Endoplasmic Reticulum Form of Calreticulin Modulates Glucocorticoid-sensitive Gene Expression*

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Calreticulin is a ubiquitously expressed Ca\(^{2+}\)-binding protein of the endoplasmic reticulum (ER), which inhibits DNA binding in vitro and transcriptional activation in vivo by steroid hormone receptors. Transient transfection assays were carried out to investigate the effects of different intracellular targeting of calreticulin on transcription mediated by glucocorticoid receptor. BSC40 cells were transfected with either calreticulin expression vector (ER form of calreticulin) or calreticulin expression vector encoding calreticulin minus leader peptide, resulting in cytoplasmic localization of the recombiant protein. Transfection of BSC40 cells with calreticulin expression vector encoding the ER form of the protein led to 40–50% inhibition of the dexamethasone-sensitive stimulation of luciferase expression. However, in a similar experiment, but using the calreticulin expression vector encoding cytoplasmic calreticulin, dexamethasone-stimulated activation of the luciferase reporter gene was inhibited by only 10%. We conclude that the ER, but not cytosolic, form of calreticulin is responsible for inhibition of glucocorticoid receptor-mediated gene expression. These effects are specific to calreticulin, since overexpression of the ER luminal proteins (BiP, ERp72, or calsequestrin) has no effect on glucocorticoid-sensitive gene expression. The N domain of calreticulin binds to the DNA binding domain of the glucocorticoid receptor in vitro; however, we show that the N+P domain of calreticulin, when synthesized without the ER signal sequence, does not inhibit glucocorticoid receptor function in vivo. Furthermore, expression of the N domain of calreticulin and the DNA binding domain of glucocorticoid receptor as fusion proteins with GAL4 in the yeast two-hybrid system revealed that calreticulin does not interact with glucocorticoid receptor under these conditions. We conclude that calreticulin and glucocorticoid receptor may not interact in vivo and that the calreticulin-dependent modulation of the glucocorticoid receptor function may therefore be due to a calreticulin-dependent signaling from the ER.

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[1] The abbreviations used are: ER, endoplasmic reticulum; DT, dystrophin tag; DMEM, Dulbecco’s modified Eagle’s medium; BiP, immunoglobulin-binding protein; BES, N,N'-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; GRE, glucocorticoid response element; PBS, phosphate-buffered saline; Dex, dexamethasone; CRAC, Ca\(^{2+}\) release-activated channel; MMTV, murine mammary tumor virus; ConA, concanavalin A; FITC, fluorescein isothiocyanate; Pipes, 1,4-piperazinediethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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In order to express the cytoplasmic form of calreticulin, cells were transfected with pcSR-L-DT vector, which encodes dystrophin-tagged calreticulin minus leader peptide (referred to throughout the paper as cytoplasmic calreticulin). To generate pcSR-C-DT vector, calreticulin cDNA was synthesized by polymerase chain reaction-driven amplification of pcR-DT using primers 5'-ATACTCGAG ATG AG GCG CCC GTC GTC ACT TCA-3' (encoding six NH2-terminal amino acids of the mature calreticulin and 5'-ATG initiation codon) and 5'-GGGATCC GAG GAG AAG CAA CAG CTC TTG TGC-3' (nucleotide sequence 1245–1264) (36 base pairs behind the stop codon of the calreticulin clone; Fliegel et al. (1989) with linking XhoI and EcoRI restriction sites, respectively. The DNA fragment was first inserted into Xhol/EcoRI restriction sites of the pBluescript to obtain a vector designated pBCR-L-DT. The Xhol/SacI fragment of pBCR-DT was inserted into XhoI/SacI restriction sites in pSVL plasmid to obtain pcSR-L-DT. To generate the pcSR-NP vector encoding the N+P domain of calreticulin (Baksh and Michalak, 1991) the BamHI DNA fragment (encoding the C domain of calreticulin) was excised and removed from the pcSR-L-DT vector. Dystrophin tagging of the N domain was carried out using synthetic oligodeoxynucleotides as described for the pcSR-DT calreticulin expression vector.

