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Overexpression of ER Resident Molecular Chaperones and Characterization of Their Interaction with Thyroglobulin in FRTL5 cells.

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Abstract

Mammalial expression vectors containing GRP94, BiP, ERp72, and PDI, were introduced into FRTL5 cells. Transfected cells were selected by neomycin resistance for exogenously overexpressed proteins in the ER. The use of a reducible cross-linker, DSP, markedly improved the ability to detect noncovalent interactions of PDI, BiP and GRP94 with newly-synthesized thyroglobulin. Under normal conditions, GRP94 was found to associate transiently with early Tg folding intermediates, displaying interaction kinetics similar to those reported for another ER chaperones of BiP.

Key words : *GRP94, BiP, ERp72, PDI, thyroglobulin*

Introduction

Cells from all kind of organisms respond to various metabolic stimulates such as temperature, heavy metals, chemical drugs and several kind of environmental factors and then increase the expression of stress proteins to protect cells through their functions in which they bind to adhesive sites on newly synthesized or stress damaged and partially unfolded structure and functional protein[22]. In the normal unstressed cell, various constitutively expressed stress proteins facilitate, as molecular chaperone, the synthesis, folding, and assembling of intracellular proteins[4]. This action prevents the formation of function-less aggregates. Damaged proteins are either presented for degradation or reconstituted by orderly disengagement from the stress protein[7]. The quality control system of proteins in the endoplasmic reticulum (ER) consist of ER resident molecular chaperones, GRP94 (a member of HSP90 family, also known as gp96 or endoplasmin), BiP (a member of HSP70 family, also known as GRP78), ERp72 (a member of HSP70 family), and a folding enzyme of PDI (protein disulfide isomerase)[3,17]. These proteins are surprisingly well conserved intracellular proteins from the mammalian to bacteria[1]. In addition to ensuring efficient folding and transport of many secretory proteins, these ER resident chaperones are postulated to perform other essential functions such as calcium regulation, ER retention and/or degradation of misfolded proteins[2,25]. Despite their obvious physiological significance in the cell, the main functions of each ER resident molecular chaperone are now becoming more clearly defined.

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The production of thyroid hormone requires efficient synthesis, folding, and assembly of its prohormone precursor, thyroglobulin (Tg), into a functional homodimer[10,11]. In thyrocytes, newly-synthesized Tg in the ER initially forms aggregates which are dissociated into monomers and then re-assembled to dimers before transport from the ER to Golgi complex associated with molecular chaperones[12-14]. In the thyrocytes isolated from a mouse showing hypothyroidism, it is also demonstrated that ER resident molecular chaperones thigtly bind to the misfolded Tg[15]. However, the function of this binding was not elucidated. Using Tg as a model protein, we have overexpressed each of the four kind of ER resident molecular chaperones in FRTL5 cells by liposome-mediated transfection in order to assess the specific function of these ER resident molecular chaperones. We have screened the cell colonies showing neomycin-resistance by Western blot analyses, and isolated moderate overexpressors of GRP94, Bip, ERp72 and PDI. In order to examine possible association of discrete Tg folding intermediates with the overexpressed GRP94, DSP was used as a reducible cross-linker which makes disulfide interaction between protein and protein *in vivo*. Our pulse-radiolabeling data from different chase times suggest that GRP94 associates directly and transiently with Tg during its biosynthesis like a molecular chaperone Bip.

Materials and Methods

Cell culture

The rat thyroid cell line FRTL5 was cultured in Coon's media containing 5% calf serum and the 4 hormones (10^{-9} M TSH, 5 $\mu g/m\ell$ transferrin, 1 $\mu g/m\ell$ insulin, 10 nm hydrocortison) at 37℃ under 5% CO_2 condition[16].

Hormones and Reagents

Bovine TSH were obtained from Dr. A. F. Parlow via the National Hormone and Pituitary Program (USA). Protease inhibitors (DFP, pepstatin, leupeptin, aprotinin, and E-64), iodoacetamide, DTT, and various basic chemical matters were purchased from Sigma Chemical Co., Zysorbin^R and Omnisorb^R were from Calbiochem Corp. (La Jolla, CA). DSP was purchased from Pierce.

