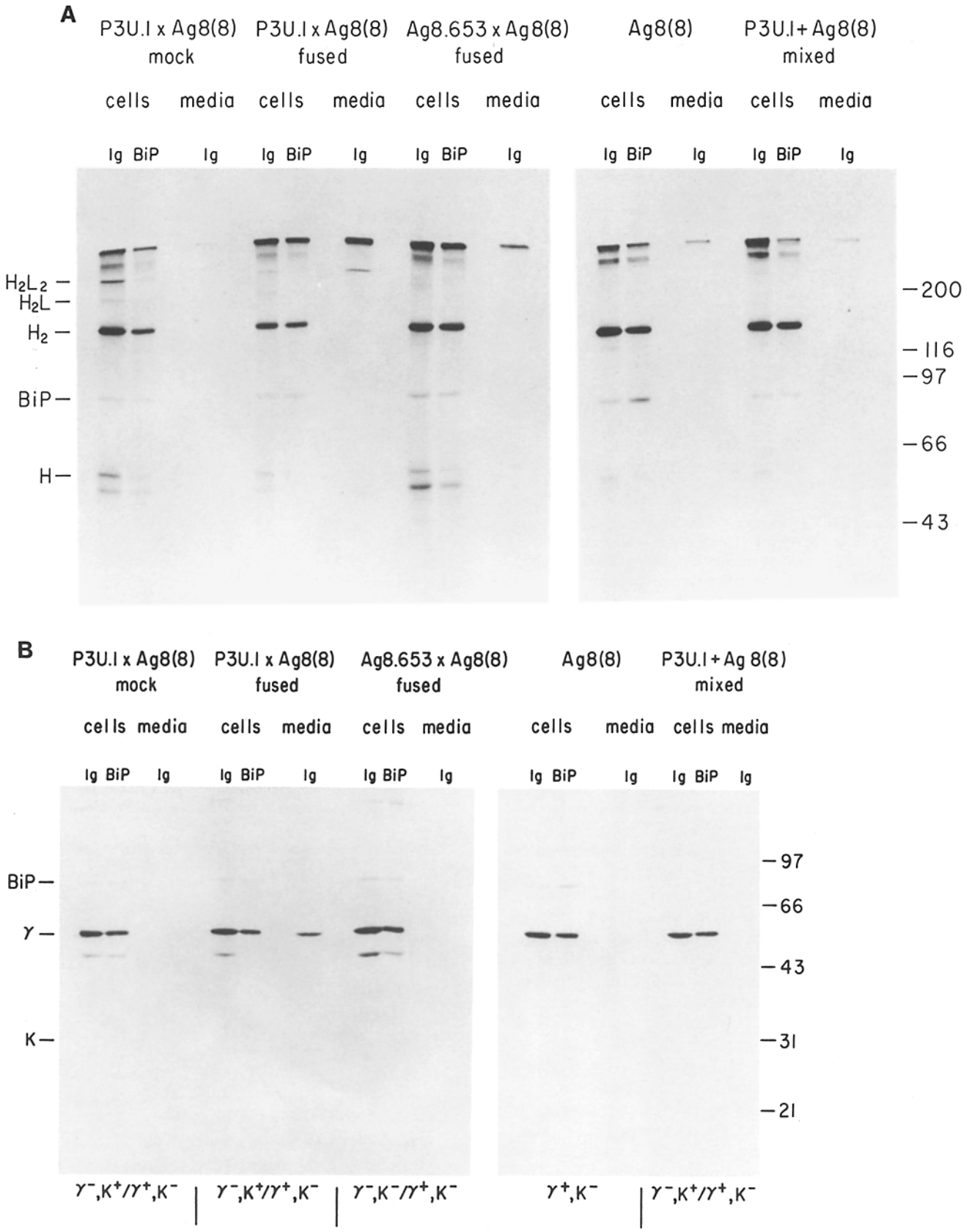


Figure 3. Dissociation of BiP/heavy chain complexes by light chain addition. Transient heterokaryons were formed according to the method of Baron and Maniatis (1986). Ag8(8) cells (4×10^7) were labeled with 300 μCi[^{35}S]methionine for 2 h, washed three times with PBS, and divided into five equal portions (8×10^6). Two portions were plated and incubated in complete medium for the duration of the experiment, at which time, one was harvested and lysed (*Ag8(8)*), and the other one was mixed with P3U.1 cells (4×10^7) just before lysing (*mixed*). The remaining three portions were plated on 100-mm dishes and incubated with 50 μg/ml of PHA for 1 h. Of the latter three, one was plated with 4×10^7 Ag8.653 cells (*Ag8.653 × Ag8(8)*) and fused, and two were plated with 4×10^7 P3U.1 cells; one of these was fused (*P3U.1 × Ag8(8) fused*) and the other was not (*P3U.1 × Ag8(8) mock*). All three cultures were incubated for 3 h more in complete media devoid of isotope, and then culture supernatants and cell lysates were analyzed for Ig assembly and secretion by immunoprecipitation with goat anti-mouse Ig and anti-BiP antibodies. Isolated proteins were divided and analyzed under both nonreducing (*A*) and reducing (*B*) conditions.

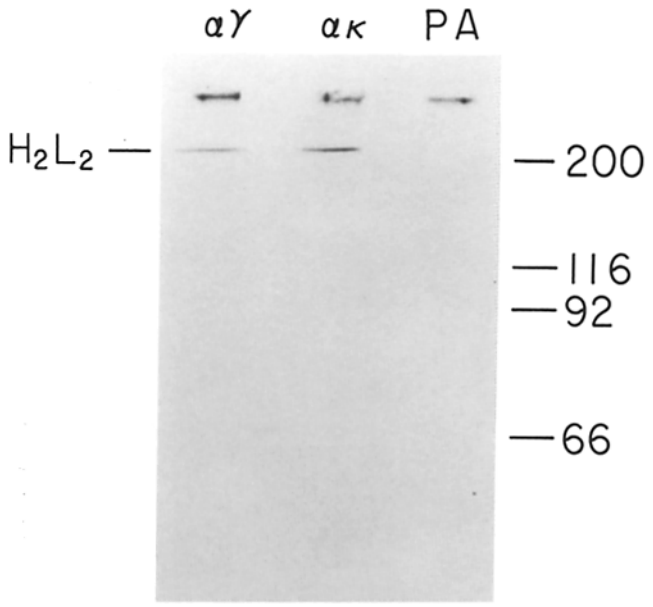


Figure 4. Composition of secreted Ig. Eight million Ag8(8) cells (γ^+,κ^-) were labeled, washed, incubated with 4×10^7 P3U.1 cells (γ^-, κ^+) and fused as in Fig. 3. After fusion, the cells were reincubated for 3 h to allow Ig secretion. The culture supernatant was divided in three tubes and immunoprecipitated with anti-γ, anti-κ, or *S. aureus* alone. The precipitated proteins were analyzed on 8% SDS-polyacrylamide gels under nonreducing conditions.

