Regulation of Calreticulin Gene Expression by Calcium

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Abstract. We have isolated and characterized a 12-kb mouse genomic DNA fragment containing the entire calreticulin gene and 2.14 kb of the promoter region. The mouse calreticulin gene consists of nine exons and eight introns, and it spans 4.2 kb of genomic DNA. A 1.8-kb fragment of the calreticulin promoter was subcloned into a reporter gene plasmid containing chloramphenicol acetyltransferase. This construct was then used in transient and stable transfection of NIH/3T3 cells. Treatment of transfected cells either with the Ca^{2+} ionophore A23187, or with the ER Ca^{2+}-ATPase inhibitor thapsigargin, resulted in a five- to sevenfold increase of the expression of chloramphenicol acetyltransferase protein. Transactivation of the calreticulin promoter was also increased by fourfold in NIH/3T3 cells treated with bradykinin, a hormone that induces Ca^{2+} release from the intracellular Ca^{2+} stores. Analysis of the promoter deletion constructs revealed that A23187- and thapsigargin-responsive regions are confined to two regions (-115 to -260 and -685 to $-1,763$) in the calreticulin promoter that contain the CCAAT nucleotide sequences. Northern blot analysis of cells treated with A23187, or with thapsigargin, revealed a fivefold increase in calreticulin mRNA levels. Thapsigargin also induced a fourfold increase in calreticulun protein levels. Importantly, we show by nuclear run-on transcription analysis that calreticulin gene transcription is increased in NIH/3T3 cells treated with A23187 and thapsigargin in vivo. This increase in gene expression required over 4 h of continuous incubation with the drugs and was also sensitive to treatment with cycloheximide, suggesting that it is dependent on protein synthesis. Changes in the concentration of extracellular and cytoplasmic Ca^{2+} did not affect the increased expression of the calreticulin gene. These studies suggest that stress response to the depletion of intracellular Ca^{2+} stores induces expression of the calreticulin gene in vitro and in vivo.

ALTERATIONS in intracellular Ca^{2+} concentration regulate a variety of diverse cellular functions including secretion, contraction–relaxation, cell motility, cytoplasmic and mitochondrial metabolism, and protein synthesis and folding (Pozzan et al., 1994). Ca^{2+} signals also trigger gene expression, promote cell cycle progression, and activate apoptosis (Schönthal et al., 1991; Little et al., 1994; Ghosh and Greenberg, 1995). The ER is considered one of the most important and metabolically relevant sources of cellular Ca^{2+} (Pozzan et al., 1994). Ca^{2+} is released from the ER by InsP$_3$ receptor/ryanodine receptor Ca^{2+} release channels and it is taken up by the Ca^{2+}-ATPase (Sorrentino and Volpe, 1993; Pozzan et al., 1994; Coronado et al., 1994). The ER contains a characteristic set of proteins, resident in the lumen, which terminates with the KDEL ER retrieval signal (Pelham, 1989) and may be involved in Ca^{2+} storage. The most extensively studied of these proteins are Grp78 (BiP), Grp94, ERp72, protein disulfide isomerase (PDI), and calreticulin (Pozzan et al., 1994). These proteins appear also to be involved in many other aspects of ER function, including protein synthesis and folding (Gething and Sambrook, 1992). Recently, Lee's group has shown that the expression of Grp78 and Grp94 is induced by various physiological stresses including glucose starvation, heat shock, and changes in intracellular Ca^{2+} concentration (Little et al., 1994).

Calreticulin is an unusual luminal ER protein. Several unique functions have been postulated for the protein, including modulation of gene expression (Burns et al., 1994; Dedhar et al., 1994; Michalak et al., 1996), a role in cell adhesion (Coppolino et al., 1995; Opas et al., 1996), and maintenance of intracellular Ca^{2+} homeostasis including control of store-operated Ca^{2+} influx (Liu et al., 1994; Camacho and Lechleiter, 1995; Bastianutto et al., 1995; Mery et al., 1996). An important recent finding is that it is the ER form of calreticulin that modulates gene expression and cell adhesiveness in mouse L fibroblasts (Michalak et al., 1996; Opas et al., 1996). Calreticulin has chaperone activity (Nigam et al., 1994; Nauseef et al., 1995; Wada et al., 1995; Peterson et al., 1995; Sapiro et al., 1996; Otteken and Moss, 1996; Van Leeuwen and Kearse, 1996; Helenius et

al., 1997) and it is similar to calnexin, an integral ER membrane protein chaperone (Bergeron et al., 1994; Michalak, 1996; Krause and Michalak, 1997). Calreticulin and calnexin are unusual as chaperones because they function like lectins and bind specifically to partially trimmed, monoglucosylated, N-linked oligosaccharides (Ware et al., 1995; Hammond and Helenius, 1995; Peterson et al., 1995; Sapiro et al., 1996; Helenius et al., 1997). Calreticulin also has an antithrombotic activity (Kubawara et al., 1995) and it is detected on the cell surface (Gray et al., 1995; White et al., 1995). The protein plays a role in long term "memory" in *Aplysia* (Kennedy et al., 1992), in cytotoxic T cell function/activation (Burns et al., 1992; Dupuis et al., 1993), in neutrophils (Stendhal et al., 1994), in viral RNA replication (Singh et al., 1994), in sperm cell function (Nakamura et al., 1993), and in autoimmunity (Sontheimer et al., 1993). To rationalize these diverse functions of calreticulin, it is important to identify and understand the mechanisms that regulate its expression. It is apparent that differential expression of calreticulin will have profound effects on these seemingly diverse cellular functions. The human calreticulin gene has been isolated, and the nucleotide sequence analysis of its promoter region has revealed several sites that might play a role in regulation of transcription (McCauliffe et al., 1992).

In the present study we describe isolation of the mouse calreticulin gene, including 2.14 kb of its promoter region. Using reporter genes we show that this promoter is sensitive to the ER Ca^{2+} store depletion. We demonstrate that, in mouse fibroblasts, both the Ca^{2+} ionophore A23187 and the ER Ca^{2+}-ATPase inhibitor thapsigargin stimulate expression of the calreticulin gene. We also show, by the nuclear run-on transcription assay, that calreticulin gene is activated by A23187 and thapsigargin in vivo. Over 4 h of continuous treatment with these drugs was required to induce calreticulin expression, and this stimulation was sensitive to cycloheximide, suggesting that the Ca^{2+} store depletion–dependent induction of calreticulin expression requires new protein synthesis. Importantly, transactivation of calreticulin promoter was also induced by bradykinin treatment of NIH 3T3 cells. Our results suggest that stress response to the depletion of intracellular Ca^{2+} stores plays a very important role in the regulation of calreticulin gene expression in vitro and in vivo.

