Induction of calreticulin expression in HeLa cells by depletion of the endoplasmic reticulum Ca^{2+} store and inhibition of N-linked glycosylation

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Calreticulin is now considered to be a multifunctional Ca^{2+} -binding protein. Its primary role is as a Ca^{2+} storage protein within the lumen of the endoplasmic reticulum (ER), where it also seems to assist in the correct folding and assembly of proteins. We have investigated whether agents that affect these processes can alter calreticulin expression in HeLa cells. Perturbation of intracellular Ca^{2+} levels by prolonged exposure to either thapsigargin or ionomycin induced calreticulin mRNA, both in the presence and absence of extracellular Ca^{2+} , consistent with the proposal that sustained depletion of the ER Ca^{2+} store can trigger these increases. The mechanism underlying the induction seems to be transcriptional up-regulation as both agents increased calreticulin promoter-driven firefly luciferase expression in transfected cells to the same degree as the observed increases in calreticulin mRNA. Experiments with a truncated

promoter construct showed that the sequences that confer this inducibility reside within the 225 bp immediately upstream of the putative major transcriptional start site. We also examined the effect of tunicamycin, which inhibits N-linked glycosylation in the ER thereby interfering with protein processing. This caused increases in calreticulin mRNA greater than those with either thapsigargin or ionomycin, but failed to transactivate the calreticulin promoter. Thus either additional *cis* sequences that reside outside our promoter region are necessary for transcriptional activation by tunicamycin, or the increases in calreticulin mRNA occur post-transcriptionally. This suggests that there are probably different mechanisms by which calreticulin expression can be induced in response to agents that affect normal ER functioning.

INTRODUCTION

Although calreticulin was initially identified over 20 years ago as a high-capacity Ca^{2+} -binding protein [1], its exact roles within the cell remain unclear. The protein is located primarily in the endoplasmic reticulum (ER), where it is thought to have a major function as a Ca^{2+} storage protein by virtue of the high-capacity, low-affinity Ca^{2+} -binding sites in its acidic C-domain [2]. Thus in NG-108-15 neuroblastoma–glioma hybrid cells, the capacity of the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} stores released in response to treatment with bradykinin correlates directly with the level of calreticulin expression [3]. However, it is becoming evident that calreticulin might perform cellular functions other than calcium storage [4]. These include modulation of steroid-inducible gene expression by binding to the DNA-binding domains of steroid receptors [5,6] and control of cell adhesion via interaction with the α -subunit of integrin receptors [7].

As well as being a major Ca^{2+} storage site within the cell, the ER is also the compartment where secretory and membrane proteins are initially folded correctly and assembled. This process requires the assistance of chaperones such as the glucose response proteins, grp78 (BiP) and grp94 (endoplasmic reticulum chaperone), as well as protein disulphide isomerase (PDI) [8]. Increasingly, evidence is emerging that calreticulin also functions as a chaperone like these other ER luminal Ca^{2+} -binding proteins. Calreticulin displays properties indicative of a Ca^{2+} -dependent chaperone *in vitro* [9], and it chaperones the biosynthesis of myeloperoxidase in myeloid cells [10]. Perturbation of intracellular Ca^{2+} by ionophores or thapsigargin induces the expression of the genes encoding grp78 and grp94 [11–14] and PDI [15]. With grp78 and grp94, the trigger for

the induction by these agents is depletion of the ER Ca^{2+} store as opposed to a rise in cytosolic $[\text{Ca}^{2+}]$. The correct folding and assembly of polypeptides in the ER requires the maintenance of the intra-organelle Ca^{2+} concentration [16–19] and it has been suggested that the induction of grp78 expression actually occurs in response to the accumulation of unfolded proteins within the compartment [20]. Other stress conditions in the ER, such as the inhibition of glycosylation by tunicamycin, also up-regulate the expression of grp78 and grp94 [13,20], again proposed to be caused by this so-called unfolded protein response [20].

The 5' flanking region of the calreticulin gene has been shown to contain regulatory elements similar to those in the promoters of the grp78, grp94 and PDI genes [21]. However, despite this and the evidence that calreticulin is a Ca^{2+} -binding protein that acts as a chaperone, there is no clear indication that its expression is similarly up-regulated. The protein levels of calreticulin increased in response to prolonged treatment with the calcium ionophore A23187 in murine plasmacytoma cells [22], but in mouse L6 myotubes neither ionophore nor tunicamycin treatment affected steady-state protein levels of calreticulin [23].

