The Dynamic Role of GRP78/BiP in the Coordination of mRNA Translation with Protein Processing*

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The role of GRP78/BiP in coordinating endoplasmic reticular (ER) protein processing with mRNA translation was examined in GH₃ pituitary cells. ADP-ribosylation of GRP78 and eukaryotic initiation factor (eIF)-2α phosphorylation were assessed, respectively, as indices of chaperone inactivation and the inhibition of translational initiation. Inhibition of protein processing by ER stress (ionomycin and dithiothreitol) resulted in GRP78 deribosylation and eIF-2 phosphorylation. Suppression of translation relative to ER protein processing (cycloheximide) produced approximately 50% ADP-ribosylation of GRP78 within 90 min without eIF-2 phosphorylation. ADP-ribosylation was reversed in 90 min by cycloheximide removal in a manner accelerated by ER stressors. Cycloheximide sharply reduced eIF-2 phosphorylation in response to ER stressors for about 30 min; sensitivity returned as GRP78 became increasingly ADP-ribosylated. Reduced sensitivity of eIF-2 to phosphorylation appeared to derive from the accumulation of free, unmodified chaperone as proteins completed processing without replacements. Prolonged (24 h) incubations with cycloheximide resulted in the selective loss of the ADP-ribosylated form of GRP78 and increased sensitivity of eIF-2 phosphorylation in response to ER stressors. Brefeldin A decreased ADP-ribosylation of GRP78 in parallel with increased eIF-2 phosphorylation. The cytoplasmic stressor, arsenite, which inhibits translational initiation through eIF-2 phosphorylation without affecting the ER, also produced ADP-ribosylation of GRP78.

Chaperones resident to the endoplasmic reticulum (ER) catalyze the folding of nascent polypeptides to tertiary structures that are competent for ER to Golgi transport. Protein folding and processing within the ER are dependent upon Ca²⁺ sequestration and the maintenance of a redox potential that permits the formation of specifically placed disulfide bonds (1–3). Protein processing is inhibited within several minutes following exposure to Ca²⁺-mobilizing or sulfhydryl-reducing agents at concentrations that do not lower ATP (4–6). Accumulation of unfolded protein in response to these agents is thought to signal a slowing of translational initiation through activation of the double-stranded RNA-stimulated eIF-2α kinase, in conjunction with eIF-2α phosphorylation and the inhibition of eIF-2 cycling (6).

Continued inhibition of processing (ER stress) results in the subsequent induction of various ER chaperones (7–9). GRP78/BiP, the chaperone induced most prominently and rapidly by ER stress, has been hypothesized to function in the correct folding and assembly of proteins during early protein processing (10), in the retention of improperly folded proteins that accumulate within the ER lumen when processing is distressed (11), and in the co-translational translocation of nascent polypeptides from the cytosol to the ER for processing (12, 13). The gene encoding GRP78 possesses a highly conserved promoter region that confers ER stress inducibility (8, 9). Induction or overexpression of the chaperone confers tolerance to translational inhibition and eIF-2 phosphorylation in response to ER stress (6, 14). In cell types overproducing proteins that translocate to the ER but that are incapable of ER to Golgi transport, GRP78 is chronically elevated, and mRNA translation is sustained upon challenge with ER stressors (6).

GRP78 is reported to undergo post-translational modification by mono-ADP-ribosylation and by phosphorylation (15, 16). Both modifications involve oligomerization of the chaperone to an inactive form. When complexed with other proteins, GRP78 is not subject to covalent modification or oligomerization (17). Post-translational modifications of GRP78 are generally observed during conditions that deplete the ER of processesible protein. For example, the fractional ADP-ribosylation of GRP78 is increased by lowered temperature, amino acid starvation, and treatments with cycloheximide or amino acid analogs (18–20). In contrast, the modification of GRP78 is suppressed by conditions that inhibit glycoprotein processing within the ER such as glucose depletion or treatments with tunicamycin, glucose analogs, or Ca²⁺-ionophores (15, 19, 21, 22). It is also suppressed by hormones that stimulate growth (22). The unmodified, unbound form of GRP78 is thought, therefore, to function as the active form of the chaperone that is available to interact with processing intermediates derived from co-translational translocation. It is this form of the chaperone that must be subject to inactivation through covalent modification. Signaling of grp78 gene expression correlates inversely with the extent to which the chaperone is covalently modified (7–9, 18, 22).

The present report describes an investigation of the relationship of GRP78 modification to protein processing and mRNA translation in GH₃ pituitary cells utilizing isoelectric focusing methodology. ADP-ribosylation of the chaperone was increased by treatments that slow the rate of mRNA translation relative to that of protein processing. By contrast, ADP-ribosylation was decreased or reversed by treatments that slow the rate of protein processing relative to that of mRNA translation. A dynamic relationship was found to prevail between the frac-
tion of ADP-ribosylation of GRP78 and the degree of eIF-2 phosphorylation that was explicable in terms of changing contents of the active monomer.

**EXPERIMENTAL PROCEDURES**

**Materials**—Monoclonal antibody to eIF-2α was the gift of Dr. Lynn O’Brien, University of Rochester. Polyclonal rabbit anti-GRP78 and recombinant GRP78 were purchased from StressGen Biotechnologies. Ampholines were purchased from Gallard Schlesinger (pH 4–8) and from ESA Inc. (pH 3.5–10). Urea was purchased from Boehringer Mannheim. [2-3H]Adenosine and [γ-32P]ATP were obtained from Amersham Pharmacia Biotech, and L-[4,5-3H]-leucine was from ICN Radiochemicals. Thapsigargin and okadaic acid were obtained from Calbiochem. Pactamycin was donated by The Upjohn Co. Ionomycin, brefeldin A, puromycin, dithiothreitol, cycloheximide, emetine, nifedipine, and calf intestinal alkaline phosphatase were purchased from Sigma.

