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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

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The 78,000-dalton glucose-regulated protein (GRP78) and the immunoglobulin heavy-chain-binding protein (BiP) were shown to be the same protein by NH_2-terminal sequence comparison. Immunoprecipitation of GRP78-BiP induced by glucose starvation and a temperature-sensitive mutation in a hamster fibroblast cell line demonstrated the association of GRP78-BiP with other cellular proteins. In both fibroblasts and lymphoid cells, GRP78-BiP was found to label with $^{32}P_i$ and [^{3}H]adenosine. Phosphoamino acid analysis demonstrated that GRP78-BiP is phosphorylated on serine and threonine residues. Conditions which induce increased production of GRP78-BiP resulted in decreased incorporation of $^{32}P_i$ and [^{3}H]adenosine into GRP78-BiP. Furthermore, we report here that the phosphorylated form of BiP resides in the endoplasmic reticulum and that BiP which is associated with heavy chains is not phosphorylated or labeled with [^{3}H]adenosine, whereas free BiP is. This suggests that posttranslational modifications may be important in regulating the synthesis and binding of BiP.

Immunoglobulin heavy-chain-binding protein (BiP) is a 78,000-dalton endoplasmic reticulum (ER) protein which was originally identified in association with the free nonsecreted heavy chains (HCs) synthesized in Abelson leukemia virus-transformed pre-B (HC^+, light chain $[LC]^-$) cell lines (10). The production of a monoclonal antibody specific for BiP led to the observation that BiP was not only associated stably with the free HCs in pre-B cells but could also be found in association with HCs in terminally differentiated, immunoglobulin (Ig)-secreting plasma cell lines (HC^+, LC^+). However, in the latter case, the association was transient and confined to incompletely assembled Ig precursors (2). It was suggested that BiP acted to prevent free HCs and assembling Ig precursors from leaving the ER, thus ensuring the transport of only completely assembled (H_2L_2) Ig molecules. Support for this hypothesis came with the examination of BiP association with Ig HC mutants. These studies demonstrated that the C_H1 domain of the HC was necessary for its association with BiP and that mutant HCs which lacked the C_H1 domain (and failed to associate with BiP) could be secreted as free and partially assembled molecules (12).

In addition to B lineage cells, BiP was also found to be synthesized by nonlymphoid cells, suggesting a more general role for BiP in protein transport (8, 9). Recent studies have shown that BiP is immunologically related to the glucose-regulated protein, GRP78. The two proteins have similar peptide maps and may in fact be identical (24). The glucose-regulated proteins are so named because their synthesis is enhanced when cells are deprived of glucose. Subsequently, glucose-regulated protein synthesis was shown to be enhanced under a variety of stress conditions (19). Like BiP, GRP78 is a nonglycosylated protein with an apparent molecular weight of 78,000 daltons and is found in the lumen of the ER of most cell types (25, 30). While the function of the glucose-regulated proteins is not presently clear, it is conceivable that during glucose starvation and other stress conditions, proteins which do not fold or assemble correctly due to their underglycosylated state would need to be retained, refolded, or degraded in the cell. The up-regulation of proteins capable of doing this would be necessary. In support of this hypothesis, the transcriptional activity of the GRP78 gene is closely correlated with a block in protein glycosylation (5, 17).

While there are many similarities between BiP and GRP78, the primary sequence of BiP has not been reported, and minor discrepancies between BiP and GRP78 have been observed (9). We demonstrate here that the amino-terminal sequences of BiP and GRP78 are identical. GRP78-BiP was demonstrated to be associated with other cellular proteins in fibroblasts during glucose deprivation and increased temperature in a mutant cell line. We further show that the incorporation of P_i and adenosine into BiP-GRP78 is restricted to its free form, whereas BiP-GRP78 which is bound to Ig HCs in myeloma cells is not modified by phosphate or adenosine.

MATERIALS AND METHODS

Cell lines. Wg1A and K12 hamster fibroblast cells were maintained in Dulbecco modified Eagle medium as described previously (18). Wg1A is the parental cell line of the temperature-sensitive mutant K12. Ag8.653 (HC^-, LC^-) and Ag8(8) (γ_1^+, LC^-) myeloma cells were maintained in RPMI 1640 medium containing 10% fetal calf serum and penicillin-streptomycin and have been described elsewhere (2, 16).

Determination of NH_2-terminal amino acid sequence. The amino-terminal sequence was determined with a gas-phase sequenator (model 470A; Applied Biosystems) by using the Edman degradation method (13). The sample was applied to a glass filter treated with polybrene to improve sample retention. Phenylthiohydantoin (PTH)-amino acids were analyzed using an automated on-line high-pressure liquid chromatograph (model 120A; Applied Biosystems) with a Chromatographics 2 data storage system (The Perkin-Elmer

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Corp., Norwalk, Conn.). PTH derivatives were identified on a C18 column (21 mm by 22 cm) (Brownlee) at 52°C by using 110 mM sodium acetate (pH 3.9) with 5% tetrahydrofuran (buffer A) and acetonitrile (buffer B) with a 19-min linear gradient from 10 to 37%.