We have investigated the effect of perturbation of intracellular Ca^{2+} levels with ionomycin or thapsigargin, and inhibition of N-linked glycosylation by tunicamycin, on calreticulin gene expression in HeLa cells. Prolonged treatment with each of these agents caused increases in calreticulin mRNA levels. The induction with thapsigargin or ionomycin occurred in both the presence and the absence of extracellular Ca^{2+} , consistent with the proposal that ER Ca^{2+} store depletion can trigger increases in calreticulin mRNA. Experiments with a firefly luciferase reporter gene driven by the calreticulin promoter showed that

Abbreviations used: CaR, calreticulin; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FCS, foetal calf serum; GAPDH, glyceraldehyde 3'-phosphate dehydrogenase; grp, glucose-regulated protein; PDI, protein disulphide isomerase; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase; UPRE, unfolded protein response element.

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The nucleotide sequence reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number X85727.

these agents induced calreticulin expression mostly via increased transcription. Tunicamycin treatment, however, failed to transactivate the calreticulin promoter, suggesting that its induction of calreticulin mRNA might occur by a post-transcriptional mechanism. The results are consistent with the proposal that calreticulin acts as a stress-inducible chaperone and indicate that there are different pathways that regulate the expression of calreticulin in response to agents that affect normal ER functioning.

MATERIALS AND METHODS

Materials

Thapsigargin and ionomycin were purchased from Sigma Chemicals (Poole, Dorset, U.K.) and tunicamycin was obtained from Calbiochem (Nottingham, Notts., U.K.). All other chemicals were the highest grade available and obtained from either Sigma or Fisons (Loughborough, Leics., U.K.).

Ultraspec reagent was obtained from AMS (Witney, Oxon., U.K.). Restriction and modifying enzymes were bought from Gibco-Life Technologies (Paisley, Scotland, U.K.) or Promega (Southampton, U.K.). Hybond N+ charged nylon membranes, Rapid-Hyb hybridization buffer, RediPrime labelling kit and [α - 32 P]dCTP (Redivue AA0005) came from Amersham International (Amersham, Bucks., U.K.). Oligonucleotides were synthesized by Severn Biotech (Kidderminster, Worcs., U.K.).

Cells and culture conditions

Tissue-culture media and supplements were obtained from Sigma. Adherent HeLa OHIO cells were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% (v/v) foetal calf serum (FCS). For treatment with thapsigargin, ionomycin or tunicamycin, cells were grown to 75–80% confluence and agents added in DMEM/10% FCS supplemented with 1 mM EGTA, pH 7.6.

For experiments that involved absolute depletion of the extracellular Ca^{2+} , HeLa OHIO cells were routinely grown and passaged in S-MEM (Sigma M8167), containing no Ca^{2+} , to which 10% FCS was added and CaCl_2 was supplemented at 1.8 mM to permit adherence. Before treatment of these cells with Ca^{2+} -perturbing agents, they were extensively washed in Ca^{2+} -free PBS plus 2 mM EGTA. The agents were then added in serum-free S-MEM, without added CaCl_2 , supplemented with 2 mM EGTA, pH 7.6.

Northern blot hybridization

Total RNA (5 μg), isolated with Ultraspec, were subjected to electrophoresis through 1% (w/v) agarose gels containing 0.66 M formaldehyde, transferred by capillary action onto Hybond N+ charged nylon membranes and cross-linked by UV irradiation with an Amersham cross-linker. Hybridizations were performed in Rapid-Hyb buffer for 1 h at 65 °C with probes randomly labelled with ^{32}P . After hybridization, membranes were washed with $0.1 \times \text{SSC}/0.1\%$ SDS at 65 °C and exposed to X-ray film or analysed with a Bio-Rad GS Phosphorimager. Where film was used, the relative mRNA levels were quantified by densitometry (Bio-Rad GS-670 densitometer). Blots were stripped and re-probed with a cDNA encoding glyceraldehyde 3'-phosphate dehydrogenase (GAPDH) (Ambion, Austin, TX, U.S.A.) to ensure equivalent loading of the RNA samples, and values for calreticulin and grp78 mRNA were normalized to those for GAPDH. Equivalent loading was also assessed by detection of ethidium bromide-stained 28 S and 18 S rRNA bands.

