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Association of the 94 KDa Glucose-regulated Protein with Immunoglobulin Heavy Chain Binding Protein (BiP)

Ho Sung Kang and Han Do Kim

Department of Molecular Biology, College of Natural Sciences, Pusan National University, Pusan 609-735, Korea

The 94 KDa glucose-regulated protein (grp 94), one of stress proteins, is a Ca^{2+}-binding protein in the endoplasmic reticulum (ER). In this study, the possible effect of Ca^{2+} on the native conformation of grp 94 was examined. When the purified grp 94 was analyzed by gel filtration in the presence of either EGTA or $CaCl_2$, it was eluted with apparent molecular weight (MW) of 100 KDa in both cases. When similarly analyzed with microsome or cell lysate, however, grp 94 was eluted with apparent MW of 200 KDa in the presence of EGTA, while with apparent MW of 100 KDa in the presence of $CaCl_2$, indicating possible association of grp 94 with one or more other proteins in the absence of $CaCl_2$. Consequently, immunoprecipitation with anti-grp 94 was carried out to determine which proteins specifically interact with grp 94. It is shown that grp 94 may interact, in a Ca^{2+}-dependent manner, with other proteins including BiP (grp 78) which is also a stress protein in the ER.

KEY WORDS: 94 KDa glucose-regulated protein, Ca^{2+}-binding protein, BiP (grp 78)

Cells highly induce the syntheses of a group of proteins, referred to as stress proteins or heat shock proteins (hsp's) when confronted with adverse changes in their environment such as increase in the growth temperature (Ashburner and Bonner, 1979; Craig, 1985; Lindquist, 1986). Most of hsps, however, are also expressed in considerable amounts in the unstressed, normal cells. This suggests hsps may play a role(s) in the very basic and essential function of normal cells. In fact, hsp 70 family members, hsp 72/73 and immunoglobulin heavy chain binding protein (BiP/grp 78) function as molecular chaperones which monitor and regulate *in vivo* folding and assembly of most, if not all, proteins (Rothman, 1989; Welch *et al.*, 1991; Goething and Sambrook, 1992). For example, hsp 72/73 interacts with cytosolic polypeptides which are being synthesized in the ribosome or which translocate across the membranes of mitochondria and yeast endoplasmic reticulum (ER) (Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Beckmann *et al.*, 1990). On the other hand, BiP (grp 78), which has sequence homology to hsp 72/73 but resides in the ER, interacts with membrane or secretory proteins such as immunoglobulin heavy chain (Bole *et al.*, 1986; Goething *et al.*, 1986; Blount and Merlie, 1991). In both cases, hsp 72/73 or BiP assist the folding and unfolding event of polypeptides in an ATP-dependent manner (Beckmann *et al.*, 1990; Flaherty *et al.*, 1990; Showra *et al.*, 1990). Hsp 90 family, another major class of hsps, also play a crucial role(s) in many aspects of cellular functions. Hsp 90 is an abundant cytosolic protein which has been identified as a component of most steroid hormone receptor complexes as well as being involved in the transport and/or regulation of various protein kinases (Catelli *et al.*, 1985;

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Sanchez *et al.*, 1985). Recent evidence has shown that hsp 90 also functions as a molecular chaperone (Picard *et al.*, 1990).

The 94 KDa glucose-regulated protein (grp 94) has amino acid sequence similar to hsp 90 but is present in the ER (Sargan *et al.*, 1986; Mazzarella and Green, 1987). The synthesis of grp 94 is enhanced concomitantly with BiP (grp 78) in the cells which are treated with Ca^{2+} ionophore or tunicamycin (Thomas *et al.*, 1982; Welch *et al.*, 1983; Lee, 1992). Grp 94 is a glycoprotein but the unglycosylated grp 94 is also accumulated by treatment of tunicamycin (Poussegur *et al.*, 1977). In contrast to data demonstrating the role of hsp 90 in various cellular functions, nothing is known about the biological function of grp 94. It is suspected that it may participate in the event of *in vivo* protein folding and assembly as most hsps do. There is also uncertainty about the topology of grp 94 within ER. Grp 94 contain a hydrophobic stretch composed of 20 hydrophobic amino acids, indicating that it might be a transmembrane protein (Mazzarella and Green, 1987). It was also suggested that grp 94 might be a lumenal protein since it has an ER lumen retention sequence, Lys-Asp-Glu-Leu (KDEL) (Munro and Pelham, 1986; Koch *et al.*, 1986). Previously we have provided some biochemical evidence that it could exist in two configurations within the ER (Kang and Welch, 1991).