Materials and Methods

Isolation and Sequencing of Genomic Clones

A mouse liver genomic library (a gift from Dr. J. Stone, University of Alberta, Canada) was constructed by partial digestion of genomic DNA (adult 129/J male) with the restriction enzyme Sau3A, followed by cloning into the BamHI site of lambda DASH (Stratagene, La Jolla, CA). Screening of the library was carried out as described by Dower et al. (1992) using GeneScreen Plus hybridization membrane (NEN, DuPont, Mississaga, Canada). DNA probes were labeled with [^{32}P]CTP (NEN, DuPont) by random priming. The first screening of the library, with a 711-bp cDNA fragment corresponding to the 5′-coding region of mouse calreticulin cDNA (nucleotides 163–874) (Smith and Koch, 1989), resulted in the isolation of a pseudogene. A second DNA probe was produced by PCR-driven amplification of mouse genomic DNA using the following primers: T1 (5′-GCGAA TTC AAA GAG CAG TTC TTG GAC GG-3′) corresponding to nucleotides 137–158 of mouse calreticulin cDNA (*underlined*) with a 5′ EcoRI restriction site, and T2 (5′-CTGGAT CCA CTC GGA AAC AGC TTC ACG-3′) corresponding to nucleotides 396–416 (*under-*

lined) and a 5′ BamHI restriction site. The PCR product was inserted between the EcoRI–BamHI restriction sites of pBluescript, and its nucleotide sequence was confirmed (see below). Screening of the mouse genomic DNA library with this probe resulted in the isolation of one clone, designated p1.3. This clone was further characterized by Southern blotting (Ausubel et al., 1989).

Various fragments of p1.3 were subcloned into pBluescript and their nucleotide sequences were determined by the double-stranded dideoxynucleotide method. Sequencing was performed in the DNA Sequencing Laboratory of the Department of Biochemistry, using DNA sequencer (model 373A; Applied Biosystems, Foster City, CA). T3, T7, or custom-made primers were used for the sequencing reactions. Synthetic oligodeoxynucleotides were made in the DNA Sequencing Laboratory of the Department of Biochemistry, using a DNA/RNA synthesizer (model 392; Applied Biosystems).

Plasmid Construction

Plasmid pCM7 was constructed by subcloning a 7-kb HindIII restriction fragment from p1.3 into the HindIII restriction site of pBluescript. This fragment contained 1.8 kb of the 5′-flanking region and the entire coding region of the calreticulin gene. The 1.8-kb promoter fragment was further subcloned into the promoterless chloramphenicol acetyltransferase (CAT)[1] reporter expression vector pCATbasic and pXP-1 (luciferase expression vector [De Wet et al., 1987] producing plasmids pCC1 and pLC1, respectively). To generate pCC1, a HindIII/StuI fragment of pCM7 (nucleotides −1,723 to +40 of the calreticulin gene; Fig. 1) was cloned into the blunt-ended XbaI/HindIII sites of pCATbasic. To generate pLC1, an SmaI/StuI fragment was subcloned into the SmaI restriction site of pXP-1.

Different restriction fragments of the calreticulin promoter were subcloned into the promoterless reporter plasmids pCATbasic (CAT expression vector) to generate promoter deletion constructs: pCC0 (2,142-bp SmaI/StuI restriction fragment of the promoter DNA), pCC1 (1,763-bp HindIII/StuI restriction fragment), pCC2 (685-bp KpnI/StuI restriction fragment), pCC3 (415-bp AflII/StuI restriction fragment), pCC4 (260-bp BamHI/StuI restriction fragment), and pCC5 (115-bp PvuI/StuI restriction fragment).

Cell Culture and Drug Treatment

All cell lines were maintained in DME supplemented with 10% calf serum at 37°C with 5% CO_2 in a humidified incubator. Cells were transferred to 10- or 2-cm tissue culture plates 1 d before drug treatment. Stock solutions of A23187, thapsigargin, BAPTA/AM (Molecular Probes, Inc., Eugene, OR), and EGTA/AM (Molecular Probes, Inc.) were prepared in 99.5% dimethyl sulfoxide and were added to the culture medium as specified in the text. Control cells were incubated with appropriate volume of 99.5% dimethyl sulfoxide. Cycloheximide and bradykinin were dissolved in water.

Transient and Stable Transfection

Plasmid DNA was purified by column chromatography (QIAGEN Inc., Chatsworth, CA). NIH/3T3 cells were grown in 10-cm dishes and transfected using the calcium phosphate method and a BES (*N,N*-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid) buffer (Ausubel et al., 1989). For transient transfection, 10 μg of reporter plasmid and 10 μg of pSVβ-gal (internal control) were used per dish. Cells were incubated with the precipitated DNA for 16–20 h. After an additional 8-h incubation, cells were treated with the appropriate drugs for the indicated times, and cell extracts were prepared and assayed for the reporter genes.

For stable transfection, NIH/3T3 cells were cotransfected with reporter plasmid (8 μg), pSVβgal (8 μg), and pNEO1 (0.5 μg). After a 24-h incubation, cells were selected for resistance to Geneticin (G418) (600 μg/ml). After 14 d of growth in the presence of G418, ∼200 clones were obtained. These G418-resistant cells were tested for expression of the reporter gene and β-galactosidase.

Cell Extraction and Reporter Assays

Cell extracts were prepared by washing cells with PBS followed by incubation for 15 min at room temperature with 100 μl per 2-cm dish of a lysis

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1. *Abbreviations used in this paper*: CAT, chloramphenicol acetyltransferase; G3PDH, glyceraldehyde-3 phosphate dehydrogenase; PDI, protein disulfide isomerase.

buffer containing 100 mM Tris, pH 7.8, 0.5% NP-40, and freshly added 1 mM DTT. Cell extracts were collected and stored at $-80°C$ until further use.

The level of CAT protein in cell extracts was determined using a CAT ELISA kit with specific anti-CAT antibodies (Boehringer Mannheim Biochemicals, Indianapolis, IN). Luciferase activity was assessed using 10 µl of cell lysate and 100 µl of luciferase assay reagent (containing 20 mM Tricine, 1.07 mM $MgCO_3$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 µM coenzyme A, 530 µM ATP, and 470 µM luciferin). CAT levels and luciferase activities in cell extracts were always normalized against β-galactosidase activity. β-Galactosidase activity was measured by incubating 20 µl of cell lysate in a covered microtiter plate, at 37°C, with 100 µl of ONPG (o-nitrophenyl-β-D-galactopyranoside) solution (0.8 mg/ml) and the OD was measured at 420 nm. Data are reported as means ± SD of four separate experiments performed in triplicate.

Nuclear Run-on Transcription Assay

Nuclei were prepared from the cells treated for 4 h with 10 µM A23187 or 100 nM thapsigargin. The elongating RNA transcripts were labeled in vitro with [^{32}P]UTP, isolated, and hybridized to membranes (GeneScreen Plus) containing slot-blotted single-stranded bacteriophage M13 DNA probes specific for mouse calreticulin gene. The probes were designed to detect either sense or antisense transcription in the gene region of interest. The following DNA probes were used in the assay, all cloned into M13mp18 and M13mp19 (Rice et al., 1995): the mouse calreticulin 5′ probe was a 700-bp (from +44 to +744) fragment of the murine calreticulin cDNA; the mouse calreticulin 3′ probe was a 630-bp (from +750 to +1,380) fragment of the murine calreticulin cDNA; the mouse Exon 1 c-myc probe was a 436-bp HindIII–BglII fragment extending from +140 to +576 of the murine c-myc gene; the mouse Intron 1 c-myc probe was an 816-bp BglII–SstI fragment from +700 to +1,516 of the murine c-myc gene; the glyceraldehyde-3 phosphate dehydrogenase (G3PDH) probe was a 979-bp fragment from +44 to +1,023 of a human G3PDH cDNA; the γ-actin 5′ probe was a 583-bp BamHI-BglI fragment from +483 to +1,083 (Exon 4–6) of a human γ-actin cDNA; the histone H2b probe was a 300-bp BstEII fragment from +110 to +412 of the chicken histone H2b gene. Radioactivity hybridizing to each probe was quantitated with a Fujix BAS1000 Phosphorimager (Fuji Photo Film Co., Ltd., Tokyo, Japan) using MacBAS imaging software.