Biosynthetic labeling of cell lines with [^{35}S]methionine. K12 cells were seeded on 60-mm tissue culture dishes in Dulbecco modified Eagle medium containing 4.5 mg of glucose per ml. When cells reached 90% confluency, one dish of cells was labeled in methionine-free labeling medium devoid of glucose at 37°C, one was labeled in methionine-free labeling medium supplemented with 2 mg of glucose per ml at 37°C, and one was labeled in methionine-free labeling medium supplemented with 2 mg of glucose per ml at 40.5°C. Each dish was labeled with 70 μCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.) per ml for 20 h as described previously (20). Ag8.653 and Ag8(8) cells were labeled with 50 μCi of [^{35}S]methionine for 3 h in 1 ml of methionine-free labeling medium at 37°C. After being labeled, cell lysates were prepared as described previously (12). Either lysates were directly immunoprecipitated with the anti-BiP monoclonal antibody followed by formalin-fixed *Staphylococcus aureus* or, in the case of Ag8(8) cells, the cell lysate was divided and one half was precipitated with anti-BiP and the other half was precipitated with anti-mouse Ig (Southern Biotechnology Associates, Birmingham, Ala.). Immunoprecipitated proteins were analyzed on sodium dodecyl sulfate (SDS)-polyacrylamide gels. The gels were treated with En^{3}Hance (Dupont, NEN Research Products, Boston, Mass.) and exposed to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.).

Biosynthetic labeling with $^{32}P_i$. K12 cells were seeded as described above. When they reached 90% confluency, one dish was shifted to 40.5°C and the other dish was left at 37°C. After 12 h, the culture medium was removed and cells were labeled for 8 h with 100 μCi of $^{32}P_i$ in 4 ml of phosphate-free labeling medium. Ag8.653 and Ag8(8) cells were labeled for 8 h with 100 μCi $^{32}P_i$ (Amersham) in phosphate-free medium at 37°C. Proteins were immunoprecipitated and analyzed as above.

Phosphoamino acids analysis. ^{32}P-labeled Wg1A cell lysate was prepared as described previously (20). Phosphoproteins were separated by two-dimensional isoelectric focusing SDS-gel electrophoresis. About 100 μg of total protein (2×10^6 cpm) was applied to each gel. The protein spot corresponding to BiP-GRP78 was excised after being stained for 30 min in 50% isopropanol, 7.5% acetic acid, and 0.25% Coomassie blue and immediately destained in 20% methanol and 7.5% acetic acid. The protein-containing gel slice was electroeluted, and BiP-GRP78 was recovered by 20% trichloroacetic acid precipitation in the presence of 150 μg of bovine serum albumin as a carrier. After centrifugation at 13,000 × *g* for 15 min, the protein pellet was suspended in 0.5 ml of 1 N NaOH and reprecipitated with 0.2 ml of 50% trichloroacetic acid. The precipitate was washed once with ethanol-ether (70:30) and once with ether.

To identify phosphoamino acids, protein precipitate isolated from four gels was hydrolyzed in 0.5 ml of 6 N HCl at 110°C for 2 h. After hydrolysis, acid was removed under reduced pressure. The released phosphoamino acids were analyzed on a cellulose thin-layer plate (20 by 20 cm) as described previously (26). Briefly, the sample was mixed with 2 μg each of unlabeled phosphoserine, phosphothreonine, and phosphotyrosine. The mixture was electrophoresed at pH 1.9 (acetic acid-formic acid-H_2O [78:25:897, vol/vol/vol]) for 90 min at 1.2 kV in the first dimension and at pH 3.5 (acetic acid-pyridine-H_2O [50:5:945, vol/vol/vol]) for 60 min at 1.0 kV in the second dimension. Individual phosphoamino acids were identified by staining the standards with ninhydrin. The plate was exposed to X-Omat AR film (Kodak) with an intensifying screen for 7 days.

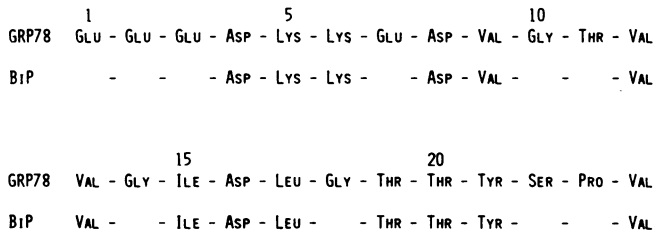

FIG. 1. Comparison of partial NH_2-terminal sequences of GRP78 isolated from K12 hamster fibroblast cells and BiP isolated from Ag8(8) murine lymphoid cells.

