

POTENTIAL INTERACTION OF CYSTEINE RICH INTESTINAL PROTEIN WITH
A PROTEIN PARTNER AND THEIR REGULATION IN IMMUNE CELLS

By

JOHN DAVID NICEWONGER II

A THESIS PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF
FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2003

Copyright 2003

by

John D. Nicewonger II

This work is dedicated to my fiancé Cynthia Rodriguez who always encourages me to be the best that I can be. She has taught me to be persistent in accomplishing my goals no matter how long it takes. I would also like to thank my family for their financial support during my time at graduate school.

ACKNOWLEDGMENTS

I would like to thank my advisor Dr. R. J. Cousins, for giving me a chance to work on this project. His funding throughout the entire two and a half years of my stay here helped me complete this project. Several times he or his staff have pointed me in the right direction whenever I needed help with a problem. He has taught me to be an independent thinker and to work hard to complete the goals that I have set for myself. I would also like to thank the members of my committee, Dr. B. McMahon and Dr. B. Langkamp-Henken, for extending their expertise to my project.

I appreciated the technical expertise of Dr. Cal Green and Dr. Ray Blanchard. Their advise with the technical issues of my project helped me immensely. I would also like to thank Virginia Mauldin for helping me make the computer do what I wanted it to do when it was being stubborn.

Finally, I would like to thank the entire University of Florida Food Science and Human Nutrition faculty for the great job they do teaching classes and running the department. I learned a vast amount of knowledge in their classes and I will make sure it gets put to good as I further my career in science.

TABLE OF CONTENTS

	<u>Page</u>
ACKNOWLEDGMENTS	iv
LIST OF FIGURES	vii
ABSTRACT	ix
CHAPTER	
1 INTRODUCTION	1
Control of Cell Proliferation and Apoptosis by Zinc.....	1
Characteristics of LIM Proteins.....	6
Characterization of Cysteine Rich Intestinal Protein.....	7
The Many Functions of Calreticulin.....	9
2 MATERIALS AND METHODS.....	13
Detection of a CRIP Protein Partner.....	13
Effects of IFN- γ , LPS, and Cell Proliferation on CRIP and CRT Expression in RAW 264.7 Cells.....	14
Real Time RT-PCR of CRIP and CRT RNA	16
Nondenaturing PAGE and Western Analysis.....	17
Immunocytochemistry	18
Statistical Analysis.....	19
3 RESULTS	20
Identification of Possible CRIP Binding Partner Using Affinity Chromatography ...	20
Effect of RAW 264.7 Cell Proliferation on CRIP, CRT, and MT mRNA	20
Effect of IFN- γ and LPS on CRIP , CRT, and TNF- α mRNA Expression and Nitric Oxide Production in RAW 264.7 Cells	21
Effect of RAW 264.7 Cell Proliferation on CRIP Protein Levels.....	22
Nondenaturing PAGE of RAW 264.7 Cell Cytosolic Extracts at 60% Confluency ..	22
Colocalization of CRIP and CRT	23