In order to express the cytoplasmic form of calreticulin, cells were transfected with pcSR-L-DT vector, which encodes dystrophin-tagged calreticulin minus leader peptide (referred to throughout the paper as cytoplasmic calreticulin). To generate pcSR-C-DT vector, calreticulin cDNA was synthesized by polymerase chain reaction-driven amplification of pcR-DT using primers 5'-ATACTCGAG ATG AG GCG CCC GTC GTC ACT TCA-3' (encoding six NH2-terminal amino acids of the mature calreticulin and 5'-ATG initiation codon) and 5'-GGGATCC GAG GAG AAG CAA CAG CTC TTG TGC-3' (nucleotide sequence 1245–1264) (36 base pairs behind the stop codon of the calreticulin clone; Fliegel et al. (1989) with linking XhoI and EcoRI restriction sites, respectively. The DNA fragment was first inserted into Xhol/EcoRI restriction sites of the pBluescript to obtain a vector designated pBCR-L-DT. The Xhol/SacI fragment of pBCR-DT was inserted into XhoI/SacI restriction sites in pSVL plasmid to obtain pcSR-L-DT. To generate the pcSR-NP vector encoding the N+P domain of calreticulin (Baksh and Michalak, 1991) the BamHI DNA fragment (encoding the C domain of calreticulin) was excised and removed from the pcSR-L-DT vector. Dystrophin tagging of the N domain was carried out using synthetic oligodeoxynucleotides as described for the pcSR-DT calreticulin expression vector.
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DNA shift assay was carried out in 1 mM EDTA, 20 mM NaCl, 0.05% bovine serum albumin, 4 mM dithiothreitol (DTT), 2 µg of poly(dI-dC), and the GRE (5'-TGC TAT TTT TGA CAG CAG TCA TGC ATT AT/AAC-3' and 5'-GTT TAT TAT AAT CCA CAG TCT TCC TTA AAA CAA CGA-3') were used in this assay. Calreticulin was isolated from canine pancreas by the ammonium sulfate precipitation procedure (Milner et al., 1991).

RESULTS

ER Form of Calreticulin Inhibits Transactivation by Glucocorticoid Receptor in Vivo—Transient transfection assays were carried out to investigate whether targeting of calreticulin to different intracellular compartments had an effect on transactivation mediated by glucocorticoid receptors. Two cell lines were used in these experiments, the BSC40 African green monkey kidney monolayer cells derived from BS-C-1 cells (Hoppins et al., 1963) and the mouse fibroblast NIH/3T3 cells. BSC40 and NIH/3T3 cells were co-transfected with different calreticulin expression vectors, MMTV-GRE-luciferase and pβgal control vector. Since the BSC40 cells do not contain an endogenous glucocorticoid receptor, they were also co-transfected with VERO plasmid encoding the glucocorticoid receptor. All results were normalized for transfection efficiency with pβgal, β-galactosidase expression vector. In the presence of 10^{-6} M Dex, BSC40 or NIH/3T3 cells co-transfected with MMTV-GRE-luciferase and the glucocorticoid receptor expression vector (VERO) exhibited >20-fold increase in the reporter gene activity over cells grown in the absence of Dex (Fig. 1). In agreement with earlier observations (Burns et al., 1994b; Winrow et al., 1995), co-transfection of these cells with MMTV-GRE-luciferase, VERO plasmid (BSC40 cells only), and calreticulin expression vector (pSCR-DT) led to significant inhibition of the Dex-sensitive stimulation of luciferase expression (Fig. 1). The effect of calreticulin on transactivation of dexamethasone-sensitive reporter gene was dose-dependent (Fig. 2). Maximal effects were observed when BSC40 cells were transfected with 10 µg of calreticulin expression vector (Fig. 2). In order to determine the level of calreticulin expression in BSC40 cells transiently transfected with calreticulin expression vector (10 µg), we performed quantitative immunological analysis of calreticulin-transfected and control cells using antibodies raised against calreticulin (Fig. 3A). The immunoblot shown in Fig. 3A was scanned, and the level of calreticulin in cells transfected with calreticulin expression vector pSCR-DT was determined to be increased approximately 4-fold over that of the endogenous protein.

