

Assembly and Secretion of Heavy Chains that Do Not Associate Posttranslationally with Immunoglobulin Heavy Chain-binding Protein

Linda Hendershot,* David Bole,‡ Georges Köhler,§ and John F. Kearney*

*Cellular Immunobiology Unit, Department of Microbiology and The Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, Alabama 35294; ‡Department of Cell Biology, Yale University, New Haven, Connecticut 06510; and §Max Planck Institute for Immunology, Freiburg, Federal Republic of Germany

Abstract. Heavy chain-binding protein (BiP) associates posttranslationally with nascent Ig heavy chains in the endoplasmic reticulum (ER) and remains associated with these heavy chains until they assemble with light chains. The heavy chain-BiP complex can be precipitated by antibody reagents against either component. To identify sites on heavy chain molecules that are important for association with BiP, we have examined 30 mouse myelomas and hybridomas that synthesize Ig heavy chains with well characterized deletions. Mutant Ig heavy chains that lack the C_H1 domain could not be demonstrated to associate with BiP, whereas mutant Ig heavy chains with deletions of the C_H2 or C_H3 domain were still able to associate with BiP. In two light chain negative cell lines that produced heavy chains with deletions of the C_H1 domain,

free heavy chains were secreted. When Ig assembly and secretion were examined in mutants that did not associate with BiP, and were compared with normal parental lines, it was found that the rate of Ig secretion was increased in the mutant lines and that the Ig molecules were secreted in various stages of assembly. In one mutant line (C_H1⁻) approximately one-third of the secreted Ig molecules were incompletely assembled, whereas the Ig molecules secreted by the parental line were completely assembled. Our data show the C_H1 domain to be important for association with BiP and that when this association does not occur, incompletely assembled heavy chains can be secreted. This implies a role for BiP in preventing the transport of unassembled Ig molecules from the ER.

IMMUNOGLOBULIN biosynthesis, assembly, and transport have been well characterized using both normal and malignant lymphoid cells (7, 27). The Ig molecule is composed of two identical heavy chain proteins and two identical light chain (LC)¹ proteins which are joined by interchain disulfide bonds. Nascent heavy and light chains contain a hydrophobic amino-terminal signal sequence which allows them to be cotranslationally translocated into the lumen of the endoplasmic reticulum (ER) (5). The addition of mannose core sugars to heavy chains occurs during this translocation (4). Very soon after translocation, heavy and light chains begin to assemble in the ER and interchain disulfide bonds are formed (24, 27). The sequence of subunit assembly appears to be determined by the heavy chain isotype. IgM and some IgG_{2b} assemble as heavy and light chain (HL) molecules and then H₂L₂ molecules, whereas IgG₁ and IgG_{2a} first form H₂ molecules, then H₂L, and finally intact H₂L₂ molecules (3). Once assembly is complete, the Ig molecule is transported to the Golgi complex for further processing of

its carbohydrate side chains (14). The mechanism for directing the Ig molecule to Golgi is not well understood. Data gathered from studying the transport of other proteins to the Golgi complex suggest the existence of inherent transport signals on protein molecules which direct their transport to the Golgi complex (10, 20). It is not clear if transport sequences are present on the heavy chain, the light chain, or both. After processing in the Golgi complex, the Ig molecule is packaged into vesicles that are targeted to the plasma membrane for secretion.

Usually, only completely assembled Ig molecules are secreted. When assembly can not occur due to the absence of light chain synthesis or is inhibited due to the underglycosylation of heavy chains in the case of IgM, the free heavy chains are not transported to the Golgi complex and are degraded internally (13, 17). A notable exception to this occurs in lymphoproliferative heavy chain disease (HCD). This disorder is characterized by the production and secretion of heavy chains in the absence of LC production (11). The secreted heavy chains are abnormal in that they contain large protein deletions that usually involve the first constant region domain (C_H1) and occasionally the variable region

1. *Abbreviations used in this paper:* BiP, heavy chain-binding protein; ER, endoplasmic reticulum; LC, light chain; HCD, lymphoproliferative heavy chain disease; HL, heavy and light chain.