PCR, cloning and sequencing

cDNAs for calreticulin (1488 bp), and grp78 (203 bp) were generated by reverse-transcription PCR and verified by sequencing to be the correct products. Amplification products were used as probes in RNA blot hybridizations directly after clean-up (Qiagen PCR preps), or cloned into the pGEM-T vector (Promega) from which appropriate fragments were excised.

The 5' flanking region of the calreticulin gene, from position –518 to +64 relative to the putative major transcriptional start site [21], was amplified by using oligonucleotides with modified 5' ends incorporating *Bam*HI recognition sites. Amplified DNA was digested with *Bam*HI and cloned upstream of the luciferase cDNA into the *Bgl*II site of the pGL-2 basic vector (Promega). Recombinant plasmids, designated pCARUS, were prepared with the Qiagen Maxi-prep system for subsequent sequencing and transfection experiments.

Sequencing was performed with the Taq Dye-deoxy Prism kit (ABI) with GL-1 and GL-2 primers (Promega) and samples were run on an ABI 373A automated sequencer.

Transfections

HeLa cells (in 60 mm dishes) were co-transfected with either pCARUS (2.5 μg) or pGL-2-promoter (2.5 μg) plasmids together with pSV β -gal plasmid (2.5 μg) (Promega) by using lipofection or calcium phosphate precipitation methods. Transfected cells were treated with thapsigargin (300 nM), ionomycin (4 μM) or tunicamycin (10 $\mu\text{g}/\text{ml}$) in DMEM/10% FCS (plus 1 mM EGTA, pH 7.6) for the appropriate period after which cell extracts were prepared by using Reporter Lysis buffer (Promega). Luciferase activity was measured in a Berthold LB9500T luminometer. To control for variations in transfection efficiency, the values were normalized to β -galactosidase activity measured with the β -galactosidase system (Promega) at 420 nm in a Beckman DU640 spectrophotometer. Parallel transfections were also performed with the pGL2 basic vector, which lacks a promoter. Background luminescence was minimal and unaffected by any of the treatments.

RESULTS

Thapsigargin and ionomycin increase calreticulin mRNA levels

Prolonged treatment with the calcium ionophore A23187 [11–13] or thapsigargin [14] has previously been shown to induce the expression of genes encoding ER luminal proteins. By Northern analysis we assessed the effect of continuous exposure to thapsigargin, or the Ca^{2+} ionophore ionomycin, on calreticulin mRNA levels in HeLa cells. We also monitored grp78 mRNA levels to ensure that its expression was being induced.

Thapsigargin is a sesquiterpene lactone that causes leakage of Ca^{2+} from the ER store by inhibition of the sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase (SERCA) pumps [24]. Induction of calreticulin mRNA in response to this agent occurred with kinetics similar to those of grp78, although the relative increases were smaller (Figure 1a). At 2 h, no change was observed for either mRNA. By 8 h, calreticulin mRNA levels had more than doubled whereas grp78 mRNA levels had risen 8-fold. These increases were just over 4-fold and 14-fold respectively by 21 h. The lag period before the appearance of induction has previously been demonstrated for thapsigargin-induced grp78 expression [14].

Because the maximal induction observed occurred at 21 h, we treated cells for this length of time with various concentrations of thapsigargin (30–1000 nM). The level of induction correlated with the increases in thapsigargin concentration for grp78,

Figure 1 Northern analysis of HeLa cells treated with thapsigargin

(a) Cells were treated with 300 nM thapsigargin for 2, 8 and 21 h in the presence of extracellular Ca^{2+} as indicated. Total RNA (5 μg) was run in each lane and transferred on to Hybond N+ membranes as described in the Materials and methods section. Membranes were probed with the ^{32}P -labelled cDNAs for calreticulin (CaR), grp78 and GAPDH. (b) Cells were treated for 21 h with 30, 300 and 1000 nM thapsigargin as indicated in the text. Experimental procedures were as outlined in (a).

Figure 2 Northern analysis of HeLa cells treated with ionomycin

(a) Cells were treated with 5 μM ionomycin for 2, 8 and 21 h in the presence of extracellular Ca^{2+} as indicated. Total RNA (5 μg) was run in each lane and transferred on to Hybond N+ membranes as described in the Materials and methods section. Membranes were probed with the ^{32}P -labelled cDNAs for calreticulin (CaR), grp78 and GAPDH. (b) Cells were treated for 21 hours with 0.2, 2 and 5 μM ionomycin. Experimental procedures were as outlined in (a).

however, equivalent levels of induction were seen in the case of calreticulin for all three concentrations used (Figure 1b). There was no discernible effect on the expression of mRNA encoding GAPDH, which remained unchanged throughout (Figures 1a and 1b) and cell viability was not significantly affected by thapsigargin treatment as assessed by staining of the cells with Trypan Blue.

