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Reversible phosphorylation of eukaryotic initiation factor 2α in response to endoplasmic reticular signaling

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Abstract

Agents, such as EGTA, thapsigargin, and ionophore A23187, that mobilize sequestered Ca^{2+} from the endoplasmic reticulum (ER) or dithiothreitol (DTT) that compromises the oxidizing environment of the organelle, disrupt early protein processing and inhibit translational initiation. Increased phosphorylation of eIF-2α (5-fold) and inhibition of eIF-2B activity (50%) occur in intact GH_3 cells exposed to these agents for 15 min (Prostko *et al. J. Biol. Chem.* 267: 16751–16754, 1992). Alterations in eIF-2α phosphorylation and translational activity in response to EGTA were reversed by addition of Ca^{2+} in excess of chelator while responses to DTT were reversible by washing. Exposure for 3 h to either A23187 or DTT, previously shown to induce transcription-dependent translational recovery, resulted in dephosphorylation of eIF-2α in a manner blocked by antinomycin D. Phosphorylation of eIF-2α in response to A23187 or DTT was not prevented by conventional inhibitors of translation including cycloheximide, pactamycin, puromycin, or verrucarin. Prolonged inhibition of protein synthesis to deplete the ER of substrates for protein processing resulted in increased eIF-2α phosphorylation, decreased eIF-2B activity, and reduced monosome content that were indicative of time-dependent blockade; these inhibitors did not abolish polysomal content. Superphosphorylation of eIF-2α occurred upon exposure of these preparations to either A23187 or DTT. Tunicamycin, an inhibitor of co-translational transfer of core oligosaccharide, provoked rapid phosphorylation of eIF-2α and inhibition of translational initiation whereas sugar analog inhibitors of glycoprotein processing did neither. A flow of processible protein to the ER does not appear to be required for the phosphorylation of eIF-2α in response to ER perturbants. We hypothesize that perturbation of the translocon, rather than suppression of protein processing, initiates the signal emanating from the ER culminating in eIF-2α phosphorylation and translational repression. (Mol Cell Biochem **127/128:** 255–265, 1993)

Key words: eIF-2 phosphorylation, eIF-2B activity, endoplasmic reticulum, sequestered Ca^{2+}, translational initiation, protein translocation and processing

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Introduction

During our formative years with Dr. Krebs at Davis, California, we (C. and M. Brostrom) were imbued with our continuing interest in the roles of Ca^{2+} and cAMP in biological control mechanisms. We remember our time with Dr. Krebs with great affection both for his intense interest and high standards in science and for his great decency in the treatment of postdoctoral fellows. We have subsequently aspired, within the constraints of our ability, to pattern our behavior in accord with this experience. Our current research involving the control of protein synthesis at mRNA translation embodies much of the familiar in protein phosphorylation with some new twists that we shall highlight in this article with our associate, Dr. Prostko.

The endoplasmic reticulum (ER) serves as the site of early protein processing, phospholipid biosynthesis, and drug metabolism as well as a repository for sequestered Ca^{2+} subject to release to the cytoplasm in response to hormonally-generated inositol trisphosphate (IP_3) [1–3]. While cytosolic free Ca^{2+} is established to function in regulating intracellular processes involved in the generation of cellular responses to various external stimuli, ER sequestered cation has not conventionally been regarded as serving a regulatory role. Recent studies, however, support the concept that Ca^{2+} sequestered by the ER functions to sustain certain early protein processing events including oligomerization of viral glycoproteins [4], folding of receptor macromolecules [5], and trimming of mannose residues of glycoproteins [6–8]. The degradation of incompletely unassembled or abnormal proteins in the ER lumen is also strongly influenced by stored Ca^{2+} [9, 10].

A large body of evidence links depletion of ER sequestered Ca^{2+} to the inhibition of translational initiation in a variety of cell types. Depletion of ER sequestered cation as measured by ^{45}Ca efflux occurs in response to (a) hormones generating IP_3, (b) chelating agents, such as EGTA, acting as extracellular extractants, (c) thapsigargin, a sesquiterpene lactone that blocks active transport of Ca^{2+} into the organelle [11], and (d) various agents fostering the passage of Ca^{2+} across the ER membrane to the cytoplasm. These latter agents include the divalent cation ionophores ionomycin and A23187, arachidonic acid, and various peptide metalloendoprotease antagonists such as Cbz-Gly-Phe-NH_2 [12–14]. In response to these agents amino acid incorporation as a function of time appears to be unaffected until the ER becomes substantially depleted of Ca^{2+}. For example, acute exposure of GH_3 pituitary cells to Ca^{2+} ionophore A23187 or EGTA has been found to result in the inhibition of amino acid incorporation, disappearance of polysomal content with accumulation of monosomes and ribosomal subunits, sharp reduction of the cellular content of 43S preinitiation complex, and the phosphorylation of eIF-2α and inhibition of eIF-2B [15–17]. Neither translational elongation nor peptide chain termination appeared to be affected in these experiments, since average ribosomal transit times and the methionylation of $tRNA_i^{met}$ were not altered. Amino acid incorporation was unaffected during the period of Ca^{2+} release prior to suppression of initiation. With the exception of thapsigargin [18] which is an irreversible inhibitor of ER Ca^{2+} accumulation, other Ca^{2+}-depleting agents were susceptible to reversal within several min by the addition of supra-physiologic Ca^{2+} concentrations to the extracellular medium under defined conditions. Reticulocytes, which lack a functional ER, do not exhibit effects of Ca^{2+} on initiation but are inhibited in translational elongation in response to influx of the cation [19]. Elongation factor 2 (EF-2) has been reported to be phosphorylated and inhibited by calmodulin-dependent protein kinase III in reticulocyte lysates [20]. Although the factor is phosphorylated in various types of intact nucleated cells in response to increased cytoplasmic free Ca^{2+}, effects on amino acid incorporation have not been demonstrated.