Northern and Western Blot Analysis

Northern blot analysis was carried out as described by Burns et al. (1992). The 711-bp, 5′ portion of mouse calreticulin cDNA was used as a probe. The blots were normalized with a human G3PDH cDNA probe (Clontech Laboratories, San Diego, CA) (Burns et al., 1992). The relative abundance of each mRNA was determined using a Fujix BAS1000 Phosphorimager. Cellular extracts were prepared for immunoblotting as described by Mery et al. (1996). Cells were directly lysed into Laemmli sample buffer followed by sonication (Mery et al., 1996). Proteins were separated by SDS-PAGE on 10% polyacrylamide gels as described by Laemmli (1970), and then transferred to nitrocellulose membranes (Towbin et al., 1979). Immunoblotting was carried out with goat anti-calreticulin as described by Milner et al. (1991).

Table I. Exons amd Introns of the Mouse Calreticulin Gene

No.	Exon	Intron	Splice junction consensus sequence	
			AGGT^G/$_A$AGT	NCAGG*
	bp	*bp*		
I	158	358	AGGTAAGG	TCAGA
II	102	184	AGGTACAA	ACAGG
III	204	897	TGGTGAGG	ATAGG
IV	95	87	AGGTGTGC	TCAGG
V	210	82	AGGTTAGT	CTAGG
VI	114	169	AGGTAGGC	ACAGG
VII	144	1030	AGGTGAGA	CCAGG
VIII	93	100	AGGTGTGT	ACAGG
IX	733	through poly (A) signal sequence		

*Mammalian consensus nucleotide sequence (Senapathy et al., 1990).

Measurements of Intracellular Ca^{2+} Concentration in Cycloheximide- and BAPTA-treated Cells

For measurement of the intracellular Ca^{2+} concentration in cycloheximide-treated (2 h with 100 µM cycloheximide) or control cells, NIH/3T3 cells (2×10^7 per ml) were loaded for 30 min with 2 µM fura-2/AM under the conditions preventing sequestration of the dye into subcellular organelles (Demaurex et al., 1992; Mery et al., 1996). The cells were washed twice and fluorescence measurements were performed while cells were continually stirred and maintained at 37°C. Fura-2 fluorescence was monitored at $\lambda_{ex} = 340$ nm. To determine effects of BAPTA on the intracellular Ca^{2+} concentration, NIH/3T3 cells were loaded with both 2 µM fura-2/AM and 20 µM BAPTA/AM as described by Muallem et al. (1990). The basal cytosolic Ca^{2+} concentrations are reported as means ± SD of four separate experiments performed in triplicate.

Results

### Isolation and Characterization of the Mouse Calreticulin Gene

To isolate a calreticulin genomic clone, we first screened a mouse liver genomic DNA library with a cDNA probe corresponding to the 5′-coding region of mouse calreticulin cDNA. Four clones were isolated and nucleotide sequence analysis revealed that each of them represented an intronless fragment of the calreticulin gene. The nucleotide sequences of these clones were identical to the nucleotide sequence of the 3′ region of calreticulin cDNA, and they were missing introns 6, 7, and 8 that were subsequently found in the calreticulin gene (see below). The 5′ regions of these clones did not align with any nucleotide sequences in the EMBL gene database. We concluded that these clones correspond to a calreticulin pseudogene. Isolation of the calreticulin genomic clone was achieved by further screening of the same library, with a genomic probe that did not hybridize to the pseudogene. Screening of >300,000 plaques resulted in the isolation of a single clone, designated p1.3. This clone has an insert of 12 kb that contains the entire calreticulin gene and 2.14 kb of the 5′-untranslated region.

The mouse calreticulin gene has nine exons and eight introns. The different lengths of these coding and noncoding regions of the gene are summarized in Table I. The exon–intron boundaries are highly homologous to the reported mammalian exon–intron consensus sequences (Table I). However, there is no typical poly(A) signal in the 3′-untranslated region. Nucleotide sequencing of the exons revealed that they are virtually identical to the mouse cDNA. Specifically, only two nucleotides differ compared with the nucleotide sequence of mouse calreticulin cDNA reported by Mazzarella et al. (1992), and only four differ compared with the sequence reported by Smith and Koch (1989). Importantly, these variations in the nucleotide sequence do not affect the amino acid sequence of the protein.

Fig. 1 shows the nucleotide sequence of the 1,723-bp promoter region of the mouse calreticulin gene. The sequence was compared with a database of transcriptional control elements using MacVector v4.5 software. The following putative regulatory elements were found: a TATA box (nucleotides -30 to -25), several AP-2 sites (nucleotides -74, -258, -300, -305, -518, -553, $-1,091$, $-1,098$, $-1,251$, and $-1,477$), GC-rich areas including SpI sites (nucleotides -76, -303, and -312), AP-1 sites (nucle-

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HindIII
AAGCTTTTAATTTTTATTTTTATTTGTATAGATGITTTGCCTGCACGTATGTCTCTGTACTATTTACACATCTGGTGCCCACAGAATAGGGCTTCAGACCC
-1720      -1700      -1680      -1660      -1640

TCTGAAACTCAAATTACCTGGTTTTGATCCTTCATGTGTGTGCTGCGGGAATAGGACTGGGGTCATTCAGAAGAGCAGCCAGTGCTCCAAA CCAAT CTCT
-1620      -1600      -1580      -1560      -1540
                                                              AP-2
CCTGTCCTTTATTTCATTGTGTTAAAGCAGTGTATCATTTAACCCAGGCTGGCCTCAAACTCACTATGCAGCTGAGAACTGAACTTTTGACCCCTGCTTC
-1520      -1500      -1480      -1460      -1440
                                          AP-1
ACTCACCGTCTTGGGTACTAGGACAAAGCAGAAGGCATCAAGCATATGTGTCAGTGGCACAGGACTGGAGGGAGGGTCAGGTCAGTCACAGGTCTGTCAA
-1420      -1400      -1380      -1360      -1340
                                                      AP-2
GATCATGTTGAACTTGGGTGTCCTCCAGTGGGACCTTCACCAGCAATGCTGAGTTCTGTACACTGCCTGGGGGACAGGGTGGAGACCCAGCTTCTTGACC
-1320      -1300      -1280      -1260      -1240

TCAAGTGTCATTGCTTGACTGAGTTCCTCTCACTGCAAAGGAAGCACGCCATTTTTGTAAGGATTCCTTCTGGGCAGTTCAATAGTCTTGTTTTCAGGGTTC
-1220      -1200      -1180      -1160      -1140
          AP-2    AP-2                                                            AP-1
CCAAT CCTCAGTTTGGAAACACCTCCCATCCCCCATCCCCTACAGGTACTGTTCTTCCTCCTCCAGGCAGCAGGCAGAGTCACATGATTTGAGTCAGAG
-1120      -1100      -1080      -1060      -1040

GACTCCAGATCCAAGGGTCCTGCTTATCTCCCTAGAACTATCACCATAGCCATCTGTGTCCTTTTTCCTCCTCAAACGGCACCCCTTGCCTTCTGCCGTT
-1020      -1000      -980       -960       -940

TATTGCAAAAGCCAAGTCGTTTGTGGGAGACCACAAGAAGTGACTCCTTTCATTCTCCAGCTTAAGAGAAAGAGAAAAAAAAAAAATCAAAAAACCCCTA
-920       -900       -880       -860       -840