Ionomycin treatment increased the levels of both grp78 and calreticulin mRNA, and, as with thapsigargin treatment, there was a lag before induction was apparent (Figure 2a). At 8 h, the calreticulin mRNA had increased 2.3-fold whereas there was a 6-fold increase for grp78 mRNA. However, by 21 h, levels for both mRNAs had dropped back to levels slightly lower than the controls, i.e. before ionomycin treatment. Dose-response experiments conducted over an 8 h time course showed that the lowest dose of ionomycin used (0.2 μM) failed to induce either calreticulin or grp78 mRNA levels (Figure 2b).

Table 1 Effect of thapsigargin or ionomycin on calreticulin mRNA levels in the absence of extracellular calcium

Northern analysis was performed, as indicated in the text, on total RNA isolated from cells that had been incubated for either 2 or 8 h in serum-free SMEM (zero calcium): control with added 1.8 mM CaCl_2 (Con2, Con8); 2 mM EGTA, pH 7.6 (+EGTA2, +EGTA8); 2 mM EGTA/300 nM thapsigargin (+Thaps2, +Thaps8); or 2 mM EGTA/5 μM ionomycin (+Iono2, +Iono8). The experiments were performed in triplicate on at least three occasions with similar results each time. The results shown are means $\pm$ S.D. compared with expression seen in untreated cells at 0 h.

Treatment	Relative calreticulin expression (%)
Untreated	100
Con2	106.11 $\pm$ 22.57
Con8	113.67 $\pm$ 11.93
+EGTA 2	98.66 $\pm$ 10.96
+EGTA 8	152.00 $\pm$ 33.85
+Thaps 2	108.68 $\pm$ 7.47
+Thaps 8	238.07 $\pm$ 32.99
+Iono 2	97.33 $\pm$ 6.81
+Iono 8	214.28 $\pm$ 13.98

As with thapsigargin treatment, GAPDH mRNA levels and cell viability were largely unchanged throughout the duration of the experiment.

Depletion of the ER Ca^{2+} store can induce calreticulin mRNA

The experiments described above were all performed with the agents added in DMEM/10% FCS containing 1.8 mM CaCl_2 to which 1 mM EGTA, pH 7.6, had been added. Under these conditions, exposure to thapsigargin (after discharge of the ER Ca^{2+} store) or ionomycin should cause an influx of Ca^{2+} into the cells leading to a sustained rise in cytosolic $[\text{Ca}^{2+}]$. Thus the induction of calreticulin mRNA might occur as a result of this increase. However, previous studies with bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid (BAPTA) to buffer increases in cytosolic $[\text{Ca}^{2+}]$ have shown that the induction of grp78 mRNA in Wg1a Chinese hamster lung fibroblasts can occur without an increase in cytosolic $[\text{Ca}^{2+}]$. Furthermore it was demonstrated that continuous treatment with ionophore in the presence of normal levels of extracellular Ca^{2+} was able to deplete the intracellular store, leading to the conclusion that the stimulus for the induction of grp78 is the depletion of Ca^{2+} from the ER rather than an increase in cytosolic $[\text{Ca}^{2+}]$ [12].

The increases in calreticulin mRNA displayed identical kinetics to that of grp78, suggesting that depletion of the intracellular Ca^{2+} store might also trigger the induction of calreticulin expression. To address this we conducted experiments under conditions designed to cause store depletion without a sustained rise in cytosolic $[\text{Ca}^{2+}]$ due to Ca^{2+} influx. Cells were extensively washed in Ca^{2+} -free PBS/2 mM EGTA before the addition of thapsigargin or ionomycin in serum-free S-MEM, containing no Ca^{2+} , supplemented with 2 mM EGTA. Prolonged elevation of cytosolic $[\text{Ca}^{2+}]$ after thapsigargin inhibition of SERCA pumps depends entirely on an influx of extracellular Ca^{2+} [25], whereas previous studies in this laboratory have shown that where extracellular Ca^{2+} was removed there was no sustained rise in cytosolic $[\text{Ca}^{2+}]$ levels in response to ionomycin, but a very transient rise lasting approx. 20 s due to emptying of the ER store [26].