**General Methodology**—GH3 pituitary cells were maintained in suspension and utilized as described (23). Cultures were normally provided with fresh growth medium for approximately 16 h before harvest. Prior to treatments, cells were equilibrated for 15 min with serum-free Ham’s F-10 medium modified to contain 0.2 mM Ca2+. Leucine pulse incorporations were measured as described previously (24) for 10- or 15-min incubations of 2 × 10^6 cells per experimental condition. One-dimensional 7.5% polyacrylamide gel electrophoresis (SDS-PAGE) of detergent-solubilized extracts of variously treated cell preparations was conducted as described previously (25).

**Determinations of the Covalent Modifications of GRP78 and of eIF-2α Phosphorylation**—Cells (10^6) were harvested by centrifugation and lysed with 300 μl of urea buffer containing 2% ampholines (pH 3.5–10, two-dimensional), 4% Triton X-100, 100 mM dithiothreitol, and 9.9 mM urea. Lysates were applied to a 6% acrylamide slab gel (30% acrylamide, 1.5% bisacrylamide) containing ampholines (1.1, pH 3.5–10 to pH 4–8) and subjected to slab gel isoelectric focusing in the presence of 9.5 mM urea to separate the modified and unmodified forms of eIF-2α and of GRP78. Gels were treated with 1 M Tris, pH 8.8, and blotted onto polyvinylidene difluoride membranes under basic conditions. Phosphorylation of GRP78 and non-phosphorylated eIF-2α were immunodetected with monoclonal antibody to eIF-2 and chemiluminescence (26). The membranes were then treated with 1% sodium dodecyl sulfate and 0.2% β-mercaptoethanol in phosphate-buffered saline at room temperature for 15–30 min to remove the secondary antibody. GRP78 was immunodetected with primary antibody (1:5000), with goat anti-rabbit antisem (1:5000) serving as the secondary antibody.

**Phosphorylation of Recombinant GRP78**—The incubation conditions for in vitro phosphorylation of GRP78 were as described previously (27), except that the reaction was conducted at 37 °C for 2 h in 250 μl of 50 mM Hepes, pH 7.0, containing 20 mM γ-[32P]ATP, 100 mM CaCl2, and 30 μg of recombinant GRP78.

**Labeling of GH3 Cells with [2-3H]Adenosine**—Cells were incubated under standard conditions (see “EXPERIMENTAL PROCEDURES”) for 30 min without addition (lane 1) or with 100 μM puromycin (lane 2), 20 μM cycloheximide (lane 3), or 0.1 μM emetine (lane 4). 0.1 μM pactamycin (lane 5), 1 μM ionomycin (lane 6), 0.2 μM thapsigargin (lane 7), or 600 μM dithiothreitol (lane 8). Aliquots were removed for protein separation by isoelectric focusing and for measurements of [3H]leucine pulse incorporation. Inhibitions of leucine incorporation by various agents ranged from 90 to 98%. Samples 2–5 were then adjusted to 1 μM ionomycin, and the incubation was continued for an additional 30 min. Aliquots were removed at 60 min for protein separation by isoelectric focusing. Western blotting: A, GRP78 at 30 min; B, eIF-2α at 30 min; C, GRP78 at 60 min; D, eIF-2α at 60 min. Samples 9 and 10, a different preparation of GH3 cells was incubated for 45 min either without addition (lane 9) or with 50 μM (lane 10) sodium arsenite. Aliquots were removed for protein separation by isoelectric focusing and for measurements of [3H]leucine pulse incorporation. Inhibitions of protein synthesis were 88% at 50 μM arsenite, respectively. Samples 9 and 10 were then adjusted to 1 μM ionomycin, and the incubation was continued for 30 min, followed by sampling for isoelectric focusing. Western blotting: A, GRP78 at 45 min; B, eIF-2α at 45 min; C, GRP78 at 75 min; D, eIF-2α at 75 min. The positions of modified (GRP78(m)) and unmodified GRP78 and of the phosphorylated (eIF2α(P)) and non-phosphorylated forms of eIF2α are indicated by the short arrows.

**RESULTS**

**Modification of GRP78 in Response to the Inhibition of mRNA Translation by Various Agents**—Good evidence supports the hypothesis that GRP78 functions in part to coordinate the respective rates of ER protein processing and mRNA translation (6). It was therefore of interest to examine GRP78 for modifications occurring in response to different classes of agents that inhibit translation. GH3 pituitary cells were chosen as a model system for the experiments in view of their extensive characterization in terms of Ca2+ homeostasis and translational control. Analyses of GRP78 modification were conducted in conjunction with determinations of amino acid incorporation into protein and measurements of the phosphorylation of eIF-2α as an index of the suppression of initiation. Relatively short incubations (30–90 min) were utilized to minimize induction of chaperone by chemical stressors that inhibit mRNA translation. Isoelectric focusing procedures permitted the separation of eIF-2α from its phosphorylated form concurrently with the separation of the unmodified and modified forms of GRP78. Both types of analyses were conducted by sequential Western blotting of the same transfer membrane.