Antibodies

Polyclonal anti-Tg antibody was raised in chicken using a synthetic peptide representing the first 15 amino acids of rat Tg[15]. Polyclonal anti-chaperone antibodies were raised in rabbit using synthetic peptide representing each C-terminals (14 residues of murine GRP94, 16 residues of murine BiP, 19 residues of rat ERp72, 18 residues of rat ER60). Rabbit antisea directed against murine PDI was obtained from Dr. T. Wileman (Pirbright Lab., Surrey, U.K.). Unpurified sera were suitable for Western blotting, affinity purified sera were used for immunoprecipitation.

Transfection

The plasmid of pcDNA3 (Invitogen^R) containing a chaperone cDNA fragment and 10 $\mu\ell$ of lipofectin^R both in OPTI-MEM^R were combined prior to pouring onto each well containing 50% confluent FRTL5 cells for 7 hrs at 37℃. Transfected cells were then exposed to media containing 4 hormones for 2 hrs to give normal conditions, prior to the addition of neomycin (400 $\mu g/m\ell$). After 1 week culture with Coon's medium containing 4 hormones and neomycin, cell colonies showing neomycin-resistance were collected using sterilized micropipette under microscope and transferred on new culture plate, which growed until 100% confluent[5].

Lysis and Cell labelling

The cells were lysed directly in a lysis buffer (ice-cold 1% Trion X-100, 0.1 M NaCl, 25 mM Tris-pH 7.5, 10 mM iodoacetamide, and protease inhibitors or boiling 2% SDS, 0.1 M NaCl, 25 mM Tris-pH 6.8, 50 mM DTT).

For the cell labelling, cells growing on 24 wells-plate were incubated for 30 min at 37℃ in Coon's media lacking methionine and cysteine. The cell was then labeled for up to 30 min with [^{35}S]amino acids (New England Nuclear, USA), and chased in the presence of excess unlabelled methionine and cysteine. For experiments in which Tg was to be coimmunoprecipitated with GRP94, cells were incubated in 50 mM PBS (pH 9.5) with DSP, a reducible cross-linker (Pierce), for 30 min at 0℃; in this case the lysis buffer omitted apyras [15].

Western blotting and Immunoprecipitation

For Western blot analyses, Amersham ECL Western blotting Kit was used according to manufacture's instruction. For immunoprecipitation, equal aliquots of cell lysates were first precleaned with Zysorbin^{R} or Omnisorb^{R} and then immunoprecipitated with each anti-chaperone antibody for 2 hrs at 4℃, before analysis by reducing SDS-PAGE[15].

Results and Discussion

A mammalian expression vector, pcDNA3, contains the CMV promoter for transcription, on our previous data promotor regions of Tg and SV40 gene were not available for effective foreign gene transcription in the FRTL5 cells (data not shown). In addition to the vector contains the neomycin resistance gene for cDNA-transfected cell screening. Various cDNA fragments encoding GRP94 (2.7 kb), ERp72 (2.3 kb), and PDI (2.5 kb) were inserted into the BamH1 site, and Bip (2.3 kb) was inserted into the Pst1-Ssp1 site of its polylinker, respectively. Each recombinant pcDNA3 vector was introduced into FRTL5 cells by lipoforin and selected for growth in the Coon's medium containing 4 hormones and 400 $\mu g/m\ell$ neomycin for 1 week at normal condition[27]. For removing death cells untransfected with cDNA in the medium, medium was changed once a day. Single transfected cell colony on the culture plate was detected from 7-day after transfection which was collected by sterilized micropipette under microscope in the clean bench and those were grown until 100% confluence after transfer to a new culture plate. Any noticeable phenotypic change were not shown between transfected and normal cells, but the growth rate of normal cells was somewhat higher than that of transfected cells.

