

The Relationship of N-linked Glycosylation and Heavy Chain-binding Protein Association with the Secretion of Glycoproteins

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Abstract. The relationship of N-linked glycosylation and association with heavy chain binding protein (BiP) to the secretion of Factor VIII (FVIII), von Willebrand Factor (vWF), and tissue plasminogen activator (tPA) was studied in Chinese hamster ovary (CHO) cells. FVIII has a heavily glycosylated region containing 20 clustered potential N-linked glycosylation sites. A significant proportion of FVIII was detected in a stable complex with BiP and not secreted. Deletion of the heavily glycosylated region resulted in reduced association with BiP and more efficient secretion. Tunicamycin treatment of cells producing this deleted form of FVIII resulted in stable association of unglycosylated FVIII with BiP and inhibition of efficient

secretion. vWF contains 17 potential N-linked glycosylation sites scattered throughout the molecule. vWF was transiently associated with BiP and efficiently secreted demonstrating that CHO cells are competent to secrete a highly glycosylated protein. tPA, which has three utilized N-linked glycosylation sites, exhibited low level association with BiP and was efficiently secreted. Disruption of N-linked glycosylation of tPA by either site-directed mutagenesis or tunicamycin treatment resulted in reduced levels of secretion and increased association with BiP. This effect was enhanced by high levels of tPA expression. The glycosylation state and extent of association with BiP could be correlated with secretion efficiency.

THE information that determines the final destination of a protein is contained in its primary structure. Structural determinants may dictate appropriate post-translational modifications necessary for correct conformation. Many of the steps in the exocytic pathway of processing and transit of membrane-spanning and secretory proteins in mammalian cells have been described (for reviews see Farquhar, 1985; Kornfeld and Kornfeld, 1985). Previous work has shown that proteins destined for the exocytic pathway are first co-translationally translocated into the lumen of the endoplasmic reticulum (ER)¹. This step is mediated by a signal sequence in most cases at or near the amino terminus of the nascent chain (Blobel and Dobberstein 1975; Walter et al., 1984). As the protein enters the ER the signal sequence is usually removed and a high mannose oligosaccharide core unit is transferred to asparagine residues located in the sequence Asn-X-Ser/Thr where X can be any amino acid except proline. The efficiency of this N-linked glycosylation is dependent on the presentation of an appropriate conformation of the peptide chain as it enters the ER since potential N-linked glycosylation sites may no longer be accessible after the protein has folded (Kornfeld and Kornfeld, 1985).

Proteins move from the ER to the Golgi apparatus where modifications such as sulfation, O-linked glycosylation, and

processing of high mannose oligosaccharide chains to complex types occur (Farquhar, 1985). Cleavage of propeptide sequences to the mature forms probably also occurs in Golgi and post-Golgi compartments (Orci et al., 1985). The movement of proteins from the ER to the Golgi apparatus has been identified as the rate limiting step in intracellular transport (Lodish et al., 1983; Fitting and Kabat, 1982). Few of the proteins resident in the ER have been extensively studied for their interaction with secretory proteins transiting that cellular compartment.

A native component of the ER, immunoglobulin heavy chain binding protein (BiP) (Haas and Wable, 1983), has been shown to be involved in the posttranslational processing of immunoglobulin heavy chains (Bole et al., 1986). BiP has been found associated with unassembled immunoglobulin heavy chains before their assembly with light chains. Unassembled heavy chains remain associated with BiP and are not transported to the Golgi apparatus (Bole et al., 1986). BiP can be released from its association with heavy chains *in vitro* by incubation in the presence of ATP (Munro and Pelham, 1986).

Recently, it has been shown that BiP is similar if not identical to GRP78 (Munro and Pelham, 1986). GRP78 was initially reported to be one of several proteins whose synthesis was induced by glucose starvation in chick fibroblasts (Shiu et al., 1977). Synthesis of GRP78 can also be increased by treatment with inhibitors of N-linked glycosylation such as

1. *Abbreviations used in this paper:* BiP, immunoglobulin heavy chain binding protein; ER, endoplasmic reticulum; FVIII, factor VIII; tPA, tissue plasminogen activator; vWF, von Willebrand factor.

tunicamycin (Olden et al., 1979; Pouyssegur et al., 1977). GRP78 has been shown to contain a carboxy-terminal tetrapeptide sequence that is involved in its retention in the ER (Munro and Pelham, 1987). BiP apparently binds glycoproteins to a greater degree when normal N-linked glycosylation or folding is blocked (Bole et al., 1986; Gething et al., 1986). These data together suggest that BiP/GRP78 may be actively involved in regulating the transport of membrane and secretory proteins from the ER to the Golgi apparatus.

We have examined the role of BiP/GRP78 in the processing and secretion of human factor VIII (FVIII), human tissue plasminogen activator (tPA), and human von Willebrand Factor (vWF) in stable Chinese hamster ovary (CHO) cell lines (Fig. 1). FVIII is synthesized as a single chain precursor of 250 kD and processed to a "heavy chain" of about 200 kD and a "light chain" doublet of ~80 kD which are associated by a metal ion bridge (Kaufman, R. J., L. C. Wasley, and A. J. Dorner, manuscript in preparation). FVIII has 25 potential N-linked glycosylation sites. 20 of these sites are located within the middle one-third of the molecule defined as the B domain (Toole et al., 1984). Carbohydrate composition analysis of FVIII secreted from CHO cells has shown that at least 75% of the N-linked sites in the B domain are occupied (Steinbrink, D. R., personal communication). In frame deletion of most of this region to remove 18 potential N-linked glycosylation sites results in higher levels of FVIII secretion with no apparent change in the procoagulant activity as assayed *in vitro* and *in vivo* (Toole et al., 1986). tPA has a molecular mass of 68 kD and contains four potential N-linked glycosylation sites, of which three are used (Pohl et al., 1984). vWF is a large glycoprotein synthesized as a 300-kD precursor that contains 17 potential N-linked glycosylation sites and 235 cysteine residues involved in intra- and intermolecular disulfide bonding (Bonthron et al., 1986). Pre-vWF forms dimers in the endoplasmic reticulum and is subsequently proteolytically processed and modified by the addition of complex N-linked and O-linked carbohydrate groups in Golgi and postGolgi compartments to produce a 220-kD mature form and 100-kD propeptide (Bonthron et al., 1986; Wagner and Marder, 1984). This group of proteins was selected for analysis because they present varying degrees of complexity of their intracellular processing and N-linked glycosylation patterns. In addition, unglycosylated forms could be derived by either tunicamycin treatment or deletion and site-directed mutagenesis.