Disruption of the oxidizing environment of the ER with dithiothreitol (DTT), which does not affect Ca^{2+} sequestration, retards the processing of proteins containing disulfide crosslinks [7, 21] and inhibits translational initiation in a rapid and comparable fashion to Ca^{2+}-mobilizing agents [7, 22]. Tunicamycin, which interferes with the lumenal transfer of core oligosaccharide during protein translocation into the ER and disrupts glycoprotein processing [23], also slows translation [8]. Exposure for several h to any of these stressing conditions provokes the induction of an ER resident protein, GRP78/BiP, that is believed to function in protein folding [24]. During this period partial recovery (60–100%) of rates of translational initiation and amino acid incorporation is routinely observed [22, 25]. Both the induction of GRP78 and the recovery of protein synthesis are blocked by actinomycin D; the recovery is also suppressed by antisense oligonucleotides directed against

the leader sequence of GRP78 mRNA. Recovery of translation requires the synthesis of new GRP78/BiP and is associated with the development of cross-tolerance to either stressor.

Increased phosphorylation of eukaryotic initiation factor 2 (eIF-2) is believed to mediate the translational repression occurring in mammalian cells subjected to various physical, chemical and nutritional stresses [26, 27]. The mechanism by which eIF-2 phosphorylation restricts translation has been well characterized. In conjunction with GTP, eIF-2 mediates binding of the initiator tRNA (Met-tRNA$_i^{Met}$) to the 40S ribosomal subunit. The resultant 43S preinitiation complex joins with the 60S ribosomal subunit to form a monosome capable of translation, whereupon eIF-2-associated GTP is converted to GDP. The binary eIF-2-GDP complex cannot function in initiation until eIF-2-GTP is reformed via the catalytic exchange of GDP for GTP. This exchange is accomplished by eIF-2B, a factor typically present at low stoichiometric ratios with respect to eIF-2. A 20–30% increase in the phosphorylation of the α-subunit of eIF-2 appears adequate for sequestration of eIF-2B into an inactive complex such that recycling of eIF-2 cannot occur [28, 29].

Acute treatments of intact GH_3 pituitary cells with various Ca^{2+}-mobilizing agents or DTT were recently found to promote an average 5-fold increase in the amount of phosphorylated eIF-2α and a 50% reduction in eIF-2B activity [17]. These changes coincided temporally with inhibition of initiation. Partial recovery from inhibition upon continued exposure to these agents was associated with the dephosphorylation of eIF-2α. It was suggested that the ER regulates rates of translational initiation through a signaling system that alters the activity of eIF-2 in a reversible fashion. The nature of such signaling, however, is poorly understood other than that it is triggered by conditions that appear to interfere with very early events in protein processing. The following study was undertaken to ascertain whether various ER perturbants could promote phosphorylation of eIF-2α in the absence of protein processing. Results are consistent with the hypothesis that perturbation of the translocon, rather than inhibition of protein processing, initiates the signal emanating from the ER that results in eIF-2α phosphorylation and suppression of mRNA translation.

Materials and methods

Materials

Pactamycin was kindly provided by the Upjohn Co. Monoclonal antibody to eIF-2α and highly purified eIF-2 were the generous gifts of Drs. Edgar Henshaw, University of Rochester, and Jaydev Dholakia, University of Louisville School of Medicine, respectively. Unless otherwise indicated, chemicals and reagents were purchased from the Sigma Chemical Co.

General procedures

GH_3 cells were propagated and maintained as described previously [15]. Harvested cells (2×10^6 per ml) were equilibrated for 30 min in serum-free Hams F-10 medium adjusted to contain 0.2 mM Ca^{2+} prior to use. Amino acid incorporation was measured as described [30] for incubations conducted in triplicate. Values for replicate samples routinely varied within 5% or less. Ribosomal and polysomal size distributions were measured by density-gradient centrifugation as described previously [15].

Determination of the phosphorylation state of the α-subunit of eIF-2

Cells were placed directly into sample buffer containing 3% ampholines (4 parts pH 4–8 and 1 part pH 3.5–10), 2% β-mercaptoethanol, 0.4% Tween-20, and 9.5 M urea. The preparations were then subjected to slab gel isoelectric focusing over a pI range of 5–7 (BDH Resolyte ampholines) in the presence of 9.5 M urea (Boehringer Mannheim) to separate the phosphorylated and unphosphorylated forms of eIF-2α [31]. Gels were treated with Tris buffer and blotted onto PVDF membranes under basic conditions, and the two species of eIF-2α were immunodetected with a monoclonal antibody as described [17]. Relative amounts of phosphorylated and unphosphorylated subunit, which differ by approximately 0.1 pI unit, were quantitated by densitometric scanning.

