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Functional Analysis of Calreticulin

by

Kimberly Burns 

A Thesis

Submitted to the Faculty of Graduate Studies and Research in partial fulfillment

of the requirements for the degree of Doctor of Philosophy

Department of Biochemistry

Edmonton, Alberta

Fall, 1994

ISBN 0-315-95156-7

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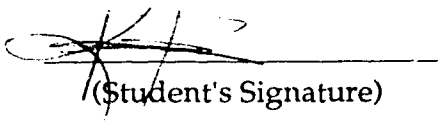

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ABSTRACT

Calreticulin is a ubiquitous protein that developed early in ontogeny and has been highly conserved throughout evolution. This suggests that the protein plays an important, yet unknown, biological role(s).

As part of this study, the expression of calreticulin was examined in resting and ConA-stimulated mouse and human T-lymphocytes. Resting lymphocytes contain very low levels of calreticulin mRNA and protein. Significant increases in both the protein and mRNA levels were observed in Con A stimulated mouse and human splenocytes. Calreticulin was also found to localize almost exclusively to the cytolytic granules in activated T-cells. Based on these studies it is possible that calreticulin is involved in the transduction of Ca^{2+}-dependent processes, in regulating intracellular Ca^{2+} concentrations, and/or in cytolytic activity in T-lymphocytes.

In a second part of this study, the ability of [^{125}I]calreticulin to bind to membrane fractions isolated from different muscle and non-muscle tissues was examined by a protein overlay technique. Specific [^{125}I]calreticulin binding proteins were detected in rat liver smooth and rough endoplasmic reticulum and Golgi, in canine pancreatic microsomes, and in rabbit skeletal muscle sarcoplasmic reticulum. [^{125}I]calreticulin binds to a 50-kDa protein and a number of lower molecular weight (20,000-38,000) endoplasmic reticulum membrane proteins and to 30-kDa protein in skeletal muscle sarcoplasmic reticulum. Calreticulin's ability to interact with many ER proteins suggests that it may have a chaperone-type function.

We have also discovered that calreticulin can bind to the DNA binding domain of the glucocorticoid receptor (GR). This interaction is likely mediated through the NH_2-terminus of calreticulin and the (K-x-F-F-K-R) in the DNA binding domain of the GR. Overexpression of calreticulin in mouse L fibroblasts inhibits GR-mediated transcriptional activation of both a glucocorticoid-sensitive reporter gene and the endogenous, glucocorticoid-sensitive gene, cytochrome P450. These results suggest that calreticulin may play an important role in gene transcription by modulating the activity of the glucocorticoid receptor.

Taken together these results suggest that calreticulin may be a multipurpose protein that subserves several fundamental biological functions.

ACKNOWLEDGEMENTS

There are many people I would like to thank for their help and guidance throughout my studies in the Department of Biochemistry. Firstly, I cannot offer enough thanks to my supervisor, Marek Michalak, for his enthusiasm, encouragement, support and guidance. I would also like to thank the members of my supervisory committee, Chris Bleackley and Bernard Lemire for their helpful comments and advice. Thanks is also extended to the members of the membrane discussion group who offered helpful comments at the Thursday morning lab meetings.

I would like to acknowledge the financial support of the Alberta Heritage Foundation for Medical Research and the University of Alberta for the Province of Alberta and the University of Alberta Dissertation Fellowships

I would also like to thank the following people for their friendship and support: Carrie Shemanko and Rachel Milner who were always willing to provide a great distraction from work, especially with Tequila; Cheryl Helgason who was a great camping companion and also for collaborative work; Cindy Miller (also a great camping companion) for helping me to get my PCR reactions working; Piers Nash for doing numerous minipreps and helping to make the calreticulin expression vector; Shairz Baksh for his 'magnetism' which made the lab an exciting place to be and also for calreticulin samples; Nasarin Mesaeli who helped me prepare picture layouts for my thesis and Thidi Vasser who provided me with an organized alternative. Many thanks to Jody Busaan who provided excellent technical assistance and who was always willing to assist in any way.

My family deserves special acknowledgment for their continued interest concern, support and encouragement throughout the years. Special thanks to my husband, Eric Atkinson, who not only generously provided me with a wealth of information, ideas and opinions but also spent many late nights helping with experiments, formatting my candidacy, with various presentations (i.e. my final 671) etc.

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ATP	adenosine triphosphate
BiP	immunoglobulin heavy-chain binding protein
bp	base pairs
BSA	bovine serum albumin
CCP	cytotoxic cell proteinase
cDNA	complementary deoxyribonucleic acid
CHAPS	(3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate
CNBr	cyanogen bromide
Con A	concanavalin A
CTL	cytotoxic T lymphocyte
CYP3A1	Cytochrome P-450
DBD	DNA binding domain
DEAE	diethylaminoethyl
DMSO	dimethyl sulfoxide
DNA	deoxynucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
EGTA	ethylene glycol bis(B-aminoethyl ether))N, N, N', N'-tetraacetic acid
ER	endoplasmic reticulum
ERp	endoplasmic reticulum protein
FCS	fetal calf serum
FF	formamide/formaldehyde
FLPC	fast protein liquid chromatography
G418	geneticin
G3PDH	glyceraldehyde 3-phosphate dehydrogenase
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GRP	glucose-regulated protein
GST	Glutathione S-tranferase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hIL	human interleukin
HPLC	high performance liquid chromatography
HRE	hormone response element

Hsp	heat-shock protein
IgG	immunoglobulin G
$InsP_3$	inositol 1,4, 5-trisphosphate
IODO-GEN	1, 3, 4, 6-tetrachloro-3a, 6a-diphenylglycouril
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobase
K_d	ligand concentration that gives half maximal binding
kDa	kilodalton
LB	Luria-Bertani
MCS	multiple cloning site
MLR	mixed lymphocyte reaction
MMTV	mouse mammary tumor virus
MOPS	4-morpholinepropanesulfonic acid
M_r	molecular weight
mRNA	messenger ribonucleic acid
PEG	polyethylene glycol 8000
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDI	protein disulfide isomerase
PMSF	phenylmethylsulfonyl difluoride
PVDF	polyvinylidene difluride
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SR	sarcoplasmic reticulum
SV40	simian sarcoma virus 40
TBS	Tris-buffered saline
TE	TRIS-EDTA
UV	ultraviolet

A	adenine
C	cytosine
G	guanine
T	thymine
U	uracil
A	alanine
C	cysteine
D	aspartate

E	glutamate
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
x	any amino acid
Y	tyrosine

CHAPTER ONE

INTRODUCTION

INTRODUCTION

Calcium ions (Ca^{2+}) play a vital role as second messengers in a wide variety of cellular processes (Carafoli, 1987). This role requires that strict Ca^{2+} homeostasis be maintained for normal cellular function. Considering that Ca^{2+} is concentrated outside the cell in the millimolar range and the cytoplasmic free Ca^{2+} concentration is about 10^{-7} M, sophisticated mechanisms are required for ensuring that intracellular Ca^{2+}concentrations are maintained at accepatable levels. This ability to buffer against excessive changes in cellular Ca^{2+} is achieved by a system of Ca^{2+} transport and storage pathways which include Ca^{2+} buffering proteins in the cytosol and in the lumen of the intracellular storage compartments.

The sarcoplasmic reticulum (SR) is one of the best understood Ca^{2+} storage compartments. This membrane has been extensively studied at the molecular level and many of the proteins involved in Ca^{2+} uptake, storage, and release have been identified and their genes cloned (Fleischer & Inui, 1989; Lytton & MacLennan, 1992). In non-muscle cells, the endoplasmic reticulum (ER) is considered to be the major Ca^{2+} storage site within the cell (Koch, 1990; Meldolesi, 1990). The Ca^{2+} sequestered within the ER is ultilized to generate local Ca^{2+} gradients that serve to stimulate a number of cellular processes. In non-muscle cells the predominant mechanism for release of stored Ca^{2+} is triggerred by a diffusible messenger, inositol 1, 4, 5-trisphosphate ($InsP_3$) (Berridge, 1993). $InsP_3$ binds to an integral ER membrane protein, the $InsP_3$-receptor, which functions as a ligand-activated Ca^{2+} release channel (Berridge, 1993). Ca^{2+} is returned to the stores by the action of a Ca^{2+}-ATPase (Pozzan *et al.*, 1992). Within the lumen of the ER, Ca^{2+} is sequestered at special high capacity, low affinity Ca^{2+} binding (storage) sites (Meldolesi, 1990; Milner *et al.*,

1992). This sequestration is important since it reduces the Ca^{2+} concentration gradient against which the ATPase must work.

The movement of Ca^{2+} into and out of the ER is regulated by a group of proteins which are analogous to the proteins of the muscle SR (see review by Cala *et al.*, 1990 & Milner *et al.*, 1992). In skeletal and cardiac muscle, calsequestrin has been established to be the major Ca^{2+} storage site in the lumen of the SR, however it is not present in non-muscle cells (MacLennan *et al.*, 1983; Cala *et al.*, 1990; Lytton & MacLennan, 1992). In contrast, in non-muscle cells, a number of lumenal ER proteins have been demonstrated to bind Ca^{2+}, but their roles in buffering ER lumenal Ca^{2+} are not clearly defined (Koch *et al.*, 1986, 1989; Macer & Koch, 1988; Nuygen Van *et al.*, 1989; Nigam & Towers, 1990; Milner *et al.*, 1991; Ozawa & Muramatsu, 1993). These proteins include calreticulin, protein disulfide isomerase (PDI), ER proteins ERp72 and ERp61, immunoglobulin binding protein (BiP), glucose regulated protein (GRP94) and reticulocalbin (alternative names of these proteins are listed in Table 1-1). Koch *et al.* (1988) determined that these proteins (BiP, GRP94, calreticulin and PDI) referred to as reticuloplasmins, accounted for a major proportion of the Ca^{2+} binding capacity (i.e. 300 nmol of Ca^{2+}/mg of protein) of the ER. The Ca^{2+} binding capabilities of GRP94 (10 mol of Ca^{2+}/mol) and calreticulin (25 mol of Ca^{2+}/mol) have been specifically examined (MacLennan *et al.*, 1972; Macer & Koch, 1988; Treves *et al.*, 1990; Baksh & Michalak, 1991). Both proteins are likely to contribute significantly to Ca^{2+} storage within the ER (Michalak *et al.*, 1992; Koch, 1990). The Ca^{2+} binding protein, calreticulin, shares certain similarites with calsequestrin (Michalak *et al.*, 1992; Milner *et al.*, 1992; Lytton & MacLennan, 1992). These are emphasized by the fact that many antibodies made against calsequestrin cross-react with calreticulin, despite that overall they share less than 10% amino acid identity. These observations together with calreticulin's

Table 1-1. Names of selected lumenal ER proteins*

Protein	Other names	References
BiP	GRP78	[1- 3]
GRP94	GP100/endoplasmin ERp99 hsp108	[4-7]
ERp72	CaBP2	[8-9]
ERp61	p5 phosphatidylinositol-specific phospholipase C GRP58	[1, 9-12]
PDI	ERp59 Prolyl 4-hydroxylase β-subunit T3BP GSBP	[13-17]
reticulocalbin	p46?	[18]
Calreticulin	see table 1.2	[19]

* Lumenal ER proteins that bind calcium and/or have putative chaperone-type roles.

References: 1. Lee *et al.*, 1981; 2. Haas & Wabl, 1983; 3. Munro & Pelham, 1986; 4. Mazzarella & Green 1987; 5. Koch *et al.*, 1985; 6. Lee *et al.*, 1984; 7. Koch *et al.*, 1985 & 1986; 8. Van *et al.*, 1993; 9. Lewis *et al.* 1986; 10. Mazzarella *et al.*, 1990; 11. Chaudhuri *et al.* 1990; 12. Bennett *et al.* 1988; 13. Edman *et al.*, 1985; 14. Srinivasan *et al.*, 1988; 15. Pihlajaniemi *et al.*, 1987; 16. Yamauchi *et al.*, 1987; 17. Geetha-Habib *et al.*, 1988; 18. Ozawa & Muramatsu, 1993; 19. Michalak *et al.*, 1992.

ability to bind Ca^{2+} with high capacity led to the suggestion that calreticulin might function as an analogue of calsequestrin in the ER (Koch, 1990; Milner *et al.*, 1992, for review see Michalak *et al.*, 1992). In view of the important role that Ca^{2+} plays in many cellular processes, studies were initiated to analyze the function of calreticulin.

CALRETICULIN

Calreticulin *alias* ------

Calreticulin was first identified in 1972 by MacLennan's group as the high-affinity Ca^{2+} binding protein (HACBP) in the SR of skeletal muscle (MacLennan *et al.*, 1972; Ostwald & MacLennan, 1974). However, when the amino acid sequences of murine and rabbit calreticulin became available in 1989 (Fliegel *et al.*, 1989; Smith & Koch, 1989) it was revealed that it had been concurrently studied in a number of other laboratories and described using a variety of different names (Fig 1-2). Other laboratories studying ER and Ca^{2+} binding proteins had identified calreticulin as CAB-63/calregulin, (Waisman *et al.*, 1985), CRP55 (Macer & Koch, 1988), ERp60 (Lewis *et al.*, 1985), CaBP3 (Peter *et al.*, 1992) and as a calsequestrin-like protein (Volpe *et al.*, 1988; Damiani *et al.*,1988). In an effort to eliminate any confusion regarding the protein's identity, in 1989 the name calreticulin was chosen to reflect both its ability to bind Ca^{2+} (Cal) and its intracellular localization to the endoplasmic and sarcoplasmic reticulum (reticulin).

A number of investigators studying rather diverse areas of cell biology have identified additional proteins that are either identical or highly similar to calreticulin based on NH_2-terminal amino acid sequence analysis (Table 1-2). McCauliffe *et al.* (1990) identified a 60-kDa protein which they proposed to be a

TABLE 1-2. Calreticulin Synonyms.

Name	# of identical N-terminal amino acid residues	Source	Identified as a:
HACBP	15 out of 15	Rabbit skeletal muscle	Ca^{2+}-binding protein
CAB-63 Calregulin	15 out of 15	Bovine liver	Ca^{2+}-binding protein
CaBP3	12 out of 15	Rat liver	Ca^{2+}-binding protein
CRP55	12 out of 15	Mouse plasmacytoma cells	Ca^{2+}-binding protein
ERp60	11 out of 15	Mouse plasmacytoma/ MOPC-315	ER resident protein
Calsequestrin-like	not determined	Rat/Human liver	Ca^{2+}-binding protein
p425	8 out of 11	Rat fibroblast	Ca^{2+}-binding protein
Ro/SS-A	14 out of 15	Human Wil-2 cell line	SLE autoantigen
C1qR	14 out of 15	Human tonsil lymphocytes	C1q-binding protein
B50	10 out of 13	Mouse melanoma cells	Melanoma antigen
Mobilferrin	11 out of 15	rat duodenal nucosa	Fe^{3+}-binding protein
RAL-1	not determined	*Onchocerca vulvulus*	Autoantigen
p407	7 out of 13	*Aplysia californica* snail	"memory molecule"

component of the Ro/SS-A autoantigen, a target of autoantibody production in systemic lupus erythematosus and Sjögren's syndrome (Lieu *et al.*, 1988). A cDNA clone for this protein, however, encodes for the human homologue of calreticulin (McCauliffe *et al.*, 1990a). Recently a 56-67-kDa plasma membrane protein was identified which binds to C1, a subcomponent of complement, and referred to as the C1q receptor (C1qR) (Malhotra *et al.*, 1993). The NH_2-terminal amino acid sequence of this receptor is identical to that of calreticulin (Table 1-1). Another group has identified a 55-kDa protein, named mobilferrin, which binds iron in the mucosa of the small intestine (Conrad *et al.*, 1990). Again this protein's NH_2-terminal amino acid sequence was found to be identical to that of calreticulin (Conrad *et al.*, 1993). Gersten *et al.* (1991) found that mice immunized with irradiated melanoma cells produce antibodies which recognize a protein designated B50. The NH_2-terminal amino acid sequence of B50 is also similar (10 out of 13 residues) to calreticulin (Gersten *et al.*, 1991). Calreticulin is, therefore, a protein with many names and, as discussed in subsequent sections, also appears to be a protein of many 'disguises'.

Characteristics of Calreticulin

General properties

The observed physicochemical properties of calreticulin are summarized in Table 1-3. Rabbit skeletal muscle calreticulin has an estimated molecular weight of 46,567 based on its deduced amino acid sequence (Fliegel *et al.*, 1989a). However, when analyzed by SDS-PAGE (Laemmli system) it migrates with an apparent molecular weight of 55,000-63,000 (Waisman *et al.*, 1985; McCauliffe *et al.*, 1990; Milner *et al.*, 1991). The molecular weight of calreticulin estimated by SDS-PAGE at neutral pH is 55,000 (Ostwald and MacLennan, 1974; Michalak *et*

Table 1-3. CHARACTERISTICS OF CALRETICULIN

MOLECULAR WEIGHT			
deduced from the nucleotide sequence		46,567	[1-6]
SDS-PAGE (Laemmli system)		60,000	
Sedimentation equilibrium		55,000	
ISOELECTRIC POINT		~ 4.65	[3, 4]
GLYCOSYLATION		+/-	[4, 7]
ION-BINDING			
Ca^{2+}-binding			
high-affinity site	K_d	1.6-11 μM	
(low capacity)	B_{max}	1 mol/mol of protein	[4, 5,
low-affinity site	K_d	0.3-2 mM	8-12]
(high capacity)	B_{max}	20-50 mol/mol of protein	
Zn^{2+}-binding	K_d	300 μM	[12]
	B_{max}	14 mol/mol of protein	
Fe^{3+}-binding	K_d	90 μM	[13]
	B_{max}	1 mol/mol of protein	
RNA-BINDING	+		[14]
KINASE ACTIVITY	+/-		[14]
PHOSPHORYLATION	+/- (Unknown kinase)		[14]

References: 1, Fliegel *et al.* (1989a); 2, Smith and Koch (1989); 3, McCauliffe *et al.* (1990a); 4, Waisman *et al.* (1985) 5, Treves *et al.* (1990); 6, Milner *et al.* (1991); ; 7, Van *et al.* (1989); 8, Baksh & Michalak (1991) 9, MacLennan *et al.* (1972); 10, Ostwald & MacLennan (1974) 11, Macer & Koch, (1988); 12, Khanna *et al.* (1986) 13, Conrad *et al.* (1993); 14, Sing *et al.* (1993).

al., 1980) which is similar to the value determined by sedimentation equilibrium also at neutral pH (Waisman *et al.*, 1985). Discrepancies between the predicted and the observed mobilities on SDS-PAGE are thought to be due to the acidic nature of calreticulin as similar anomolous migration has been reported for other acidic proteins (MacLennan *et al.*, 1983). In fact, calreticulin contains 109 acidic amino acids residues. Its acidic nature is reflected in its isoelectric point which is calculated to be 4.14 (Fliegel *et al.*, 1989a; McCauliffe *et al.*, 1990a) but experimentally determined to be 4.65-4.67 (Waisman *et al.*, 1985; McCauliffe *et al.*, 1990a). Based on sedimentation equilibrium analysis calreticulin is reported to be a monomer (Waisman *et al.*, 1985). However, detergent-solubilized C1qR (calreticulin?) behaves as an elongated dimer with a molecular mass of 115,000 ± 7000 (Malhotra *et al.*, 1993). Recent high resolution STEM (scanning tunneling electron microscopy) analysis has also suggested that calreticulin may aggregate to form dimers and/or tetramers (D. Andrews, S. Baksh, and M. Michalak, unpublished observations).

Ion binding

It is well documented that calreticulin binds Ca^{2+} (MacLennan *et al.*, 1972; Ostwald & MacLennan, 1974; Waisman *et al.*, 1985; Van *et al.*, 1989; Treves *et al.*, 1990; Milner *et al.*, 1991; Michalak *et al.*, 1991; Baksh & Michalak, 1991) (Table 1-3). Waisman *et al.* (1987) and Van *et al.* (1989) reported only high affinity Ca^{2+} binding to calreticulin, whereas Macer and Koch (1988) and Treves *et al.* (1990) showed only low affinity Ca^{2+} binding. These reports contrast the observations of Ostwald and MacLennan (1974) and Baksh & Michalak (1991) who demonstrated that calreticulin contains both high affinity/low capacity and low affinity/high capacity Ca^{2+} binding sites. The discrepancies in the reported number of Ca^{2+} binding sites are most likely a result of the different methods

used by the investigators to measure Ca^{2+} binding. Macer and Koch (1988) and Treves *et al.* (1990) studied Ca^{2+} binding to calreticulin only at relatively high concentrations of Ca^{2+}, and consequently did not detect any high affinity Ca^{2+} binding. Waisman *et al.* (1987) and Van *et al.* (1989) studied Ca^{2+} binding to calreticulin in the presence of millimolar concentrations of Mg^{2+} which has subsequenctly been reported to reduce Ca^{2+} binding to the low affinity sites by 60% (Baksh & Michalak, 1992). Calreticulin, therefore, also binds Mg^{2+} *in vitro*.

Thirty-seven of the last 52 amino acids of calreticulin are glutamic or aspartic acid residues. This cluster of acidic residues in the carboxyl-terminus of calreticulin has been demonstrated to be involved in its high capacity low affinity Ca^{2+} binding (Baksh and Michalak, 1992). Similar stretches of acidic residues have been implicated in Ca^{2+} binding to calsequestrin (Ohnishi and Reithmeier, 1987). Although calreticulin binds 1 mole of Ca^{2+}/mole of protein (K_d 6-11 μM) with high affinity, it does not contain the EF hand consensus sequence found in other high affinity Ca^{2+} binding proteins (Kretsinger *et al.*, 1988). The region from amino acids 187-273 is, however, predicted to form a helix-loop-helix motif similar to that of the EF-hand (S. Baksh, R. Hodges, M. Michalak, unpublished observations).

Calreticulin also binds 14 moles of Zn^{2+}/mole of protein, with relatively low affinity (~300 μM) (Khanna *et al.*, 1986 & 1987). In contrast to Ca^{2+} binding, Zn^{2+} binding induces dramatic conformational changes in calreticulin which result in increased hydrophobicity of the protein (Ostwald *et al.*, 1974; Khanna *et al.*, 1986 & 1987; Van *et al.*, 1989; Baksh *et al.*, 1992). Since calreticulin does not have a "Zn^{2+}-finger" consensus amino acid sequence the location of Zn^{2+} binding to the protein is not clear at present. The NH_2-terminus of the protein is, however, enriched in residues (Cys and His) that are implicated in Zn^{2+} binding to other proteins (Berg, 1990).

Calreticulin/mobilferrin is an iron binding protein (Conrad *et al.*, 1991). Mobilferrin binds 1 mol of iron/mol of protein with relatively high affinity (K_d 9 $x10^{-5}$) (Conrad *et al.*, 1990). In addition to iron, mobilferrin binds other metals (zinc > cobalt > copper > lead) but, with lower affinity than iron. Conrad *et al.* (1993) compared the ability of purified preparations of calreticulin and mobilferrin to bind iron and Ca^{2+}. They found that both proteins bound iron with greater affinity than Ca^{2+}, but that Ca^{2+} at high concentrations could inhibit binding of radioactive iron to mobilferrin and calreticulin (Conrad *et al.*, 1993).

Glycoslation

All mammalian calreticulins have a potential glycosylation site at residue #326. Despite this, glycosylation appears to be tissue and species specific (Michalak *et al.*, 1992). For example, chicken and rabbit liver calreticulin apparently contain no carbohydrate, while bovine liver calreticulin has been shown to be glycosylated (Waisman *et al.*, 1985; Matsuoka *et al.*, 1994). The rat liver protein is also glycosylated, but it is reported to have a complex hybrid type of oligosaccharide with a terminal galactose residue (Van *et al.*, 1989). This is an unusual type of glycosylation for an ER protein and would predict that calreticulin moves to the trans Golgi before being retrieved to the lumen of the ER. The C1qR in human endothelial cells is also a glycoprotein (15-20% carbohydrate) but has a higher sugar content than other glycosylated calreticulins (Malhotra *et al.*, 1993). A carbohydrate moiety has not been detected on skeletal or smooth muscle, human, murine, chicken liver, rabbit pancreas or liver calreticulin (Milner *et al.*, 1991; Fliegel *et al.*, 1989a). Further studies are required to establish why mammalian calreticulins are not universally glycosylated. Interestingly, nematode calreticulins do not have a consensus

amino acid sequence for N-linked glycosylation (Unnasch *et al.*, 1988; Khalife *et al.*, 1993).

RNA binding

In infected kidney cells, calreticulin has recently been found to bind to the 3' cis acting element (3' (+) SL) of Rubella virus RNA as an essential component of the virus replication complex (Nakhasi *et al.*, 1990; Singh *et al.*, 1993; Pogue *et al.*, 1993). Calreticulin binds via its amino terminus (residues 1-19) to a stem-loop structure present on the 3' end of rubella viral genomic RNA (Singh *et al.*, 1994). Although, calreticulin does not have the conventional ribonucleoprotein (RNP) binding motif found in many nuclear and cytoplasmic RNA binding proteins, residues 3-6 of calreticulin (A-V-Y-F) are present in the RNA recognition motif of human splicing factor ASF/SF2 (Singh *et al.*, 1994).

Phosphorylation/kinase activity

Calreticulin has a number of putative recognition sequences for phosphorylation by protein kinase C (clustered at the NH_2-terminus of the protein; residues 17-19, 36-38, 61-63, 68-70, 79-81 and 124-126) casein kinase II (residues 51-54, 172-175, 178-181, 196-200, 204-408, 307-311 and 316-319) and tyrosine kinase (residues 261-268). However, attempts to phosphorylate purified protein by these kinases have not been successful (Michalak *et al.*, 1992). Despite this, Singh *et al.*(1994) have demonstrated that simian calreticulin is a phosphoprotein. Phosphorylation of calreticulin was found to be essential for an interaction with Rubella virus RNA and the degree of phosphorylation increased with viral infection without a concomitant change in the protein levels.

Singh *et al.* (1994) also demonstrated that simian calreticulin is autophosphorylated, possibly at Thr^{17}. Both simian and human calreticulin, but

not other mammalian calreticulins, have sequence elements at the NH_2-terminus which show significant similarity with the catalytic domain of protein kinases (Singh *et al.*, 1994; Hanks *et al.*, 1988). Human calreticulin has not yet been tested for kinase activity. Other calreticulins also have an amino acid sequence that is similar to the active site of protein kinase C (residues 215-224) (Hanks *et al.*, 1988), but Waisman *et al.* (1985) were unable to detect any kinase activity associated with purified bovine liver calreticulin.

The Amino Acid Sequence of Calreticulin

The amino acid sequence

The amino acid sequence of rabbit skeletal muscle calreticulin is shown in Fig. 1-1. Calreticulin is made as a 418 amino acid precursor from which its 17 amino acid NH_2-terminal signal peptide is cleaved upon translocation into the lumen of the ER (Fliegel *et al*, 1989a; Rokeach *et al.*, 1991). Its hydrophobic NH_2-terminal signal sequence is somewhat atypical in that it lacks a basic residue near its NH_2-terminus (Fig. 1-1). There are no long hydrophobic segments capable of spanning the membrane bilayer (Fliegel *et al.*, 1989a). Secondary structure predictions suggest that calreticulin can be divided into three domains referred to as the N-, P- and C-domains (Fliegel *et al.*, 1989a; Smith & Koch, 1989) (Fig. 1-1). These domains have been schematically represented in Figure 1-2. The N-domain encompasses the first half of the molecule (residues 1- 200) and is predicted to form a globular domain of 8 anti-parallel β-strands with a helix-turn-helix motif at the extreme NH_2-terminus. The next one-third of the sequence is proline-rich (amino acid 187 -317) and is referred to as the P-domain. A portion of the P-domain from residues 266-295 contains a number of proline residues spaced every four or five amino acids. Within this region there is a

Fig. 1-1. The amino acid sequence of rabbit skeletal muscle calreticulin.

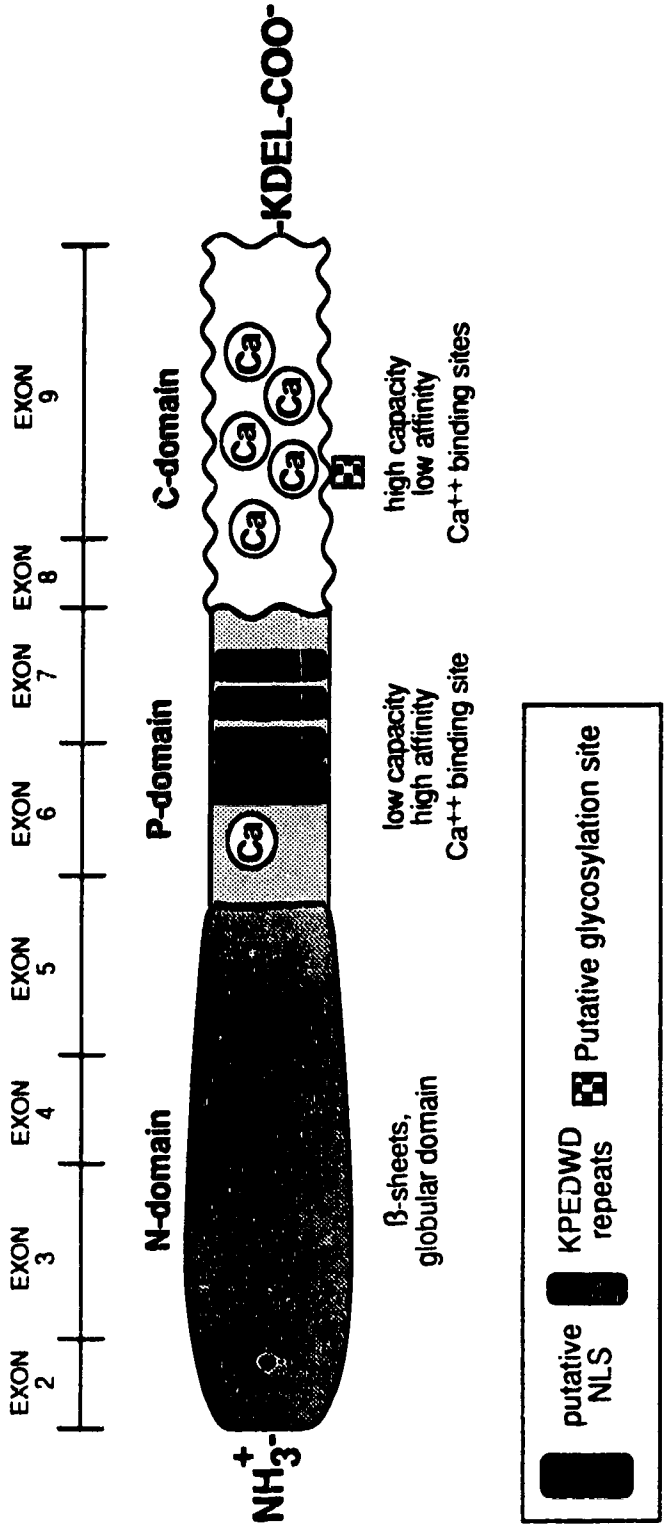
The amino acid sequence has been deduced from the nucleotide sequence of the rabbit skeletal muscle clone (Fliegel *et al.*, 1989a). Mature calreticulin contains 401 amino acids. It is synthesized as a precursor protein with a 17 amino acid ER signal sequence. Based on secondary structure predictions calreticulin can be divided into three distinct structural regions: the N-domain: the P-domain and the C-domain. Within the N-domain there is a putative nuclear localization signal (shaded box) and the sequence KPEDWD (boxed) is repeated three times. The acidic residues in the C-domain are underlined. The asterisk indicates a possible glycosylation site. Calreticulin terminates with the KDEL ER retention tetrapeptide.

-17 signal sequence
MLLPVPLLLGLLGLAAA

```
        10         20         30         40         50         60         70         80
EPVVYFKEQF LDGDGWTERW IESKHKSDFG KFVLSSGKFY GDQEKDKGLQ TSQDARFYAL SARFEPFSNK GQPLVVQFTV
                                                N-domain

        90        100        110        120        130        140        150        160
KHEQNIDCGG GYVKLFPAGL DQKDMHGDSE YNIMFGPDIC GPGTKKVHVI FNYKGKNVLI NKDIRCKDDE FTHLYTLIVR
                                                N-domain

       170        180        190        200        210        220        230        240
PDNTYEVKID NSQVESGSLE DDWDFLPPKK IKDPDASKPE DWDERAKIDD PTDSKPEDWD KPEHIPDPDA KKPEDWDEEM
      N-domain                                          P-domain

       250        260        270        280        290        300        310        320
DGEWEPPVIQ NPEYKGEWKP RQIDNPDYKG TWIHPEIDNP EYSPDANIYA YDSFAVLGLD LWQVKSGTIF DNFLITNDEA
                          P-domain                                C-domain

     * 330        340        350        360        370        380        390        401
YAEEFGNETW GVTKTAEKQM KDKQDEEQRL KEEEEEKKRK EEEEAEEDEE DKDDKEDEDE DEEDKDEEEE EAAAGQAKDEL
                                                C-domain
```

Fig. 1-2. Model of calreticulin domains.

This is a schematic illustration representing the various domains of calreticulin The localization of KPEDWD repeats, a putative nuclear localization signal (NLS), a putative glycosylation site and the Ca^{2+} binding sites identified by Baksh and Michalak (1992) are indicated. The exon-intron boundaries of the human calreticulin gene (isolated by McCauliffe *et al.*, 1992) are shown. Exon 1, encoding the signal sequence of calreticulin, is not shown.


EXON 2
EXON 3
EXON 4
EXON 5
EXON 6
EXON 7
EXON 8
EXON 9
N-domain
P-domain
C-domain
KDEL-COO-
NH3+
Ca
β-sheets, globular domain
low capacity high affinity Ca++ binding site
high capacity low affinity Ca++ binding sites
putative NLS
KPEDWD repeats
Putative glycosylation site

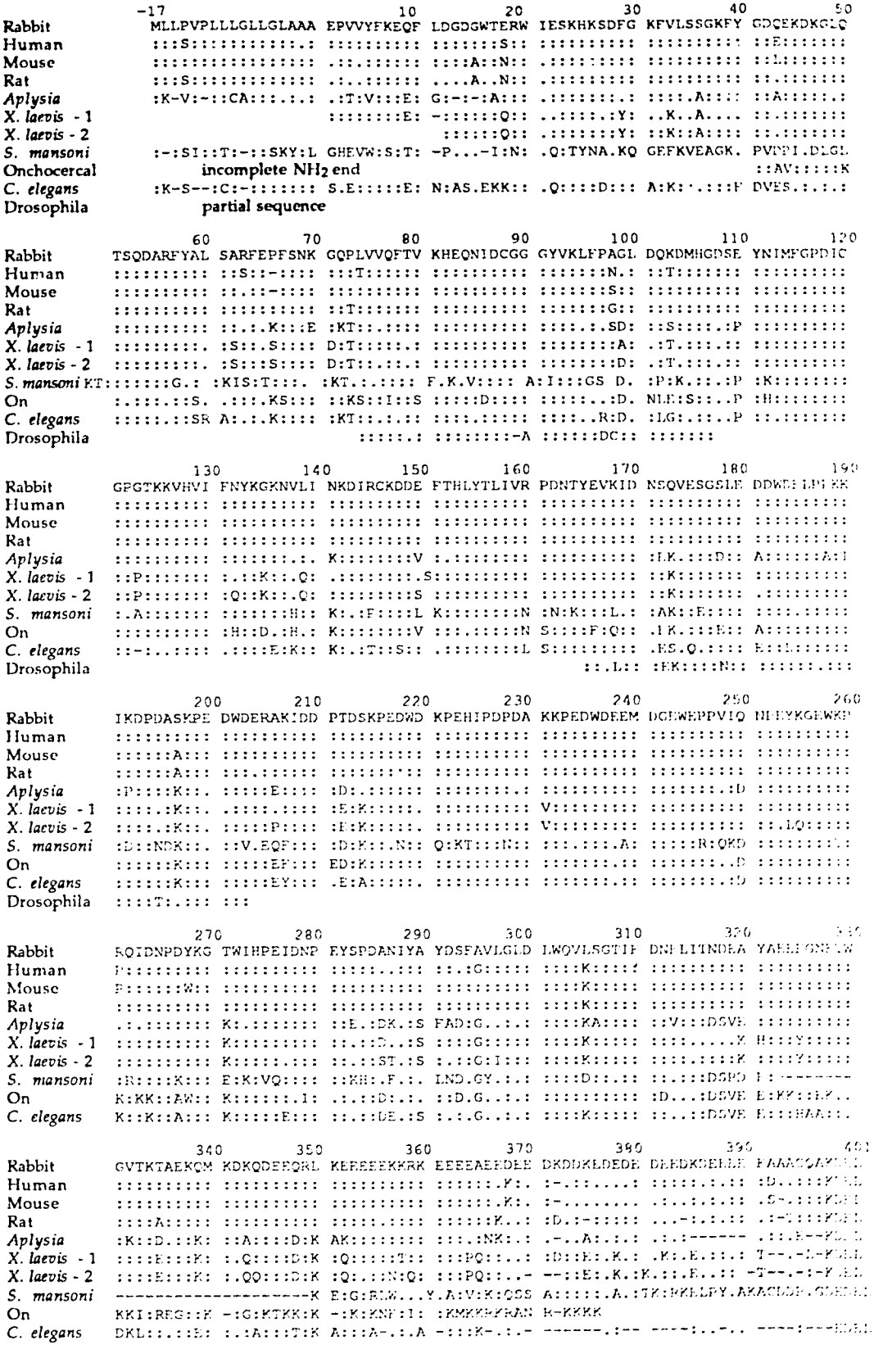
putative nuclear localization signal (P-P-K-K-I-K-P-D-P; residues 187-195) (Michalak *et al.*, 1992). Another interesting feature of this proline rich region is the amino acid sequence K-P-E-D-W-D which is repeated three times (Fig. 1-1, 1-2). It has been suggested that this region might contain a repeating, rigid turn structure which separates the globular head of the protein from the acidic C-domain (residues 310-401). The C-domain may exist in an extended conformation due to long stretches of acidic residues interdispersed with basic residues and terminates with the K-D-E-L ER retention tetrapeptide.