It was shown previously that grp 94 is a major Ca^{2+} binding protein in ER (Van *et al.*,1989; Kang and Welch, 1991). In this study, the possible effect of Ca^{2+} on the native conformation of grp 94 was examined. It is shown that grp 94 associates, in a Ca^{2+}-dependent manner, with other proteins including BiP (grp 78).

Materials and Methods

Cell culture and metabolic labeling

HeLa cells were grown in Dulbecco's modified Eagles medium (DMEM) or Jocklick's modified DMEM plus 10% calf serum. For ^{35}S-methionine labeling, the cells were washed in methionine-free DMEM once and labeled in methionine-free DMEM plus ^{35}S-methionine supplemented with 2% dialyzed calf serum. The cells were harvested in appropriate buffers for next experiments.

Purification of microsomes and heat shock proteins

For the purification of microsomes, HeLa cells were washed with cold phosphate-buffered saline (PBS) and swelled in cold hypotonic buffer (10 mM Tris-acetate, pH 7.4). After homogenization, cell lysate was immediately adjusted to 0.25 M sucrose, 1 mM $MgCl_2$ and centrifuged at 1,000 $\times$ g for 10 min at 4°C. The supernatant was centrifuged at 10,000 $\times$ g for 20 min and the 10, 000 $\times$ g supernatant was centrifuged at 100,000 $\times$ g for 90 min at 4°C. The 100,000 $\times$ g pellet, representing microsomes, was resuspended in buffer M (10 mM Tris-acetate, pH 7.4, 0.25 M sucrose and 1 mM EDTA) and solubilized by gentle dounce homogenization. Grp 94, hsp 90, grp 75, BiP (grp 78), and hsp 72/73 were purified from HeLa cells as described previously (Welch and Feramisco, 1982 and 1985; Mizzen *et al.*, 1989; Kang and Welch, 1991).

One dimensional and two dimensional gel electrophoresis

The cells were harvested and solubilized in SDS-Laemmli sample buffer. The analysis of proteins was done by either one dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) or by two dimensional gel electrophoresis employing pH 5-7 (80%), 3-10 (20%) ampholines in the isoelectric focusing dimension, and 12.5% SDS-PAGE in the second dimension (Laemmli, 1970; Garrels, 1979), followed by fluorography.

Cross-linking of microsomal proteins

For the cross-linking experiment, microsomes in buffer M were treated with varying amounts of dithiobissuccinimidyl propionate (DSP) and incubated for 7 min at room temperature and then the reaction terminated by adding glycine at a final concentration of 100 mM. The cross-linked proteins were boiled in SDS-Laemmli sample buffer with or without β-mercaptoethanol and the proteins were analyzed by SDS-PAGE.

Gel filtration analysis

For gel filtration studies, cell lysates, crude microsomes, or purified grp 94 were solubilized in buffer BT (20 mM Tris-acetate, pH 7.4, 20 mM NaCl, 0.1 mM EDTA, 10 mM β-mercaptoethanol and 1% Triton X-100) and immediately adjusted to either 5 mM EGTA or 3 mM $CaCl_2$, clarified by centrifugation at 25,000 × g for 20 min at 4°C and then applied to an Ultrogel ACA-34 column (1.2 × 90 cm) equilibrated in buffer BT containing either 5 mM EGTA or 3 mM $CaCl_2$. The column was developed in buffer BT (plus EGTA or $CaCl_2$), 100 fractions were collected and the proteins eluting off the column were analyzed by SDS-PAGE. Calibration of the column to determine relative stokes radius was done by using standard proteins.

Western blotting and immunoprecipitation

Antibodies for Western blotting and immunoprecipitation included a monoclonal anti-grp 94, 9G10, provided by Dr. Edwards and polyclonal anti-grp 94 which was prepared in the rabbit against purified grp 94 as described previously (Kang and Welch, 1991). Other antibodies such as anti-hsp 72/73 (N27) and anti-hsp 90 were provided by Dr. Welch (U. of California, S.F.).

Western blotting was done essentially as described by Towbin *et al.* (1979). The proteins were separated by SDS-PAGE and transferred to nitrocellulose paper. After incubation in 3% BSA in PBS, the paper was treated with appropriate antibody and then alkaline phosphatase-conjugated secondary antibody. After extensive washing, color development of the blots was performed.