Our analysis of the secretion of FVIII, tPA, and vWF suggests that the efficiency of secretion can be inversely correlated with the extent of BiP association. We propose that the utilization of N-linked glycosylation sites on a protein may influence the extent and stability of an association with BiP/GRP78 and this can be correlated with the efficiency of secretion. In the absence of normal N-linked glycosylation efficient secretion is impaired. The unglycosylated protein displays increased association with BiP and this complex is stable. We show that a block in efficient secretion is dependent in some cases on expression level.

Materials and Methods

Cell Culture and Cell Lines

All CHO cell lines are derived from the dihydrofolate reductase (DHFR)

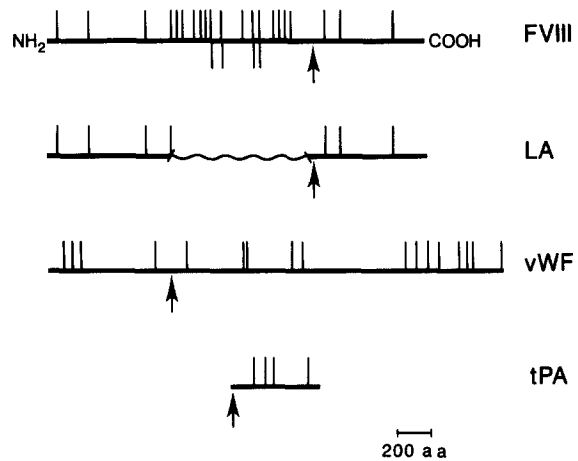


Figure 1. N-linked glycosylation pattern of secretory proteins. Schematic representation shows the positions of potential N-linked glycosylation sites of the type Asn-X-Ser/Thr. Proteins are represented as the single chain precursor form including propeptides as deduced from published nucleotide sequences. Wavy line in LA represents region of wild type FVIII deleted in LA. Arrows indicate sites of proteolytic processing that generate mature forms of the proteins. aa, amino acids.

deficient cell line DUKX-B11 (Urlaub and Chasin, 1980). Wild type tPA-producing CHO cell line, designated Clone 5, was obtained by cotransformation with a DHFR gene and coamplification as described (Kaufman et al., 1985) and produces 10 mU/cell/d tPA activity. vWF-producing CHO cell line PM5F was obtained by DNA cotransformation and coamplification of a vWF expression plasmid containing an adenosine deaminase marker gene as described (Bonthron et al., 1986). PM5F was selected to 0.03 μ M deoxycoformycin and produces 1–2 μ g/ml/d vWF antigen. tPA3x-producing cell lines were developed using vectors and procedures similar to those described for Clone 5. tPA3x-4 was selected for resistance to 0.02 μ M methotrexate and produces 0.006 mU/cell/d tPA activity. tPA3x-13 was selected for resistance to 0.02 μ M methotrexate and produces 1.23 mU/cell/d tPA activity. FVIII-producing CHO cell line (clone 10A1) was selected for resistance to 1 mM methotrexate and produces 1 U/ml/d FVIII activity. LA-producing CHO line (clone LA3-5) was selected for resistance to 1 μ M methotrexate and produces 1–2 U/ml/d. Assays for tPA, vWF, and FVIII have been described previously (Bonthron et al., 1986; Kaufman et al., 1985; Toole et al., 1984).

Radiolabeling and Immunoprecipitation

Approximately 2×10^6 cells per 10-cm dish were labeled in 1 ml of methionine-free medium containing 0.5 mCi [35 S]methionine (New England Nuclear, Cambridge, MA) and 1% aprotinin (Sigma Chemical Co., St. Louis, MO). Chase was done in the cell line's normal growth medium containing excess unlabeled methionine and aprotinin. Tunicamycin (Sigma Chemical Co.), used at a concentration of 10 μ g/ml in the cell line's normal growth medium, was added 1 h before pulse labeling and maintained throughout the pulse/chase. Cell lysates were prepared in 1 \times lysis buffer (0.15 M NaCl, 0.05 M Tris-HCl, pH 7.5, 0.05% SDS, 1% NP-40) on ice. Equivalent volumes of cell extract or conditioned medium were immunoprecipitated at 4°C with either rabbit anti-vWF antibody (Behring Diagnostics, American Hoechst Corp., San Diego, CA), anti-BiP monoclonal antibody culture supernatant (Bole et al., 1986), anti-tPA monoclonal ascites fluid (provided by G. Larsen, Genetics Institute), monoclonal antibody specific for the 80-kD light chain of FVIII (Hybridtech Inc., San Diego, CA), or monoclonal antibody specific for the heavy chain of FVIII (provided by W. Foster, Genetics Institute). Rabbit anti-mouse IgG (CooperBio-medical, Malvern, PA) or staphylococcal protein A immobilized on Sepharose beads was used as the precipitating agent. Precipitated protein was analyzed on 5–12% polyacrylamide gradient gels, or 5 or 6% polyacrylamide gels using the Laemmli buffer system (Laemmli, 1970), followed by treatment with EN 3 HANCE (New England Nuclear), and drying. Band intensity on autoradiograms was quantitated using an ultrascan laser densitometer (Model No. 2202; LKB Instruments, Gaithersburg, MD).

Results

Association of Factor VIII with BiP

The association of FVIII with BiP was measured by comparing the amount of FVIII detected by immunoprecipitation of cell lysates using a monoclonal antibody specific for BiP versus that detected by immunoprecipitation using a monoclonal antibody specific for FVIII. A stable FVIII-producing CHO cell line was labeled for 1 h with [³⁵S]methionine, lysed, and immunoprecipitated as described in Materials and Methods. PAGE revealed a major band migrating with a molecular mass of 250 kD, immunoprecipitated with anti-FVIII antibody that represents the single chain precursor FVIII (Fig. 2, lane 1). Immunoprecipitation with anti-BiP antibody revealed BiP with a molecular mass of 78 kD and a 250-kD band migrating with the same mobility as single chain FVIII (Fig. 2, lane 2). As measured by densitometry, ~85% of the FVIII was detected in a complex with BiP. Only the 250-kD single chain form was found associated with BiP. No processed 80-kD form was coprecipitated by the anti-BiP