In order to test if calreticulin’s effects on glucocorticoid receptor function are due to the cytoplasmic protein, we generated a calreticulin expression vector encoding calreticulin minus leader peptide (pSCR-L-DT). Fig. 1 shows that co-transfection of either BSC40 or NIH/3T3 cells with MMTV-GRE-luciferase, VERO plasmid encoding glucocorticoid receptor (BSC40 cells only), and pSCR-DT led to >50% decrease in less than 10% inhibition of Dex-stimulated activation of the luciferase reporter gene. This inhibition was not statistically significant (p > 0.42). Co-transfection of BSC40 cells with yene glycol 8000 for 3 min, washed in PBS for 10 min, and then precleared with primary antibodies followed by secondary antibodies. The secondary antibodies were FITC-conjugated donkey anti-goat (diluted 1:30 in PBS) and Texas Red-conjugated donkey anti-rabbit (used at 1:30 dilution). For double labeling, all incubations were done sequentially. After the final wash (3 times for 5 min), the slides were mounted in Vialis containing 0.25% 1,4-diazabiciclo-(2,2,2)-octane and 0.002% p-phenylenediamine to prevent photobleaching. A Bio-Rad model MRC-600 confocal fluorescence microscope equipped with a krypton/argon laser was used. To maintain pixel registration necessary for subtractive imaging, fluorescence images were collected using a simultaneous double labeling K1/K2 filter set. However, the images were recorded using single excitation wavelengths in order to avoid any bleed-through of emissions. Furthermore, no appreciable specific signal could be detected if any of the primary antibodies were omitted from the staining procedure. No signal could be detected when both the primary and secondary reagents were omitted. Finally, staining of mock-transfected cells with the anti-DT antibody did not produce any appreciable specific labeling. Subtractive imaging was performed by arithmetic subtraction of pixel-registered images of two labels in the same cells using the Image-1 program (West Chester, PA).
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FIG. 1. Calreticulin represses the in vivo transactivation of the glucocorticoid-sensitive gene expression in BSC40 and NIH/3T3 cells. BSC40 or NIH/3T3 cells were co-transfected with MMTV-GRE-luciferase vector, pβGAL control vector, VERO plasmid expressing glucocorticoid receptor (BSC40 cells only), and different calreticulin expression vectors as indicated. Cellular extracts were prepared and assayed for luciferase and β-galactosidase expression as described under “Experimental Procedures.” Cells were incubated in DMEM containing 10% charcoal-treated calf serum for 12 h followed by incubation for 24 h with DMEM alone (Dex -) or in DMEM containing 10^{-6} dexamethasone (Dex +). The values shown are relative activities from four independent transfections done in triplicate and normalized for β-galactosidase activity. The means ± S.D. are given.

FIG. 2. Dose-dependent effects of calreticulin on the in vivo transactivation of the glucocorticoid receptor in BSC40 cells. BSC40 cells were co-transfected with MMTV-GRE-luciferase vector, pβGAL control vector, VERO plasmid expressing glucocorticoid receptor, and different amounts of the calreticulin expression vector (pSVL-CRT) as indicated. Cellular extracts were prepared and assayed for luciferase and β-galactosidase expression as described under “Experimental Procedures.” Cells were incubated in charcoal-treated calf serum for followed by incubation for 24 h with DMEM alone (Dex -) or in DMEM containing 10^{-6} dexamethasone (Dex +). The values shown are relative activities from three independent transfections done in triplicate and normalized for β-galactosidase activity. The means ± S.D. are given.

FIG. 3. Immunological identification of calreticulin and calreticulin domains in BSC40 cells. BSC40 cells were transfected with expression vectors encoding the ER form of calreticulin (A), the cytoplasmic form of calreticulin (B), or the cytoplasmic N-P domain of calreticulin (C) as described under “Experimental Procedures.” Cellular proteins (equal to 200,000 cells/lane) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose filters, and incubated with goat anti-calreticulin (A and B) or anti-DT (C) antibodies. Lanes 1, mock-transfected cells; lanes 2, BSC40 cells transfected with plasmid encoding the ER form of calreticulin (A), the cytoplasmic form of calreticulin (B) or the dystrophin-tagged cytoplasmic N-P domain of calreticulin (C).