eIF-2B (GEF) assays

Crude cell lysates were prepared as published elsewhere

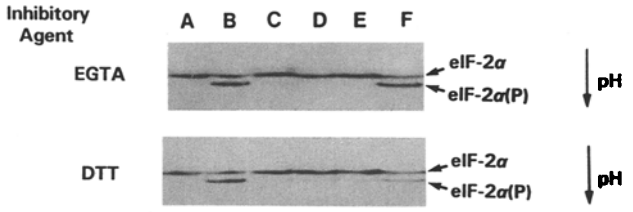

Fig. 1. Rapid reversibility of phosphorylation of eIF-2α in GH_3 cells treated with EGTA or dithiothreitol. Upper panel: Reversibility of the effects of EGTA on eIF-2α phosphorylation. Cells suspended in serum-free Ham's F-10 medium were incubated at 37° C for 30 (A), 60 (C), or 90 (E) min. A separate suspension from the same cell preparation was treated for 30 min in medium containing 2 mM EGTA (B), was then adjusted with 3 mM Ca^{2+} (1 mM in excess of chelator) and the incubation continued for an additional 30 min (D), and then re-adjusted with 5 mM EGTA for the final 30 min of incubation (F). At the end of each treatment, aliquots were removed for measurements of eIF-2α phosphorylation and leucine incorporation (Table 1). Lower panel: Reversibility of the effects of dithiothreitol (DTT) on eIF-2α phosphorylation. Untreated preparations in F-10 medium were incubated at 37° C for 15 min (A), and were then washed twice with buffered saline, resuspended in fresh medium, and incubated for an additional 15 (B) or 30 (C) min. A separate aliquot from the same cell preparation was treated for 15 min with 1.5 mM DTT (B), washed twice with buffered saline, resuspended in fesh medium lacking DTT and incubated for an additional 15 min (D), and then re-adjusted with DTT (1.5 mM) for the final 15 min of incubation (F). At the end of each treatment, samples were taken for measurements of eIF-2α phosphorylation and leucine incorporation (Table 1).

[32]. eIF-2B activity of lysates was measured as the ability to dissociate (^{3}H)GDP from preformed eIF-2·[^{3}H]GDP [29, 32]. Units of activity were calculated as previously described [17], except that standard 200 µl reactions contained 0.35 A_{260} units of cell extracts (approximately 75 µg of total protein).

Results

Rapid reversibility of the effects of ER perturbants on eIF-2α phosphorylation and inhibition of protein synthesis

In initial experiments EGTA and DTT, typifying two classes of ER perturbing agents, were examined for reversible actions of the phosphorylation of eIF-2α and the inhibition of amino acid incorporation in intact GH_3 pituitary cells (Fig. 1 and Table 1). In accord with previous findings [30, 33], EGTA was a highly effective inhibitor of amino acid incorporation with effects that were fully reversed by the addition of Ca^{2+} in excess of chelator and that could be reimposed upon addition of EGTA in excess of Ca^{2+} (Table 1). Similarly, the marked inhibition of incorporation by DTT was reversible by washing and resuspending the cells in fresh medium lacking the reducing agent and was quickly reinstituted by readdition of DTT. The relative amount of phosphorylated eIF-2α subunit [eIF-2α(P)] in untreated control preparations carried throughout the experiments was typically very low, approximating less than 2% of the total eIF-2 molecules (Fig. 1A, C, and E, top and bottom). Treatment with EGTA in excess of Ca^{2+} resulted in a marked increase in eIF-2α(P) with more than 50% of

Table 1. Reversible inhibition of leucine incorporation and phosphorylation of eIF-2α in GH_3 cells treated with EGTA or dithiothreitol (DTT). Conditions A–F for experiments I and II are as described in the legend to Fig. 1. Leucine incorporation is expressed the mean± range of values obtained for triplicate samples incubated for 15 min at 37° C. Densitometry was performed on the immunoblots displayed in Fig. 1 to quantitate the percentage of eIF-2α in the phosphorylated form

	Leucine incorporation (pmol/10^6 cells)	eIF-2α(P) (%)
Experiment I		
A Control	565± 35	1
B EGTA (2 mM)	23± 3	57
C Control, no additions to A	574± 21	1
D Ca^{2+} (3 mM) added to B	566± 74	1
E Control, no additions to C	400± 42	1
F EGTA (5 mM) added to D	25± 11	62
Experiment II		
A Control	210± 13	1
B DTT (1.5 mM)	39± 11	64
C Control, washed twice	192± 10	2
D B, washed twice	176± 11	2
E Control, no additions to C	201± 8	2
F DTT (1.5 mM) re-added to D	18± 1	41

the total subunit being phosphorylated (B, top). Ca^{2+} added in 1 mM excess of chelator returned eIF-2α phosphate content to control values within 30 min (C and D, top). Subsequent addition of EGTA in excess of Ca^{2+} resulted in increased levels of eIF-2α phosphorylation (F, top). In agreement with previous findings [17], the imposition of reducing conditions with DTT resulted in a 64% increase in the amount of eIF-2α(P) (B, bottom). Phosphorylation of the protein was reversed to control values following removal of the agent (D, bottom) and restored by re-challenging with freshly added DTT (F, bottom). Concurrent measurements of amino acid incorporation confirmed that translational arrest occurred during elevated eIF-2α phosphorylation (Table 1).

