Induction of calreticulin expression in response to amino acid deprivation in Chinese hamster ovary cells

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The role of calreticulin as a stress-induced molecular chaperone protein of the endoplasmic reticulum is becoming more apparent. We characterize here the induction of calreticulin in response to complete amino acid deprivation in Chinese hamster ovary cells. Amino acid deprivation caused a 4-fold increase in calreticulin protein levels over a period of 4–10 h. In addition to an overall increase in protein levels, the glycosylation of calreticulin was increased. This glycosylation event was blocked by tunicamycin and was not required for the increase in calreticulin protein levels. Immunofluorescence studies localized calreticulin to the ER of CHO cells, and no significant change was observed after amino acid deprivation. Northern-blot analysis showed that calreticulin mRNA levels were increased approx. 10-fold in response to complete amino acid deprivation. The response was sensitive to actinomycin D and α-amanitin, implying that regulation is primarily at the level of transcription. These results are similar to the large increases in asparagine synthetase mRNA observed in response to amino acid deprivation, but the amino acid-deprivation-response element identified to be involved in asparagine synthetase induction is absent from the calreticulin promoter.

INTRODUCTION

Calreticulin is a ubiquitous protein found in the lumen of the endoplasmic reticulum (ER) of a wide variety of different cell types, including higher plants (for a review, see [1]). It was initially identified as a high-capacity Ca^{2+}-storage protein and has now been shown to be a multifunctional protein with a number of diverse roles in the cell.

Calreticulin has been shown to be one of the major Ca^{2+}-binding proteins of the ER [2]. Two distinct Ca^{2+}-binding sites are present in the protein, one high-affinity/low-capacity site and one low-affinity/high-capacity site [3]. The role of calreticulin in Ca^{2+} homoeostasis has been shown through studies on the release of Ins(1,4,5)P_3-sensitive Ca^{2+} stores in response to bradykinin [4], and through the use of ionomycin and thapsigargin, which cause perturbations in intracellular Ca^{2+} levels [5].

The production and maturation of secretory and membrane proteins is one of the major tasks performed by the ER. Inside the lumen of the ER a subset of proteins are present, the molecular chaperone proteins, which assist in protein-folding events. The ability of calreticulin to act as a molecular chaperone was demonstrated by its ability to facilitate myeloperoxidase biosynthesis in myeloid cells [6]. Studies on glycoprotein maturation (reviewed in [7]) have revealed that calreticulin acts in a lectin-like manner, binding to trimmed oligosaccharides, and aiding protein folding. The similarity between calreticulin and other ER chaperone proteins extends to the 5′ upstream regions of these genes [8]. A comparison of calreticulin, Grp78, Grp94 and protein disulphide-isomerase reveals a number of highly conserved elements in their promoters.

It has been postulated that the regulation of molecular chaperone proteins in response to cellular stress is mediated through an unfolded protein response element [9]. Cell stresses that deplete ER Ca^{2+} stores, affect protein glycosylation or result in increased protein denaturation produce a corresponding increase in the level of unfolded proteins in the lumen of the ER. This trigger induces increased synthesis of the chaperone proteins, which protect the cell by preventing protein aggregation. Preliminary evidence shows that calreticulin expression is increased in response to thapsigargin and tunicamycin treatments [5]. Calreticulin has been shown to associate with the cytoplasmic domains of integrin α-subunits [10] and bind to nuclear hormone receptors such as the glucocorticoid receptor [11]. These findings imply that calreticulin may be involved in transcriptional regulation and the regulation of cell shape, motility, growth and differentiation.