Owing to the lack of serum and Ca^{2+} in the culture medium, these experiments were carried out over an 8 h time period at the end of which treated cells had started to detach from the culture

TCCCATCCCAGGCGGGG - 501
GGGGGAGCAGCAGTGGGGTGGTCTCTCAATGCAAGATAAGAGCTGG - 451
TTAAGAAAGCCTTCCCAGCCCCCTCCACTAGAGGGAATGGGAGGAGAG - 401
AAGCTGAGGGCAGGGTCCCGTCCCGCTGGAGACAGCTCGCTCCCGCG - 351
GTTTCTTTAAACGCCAGATGGGCAACGACGCGCGGACGAGGGCGGGG - 301
TTGGGTTGAGGTCTGGTCAATGACCTGGCCTGAGGTGCTCGCGCCCCC - 251
ACCCACCACTGAGGCGTCCCCCCACGGTGGTGGACCATC**ATTGG**TCGG - 201
TGGTGAGG**CCAAT**AGAAATCGGCCATCTGGGAACCCAGCGTTCGAGGGCG - 151
CAGCCTAACATAGTGAACCGACGAAGGT**CCAAT**GGAAAAAGACGGCCATG - 101
GGCATAGA**CCAAT**GACAAAGTGGCAGGGGCGGGCCCAAGGGCTGGGTGAG - 51
GTTGGTTTGGAGGCGGGTGGGTATAAAAGTGCAAGGCGGGCGGCGGT - 1
CGGTCCGTACTGCAGAGCCGCTGCCGAGGGTGGTTTAAAGGCCCGCCG + 50
CGTGGCCGCCCTCGG

Figure 3 Promoter region of the human calreticulin gene

The 5' flanking region of the human calreticulin gene was isolated from HeLa cell DNA by PCR with modified 27mer oligonucleotides at positions -518 (sense) and +64 (anti-sense) derived from the published T-cell clone sequence [21]. All clones examined gave identical sequences in both directions. The TATA (bold) and CCAAT (bold italics) boxes, and the major transcriptional start site (with the consequent sequence numbering) are as designated previously [21]. The *Mlu*I site used to create the truncated promoter construct is underlined.

flask. As shown in Table 1, no changes were seen in the levels of calreticulin mRNA at 2 h in either control or treated cells in response to 300 nM thapsigargin or 5 µM ionomycin. At 8 h, calreticulin mRNA levels had more than doubled with both agents, these increases being comparable to those seen with cells similarly exposed to thapsigargin or ionomycin for the same periods in the earlier experiments with extracellular Ca²⁺ present (Figures 1 and 2). A smaller 1.5-fold increase was also observed in cells that had been exposed to Ca²⁺-free medium to which 2 mM EGTA had been added, which we predict should also cause a sustained drop in the ER Ca²⁺ store. No effect was observed on GAPDH mRNA levels. To rule out any effect of a transient rise in cytosolic [Ca²⁺], we incubated cells for 5 min with 300 nM thapsigargin or 5 µM ionomycin in the presence of S-MEM supplemented with 1.8 mM CaCl₂ before washing the cells with PBS/EGTA as outlined above and adding Ca²⁺-free S-MEM. Under these conditions no increase in either calreticulin or grp78 mRNA was seen (results not shown). We therefore conclude that sustained depletion of the ER Ca²⁺ store can trigger an increase in the levels of calreticulin mRNA.

Thapsigargin and ionomycin induce calreticulin expression primarily through transcriptional activation of the promoter

It has previously been shown that the induction of grp78 expression by A23187 [12] and thapsigargin [14] occurs as a result of transcriptional activation of the grp78 gene promoter. To see whether this was also true for the increases in calreticulin mRNA, we constructed a reporter plasmid in which luciferase expression was driven by the 5' flanking region of the calreticulin gene. PCR was used to isolate a 582 bp fragment from the calreticulin 5' flanking region from positions -518 to +64 relative to the putative major transcriptional start site [21]. This was cloned upstream of the luciferase cDNA in the pGL-2-basic vector and designated pCARUS. Three different clones were sequenced in both directions and were identical, actually comprising 585 bp in total (Figure 3). The sequence showed a number

Table 2 Effect of thapsigargin, ionomycin or tunicamycin on luciferase expression driven by the calreticulin promoter