GH3 cells were initially exposed for 30 min to various direct-acting inhibitors of translational elongation (puromycin, cycloheximide, and emetine) or to pactamycin, an acknowledged inhibitor of translational initiation (Fig. 1). All of these agents...
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were strongly inhibitory to amino acid incorporation. None of them promoted eIF-2 phosphorylation (B, lanes 2–5) with respect to an untreated control (B, lane 1), but all generated a separable modified form of GRP78 (A, lanes 2–5) as compared with an untreated control (A, lane 1). The ER Ca\(^{2+}\)-mobilizing agents, ionomycin and thapsigargin, and the reducing agent, dithiothreitol, strongly inhibit both ER protein processing and translational initiation. These agents increased eIF-2 phosphorylation (B, lanes 6–8) without generating modified GRP78 (A, lanes 6–8). During longer term exposures, these agents act as ER stressors that induce the synthesis of GRP78 but not heat shock proteins. Sodium arsenite, in contrast, modifies the sulfhydryl groups of cytoplasmic proteins. This event produces cytoplasmic stress associated with the complexing of cytoplasmic stressors that induce the synthesis of GRP78 but not heat shock proteins. Sodium arsenite, in contrast, modifies the sulfhydryl groups of cytoplasmic proteins. This event produces cytoplasmic stress associated with the complexing of cytoplasmic chaperones, the activation of double-stranded RNA-activated eIF-2\(^{a}\) kinase, and the inhibition of translational initiation, and the subsequent induction of the heat shock proteins but not GRP78 (28). Arsenite treatment produced phosphorylation of eIF-2 (B, lane 10) in conjunction with GRP78 modification (A, lane 10). The modification of GRP78 therefore differed according to the class of agent producing translational inhibition.

During the subsequent 30 min of incubation the fractional modification of GRP78 increased in the nontreated controls (C, lane 1), a typical finding associated with longer term incubations without serum. Addition of ionomycin to mobilize sequestered Ca\(^{2+}\) reversed this modification (C, lane 9). Replicate samples undergoing treatments that inhibited protein synthesis (C and D, lanes 2–8, and 10) were also adjusted with ionomycin to ascertain whether the cells remained responsive to an ER stressor. Ionomycin treatment decreased pre-existing modification of GRP78 resulting from conventional inhibitors of translation at either elongation (C, lanes 2–4) or at initiation (C, lane 5). The modification of GRP78 in response to arsenite was also abolished by ionomycin (C, lane 10). Cells that were pretreated with ER stressors (C and D, lanes 6–8) were not further affected by the addition of ionomycin. Phosphorylation of eIF-2\(^{a}\) developed following the addition of ionomycin to a previously untreated sample (D, lane 9) but not in an untreated control (D, lane 1) as well as in samples containing conventional elongation inhibitors (D, lanes 2–4). Pactamycin treatment invariably precluded eIF-2\(^{a}\) phosphorylation in response to ER stressors (D, lane 5). Cells treated with arsenite displayed intensified eIF-2\(^{a}\) phosphorylation in response to ionomycin (D, lane 10).

Both the modified and unmodified forms of GRP78 focused as discrete protein bands without evidence of multiple banding, as would have been anticipated if multiple sites were covalently modified or if more than one type of modification was occurring. Migration of the modified GRP78 to a more acidic isoelectric point was then tested by the addition of [\(2^{2}\)-H]adenosine into the modified form of GRP78. Cells (4 \( \times \) 10\(^{6}\) m\(^{-1}\)) were incubated as described under “Experimental Procedures” with [\(2^{2}\)-H]adenosine (100 \( \mu \)g/ml) for 2 h. Cells were then diluted 2-fold with fresh [\(2^{2}\)-H]adenosine-containing medium with or without 50 \( \mu \)g cycloheximide (CHX). After an additional hour of incubation, samples were collected for separation of proteins by isoelectric focusing. The same blot was used for analysis of GRP78 by Western blotting (left) and autoradiography (1 month, right). B, stability of the modified form of GRP78 to alkaline phosphatase. Cells were incubated with 50 \( \mu \)g cycloheximide for 2 h. Lysates (200 \( \mu \)l) and/or recombinant [\(32\)-P]GRP78 (1 \( \mu \)g) were incubated for 2 h either without further addition or with calf intestinal alkaline phosphatase (Alk. P’tase, 10 units), okadaic acid (1 \( \mu \)M), or trypsin (0.001%). Samples were treated for 18 h with 5 volumes of ice-cold acetone, and the precipitates were collected, dried, and resuspended in 100 \( \mu \)l urea buffer, 30 \( \mu \)l of which was subjected to isoelectric focusing. Lower, Western blots of GRP78; upper, autoradiography of the same blots. C, inhibition of GRP78 modification by inhibitors of ADP-ribosylation. Cells were incubated for 30 min without addition, with 0.5 or 1 \( \times \) novobiocin (Novo), or with 20, 40, or 80 \( \mu \)g nicotinamide (Nico). Samples were then divided into two portions, one of which received cycloheximide (20 \( \mu \)M), trypsin (0.001%). All samples were incubated for an additional 90 min and analyzed for GRP78 by isoelectric focusing followed by Western blotting. Upper blots, without cycloheximide; lower blots, with cycloheximide. Short arrows in A–C indicate the positions of modified (GRP78(m)) and unmodified GRP78.