For the immunoprecipitation, the labeled cells were solubilized in RIPA (+) buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS in PBS). Following preabsorption in protein A-Sepharose at 4°C for 30 min, the cell lysate was incubated in appropriate antibody. After 2 hour incubation at 4°C, the immune complexes were captured by protein A-Sepharose. The immunoprecipitate was washed with RIPA (+) 5 times and the proteins were then released by boiling in Laemmli sample buffer and analyzed by SDS-PAGE and fluorography.

Ca^{2+} overlay technique

Analysis of Ca^{2+}-binding of grp 94 and other hsps was carried out as described by Kang and Welch (1991). The proteins were analyzed by SDS-PAGE and then transferred to nitrocellulose paper. The nitrocellulose paper was washed in 10 mM Tris-acetate, pH 7.4, 50 mM NaCl and 5 mM $MgCl_2$ and then washed in 10 mM Tris-acetate, pH 7.4, 50 mM NaCl. The paper was overlaid with $^{45}CaCl_2$ in the above buffer for 15 min at room temperature. The radiolabel was removed, the paper washed extensively with the buffer, and then washed with 50% ethanol. After drying the paper was placed on film. Following autoradiography, the position of proteins on the nitrocellulose paper was revealed by amido black staining.

Results and Discussion

The synthesis of grp 94 is enhanced in the cells which are exposed to Ca^{2+} ionophore (A23187) preventing intracellular Ca^{2+} homeostasis (Welch *et al.*, 1983). Grp 94 has highly negative charged amino acids and resides in the ER which is known to be an intracellular Ca^{2+} store (Munro and Pelham, 1986). As shown in Fig. 1 and demonstrated previously (Van *et al.*, 1989; Kang and Welch, 1991), grp 94 and its cytosolic counterpart, hsp 90, bind $^{45}Ca^{2+}$ (lanes 6-7). On the other hand, hsp 70 family proteins, hsp 72/73, grp 78 (BiP) and grp 75 (dnaK homolog) did not bind $^{45}Ca^{2+}$ (lanes 8-10). There are small set of Ca^{2+}-binding proteins in the intracellular organelles (lanes 1-5). The microsomal Ca^{2+}-binding proteins were similar to the ER Ca^{2+}-binding proteins reported by Van *et al.* (1989). Grp 94 was one of major Ca^{2+}-binding proteins in the microsome which include the ER, a Ca^{2+} store.

Many Ca^{2+}-binding proteins such as calmodulin and troponin C are known to exhibit conformational changes in the presence of Ca^{2+}. Gel filtration analysis was adopted to examine if Ca^{2+} cause conformational changes of grp 94. Purified grp 94 was analyzed by gel filtration in

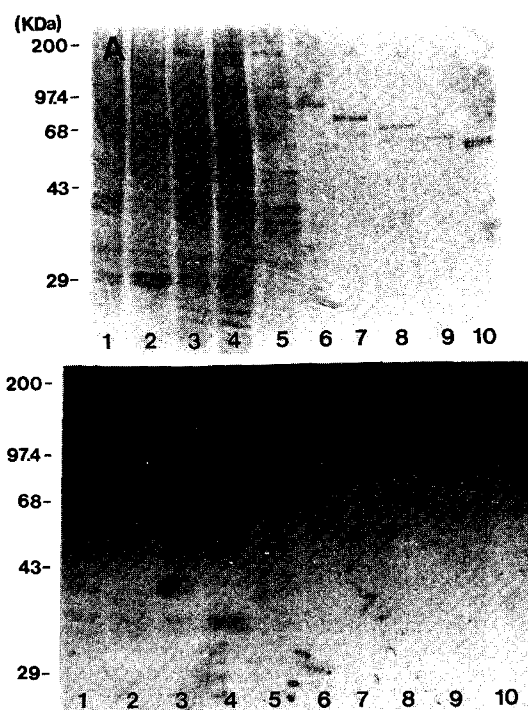

Fig. 1. Ca^{2+}-binding of grp 94 and other stress proteins. Grp 94, hsp 90, BiP (grp 78), grp 75 (dnaK homolog) and hsp 72/73 were purified as described previously (Welch and Feramisco, 1982 and 1985; Mizzen *et al.*, 1989; Kang and Welch, 1991). For the subcellular fractionation, HeLa cells were washed with cold PBS and swelled in cold hypotonic buffer (10 mM Tris-acetate, pH 7.4). After homogenization, subcellular fractionation was carried out as described in Materials and Methods. The proteins were analyzed by SDS-PAGE and then transferred to nitrocellulose paper and Ca^{2+}-binding assay was performed (Panel B). Following autoradiography, the position of proteins on the nitrocellulose paper was revealed by amino black staining (Panel A). HeLa cell lysate (lane 1), 1,000 × g pellet (land 2), 10,000 × g pellet (lane 3), 100,000 × g pellet (land 4), 100,000 × g supernatant (lane 5), grp 94 (lane 6), hsp 90 (lane 7), BiP (grp 78; lane 8), grp 75 (lane 9) and hsp 72/73 (lane 10).