In vitro translation. Cellular mRNA was isolated from 5×10^7 Ag8(8) cells by the phenol-chloroform method of Lizardi (22). RNA (1 μg) was translated in a rabbit reticulocyte system (Bethesda Research Laboratories, Gaithersburg, Md.) which was supplemented with [^{35}S]methionine for 90 min. The labeled lysate was diluted with lysing buffer and immunoprecipitated with the anti-BiP antibody.

Subcellular fractionation. Labeled Ag8(8) (2×10^7) cells were allowed to swell on ice for 10 min in 0.25 M sucrose buffered with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4. The cells were then mechanically disrupted in a Dounce homogenizer, and membranes were fractionated on a 10 to 30% continuous sucrose gradient (23). After centrifugation at 36,000 rpm (Beckman ultracentrifuge; SW41 rotor) for 3 h, 16 fractions were collected from the bottom of the tube. The fractions were diluted with lysing buffer, divided in half, and immunoprecipitated with anti-BiP or anti-γ antibodies.

RESULTS

Partial NH_2-terminal sequence of BiP and GRP78. To determine if BiP and GRP78 were indeed the same protein, the NH_2-terminal sequence of murine BiP was determined and compared with the previously published sequence of GRP78 (20, 24). BiP was isolated from the mouse myeloma cell line Ag8(8), which produces γ_1 HCs but no LC, by immunoprecipitation with the anti-BiP monoclonal antibody. The precipitated BiP and γ_1 HCs were separated on SDS-polyacrylamide gels. The portion of the gel containing BiP was eluted from the gel by overnight incubation in 0.01% SDS at room temperature. The eluted material was lyophilized and acetone precipitated. A sample of the recovered protein was analyzed by SDS-polyacrylamide gel electrophoresis for purity and yield. Approximately 50 μg of BiP was obtained with no detectable contamination. The NH_2-terminal sequence was determined by using a gas-phase protein sequenator. Figure 1 shows the NH_2-terminal sequence obtained for murine BiP and the previously reported sequence for hamster GRP78 (20). In every case in which the amino acid sequence could be unambiguously determined for BiP, it matched the reported sequence of GRP78. We were unable to determine Gly and Glu residues due to a high background accompanying these particular residues during liquid chromatography. In almost every case, the undetermined amino acids in BiP correspond to a Glu or Gly residue in GRP78. Thus, BiP and GRP78 have the same amino-terminal sequences.

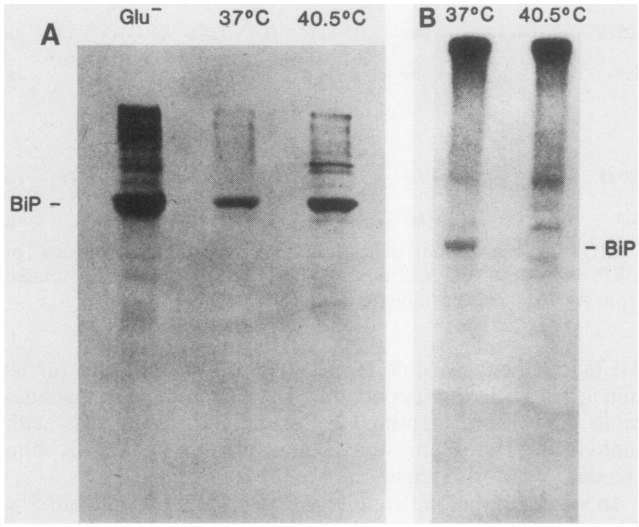

FIG. 2. Induction of BiP by glucose starvation and increased temperature. (A) Three confluent 60-mm plates of K12 cells were labeled with 70 μCi of [^{35}S]methionine for 20 h in methionine-RPMI 1640 medium. One plate was at 37°C without glucose (Glu$^-$), one plate was at 37°C with 4.5 g of glucose per liter (37°C), and one plate was at 40.5°C with 4.5 g of glucose per liter (40.5°C). Labeled lysates from equal numbers of cells were immunoprecipitated with anti-BiP and analyzed by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels under reducing conditions. (B) One 60-mm plate of K12 cells was preincubated at 40.5°C, and one was incubated at 37°C for 12 h and then labeled with 100 μCi of $^{32}P_i$ for 8 h. Cell lysates were prepared, immunoprecipitated with anti-BiP, and analyzed by SDS-polyacrylamide gel electrophoresis on 8% polyacrylamide gels under reducing conditions.