FIG. 4. Cytoplasmic form of calreticulin does not modulate dexamethasone-sensitive gene expression. BSC40 cells were co-transfected with MMTV-GRE-luciferase vector, pβGAL control vector, different calreticulin expression vectors, and different amounts of the VERO plasmid expressing glucocorticoid receptor as indicated. Cellular extracts were prepared and assayed for luciferase and β-galactosidase expression as described under “Experimental Procedures.” Cells were incubated with DMEM alone (Dex -) or in DMEM containing 10^{-6} dexamethasone (Dex +). The values shown are relative activities from three independent transfections done in triplicate and normalized for β-galactosidase activity. The means ± S.D. are given.

was not due to a limited number of glucocorticoid receptor molecules in BSC40 cells. Immunoblot analysis with antibodies raised against calreticulin showed that BSC40 cells transfected with pSCR-L-DT had a ~2.5-fold increase in the level of calreticulin (Fig. 3B).

We have previously shown that the in vivo interaction between the DNA binding domain of glucocorticoid receptor and calreticulin is confined to the N domain and N-P domain of the protein (Burns et al., 1994b). To test the in vivo role of the N domain of calreticulin in modulation of glucocorticoid receptor-sensitive gene expression BSC40 cells were co-transfected with plasmids encoding the N-P domain (pSCR-NP). The pSCR-NP vector was chosen for these experiments, since it encodes calreticulin minus the C domain, a high capacity Ca^{2+} binding region of the protein (Baksh and Michalak, 1991). This was to avoid any nonspecific effects of cytoplasmic Ca^{2+} sequestration by calreticulin on the function of the glucocorticoid receptor. In addition, this expression vector encoded calreticulin domains minus leader peptide, resulting in the targeting of the recombinant protein to the cytoplasm. Furthermore, the DT was introduced into the C terminus of the N-P domain to enable monitoring expression and intracellular localization of the recombinant protein. Fig. 1 shows that expression of N-P domain in either BSC40 or NIH/3T3 cells transfected with MMTV-GRE-luciferase had no effect on Dex-sensitive luciferase expression. The presence of the N-P domain of calreticulin in BSC40 cells transfected with the N-P domain expression vector was established using the anti-DT antibodies (Fig. 3C).

Intracellular localization of the recombinant calreticulin and pSCR-L-DT cytoplasmic calreticulin expression vector and an increasing amount of glucocorticoid receptor expression vector did not have any additional effect on the glucocorticoid receptor-sensitive expression of the luciferase reporter gene (Fig. 4), ruling out the possibility that the observed lack of inhibition
FIG. 5. Co-localization of calreticulin, recombinant calreticulin, N+P domain of calreticulin, and ER membrane markers in BSC40 transfected cells by confocal fluorescence microscopy and subtractive imaging. A, co-localization of the ER marker, FITC-ConA (ER

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its fragments was examined in the transfected cells by immunofluorescence microscopy using antibodies against the DT. The recombinant proteins were co-localized with an ER marker, ConA (Virtanen et al., 1980; Tartakoff and Vassalli, 1983) (Fig. 5A), or endogenous calreticulin (Fig. 5B). As expected, in cells transfected with the pSCR-DT calreticulin expression vector the full-length protein was found in the ER as demonstrated by both the ConA fluorescence (Fig. 5A) and labeling of the endogenous calreticulin (Fig. 5B). In cells transfected with the pSCR-L-DT cytoplasmic calreticulin expression vector, the recombinant protein was localized to the cytoplasm (Fig. 5A). The recombinant N+P domain of calreticulin lacking leader peptide also localized to the cytoplasm (Fig. 5, N+P domain). Subtractive imaging (difference) of both the cytoplasmic calreticulin and the N+P domain against the ER clearly demonstrated cytoplasmic targeting of these recombinant molecules. This was further confirmed by subtractive imaging of DT on the recombinant proteins against the anti-calreticulin label (Fig. 5B). In the pSCR-L-DT-transfected cells (Cytoplasmic CRT) the goat antibody detected calreticulin in both the ER and the cytoplasm; thus, the differential image exposed the ER-localized protein (Fig. 5B, Cytoplasmic CRT, difference). In contrast, since this antibody recognizes an epitope close to the C terminus of calreticulin, it did not label the N+P domain in the pSCR-NP-transfected cells, and the subtractive imaging yielded familiar “ER exclusion” pattern in the differential image (Fig. 5B, N+P domain, difference). Based on the transfection experiments (Fig. 1) and the immunological localization of the recombinant proteins (Fig. 5), we concluded that the ER, but not the cytosolic form of calreticulin inhibited glucocorticoid receptor-dependent activation of the luciferase reporter gene in both BSC40 and NIH/3T3 cells.