The role of phosphorylation in the generation of modified GRP78 was examined in more detail. Purified recombinant GRP78 was autophosphorylated with [\(\gamma^{32}\)-P]ATP. The stability of the phosphate moiety was then tested by the addition of a cell lysate derived from GH\(_3\) cells that had been pretreated with cycloheximide to promote modification of GRP78. Isoelectric focusing and Western blotting procedures revealed one major band of recombinant GRP78 migrating to the same position as the unmodified GRP78 derived from the cell lysates (Fig. 2B, lower lanes). The radiolabeled recombinant protein (Fig. 2B, upper lanes), as determined by radioautography, displayed the same isoelectric behavior as the modified form of GRP78 detected in cell lysates by Western blotting. Treatment of lysates with alkaline phosphatase did not affect the propor-

![Figure 2](image)

**Fig. 2.** Evidence that GRP78 of GH\(_3\) cells is modified by ADP-ribosylation rather than by phosphorylation. A, incorporation of adenine into the modified form of GRP78. Cells (4 \( \times \) 10\(^{6}\) m\(^{-1}\)) were incubated as described under “Experimental Procedures” with [\(2^{2}\)-H]adenosine (100 \( \mu \)g/ml) for 2 h. Cells were then diluted 2-fold with fresh [\(2^{2}\)-H]adenosine-containing medium with or without 50 \( \mu \)g cycloheximide (CHX). After an additional hour of incubation, samples were collected for separation of proteins by isoelectric focusing. The same blot was used for analysis of GRP78 by Western blotting (left) and autoradiography (1 month, right). B, stability of the modified form of GRP78 to alkaline phosphatase. Cells were incubated with 50 \( \mu \)g cycloheximide for 2 h. Lysates (200 \( \mu \)l) and/or recombinant [\(32\)-P]GRP78 (1 \( \mu \)g) were incubated for 2 h either without further addition or with calf intestinal alkaline phosphatase (Alk. P’tase, 10 units), okadaic acid (1 \( \mu \)M), or trypsin (0.001%). Samples were treated for 18 h with 5 volumes of ice-cold acetone, and the precipitates were collected, dried, and resuspended in 100 \( \mu \)l urea buffer, 30 \( \mu \)l of which was subjected to isoelectric focusing. Lower, Western blots of GRP78; upper, autoradiography of the same blots. C, inhibition of GRP78 modification by inhibitors of ADP-ribosylation. Cells were incubated for 30 min without addition, with 0.5 or 1 \( \times \) novobiocin (Novo), or with 20, 40, or 80 \( \mu \)g nicotinamide (Nico). Samples were then divided into two portions, one of which received cycloheximide (20 \( \mu \)M), trypsin (0.001%). All samples were incubated for an additional 90 min and analyzed for GRP78 by isoelectric focusing followed by Western blotting. Upper blots, without cycloheximide; lower blots, with cycloheximide. Short arrows in A–C indicate the positions of modified (GRP78(m)) and unmodified GRP78.

![Diagram](image)
tion of GRP78 in the modified form, whereas the \(^{32}\)P-labeled recombinant protein was stripped of radioactivity. Dephosphorylation of recombinant GRP78 also occurred in incubations with lysate but without okadaic acid or alkaline phosphatase (not shown). Both the modified and unmodified forms of GRP78 were fully accessible and susceptible to proteolysis by trypsin. ADP-ribosylation of various proteins has been reported to be inhibited by novobiocin and nicotinamide (29). The small amount of GRP78 that was modified in control cells was found to be abolished by either agent (Fig. 2C, upper lanes). The much larger fractional modification of GRP78 in response to cycloheximide was also completely suppressed by either novobiocin or nicotinamide (Fig. 2C, lower lanes). Novobiocin at 50 \(\mu M\) was sufficient for full suppression of ADP-ribosylation (not shown). Collectively, these findings emphasize that the covalent modification of GRP78 occurring in intact GH\(_3\) cells is consistent with ADP-ribosylation of the protein as described by others (15–22).

ADP-ribosylation of GRP78 and the Phosphorylation of eIF-2\(\alpha\) in Response to ER Stressors—The time dependence of the ADP-ribosylation of GRP78 in response to cycloheximide was examined over 90 min (Fig. 3). ADP-ribosylation did not change in untreated controls throughout this period (lanes A, left). Cells exposed to cycloheximide developed increasing degrees of ADP-ribosylation within 15 min that approached maximal values at 60 min (lanes B, left). Neither of these conditions affected eIF-2\(\alpha\) phosphorylation (lanes A and B, right). Cycloheximide-treated cells were then washed to remove the drug such that amino acid incorporation was no longer inhibited, and the cells were analyzed for the reversal of the ADP-ribosylation of GRP78. Reversal occurred with the same time dependence as seen for the development of ADP-ribosylation of GRP78 (lanes C, left) without eIF-2\(\alpha\) phosphorylation (lanes C, right). The reversal of ADP-ribosylation was accelerated approximately 2-fold by either ionomycin (lanes D, left) or by dithiothreitol (lanes E, left) at concentrations established to suppress protein processing and amino acid incorporation.

Phosphorylation of eIF-2 in response to either agent became maximal within 15 min (lanes D and E, right). It was therefore apparent that eIF-2\(\alpha\) phosphorylation occurred more rapidly than either ADP-ribosylation or deribosylation of GRP78.