C TTCTCACCCAAGAAAAACACGCTTACACTCACACCCGAGACCTCTGTGTTTCCTGCTCTTGAACCGTGGACAGCCCTAAAAGGTTTAAAAGCATTTCTGT
-820       -800       -780       -760       -740
                                                                                      PstI
CAAGATAGCTCAGGGTCACCCCTGTTGGCTTCTGACATTTCAGGGAGGCGAAATGACTTGTATCATGTCACACAGGCTGCAGGGAACTACGGGACGAGAG
-720       -700       -680       -660       -640
                                                                    AP-2
GAGAAAGCTGGTACGTTCTCTATGGCCTGAACAACTGTGGAACCAGAATGCAGCGAAACCTCAGGATCCTGGCTGGCCCCTTGACCCTTATCCTGAATAGGA
-620       -600       -580       -560       -540
           AP-2
AACGCTCGCCATCGGTGGGCGTTCCTAGGTGCAGGACAGACGGAACGTGAAAGTTGCAAATAATCCTTACTTCTTCCCTCTGACCAGAGAGGATGGGAA
-520       -500       -480       -460       -440
             SIF                         KpnI
AGGGCCGAAGCTAAGCACCCCTCTCGGTCCCGCACCGCACGGTTAACACCTGGTACCGCTCGCGCGGATTCTTTAAACGACTCCTAGCGAGCCAGAGACT
-420       -400       -380       -360       -340
        Sp1    Sp1                                                    AP-2
CTCAGCAGCCAAGGGCGGGGTTGGGCTGAGGTTCAGTCACGTGACCGTGCCTGAGTGGGCTAGCGGCCCCCACCCCACCAGGGGGCGTCCCCCACAACGCG
-320       -300       -280       -260       -240
        AP-2  Sp1
SalI                          H4TF-1
TGGTCGACCCTC ATTGG CCCATAGTGCGA CCAAT AGAAATCAGCCATCTGGGATCCCAGCGTTCCGAGCCACAGCCTAACTTGCTGAGCCGACTGGGAAG
-220       -200       -180       -160       -140
                                      AP-2   Sp1
CAATGGAAAGGGACAGCTGTAGGTCTAAACCAGTCAAAAGGACCGAGGGGCGGGCTCAGCGGCCGTGTCAGGTTCGGGTGAGAGGTAGGTGAA TATAAAT
-120       -100       -80        -60        -40
                                             BanII      StuI
TGAAGCGGCGGTGGCCGCGTCCGTCAATACCGCAGAGCCGCTGCCTGAAGATCGTTTTAAAAGGCCTGTGTGCCGCCGCCCCCTCGGACCGCC ATG
-20        0          20         40         60
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Figure 1. The nucleotide sequence of the promoter region of the mouse calreticulin gene. The nucleotides are numbered with the putative transcriptional initiation site of the mouse calreticulin gene at +1 (Smith and Koch, 1989). (*Horizontal lines*) Putative binding sites for the DNA binding proteins: AP-2, Sp1, AP-1 and H4TF-1, and SIF. CCAAT sites (*boxed*). TATA box (*underlined*). ATG initiation codon (*bold*). These sequence data are available from GenBank/EMBL/DDBJ under accession number U38249.

Figure 2. DNA dot matrix comparison of the mouse and the human calreticulin gene. DNA dot matrix analysis was carried out using MacVector v4.5 software with the following settings: windows size 30, minimum score 65%, and hash value 6. The nucleotide sequence −526 to +5,023 of the mouse gene was compared with nucleotide sequence −529 to +4,418 of the human gene. (*Bottom*) The intron–exon configuration of the mouse calreticulin gene. (*Empty boxes*) Exons, numbered from 1 to 9. (*Filled box*) The 526-bp promoter region of the mouse gene.

otides −1,034 and −1,378), an SIF PDGF binding site (nucleotide −404), an H4TF-1 site (−183), and four CCAAT sequences (nucleotides −194, −207, −1,123, and −1,532), three of which are oriented in the forward direction. AP-2 and H4TF-1 recognition sequences are typically found in genes that are active during cellular proliferation, and this is consistent with the finding that calreticulin expression is increased in stimulated T cells (Burns et al., 1992).

Comparison of the Mouse and Human Calreticulin Genes

The genomic organization and nucleotide sequence of the mouse calreticulin gene are very similar to those reported for the human gene (McCauliffe et al., 1992). The nucleotide sequences of the mouse and the human gene show >70% identity (calculated by BESTFIT; Genetics Computer Group software, Madison, WI), with the exception of introns 3 and 6. There are also remarkable similarities in the lengths of the exons and introns of both genes. Fig. 2 shows a DNA dot matrix analysis of the mouse calreticulin gene compared with the human gene. The coding regions, especially, show a high degree of identity, and the only significant differences between the two genes are centered around introns 3 and 6. In the mouse gene these introns are approximately twice the size of the corresponding introns in the human gene (897 bp vs 421 bp for intron 3, and 169 bp vs 88 bp for intron 6).

Several putative regulatory elements are found in the promoter region of both genes. However, there are differences between the two promoters in the position and number of these sites. For example, in the 526-bp promoter region, there are only two CCAAT sites found in the mouse gene, but four in the human gene (Fig. 1). Three Sp-1 sites are located in the first 500 bp of the mouse promoter compared with only two in the human gene. In this same region, there are five AP-2 sites in the mouse promoter and only one in the human gene (McCauliffe et al., 1992). No SIF PDGF binding site is found in the human gene, but both promoters contain several poly G-rich sequences.

The Calreticulin Promoter Is Activated by Changes in Intracellular Ca^{2+} Concentration

The availability of the promoter from the mouse calreticulin gene has allowed us to study how it might regulate transcription. To do this, two different reporter gene systems were used. The 1.8-kb calreticulin promoter region was cloned into CAT and luciferase expression plasmids, as described in Materials and Methods, to generate plasmids pCC1 and pLC1, respectively. These plasmids were then used for transient and stable transfection of NIH/3T3 cells. pSVβ-galactosidase was used as an internal control. Basal levels of CAT protein and luciferase activity were observed in both stably and transiently transfected NIH/3T3 cells, whereas cells transfected with promoterless control plasmids showed no detectable CAT protein or luciferase activity (data not shown). Once cells had been transfected, we investigated whether alteration of intracellular Ca^{2+} levels, using either A23187 or thapsigargin, affected the activity of the calreticulin promoter. A23187, a Ca^{2+} ionophore, equilibrates any Ca^{2+} gradient across membranes. Thapsigargin, however, leads to the depletion of the ER Ca^{2+} stores by inhibiting the ER Ca^{2+}-ATPase (Thastrup et al., 1990; Ghosh et al., 1991).

Fig. 3 shows that NIH/3T3 cells stably transfected with pCC1 and pSVβ-galactosidase produced five- to sevenfold more CAT protein after treatment for 16 h with 7 μM A23187 or 100 nM thapsigargin. NIH/3T3 cells were also stably transfected with pLC1 and pSVβ-galactosidase. When these cells were treated with 7 μM A23187 or 100 nM thapsigargin, a threefold increase in luciferase activity was observed (Fig. 3 *A*). Similar results were obtained with mouse fibroblasts that were transiently transfected with pCC1 or pLC1. The reason for this difference between the CAT and luciferase reporter systems is not

clear, but it may be related to an inhibitory effect of Ca^{2+} on luciferase activity (unpublished observations).