Association of GRP78-BiP with other proteins in fibroblasts. To further substantiate the identity of BiP and GRP78 and to determine whether GRP78-BiP in fibroblasts has protein-binding affinities as demonstrated for BiP in lymphoid cells, the synthesis and binding properties of GRP78-BiP were examined in a temperature-sensitive mutant fibroblast line, K12. When K12 cells are shifted to 40.5°C, GRP78 and two other glucose-regulated proteins are overproduced (18). The defect in the K12 cell lines at the nonpermissive temperature involves a reduced ability to transfer oligosaccharides from the dolichol-linked intermediate to nascent polypeptides, and as a consequence, protein glycosylation is significantly reduced (21). K12 cells were grown to confluency at 37°C, and then three flasks of these cells were labeled with [^{35}S]methionine for 20 h, with one flask at 37°C (the permissive temperature), one flask at 40.5°C (the nonpermissive temperature), and one flask without glucose at 37°C. Cell lysates were prepared and immunoprecipitated with anti-BiP. While easily detectable amounts of BiP were present at 37°C, the synthesis of BiP was greatly enhanced at 40.5°C as well as in the absence of glucose (Fig. 2A). Thus, the induction of BiP is identical to that previously reported for GRP78 in the K12 mutant cell line. Further, a number of coprecipitating proteins could be observed in association with GRP78-BiP in the lysates from cells incubated without glucose or at the nonpermissive temperature (Fig. 2A). Therefore, in stressed fibroblasts in which GRP78 is induced, GRP78-BiP is complexed to other proteins.

When K12 cells were incubated at 37 or 40.5°C for 12 h, labeled with ^{32}P-orthophosphate for 8 h, and immunoprecipitated with anti-BiP, only the BiP-GRP78 synthesized at 37°C was labeled with ^{32}P (Fig. 2B). The GRP78-BiP isolated from the K12 cells and incubated at 40.5°C (a condition which was shown in Fig. 2A to result in the increased production and association of GRP78-BiP with cellular proteins) was not labeled with $^{32}P_i$ (Fig. 2B).

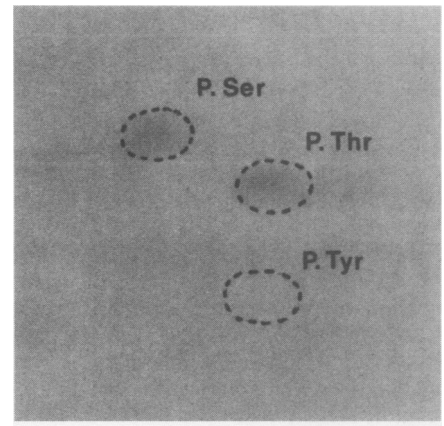

FIG. 3. Identification of phosphoamino acids in GRP78. Wg1A cells were labeled with $^{32}P_i$ at 35°C for 8 h. Isolation and acid hydrolysis of GRP78 and two-dimensional electrophoretic separation of the phosphoamino acids are described in Materials and Methods. Only the central part of the autoradiogram is shown. The origin was in the lower right-hand corner of the plate. The positions of the stained marker phosphoamino acids are indicated by broken lines. P.Ser, Phosphoserine; P.Thr, phosphothreonine; P.Tyr, phosphotyrosine.

Phosphoamino acid analysis of GRP78-BiP. To determine which amino acid(s) is covalently modified by phosphorylation, $^{32}P_i$-labeled GRP78 from Wg1A cells (the nonmutant parental cell line of K12) was purified from two-dimensional acrylamide gels and subjected to phosphoamino acid analysis. As shown in Fig. 3, GRP78 isolated from Wg1A cells contained phosphoserine and phosphothreonine, and the amount of label detected in serine and threonine was approximately equivalent. Examination of the phosphoamino acid composition of GRP78-BiP in K12 cells showed that identical residues (serine and threonine) were labeled (data not shown). To monitor for contamination from the closest ^{32}P-labeled spot (a 90,000-dalton protein [20]), identical phosphoamino acid analysis was carried out for this protein, and a different pattern of phosphorylated amino acid residues was obtained (J. Ting and A. S. Lee, unpublished results).

It should be noted that while the $^{32}P_i$ can be incorporated into various forms of phosphate groups, after acid hydrolysis only phosphate groups directly linked to the amino acid residues through a phospho-monoester bond will comigrate with the unlabeled phosphoamino acid markers and be detected. In the case of modification by ADP ribosylation, the radiolabeled phosphate groups are indirectly linked to amino acid residues different from serine and threonine in the form of ADP through a second ribosyl moiety (29). Such modified amino acids would not be expected to comigrate with the phosphoamino acid markers. Thus, these experiments provide evidence for direct phosphorylation of serine and threonine residues in GRP78-BiP.