membranes, initiation of new light chain synthesis on ER membranes containing γ heavy chains should be necessary in order to provide a pool of light chains that gains access to preexisting heavy chain/BiP complexes. To test the requirement for protein synthesis, biosynthetically labeled Ag8(8) cells (γ^+, κ^-) were incubated with unlabeled P3U.1 cells (γ^-, κ^+) for 1 h and then treated with cycloheximide just before fusing the cells. After fusion (or washing for mock-fused cultures), cells were recultured for 3 h with cycloheximide. Control cultures were set up and analyzed at the same time. Cell lysates and culture supernatants were immunoprecipitated with anti-mouse Ig antibodies. Immunoprecipitates were divided and analyzed under either reducing or nonreducing conditions. In both the reduced and nonreduced gel, there were only trace amounts of Ig secreted in the cycloheximide-treated cultures (Fig. 8). This is in contrast to the Ig secretion observed in the control fused cultures. Thus, in the absence of new protein synthesis, there was virtually no assembly or secretion of Ig (Fig. 8), demonstrating a need for new light chain synthesis. Once again, H_2L_2 molecules can be detected in the cell lysates of both fused and mock-fused cells examined under nonreducing conditions (Fig. 8 *B*). However, the presence of H_2L_2 molecules in the cell lysates of fused and mock-fused cycloheximide-treated cultures is much diminished (Fig. 8 *B*). The κ light chains synthesized by the P3U.1 cells have a half-life of ~1 h. Thus, at the time of cell fusion, there are the same number of light chains present in both the control and cycloheximide-treated cultures. But, at the time of cell lysis (3 h later) there are only about one-eighth the number of light chains present in the cycloheximide cultures as in the control cultures which is analogous to the decreased amount of H_2L_2 molecules found in the cycloheximide cultures. This finding is consistent with the theory that these H_2L_2 molecules are formed at the time of cell lysis.