the presence of either 5 mM EGTA or 3 mM $CaCl_2$. In both cases, grp 94 was eluted in a similar fashion, with stokes radius 45Å and apparent molecular weight (MW) of 100 KDa (data not shown). However, different results were obtained in the gel filtration analysis using cell lysates or microsomes as a starting material. Microsomes were treated by either 5 mM EGTA or 3 mM $CaCl_2$ and then applied to gel filtration columns. The eluted proteins were separated by SDS-PAGE and the position of grp 94 determined by Western blotting using anti-grp 94. The position of hsp 72/73 and hsp 90 was also examined by Western blotting using anti-hsp 72/73 (N27) and anti-hsp 90. As shown in Fig. 2, most polypeptides exhibited no differences with respect to their elution profile by treatment of $CaCl_2$. As shown in Fig. 3, hsp 90 and hsp 70 were eluted with apparent MW of 180 and 140 KDa, respectively, which represent homodimeric states as reported by Welch and Feramisco (1982). The gel filtration profile of hsp 72/73 and hsp 90 did not change as a function of Ca^{2+}. However, as shown in Figs. 2 and 3, the elution profile of grp 94 were different according to the presence of either EGTA or $CaCl_2$. Grp 94 was eluted relatively earlier off the column in the presence of EGTA (peak fractions 16 to 28) than in the presence of $CaCl_2$ (peak fractions 32 to 56). Grp 94 were eluted in the presence of EGTA with stokes radius 70Å and apparent MW of 200 KDa, whereas eluted in the presence of $CaCl_2$ with stokes radius 45Å and apparent MW of 100 KDa. Since no significant changes as a function of Ca^{2+} were observed with the purified grp 94, we suspect that in the presence of EGTA, where grp 94 elutes earlier off the sizing column, the protein may be interacting with one or more other proteins thereby influencing its apparent size. In the presence of Ca^{2+}, such interaction may not occur or be reduced.

In vitro cross-linking experiment was carried out to test if grp 94 physically associates with other proteins. Microsome was purified from HeLa cells and treated with varying amounts of a cleavable cross-linking agent, DSP. The cross-linked samples were boiled in SDS-Laemmli sample buffer with or without β-mercaptoethanol. The proteins were analyzed by 5% SDS-PAGE and Western blotting using anti-grp 94. As shown in Fig. 4, cross-linked grp 94 was heterogenous and has MW's higher than 200 KDa, indicating again the possible association of grp 94 with other proteins.

To determine further which proteins specifically interact with grp 94, immunoprecipitation with