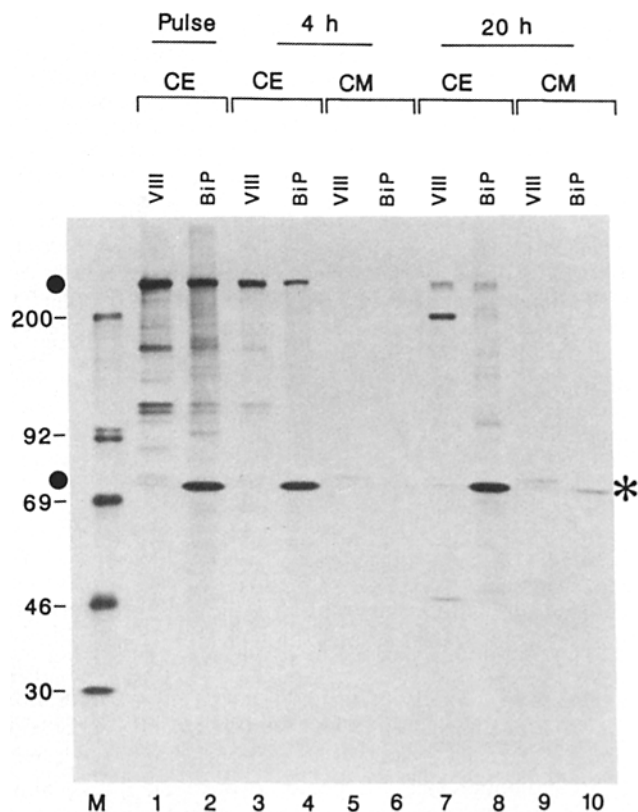


Figure 2. Association of wild type FVIII with BiP. Wild type FVIII-producing CHO cells were pulse labeled for 1 h with [³⁵S]-methionine followed by a 4- and 20-h chase with medium containing excess unlabeled methionine. Cell extracts (CE) and conditioned medium (CM) were prepared and immunoprecipitated with anti-FVIII light chain-specific monoclonal antibody (VIII) or anti-BiP monoclonal antibody (BiP). The FVIII antibody recognizes the 80-kD light chain doublet and 250-kD single chain forms. An autoradiogram of a 6% polyacrylamide gel is shown. Solid circles indicate positions of single chain and light chain FVIII and the asterisk indicates position of BiP.

antibody although the 80-kD doublet can be detected in the cells by immunoprecipitation using anti-FVIII antibody. The other bands present in the immunoprecipitations most probably represent degradation products of FVIII, which is very sensitive to proteolytic activity.

After a 4-h chase mature heavy chain, migrating as a broad smear around 200 kD, and a 80-kD doublet representing light chain were detected by immunoprecipitation of the conditioned medium with anti-FVIII antibody (Fig. 2, lane 5). It is difficult to visualize and quantitate the 200-kD species in the medium because of its heterogeneity and since the precipitating antibody is specific for the light chain, only heavy chain associated with light chain will be detected. Immunoprecipitation of conditioned medium with anti-BiP antibody detected a slight amount of BiP that was not associated with mature FVIII (Fig. 2, lane 6). The amount of intracellular FVIII associated with BiP decreased to <50% of the total intracellular amount after a 4-h chase (Fig. 2, compare lanes 3 and 4). After a 20-h chase the intracellular single chain FVIII has begun to degrade as indicated by the smearing of the 250 kD band. Most of the FVIII was coprecipitated using anti-BiP antibody, indicating that most of the remaining intracellular FVIII was associated with BiP (Fig. 2, compare lanes 7 and 8). Little intracellular 80-kD light chain was detected by immunoprecipitation with anti-FVIII antibody indicating that none remains in the cell (Fig. 2, lane 7). The absence of the 80-kD doublet permits the detection of a band migrating with the mobility of BiP by anti-FVIII antibody immunoprecipitation (Fig. 2, lane 7). The band of roughly 200 kD is an artifact since it is also present in the LA and vWF immunoprecipitations described below and is most likely an endogenous CHO protein. With time the amount of BiP had increased in the conditioned medium but an association with secreted FVIII was not detected (Fig. 2, lanes 9 and 10). A small amount of BiP is either released from damaged cells or secreted at a low rate.

The association of BiP with the deleted form of FVIII called LA was examined in a similar manner. LA has only seven potential N-linked sites compared with 25 on wild type FVIII and is processed and efficiently secreted in a biologically active form by CHO cells (Toole et al., 1986). After a 1-h pulse with [³⁵S]methionine ~60% of intracellular single chain LA was associated with BiP based on densitometry (Fig. 3, compare lanes 1 and 2). Single chain LA migrates as a doublet with a molecular mass of 150 kD and the light chain migrates as a doublet with a molecular mass of 80 kD. As observed with FVIII the single chain form but not the 80-kD light chain was detected in a complex with BiP by coprecipitation using anti-BiP antibody.

After a 4-h chase the association of LA with BiP was decreased significantly compared to the earlier time point (Fig. 3, compare lanes 3 and 4). Approximately 10% of the intracellular single chain LA was associated with BiP based on densitometry. In the conditioned medium, single chain form of 150 kD, processed heavy chain form migrating as a diffuse band around 90 kD, and 80-kD light chain doublet were detected by immunoprecipitation with anti-FVIII antibody (Fig. 3, lane 5). These are the expected secreted forms of LA. Immunoprecipitation with anti-BiP antibody detected some BiP in the conditioned medium but it was not associated with LA (Fig. 3, lane 6). After a 20-h chase only a small amount of LA remained in the cell and the proportion

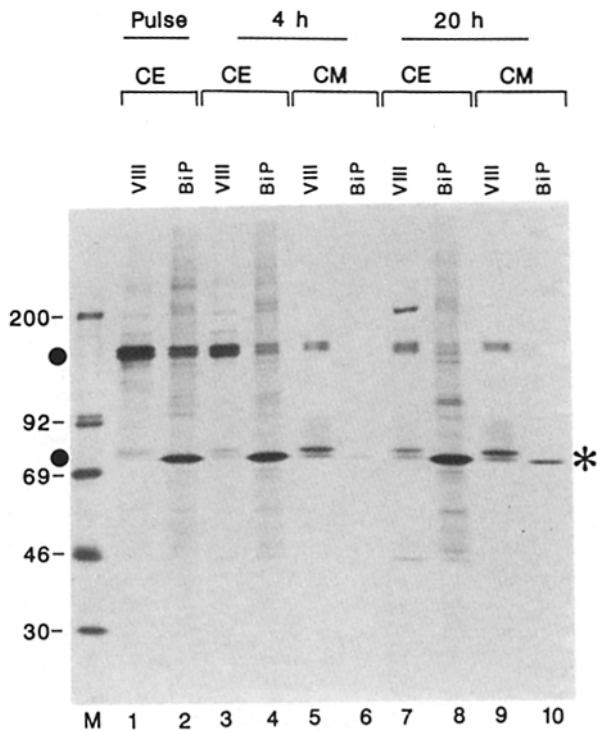


Figure 3. Association of LA with BiP. LA-producing CHO cells were pulse labeled for 1 h with [³⁵S]methionine followed by a 4- and 20-h chase with medium containing excess unlabeled methionine. Cell extracts (CE) and conditioned medium (CM) were prepared and immunoprecipitated with anti-FVIII light chain-specific monoclonal antibody (VIII) or anti-BiP monoclonal antibody (BiP). An autoradiogram of a 6% polyacrylamide gel is shown. Solid circles indicate positions of single chain and light chain LA and the asterisk indicates position of BiP.

of this LA which was associated with BiP was slight (Fig. 3, compare lanes 8 and 7).