To identify regions in the calreticulin promoter that may be responsible for the A23187- and thapsigargin-dependent activation of the calreticulin gene, we have generated constructs containing several deletions in the calreticulin promoter. Fig. 3 *B* shows that the first 115-bp DNA fragment of the calreticulin promoter was not activated by A23187 or thapsigargin. The region encompassing −115 to −260 was responsible for two- to threefold A23187- and thapsigargin-dependent induction of the calreticulin promoter (Fig. 3 *B*). Additional activation of the promoter by A23187 and thapsigargin was observed within the second region of calreticulin promoter localized between −685 and −1,763 (Fig. 3 *B*). These two regions of calreticulin promoter contain the CCAAT nucleotide motif that, at least in part, may be responsible for the Ca^{2+} store depletion–dependent activation of the calreticulin gene as shown for transactivation of the Grp78 (BiP) promoter (Wooden et al., 1991; Roy and Lee, 1995; Roy et al., 1996).

Figure 3. Activation of the calreticulin promoter by A23187 and thapsigargin treatment. (*A*) NIH/3T3 were stably transfected with either pCC1 or pLC1 and pSVLβ-galactosidase as described in Materials and Methods. Cells were incubated for 16 h with 7 μM A23187, 100 nM thapsigargin, or an appropriate volume of DMSO (control cells), as described in Materials and Methods. (*B*) Different fragments of the calreticulin promoter were subcloned into the promoterless reporter plasmids pCATbasic (CAT expression vector) as described in Materials and Methods. Vectors pCC0, pCC1, pCC2, pCC3, pCC4, and pCC5 contained the 2,142-, 1,763-, 685-, 415-, 260-, and 115-bp DNA fragments of the calreticulin promoter, respectively. NIH/3T3 were transiently transfected with different pCC vectors and pSVLβ-galactosidase as described in Materials and Methods. Cells were incubated for 16 h with 7 μM A23187 (*open bars*) or 300 nM thapsigargin (*filled bars*) and lysed, and reporter enzyme assays were carried out. *n/d*, not detectable. Data shown are means ± SD of four separate experiments performed in triplicate.

The Expression of Calreticulin mRNA and Protein Is Induced by A23187 and Thapsigargin in NIH/3T3 Cells

Treatment of nontransfected cells with A23187 and thapsigargin also led to altered expression of the endogenous calreticulin gene. Specifically, we used Northern blot analysis to measure the relative mRNA levels in NIH/3T3 cells treated with these drugs. Fig. 4 *A* shows that an approximately four- to fivefold increase in the abundance of calreticulin mRNA was observed in cells incubated with these drugs. There was an approximately fourfold increase in calreticulin protein in NIH/3T3 cells incubated with thapsigargin (Fig. 4 *B*). This suggests that changes in the level of calreticulin mRNA resulted in changes in calreticulin expression.

The Endogenous Calreticulin Gene Is Activated by A23187 and Thapsigargin in NIH/3T3 Cells

To measure transcription rates of the endogenous calreticulin gene and to determine if the accumulation of calreticulin mRNA was the result of increased transcription due to A23187 and thapsigargin, nuclear run-on transcription assays were carried out. NIH/3T3 cells were treated for 4 h with either A23187 or thapsigargin. Nuclei were prepared from the drug-treated cells and RNA transcripts initiated in vivo were elongated in vitro in the presence of [^{32}P]UTP. The radiolabeled run-on transcripts were hybridized to single-stranded DNAs complementary to either specific calreticulin or control mRNAs (sense probes) or to antisense RNAs from the same regions (antisense probes). The probes used detected two regions of the mouse calreticulin gene (5' and 3' regions). Single-stranded probes for the two regions of the γ-actin gene, G3PDH gene, histone H2b gene, and c-*myc* gene (intron 1 and exon 1 regions) were included as controls for levels of transcription. Fig. 5 shows that both A23187 and thapsigargin induced transcription of calreticulin gene and that the transcription pattern was consistent with A23187- and thapsigargin-dependent accumulation of calreticulin mRNA (Fig. 5). The relative abundance of the calreticulin signal was determined using Phosphorimager analysis of the
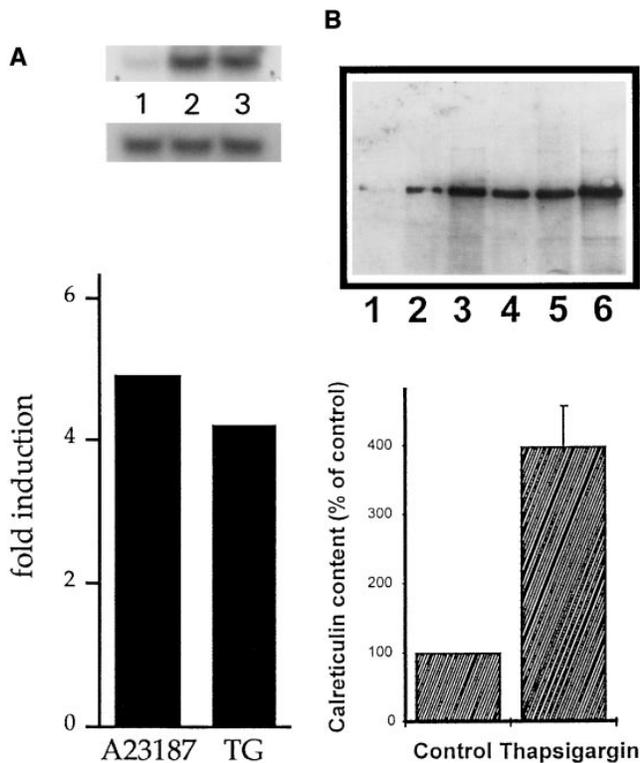
Figure 4. A23187- and thapsigargin-dependent increase in calreticulin mRNA levels (*A*) and protein levels (*B*). (*A*) Nontransfected NIH/3T3 cells were incubated for 16 h with 7 µM A23187, 100 nM thapsigargin, or DMSO. Total RNA was then isolated and electrophoretically separated on a formaldehyde-agarose gel, blotted onto Hybond N nylon membrane, and hybridized with cDNA probes encoding mouse calreticulin and G3PDH, as described in Materials and Methods. (*Top*) Autoradiogram of a Northern blot probed with calreticulin and G3PDH cDNA. (Lane *1*) Control cells; (lane *2*) A23187-treated cells; (lane *3*) thapsigargin-treated cells. (*Bottom*) The abundance of calreticulin mRNA (calreticulin mRNA/G3PDH mRNA ratio) was determined using Phosphorimager analysis of Northern blots. (*B*) NIH/3T3 cells were incubated for 16 h with 100 nM thapsigargin and cellular extracts were prepared as described by Mery et al. (1996). The proteins were separated by SDS-PAGE, transferred electrophoretically to nitrocellulose membranes, and incubated with goat anti-calreticulin antibody as described by Milner et al. (1991). For each condition, three different amounts of cells were analyzed (lanes *1* and *4*, 30,000 cells per well; lanes *2* and *5*, 100,000 cells per well; lanes *3* and *6*, 150,000 cells per well), and the immunoblots were scanned by densitometry. (*Top*, lanes *1*, *2*, and *3*) Control, untreated cells; (lanes *4*, *5*, and *6*) thapsigargin-treated cells. (*Bottom*) The slope of the cell number as a function of OD was obtained by a linear fit to give a relative cellular content of calreticulin (Mery et al., 1996). *TG*, thapsigargin.