A 585 bp fragment of the 5' flanking region of the human calreticulin gene (shown in Figure 3) was cloned in front of the luciferase reporter gene in the pGL2 basic vector (pCARUS518). A truncated promoter construct was also created by digestion with *Mlu*I to remove the distal 293 bp (pCARUS225). HeLa cells were transfected with these constructs, as well as the control pGL2P vector in which luciferase is driven by the SV40 early promoter and the pGL2 basic promoterless vector. Transfected cells were either left untreated or treated with 300 nM thapsigargin for 21 h, 5 µM ionomycin for 8 h or 10 µg/ml tunicamycin for 21 h. These times were chosen because they corresponded to the times at which mRNA induction was greatest for each agent. All plasmids were co-transfected with pSV-βgal to control for variations in transfection efficiency and luciferase values were normalised to the β-galactosidase activity. The activities of the each constructs, or the pGL-2 P control vector, in medium with no added agents were given arbitrary values of 1. All experiments were performed in triplicate on at least three occasions. The values shown represent means ± S.D.

Construct		Relative induction in luciferase expression			
		Medium alone	Thapsigargin	Ionomycin	Tunicamycin
pGL-2P	1		1.21 ± 0.18	1.16 ± 0.11	1.05 ± 0.16
pCARUS 518	1		3.54 ± 0.20	1.97 ± 0.19	0.94 ± 0.20
pCARUS 225	1		3.45 ± 0.37	2.01 ± 0.26	1.35 ± 0.43

of minor differences from the previously published sequence derived from a T-cell line clone [21], the biggest deviation from which was a 5 bp difference that eliminates the most distal published *Sp1* site. These alterations were also seen in clones derived from HL60 DNA (F. N. Sheikh and D. H. Llewellyn, unpublished work).

HeLa cells transfected with full-length pCARUS constructs (pCARUS 518) and treated with 300 nM thapsigargin in the presence of extracellular Ca²⁺ for 21 h showed a 3.5-fold increase in luciferase activity compared with untreated, similarly transfected cells (Table 2). In cells transfected with the pGL-2-promoter vector, in which luciferase expression is driven by the SV40 early promoter, there was only a 1.2-fold increase in emitted light from thapsigargin-treated cells compared with untreated cells. To locate more closely the region of the promoter that confers thapsigargin inducibility, we conducted parallel experiments with a pCARUS construct in which the most distal 293 bp of the calreticulin 5' flanking region had been removed by *Mlu*I digestion. Cells transfected with this truncated promoter construct (pCARUS 225) demonstrated similar levels of increased luciferase activity in response to thapsigargin to those transfected with the full-length construct (Table 2). Approximately 2-fold increases in luciferase expression were observed with pCARUS 518 and pCARUS 225 on treatment of the cells for 8 h with 5 µM ionomycin (Table 2). As with thapsigargin, the increases were commensurate with those seen in the mRNA levels in the Northern analyses. This suggests that the increases in calreticulin mRNA in response to these agents arise largely, if not wholly, through enhanced transcription with most, if not all, of the *cis* transcriptional elements required for induction residing in the 225 bp immediately upstream of the major transcriptional start site.

Tunicamycin treatment induces calreticulin mRNA

Abnormal glycosylation can lead to the incorrect folding of proteins in the ER. The induction of grp expression as a result of inhibition of glycosylation is thought to occur as a result of the accumulation of unfolded proteins within the compartment [20]. We therefore investigated the effect of tunicamycin, an antibiotic

Figure 4 Northern analysis of HeLa cells treated with tunicamycin

Cells were treated with 10 μ g/ml tunicamycin for 3, 8 and 21 h as indicated. Total RNA (5 μ g) was run in each lane and transferred on to Hybond N+ membranes as described in the Materials and methods section. Membranes were probed with 32 P-labelled cDNAs for calreticulin (CaR), grp78 and GAPDH. Full experimental details are given in the Materials and methods section.

that inhibits N-linked glycosylation of proteins in the endoplasmic reticulum [27]. The results are shown in Figure 4. Increases in both calreticulin and grp78 mRNA of 3-fold and 5.9-fold respectively were observed after 3 h of exposure. By 8 h these had risen further to 4.3-fold and 15.2-fold. The increase in calreticulin mRNA levels by 21 h was 7.2-fold. However, the levels of grp78 mRNA had started to return towards control levels although remaining elevated. As with ionomycin and thapsigargin treatment, GAPDH mRNA levels remained unchanged throughout and no significant effect was observed on cell viability with tunicamycin.