Amino acid deprivation represents a form of metabolic stress which has received little attention, despite its potential physiological relevance. Mammalian cells require a sufficient supply of amino acids for protein and nucleotide biosynthesis. When mammalian cells are cultured in limiting amino acid concentrations, the overall rate of protein synthesis falls. Against this general decline in metabolic activity, a number of proteins are induced. These include asparagine synthetase [12], amino acid transporter systems such as System A and System X_{AG^-} (for a review, see [13]), and the stress proteins Grp78 [14] and Grp75 [15]. These proteins act as a cellular defence mechanism maintaining intracellular amino acid concentrations and protecting cells from the accumulation of malfolded proteins. In a previous preliminary study [14] it was shown, by metabolic labelling with [^{35}S]methionine and microsequencing, that calreticulin was induced in response to amino acid deprivation in renal epithelial cells. In the present paper we characterize in detail the response of calreticulin to amino acid deprivation in Chinese hamster ovary (CHO) cells.

MATERIALS AND METHODS

Materials

Tunicamycin, isopropyl β-D-thiogalactoside and goat anti-rabbit antibody conjugates were purchased from Sigma Chemicals (Poole, Dorset, U.K.). Restriction enzymes were obtained from Boehringer-Mannheim (Lewes, Sussex, U.K.).

Abbreviations used: ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3′-phosphate dehydrogenase; GDH, glutamate dehydrogenase; CHO, Chinese hamster ovary.

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Cell culture

CHO-K1 cells were cultured in Ham's F12 nutrient medium supplemented with 10 % foetal bovine serum, 2 mM glutamine, 10 mM Hepes, 100 units/ml penicillin G, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B (Gibco). For amino acid-deprivation experiments, cells were washed in PBS and cultured in the following medium: Ham's F12 salts supplemented with 10 mM glucose, 0.1 % BSA, 0.01 % Phenol Red, pH 7.4, 100 units/ml penicillin G, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B. Total cell protein was prepared by washing the cells in 5 ml of PBS before lysing in 0.25 ml of 20 mM Tris/HCl, pH 7.4, containing 150 mM NaCl and 1 % Triton X-100. After clarification by centrifugation, 10–15 μg of total protein was used for SDS/PAGE.

Production of a polyclonal antibody to calreticulin fusion protein

Using the published mouse calreticulin cDNA sequence [16], the predicted amino acid sequence was subjected to antigenicity analysis (DNA* programs) to define regions suitable for antibody production. It was decided to concentrate on the region spanning amino acids 154–347. Exact-match oligonucleotide primers were designed to the amino acid sequence VLINKDIR, spanning amino acids 154–162, and to EEFGNETW, spanning amino acids 339–347. These were used in PCRs with CHO first-strand cDNA template. The forward primer was 5′-TTTTGGATCC-GTGCTGATCAACAAGGATATCCGG-3′ and the reverse primer was 5′-TTTTGGATCCCACGTCTCATTGCCAAACT-CCTC-3′. The single 597 bp PCR product was cloned into the pGEX-2T vector (Pharmacia). Clones were isolated on the basis of fusion protein production under isopropyl β-D-thiogalactoside (100 mM) induction, and sequenced using the pGEX forward and reverse primers (Pharmacia). Sequencing of the 589 bp fragment revealed the DNA sequence to be 86 % identical with the published sequence of the mouse clone [16]. The fusion protein was purified using GSH–Sepharose beads (Pharmacia) and checked by SDS/PAGE analysis. Aliquots (50 μg) were used in conjunction with Freund's adjuvant in a series of rabbit injections. The resultant polysera were highly specific for the fusion protein and produced a single band at approx. 60 kDa in Western-blot analysis of DEAE-purified calreticulin from rat liver and total CHO cell lysates.

SDS/PAGE and Western blotting

Total cell proteins were separated on SDS/7–10 % polyacrylamide gels, as described by Laemmli [17], and transferred to nitrocellulose using a wet-blot apparatus (Bio-Rad). Western blotting was performed by the method of Towbin et al. [18] except that nitrocellulose filters were initially blocked in a 5 % milk/PBS solution. The rabbit anti-calreticulin antibody was used at 1:300 dilution and incubated for 1 h at room temperature. The secondary antibody was used at 1:5000 dilution in 5 % milk/PBS, and blots were developed using the enhanced chemiluminescent system from Amersham as directed. Anti-[glutamate dehydrogenase (GDH)] antibody was a rabbit polyclonal antibody from Biogenesis (Poole, Dorset, U.K.) Blots were quantified using an Image Quant densitometer from Molecular Dynamics.

