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The 170-kDa Glucose-regulated Stress Protein Is an Endoplasmic Reticulum Protein that Binds Immunoglobulin

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Anoxia, glucose starvation, calcium ionophore A23187, EDTA, glucosamine, and several other conditions that adversely affect the function of the endoplasmic reticulum (ER) induce the synthesis of the glucose-regulated class of stress proteins (GRPs). The primary GRPs induced by these stresses migrate at 78 and 94 kDa (GRP78 and GRP94). In addition, another protein of ~150–170 kDa (GRP170) has been previously observed and is coordinately induced with GRP78 and GRP94. To characterize this novel stress protein, we have prepared an antisera against purified GRP170. Immunofluorescence, Endoglycosidase H sensitivity, and protease resistance of this protein in microsomes indicates that GRP170 is an ER lumenal glycoprotein retained in a pre-Golgi compartment. Immunoprecipitation of GRP170 with our antibody coprecipitates the GRP78 (also referred to as the B cell immunoglobulin-binding protein) and GRP94 members of this stress protein family in Chinese hamster ovary cells under stress conditions. ATP depletion, by immunoprecipitation in the presence of apyrase, does not affect the interaction between GRP78 and GRP170 but results in the coprecipitation of an unidentified 60-kDa protein. In addition, GRP170 is found to be coprecipitated with immunoglobulin (Ig) in four different B cell hybridomas expressing surface IgM, cytoplasmic Ig light chain only, cytoplasmic Ig heavy chain only, or an antigen specific secreted IgG. In addition, in IgM surface expressing WEHI-231 B cells, anti-IgM coprecipitates GRP78, GRP94, as well as GRP170; antibodies against GRP170 and GRP94 reciprocally coprecipitate GRP94/GRP170 as well as GRP78. Results suggest that this 170-kDa GRP is a retained ER lumenal glycoprotein that is constitutively present and that may play a role in immunoglobulin folding and assembly in conjunction or consecutively with GRP78 and GRP94.

INTRODUCTION

The heat shock proteins or stress proteins are a set of proteins that have been implicated in a number of stress-protective functions (reviewed in Pelham, 1986; Lee, 1987; Lindquist and Craig, 1988; Rothman, 1989; Gething, 1991). A subcategory of stress proteins in eukaryotes are the glucose-regulated proteins, which were originally described as being induced under conditions of glucose deprivation (Pouyssegur *et al.*, 1977; Shiu *et al.*, 1977). In higher eukaryotes the glucose-regulated proteins (GRPs) do not appear to be readily inducible by heat but are sensitive to stresses that have a deleterious effect on the function and/or integrity of the endoplasmic reticulum (ER). These include agents that affect calcium homeostasis such as ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (Lamarche *et al.*, 1985), thapsigargin (Price *et al.*, 1992), and calcium ionophore A23187 (Welch *et al.*, 1983), reducing agents such as 2-mercaptoethanol that retard the formation of disulfide bonds (Whelen and Hightower, 1985; Kim and Lee, 1987), agents that perturb protein glycosylation (Lee, 1987), and anoxia (Sciandra and Subjeck, 1984). The principal members of this family of stress proteins

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are ER resident proteins of ~78 and 94 kDa (GRP78 and GRP94, respectively). GRP78 is a lumenal protein that is required for the transport of proteins into the ER in yeast, and its association with partially assembled immunoglobulin (Ig) and misfolded or mutant proteins has been well documented (reviewed in Hartl *et al.*, 1992). GRP78 is also widely referred to as the B cell immunoglobulin-binding protein or BiP (Haas and Wabl, 1983). GRP94 has been found to be a major glycoprotein of the ER lumen that has been purified (Kang and Welch, 1991) and is found complexed with BiP and with unassembled Ig subunits (Melnick *et al.*, 1992). In addition to GRP78 and GRP94, reports have described the coinduction during stress of a third GRP of ~150–170 kDa (Olden *et al.*, 1978; Sciandra and Subjeck, 1983; Shen *et al.*, 1987). This member of the GRP family has not been previously studied; we describe here the first analysis of this stress protein. We find that GRP170 is an ER lumenal glycoprotein that is associated with GRP78 and GRP94 by coprecipitation studies. In addition, GRP170 is associated with Ig and/or components thereof in various B cell hybridomas as well as being present in all murine tissues examined. It is possible that like GRP78, GRP170 may cooperate in facilitating protein folding and assembly in the ER, representing a new element in this pathway.

MATERIALS AND METHODS

Cell Culture

The Chinese hamster ovary (CHO) fibroblast cell line was initially obtained from R. Tobey, Los Alamos National Laboratory, and was maintained as a monolayer culture at 37°C cultured in Ham's F-10 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 15% (vol/vol) newborn calf serum. C3H mouse embryo 10T1/2 fibroblasts were initially obtained from Dr. John Bertram (University of Hawaii). These cells were carried as monolayers at 37°C in Eagle's basal medium with Earle's salts (GIBCO) containing 10% fetal bovine serum (FBS). The membrane IgM bearing immature B lymphocyte hybridoma WEHI-231 was grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 5×10^{-5} M 2-mercaptoethanol. The IgG-secreting B lymphocyte hybridoma (1G1B6), a kind gift of Dr. R. B. Bankert, was grown and maintained in HAT medium. The NS-1 cell line is a nonsecreting, heavy chain negative, light chain positive B cell line that was maintained in DMEM supplemented with 20 μg/ml 8-azaguanine and 20% FBS as described (Kohler and Milstein, 1976). The HB-34 cell line is a nonsecreting mu heavy chain positive, light chain negative B cell line that was maintained in RPMI1640 medium supplemented with 20% FBS, 10 U/ml penicillin, 10 μg/ml streptomycin, 2 mM L-glutamine, and 2.5×10^{-5} M 2-mercaptoethanol.

Induction of GRPs in CHO Cell Cultures

The induction of GRPs in CHO cell cultures was described previously (Shen *et al.*, 1987). Briefly, to achieve glucose depletion, 10^6 cells were seeded in a 100-mm dish and incubated for 12 h to allow the cells to attach. At this time the medium was replaced with 5 ml of glucose-deficient F-10 medium (GIBCO) with 15% (vol/vol) newborn calf serum. The Sigma (St. Louis, MO) glucose calorimetric assay was used to monitor glucose levels. Glucose was entirely consumed by 20 h with GRPs first seen at ~34 h. For the calcium ionophore A23187 treatment, 10^6 cells were seeded and incubated for 36 h, after which A23187 was added to a concentration of 10 μM. GRPs were induced within 2–4 h. For glucosamine, 2-deoxyglucose and tunicamycin treatment, cells were seeded as just described and incubated for 36 h, at which time glucosamine, 2-deoxyglucose, and tunicamycin were added to final concentrations of 10 mM, 10 mM, and 2 mM, respectively. GRPs were again induced within 2–4 h. For anoxia experiments, cells were seeded as described above, and cells were incubated for 36 h. At this point the medium was changed to 10 ml of medium containing 200 μl of 7.5% (wt/vol) sodium bicarbonate. Cells were then exposed to anoxia in a Brewer jar as described (Sciandra and Subjeck, 1984). GRPs were induced within 4–8 h. Anoxia was also achieved using a flow through chamber, fed into a water trap, and maintained under positive pressure by 95% N_2 and 5% CO_2 with similar results. In each treatment the concentration/time used were chosen for a strong induction of GRPs while being essentially nonlethal (Shen *et al.*, 1989).