4	DISCUSSION	34
	LIST OF REFERENCES	43
	BIOGRAPHICAL SKETCH	52

LIST OF FIGURES

<u>Figure</u>	<u>page</u>
3-1 Silver stained 10% SDS tris-glycine polyacrylimide gels of proteins eluted from the CRIP affinity purification column. (A) Silver staining of thymus cytosolic proteins eluted off the column identified a 55 and 80 kD band. (B) Silver staining of spleen extracts eluted off the column also identified a 55 and 80 kD band. (C) Silver staining of intestinal extracts eluted off the column identified only a 55 kD band. (D) Salt was removed from the column eluate prior to electrophoresis. Note the improved apparent resolution of the 55 and 80 kD bands. M stands for marker and E# stands for elution number.	24
3-2 Western analysis of thymic (left) and intestinal (right) proteins eluted from the CRIP column performed with a calreticulin (CRT) antibody (Santa Cruz Biotechnology) revealed that the 55 kD band that bound to the column in these samples was CRT.	25
3-3 Silver stained 10% SDS tris-glycine polyacrylimide gels of proteins that eluted off of the control column. The molecular weight of the 50 and 75 kD marker bands are labeled. (A) Silver staining of thymus cytosolic proteins eluted off the column identified no bands (B) Silver staining of spleen extracts eluted off the column identified no bands (C) Silver staining of intestinal extracts eluted off the column identified no bands.	26
3-4 Effect of RAW 264.7 cell proliferation on CRIP (A), CRT (B), and MT (C) mRNA levels relative to 18S RNA as measured by real time RT PCR and approximate confluence (D) at 0, 8, 12, 15, and 24 h after plating cells at a 1:3 ratio.	27
3-5 Effects of LPS and IFN- γ treatment of RAW 264.7 cells at 80% confluency on CRIP (A) and CRT (B) mRNA levels relative to 18S RNA as measured by real time RT PCR at 0, 3, and 9 h after treatment.	28
3-6 Effects of LPS and IFN- γ treatment of RAW 264.7 cells at 80% confluency on (A) TNF- α mRNA levels relative to 18S RNA as measured by real time RT PCR and (B) nitric oxide production as measured by addition of Greiss reagent to medium at 0, 3, and 9 h after treatment.	28

3-7	(A) Western analysis of RAW cell cytosolic CRIP protein levels up to 32 h after plating. (B) Densitometric measurement of CRIP protein increased from initial levels up till 10 h. At 22 h CRIP protein expression was back at initial levels.....	29
3-8	Western analysis of RAW 264.7 cell cytosol protein extracts analyzed in a nondenaturing 7.5% polyacrylide gel and transferred to PVDF membrane (0.1 um) for analysis with CRIP and CRT antibodies.....	30
3-9	RAW 264.7 cells were incubated with polyclonal rabbit anti-CRIP (UF 83) and anti-rabbit conjugated FITC antibodies (Sigma) and polyclonal goat anti-CRT (Santa Cruz Biotech) and anti-goat conjugated Texas Red antibodies (Santa Cruz Biotech) 12 and 24 h after plating on slides images were obtained using confocal microscopy. (A) CRIP (green) localization in cells at 12 h at 900 X (B) CRT (red) localization in cells at 12 h at 900 X (C) CRIP and CRT (yellow) colocalization in cells at 12 h at 900 X (D) CRIP (green) localization in cells at 12 h at 3000 X.....	31
4-1	Model of potential CRIP interaction with CRT and PKC which would cause translocation of CRT to integrins at the cell membrane and block translocation of CRT into the endoplasmic reticulum.	42

Abstract of Thesis Presented to the Graduate School of the University of Florida in
Partial Fulfillment of the Requirements for the Degree of Master of Science

POTENTIAL INTERACTION OF CYSTEINE RICH INTESTINAL PROTEIN WITH
A PROTEIN PARTNER AND THEIR REGULATION IN IMMUNE CELLS

By

John David Nicewonger II

May 2003

Chair: Dr. Robert J. Cousins

Major Department: Food Science and Human Nutrition

Cysteine rich intestinal protein (CRIP) is an 8.5 kD protein which contains a distinct LIM domain consisting of two zinc coordinating fingers in close proximity to one another. There are several facts known about CRIP, but the function of the protein is still not well understood. CRIP mRNA is most abundant in macrophages, PBMCs (peripheral blood mononuclear cells), and the intestine. It is also very abundant in thymus, spleen, heart, and lungs. Overexpression of CRIP in transgenic mice leads to increased secretion of Th2 (T helper 2) cytokines (interleukin 6, IL-6 and interleukin 10, IL-10) and decreased secretion of Th1 (T helper 1) cytokines (interferon- γ , IFN- γ and interleukin-2, IL-2) after challenge of mice with LPS (lipopolysaccharide) or virus, or mouse splenocytes with PHA (phytohemagglutinin). In order to better understand the function of CRIP we designed a CRIP affinity purification column to identify possible protein partners. Using the column we identified calreticulin (CRT) as a possible protein partner. CRT was first identified as a calcium binding chaperone in the endoplasmic reticulum,