Inhibition of Glucocorticoid Receptor Function Is Specific to Calreticulin—In order to test if the observed inhibition of glucocorticoid receptor-sensitive gene expression is calreticulin-specific or if it could be mediated by other luminal ER proteins, we performed a series of co-transfection experiments. BSC40 cells were transfected with MMTV-GRE-luciferase and expression plasmid encoding BiP, ERp72, or with calseaquerin the sarcoplasmic reticulum luminal Ca$^{2+}$-binding protein. Fig. 6 shows that overexpression of BiP or ERp72 did not have any effect on glucocorticoid receptor-dependent expression of luciferase reporter gene. However, transient co-transfection of BSC40 cells with both calreticulin (pSCR-DT) and ERp72 expression vectors led to 50% inhibition of the Dex-dependent luciferase activity. The means ± S.D. are given.
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Luciferase activity (Fig. 6). Overexpression of cardiac calsequestrin, a Ca^{2+}-binding protein of similar physicochemical properties to calreticulin (Scott et al., 1988), also did not have any effect on the Dex-sensitive expression of luciferase (Fig. 6). To confirm that BiP, ERp72, and calsequestrin were expressed in transfected cells, we carried out Western blot analysis of cellular extracts (Fig. 7). Since BiP expression vector encoded BiP tagged with specific c-myec epitope, we detected expression of this protein using the anti-c-myec antibodies (Fig. 7A). Fig. 7B shows that cells transfected with the ERp72 expression vector had a 3-fold increased level of the immunoreactive ERp72. Finally, using anti-calsequestrin antibodies, we showed that the protein was expressed in BSC40 cells transfected with the calsequestrin expression vector (Fig. 7C). These results suggest that calreticulin-dependent modulation of the Dex-sensitive luciferase reporter gene was specific to calreticulin and that it was not a result of protein overexpression in the lumen of the ER or increased sequestration of ER lumenal Ca^{2+} as documented by overexpression of calsequestrin.