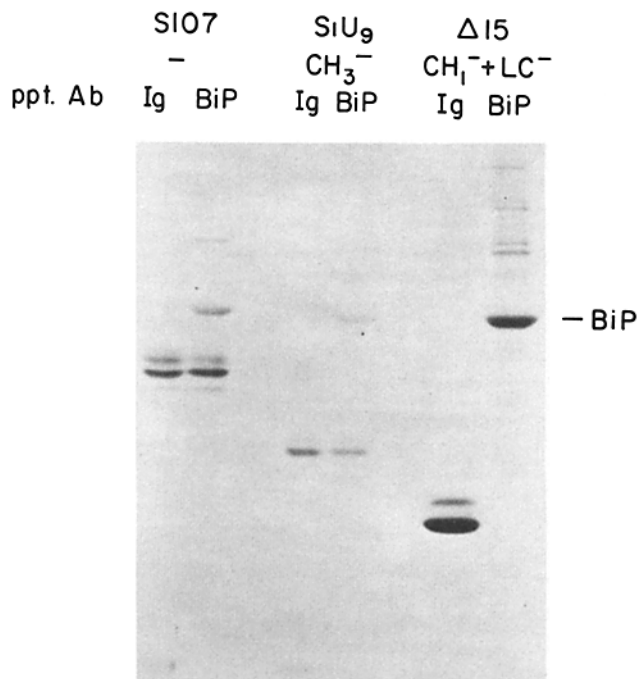


Figure 2. S107, S₁U₉, and Δ15 cells were pulse labeled for 10 min with [³⁵S]methionine. Cell lysates were divided and one half was precipitated with anti-IgA and the other half with anti-BiP. The precipitated proteins were analyzed on 10% SDS PAGE under reducing conditions.

to the fact that Ig in this cell line also assembles along the H → HL → H₂L₂ pathway, thus leaving fewer free heavy chains to associate with BiP. It is also possible that this line has a larger unlabeled intracellular pool of BiP which is associating with the γ_{2b} heavy chains. In the anti-IgG_{2b} precipitated material from both ArM16 and ArM1, a faint band

is also observed which migrates slightly slower than BiP in the case of ArM1 and slightly faster than BiP in the case of ArM16. These do not appear to be alternate forms of BiP as they are not precipitated with anti-BiP. It is possible that they represent some incompletely reduced IgG_{2b}. When each of the lysates were immunoprecipitated with anti-BiP, a band migrating at 78 kD was obtained. In both of the parent lines (Ar13.4 and 45.6), normal γ heavy chains coprecipitated with BiP (Fig. 1). Examination of the mutant sublines showed that the heavy chains missing the C_{H2} and C_{H3} domains (ArM16 and ArM1) were also coprecipitated with BiP. However, heavy chains lacking the C_{H1} domain from the 10.1 subline were not coprecipitated with BiP. Therefore, deletions of the C_{H2} or C_{H3} domain did not appear to affect significantly the association of γ_{2b} heavy chains with BiP whereas the C_{H1} domain seemed to be necessary for this association.

Association of Mutant IgA Heavy Chains with BiP

Three IgA-producing lines were examined for the ability of their heavy chains to associate with BiP. S107 cells produce IgA immunoglobulin with specificity for phosphorylcholine (30). S₁U₉ is a subline of S107 and produces α heavy chains which lack the C_{H3} domain. The Δ15 line, which produces α heavy chains missing the C_{H1} domain, is a subline of W3129 which recognizes α1 → 6 dextran (8). The cells were pulse labeled and immunoprecipitated with anti-IgA or anti-BiP antibodies. The α heavy chains were precipitated from each of the cell lines with anti-IgA (Fig. 2). When lysates were immunoprecipitated with anti-BiP, BiP could be precipitated from each of the cell lines. In the case of S107 and S₁U₉ (C_{H3}⁻) cells, α heavy chains were coprecipitated with BiP. However, no α heavy chains from the Δ15 cells (C_{H1}⁻) could be demonstrated to coprecipitate with BiP (Fig. 2). Therefore, as in the γ_{2b} heavy chains, the C_{H1} domain appeared to be necessary for the association of α heavy