but recently, CRT has been shown to be necessary for cell adhesion and spreading because of its ability to bind integrins in the cell membrane and regulate integrin-mediated cell adhesion. CRT has also been localized to the nucleus where it binds to nuclear steroid hormone receptors, inhibiting their ability to bind to DNA. In order to further explore the relationship between CRIP and CRT, their mRNAs were measured during RAW 264.7 cell proliferation and upon treatment of cells with LPS +/- IFN- γ which led to apoptosis in this cell type. During cell proliferation both CRIP and CRT mRNA expression increased significantly and upon treatment with LPS +/- IFN- γ mRNA expression of both proteins decreased significantly. Immunocytochemistry using confocal microscopy revealed that both proteins colocalized during cell proliferation. The binding of CRIP to CRT may shift localization of CRT from the endoplasmic reticulum to sites of cell adhesion and/or the nucleus. Since CRT is an important chaperone for IFN- γ (a Th1 cytokine) and MHC-I (major histocompatibility 1) complexes, this may also explain why overexpression of CRIP leads to increased secretion of Th2 cytokines and decreased expression of Th1 cytokines.

CHAPTER 1 INTRODUCTION

Zinc is a divalent cation that engages in structural, catalytic, and regulatory roles within organisms. Many proteins rely on zinc for both their structure and/or function. For example, zinc atoms are an important part of the structure of the zinc fingers at the amino terminus of the enzyme protein kinase C (PKC) (Hubbard et al., 1991). Yet, increased free intracellular zinc levels also activate PKC without affecting the zinc coordinated in the zinc fingers of PKC (Csermely et al., 1988a and 1988b and Zalewski et al., 1990). Zinc is also an important part of metalloenzymes like carbonic anhydrase. In carbonic anhydrase catalyzed reactions, the zinc atom acts as a Lewis acid, stabilizing the negative charge and allowing ionization of water and formation of bicarbonate (Voet & Voet, 1995). In addition, zinc has been shown to regulate transcription of genes. For example, the transcription factor MTF-1 (metal response element-binding transcription factor 1) has six zinc fingers in its structure. Binding of zinc to the sixth zinc finger causes translocation of MTF-1 to the nucleus, where it activates transcription of several genes by binding to metal response elements in their promoters (Smirnova et al., 2000).

Control of Cell Proliferation and Apoptosis by Zinc

The mineral zinc is essential for cell proliferation. In many cell types zinc deficiency and excess results in apoptosis. Intracellular free zinc concentrations are very low, in the range of 1 nM (Atar et al., 1995). The intracellular distribution of loosely bound zinc may be much higher, micromolar concentrations (Coyle et al., 1994). Zinc is part of the structure of many proteins, including enzymes of cellular signaling pathways

and transcription factors. Related to these roles, zinc regulates cell signaling on many levels, including modifying the activities of PKC, mitogen activated protein kinases (MAPK), and MTF-1. These signal cascades are important in cell proliferation or apoptosis (Beyersmann & Haase, 2001).

There are over one thousand transcription factors containing zinc finger domains (Berg & Shi, 1996). Therefore, zinc is essential for the proper regulation of transcription of many genes, including numerous genes important for the stimulation of growth. All of the nuclear steroid hormone receptors contain zinc fingers. Upon ligand binding, these proteins bind to steroid response elements in the promoters of genes involved in proliferation and differentiation (Berg et al., 1996). Also, the most well recognized zinc activated transcription factor is MTF-1. This protein contains six zinc finger motifs. Binding of zinc to the sixth zinc finger of MTF-1 triggers the translocation of MTF-1 from the cytoplasm to the nucleus (Smirnova et al., 2000). Once in the nucleus MTF-1 binds to metal response elements in the promoters of the genes that code for proteins such as the zinc transporter ZnT-1, the zinc-donor/acceptor protein metallothionein (Langmade et al., 2000), calreticulin (Nguyen et al., 1996), and a growing list of other proteins (Lichtlen et al., 2001). Recently, calreticulin has been characterized as a protein that binds to integrins in the cell membrane, and has been shown to be essential for cell adhesion and spreading (Coppolino et al., 1998). Therefore, it is not out of place for expression of this protein to be upregulated by zinc. Because of its relevance to this project, this protein will be discussed more in a later section of this introduction.