blots shown in Fig. 5. Comparison of the levels of calreticulin gene transcription between control and drug-treated cells relative to that observed for G3PDH revealed an approximately twofold increase in the calreticulin signal (Fig. 5). When levels of calreticulin gene transcription were compared between control and drug-treated cells relative to γ-actin, H2b, and c-*myc* gene transcription, an approximately sixfold increase in calreticulin signal was observed. Control genes (G3PDH, H2b, and c-*myc*) were also induced in the presence of A23187 and thapsigargin
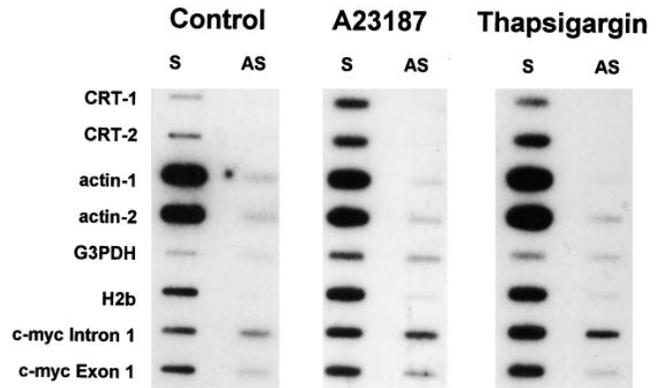
Figure 5. Nuclear run-on analysis of the calreticulin gene in NIH/3T3 cells. Nuclei were isolated from cells treated for 4 h with DMSO (*Control*), 10 µM A23187 (*A23187*), or 100 nM thapsigargin (*Thapsigargin*), and transcription was allowed to proceed in the presence of [^{32}P]UTP as described in Materials and Methods. RNA products from equal numbers of nuclei per sample were hybridized to immobilized single-stranded DNA probes that detect sense (*S*) or antisense (*AS*) transcript arising from calreticulin, actin, G3PDH, H2b, and c-*myc* genes. *CRT-1*, calreticulin 5″ probe; *CRT-2*, calreticulin 3″ probe; *actin-1*, γ-actin 3″ probe; *actin-2*, γ-actin 5″ probe.

but to a lesser extent than that observed for calreticulin gene (Fig. 5). These results indicate that treatment of the cells with the Ca^{2+} ionophore A23187, or with thapsigargin, induces the expression of calreticulin at the transcriptional level in vivo.

The Kinetics of Activation of the Calreticulin Promoter

A23187 and thapsigargin modulate intracellular Ca^{2+} concentration within seconds. To determine the kinetics of the Ca^{2+}-dependent activation of calreticulin promoter, time-dependent expression of CAT was measured in NCB1 cells (NIH/3T3 cells stably transfected with pCC1 and pSVβ-galactosidase). Two experimental protocols were used for this analysis. First, the cells were incubated for 2, 4, 8, 12, and 16 h with A23187 or thapsigargin followed by measurement of CAT expression (Fig. 6, *open bars*). In the second protocol, the cells were incubated for 2, 4, 8, and 12 h with the drugs followed by incubation in a drug-free medium to a total of 16 h of incubation for each data point (Fig. 6, *hatched bars*). Fig. 6 shows that for both protocols the overall kinetics and magnitude of induction of CAT expression by A23187 and thapsigargin were similar. Maximal induction of CAT expression in the NCB1 cells required 16 h of continuous incubation with both drugs (Fig. 6), or exposure to the drugs for 4–8 h followed by incubation in a drug-free medium to a total of 16 h (Fig. 6). These results indicate that the Ca^{2+} store depletion–dependent activation of the calreticulin promoter is very slow and that it may require de novo protein synthesis.

In previous experiments with untransfected NIH/3T3 cells (Fig. 4), we found that treatment with A23187 or thapsigargin induced expression of calreticulin mRNA. To assess whether or not new protein synthesis is required to mediate this change, NIH/3T3 cells were pretreated with 100 µM cycloheximide before the addition of either 7 µM A23187 or 100 nM thapsigargin. Total RNA was then iso-
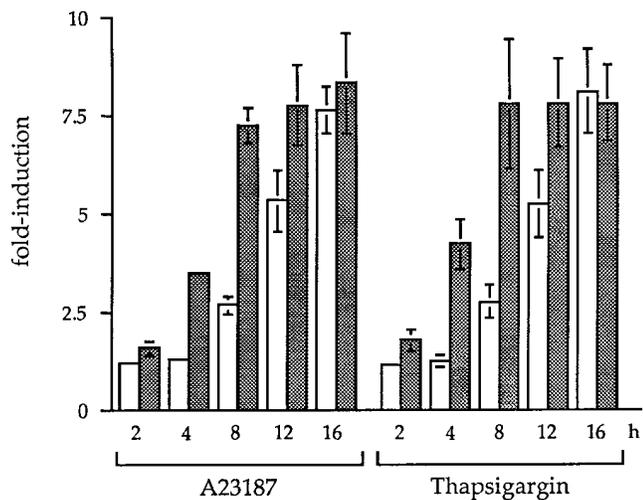
Figure 6. The kinetics of activation of the calreticulin promoter by A23187 and thapsigargin treatment. NCB1 cells (stably expressing CAT under control of the calreticulin promoter and β-galactosidase) were incubated with 7 μM A23187, 100 nM thapsigargin, or DMSO (control cells) for the times indicated. At different time points, cells were either harvested (*open bars*) or washed with PBS and then incubated in drug-free media to a total of 16 h of incubation (*cross-hatched bars*). CAT protein levels and β-galactosidase activity were measured as described in Materials and Methods. Data are reported as a mean ± SD of four separate experiments performed in triplicate.

Figure 7. Cycloheximide inhibits the A23187- and thapsigargin-dependent increase of calreticulin mRNA levels. Total RNA was isolated from nontransfected NIH/3T3 cells that had been incubated for 16–18 h with 7 μM A23187 or 100 nM thapsigargin, and either with or without cycloheximide. The RNA was separated electrophoretically on a formaldehyde-agarose gel, blotted onto Hybond N nylon filters, and hybridized with cDNA probes encoding mouse calreticulin and G3PDH as described in Materials and Methods. (*Top*) Autoradiogram of a Northern blot probed with calreticulin and the G3PDH cDNAs. (Lane *1*) Control cells; (lane *2*) cells incaxxubated with 100 μM cycloheximide; (lane *3*) A23187-treated cells; (lane *4*) A23187- and cycloheximide-treated cells; (lane *5*) thapsigargin-treated cells; (lane *6*) thapsigargin- and cycloheximide-treated cells. (*Bottom*) The abundance of calreticulin mRNA (calreticulin mRNA/G3PDH mRNA ratio) was determined using Phosphorimager analysis of the Northern blots. (*Filled bars*) Cells treated with A23187 or thapsigargin in the absence of cycloheximide; (*open bars*) cells pretreated with 100 μM cycloheximide for 2 h followed by incubation with either A23817 or thapsigargin. *TG*, thapsigargin.

lated and relative levels of calreticulin mRNA were assessed using Northern blot analysis. Fig. 7 shows that treatment with cycloheximide reduced the A23187- and thapsigargin-dependent increase in calreticulin mRNA levels by ~75%, suggesting that new protein synthesis was involved in the response. To rule out that these effects may be due to the cycloheximide-dependent changes in the intracellular Ca^{2+} concentrations, we measured cytosolic Ca^{2+} concentration in the cycloheximide-treated cells. The basal cytosolic-free Ca^{2+} concentration was not changed in the cycloheximide-treated cells and was determined to be 100 ± 8 nM ($n = 4$) and 110 ± 6 nM ($n = 4$) in control and cycloheximide-treated cells, respectively.