Table I. Summary of Mutant Cell Lines and Their Reactivity with BiP

Cell lines	Isotype	Parent line	Deletion	Assembly	Reactivity with BiP
Ar13.5 (29)	IgG _{2b}	-	-	H ₂ L ₂	+
ArM16 (29)	IgG _{2b}	Ar13.5	CH ₂	H ₂ L ₂	+
ArM1 (29)	IgG _{2b}	Ar13.5	CH ₃	HL	+
45.6 (21)	IgG _{2b}	MPC11	-	H ₂ L ₂	+
10.1 (21)	IgG _{2b}	45.6	CH ₁	H ₂ L ₂ (noncov L)	-
G403 (21)	IgG _{2b}	10.1	CH ₁ + LC ⁻	H	-
S107 (30)	IgA	-	-	H ₂ L ₂ (noncov L)	+
S ₁ U ₉ (30)	IgA	S107	CH ₃	HL	+
W3129 (8)	IgA	-	-	H ₂ L ₂ (noncov L)	+
R15 (8)	IgA	W3129	LC ⁻	H ₂	+
Δ15 (8)	IgA	R15	CH ₁ + LC ⁻	H	-
RP3	IgG ₁	3665	-	H ₂ L ₂	+
RP4	IgG ₁	RP3	CH ₂	H ₂ L ₂	+
24F3	IgG ₁	3665	CH ₃	H ₂ L ₂	+
26C2	IgG ₁	3665	CH ₂	H ₂ L ₂	+
th3	IgG ₁	3665	CH ₁	H ₂ L ₂ (noncov L)	-
Ag8(8) (6)	IgG ₁	P3X63Ag8	LC ⁻	H ₂	+
K25.1 (22)	IgG _{2b}	-	-	H ₂ L ₂ (noncov L)	-
PC700 (2)	IgM	-	-	(H ₂ L ₂) ₅	+
208 (2)	IgM	PC 700	CH _{1,3}	HL	-
562 (2)	IgM	PC 700	Carboxyterm	H ₂ L ₂	+
574 (31)	IgM	PC 700	LC	H ₂	+
482 (18)	IgM	Sp6	CH _{3,4}	H ₂ L ₂	+
662 (18)	IgM	Sp6	CH _{3,4}	H ₂ L ₂	+

chains with BiP. Deletion of the C_{H3} domain of the α heavy chain did not appear to affect this association.

Summary of Mutant Cell Lines and Their Reactivity with BiP

Altogether 30 mouse myeloma and hybridoma cell lines, including 6 cell lines producing IgG_{2b}, 4 producing IgA, 1 producing IgG_{2a}, 10 producing IgG₁, and 9 synthesizing IgM, were examined for association of heavy chains with BiP (Table I). The Ig subclass, degree of Ig assembly, and the extent of the heavy chain deletion for each line has been characterized previously and are summarized in Table I. For most of the isotypes, lines were obtained that produced heavy chains with deletions of each of the C_H domains. Six cell lines were found that produced heavy chains that did not associate with BiP as judged by the inability of anti-Ig to coprecipitate BiP or anti-BiP to coprecipitate heavy chains. These included two cell lines that synthesized γ_{2b} heavy chains, one cell line making γ_1 heavy chains, one producing γ_{2a} heavy chains, one synthesizing α heavy chains, and one producing μ heavy chains. In each case these heavy chains lacked the C_{H1} domain. The deletion of other C_H domains did not appear to affect the posttranslational association of BiP with heavy chains.