Amino acid sequence conservation

Full length amino acid sequences have now been deduced from the cDNAs for numerous calreticulins including human, mouse, rabbit, rat, *Xenopus, Aplysia, Drosophila, C. elegans,* and *S. manosi* (Fliegel *et al.*, 1989; Smith & Koch, 1989; McCauliffe *et al.*, 1990a; Murthy *et al.*, 1990; Rokeach *et al.*, 1991; Kennedy *et al.*, 1992; Mazzarella *et al.*, 1992; Smith, 1992; Treves *et al.*, 1992; Waterston *et al.*, 1992; Nakamura *et al.*, 1993; Liu *et al.*, 1993; Hawn *et al.*, 1993). As well a full-length cDNA for the *Onchocerca vulvulus* homologue of calreticulin, RAL-1, has been obtained (Unnasch *et al.*, 1988). All calreticulin cDNAs cloned so far have hydrophobic NH_2-terminal signal sequences and except RAL-1 terminate with the K-D-E-L/H-D-E-L ER retention signal. In Figure 1-3, calreticulin sequences have been listed to illustrate the conserved nature of this molecule from mammals to non-vertebrates including insects and parasites. Rabbit, mouse, human and rat calreticulins, for example, share over 90% amino acid identity. Although there are slight variations in the amino acid sequences of the different calreticulins, several areas of the sequence are invariant (Fig. 1-3). These areas may well be areas of greatest structural and functional importance. The area of least homology is at the carboxyl-terminal. In RAL-1 which shares 64.4% identity

Fig. 1-3. Amino acid sequences of different calreticulins.

Comparison of rabbit, human, mouse, rat, *Aplysia,Xenopus, S. mansoni,* Onchocercal (On), *C. elegans,* and the Drosophila sequences is shown. (:) represent conserved residues and (-) represent a gap in the sequence. All other amino acids are listed. The amino acid sequences are those deduced from the cDNAs encoding the proteins (Fliegel, *et al.,* 1989a: Smith & Koch, 1989; McCauliffe *et al.,* 1990a, b,: Murthy *et al.,* 1990; Unnasch *et al.,* 1988; Treves *et al.,* 1992; Waterson *et al.,* 1992; Khalife *et al.,* 1993).


-17 10 20 30 40 50
Rabbit MLLPVPLLLGLLGLAAA EPVVYFKEQF LDGDGWTERW IESKHKSDFG KFVLSSGKFY GDQEKDKGLQ
Human
Mouse
Rat
Aplysia
X. laevis - 1
X. laevis - 2
S. mansoni
Onchocercal
C. elegans
Drosophila
incomplete NH2 end
partial sequence
60 70 80 90 100 110 120
Rabbit TSQDARFYAL SARFEPFSNK GQPLVVQFTV KHEQNIDCGG GYVKLFPAGL DQKDMHGDSE YNIMFGPDIC
Human
Mouse
Rat
Aplysia
X. laevis - 1
X. laevis - 2
S. mansoni
On
C. elegans
Drosophila
130 140 150 160 170 180 190
Rabbit GPGTKKVHVI FNYKGKNVLI NKDIRCKDDE FTHLYTLIVR PDNTYEVKID NSQVESGSLE DDWDFLPPKK
Human
Mouse
Rat
Aplysia
X. laevis - 1
X. laevis - 2
S. mansoni
On
C. elegans
Drosophila
200 210 220 230 240 250 260
Rabbit IKDPDASKPE DWDERAKIDD PTDSKPEDWD KPEHIPDPDA KKPEDWDEEM DGEWEPPVIQ NPEYKGEWKP
Human
Mouse
Rat
Aplysia
X. laevis - 1
X. laevis - 2
S. mansoni
On
C. elegans
Drosophila
270 280 290 300 310 320 330
Rabbit RQIDNPDYKG TWIHPEIDNP EYSPDANIYA YDSFAVLGLD LWQVLSGTIF DNFLITNDEA YAEEFGNETW
Human
Mouse
Rat
Aplysia
X. laevis - 1
X. laevis - 2
S. mansoni
On
C. elegans
340 350 360 370 380 390 400
Rabbit GVTKTAEKQM KDKQDEEQRL KEEEEEKKRK EEEEAEEDEE DKDDKEDEDE DEEDKDEELE EAAAEGAYEE
Human
Mouse
Rat
Aplysia
X. laevis - 1
X. laevis - 2
S. mansoni
On
C. elegans

with human calreticulin the carboxyl-terminus is also highly charged but is positively, rather than negatively, charged (Unnasch *et al.*, 1988; McCauliffe *et al.*, 1990b). Many of the *Drosophila* residues that differ from the human, mouse, rat and rabbit sequences are identical to the *Onchocerca* residues. The sequence conservation is quite striking considering that mammals diverged phylogenetically approximately 75 million years ago and *Drosophila* and *Onchocerca* diverged over 350 million years ago (Barrett *et al.*, 1986). Calreticulin's development early in ontogeny and its conservation throughout evolution are highly suggestive that it plays an important biological role(s).

Proteins with sequence similarities to calreticulin

The amino acid sequence of calreticulin has limited similarity with the sequences of other ER lumena proteins including PDI, BiP, ERp72, GRP94, and ERp61 (Fliegel, *et al.*, 1989b; Michalak *et al.*, 1992). These proteins all contain relatively large numbers of acidic residues (generally clustered in the carboxyl-terminus), have hydrophobic ER leader peptides and terminate with the C-terminal retention peptide K-D-E-L or the related sequences K-E-E-L (ERp72), Q-E-D-L (ERp61) (Munro & Pelham, 1987; Mazzarella *et al.*, 1990; Haugejorden *et al.*, 1991).

Interestingly, calreticulin and calsequestrin have limited sequence similarity (Fliegel *et al.*, 1987; Michalak *et al.*, 1992). Their general similarity is emphasized by the fact that some antibodies raised against calsequestrin cross-react with calreticulin (Damiani *et al.*, 1988; Volpe *et al.*, 1988; Krause *et al.*, 1990; Treves *et al.*, 1990). This cross-reactivity is thought to occur because they have similar long stretches of acidic residues in their carboxyl-terminal regions although overall these proteins share less than 10% amino acid identity (Fliegel *et al.*, 1987; Scott *et al.*, 1988)

Calreticulin also shares certain regions of sequence similarity, ranging from 42% to 78% identity, with an integral ER membrane protein named calnexin that is believed to function as an ER chaperone (Wada *et al.*, 1991; reviewed in Bergeron *et al.*, 1994). Interestingly, the highly conserved K-P-E-D-W-D sequence found repeated in calreticulin three times is repeated twice in calnexin. The sequence similarites between these molecules suggests the possiblity that they may also have functional similarities, as discussed in Chapter 3.

Proteins with calreticulin-like amino acid sequences

There is a group of proteins which exhibit an unusual type of homology (identity) to calreticulin for which there is no real precedent (Malhotra *et al.*, 1993). These proteins appear to have blocks of identical sequence to calreticulin and blocks of completely unrelated sequence. In the C1qR, for example, nine out of twelve peptides sequenced were found to have an identical amino acid sequence to calreticulin (Malhotra *et al.*, 1993). Two of the remaining three peptides sequenced were similar to calreticulin sequences, but the third was not present in calreticulin (Malhotra *et al.*, 1993). Although the amino acid composition of the C1qR is considerably similar to that of calreticulin, slight differences have also been identified. Specifically, the cysteine content was found to be slightly higher in the C1qR than in calreticulin (3% compared with 1%).

A second protein which also appears to be related to calreticulin/C1qR was isolated from human spleen (Malhotra *et al.*, 1993). Intriguingly this protein was found to migrate in SDS-PAGE at the same mobility as C1qR, to have an identical NH_2-terminal amino acid sequence, to be recognized by C1qR antibodies and anti Ro/SS-A antibodies but have a different overall charge than C1qR/Ro/SS-A (Malhotra *et al.*, 1993).

Another protein was recently isolated from bovine brain that also exhibits rather unusual amino acid sequence similarity to calreticulin (Johnson *et al.*, 1992). Although the NH_2-terminal amino acid sequence of this protein is different from calreticulin, its pI, amino acid composition and immunological characteristics are very similar to calreticulin (Johnson *et al.*, 1992; Liu *et al.*, 1993). Recently a cDNA clone was obtained from a cDNA library which may encode for this protein (Liu *et al.*, 1993). This clone encodes a protein of 387 amino acids with a potential leader peptide of 34 amino acids and a carboxyl-terminal K-D-E-L tetrapeptide (Liu *et al.*, 1993). Although the deduced amino acid sequence of this protein is highly similar in the carboxyl-terminal 318 amino acids to mouse calreticulin, a block of 69 amino acids in the NH_2-terminus is completely divergent. A 3.75 kb mRNA species was detected on Northern blots probed with a cDNA fragment encoding this divergent region (Liu *et al.*, 1993). A similarly sized transcript, that hybridizes with calreticulin cDNA, has previously been detected by Northern blot analysis of mRNA isolated from fast- and slow-twitch muscles (Fliegel *et al.*, 1989a). However, a cDNA corresponding to this 3.75 kb mRNA species has not yet been isolated. Liu *et al.* (1993) suggest that the 3.75 kb mRNA is the transcript for the bovine brain isoform of calreticulin. However, it is not clear why this mRNA species was only weakly detected on Northern blots probed with a full-length cDNA fragment (i.e. containing both divergent and similar nucleotide sequences to calreticulin). Further work is required to clarify the identity of the 3.75 kb mRNA species.

cDNAs encoding two additional isoforms of calreticulin have been isolated from a *Xenopus laevis* central nervous system library (Treves *et al.*, 1992). The amino acid and nucleotide sequences of these clones are 93% and 89% identical, respectively. The regions of non-identity, however, are not localized to

specific regions but occur throughout these molecules, suggesting that there may be two genes for calreticulin in *Xenopus*.

The Distribution and Cellular Localization of Calreticulin

Widespread tissue and species distribution

Using a radioimmunoassay, Khanna & Waisman (1986) quantitated the amount of calreticulin in various bovine tissues. Calreticulin was found in particularly high concentrations (200-500 μg/g of tissue) in pancreas, liver, and testis. In contrast, kidney, spleen, adrenals and parathyroid had only moderate amounts (100 μg/g of tissue) and the cerebral cortex and muscle tissues had relatively low amounts (~ 20 μg/g of tissue). Calreticulin has now been found in a wide variety of cell types and shown to be present in a number of extremely diverse species extending from mammals to non-vertebrates (Michalak *et al.*, 1992). In fact, calreticulin has been identified in all eukaryotic cells so far studied with the exception of yeast and erythrocytes (Khanna & Waisman, 1986). Recently calreticulin has also been detected in the ER of higher plants (Menegazzi *et al.*, 1993).

ER/SR localization of calreticulin

Calreticulin was orginally purified from the lumen of the SR, although only small quantities of it actually exist there (Ostwald & MacLennan, 1974). Subsequently, calreticulin has been found to be a major lumenal component of the ER (Lewis *et al.*, 1985; Opas *et al.*, 1988; Koch & Macer , 1989). The localization of calreticulin to both ER and SR in smooth muscle, muscle, and non-muscle cells has been demonstrated using a variety of biochemical and immunological techniques (Michalak *et al.*, 1980; Lewis *et al.*, 1985, 1986; Koch & Macer, 1988;

Opas *et al.*, 1988, 1991; Treves *et al.*, 1990; Milner *et al.*, 1991; Tharin *et al.*, 1992). Calreticulin appears to be confined to, or enriched in the rough ER (Lewis *et al.*, 1985; Opas *et al.*, 1991; Peter *et al.*, 1992). The typical distribution (characteristic ER staining) of calreticulin in a fibroblast stained with anti-calreticulin antibodies is shown in Fig. 1-4. That calreticulin is distributed in the ER lumen of these cells is supported by its sequence which contains a NH_2-terminal ER leader peptide and a carboxyl-terminal K-D-E-L ER retention peptide. Proteins with K-D-E-L-like retention tetrapeptides are believed to be maintained within the ER by a K-D-E-L-dependent retrieval process that is mediated by 22-kDa receptors which are localized in a post-ER compartment (Lewis & Pelham, 1990; Lewis & Pelham, 1992a, b). Although these signal peptides would suggest that calreticulins reside exclusively in the ER, calreticulin or calreticulin-like molecules have now been shown to be present outside of the ER.

Acrosomes/granules localization of calreticulin

One location outside the ER that calreticulin has been found is in the acrosome of sperm cells. It was proposed that calreticulin becomes acrosomal during spermatogenesis by incorporation into these vesicles from the Golgi apparatus (Nakamura *et al.*, 1993). In cytotoxic T-lymphocytes (CTL) calreticulin has also been found outside the ER, in the lytic granules (Dupuis *et al.*, 1993). During T-cell activation and during spermatogenesis calreticulin would presumably have to bypass the K-D-E-L receptor in order to appear in those vesicles. Calreticulin isolated from granules appears to have a K-D-E-L retention tetrapeptide (R. C. Bleackley and M. Michalak, unpublished observations). Other lumenal ER proteins have not been detected in the cytolytic granules suggesting that the retention system is functional (Bleackley *et al.*, 1994). Therefore, it is presently unclear how calreticulin becomes localized to the granules.

Fig. 1-4. Intracellular localization of calreticulin.

In (A) pancreatic fibroblasts have been immunolabelled with specific anti-calreticulin antibodies and examined by confocal scanning laser microscopy. The dark gray regions correspond to specific calreticulin staining which closely reflects the distribution of the lumenal endoplasmic reticulum. In (B) L6 myoblast have been immunostained with anti-calreticulin antibodies. Shown is a confocal optical section through the nucleus. Optical sectioning of the nuclei reveals that the calreticulin-like antigen(s) localize to discrete foci within the nucleoli. These pictures were taken by M. Opas (University of Toronto).

Cell surface/secretion

Cell surface localization of calreticulin has not been specifically reported. However, B50 and the ClqR, which have NH_2-terminal amino acid sequence identity to calreticulin are localized to the cell surface (plasma membrane) (Gersten *et al.*, 1991; Malhotra *et al.*, 1993). The ClqR has been detected on a wide range of cells including peripheral blood B cells, T cells, monocytes, platelets, tissue macrophages, fibroblasts and endothelial cells (Peerschke *et al.*, 1993; Malhotra *et al.*, 1993). B50 was originally thought to be a melanoma specific antigen but has recently been detected on the surface of 29 out of 29 cell lines tested (Gersten *et al.*, 1979; Gersten, *et al.*, 1991). Confirmation that calreticulin is also localized to the plasma membrane requires further analysis.

Calreticulin has been isolated from human serum and tick saliva suggesting that it may also be secreted (Sueyoshi *et al.*, 1991; G. Needham, personal communication)

Cytoplasmic localization of calreticulin

Cytoplasmic localization of calreticulin has been postulated based on its similarity (identity?) to the cytoplasmic iron binding protein, mobilferrin, and on its proposed interactions with the α-subunit of integrin (Rojiani *et al.*, 1991; Conrad *et al.*, 1993). Mobilferrin has been found in relatively high concentrations in the apical cytoplasm of human and rat duodenal cells. Immunostaining for mobilferrin was less intense in specimens from the distal small intestine and was not detected in other regions of the intestine or in rat liver, spleen, lung, pancreas, kidney, or brain (Conrad *et al.*, 1990). This is suprising considering that calreticulin is relatively abundant in these tissues (Khanna *et al.*, 1986) and that anti-mobilferrin antibodies cross-react with calreticulin (Conrad *et al.*, 1991). Despite this apparent discrepancy, calreticulin and mobilferrin have

independently been demonstrated to co-localize with the α-subunits of integrin (Conrad *et al.*, 1993; Leung-Hagesteijn *et al.*, 1994). It is also proposed that some calreticulin may be cytoplasmically localized based on the observation that calreticulin interacts with members of the nuclear steroid receptor family (discussed in Chapter 5). We (Burns *et al.*, 1994) have suggested that calreticulin associates with these receptors in the nucleus, but that it most likely becomes nuclear via movement from the cytosol to the nucleus.

Nucleus and nuclear envelope localization of calreticulin

Calreticulin has been detected in the nucleus of some cells (Opas *et al.*, 1991; Dedhar *et al.*, 1994, Söling, personal communication). Notably, the protein possesses a putative nuclear localization signal (NLS) (Michalak *et al.*, 1992). Recently, Hatcher's group has shown that calreticulin is transported to the nucleus in a nuclear transport assay suggesting that the NLS may be functional *in vivo* (personal communication). Calreticulin is not readily obvious in the nucleus of many cells examined by immunocytochemistry (K. Burns & M. Michalak, unpublished observations). It is possible that calreticulin may evade detection if only a small amount of the protein is localized in the nucleus or is only transiently transported there.

In proliferating rat L6 cells in addition to its ER localization, calreticulin has also been immunolocalized to the nucleoli (Opas *et al.*, 1991). Interestingly when myoblast fusion was inhibited in these cells the intranuclear staining disappeared, although the ER staining remained unchanged. In differentiated myotubes both intracellular and intranuclear immunoreactivity with anti-calreticulin antibodies was absent (Opas *et al.*, 1991). The reason for calreticulin in 'nucleoli' structures is not clear at present.

In some cell types, calreticulin can also be detected in the nuclear envelope. This is not surprising as the ER is continuous with the outer nuclear membrane, however, it also remains unclear why this pattern of staining is not seen universally.

In summary, calreticulin or calreticulin-like molecules appear to be subject to rather unique intracellular trafficking (Fig. 1-5).

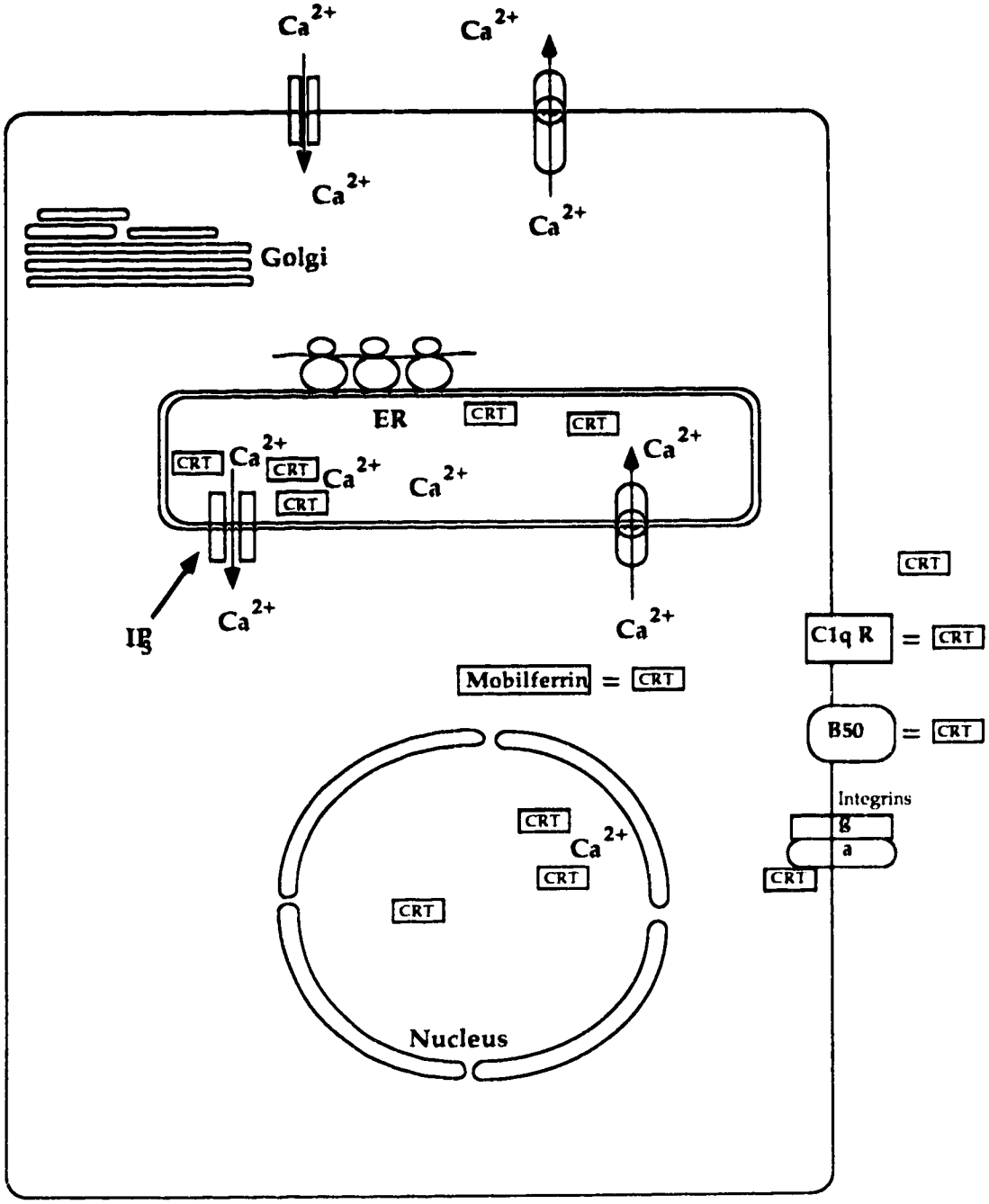
Expression of Calreticulin

The human and mouse genes for calreticulin have recently been isolated (McCauliffe *et al.*, 1992; Waser & Michalak, unpublished observation). The human gene has been localized to chromosome 19 and is not highly polymorphic (McCauliffe *et al.*, 1990a). Both genes are approximately 4 kb in size, contain 9 exons, 8 introns, and several hundred base pairs 3' of a polyadenylation sequence. In total, the introns contribute about 2.4 kb to the gene, but four of the eight are shorter than 100 bp (McCauliffe *et al.*, 1992). The location of the intron-exon boundaries in relation to the amino acid sequence of full length mature calreticulin is shown in Fig. 1-3. Interestingly, the human and mouse genes show a high degree of similarity that even extends to intron size and nucleotide sequence (Waser and Michalak, unpublished observations).

Very little is known about how the expression of calreticulin is regulated. However, a number of potential regulatory sites have been identified in the promoter region of the calreticulin gene (McCauliffe *et al.*, 1992). This region of the calreticulin gene contains AP-2 (Imagawa *et al.*, 1987) and H4TF-1 (Dailey *et al.*, 1986) recognition sequences, which are typically found in genes that are active during cellular proliferation. The calreticulin promoter also contains a number of potential regulatory sites that are found in the genes for other ER proteins including human BiP, GRP94, PDI, and ERp72 (McCauliffe *et al.*, 1992

Fig. 1-5. Calreticulin may undergo unique intracellular trafficking.

Calreticulin is shown in the lumen of the ER, in the nucleus, and in lytic granules. Calreticulin and/or calreticulin-like molecules may also be present in the cytoplasm and on the plasma membrane. Calreticulin has been proposed to interact with the cytoplasmic domain of the α-integrin subunits and may also be the same protein as mobilferrin (an iron-binding cytoplasmic protein) suggesting that it may exist in the cytosol. The B50 antigen and the C1q receptor (cell surface proteins) have NH_2-terminal amino acid sequences which are highly similar (identical) to calreticulin (Table 1-2) suggesting that calreticulin may also be trafficked to the plasma membrane.


Ca2+
Ca2+
Ca2+
Ca2+
Golgi
ER
CRT
CRT
CRT
Ca2+
CRT
Ca2+
Ca2+
CRT
Ca2+
IP3
Ca2+
Mobilferrin = CRT
CRT
C1q R = CRT
B50 = CRT
Integrins
β
a
CRT
CRT
Ca2+
CRT
CRT
Nucleus

and references therein; Srinivasan *et al.*, 1993). These sites include multiple Sp1 and CCAAT consensus sequences, an AP-2 recognition sequence (absent from PDI), and multiple GC-rich areas.

Expression of many of the lumenal ER proteins (BiP, ERp72, GRP94, and ERp61) is upregulated in response to cellular stress, such as glucose starvation or Ca^{2+} ionophore treatment (for review, see Lee 1987; Dorner *et al.*, 1990; Lee, 1992). Increased traffic through the ER or accumulation of incompletely assembled or incorrectly folded proteins in the ER leads to the induction of these proteins (Lewis *et al.*, 1985; Kozutsumi *et al.*, 1988; Dorner *et al.*, 1989; Lenny & Green, 1991). Elements in the promoter of the rat BiP and murine ERp72 genes which are responsible for their induction due to stress or the accumulation of unfolded proteins in the ER have been identified (Wooden *et al.*, 1991; Srinivasan *et al.*, 1993). The calreticulin promoter has a region homologous to the sequence identified in BiP, suggesting that it may also be regulated by cellular stress (Nigam *et al.*, 1994). However, it is not yet clear whether calretiuclin is also a stress induced protein. For example, treatment of Ló cells with the Ca^{2+} ionophore A23187 for periods up to 8 hours had no effect on calreticulin protein levels as determined by Western blot and immunocytochemical analysis (Opas *et al.*, 1991). Similarly, in another study, the levels of calreticulin were reported to be unchanged following glucose starvation for 20 hours or treatment of cells with tunicamycin (Nguyen Van *et al.*, 1993). However increased levels of calreticulin were observed in MOPC-315 cells treated wtih Ca^{2+} ionophore or tunicamycin for extended periods (Macer & Koch, 1988). As well, calreticulin mRNA levels were found to be significantly increased in L-fibroblasts treated with Ca^{2+} ionophore for 24 hours (P. Nash, K. Burns, M. Michalak, unpublished observations). In cultured *Aplysia* neural cells calreticulin mRNA and protein levels were also shown to be increased following glucose starvation experiments

(Kennedy *et al.*, 1992). In these experiments calreticulin was induced with similar kinetics to BiP. However, in contrast to BiP, calreticulin was not induced in *Aplysia* cells by heat shock, although in endothelial cells its expression was significantly induced by a similar treatment (Conway, unpublished observations).

In the marine snail *Aplysia californica* the steady state level of calreticulin mRNA was shown to be significantly increased during the maintenance phase of long-term sensitization (an elementary form of nonassociative learning) by behavioral training (Kennedy *et al.*, 1992). Long term sensitization requires protein synthesis and is associated with neuronal growth (Castellucci *et al.*, 1989; Glanzman *et al.*, 1990). Only one other protein, the Aplysia form of BiP, was induced during the maintenance phase of long-term sensitization (Kennedy *et al.*, 1992). Calreticulin was also found to be significantly induced in mouse C127 fibroblasts infected with bovine papillomavirus type 1 (BPV-1) (O'Banion *et al.*, 1993). The expression and synthesis of calreticulin are also reported to be elevated in some proliferating cells (Gersten *et al.*, 1990; Opas *et al.*, 1991; McCauliffe *et al.*, 1992; Burns *et al.*, 1992), however, the levels of calreticulin were not found to be induced in mouse splenocytes stimulated with lipopolysaccharide (Lewis *et al.*, 1985).

RATIONALE

Calreticulin is an ubiquitous protein that has a highly conserved amino acid sequence. The widespread distribution and striking sequence conservation of the protein strongly suggests that it is involved in key cellular function(s) (Michalak *et al.*, 1992). Further, its localization to disparate regions within the cell (including the nucleus), its physical characteristics (including interaction with different cellular proteins) and its putative domain structure suggest that calreticulin may have multiple cellular roles. Given these intriguing characteristics of the protein these studies were initiated to analyze the function(s) of calreticulin.

Calreticulin had only recently been cloned in 1989 when I began these studies (Fliegel *et al.*, 1989a; Macer & Koch, 1989). With the availability of the cDNA and the deduced amino acid sequence, it was soon realized that the protein had been studied under a number of different guises by a number of laboratories. During the last five years, it has been isolated in experimental systems spanning very diverse areas of cell biology. Many observations have provided clues to the cellular function (s) of calreticulin and ultimately helped to shape the course of these studies. These observations suggested that calreticulin has the potential to be far more than a Ca^{2+} binding protein. This study has evolved accordingly to access potential roles for calreticulin. The specific objectives of this thesis were as follows:

i) to examine the synthesis and expression of calreticulin in cytotoxic T-lymphocytes;

ii) to determine if calreticulin could interact with other cellular proteins;

iii) to provide evidence that calreticulin specifically associates with the glucocorticoid receptor (GR).

CHAPTER TWO

EXPERIMENTAL PROCEDURES

A section of the Chapter has been published:

Baksh, S, Burns, K, Busaan, J, and Michalak, M. 1992. **Expression and purification of recombinant and native calreticulin.** Prot. Exp. Pur. 3: 322-331.

MATERIALS

Secondary antibodies

Peroxidase-conjugated rabbit anti-goat IgGs and goat anti-rabbit IgGs were obtained from Bio-Rad, (Hercules, California), and Boehringer Mannheim (Laval, Quebec), respectively. Cyc^{TM}- conjugated anti-goat IgG was obtained from Bio/Can Scientific (Mississauga Ontario).

Molecular biological reagents

Restriction endonucleases, DNA modifying enzymes, and Taq DNA polymerase were obtained from Boehringer Mannheim and/or Gibco/BRL Life Technologies (Burlington, Ontario). All enzymes were used in buffers provided by the manufacturer.

Cell culture materials

COS-1 cells and L-fibroblasts were kind gifts of Drs. D. H. MacLennan (University of Toronto) and D. Scraba (University of Alberta), respectively. New born calf serum, calf serum, penicillin/streptomycin and geneticin G-418 sulfate (G418) were obtained from Gibco/BRL. Charcoal stripped calf serum was obtained from Sigma (St. Louis, Missouri).

Radiochemicals

$\alpha[^{32}P]$-dCTP [deoxycytidine 5'-triphosphate tetra-(triethylammonium) salt] and $\gamma[^{32}P]$-ATP [deoxyadenosine 5'-triphosphate tetra-(triethylammonium) salt] were obtained from New England Nuclear (Lachine Quebec). $[^{125}I]$Na (100 mCi/ml) was purchased from Amersham (Oakville, Ontario).

Protein separation, isolation and detection

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents were from Bio-Rad. Nitrocellulose and Immobilon (polyvinylidene difluoride) membrane filters were from Schleicher and Schuell (Keene, NH) and Millipore, (Mississauga, Ontario), respectively. The protease inhibitors, referred to throughout as a "protease cocktail", were obtained from Boehringer Mannheim. The protease cocktail contains the following inhibitors: aprotinin, phosphoramidone, l-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride, L-chloro3-(4-tosylamido)-4-pheny-2-butanone, (4-amidonphenyl) methanesulfonyl fluoride, N-[N-(l-3-trans-carboxyoxirane-2-carbonyl)-L-leycyl] agmatine), leupeptin, and pepstatin. Dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF) and benzamidine were from Sigma. The Mono Q FPLC column, Glutathione-Sepharose 4B, Cyanogen bromide (CNBr)-activated Sepharose, and DEAE-Sephadex G50 were obtained from Pharmacia (Baie d'Urfe, Quebec) and Bio-Gel hydroxylapatite beads were from Bio-Rad. Dialysis bags (6.4 mm in diameter) were from Spectra/Por, Fisher Scientific. CHAPS , Triton-X-100, Tween 20, Nonident P-40 and sodium deoxycholate (DOC) were from Sigma.

Other

X-ray film was obtained from Kodak (Rochester, New York). The BNN103 *E. coli* host was a generous gift of Dr. J. H. Weiner (University of Alberta). The MMTV-GRE-luc reporter plasmid (pJA358), described by Drouin *et al.* (1992), was a kind gift of Dr. M. Nemer (Clinical Research Institute of Montreal, Montreal Quebec). Cytochrome P-450 cDNA was a kind gift of Dr. F. J. Gonzalez. All chemicals used were of the highest grade commerially available.

Concanavalin A (ConA) and dexamethasone (DEX) were obtained from Sigma and stored in solution at -20°C. Con A was stored as an aqueous solution and DEX was prepared as a stock solution (10 mM) in methanol.

METHODS

Production of Antibodies

The polyclonal goat anti-rabbit calreticulin antibodies used in this study have previously been characterized (Milner *et al.*, 1991). The rabbit anti-cytochrome P-450, (CYP3A1) antibodies were a gift of Dr. R. Edwards. Polyclonal rabbit anti-PDI antibodies were raised in New Zealand White rabbits. The rabbits were injected subcutaneously with 1 mg of bovine liver PDI emulsified in Freund's complete adjuvant. Two weeks later the immunization was repeated with additional 1.0 mg of the protein emulsified in Freund's incomplete adjuvant.

A synthethic peptide representing amino acids residues #3668-#3679 (NH_2-P-S-S-R-G-N-T-P-G-K-P-COOH) in the carboxyl-terminus of dystrophin was used for the production of anti-DT antibodies as described by Milner *et al.*(1992). The specificity of these antibodies had previously been characterized in the laboratory (Milner *et al.*, 1992). Affinity purified anti-DT antibodies were used in these studies for the identification of dystrophin-tagged calreticulin (see Chapter 5). The anti-DT antibodies were affinity purified via a modified solid-phase binding assay. As an antigen for this procedure, the GST-CT-1 fusion protein was used. GST-CT-1 is a GST fusion protein that encodes the carboxyl-terminal region of dystrophin containing the amino acids of the DT peptide. Nitrocellulose strips containing GST-CT-1 (a gift of Jody Busaan) were incubated with sera for 2 hours in blocking buffer containing 1% milk powder in PBS (137

mM NaCl, 2.7 mM KCl, 5.4 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.2). Anti-DT antibodies were eluted from the strips in 200 mM glycine (pH 2.0) for 2 min followed by neutralization with 1.0 M Tris (pH 7.0).

SDS-Polyacrylamide Gel Electrophoresis and Western Analysis

SDS-PAGE was carried out as described by Laemmli (1970) using 10% polyacrylamide gels. The molecular weight protein markers used on each gel were the following low range protein markers (Bio-Rad) : phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (42,700), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme, (14,400). After gel electrophoresis the gels were stained with Coomassie blue or electrophoretically transfered onto either nitrocellulose membrane (Towbin *et al.*, 1979) for Western analysis or onto Immobilon membrane (Matsudaira, 1987) for NH_2-terminal amino acid analysis.

After transfer to nitrocellulose, the membranes were stained with Ponceau S (Sigma) to ensure that efficient protein transfer had occured. The membranes were then blocked in PBS containing 5% milk powder for 1 hour. Following, blocking the membranes were incubated with the primary antibody for 1 to 3 hours. All antibodies were diluted in PBS containing 1% milk powder. Tween-20 (0.05%) was also added to the PBS/milk powder solution to reduce background staining on blots incubated with anti-CYP3A1 or anti-PDI antibodies. Antibodies were diluted as follows: goat anti-calreticulin, 1:300; affinity purified rabbit anti-DT, 1:100 dilution; rabbit anti-PDI, 1:500 dilution and rabbit anti-CYP3A1, 1:1000 dilution. After incubation with the appropriate antibody, the blots were washed twice (10 min) in PBS containing 0.05% Tween-20, followed by a final 10 min wash in PBS. The blots were then incubated for 40 to 90 minutes with the appropriate peroxidase-conjugated secondary antibody (i.e. anti-goat IgG or anti-

rabbit IgG) diluted 1:2000 in PBS containing 1% milk powder. The ECL detection system (Amersham), was used for the detection of peroxidase-labelled antibodies.