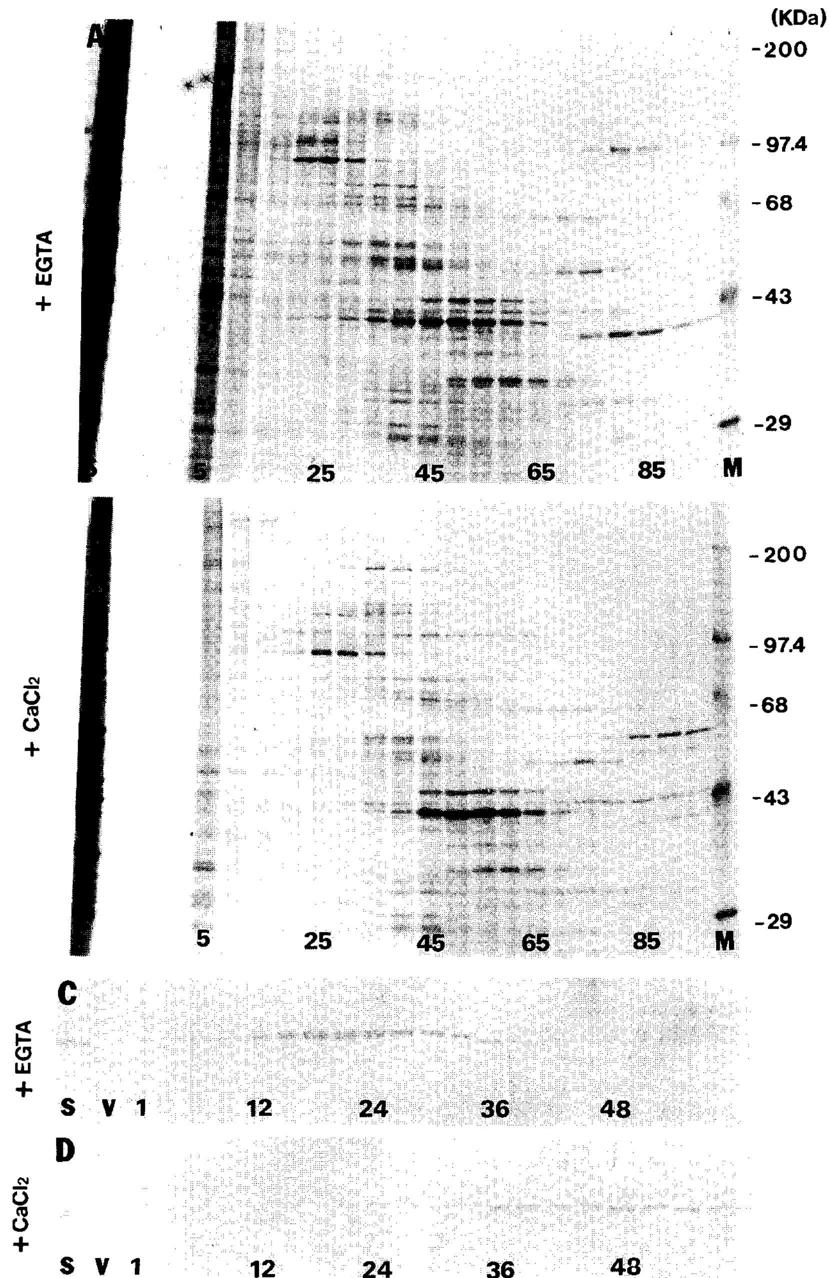

Fig. 2. Effects of $CaCl_2$ and EGTA on the gel filtration profile of grp 94. Microsomes were prepared from HeLa cells, solubilized in the buffer BT and immediately treated by either 5 mM EGTA or 3 mM $CaCl_2$ and applied to an ACA-34 gel filtration column. The column was developed in buffer BT containing either 5 mM EGTA (Panels A and C) or 3 mM $CaCl_2$ (Panels B and D). 100 fractions were collected and an aliquot of fractions analyzed by SDS-PAGE and Western blotting using anti-grp 94. Shown in panels A and B are the Coomassie blue stained gels and in panels C and D the corresponding Western blots using the monoclonal anti-grp 94 (9G10). Lane S represents the crude microsomal extract applied to the column, lane V represents void volume and the numbers below represent the fractions eluting off the columns. Shown in lane M is molecular weight markers (in descending order are myosin (200 KDa), phosphorylase b (97.4 KDa), bovine serum albumin (68 KDa), ovalbumin (43 KDa) and carbonic anhydrase (29 KDa)).