Phosphorylation of BiP in lymphoid cells. Because previous studies on lymphoid cells had suggested a function for BiP in associating with free HCs and preventing their transport, and because we were able to directly examine BiP in both the free and bound forms, we wished to examine the phosphorylation profile to BiP in these cells. For these

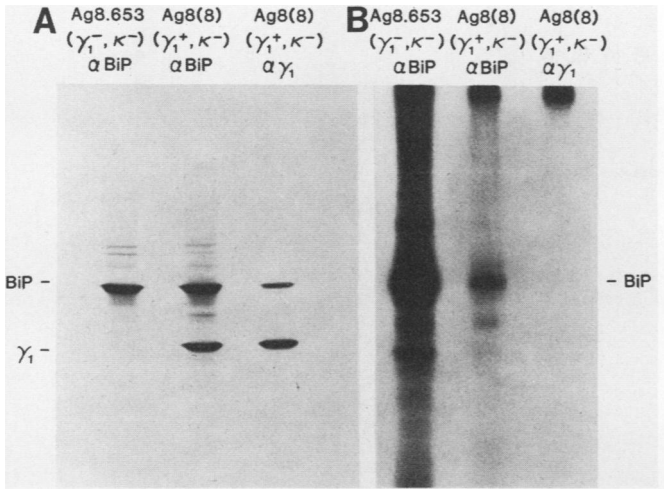

FIG. 4. Incorporation of [^{35}S]methionine and ^{32}P-orthophosphate into BiP synthesized by lymphoid cells. (A) Ag8.653 cells and Ag8(8) cells (5×10^6 of each) were labeled with 50 μCi of [^{35}S]methionine for 3 h, and cell lysates were prepared. All of the Ag8.653 lysate was immunoprecipitated with anti-BiP. The Ag8(8) lysate was split; one half was immunoprecipitated with anti-BiP, and one half was immunoprecipitated with anti-γ antibodies. (B) Ag8.653 cells and Ag8(8) cells (5×10^6 of each) were labeled with 100 μCi of $^{32}P_i$ for 8 h. Cell lysates were prepared and immunoprecipitated as described for panel A.

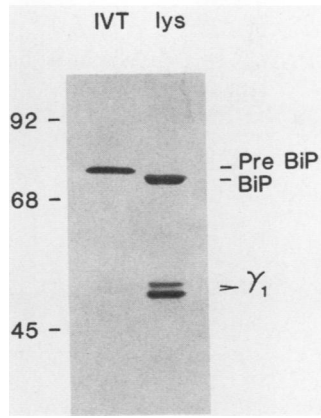

FIG. 5. Demonstration of a single precursor form of GRP78 BiP. Total cellular RNA from Ag8(8) cells (5 μg) was translated in a rabbit reticulocyte system supplemented with [^{35}S]methionine for 90 min. The translated material was diluted with lysing buffer, immunoprecipitated with anti-BiP (lane IVT) and compared with ^{35}S-labeled BiP synthesized in tunicamycin-treated Ag8(8) cells (lane lys). Both samples were analyzed by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels under reducing conditions.

experiments, Ag8.653 cells, which produce BiP but no longer produce HCs or LCs (16), as well as Ag8(8) cells, which produce BiP and γ_1 HCs but no LCs (2), were used. A significant amount of the BiP in Ag8(8) cells has previously been shown to exist in stable association with γ1 HCs. Equal numbers of cells from both lines were first labeled with [^{35}S]methionine, and cell lysates were immunoprecipitated with anti-BiP in the case of Ag8.653 cells or split and immunoprecipitated with anti-BiP or anti-Ig in the case of Ag8(8) cells. Since similar quantities of incorporated radioactivity were found in the BiP band from both Ag8.653 cells and Ag8(8) cells and the material immunoprecipitated with anti-BiP from the Ag8.653 cells was derived from twice the number of cells as was used for the anti-BiP precipitation of the Ag8(8) cell line, it appeared that the Ig$^-$ Ag8.653 cells synthesize about half the amount of BiP as the HC$^+$ sister line, Ag8(8) (Fig. 4A). The anti-BiP-precipitated material from Ag8(8) cells contained BiP and coprecipitating γ_1 HCs. When the same lysate was immunoprecipitated with anti-Ig, both γ_1 HCs and BiP were again observed (Fig. 4A). However, not all the BiP could be precipitated in association with γ_1 HCs, implying the existence of a pool of free BiP in these cells.

The same cell lines were labeled with $^{32}P_i$ for 8 h and immunoprecipitated as described above. ^{32}P was incorporated into the anti-BiP-precipitated material from both Ag8.653 cells and Ag8(8) cells (Fig. 4B). However, even though nearly equal amounts of BiP were present in these lanes (Fig. 4A), the incorporation of $^{32}P_i$ into BiP synthesized by Ag8.653 cells was much greater. When the anti-Ig-precipitated material from Ag8(8) cells was analyzed, no ^{32}P-incorporation could be detected in the associated BiP (Fig. 4B). Thus, the BiP which is associated with HCs appears to exist in an unphosphorylated state. The free or unassembled BiP, however, is phosphorylated. This is supported by the finding that preclearing cell lysates with anti-Ig, which also removes about one-third of the BiP, did not diminish ^{32}P incorporation into residual BiP (data not shown). This may explain the intense ^{32}P labeling of BiP in the Ag8.653 cells, in which BiP must exist primarily in the free form.