Shorter term incubations were designed to minimize deactivation of GRP78 from ADP-ribosylation in response to cycloheximide while providing sufficient time (10 min) for eIF-2\(\alpha\) phosphorylation in response to ER stressors. It was anticipated that slowed rates of translational elongation should result in the depletion of ER-processible protein as proteins completed processing in the absence of continuing translocation. Some accumulation of the active (unmodified) form GRP78 dissociating from proteins completing processing would be anticipated in view of the relatively slow rate of inactivation of GRP78 by ADP-ribosylation. The relationship of protein synthetic rates to the ADP-ribosylation of GRP78 and the phosphorylation of eIF-2\(\alpha\) was examined at a series of cycloheximide concentrations (0.2, 0.75, and 10 \(\mu M\)) (Fig. 4). These concentrations produced graded inhibitions of amino acid incorporation ranging from 50 to 93%. Over 30 min ADP-ribosylation was increased by each cycloheximide concentration (lanes 1, B–D, left) as compared with that of the untreated control (lane 1A, left). Phosphorylation of eIF-2\(\alpha\) was not altered at any cycloheximide concentration in incubations without ER stressor (lanes 1, right). Phosphorylation of eIF-2\(\alpha\) in response to a 15-min exposure to either ionomycin (1 \(\mu M\), lanes 2, right) or dithiothreitol (600 \(\mu M\), lanes 3, right) was also examined in cells that had been pretreated with cycloheximide for 15 min. Under these conditions eIF-2\(\alpha\) phosphorylation in response to stressors was sharply reduced by increasing concentrations of cycloheximide (lanes 2 and 3, right). Phosphorylation of eIF-2\(\alpha\) in response to ER stressors was completely suppressed at cycloheximide concentrations that largely inhibited translation (lanes D, right). Modest reductions of ADP-ribosylation of GRP78 occurred in response to ionomycin (lanes 2, left) or dithiothreitol (lanes 3, left) as compared with incubations with cycloheximide alone (lanes 1, B–D, left).

The effect of cycloheximide on the sensitivity of eIF-2\(\alpha\) phosphorylation to ER stressors was examined at various times of incubation (0–90 min) at a high concentration of the elongation inhibitor (20 \(\mu M\)) (Fig. 5). Samples for each incubation time were adjusted with either ionomycin or dithiothreitol 15 min...
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**Fig. 5.** Sensitivity of eIF-2α phosphorylation to ER stressors as a function of time following inhibition of mRNA translation by cycloheximide. GH<sub>3</sub> cells were suspended for incubation under standard conditions. Initial samplings (A) were conducted at the beginning of the incubation, and the remaining cells were adjusted to 20 μM cycloheximide for samplings immediately (B), and at 15, 30, 45, and 90 min of incubation (lanes C–F, respectively). The samplings were either left unadjusted (lane 1) or immediately adjusted with 0.2 μM ionomycin (lane 2), 1 μM ionomycin (lane 3), 200 μM dithiothreitol (lane 4), or 600 μM dithiothreitol (lane 5). Cells were then incubated for 15 min with these adjustments and prepared for isoelectric focusing and Western blotting of GRP78 and eIF-2α. As in Fig. 1, the modified form of GRP78 and the phosphorylated form of eIF-2α migrated to slightly more acidic positions. Prior to addition of cycloheximide (as in A), samples were also removed for measurements of [3H]Leucine pulse incorporation after 15 min with or without stressor. Inhibitions of incorporation by 0.2 and 1 μM ionomycin or by 200 or 600 μM dithiothreitol were 77, 92, 89, and 95%, respectively.

before collection. Concentrations of each agent were tested that were known to produce either submaximal or maximal degrees of eIF-2α phosphorylation. Cells incubated as controls without cycloheximide displayed high degrees of eIF-2 phosphorylation with either concentration of either stressor (lanes A, 2–5, right) as compared with the unstimulated control (lane A1, right) which did not change throughout the course of the experiment (lanes 1, right). Samples adjusted simultaneously with cycloheximide and ER stressors for 15 min displayed much less eIF-2 phosphorylation (lanes B, 2–5, right). Samples pretreated with cycloheximide for 15 min before the addition of stressors (lanes C, 2–5, right) displayed minimal degrees of phosphorylation of eIF-2. Phosphorylation in samples adjusted at 30 min of cycloheximide treatment (lanes D, 2–5, right) was depressed to a slightly lesser extent. By 45 min of cycloheximide treatment before adjustment with stressor, eIF-2 phosphorylation had nearly recovered (lanes E, 2–5, right) to the original values observed without cycloheximide (lanes A, right). This pattern was maintained at 90 min (lanes F, 2–5, right). The simultaneous analysis of ADP-ribosylation of GRP78 was also conducted. GRP78 was not significantly modified in controls lacking cycloheximide (lanes A, 1–5, left). ADP-ribosylation of GRP78 increased with time of incubation in cells exposed to cycloheximide without ER stressors (lanes A, B–F, left) to an approximately 50% at the longest treatment period with cycloheximide (lane 1, F, left). At each time the addition of either ionomycin or dithiothreitol lowered the fraction of GRP78 that was ADP-ribosylated. Marked reductions of ADP-ribosylation occurred during shorter pretreatment periods with cycloheximide (0–30 min) (lanes B–D, 2–5, left). Cells treated for 45 or 90 min with cycloheximide before the addition of stressors exhibited more modest reversals of ADP-ribosylation of GRP78 (lanes E–F, 2–5 left). Cycloheximide-treated samples with high degrees of ADP-ribosylation of GRP78 were invariably more subject to eIF-2 phosphorylation in response to ER stressors.