Cellular Fractionation

The procedures described below were performed on ice or at 4°C, unless otherwise stated. All purification procedures were carried out in the presence of a mixture of protease inhibitors. A 'protease cocktail' was prepared as a 2000X stock solution in 100% dimethyl sulfoxide and contains the following protease inhibitors which were used at the indicated concentrations: 0.01 mg/ml of aprotinin, phosphoramidone, l-1-chloro-3-(4-tosyamido)-7-amino-2-heptanone hydrochloride, L-chloro3-(4-tosylamido)-4-pheny-2-butanone, (4-amidionphenyl) methanesulfonyl fluoride, N-[N-(l-3-trans-carboxyoxirane-2-carbonyl)-L-leycyl] agmatine): 0.05 mg/ml leupeptin; and 0.01 mg/ml pepstatin. As well 0.5 mM benzamidine hydrochloride and 0.5 mM PMSF were added to all buffers to inhibit protease acivity. These inhibitors were made as 200X concentrated stock solution in 100% ethanol. All inhibitors were added to the buffers just before use.

Preparation of membrane fractions

Pancreatic microsomes were isolated by the procedure of Walter and Blobel (1983). Fresh dog pancreas (a gift of Dr. B. Jugdatt, University of Alberta) was homogenized in 4 ml of buffer A (250 mM sucrose, 50 mM triethanolamine, pH 7.5, 50 mM KOAc, 6 mM $Mg(OAc)_2$, 1 mM EDTA, 1 mM DTT) per gram of tissue. The homogenate was centrifuged at 1000 xg for 10 min. Floating fatty material was removed from the supernatant by aspiration and the supernatant was recentrifuged for 10 min. at 10,000 xg. Crude rough microsomes were prepared by centrifugation of the 10,000 xg supernatant for 2.5 hours at 140,000

xg (rotor) through a 1.3 M sucrose cushion. The pellet containing the pancreatic microsomes was resuspended at a concentration of ~10 mg/ml in buffer B (250 mM sucrose, 50 mM triethanolamine, 1 mM DTT, pH 7.5) using 3 strokes in a hand homogenizer. Isolated membrane vesicles were frozen in liquid nitrogen and stored at -70°C.

Preparation of smooth and rough ER and Golgi

Smooth and rough ER and Golgi membrane fractions were kind gifts of Dr. J. Vance (University of Alberta). These fractions were isolated in her laboratory from rat liver as described by Croze and Morre (1974), and extensively characterized (Milner *et al.*, 1991).

Preparation of nuclei and cytosolic fractions

Samples of rabbit liver nuclei were a gift of Dr. K. Famulski (University of Alberta). These nuclei were prepared from fresh liver tissue homogenized (1:5 w/v) in 320 mM sucrose containing, 10 mM Tris-Cl, pH 7.4, 3 mM $MgCl_2$, and protease cocktail in a Waring blendor (2 x 30 sec). The homogenate was centrifuged at 3,000 xg for 10 min and the supernatant saved for preparation of the cytosolic fraction, described below. The pellet was blended (30 sec) in 250 mM sucrose containing 3 mM $MgCl_2$, 10 mM Tris, pH 7.4 and protease cocktail, filtered, and recentrifuged. The washed pellet was resuspended in 2.2 M sucrose containing 1 mM $MgCl_2$ and 10 mM Tris, pH 7.4 by homogenization for 15 seconds and then centrifuged at 25,000 rpm for 60 min in SW 28 Beckman rotor. The resulting white nuclear pellet was washed and resuspended in 250 mM sucrose containing 3 mM $MgCl_2$, 10 mM Tris, pH 7.4, protease cocktail and 1 mM DTT. Nuclei were stored at -70°C or used directly.

A cytosolic fraction was prepared using the supernatant obtained after the initial spin in the preparation of liver nuclei, described above. The supernatant was adjusted to 140 mM KCl by the addition of solid KCl. It was then passed through glass wool and spun for 1 hour at 100,000 xg using a 70Ti rotor. Following centrifugation the supernatant was dialyzed overnight in buffer containing 20 mM Hepes, pH 7.5, 100 mM KCl, 0.2 mM EDTA and 20% glycerol. PMSF and benzamidine were added to the dialysis buffer at a concentration of 0.2 mM. Cytosolic fractions were frozen in liquid nitrogen and stored at -70°C.

Preparation of pancreatic calreticulin

Calreticulin was purified from fresh canine pancreatic tissue by a selective ammonium sulfate precipitation method (Baksh *et al.*, 1992). The tissue was homogenized in 4 vol of buffer containing (100 mM KH_2PO_4, pH 7.1, containing 2.66 M $(NH_4)_2SO_4$ (55% saturation), 1 mM EDTA and the protease cocktail) at high speed in a Waring blender for 1 min. The homogenate was spun at 12,000 xg for 30 min using a Sorvall GSA rotor. The supernatant was filtered through cheesecloth and saved. The pellet was rehomogenized in the same buffer for 1 min using half the original volume. The homogenate was centrifuged as before, and the supernatant was filtered and combined with the first supernatant: 200 g of solid $(NH_4)_2SO_4$ was added per litre of supernatant to achieve 85% saturation of $(NH_4)_2SO_4$, and the suspension was stirred for 30 min. The pH was adjusted to 4.4 using phosphoric acid, the suspension was stirred for 3 h in the cold room and centrifuged at 12,000 xg for 30 min using a Sorvall GSA rotor. The pellet was dissolved in 100 mM KH_2PO_4, buffer, pH 7.1, containing 1 mM EGTA, and dialyzed for 48 h against the same buffer containing 50 mM NaCl. The solution was clarified by centrifugation at 12,000 xg for 30 min. The supernatant was applied directly to a DEAE-Sephadex A-50 column (3 cm x 12 cm; 60 ml bed

volume; flow rate 0.6 ml/min; capacity 200 mg) equilibrated with 100 mM potassium phosphate, pH 7.1, containing 1 mM EGTA and 50 mM NaCl. The column was washed with 5 volumes of the phosphate buffer, and then a linear gradient of NaCl from 50 mM to 800 mM was applied. Five ml fractions were collected, and the protein content of each fraction was monitored by the absorption at 280 nm, by the Bradford protein assay, by SDS-PAGE and by reactivity with anti-calreticulin antibodies. Fractions containing calreticulin were pooled and dialyzed for 16 h against 10 mM KH_2PO_4,, pH 7.0. Further purification of calreticulin was achieved by hydroxylapatite chromatography as described by MacLennan (1974). The sample was loaded onto a hydroxylapatite column (2 cm x 15 cm; 50 ml bed volume; flow rate 0.5 ml/min; capacity 20 mg) and unbound protein was washed out with 150 ml of 10 mM KH_2PO_4, pH 7.0. The bound proteins were eluted with a 400 ml linear gradient of 10 mM to 1000 mM KH_2PO_4 pH 7.0. Five ml fractions were collected and analyzed for the presence of calreticulin by SDS-PAGE and reactivity with anti-calreticulin antibodies. Peaks containing calreticulin were pooled, dialyzed against 10 mM ammonium bicarbonate and freeze-dried. Calreticulin was further purified for use in iodination experiments by centrifugation through a 10%-40% linear sucrose gradient spun at 100, 000 xg (SW 50.1 rotor) for 16.5 hours.

Preparation of light and heavy SR

SR membrane fractions were isolated from rabbit skeletal muscle by the method of Meissner (1984).

Purification of PDI

PDI was purified from bovine liver as described by Lambert and Freedman (1983) with minor modifications. Frozen bovine liver was thawed

overnight at 4°C and homogenized in a buffer containing 100 mM sodium phosphate, pH 7.5, 5 mM EDTA, 1% v/v Trition X-100, the protease cocktail, 0.5 mM PMSF and 0.5 mM benzamidine. The homogenate was centrifuged and the supernatant was then placed in a water bath at 70°C with constant stirring and the temperature of the extract allowed to reach 54°C, where it was maintained for 15 min, cooled on ice and then centrifuged. The pellet was discarded and the supernant fractionated by selective ammonium sulfate precipitation. Following addition of ammonium sulfate to obtain 85% saturation, the sample was centrifuged and the supernatant discarded. The pellet was resuspended in 25 mM sodium citrate buffer, pH 5.3 and dialysed overnight against the same buffer. For further purification a phosphocellulose column was then used instead of the CM-Sephadex C-50 column described by Lammbert and Freedman (1983). The sample was loaded onto a phosphocellulose column (2 x 15 cm/ 50 ml bed volume; flow rate. 0.4 ml/min; capacity, 20 mg) previously equilibrated with the 25 mM citrate buffer and ... ed with the same buffer at a flow rate of 3-4 ml/min. Eluted protein was collected and then precipitated by addition of ammonium sulfate to achieve 100% saturation. The sample was then centrifuged at 18,000 xg for 30 min., the supernant discarded and the pellet resuspended in 20 mM sodium phosphate buffer pH 6.3 and dialysed extensively against this buffer. Following dialysis the sample was applied to a MonoQ FPLC column equilibrated in 20 mM Na_2PO_4, pH 6.3. Proteins were eluted with a linear salt gradient from 0 to 700 mM NaCl in the same buffer and 2.5-ml fractions (0.33 ml/min) collected. Three peaks of protein were detected by absorbance at 280 nm. The middle peak which elutes at ~350 mM NaCl contained PDI.

Determination of Protein Concentration

Protein concentrations were determined by the method of Lowry *et al.* (1951), or Bradford (1976). For NH_2-terminal sequence analysis the protein was electroblotted to Immobilon membranes (Mastudaira, 1987). NH_2-terminal sequence analysis was carried out at the Department of Biochemistry, University of Victoria, B.C. The automated analyses were performed with an Applied Biosystems Model 470A gas-liquid-phase protein sequencer connected on-line to an Applied Bio-systems Model 120A HPCL, using current protocols of Applied Biosystems.

Calreticulin Affinity Chromatography

A calreticulin affinity column was synthesized by coupling purified recombinant calreticulin to activated CNBr Sepharose 4B (Pharmacia) by the protocol recommended by the manufacturer. Two mg of calreticulin was coupled to 1 ml of activated CNBr-Sepharose 4B by rotation in coupling buffer (100 mM $NaHC0_3$, pH 8.3 and 500 mM NaCl) for 16-18 hours at 4°C. Unbound calreticulin was removed by sequential washes in coupling buffer. Remaining active groups were then blocked for 1 hour at room temperature by incubation in a solution containing 1 M ethanolamine and 0.1 M $NaHCO_3$, pH 9.0. The column was further washed to remove any excess absorbed protein by three consecutive washes in a low pH buffer (100 mM sodium acetate, 500 mM NaCl, pH 4.0) followed by high pH buffer (100 mM Tris and 500 mM NaCl, pH 8.0). Efficiency of coupling was determined to be greater than 95%. A pre-column was prepared by incubation of CNBr activated Sepharose 4B with 1 M ethanolamine and 100 mM $NaHCO_3$, pH 9.0. The calreticulin affinity column was subsequently used to purify calreticulin binding proteins from pancreatic microsomes and to

demonstrate an interaction between calreticulin and the DNA binding domain (DBD) of the GR.

Purification of calreticulin binding proteins in pancreatic microsomes

Samples of pancreatic microsomes containing ~20 mg of protein were thawed at 37°C and then cooled on ice, followed by centrifugation at 100,000 xg for 30 min using a Ti70.1 rotor. The membrane pellet was resuspended by dounce homogenization in 800 µl of buffer A (10 mM MOPS, 100 mM KCl, 2 mM $MgCl_2$, 0.5 mM EGTA, pH 7.0). The microsomes were then solubilized with 1% Trition-X-100 and 0.5 % DOC by incubation on ice 30 min. Solubilized microsomes were centrifuged at 100,000 xg for 30 min. using a Ti70.1 rotor. The soluble extract was diluted 10 fold with buffer A and either Ca^{2+}, Zn^{2+}, or EGTA was added to give a final concentration of 2 mM, 2 mM, or 5 mM, respectively. The extracts were first applied onto a pre-column to remove any proteins that non-specifically associate with the matrix. The flow-through (~2 mg/ml of protein) was then directly applied to the calreticulin affinity column. The column was washed with 15 ml of buffer A containing 0.1% Triton X-100 and 0.05 % DOC. Bound proteins were eluted with a 25-ml linear gradient of 100 to 750 mM KCl at a flow rate of 0.2 ml/min. Bound proteins were concentrated using an Amicon concentrator, separated by SDS-PAGE and electrophoretically transfered to Immobilon for NH_2-terminal sequencing (Matsudaira, 1987) or to nitrocellulose for Western analysis.

Iodination of Calreticulin and Ligand Blotting

Dog pancreatic or recombinant calreticulin were iodinated using 1, 3, 4, 6-tetrachloro-3α,6α-diphenylglycouril (IODO-GEN, Sigma). IODO-GEN (200 µg) dissolved in acetone was added to a glass scintillation vial, and allowed to

evaporate. Pancreatic (133 μg) or recombinant calreticulin (1.6 mg) was added to an IODO-GEN coated vial. [^{125}I]Na (100 mCi/ml, Amersham) 5 μl/mg of protein was added and gently mixed on a rocking platform for 20 min. at room temperature. The sample was dialysed against 50 mM Tris pH 7.6, 100 mM NaCl, 2 mM $CaCl_2$, to remove unincorporated [^{125}I] Na.

For ligand blotting, protein samples were separated by SDS-PAGE (10% acrylamide) and transferred electrophoretically onto nitrocellulose membranes. Transferred proteins were visualized with Ponceau-S prior to ligand blotting which did not affect [^{125}I]calreticulin binding. The nitrocellulose blots (strips) were preincubated for 1 hour in blocking buffer containing 20 mM Tris, 150 mM KCl, 5% bovine serum albumin and either 2 mM $ZnSO_4$, 5 mM $CaCl_2$ or 5 mM EGTA. The strips were then incubated for 2 hours at room temperature in fresh blocking buffer containing [^{125}I]calreticulin (10^6 cpm/ml). The blots were rinsed 3 times with blocking buffer, followed by two 30 min. washes in the same buffer. Autoradiographs were obtained by exposing the dried blots to Kodak AR film at -70°C overnight unless otherwise indicated.

Synthesis of oligonucleotides and DNA sequencing

Oligonucleotides used in this study were synthesized either in the Department of Biochemistry using an Applied Biosystems 392 DNA/RNA synthesizer or in the Department of Microbiology using model ABI391.

Templates were prepared for DNA sequencing using the modified mini alkaline lysis procedure recommended by Applied Biosystmes, Inc. Sequencing was carried out in the the DNA synthesis facility in the Department of Biochemistry, University of Alberta.

Preparation of Plasmid DNA

DNA fragments used in the construction of plasmids were isolated from agarose gel slices by glass powder elution (Davis *et al.*, 1986) or from polyacrylamide gel slices using the "crush and soak" procedure (Sambrook *et al.*, 1989). Vector preparation (including alkaline phosphatase treatment) and cloning procedures were carried out according to published protocols (Sambrook *et al.*, 1989; Ausubel *et al.*, 1992). Following a ligation, a portion of the reaction was diluted 100 fold in water, transformed into DH5α, and plated onto LB plates containing 100 μg/ml ampicillin. For the expression of GST-fusion proteins *E. coli*, strain BNN103 was used. BNN103 cells were made competent and tranformed as described by Miller (1987). DNA was isolated using the alkaline lysis method from individual colonies that had been picked and grown overnight in LB/ampicillin broth. Desired recombinants were identified by restriction endonuclease analysis and when required by DNA sequencing.

PCR reactions unless specified were carried out in 1X PCR buffer supplied by Gibco/BRL. Two hundred μM of each dNTP, 2.5 units of Taq polymerase, 100 pmol of each primer and 20 ng of template were used per reaction.

To obtain the high purity covalently closed-circular (CCC) plasmid DNA required for efficient transfections, DNA was prepared using the alkaline lysis method followed by centrifugation through a cesium chloride-ethidium bromide density gradient (Ausubel *et al.*, 1992). Following purification, the DNA was precipitated at -70°C for 15 min. with 1/10 volume of 3 M sodium acetate-pH 5.2 and two volumes of 95% ethanol. The DNA was pelleted by centrifugation at 10,000 xg (Eppendorf centrifuge) for 10 min and the DNA pellet washed twice with 70% ethanol. After the second ethanol wash the pellet was allowed to dry under sterile conditions. It was resuspended in the appropriate volume of sterile TE, pH 7.05. DNA was quantitated by ethidium bromide fluorimetry (Morgan *et*

al., 1979) at pH 12.0 and the percentage of CCC calculated by taking a second reading after denaturation (2 min) and renaturation (2 min at 25°C) in the ethidium bromide solution at pH 12.

Nomenclature for recombinant plasmids

The recombinant calreticulin plasmids discussed below are referred to using the conventions illustrated by the following example:

pRCR-DT-1

The first two letters designate the parental plasmid into which a particular DNA fragment has been cloned. In this example, pR, refers to the parental plasmid pRc/CMV (Invitrogen). Other vectors used in this study are referred to as follows: pS for pSVL (Pharmacia), and pB for pBluescript (Stratagene). The next two letters are used to indicate the nature and source of the cDNA insert. In this example C refers to calreticulin and R to rabbit indicating that this construct contains cDNA for rabbit calreticulin. Any non-calreticulin sequences that have been incorporated into a particular construct are indicated following a dash. For example, DT refers to a dystrophin specific 12 amino acid peptide that has been used to epitope tag calreticulin.

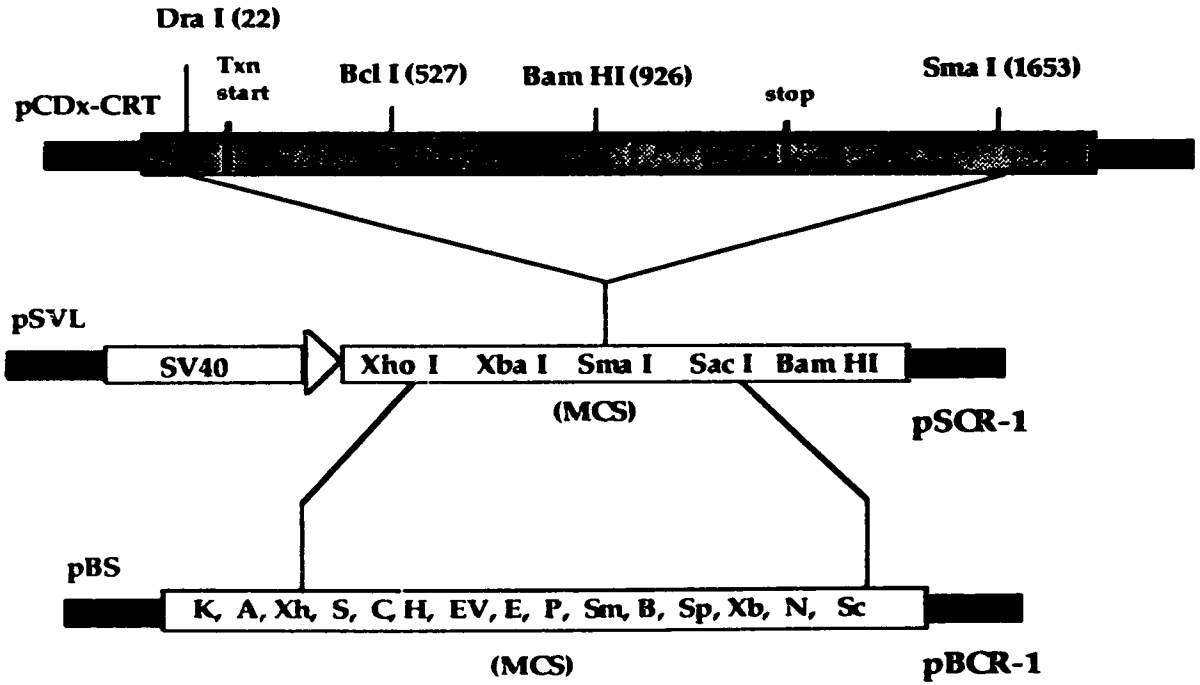
Construction of eukaryotic expression vectors :

The eukaryotic expression vector pSVL was used to obtain high level expression in eukaryotic cells. It contains an SV40 promoter, and has an SV40 origin of replication. To construct the calreticulin expression vector designated pSCR-1, the Dra/Sma fragment (nucleotides 20 - 1653) of pcDx-CRT was inserted into Sma I digested pSVL (Fig. 2-1). Epitope tagging of calreticulin was carried out in order to distinguish recombinant calreticulin from the native protein in transfected cells. A unique epitope was engineered into the C-terminus of

Fig. 2-1. Structure of plasmids pSCR-1 and pBCR-1.

The eukaryotic expression vector pSVL was used to obtain high level expression in eukaryotic cells. It contains an SV40 promoter, and has an SV40 origin of replication that is functional in COS cells. To construct the calreticulin expression vector designated pSCR-1, the Dra I/Sma I fragment (nucleotides 20 - 1653) of pcDx-CRT (Fliegel *et al.*, 1989) was inserted into Sma I digested pSVL in the correct orientation. pBCR-1 was made to facilitate epitope tagging of calreticulin (Fig. 2-2). pBCR-1 was constructed by removing the Xho I/Sac I fragment from pSCR-1 and inserting into pBluescript digested with Xho I/Sac I. Restriction endonucleases: K-Kpn I, A-Ava I, Xh-Xho I, S-Sal I, C-Cla I, H-Hind III, EV-Eco RV, E-Eco RI, P-Pst I, Sm-Sma I, B-Bam HI, Sp-Spe I, Xb-Xba I, N-Not I, Sc-Sac I. Other abbreviations: MCS-multiple cloning site; Txn-translation start site; stop-refers to translation stop site.

Fig. 2-1. Structure of plasmids pSCR-1 and pBCR-1



calreticulin. The epitope encoded for a 12 amino acid sequence unique to the Carboxyl-terminus of the muscular dystrophy gene product, dystrophin (Milner *et al.*, 1992). Recombinant calreticulin containing this dystrophin specific peptide is referred to as DT-(Dystrophin tagged) calreticulin. To facilitate insertion of the cDNA for the dystrophin tag, pBCR-1 was constructed by removing the Xho I/Sac I fragment from pSCR-1 and inserting it into Xho I/Sac I cut pBluescript (Fig. 2-1).

Two complementary oligodeoxynucleotides, KBU 2 and KBU 3 (Table 2-1) were synthesized which encode the DT. KBU2 and 3 were designed so that Not I and Sty I restriction sites are formed at the 5' and 3' ends, respectively when the two oligodeoxynucleotides are annealed. The oligodeoxynucleotides were annealed and then treated with T4 polynucleotide kinase for one hour at 37°C (Sambrook *et al.*, 1989). cDNA encoding the DT was inserted into pBCR-1 which had been digested with Not I and Sty I to form pBCR-DT-1 (Fig. 2-2). Insertion of the DT into the Not I/Sty I digested pBCR-1 deletes nucleotides #1290-1299 resulting in the loss of amino acids #394-#396 and the addition of 12 new amino acids (P-S-S-R-G-R-N-T-P-G-K-P) to the calreticulin sequence. The Xho I/Sst I fragment from pBCR-DT-1 was directionally cloned into Xho I/Sst I cut pSVL to form pSCR-DT-1 (Fig. 2-2).

The eukaryotic expression vector pRc/CMV was used for the generation of stably transfected eukaryotic cells. Genes inserted into its multiple cloning site are expressed from the first ATG using the CMV promoter and enhancer. pRc/CMV contains the neomycin resistance gene which was used for selection of stable transformants with geneticin (G418). To order to construct pRCR-DT-1 and pRCR-DT-2 pBCR-DT-2 was first made. pBCR-DT-2 was made by ligating the Xba I/Sma I fragment from pBCR-DT-1 and inserting it into Xba I/Sma I cut pBluescript (Fig. 2-3). The XbaI fragment was then removed from pBCRDT-2

Table 2-1. Oligonucleotide Primers

Primer Name	Sequence	Restriction Site	Position
* MMI 1	atgaattcGGAGCCCGTCGTCTACTTCAA	EcoRI	112-131
* MMI 5	gggaattcACTACCAGTCATCCTCCAGGGA	EcoRI	643-660
* MMI 11	gggaattcAGAGACATTATTTGGCTCTGCG	EcoRI	1322-1376
■ KBU 2	ggccgccCCTAGTTCAAGAGGAAGAAATA CCCCTGGAAAGCC	NotI	11210-11243
■ KBU3	cttgggCTTTCCAGGGGTATTTCTTCCTCT TGAACTAGGGGC	StyI	11210-11243
• A	atggatcCTCTGCCTGGTGTGCTCTGATGAA	BamHI	1389-1403
• B	atggatccTCCTGTAGTGGCCTGCTGAATTCC	BamHI	1626-1650

* calreticulin primers

■ dystrophin-tag primers

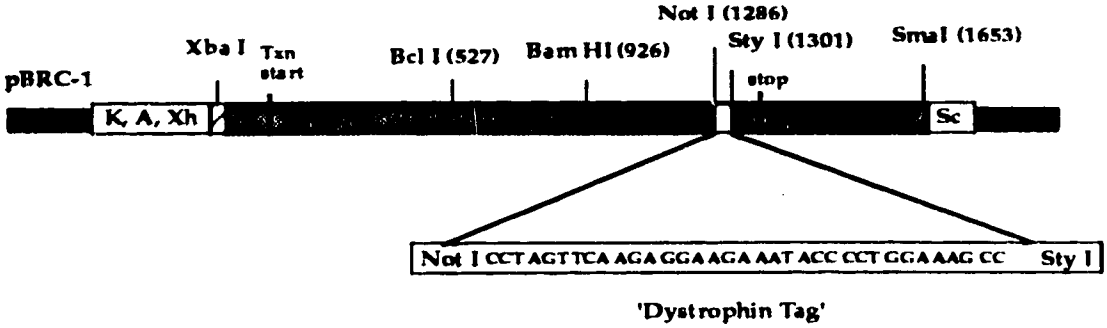
• glucocorticoid receptor primers

Table 2-1. The primer sequences are shown in the 5' to 3' orientation. At the 5' ends additional nucleotides were added to create restriction sites (underlined) or stop sites (shadowed). The first and last nucleotides (written in capital letters) correspond to the positions given in the last column for the rabbit calreticulin nucleotide sequence (Fliegel *et al.*, 1989), the dystrophin nucleotide sequence (Milner *et al.*,. 1992) or the glucocorticoid receptor nucleotide sequence (Hollenberg *et al.*, 1985).

Fig. 2-2. Structure of pBCR-DT-1 and pSCR-DT-1.

Two complimentary oligodeoxynucleotides, KBU 2 and KBU 3 (Table 2-1) were synthesized which encode the DT, the dystrophin specific peptide (residues #3668-3679 of human dystrophin) used to epitope tag calreticulin (Milner *et al.*, 1992). Epitope-tagging was carried out to facilitate identification of recombinant calreticulin in transfected cells. In order to construct the eukaryotic expression vector pSCR-DT-1, pBCR-DT-1 was first made. In (A) the DT was inserted into Not I/Sty I digested pBCR-1 to form pBCR-DT-1. Insertion of the DT into the Not I/Sty I site of pBCR-1 deletes nucleotides #1290-1299 resulting in the loss of amino acids #394-#396 and the addition of 12 new amino acids (PSSRGRNTPGKP) to the calreticulin sequence. Nucleotides are numbered based on Fliegel *et al.*, 1989. In (B) the Xho I/Sst I fragment from pBCR-DT-1 was directionally cloned into Xho I/Sst I cut pSVL to form pSCR-DT-1. Restriction endonucleases: K-Kpn I, A-Ava I, Xh-Xho I, Sc-Sac I. Other abbreviations: MCS-multiple cloning site; Txn-translation start site; stop-refers to translation stop site.

A. Structure of pBCR-DT-1



B. Structure of pSCR-DT-1

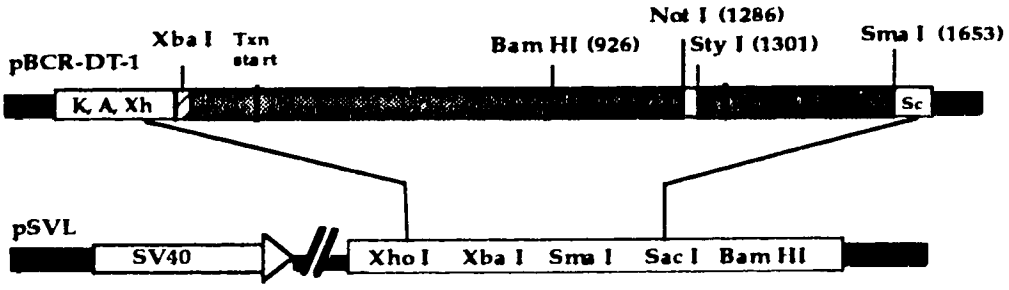
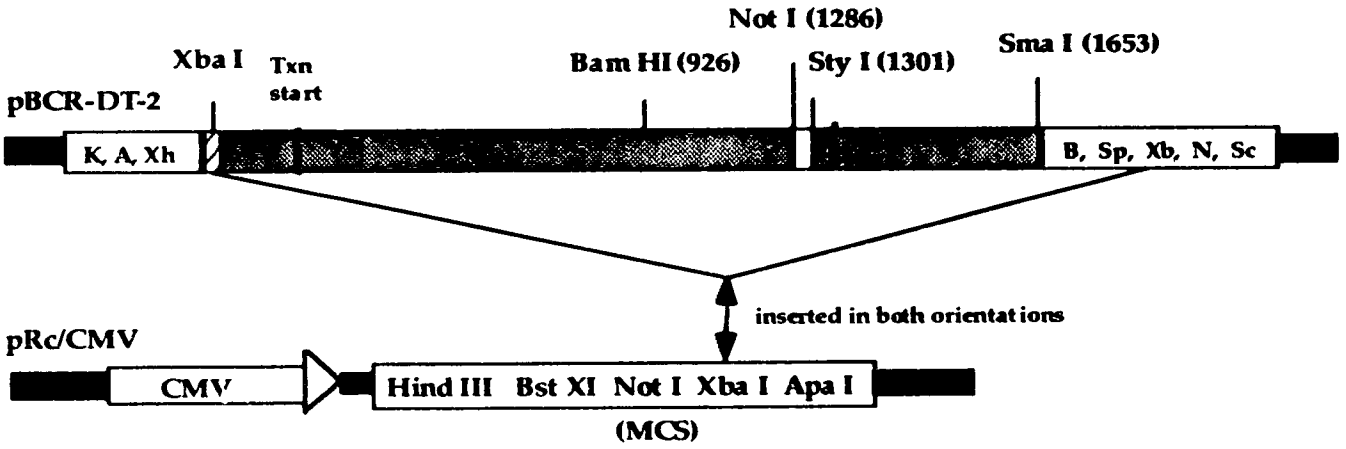


Fig. 2-3. Structure of pRCR-DT-1 and pRCR-DT-2.

The parental plasmid pRc/CMV was used to construct the expression vectors designated pRCR-DT-1 and pRCR-DT-2. Genes inserted into the multiple cloning site of pRc/CMV are expressed from the first ATG codon using the CMV promoter and enhancer. pRc/CMV contains the neomycin resistance gene which allows for selection of stable transformants with geneticin (G418). pRCR-DT-1 and -2 were constructed by inserting the Xba I fragment from pBCR-DT-2 into Xba I linearized pRc/CMV in the sense and anti-sense orientations, respectively. These vectors contain the entire coding sequence for calreticulin and an additional 36 nucleotides that code for the DT shown in Fig. 2-2. Restriction endonucleases: K-Kpn I, A-Ava I, Xh-Xho I, B-Bam HI, Sp-Sph I, Xb-Xba I, N-Not I, Sc-Sac I. Other abbreviations: MCS-multiple cloning site; Txn-translation start site; stop-refers to translation stop site.

Fig. 2-3. Structure of pRCR-DT-1, 2



and inserted into Xba I cut pRc/CMV to form pRCR-DT-1 and -2 which contain calreticulin cDNA in the sense and anti-sense orientations, respectively (Fig. 2-3). These vectors were used to stably transfect L-fibroblast cell-lines (described below) to create cell-lines expressing elevated or reduced levels of calreticulin.

The Glutathione-S-transferase Fusion System

The Glutathione S-transferase (GST) fusion system (Pharmacia) was used so that sufficient quantities of protein could be produced and easily isolated from bacteria for functional studies. In addition, this system enabled us to obtain fusion proteins representing distinct regions (domains) of calreticulin or the GR. This system utilizes the prokaryotic expression vector pGEX-3X (Smith and Johnson, 1988) . pGEX-3X codes for GST followed by a Factor Xa cleavage site (I-G-C-R), a multiple cloning site and a stop codon. The Factor Xa cleavage site allows for the site-specific proteolysis of fusion proteins from GST.

The mature form of calreticulin or various regions of the protein including (the N-, P-, C- domains) were inserted into pGEX-3X to form pGEX-CRT, pGEX-N, pGEX-P, and pGEX-C, respectively. pcDx-CRT containing cDNA encoding rabbit skeletal muscle calreticulin (Fliegel *et al.*, 1989) was used for construction of these vectors. cDNA fragments encoding the mature form of calreticulin (i.e minus a 17 amino acid leader peptide) (Fig. 2-4) and for the N-domain (amino acid #1-200) (Fig. 2-5) were PCR copied from Sal I linearized pcDx-CRT. MMI 1 (Table 2.1) was used as the sense primer for both PCR reactions and MMI 11 and MMI 5 (Table 2.1) were used as the antisense primers for PCR synthesis of mature and N-domain fragments, respectively. The PCR reactions were carried out for 25 cycles consisting of 1 min. at 94°C, 1 min. at 57°C (mature calreticulin) or 52°C (N-domain) and 2 min. at 72°C. Amplication was completed by a final incubation at 72°C for 10 min. Both PCR fragments were digested with Eco RI

Fig. 2-4. Structure of pGEX-CRT.

The Gluthathione S-transferase (GST) fusion system was used for the expression of calreticulin fusion proteins. This system utilizes the prokaryotic expression vector pGEX-3X (Smith and Johnson, 1988) which codes for GST followed by a Factor Xa cleavage site (I-G-C-R), a multiple cloning site and a stop codon. The Factor Xa cleavage site allows for the site-specific proteolysis of fusion proteins from GST. To construct pGEX-CRT a cDNA fragment encoding for the mature form of calreticulin (i.e minus a 17 amino acid leader peptide) was PCR copied from Sal I-linerized pcDx-CRT (Fliegel *et al*, 1989). MMI 1 and MMI 11 (Table 2-1) were used as the sense and as the antisense primers, respectively, for PCR. The PCR product was digested with Eco RI and inserted into pGEX-3X previoulsy linearized with Eco RI and treated with alkaline phosphatase. The nucleotide sequence of pGEX-CRT was confirmed by sequencing as discussed in the text. Abbreviations: PCR-polymerase chain reaction; MCS-multiple cloning site.