Overexpression of chaperones in transfected FRTL5 cells was analyzed by Western blotting. Lystes of transfected monolayer cells were normalized to DNA content, which were separated on 8% reducing SDS-PAGE followed by immunoblotting with each antibody against GRP94, ERp72, Bip, and PDI. More than 100 clones were screened in each case, and the resulting spots of Western blotting were quantitatively estimated by Imageanalyser which is shown in the Fig. 1. In case of GRP94, 3-fold overexpressing cell compared its normal cell was detected in the upper-left panel indicated by 3X, 4-fold overexpressing cell of ERp72 was indicated in the upper-right panel, 2-fold overexpressing cell of Bip was also represented in the lower panel. While 4-fold overexpressing transfected cell which was identified using polyclonal rabbit anti-PDI antibody which recognized only exogenous rat PDI indicated by black arrow in the left panel, no significant differences in the both exo-and endogenous PDI using monoclonal mouse anti-PDI antibody which recognize both foreign mouse PDI and endogenous PDI is shown in the right panel. This results indicate that exogenous cDNAs of GRP94, ERp72, Bip and PDI are stably overexpressed in the FRTL5 cell by CMV promoter instead of Tg and SV40 promoters.

We have improved a method to detect noncovalent interactions between chaperones and nascent Tg molecule using DSP *in vivo*[17]. In experiment, it is important to make cross-linking solution freshly in PBS (0.2 mM DSP in fresh 50 mM DMSO, 20 mM NEM in fresh 3 M EtOH), and DSP added just before using to the freshly

