The Stress Response in Chinese Hamster Ovary Cells

REGULATION OF ERp72 AND PROTEIN DISULFIDE ISOMERASE EXPRESSION AND SECRETION*

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Expression of the glucose-regulated proteins (GRPs), GRP78 and GRP94, is induced by a variety of stress conditions including treatment of cells with tunicamycin or the calcium ionophore A23187. The stimulus for induction of these resident endoplasmic reticulum (ER) proteins appears to be accumulation of misfolded or underglycosylated protein within the ER. We have studied the induction of mRNAs encoding two other resident ER proteins, ERp72 and protein disulfide isomerase (PDI), during the stress response in Chinese hamster ovary cells. ERp72 shares amino acid sequence homology with PDI within the presumed catalytic active sites. ERp72 mRNA and, to a lesser degree, PDI mRNA were induced by treatment of Chinese hamster ovary cells with tunicamycin or A23187. These results identify ERp72 as a member of the GRP family. Stable high level overproduction of ERp72 or PDI from recombinant expression vectors did not alter the constitutive or induced expression of other GRPs. High level overexpression resulted in secretion of the overproduced protein specifically but not other resident ER proteins. This suggests that the ER retention mechanism is mediated by more specific interactions than just KDEL sequence recognition.

Native disulfide bond formation occurs in the endoplasmic reticulum (ER) during and shortly after translocation of secretory proteins into that compartment. A resident protein of the ER, protein disulfide isomerase (PDI), is involved in the formation of disulfide bonds (for review, see Freedman (1984)). In vitro experiments using reduced and denatured proteins have shown that PDI increases the rate of formation of native disulfide bonds and catalyzes the rearrangement of non-native bonds (for review, see Freedman et al., 1989). Recently, it has been shown that the presence of PDI within dog pancreas microsomes during in vitro protein synthesis is necessary for disulfide bond exchange reactions to occur (Bulleid and Freedman, 1988).

Other resident ER proteins may also be involved in the processing and transport of secretory proteins within that compartment. Proteins which are major components of the ER of murine plasmacytoma cells have been identified (Lewis et al., 1985, 1986). Among them, GRP94 and PDI were characterized by molecular cloning and sequence analysis (Mazzarella and Green, 1987; Mazzarella et al., 1990). GRP94 and two other proteins, GRP78 and GRP58, are members of the glucose-regulated protein (Lee, 1981, 1987). GRP78 and GRP94 are resident ER proteins induced by a variety of stress conditions associated with increased secretory protein traffic (Donner et al., 1989) or the accumulation of misfolded or unglycosylated protein in the ER (Chang et al., 1987; Kozutsumi et al., 1988; Watowich and Morimoto, 1988). GRP78, also known as immunoglobulin-binding protein (Munro and Pelham, 1986), exhibits both transient and stable association with secretory proteins transiting the ER (Donner et al., 1987). Stable association of GRP78 with misfolded, unglycosylated, or incompletely assembled proteins correlates with a block in efficient secretion (Bolc et al., 1986; Donner et al., 1987; Hurtley et al., 1989), while transient association may be part of the normal secretory pathway (Kaufman et al., 1989; Machamer et al., 1990; Ng et al., 1989).

The amino acid sequence KDEL present at the carboxyl terminus of many resident ER proteins is required for efficient retention of these proteins within that compartment (Munro and Pelham, 1987). GRP78, GRP94, and PDI have KDEL at their carboxyl termini, while ERp72 has the related sequence KEEL (Mazzarella and Green, 1987; Mazzarella et al., 1990; Munro and Pelham, 1986). The presence of this sequence may serve as a generalized signal for retention in the ER. However, the retention mechanism is not absolute for all KDEL-containing proteins (Zagouras and Rose, 1989).

Since GRPs are postulated to interact with proteins transiting the ER, especially under conditions of misfolding or aggregation, it is possible that other resident ER proteins may also be members of the GRP family. It has been suggested but not proven that GRP58 may be the same protein as PDI (Whelan and Hightower, 1986). ERp72, a luminal ER protein of murine plasmacytoma cells, has been shown to share amino acid sequence homology with PDI at regions believed to constitute the active sites of PDI (Mazzarella et al., 1990). The induction of proteins with disulfide isomerase activity, along with the induction of GRP78 and GRP94, may be part of the cellular response to the presence of misfolded protein in the ER.