Association of BiP with Heavy Chains in LC⁻ Lines

Four cell lines that produce heavy chains but do not synthesize LC (H⁺, LC⁻) were examined for posttranslational association of heavy chains with BiP and heavy chain secretion. Ag8(8) (γ_1^+ , LC⁻) and 574 (μ^+ , LC⁻) cells produce complete heavy chains that are not secreted (6, 28a). The $\Delta 15$ (α^+ , LC⁻) and G403 (γ_{2b}^+ , LC⁻) cells produce heavy chains that lack the C_{H1} domain and are secreted in the absence of LC synthesis (8, 21). The cell lines were labeled for 90 min and after harvesting, both cell lysates and culture supernatants were analyzed. Cell lysates were immunoprecipitated with either anti-heavy chain isotype specific or anti-BiP an-

tibodies, and the culture supernatants were precipitated with anti-isotype specific antibodies. Anti-heavy chain precipitation of Ag8(8) and 574 cell lysates resulted in the precipitation of γ_1 and μ heavy chains, respectively, as well as coprecipitation of BiP (Fig. 3). Anti-BiP precipitation of these cell lysates demonstrated two bands: one representing BiP and the other representing γ_1 heavy chains in the case of Ag8(8) cells and μ in the case of 574 cells. The heavy chains were not secreted from these cells as determined by our inability to precipitate them from the culture supernatants (Fig. 3). When $\Delta 15$ and G403 cells were analyzed in the same manner, the anti-heavy chain antibodies were shown to precipitate only α heavy chains ($\Delta 15$) or γ_{2b} -heavy chains (G403). We were unable to detect any coprecipitating BiP in either of these lines. Likewise, anti-BiP precipitated only BiP from $\Delta 15$ and G403 cells with no coprecipitating bands representing α or γ_{2b} heavy chains. When culture supernatants from these two lines were examined for the secretion of heavy chains, C_{H1}-deleted α chains, in the case of $\Delta 15$ cells, and C_{H1}-deleted γ_{2b} chains, in the case of G403 cells, could be found (Fig. 3). These experiments suggest that the secretion of free heavy chains occurs when the heavy chains fail to associate with BiP.

Assembly of Secreted Ig Molecules in C_{H1}⁻ Mutant

Secreted Ig from a cell line producing γ heavy chains with a C_{H1} domain deletion was compared with normal secreted IgG to determine if the inability of heavy chains to associate with BiP affected their assembly and secretion. Four cell lines that produce normal γ heavy chains as well as 10.1 cells, which produce γ heavy chains with a C_{H1} domain deletion, were labeled for 2 h. The culture supernatant was immunoprecipitated with anti-Ig and analyzed under nonreducing conditions by SDS PAGE. The four cell lines that synthesize normal γ heavy chains secreted them as completely polymerized H₂L₂ molecules (Fig. 4 a, Ig). When secreted Ig from the 10.1 cell line was examined in the same manner, three major bands were precipitated with anti-Ig (Fig. 4 a, Ig). They represent H₂, H, and LC molecules. This agrees with previously reported data on this cell line (21).

When the C_{H1} domain is missing, LC are attached to the C_{H2} or C_{H3} domain of the heavy chain by noncovalent bonds (21). After SDS denaturation of these noncovalent bonds, it was impossible to tell if the H₂ band was derived from H₂L₂, H₂L, H₂ molecules, or all three and whether the H band came from H or HL molecules (Fig. 4 b). To clarify this, the labeled culture supernatant from 10.1 cells was first immuno-absorbed with monoclonal anti- κ conjugated sepharose beads to precipitate all LC-containing molecules. The remaining Ig molecules (H and H₂) were then precipitated with anti-Ig. When the precipitated material was examined by SDS PAGE, we found that the anti- κ antibody precipitated all of the LC band, all of the H band (HL), and about half of the H₂ band (H₂L₂, H₂L) (Fig. 4 a, k'). The remaining H₂ molecules were precipitated with anti-Ig (Fig. 4 a, Ig'). Therefore, the three bands shown in the secreted material from 10.1 cells represent H₂L₂, H₂L, H₂, HL, and LC molecules. When th3 (γ_1 , C_{H1}⁻) and K25 cells (γ_{2a} , C_{H1}⁻) were examined in the same way, they were also found to secrete unassembled Ig molecules (data not shown).