Zinc is important for the stimulation of cell proliferation and growth. The evidence for this has been derived from numerous experimental approaches. Chelation of zinc in

mammalian cell cultures causes decreased growth and DNA synthesis (Grummt et al., 1986). Zinc and calcium synergistically initiate DNA synthesis and mitogenic signaling in murine fibroblasts (Huang et al., 1999). Chesters et al. (1989 and 1990) reported the zinc chelator diethylenetriaminepenta-acetic acid (DTPA) reduced thymidine incorporation into DNA and impaired transcription of the thymidine kinase gene in Swiss 3T3 cells. Roth & Kirchgessner (1994) found that zinc deficient rats secreted less growth hormone from the pituitary gland. Zinc deficiency led to decreased expression of growth hormone and insulin-like growth factor-1 (IGF-1) in the livers of both humans (Cossack, 1991) and rats (McNall et al., 1995). IGF-1 is important for cell growth because it has been shown to mediate the transition from the G1- to the S-phase in cultured cells. In addition, a translocation of metallothionein (MT) into the nucleus has been detected during the early S-phase of growth factor-stimulated primary rat hepatocytes (Tsujikawa et al., 1991), in regenerating rat liver (Tsujikawa et al., 1994), certain tumors (Kuo et al., 1994; Nartey et al., 1987; Woo & Lazo, 1997), and rat keratinocytes after irradiation with UV-B (Hanada et al., 1998). Apostolova & Cherian (2000) demonstrated that blocking DNA synthesis with aphidicolin in order to trap cells in the G1- to S-transition, caused zinc and MT to remain in the nucleus. Only after removal of aphidicolin did cells reenter the S-phase, and both zinc and MT were translocated back to the cytoplasm. The nuclear translocation of MT could be important for donation of zinc to transcription factors important in cell proliferation.

Zinc is also an important mediator of cell migration and proliferation. Zinc has been shown to modify integrin expression and cause increased cell migration in human keratinocyte cell cultures (Tenaud et al., 2000). Prior to this, Dreno (1996) demonstrated

that zinc stimulates proliferation of keratinocytes and fibroblasts in wounds. Zinc is an essential structural element in the zinc fingers of the LIM domains in a family of proteins known as LIM proteins. LIM domains mediate protein-protein interactions. These proteins have been shown to be important in cell fate determination, cytoskeleton reorganization, and growth control (Bach, 2000; Dawid et al., 1998). Because of their relevance to this study, these proteins will be discussed further in a latter section of this introduction as well.

Zinc is also part of the integral structure of many enzymes and zinc also has been shown to influence the catalytic activity of other enzymes. In murine fibroblasts increased extracellular zinc triggered protein tyrosine phosphorylation and MAPK activity (Hansson, 1996). Wu et al. (1999) reported zinc activated epidermal growth factor receptor phosphorylation and MAPK activity in human bronchial epithelial cells. In addition, Samet et al. (1999) showed that zinc blocked several protein tyrosine phosphatases in human airway epithelial cells. Zinc has also been shown to be a structural part of many isoforms of PKC (Parker et al., 1986). Hubbard et al. (1991) reported that EXAFS spectra of PKC-beta-I confirmed complexation of four zinc atoms each by three cysteines and one histidine residue. The four zinc atoms are coordinated within four zinc fingers at the amino terminus of PKC. Nanomolar concentrations of zinc can activate PKC and cause translocation of the enzyme to the plasma membrane (Csermely et al., 1988a and 1988b). In addition, Zalewski et al. (1990) demonstrated that zinc regulates translocation of PKC to the cytoskeleton. Moreover, it was revealed that a chelatable pool of intracellular zinc enhances the binding of the PKC activator phorbol dibutyrate (Forbes et al., 1990). Zinc atoms bound in the finger domains of PKC could

not be removed by treatment of PKC with high affinity heavy metal ion chelators (Hubbard et al., 1991). Additionally, the zinc chelators N,N,N',N'-terakis-(2-pyridylmethyl)ethylenediamine (TPEN) (Csermely et al., 1988b) and 1,10-phenanthroline (Forbes et al., 1990) were shown to hinder PKC activation. Therefore, the zinc bound in zinc fingers of PKC does not appear to influence activation of PKC.