Calreticulin Is Not a Resident Nuclear Protein—As shown in Fig. 5, calreticulin was not detected in the nucleus of transfected cells, yet we observed significant inhibition of the glucocorticoid receptor-dependent expression of the reporter gene (Fig. 1). However, we and others have reported that a calreticulin-like antigen is detected in the nuclei of L6 cells (Opas et al., 1991) and in the isolated nuclei from P19 cells (Dedhar et al., 1994a). In order to determine if calreticulin is a resident nuclear protein, we have immunolabeled isolated nuclei with two different anti-calreticulin antibodies. Furthermore, we have fractionated rat liver nuclei to determine if there is any association of calreticulin with the nuclei and/or the nuclear membranes. Fig. 8 shows staining of the isolated nuclei either with goat anti-calreticulin (B) or rabbit anti-calreticulin (D) antibody. The goat anti-calreticulin antibody gave no nuclear staining of the isolated nuclei, whereas the rabbit anti-calreticulin showed some patches of nuclear staining. The reason for the different staining by these two antibodies is not clear at present, but it might be due to a different degree of contamination of the isolated nuclei with a nuclear envelope membrane. To further test for the presence of calreticulin in the nucleus, we have fractionated rat nuclei followed by immunological analysis of different fractions with the goat and the rabbit anti-calreticulin antibodies. As a control, we employed the anti-lamin B receptor antibodies (Ye and Worman, 1994; Lin et al., 1996). Fig. 9 shows that a 60-kDa calreticulin was present in the isolated, intact nuclei (lane 3). Extraction of purified nuclei with 2% Triton X-100 leads to separation of the detergent-soluble outer nuclear membrane from the nuclear content surrounded by inner nuclear membrane (Blobel and Potter, 1966). Resident nuclear proteins are associated with the Triton-insoluble nuclear fractions, whereas the Triton-soluble fractions contain proteins of the ER origin. Fig. 9, A and B, lane 4 shows that calreticulin was associated with the Triton-soluble outer nuclear membrane and not with the nuclei (lane 6). An identical pattern of immunological reactivity of different nuclear fractions was obtained using either goat or rabbit anti-calreticulin antibodies (Fig. 9, A and B). The same protein blot was also tested for the reactivity with the antibodies against the lamin B receptor, a specific integral inner nuclear membrane protein (Ye and Worman, 1994; Lin et al., 1996). As expected, a 60-kDa lamin B receptor was present in the intact nuclei and in the Triton-insoluble fractions containing nuclei content surrounded by inner nuclear membrane (Fig. 9C). There was no immunoreactive lamin B receptor in the Triton-soluble fraction (Fig. 9C, lane 4), which contained calreticulin (Fig. 9, A and B, lane 4). Taken together, these results suggested that calreticulin is not a resident nuclear protein and, therefore, that calreticulin may not inhibit function of the glucocorticoid receptor by direct interaction with its DNA binding domain in the cytoplasm or the nucleus. This conclusion is further supported by the in vitro DNA mobility shift experiments. Fig. 10 shows that, as reported earlier (Burns et al., 1994b), calreticulin inhibits glucocorticoid receptor binding to the GRE in vitro. However, when glucocorticoid receptor was first bound to the GRE prior to incubation with calreticulin, the protein no longer inhibited glucocorticoid receptor interaction with the GRE (Fig. 10). This suggests that in vitro the DNA bound (nuclear form) of the glucocorticoid receptor does not interact with calreticulin.

Calreticulin Does Not Interact with the DNA Binding Domain of the Glucocorticoid Receptor in the Yeast Two-hybrid System—Finally, we employed the yeast two-hybrid system (Fields and Song, 1989; Chien et al., 1991) to test if the DNA binding domain of the glucocorticoid receptor and calreticulin interact under the in vivo cellular conditions. For these experiments, calreticulin and the N domain of calreticulin (amino acid residues 1–174) were fused to the GAL4-DNA binding domain. Calreticulin and the N domain fusion proteins did not activate reporter gene expression when co-expressed with the GAL4 transcriptional activation domain. However, in a control experiment (Fig. 11, filter 1) expression of GAL4 transcription factor induced expression of β-galactosidase. Next the DNA binding domain of the glucocorticoid receptor and calreticulin interact under the in vivo cellular conditions. These experiments, calreticulin and the N domain fusion proteins did not activate reporter gene expression when co-expressed with the GAL4 transcriptional activation domain. This fusion protein did not activate the reporter gene when co-expressed with the GAL4-DNA binding domain (Fig. 11, filters 2 and 5). When jointly expressed as GAL4 fusion proteins, full-length calreticulin, or the N domain with the DNA binding domain of GR (Fig. 11, filters 4 and 7), they also did not activate expression of β-galactosidase, indicating that the two proteins did not interact under these conditions.

Discussion

In order to examine a role of calreticulin in regulation of the steroid-sensitive gene expression, the protein was targeted either to the lumen of the ER or to the cytoplasm followed by...
analysis of the effect of calreticulin on the glucocorticoid receptor-dependent reporter gene expression. We show that the ER, but not cytosolic, form of calreticulin is the most effective in inhibition of the glucocorticoid receptor-mediated gene expression. This effect is specific to calreticulin, since transfection of BSC40 cells with expression vectors for ER luminal proteins such as BiP, ERp72, or calsequestrin did not have a significant effect on the glucocorticoid-sensitive gene expression. Furthermore, we show that the N + P domain of calreticulin, when synthesized without the ER signal sequence, also does not inhibit glucocorticoid receptor function. The N domain of the protein interacts with the DNA binding domain of the glucocorticoid receptor in vitro (Burns et al., 1994b). Furthermore, expression of either calreticulin or the N domain of calreticulin and the DNA binding domain of glucocorticoid receptor as fusion proteins with GAL4 in the yeast two-hybrid system revealed that they do not interact with glucocorticoid receptor under these in vivo conditions. We conclude that calreticulin may not interact with steroid receptors in vivo but that it may function as a “signaling” molecule from the lumen of the ER.