Depletion of Ca^{2+} from the Lumen of the ER Activates the Calreticulin Promoter

To test whether extracellular concentrations of Ca^{2+} affect the drug-mediated activation of the calreticulin promoter, we incubated NCB1 cells in a Ca^{2+}-depleted medium supplemented with EGTA. We found that A23187- and thapsigargin-dependent activation of the promoter was independent of changes in the extracellular Ca^{2+} concentration (Fig. 8). These results further indicate that depletion of Ca^{2+} from ER stores is involved in the activation of the calreticulin promoter.

The Effects of BAPTA/AM and EGTA/AM on Activity of the Calreticulin Promoter

To test whether activation of the calreticulin promoter by A23187 and thapsigargin is affected by changes in the cytoplasmic Ca^{2+} concentration, NCB1 cells were treated with the membrane-permeable Ca^{2+} chelators BAPTA/

AM (10 μM) or EGTA/AM (10 μM) before treatment with the drugs. Once BAPTA/AM or EGTA/AM enter the cytosol, they are cleaved to membrane-impermeable BAPTA or EGTA. They significantly reduce and maintain a low (resting or below) cytoplasmic Ca^{2+} concentration (Muallem et al., 1990; Preston and Berlin, 1992). For example, we showed that the basal cytosolic Ca^{2+} concentration in the BAPTA-treated cells was reduced to 65 ± 9 nM ($n = 4$) as compared with the control cells (100 ± 5 nM ($n = 4$)). NCB1 cells were loaded with BAPTA/AM or EGTA/AM for 30 min followed by 16-h incubation with either A23187 or thapsigargin, in normal or Ca^{2+}-depleted medium. Fig. 8 shows that in BAPTA-treated cells the A23187- and thapsigargin-dependent activation of the calreticulin promoter was reduced by 50 and 30%, respectively. Depletion of extracellular Ca^{2+} further enhanced these effects, especially for cells treated with thapsigargin (Fig. 8). In contrast, loading of NCB1 cells with EGTA/AM, a cytoplasmic Ca^{2+} chelator, had no effect on A23187- or thapsigargin-dependent activation of the calreticulin promoter (Fig. 8).

The Effects of Bradykinin on Activity of the Calreticulin Promoter

To investigate if there is any transactivation of the calreticulin promoter under more physiological conditions of Ca^{2+} store depletion, we treated the NCB1 cells with
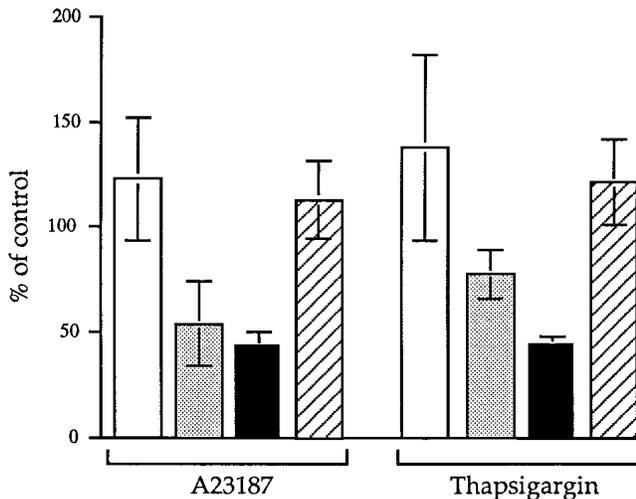
Figure 8. Effects of BAPTA on A23187- and thapsigargin-dependent activation of calreticulin promoter. NCB1 cells were loaded with 10 μM BAPTA/AM for 30 min in a media containing different drugs for 16 h and lysed, and reporter gene analysis was carried out as described in Materials and Methods. (*Open bars*) Cells grown and incubated with 7 μM A23187 or 100 nM thapsigargin in DME supplemented with 100 μM EGTA; (*dotted bars*) cells loaded with BAPTA/AM followed by incubation with either A23187 or thapsigargin (control cells were not treated with BAPTA/AM); (*filled bars*) cells loaded with BAPTA/AM and incubated with either A23187 or thapsigargin in Ca^{2+}-depleted DME. (*Cross-hatched bars*) Cells loaded with 10 μM EGTA/AM for 30 min followed by 16-h incubation with either A23187 or thapsigargin in DME (control cells were not treated with EGTA/AM); data are shown as a mean ± SD.

Figure 9. Effects of bradykinin on activation of calreticulin promoter. NCB1 cells were incubated with either 100 nM thapsigargin (*TG*) or 200 nM bradykinin (*BK*) in Ca^{2+}-depleted DME supplemented with EGTA. After 16 h the cells were lysed, and then reporter gene analysis was carried out as described in Materials and Methods. Data are shown as a mean ± SD.

bradykinin in Ca^{2+}-free DME supplemented with EGTA. Bradykinin induces Ca^{2+} depletion of the intracellular Ca^{2+} stores in the NIH/3T3 cells (Hashii et al., 1993). Fig. 9 shows that prolonged treatment of the NCB1 cells with bradykinin in the absence of the extracellular Ca^{2+} resulted in a fourfold activation of the calreticulin promoter, suggesting that bradykinin-dependent Ca^{2+} depletion of Ca^{2+} stores activates transcription of the calreticulin gene. In contrast, incubation of the NCB1 cells with bradykinin for a shorter period of time (4 h), either in the absence or presence of the extracellular Ca^{2+} followed by incubation in bradykinin-free media, did not activate the promoter (data not shown).

Discussion

In this study we have isolated the mouse calreticulin gene and determined its genomic organization. Using a reporter gene assay system, we demonstrated that the calreticulin gene is activated by either thapsigargin-, A23187-, or bradykinin-dependent Ca^{2+} depletion of intracellular Ca^{2+} stores both in vitro and in vivo. Importantly, run-on experiments documented that depletion of Ca^{2+} stores also activate endogenous calreticulin gene.[2] Finally, we showed

2. Since submission of this manuscript, publications by Lewellyn et al. (1996) and Nguyen et al. (1996) showed Ca^{2+}-dependent induction of expression of calreticulin.

that stress response to ER Ca^{2+} store depletion results in increased calreticulin mRNA and protein levels, and that this increased expression of the calreticulin gene requires de novo protein synthesis.

To initiate this study we first isolated and characterized a 12-kb genomic DNA fragment containing the entire mouse calreticulin gene and 2.14 kb of its promoter region. This allowed us, for the first time, to compare the nucleotide sequence of the two genes encoding calreticulin. The mouse gene is highly homologous to the human gene (McCauliffe et al., 1992). With the exception of two introns, which, in the mouse, are twice the size of their human counterparts, the exon–intron organizations of these genes are basically identical. This high degree of conservation at the level of gene organization and its nucleotide sequence is in keeping with earlier observations that the amino acid sequences of calreticulin from different species are also highly conserved (Nash et al., 1994; Michalak, 1996). For example, the amino acid sequence identity of mouse and human calreticulins is >95% (Smith and Koch, 1989; McCauliffe et al., 1992).