Discussion

The retention of many abnormal proteins in the ER appears

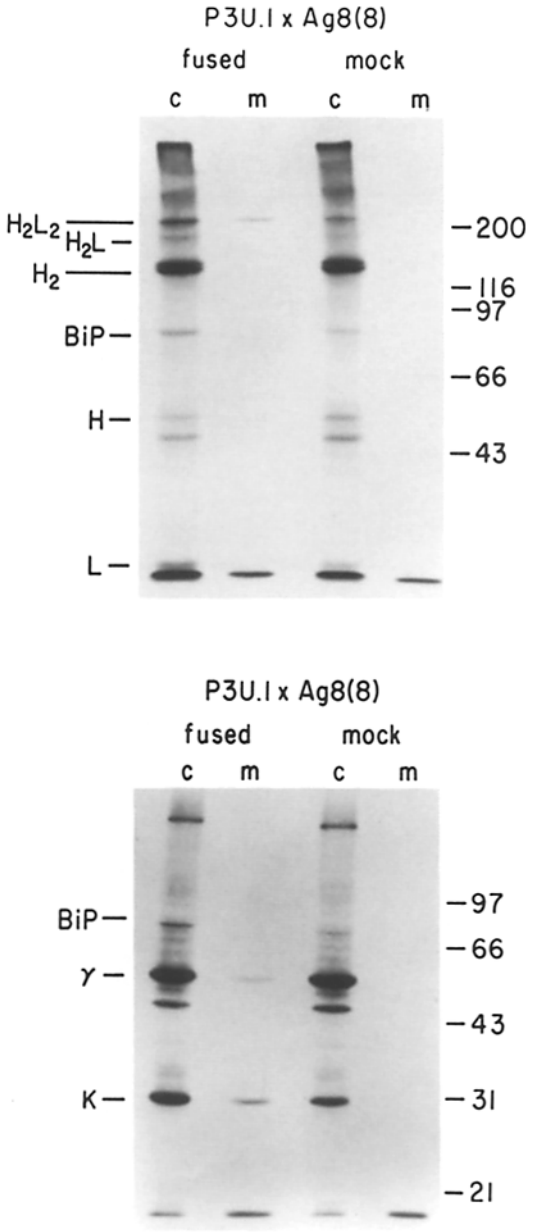


Figure 5. Heavy and light chains are labeled equally well in secreted Ig monomers labeled after fusion. Equal numbers of Ag8(8) (γ^+, κ^-) and P3U.1 (γ^-, κ^+) cells (8.5×10^6) were mixed and incubated with PHA on 60-mm tissue culture plates for 1 h. After fusion, or just incubation (*mock*), cultures were labeled for 3 h with 100 μCi [^{35}S]methionine. Both culture supernatants and cell lysates were immunoprecipitated with goat anti-mouse Ig antibodies, and precipitated proteins were separated on SDS-polyacrylamide gels under nonreducing (*top*) or reducing (*bottom*) conditions.