Subcellular location of phosphorylated BiP-GRP78. Because BiP-GRP78 was shown to be phosphorylated on serine and threonine residues, a modification which normally takes place on the cytoplasmic face of the cellular membranes, and because only free BiP was phosphorylated, we determined whether a cytosolic, phosphorylated form and an ER, unphosphorylated form of BiP were being synthesized. The two forms could conceivably derive from differential splicing of GRP78 mRNA, as has been reported for yeast invertase (3). To test this hypothesis, total RNA from Ag8(8) cells was translated in vitro in a rabbit reticulocyte system. The in vitro-translated material was immunoprecipitated with anti-BiP and compared with ^{35}S-labeled BiP synthesized in tunicamycin-treated Ag8(8) cells. A single form of BiP was isolated from the in vitro-translated material (Fig. 5). This species migrated slightly more slowly than BiP isolated directly from the cell line (Fig. 5), consistent with the presence of a cleavable leader sequence on nascent BiP. There was no evidence of a leaderless form of BiP which could give rise to a cytosolic protein.

To determine the cellular location of the phosphorylated BiP, Ag8(8) cells were labeled with either [^{35}S]methionine or $^{32}P_i$. Cells were washed and homogenized, and membranes were fractionated on a 10 to 30% continuous sucrose gradient. Fractions were collected, divided in half, and precipitated with anti-BiP or anti-γ. Examination of [^{35}S]methionine-labeled material showed that BiP and γ HCs were found in the first and second fractions representing the ER and at the top of the gradient (fractions 13 to 16) containing cytosolic proteins (Fig. 6A and B). Because BiP is synthesized as a single precursor form, the most logical interpretation is that BiP is translocated into the lumen of the ER and that the cytosolic form of BiP seen in these experiments is due to leakage of soluble proteins from the ER during membrane preparation. This is supported by the finding that γ HCs are also found in the cytosolic fraction (Fig. 6A). The ^{32}P-labeled cells were prepared in the same way. Fractions 1

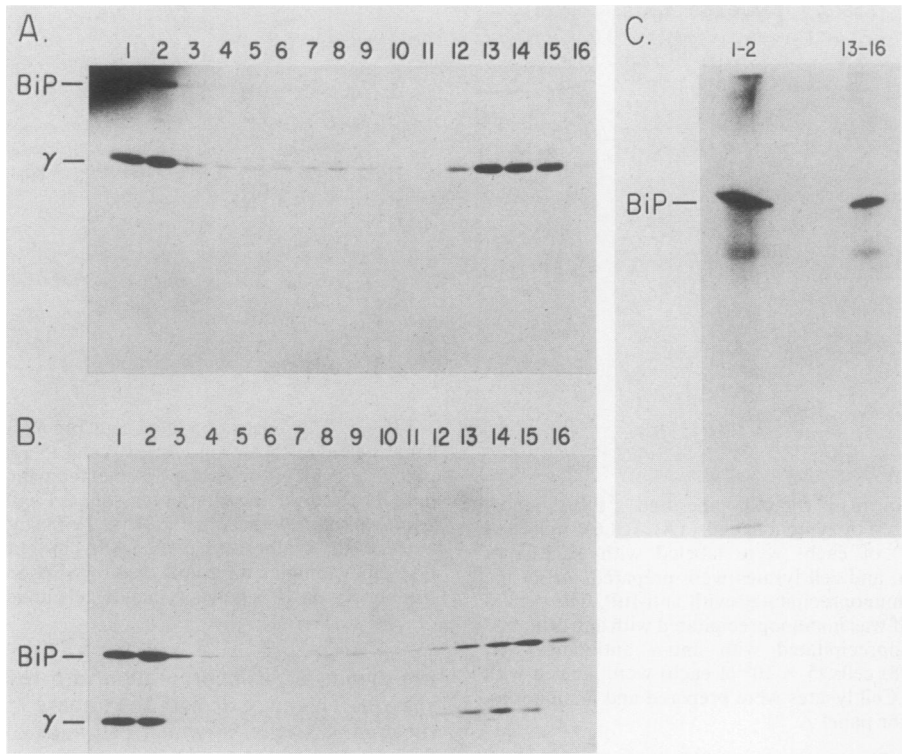

FIG. 6. Subcellular location of GRG78-BiP. [^{35}S]methionine-labeled Ag8(8) cells were Dounce homogenized, and membranes were separated on a sucrose gradient. Sixteen fractions were collected from the bottom of the tube, with fraction 1 representing the bottom and fraction 16 representing the top. Each fraction was diluted with lysing buffer, divided, and immunoprecipitated with anti-γ (A) or anti-BiP (B). $^{32}P_i$-labeled Ag8(8) cells were prepared and fractionated as for panel B. Fractions 1 and 2 were pooled, fractions 13 to 16 were pooled, and both were immunoprecipitated with anti-BiP (C).

and 2 containing the ER membranes and fractions 13 to 16 were pooled, and both pooled fractions were immunoprecipitated with anti-BiP. The BiP in both fractions was phosphorylated (Fig. 6C). The relative incorporation of ^{32}P into BiP in each fraction is similar to the incorporation of [^{35}S]methionine into BiP in each fraction, suggesting that there is no inherent difference in phosphorylation between the two pools and that both the phosphorylated and nonphosphorylated form of BiP are present in the ER. These data demonstrate that BiP is synthesized as a single precursor form which is translocated into the lumen of the ER, where it exists in both a phosphorylated and an unphosphorylated form.