Preparation of Anti-GRP170 Antisera

Purified GRP170 was obtained by two-dimensional (2-D) gel electrophoresis as previously described (O'Farrell, 1975) using lysates of CHO cells treated with calcium ionophore A23187 for 12–16 h. One hundred micrograms of protein was loaded per gel, as determined using the Bradford method (Bradford, 1976, supplied by Bio-Rad, Richmond, CA). Gels were stained by Coomassie blue, and the GRP170 spots were excised and homogenized in complete Freund's adjuvant for the primary injection and incomplete Freund's adjuvant for subsequent immunizations. Intradermal injections were given at 4-wk intervals after a 1-wk interval from the first injection. Bleeds were taken from the ear vein, and the derived serum was precipitated with 50% ammonium sulfate and stored below 0°C. Bleeds were tested for anti-GRP170 reactivity by Western blot analysis.

Protein Radiolabeling and Western Immunoblot Analysis

[^{35}S]methionine (>800 μCi/mmol) was obtained from Amersham International (Amersham, England) and added to methionine-free media (GIBCO) at a final level of 10 μCi/ml for use in pulse-labeling experiments. After stress, cells were washed and resuspended in the labeling medium, incubated for various times, and then washed twice with media without serum at 4°C. Cells were then resuspended in Hanks balanced salt solution (Grand Island Biol, Grand Island, NY) without calcium and magnesium containing 1 mM tosyl-L-arginine methyl ester, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 5 mM EDTA (Sigma) and sonicated at 4°C. Protein determinations for gel loading were performed on lysates as described above. Sodium dodecyl sulfate (SDS) sample buffer (4×) was then added to a 1× final concentration. The final sample solution containing 5% 2-mercaptoethanol was boiled for 5 min. Discontinuous SDS-polyacrylamide gel electrophoresis ([PAGE] usually 10% polyacrylamide) was performed using the Laemmli buffer system (Laemmli, 1970). Gels were stained and exposed to XAR5 X-ray film (Eastman Kodak, Rochester, NY), or the proteins were transferred to nitrocellulose filters. In the latter case, the gels were stained with Coomassie brilliant blue after the transfer to confirm the uniformity of the transfer. The filters were incubated overnight at room temperature with a 1:1000 dilution of the antibody to GRP170. The immunoblots were reacted with goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Boehringer Mannheim Biochemicals, Indianapolis, IN) and detected colorimetically (Figure 2) as described previously (Shen *et al.*, 1987) or detected by enhanced chemiluminescence (Amersham) (Figures 3 and 9). Western blots for GRP78, GRP94, or protein disulfide isomerase (PDI) were performed using rabbit anti-GRP78 (1:1000), rat anti-GRP94 (1:1000), or rabbit anti-PDI (1:1000), respectively (StressGen, Sidney, British Columbia, Canada).

Immunofluorescence

10T1/2 cells were allowed to adhere to glass coverslips in a Petri dish for 24–36 h. Cells on slips were then induced by A23187, glucose deprivation, or 2-deoxyglucose as described above. Cells were then fixed in 2% formaldehyde (Fisher, Pittsburgh, PA) for 10 min and permeabilized with 0.5% Triton X-100 (Sigma) in phosphate-buffered saline (PBS) (vol/vol) for 5 min. The coverslips were blocked with a 1:400 dilution of normal goat serum for 30 min and then rinsed. The primary antibody, rabbit anti-GRP170, was added at 1:100, allowed to bind for 30 min, and rinsed in PBS before addition of the secondary antibody, fluorescein isothiocyanate-conjugated goat anti-rabbit Ig (Kierkegaard and Perry, Gaithersburg, MD). After binding, coverslips were rinsed well in PBS and rinsed with distilled water before mounting with aqua-mount mounting medium (Polysciences, Warrington, PA). Control for nonspecific binding was carried out with PBS in lieu of rabbit-anti-GRP170.

Immunoprecipitation

Cell lysates for immunoprecipitations were made by rinsing cells twice in PBS and then resuspending to 10^7 cells per ml of lysis buffer containing 1% Triton X-100, 1% bovine hemoglobin, 10 mM iodoacetamide, 5 mM PMSF, 25 mg/ml aprotinin, and 10 mM leupeptin (Sigma) in TSA (0.01 M tris(hydroxymethyl)aminomethane [Tris-HCl] pH 8.0, 0.14 M NaCl, 0.025 M NaN_3). Lysates were clarified by centrifugation at 15 000 × *g* for 20 min. The supernatant was adjusted to 1% sodium deoxycholate and 0.1% SDS. The samples were then vortexed and solubilized for 30 min at 4°C. For direct immunoprecipitation, 100 μl of cell lysate was incubated with 10 μl of rabbit anti-GRP170 antiserum or rat anti-GRP94 antiserum (StressGen) and allowed to bind 2 h with rocking at 4°C. After 2 h, 20 μl of preactivated protein A sepharose CL-4B (Sigma) was added and continuously mixed at 4°C for 1 h. To immunoprecipitate Ig directly, goat-anti-mouse Ig-sepharose or goat anti-mouse IgM antibody (Sigma) was used at 10 μl per 100 μl cell lysate. Precipitates were washed twice in dilution buffer containing 0.1% Triton X-100, 0.1% bovine hemoglobin, 0.01 M Tris-Cl (pH 8.0), 0.14 M NaCl, and 0.025% NaN_3, and once with buffer containing 0.01 M Tris-Cl, 0.14 M NaCl, and 0.025% NaN_3 before resuspension in SDS-sample buffer followed by boiling for 3 min. Gel electrophoresis was performed as described above. Gels were stained with Coomassie blue, dried down, and exposed to XAR5 X-ray film (Eastman Kodak), or the proteins were transferred to nitrocellulose filters for Western blotting as described above. Treatment with apyrase/ATP were performed as described (Beckmann *et al.*, 1990).

Microsomal Preparation

The microsomal fraction of CHO cells was prepared using a modified procedure of Adelman *et al.* (1973). In brief, [^{35}S]methionine-labeled CHO cells were suspended in the cold buffer containing 10 mM KCl and 1 M sucrose and homogenized in a Dounce homogenizer with a B paddle. After 50 shakes, the homogenate was centrifuged to precipitate the nuclei. The postnuclear homogenate was diluted in 0.1 M sucrose and ultracentrifuged for 30 min to precipitate the membrane fraction containing microsomes and free ribosomes. The precipitate was rinsed once, resuspended in digestion buffer, and aliquoted into three portions, treated with either 0.1% SDS, proteinase K, or both. The digest was then sonicated and immunoprecipitated with Protein-A sepharose beads precoated with anti-170 antiserum.