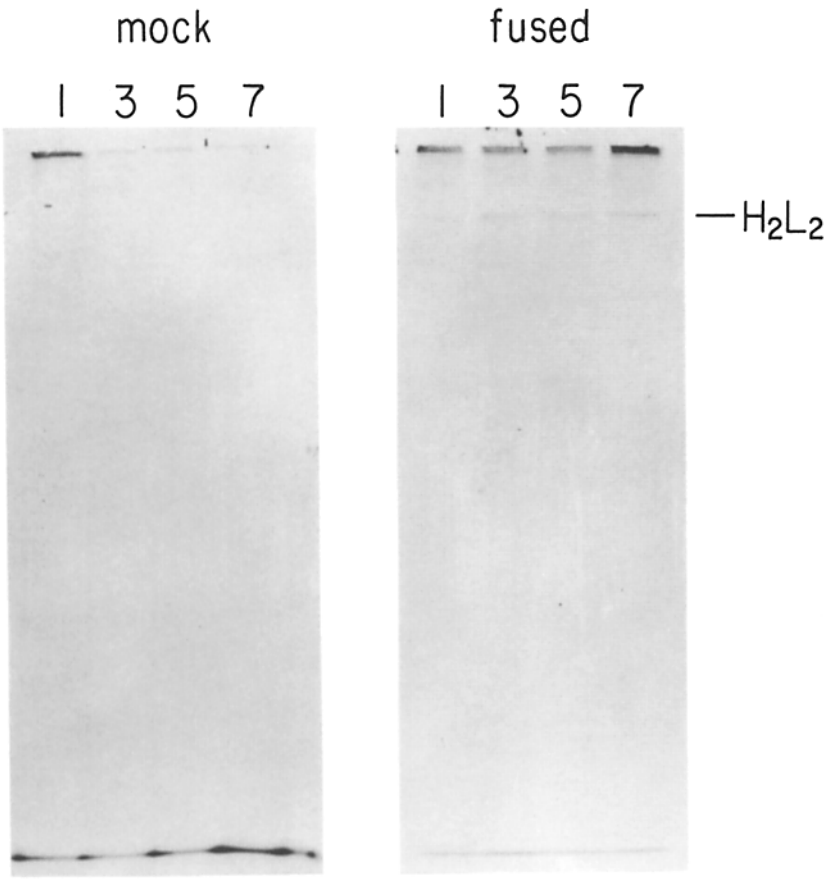


Figure 6. Kinetics of Ig secretion after fusion. 24,000,000 Ag8(8) cells (γ^+, κ^-) were labeled overnight with 200 μCi of [^{35}S]methionine, washed, mixed with 1.2 × 10^8 P3U.1 cells (γ^-, κ^+) and distributed into eight 60-mm dishes. After 1 h, four plates were fused and four served as mock-fused controls. Culture supernatants at 1-, 3-, 5-, and 7-h postfusion were harvested and reacted with anti-Ig antibodies. Precipitated proteins were analyzed on 8% polyacrylamide gels under nonreducing conditions.