Fig. 2-4. Structure of plasmid pGEX-CRT

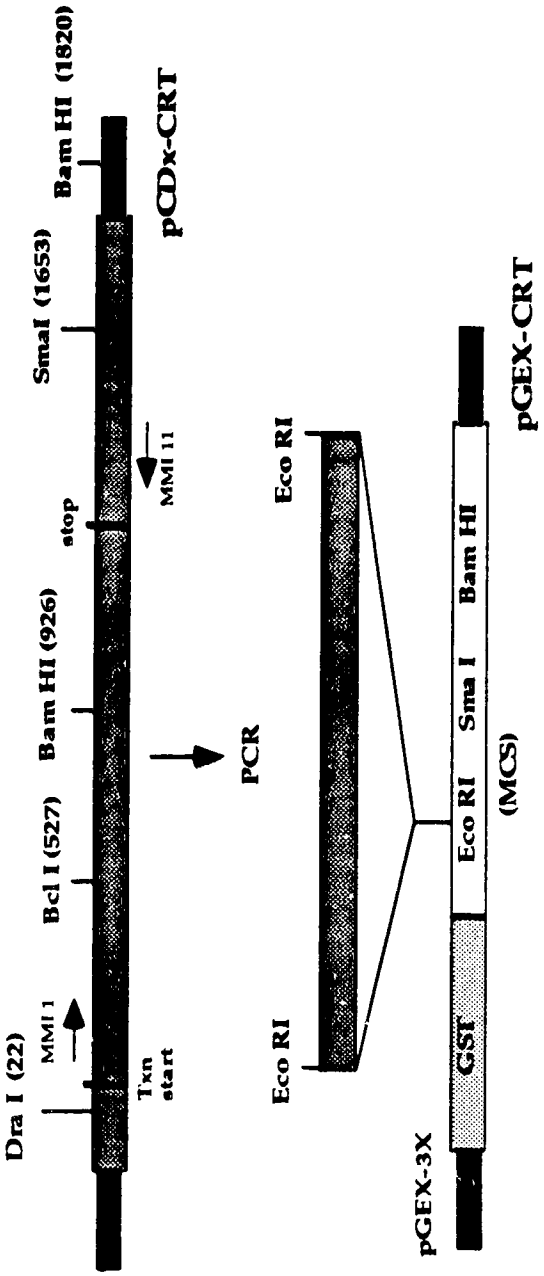
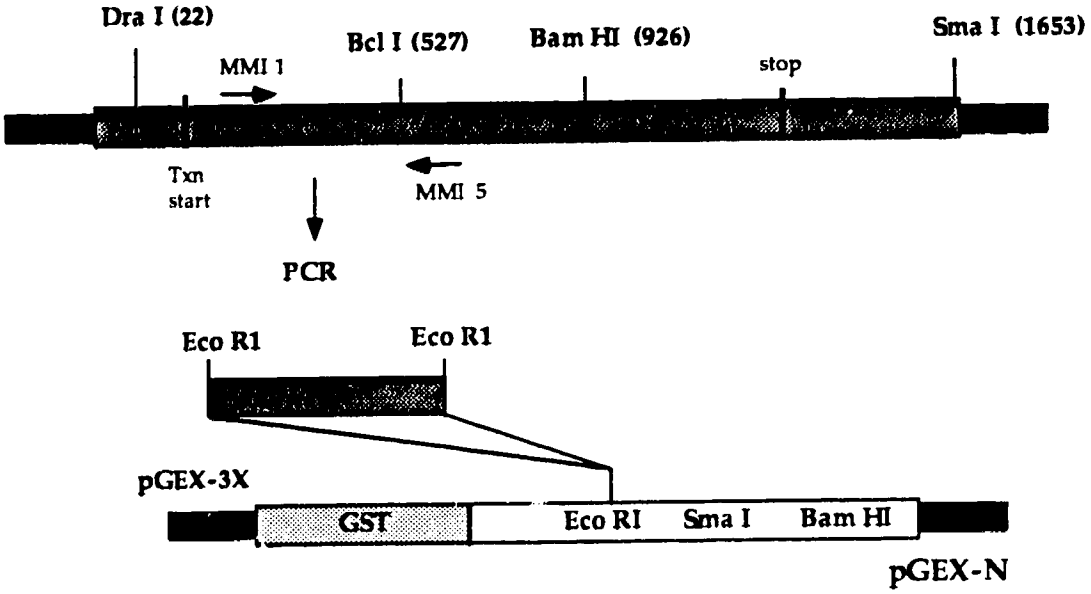


Fig. 2-5. Structure of pGEX-N

pGEX-N was used to express the region of calreticulin referred to as the N-domain (amino acid #1-200) in *E. coli* as a GST-fusion protein. A cDNA fragment encoding the N-domain (amino acid #1-200) was PCR copied from Sal I linearized pcDx-CRT (Fliegel *et al.*, 1989). MMI 1 and MMI 5 (Table 2-1) were used as the sense and antisense primers, respectively. The PCR product was digested with Eco RI and inserted into pGEX-3X linerized with Eco RI and treated with alkaline phosphatase to form pGEX-N. Abbreviations: MCS-multiple cloning site; PCR-polymerase chain reaction.

Fig. 2-5. Structure of pGEX-N



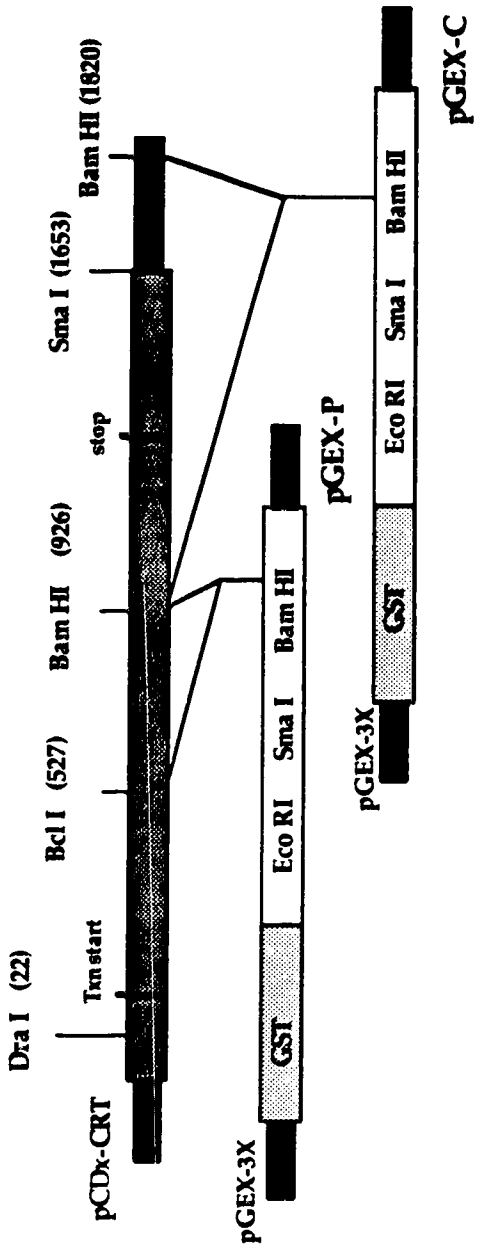
and inserted into pGEX-3X linearized with Eco RI and treated with alkaline phosphatase (Fig. 2-4, 2-5). pGEX-CRT was sequenced to determine if there were any base changes as a result of PCR. Five base changes were generated by PCR but only one change, at postion #1286 (C-A), created an amino acid substitution from (alanine to glutamate) at residue #391-#392. This change was considered to be a conservative one, since human calreticulin has an aspartate at this position (see Fig. 1-1). pGEX-P encoding GST-P domain fusion protein was made by isolation of the Bcl I/Bam HI fragment from pcDx-CRT (nucleotides 416-815) followed by its insertion into Bam HI digested, alkaline phosphatase-treated pGEX-3X (Fig 2-6). pGEX-C which encodes for residues #310-401 of the C-terminus of calreticulin was made by excising the Bam HI/Bam HI fragment (nucleotides 815 -1865) from pcDx-CRT and ligating it into Bam HI cut pGEX-3X (Fig. 2-6).

GST or all GST-fusion proteins were expressed in the protease-deficient *E. coli* strain, BNN103, grown in Luria-Bertani (LB) medium containing 50 μg of ampicillin/ml. Cultures were grown to mid-log phase (A_{600} = 0.6-1.0) and then 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG, Boehringer Mannheim) was added to induce expression of the fusion proteins. Four hours after induction the cells were harvested by centrifugation at 3,500 x g for 15 min at 4°C, resuspended in PBS containing 1.0% Titon X-100 and lysed using a French Press set at 1,000 p.s.i. The lysates were then spun at 10,000 x g for 10 min to remove insoluble matter. The GST-calreticulin, -P and -C fusion proteins were found in the high speed supernatant fraction. Although, GST-N was less soluble than the other fusion proteins, approximately 20% of it was recovered in the supernatant fraction. The solubility of these proteins suggests that they might be properly folded. The fusion proteins made up approximately 15-18% of the *E. coli* lysate (25-30 μg of fusion protein was obtained/ml of *E. coli* culture).

Fig. 2-6. Structure of the plasmids pGEX-P and pGEX-C.

The pGEX-P and pGEX-C constructs were used to express the P-domain (amino acids residues #182- X) and C-domain (amino acid residues #310-401) of calreticulin in E. coli as GST-fusion proteins. A cDNA fragment encoding the P-domain was obtained by digestion of pCDX-CRT (Fliegel *et al.*, 1989) with Bcl I/Bam HI. The Bcl I/Bam HI fragment (nucleotides 416-815) was inserted into Bam HI digested and alkaline phosphatase-treated pGEX-3X to form pGEX-P. A cDNA fragment encoding the C-domain of calreticulin was made by excising the Bam HI/Bam HI fragment (nucleotides 815 -1865) from pcDx-CRT and ligating it into Bam HI cut and alkaline phosphatase-treated pGEX-3X. Abbreviations: MCS-multiple cloning site.

Fig. 2-6. Structure of plasmids pGEX-P and pGEX-C



Glutathione-Sepharose 4B affinity chromatography was used as the first step in the purification of GST and the GST-fusion proteins. Samples of the *E. coli* extracts in PBS containing 0.1% Triton X-100 were applied to a glutathione-Sepharose 4B column (2 cm x 12 cm: 40-ml bed volume) equilibrated with PBS containg 0.1% Trition X-100. The column was extensively washed in the same buffer. Fusion proteins were eluted from this column using a buffer containing 5 mM-glutathione and 50 mM-Tris/HCl, pH 8.0. After chromatography the fractions containing the fusion proteins were concentrated using an Amicon concentrator, dialysed against appropriate buffer and stored at -70°C until required.

Using this chromatography procedure, the fusion proteins were purified to near homogenity (Fig. 2-7, A, B, C, D). Similar to native calreticulin the GST-calreticulin fusion protein ran anomalously in SDS-PAGE. It migrated with a mobility corresponding to a polypeptide of 80-kDa rather than 72-kDa (46-kDa calreticulin + 26-kDa recombinant GST) (Fig. 2-7, A & 2-8, A lane 1). The identities of GST-calreticulin and the GST-P and -C fusion proteins were confirmed by their reactivity with goat anti-calreticulin antibodies (Fig. 2-7 A, C, D). The identity of the NH_2-terminal domain was confirmed with antibodies made against a synthethic peptide corresponding to in the NH_2-terminus of calreticulin (Fig. 2-7, B). The functional analysis of the fusion proteins suggested that they may be folded correctly (Baksh & Michalak, 1991; and as discussed in Chapter 4 and 5).

In some experiments, the GST moiety was removed from GST-calreticulin by factor Xa to produce full-length mature calreticulin, referred to throughout as recombinant calreticulin (Fig. 2-8, A lane 4). Purified GST-calreticulin was cleaved with factor Xa (Boehringer Mannheim) diluted 1:500 (w:v) in a buffercontaining 100 mM NaCl, 1 mM $CaCl_2$ and 50 mM Tris-HCl, pH 8.0 at

Fig. 2-7. Calreticulin GST-fusion proteins.

The Glutathione S-transferase (GST) fusion system was used to produce and isolate fusion proteins encoding the mature form and distinct regions (domains) of calreticulin. The GST-fusion proteins were expressed in the protease-deficient *E. coli* strain, BNN103, grown in Luria-Bertani (LB) medium containing 50 μg of ampicillin/ml. Cultures were grown to mid-log phase (A_{600} = 0.6-1.0) and then IPTG was added to induce expression of the fusion proteins. The cells were harvested four hours later by centrifugation and then lysed. The GST-fusion proteins were purified from the soluble fractions by Glutathione-Sepharose 4B affinity chromatography. The upper panel shows the SDS-PAGE of cellular extracts containing the fusion proteins and of the purified fusion proteins. In the lower panel these proteins were electrophoretically transferred to nitrocellulose strips for immunostaining. The nitrocellulose strips were incubated with goat anti-calreticulin (1:300) antibody in PBS containing 1% milk powder followed by incubation with anti-rabbit IgG (1:2000) conjugated to horse-radish peroxidase. The peroxidase reaction was developed using chloronaphthol as a substrate. In (A) lane 1 Molecular weight standards: phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (42,700), bovine carbonic anhydrase (31,000) and soybean trypsin inhibitor (21,500); lane 2 purified pancreatic calreticulin; lane 3 cellular extract; lane 4 GST-calreticulin. In (B) lane 1 cellular extract; lane 2 GST-N-domain. In (C) lane 1 cellular extract; lane 2 GST-P-domain. In (D) lane 1 cellular extract lane 2 GST-C-domain. The arrows indicate the purified fusion proteins detected by Coomassie blue or immunostaining.


N-domain
P-domain
C-domain
NH3+
KDEL-COO-

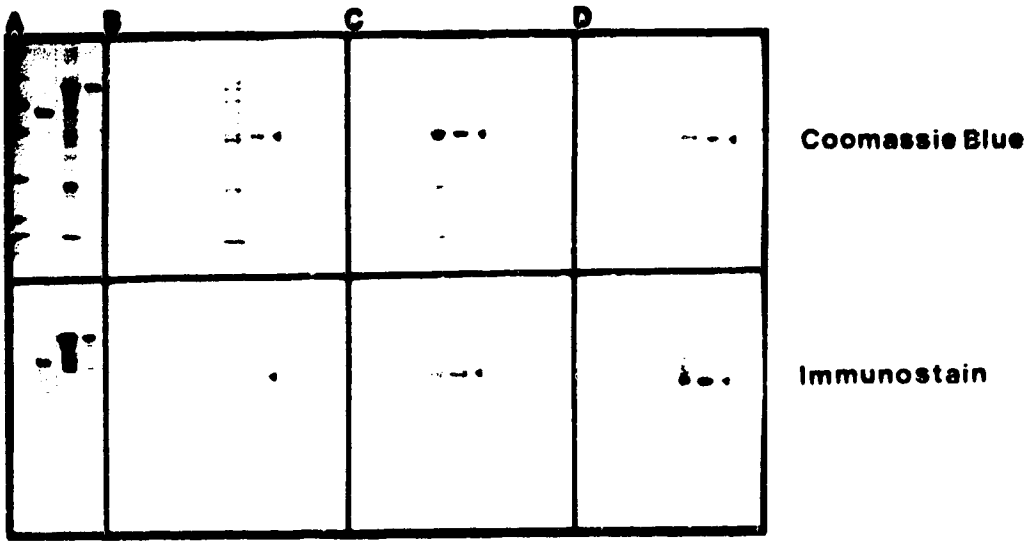
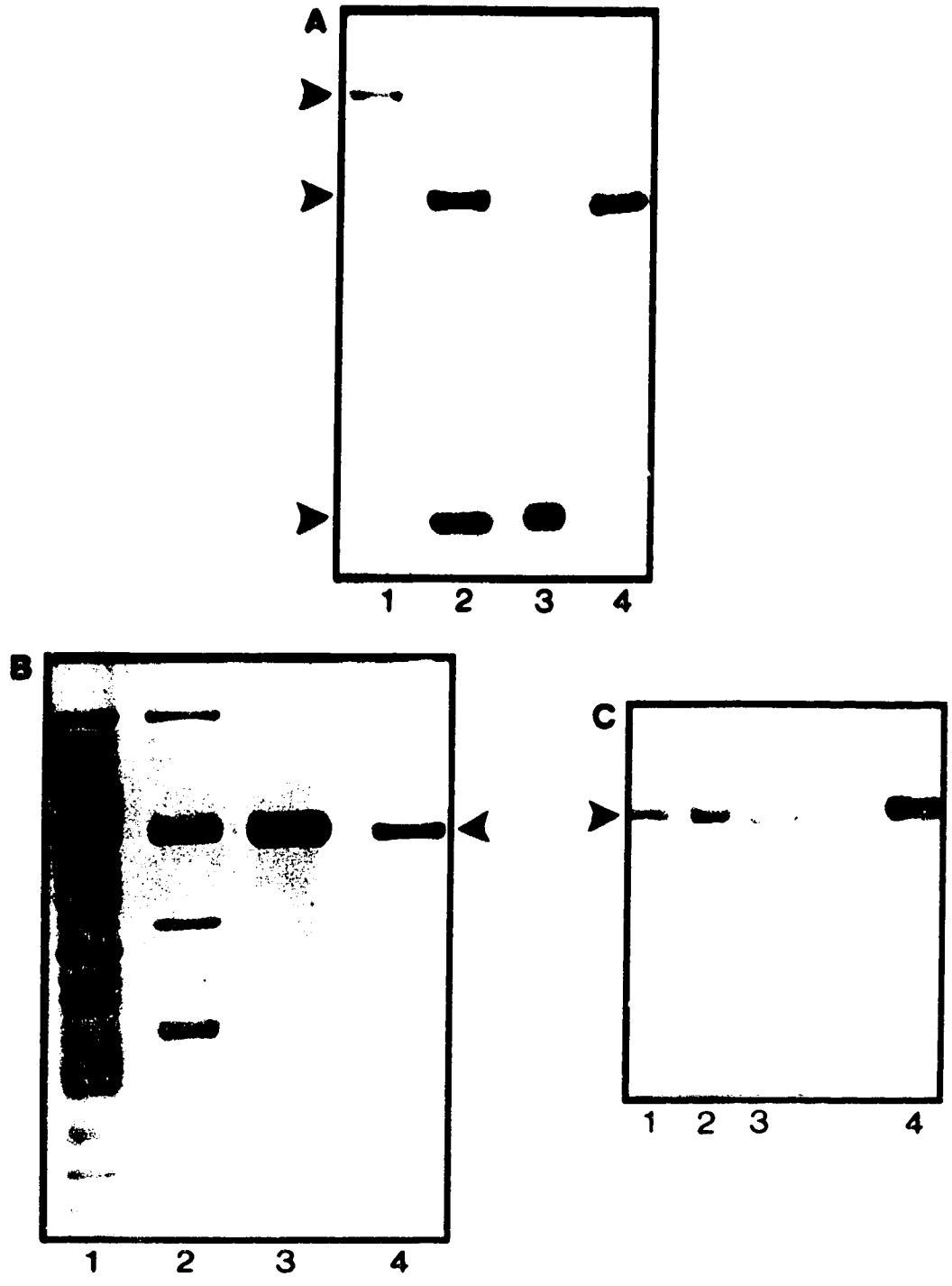

A
B
C
D
Coomassie Blue
Immunostain

Fig. 2-8. Recombinant and native calreticulin.

Calreticulin was expressed as a GST-fusion protein in *E. coli*. The GST-fusion protein was purified to near homogenity from a cellular extract by Glutathione-Sepharose 4B chromatography. In (A) Coomassie blue stained SDS-PAGE of lane 1, GST-calreticulin purified on Glutathione-Sepharose 4B; lane 2, GST-calreticulin fusion protein cleaved with factor Xa; lane 3, recombinant GST; lane 4, recombinant calreticulin purified by Mono Q FPLC. The position of molecular weight marker proteins is indicated by arrow heads (phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (42,700), bovine carbonic anhydrase (31,000) and soybean trypsin inhibitor (21,500). In (B) Coomassie blue stained SDS-PAGE of protein samples taken at various stages in the purification of native reticulin by a selective ammonium sulfate precipation procedure lane 1, 85% ammonium sulfate precipitate; lane 2, DEAE-Sephadex purified calreticulin; lane 3, hydroxylapatite purified native calreticulin; lane 4, purified recombinant calreticulin. The arrow head indicates calreticulin. In (C) the protein samples in (B) were separated by SDS-PAGE and electrophoretically transferred to a nitrocellulose filter. The blots were incubated with goat anti-calreticulin antibodies (1:300) in PBS containing 1% milk powder, followed incubation with anti-rabbit IgG conjuaged to horse-radish peroxidase (1:2000). The peroxidase reaction was developed using chloronaphthol as a substrate. The arrow head indicates calreticulin.


A
1 2 3 4
B
1 2 3 4
C
1 2 3 4

room temperature for 16-18 hours (Fig. 2-8, A lane 2). The reaction was stopped by the addition of PMSF at a final concentration of 2.5 mM. To separate recombinant calreticulin from GST, digested sample was loaded onto the gluthathione-Sepharose 4B column and the flow-through, containing recombinant calreticulin was collected, concentrated, and dialyzed against 50 mM NaCl, 100 mM KH_2PO_4, 1 mM EDTA, pH 7.1. Dialysed sample was applied directly onto a Mono Q FPLC column equilibrated in diaylsis buffer. The column was extensively washed and then bound protein was eluted with a linear salt gradient (0-750 mM NaCl) applied at a flow rate of 1 ml/min for 30 min. The protein eluted in a sharp peak at approximately 300 mM NaCl. SDS-PAGE of the Mono Q FPLC purified protein is shown in Fig. 2-8 (A, B) lane 4. Analysis of purified protein by reverse phase HPLC chromatography revealed only one protein peak further indicating that a high level of purity was achieved (Baksh *et al.*, 1992). Recombinant calreticulin eluted from the Mono Q FPLC column at the same salt concentration as the native protein (Milner *et al.*, 1991) and they had the same mobility in SDS-PAGE, corresponding to an apparent molecular weight of 60,000 (Fig 2-8, B lanes 3, 4). The identity of recombinant calreticulin was further confirmed by NH_2-terminal amino acid sequence analysis. The NH_2-terminal amino acid sequence obtained for the recombinant protein was NH_2-G-I-P-G-N-S-E-P-V-V-Y-F-K-E-Q-F-, where the underlined amino acid sequence corresponds to the amino acid sequence of mature calreticulin (Fig. 1-1) and the first six amino acids (NH_2-G-I-P-G-N-S-) correspond to the amino acid sequence of the pGEX-3X multiple cloning. The recombinant protein also reacted with rat anti-rabbit calreticulin antibodies by Western blot analysis (Fig. 2-8, C lane 4) and bound Ca^{2+} with the same capacity as the native protein (Baksh and Michalak, 1991; Baksh *et al.*, 1992). That the recombinant calreticulin is folded correctly has been supported by C.D. spectrum analysis of recombinant calreticulin. Virtually

identical C.D. spectra were obtained for the tissue purified and recombinant calreticulins (W. McBlain, C. Kay, S. Baksh, M. Michalak, unpublished observations).

Expression and purification of GST-GR

The DNA binding domain (DBD) of the GR was expressed as a GST-fusion protein for the calreticulin:GR studies. cDNA for this region of the receptor (amino acid residues #420 to #506) was synthesized by PCR driven amplication of pOB10 which encodes for the β-form of the human GR. Primers A and B (see Table 2.1) were used as sense and antisense primers, respectively, for PCR. The reaction was carried out for 25 cycles consisting of 1 min. at 94°C, 1 min. at 57°C and 2 min. at 72°C. Amplication was completed by a final incubation at 72°C for 10 min. The PCR product was digested with Bam HI and Eco RI and directionally cloned into Bam HI/Eco RI linearized pGEX-3X to form pGEX-GR.

The GST-GR fusion protein was purified by glutathione-Sepharose 4B affinity chromatography as described for the calreticulin GST fusion protein. Fractions from the glutathione affinity column containing GST-GR fusion proteins were first concentrated using the Amicon concentrator and were then dialysed against a buffer containing 100 mM NaCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 40 mM Tris, pH 7.2. For further purification the GST-GR sample was loaded onto the calreticulin affinity column in the same buffer. The column was washed in buffer containing 5 mM EDTA, 100 mM NaCl, and 40 mM Tris, pH 7.2 and then bound proteins were eluted by a high salt buffer elution (20 mM EDTA, 750 mM NaCl, and 40 mM Tris, pH 7.2).

Cell Lines and Culture Conditions

All cell-lines were maintained in 75 cm^2 tissue culture flasks (Corning, Corning N.Y.) at 37°C with 5% CO_2 in a humidified incubator. COS-1 cells were used to examine the intracellular localization of calreticulin following transient transfection with various calreticulin constructs. The cells were grown in Dulbecco's Modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% fetal calf serum, 5 mM glutamine, 100 µg/ml streptomycin and 100 IU/ml penicillin.

Mouse L-fibroblasts, which express the GR, were used to study the interaction between calreticulin and the receptor. The L-cells were maintained in DMEM supplemented with 10% calf serum, 100 µg/ml streptomycin and 100 IU/ml penicillin. In some experiments the L-cells were stimulated for 24 hours with 1 µM DEX which was added in fresh media containing 10% charcoal-stripped calf serum (i.e. steroid depleted serum). Prior to stimulation the L-cells were grown in DMEM supplemented with 10% charcoal-stripped calf serum for 24-48 hours.

Mouse splenocytes and human peripheral blood lymphocytes were prepared by Cheryl Helgason. The mouse splenocytes were suspended to a density of 5 x 10^5 cells/ml in RHFM buffer consisting of RPMI 1640 (Gibco BRL) supplemented with 10% fetal bovine serum, 20 mM HEPES; pH 7.4, 100 µM 2-mercaptoethanol, 100 µg/ml streptomycin, 100 IU/ml penicillin, 20 units/ml recombinant human interleukin-2 (rhIL-2). The splenocytes were stimulated for up to 7 days by the addition of ConA at a concentration of 10 µg/ml. Cells were removed every day for immunological analysis of protein and for Northern blot analysis of calreticulin mRNA. In separate experiments splenocytes isolated from C57Bl/6 mice were stimulated for a period of 7 days with irradiated Balb/c splenocytes (1:1). This type of stimulation is is referred to as mixed lymphocyte

reaction or MLR. Aliquots were removed on a daily basis for Western blot analysis. On day 6 of MLR stimulation a sample was removed for immunocytochemical analysis (described in a subsequent section). Human peripheral blood lymphocytes were isolated by centrifugation over Ficoll-Pague and resuspended in RHFM buffer as described above except with 500 units/ml of rhIL-2. These lymphocytes were stimulated with Con A for up to 3 days. Each day samples were also taken for Western and Northern blot analyses.

Transfection Procedures

COS-1 cells were transfected with appropriate plasmids using the standard DEAE-dextran (Diethylaminoethyl-Dextran, M.W. 500,00, Sigma) procedure described by Kriegler, (1990). Cells were grown to 75% confluency and seeded in six-well tissue culture dish at a density of 1.92 x10^5 cells/well. The following day, the cells were washed with PBS and then incubated with DNA (0.5 μg)/DEAE Dextran (to give a final concentration of 0.04%) mixture at room temperature for 1 hour. The cells were washed with 3 ml of TBS (137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 25 mM Tris, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$) and 3 ml of PBS. The cells were treated with chloroquine (100 μM) in growth media for 4 hours at 37°C followed by incubation in growth media without chloroquine. Forty-eight hours later the cells were harvested for analysis.

L-fibroblasts were transfected using a Ca^{2+} phosphate procedure (Ausbel *et al.*, 1988). Cells in log phase were seeded onto 10 cm^2 tissue culture dishes at a concentration of 2.5 x10^5 cells/dish and allowed to reattach overnight in DMEM containing 10% calf serum. The next morning the cells were placed in DMEM containing 10% charcoal-stripped serum for 3-4 hours. For each transfection 10 μg of DNA (purified twice by CsCl gradient centrifugation and sterilized by ethanol precipation) was used per 10-cm^2 plate. A Ca^{2+} phosphate-DNA

precipitate was formed in 2X HEPES-buffered saline (280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM dextrose, and 50 mM HEPES) as follows: the DNA was resuspended in 450 µl of sterile water and mixed with 50 µl of 2.5 M $CaCl_2$. The mixture was then added dropwise with bubbling to 500 µl of 2X HEPES-buffered saline in a sterile 15-ml concical tube. The precipitate was allowed to form for 20 min at room temperature and then added dropwise to the L-fibroblasts. The cells were incubated with DNA for 6 hours and then glycerol shocked as follows: the media was removed and 5 ml of a 10% glycerol solution in PBS added to each plate for 2 min. The cells were washed 3 X with 5 ml of PBS and harvested for analysis ~48 hours later.

Electroporation was used for stable transformation of L-fibroblasts. pRC-DT-1 and -2 were electroporated into L-cells to create stable cell lines expressing elevated or reduced amounts of calreticulin. Prior to electroporation the vectors were linearized with Pvu I, sterilized by ethanol precipation and resuspended in a TE. The procedure used for electroporation has been described by Ausbel *et al.* (1988). L-cells were harvested, washed in PBS and resuspended at at density of $1x10^7$ cells/ml in ice-cold 1X HEPES-buffered saline. A 500 µl aliquot of cells was then added to a pre-chilled electroporation cuvette (Bio-Rad electroporation cuvettes #168-2088) together with 10-30 µg of a linearized vector. The DNA/cell mixture was incubated on ice for 5 minutes and then electroporated using the Bio-Rad Gene Pulser at 1500V/cm and a capacitance of 25 µF. Following electroporation the cell/DNA mixture was incubated on ice for 10 min and then added dropwise to a 10 cm^2 tissue culture dish containing 10 mls of non-selective growth medium. The cells were grown for 48 hours in non-selective medium then transfered to media containing 400 µg/ml of G418. G418 was prepared as a 100 mg/ml stock solution in PBS, filter sterilized and stored in 250 µl aliquots at -20°C.

Prior to transfection experiments, G418 sensitivity of L-cells was established. L-cells were seeded at a concentration $1x10^4$ cells/well in a 96 well tissue culture dish and grown in media containing G418 at concentrations ranging from 0 - 1 mg/ml. The viability of these cells was monitored for up to two weeks. The concentration required for selection was determined to be the minimal concentration of G418 required to kill all cells.

Preparation of Cellular Extracts

Cellular extracts were prepared by detergent lysis as described by Harlow and Lane (1988). The cells were washed in PBS, scrapped from the tissue culture dish and collected by centrifugation at 400 xg (Eppendorf centrifuge) for 10 min. The pellet was placed on ice and and resuspended in 1.0 ml (per 10^7 cell) of prechilled lysis buffer containing 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris, pH 7.5, 0.1 mM benzamidine, 0.1 mM PMSF and 1X protease cocktail. The cell suspension was incubated on ice for 30 min with occasional mixing and then spun at 10,000 xg for 10 min at 4°C. The lysate was removed to a fresh tube, frozen quickly and and stored at -20°C until further use.

Luciferase Assays

The cells were harvested for luciferase assays 48 hours after transfection as follows: the cells were washed 2 X with 5 ml of PBS, scraped with a rubber policeman into 1 ml of PBS and pelleted by centrifugation at 400 xg for 5 min. The cells were then lysed for 10 min at room temperature in 150 µl of lysis buffer containing 1% Triton X -100, 25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA and 1 mM DTT (freshly added) (Brasier *et al.*, 1989). The cellular lysate was spun at at 10,000 xg for 5 min at 4° C. The supernatant was collected and covered with foil to prevent inactivation of the light-sensitive luciferase. An

aliquot of the supernatant (10-20 µl) was placed into a Lumat LB 9501 Luminometer (Berthold) and the enzymatic reaction started by addition of 100 µl of a solution containing 470 µM D-luciferin (Sigma), 20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 530 µM ATP and 270 µM Coenzyme A. Light output was measured for 20 sec at 25°C.

RNA Isolation and Northern Analysis

Total cellular RNA was isolated either by a guanidine thiocyanate-cesium chloride gradient method (Chirgwin *et al.*, 1979) or using TRI REAGENT (MRC, Molecular Research Center, Inc.) as directed by the manufacturer.

Northern blots were prepared by denaturing up to 10 µg of RNA for 15 min at 65°C in two volumes of FFMOPS [50% formamide, 6.5% formaldehyde, in a MOPS buffer, (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0)]. Tracker dye was then added and the RNA was size fractionated by electrophoresis in MOPS running buffer through 1% agarose gels containing 0.7% formaldehyde and 0.33 µg/ml ethidium bromide. RNA was transferred for 16-18 hour by capillary action onto Hybond-N nylon membrane (Amersham). The gel was placed face-down on a paper towel in a small volume (the volume used did not exceed half the depth of the gel) of 20X SSC (1X SSC, pH 7.0: 150 mM NaCl, 15 mM sodium citrate) for transfer. A membrane (Hybond-N nylon) was then placed on top of the gel, followed by two sheets of Whatman 3MM paper, two inches of paper towels, a glass plate and a 1 kg weight. After transfer, the membranes were cross-linked in a UV Stratalinker™ 1800 using the autocrosslink setting.

Filters were prehybridized for 16-18 hours at 42°C in hybridization buffer containing 20% or 50% formamide, 5X SSC, 5X Denhardt's (100X Denhardt's: 1% Pharmacia Type 400 Ficoll, 1% polyvinylpyrrolidone, 1% BSA), 50 mM $NaPO_4$,

pH 6.5, 1 mM NaP_4PO_4, 0.1% SDS, 100 μM ATP, 2.5 mM EDTA. Hybridizations were carried out at 42°C in the same buffer for 12 to 18 hours. Unless indicated otherwise, the blots were washed to a stringency of 0.1X SSC, 0.1% SDS at 55°C. Prior to reprobing the filters were stripped in a 0.1% SDS solution at 95°C which was allowed to cool gradually to room temperature.

The DNA probes used for Northern analysis were labeled using the Random Primer DNA Labelling System (BRL) following the recommended protocol. Approximately 50 ng of DNA was labeled at room temperature for 3 to 18 hr with 500 μCi α [^{32}P]-dCTP (specific activity 3000 Ci/mmol). Unincorporated nucleotide was removed using gel filtration. A column was made by plugging a pasteur pipette with siliconized glass wool and adding ~1.5 ml of Sephadex G-50 (medium) equilibrated in TRIS-EDTA buffer, pH 7.4. Sample was eluted from the column with TRIS-EDTA buffer and 200 μl fractions were collected. Fractions 4, 5, and 6 containing radiolabelled DNA were pooled and used without further purification.

As probes for identification of calreticulin mRNA, the ~1.2 kb EcoR1 fragment of pGEX-CRT (Fig. 2-4) or the ~ 700 kb SacI/BamHI fragment of pTZ18μ-CRT (a kind gift of Dr. D. McCauliffe) containing the 5′ portion of the mouse calreticulin cDNA were used. RNA levels were normalized by probing the same blot with either γ-actin, 28S ribosomal RNA (Triemeier *et al.*, 1977), ribosomal protein L32 cDNA (Dudov and Perry, 1984) or human glyceraldehyde-3-phoshate dehydrogenase (G3PDH) cDNA. A plasmid containing C3PDH cDNA was obtained from Dr. Fliegel (University of Alberta). The ~700 bp Xba I fragment of G3PDH was removed and used as a probe. For analysis of cytochrome P450 mRNA, the ~ 1.3 kb Eco RI fragment excised from the plasmid described by Harwick *et al.*(1983) was used.

DNA Mobility Shift Assays

The DNA mobility shifts assays were performed by Brenda Duggan. Two complementary oligodeoxynucleotides containing the GRE were synthesized and used in this assays: 5'-TCC TTG TTT TAA GAA CAG TTA TCG ATT ATA AAC-3' and 5' GTT TAT AAT CGA TAA CGT TTC TTA AAA CAA GGA. The assays was carried out in 20 mM Tris, pH 7.5, 1 mM EDTA, 20 mM NaCl, 0.05% bovine serum albumin, 4 mM DTT, 10% glycerol and 2 μg of Poly-dI:dC12. The proteins and buffer were added together and incubated at 25°C for 15 min. The radiolabeled GRE was then added, the mixture was allowed to incubate for a further 10 min and then the mixture was loaded onto a polyacrylamide gel (5% acrylamide) in 22 mM Tris, pH 7.5, 22 mM boric acid and 0.5 mM EDTA.

Immunofluorescence Microscopy

For immunostaining, (COS-1, L-cells) were plated onto 1 mm coverslips (Fisher) and grown in complete medium overnight or to a density of about ~60%. Cytotoxic T-lymphocytes (CTL) were attached to coverslips previously coated with in a 1 mg/ml poly-L-lysine solution (Harlow & Lane, 1988). For immunostaining of transfected cells, transfections were performed on cells that had been plated onto coverslips. The cells were fixed for 12 min with 3.8% formaldehyde in PBS and then washed with PBS. COS-1 cells and CTL were permeablized with 0.1% saponin (Sigma) in PBS (2 X 5 min) followed by incubation in PBS/0.1% saponin/2% milk powder (PBS-S-M) buffer for 30 min. L-cells were permeabilized by incubation in 0.1% Triton-X-100, 100 mM PIPES, 1 mM EGTA, 4% [w/v] polyethylene glycol 8000, pH 6.9 for 3 min. The cells were then incubated with primary antibodies in the PBS containing 1% milk powder (PBS-M) for 1 hour at room temperature or for 30 min at 37°C. Polyclonal goat anti-calreticulin antibodies and anti-DT antibodies were used at a 1:50 and 1:20

dilutions, respectively. Following incubation with primary antibodies, the cells were washed in PBS-M and incubated for 1 hour with Cyc™- labelled secondary antibodies diluted 1:70 in PBS-M. The cells were washed 3X in PBS (5 min) and mounted onto glass slides in TESTOG™ FITC-guard (Testog Inc., Chicago, IL) to prevent photobleaching. The edges of the coverslips were sealed with nail polish for storage of the slides at 4°C.

A Zeiss Photomicroscope III equipped with an epifluorescence condenser and selective FITC/TRITC filter combinations was used for observation and photography. Photographs were recorded on TMax-400 negatives (Kodak). Confocal microscopy was carried out by Dr. M. Opas (University of Toronoto) using Bio Rad MRC-600 microscope.

CHAPTER THREE

CALRETICULIN IN T-LYMPHOCTYES

A version of this Chapter has been published:

Burns, K., Helgason, C. D., Bleackley, R. C. and Michalak, M. 1992. **Calreticulin in T-lymphocytes**. J. Biol. Chem. 267:19039-19042.