Protein determination

Protein was quantified using Bradford's reagent [19]. Equal protein loading was ensured by probing blots with an antibody to GDH and using scans of these blots as equal-intensity loading guides. GDH is a mitochondrial enzyme that is not induced in response to cell stress.

Immunofluorescence

CHO-K1 cells were seeded on to ethanol-sterilized coverslips (50–80 % confluence), and, after 2 days in normal culture conditions, coverslips were transferred to amino acid-free culture medium and incubated for a further 16 h. Control coverslips were maintained in normal culture medium. Cells were washed in 5 ml of 5 mM $MgCl_2$ in PBS, and fixed for 2 min in methanol which had been stored at −20 °C. Coverslips were air-dried, and BSA (1 % in PBS) was added. After a 1 h incubation at room temperature, coverslips were dried and antibody solution was added (1:100 dilution in 1 % BSA/PBS). After a further 1 h at room temperature, coverslips were washed three times in PBS before the addition of secondary antibody conjugated to FITC (1:200 dilution in 1 % BSA/PBS) and incubated overnight. Coverslips were washed three times in PBS, dried and mounted on a glass microscope slide [using Mowiol plus 2.5 % 1,4-diazabicyclo-(2,2,2)octane]. Images were taken using a confocal microscope (Zeiss).

Calreticulin cDNA probe

A cDNA probe was constructed corresponding to nucleotides 533–1110 of the published mouse calreticulin cDNA sequence [16]. The PCR product was cloned into the pGEM vector and sequenced to check its integrity. The insert was excised with *Bam*HI and gel-purified (QIAex; Qiagen) for radioactive labelling.

Total RNA extraction

Total RNA was extracted from 35 mm dish cultures using TRI-Reagent (Sigma). RNA was stored under propan-2-ol at −80 °C until the day of use, when the RNA was pelleted (15 min; Microfuge), washed in 75 % ethanol and resuspended in water. RNA samples were quantified by measuring the A_{260}, and equal amounts of RNA were used in Northern-blot analysis. A_{260}/A_{280} ratios were consistently in the range 1.75–1.9.

Northern-blot analysis

Total RNA was denatured in formamide/formaldehyde/Mops (65 °C; 15 min) and separated on a 0.8 % agarose/formaldehyde gel [20]. The RNA was transferred (16 h) to nylon membrane (Hybond N; Amersham International, Amersham, Bucks., U.K.) according to the manufacturer's instructions. In addition, a separate lane contained total RNA, cut out before transfer, and stained with ethidium bromide to reveal the 28 S rRNA and 18 S rRNA positions. The membrane was baked at 80 °C for 2 h and cross-linked (1200 J/cm^2; Stratalinker) before being prehybridized (4 h; 42 °C) in solution composed of 4 × SSPE (1 × SSPE contains 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4, 1 mM EDTA), 5 × Denhardt's solution (1 × Denhardt's solution contains 0.02 % Ficoll 400, 0.02 % polyvinylpyrrolidone and 0.02 % BSA), 0.1 % SDS, 4.5 % formamide, 0.2 mg/ml salmon sperm DNA and 0.5 % dextran sulphate. The denatured label was prepared using the Prime-a-gene kit (Promega) and left to hybridize overnight at 42 °C. The post-hybridization washes were 1 × SSPE/0.1 % SDS (15 min; room temperature; × 2) and 0.2 × SSPE/0.1 % SDS (5 min; room temperature). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and actin probes were used as described previously [21].