We have examined the induction of ERp72 and PDI mRNAs following exposure of Chinese hamster ovary (CHO) cells to tunicamycin or the calcium ionophore A23187, treatments which induce GRP expression (Lee, 1987). ERp72 mRNA and protein were induced following treatment with A23187 or inhibition of N-linked glycosylation with tunicamycin. PDI mRNA was also induced by both treatments but at a much reduced level compared with ERp72. These results suggest that regulation of expression of ERp72 and perhaps...
PDI is similar to that of GRP78 and GRP94 and indicate that ERp72 is a member of the GRP family.

To establish the role of increased expression of ERp72 or PDI, these proteins were constitutively expressed at high levels in CHO cells. Overexpression of either protein did not alter the expression or stress-mediated induction of other GRPs. Interestingly, overexpression of PDI or ERp72 resulted in detectable secretion only of the overexpressed protein rather than of all KDEL-containing proteins. This result suggests that retention of resident ER proteins may involve protein-specific signals in addition to the KDEL retention mechanism. Further studies will utilize these overexpressing cells to help define the roles of ERp72 and PDI in secretion and the stress response independently of increased expression of the other GRPs.

**MATERIALS AND METHODS**

**Expression Vectors**—A 1979-base pair fragment containing the entire coding region for murine ERp72, isolated by digestion of pCD72-1 (Mazzarella et al., 1990) with PstI and SpeI followed by treatment with the Klenow fragment of DNA polymerase I. A 1307-base pair fragment containing the entire coding region for murine PDI was isolated by digestion of pCD59-11 (Mazzarella et al., 1990) with AatI and BglII followed by treatment with the Klenow fragment. These vectors generate a dicistronic mRNA with the 5' open reading frame encoding PDI or ERp72 and the 3' open reading frame encoding dihydrofolate reductase (DHFR) (Kaufman et al., 1989). All restriction enzymes were obtained from New England Biolabs.

**Cell Lines**—The DHFR-deficient cell line CHO DUKX-B11 designated CHO which requires nucleosides in the medium for growth has been described elsewhere (Urlaub and Chasin, 1980). The DHFR-deficient CHO DUKX cell line expressing the glucocorticoid receptor designated CHO-GRA was derived as previously described (Israel and Kaufman, 1990). Since BiP and GRP78 are identical proteins (Munro and Pelham, 1989), GRP78 was isolated by digestion of pCD72-1 (Mazzarella et al., 1990) as described elsewhere (Dorner and Kaufman, 1990). Alternatively, the nitrocellulose blot was immunoblotted with a mixture of both anti-ERp72 and anti-PDI antisera and antiserum directed against the carboxyl-terminal 16 amino acid residues of murine PDI or anti-ERp72 rabbit serum directed against the carboxyl-terminal 16 amino acid residues of murine ERp72. The blot was then incubated with 125I protein A (Amersham Corp.) (Dorner and Kaufman, 1990). The band corresponding to GRP78 was detected as a single band of approximately 78 kDa in CHO-GRA cells infected with vSV401 DNA extracted from the NIH-3T3 cell line (Singh et al., 1985) and conditioned medium from CHO cell lines expressing GRP78 and GRP94 (Chang et al., 1987; Olden et al., 1979).

**Cell Line Derivation**—CHO-GRA cells were transfected with pMT2-PDI or pMT2-72 by the calcium phosphate precipitation procedure and selected for DHFR expression in increasing concentrations of methotrexate (Kaufman, 1989). Individual DHFR-positive colonies were analyzed for PDI or ERp72 expression by Northern analysis and Western protein analysis. The highest expressing line for PDI and ERp72 is designated CHO which requires nucleosides in the medium for growth has been described elsewhere (Urlaub and Chasin, 1980). The DHFR-deficient CHO DUKX cell line expressing the glucocorticoid receptor designated CHO-GRA was derived as previously described (Israel and Kaufman, 1990).