This demonstrates that in the absence of posttranslational association with BiP, heavy chains and all of the assembly

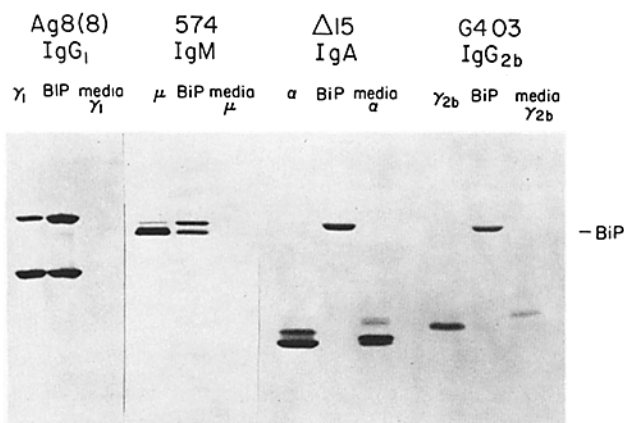


Figure 3. Ag8(8) (γ_1), 574 (μ), $\Delta 15$ (α), and G403 (γ_{2b}) cells were labeled for 90 min with 25 μ Ci of [³⁵S]methionine. Cell lysates were split and immunoprecipitated with either anti-heavy chain specific antibodies or anti-BiP. Culture supernatants were immunoprecipitated with anti-heavy chain-specific antibodies to detect any secreted heavy chains. Precipitated proteins were analyzed on 10% SDS PAGE under reducing conditions.

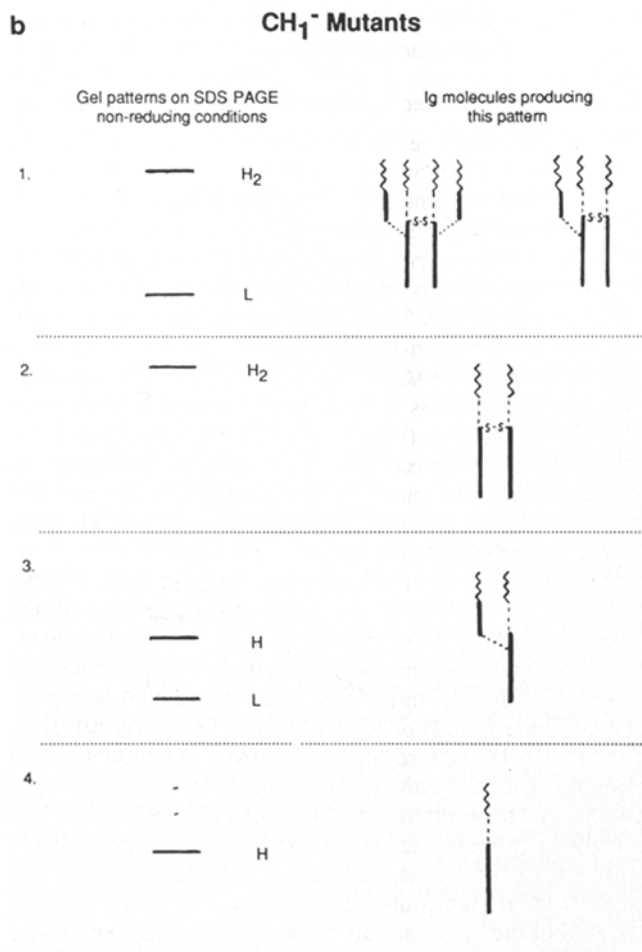
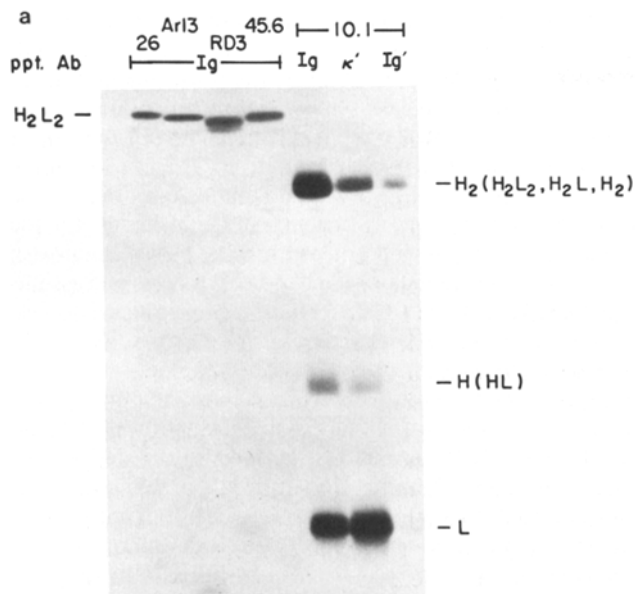