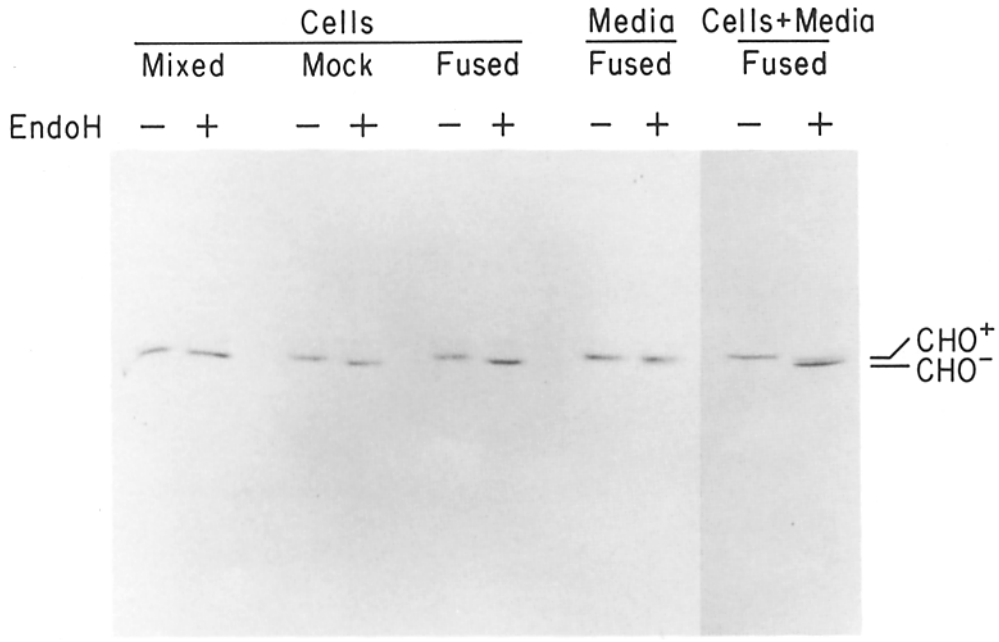


Figure 7. Endo H sensitivity of H_2L_2 molecules. 24,000,000 Ag8(8) cells (γ^+, κ^-) were labeled with 300 μCi of [^{35}S]methionine for 2 h and mixed, fused, or mock fused to fivefold excess P3U.1 cells (γ^-, κ^+) as in Fig. 3. 3-h cell lysates and tissue culture supernatant from the fused culture were immunoprecipitated with anti-Ig antibodies. The precipitated material was divided in two and one-half was treated with Endo H and the other half served as a control. The proteins were then separated on an 8% SDS gel under nonreducing conditions. The portion of the gel containing the H_2L_2 molecules was cut from the gel, equilibrated in SDS sample buffer containing 2-β-mercaptoethanol and rerun on a 10% SDS gel under reducing conditions. A second fusion experiment was set up exactly like the one described above, but the anti-Ig precipitated proteins from the cell lysate and culture supernatant were mixed together, divided into two portions, treated with Endo H, and analyzed in the same way as the other samples.