Endoglycosidase H (Endo H) Treatment

Endo H digestion was carried out as described previously (Tai *et al.*, 1975). Briefly, lysate immunoprecipitated with anti-GRP170 antibody as described above was incubated with 10 μg/ml Endo H in 50 mM sodium citrate buffer pH 5.5 with 0.1% bovine serum albumin, for 22 h at 37°C in 5% CO_2. Sample was then resuspended in SDS-sample buffer and prepared for electrophoresis as described.

Preparation of Murine Tissues

Cell lysates were prepared using an Omni International 2000 tissue homogenizer (Waterburg, CT). Cells were washed three times in PBS, resuspended in nonreducing SDS-sample buffer containing 2-mercaptoethanol, boiled, sonicated, and centrifuged before electrophoresis. Loading of samples was performed to equivalent mass as described above. The blot was probed with the GRP170 antibody followed by HRP-conjugated antibody. Detection was performed by enhanced chemiluminescence (Amersham).

RESULTS

It has been well documented that GRP78 and GRP94 are coinducible by a variety of conditions in numerous cell lines (Lee, 1981, 1987; Lee *et al.*, 1983; Shen *et al.*, 1987). In addition, there are also reports of the coinduction of a third, higher molecular weight GRP (Olden *et al.*, 1978; Shen *et al.*, 1987) of ~150–170 kDa. Figure 1 shows the induction of GRPs in CHO cells metabolically labeled with [^{35}S]methionine, followed by 2-D isoelectric focusing/SDS-PAGE. The induction of a higher molecular weight species of a calculated molecular mass of 164 kDa (henceforth designated GRP170) by glucose starvation is evident in Figure 1B (arrow) as can be seen by comparison with Figure 1A. The induction of GRPs 78 and 94 can also be seen (stars). Pulse labeling studies using several stress conditions known to induce GRPs 78 and 94 including anoxia, treatments with calcium ionophore A23187, 2-deoxyglucose, glucosamine, thapsigargin, and tunicamycin demonstrate a similar induction of GRP170 in CHO cells with induction kinetics paralleling GRP78 and GRP94 (tunicamycin and 2-deoxyglucose yield a slightly different pattern, see below). Moreover, similar responses are obtainable in mouse embryo 10T1/2 cells. As seen in Figure 1A, the expression of GRP170 is notably less in unstressed cells than is the expression of either GRP78 or GRP94. As described in MATERIALS AND METHODS, GRP170 spots were excised from Coomassie blue–stained 2-D gels of stressed cells, homogenized, and used as immunogen. A Western blot analysis of the resultant rabbit antibody is presented in Figure 2, illustrating the accumulation of GRP170 in control cells and in cells subjected to several GRP-inducing stresses. In the cases of 2-deoxyglucose and tunicamycin (Figure 2, lanes 5 and 8), cross-reactivity with two bands is evident. This is consistent with the fact that these inducers lead to the presence of a nonglycosylated form of GRP170 of Mr 150 kDa, whereas the other conditions do not inhibit glycosylation as indicated by gel migration (see below). All inducing conditions except glucose starvation lead to an increased amount of GRP170 (in the case of tunicamycin and 2-deoxyglucose, this is indicated by the presence of two bands instead of one). The increase observed after 14 h of glucose starvation is slight, probably because of the fact that this time was inadequate to allow a more significant accumulation. Figure 2, lane 1 is a preimmune control.

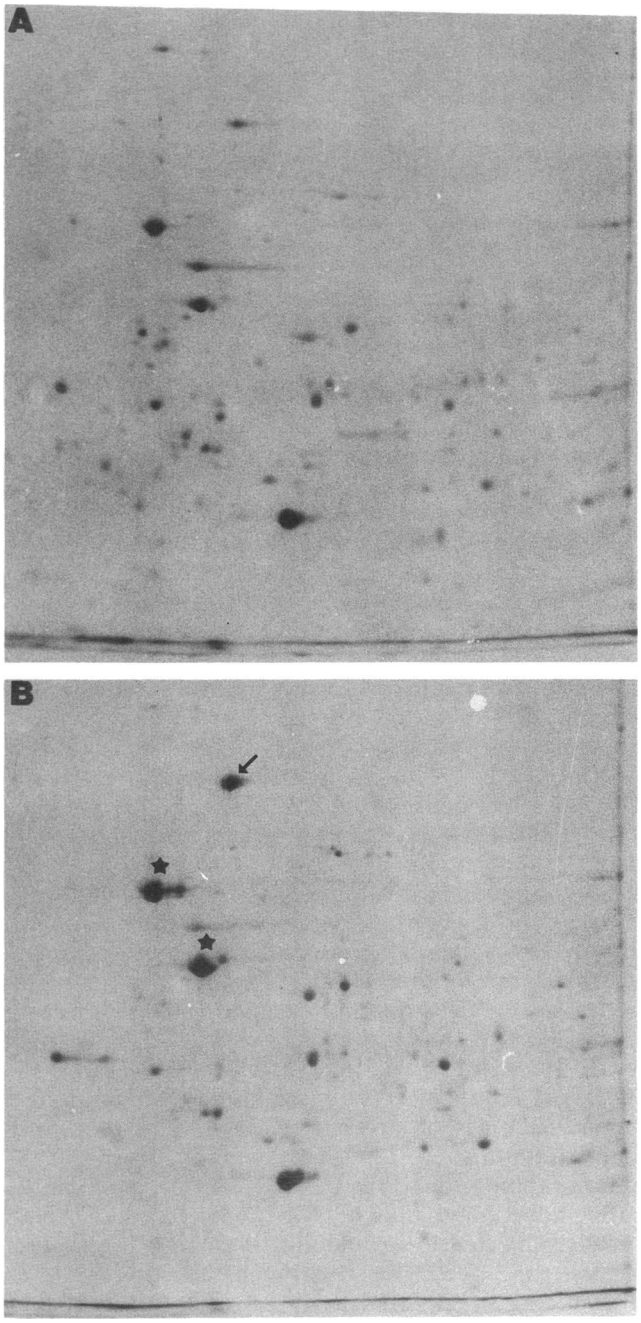

Figure 1. GRPs 78, 94, and 170 are coinduced by glucose starvation. CHO cells were exposed to 14 h of glucose starvation (or cultured in normal glucose containing medium) after which they were labeled with [^{35}S]methionine for 1 h. Lysates were analyzed by isoelectric focusing/SDS-PAGE followed by autoradiography. (A) Protein synthesis pattern of control CHO cells. (B) Protein synthesis pattern of glucose-starved CHO cells. In B the induction of GRP78/BiP and GRP94 are visible (★). The induction of GRP170 in this figure is indicated by the arrow.