**Treatment Conditions and RNA Analysis**—Cells were plated 36 h before treatment in a medium containing 10% fetal calf serum and 10 μg/ml each of adenosine, deoxyadenosine, and thymidine. Cells were fed fresh medium containing 10 μg/mL tunicamycin (Sigma) or 7 μM A23187 (Sigma). Total RNA was isolated by the guanidinium thiocyanate-phenol procedure (Chirgwin et al., 1979). RNA (6 μg) was electrophoresed on 1% agarose gels containing 7.4% formaldehyde and 20 mM sodium phosphate, pH 6.5, transferred to nitrocellulose, and hybridized to specific DNA probes as indicated. Exposure times are different for each DNA probe. Portions of autoradiograms containing all hybridizable RNA species are shown.

**RESULTS**

**mRNA Induction after Tunicamycin or A23187 Treatment**—Treatment of animal cells with tunicamycin inhibits N-linked glycosylation and induces transcription of the genes for GRP78 and GRP94 (Chang et al., 1987; Olden et al., 1979). To ascertain whether PDI or ERp72 mRNAs were also induced by this stress condition, CHO cells were treated with tunicamycin for 8 or 24 h. Total cellular RNA was subjected to Northern analysis, and the levels of mRNAs for GRP78, GRP94, PDI, and ERp72 were determined. The level of GRP78 mRNA increased 33-fold after 8 h of treatment with tunicamycin and 28-fold after 24 h of treatment as measured by hybridization to a GRP78-specific probe (Fig. 1A). GRP94 mRNA levels, as detected with a GRP94-specific probe, increased 16- and 29-fold following 8- and 24-h tunicamycin treatment, respectively (Fig. 1B).

ERp72 mRNA increased 10-fold after 8-h treatment and 13-fold after 24-h treatment as measured by hybridization to a GRP78-specific probe (Fig. 1A). GRP94 mRNA levels, as detected with a GRP94-specific probe, increased 16- and 29-fold following 8- and 24-h tunicamycin treatment, respectively (Fig. 1B).

**Immunoblot Protein Analysis**—Following 24 h of growth in fresh medium cell extracts were prepared (Dorner et al., 1987) and conditioned medium harvested. Protein concentration of cell extracts was determined by the method of Bradford (Bradford, 1976) (Bio-Rad protein assay). Cell extract (23 μg) and volumes of 24-h conditioned medium corresponding to 50% of the cell extract amount were electrophoresed on a 10% polyacrylamide-sodium dodecyl sulfate gel and transferred onto nitrocellulose. The nitrocellulose blot was immunoblotted with anti-PDI rabbit serum directed against the carboxyl-terminal 16 amino acid residues of murine PDI or anti-ERp72 rabbit serum directed against the carboxyl-terminal 16 amino acid residues of murine ERp72. The blot was then incubated with 125I protein A (Amersham Corp.) (Dorner and Kaufman, 1990). The band corresponding to GRP78 was detected as a single band of approximately 78 kDa in CHO-GRA cells infected with vSV401 DNA extracted from the NIH-3T3 cell line (Singh et al., 1985) and conditioned medium from CHO cell lines expressing GRP78 and GRP94 (Chang et al., 1987; Olden et al., 1979).

**Fig. 1. Induction of mRNA after tunicamycin treatment.** Total cellular RNA was purified from CHO cells untreated (lane 1) and treated with 10 μg/ml tunicamycin for 8 h (lane 2) or 24 h (lane 3). RNA was electrophoresed on a formaldehyde-agarose gel, blotted onto nitrocellulose, and hybridized to specific DNA probes as indicated. Exposure times are different for each DNA probe. Portions of autoradiograms containing all hybridizable RNA species are shown.
a specific probe (Fig. 1C). Hybridization with a PDI-specific probe showed an increase in PDI mRNA of only 3-fold after 8 h and 4-fold after 24 h treatment with tunicamycin (Fig. 1D). Actin mRNA levels, measured by hybridization to an actin-specific probe, were used to quantitate the equivalence of the gel load and normalize quantitation of the level of induction. Actin mRNA levels were equivalent except for a slight decline at the 24-h tunicamycin time point (data not shown). These results showed that ERp72 mRNA and, to a lesser extent, PDI mRNA were induced by tunicamycin treatment as were GRP78 and GRP94 mRNAs (Table I).