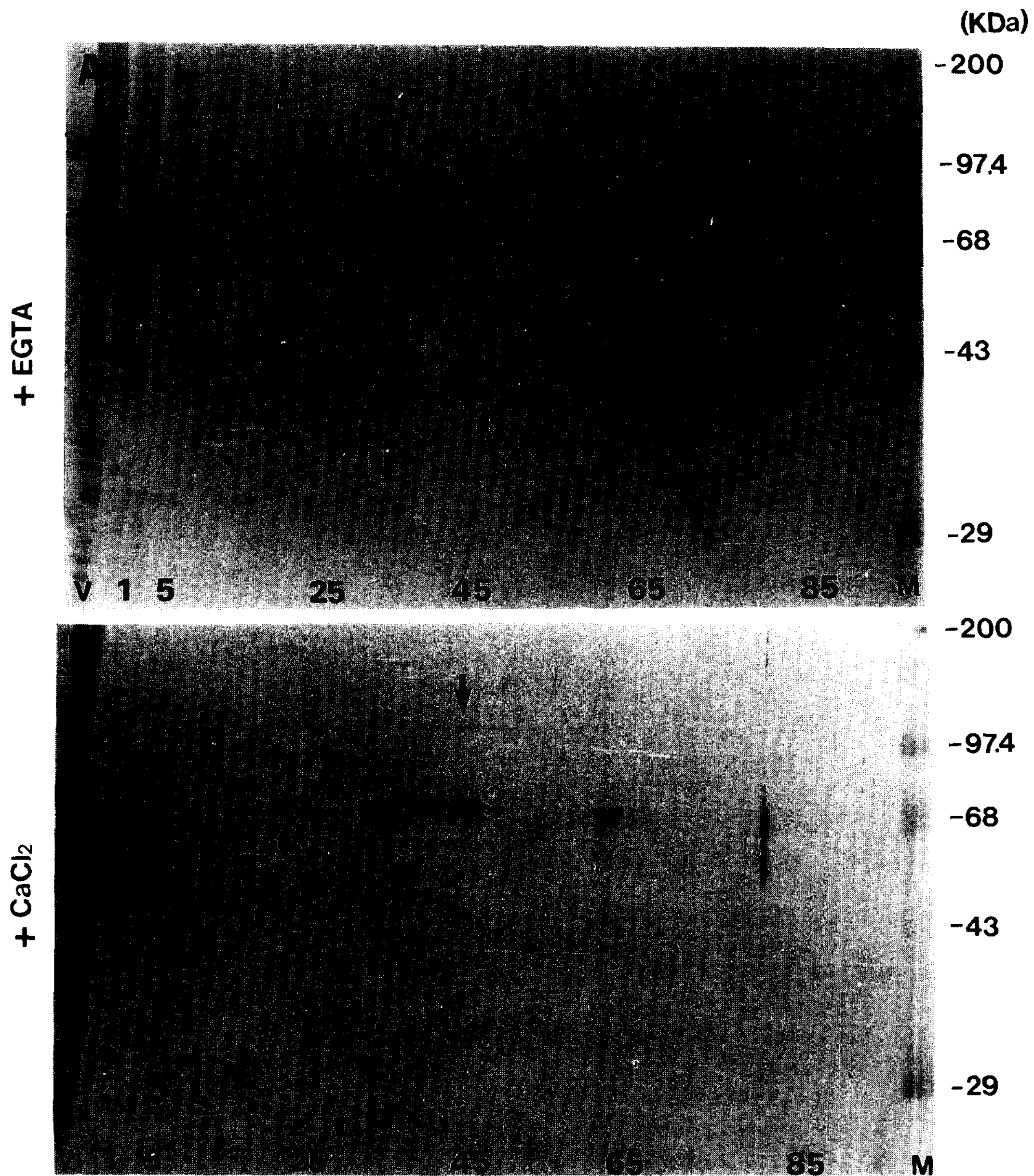

Fig. 3. Comparision of gel filtration elution profiles of grp 94, hsp 72/73 and hsp 90. As described in the legend of Fig. 2, microsomal proteins were analyzed by gel filtration in the presence of either EGTA (Panel A) or $CaCl_2$ (Panel B). An aliquout of every fifth fraction was analyzed by SDS-PAGE and the positions of grp 94, hsp 90 and hsp 72/73 (indicated by thick arrow, arrow head, and thin arrow, respectively) determined by Western blotting using a mixture of anti-hsp 90, anti-hsp 72/73 and anti-grp 94. Lane V represents void volume and the numbers below represent the fractions eluting off the columns. Shown in lane M is molecular weight markers.

anti-grp 94 was performed. The HeLa cells were preincubated in the either presence or absence of tunicamycin which can induce the synthesis of grp 94 and BiP (Poussegur *et al.*, 1977; Mizzen *et al.*, 1989). Then the cells were labeled in ^{35}S-methionine and lysed in the RIPA (+) buffer which