RESULTS

Response of calreticulin to cellular stress

The response of calreticulin to cellular stress in CHO cells was analysed by Western blotting on total protein lysates. Blots were

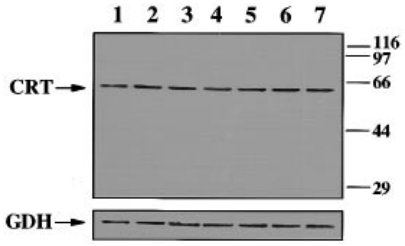

Figure 1 Response of calreticulin to stress in CHO cells

A representative Western blot of protein from CHO-K1 cells subjected to various cellular stresses is shown. About 10–20 μg of total protein was separated by SDS/PAGE, blotted on to nitrocellulose, and probed with an antibody to calreticulin. Blots were quantified and corrected for equal loading by probing with an anti-GDH antibody. Lane 1, normal medium; lane 2, normal medium + 1 μg/ml tunicamycin, 16 h; lane 3, normal medium + 200 mM sucrose, 16 h; lane 4, normal medium + 2-mercaptoethanol (0.01%), 3 h; lane 5, heat shock (42 °C; 30 min); lane 6, UV light (UVC; 90 s followed by incubation in normal medium for 30 min); lane 7, glucose-free medium, 16 h. CRT, calreticulin. Molecular-mass markers (kDa) are shown on the right.

Table 1 Induction of calreticulin in response to cellular stress in CHO cells

CHO-K1 cells were subjected to various cellular stresses. Total protein was prepared, separated using SDS/PAGE, blotted, and probed with the calreticulin antibody. To correct for protein loading, blots were probed with an anti-GDH antibody. The enhanced chemiluminescence films were scanned and corresponding bands quantified. The results are means ± S.E.M. for at least three different incubations in each case. * $P < 0.05$ (significant), ** $P < 0.01$ (very significant) when compared with cells grown in normal medium.

Culture conditions	Relative amount of calreticulin
Control medium	1.00
Amino acid-free medium, 16 h	3.80 ± 0.45*
Control medium + 1 μg/ml tunicamycin, 16 h	2.00 ± 0.10*
Control medium + 200 mM sucrose, 16 h	1.90 ± 0.05**
Control medium + 0.01% 2-mercaptoethanol, 3 h	0.85 ± 0.10
UV light, 90 s UVC exposure followed by 30 min in normal medium	2.30 ± 0.15*
Heat shock, 30 min at 42 °C	2.0 ± 0.10*
Glucose-free medium, 16 h	1.90 ± 0.15*

probed with an anti-(fusion protein) antibody specific for calreticulin (see the Materials and methods section) and quantified. Equal loading was ensured by subsequent probing of the same blots with anti-GDH antibodies. Figure 1 is a representative blot showing the response of calreticulin to different forms of cell stress. Results from a number of similar experiments are quantified in Table 1. Calreticulin protein levels were increased in response to hyperosmotic shock, heat shock, exposure to UV light, and also in response to perturbants of glycosylation such as tunicamycin and glucose deprivation. In contrast, the protein denaturant 2-mercaptoethanol had little or no effect on calreticulin protein levels in CHO cells.

Expression of calreticulin in response to amino acid deprivation

Previous preliminary work in our laboratory based on metabolic labelling and microsequencing has shown that amino acid deprivation causes induction of calreticulin in the renal epithelial cell line NBL-1 [14]. The results presented here (Figure 2a) show that this effect was observed also in CHO cells. When CHO cells were cultured in the absence of amino acids for 16 h there was a 4-fold increase in the level of calreticulin protein. This effect was reversible, and 6 h after all the amino acids were added back to the amino acid-free medium calreticulin protein levels returned to normal (Figure 2b). This shows that the effect was due to removal of amino acids rather than the replacement of serum by albumin in the amino acid-free medium.

Figure 2(c) shows a time course for the induction of calreticulin in response to complete amino acid deprivation. Calreticulin protein levels were increased after 4 h of culture in amino acid-free medium and reached a maximum after 10 h.