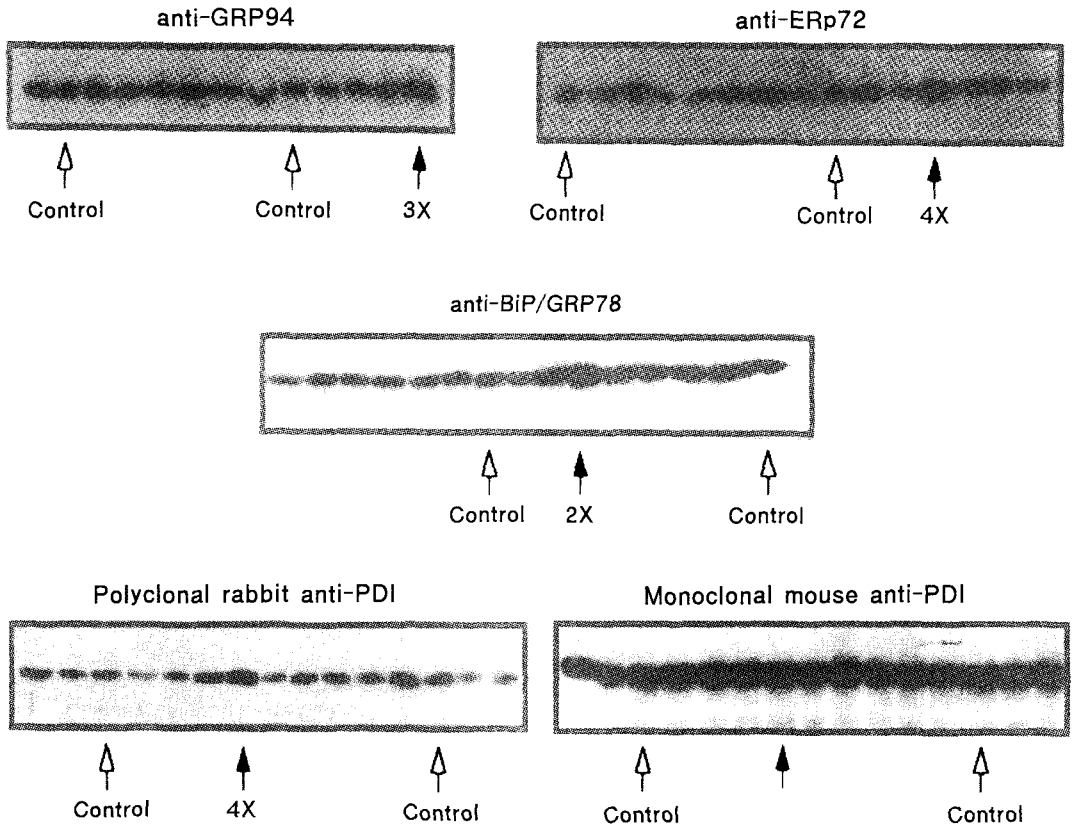

Fig. 1. An Western blot analysis of molecular chaperone expression in transfected FRTL5 cells.
Each complete cDNA fragment for murine GRP94, ERp72, or PDI and chinese hamster Bip were ligated into the expression vector pcDNA3. Transfected monolayers normalized to DNA content were analyzed by 8% reducing SDS-PAGE followed by immunoblotting using affinity-purified antibodies. More than 100 cell clones were screened in each case. Clones expressing transfected mouse PDI were identified using polyclonal antibody which recognized both mouse and endogenous rat PDI. When the same lysates were re-examined using monoclonal anti-PDI antibody which does not recognize the foreign mouse PDI, no significant differences in endogenous rat PDI levels were noted.

confluent monolayer cells, but not to old-cultured cells. As shown in the Fig. 2., in absence of DSP, there are no notable interacting proteins with Tg and appearing only a Tg band under both reducing (without DTT) and nonreducing (with DTT) conditions. However, in pre sence of DSP, a significantly amount of aggregated form were seen on the top of nonreducing gel, indicating that Tg molecule strongly interacts with some proteins through DSP. If DTT is added to the DSP-treated cell lysate, Tg-some proteins of noncovalent interactions were broken, and Tg and some proteins were separated on the reducing gels. This result demonstrated that Tg interacts with some unknown proteins noncovalently *in vivo*, and that experiments using DSP as a cross-linker is a very useful method to study protein-protein interaction *in vivo*.

Next, we have examined the interaction between Tg and chaperones in the FRTL5 cell. Cells was pulse-

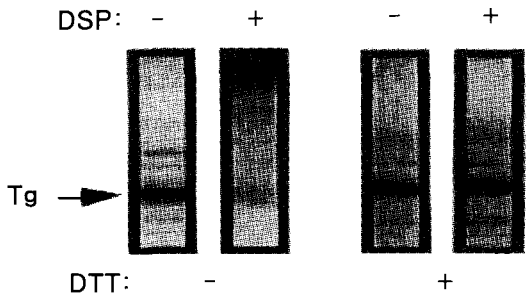

Fig. 2. Cross-linking of intractellular proteins using DSP. No overexpressed bands in the all panels were indicated without arrows. Confluent monolayers of FRTL5 cells pulse-labeled, chased, alkylated, exposed to DSP and NEM for 30 min at 4℃, and lysed under nondenaturing conditions. This processing is described in Materials and Methods and reference[14]. The samples were then analyzed by 4% SDS-PAGE under nonreducing [DTT(-)] and reducing conditions [DTT(+)]. The resulting gel was exposed on the X-ray film for one day in -80℃.

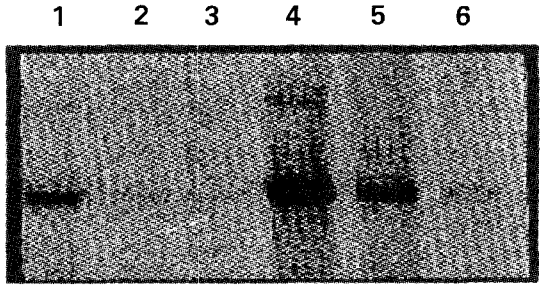

Fig. 3. Detection of Tg-chaperone interactions using the cross-linking method.
Radiolabelled FRTL5 cells according Fig. 2 were subjected to the cross-linking protocol as in Fig. 2 and lysed under nondenaturing conditions. When lysates were immunoprecipitated using antibodies generated against different chaperones (lane 1-6: PDI, ERp72, calnexin, Bip, GRP94, and ER60, respectively) and analyzed by 4% reducing SDS-PAGE, varying amounts of Tg were co-precipitated with each of the them. The resulting bands were shown on the X-ray film.

labelled with ^{35}S-methionine, subjected to the cross-linking protocol as in Fig. 2, and lysed under non-denaturing conditions. Lysates were subjected to immunoprecipitation with antibodies against different chaperones which were able to recognize each chaperones in the Western blotting (Fig. 3. lane 1-6: PDI, ERp72, calnexin, Bip, GRP94, and ER60, respectively) and the immunoprecipitate was analyzed on the 4% reducing SDS-PAGE to detect Tg molecules. Tg was co-precipitated with each antibodies against PDI, Bip and GRP94 (Fig. 3. lane 1, 4 and 5). However, no Tg molecule was detected with antibodies against ERp72, calnexin and ER60 (Fig. 3. lane 2, 3 and 6).