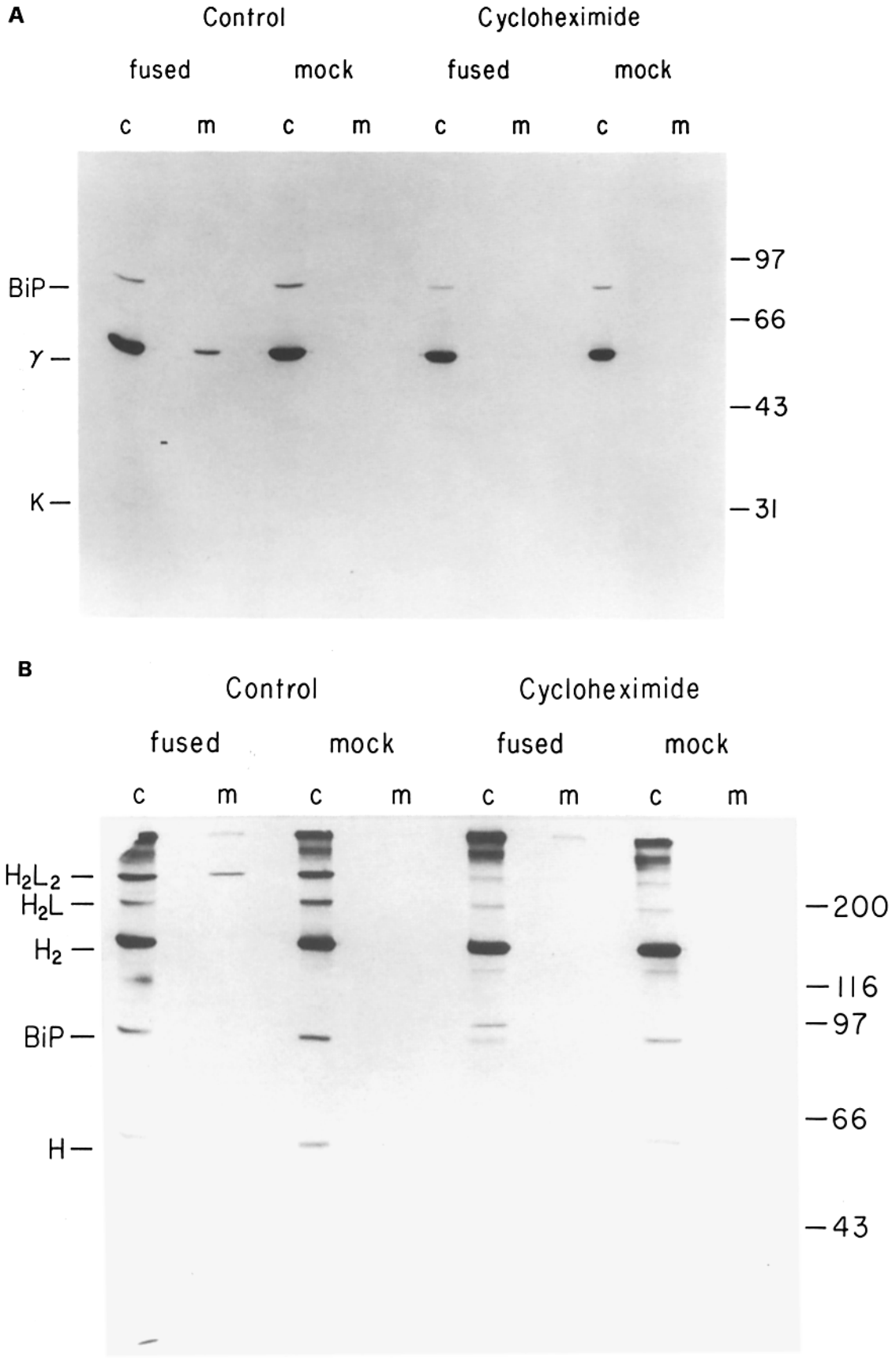


Figure 8. Requirement of new protein synthesis for assembly and transport of prelabeled heavy chains. Ag8(8) cells (γ^+, κ^-) were prelabeled with [^{35}S]methionine, washed, and plated with P3U.1 cells (γ^-, κ^+) as in Fig. 3. 5 min before fusion, 100 μM cycloheximide was added to two of the cultures. One control and one cycloheximide-treated culture was fused. The cultures were washed and reincubated for 3 h in complete medium. Cycloheximide-treated cultures were maintained in cycloheximide. Ig was isolated and analyzed as in Fig. 2 under reducing (*A*) or nonreducing (*B*) conditions.

to be due to their association with BiP (Bole et al., 1986; Gething et al., 1986; Dorner et al., 1987). Attempts to demonstrate the association of normal nascent proteins with BiP have shown that only small amounts of the intracellular pool of these proteins appear to be bound to BiP. It has been difficult to clearly demonstrate that these proteins represent intermediates in the normal folding and assembly pathway and are ultimately secreted. This has led some to suggest that the small pool of "normal" proteins transiently associated with BiP may actually represent malformed polypeptides which are not secreted but ultimately degraded (Lodish, 1988). As such, the function of BiP would be to search out defective proteins and target them for degradation. The results presented here show that combination of BiP with Ig heavy chains is reversible in vivo. This is the first definitive evidence that a protein which is bound to BiP can be released and transported from the cell, thus demonstrating that normal "transportable" proteins can be initially associated with BiP.

While it is very clear that prelabeled BiP-bound heavy chains can combine with light chains, dissociate from BiP, and be secreted, it was difficult to demonstrate a decrease in the intracellular pool of γ heavy chains bound to BiP. Given the fact that only 3–5% of the heavy chain containing cells fuse with light chain–producing cells and that the labeled heavy chains must complete with newly synthesized heavy chains for light chains, this is not surprising. Perhaps under these circumstances it is more surprising that any assembled Ig molecules are detected and suggests that the efficiency of BiP dissociation from heavy chains and the concordant assembly of the released heavy chains must be very high.

BiP has been identified as a member of the highly conserved heat shock family of proteins (Munro and Pelham, 1986; Hendershot et al., 1988; Haas and Meo, 1988). Therefore, the finding that BiP can release proteins in a transport-competent state has bearing on the function of other heat shock proteins. They have been hypothesized to repair or disaggregate proteins damaged by heat (Pelham, 1986; Linquist, 1986) or to aid in translocation, folding and assembly of nascent proteins in other organelles under physiological conditions (Bulleid and Freedman, 1988; Deshaies et al., 1988; Chirico et al., 1988; Cheng et al., 1989). Clearly, the binding of heat shock proteins must be reversible if they function in this capacity. Like other heat shock proteins, a consensus ATP-binding site has been identified on BiP (Hendershot et al., 1988) and ATPase activity has been demonstrated (Kassenbrock and Kelly, 1989). This information, coupled with the observation that ATP mediates BiP release from heavy chains and peptides in vitro (Munro and Pelham, 1986; Flynn et al., 1989), suggest that BiP might bind to nascent proteins, and through utilization of ATP, promote their folding and assembly.

While the data presented here show that an association of proteins with BiP does not necessarily result in their intracellular degradation, BiP has primarily been detected in association with aberrant proteins. This may reflect the inability of BiP to aid in their folding or assembly and to dissociate from them. In contrast, BiP binding to normal proteins may usually be very transient and may often escape detection.