Zinc has also been shown to affect cGMP (cyclic guanine monophosphate) content of cells by modifying the activity of cGMP degrading cyclic nucleotide phosphodiesterases. In addition, cGMP has been shown to control zinc uptake. After incubation of C6 rat glioma cells with 150 μ M zinc chloride, zinc uptake reached maximal levels after 1 h. This increase was not observed when the cells were treated with specific guanylate cyclase inhibitors, but was accelerated by nitric oxide, an activator of guanylate cyclase. The rise in intracellular cGMP (cells treated with guanylate cyclase inhibitors) inhibited zinc import, while lower intracellular concentrations of cGMP (cells treated with nitric oxide) accelerated zinc import (Haase & Beyersmann, 1999).

Studies have shown that in zinc deficient animals display more apoptotic cells in several tissues and organs, including the intestinal and retinal pigment epithelium, skin, thymic lymphocytes, testis and pancreatic acinar cells of adult animals (Duvall & Wyllie, 1986; Zalewski et al., 1990), and the neuroepithelium of fetal rats (Rogers et al., 1995). Apoptosis induced by zinc-deficiency has been shown to cause DNA and nuclear fragmentation, chromatin condensation, and apoptotic body formation (Truong-Tran, 2001). In addition, this type of apoptosis results in increased caspase-3 activity and caspase-3 inhibitors have been shown to partially block apoptosis (Chai et al., 2000).

Moreover, studies by Troung-Tran et al. (2000) have revealed that labile pools of zinc are associated with precursor forms of caspase-3 in the mitochondria and microtubules. They suggested that decreased intracellular zinc concentrations may disrupt these zinc pools and trigger apoptosis. Furthermore, Cui et al. (1999 and 2000) have demonstrated increased oxidative stress due to enhanced inducible nitric oxide synthase (iNOS) activity and nitric oxide (NO) production, increased tissue damage, and increased apoptosis in the skin and intestinal villi of zinc deficient rats.

In conclusion, zinc has been shown to be very important in controlling life or death of the cell. Not only is zinc a structural component of many proteins, including transcription factors, enzymes, and adapter proteins, zinc also regulates the control of many cell signaling cascades involved in proliferation and apoptosis.

Characteristics of LIM Proteins

The LIM domain consists of a cysteine rich, zinc-coordinating domain that forms a double zinc finger motif and exists in proteins that are involved in determination of cell fate, growth control, or cytoskeleton organization (Bach, 2000 and Dawid et al. 1998). LIM proteins that have been identified to date are localized to the nucleus, cytoplasm, or some are shuttled between both compartments (Bach, 2000). LIM proteins contain one or multiple LIM domains, where the fingers always occur in tandem. In many of these proteins, the LIM domain has been identified as an important structure in mediating protein:protein interactions and the formation of multiprotein complexes (Bach, 2000).

LIM proteins that are localized to the nucleus include LHX (LIM homeodomain X) and LMO (LIM-only) proteins. LHX proteins are transcription factors that bind to DNA via their homeodomains. LMO proteins are transcriptional cofactors that are components of multiprotein regulatory elements (Bach, 2000).

Several LIM proteins that are localized to the cytoplasm and/or the nucleus include LIMK (LIM kinase), CRIP, CRP (cysteine rich protein), enigma, zyxin, paxillin, and PINCH (particularly interesting new cysteine-histidine rich protein). LIMK proteins have LIM domains that are repeated in tandem, but they additionally have a kinase domain. The LIMK proteins have been localized to both the cytoplasm and nucleus. CRIP and CRP are proteins that have one and two LIM domains, respectively, but no apparent additional functional unit (Dawid et al., 1998). CRIP has been localized to the cytoplasm, but its function is still unknown (Fernandes et al., 1995). CRPs are associated with the cytoskeleton, and are involved in muscle differentiation (Harper et al., 2000). Enigma, zyxin, paxillin, and PINCH are proteins that contain different numbers of LIM domains located at the C-terminus (Dawid et al. 1998). These proteins have been reported to have important functions in cytoskeleton organization and cellular adhesion (Borrello et al., 2002; Hobert et al., 1999; Nix & Beckerle, 1997; Rearden, 1994; Sadler et al., 1992; Turner et al., 1990).