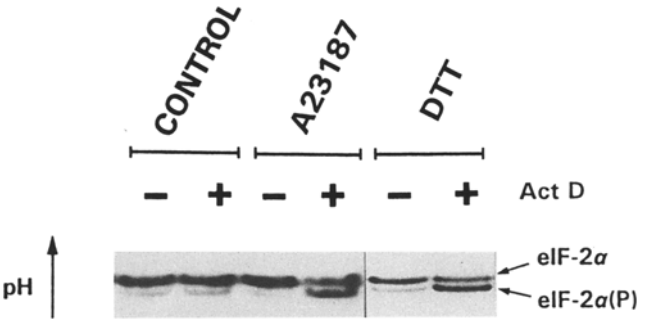

Fig. 2. Dephosphorylation of eIF-2α during chronic exposure of GH_3 cells to A23187 or dithiothreitol and blockade of the dephosphorylation by actinomycin D. Cells in F-10 medium were treated for 3 h in the absence or presence of 3 μg/ml actinomycin D (control) or in the presence of 0.6 μM phorbol 12-myristate 13-acetate, 0.3 μg/ml forskolin and either 1 μM A23187 or 1 mM dithiothreitol (DTT) and without or with actinomycin D as indicated. Samples were taken at the end of each incubation for measurements of eIF-2α phosphorylation.

Reversibility of eIF-2α phosphorylation during translational accommodation to ER perturbants

Various cell types adapt over several h to Ca^{2+}-mobilizing or reducing agents with partial to almost complete recovery of rates of amino acid incorporation and translational initiation. This accommodation depends on active synthesis of new mRNA and, in certain cell types including GH_3, requires cAMP elevation and/or the presence of a phorbol ester [22, 25]. The fraction of eIF-2 phosphorylated on the α-subunit was examined after 3 h of incubation with either A23187 or DTT (Fig. 2) under conditions wherein GH_3 cells exhibited significant translational accommodation to these agents (not shown). During the longer incubations without ER perturbant or actinomycin D, or with actinomycin D alone, the content of eIF-2α(P) remained at the basal values routinely observed during short incubations. For preparations incubated for 3 h with either A23187 or DTT, eIF-2α(P) content was observed to decline from the elevated values obtained during brief incubations with each stressor to basal, control values. When actinomycin D was included in the extended incubations with ionophore or DTT, however, phosphorylated eIF-2α remained at an elevated level.

Effects of RNA and protein synthetic inhibitors on the phosphorylation of eIF-2α in response to ER perturbants

Since both Ca^{2+} depletion [4–10] and reducing conditions [7, 12] disrupt ER processing of various proteins, accumulation within the organelle of underprocessed polypeptides in response to such conditions could hypothetically trigger the phosphorylation of eIF-2α and the inhibition of translational initiation. The possibility that the phosphorylation of eIF-2α in response to ionomycin or DTT depended on synthesis of new polypeptides was therefore examined and the hypothesis tested that communication between the ER and the translational apparatus is disrupted when protein synthesis is shut off. Translational blockade was imposed with pactamycin, an inhibitor of initiation, or with cycloheximide, puromycin or verrucarin, each of which inhibits elongation in a different manner [34]. The RNA synthesis inhibitor actinomycin D was also included in this study. With the exception of actinomycin D, each of these inhibitors caused almost complete inhibition of amino acid incorporation within 30 min (Table 2). None of the inhibitors, including pactamycin, abolished polysomal content during either 30 min or 12 h of treatment (Fig. 3). Prominent increases in 80S monosomal content occurring during 30 min exposure to pactamycin (trace D), puromycin (trace E), or verrucarin (trace F) disappeared by 12 h, an unexpected finding suggestive of a time-dependent alteration in initiation.

Untreated preparations displayed typical increases in eIF-2α(P) following a 12 min challenge with Ca^{2+} ionophore A23187 or DTT (Fig. 4A, top). Pretreatment for 30 min with actinomycin D (B), cycloheximide (C), pactamycin (D), puromycin (E), or verrucarin A (F) did not qualitatively abolish the response to either A23187 or DTT. However, the relative amount of eIF-2α phosphorylation that was ultimately achieved in response to