The calcium ionophore A23187 affects the permeability of cellular membranes to calcium. Previous studies have shown that the GRP78 and GRP94 genes are activated at the transcriptional level by treatment with A23187, resulting in increased levels of mRNA for these proteins (Resendez et al., 1985). We examined whether PDI and ERp72 mRNA levels could also be elevated by A23187 treatment. CHO cells were treated for 6 or 12 h with A23187. Northern analysis of total RNA was performed. Hybridization with a GRP78-specific probe revealed a 20- and 30-fold increase in the level of GRP78 mRNA after 6 and 12 h of treatment, respectively (Fig. 2A). The GRP94 mRNA level was increased 5-fold after 6 h and 11-fold after 12 h of A23187 treatment as detected by hybridization with a GRP94-specific probe (Fig. 2B).

ERp72 mRNA levels showed 3.6- and 5-fold increases after 6- and 12-h treatment, respectively (Fig. 2C). After 6-h treatment, PDI mRNA levels were not increased and a 2-fold induction of PDI mRNA occurred after 12-h treatment (Fig. 2D). Thus, ERp72 mRNA was induced similarly to GRP78 and GRP94 mRNAs after tunicamycin or A23187 treatment, while PDI mRNA showed only moderate induction after tunicamycin treatment but not A23187 treatment (Table I).

**Table I**

| mRNA  | Tunicamycin | A23187 |
|-------|-------------|--------|
|       | 8 h | 24 h | 6 h | 12 h |
| GRP78 | 33  | 28  | 20  | 30  |
| GRP94 | 16  | 29  | 5   | 11  |
| ERp72 | 10  | 13  | 3.6 | 5   |
| PDI   | 3   | 4   | 1   | 2   |

**Fig. 2. Induction of mRNA after A23187 treatment.** Total cellular RNA was purified from CHO cells untreated (lane 1) or after treatment with 7 μM A23187 for 6 h (lane 2) or 12 h (lane 3). RNA was electrophoresed on a formaldehyde-agarose gel, blotted onto nitrocellulose, and hybridized to specific DNA probes as indicated. Exposure times are different for each DNA probe. Portions of autoradiograms containing all hybridizable RNA species are shown.

**Fig. 3. Elevated protein levels after A23187 or tunicamycin treatment.** Untreated CHO cells (lanes 1 and 4) and CHO cells treated with 7 μM A23187 for 12 h (lanes 2 and 5), or tunicamycin for 24 h (lanes 3 and 6) were pulse-labeled for 30 min with [35S]methionine and cell extracts prepared. Equal trichloroacetic acid-precipitable counts were immunoprecipitated using a monoclonal antibody directed against BiP (GRP78) (lanes 1 and 3) or a rabbit polyclonal antibody directed against ERp72 (lanes 4–6). M, molecular weight markers. Molecular sizes (>1000) are indicated on the left. A portion of an autoradiogram of an 8% polyacrylamide gel is shown.

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secretion of these proteins. In the conditioned medium of 72A1 cells only ERp72 was detected and not GRP78, GRP94, or PDI (Fig. 4B, lane 5). Similarly, only PDI was detected in the conditioned medium of 59A6 cells (Fig. 4B, lane 4). No KDEL-containing proteins were detected in the conditioned medium of CHO-GRA cells (Fig. 4B, lane 6). While secretion of all GRPs was not observed, overexpression resulted in detectable secretion specifically of the overexpressed protein.

**PDI Activity in 59A6 and 72A1 Cells**—Disulfide isomerase activity can be measured by the disruption of the disulfide bonds between the two chains of insulin by the release of the $\beta$ chain as trichloroacetic acid-soluble material (Tomizawa, 1962). Homogenates of 72A1, 59A6, and CHO cells were mixed with $^{125}$I-labeled insulin and the release of $\beta$ chain monitored by Northern blotting. Hybridization to a GRP78-specific probe showed a 14-fold induction of GRP78 mRNA in CHO-GRA cells (Fig. 5A, lanes 1 and 2), a 12-fold induction in 59A6 cells (Fig. 5A, lanes 3 and 4), and an 11-fold induction in 72A1 cells (Fig. 5A, lanes 5 and 6). Hybridization to a GRP94-specific probe showed that the level of induction of GRP94 mRNA was also similar in 50A6 cells (4.6-fold) (Fig. 5A).