Immunoprecipitation with a control antibody precipitated little wild type FVIII or LA indicating that the association detected with BiP was not the result of nonspecific precipitation (data not shown). After precipitation with anti-BiP antibody, complexes of BiP with FVIII or LA were dissociated to a slight degree by incubation in the presence of 1 mM ATP (data not shown). The dissociated protein can be recovered by immunoprecipitation with anti-FVIII antibody. This result proves that the protein associated with BiP is FVIII since the released material was recognized by FVIII-specific antibodies.

These experiments demonstrate that LA exhibits a transient association with BiP in contrast to wild type FVIII. LA is not retained intracellularly for as long a time in a complex with BiP as wild type FVIII. Deletion of the highly glycosylated region to produce LA results in a protein which is associated with BiP to a lesser degree than wild type FVIII. This suggests that the complexity of N-linked glycosylation of FVIII may influence the degree of BiP association.

Effect of Tunicamycin on the Association of LA with BiP

It has been previously reported with influenza hemagglutinin (Gething et al., 1986) and immunoglobulin heavy chain

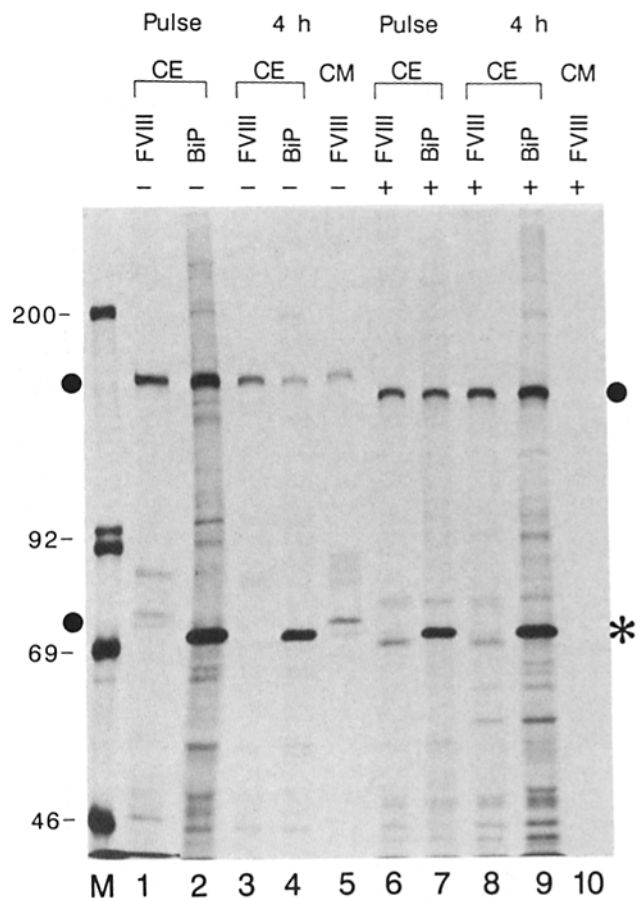


Figure 4. Association of LA with BiP in tunicamycin-treated (+) or untreated (-) cells. LA-producing CHO cells were pretreated for 1 h with 10 µg/ml tunicamycin and pulse labeled for 30 min with [³⁵S]methionine followed by a 4-h chase as described in the text. Cell extracts (CE) and conditioned medium (CM) were prepared and immunoprecipitated with anti-FVIII heavy chain specific monoclonal antibody (VIII) or anti-BiP monoclonal antibody (BiP). Lanes 1-5, untreated cells; lanes 6-10, tunicamycin-treated cells. An autoradiogram of a 6% polyacrylamide gel is shown. Solid circles indicate positions of single chain and light chain LA and the asterisk indicates position of BiP.

(Bole et al., 1986) that disruption of the N-linked glycosylation results in greater association with BiP. To examine this effect with LA, CHO cells producing LA were treated with tunicamycin, which prevents N-linked glycosylation by blocking the formation of the lipid-linked oligosaccharide donor (Takatsuki et al., 1975). After a 30-min pulse with [³⁵S]methionine and 4-h chase, extracts of untreated or tunicamycin-treated cells were prepared and immunoprecipitated with anti-FVIII antibody or anti-BiP antibody. In untreated cells most of the 150-kD single chain LA was associated with BiP after the 30-min pulse (Fig. 4, compare lanes 1 and 2). This association decreased after a 4-h chase (Fig. 4, compare lanes 3 and 4). At this time the secreted forms with molecular mass of 150, 90, and 80 kD were detected by immunoprecipitation of the conditioned medium with anti-FVIII antibody (Fig. 4, lane 5). In the presence of tunicamycin the molecular mass of single chain LA was reduced consistent with the absence of N-linked glycosyla-