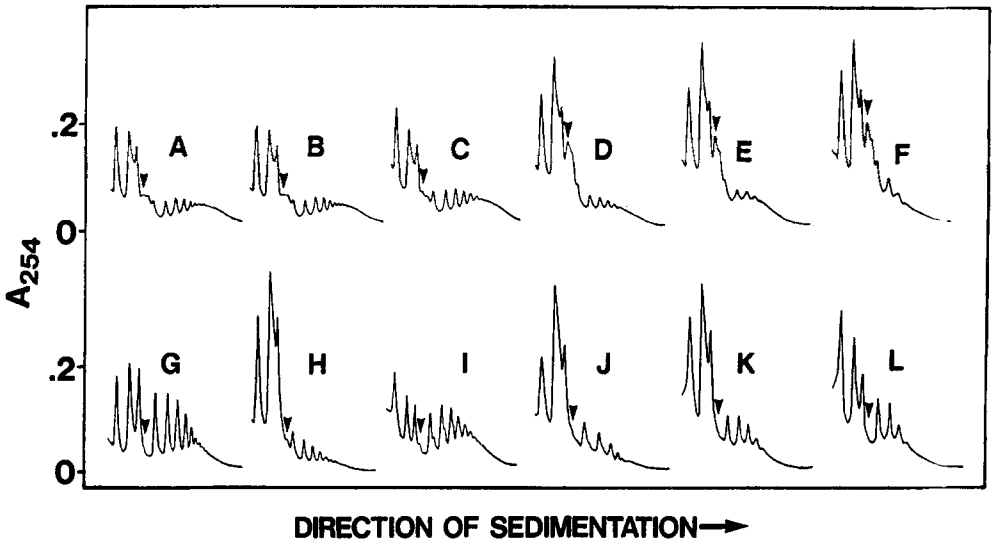

Fig. 3. Ribosomal density profiles of GH_3 cells after brief or prolonged incubation with transcriptional or translational inhibitors. Cells in serum-enriched F-10 medium received no treatment (A, G), 3 μg/ml actinomycin D (B, H), 20 μM cycloheximide (C, I), 0.1 μM pactamycin (D, J), 100 μM puromycin (E, K), or 0.5 μg/ml verrucarin A (F, L). Preparations were incubated at 37° C for 30 min (A–F) or for 12 h (G–L). Lysates of variously treated preparations were then subjected to sucrose-density-gradient centrifugation for analysis of ribosomal size. The arrow denotes the position of the 80S monosomal peak.

DTT was often less than that with A23187 (Table 2). Variation in the extent of eIF-2α phosphorylation ranging from 12–55% was observed across experiments.

The ability of ER perturbants to provoke eIF-2α phosphorylation was examined following prolonged incubations (12 h) with protein synthesis inhibitors such that post-translocational protein substrates should have been substantially depleted (Fig. 4, bottom, Table 2). The inhibition of incorporation was maintained throughout this period with each inhibitor (Table 2). Unexpectedly, increased eIF-2α(P) content relative to control cells was observed to occur from protracted treatment with each of these inhibitors that ranged from a low of 10% with pactamycin to as much as 75% with

Table 2. Leucine incorporation and eIF-2α phosphorylation following brief or extended treatments of GH_3 cells with transcriptional or translational inhibitors. Treatments are as described in the legend to Fig. 4. Leucine incorporation is expressed the mean ± range of values obtained for triplicate samples incubated for 15 min at 37° C. Densitometry was performed on the immunoblots displayed in Fig. 4 to quantitate the percentage of eIF-2α in the phosphorylated form after the various treatments and a subsequent challenge with A23187 or dithiothreitol (DTT)

	Leucine incorporation (pmol/10^6 cells)	eIF-2α(P) (%) 2nd challenge		
		None	A23187	DTT
30 min treatment				
A Control	253 ± 14	1	53	51
B Actinomycin D	230 ± 17	1	44	39
C Cycloheximide	12 ± 2	1	55	35
D Pactamycin	11 ± 2	2	52	19
E Puromycin	13 ± 4	1	38	12
F Verrucarin A	8 ± 4	2	40	16
12 h treatment				
A Control	154 ± 23	1	39	37
B Actinomycin D	21 ± 7	7	90	85
C Cycloheximide	6 ± 4	15	93	79
D Pactamycin	7 ± 4	10	52	19
E Puromycin	14 ± 10	75	98	95
F Verrucarin A	7 ± 4	45	92	84

puromycin (Fig. 4, bottom). Acute treatment of each preparation of cells with either ionomycin or DTT resulted in further phosphorylation of eIF-2α, with almost all of the α-subunit becoming phosphorylated in the preparations pretreated with cycloheximide, puromycin, or verrucarin A. To our knowledge such heavy degrees of eIF-2α phosphorylation have not been previously reported.

Cells treated for 12 h with actinomycin D developed strong inhibition of amino acid incorporation that was not seen at 30 min and approximately 7% residual phosphorylation of eIF-2α. Upon acute exposure of these cells to either A23187 of DTT eIF-2α became heavily phosphorylated (Fig. 4, bottom).