Figure 4. (a) Four cell lines producing normal γ heavy chains (26, Ar13.4, RD3, and 45.6) and one cell line producing γ heavy chains with a C_{H1} domain deletion (10.1) were labeled for 2 h with $[^{35}\text{S}]$ -methionine. Culture supernatants were precipitated with anti-Ig and separated on 8% SDS PAGE under nonreducing conditions (Ig). To determine the identity of the three bands precipitated from the 10.1 cells, the supernatant was reacted first with anti- κ (lane κ') to precipitate LC-containing molecules, followed by precipitation with anti-Ig (lane Ig') to isolate remaining heavy chains. (b) A

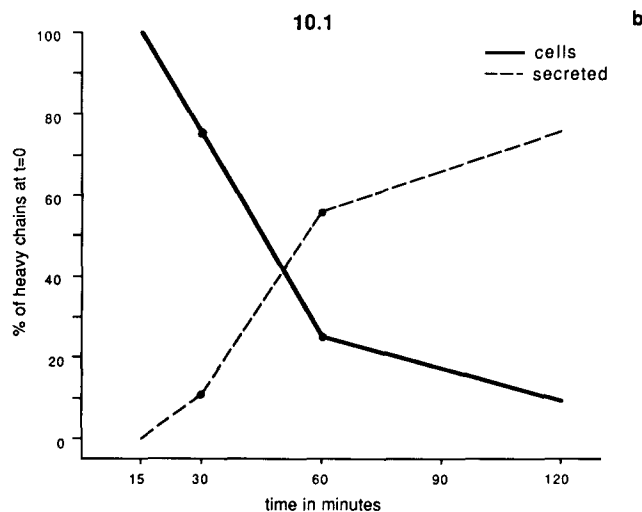
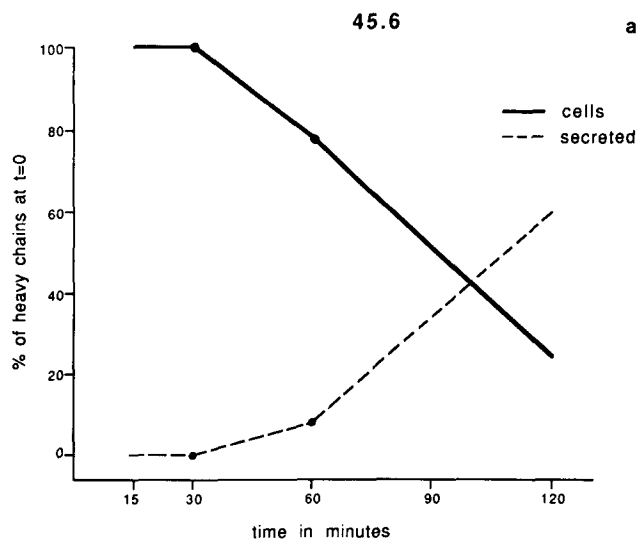