An immunoprecipitation analysis of metabolically labeled CHO cells using this antibody is presented in Figure 3. Figure 3A shows an autoradiogram of immunoprecipitates of calcium ionophore A23187–treated cells, where the precipitation is performed with either preimmune or immune serum (Figure 3A, lanes 1 and 2, respectively). Figure 3A, lane 2 shows that other bands also coprecipitate with GRP170, the most prominent migrating at 78 kDa (arrow). Other coprecipitating proteins are also evident in this panel, one of which migrates at 94 kDa (star). In a separate experiment using tunicamycin as the inducer, anti-GRP170 immunoprecipitates were analyzed on a 2D gel (Figure 3B) and compared with a Coomassie blue stained gel of total cellular protein prepared at the same time (Figure 3C). By comparison of these figures and Figure 1, it is evident that the coprecipitated protein (arrow) is GRP78/BiP. Although not readily detectable in Figure 3B, a small amount of GRP94 is also coprecipitated. Finally, anti-GRP170 immunoprecipitates of unstressed or calcium ionophore A23187–treated CHO cells were transferred and probed with antibodies against GRP78, GRP94, or GRP170. This data, presented in Figure 3D, demonstrates the coprecipitation of GRP78 and GRP94 with GRP170 in ionophore treated cells (Figure 3D, lanes 2 and 5, respectively). Controls showing reactivity with total cell lysate (Figure 3D, lanes 3 and 6) and self reactivity with anti-GRP170 precipitates (Figure 3D, lanes 7–9) are also shown. The anti-GRP78 and anti-GRP94 antibodies fail to react with anti-GRP170 precipitates of unstressed samples. In Figure 3D, the amount of GRP94 detected in the anti-GRP170 precipitates (lane 5) is much less than that in the total cell lysate (lane 6), whereas the amount of coprecipitated GRP78 (lane 2) is less than, but more comparable to, the amount detected in the cell lysate (lane 3). This difference is in line with data presented in Figure 3B and with Figure 1 where both GRP78 and GRP94 are present in approximately equivalent quantities (the amounts of GRP78 and GRP94 cannot be compared because the data result from reactions with different antibodies). In all of the precipitation studies, some unidentified low molecular weight bands are also seen. Thus, at any given time, some of the GRP78 and GRP94 present in the stressed cell is complexed with some of the GRP170. However, a larger proportion of total cell GRP78 is complexed with GRP170, compared with the proportion of GRP94 complexed with GRP170.

Reports of ATP-binding activity of GRPs 78 and 94 (Welch and Feramisco, 1985; Clairmont *et al.*, 1992), and the ATP dependent release of bound proteins from GRP78 (Munro and Pelham, 1986) led us to examine whether GRP170 coprecipitated proteins would be affected by ATP depletion by incubation with apyrase or ATP augmentation. Control CHO cells and cells stressed sufficiently to result in GRP induction were labeled with [^{35}S]methionine for 30 min, and lysates were prepared

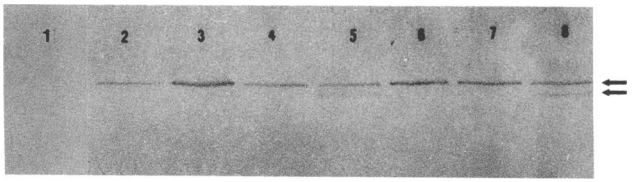

Figure 2. Western blot analysis using the rabbit antibody raised against GRP170. The rabbit antisera prepared against purified GRP170 was used to probe blots of control (lane 2) and stressed CHO cells (anoxia for 24 h, lane 3; glucose starvation for 14 h, lane 4; 2-deoxyglucose for 12 h, lane 5; glucosamine for 12 h, lane 6; calcium ionophore for 12 h, lane 7; and tunicamycin for 12 h, lane 8). Concentrations and conditions of treatment are described in MATERIALS AND METHODS. The antisera reacts with a band at ~170 kDa in all instances, except lanes 5 and 7, where a secondary reaction with a 150-kDa band is also visible (arrows, right margin). The lower 150-kDa band represents the unglycosylated form of GRP170 (see text). Lane 1 shows a blot of A23187-treated CHO cells probed with preimmune serum.

in the presence of either 10 units/ml apyrase or 2.5 mM ATP. Immunoprecipitation was then performed with the GRP170 antibody. Figure 4 shows the effect of these treatments on the coprecipitation of other proteins. Comparison of Figures 4, A and B indicates that apyrase treatment results in the coprecipitation of an unidentified 60-kDa protein (arrow, Figure 4B) not seen in ATP augmented samples. In addition, a slightly enhanced coprecipitation of other proteins is also obtained in the apyrase-treated lysates. These results indicate a very slight ATP-dependence of the interaction between GRP170 and other proteins, excepting the interaction with the unidentified 60-kDa species where the dependence is obvious. Notably, the coprecipitation of GRP78 with GRP170 is not dependent upon ATP.

Because Figure 2 suggested that tunicamycin treatment leads to a more rapidly migrating GRP170 species, correspondingly suggesting that the protein is a glycoprotein, we examined the sensitivity of GRP170 to Endo H, a glycosidase that cleaves sugars that have not been further processed in the Golgi compartment. Figure 5 shows the Endo H digestion of pulse-labeled immunoprecipitates. When cells are pulse labeled for 15 min and chased for ≤240 min, the sensitivity of GRP170 to Endo H remains unchanged. Sensitivity of radiolabeled immunoprecipitates to Endo H is also observed under different GRP-inducing conditions such as treatment with ionophore A23187, anoxia, or glucose starvation but not after treatment with tunicamycin or 2-deoxyglucose. This is consistent with the data presented in Figure 2. Again, the coprecipitation of GRP78/BiP is evident in Figure 2 (arrow). Therefore, GRP170 is a glycoprotein that is not further processed in the Golgi, implying its retention in the pre-Golgi, ER.

The localization of GRP170 was studied in mouse embryo 10T1/2 cells using the technique of indirect immunofluorescence under control and stress conditions. It is seen in Figure 6 that GRP170 displays a perinuclear, reticular pattern, characteristic of an ER distribution. The intensity of the fluorescence is clearly increased upon stress induction of this protein (Figure 6, B–D). Treatment of cells with the calcium ionophore A23187 results in severe structural alterations as reflected in the distinct swelling and vacuolization of the ER (Figure 6D). The concentration of this protein in the nuclear envelope is notable in many instances. The increased fluorescence obtained under stress conditions is not seen when various irrelevant rabbit antisera are used. In addition, nonpermeabilized cells did not react with the antisera, and analysis of supernatants from the metabolically labeled IgG-secreting 1G1B6 hybridoma indicated that whereas IgG was detectable in the supernatant, GRP170 was not.