INTRODUCTION

Changes in the levels of free Ca^{2+} have been shown to be important in signal transduction in a variety of cellular systems. Many cellular stimuli exert their effects, at least in part, by raising the Ca^{2+} concentration in the cytoplasm. This leads to activation of a variety of Ca^{2+}-dependent enzymes that control many cellular responses (Carafoli, 1987). Similarly, when the antigen receptors on T-lymphocytes are stimulated by either mitogen, cross-linking antibodies or their cognate ligands, a rise in cytosolic Ca^{2+} is also observed (Hesketh *et al.*, 1983; Gelfand *et al.*, 1984; for review see Gardner, 1989). This increase of intracellular Ca^{2+} concentration is believed to be a key step in T-lymphocyte stimulation because it results in the activation of a variety of Ca^{2+}-dependent kinases and phosphatases. Depending on its state of maturation, a cytotoxic T-lymphocyte (CTL) is activated to proliferate or to lyse target cells.

Antigen binding to precursor T-cells initiates the signals required to induce proliferation and maturation in the presence of the appropriate growth factors. Phospholipase C becomes activated which hydrolyses phosphatidylinositol 4, 5-bisphosphate into diacylglycerol (DAG) and $InsP_3$ (Gardner, 1989; Taylor *et al.*, 1984; Berridge, 1990 & 1993). DAG stimulates protein kinase C resulting in the phosphorylation of a number of intracellular proteins. $InsP_3$ induces a release of Ca^{2+} from the ER-associated intracellular store and may also lead to the enhanced Ca^{2+} influx via plasma membrane Ca^{2+} channels resulting in elevated Ca^{2+} levels. Recent work from several laboratories suggests that the Ca^{2+} content of an internal Ca^{2+} store may affect the Ca^{2+} permeability of the plasma membrane (Putney, 1990; Mason *et al.*, 1991; Clementi *et al.*, 1992; Demaurex *et al.*, 1992). This coupling mechanism may be important in mediating the effects of mitogen and other agonists in the control of

Ca^{2+} homeostasis in T cells. Therefore, intracellular Ca^{2+} stores appear to be important sources of Ca^{2+} during T cell activation. Ca^{2+}-binding proteins, especially those associated with intracellular Ca^{2+} stores, would be expected to play a major role in controlling intracellular Ca^{2+} concentrations.

In addition to its role in CTL signal transduction, Ca^{2+} is also thought to be important in the actual process of target cell destruction (Berke *et al.*, 1989; Henkart *et al.*, 1988). As a T cell matures, it acquires cytolytic effector molecules which become localized to lytic granules within the cytoplasm of the CTL. The vectoral release of these granules towards a bound target cell is strictly Ca^{2+} dependent, as is the polymerization of the pore forming protein perforin, a granule protein believed to be important in target cell lysis (reviewed in Tschopp *et al.*, 1990).

The importance of Ca^{2+} in the signal transduction process of CTL, as well as in various stages of the lytic pathway suggests that Ca^{2+}-binding proteins would be expected to be of fundamental importance to the normal functioning of these cells. As calreticulin is believed to be a major Ca^{2+}-binding protein in many cell types, the experiments described in this chapter were designed to investigate the expression of this protein during the activation of T lymphocytes.

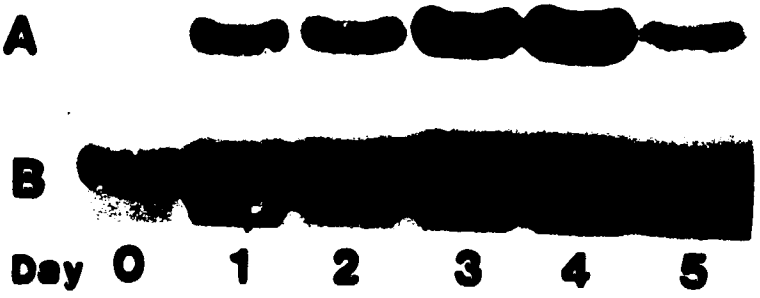
RESULTS

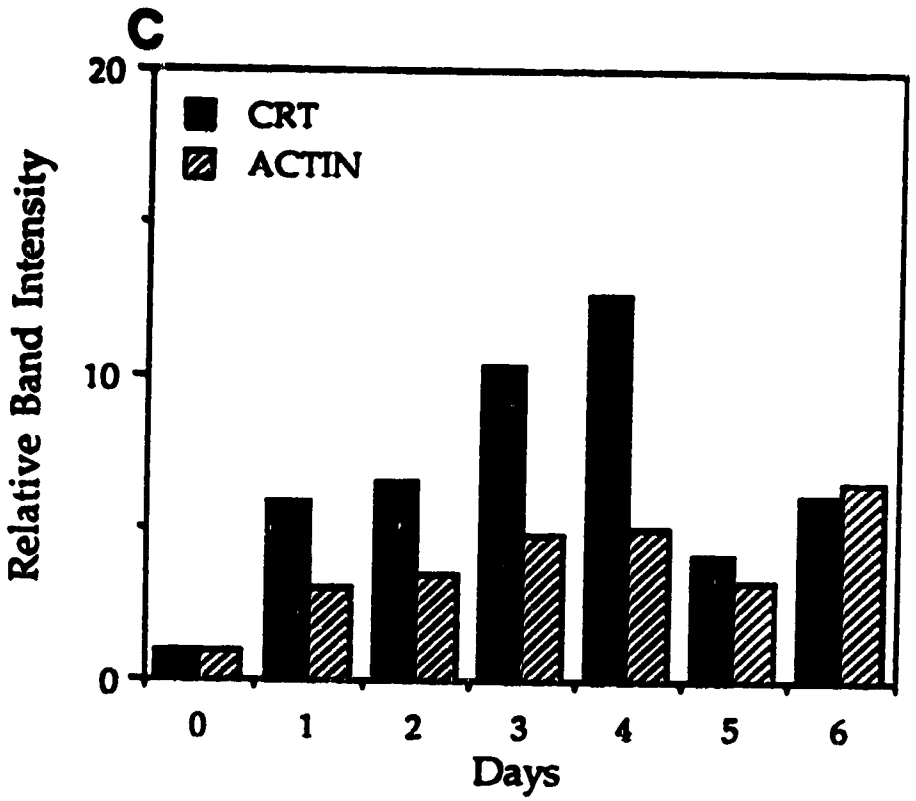
Expression of calreticulin in mouse splenocytes stimulated with ConA

Each day over a period of 6 days, total cellular RNA was isolated from ConA stimulated mouse splenic cells and subjected to Northern blot analysis. Resting lymphocytes contain very low to undetectable levels of calreticulin mRNA (Fig. 3-1, A Day 0). However, in stimulated splenocytes the level of calreticulin mRNA steadily increased over a period of days (Fig. 3-1, A Day1-5).

Fig. 3-1. Induction of calreticulin mRNA in mouse splenocytes stimulated with ConA.

Total cellular RNA was purified from resting (lane 1) and ConA stimulated (lanes 2-6) lymphocytes. RNA was electrophoretically separated on a formaldehyde-agarose gel, blotted onto Hybond-N nylon filters and hybridized to cDNA encoding full-length rabbit calreticulin at 42°C in FFMOPS buffer containing 20% formaldehyde for 18 hours (**A**). The filter was washed in 0.1X SSC, 0.1% SDS at 55°C, then stripped and reprobed with γ-actin (**B**). The conditions used for hybridization and washing of the γ-actin cDNA probe were the same as those used for the calreticulin cDNA probe. The region of the autoradiogram shown contains all hybridizable RNA species. The abundance of calreticulin and γ-actin mRNA was determined by densitometry scanning of blots (**A**) and (**B**) and is shown in (**C**).


A
B
Day 0 1 2 3 4 5


C
20
10
0
CRT
ACTIN
Relative Band Intensity
0 1 2 3 4 5 6
Days

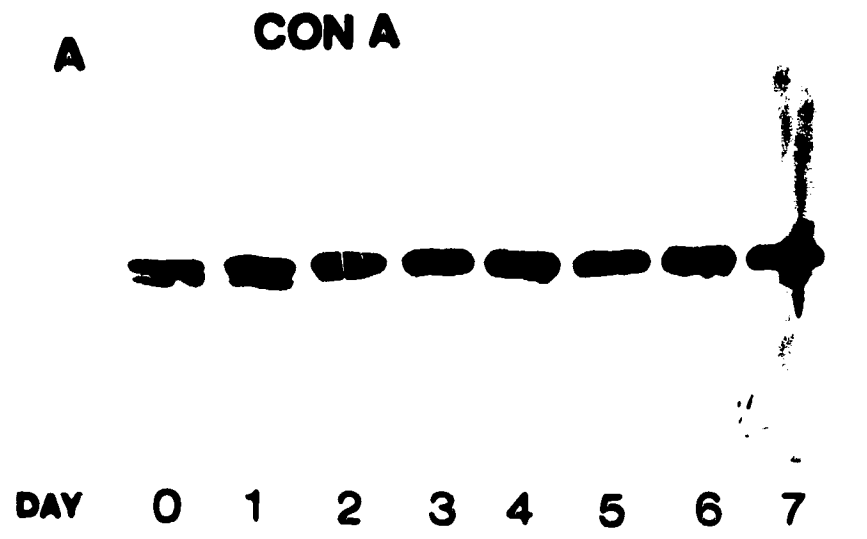
The expression of calreticulin mRNA was maximal by Day-4 and then sharply declined by Day-5 (Fig. 3-1, A). The blots were also probed with γ-actin cDNA so that the relative amounts of the calreticulin mRNA could be compared. (Fig. 3-1, B). The relative abundance of calreticulin and γ-actin mRNA was quantitated by densitometry. Figure 3-1 (C) shows that the mRNA encoding calreticulin was increased in stimulated cells (Day 2-5) and that the expression of this mRNA was maximal by Day-4. There was approximately a 2-fold increase in calreticulin mRNA from Day-1 (Day-0 levels of calreticulin mRNA were almost undetectable) to Day-4 (Fig. 3-1, C). In three separate experiments this increase was found to be 3.2 ± 1.3-fold. In contrast the γ-actin mRNA increased by less than 1.6-fold in the same period of time. In a separate experiment, in addition to calreticulin and γ-actin, a Northern blot was probed with 28S ribosomal RNA, ribosomal protein L32 cDNA and G3PDH cDNA probes (data not shown). When the mRNA levels for each of these were compared with those for calreticulin on Day-1 and Day-4 of stimulation, calreticulin mRNA was found to be increased 6-fold compared with only 2-, 3-, 3- and 4-fold for γ-actin, 28S ribosomal RNA, ribosomal protein L32 and G3PDH mRNAs, respectively.

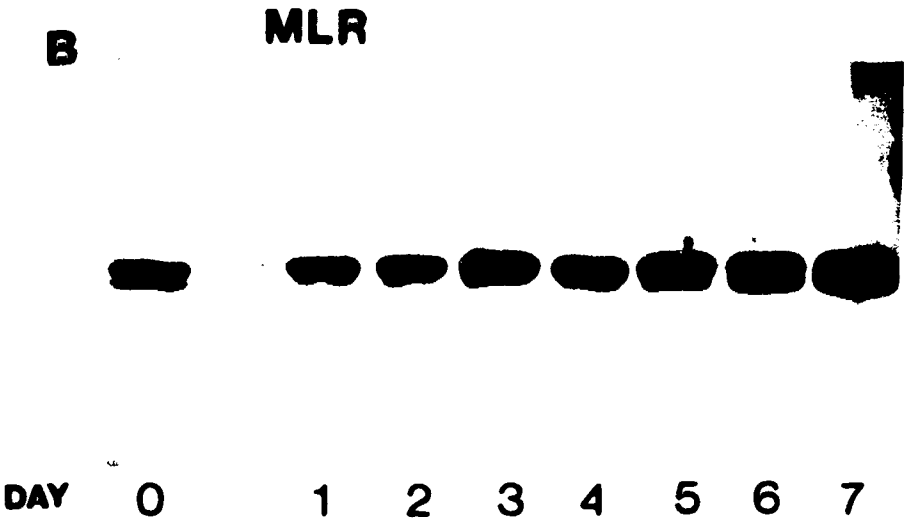
Immunoblotting analysis of calreticulin

In order to establish if the increased levels of the calreticulin mRNA lead to a concomitant increase in the amount of the protein synthesized Western blot analysis was employed. Cellular extracts from resting and stimulated mouse splenocytes were analyzed for the presence of calreticulin using specific goat anti-calreticulin antibodies. 60-kDa and ~62-kDA immunoreactive proteins were detected in lymphocytes probed with anti-calreticulin antibodies (Fig. 3-2, A Day 0-1). When mouse lymphocytes were stimulated with ConA, a significant increase in the amount of immunoreactive calreticulin was observed (Fig. 3-2, A

Fig. 3-2. Western blot analysis of calreticulin in resting and stimulated mouse splenocytes.

Cellular extracts of resting and ConA (**A**) or MLR (**B**) stimulated mouse splenocytes were prepared by detergent lysis. The protein concentration of each extract was determined by the method of Lowry *et al.* (1951). Cellular extracts (20 μg/lane) were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. The membranes were incubated with goat anti-calreticulin antibody (1:300 dilution) in PBS containing 1% milk powder followed by incubation with rabbit anti-goat IgG (1:2000 dilution) conjugated with horseradish peroxidase. The Western blots were developed using the Amersham ECL detection system. In (**A**) Day O, resting lymphocytes; Day 1-7, Con A stimulated lymphocyte. In (**B**) Day 0, resting lymph[illegible] Day 1-7 MLR stimulated lympyhocytes.


A
CON A
DAY 0 1 2 3 4 5 6 7


B
MLR
DAY 0 1 2 3 4 5 6 7

Day 1-7). The amount of immunoreactive protein in stimulated cells was estimated by densitometry at day 4 (Fig. 3-2, A) (maximal level of mRNA) to contain ~4.0 ± 2.0 (3) fold greater levels of proteins than resting cells (Day 0). Only the 60-kDa protein was induced over the seven day time course of stimulation with ConA. (Fig. 3-2, A Day 1-7).

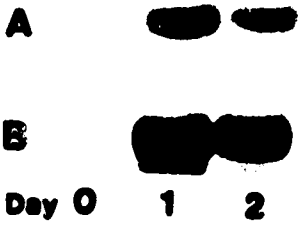
Cellular extracts from splenocytes that had been stimulated by mixed lymphocyte reaction (MLR) were also analyzed for the presence of calreticulin (Fig. 3-2, B). Both in resting (Fig. 3-2, B Day 0) and stimulated cellular extracts (Fig. 3-2, B Day 1-7) two immunoreactive bands were detected. Although there was an increase in both the upper and lower bands relative to unstimulated cells the lower band was the major species induced.

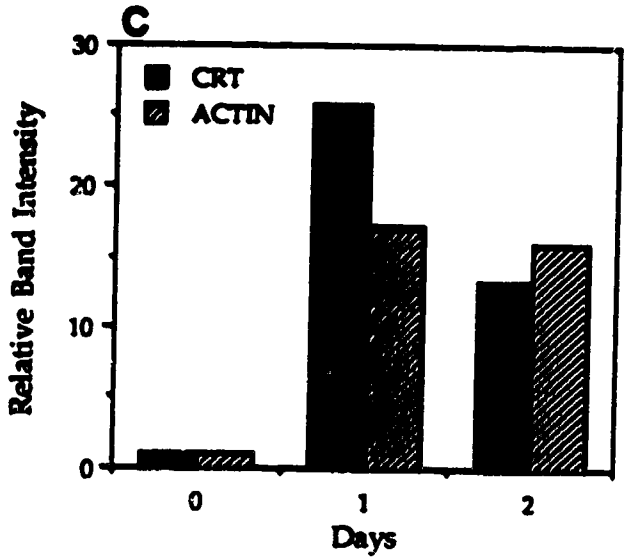
Expression of calreticulin in human T cells

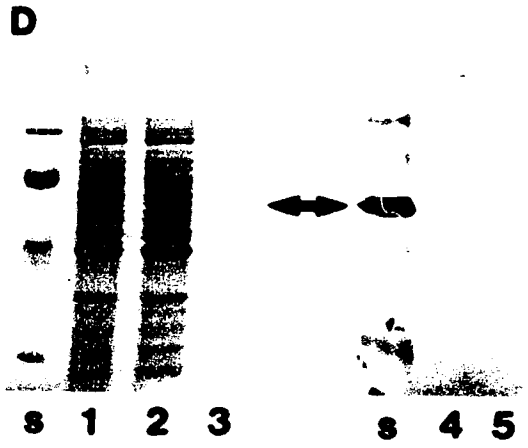
Resting and stimulated human peripheral blood lymphocytes were also tested for the presence of calreticulin mRNA and protein. In unstimulated human peripheral blood lymphocytes (Day-0) calreticulin mRNA was virtually undetectable (Fig. 3-3, A & C), however, when the lymphocytes were cultured with ConA significant levels of the calreticulin mRNA became apparent (Fig. 3-3 A & C Day 1, 2). The induction profile was relatively rapid as calreticulin mRNA was decreased slightly by Day-2 (Fig. 3-3, A & C). Comparison of the levels of calreticulin mRNA in Day-0 (levels were almost undetectable) with those in Day-1 revealed approximately 17.6 ± 5.6-fold increase (n=3). The increased level of calreticulin mRNA was observed in the stimulated human T cells regardless of whether blots were normalized with γ-actin (Fig. 3-3, B), 28S ribosomal RNA, ribosomal protein L32 cDNA or G3PDH cDNA probes (not shown). Synthesis of calreticulin protein in stimulated human peripheral blood lymphocytes, as

Fig. 3-3. Induction of calreticulin mRNA and protein in Con A stimulated human lymphocytes.

Total cellular RNA was isolated from resting (Day O) and ConA stimulated lymphocytes (Day 1 and 2). RNA was electrophoretically separated on a formaldehyde-agarose gel, blotted onto Hybond-N nylon filters and probed with rabbit full-length calreticulin cDNA (**A**) at 42 °C in FFMOPS buffer containing 20% formaldehyde for 18 hours. The filter was washed at 55°C in 0.1X SSC, 0.1% SDS and then stripped prior to reprobing with γ-actin cDNA (**B**). The conditions used for hybridization and washing of the γ-actin cDNA probe were the same as those used for the calreticulin cDNA probe. The portion of the autoradiogram shown contains all hybridizing RNA species. The abundance of calreticulin and γ-actin mRNA was determined by densitometry scanning of blots (**A**) and (**B**) and is shown in (**C**). In (**D**) cellular extracts of resting and stimulated lymphocytes were prepared by detergent lysis and the protein concentration of each extract was determined by the method of Lowry *et al.*, (1951). The cellular extracts (20 μg of protein/lane) were separated by SDS-PAGE. Gels were stained with Coomassie Blue (lanes 1-3) or transferred electrophoretically to nitrocellulose membranes for Western blot analysis (lanes 4-5). The blots were incubated with goat anti-calreticulin antibodies (1:300 dilution) in PBS containing 1% milk powder followed by incubation with rabbit anti-goat antibodies (1:2000). The peroxidase reaction was developed using chloronaphthol as a substrate. Lane S, molecular weight marker proteins phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (42,700), carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500). Lane 1, 4, resting lymphocytes; lane 2, 5, ConA stimulated T-lymphocytes, Day-2; lane 3 purified pancreatic calreticulin (2 μg), and in lane S for Western blot analysis- purified calreticulin was co-electrophoresed with the molecular weight protein standards. The arrow indicates calreticulin.


A
B
Day 0 1 2


C
CRT
ACTIN
Relative Band Intensity
30
20
10
0
0
1
2
Days


D
s 1 2 3
s 4 5

determined by Western blotting, was also increased (3.1 ± 1.2-fold; n=3) (Fig. 3-3, D lane 5).

Immunocytochemical localization of calreticulin in CTL

The intracellular localization of calreticulin was examined on day 6 of MLR stimulation of CTL. Confocal microscopy of CTL stained with anit-calreticulin antibodies revealed that calreticulin was localized almost exclusively to the lytic granules (Fig. 3-4).

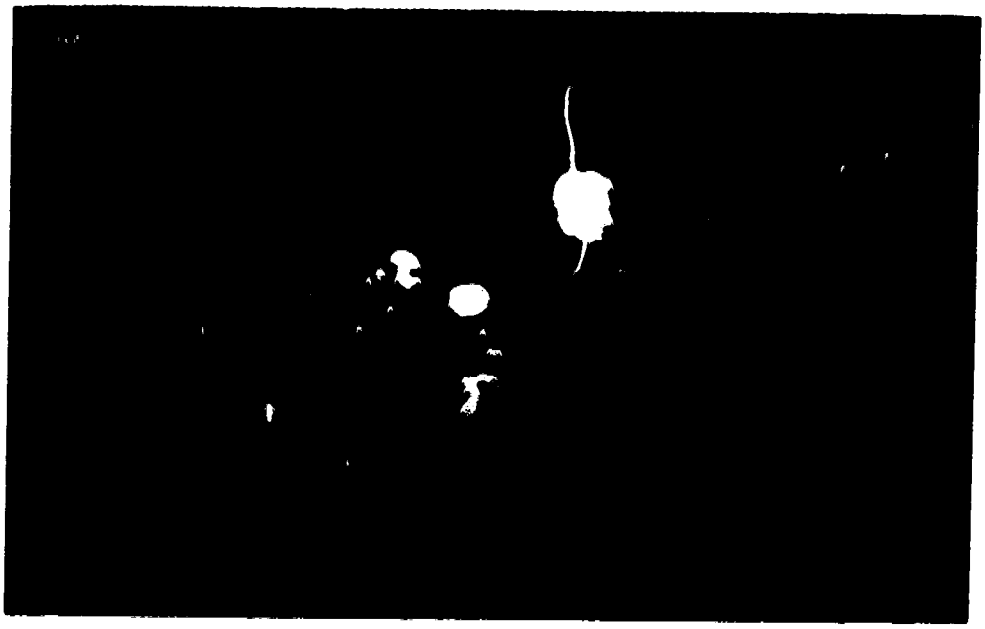
DISCUSSION AND CONCLUSIONS

Calreticulin mRNA and protein are present in mouse and human T-lymphocytes and are significantly induced in cells stimulated with ConA or MLR. Immunocytochemistry of MLR stimulated mouse splenocytes with anti-calreticulin antibody revealed the presence of an immunoreactive protein in the lytic granules of these cells. Interestingly, by immunocytochemistry calreticulin appeared to be solely in the lytic granules and virtually absent from the ER.

Synthesis of specific mRNA and protein is one of the major biochemical events during the lymphocyte activation process (Cooper and Braverman, 1981; Ashman, 1984). In cell lines representing various stages of B-lymphocyte development (Munro and Pelham, 1986) or in B-cells induced to differentiate, elevated expression of several proteins of the K-D-E-L family has been reported (Haas and Wabl, 1983; Lewis *et al.*, 1985; Mazzarella and Green, 1987; Mazzarella *et al.*, 1990). No comparable information has previously been available concerning expression of K-D-E-L proteins in resting or stimulated T-lymphocytes. The increased synthesis of calreticulin reported here may be related to a number of possible roles for calreticulin in T-cells.

Fig. 3-4. Immunocytochemical localization of calreticulin to cytolytic granules.

Mouse splenocytes were stimulated by MLR. On day 6 of stimulation, cells were removed and placed onto a poly-lysine coated glass coverslip. The attached cells were fixed in 3.7% formaldehyde for 10 min, followed by permeablization with 0.1% saponin in PBS containing 1% milk powder. The cells were incubated with goat anti-calreticulin antibodies (1:50 dilution) followed by incubation with rabbit anti-goat Cyc™ conjugated secondary antibody (1:75 dilution). Confocal microscopy was carried out by Dr. M. Opas (University of Toronoto) using Bio Rad MRC-600 microscope. The upper photograph has been taken using phase contrast and the lower panel shows immunoreactive calreticulin staining localized to the granules.



Arber *et al.* (1992) have shown that calreticulin and s-cyclophilin co-localize in the same intracellular compartment. Cyclophilin has been shown recently to interact with the Ca^{2+}-binding polypeptides calcineurin (Ca^{2+}/calmodulin-activated phosphatase) and calmodulin (Liu *et al.*, 1991; Friedman *et al.*, 1991). Calreticulin together with these proteins may thus play a critical role in signal transduction in stimulated T cells.

The mitogen induced increases in calreticulin mRNA and protein may relate to calreticulin's potential Ca^{2+} storage role. Stimulation of CTL leads to elevated intracellular Ca^{2+} levels. Changes in intracellular Ca^{2+} concentration appear to be an obligatory step in the metabolic cascade leading eventually to DNA synthesis and cell division (Hesketh *et al.*, 1983; Gelfand *et al.*, 1984; Lederman *et al.*, 1984; Ashman, 1984). Therefore, increased amounts of calreticulin could conceivably increase the Ca^{2+} storage capacity of the ER and in turn modulate activities of both the ER Ca^{2+}-ATPase and $InsP_3$-sensitive Ca^{2+} release pathways. Rapid and efficient sequestration of Ca^{2+} in the lumen of the ER by calreticulin could lead to the increased Ca^{2+} uptake by ER Ca^{2+}-ATPase, dramatically enhancing the removal of Ca^{2+} from the cytoplasm. Increased Ca^{2+} capacity of internal stores may have additional consequences for Ca^{2+} homeostasis in T cells. As discussed above, studies in various cell types, including T-lymphocytes, have suggested a correlation between filling of intracellular Ca^{2+} pools and Ca^{2+} entry across the plasma membranes (Clementi *et al.*, 1992). Thus, according to this model of Ca^{2+} entry, the filling state of the intracellular Ca^{2+} stores determines the permeability of the plasma membranes. The Ca^{2+} capacity of internal Ca^{2+} stores could depend on the availability of calreticulin. Therefore, it is possible that the expression of calreticulin may be directly related to lymphocyte proliferation and/or activation both of which are signalled by increases in intracellular Ca^{2+} concentrations.

Although virtually undectable at day 0 in mouse splenocytes, calreticulin mRNA increased dramatically with mitogen stimulation and steadily increased over a period of days, plateaued and then declined. Corresponding increases in the protein were observed with mitogen stimulation, however the protein levels remained elevated despite the decline in mRNA level at day 5. Two immunoreactive protein bands were detected by Western blot analysis in cellular extracts from resting cells and day 1 and 2 ConA stimulated cells. Elevated levels of calreticulin were also detected by Western blot analysis in cellular extracts from mouse splenocytes stimulated by MLR. Interestingly, in cellular extracts from MLR stimulated mouse splenocytes, the two immunoreactive calreticulin protein bands were differentially induced. In contrast to ConA stimulated cells, both the upper and lower protein bands were significantly induced over the time course of MLR stimulation.

The identity of these two immunoreactive bands is not clear at present. Dupuis *et al.* (1993) also detected two protein bands in granules isolated from human lymphokine activated killer cells and mouse CTL cell lines which were immunoreactive with anti-calreticulin antibodies raised against a peptide encompassing the NH_2-terminus of calreticulin. It is possible that the two protein bands represent differentially modified forms of calreticulin. For example, the bands could represent differentially glycosylated forms of calreticulin. Mammalian calreticulins have one potential site for N-linked glycosylation and in rat liver calreticulin is reported to have a complex type of oligosaccharide moiety (discussed in Chapter 1). Dupuis *et al.* (1993) has shown that calreticulin interacts with perforin, an important component of lytic granules. Based on its complex pattern of glycosylation, perforin is believed to pass through the trans-Golgi en route to lytic granules (Tschopp *et al.*, 1990; Podack *et al*, 1991). Therefore, it is conceivable that if calreticulin interacts with

perforin in the ER and migrates with it through the secretory pathway, including the trans Golgi, that calreticulin may also acquire a complex type of oligosaccharide moiety. Analysis of possible post-translational modification(s) of calreticulin requires further experiments.

An exciting observation is the recent localization of calreticulin to the lytic granules of stimulated CTL (Dupuis *et al.*, 1993). Like many of the other molecules that are packaged within granules, calreticulin's synthesis may be specifically upregulated and concomitant with the ability of the CLT to lyse its target cell. Calreticulin may thus be synthesized as a key component of the granules which is important for their cytotoxic activity (Bleackley *et al.*, 1994).

A current view of target cell destruction is that the CTL delivers a lethal hit by vectorially secreting the contents of its granules into the intracellular space at the site of contact with a bound target cell (Henkart *et al.*, 1988; Young *et al.*, 1989; Podack *et al.*, 1991). Packaged within the granules are a number of proteins including typical lysosomal enzymes, a pore forming protein called perforin, a family of serine proteinases and proteoglycans such as chondroitin sulfate A Perforin is the only molecule within the granules for which independent lytic potential has been directly demonstrated (Shiver and Henkart, 1991). In the presence of Ca^{2+}, perforin is thought to effect target lysis by polymerizing on target cell membranes into cylindrical amphipathic pore structures (reviewed in Podack *et al.*, 1991). Calreticulin is associated with perforin in equimolar amounts, suggesting that it is also relatively abundant in the lytic granules (Dupuis *et al.*, 1993). Like other components of the granules, which are excocytosed toward the target cell, calreticulin was also detected in the media after activation of CTL (Dupuis *et al.*, 1993).

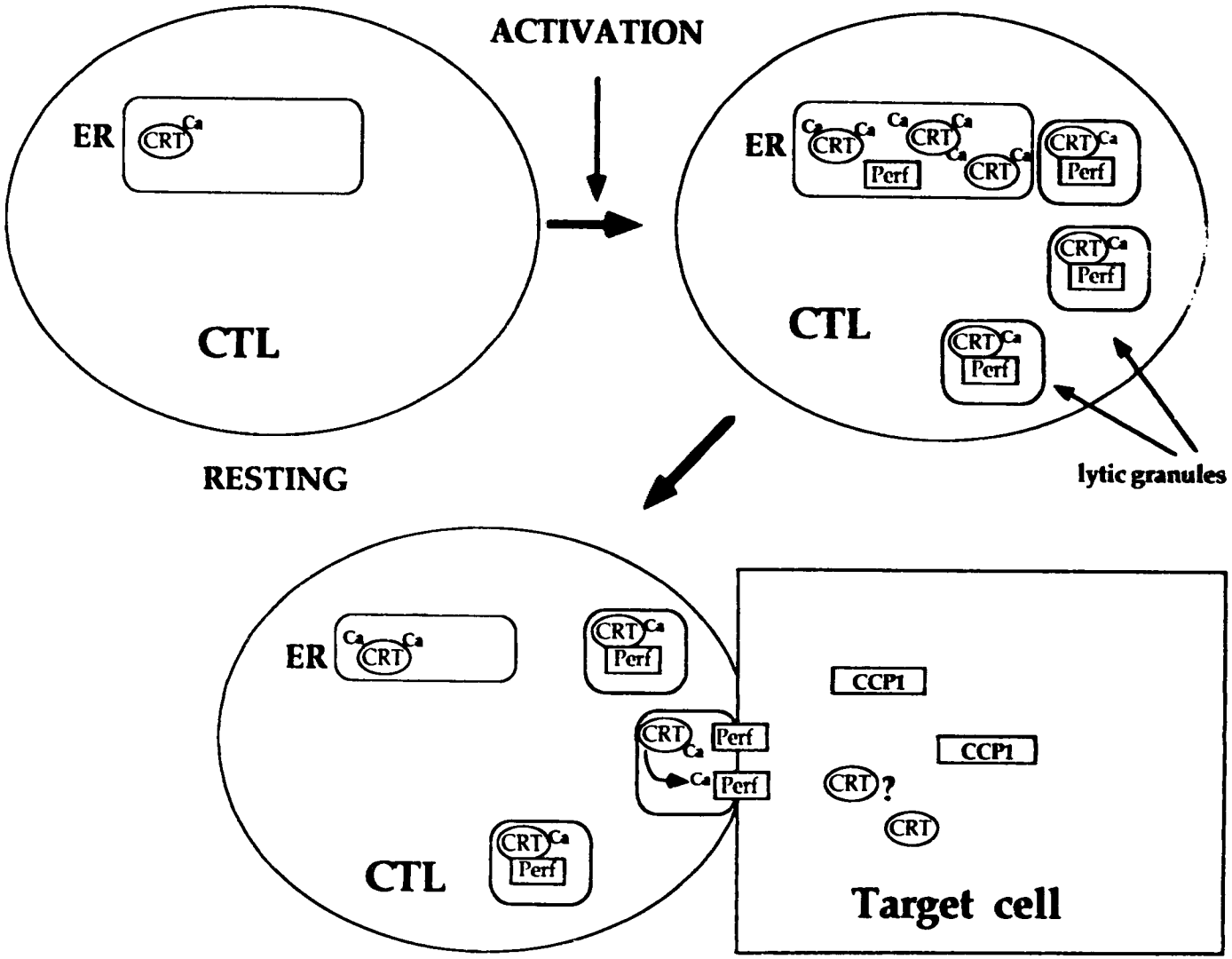
Association between calreticulin and perforin may play a critical role in perforin synthesis and function. Dupuis *et al.* (1993) and Bleackley *et al.* (1994)

proposed that calreticulin may play a dual function in CTL: it may function as a chaperone for perforin and it may sequester Ca^{2+} to prevent inappropriate polymerization and pore formation of perforin during synthesis or transport through the secretory pathway to the granules. Bleackley *et al.* (1994) also proposed that calreticulin may help protect the CTL from perforin lysis by sequestering any excess unpolymerized perforin that might subsequently polymerize and damage its own cellular membrane. Finally, calreticulin may also be an initial source of "activated" Ca^{2+} for perforin function, as perforin polymerization in strictly Ca^{2+} dependent.

We have established that the expression and synthesis of calreticulin becomes elevated in stimulated mouse and human lymphocytes and that calreticulin becomes localized to lytic granules in mature CTL, as schematically illustrated in Fig. 3-5. Although the Ca^{2+}-binding/storage properties of calreticulin likely contribute to Ca^{2+} homeostasis in, and proliferation and maturation of CTL, we believe that the increased synthesis and expression of calreticulin in T-cells may play additional roles. Calreticulin's localization to the granules and its association with perforin is more consistent with an important role for the protein in mediating cytolytic activity. As perforin has been clearly demonstrated to function in the lytic process, calreticulin may have a role in mediating target cell death by ensuring that perforin is able to carry out this role. As discussed above, calreticulin may act as a chaperone for perforin and/or as an initial source of Ca^{2+} required for perforin polymerization and its subsequent integration into the target cell membrane. Following release of the contents of the granules toward the target cell, an intriguing further possibility is that calreticulin may gain access to cytoplasm of the target cell by being injected directly into it (Fig. 3-5). In this way the Ca^{2+} binding properties of calreticulin could be exploited by CTL to participate in the destruction of target cells, a

Fig. 3-4. Model for the role of calreticulin in cytotoxic T-cells.

In resting T-cells, calreticulin is localized to the ER. When these cells are activated the protein is overexpressed and trafficked into cytolytic granules. In the granules calreticulin is proposed to be associated with perforin (Dupuis *et al.*, 1993). The association between calreticulin and perforin suggests at least two possible roles for calreticulin in the lytic process: calreticulin may act as a chaperone for perforin and/or as an initial source of Ca^{2+} required for perforin polymerization and its subsequent integration into the target cell membrane. It is possible that calreticulin along with other components of the lytic granules, such as the serine proteinases, may gain access to cytoplasm of the target cell by being injected directly into it. In this way the Ca^{2+} binding properties of calreticulin could be exploited by CTL to participate in the destruction of target cells, a process known to be Ca^{2+} dependent. Additionally, calreticulin could facillitate target cell death by interacting with and possibly inhibiting the activities of cellular proteins in the target cell, including for example proteins such as the steroid receptors (see Chapter 5).


ACTIVATION
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CHAPTER FOUR

DETECTION OF CALRETICULIN-BINDING PROTEINS

A version of this Chapter was published:

Burns, K., and M. Michalak. 1993. **Interactions of calreticulin with proteins of the endoplasmic and sarcoplasmic reticulum membranes**FEBS Lett. 318: 181-185.

INTRODUCTION

It has been suggested that calreticulin may function as a major Ca^{2+} binding protein in the lumen of the ER (Koch, 1990; Milner *et al.*, 1991). Localization of the protein to the ER (the site of synthesis and processing of membrane and secretory proteins) might also allow calreticulin to interact with other cellular proteins and perform other functions in addition to Ca^{2+} binding/ storage in the lumen of the ER. Therefore is calreticulin more than just a Ca^{2+} binding protein in the ER?

Support for the idea that calreticulin might interact with other ER proteins was orginally provided by the observation of Guan *et al.* (1991). While examining the properties of lung flavin-contaning mono-oxygenase (FMO), an enzyme that catalyses the NADPH-dependent oxidation of drugs, chemicals, and pesticides containing nitrogen, sulphur, and phosphorus, they discovered that FMO formed a tight complex with calreticulin.