Figure 5. 45.6 cells (a) and 10.1 cells (b) were pulse labeled for 10 min with $[^{35}\text{S}]$ methionine, resuspended in complete RPMI 1640, and chased for 0, 15, 30, 60, and 120 min. Cell lysates and culture supernatants were immunoprecipitated with anti-Ig and analyzed on 10% SDS PAGE under reducing conditions. The γ heavy chains from each time point on the autoradiograph were quantitated by densitometer tracing and are represented as percent of cell-associated heavy chains at $t = 0$.

intermediates of Ig are secreted. This is in direct contrast to heavy chains containing the C_{H1} domain that associate with BiP and are not secreted except as completely assembled molecules

Rate of Secretion of Ig in the Absence of Association with BiP

To determine whether the association of BiP with heavy chains affected the rate of Ig transport, the kinetics of Ig secretion were examined in 10.1 cells (C_{H1}^-) and the parent line 45.6. Cells were pulse labeled with $[^{35}\text{S}]$ methionine for 15 min and chased for 0, 15, 30, 60, and 120 min. Ig was

schematic drawing to show the assembly of Ig molecules when the C_{H1} domain is missing, and the gel patterns observed when this molecule is analyzed on SDS PAGE under nonreducing conditions.

precipitated from both the cell lysates and culture supernatants. Immunoprecipitated proteins were analyzed by SDS PAGE, and cellular and secreted Ig was quantitated by densitometer tracings of the autoradiograph. Densitometer tracings of each time point were calculated as percent of cell-associated heavy chain at $t = 0$ (Fig. 5, *a* and *b*). The $t_{1/2}$ for transport of heavy chains from the 45.6 cells was calculated to be 100 min, whereas the $t_{1/2}$ for transport of heavy chains from 10.1 cells was only 50 min. The C_{H1}^- heavy chains from the 10.1 cells that did not associate with BiP were secreted at twice the rate of their normal counterparts synthesized by 45.6 cells, which do associate with BiP.

Discussion

In this study we have compared the posttranslational association of mutant and wild type heavy chains with BiP. Six cell lines were found which produced heavy chains that did not appear to associate with BiP as judged by the inability of anti-Ig to coprecipitate BiP or anti-BiP to coprecipitate heavy chains. These cell lines produced heavy chains representing five different isotypes. In each case these heavy chains lacked the C_{H1} domain. The deletion of other C_H domains did not appear to prevent the posttranslational association of BiP with heavy chains. This implies that the C_{H1} domain contains or contributes to a site necessary for BiP association. It is of interest that LC are covalently attached to heavy chains at the C_{H1} domain. It is possible that BiP interacts with a portion of the heavy chain that is involved in the association with LC.

When LC^- cell lines were examined, a direct correlation was found between the lack of association of BiP with heavy chains and the ability of these free heavy chains to be secreted. Two LC^- cell lines ($\Delta 15$ and G403) were examined here that produced heavy chains that did not associate with BiP and were secreted. In both cases, the heavy chains contained deletions of the C_{H1} domain. This situation closely parallels that seen in human HCD where free mutant heavy chains are secreted. These mutant heavy chains contain large deletions that usually include the C_{H1} domain (25). We have shown that human lymphoid cell lines also produce BiP which associates with heavy chains in much the same fashion as in mouse cell lines (Hendershot, L., D. Bole, and J. F. Kearney, manuscript submitted for publication). It is, therefore, very tempting to suggest that the inability of HCD proteins to associate with BiP is responsible for the secretion of free heavy chains in this disease.