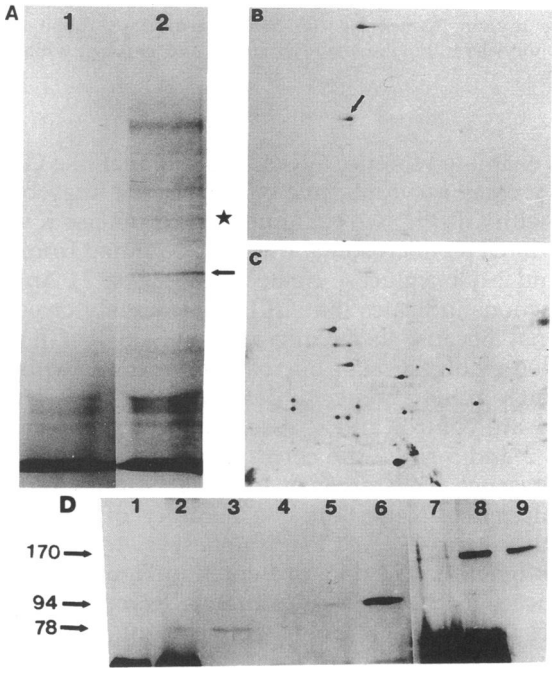

Figure 3. Immunoprecipitation analysis of CHO cells with anti-GRP170 antibody: coprecipitation of GRP78 and GRP94. (A) The immunoprecipitation of A23187, 12 h-treated CHO cells that were pulse labeled and immunoprecipitated with anti-GRP170 (lane 2) or with preimmune serum (lane 1) and analyzed by gel electrophoresis and autoradiography. The coprecipitation of other proteins is visible in lane 2 at 78 kDa (arrow) and 94 kDa (★). (B) A similar analysis of cells treated for 12 h with tunicamycin, pulse labeled, and analyzed by 2-D gel electrophoresis and autoradiography. (C) A parallel gel stained with Coomassie blue. By comparing B and C and Figure 1, the 78-kDa coprecipitated protein (arrow) seen in A, lane 2, is GRP78. Although not clearly evident, a spot migrating in the position of GRP94 was also observed in B. (D) Results of an immunoprecipitation with anti-GRP170 of control (lanes 1, 4, and 7) and A23187-treated (lanes 2, 5, and 8) CHO cells followed by blotting and probing with anti-GRP78 (lanes 1–3), anti-GRP94 (lanes 4–6), or anti-GRP170 (lanes 7–9). Lanes 3, 6, and 9 are blots of lysates of A23187-treated CHO cells. Lanes 2 and 5 again indicate that GRP78 and GRP94 are coprecipitated with GRP170-stressed CHO cells. Left lane: position of GRPs.

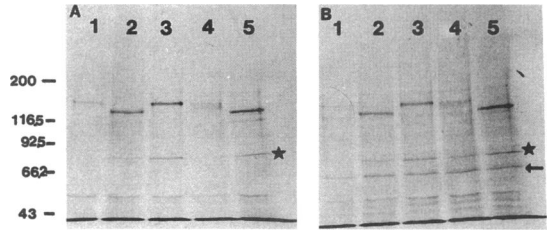

Figure 4. The effect of ATP depletion on coprecipitation of other proteins with anti-GRP170. CHO cells were stressed with 2-deoxyglucose (lane 2), calcium ionophore A23187 (lane 3), glucosamine (lane 4), or tunicamycin (lane 5) for 12 h as described, and untreated cells served as control (lane 1). All treatments were followed by labeling with [^{35}S]methionine for 15 min. Immunoprecipitation was performed in the presence of ATP (A) or apyrase (B). The precipitation of a 60-kDa protein is enhanced upon ATP depletion (arrow, right margin). The coprecipitation of GRP78 is again observed (★). Other than for the 60-kDa protein, the effect of apyrase appears slight. Molecular weight standards in kDa (left margin, top to bottom): rabbit skeletal muscle myosin, *Escherichia coli* beta-galactosidase, rabbit muscle phosphorylase b, bovine serum albumin, and hen egg white ovalbumin.

To examine whether GRP170 is lumenal like GRP78 or expresses a cytoplasmic component as may be the case with GRP94, we examined its Proteinase K sensitivity in CHO microsomal fractions obtained from control and 2-deoxyglucose-treated cells (Figure 7). Analysis of fractions indicates that GRP170 is largely or wholly lumenal, because its gel migration in SDS-PAGE is not affected by digestion of microsomal fractions with Proteinase K in the absence of detergent (Figure 7, lanes 1 and 4 are control and stressed samples, respectively; lanes 2 and 5 show the corresponding precipitation in the presence of detergent). Although the migration is not altered by digestion, the quantity of GRP170 is slightly decreased in the Proteinase K–treated lanes. Taken together, the data presented in Figures 5, 6, and 7 indicate that GRP170 is a lumenal glycoprotein that resides in the ER/cis-Golgi compartment.

Because GRP170 was observed in hamster and mouse cell lines, we decided to examine the distribution of this protein in various mouse tissues. Heart, skeletal muscle, liver, kidney, brain, spleen, lymph node, and Peyer's patch were excised from Balb/c mice, and cell suspensions were prepared, electrophoresed, and transferred for Western blot analysis. The results are presented in Figure 8. To varying degrees, all tissues were positive for GRP170. Liver (Figure 8, lane 4), an organ involved in the secretion of a variety of proteins, showed a large quantity of GRP170.

Because GRP170 was coprecipitated with GRP78/BiP in CHO cells (a protein recognized as an Ig heavy chain binding protein in B lymphocytes) and because of its presence in the lymphoid tissues presented in Figure 8, it was of interest to determine if GRP170 might also exist in B cells and in association with Ig. To pursue this, we examined the association of GRP170 with Ig components in HB-34 cells, a murine mu heavy chain positive, light chain negative B cell line; WEHI-231, a murine membrane IgM-bearing B cell line; NS-1, a murine nonsecreting, light chain positive, heavy chain negative B cell line; as well as 1G1B6, a murine antigen-specific IgG-secreting B cell hybridoma, using the technique of immunoprecipitation with the appropriate anti-Ig antibodies followed by examination of the precipitate for the presence of GRP170. Lysates of HB-34 cells and WEHI-231 cells were immunoprecipitated with anti-IgM antibody and protein A-sepharose (Figure 9A, lanes 3 and 5, respectively); lysates of NS-1 and 1G1B6 cells were immunoprecipitated by addition of anti-IgG antibody-sepharose alone (Figure 9A, lanes 6 and 7, respectively). Results indicate that GRP170 is coprecipitated with the Ig component in all B cell hybridomas examined. Immunoprecipitation of lysates of WEHI-231 cells with *irrelevant rabbit serum* and protein A-sepharose or protein A-sepharose alone that were blotted and probed with GRP170 antibody demonstrated a complete lack of GRP170 signal in the absence of anti-IgM (Figure 9A, lane 4 shows the result obtained by precipitation with the irrelevant serum and protein A sepharose). A corresponding analysis of HB-34 cells using irrelevant serum and protein A-sepharose also failed to precipitate GRP170 (Figure 9A, lane 2). This indicates that anti-IgM antibody is a necessary element in the immuno precipitation required for the coprecipitation of GRP170 in WEHI-231 and HB-34 cells. Figure 9A, lane 1, a positive control, represents a Western analysis of total lysate from ionophore A23187–treated CHO cells showing the migration of GRP170. This analysis indicates that GRP170 can be complexed with either heavy or light chain alone in hybridomas expressing only these com-