Calreticulin is only one of a group of proteins, as discussed previously, that is reported to bind Ca^{2+} (Macer & Koch, 1988, Nuygen Van *et al.*, 1989; Milner *et al.*, 1991). Many of the other proteins (BiP, GRP94, ERp72, ERp61 and PDI) have also been implicated in additional ER functions (Gething & Sambrook, 1992; Noiva & Lennarz, 1992; Rupp *et al.*, 1994). For example, BiP is known to associate with nascent proteins and assist in their assembly and folding (reviewed in Gething & Sambrook, 1992). It can also block the transport of improperly assembled or folded proteins from the ER by binding stably to them (Kassenbrock *et al.*, 1988; Suzuki *et al.*, 1991). PDI is believed to catalyze disulfide bond formation of newly synthesized proteins and to be involved in various other activities within the ER (reviewed in Freedman, 1989; Noiva & Lennarz, 1992). ERp61 and ERp72 are recently identified members of the PDI

family of proteins (Lewis *et al.*, 1986; Mazzarella *et al.*, 1990). In addition to PDI-like activity, these proteins may also associate with other proteins to perform additional cellular roles (Johnson *et al.*, 1992; Gunther *et al.*, 1993; Schaiff *et al.*, 1992; Rupp *et al.*, 1994).

Growing evidence suggests that Ca^{2+} binding may be intimately related to the ability of these lumenal ER proteins to bind to and dissociate from other cellular proteins. For example, BiP's interaction with T-cell receptor α chain variants, which are stably retained within the ER, is Ca^{2+} dependent (Suzuki *et al.*, 1991). As well, Nigam *et al.* (1994) have recently shown that PDI, ERp72, ERp61 and calreticulin bind to unfolded proteins *in vitro*, and that their release from these proteins is Ca^{2+} and ATP dependent. Calnexin a recently identifed ER chaperone is also reported to be a major Ca^{2+} binding protein (Wada *et al.*, 1991). Interestingly, calnexin has certain regions of amino acid sequence identity with calreticulin (Wada *et al.*, 1991). Based on these observations, it is possible that calreticulin might also have a dual role in the ER: Ca^{2+} binding and Ca^{2+} dependent control of folding of newly synthesized proteins.

Experiments were designed to explore the possiblity that calreticulin might associate with other cellular proteins. Through identification of calreticulin-binding proteins, we reasoned that clues to the function of calreticulin in the ER might be obtained. To investigate calreticulin-protein interactions, ligand blotting and calreticulin affinity chromatography were employed.

RESULTS

Detection of calreticulin binding proteins by ligand blotting

Initially, ligand blotting was used to investigate association of calreticulin with ER proteins. Ligand blotting is essentially a modification of Western blot analysis, in which radiolabeled protein rather than antibodies is used as a probe. In order to identify calreticulin binding proteins, membrane fractions (80 μg of protein) or samples of purified protein (2-5 μg of purified protein) were separated by SDS-PAGE, followed by electrophorectic transfer to nitrocellulose membrane and incubation with [^{125}I]calreticulin. After extensive washing to remove uncomplexed calreticulin, [^{125}I]calreticulin-binding proteins were detected by autoradiography.

Canine pancreatic microsomes, which are highly enriched in ER membrane, were examined for calreticulin binding proteins. Using ligand blotting, seven (major) proteins were detected in these membranes which consistently interacted with either native (Fig. 4-1) or recombinant (data not shown) [^{125}I]calreticulin: a protein doublet of 50-and 52-kDa, and a series of lower molecular mass proteins ranging from 38-kDa to 20-kDa (Fig. 4-1, lanes 2, arrowheads). Lower amounts of higher molecular weight proteins, in particular an 80-kDa protein, were also observed with longer exposure times (Fig. 4-2, -3, A). These higher molecular weight proteins are likely less abundant and/or less reactive with calreticulin. When ligand blotting was carried out in buffers containing millimolar concentrations of either Ca^{2+}, Zn^{2+} or EGTA there were no apparent differences in the [^{125}I]calreticulin binding pattern (data not shown) suggesting that the observed protein-protein interactions were ion-independent.

The specificity of calreticulin's interaction with the microsomal protein was examined using competition experiments. Duplicate nitrocellulose strips

Fig. 4-1. SDS-PAGE and ligand blotting of pancreatic membrane proteins with [^{125}I]labeled calreticulin.

Dog pancreatic calreticulin was purified by selective ammonium sulfate precipation and iodinated using IODO-GEN. Pancreatic microsomal membranes were subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose membrane and analyzed by ligand blotting with [^{125}I]calreticulin. The strips were incubated with [^{125}I]calreticulin (10^6 cpm/ml) in buffer containing 20 mM Tris, 150 mM KCl, 5% bovine serum albumin and 5 mM EGTA followed by washes in the same buffer. Autoradiographs were obtained by exposing the dried blots to Kodak AR film at -70°C. Lane S, Bio-Rad low range molecular weight markers; lane 1, Coomassie Blue staining of proteins of pancreatic microsomes; lane 2, autoradiograph of the [^{125}I]calreticulin binding proteins in pancreatic microsomes. Lane 1, 30 μg of protein, lanes 2, 80 μg of protein. Arrow heads indicate calreticulin binding proteins.

Fig. 4-2. Excess of unlabeled calreticulin inhibits binding of [^{125}I]calreticulin to pancreatic microsomes.

Different amounts of pancreatic microsomal proteins (10-80 μg) were subjected to SDS-PAGE, electrophoretically transfered to nitrocellulose membrane and probed with [^{125}I]calreticulin in the absence (A) or presence (B) of unlabeled calreticulin (1 mg/ml; 1000 fold excess). The positions of Bio-Rad prestained molecular mass standards are indicated.

Fig. 4-3. C-domain of calreticulin inhibits binding of [^{125}I] calreticulin to pancreatic microsomes.

Pancreatic microsomes (80 μg of protein) were subjected to SDS-PAGE followed by electrophoretic transfer to nitrocellulose membrane. The regions of calreticulin designated the N-, P-, and C- domains of calreticulin were expressed and isolated from *E. coli* as GST fusion proteins. Lane A, ligand blot with a [^{125}I]calreticulin; lanes B, C and D, [^{125}I]calreticulin overlays carried out in the presence of unlabeled GST-N-domain, GST-P-domain and GST-C-domain, respectively (1 mg/ml). Lane E, [^{125}I]calreticulin overlay in the presence of GST (1 mg/ml). The positions of Bio-Rad prestained molecular mass standards are indicated.

Fig. 4-1

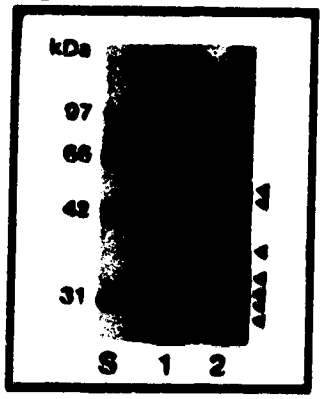


Fig. 4-2

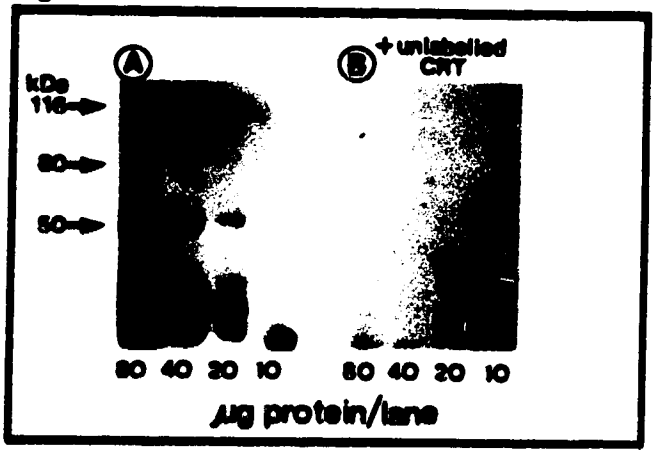
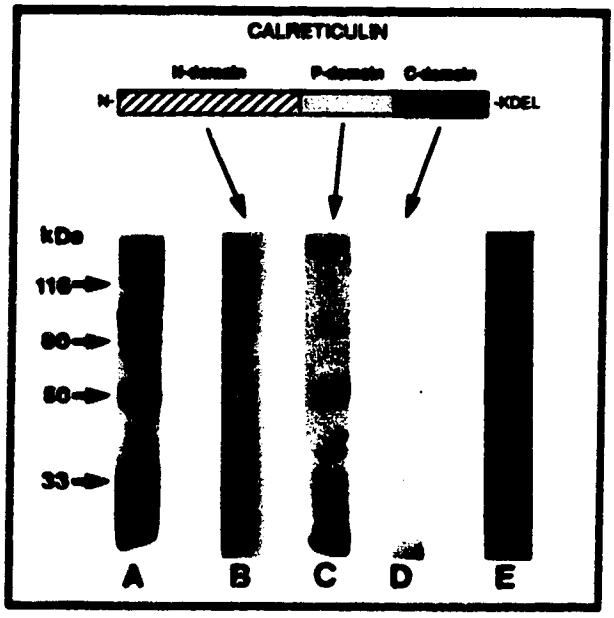


Fig. 4-3



were prepared containing microsomal proteins at a range of concentrations (10-80 µg/lane). These blots were incubated with [^{125}I]calreticulin in the absence (Fig. 4-2, A) or presence (Fig. 4-2, B) of excess unlabeled calreticulin (1000 fold excess). Excess unlabeled calreticulin abolished [^{125}I]calreticulin binding, indicating the specificity of the ligand blotting procedure.

[^{125}I]Calreticulin-protein interactions are mediated through the C-domain

In order to establish which region(s) of calreticulin is involved in the observed interactions, additional competition experiments were carried out. Excess unlabelled GST fusion proteins for the N-, P-, and C- domains were used in these experiments. The GST-C-domain (Fig 4-3, D), but not GST (Fig 4-3, E), efficiently abolished [^{125}I]calreticulin binding to pancreatic microsomal proteins. The GST-N-domain partially inhibited binding to all microsomal proteins (Fig. 4-3, B) and the GST-P-domain only partially inhibited binding to the 50-kDa protein doublet (Fig 4-3, C). We conclude that calreticulin interacts with these ER proteins mainly via its carboxyl-terminal region (C-domain).

[^{125}I]calreticulin interacts with proteins of the ER

Although pancreatic microsomes are a rich source of ER membranes they may be contaminated with other intracellular membranes (i.e Golgi and Plasma membrane). Therefore, purified preparations of rough ER, smooth ER, Golgi and mitochondria (kind gifts of Dr. J. Vance) were also tested. Figure 4-4 shows that calreticulin interacts with a set of proteins in rat liver smooth and rough ER (lanes 2 and 3) and in a purified Golgi fraction (lane 4) similar to those detected in pancreatic microsmes (Fig. 4-1).

Guan *et al.* (1991) showed that calreticulin interacts with FMO (flavin mono-oxygenase), an ER protein found in rat liver and lung. Therefore, rat liver

Fig. 4-4. Binding of [^{125}I]calreticulin to ER proteins.
In lane 1, purified FMO (2 μg); lane 2, rat liver smooth ER (80 μg); lane 3, rat liver rough ER (80 μg); lane 4, rat liver Golgi membranes (80 μg) were separated by SDS-PAGE and transferred to nitrocellulose membrane for ligand blotting with [^{125}I]labeled calreticulin. The positions of Bio-Rad prestained low range molecular mass standards are indicated: phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (42,700), bovine carbonic anhydrase (31,000) and soybean trypsin inhibitor (21,500).

Fig. 4-5. [^{125}I]Calreticulin binds to SR and nuclear proteins.
Skeletal muscle SR fractions and a nuclear fraction (80 μg) were prepared and subjected to SDS-PAGE followed by transfer to nitrocellulose membrane for ligand blotting with [^{125}I]calreticulin. Lane 1, rabbit skeletal muscle light SR membrane; lane 2, rabbit skeletal muscle heavy SR membrane; lane 3, rabbit skeletal muscle microsomes, lane 4, rat liver nuclei. The positions of the Bio-Rad prestained low range molecular mass standards are indicated by arrow heads.

Fig. 4-6. Cellular fractions that do not contain [^{125}I]calreticulin-binding proteins.
Proteins from various cellular fractions were prepared (80 μg) and separated by SDS-PAGE and electrophoretically tranferred to nitrocellulose membrane for ligand blotting with [^{125}I]calreticulin. Lane 1, pancreatic microsomes; lane 2, rat liver mitochondria; lane 3, rat liver cytosolic proteins (100,000 xg supernatant); lane 4, purified, recombinant calreticulin; lane 5, *E. coli* cellular extract.

Fig. 4-7. PDI is retained on a calreticulin-affinity column.
A calreticulin affinity column was made by coupling recombinant calreticulin to CNBr-activated Sepharose 4B. Samples of pancreatic microsomes (~20 mg) were solubilized with 1% Trition-X-100 and 0.5 % DOC in a buffer containing 10 mM MOPS, 100 mM KCl, 2 mM $MgCl_2$, 0.5 mM EGTA, pH 7.0. The soluble extract was diluted 10 fold with the same buffer and applied onto a pre-column to remove any proteins that may non-specifically associate with the matrix. The flow-through (~2 mg/ml of protein) was then directly applied to the calreticulin affinity column. The column was extensively washed and bound proteins were eluted by a gradient of 100 to 750 mM KCl. Fractions from the column were separated by SDS-PAGE, followed by silver staining. The N-terminus of the protein band detected here was sequenced and determined to be PDI. Lane S, Biorad molecular weight protein marker, lane 2 applied sample; lane 3 eluted proteins (PDI).

Fig.4-4

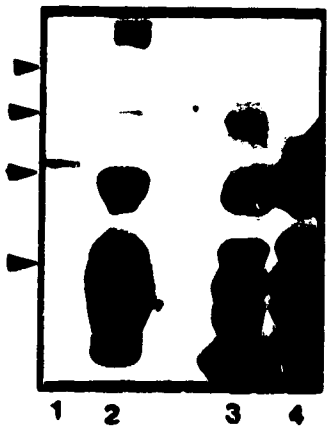


Fig.4-5

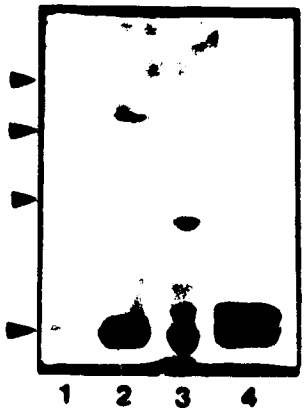


Fig.4-6

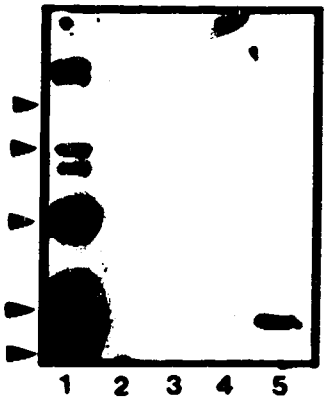
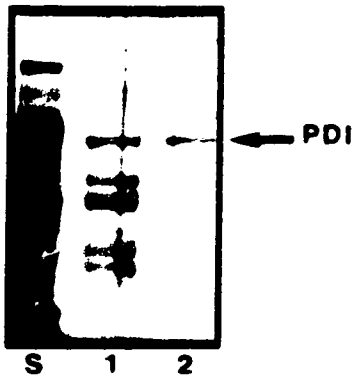


Fig.4-7



microsomes and purified FMO were tested for their interaction with [^{125}I]calreticulin. In rat liver microsomes, the [^{125}I]calreticulin binding pattern was similar to that detected in pancreatic microsomes despite the tissue and species differences (not shown). [^{125}I]calreticulin bound to the purified FMO sample (Fig. 4-4, lane 1), however, this interaction was relatively weak, considering that there was ~100X more FMO loaded onto SDS-PAGE than the other microsomal proteins.

Calreticulin binding proteins in the SR and nucleus

Calreticulin has also been localized to the SR, nuclear envelope and in L6 cells to the nucleus (Michalak *et al.*, 1980; Opas *et al.*, 1991). Samples corresponding to these intracellular fractions were, therefore, also analyzed for the presence of any [^{125}I]calreticulin binding proteins. Figure 4-5, lanes 1-3 shows binding of [^{125}I]calreticulin to skeletal muscle SR membrane proteins. A protein band of 30-kDa was the major [^{125}I]calreticulin binding protein in the heavy and light SR membranes. Smaller quantities of a 90-kDa and 50-kDa protein also bound [^{125}I]calreticulin in these SR membrane fractions (Fig. 4-5 lanes 2-3). In the nucleus three proteins of 35-kDa, 33-kDa and 30-kDa were detected (Fig. 4-5 lane 4). The calreticulin binding proteins of the SR and nucleus, therefore, sharply contrast those found in the ER and Golgi membranes of non-muscle cells.

Using ligand blotting we also showed that [^{125}I]calreticulin does not bind to rat liver mitochondria (Fig. 4-6, lane 2) or cytosolic (Fig. 4-6 lane 3) proteins. In addition, there was only one protein (~33-kDa) detected when a large amount (> 200 μg protein/lane) of *E. coli* cellular extract was tested for [^{125}I]calreticulin binding (Fig. 4-6 lane 5). [^{125}I]calreticulin did not bind to unlabeled calreticulin (Fig. 4-6 lane 4).

Protein disulphide isomerase (PDI) binds to calreticulin

In order to further purify and characterize the [^{125}I]calreticulin-binding proteins, experiments were carried out using calreticulin affinity chromatography. This led to an unexpected discovery: only one protein band was specifically adsorbed by the calreticulin afffinity column as observed on a silver stained gel (Fig. 4-7 lane 3). NH_2-terminal amino acid analysis of this protein was A-P-E-E-D which corresponds to the amino acid sequence of PDI (Edman *et al.*, 1985). The identity of the eluted protein as PDI was confirmed by its reactivity with anti-PDI antibodies (data not shown). Interestingly, the association between calreticulin and PDI was observed in the presence of EGTA but not in the presence of Ca^{2+} and Zn^{2+}. PDI was tested by ligand blotting for an interaction with calreticulin, but [^{125}I]calreticulin did not associate with immobilized PDI (data not shown).

DISCUSSION AND CONCLUSIONS

Ligand blotting is a simple yet powerful technique for the detection of protein-protein interactions. The main advantages of this technique is that it is capable of identifying binding proteins with high specificity using small quantities of probe (i.e. 1 μg) (Carr & Scott, 1992). In this study a number of low molecular weight ER proteins were found to specifically associate with [^{125}I]calreticulin via this technique.

Ligand blotting has been used to identify various other protein-protein interactions including the calmodulin binding proteins (Lottenberg *et al.*, 1981; Bachs & Carafoli, 1987), the low density lipoprotein receptor (Daniel *et al.*, 1983) the calsequestrin binding proteins (Damiani & Margreth, 1990; Mitchell *et al.*, 1988), and the type II cAMP dependent protein kinase binding proteins (Carr &

Scott, 1992). Many of the proteins which bind to calmodulin and to cAMP dependent protein kinase by ligand blotting have also been demonstrated to associate with these proteins using independent techniques (Carr & Scott, 1992; Slaughter *et al.*, 1987). It is believed that many proteins retain their ability to bind to other proteins after transfer to nitrocellulose due in part to the ability of the immobilized protein to renature somewhat in the blocking solution.

Calreticulin affinity chromatography was used in an effort to isolate and purify the [^{125}I]calreticulin binding proteins from pancreatic microsomes. Only one protein of ~ 55-kDa was successfully isolated using this procedure and was identified as PDI based on its NH_2-terminal amino acid sequence analysis. An association between calreticulin and PDI has since been supported by other work in the laboratory (S. Baksh & M. Michalak, unpublished observations). It was surprising that the proteins identified by ligand blotting did not interact with calreticulin under the conditions employed for calreticulin affinity chromatography and that PDI bound to the calreticulin affintity column but did not interact with [^{125}I]calreticulin by ligand blotting. There are a number of possible explanations for these results: (i) Protein-protein interactions that occur through contact at several sites on the surface of the target protein are less likely to be detected by ligand blotting due to the harsh condtions used to separate and transfer proteins to nitrocellulose. Only about 50% of the calmodulin binding proteins can be detected by overlay methods. For this reason PDI and perhaps FMO were not detected by ligand blotting. (ii) The interaction between calreticulin and the proteins detected by overlay is mediated primarily through the carboxyl-terminus of calreticulin. The C-domain of calreticulin when immobilized to the CNBr activated sepharose may not be accessible for interaction with these proteins. This is further supported by the recent observation in the laboratory that PDI interacts with the P-and N-domains of

calreticulin but not with the C-domain. (iii) Calreticulin may interact with regions of the ER proteins that are not accessible in the native confirmation (i.e. calreticulin may only bind to these proteins in an unfolded or partly unfolded state). (iv) Preliminary results suggest that the low molecular weight [^{125}I]calreticulin binding proteins may be integral membrane proteins. It is therefore, possible that under the conditions used for solubilization of microsomal membranes, prior to calreticulin affinity chromatography, the [^{125}I]calreticulin binding proteins do not have their binding sites accessible for interaction with calreticulin immobilized to CNBr sepharose. This may be related to the type of micelles formed when the membrane vesicles are solubilized with detergent.

In this study the potential for calreticulin to interact with proteins of the ER has been established (Fig. 4-8). These results suggest that in addition to its potential Ca^{2+} binding/storage role in the ER calreticulin may have other ER functions. Recent observations suggest at least two possibilities for the functions of calreticulin in the ER: as a molecular chaperone and as a "ER signal transduction molecule". Many molecular chaperones have been characterized by their ability to bind selectively to unfolded proteins (reviewed in Gething and Sambrook, 1992). Due to the harsh conditions used to separate and transfer proteins onto nitrocellulose many of these proteins are likely unfolded, suggesting that calreticulin may also interact with unfolded proteins (Carr & Scott, 1992). The potential for calreticulin to interact with unfolded protein has recently been established in a series of elegant experiments carried out by Nigam *et al.* (1994). The investigators showed that calreticulin like other lumenal ER proteins including BiP, PDI, GRP94, p50 (ERp61), and p46, bound selectively to an affinity column of denatured histone. These proteins, including calreticulin,

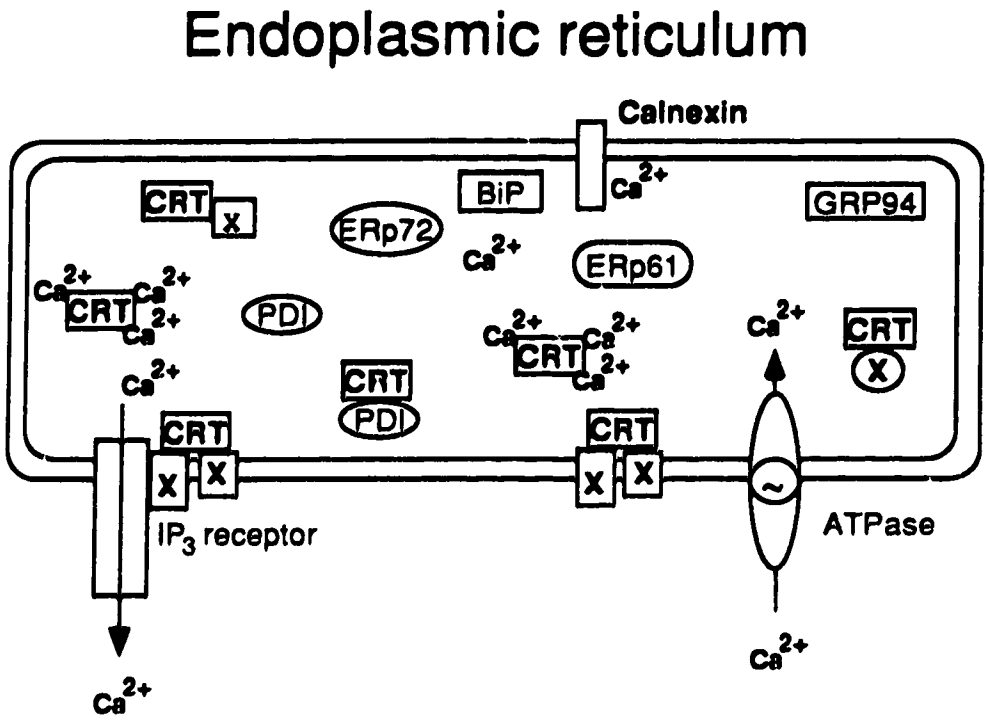


Fig. 4-8. Calreticulin binding proteins in the ER.

A number of proteins were detected which interact with [^{125}I]calreticulin by ligand blotting or with calreticulin immobilized to CNBr activated sepharose. PDI was the only calreticulin-binding protein which was identified. The identities of the other calreticulin-binding proteins (designated as proteins X) are not yet known. Many of the proteins which interact with calreticulin were relatively small (~20-50 kDa). Some of the calreticulin-binding proteins may be membrane associated as illustrated. Calreticulin may interact with these proteins to facilitate Ca^{2+} release from the ER, for example. Calreticulin might also act as a chaperone in the ER.

could be specifically eluted from the column in the presence of ATP. With the exception of BiP and p46, Ca^{2+} was essential for maximal ATP-dependentdissociation of these proteins from the column suggesting that they may function as Ca^{2+} dependent chaperones. A number of matrixes were prepared using various denatured secretory proteins. Calreticulin and GRP94 displayed greater substrate specificity for the denatured proteins than the other lumenal proteins suggesting that they may act as chaperones for a specific subset of proteins (Nigam *et al.*, 1994). Chaperone function for calreticulin is further supported by its amino acid sequence similarities to calnexin (Wada *et al.*, 1991). Calnexin is an integral membrane protein that is proposed to function as a chaperone for a specific subset of proteins (i.e. specifically glycoproteins) (Ahluwalia *et al.*, 1992; reviewed in Hammond & Helenius, 1993; Bergeron *et al.*, 1994).

The observed pattern of calreticulin binding proteins in the ER is surprisingly similar to the pattern of binding proteins detected for calsequestrin in the SR also by ligand blotting. Mitchell *et al.* (1988) detected a 26-kDa calsequestrin-binding protein and Damiani and Margreth (1990) detected a set of calsequestrin binding proteins in skeletal muscle SR (47-kDa; 31-30-kDa, 29-kDa and 26-25-kDa) in skeletal muscle SR. The 30-kDa [^{125}I]calreticulin binding protein identified in the SR of this study may correspond to the 26-kDa calsequestrin binding protein identified by Mitchell *et al.* (1988). Calsequestrin interacts with these proteins in a Ca^{2+}-dependent manner (Mitchell *et al.*, 1988; Damiani & Margreth, 1990). Through these interactions it was proposed that calsequestrin may also be involved in Ca^{2+} release from the SR via the ryanodine receptor (Damiani & Margreth, 1990). Many similarites exist between the Ca^{2+} release and uptake processes of the ER and SR (Mignery, *et al.*, 1989; Walz & Baumann, 1989; reviewed in Milner *et al.*, 1992). Calreticulin is similar in size and

has a number of diagnostic features common to calsequestrin (Michalak *et al.*, 1992). However, the most striking similarity between these two molecules is their ability to bind large amounts of Ca^{2+} (calreticulin binds 25 moles of Ca^{2+}/mole of protein vs calsequestrin which binds 50 moles of Ca^{2+}/mole of protein). Considering the striking similarity between Ca^{2+} release channels of the ER and SR, the ryanodine and the $InsP_3$ receptors, respectively, and the similarites between calreticulin and calsequestrin it is tempting to speculate that calreticulin might interact with other cellular proteins to facilitate Ca^{2+} release from the ER. Calreticulin through interaction with other cellular protein could be involved in the generation of a Ca^{2+} induced signal from the ER.

Based on TX-114 extraction of the pancreatic microsomes, many of the low molecular weight calreticulin binding proteins may be integral membrane proteins. Interestingly, many similar sized proteins in the ER have been identified which are believed to be involved in translocation and/or in vesicular transport (Gorlich *et al.*, 1992). Although highly speculative, calreticulin could be involved in ER translocation or vesicular traffic through association with some of the low molecular weight proteins.

Future work is required to clearly establish calreticulin's ER function. Through identification of the proteins detected in this study important clues to the role for calreticulin in the ER may be obtained. Future approaches to identify the calreticulin binding proteins are discussed in Chapter 6.

CHAPTER FIVE

CALRETICULIN AND MODULATION OF GENE EXPRESSION

Versions of this Chapter have been published:

Burns, K., Duggan, B., Atkinson, E. A., Famulski, K. S., Nemer, M., Bleackley, R. C., Michalak, M. 1994. **Modulation of gene expression by calreticulin binding to the GR**. Nature. 367: 476-480.

Burns, K., Atkinson, E. A., Bleackley, R. C., and Michalak, M. 1994. **Calreticulin: from Ca^{2+} binding to control of gene expression**. Trends in Cell Biology. 4: 152-154.

INTRODUCTION

Two important observations led to the initiation of this study: (i) calreticulin was found localized to the nucleus in some cells suggesting potential role for it specific to this cellular compartment and (ii) calreticulin was demonstrated to interact with a peptide, K-L-G-F-F-K-R, which is very similar to the peptide found in the DNA binding domain of the nuclear receptors (KXGFFKR). These observations suggested to us that calreticulin may interact with the DNA binding domain of the GR and other steroid receptors and may thus have a potential role in modulating gene transcripition controlled by these receptors.

The GR belongs to the nuclear hormone receptor superfamily which includes a group of proteins that are functionally and structurally related (Evans, 1988; Laudet *et al.*, 1992). Other receptors which belong to this family include receptors for steroid horomones, such as progesterone, androgen and estrogen, as well as receptors for vitamin A and D, thyroid hormone and retinoic acid, and also a group of proteins without known ligands, the "orphan" receptors (Table 5-1). The nuclear receptors function as ligand dependent transcription factors that control a diversity of cellular processes by regulating the expression of their target genes through an interaction with specific DNA response elements that are associated with these genes (Evans, 1988).

A receptor that is not bound to its ligand is maintained in an inactive state as part of a heterooligomeric complex (reviewed in Muller & Renkawitz, 1991). Other proteins which are part of these complexes include the heat shock proteins hsp90, hsp 56/p59 (an immunophilin which binds the immunosuppressive agent FK506) and in some cases hsp70 (reviewed in Pratt, 1993). These hsp proteins are proposed to have roles in regulating the function, folding and trafficking of the

Table 5-1. Members of the nuclear receptor superfamily.

The amino acid sequences are taken from Laudet *et al.* (1991). The highly conserved peptide (consensus amino acid sequence KxFF(K/R)R) is found in the DNA binding domain of the receptors. This peptide is likely mediates an interaction between calreticulin and the steroid receptors.

Table 5-1. Members of the nuclear receptor superfamily.

Glucocorticoid receptor	Human	K V F F K R
Thyroid hormone receptor a	Human	K G F F R R
Thyroid hormone receptor ß	Human	K G F F R R
Retinoic acid receptor a	Human	K G F F R R
Retinoic acid receptor ß	Human	K G F F R R
Oestrogen receptor	Human	K A F F K R
Progesterone receptor	Human	K V F F K R
Mineralocorticoid receptor	Human	K V F F K R
Androgen receptor	Human	K V F F K R
Vitamin D receptor	Human	K G F F R R
Orphan receptor	Human	K G F F K R
Orphan receptor	Human	K G F F K R
Orphan receptor	Human	K A F F K R
Orphan receptor	Human	K A F F K R
Orphan receptor	Human	K G F F K R
Orphan receptor (*ear3*)	Human	K G F F K R
Orphan receptor	Human	K G F F K R
Orphan receptor (*Rev-ErbA*)	Human	K G F F R R
Orphan receptor	Rat	K G F F R R
Orphan receptor	Rat	K G F F K R
Orphan receptor (*nur77=N10*)	Rat	K G F F K R
Retinoic acid receptor g	Mouse	K G F F R R
Orphan receptor	Mouse	K G F F R R
Thyroid hormone receptor a	Chicken	K G F F R R
v-*erbA* gene (erythroblastosis virus)	Chicken	K S F F R R
Thyroid hormone receptor ß	Chicken	K G F F R R
Thyroid hormone receptor a	*Xenopus*	K G F F R R
Thyroid hormone receptor a	*Xenopus*	K G F F R R
Thyroid hormone receptor ß	*Xenopus*	K G F F R R
Thyroid hormone receptor ß	*Xenopus*	K G F F R R
Retinoic acid receptor a	*Xenopus*	K G F F R R
Retinoic acid receptor g	*Xenopus*	K G F F R R
Ecdysone receptor	*Drosophila*	K G F F R R
Orphan receptor (*Ultraspiracle*)	*Drosophila*	K G F F K R
Orphan receptor (*knirps*)	*Drosophila*	K S F F G R
Orphan receptor (*knirps-related*)	*Drosophila*	K S F F G R
Orphan receptor (*tailless*)	*Drosophila*	A G F F K R
Orphan receptor (*sevenup*)	*Drosophila*	K G F F K R
Orphan receptor	*Drosophila*	K G F F R R
Orphan receptor	*Drosophila*	K G F F K R
Orphan receptor	*Drosophila*	K S F F G R
CONSENSUS		K x F F K/R R

receptors (Pratt, 1993). The activity of the receptors is also regulated by protein phosphorylation (Orti *et al.*, 1992).

Hormone binding to the receptor leads to a rapid transformation from a nonactivated complex to an activated state characterized by the ability of the receptor to bind DNA (Pratt, 1987; Muller & Renkawitz, 1991). It is believed that upon hormone binding the receptor undergoes a conformational change which leads to dissociation of the hsp proteins and subsequently to dimerization. This process is schematically illustrated for the GR in Fig. 5-1. The GR is translocated to the nucleus in its active state where it associates with specific DNA sequences referred to as glucocorticoid response elements, or GREs, that act as enhancer elements in target genes (Beato, 1989). Dimer formation is important for binding of the receptors to their specific DNA sequences, collectively referred to as hormone responsive elements (HREs). The HREs are imperfect palindromic binding sites consisting of two six bp consensus half sites with three intervening base pairs. The steroid receptors such as the GR generally form homodimers. In contrast the vitamin D3, thyroid hormone and retinoic acid receptors can form functional heterodimers with other members of the nuclear receptor family (Truss & Beato, 1993).

Most receptors are believed to be loosely associated and then to become tightly associated with the nucleus upon ligand binding (Truss & Beato, 1993). However, the glucocorticoid and mineralocorticoid receptors appear to be exceptions as they are found predominantly in the cytosol in the absence of hormone (Wikstrom *et al.*, 1987; Muller & Renkawit, 1991). It is still unclear whether cellular localization is an intrinsic property of each type of receptor or is contingent on underlying variables such as the relative rates of transport in an out of the nucleus (Bodwell and Munck, 1992). For example, in cells which have been heat shocked or in which GR is overexpressed, the unliganded receptors are

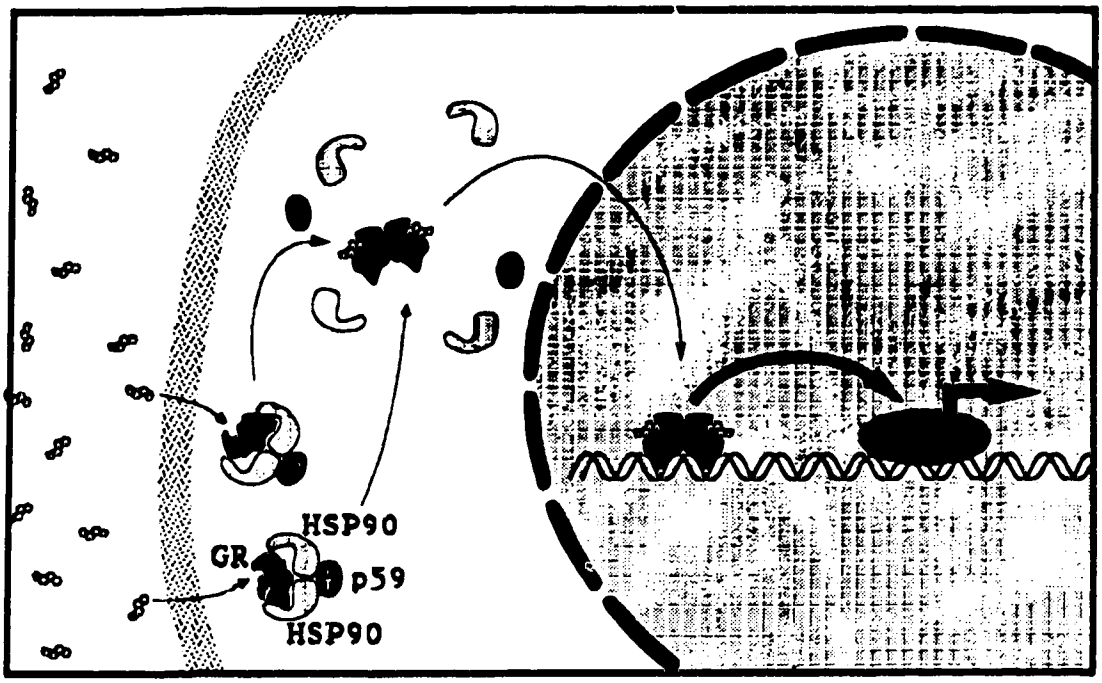


Fig. 5-1. Gene induction by glucocorticoid hormones: Activation of the glucocorticoid receptor

In the absence of hormone, the glucocorticoid receptor exists in the cytosol as part of a heterooligomeric complex. Hormone binding to the receptor leads to a rapid transformation from a nonactivated complex to an activated state characterized by the ability of the receptor to bind DNA. The complex dissociates and gives rise to its components: two heat shock proteins, hsp90, and p59/hsp56. The hormone-bound receptor assembles to form homodimers. The homodimer is transported to the nucleus where it associates with specific DNA sequences referred to as glucocorticoid response elements, or GREs, that act as enhancer elements in target genes. Finally, the activated receptor dimer interacts with the transcription machinery (shaded oval) to induce transcription of glucocorticoid-sensitive target genes. Adapted from Muller and Renkawitz (1991).

predominantly nuclear (Sanchez, 1992; Brink *et al.*, 1992). Both the progesterone receptor and the GR have been demonstrated to continuously shuttle in an energy dependent fashion into and out of the nucleus (Guiochon-Mantel *et al.*, 1991; Truss & Beato, 1993).