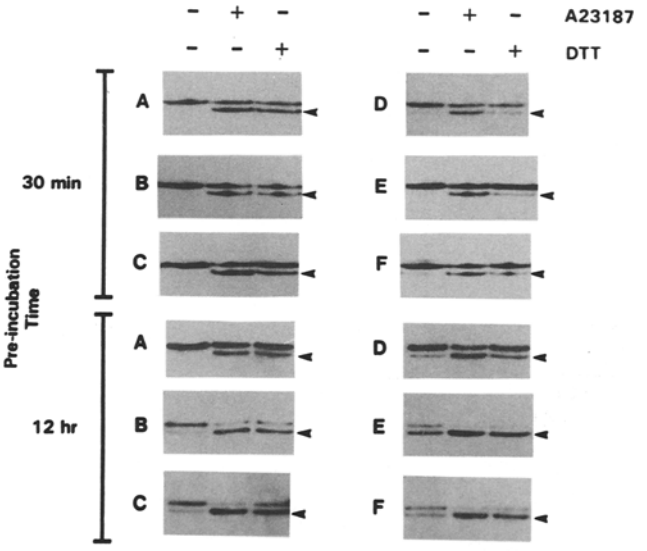

Fig. 4. Extent of eIF-2α phosphorylation following brief or prolonged incubation with transcriptional or translation inhibitors: effects of subsequent challenge with A23187 or dithiothreitol. GH_3 cells in serum-enriched F-10 medium received no treatment (A), 3 μg/ml actinomycin D (B), 20 μM cycloheximide (C), 0.1 μM pactamycin (D), 100 μM puromycin (E), or 0.5 μg/ml verrucarin A (F). After 30 min (top panel) or 12 hr (lower panel) of incubation at 37° C, cells were collected by centrifugation and resuspended in serum-free medium with freshly added drug. Aliquots were removed for measurements of leucine incorporation (Table 2). The remainder of each preparation was then challenged for 12 min either with no further additions, with 1 μM A23187, or with 1.5 mM dithiothreitol (DTT) as indicated. Samples were taken immediately for measurements of eIF-2α phosphorylation. The position of eIF-2α(P) is indicated by the arrow.

The effect of residual eIF-2α phosphorylation on eIF-2B activity

As previously reported, large reductions (approximately 50%) in eIF-2B nucleotide exchange activity occur when only 12–15% of eIF-2α is phosphorylated [17]. The activity of eIF-2B was determined for GH_3 cells exposed for 12 h to puromycin or verrucarin A to establish (a) whether the relatively high residual phosphorylation of eIF-2α consequent to such treatments affected GTP for GDP exchange and (b) whether the superphosphorylation introduced with A23187 had functional significance (Table 3). Control cells that had not been treated with inhibitors responded to ionophore with a relative increase in eIF-2α(P) of 39% and a corresponding 67% reduction in eIF-2B activity. The residual phosphorylation occurring from treatment with either protein synthesis inhibitor resulted in almost complete inhibition of eIF-2B activity. Very low values for eIF-2B activity were obtained as eIF-2α(P) approached 50%. Phosphorylation of eIF-2α in excess of 50%, as was observed with puromycin alone or upon addition of A23187 to puromycin or verrucarin A-treated samples, suppressed GDP displacement so completely that measured activity values were not significantly different from that of controls lacking cell extract.

Phosphorylation of eIF-2α during suppression of glycoprotein synthesis or early glycoprotein processing

Upon the co-translational transfer of core oligosaccharide to an acceptor polypeptide, a series of enzymatic trimming reactions is initiated within the ER [35–37]. The removal of glucose residues by glucosidases I and II is immediately followed by removal of mannose residues by α1,2 mannosidases. Tunicamycin blocks production of core oligosaccharide such that proteins cannot be glycosylated during their translocation into the ER [23]. Castanospermine, 1-deoxynojirimycin, and 1-N-methyldeoxynojirimycin inhibit the ER glucosidases whereas 1-deoxymannojirimycin inhibits α1,2 mannosidases present in both ER and Golgi [23, 38]. Although tunicamycin was found to reduce methionine incorporation in HepG2 cells by approximately 50%, a variety of traditional inhibitors of glycoprotein processing including the ER glucosidase inhibitors and 1-deoxymannojirimycin had no effect on incorporation in these cells [8]. Acute treatment of GH_3 cells with tunicamycin dramatically suppressed incorporation while concurrently decreasing polysome content (Fig. 5), findings indicative of inhibition of initiation. The degree of initiation block produced by tunicamycin was comparable to that observed with A23187 or DTT (Fig. 5). In accord with these

Table 3. eIF-2α phosphorylation and eIF-2B activity of GH_3 cells after prolonged exposure to puromycin or verrucarin and subsequent challenge with A23187. GH_3 cells in serum-enriched F-10 medium received no treatment, 100μM puromycin, or 0.5μg/ml verrucarin A for 12h. Portions of each preparation were then challenged for 12min with 1μM A23187. eIF-2α(P) values were obtained from Table 2. eIF-2B activity is expressed as the mean± range of values for triplicate incubation samples from a representative experiment

Condition	eIF-2α(P) (%)	eIF-2B activity (units)
Control	1	8.37± 0.13
Control+ A23187	39	2.75± 0.38
Puromycin	75	ND*
Puromycin+ A23187	98	ND*
Verrucarin A	45	1.60± 1.10
Verrucarin A+ A23187	92	ND*

*ND = not detectible.

findings, phosphorylation of eIF-2α increased during treatment of GH_3 cells with tunicamycin but not with 1-deoxymannojirimycin (Fig. 6), which did not inhibit incorporation (not shown).

Discussion

An increasing body of evidence supports the proposition that protein synthesis is regulated at mRNA translation by phosphorylation of various proteins associated with the translational apparatus [26, 27]. The phosphorylation of the α-subunit of eIF-2 which binds to and inhibits the guanine nucleotide exhange factor, eIF-2B, is a central feature of most regulatory schemes for translational initiation. As emphasized in the present report, eIF-2 phosphorylation in intact GH_3 pituitary cells appears to depend on an ER based signaling system [17]. Increased phosphorylation of the factor occurred in response to three distinct types of ER perturbants including (a) depletors of sequestered Ca^{2+}, (b) DTT and (c) tunicamycin. Each of these types of perturbants also inhibits amino acid incorporation and translational initiation, induces the synthesis of the ER resident chaperonin GRP78, and impedes early protein processing. In some respects DTT and Ca^{2+}-depleting agents behave as minor stressors (see 17 for discussion); neither lowers ATP or viability over a period of several h.