The ADP-ribosylation of GRP78 and the phosphorylation of eIF-2 in GH<sub>3</sub> cells were examined for coordination during a restricted time frame (Fig. 6). Additions of cycloheximide were staged at various times with respect to adjustments with ionomycin over a total incubation period of 30 min. The 30-min control for untreated cells possessed a marginal degree of ADP-ribosylation (A, lane 1) and undetectable eIF-2 phosphorylation (B, lane 1). Samples exposed to cycloheximide alone for 30 min developed moderate ADP-ribosylation (A, lane 2) without eIF-2 phosphorylation (B, lane 2). In contrast, samples treated with ionophore for 30 min displayed minimal ADP-ribosylation of GRP78 (A, lane 3) in conjunction with increased eIF-2 phosphorylation (B, lane 3). Some samples that were incubated with ionomycin for a total of 30 min were challenged at 10 min (lane 4) or 5 min (lane 5) of incubation with cycloheximide, providing 20 and 25 min of drug overlap, respectively. These incubations provided an index of how readily cycloheximide interdicted the actions of ionomycin. Cycloheximide, when added 10 min after ionomycin, produced little, if any, alteration in GRP78 ADP-ribosylation (A, lane 4) or eIF-2 phosphorylation (B, lane 4) from that observed in the ionomycin control (lanes 3). When added 5 min after ionomycin, however, cycloheximide suppressed eIF-2 phosphorylation about 50% (B, lane 5) without much increase in ADP-ribosylation of GRP78 (A, lane 5). It was therefore apparent that the effects of ionophore on eIF-2 phosphorylation became refractory to modification by cycloheximide within 10 min. This time frame corresponds closely with that required for ionomycin to generate eIF-2 phosphorylation in GH<sub>3</sub> cells. Phosphorylation is half-maximal at 4 mm and approaches maximal values by 8 mm (24). The ability of ionomycin to reverse cycloheximide-generated changes was assessed in additional incubations. Samples were treated with cycloheximide for 30 min with ionophore being added at 2, 5, and 10 min of incubation corresponding to overlap periods of 28, 25, and 20, respectively. With these increasingly later times of ionomycin addition, graded increases in ADP-ribosylation (A, lanes 6–8) and graded decreases in eIF-2 phosphorylation (B, lanes 6–8) were observed. The 2-mm delay in ionophore addition reduced eIF-2 phosphorylation about 50% (B, lane 6) as compared with the ionomycin control (B, lane 7), while a 10-mm delay in the addition almost abolished phosphorylation (B, lane 8). Only a brief exposure to cycloheximide, therefore, was needed to arrest eIF-2 phosphorylation in response to the subsequent addition of ionomycin. These data are consistent with the time required for maximal inhibition of amino acid incorporation in response to cycloheximide (1–2 min).

**Phosphorylation of eIF-2 and the ADP-ribosylation of GRP78 in Longer Term Incubations**—The preceding results collectively...
emphasize that a continuing flow of processible protein to the ER is required to generate high degrees of phosphorylation of eIF-2 in response to stressors that retard protein folding. Folding intermediates that accumulate in response to ER stressors provide high affinity sites that sequester GRP78 and retard recycling of the chaperone. In contrast inhibitors of mRNA translation, such as cycloheximide, abolish the provision of new processible protein to the ER while permitting completion of protein processing already in progress within the organelle. As proteins complete processing, GRP78 accumulates in the catalytically active form that is subject to ADP-ribosylation and inactivation during the following 90 min. The fractional ADP-ribosylation of GRP78 in response to cycloheximide therefore represents a measure of the chaperone that had formerly been catalytically active in protein processing.

Brefeldin A inhibits ER to Golgi traffic while permitting the retrograde coalescence of cis-, medial-, and trans-Golgi components into the ER. Protein synthesis continues unabated for several hours but thereafter declines modestly in conjunction with increasing phosphorylation of eIF-2 (30, 31). It was of interest to determine whether ER protein retention in response to brefeldin A affected the ADP-ribosylation of GRP78 observed in response to cycloheximide treatment. Replica samples of GH3 cells in replicate samples were incubated for 3 h in Ham's F-10 medium with 10% fetal calf serum. Brefeldin A (5 μg/ml) was added at various times (0, 1.5, and 2.5 h of incubation). At the end of the 3-h incubation, the cells were collected and resuspended under standard conditions either with (B) or without (A) adjustment to 20 μM cycloheximide. Samples previously adjusted with brefeldin A received additional drug. The incubation was then continued and additional replicates were adjusted with brefeldin at 3.5 h and at 4.5 h total incubation time. All samples were then collected for analysis of GRP78 and eIF-2α by isoelectric focusing and Western blotting. Time (h) with brefeldin A is indicated. As in Fig. 1, the modified form of GRP78 and the phosphorylated form of eIF-2 migrated to slightly more acidic positions.

FIG. 7. Effect of brefeldin A on eIF-2α phosphorylation and modification of GRP78. GH3 cells in replicate samples were incubated for 3 h in Ham's F-10 medium with 10% fetal calf serum. Brefeldin A (5 μg/ml) was added at various times (0, 1.5, and 2.5 h of incubation). At the end of the 3-h incubation, the cells were collected and resuspended under standard conditions either with (B) or without (A) adjustment to 20 μM cycloheximide. Samples previously adjusted with brefeldin A received additional drug. The incubation was then continued and additional replicates were adjusted with brefeldin at 3.5 h and at 4.5 h total incubation time. All samples were then collected for analysis of GRP78 and eIF-2α by isoelectric focusing and Western blotting. Time (h) with brefeldin A is indicated. As in Fig. 1, the modified form of GRP78 and the phosphorylated form of eIF-2 migrated to slightly more acidic positions.