Characterization of Cysteine Rich Intestinal Protein

Cysteine Rich Intestinal Protein (CRIP) is a small 8.5 kDa protein with one LIM domain. It is abundant in immune cells and organs, including the intestine. It's identification as a zinc binding intestinal protein using high resolution chromatography has been reviewed in detail by Khoo & Cousins (1993).

Many proteins containing LIM domains are able to mediate multiple protein-protein interactions through these domains (Dawid et al. 1998). For example, zyxin, a protein containing three LIM domains, has been shown to bind to both H-warts/LATS1 which is involved in regulating mitotic progression and members of the CRP family which stabilize actin-rich structural elements in muscles. Zyxin seems to be important in

actin assembly and organization because it localizes to sites of cell adhesion and leading lamellipodia and its LIM domains interact with proteins important in these processes. It has also been shown that upon mislocalization of zyxin, one member of the Ena/VASP family is also displaced, and the organization of the actin cytoskeleton is perturbed (Nix & Beckerle, 1997; Nix et al., 2001; Sadler et al., 1992). Another LIM domain protein, DRAL/FHL2, has been shown by deletion analysis of particular LIM domains to bind to the cytoplasmic domain of several alpha and beta integrin chains. Particular LIM domains or LIM domain combinations of DRAL/FHL2 bound to these proteins. The fact that DRAL/FHL2 is found in cell adhesion complexes suggests that it is an adaptor/docking protein involved in integrin signaling pathways (Wixler et al., 2000). LIM proteins with only short sequences beyond their LIM motifs, such as CRP and LMO, function as adapter proteins to bring multiprotein complexes together (Dawid et al. 1998). Although the function of CRIP is unknown, CRIP also has only a short sequence beyond its LIM domain, and therefore it may function in an analogous fashion as an adapter protein.

While little is known about CRIP's function, its tissue localization has been well characterized. CRIP mRNA is most abundant in macrophages, PBMCs, and the intestine. It is also very abundant in thymus, spleen, heart, and lungs (Hallquist et al., 1996). The organ in which CRIP mRNA seems to be least abundant is the liver. However liver CRIP mRNA increases upon carbon tetrachloride induced inflammation and CRIP was shown by immunohistochemistry to be localized to hepatocytes near the blood supply (Khoo et al., 1996). It is reasonable to conclude that CRIP has an important function in cell

proliferation or differentiation because CRIP is most abundant in organs with high cellular turnover rates and increases in organs that are damaged.

In addition, CRIP mRNA and protein levels increase in the rat small intestine throughout the suckling period, reaching highest levels by the late weanling stage. Because the CRIP gene promoter has several glucocorticoid response elements (GREs) it was hypothesized CRIP was developmentally regulated by glucocorticoids in the small intestine (Levenson et al., 1994). This was proven by administration of dexamethasone to both rat neonates and adults. CRIP mRNA and protein levels increased in the neonates, but in the adults they remained constant (Levenson et al., 1993). Since CRIP seems to be important in cell proliferation or differentiation it seems logical that it reaches maximum levels in the intestine by the weanling age when the rats switch from breast milk to solid food (Levenson et al., 1993). At this time development of rat immune defenses is taking place in the intestine. CRIP may be involved in differentiation or proliferation of these cells. Also, immunohistochemical localization of CRIP in the rat small intestine showed it to be localized to the Paneth cells, as well as enterocytes (Fernandes et al., 1995). Paneth cells are important in host defense, through production of lysozyme, defensins, and other proteins with antimicrobial activities (Erlandsen et al., 1974; Ouellette et al., 1992).

The Many Functions of Calreticulin

The protein calreticulin (CRT) was first identified as a calcium binding chaperone in the endoplasmic reticulum (ER) (Ostwald & MacLennan, 1974). Recently, CRT was identified in other areas of the cell, and has also been shown to both inhibit cell proliferation by binding nuclear steroid hormone receptors and to be necessary for cell

adhesion and spreading because of its ability to bind integrins in the cell membrane and regulate integrin-mediated cell adhesion and calcium signaling (Coppolino et al., 1998).