Relatively modest degrees of eIF-2α phosphorylation are sufficient to inhibit eIF-2B and thereby suppress the formation of the eIF-2·GTP·Met-$tRNA_i^{Met}$ ternary complex required in assembling the 43S preinitiation complex. Phosphorylation of approximately 20–30% of the total eIF-2α molecules is sufficient to completely bind and inhibit the activity of eIF-2B in reticulocytes since the exchange factor is present in relatively low abun-

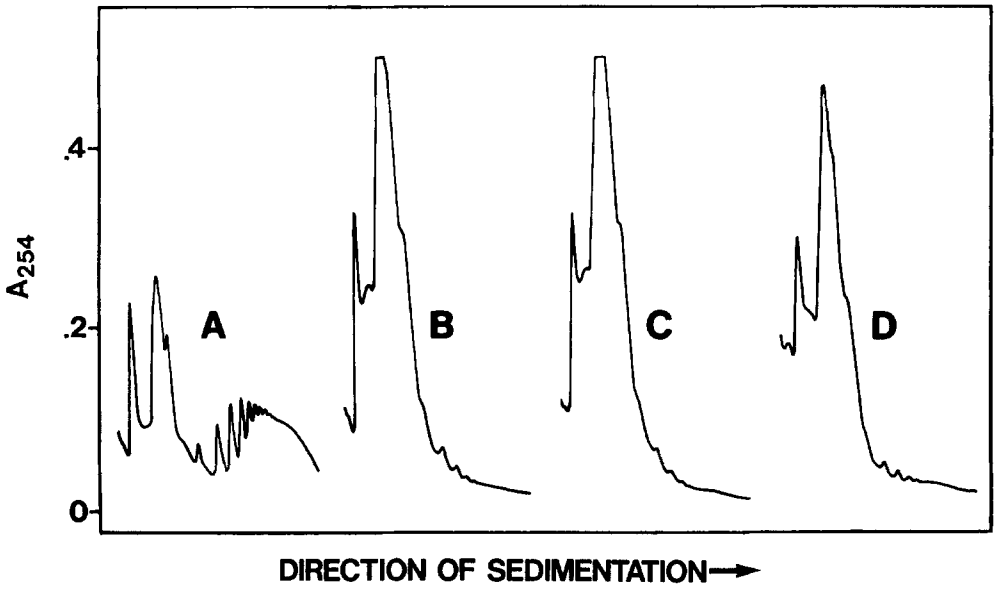

Fig. 5. Ribosomal density profiles of GH_3 cells treated with ionophore A23187, dithiothreitol, or tunicamycin. Cells in F-10 medium were incubated for 15 min with no further additions (A), with 1μM A23187 (B), or with 1mM dithiothreitol (C), and for 30 min with 20μg/ml tunicamycin (D). Lysates were then prepared and subjected to sucrose-density-gradient centrifugation for analysis of ribosomal size. Aliquots were also removed at the end of each treatment for measurements of leucine incorporation. Corresponding incorporation values (pmol leucine incorporated/10^6 cells), expressed as mean± range of values obtained for triplicate samples incubated for 15 min, were: A, 352± 28; B, 23± 1; C, 42± 11; D, 23± 11.

dance [28]. Ehrlich ascites cells, which possess a higher eIF-2B/eIF-2 ratio and a higher basal level of eIF-2α phosphorylation, reportedly require a higher fractional phosphorylation of eIF-2α for marked inhibition of translation [32]. GH_3 cells exhibit some variability from one experiment to another in the degree of eIF-2B inhibition with increasing degrees of eIF-2α phosphorylation. We suspect that this variation derives in part from inherent difficulties in the assay for eIF-2B activity and perhaps in part from relatively subtle differences in cell nutritional status and handling. Some variability is also encountered in that densitometry was involved in measuring eIF-2α. At approximately 15% fractional phosphorylation of eIF-2α, eIF-2B is inhibited 50%. At 40% fractional phosphorylation the inhibition is in the range of 70%. It is likely that these are minimal values of inhibition of eIF-2B activity in that the corresponding inhibitions of amino acid incorporation measured under comparable conditions with either Ca^{2+}-mobilizing agents or DTT are in the range of 90–95%.

The fraction of eIF-2α that is phosphorylated in freshly harvested GH_3 cells is almost undetectable. With protracted exposure to conventional inhibitors of translation, however, phosphorylation of the factor increases steadily with time. At 12 h of treatment these residual degrees of phosphorylation ranged from 10% with pactamycin to 75% with puromycin (Table 2, Fig. 4) and were strongly inhibitory to eIF-2B (Table 3). Exposure of such samples to either A23187 or DTT for a brief period converted almost all of the eIF-2α to the phosphorylated form. It seems clear from these results that the fractional phosphorylation of eIF-2α can substantially exceed the amounts required for the full inhibition of eIF-2B. Almost all of the eIF-2 should have been in the GDP-binding state following prolonged puromycin treatment since eIF-2B was completely inhibited. In contrast, in untreated control cells eIF-2 would mostly reside in the GTP-binding state. The fractional phosphorylation of eIF-2α was readily increased in either preparation in response to ER perturbants. The phosphorylation of eIF-2α must therefore proceed independently of the functional status of eIF-2B and eIF-2 guanine nucleotide binding.