Tunicamycin treatment fails to transactivate the calreticulin promoter

To see whether the effect of tunicamycin upon calreticulin mRNA was mediated through increased transcription, we again performed transfection experiments with the pCARUS constructs. These showed that, in contrast with the thapsigargin and ionomycin results, treatment of the transfected cells with tunicamycin for 21 h failed to induce any real increase in luciferase activity from either the full-length or truncated promoter (Table 2). Experiments were also conducted over a 8 h period and again failed to demonstrate any transactivation (results not shown). Thus it seems that either different or additional *cis* sequence elements from those necessary for induction by thapsigargin are required for transactivation of the calreticulin gene by tunicamycin; alternatively, treatment with the antibiotic increases calreticulin mRNA levels by a post-transcriptional mechanism, presumably by increasing mRNA stability.

DISCUSSION

The endoplasmic reticulum is the major storage site within the cell for Ca^{2+} , which can be released on stimulation of the appropriate receptors by $\text{Ins}(1,4,5)\text{P}_3$ and possibly cADP-ribose. It is also the initial site for the correct folding and assembly of membrane-bound and secretory proteins. Located primarily in the lumen of the ER, calreticulin acts as a major Ca^{2+} storage protein [2,3] and is being increasingly implicated as a chaperone involved in processing proteins within the organelle (9,10). The results presented here demonstrate clearly that prolonged treatment of HeLa cells with agents that either perturb intracellular Ca^{2+} levels, i.e. thapsigargin or ionomycin, or inhibit the N-

linked glycosylation of proteins thereby disrupting their correct folding, i.e. tunicamycin, are able to induce calreticulin mRNA.

Previous studies have investigated the effects of another calcium ionophore, A23187, and tunicamycin on calreticulin protein levels in other cell types. In L6 mouse myotubes no effect was observed with either agent [23], whereas tunicamycin failed to increase calreticulin in myeloid cells [10]. The apparent discrepancies between these observations and our own might be attributable to differences in the concentrations used and/or the times for which the cells were exposed to the agents. It might also be that increases in mRNA are not exactly mirrored by those in protein levels, for which the alterations might be smaller and not as easily detected. However, A23187 significantly increases calreticulin protein levels in murine plasmacytoma cells [22], possibly indicating that the response is cell-type-specific.

One of the major considerations of our results concerns the mechanism(s) by which changes in intracellular Ca^{2+} , in response to thapsigargin or ionomycin, induce calreticulin mRNA. The initial experiments were performed in the presence of relatively high extracellular Ca^{2+} levels, well in excess of the cytosolic $[\text{Ca}^{2+}]$. Under these conditions both thapsigargin and ionomycin should cause a prolonged rise in cytosolic $[\text{Ca}^{2+}]$ as a result of influx into the cell. Thus the induction could simply result from this elevation of cytosolic $[\text{Ca}^{2+}]$. However, increases in gene expression resulting from rises in cytosolic $[\text{Ca}^{2+}]$, such as those for *c-fos* and *c-jun*, usually occur rapidly [28]. In contrast, the lag before calreticulin mRNA induction was apparent, paralleling that seen for grp78 mRNA expression, suggested that the trigger might be more than simply a rise in cytosolic $[\text{Ca}^{2+}]$. The experiments we conducted with thapsigargin or ionomycin, where extracellular Ca^{2+} was absent such that there was no sustained elevation in cytosolic $[\text{Ca}^{2+}]$, showed that calreticulin mRNA still increased. Thus we conclude that prolonged depletion of the ER Ca^{2+} store is able to induce the levels of calreticulin mRNA. Strong corroborating evidence for this proposal comes from previous studies by Lee and co-workers, who have demonstrated that the stimulus for the increase in grp78 and grp94 expression is in fact the emptying of the intracellular Ca^{2+} store as opposed to a rise in cytosolic $[\text{Ca}^{2+}]$ [12]. However, it might be that long, sustained increases in cytosolic $[\text{Ca}^{2+}]$ can also trigger such increases, perhaps serving a protective role against abnormally high cytosolic $[\text{Ca}^{2+}]$ by providing extra buffering capacity in the store. Down-regulation of calreticulin expression as a result of antisense oligonucleotide treatment of NG-108-15 neuroblastoma–glioma hybrid cells led to an increased sensitivity in ionomycin-induced cytotoxicity [3]. We are currently addressing these aspects further by monitoring changes in the ER and the cytosolic $[\text{Ca}^{2+}]$ as a result of chronic treatment with thapsigargin and ionomycin by using targeted indicators for aequorin [26].