Amino acid-deprivation-induced glycosylation of calreticulin

In addition to an overall increase in calreticulin levels in response to amino acid deprivation, there was also a slight increase in the molecular mass of the protein from 62 to 64 kDa (Figure 3). The addition of the glycosylation inhibitor tunicamycin, at concentrations known to block glycosylation in other cell types, resulted in an induced form of calreticulin of a lower molecular mass, 60 kDa. A band corresponding to the normal form of calreticulin was also observed (Figure 3, lane 3). In the presence of tunicamycin, protein levels were induced 3.60 ± 0.15-fold in response to amino acid deprivation, which represents an induction of 86% compared with the level of induction observed in response to amino acid deprivation alone. Treatment of CHO cells with tunicamycin alone represents a form of cellular stress, but this only produces a 2-fold increase in calreticulin protein levels (Table 1). The fact that induction by amino acid deprivation in the presence of tunicamycin exceeds that caused by tunicamycin alone indicates that the amino acid-sensing mechanism does not have a requirement for glycosylation *per se*. The induced form of calreticulin after tunicamycin treatment had a lower molecular mass than that in control conditions. This implies that calreticulin in normal cells is partially glycosylated. Since only a single band was observed in response to amino acid deprivation, the endogenous form of the protein is further glycosylated under these conditions. Glycosylation of calreticulin has also been shown to be increased in response to heat shock both *in vitro* and *in vivo* [22].

Localization of calreticulin in response to limiting amino acid concentrations

To date most of the direct localization studies have shown that calreticulin resides predominantly in the ER. Figure 4 shows immunofluorescence studies on calreticulin localization in CHO cells cultured under control and amino acid-free conditions. Calreticulin was located in the ER of control CHO cells and remained predominantly localized in the ER in response to amino acid deprivation.

Induction of calreticulin in response to amino acid deprivation is regulated at the level of transcription

Northern-blot analysis was performed on total RNA from CHO cells using a cDNA probe specific for CHO calreticulin mRNA. Figure 5(a) shows a representative time course for calreticulin mRNA levels in CHO cells incubated in the complete absence of amino acids. Blots were standardized using cDNA probes for the housekeeping genes GAPDH and actin as equal-intensity loading guides. In response to complete amino acid deprivation there was a 10-fold increase in calreticulin steady-state mRNA levels over a time course which corresponds to a 4-fold increase in the level of the protein (Figure 5b). Calreticulin mRNA increased significantly after 4 h of incubation in amino acid-free medium, and reached a maximum at around 6 h.