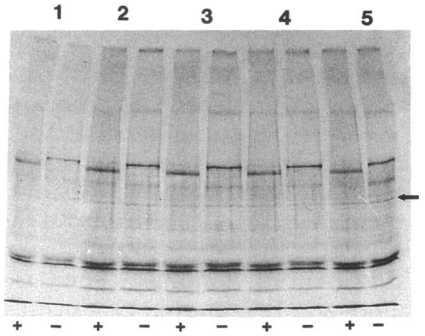

Figure 5. Endo H digestion of pulse-chase labeled anti-GRP170 immunoprecipitates. CHO cells were treated with calcium ionophore A23187 for 12 h as described and pulsed with [^{35}S]methionine for 30 min. After wash, cells were chased with cold medium for the hours as indicated. Lane 1, 0 h; lane 2, 1 h; lane 3, 2 h; lane 4, 3 h; and lane 5, 4 h. The anti-GRP170 immunoprecipitates were treated with Endo H as described in MATERIALS AND METHODS. +, −: indicating the presence or absence of Endo H. GRP170 remains sensitive to Endo H digestion over a period of 4 h, characteristic of proteins that have not traversed the medial/trans-Golgi. Coprecipitated GRP78/BiP is also evident, as indicated by arrow (right margin).

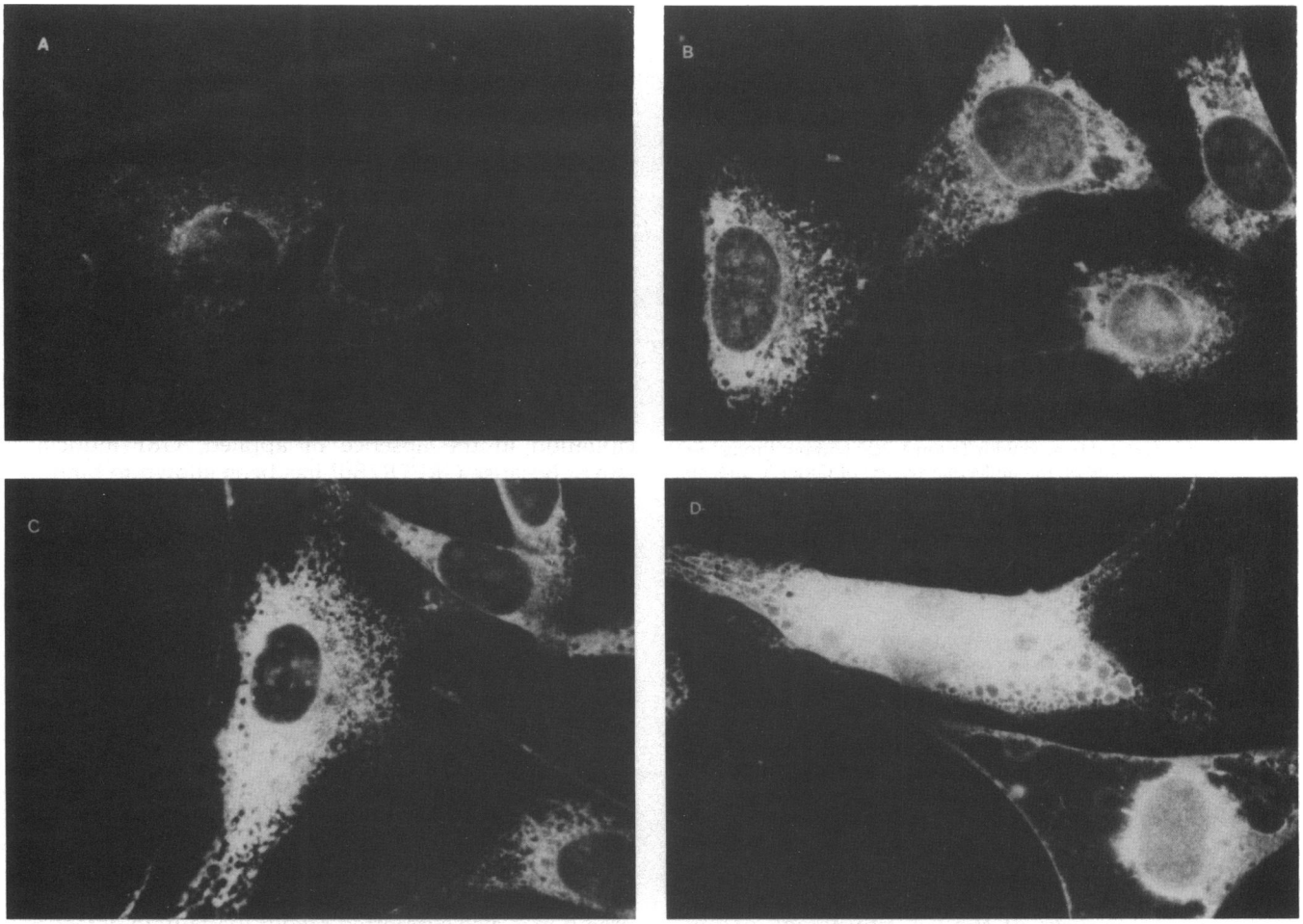

Figure 6. GRP170 induction in stressed cells is evident by immunofluorescence. Mouse embryo fibroblast 10T1/2 cells were treated as follows: A, control; B, 20 h of glucose deprivation; C, 12 h of 2-deoxyglucose; and D, 12 h of A23187. GRP170 is seen in a perinuclear and reticular staining pattern and is increased by stress. A23187 treatment causes structural damage to ER membrane (see text). Photographic exposure times are identical for each panel.

ponents and furthermore that coprecipitation of GRP170 is also obtained in lines producing functional IgG and IgM, indicating that GRP170 can be associated with these Igs and/or their components in each case. It is important to emphasize that this data does not demonstrate association with fully assembled IgG or IgM, because unassembled forms of these Igs will also be present. Figure 9B presents an immunoprecipitation analysis of lysates from the same four lymphoid cell lines with the same antibodies described in Figure 9A, followed by transfer and reaction with an antibody against PDI, an abundant ER-localized protein (Edman *et al.*, 1985). Although PDI is detectable in total cell lysate (WEHI-231 cell lysate, Figure 9B, lane 1), it is not detected as a coprecipitating protein with Ig. Figure 9, C–E presents a series of immunoprecipitation studies using WEHI-231 cells. In Figure 9C, anti-IgM and protein A sepharose immunoprecipitates of WEHI-231 cell lysates are blotted and probed with anti-GRP78 (lane 1), anti-GRP94 (lane 2), or anti-GRP170 (lane 3). This indicates that all three GRPs co-precipitate with IgM in WEHI-231 cells. In Figure 9D, corresponding WEHI-231 cell lysates immunoprecipitated with anti-GRP94 antibody are blotted and probed with anti-GRP78 (lane 1) or anti-GRP170 (lane 2). It is seen here that precipitation of GRP94 coprecipitates both GRP170 and GRP78. Lastly, Figure 9E shows the immunoprecipitation of WEHI-231 cells with anti-GRP170 antibody, followed by transfer and reaction with anti-GRP78 (lane 1) or anti-GRP94 (lane 2). As was seen to be the case in CHO cells (Figure 3), anti-GRP170 coprecipitates both GRP78 and GRP94. Precipitations with anti-GRP78 antibodies could not be performed because all available antibodies to this stress proteins are not readily applicable for such studies.