**Stress Response of Overexpressing Cells**—We next examined whether constitutive overexpression of a single GRP would alter the induction of the other GRPs following stress elicited by tunicamycin treatment. 72A1, 59A6, and CHO-GRA cells were treated for 6 h with tunicamycin and total cellular RNA analyzed by Northern blotting. Hybridization to a GRP78-specific probe showed a 14-fold induction of GRP78 mRNA in CHO-GRA cells (Fig. 5A, lanes 1 and 2), a 12-fold induction in 59A6 cells (Fig. 5A, lanes 3 and 4), and an 11-fold induction in 72A1 cells (Fig. 5A, lanes 5 and 6). Hybridization to a GRP94-specific probe showed that the level of induction of GRP94 mRNA was also similar in 50A6 cells (4.6-fold) (Fig. 5A).

**TABLE II**

| Cell line | Insulin degraded | Specific activity |
|-----------|-----------------|------------------|
| CHO-GRA   | 1.63            | 0.27             |
| 72A1 (ERp72)* | 2.16            | 0.36             |
| 59A6 (PDI) | 8.32            | 1.39             |
| PDF*      | 4.22            | 0.70             |

* Overproduced protein is indicated in parentheses.

* Purified bovine PDI (0.06 $\mu$g) was used as positive control.

**FIG. 5. Tunicamycin treatment of 72A1 and 59A6 cells.**

Total cellular RNA was purified from cells after treatment with tunicamycin for 6 h, electrophoresed on a formaldehyde-agarose gel, blotted onto nitrocellulose, and hybridized to probes specific for GRP78 (panel A), GRP94 (panel B), or actin (panel C). Exposure times are different for each probe. Portions of autoradiograms containing all hybridizable species are shown.
5B, lanes 3 and 4) and 72A cells (3.7-fold) (Fig. 5B, lanes 5 and 6) compared with CHO-GR can cells (4-fold) (Fig. 5D, lanes 1 and 2). The level of actin RNA was used to quantitate the induction level (Fig. 5C). Thus, stable overexpression of ERp72 or PDI had no effect on the level of induction of either GRP78 or GRP94. Constitutive mRNA levels for GRP78 and GRP94 were similar between all cell lines except for a less than 2-fold increase in GRP78 mRNA observed in 72A cells.

DISCUSSION

Expression of GRPs is increased by conditions which block normal processing and secretion of proteins (Lee, 1987). These conditions result in the accumulation of misfolded or aggregated protein in the ER. We therefore examined whether other resident ER proteins, PDI and ERp72, were coordinately induced with GRP78 and GRP94. PDI facilitates disulfide bond interchange and thus is involved in protein folding in the ER. PDI contains two homologous regions related to amino acid sequences of thioredoxin (Edman et al., 1985). These regions of PDI are believed to constitute the active sites involved in disulfide bond interchange (Freedman et al., 1989). ERp72 contains three copies of this conserved sequence, and the spacing between two copies is nearly identical to that found in PDI (Mazzarella et al., 1990). While a disulfide isomerase activity has not yet been demonstrated for ERp72, this homology is suggestive that it may also facilitate disulfide bond formation to promote protein folding or unfolding.

Since PDI and ERp72 may play a role in protein folding in the ER, we studied whether they are induced by stress conditions which result in the accumulation of misfolded protein in the ER. Treatment of CHO cells with tunicamycin or the calcium ionophore A23187 produced a significant increase in ERp72 mRNA levels. The -fold induction of ERp72 mRNA was comparable with that observed for GRP94 mRNA. We observed that, while PDI mRNA levels were moderately increased after tunicamycin treatment, little induction was observed after A23187 treatment. Previously it has been reported that PDI protein levels are not increased after A23187 treatment (Macer and Koch, 1988). Increases in protein levels are usually disproportionately less than the induction level of mRNA for the GRPs (Watowich and Morimoto, 1988), and so the -fold mRNA induction after A23187 treatment which we observed may not produce a detectable increase in PDI protein. We did observe increased ERp72 protein synthesis levels after A23187 and tunicamycin treatment. Thus, in the case of ERp72, induction of mRNA correlated with increased levels of protein.