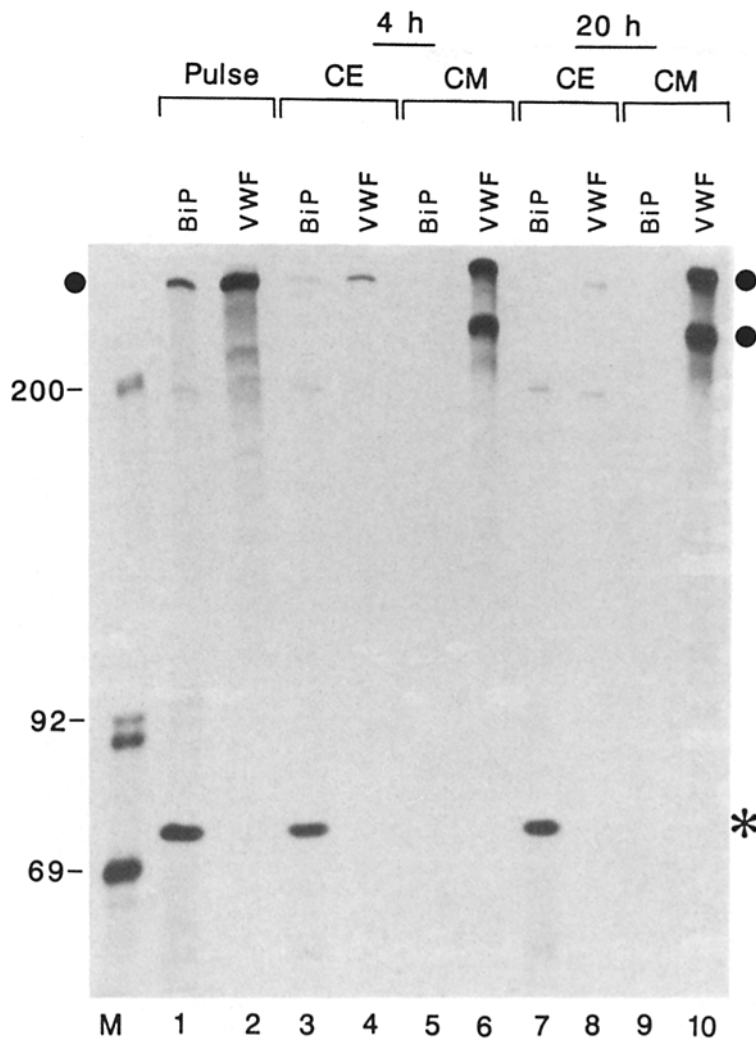


Figure 5. Association of vWF and BiP. vWF-producing CHO cells were pulse labeled for 1 h with [³⁵S]-methionine followed by a 4- and 20-h chase with unlabeled medium. Cell extracts (CE) and conditioned medium (CM) were prepared and immunoprecipitated with anti-vWF antibody (vWF) or anti-BiP monoclonal antibody (BiP) as described in the text. An autoradiogram of a 5% polyacrylamide gel is shown. Solid circles indicate positions of precursor and processed forms of vWF and the asterisk indicates position of BiP.

tion, and all of this unglycosylated protein was associated with BiP after a 30-min pulse labeling (Fig. 4, compare lanes 6 and 7). The proportion of intracellular LA associated with BiP was unchanged after a 4 h chase (Fig. 4, lanes 8 and 9) and little secretion of LA was detected (Fig. 4, lane 10). These observations indicate that disruption of N-linked glycosylation reduced efficient secretion of LA and increased its association with BiP compared with the normally glycosylated molecule. Thus, molecules that were not competent for secretion in the absence of N-linked glycosylation were found in a complex with BiP. By analogy, the incomplete occupancy of the multiple potential glycosylation sites on wild type FVIII might influence the extent and stability of its association with BiP.

Association of vWF with BiP

It was possible that CHO cells are deficient in some aspect of the secretory pathway and therefore cannot properly process a complex glycoprotein. To rule out this possibility, we examined the processing of vWF. The precursor form of vWF has 17 N-linked glycosylation sites distributed along the molecule. After a 1-h pulse labeling a major band migrating with a molecular mass of 260 kD was immunoprecipitated

from a cell lysate by anti-vWF antibody (Fig. 5, lane 2). This band represents the precursor form of vWF. Immunoprecipitation with anti-BiP antibody detected a 78-kD band representing BiP and a band that comigrates with the vWF precursor (Fig. 5, lane 1). Less than 10% of the intracellular vWF was associated with BiP. vWF was efficiently secreted such that after a 4-h chase little of the 260-kD precursor form remained in the cell (Fig. 5, lane 4). Immunoprecipitation of conditioned medium with anti-vWF antibody revealed processed forms of vWF with molecular mass of 275 and 220 kD (Fig. 5, lane 6), while immunoprecipitation with anti-BiP antibody did not precipitate any vWF (Fig. 5, lanes 5 and 7). Little if any of these processed forms were observed intracellularly, which is consistent with previous observations (Bonthron et al., 1986; Wasley, L. C., and R. J. Kaufman, unpublished data). After a 4-h chase only 4% of intracellular vWF was associated with BiP (Fig. 5, compare lanes 3 and 4). Little if any vWF-BiP complex was observed after a 20-h chase (Fig. 5, compare lanes 7 and 8). Immunoprecipitation with a control antibody precipitated little vWF, ruling out nonspecific precipitation (data not shown). Thus, vWF, a large complex glycoprotein, displays a low level of association with BiP and is efficiently secreted from CHO

cells. This is in contrast to the situation with FVIII and indicates that CHO cells are competent to efficiently process and secrete a highly glycosylated glycoprotein.

Association of tPA with BiP

To further analyze the role of glycosylation on protein secretion and BiP association, we examined the processing of glycosylated and unglycosylated forms of tPA in CHO cells. tPA has four potential N-linked glycosylation sites, of which three are used (Pohl et al., 1984). PAGE resolves tPA as a doublet of roughly 68 kD due to variability in the utilization of one of the glycosylation sites. tPA3x is a genetically engineered mutant in which the three normally used N-linked glycosylation sites have been abolished by asparagine to glutamine codon changes in the canonical recognition site sequence (Larsen, G. R., M. Metzger, P. Horgan, Y. Blue, and K. Henson, manuscript in preparation).

Glycosylated wild type tPA (tPAwt) is efficiently processed and secreted at high levels by a CHO cell line designated Clone 5 (Kaufman et al., 1985). After a 30-min pulse with [³⁵S]methionine tPAwt exhibited a slight association with BiP. Less than 5% of the intracellular tPA that was precipitated using anti-tPA antibody (Fig. 6 A, lane 1) was coprecipitated using anti-BiP antibody (Fig. 6 A, lane 2). After a 1- or 3-h chase some tPAwt remained in the cell as detected by immunoprecipitation with anti-tPA antibody (Fig. 6 A, lanes 3 and 5). No tPA was coprecipitated using anti-BiP antibody (Fig. 6 A, lanes 4 and 6). Thus, at high expression levels tPAwt is correctly processed and secreted with low detectable association with BiP. Immunoprecipitation of cell extracts with a control antibody precipitated little tPA compared with that immunoprecipitated with anti-BiP antibody (data not shown). Therefore, the small amount of tPA associated with BiP is not due to nonspecific coprecipitation. When Clone 5 cells were subjected to a pulse labeling of only 2 min the amount of intracellular tPA associated with BiP was ~17% compared with <5% after a 5-min pulse labeling (data not shown). This result suggests that a higher proportion of tPA is associated with BiP at an early stage after synthesis compared with longer pulse-labeling periods.

The processing of tPA3x in a cell line called tPA3x-4 was examined to determine if the absence of N-linked glycosylation would prevent the efficient secretion of tPA. This unglycosylated form of tPA displayed little association with BiP (Fig. 6 B, compare lanes 2 and 3) and was efficiently secreted into the medium (Fig. 6 B, lanes 8 and 9). The time course of its transit through the cell was similar to that observed for tPAwt. Less protein was detected in the cell after a 1- and 3-h chase (Fig. 6 B, lanes 4 and 6), indicating that tPA3x does not experience a block in its secretion due to the absence of N-linked glycosylation. Unglycosylated tPA is apparently in a secretion competent form which displays little association with BiP in this cell line.