Incorporation of [^{3}H]adenosine into BiP in lymphoid cell lines. GRP78 has also been reported to be modified post-translationally by ADP ribosylation and can be labeled with [^{3}H]adenosine (4). However, the incorporation of [^{3}H]adenosine into GRP78 is greatly diminished under conditions which lead to its enhanced synthesis (4). To determine if the same phenomenon is observed with BiP in murine B cells, and if the degree of adenosine incorporation is also related to the bound versus the free form, the Ag8.653 cells and Ag8(8) cells were labeled with [^{3}H]adenosine, lysed, and immunoprecipitated as described for Fig. 4. The anti-BiP-precipitated material in Ag8.653 (γ^-, LC$^-$) cells was readily labeled with [^{3}H]adenosine, demonstrating that it was either adenylated or ADP-ribosylated (Fig. 7). Examination of equivalent amounts of BiP from Ag8(8) (γ^+, LC$^-$) cells showed detectable amounts of [^{3}H]adenosine incorporation into BiP but at much reduced levels compared with Ag8.653

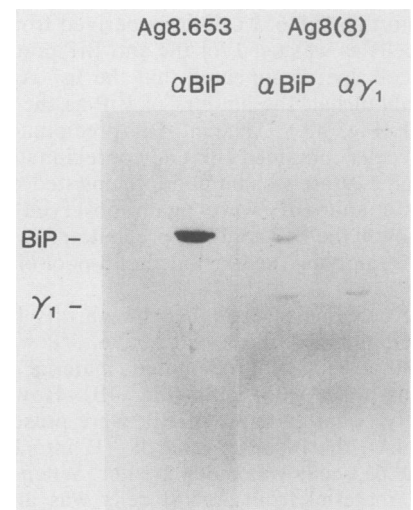

FIG. 7. Labeling of BiP with [^{3}H]adenosine. Ag8.653 and Ag8(8) cells (10×10^6 of each) were preincubated with 20 μg of actinomycin D per ml and 10 μM 1-β-D-arabinofuranosylcytosine for 30 min and then labeled with 200 μCi of [2,8,5-^{3}H]adenosine for 6 h. Cell lysates were prepared and precipitated with anti-BiP in the case of Ag81653 cells and with anti-γ or anti-BiP in the case of Ag8(8) cells.

cells (Fig. 7). Immunoprecipitation with anti-γ revealed no detectable incorporation of [^{3}H]adenosine into HC-associated BiP, but surprisingly, the associated γ HCs were labeled. Very similar results were found when μ^+, LC^- cells were examined in the same way (data not shown). In these cells, free BiP was labeled with [^{3}H]adenosine, but BiP which was precipitated in association with μ heavy chains was not labeled. In both anti-BiP-precipitated material and anti-μ-precipitated material, we found the μ HCs to be labeled with [^{3}H]adenosine. These results suggest that free BiP contains phosphorylated and adenylated moieties, whereas bound BiP does not.

DISCUSSION

BiP and GRP78 were shown to be the same protein by NH_2-terminal sequence, by examination of the induction of GRP78 and BiP in a mutant fibroblast cell line, and by detection of identical posttranslational modifications. This conclusion is further supported by the isolation and sequence analysis of the single-copy gene coding GRP78 in rats (S. K. Wooden, R. P. Kapur, and A. S. Lee, Exp. Cell Res., in press) and humans (28). A recent flurry of reports (6, 7, 11, 15) have speculated that BiP, GRP78, and the heat-shock cognates may act as a family of "foldases" or "molecular chaperons" to ensure that proteins fold and assemble correctly. It has further been suggested that during stress, nascent polypeptides would be synthesized which would have difficulty folding and assembling correctly and that a protein such as BiP or GRP78 would aid in refolding and assembling these proteins. We now provide the first evidence that, under conditions which up-regulate the stress protein GRP78, it is in fact associated with many other cellular proteins. This conclusion follows from direct coprecipitation of cellular proteins with stress-induced GRP78-BiP and, indirectly, from the demonstration that the stress-induced, nonphosphorylated, non-ADP-ribosylated form of GRP78 is the bound form.

The finding that GRP78 is phosphorylated on serine and threonine residues indicates that BiP-GRP78 is posttranslationally modified both by direct phosphorylation as well as through acquisition of adenosine groups, since mono-ADP-ribosylation usually occurs on arginine, asparagine, and lysine residues, and poly-ADP-ribosylation usually occurs on glutamic acid residues (29). It is therefore likely that specific serine-threonine protein kinases are involved in the phosphorylation of GRP78 and regulate its function. While protein kinases generally act on cytoplasmic portions of protein molecules, casein, a secreted protein, is phosphorylated on both serine and threonine residues (27).