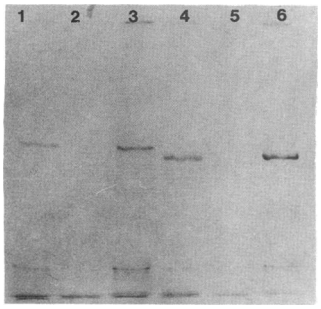

Figure 7. Proteinase K digestion of microsomes does not change the mobility of GRP170. Immunoprecipitates of crude microsomal fractions of control (lanes 1–3) and 2-deoxyglucose–treated (lanes 4–6) CHO cells were subjected to Proteinase K digestion in the presence (lanes 2 and 5) or absence (lanes 1 and 4) of 0.2% Triton X-100. Lanes 3 and 6 are precipitates treated with Triton X-100 without Proteinase K. By SDS-PAGE analysis, the molecular weight of GRP170 appears unchanged, suggesting that GRP170 lacks a cytoplasmic component.

DISCUSSION

When cells are subjected to a variety of conditions, all apparently perturbing the function of the ER/nuclear envelope compartment, the syntheses of two GRPs, GRP78 and GRP94, are greatly enhanced. In addition to these widely recognized GRPs, there is a third species of this stress protein group of ~170 kDa that is also seen to be coordinately upregulated. Although this member of the GRP family of stress proteins has been long observed (Olden *et al.*, 1978; Sciandra and Subjeck, 1983; Shen *et al.*, 1987), the present study describes the first extensive analysis of the cell physiology of this protein. In different respects we find that GRP170 exhibits characteristics that parallel both GRP78 and GRP94. We have found that, like GRP94, GRP170 is a glycoprotein that is Endo H sensitive in a pulse chase study for times <4 h (the longest time interval examined) indicating that it is not further processed in the Golgi and therefore, like GRP78 and GRP94 (in general) remains as a resident ER-localized protein. The ER locale of GRP170 is further suggested by the immunofluorescence patterns presented in Figure 6. An unusual result is seen in Figure 6D that shows that A23187, unlike the other GRP inducers presented in this figure that do not interfere with the reticular structure of this organelle, leads to the structural disintegration of the ER resulting in a vacuolization and in some instances a collapse of ER structure about the nucleus, which is represented by the two cells seen in this figure. That GRP170 appears to be *over and around* the nucleus rather than in the nucleus is indicated by the halo-like image outlining the nucleus as seen in the lower right hand cell in Figure 6D. A disruptive effect of A23187 on the ER network has been described previously in conjunction with the release of GRP94 throughout the cytoplasm (Booth and Koch, 1989). Interestingly, in the present studies with A23187, regardless of the apparent degree of ER destruction, GRP170 is *not* released throughout the cytoplasm but appears to remain closely associated with the ER membrane. However, digestion of microsomal vesicles with Proteinase K does not affect this protein, indicating that in its mature form GRP170 does not express a distinguishable cytoplasmic component. That GRP170 is retained in the ER implies that like the other GRPs it has a specific function in the lumen of the ER/nuclear envelope. What this function might be, can begin to be inferred from the immunoprecipitation studies performed in the present study.

We have described in Figure 3 the coprecipitation of GRP78 and GRP94 with GRP170 in CHO cells during stress. However, we were unable to detect this coprecipitation in the absence of applied, GRP-inducing stress. Because GRP78/BiP has been shown to bind to partially assembled/folded or mutant proteins, does this association in CHO cells represent a stress-related event whereby some structural and/or glycosylation change in GRP170 leads to this interaction? In this instance, this ensemble of GRPs in CHO cells would represent a stress-induced structure without anticipated function at 37°C. This would not seem to be the case, because GRP170 is also found associated with other GRPs and IgM in WEHI-231 cells as well as with Igs in general in other B-cell hybridomas in the *absence* of stress (Figure 9). It is likely that the immunoprecipitation procedure utilized here may interfere sufficiently with protein-protein associations such that, at low protein concentrations (as may be the case in unstressed CHO cells), these interactions become difficult to detect. This interpretation is also supported by our inability to detect an interaction between PDI and Ig (discussed below). In addition, there are some interesting observations in CHO cells that bear on this point. First, exposure of CHO cells to all inducers of GRPs examined here, excepting 2-deoxyglucose and tunicamycin, resulted in *no*

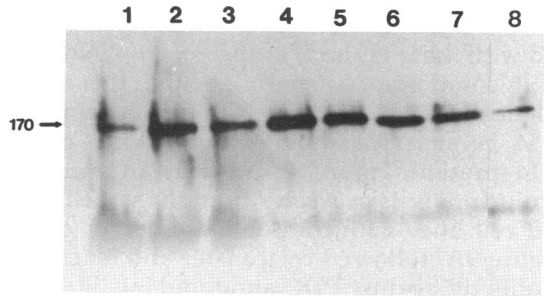

Figure 8. Identification of GRP170 in various murine tissues. Heart (lane 1), skeletal muscle (2), brain (3), liver (4), kidney (5), spleen (6), lymph node (7), and Peyer's patch (8) were excised from Balb/c mice. Cell suspensions were prepared, electrophoresed, and transferred for Western blot analysis using the GRP170 antibody. To varying degrees, GRP170 is present in each tissue. Equal masses of protein were loaded in each lane.