Within the ER, CRT functions as a free floating lectin-like molecular chaperone for many different proteins. Along with other ER chaperones, such as the membrane bound lectin-like chaperone calnexin, CRT maintains proper folding of many glycosylated proteins (Michalak et al., 1999). A good example of how calnexin and CRT maintain this folding is the formation of the Class I MHC protein complex that presents antigens to T cells in order to activate the immune system against intracellular invaders. The MHC I complex is made up of an alpha chain, with constant and variable domains, which noncovalently associates with beta2 microglobulin (β 2-m), and in the process of folding binds an eight to eleven amino acid antigenic peptide that it will present to CD8⁺ T cell receptors when it reaches the cell membrane. In the ER the alpha chain associates with calnexin which maintains proper folding of the alpha chain and allows it to bind to B2-m. After binding, the alpha chain: β 2-m complex dissociates from calnexin and becomes associated with CRT. Correct folding is maintained by CRT until the proper antigenic peptide is placed in a groove within the alpha chain. Then the MHC I complex is secreted to the cell surface for interaction with the proper CD8⁺ T cell receptor (Sadasivan et al., 1995 and 1996).

At the C terminal end of CRT there is a carboxy-terminal ER retention sequence (KDEL). This sequence directs CRT to the ER, where it functions as a chaperone. Yet, CRT has been identified outside the ER (Dedhar, 1994). Specifically, CRT has been shown to colocalize and copurify with integrin subunits in the cell membrane (Leung-Hagesteijn et al., 1994), and CRT has been shown to bind to steroid hormone

receptors *in vitro* and *in vivo*. The question of how CRT escapes the ER has not yet been clearly answered. It has been suggested that this happens through saturation of the KDEL receptors on the ER because CRT that was associated with integrin subunits in the cell membrane was recognized by an anti-KDEL antibody (Coppolino & Dedhar, 1998). Later experiments revealed the presence of a KDEL receptor in the cell membrane near the integrins to which CRT was found bound (Zhu et al., 1997). Therefore, it is still unknown how CRT is selectively targeted to KDEL receptors on the ER and/or those on cell membrane. It seems more likely that CRT may have a binding partner that directs it away from the ER. Binding of zinc, calcium, or phosphorylation of CRT has been shown to change the conformation of the protein (Khanna et al., 1986; Li et al., 2001; Michalak et al., 1999). Therefore, binding of a partner protein to CRT may change its conformation, rendering the KDEL sequence inoperable until the protein is released. Alternatively, binding of a protein partner may cause transport of CRT to the cell membrane, rather than the ER. In addition, other KDEL sequence-containing proteins have been shown to localize outside the ER, including protein disulfide isomerase (Yoshimori et al., 1990), endoplasmic reticulum chaperone, and the heavy chain binding protein (BiP/GRP78) (Takemoto et al., 1992), therefore it is conceivable that CRT can escape the ER.

CRT has been shown to bind to the KXFFKR sequence in between the two zinc fingers of all known nuclear steroid hormone receptors (Dedhar, 1994). Specifically, CRT has been reported to bind to glucocorticoid receptor (Burns et al., 1994), androgen receptor, retinoic acid receptor (Dedhar, 1994), retinoic X receptor (Shago et al., 1997), and vitamin D receptor (St. Arnaud et al., 1995) *in vitro* and *in vivo*, and to inhibit the binding of these receptors to their response elements in DNA. In addition, CRT has been

localized to the nucleus in several cell types (Dedhar, 1994; Ramsamooj et al., 1995; Roderick et al., 1997). By obstructing the binding of steroid hormone receptors to their steroid response elements, CRT has been shown to inhibit retinoic acid-sensitive neuronal differentiation (Shago et al., 1997) and vitamin D-induced mineralization in osteoblasts (St. Arnaud et al., 1995). Therefore, CRT can control steroid hormone-induced cell responses.