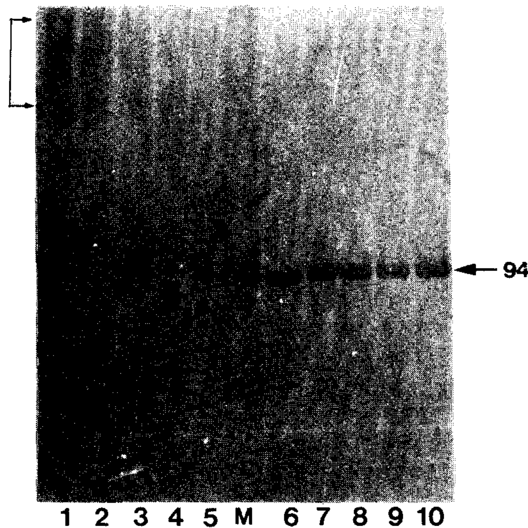

Fig. 4. Cross-linking of microsomal proteins. For the cross-linking experiment, microsomes purified from HeLa cells were incubated in buffer M with DSP at concentrations of 2 (lanes 1 and 6), 4 (lanes 2 and 7), 6 (lanes 3 and 8), 8 (lanes 4 and 9) and 10 mM (lanes 5 and 10) for 7 min and the reaction terminated by addition of 100 mM glycine. The cross-linked proteins were boiled in Laemmli sample buffer without (lanes 1-5) or with (lanes 6-10) β-mercaptoethanol and the proteins analyzed by 5% SDS-PAGE and Western blot using anti-grp 94. Shown in lane M is molecular weight markers. Unreduced and reduced grp 94 are indicated by thin and thick arrow, respectively.

does not disrupt the specific interaction between proteins. The resulting cell lysate was immunoprecipitated with either monoclonal anti-grp 94 (9G10) or polyclonal anti-grp 94, which both were previously shown to be highly specific to grp 94 (Kang and Welch, 1991) and shown in Figs. 2 and 3 in this study. As expected, treatment of tunicamycin to cells results in the induction of unglycosylated grp 94 (Fig. 5, indicated by arrow head). The glycosylated and unglycosylated grp 94 were precipitated with either monoclonal or polyclonal anti-grp 94. As shown in Fig. 5, a number of proteins were specifically coprecipitated with grp 94, although some of coprecipitated proteins were sightly different according to types of used antibodies. Thus, grp 94 appears to associate with other proteins. However, few proteins were coprecipitated with grp 94 when cells were lysed in the presence of $CaCl_2$ (data not shown), indicating that the association of grp 94 with other proteins is Ca^{2+}-dependent. Among polypeptides interacting with grp 94, of particular interest was the 80 KDa protein (indicated by thin arrow) which has a similar MW to BiP, another ER stress protein, and was more coprecipitated with unglycosylated grp 94. To examine whether the 80 KDa protein is BiP (grp 78), ATP-agarose binding assay was performed with an aliquout of cell lysates used in the immunoprecipitation, since BiP is one of ATP-agarose binding hsp 70 family proteins (Welch and Feramisco, 1985). As shown in Fig. 5, BiP (grp 78), grp 75 and hsp 72/73 (indicated thin arrow, open arrow head and asterisk, respectively) were shown to be induced by treatment of tunicamycin. The 80 KDa protein was shown to comigrate with BiP in SDS-PAGE, indicating it may be a BiP. To further confirm their identity, the immunoprecipitates were analyzed by two dimensional gel electrophoresis. As shown in Fig. 5 (Panels B and C), the 80 KDa proteins has same isoelectric point as that of BiP. Superimposition of autoradiograms showed that the overall migration of the 80 KDa was same as that of BiP. Based on these results, we suggest that grp 94 specifically associates, in a Ca^{2+} dependent manner, with BiP and other proteins.

The biological role of the complex of grp 94/BiP/other proteins is not yet understood. However, two explanations are possible. First, grp 94 might be a component of molecular chaperone complex along with BiP. Some molecular chaperones are known to form a large complex. For example, hsp 72/73 physically associates with complex of hsp 90 and steroid hormone receptor, although the precise role of hsp 72/73 is not clear (Sanchez *et al.*, 1990). Grp 94 might function with BiP to assist the folding and assembly of polypeptides, since it is induced concomitantly with BiP by the accumulation of unfolded proteins in the ER and has sequence homology to hsp 90, a molecular chaperone. However, since the unglycosylated grp 94 was more associated with BiP (Fig. 5), another explanation is also possible that grp 94 might be a substrate of BiP, which interacts with unglycosylated, misfolded proteins. It remains to determine the exact biological function