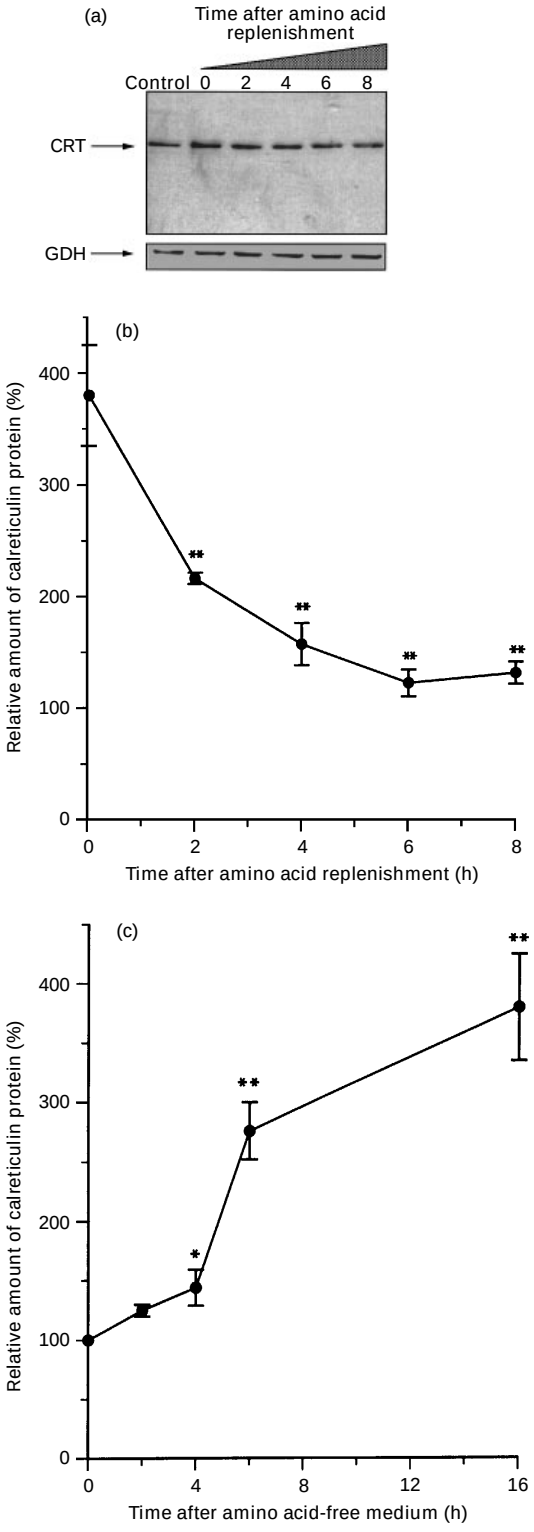

Figure 2 Expression of calreticulin in response to amino acid deprivation

(**a**) A typical Western blot showing the response of calreticulin to amino acid deprivation in CHO-K1 cells is illustrated. After a 16 h incubation in amino acid-free medium, CHO cells were switched to amino acid-free medium to which all the amino acids had been added back at 2 mM. Incubation was continued for the times indicated, after which total protein was prepared and Western-blot analysis performed. The basal level of calreticulin was determined by measurement of the level of protein in CHO cells incubated in normal medium (Control). CRT, calreticulin. (**b**) Quantified analysis of the effect of amino acid replenishment on calreticulin protein levels. Three separate blots were probed with anti-calreticulin, and GDH protein levels were used as an equal-intensity loading guide. (**c**) CHO-K1 cells were incubated for 0, 2, 4, 6 and 16 h in amino acid-free medium. Protein was prepared for each condition and analysed by Western blotting. Blots were quantified corrected for protein loading. Graphical data shown represent values from at least three individual incubations shown as means ± S.E.M., with data for control cells taken as 100%. $^{*}P < 0.05$ (significant), $^{**}P < 0.01$ (very significant) by Student's *t* test.

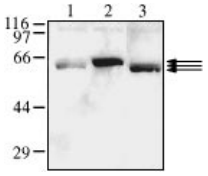

Figure 3 Western blot showing increased glycosylation of calreticulin in response to amino acid deprivation

CHO-K1 cells were incubated for 16 h in normal medium (lane 1), amino acid-free medium (lane 2) and amino acid-free medium + 1 μg/ml tunicamycin (lane 3). Protein was separated by SDS/PAGE (8% gel), blotted on to nitrocellulose, and probed with the calreticulin antibody. Shown here is a representative blot from three separate experiments. Molecular-mass markers (kDa) are shown on the right.

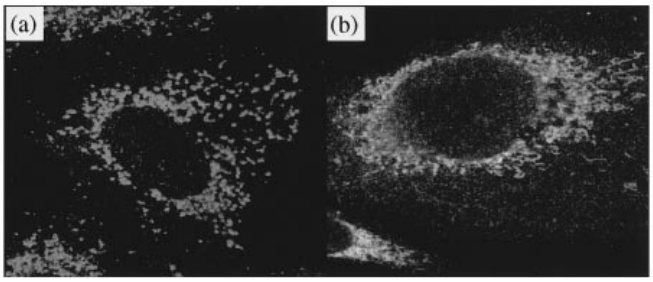

Figure 4 Localization of calreticulin in response to amino acid deprivation

Localization of calreticulin in response to amino acid deprivation was studied by immunofluorescence in CHO-K1 cells. Cells were fixed in methanol and stained using rabbit anti-calreticulin antibody. Subcellular localization was revealed using an FITC-conjugated secondary antibody. (**a**) Normal medium, 16 h; (**b**) amino acid-free medium, 16 h. Analysis of at least three separate coverslips gave the same result as shown here.