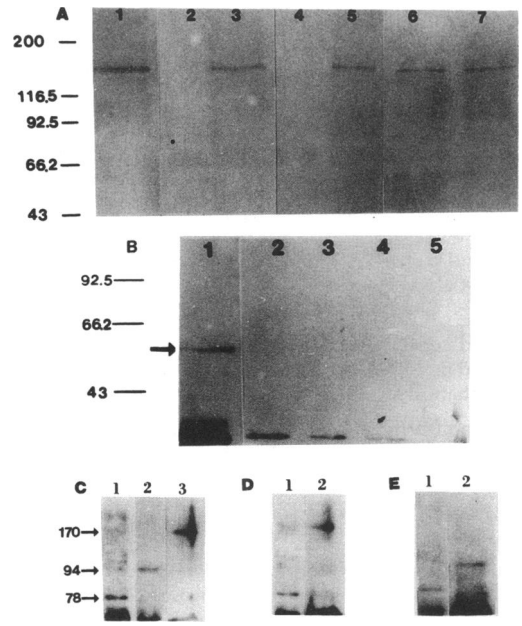

Figure 9. Coprecipitation of GRP170, GRP78 and GRP94, and Ig in B cell lines. (A) Cell lysates were immunoprecipitated with appropriate anti-Ig conjugated to sepharose, electrophoresed, and probed with anti-GRP170 as described. Lane 1, CHO cells treated with 10 μM A23187 as positive control; lane 2, lysate of Ig mu heavy chain positive and Ig light chain negative HB-34 cells immunoprecipitated with irrelevant rabbit serum and protein A sepharose; lane 3, lysate of HB-34 immunoprecipitated with anti-IgM antibody and protein A sepharose; lane 4, lysate of membrane IgM bearing WEHI-231 cells immunoprecipitated with irrelevant rabbit serum and protein A sepharose; lane 5, lysate of WEHI-231 cells immunoprecipitated with anti-IgM and protein A sepharose; lane 6, lysate of Ig light chain positive and Ig heavy chain negative NS-1 cells immunoprecipitated with anti-IgG sepharose; and lane 7, lysate of IgG-secreting 1G1B6 cells immunoprecipitated with anti-IgG sepharose. In all B cell hybridomas examined, GRP170 coprecipitated with anti-Ig. (B) Lysates of HB-34, WEHI-231, NS-1, and 1G1B6 cells (lanes 2–5) were immunoprecipitated as described in A and blotted and probed with antibody against PDI. Lane 1, total lysate control from WEHI-231 cells; lane 2, HB-34; lane 3, WEHI-231; lane 4, NS-1; and lane 5, 1G1B6. PDI is not detectable as a coprecipitate with Ig using the techniques employed here. (C) WEHI-231 cell lysates were immunoprecipitated with anti-IgM antibody and protein A sepharose and blotted and probed with anti-GRP78 (lane 1), anti-GRP94 (lane 2), or anti-GRP-170 (lane 3). In this cell line all three GRPs coprecipitate with IgM. (D) WEHI-231 cell lysates were immunoprecipitated with anti-GRP94 antibody and protein A sepharose and blotted and probed with anti-GRP78 (lane 1) or anti-GRP170 (lane 2). GRP78 and GRP170 coprecipitate with GRP94. (E) WEHI-231 cell lysates were immunoprecipitated with anti-GRP170 antibody and protein A sepharose and blotted and probed with anti-GRP78 (lane 1) or anti-GRP94 (lane 2). GRP78 and GRP94 coprecipitate with GRP170. In each panel, molecular weight standards are as in Figure 4. C, left margin: position of GRPs.

change in either the charge or molecular size of induced GRP170 as determined by the criteria of isoelectric focusing and $NaDodSO_4$, PAGE, suggesting that in conditions such as glucose starvation, there are no obvious changes that might trigger the observed binding by GRP78. Second, the association of GRP170 with GRP78 in CHO cells is not notably altered by the presence or absence of ATP, suggesting that this interaction is not stress related and that it occurs on region of GRP78 distinct from that responsible for the binding of damaged or unglycosylated proteins. These GRP ensembles may perform a function in stressed CHO cells that is probably similar to the function they perform in WEHI-231 cells, i.e., presumably involving the assembly of IgM. The increase in quantity of ensembles seen in stressed CHO cells, relative to unstressed CHO cells, may have as substrates *other* proteins/ER structures that are directly affected or damaged in some manner by the GRP-inducing stress.

Associations between GRP78 and Ig, GRP94 and Ig, and between GRP78 and GRP94 have already been described (Bole *et al.*, 1986; Hendershot *et al.*, 1987; Melnick *et al.*, 1992) and the present study adds GRP170 to this composite. Conversely, it is also interesting that another major ER protein known to be required for Ig assembly, PDI, fails to detectably coprecipitate with Ig in the four B cell hybridomas examined because it has been shown previously that PDI can be cross-linked in vivo to Igs (Roth and Pierce, 1987). This would indicate that the complex between PDI and Ig is simply not being detected by the approach used here for identifying protein associations. This also suggests that the associations between GRPs and Ig differs in some manner from the association between PDI and Ig, perhaps reflective of a different half-life of association in Ig assembly. Finally, an unidentified protein of 60 kDa was observed in the present study to be coprecipitated with anti-GRP170 antibody in CHO cells in the presence of apyrase. This unique coprecipitation in the presence of apyrase may be reflective of the ATP dependent function of this 60-kDa protein itself, rather than of GRP170.

The 78- and 94-kDa GRPs described in this study are members of the 70- and 90-kDa heat shock gene families (Munro and Pelham, 1986; Mazzarella and Green, 1987), respectively, and available evidence indicates that members of each family express analogous functions and physico-chemical characteristics (Lindquist and Craig, 1988; Hartl *et al.*, 1992). GRPs do, however, differ from the heat shock proteins, which are nuclear/cytoplasmic proteins, in that they appear to be localized in the ER and nuclear envelope. There are numerous reports on GRP78 and its role in transport, folding, and assembly of proteins in the ER (Hass and Wabl, 1983; Bole *et al.*, 1986; Gething *et al.*, 1986; Hendershot *et al.*, 1987). GRP94 has also been found to be associated with both free light or heavy chains as well as with GRP78 in a B-cell myeloma (Melnick *et al.*, 1992). In the present study we have shown that GRP170 is observed in all murine tissues examined, including a variety of lymphoid tissues. Moreover, like GRP78 and GRP94, GRP170 also associates with free light and heavy chains as well as with Ig or *subcomponents thereof* in B-cell hybridomas producing fully assembled Ig. We have also

shown that GRP170 is directly or indirectly associated with GRP78 and GRP94 in stressed CHO cells as well as in the WEHI-231 B cell line under normal growth conditions. Because GRP78 and GRP94 are two molecular chaperones known or considered (in the case of GRP94) to be involved in Ig processing, the present data implies that GRP170 may also function as a molecular chaperone affecting the processing of other proteins traversing the ER. The precedents for heat shock proteins interacting together in protein folding and assembly in the mitochondria have been well described (reviewed in Gething, 1991). It is possible that these three glucose regulated proteins, GRP78, GRP94, and GRP170, interact together in an analogous manner, either simultaneously or sequentially, comprising a protein processing pathway in the ER. Alternatively, GRP170 may exhibit function(s) overlapping those of GRP78 and/or GRP94, perhaps replacing one or both chaperons in some circumstances. Similarities in associations, regulation, and localization of GRP170 with GRP78 and GRP94 suggests that GRP170 may represent a new and previously unstudied component of the protein processing pathway in the ER.

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