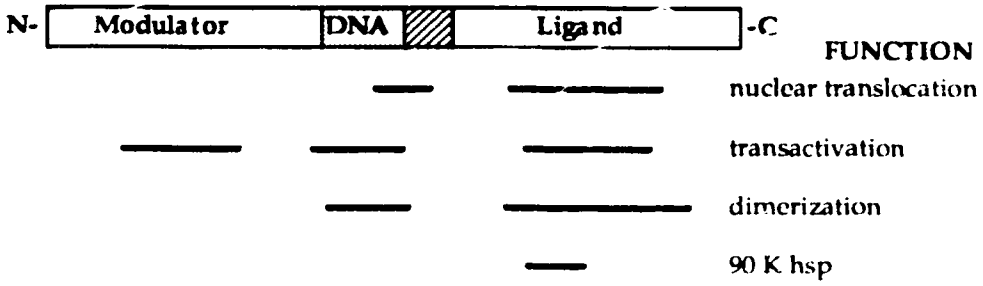
The steroid receptors can be divided into three principal domains as schematically illustrated in (Fig. 5-2): the NH_2-terminal domain, the DNA binding domain (DBD) and the ligand binding domain (Beato, 1989). The NH_2-terminal is the least conserved domain and can vary in length from more than 600 amino acids for the mineralocorticoid receptor to approximately 25 amino acids for the vitamin D receptor (Beato, 1989). The DBD lies in the central basic region of the molecule and the ligand binding domain lies at the carboxyl-terminus. The domains are modular in that they retain their functions when fused to other proteins (Evans, 1988). For example, protein fragments containing the GR DBD expressed in *E. coli* exhibit sequence-specific binding to GREs (Freedman *et al.*, 1988; Dahlman *et al.*, 1989).

The DBD of the nuclear receptors is organized into two zinc finger motifs as schematically illustrated for the GR in Figure 5-2 (Freedman *et al.*, 1988; Evans & Hollenberg, 1988). This region is highly conserved and contains sequences which are invariant among all members of the family (Fig. 5-2). One such sequence K-x-F-F-K-R (consensus sequence K-x-F-F--(K/R-R) is situated between the two zinc fingers. Crystallography of the DNA binding domain of the GR complexed with its GRE revealed that this peptide (K-V-F-F-K-R) forms an alpha helix which is in direct contact with DNA (Luisi *et al.*, 1991). Other members of the nuclear receptor family are proposed to interact with their HREs in a similar manner via an association mediated through their respective K-x-F-F-K-R peptides (Luisi *et al.*, 1991; Schwabe *et al.*, 1993). Can calreticulin interact with this peptide in the DNA binding of the receptors and affect their function?

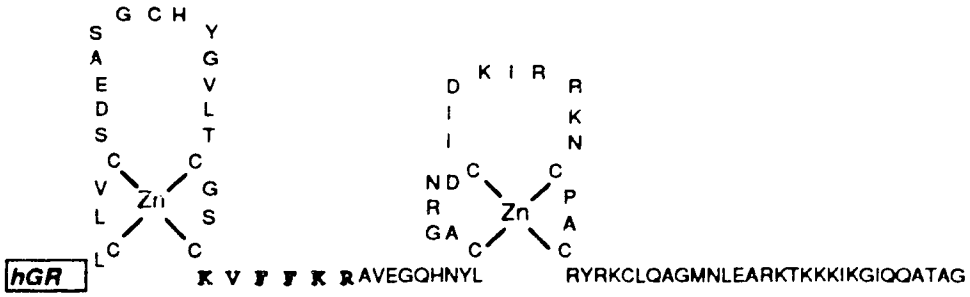
Fig. 5-2. Structural and functional organization of nuclear receptors.

(**A**) The steroid receptors can be divided into three principal domains: the N-terminal domain, the DNA binding domain (DBD) and the ligand binding domain. The domains are modular in that they retain their functions when fused to other proteins. The N-terminal is the least conserved domain and can vary in length from more than 600 amino acids to 25 amino acids. The DBD lies in the central basic region of the molecule and the ligand binding domain lies at the C-terminus. Within the general domain structure of the glucocorticoid receptor (GR) several regions have been identified which are associated with specific actvities (Beato *et al.*, 1989). Regions within the N-terminal and DBD domains have been found to be necessary for full transcriptional enhancer activity. Regions within the DBD and ligand binding domains are important for dimerization and nuclear localization of the GR (Picard & Yammamoto, 1987). The region of the receptor that interacts with hsp90 has been mapped to the ligand binding domain (Howard *et al.*, 1990). (**B**) The 86-residue DNA binding domain of the human glucocorticoid receptor is shown in the classical zinc-finger representation (Evans *et al.*, 1988). Each zinc ion is tetrahedrally ligated by four conserved cysteine residues (Freedman *et al.*, 1988). This region contains the conserved peptide KXFFKR (in bold) which is similar to the calreticulin-binding peptide described by Rojiani *et al.*, 1991.

A


N-
Modulator
DNA
Ligand
-C
FUNCTION
nuclear translocation
transactivation
dimerization
90 K hsp

B


G C H
S A E D S C V L C
Y G V L T C G S C
Zn
K I R
D I I N D R G A C
R K N C P A C
Zn
hGR
K V F F K R AVEGQHNYL
RYRKCLQAGMNLEARKTKKKIKGIQQATAG

To test this hypothesis we chose specifically to determine whether calreticulin could bind to the GR. The studies in this chapter show that calreticulin can interact with the DBD of the GR and modulate its function *in vitro* and *in vivo* .

RESULTS

Calreticulin interacts with the DBD of the glucocorticoid receptor

Calreticulin affinity chromatography was employed to determine whether calreticulin could interact with the DBD of the GR. The DBD of GR, as shown in Figure 5-2 (B), was expressed in *E. coli* as a GST fusion protein, GST-GR, and assayed for binding to immobilized calreticulin. GST did not bind (Fig. 5-3, A), however GST-GR was specifically adsorbed by the calreticulin-affinity column (Fig. 5-3, A & B, fractions #8 and 9) demonstrating that calreticulin and the receptor could associate.

The calreticulin/GR interaction interferes with DNA binding

Using gel mobility shift assays, Brenda Duggan tested whether this association had any effect on the ability of GR to interact with its GRE. Figure 5-4 (A) shows that the GST-GR fusion protein interacts with a synthetic GRE (lane 2) and that neither the GST nor calreticulin alone bind to this sequence (lanes 1 and 6). The interaction of GST-GR with the GRE was blocked by the addition of 2 μg of calreticulin (lane 5) and this inhibition could be reversed in the presence of competing K-V-F-F-K-R peptide (lane 3). No significant influence of Ca^{2+} on the interaction was observed. These results suggest that calreticulin interacts with the DNA binding domain of the receptor and prevents the receptor from interacting with the GRE.

Fig. 5-3. The GST-GR fusion protein binds specifically to a calreticulin affinity column.

The DNA binding domain of glucocorticoid receptor (amino acid residues #420 to #506) was expressed in *E. coli* as a Glutathione S-transferase (GST) fusion protein. The exact region of the glucocorticoid receptor used in this study is shown in Figure 5-2. *E. coli* transformed with pGEX-GR were grown to mid-log phase and then induced to express the fusion protein with 0.1 mM IPTG. GST-GR was purified by step chromatography: first by a Glutathione-Sepharose 4B affinity column and then by a calreticulin-affinity column. Chromatography was carried out using the FPLC system (Pharmacia). In (A) two mg of Glutathione-Sepharose purified GST (broken line) or GST-GR (solid line) were applied onto a calreticulin affinity column equilibrated with a buffer containing 100 mM NaCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 40 mM Tris, pH 7.2. The column was then washed with a buffer containing 100 mM NaCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 40 mM Tris, pH 7.2. This was followed by (a) low salt buffer elution (5 mM EDTA, 100 mM NaCl, and 40 mM Tris, pH 7.2) and (b) high salt buffer elution (20 mM EDTA, 750 mM NaCl, and 40 mM Tris, pH 7.2). Fractions were analyzed by absorbance at 280 nm (A). In (B) the SDS-PAGE of the protein fractions obtained from the GST-GR separation in A is shown. S - low range Bio-Rad molecular weight standards: phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (42,700), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400). GST-GR in (B) refers to the Glutathione-Sepharose purified GST-GR fusion protein. Arrow head indicates GST-GR.

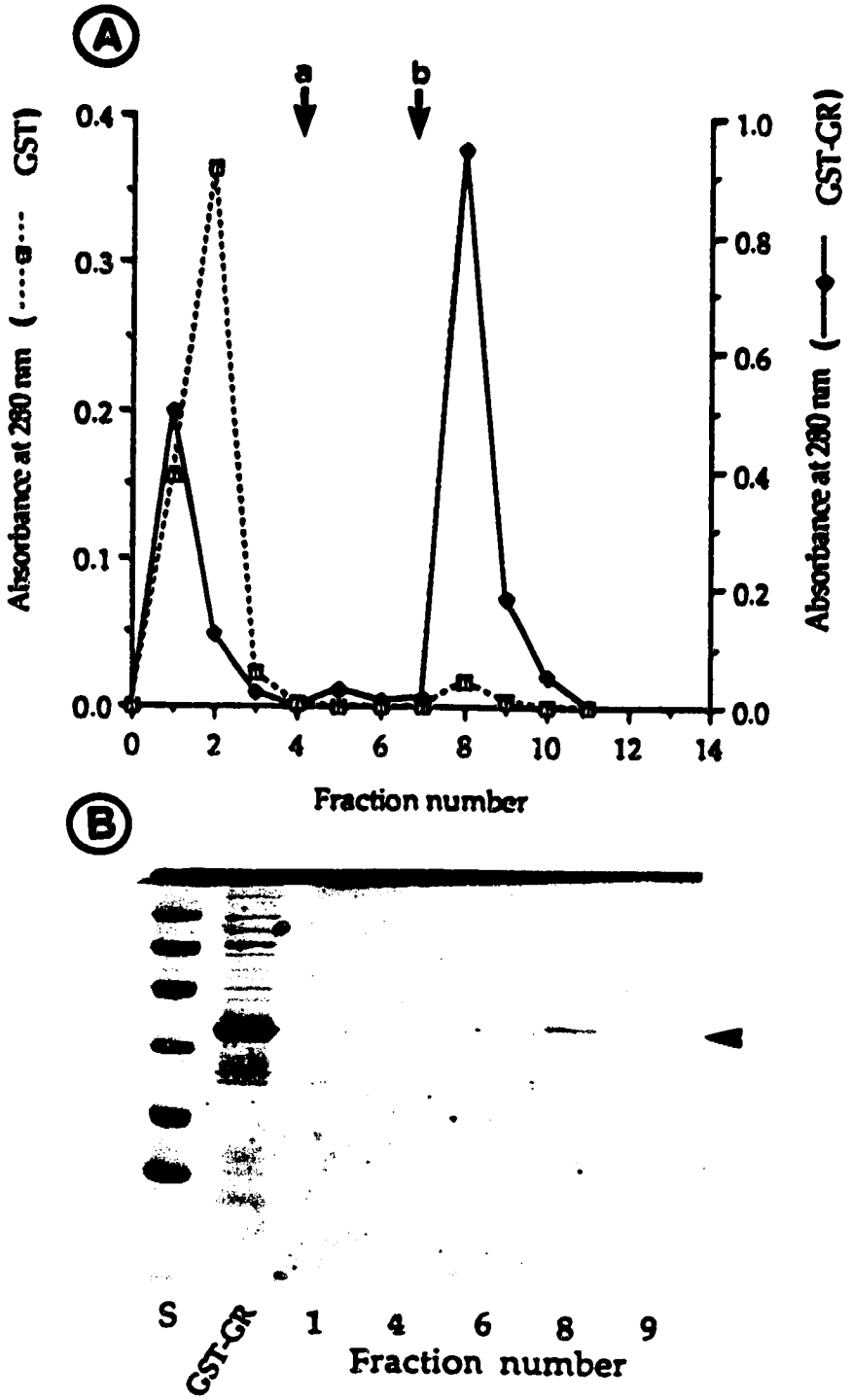
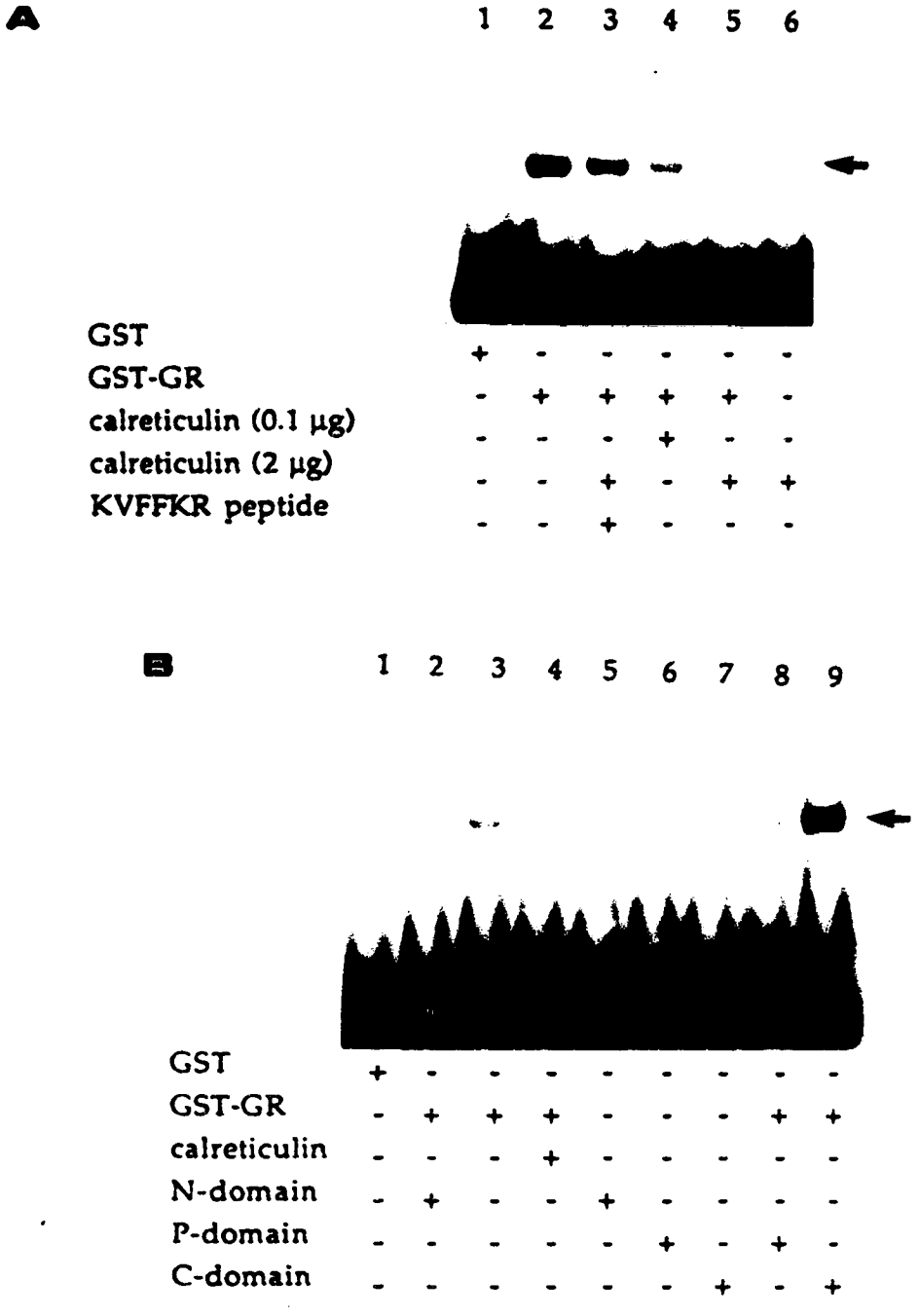

A
a
b
0.4
0.3
0.2
0.1
0.0
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
14
Absorbance at 280 nm (----g--- GST)
Absorbance at 280 nm (—●— GST-GR)
Fraction number
B
S
GST-GR
1
4
6
8
9
Fraction number

Fig. 5-4. Interactions of the glucocorticoid receptor DNA binding domain with the GRE is abolished in the presence of calreticulin .

The DNA mobility shift assays were carried out in 20 mM Tris, pH 7.5, 1 mM EDTA, 20 mM NaCl, 0.05% bovine serum albumin, 4 mM DTT, 10% glycerol and 2 μg of Poly-dI:dC. The proteins (pancreatic calreticulin and GST-GR) and buffer were added together and incubated at 25°C for 15 min. The radioactive GRE (5'-TCC TTG TTT TAA GAA CAG TTA TCG ATT ATA AAC-3') was then added, the mixture was allowed to incubate for a further 10 min., and then was loaded onto a polyacrylamide gel (5% acrylamide) in 22 mM Tris, pH 7.5, 22 mM boric acid and 0.5 mM EDTA. . In (A) Lane 1, GST (2 μg); lane 2, GST-GR (2 μg); lane 3, GST-GR in the presence of calreticulin and the KVFFKR synthetic peptide (100 μg); lane 4, GST-GR in the presence of 0.1 μg of calreticulin; lane 5, GST-GR in the presence of 2 μg of calreticulin; lane 6, calreticulin alone (2 μg). In (B) The N-P- and C-domains were expressed in *E. coli* as GST-fusion proteins and purified by Glutathione S-sepharose affinity chromatography. The fusion proteins were then assayed for their ability to inhibit GST-GR from interacting with the GRE. Lane 1, GST (2 μg); lane 2, GST-GR in the presence of 2 μg of GST-N-domain; lane 3, GST-GR (2 μg); lane 4, GST-GR in the presence of 2 μg of calreticulin; lane 5, GST-N-domain (2 μg); lane 6, GST-P-domain (2 μg); lane 7, GST-C-domain (2 μg); lane 8, GST-GR in the presence of GST-P-domain (2 μg); lane 9, GST-GR in the presence of GST-C-domain (2 μg). Arrow indicates the position of the protein/DNA complex .


A
1 2 3 4 5 6
GST
GST-GR
calreticulin (0.1 µg)
calreticulin (2 µg)
KVFFKR peptide
+ - - - - -
- + + + + -
- - - + - -
- - + - + +
- - + - - -
B
1 2 3 4 5 6 7 8 9
GST
GST-GR
calreticulin
N-domain
P-domain
C-domain
+ - - - - - - - -
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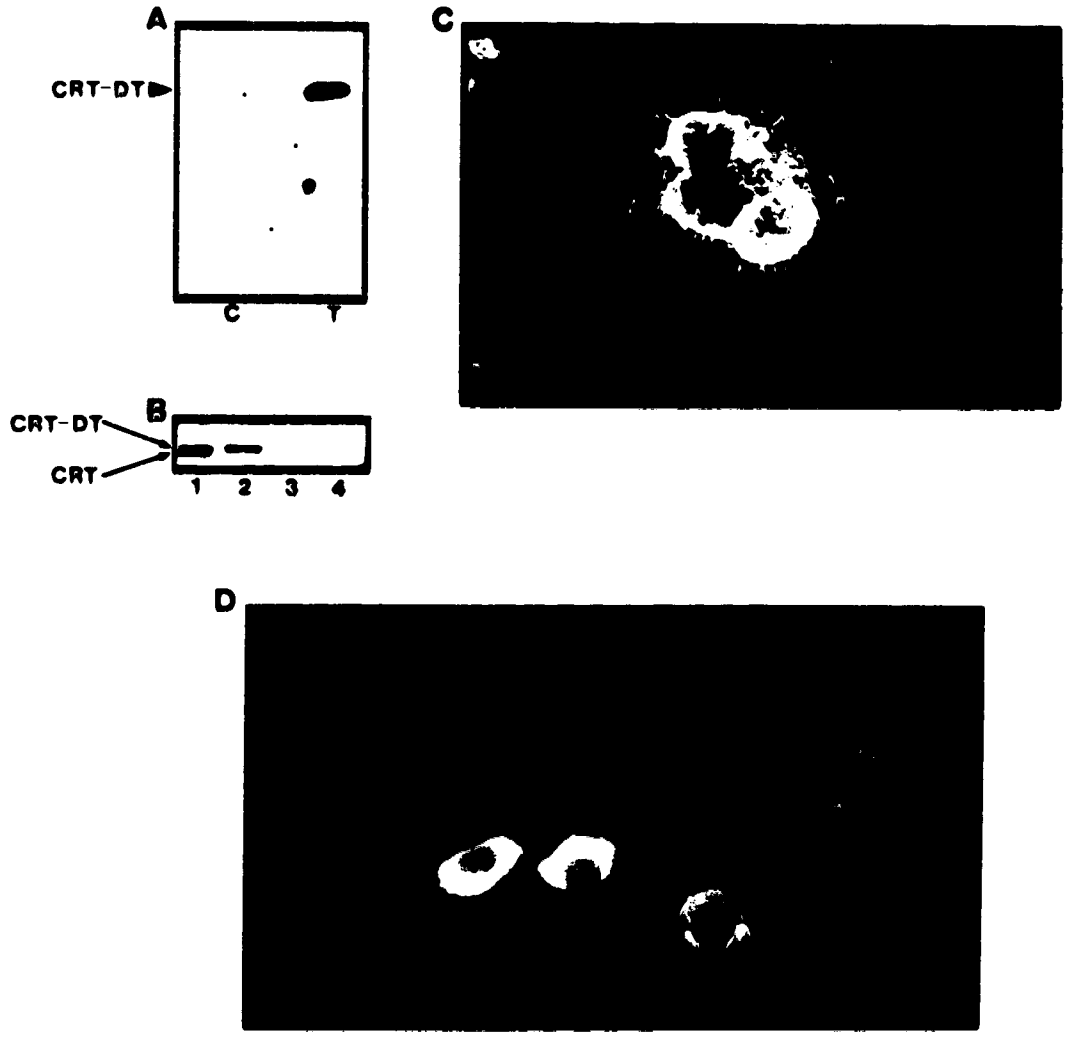
DNA mobility shift assays were also used to determine which region of calreticulin interacts with the GST-GR. For use in these assays the domains of calreticulin were expressed and purified as GST-fusion proteins (as described in Chapter 2). The N-domain fusion protein blocked the binding of GST-GR with DNA in a manner similar to that observed for the full length calreticulin (Fig. 5-4 (B) lane 2 & 4). In contrast, the P-domain had very little effect on the binding (Fig. 5-4 (B) lanes 8). Surprisingly the binding of GST-GR to the GRE was enhanced in the presence of the C-domain (Fig. 5-4 (B) lane 9). The reason for this enhanced binding is not clear at present. However, it is not due to the C-domain binding to DNA since the purified N-, P- and C-domains alone did not bind to the GRE (Fig. 5-4 (B) lanes 5-7). It would appear, therefore, that the interaction between calreticulin and the GR is mediated through the N-domain.

Expression of DT-tagged calreticulin

The calreticulin expression vector, pSCR-DT-1, was utilized to study the effects of calreticulin on GR activity *in vivo*. As all mammalian cells examined so far express significant amounts of calreticulin, this vector was designed to encode an epitope tagged version of calreticulin so that exogenously expressed calreticulin could be detected. For epitope tagging a unique amino acid sequence was employed. This amino acid sequence corresponds to amino acids #3668 to #3679 of the carboxyl-terminal region of human dystrophin (Milner *et al.*, 1992b). Polyclonal antibodies were made against this synthetic peptide which recognize dystrophin and dystrophin-tagged calreticulin (Fig. 5-5). The isoform of dystrophin recognized by this antibody is present only in muscle cells (Milner *et al.*, 1992b). A complementary oligodeoxynucleotide encoding the 12 amino acid peptide was inserted into unique restriction sites in calreticulin cDNA. This resulted in the loss of amino acids #394-396 in the C-terminus of calreticulin, and

Fig. 5-5. Expresion of dystrophin-tagged calreticulin.

In (A) COS cells were mock transfected (C) or transfected with pSCR-DT-1 (0.5 μg/ 2 mm^2 dish) (T) using a standard DEAE-dextran procedure. Cells were harvested 48 hours later for Western blot analysis. Cellular extracts were obtained by detergent lysis, separated by SDS-PAGE (20 μg/lane) and electrophoretically transferred to a nitrocellulose membrane. The membrane was probed with affinity purified anti-DT antibodies diluted 1:100 in PBS containing 1% milk powder, followed by incubation with anti-rabbit IgG conjugated to horseradish peroxidase. The peroxidase reaction was developed using Amersham's ECL detection system. In (B) COS cells were either transfected with 0.5 μg (lane 1) or 1 μg/2 mm^2 (lane 2) of pSCR-DT-1 or mock transfected (lanes 3, 4). Cellular extracts were obtained from these cells, separated by SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. The membrane was probed both with affinity purified anti-DT antibodies, as described above, and with anti-calreticulin antibodies diluted 1:300 in PBS containing 1% milk powder. The immunoreactive bands were detected by ECL and are indicated by the arrows. In (C) COS cells transfected with pSCR-DT-1 were analyzed for expression of DT-calreticulin by immunocytochemistry. The cells were fixed with formaldehyde (3.7%) and permeablized by incubation in PBS containing 1% saponin. The permeabilized cells were incubated with affinity purified anti-DT antibodies (1:20 dilution) followed by incubation with anti-rabbit IgG conjugated with the fluorescent-tag, Cyc ™ (1:75 dilution). Confocal microscopy was carried out by Dr. M. Opas (University of Toronto) using Bio Rad MRC-600 microscope. In (D) L-cells were transfected with pSCR-DT-1 using a Ca^{2+} phosphate procedure. The cells were fixed with formaldehyde (3.7%) and permeablized by incubation in buffer containing 0.1% Triton-X-100, 100 mM PIPES, 1 mM EGTA, 4% [w/v] polyethylene glycol 8000, pH 6.9 for 3 min. The cells then immunostained as described above. A Zeiss Photomicroscope III equipped with an epifluorescence condenser and selective FITC/TRITC filter combinations was used for observation and photography. Photographs were recorded on TMax-400 negatives.


A
CRT-DT
C
T
B
CRT-DT
CRT
1 2 3 4
C
D

therefore extended the sequence of calreticulin by nine amino acids. The C-terminus was chosen as a site for this insertion based on secondary structural predictions which suggest that it exists in an extended conformation (Fliegel *et al*, 1989a). In such a conformation the epitope would be predicted to be exposed and thus detected in the cell by immunocytochemistry.

The specificity of this system was initially established by transfecting COS cells with pSCR-DT-1. Forty-eight hours following transfection, cellular extracts were prepared and analysed for expression of DT-calreticulin by Western blot analysis (Fig. 5-5, A, B) or by immunocytochemistry (Fig. 5-5, C). Using goat anti-calreticulin antibodies and affintity purified anti-DT antibodies, both recombinant and native calreticulin could be distinguished in these cells (Fig. 5-5, B).

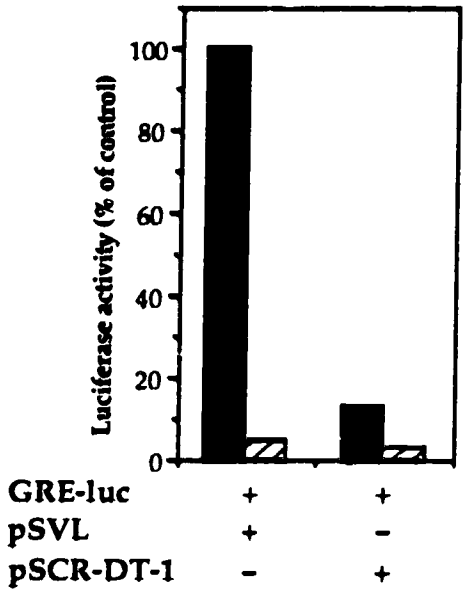
Calreticulin modulates GR-sensitive gene expression

To assess the *in vivo* significance of the GST-GR-calreticulin interaction mouse L fibroblasts were transfected with a GRE-luciferase reporter plasmid (GRE-Luc) (Drouin *et al.*, 1992), and asked whether cotransfection with a calreticulin expression vector (pSCR-DT) modulated GR-mediated activation of the luciferase reporter gene. Expression of DT-tagged calreticulin was confirmed in these cells by immunocytochemistry (Fig. 5-5, D). In the presence of a control plasmid (pSVL) the luciferase activity increased 20-fold upon addition of DEX (Fig. 5-6, A). This glucocorticoid-dependent increase in reporter gene expression was reduced ~85% when the calreticulin expression vector was included in the transfection. To demonstrate that the calreticulin effects are specific to GR-mediated transcription, Mona Nemer co-transfected L fibroblasts with glucocorticoid-insensitive reporter genes (luciferase and growth hormone) under the control of GRE-deleted MMTV and RSV promoters. Co-transfection with

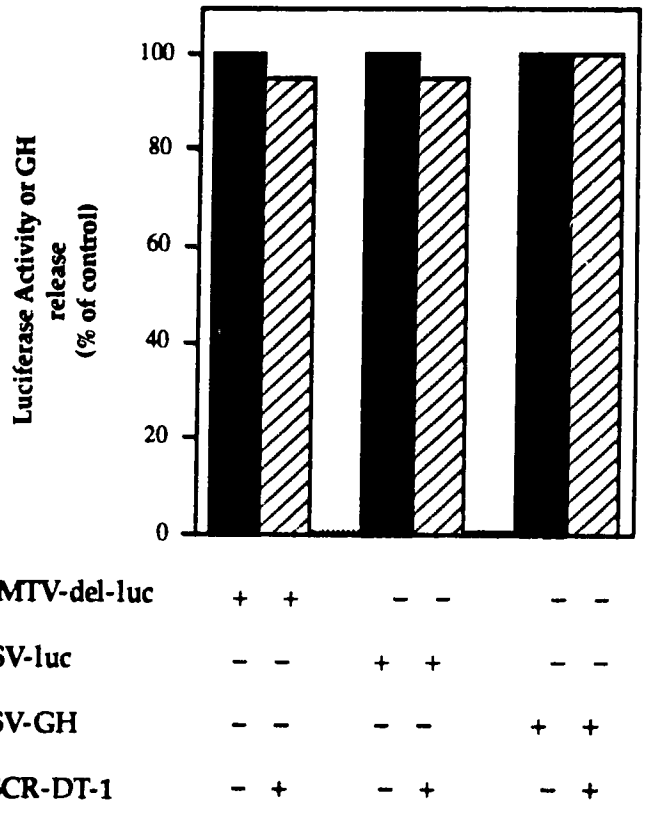
Fig. 5-6. Calreticulin represses GR-mediated transcription.

In (**A**) mouse L fibroblasts were cotransfected with the MMTV-GRE-luc reporter plasmid (pJA358) and either control plasmid (pSVL) or the calreticulin expression vector (pSCR-DT-1) (5 μg of each) using a calcium phosphate procedure. The cells were incubated in DMEM containing 10% charcoal-treated calf serum for 12 hours followed by incubation for 24 hours either in DMEM alone (striped bars; -DEX) or DMEM with 10^{-6} M dexamethasone (black bars; +DEX). The cells were harvested and assayed for luciferase activity. 100% activity corresponds to the luciferase activity of L cells cotransfected with GRE-luc reporter plasmid and the pSVL vector and induced with 10^{-6} M DEX for 24 hours. In (**B**) L cells were cotransfected by Mona Nemer with one of the following vectors (5 μg of each) and with pSCR-DT-1 (striped bars) or pSVL (black bars): GRE-deleted MMTV-luciferase (MMTV-del-luc), RSV-luciferase (RSV-luc) or RSV-human growth hormone (RSV-GH). Transfected cultures were grown in DMEM containing 10^{-6} M DEX, harvested and assayed for luciferase activity or levels of secreted growth hormone.

A


Luciferase activity (% of control)
100
80
60
40
20
0
GRE-luc + +
pSVL + -
pSCR-DT-1 - +
+ DEX
- DEX

B


Luciferase Activity or GH release (% of control)
100
80
60
40
20
0
MMTV-del-luc + + - - - -
RSV-luc - - + + - -
RSV-GH - - - - + +
pSCR-DT-1 - + - + - +

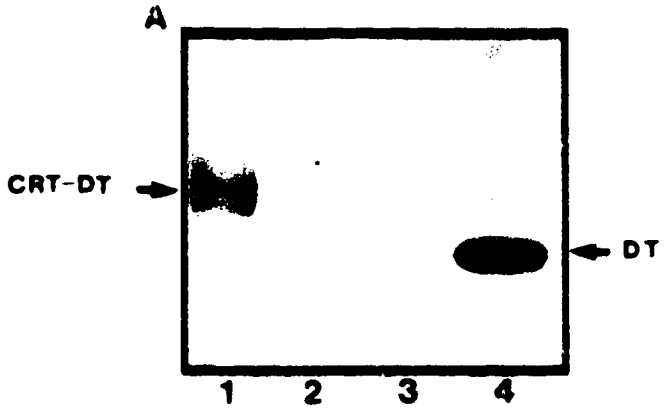
pSVL-CRT (Fig. 5-6, B) had no effect on the activities of these promoters indicating that calreticulin did not sequester any general factors and/or DNA binding proteins that are involved in their activation. Identical results were also obtained in HeLa cells (data not shown).

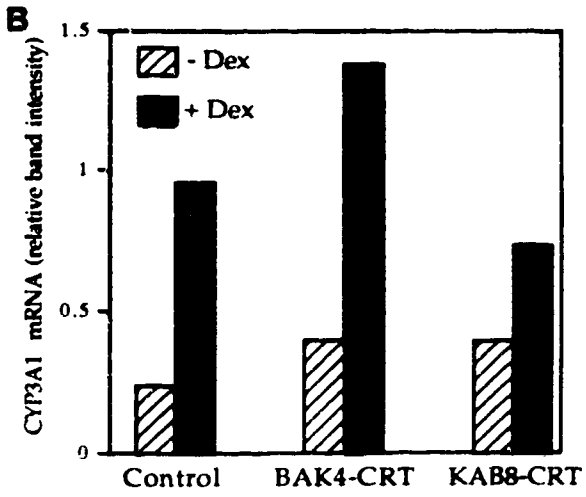
Mouse cell lines expressing elevated or reduced levels of calreticulin were used to analyze the physiological significance of these findings. These cell lines were made by stable transfection of mouse L-cells with plasmids encoding DT-tagged calreticulin either in the sense (pRCR-DT-1) or anti-sense orientations (pRCR-DT-2), respectively. A number of different clones were examined for calreticulin expression by Western analysis. Most stably transfected cells expressed levels of calreticulin comparable to those of control cells. However two cell-lines designated KAB-8 and BAK-4 were chosen for further analysis because they were determined to express slightly elevated (about 2.0 fold) and reduced (1.5 fold) levels of calreticulin. To confirm that elevated levels of calreticulin were expressed in KAB-8 cells, cellular extracts were examined by Western blot analysis with anti-DT antibodies (Fig. 5-7, A).