To examine if the quantitative level of tPA expression affected the degree of BiP association with tPA, cell lysates from cell lines producing different levels of tPA activity were compared by immunoprecipitation. tPA3x-13 produces 200-fold higher levels of tPA activity than tPA3x-4 as described in Materials and Methods. tPA3x-13 cells were labeled for 30 min with [³⁵S]methionine followed by 1- and 3-h chases. Cell lysates were prepared and immunoprecipitated with

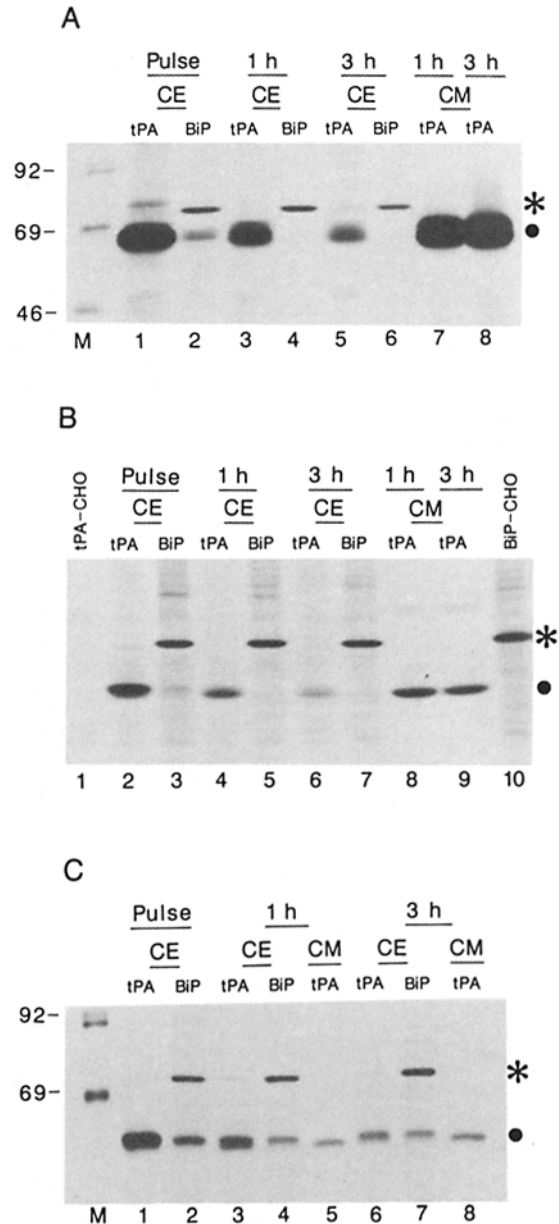


Figure 6. Association of tPA with BiP. (A) Wild type tPA-producing CHO cells were pulse labeled with [³⁵S]methionine for 30 min followed by a 1- and 3-h chase with unlabeled medium. Cell extract (CE) and conditioned medium (CM) were prepared and immunoprecipitated with anti-tPA antibody (tPA) or anti-BiP monoclonal antibody (BiP). A portion of an autoradiogram of a 5–12% gradient polyacrylamide gel is shown. (B) tPA3x-producing CHO cells at low expression level were pulse labeled for 30 min with [³⁵S]methionine followed by a 1- and 3-h chase. Cell extract (CE) and conditioned medium (CM) were prepared and immunoprecipitated with anti-tPA antibody (tPA) or anti-BiP monoclonal antibody (BiP). A portion of a 5–12% gradient polyacrylamide gel is shown. (C) tPA3x-producing CHO cells at high expression level were pulse labeled for 15 min with [³⁵S]methionine followed by a 1- and 3-h chase. Cell extracts (CE) and conditioned medium (CM) were prepared and immunoprecipitated with anti-tPA antibody (tPA) or anti-BiP monoclonal antibody (BiP). A portion of an autoradiogram 10% polyacrylamide gel is shown. In all panels asterisks indicate position of BiP and solid circles indicate position of tPA.

anti-tPA antibody (Fig. 6 C, lanes 1, 3, and 6) or anti-BiP antibody (Fig. 6 C, lanes 2, 4, and 7). In this case tPA3x exhibited an association with BiP (Fig. 6 C, compare lanes 1 and 2) which was greater than that observed for either wild type tPA (Fig. 6 A) or the tPA3x-4 line (Fig. 6 B). Based on densitometry, ~14% of the intracellular tPA3x in the high expression line was associated with BiP after a 30-min pulse and a 1-h chase (Fig. 6 C, compare lanes 1-4). Strikingly, after a 3-h chase ~40% of the tPA3x which remained in the cell was detected in a complex with BiP (Fig. 6 C, compare lanes 6 and 7). Immunoprecipitation with anti-tPA antibody of conditioned medium from these cells revealed secreted tPA3x (Fig. 6 C, lanes 5 and 8). However, a population of the intracellular tPA was not competent for efficient secretion and was detected in a stable complex with BiP. These observations suggest that the efficiency of secretion and the extent of association with BiP of unglycosylated tPA is influenced by expression level.

Effect of Tunicamycin on the Association of tPA with BiP

Another way to examine unglycosylated forms of tPA is to inhibit N-linked glycosylation by tunicamycin treatment as described in Materials and Methods. Immunoprecipitated unglycosylated tPA appears as a doublet (see Fig. 7, lanes 3 and 8, for example). Since tPA is synthesized with an amino-terminal propeptide sequence (Pennica et al., 1983) most probably the higher molecular mass band represents the uncleaved pro-tPA precursor form while the lower band represents the mature form which has been processed to remove the propeptide. Only the pro-tPA precursor form should be present in the same compartment with BiP since propeptide cleavage probably occurs in Golgi or post-Golgi compartments (Orci et al., 1985) and BiP has been localized to the ER (Bole et al., 1986; Dorner, A. J., and R. J. Kaufman, unpublished data). Consistent with this interpretation is the observation that only the higher molecular mass form was found associated with BiP (Fig. 7, lanes 10 and 11) while only the lower molecular mass form was detected in the conditioned medium (Fig. 7, lane 14).