A less likely possibility is that BiP-GRP78 undergoes a conformational change when it is the free state, resulting in the activation of an intrinsic kinase activity that leads to its autophosphorylation. Examination of the rat GRP78 amino acid sequence (24) by using the IntelliGenetics Database Searching Program showed a near consensus sequence beginning at amino acid 425 with the reported protein kinase ATP-binding region sequence (LIV)GXGX(GY)GX(LIV) (1). The corresponding BiP-GRP78 sequence beginning at amino acid 425 is LGIETVGGV, with a glutamic acid in position 428 replacing the glycine in the consensus sequence. While this sequence has been used to identify both tyrosine kinases and cyclic AMP-dependent kinases, it is also found in other, ATP-binding proteins, such as alcohol dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, and glutathreonine reductase (14). Previous reports have shown GRP78 to be an ATP-binding protein (24). To further test the possibility that free BiP might act as a kinase, immune complex kinase assays were performed. BiP was immunoprecipitated from Ag8.653 cells with anti-BiP and from Ag8(8) cells with both anti-γ and anti-BiP. We were unable to detect any evidence that the precipitated BiP was able to phosphorylate either itself or exogenously added substrates, such as casein or histones, in the kinase assay (data not shown). Thus, while BiP-GRP78 does contain an ATP-binding domain, we have no evidence that it is able to autophosphorylate itself either as a consequence of releasing proteins or as a way of releasing associated proteins.

The previous report showing GRP78 to be ADP ribosylated (4) suggests that adenosine labeling of BiP is due to ADP ribosylation. We do not know, however, if the labeling of HCs with [^{3}H]adenosine is due to ADP ribosylation or adenylation. Separate experiments have shown that HCs which are associated with LC as well as HC mutants which do not associate with BiP are labeled with [^{3}H]adenosine, suggesting that the labeling of HCs is not related to their association with BiP (L. M. Hendershot, unpublished data).

BiP-GRP78 is posttranslationally modified by phosphorylation and ADP ribosylation, and both the unmodified and modified forms coexist in the same compartment of the cell. In lymphoid cell lines, BiP bound to HCs was not labeled with either radioactive phosphate or adenosine, whereas free BiP was labeled with both. Similarly, when GRP78 was induced in fibroblasts by glucose starvation and found to be in association with other cellular proteins, the ratio of unmodified to modified forms was increased. Thus, the ratio of unmodified to modified forms appears to be indicative of the relative amounts of bound and free BiP-GRP78. While the modified form of BiP-GRP78 was not complexed to other proteins, we cannot exclude the possibility that a portion of the unmodified protein is present in an unbound form. Nor has a kinetic relationship, if any, between the bound and free pools of BiP-GRP78 been determined. GRP78 is a stable protein with a half-life of more than 48 h, and the turnover of total [^{35}S]methionine-labeled molecules is not detectably accelerated under stress conditions, even though phosphorylation and ADP-ribosylation are greatly diminished (unpublished results), suggesting that the pools of modified and unmodified protein may be interconvertible. Therefore, if unmodified BiP-GRP78 is the only species able to form complexes with other proteins, phosphorylation and adenylation would have to occur during or after their dissociation. Alternatively, modified forms of BiP-GRP78 might be dephosphorylated and deadenylated during complex formation.

In lymphoid cell lines, the total levels of BiP-GRP78 were highest in cells synthesizing nonsecreted HCs which remained stably associated with BiP. Cells which did not produce HCs synthesized BiP at a lower rate, but the relative amount of the modified form was higher. Similarly, under nonstress conditions, BiP-GRP78 associates transiently with nascent proteins (2, 8, 9), but when underglycosylated or aberrantly folded proteins are synthesized during stress, they appear to form stable complexes with unmodified BiP-GRP78. The relative proportion of the modified form is decreased at a time when the rate of synthesis of BiP-GRP78 is increased. Thus, in both cases there is a correlation between a high rate of synthesis of BiP-GRP78 and a decreased ratio of modified to nonmodified forms. The up-regulation of mRNA encoding BiP-GRP78 observed during glucose starvation occurs only in the presence of protein synthesis (20), suggesting that perhaps depletion of the free

BiP-GRP78 pool through complex formation and the concomitant decrease in the ratio of modified to unmodified forms precedes induction of the BiP-GRP78 gene. Thus, transcription of BiP-GRP78 RNA may be autoregulated through a sensing mechanism that monitors the ratio of the unmodified to modified protein. Experiments are under way to examine this hypothesis.

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ADDENDUM IN PROOF

The gene for BiP has been cloned and sequenced and is a direct match with the reported sequence for GRP78 (I. Haas and T. Meo, Proc. Natl. Acad. Sci. USA **85**:2250–2254, 1988).

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