Biosynthetic studies of pre-B hybridomas and LC^- myelomas have demonstrated that free heavy chains are not normally secreted from lymphoid cells (17, 23). It has been suggested that heavy chains do not contain transport signals necessary for targeting them to the Golgi and that the transport signals are on the LC (28). Only after combination with LC would heavy chains be transported. However, the secretion of mutant heavy chains in HCD and in certain LC^- cell lines suggests that heavy chains do contain transport signals (21, 25). The two LC^- cell lines examined here ($\Delta 15$ and G403), which secrete free heavy chains, produce heavy chains that do not associate with BiP. This strengthens our hypothesis that BiP either obscures the transport signals inherent to heavy chains or prevents the formation of cryptic transport signals (6). Only after BiP is displaced by LC or

when it is unable to bind (in the case of C_{H1} domain mutants) would heavy chain transport signals be exposed, resulting in the transport of heavy chains to the Golgi.

The examination of Ig assembly and secretion in 10.1 cells (C_{H1}^- , LC^+) contributes to our understanding of the role of BiP in preventing heavy chain secretion. The fact that some complete assembly of Ig occurred demonstrates that BiP is not necessary for assembly of heavy and light chains. But the finding that unassembled and assembling heavy chains can be secreted, even in the presence of LC synthesis and assembly, is perhaps the most compelling evidence that BiP acts to prevent the secretion of unassembled heavy chains. In most cases, heavy chains are only secreted as intact H_2L_2 molecules. It is noteworthy that a trace amount of HL molecules are secreted from 45.6 cells. A portion of Ig assembly in this cell line follows the $H \rightarrow HL \rightarrow H_2L_2$ pathway. We have found that the HL molecule in this cell line is not associated with BiP (unpublished data). Previous studies have shown that mutant IgA molecules that cannot form interchain H-H bond are secreted as HL molecules (30). This might be interpreted to suggest that any Ig intermediate not associated with BiP is capable of being secreted. It is also noteworthy that the heavy chains from the 10.1 cell line, which do not associate with BiP, are secreted at a faster rate than their normal counterparts. This suggests that association with BiP might delay heavy chain transport until assembly with LC is complete.

When examining IgM mutants, we found that the absence of the C_{H1} domain did not entirely prevent the association of μ heavy chains with BiP. A very weak association of μ heavy chains with BiP remained in 427 (18) and 128 (2) cells, which are missing the C_{H1} and C_{H2} domains, and 43 (18) cells, which are lacking the C_{H1} domain. This suggests a second domain with the ability to associate weakly with BiP. This association could only be observed after overexposure of gels even in LC^- mutants (unpublished data). It is interesting that only the μ heavy chains appear to have a second (albeit weak) site for BiP association. When the C_{H1} and C_{H2} domain of μ chains were absent, LC were able to form disulfide bonds with the C_{H3} or C_{H4} domains (18). However, when the C_{H1} domain is missing from α or γ heavy chains, the LC can only form noncovalent bonds with the C_{H2} or C_{H3} domain. It is possible that BiP interacts with either the free sulfhydryl involved in H-L bond formation or a hydrophobic region of the C_{H1} domain, which is eventually covered by the constant domain of the LC (9, 16). Both of these must be duplicated in the μ heavy chains since there is a second site for LC attachment. However, the fact that BALB/c α heavy chains (1) and human α_2 heavy chains (15) both have mutations, which change the heavy chain cysteine residue involved in H-L formation but are still able to associate with BiP, suggests that this sulfhydryl is not involved in BiP association.

The data presented here demonstrate that the C_{H1} domain of heavy chains is necessary for their association with BiP. In the absence of association with BiP, free heavy chains and assembling intermediates of Ig can be secreted, whereas heavy chains that associate with BiP are secreted only as completely assembled Ig molecules.

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