FIG. 8. Selective loss of the modified form of GRP78 during prolonged incubation of GH3 cells with cycloheximide. A, disappearance of modified GRP78. Cultures at 7 days growth were divided into two portions, one of which was adjusted to 20 μM cycloheximide (CHX). Both portions were adjusted with 10% fresh growth medium and incubated for 2 h to lower the fractional phosphorylation eIF-2α and fractional modification of GRP78. Culturing of the cells was then continued for 26 h with periodic sampling for isoelectric focusing and analysis of GRP78 and eIF-2α by Western blotting. B, decrease in total GRP78 content. Urea sample preparations from A above were diluted with two parts of 5× Laemmli's sample buffer. Samples were subjected to SDS-PAGE (7.5%) followed by Western blotting onto polyvinylidene difluoride membranes and immunostaining with anti-GRP78 antibodies as described under "Experimental Procedures."
ADP-ribosylation of GRP78 and mRNA Translation

Fig. 9. Phosphorylation of eIF-2α after prolonged incubation of GH3 cells with cycloheximide. Cells cultured as described in the legend to Fig. 8 were adjusted with 10% fresh growth medium and incubated for 23 h with or without 20 μM cycloheximide (CHX). The cells were then resuspended under standard conditions; replicates were immediately challenged for 20 min with the indicated concentrations of ionomycin (IM); and samples were taken for isoelectric focusing and analysis of GRP78 and eIF-2α by Western blotting. As in Fig. 1, the modified form of GRP78 and the phosphorylated form of eIF-2α migrated to slightly more acidic positions.

Fig. 10. The dynamics of GRP78/BiP. Solid arrows indicate the immediate interactions of free unmodified chaperone, and dashed arrows indicate consequences of these interactions. The – and + symbols indicate suppressive and stimulatory actions, respectively, of the free unmodified form of GRP78/BiP. For further details, see “Discussion.”

ADP-ribosylation of the ER chaperone, GRP78, was analyzed in conjunction with eIF-2 phosphorylation during challenge with agents that inhibit mRNA translation by various mechanisms. Phosphorylation/dephosphorylation of eIF-2 provided a sensitive index for monitoring rapid changes in translational initiation capabilities even when amino acid incorporation was strongly inhibited. This parameter also provided a functional marker for assessing whether alterations in the availability of GRP78 affected translational initiation. Direct-acting inhibitors of mRNA translation such as pactamycin, cycloheximide, and emetine that depressed protein flow to the ER produced ADP-ribosylation of GRP78 without the phosphorylation of eIF-2 (Fig. 1). This observation is explicable in terms of the ADP-ribosylation and inactivation of GRP78 following its release from proteins completing processing in the ER. Under conditions where new protein influx is suppressed at mRNA translation, ER processing requirements for GRP78 are sharply reduced. Sodium arsenite, which modifies the sulfhydryl groups of cytoplasmic proteins such that binding to heat shock proteins occurs, facilitated eIF-2 phosphorylation in conjunction with the ADP-ribosylation of GRP78. This observation is in accord with the finding that arsenite does not directly affect ER protein processing other than by depressing the flow of protein to the organelle (28). Ca2+-mobilizing agents and dithiothreitol, however, prevented ADP-ribosylation of GRP78 while producing eIF-2 phosphorylation. These changes were consistent with the inhibition of mRNA translation secondary to the inhibition of protein processing. Under this circumstance free GRP78 would be anticipated to decline in response to increased binding opportunities on protein folding intermediates. ADP-ribosylated GRP78 values fell sharply during application of ER stressors.

In assessing the potential role of GRP78 in regulating eIF-2 phosphorylation, several time-dependent parameters must be considered. Cycloheximide maximally inhibits amino acid incorporation within 1–2 min. Clearing the ER of processible protein, however, proceeds relatively slowly and varies from protein to protein. Small proteins such as α1-antitrypsin are largely cleared within 30 min, whereas large proteins such as thyroglobulin require several hours (32, 33). ADP-ribosylation of GRP78 would depend upon the release of the active form of the chaperone from proteins completing ER processing. ADP-ribosylation of GRP78 in response to cycloheximide treatment increased rapidly for 60 min (Fig. 3) rising by 4 h to maximal values of approximately 60% (Fig. 8). This value provides an indication of the fraction of the GRP78 that was engaged catalytically in protein folding at the start of the incubation. The ADP-ribosylation of GRP78 in cells treated for 90 min with cycloheximide was readily reversible upon removal of the inhibitor by washout as monitored by complete restoration of rates of amino acid incorporation (Fig. 3). The onset and offset of the modification, which respectively reflect the depletion and repletion of ER protein folding intermediates, appeared to proceed at approximately the same rate in cells that were not treated further with ER stressors. Deribosylation was accelerated by the addition of either ionomycin or dithiothreitol and was complete by 90 min. In contrast, the eIF-2 phosphorylation that occurred in response to these agents achieved maximal values within 15 min that were subsequently sustained throughout the incubation. Phosphorylation of eIF-2 did not appear to be directly dependent upon either deribosylation (Fig. 3) or ADP-ribosylation (Fig. 5) of GRP78.