In conclusion, the finding that BiP-associated heavy chains can be released from BiP and combine with light chains offers compelling evidence that BiP can function to keep protein subunits on, or return them to, the normal exocytic transport pathway instead of targeting them for degradation. As such, the function of BiP may be to monitor protein folding and assembly in the ER, perhaps aiding in these processes, and block protein transport until a correct conformation is achieved.

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References

Baron, M. H., and T. Maniatis. 1986. Rapid reprogramming of globin gene expression in transient heterokaryons. *Cell.* 46:591–602.

Blau, H. M., C. P. Chiu, and C. Webster. 1983. Cytoplasmic activation of human nuclear genes in stable heterokaryons. *Cell.* 32:1171–1180.

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

Bulleid, N. J., and R. B. Freedman. 1988. Defective co-translational formation of disulphide bonds in proteins disulphide-isomerase-deficient microsomes. *Nature (Lond.).* 335:649–651.

Cheng, M. Y., F. U. Hartl, J. Martin, R. A. Pollock, F. Kalousek, W. Neupert, E. M. Hallberg, R. L. Hallberg, and A. L. Horwich. 1989. Mitochondrial heat-shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria. *Nature (Lond.).* 337:620–625.

Chirico, W. J., M. G. Waters, and G. Blobel. 1988. 70 K heat shock related proteins stimulate protein translocation into microsomes. *Nature (Lond.).* 322:805–810.

Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig, and R. Schekman. 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature (Lond.).* 332: 800–805.

Dorner, A. J., D. G. Bole, and R. J. Kaufman. 1987. The relationship of N-linked glycosylation and heavy chain-binding protein association with the secretion of glycoproteins. *J. Cell Biol.* 105:2665–2674.

Flynn, G. C., T. G. Chappell, and J. E. Rothman. 1989. Peptide binding and release by proteins implicated as catalysts of protein assembly. *Science (Wash. DC).* 245:385–390.

Gething, M.-J., K. McCammon, J. Sambrook. 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: The role of folding in intracellular transport. *Cell.* 46:939–950.

Haas, I., and T. Meo. 1988. cDNA cloning of the immunoglobulin heavy chain binding protein. *Proc. Natl. Acad. Sci. USA.* 85:2250–2254.

Haas, I. G., and M. Wabl. 1983. Immunoglobulin heavy chain binding protein. *Nature (Lond.).* 306:387–389.

Hendershot, L. M., J. Ting, and A. S. Lee. 1988. Identity of the immunoglobulin heavy-chain-binding protein with the 78,000-dalton glucose regulated protein and the role of posttranslational modifications in its binding function. *Mol. Cell Biol.* 8:4250–4256.

Horibata, K., and A. W. Harris. 1970. Mouse myelomas and lymphomas in culture. *Exp. Cell Res.* 60:61–70.

Kassenbrock, C. K., and R. B. Kelly. 1989. Interaction of heavy chain binding protein (BiP/GRP78) with adenosine nucleotides, *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1461–1467.

Kassenbrock, C. K., P. D. Garcia, P. Walter, and R. B. Kelly. 1988. Heavy-chain binding protein recognizes aberrant polypeptides translated *in vitro*. *Nature (Lond.).* 333:90–93.

Kearney, J. F., A. Radbruch, B. Liesegang, and A. Rajewsky. 1979. A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. *J. Immunol.* 23:1548–1550.

Linquist, S. 1986. The heat-shock response. *Annu. Rev. Biochem.* 55:1151–1191.

Lodish, H. F. 1988. Transport of secretory and membrane glycoproteins from the rough endoplasmic reticulum to the Golgi. *J. Cell Biol.* 263:2107–2110.

Munro, S., and H. R. B. Pelham. 1986. An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell.* 46:291–300.

Pelham, H. R. B. 1986. Speculations on the functions of the major heat shock and glucose-regulated proteins. *Cell.* 46:959–961.

Pfeffer, S. R., and J. E. Rothman. 1987. Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Annu. Rev. Biochem.* 56:829–852.

Yelton, D. E., B. A. Diamond, S. P. Kwan, and M. D. Scharff. 1978. Fusion of mouse myeloma and spleen cells. *Curr. Top. Microbiol. Immunol.* 81:1–7.