Tunicamycin treatment results in the accumulation of tPA in the ER as monitored by immunofluorescence using anti-tPA antibody (Wasley, L. C., and R. J. Kaufman, unpublished data). Treatment of Clone 5 cells with tunicamycin resulted in increased association of unglycosylated tPA with BiP compared with the glycosylated molecule. After a 15-min pulse label the higher molecular mass form of tPA was coprecipitated with BiP (Fig. 7, compare lanes 3 and 4) to a greater degree than the glycosylated form (Fig. 7, compare lanes 1 and 2). After a 1-h chase most of this precursor form that remains in the cell was detected in a complex with BiP (Fig. 7, compare lanes 5 and 6). Tunicamycin treatment of tPA3x-13 cells did not alter the amount of tPA3x associated with BiP compared with untreated cells. After a 15-min pulse label approximately equivalent percentages of tPA were associated with BiP in the presence or absence of tunicamycin (Fig. 7, compare lanes 8-11). After a 1-h chase ~14% of the intracellular tPA3x remained associated with BiP and this population represents most of the remaining precursor form (Fig. 7, lanes 12 and 13). This profile of protein processing in tunicamycin-treated cells is similar to that observed for

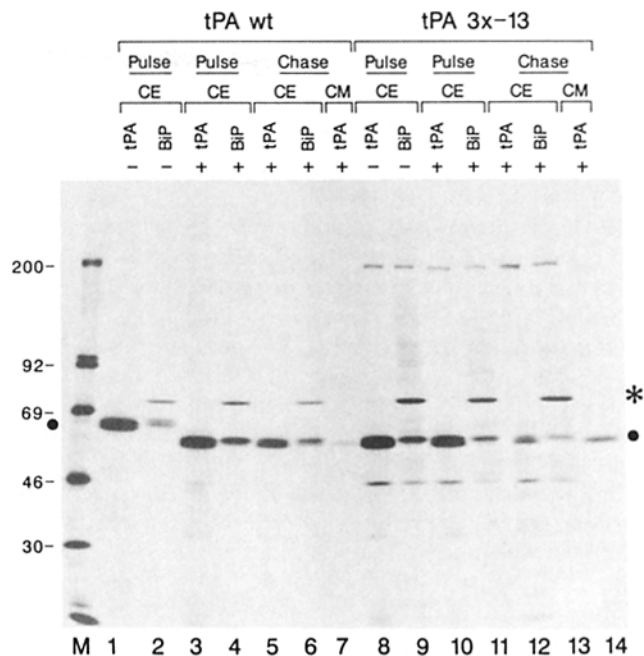


Figure 7. Association of tPA with BiP in tunicamycin-treated (+) cells. Wild type tPA-producing cells (lanes 3-7) or tPA3x-producing CHO cells at high expression level (lanes 10-14) were pretreated for 1 h with 10 μ g/ml tunicamycin and pulse labeled with [³⁵S]methionine for 15 min followed by a 1-h chase. Cell extracts (CE) and conditioned medium (CM) were prepared and immunoprecipitated with anti-tPA antibody (tPA) or anti-BiP monoclonal antibody (BiP). Lanes 1 and 2 are cell extracts of a pulse label of untreated (-) wild type tPA producers. Lanes 8 and 9 are cell extracts of a pulse label of untreated (-) tPA3x high level producers. An autoradiogram of a 10% polyacrylamide gel is shown. Solid circles indicate position of tPA and the asterisk indicates position of BiP.

untreated tPA3x-13 cells (Fig. 6 C). There is an increase of intracellular mature form in tunicamycin-treated cells which may be due to a slower rate of secretion. However, the overall similarity between treated and untreated tPA3x-13 cells indicates that the effect of tunicamycin on the secretion of wild type tPA is due to the absence of N-linked glycosylation on tPA rather than an indirect effect of the drug.

It is striking that treatment of Clone 5 cells with tunicamycin (Fig. 7) produced a pattern of unglycosylated tPAwt processing that is similar to that observed for tPA3x at high expression levels (Fig. 6 C). At lower expression levels, tPA3x showed little association with BiP (Fig. 6 B). We have also observed that in a cell line expressing lower levels of tPA than that obtained in Clone 5 cells, the association of tPAwt with BiP was less affected by tunicamycin treatment (data not shown). Thus, as the result of tunicamycin treatment or mutagenesis, the degree of association of unglycosylated tPA with BiP is influenced by the expression level of tPA.

Discussion

The association of three secreted glycoproteins with BiP, a resident protein of the ER, was studied. The degree of association of a glycoprotein with BiP can be correlated with the efficiency of secretion and the occupancy of N-linked glycosylation sites. FVIII is a highly glycosylated glycopro-

tein in which 20 out of the 25 potential N-linked sites are clustered within the middle one-third of the protein. On the secreted form of FVIII a majority of these sites are occupied. We have observed a high degree of association between FVIII and BiP. Strikingly, we have detected a population of FVIII molecules which are apparently retained inside the cell in a stable complex with BiP. This material may be composed of secretion-incompetent molecules which remain associated with BiP in the ER. We have observed that secretion-competent FVIII appears in the conditioned medium 6 h after a pulse label, after which little more FVIII is secreted (Wasley, L. C., and R. J. Kaufman, unpublished data). This further suggests that FVIII retained in a complex with BiP is not destined for secretion. Our study is the first description of the intracellular processing of FVIII and suggests a role for BiP in the secretion pathway of FVIII.

In-frame deletion of most of the highly glycosylated internal domain of FVIII produces a protein designated LA which retains procoagulant activity and displays higher levels of secretion compared with wild type FVIII (Toole et al., 1986). The degree of BiP association with LA, which has only seven potential N-linked sites, was reduced compared with wild type FVIII. Furthermore, the association of LA with BiP was transient, while the association of wtFVIII with BiP was stable after a 20-h chase. Wild type FVIII that remained in the cell was not released from its association with BiP and apparently failed to exit the ER. Disruption of the N-linked glycosylation of LA by tunicamycin treatment promoted the association of LA with BiP and blocked its efficient secretion. We have also observed that inhibition of N-linked glycosylation of wtFVIII by tunicamycin treatment blocked its secretion (data not shown). We conclude that the occupancy of the N-linked glycosylation sites on FVIII plays a role in its efficient secretion and in the extent and stability of its association with BiP.