The basis for the increase in eIF-2α phosphorylation that occurs during extended translational inhibition is unclear. We hypothesize that one or more critical proteins that suppress eIF-2α phosphorylation may become limiting with time from continuing protein degradation without resynthesis. This concept is supported by the observation that superphosphorylation of eIF-2α does not occur in cells inhibited for 30 min with conventional inhibitors and subsequently exposed to ER perturbants. It is also consistent with data derived from cells treated with the transcription inhibitor actinomycin D. This inhibitor produces inconsequential effects on amino acid incorporation, polysomal contents, and eIF-2α phosphorylation at 30 min but strong inhibition of incorporation, reduced polysomal contents, and 7% eIF-2α phosphorylation at 12 h. The decrease in amino acid incorporation and reduced polysomal content may arise from the increased content of eIF-2α(P). Longer term actinomycin D treatment is also associated with superphosphorylation of eIF-2α upon addition of ER perturbants. Putative proteins whose decline could produce slowly increasing eIF-2α phosphorylation would include an inhibitor of eIF-2α kinase or an eIF-2α phosphatase.

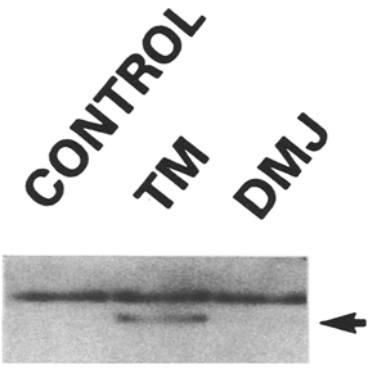

Fig. 6. Effects of tunicamycin and 1-deoxymannojirimycin on the phosphorylation of eIF-2α. GH_3 cells were incubated for 3 h in medium with no further additions, with 20 μg/ml tunicamycin, or with 4 mM 1-deoxymannojirimycin, and samples were prepared for measurements of eIF-2α phosphorylation. The position of eIF-2α(P) is indicated by the arrow.

Currently the signaling system through which the ER alters the phosphorylation of eIF-2α is poorly understood other than that it is produced by agents that interfere with very early events in protein processing and that induce GRP78. The properties and regulation of the responsible protein kinase and/or phosphatase activities remain to be identified. Decreased phosphatase activity has been suggested as responsible for phosphorylation of eIF-2α in perfused liver deprived of a single essential amino acid [39], whereas activation of eIF-2α kinases has been proposed to regulate phosphorylation of the protein in other systems [27]. In preliminary experiments we believe that we have identified an eIF-2 kinase activity that is activated in intact cells in response to ER perturbants and is detectible in cell lysates (C. Prostko, unpublished results). GRP78, a member of the

70 kD family of stress proteins, appears to function importantly in the signaling system in an as yet undefined manner. Various recent reports suggest that the yeast homologue of GRP78 may function as part of a translocation system for peptide passage through the ER membrane [40, 41]. The transcription-dependent induction of GRP78 in response to ER perturbants, including tunicamycin (M. Brostrom, unpublished results), is invariably associated with the dephosphorylation of eIF-2α, reactivation of eIF-2B, and restoration of amino acid incorporation. Newly induced GRP78, as opposed to a large existing pool of the protein within the ER, appears able to reverse the effects of three divergent types of perturbants.

Major aspects of ER protein disposition include the docking and entry of nascent peptides through a membrane pore frequently termed the 'translocon', early processing including such events as signal peptide cleavage, folding, disulfide bonding, proline hydroxylation, and modification of glycosyl residues, and vesicular transport to the *cis* Golgi. Tunicamycin, Ca^{2+}-mobilizing agents, and DTT exert differential effects on ER protein processing. As noted earlier, tunicamycin depresses the transfer of core oligosaccharide in glycoprotein formation during protein translocation. It is the only conventional inhibitor of processing that we have examined that affects amino acid incorporation [8]. Ca^{2+} depletion retards later luminal glycoprotein trimming by mannosidase reactions but does not inhibit the processing and export of non-glycosylated proteins such as albumin [6, 7]. A variety of other inhibitors of various glycosyl trimming reactions do not inhibit translational initiation nor does brefeldin A, which coalesces the *cis* Golgi with the ER compartment [8]. DTT inhibits albumin processing but does not affect glycoprotein processing and export [7]. Post-translocational protein processing of specific proteins such as α_1-antitrypsin is not affected by conventional inhibitors of translation [8]. Protracted inhibition of protein synthesis with conventional translational inhibitors such that the ER should have been cleared of processible protein did not, however, affect the phosphorylation of eIF-2α in response to ER perturbants (Fig. 4). None of these inhibitors, including pactamycin, puromycin, and verrucarin A, abolished polysomal content such that possible threading of the translocon with arrested peptide could be dismissed. In contrast to these agents Ca^{2+}-mobilizing drugs, DTT, and tunicamycin provide much more complete removal of polysomes. The emerging picture from a large body of information is that post-translocational events do not impact significantly on eIF-2α phosphorylation and translational initiation. Rather, accumulating evidence favors the conclusion that ER perturbants promoting these events act at a common site of action involving passage of proteins into the ER. In this regard, the properties of the translocon as reviewed recently by Sanders and Shekman [42] appear to include a sulfhydryl requirement and measurable ion flow during active protein transport.

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