Increases in the expression of ER luminal proteins due to emptying of the ER Ca^{2+} store might be an attempt to sequester and retain Ca^{2+} within the ER because they are Ca^{2+} -binding proteins that, certainly in the case of calreticulin, are involved in Ca^{2+} storage. Thapsigargin-induced emptying of the store, however, is apparently quick, occurring maximally within about 10 min [29], whereas the kinetics of the induction of grp78 and calreticulin are slow. If the aim were to try to retain Ca^{2+} within the organelle it might be reasonable to predict a more rapid induction. It is now clear that the correct folding and assembly of polypeptides requires maintenance of the intraluminal Ca^{2+} concentration [16–19], with the induction of grp78 expression by A23187 suggested to be due to the resultant accumulation of unfolded or misfolded proteins within the ER after depletion of the Ca^{2+} store [20]. This might also be true for the induction

of calreticulin mRNA, a proposal supported by the observed increases with tunicamycin, which interferes with correct protein folding in the ER. However, in myeloid cells, where relatively short incubations with tunicamycin seemingly fail to increase calreticulin protein levels, calreticulin chaperones the synthesis of glycosylated precursors and is unable to interact with non-glycosylated precursors [10]. If this specificity of interaction also holds true in HeLa cells, it is not clear why the accumulation of unfolded, unglycosylated proteins should increase calreticulin mRNA. It might be that, under severe stress conditions, calreticulin is able to bind unglycosylated, misfolded proteins with which it does not normally interact. Alternatively it might be that the interaction with glycosylated precursors is restricted to certain cell types or that the elevation occurs indirectly as a result of tunicamycin-induced increases in the levels of other ER luminal proteins with which calreticulin interacts.

The increase in the steady-state levels of calreticulin mRNA in response to thapsigargin or ionomycin seems to occur primarily through enhanced transcription. The human calreticulin gene promoter shares similarity with those of the *grp78* and *grp94* genes [21]. It is possible that the same transcription factors and *cis*-acting elements are involved in the up-regulation of both calreticulin and *grp78* in response to thapsigargin or ionophore. The control elements that confer thapsigargin and A23187 inducibility upon the mammalian *grp78* gene promoter are CCAAT boxes surrounded by GC sequences [14]. Recently a model has been proposed in which thapsigargin inducibility of the mammalian *grp78* promoter is transduced via Ca^{2+} -dependent binding of the transcriptional factor C1F to the C1 element that contains its proximal CCAAT box [30]. Most, if not all, of the *cis* elements responsible for the inducibility of the calreticulin gene promoter in response to thapsigargin or ionomycin reside in the 225 bp immediately upstream of the proposed major transcriptional start site, a region that contains all the CCAAT boxes present in the 5' flanking sequence. In yeast the so-called unfolded protein response transactivates the promoter of the yeast *grp78* homologue, *KAR2*, via a 22 bp unfolded protein response element (UPRE) [31]. It has been suggested that a similar sequence to the yeast UPRE exists in the calreticulin promoter [9]. This is found at position -191 to -170 in our sequence (Figure 3) and is thus contained within our truncated promoter construct. However, we calculate that only 5 out of the 22 bases are homologous with the yeast UPRE. The inability of tunicamycin to transactivate the calreticulin promoter indicates that if it increases calreticulin mRNA by transcriptional up-regulation, then additional *cis*-acting elements that reside outside our promoter region are necessary. Alternatively, a post-transcriptional mechanism increasing the stability of the mRNA might be involved, which should be clarified by nuclear run-on experiments. Interestingly, blockage of transport from the ER to the Golgi by Brefeldin A up-regulates *grp78* expression in human hepatoma cells and hamster K12 cells by post-transcriptional and transcriptional routes respectively [32]. Thus the same process can up-regulate expression of the same gene by different mechanisms, albeit in different cells.

The results presented here support the proposal that calreticulin acts as a stress-induced chaperone and demonstrate that modulation of its expression at the level of mRNA can probably occur by a number of different mechanisms. Despite the increasing number of functions being ascribed to calreticulin in a

wide variety of processes, relatively little is known about the regulation of its expression. It is thus imperative to investigate this further and to ascertain how alterations in its expression might affect the processes in which it is involved.

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