To provide evidence that GRP94, another endoplasmic reticulum stress protein[18] homologous to HSP90[20], mediates newly synthesized Tg in the ER, ^{35}S-methionine labelled cells were incubated with DSP in the same condition as in Fig. 2. After 50 min chase, cell lysate was subjected to immunoprecipitation with anti-GRP94 antibody and the precipitate was separated on a 4% reducing SDS-PAGE (Fig. 4). In zero chase time, only minimal amount of Tg was present for its early folding. However, in chase times of 10 min and 20 min, the association of GRP94 with Tg was strongest, and its association is limited from chase time 30 min. These results indicate that GRP94 is transiently involved in

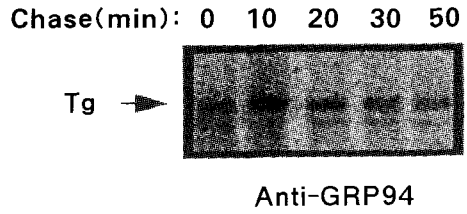

Fig. 4. Transient association of GRP94 with Tg folding intermediates.
Cell were pulse-labelled, chased for 50 min, alkylated, subjected to cross-linking, lysed, immunoprecipitated using anti-GRP94 antibody and with Zysorbin^{R} or Omnisorb^{R} at 4℃ for 2 hrs. The precipitates were separated on 4% reducing SDS-PAGE and gel was exposed on the X-ray film for one day in -80℃.

association Tg folding during its synthesis, and provide evidence for GRP94's role in Tg biosynthesis, that is, it acts as a chaperone like Bip which associate with nascent polypeptides during early stage of folding process[12]. Considering that the dissociation time of Bip from nascent Tg is about 1 hour in FRTL-5 cells[12], it is clear that GRP94 interacts with newly synthesized Tg Just before dissociation of Bip from nascent Tg. While one report showed that Bip preferentially binds an early immunoglobulin rather than GRP94 in the ER which sequentially interacts with Bip[21], another result demonstrated that, on study of recombinant nonmutant Tg expression in CHO cells, GRP94 was involved in Tg folding and exporting[24]. It can be suggested that different folding condition need different associational order of chaperone for folding in the ER. Based on our present data, we conclude that, in FRTL5 cells, GRP94 itself is molecular chaperone which interacts, just after dissociation of Bip from nascent Tg, directly with Tg during its synthesis in the ER. Indeed, overproduction of another chaperone of Hsp72 leads to a dramatic protection against severe heat shock, UV irradiation, H_2O_2, and other harmful conditions and factors[6,19,23, 26], and also to reduction of stress-induced denaturation and aggregation of certain proteins[8,9]. Further studies are necessary for its detailed folding mechanism and other phenomena after overexpression of GRP94 in the FRTL5.

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Overexpression of ER Resident Molecular Chaperones and Characterization of Their Interaction with Thyroglobulin in FRTL5 cells.

초록 : GRP94는 thyroglobulin의 folding에 관여한다.

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Endoplasmic reticulum (ER)내에 단백질의 folding과 안정화에 관여하는 단백질을 molecular chaperone이라고 한다. GRP94 역시 ER내에 존재하는 molecular chaperone으로 알려지고 있지만 갑상선세포에서 단백질의 folding에 관여한다는 증거는 아직 불충분하다. 본 실험은 molecular chaperone을 세포내에서 overexpression시킬 수 있는 system을 확립하였다. 그 중에서 GRP94가 단백질의 folding에 직접적으로 관여한다는 증거를 얻기 위하여, endogenous GRP94를 code한 cDNA를 overexpression vector에 의해서 forced expression시킴으로 신생thyroglobulin의 folding에 직접적으로 관여하는 증거를 immunoprecipitation으로 증명하였다.