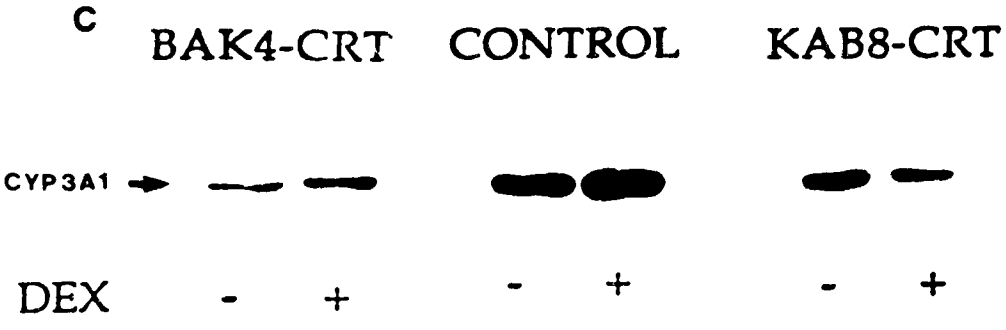
Both BAK4 and KAB8 cells were used to examine if calreticulin could influence expression of an endogenous glucocorticoid-sensitive gene, cytochrome P450 (CYP3A1) (Yanagimoto *et al.*, 1992). BAK4, KAB8 and control L-cells designated PGK-4, were plated in duplicate at equal densities in media containing charcoal-filtered fetal calf serum. 24-48 hours later half the plates were stimulated with DEX for an additional 24 hours. From DEX treated and untreated cells mRNA and protein was isolated and subjected to Northern and Western blot analysis. Dex treated BAK4 cells exhibited enhanced levels of CYP3A1 mRNA (Fig. 5-7, B) and protein (Fig. 5-7, C) relative to control cells. In contrast, the DEX-dependent increase in CYP3A1 mRNA (Fig. 5-7, B) and protein (Fig. 5-7, C) was inhibited in KAB8 cells relative to control cells. These data

Fig. 5-7. Calreticulin represses GR-mediated induction of Cytochrome P-450.

Mouse L fibroblasts were transfected by electroporation (1500 V/cm; 25 μF) with pRCR-DT-1 or pRCR-DT-2 (20 μg) (i.e. vectors containing calreticulin cDNA in the sense or anti-sense orientations, respectively). Cells were selected for stable integration of plasmid DNA in the presence of G418 (~400 μg/ml). A control cell-line (PGK-4 cells) and cell-lines expressing elevated (KAB-8 cells, ~2.0 fold increase) or reduced levels (BAK-4 cells, ~ 1.5 fold decrease) of calreticulin were selected for further analysis. In (A) cellular extracts from PGK-4, KAB-8 and BAK-4 cell-lines were prepared by detergent extraction and examined by Western blot anlaysis for the expression of DT-tagged calreticulin. The cellular extracts (15 μg/lane) were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose filters. Lane 1-KAB-8, lane 2, PGK-4, lane 3-BAK-4 and lane 4 GST-CT-1 (control: C-terminus of dystrophin). The membrane was probed with anti-rabbit DT antibodies diltued 1:100 in PBS containing 1% milk powder followed by incubation with anti-rabbit IgG diluted 1:2000 in the same buffer. Immunoreactivity was detected using Amersham's ECL detection system. In (B) the cell-lines were grown in DMEM containing 10% charcoal-treated calf serum for 24 hours followed by incubation for 24 hours in DMEM alone (black bars) or DMEM with 10^{-6} M DEX (striped bars). Total cellular RNA was isolated using the TRI REAGENT, separated by formaldehyde gel electrophoresis, and transferred to Hybond nylon membrane by capillary action. The blot was probed with CYP3A1 cDNA in FFMOPS buffer containing 20% formaldehyde at 42°C for 18 hours and then washed at 55°C in 0.1X SSC, 0.1% SDS. CYP3A1 mRNA levels were normalized by comparison to ribosomal protein L32 and glyceraldehyde-3-phosphate dehydrogenase mRNAs. The relative abundance of each mRNA was determined using Fujiex BAS1000 Phosphoimager. In (C) the expression of cytochrome P450 was determined in cells grown in the presence or absence of 10^{-6} M DEX by immunoblotting in the presence of 0.05% Tween 20 using 1:1,000 dilution of anti-rabbit CYP3A1 antibody in PBS containing 1% milk powder followed by incubation with anti-rabbit IgG conjugated to horseradish peroxidase. The peroxidase reaction was developed using Amersham's ECL detection system.


A
CRT-DT
DT
1
2
3
4


B
CYP3A1 mRNA (relative band intensity)
1.5
1
0.5
0
- Dex
+ Dex
Control
BAK4-CRT
KAB8-CRT


C
BAK4-CRT
CONTROL
KAB8-CRT
CYP3A1
DEX
-
+
-
+
-
+

further demonstrate that changes in the expression of calreticulin can modulate transcriptional activity of glucocorticoid-sensitive genes *in vivo*.

DISCUSSION AND CONCLUSIONS

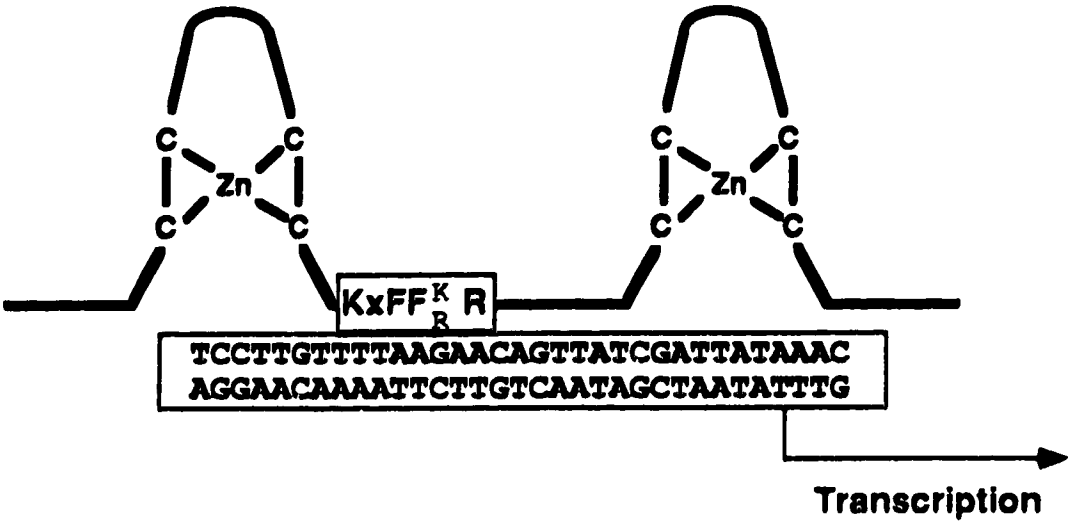
The experiments described in this chapter were designed to assess the potential for nuclear calreticulin to function as a transcriptional modulator. We have discovered that calreticulin can perform such a role by establishing that it interacts with and modulates the transcriptional activity of the GR *in vitro* and *in vivo* .

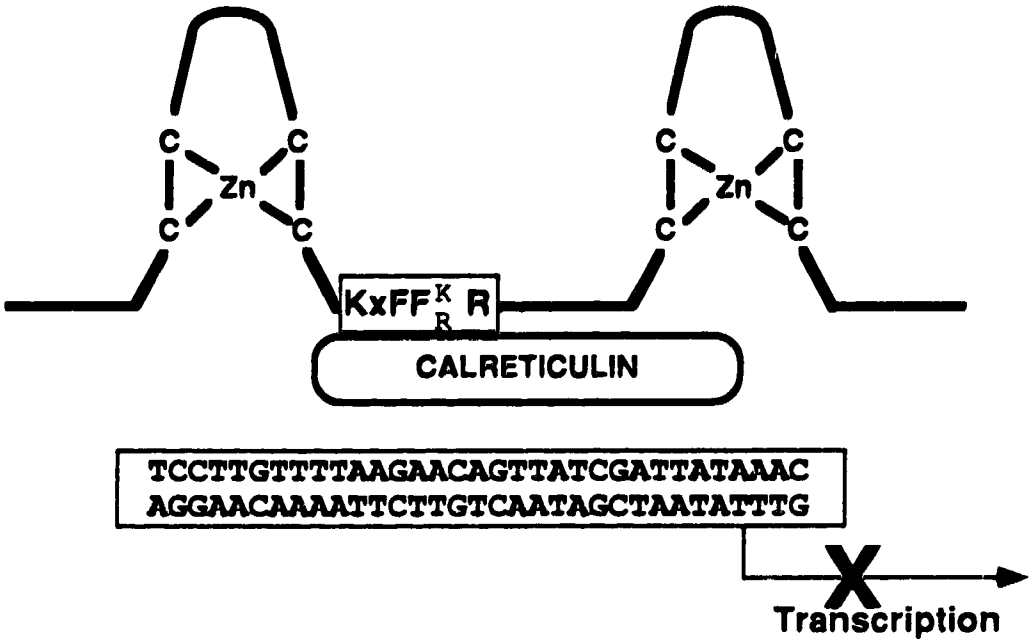
Simultaneously, Dedhar's group (Dedhar *et al.*, 1994) showed that calreticulin also interacts with the androgen receptor and that it modulates retinoic acid dependent differentiation of P19 cells. Recently, Rachubinski's group has shown that calreticulin also modulates the function of peroxisome proliferator-activated receptor (PPRAP), another transcription factor which also contains the K-x-F-F-K-R peptide (unpublished observations). Together these observations indicate that calreticulin may be important in gene transcription, regulating not only the GR, but also other members of the nuclear receptor superfamily.

Our interpretation of these findings is that in the presence of calreticulin, steroid receptors cannot interact with their HREs because their DBDs are masked (Fig. 5-8). We believe that calreticulin and the steroid receptors may co-localize in the nucleus in order to associate with each other. Although calreticulin has been detected in the nucleus (Opas *et al.*, 1991; Michalak *et al.*, 1992; Dedhar *et al.*, 1994) this localization is difficult to reconcile with the fact that all known calreticulins have an NH_2-terminal signal sequence and a carboxyl-terminal K-D-E-L ER retention signal. Calreticulin also possesses a putative nuclear

Fig. 5-8. Calreticulin interacts with the DNA-binding domain of steroid receptors.

The zinc fingers motif in the DNA-binding domain of the steroid receptors is shown. Our results suggest that in the presence of calreticulin, steroid receptors cannot interact with the hormone responsive elements of their target genes, leading to inhibition of steroid-sensitive transcription. This interaction between the steroid receptors and calreticulin may be mediated through the consensus amino acid sequence (KXFF[K/R]R) and the N-domain of calreticulin.


C
C
Zn
C
C
KxFF K R
R
TCCTTGTTTTAAGAACAGTTATCGATTATAAAC
AGGAACAAAATTCTTGTCAATAGCTAATATTTG
Transcription


C
C
Zn
C
C
KxFF K R
R
CALRETICULIN
TCCTTGTTTTAAGAACAGTTATCGATTATAAAC
AGGAACAAAATTCTTGTCAATAGCTAATATTTG
X
Transcription

localization signal (NLS) (Michalak *et al.*, 1992). However, in order for this NLS to target calreticulin to the nucleus, the protein must first presumably gain access to the cytoplasm.

The conundrum then becomes, how does calreticulin initially gain access to the cytoplasm? There are at least four conceivable mechanisms that could be utilized in order to allow calreticulin to become cytoplasmic: (i) two isoforms of calreticulin could be synthesized, one possessing ER targeting and retention peptides and the other devoid of these signals: (ii) calreticulin could be inefficiently translocated into the ER, and therefore remain in the cytosol (iii) calreticulin could gain access to the cytosol directly from the ER; (iv) calreticulin could be introduced into the cytosol from outside of the cell.

The most straightforward mechanism for generating cytoplasmically-localized calreticulin would be to synthesize an isoform that was never translocated into the ER. This could be achieved either by alternative splicing of the primary transcript and/or utilization of an alternate translation start site, or by the existence of mutliple genes encoding different isoforms of calreticulin. As discussed previously, cDNAs encoding different isoforms of calreticulin have recently been isolated from libraries generated from bovine brain and the central nervous system of *X. lacvis* (Liu *et al.*, 1993; Treves *et al.*, 1992) but it is not clear what mechanism is employed in the generation of these transcripts. Although the different cDNAs do include potential ER signal sequences, their existence indicates that some plasticity in calreticulin coding sequences does occur. Despite the current lack of direct genetic evidence, it therefore remains quite feasible that calreticulin mRNAs are sometimes synthesized that lack the coding sequences for the ER signal peptide.

It is also possible that some calreticulin is translated in the cytoplasm despite the presence of a signal sequence due to inefficient co-translational

translocation of the protein into the ER. As a polypeptide destined for the ER emerges from a ribosome it is recognized by a signal recognition particle which facilitates its delivery to the ER membrane (reviewed in Rapaport, 1992). Overloading of the translocation apparatus may occur during periods of high protein synthesis (overexpression of calreticulin) resulting in the cytoplasmic localization of the protein.

Another possibility is that calreticulin somehow escapes from the ER and moves directly into the cytoplasm. There is at present no evidence to support the notion that proteins can move from the lumen of the ER to the cytoplasm, although this idea has been recently considered (Pelham *et al*, 1992). For example, it has been proposed that certain bacterial toxins containing carboxyl-terminal K-D-E-L-like tetrapeptides may be endocytosed into a cell and then transported into the ER via retrograde transport (Pelham *et al*, 1992). Once in the ER, it has been hypothesized that that these proteins can escape into the cytoplasm. Although this would appear to be a less likely mechanism for calreticulin to be introduced in the cytoplasm, there is presently no evidence that would force us to exclude it as a possibility.

Finally, it is possible that calreticulin could be targeted out of the ER in one cell and delivered, by exocytosis, into the cytoplasm of another cell. As discussed previously, calreticulin may gain access to the cytoplasm of a target cell by being injected directly into it from the lytic granules of CTL (Chapter 3 Fig. 3-5). Even more intriguing is the possibility that calreticulin may be delivered into a target cell in order to modulate steroid receptor-dependent gene transcription that may be involved in the target cell response to the damage inflicted by perforin and other granule-localized lytic mediators.

The physiological circumstances under which calreticulin regulates glucocorticoid-sensitive transcription are not yet known. Regulation of genes by

the steroid receptors is the result of a cascade of interactions between the receptor and its HRE, the receptor and other DNA sequences and the receptor and other transcription factors (reviewd in Truss & Beato, 1993). A particular gene can be induced or repressed by a hormone-receptor complex in a certain cell type, not regulated in another cell type, and may even be inversely regulated in a third type of cell. Tuss and Beato (1993) suggest that cell specificity of receptor activity could be explained in part by the the existence of different sets of cell-specific factors. Although calreticulin is not a cell-specific factor (i.e., found in one cell and not another) it may function to regulate the activity of the receptors in a cell specific manner (i.e. through cell specific translocation to the nucleus).

In certain breast cancers, for example, cell sensitivity to steroids changes as the cancer progresses, despite the fact that the number of functional steroid receptors have not apparently changed in these cells (Van Der Burg *et al.*, 1991; Thompson *et al.*, 1991). Is calreticulin the 'unexplained modulator' of steroid receptor activity? It would be important to test if these changes in steroid sensitivity correlate with differential expression and/or localization of calreticulin in these cells. Interestingly, another ER protein, ERp61 has similarly been suggested to affect the response of cancer patients to treatment. In leukemia cells from patients with chronic myelogenous leukemia (CML), ERp61 has been demonstrated to alter complex formation between nuclear proteins and regulatory regions of interferon-inducible genes (Johnson *et al.* 1992). CML is a disease in which unregulated growth results in a relative increase in the numbers of late myeloid progenitor cells (Strife, *et al.*, 1990). In about 15% of CML patients interferon-α (IFN-α) induces complete cytogenetic remission, however most patients are not sufficiently sensitive to IFN-α to develop complete remission and some are totally resistant to IFN-α (Talpaz, *et al.*, 1986). Down-regulation of ERp61 occurs in CML cells in repsonse to IFN-α and correlates with clinical

sensitivity to IFN-α, suggesting that ERp61 may have a role in interferon-induced cytogenic remisssions in CML (Johnson *et al.*, 1992). These results also suggest that calreticulin's nuclear activities may not be unique and that at least one other ER protein may have potential effects on gene transcription.

In conclusion, various physiological conditions may exist in which calreticulin co-localizes with steroid hormone receptors in the nucleus to modulate their function. The key to understanding calreticulin's role as a transcriptional requlator may be derived from future experiments designed to determine how and when calreticulin becomes nuclear.

CHAPTER SIX

GENERAL DISCUSSION AND FUTURE DIRECTIONS

POTENTIAL CELLULAR ROLES FOR CALRETICULIN

In this study I have shown that: (i) the synthesis of calreticulin is elevated in stimulated T-cells and that the protein is targeted to lytic granules; (ii) calreticulin interacts with a set of ER associated proteins; and (iii) calreticulin binds to the DBD of the GR and modulates its activity*in vitro* and *in vivo*. Based on the present work and observations from other laboratories that encompass very diverse areas of cell biology, calreticulin appears to be a multifunctional protein (Fig. 6-1).

Calreticulin in T-lymphocytes - Is it a Ca^{2+} storage protein, chaperone and/or effector?

The expression and synthesis of calreticulin is elevated in stimulated mouse and human T-cells. This may be directly related to T-cell Ca^{2+} homeostastis, and/or their proliferation and maturation. Localization of calreticulin to the lytic granules in mature CTL suggests a possible role for calreticulin in the cytolytic activity of CTL. Calreticulin may be a chaperone for perforin, and/or a Ca^{2+} storage protein of cytolytic granules. To clarify calreticulin's role in T-lymphocytes an important future question will be to determine if calreticulin is actively targeted to the granules (i.e. does calreticulin become localized to the granules in the absence of perforin?).

Calreticulin in the ER - Is calreticulin a chaperone and/or signal transducer?

Using ligand blotting, a set of ER proteins was found to specifically associate with [^{125}I]calreticulin. These proteins include: a 50-and 52-kDa protein doublet and a number of lower molecular weight (38-20-kDa) proteins. The

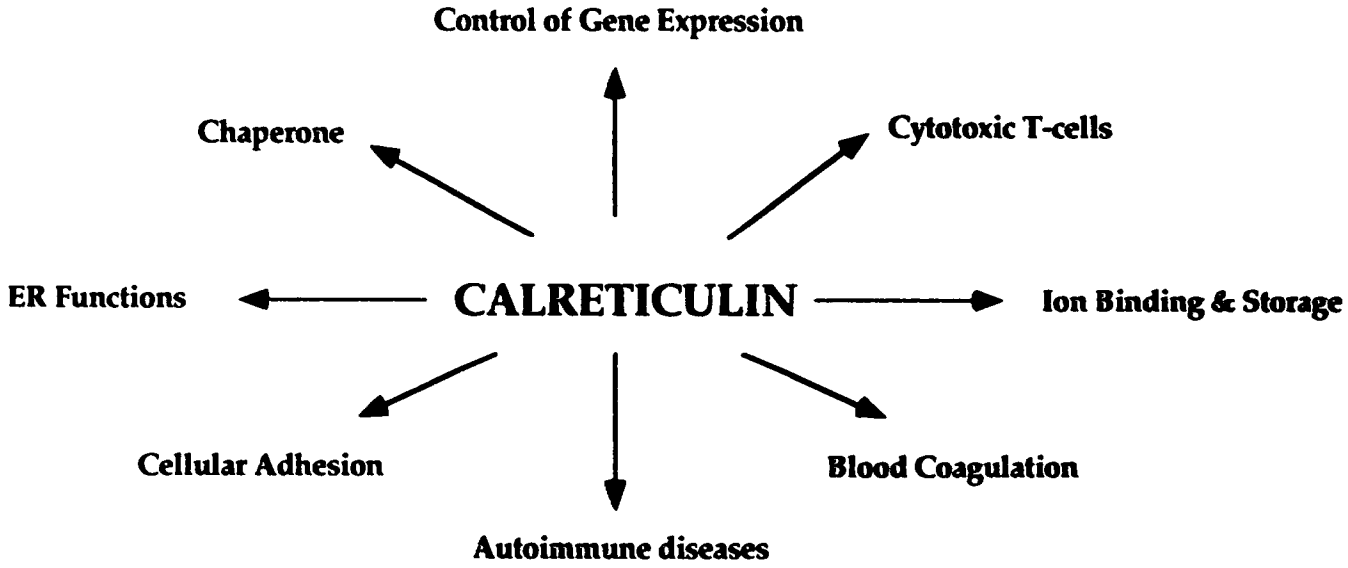


Fig. 6-1. Putative functions for calreticulin

number of proteins found to interact with calreticulin via this technique was initially suprising. However, multiple protein/protein interactions might be expected for if calreticulin acts as a molecular chaperone. Many of the low molecular weight proteins found to interact with calreticulin by ligand blotting may be membrane associated. It is possible that through interaction with these proteins calreticulin may be involved in regulating membrane associated events such as Ca^{2+} release from the ER. This would be similar to calsequestrin in muscle cells (Damiani *et al.*, 1990; Mitchell *et al.*, 1988). Future identification of calreticulin binding proteins should shed more light on the function of calreticulin in the ER.

Calreticulin - Does it interact with PDI?

PDI was retained on a calreticulin affinity column and subsequently found to interact with calreticulin in independent experiments carried out in the laboratory. Calreticulin and PDI may associate as part of an intracellular network in the ER (Koch *et al.*, 1987). Calreticulin and PDI may also associate to modulate their respective activities *in vivo*. Sharaiz Baksh has observed that calreticulin/PDI association affects the high Ca^{2+} binding capacity of calreticulin as well as the isomerase acitvity of PDI *in vitro*. Calreticulin/PDI complexes may exhibit additional, though not yet identified activities involved in post-translational modification of proteins.

Calreticulin in the nucleus - Is calreticulin a transcriptional regulator of steroid-sensitive gene expression?

Perhaps the most suprising finding in this study is that calreticulin can function as an inhibitor of steroid-sensitive gene expression. *In vitro*, calreticulin was shown to directly associate with the DNA binding domain of the GR. As

demonstrated by gel mobility shift assays, this interaction prevents the GR from interacting with DNA. The calreticulin/GR interaction is likely mediated through the K-V-F-F-K-R peptide in the DBD of the receptor and through the N-domain of calreticulin, suggesting a fundamental importance for this region of the protein.

In co-transfection experiments glucocorticoid-sensitive gene expression of a luciferase reporter plasmid was specifically inhibited by overexpression of calreticulin demonstrating that calreticulin can modulate glucocorticoid-sensitive gene expression *in vivo*. Expression of the glucocorticoid-sensitive endogenous gene cytochrome P-450 was inhibited or induced (relative to control cells) in L-fibroblast cell-lines expressing elevated or reduced levels of calreticulin, respectively. This modulation of receptor activity observed *in vivo* is postulated to occur in the nucleus through an interaction of calreticulin with the GR which prevents the receptor from interacting with its GRE.

Does calreticulin interact with proteins containing KXFFKR-like peptides?

In studies designed to identify proteins which interact with the α-integrins, Rojiani *et al.* (1991) demonstrated that calreticulin binds to the KLGFFKR peptide, a highly conserved sequence found in all α–integrins. Calreticulin and its cytoplasmic homologue mobilferrin have subsequently been shown to coimmunoprecipate with α–integrins (Leung-Hagesteijn *et al.*, 1994; Conrad *et al.*, 1993). As discussed previously, we and Dedhar *et al.* (1994) have demonstrated that the interaction of calreticulin with steroid receptors is likely mediated through a similar peptide (K-X-F-F-K-R). Interestingly, a very similar peptide, is also present in the amino acid sequences of perforin, cyclophilins, the $InsP_3$ receptor and other transcription factors (Haendler *et al.*, 1987; Shinhai *et al.*, 1988; Furuichi, *et al.*, 1989). Structural and possibly functional association of

calreticulin with some of these molecules has been proposed (Dupuis *et al.*, 1993; Bleackley *et al.*, 1994). The widespread nature and distribution of molecules which contain this peptide suggests the intriguing possibility that calreticulin might be involved in regulating cellular activities of different proteins from the cell surface to the nucleus.

Calreticulin and integrin - A role in cellular adhesion?

Calreticulin is also proposed to have a role in cell-extracellular matrix interactions (Rojiani *et al.*, 1991; Leung-Hagesteijn *et al.*, 1994). Leung-Hagesteijn *et al.* (1994) demonstrated that attachment and spreading of cells cultured in the presence of fetal bovine serum was specifically inhibited in cells in which the expression of calreticulin was downregulated by treatment with calreticulin specific antisense oligodeoxyonucleotides. Calreticulin may mediate an effect on cellular adhesion through interactions with the highly conserved peptide (K-X-L-G-F-F-K-R) found in the cytoplasmic domain of α-integrin subunits (Rojiani *et al.*, 1991; Leung-Hagesteijn *et al.*, 1994). Recently, O'Toole *et al.* (1994) showed that this peptide is important for affinity modulation of the receptor. A truncation which eliminated a domain containing this peptide resulted in a constitutively active receptor, whereas a truncation that preserved the motif did not. It is conceivable that calreticulin may associate with the integrin K-X-L-G-F-F-K-R peptide *in vivo* to set the default affinity state of ligand binding (O'Toole *et al.*, 1994).

Ion/Metal Binding (Storage Protein) ?

Calreticulin has been proposed to be the major Ca^{2+} binding (storage) protein in the ER (Milner *et al.*, 1991; Koch, 1990). In additon to Ca^{2+}, calreticulin also bind Ca^{2+}, Mg^{2+}, Zn^{2+} and Fe^{3+} *in vitro* (Khanna *et al.*, 1986; Baksh and

Michalak, 1991; Conrad *et al.*, 1993). It has been suggested that in the ER Mg^{2+} may balance charge movement related to Ca^{2+} release (Baumann *et al.*, 1991) and therefore it is possible that, *in vivo*, calreticulin might have some role in binding Mg^{2+} within the lumen of the ER. Calreticulin may also play a role in the regulation of cellular Zn^{2+} concentrations. Zn^{2+} has been implicated to have a potential neuromodulatory role in synaptic vesicles (Friedman & Price, 1984; Holm *et al.*, 1988; Xie & Smart, 1991) which may contain increased amounts of calreticulin (Johnson *et al.*, 1991). Calreticulin, therefore, may act to regulate both Zn^{2+} and Ca^{2+} levels in synaptic vesicles. Further, mobilferrin/calreticulin has been postulated to play a role in the absorption and intracellular metabolism of iron and other transitional metals (i.e. copper, cobalt, lead) in the duodenal mucosa (Conrad *et al.*, 1993). Calreticulin may thus have a role in regulating intracellular concentrations of various ions including Ca^{2+}, Mg^{2+}, Zn^{2+} and Fe^{3+}.

Calreticulin in blood - Is it an antithrombotic agent?

Calreticulin has been isolated from bovine serum (Sueyoshi *et al.*, 1991) suggesting that it may be secreted into the blood stream. Calreticulin was demonstrated to bind through its C-domain to the vitamin K-dependent coagulation factors, IX, X and prothrombin *in vitro* , suggesting that it may play some role in blood coagulation. Subsequently, it was shown that intracoronary infusion of calreticulin had a potent antithrombotic effect in a canine thrombosis model (Kuwabara *et al.*, 1993). Further experiments carried out at Genentech confirmed these observations and suggested that calreticulin may be an antithrombotic agent with significant advantages over currently available agents. It is not yet clear how calreticulin elicits this effect but it has been suggested that calreticulin may interact with the endothelium to stimulate or interfere with the clotting mechanism (Kuwabara *et al.*, 1993).

Calreticulin - A Role in immune and autoimmune responses?

In addition to its localization to cytotoxic T cell granules (Dupuis *et al.*, 1993) several observations also suggest that calreticulin may have a multifaceted role in the immune response: (i) A peptide corresponding to the calreticulin leader sequence has been identified in the antigen-binding groove of class I molecules expressed by antigen processing mutant cell lines (Henderson, *et al.*, 1992); (ii) Mice immunized with irradiated melanoma cells produce antibodies which recognize B50 (Gersten *et al.*, 1979, 1991); (iii) The C1qR which appears to be highly homologous to calreticulin is expressed on B-cells, neutrophils and endothelial cells and is believed to be involved in immunoglobulin secretion and clearance of immune complexes from the circulation (Malhotra *et al.*, 1993).

Calreticulin has been suggested to be the Ro/SS-A antigen (Lieu *et al.* 1988 & 1989; Collins *et al.*, 1989; McCauliffe *et al.*, 1990a & 1990b; Rokeach *et al.*, 1991). Antibodies directed against Ro/SS-A (calreticulin?) are found in the majority of patients with primary Sjögren's Syndrome and with systemic lupus erythematosus (SLE). These antibodies also occur in mothers of infants with neonatal lupus and congenital heart block (Tan, 1989). Despite the reports that calreticulin is the Ro/SS-A antigen Rokeach *et al.* (1991) were not able to immunoprecipitate Ro/SS-A RNA with anti-calreticulin antibodies. They also found that the antibodies of patients did not cross-react with recombinant calreticulin which suggests that calreticulin is not the Ro/SS-A autoantigen. To explain these discrepancies Sontheimer & Capra (1994) postulate that phosphoryation or another post-translational modification of calreticulin is required for anti-Ro/SS-A antibodies to bind calreticulin. Despite this, there is growing evidence to suggest that calreticulin is an autoantigen. Antibodies reacting with calreticulin can be found in the sera of patients with systemic lupus

erythematosus (Lieu *et al.*, 1988). As well, calreticulin is suggested to be a rheumatic disease-associated autoantigen (Lieu *et al.*, 1989; Routsias *et al.*, 1993). Autoantibodies against calreticulin are also found in onchocerciasis patients (Rokeach *et al.*, 1991; Lux *et al.*, 1992). Onchocerciasis or red river blindness is caused by the filarial nematode *Onchocerca volvulus*. The ocular pathology leading to blindness is believed to develop through autoimmunological reactions resulting from crossreactivity between parasite antigens and components of eye tissues. *Onchocerca volvulus* expresses the RAL-1 antigen, the *O. volvulus* analogue of calreticulin (Unnasch *et al.*, 1988; McCauliffe *et al.*, 1990b; Murthy *et al.*, 1990).

Calreticulin has also been implicated in halothane hepatitis. Halothane hepatitis is a rare, often fatal, idiosyncratic reaction to halothane which may have an immunological basis (Neuberger, *et al.*, 1987; Pumford *et al.*, 1993). Serum antibodies are directed against liver microsomal proteins that become covalently trifluoroacetylated (Kenna *et al.*, 1988). Calreticulin is one of a number of microsomal proteins that becomes modified by the trifluoroacetyl halide metabolite of halothane. Serum antibodies from patients with halothane hepatis reacted with both modified and native protein to a greater extent than did serum antibodies from control patients (Butler *et al.*, 1992). Interestingly, ERp72, another lumenal ER protein, also become modified by the trifluoroacetyl halide metabolite of halothane (Kenna *et al.*, 1988; Pumford *et al.*, 1993). Calreticulin and other lumenal ER may therefore become autoantigens in halothane hepatis patients.

Calreticulin - Is it the C1qR?

As discussed previously, the C1qR is a protein which appears to be very similar to calreticulin. In addition to C1q, this plasma membrane receptor binds

to conglutinin, lung surfactant protein A, and mannan binding protein (Malhotra *et al.*, 1993). Various ClqR-mediated functions have been described, including enhancement of phagocytosis, stimulation of oxygen radical generation and immunoglobulin secretion, and clearance of immune complexes from the circulation. In addition, ClqR has been shown to act as a transient component of the extracellular matrix where it may provide additional signals for ClqR endowed cells to localize and regenerate damaged tissue. Future identification of calreticulin as the C1qR would implicate calreticulin in these putative additional diverse functions of the C1qR.

Calreticulin - A protein with distinct functional domains?

In various aspects of this study and in studies done by other laboratories fusion proteins representing distinct structural regions of calreticulin (N-, P-, C-domains) were used to assess the relative contributions of each of these regions to the functions of the protein. The C-domain and to a lesser extent the P-domain are involved in mediating the interactions between calreticulin and other cellular proteins as demonstrated by ligand blotting. Both the P-and C-domains have been established to bind Ca^{2+} (Baksh & Michalak, 1991). The high affinity/low capacity and the low affinity/high capacity Ca^{2+} binding sites were localized to the P- and C-domains, respectively (Baksh & Michalak, 1991). The N-domain is involved in the interaction between calreticulin and the GR (Burns *et al.*, 1994), calreticulin and perforin (M. Michalak, unpublished observations) and calreticulin and α-integrin (Leung-Hagesteijn *et al.*, 1994). As summarized in Figure 6-2 the distinct structural regions of calreticulin may also represent functional domains.

In summary, that calreticulin may have distinct functional domains, taken together with the observations that calreticulin is involved in the diverse

N-domain	P-domain	C-domain
amino acid sequence unique to calreticulin; highly conserved	amino acid sequence similar to calnexin; highly conserved	amino acid sequence similar to calsequestrin & other "KDEL" proteins
		glycosylation site (?)
Zn binding sites (?)	high affinity Ca^{2+} binding site	low affinity Ca^{2+} binding sites
interacts with the DNA binding domain of steroid receptors	interacts with PDI	interacts with ER & SR membrane proteins
interacts with synthetic peptide KLGFFKR		binds to coagulation factors:
interacts with α-subunit of integrin		interacts with KDEL receptor

Fig. 6-2. Putative functions of calreticulin domains.

biological functions proposed above, strongly suggests that calreticulin is a multifunctional protein.

FUTURE DIRECTIONS

How can one protein perform so many different functions? To address this complex question an important future goal will be to generate calreticulin gene knock-out mice. These mice (providing the knock-out is not lethal) would provide an experimental model for examining the various functions of calreticulin and may provide insight into calreticulin's cellular roles. Calreticulin null mice would certainly address the 'burning' question asked in the laboratory over the years: can there really be life without calreticulin?

What is the precise role of calreticulin during maturation of T-cells? Perforin knockout mouse have recently been generated (reported in Doherty, 1993). Splenocytes isolated from these mice would provide an excellent model system to examine the role of calreticulin in T-cells. For example, analysis of these cells will determine whether an ass ociation with perforin is required for trafficking of calreticulin to the granules. Cotransfection of cDNA encoding perforin into perforin minus splenocytes may also help to establish the nature of the calreticulin/perforin association. Splenocytes from calreticulin minus mice or downregulation of calreticulin in normal CTL by treatment with calreticulin specific antisense oligodeoxynucleotides could also provide further insight into calreticulin's putative roles in the biogenesis of lytic granules and the cytolytic activity of T-cells.

What are the functions of calreticulin in the ER? Before this question can clearly be addressed, further experiments are required to establish the identity of the [^{125}I]calreticulin-binding proteins. Multiple experimental approaches are

then necessary to verify that the binding of calreticulin to the proteins by overlay is specific and of high affinity, and if possible to determine if the interaction with calreticulin has an effect on the function of the protein. Purification of the [^{125}I]calreticulin-binding proteins could be attempted using various chromatographic procedures (i.e. ion-exchange columns, lectin chromatography), followed by analysis of flow through and eluted fractions for [^{125}I]calreticulin-binding proteins by ligand blotting. If purification is successful it may be possible to establish their identities by NH_2-terminal amino acid sequence analysis. Additionally, an expression cloning strategy using radiolabeled calreticulin as a probe could be used to isolate cDNAs encoding the calreticulin-binding proteins. As discussed previously, the nature of the association between calreticulin and the ER proteins is unknown. Denaturation of the proteins with 6 M urea and DTT prior to application on a calreticulin affinity column would help to establish whether calreticulin binds these proteins through regions exposed in the unfolded protein. It would also be interesting to compare the [^{125}I]protein-binding patterns of BiP (an ER chaperone) obtained by ligand blotting with the [^{125}I]calreticulin binding pattern observed in this study.

Is there a cytosolic/nuclear form of calreticulin involved in control of steroid-sensitive gene expression? Trafficking studies in eukaryotic cells are required to understand how calreticulin is targeted to the nucleus and may ultimately provide insight into the physiological conditions under which calreticulin regulates control of glucocorticoid-sensitive gene expression. To address which regions of calreticulin's amino acid sequence are involved and/or required for directing the protein to the nucleus various constructs tagged with DT could be generated (discussed in Chapter 5). For example, a vector containing a version of calreticulin, minus its leader sequence, could be transfected into eukaryotic cells to determine if a cytosolic form of calreticulin is

targeted to the nucleus (i.e. that the putative NLS is operational)? The intracellular localization of this and other calreticulin mutants could be examined immunocytochemically or by sub-cellular fractionation using DT-specific antibodies.

In summary, calreticulin or highly homologous gene products are associated with a number of rather diverse biological functions that take place in different subcellular compartments. The mechanisms which allow this molecule to participate in such disparate cellular roles will undoubtedly be the subject of future debate and investigation. If research continues on calreticulin at the present pace the future holds much insight into this intriguing protein.

CHAPTER SEVEN

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