To investigate the role of the ER Ca^{2+} stores in activation of calreticulin gene expression, we used two different agents, the Ca^{2+} ionophore A23187 and the ER Ca^{2+}-ATPase inhibitor thapsigargin. We found that these drugs are associated with activation of the calreticulin promoter in vitro and in vivo, as well as with increased expression of calreticulin mRNA and protein. Activation of the calreticulin promoter by these drugs is independent of changes in extracellular Ca^{2+} concentration. Most importantly, we show that transactivation of calreticulin promoter is increased in cells treated with bradykinin, a hormone that induces Ca^{2+} release from Ca^{2+} stores in NIH/3T3 cells (Fu et al., 1992; Hashii et al., 1993). Bradykinin stimulation of cells leads to activation of protein kinase C. However, the bradykinin-dependent activation of calreticulin promoter was not due to the activation of protein kinase C since phorbol esters had no effect on transactivation of the calreticulin promoter as measured using the reporter gene assay system (unpublished observations).

In this study we used a relatively large fragment of the calreticulin promoter, which allowed us to identify two regions (−115 to −260 and −685 to −1,763) in the promoter

containing unique CCAAT nucleotide motifs that may play a role in the Ca^{2+} depletion–dependent activation of the gene. Similar regions have been identified on the Grp78 promoter and shown to be responsible for the thapsigargin- and Ca^{2+} ionophore–mediated transactivation of the gene (Wooden et al., 1991; Li et al., 1993; Roy and Lee, 1995; Roy et al., 1996). This motif may therefore play a specific role in Ca^{2+}-sensitive regulation of calreticulin genes. It is important to note, however, that CCAAT element alone may not be sufficient for promoter activation since another cellular promoter, the $\alpha2(I)$ collagen promoter, which contains a similar motif, is only weakly inducible by Ca^{2+} depletion signal (Roy and Lee, 1995; Roy et al., 1996). It is unlikely, therefore, that there is a single element responsible for Ca^{2+} depletion–dependent activation of gene expression. We are currently investigating, using gel retardation and site-specific mutagenesis techniques, a precise role of the two regions in the calreticulin promoter in Ca^{2+} store depletion–dependent activation of the calreticulin gene. Increases in expression of the calreticulin gene required prolonged exposure to Ca^{2+} ionophore, thapsigargin, or bradykinin ($\sim$4 h), and it was inhibited by cycloheximide, indicating that the calreticulin gene belongs to a group of "delayed response" genes that are activated slowly and typically require new protein synthesis for their expression. The mechanism(s) responsible for the Ca^{2+} depletion–dependent activation of calreticulin and other genes is not yet known. One possibility is that the treatment of cells with A23187, thapsigargin, or bradykinin leads to a brief Ca^{2+} elevation in the cytoplasm, which may be sufficient to activate long-term effects on calreticulin gene expression several hours later. However, this is unlikely since the short-term exposure of cells to A23187, thapsigargin, or bradykinin either in the presence or absence of the extracellular Ca^{2+} has no effect on the transactivation of the calreticulin gene. Whether or not changes in the nuclear-free Ca^{2+} or nuclear Ca^{2+} binding proteins (Bachs et al., 1992) are involved remains to be determined. Thus, we conclude that induction of the calreticulin gene is likely due to a stress response upon ER Ca^{2+} store depletion.

Recently, Nguyen et al. (1996) and Llewellyn et al. (1996) reported activation of the human calreticulin gene by Ca^{2+} and/or Ca^{2+} store depletion. Llewellyn et al. (1996) and Nguyen et al. (1996) used a relatively short fragment of the human calreticulin promoter (585 and 504 bp, respectively) and therefore observed only threefold induction of expression of the calreticulin gene. This is likely due to transactivation of the first region of the promoter identified in the present study. An interesting observation is that calreticulin promoter is also activated by Zn^{2+} (Nguyen et al., 1996). We show that BAPTA, a chelator of Ca^{2+} and heavy metals (including Zn^{2+}), partially inhibits activation of the calreticulin promoter elicited by A23187 or thapsigargin treatment. In contrast, EGTA/AM, which has a higher specificity for Ca^{2+} than BAPTA, had no effect on A23187- and thapsigargin-dependent activation of the gene. This indicates that cytoplasmic Ca^{2+} may not play a significant role in the A23187- or thapsigargin-dependent activation of the calreticulin gene. It is tempting to speculate, however, that alterations in cytoplasmic heavy metals (perhaps Zn^{2+} concentration) may play a

role in transactivation of the calreticulin gene. Baksh et al. (1995) discovered that Zn^{2+} and Ca^{2+} profoundly affect interactions between calreticulin and PDI, the two ER-resident proteins. In the presence of Ca^{2+} or Zn^{2+}, the two proteins do not associate (Baksh et al., 1995). Therefore, change in the Zn^{2+} concentration may not only transactivate calreticulin gene but, if elevated in the lumen of the ER, it may also modulate levels of "free" calreticulin and/or PDI in the lumen of the ER (Krause and Michalak, 1997). Understanding the significance of Zn^{2+}-dependent transactivation of calreticulin gene awaits further investigation.

What is the significance of Ca^{2+} store depletion–dependent transactivation of calreticulin gene? Calreticulin is a multifunctional protein: it modulates steroid-sensitive gene expression (Burns et al., 1994; Dedhar et al., 1994), cellular adhesiveness (Coppolino et al., 1996; Opas et al., 1996), and the store-operated Ca^{2+} influx (Bastianutto et al., 1995; Mery et al., 1996). Furthermore, the protein is a unique and unusual chaperone as it interacts specifically with glucosylated proteins (Hammond and Helenius, 1995; Helenius et al., 1997). It remains to be determined why cells upregulate the expression of calreticulin under these conditions. It is conceivable that overexpression of calreticulin, a Ca^{2+} binding protein (Bastianutto et al., 1995; Mery et al., 1996), may be required to overcome depletion of the ER Ca^{2+} stores. Alternatively, changes in the intraluminal ER Ca^{2+} concentration will affect protein translation, folding, and posttranslational modification, and increased expression of calreticulin might be necessary to fulfill requirements for chaperone activity. In this study we show Ca^{2+} store depletion–dependent regulation of expression of calreticulin, a new mechanism responsible for the control of expression of the protein. This will likely have an important role in the regulation of many cellular processes that are under the control of calreticulin.

We have recently established that the ER form of calreticulin is responsible for the modulation of steroid-sensitive gene expression (Michalak et al., 1996) and for the regulation of cellular adhesiveness via upregulation of expression of vinculin (Opas et al., 1996). Thus, we proposed that calreticulin may function as a "signaling" molecule from the lumen of the ER (Mery et al., 1996; Opas et al., 1996; Michalak et al., 1996; Krause and Michalak, 1997). This may be similar to the BiP-dependent ER-nuclear signal transduction described in the yeast (Mori et al., 1993, 1996; Cox et al., 1993; Cox and Walter, 1996; Sidrauski et al., 1996); control of cellular cholesterol homeostasis by SREBP, an ER integral membrane protein (Wang et al., 1994); or ER-dependent activation of the NF-κB (Pahl and Baeuerle, 1995; Pahl et al., 1996; for review see Pahl and Baeuerle, 1997). It is conceivable, therefore, that depletion of Ca^{2+} stores not only acts as an ER–nucleus signaling pathway but it may also, via upregulation of expression of calreticulin as documented in this work, affect the calreticulin-dependent ER–nucleus/cytoplasm signaling. This may be a new mechanism of Ca^{2+}-dependent modulation of numerous biological and pathophysiological processes.

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