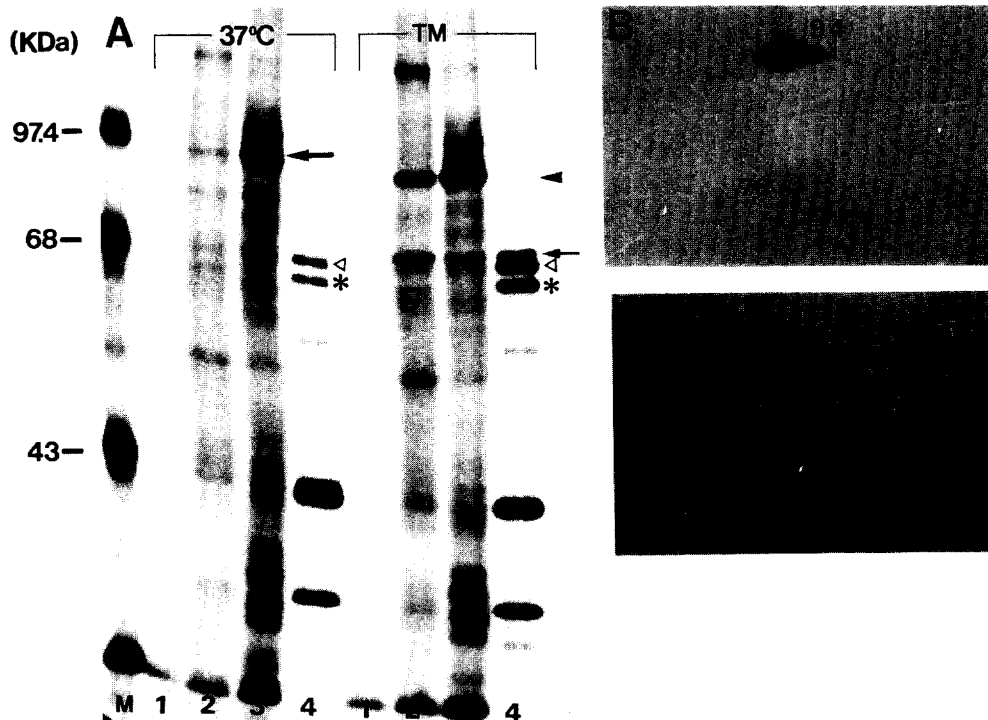

Fig. 5. Immunoprecipitation of ^{35}S-methionine labeled proteins with anti-grp 94. HeLa cells were grown at 37°C in DMEM containing 10% calf serum. The cells were then exposed to normal growth medium (37°C) or 3 μg/ml tunicamycin (TM) for 3 hours. The cells were then labeled with ^{35}S-methionine either in the absence or presence of tunicamycin for 6 hours. Following labeling, the cells were lysed in RIPA (+) buffer and the resulting cell lysates were then immunoprecipitated with no antibody (lane 1), monoclonal anti-grp 94 (9G10, lane 2) or polyclonal anti-grp 94 (lane 3). In parallel, an aliquout of labeled cell lysates was incubated with an excess of ATP-agarose to bind hsp 70 family proteins, hsp 72/73, grp 75, BiP (grp 78) according to Welch and Feramisco (1985) (lane 4). The resulting immunoprecipitates and ATP-agarose binding proteins were analyzed by SDS-PAGE and fluorography. Shown in Panels B and C are the two dimensional gel electrophoretic patterns of immunoprecipitates (Panel A, lane 2) of 37°C and TM samples, respectively. Glycosylated grp 94, unglycosylated grp 94, BiP (grp 78), grp 75 and hsp 72/73 are indicated by thick arrow, arrow head, thin arrow, open arrow head and asterisk, respectively. Shown in lane M is molecular weight markers (in descending order are phosphorylase b (97.4 KDa), bovine serum albumin (68 KDa), ovalbumin (43 KDa)).

of the complex of grp 94/BiP/other proteins.

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94 KDa Glucose-regulated Protein의 BiP과의 결합

강호성 • 김한도(부산대학교 자연과학대학 분자생물학과)

stress proteins의 일종인 94 KDa glucose-regulated protein(grp 94)는 소포체의 Ca^{2+}-binding protein이다. 본 연구에서는 grp 94의 구조에 미치는 Ca^{2+}의 영향을 조사하였다. 순수분리한 grp 94에 Ca^{2+}이나 EGTA를 첨가하여 gel filtration으로 분석한 결과, grp 94는 분자량이 100 KDa되는 부분에서 유출되었다. 그러나 cell lysate나 microsome을 gel filtration으로 분석한 결과, EGTA가 첨가된 경우 grp 94는 분자량이 200 KDa되는 부분에서, Ca^{2+}이 첨가된 경우는 분자량이 100 KDa되는 부분에서 유출되었다. 이는 Ca^{2+}이 없을때 grp 94가 다른 단백질과 결합한다는 것을 시사하므로, 이를 immunoprecipitation 실험으로 조사하였다. 본 연구에서 grp 94는 다른 단백질과 Ca^{2+}의존성 결합을 하며, 소포체의 또 다른 stress protein인 BiP과 결합한다는 것을 시사하는 결과를 보인다.