The relatively slow rate of ADP-ribosylation of GRP78 during cycloheximide treatment raised the possibility that the free active, monomeric form of the chaperone was accumulating prior to its modification. In the event that this form of GRP78 functions in a mass action manner to suppress eIF-2 kinase activity, alterations would be expected to occur in the sensitivity of eIF-2 phosphorylation in response to ER stressors. Such changes were indeed observed. Cells adjusted to graded rates of amino acid incorporation with cycloheximide and then exposed for 15 min to ionomycin or dithiothreitol displayed remarkable reductions in eIF-2 phosphorylation as amino acid incorporation was increasingly inhibited (Fig. 4). Phosphorylation was largely unaffected by 50% inhibitions of protein synthesis, markedly reduced at 70% inhibitions, and abolished by inhibitions exceeding 90%. These changes were accompanied by relatively marginal alterations in the content of the ADP-ribosylated form of GRP78. The sensitivity of eIF-2 phosphorylation in response to stressors also varied as a function of increasing pretreatment times with cycloheximide at full inhibitory doses (Fig. 5). Phosphorylation declined sharply within 15 min reaching minimal values at 30 min of pretreatment. At longer incubation periods sensitivity to stressors recovered and approached control values by 90 min. These later periods were associated with increasing contents of ADP-ribosylated GRP78. Collectively these results appear to reflect an early increase followed by a decline in the active form of GRP78 during cycloheximide treatment.

ADP-ribosylation of GRP78 occurred spontaneously during longer term incubations without concurrent eIF-2 phosphorylation. Cells were normally provided with fresh growth medium 16 h before harvesting. Under this condition basal contents of the ribosylated protein were low and frequently negligible. Spontaneous ADP-ribosylation of GRP78 began to appear after...
1–2 h in incubations without serum. High cell density facilitated the fractional modification of GRP78 presumably via a more rapid exhaustion of nutrients. The ADP-ribosylated form of GRP78 invariably accumulated as rates of protein synthesis declined relative to ER processing capability. In effect, the ADP-ribosylation of the chaperone appeared to provide a buffer system by which the rate of protein processing could be balanced with that of protein synthesis. The induction of grp78 mRNA only occurs when protein processing capacity is lower than synthetic capability, as would be exemplified in cells provided with fresh medium (31). The ability of cycloheximide and other direct inhibitors of mRNA translation to block grp78 mRNA induction (34, 35) therefore becomes readily explicable.

GRP78 appears to differ from those rapidly inducible proteins that are also rapidly degraded upon removal of the inducer. GH3 cells exposed to Ca\(^{2+}\)-mobilizing agents induce grp78 mRNA to nearly maximal degrees within 3–4 h in conjunction with expression of the chaperone and the development of resistance to the stressor (31, 36). This resistance is maintained for at least 4 h following removal of the stressor without a perceptible fall in total GRP78. The results of the present report indicate that ADP-ribosylation provides an alternative to degradation for rapidly decommissioning excess chaperone. This alternative is reversible for several hours. It is also apparent that ADP-ribosylation of excess GRP78 would complicate efforts to develop mutants that overexpress the active form of GRP78 in response to a promoter.

During extended incubations with cycloheximide, approximately 40% of the total GRP78 pool was resistant to modification by ADP-ribosylation (Fig. 8). The modified chaperone was selectively lost, either through degradation or secretion, over 24 h. These observations imply that a large fraction of the total GRP78 in GH3 cells is not subject to catalytic recycling. The GRP78 of this fraction was indistinguishable from the catalytically active form of the chaperone. The long term stability of this pool of GRP78 is consistent with the existence of a large pool of high affinity binding sites for the chaperone. Conceivably such sites could be created by interactions of GRP78 with misfolded proteins incapable of ER to Golgi transport, by complexing of GRP78 to translocation intermediates during elongation or, by strong interactions with ER resident proteins. The stability of this pool of GRP78 appears to exceed that of ER protein folding intermediates reported to undergo posttranslational degradation. Cells that were treated for several hours with brefeldin A, which expands the protein content of the ER without inhibiting processing, displayed reduced ADP-ribosylation of GRP78 and increasing eIF-2 phosphorylation when challenged with cycloheximide (Fig. 7). These results are compatible with the conclusion that GRP78 binds well to proteins that have largely completed maturation.

The diagram displayed in Fig. 10 models the various mass-action interactions of GRP78 that are supported by this report in conjunction with the literature. In this scheme the free monomeric, active form of GRP78 serves as a multifunctional modulator of various ER-supported processes including regulation of eIF-2 kinase and mRNA translation, regulation of grp78 expression, and the catalysis of protein folding, as well as, potentially, the targeting of misfolded proteins for degradation (37). In cells with high rates of protein synthesis, the bulk of the GRP78 in the non-modified form (approximately 60%) is complexed with protein folding intermediates. Any slowing of protein synthesis relative to protein processing capability would result in the short term accumulation of the free, active form of GRP78 which is subject to subsequent inactivation by ADP-ribosylation. The ADP-ribosylated form of the chaperone provides a buffering system permitting rates of protein processing to be balanced with protein synthesis. The remaining 40% of the non-modified GRP78 recycles poorly, and its function is unknown. An accumulation of protein folding intermediates would lower the free, monomeric pool of GRP78 while facilitating deribosylation of the modified chaperone and the dissociation of the chaperone from transmembrane components that are responsible for feedback regulation of mRNA translation and grp78 induction. Induction of GRP78 would be anticipated to foster eIF-2 dephosphorylation and resumption of amino acid incorporation, in accord with experimental observation.

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