Our data indicate that ERp72 mRNA levels may be regulated in a similar manner to GRP78 and GRP94 after tunicamycin or A23187 treatment. These observations indicate that ERp72 is a member of the GRP family. Since induction by calcium ionophore treatment is a distinctive feature of GRPs (Lee, 1987), we cannot definitively include PDI in the GRP family. PDI levels are elevated in normal cells engaged in high levels of synthesis of secreted proteins (Freedman, 1989), but we observed little stress induction. This suggests that PDI functions during synthesis of normal proteins to form correct disulfide bonds but that increased levels of PDI are not part of the cellular response to the accumulation of misfolded protein. In contrast, ERp72 is induced similarly to other GRPs by stress conditions. Since ERp72 displays PDI homology, we speculate that ERp72 may interact with misfolded or denatured protein in the ER as part of a refolding or unfolding process after stress.

To further identify the functions and activities of ERp72 and PDI, we have generated CHO cell lines which overexpress the murine proteins individually. As measured by the ability to disrupt the disulfide bonds of the insulin heterodimer and release the b chain (Tomizawa, 1982), overexpression of ERp72 in 72A cells did not result in increased insulin reduction activity in cell homogenates. We expect that a disulfide isomerase activity of ERp72 may be observed under different assay conditions and exhibit specificities different than PDI. Alternate assay conditions are presently being evaluated to measure ERp72 activity. Homogenates of 59A6 cells displayed increased levels of insulin reduction activity which correlated with the increased level of PDI protein. This shows that the expressed PDI has an appropriate activity following overexpression in CHO cells.

With these cell lines we have initiated studies on the effect of overexpression of ERp72 or PDI in the absence of increased levels of the other GRPs. As detected by immunoblot analysis, murine ERp72 was expressed at a very elevated level in 72A1 cells compared with the endogenous CHO protein. This high level of ERp72 did not result in increased expression of GRP78, GRP94, or PDI. In addition, high levels of ERp72 do not appear to be toxic and can be maintained by the cell in the absence of stress conditions. Immunoblot analysis of 59A6 cells showed high constitutive levels of PDI could also be maintained by the cell without inducing a stress response. Thus, stable overexpression of a single resident ER protein does not signal the cell to increase expression of other resident proteins. In particular, overexpression of ERp72, a GRP, did not induce expression of other GRPs. This suggests that the level of individual stress proteins in the ER is not part of the induction signal. Furthermore, the presence of high levels of ERp72 or PDI did not trigger an induction of GRPs due to misfolding or disruption of normal ER processes.

It is of interest that only ERp72 could be detected in the conditioned medium of 72A1 cells and only PDI detected in the conditioned medium of 59A6 cells. It is possible that high level expression of a protein normally retained in the ER results in saturation of the retention mechanism and a low level of secretion of all KDEL-containing proteins. If the retention mechanism were a generalized one involving all KDEL-containing proteins (Monro and Felham, 1987), then it would follow that saturation by overexpression of ERp72 or PDI would also result in a secretion of a proportion of GRP78 and GRP94. Instead we have observed only secretion of the overexpressed protein. This suggests that the retention mechanism for ER-localized proteins may be more selective than just recognition of the KDEL sequence. This is consistent with a recent study in which addition of the KDEL sequence to two secretory proteins resulted in a retardation but not inhibition of secretion (Zagouras and Rose, 1989). Additional control over retention and secretion may also involve protein-protein interactions or other signals specific for each protein.

Stable overexpression of a GRP has not previously been reported. We examined whether elevated levels of ERp72 or PDI would have an effect on the strength of the cellular stress response by measuring RNA levels for GRP94 and GRP78 following tunicamycin treatment. If an elevated level of PDI or ERp72 could reduce the amount of misfolded protein, then the induction of GRPs might be reduced. The induction levels of GRP94 and GRP78 were similar for the overexpressing cells compared with the parental CHO cells. This suggests that increased levels of either PDI or ERp72 alone could not mitigate the stress of blocking N-linked glycosylation by tunicamycin treatment. In addition, overexpression did not

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perturb the normal signaling mechanism which induces GRP expression. These cell lines will be further utilized as hosts for the expression of mutant and normal secreted proteins to determine the functions of ERp72 and PDI in the cell’s secretory pathway under normal and stress conditions.

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