Calreticulin-like immunoreactivity was detected in the nucleus of some cells (Opas et al., 1991; Dedhar et al., 1994a, 1994b), suggesting that the nuclear antigen, if it was calreticulin, could have been responsible for affecting gene expression. Despite the apparent presence of calreticulin in intact cells, a calreticulin-like antigen is not consistently found by immunofluorescence in the isolated nuclei, and the immunostaining for calreticulin is not reproducible when different antibodies are used. Calreticulin also fractionates away with the outer nuclear membrane, but not with the nuclear matrix or with the inner nuclear membrane (containing lamin B receptor, a nuclear membrane marker), indicating that calreticulin is associated with the ER but not with the nucleus. We were unable to detect any nuclear and cytoplasmic calreticulin by immunofluorescence analysis of the BSC40 and NIH/3T3 cells overexpressing calreticulin, using two different anti-calreticulin antibodies. This is similar to other cells overexpressing calreticulin including HeLa, mouse L fibroblasts, A10, and COS-7 cells (Sonnichsen et al., 1994; Bastianutto et al., 1995; Mery et al., 1996). It is possible that levels of calreticulin in the cytoplasm or the nucleus may be too low for the detection methods used in this study. This is unlikely, however, since overexpression of cytoplasmic calreticulin did not significantly affect glucocorticoid receptor function.

M. Michalak, K. Burns, C. Andrin, N. Mesaeli, J. L. Busaan, and M. Opas, unpublished observations.
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We concluded that calreticulin may not be a nuclear resident protein and that, in vivo, calreticulin may not interact directly with the DNA binding domain of steroid receptors, but more likely the protein inhibits their activation of transcription indirectly, from the lumen of the ER. This is further supported by experiments using the yeast two-hybrid system. Under these conditions we also did not observe any interaction between the DNA binding domain of the glucocorticoid receptor (fused to the GAL4 transcriptional activation domain) and full-length calreticulin or its N domain (fused to the GAL4-DNA binding domain).

Calreticulin modulates steroid-sensitive gene expression (Burns et al., 1994a, 1994b; Dedhar et al., 1994a, 1994b). The protein in vitro binds to the conserved amino acid sequence KXXF/K/R/R found between the two Zn²⁺ fingers in the DNA binding domain of steroid receptors and prevents their interaction with the DNA-responsive element (Burns et al., 1994b; Dedhar et al., 1994b). Overexpression of calreticulin in mouse L cells and Vero fibroblasts inhibited transcriptional activation by glucocorticoid receptor and androgen receptor in vivo, respectively (Burns et al., 1994b; Dedhar et al., 1994b). Furthermore, overexpression of calreticulin inhibits retinoic acid-induced differentiation of P19 cells (Dedhar et al., 1994) and decreases the induction of protein kinase C by retinoic acid (Desai et al., 1996). Calreticulin also binds, in vitro, to other transcription factors that contain the KXXF/K/R/R consensus amino acid sequence: the peroxisome proliferator-activated receptor-retinoid X receptor heterodimers, the hepatocyte nuclear factor-4, the chicken ovalbumin upstream promoter-transcription factor (Winrow et al., 1995), and the vitamin D₃ receptor (Wheeler et al., 1995; St-Arnaud et al., 1995). Surprisingly, in vivo calreticulin inhibits transcriptional activation by the vitamin D₃ receptor, retinoic acid receptor, androgen receptor, and glucocorticoid receptor (Dedhar et al., 1994; Burns et al., 1994; Wheeler et al., 1995; St-Arnaud et al., 1995; Desai et al., 1996) but not by the peroxisome proliferator-activated receptor-retinoid X receptor heterodimers (Winrow et al., 1995). All of these experiments were carried out using the ER form of calreticulin, further supporting the conclusion of the present work.