Addition of the N-linked oligosaccharide core unit occurs as the protein translocates into the lumen of the endoplasmic reticulum during the process of protein folding (Kornfeld and Kornfeld, 1985). Once the protein has folded, potential glycosylation sites are no longer accessible (Kornfeld and Kornfeld, 1985). Consequently, the 20 N-linked sites clustered in the B domain of wtFVIII may be presented in an appropriate conformation for too short a period of time for efficient glycosylation. Some molecules may, therefore, exhibit incomplete N-linked glycosylation. Since we have observed that inhibition of N-linked glycosylation enhances association with BiP; incomplete glycosylation of FVIII may result in stable association with BiP and retention in the cell. Carbohydrate analysis has indicated that on secreted FVIII most of these sites are occupied suggesting that only heavily glycosylated FVIII is secreted. Deletion of the B domain in LA removed the peptide sequences containing the clustered glycosylation sites and resulted in transient association with BiP. This is consistent with a requirement for proper B domain processing in efficient FVIII secretion. Thus, BiP may be involved in an early processing step for FVIII. An alternative hypothesis is that due to the clustering of N-linked sites the B domain of FVIII presents an unusual conformation that BiP recognizes even if N-linked glycosylation is complete. Detailed analysis of BiP-associated FVIII should provide insight into which hypothesis is correct.

vWF is efficiently processed and secreted from CHO cells. We observed that vWF was only transiently associated with BiP. vWF has 235 cysteine residues involved in intra- and intermolecular bonding suggesting that the complexity of the disulfide bonding does not influence association with BiP. 17 potential N-linked glycosylation sites are spread throughout the molecule rather than clustered. These observations show that CHO cells are competent to efficiently secrete a highly glycosylated glycoprotein and further point to the unusual nature of the clustered sites on FVIII.

Wild type tPA was also transiently associated with BiP. The proportion of the intracellular population detected in a complex with BiP was at least 10-fold less than that observed for FVIII. tPA is apparently processed and transits through the ER with little detectable interaction with BiP. The proportion of tPA associated with BiP was greater early after synthesis as detected after a very short pulse labeling. This result suggests that association of tPA with BiP occurs during or immediately after its synthesis. The low level of tPA associated with BiP may reflect the time of an initial processing event in the ER. However we cannot rule out that tPA associated with BiP is not competent for secretion. Disruption of glycosylation by tunicamycin treatment resulted in increased association of the unglycosylated tPA with BiP and inhibition of efficient secretion.

Of particular interest was the observation that tPA3x, in which the three normally used glycosylation sites have been removed by asparagine to glutamine codon changes, exhibited only a slight association with BiP at low expression levels. Since tPA3x should be structurally similar to unglycosylated tPAwt synthesized in the presence of tunicamycin, it was unexpected that tPA3x was not associated with BiP. However, at expression levels of tPA3x approaching that obtained for tPAwt, we observed an association of tPA3x with BiP that was highly reminiscent of that observed for unglycosylated tPAwt synthesized in the presence of tunicamycin. This result indicates that differences in BiP association are not due to variation between cell lines since similar degrees of association were obtained for unglycosylated tPA obtained by either tunicamycin treatment or site-specific mutagenesis in different cell lines.

We conclude that the retention of unglycosylated tPA in a complex with BiP is dependent on the expression level of tPA. Consistent with this conclusion is our observation that in a cell line producing 25-fold less tPA activity than Clone 5 the effect of tunicamycin treatment is less pronounced than in Clone 5. A possible explanation is that unglycosylated tPA may aggregate when present at high concentration in the ER leading to a block in its secretion and consequent association with BiP. In this case BiP may both transiently interact with nascent tPA and bind aggregated protein.

It has been reported that N-linked glycosylation can be crucial in the attainment of the native conformation of a protein and that improper folding may result in protein aggregation and decreased solubility (Gibson et al., 1979, 1981; Leavitt et al., 1977). Each glycoprotein probably has a different oligosaccharide requirement for proper polypeptide folding and solubility. A previous study on influenza hemagglutinin synthesis has shown that unfolded molecules are specifically associated with BiP and that disruption of N-linked glycosylation with tunicamycin results in increased associa-

tion of unfolded hemagglutinin with BiP and slowed intracellular transport (Gething et al., 1986). Prevention of the glycosylation of immunoglobulin heavy chain delays assembly with light chain and enhances BiP association perhaps by disrupting normal protein folding (Bole et al., 1986). Our results also suggest that the absence of appropriate glycosylation may cause retention of proteins in the ER in a complex with BiP. We postulate that this block in efficient secretion may be due to aggregation or insolubility of improperly folded protein. In this case BiP may function to clear such aggregated protein from the cell (Pelham, 1986).

Coprecipitation of intracellular secretory proteins with BiP was predominantly observed when anti-BiP antibody was used as the precipitating antibody. Little radiolabeled BiP was observed to coprecipitate when antibody against the secretory protein was used. This observation suggests that under these labeling conditions a population of unlabeled BiP exists in the ER which can associate with nascent proteins as they translocate into the ER. Newly synthesized BiP has not entered this functional population and so little radiolabeled BiP is found in complexes. The formation of a complex may occur during or immediately after translocation. We conclude that the factors that determine the extent of BiP association and retention in the ER are active at an early stage of protein processing.

Our results suggest that BiP/GRP78 plays an important role in the secretory pathway. BiP may be involved in an early step of the secretion pathway and so generally interacts with proteins as they move through the ER. The association of BiP with unglycosylated proteins suggests that BiP may monitor the conformation of newly synthesized proteins in the ER. When an N-linked site is not appropriately occupied, an incorrectly folded protein may result and BiP remains associated. Protein insolubility and aggregation could be influenced by the appropriate utilization of N-linked glycosylation sites on a protein and by the concentration of the protein in the ER. However, we cannot completely rule out that the association of nonglycosylated proteins with BiP reflects a common localization and aggregation of these proteins and BiP. When glycosylation and protein folding occur properly, the association with BiP is transient and the secretory pathway to the Golgi apparatus is traversed. If this association is transient and occurs early after synthesis, the detection of complexes that represent secretion-competent protein will require very short pulse-labeling periods. Our observation that a significantly higher percentage of tPA is associated with BiP after a very short pulse labeling (17%) compared with longer time periods (<5%) is consistent with this idea. However, we cannot rule out the possibility that protein associated with BiP is not secretion competent and may be destined for degradation. Since approximately 30 min is required for the secretion of tPA from CHO cells, the association and dissociation of complexes with BiP must occur very soon after synthesis. This suggests that tPA remains in the ER for some time after release from the complex. This is consistent with the report that trimeric forms of hemagglutinin are found in the ER after dissociation of the monomeric forms from a complex with BiP (Gething et al., 1986).

Our experiments show that, in the case of FVIII, deletion of the heavily glycosylated region by removal of protein sequence resulted in reduced association with BiP. When

N-linked glycosylation was prevented by tunicamycin treatment or mutagenesis, association with BiP was increased and secretion decreased. This may be due to the inability of the unglycosylated protein to achieve native conformation or solubility necessary for transit through the ER. Under conditions of appropriate glycosylation, proteins that are efficiently secreted may only transiently associate with BiP. BiP/GRP78 may function to allow only correctly folded protein to continue along the secretory pathway and to target aberrant protein for destruction.

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