Figure 6 shows that addition of the inhibitors actinomycin D (an inhibitor of mRNA synthesis) and α-amanitin (a specific inhibitor of RNA polymerase II) at concentrations that specifically block transcription in other systems completely blocked calreticulin induction in CHO cells. Cycloheximide, which blocks translation in the cell, also abolished calreticulin induction. Data from three separate experiments showed that the increase in calreticulin protein caused by amino acid deprivation was $100 \pm 16\%$, and was reduced to $10 \pm 5\%$, $7 \pm 3\%$ and $18 \pm 4\%$ in the presence of cycloheximide, actinomycin D and α-amanitin respectively. These findings imply that the molecular mechanism involved in the response of calreticulin to amino acid deprivation is predominantly at the level of transcription.

DISCUSSION

The results in this paper show that calreticulin is induced in response to a number of different stress situations in CHO cells. Induction of calreticulin by hyperosmotic stress and exposure to UV has, to our knowledge, not been shown previously. Treatment with tunicamycin or glucose-free medium, known to affect

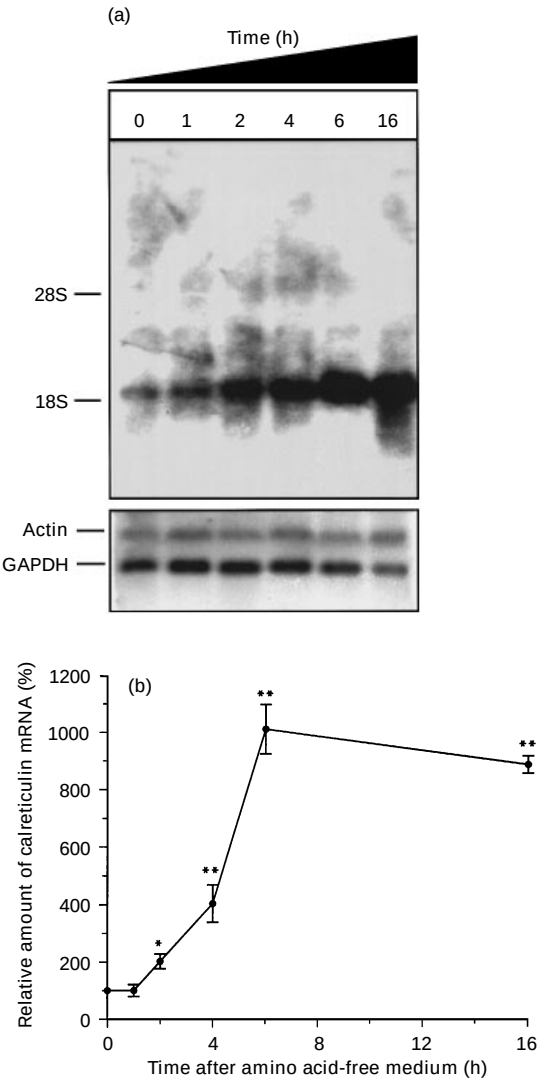

Figure 5 Northern-blot analysis showing induction of calreticulin mRNA levels

(**a**) A representative Northern blot of CHO-K1 RNA is shown. Total RNA was prepared from CHO-K1 cells incubated for various times in amino acid-free medium. RNA was separated on a 0.8% agarose/formaldehyde gel and blotted on to nitrocellulose. Blots were probed with a cDNA probe specific for calreticulin (CRT). Actin and GAPDH probes were used as equal-intensity loading guides. (**b**) Quantified analysis of four separate Northern blots. Values shown are means ± S.E.M. $^{*}P < 0.05$ (significant), $^{**}P < 0.01$ (very significant) when compared with cells grown in normal medium.