How can cells respond to signals from the lumen of the ER? For example, cells respond to ER stress by inducing novel gene expression (McMillan et al., 1994). Consequently, a signal must be transduced from the lumen of ER to the nucleus, to the plasma membrane, and to the cytoplasm to activate a specific response (adhesion, gene expression, or ion fluxes). Very little is known about "ER signaling" in mammalian cells. This pathway has been studied extensively in yeast (McMillan et al., 1994). An ER-resident protein kinase Ire1-1/Ern1-1 was identified in yeast whose kinase activity is essential for ER-nuclear translocation (Cox et al., 1993; Mori et al., 1993). However, there is no evidence that these kinases function in the mammalian cells (Cao et al., 1995). Cellular cholesterol homeostasis is controlled by ER-nuclear signaling via sterol-regulated proteolysis of ER membrane-bound transcription factors called sterol regulatory element-binding proteins (Wang et al., 1994). In sterol-deprived cells, a protease cleaves sterol regulatory element-binding protein, which is an integral ER membrane protein, and releases an N-terminal fragment of the protein that enters the nucleus and acts as a transcription factor that activates the genes for the low density lipoprotein receptor (Sato et al., 1994; Wang et al., 1994). Sterol regulatory element-binding proteins are also cleaved by CPP32 protease during programmed cell death (apoptosis) (Wang et al., 1996).

Recently, a new ER-nucleus pathway has been described involving NF-κB (Pahl and Baeuerle, 1995; Pahl et al., 1996). Accumulation of proteins in the lumen of ER induces NF-κB DNA binding and NF-κB-dependent gene expression (Pahl and Baeuerle, 1995; Pahl et al., 1996). If calreticulin activates NF-κB, the protein may affect several aspects of cell function via NF-κB-dependent activation of expression of a large number of genes including interferons, cytokines, cell adhesion molecules, and growth factors (Baeuerle and Henkel, 1994). Similar to calreticulin, gene expression is also regulated by ERp61, an ER protein that belongs to a family of protein disulfide isomerase proteins (Johnson et al., 1992). In leukemia cells from patients with chronic myelogenous leukemia, ERp61 has been demonstrated to alter complex formation between nuclear proteins and regulatory regions of interferon-inducible genes (Johnson et al., 1992). It is not clear at present if calreticulin is involved in the activation of any of these pathways.

Ca²⁺ may play an important role in many calreticulin-dependent cellular functions. Calreticulin is an ER Ca²⁺-binding protein, and overexpression of the protein results in increased Ca²⁺ concentration in the thapsigargin-sensitive intra- and extracellular Ca²⁺ stores (ER) (Bastianutto et al., 1995; Mery et al., 1996). The protein might also alter Ca²⁺ concentrations not only in the lumen of ER but also in the cytoplasm (Camacho and Lechleiter, 1995). Depletion of Ca²⁺ from the lumen of the ER (Greber and Gerace, 1995) or nuclear envelope (Stehno-Bittler et al., 1995), as well as modulation of the cytoplasmic Ca²⁺ concentrations (Macaulay and Farbes, 1996), regulates transport of molecules into the nucleus. Therefore, calreticulin-dependent Ca²⁺ fluctuations either in the luminal ER/nuclear envelope or in the cytoplasm may also affect steroid receptor function and/or nuclear compartmentalization.

Calreticulin, from the lumen of the ER, may modulate other cellular processes. Bastianutto et al. (1995) and Mery et al. (1996) showed that overexpression of calreticulin in the lumen of the ER results in inhibition of the store-operated Ca²⁺ influx via plasma membrane Ca²⁺ release-activated channel (CRAC) (Hoth and Penner (1992); reviewed in Bennett et al. (1995) and Berridge (1995)). Entry of Ca²⁺ through the CRAC is increased when the internal ER stores are drained of their Ca²⁺ content (Putney, 1986; Hoth and Penner, 1992). Calreticulin may modulate CRAC function from the lumen of the ER by a similar mechanism to the inhibition of steroid-sensitive gene expression. Calreticulin also contributes to the regulation of cell adhesiveness (Leung-Hagesteijn et al., 1994; Coppolino et al., 1995). It will be important to test the effects of the cytoplasmic versus the ER form of calreticulin on cell adhesiveness and Ca²⁺ influx to establish if calreticulin may also affect these processes indirectly from the lumen of the ER.

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