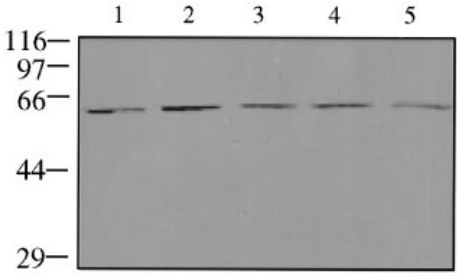

Figure 6 Inhibitor study of calreticulin induction in response to amino acid deprivation

CHO-K1 cells were cultured for 16 h in control medium (lane 1), amino acid-free medium (lane 2) and amino acid-free medium plus actinomycin D (10 μg/ml) (lane 3), α-amanitin (1 μg/ml) (lane 4) or cycloheximide (10 μg/ml) (lane 5). Similar results were obtained from three separate experiments.

glycosylation in the cell, produced an increase in calreticulin protein levels. This is consistent with previous findings on HeLa cells [5] but disagrees with the work on mouse myeloid cells [6]. Calreticulin has been shown to be important in glycoprotein maturation, and it is likely therefore that increased expression of calreticulin prevents aberrant protein-folding events that may occur when glycosylation is impeded. The response of calreticulin to heat shock is consistent with previous work on human umbilical vein endothelial cells [22], and other studies have shown an increase in response to heavy-metal treatment [23] and perturbation of cellular Ca^{2+} levels [5]. Various inconsistencies in the published findings may indicate that the response varies in different cell types.

Calreticulin is an extremely sensitive indicator of limiting amino acid concentrations in CHO cells. When CHO cells were cultured for 16 h in amino acid-free medium, calreticulin protein levels were induced 4-fold. Two independent mechanisms operate in the response of calreticulin to amino acid deprivation in CHO cells. In addition to an overall increase in calreticulin protein levels there was also an increase in glycosylation of the protein. The increased glycosylation was not necessary for protein induction, since calreticulin protein levels were increased by comparable levels in response to amino acid deprivation regardless of whether tunicamycin was present or not. Other stresses such as 'acute' heat shock [24] and hyperthermic stress [22] produce an increase in glycosylation of calreticulin, with induction of calreticulin protein occurring only in response to hyperthermic stress.

Calreticulin has been observed in the nucleus of some cells [25], and more recent findings have implied a cytoplasmic location [10]. In our studies calreticulin was found to reside in the ER of CHO cells, and no significant change in localization was observed in response to amino acid deprivation.

The response of calreticulin to amino acid deprivation is regulated primarily at the level of transcription. A rapid and sustained increase in calreticulin mRNA levels that preceded the increase in calreticulin protein levels was observed when CHO cells were cultured in amino acid-free medium. Addition of the transcriptional inhibitors α-amanitin and actinomycin D completely abolished induction of the protein. These results are similar to those observed in the response of asparagine synthetase to amino acid deprivation in Fao hepatoma cells [26]. Asparagine synthetase mRNA levels are induced 10-fold in response to complete amino acid deprivation by a mechanism sensitive to transcriptional inhibitors. The promoter region of the asparagine synthetase gene has been cloned and characterized. Both *cis* and *trans* elements have been isolated that confer amino acid sensitivity, and it has been shown that the sequence 5′-CATGATC-3′ is necessary for the amino acid-starvation response [27]. The promoter region of the hamster calreticulin gene has not yet been sequenced. However, we have shown that induction of calreticulin by amino acid deprivation also occurs in the human embryonic kidney cell line HEK (R. Heal, unpublished work). The calreticulin 5′-flanking region of the human calreticulin gene [8] does not contain this sequence, suggesting that a novel amino acid response element may be involved in this case.

Asparagine synthetase and calreticulin respond to complete amino acid deprivation by increasing the level of transcription of

the corresponding gene. In contrast, induction of Grp75, a mitochondrial stress protein, does not involve an increase in the steady-state levels of Grp75 mRNA. Rather, there is an increase in translation of Grp75 mRNA [21]. A putative amino acid-binding activator protein regulates the activity of the glutamate transporter, System X_{AG-}, and responds specifically to glutamate levels in the cell [28]. These findings indicate that the amino acid-sensing system of mammalian cells is very complex and a number of different molecular mechanisms may be involved.

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