CRT has been shown to co-localize with integrins by immunofluorescence and to co-purify with integrins within cell adhesion complexes using native gels, and to bind to the KXGFFKR sequence in the integrin alpha-subunit cytoplasmic domains by mutational analysis (Coppolino et al., 1995). Furthermore, several studies have shown blocking CRT function by downregulating CRT mRNA levels or treating with anti-CRT antibodies, inhibits adhesion and spreading of cells on an extra cellular matrix (ECM) (Leung-Hagesteijn et al., 1994 and Coppolino et al., 1995). It is thought that the binding of CRT to the alpha subunit of integrins stabilizes them in an activated confirmation to allow binding to the ECM and cell spreading (Coppolino et al., 1995). In addition, Coppolino et al. (1997) showed that the binding of surface integrins by CRT elevated cytosolic calcium concentrations owing to an influx of extracellular calcium, and that this transient calcium flux was absent in CRT-deficient embryonic stem cells. This calcium influx has been shown to be essential for integrin-mediated cell adhesion (Sjaastad et al., 1996). It has been suggested that the influx of calcium permits the development of full adhesive sites around the integrins (Coppolino & Dedhar, 1998). Therefore, CRT may control cell adhesion and spreading by modifying integrin function.

CHAPTER 2 MATERIALS AND METHODS

Detection of a CRIP Protein Partner

Spleen, thymus, and intestinal samples from B6SJL mice (Jackson Laboratory, Bar Harbor, ME) were homogenized in S-12 buffer (10 mM Tris, 150 mM NaCl, 10 mM MgSO₄ * 7H₂O, pH 8.0) using a Potter-Elvehjem homogenizer and centrifuged at 40,000 g for 30 min. The supernatant was then transferred to polycarbonate tubes and centrifuged at 100,000 g for 60 min. The supernatant was removed and stored as a cytosol preparation at -80 ° C. Protein concentrations were measured using the Markwell method (97). The samples were then analyzed by 10% SDS PAGE and the gel was stained with Coomassie blue to determine if protein degradation had occurred.

A CRIP affinity purification column was made using Pierce's EDC/Diaminodipropylamine Immobilization Kit (Pierce Biotechnology, Rockford, IL) to cross-link CRIP (0.7 mg) to the column following the manufacturer's protocol. Binding of CRIP to the column was confirmed by measuring the zinc content by atomic absorption spectrophotometry of the flow through eluate before and after conjugation. Next, cytosol from the intestine, thymus, and spleen were individually loaded onto the CRIP affinity purification column and equilibrated at 4 ° C overnight. The column was washed first with S-12 buffer to remove any non-bound proteins from the column, and then 1 M NaCl was used to elute any proteins that specifically bound to the column. Identical amounts of cytosol were added to a control column with no protein bound to it, which was then treated in the same manner. Fractions were collected. Aliquots of each

fraction were analyzed by 10% SDS-PAGE. The gel was then stained using a silver staining detection kit (Biorad, Hercules, CA) to determine if any proteins bound to the CRIP or control columns. Thymus and intestinal cytosolic extracts were applied to the CRIP affinity purification column for comparative purposes. The samples were purified as above, analyzed by 10% SDS-PAGE, and transferred to a 0.45 um PVDF membrane for Western analysis using a CRT antibody purchased from Santa Cruz Biotech (Santa Cruz, CA).

Larger quantities of potential partner proteins were generated from spleens of mice of the same genotype as above. Spleens were harvested from eight mice and the cytosol extracts were obtained and individually loaded onto the CRIP affinity purification column as described earlier. The eluted fractions which contained the potential partner proteins were pooled, washed, and concentrated with S-12 buffer in Centricon-3 tubes (Amicon, MA). The concentrated sample was further resolved by 10 % SDS-PAGE and transferred to 0.45 um PVDF membrane. The membrane was stained using 0.02% Coomassie blue. A 55 kD and 80 kD band were identified and removed for sequencing at University of Florida's ICBR.

Effects of IFN- γ , LPS, and Cell Proliferation on CRIP and CRT Expression in RAW 264.7 Cells

RAW 264.7 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM supplemented with 2 mM glutamine, 10% heat inactivated fetal bovine serum (FBS), and antibiotic-antimycotic (ABAM). The cells were grown in T-75 flasks and as needed for experiments cells were transferred to 100 mm culture plates and allowed to adhere and grow to approximately 80% confluence over the span of 24 h. At 80% confluence the old medium was removed and

10 mL of fresh medium containing either no treatment, rm (recombinant murine) IFN- γ (100 U/ml, purchased from Peprotech, Rocky Hill, NJ), LPS (1 ug/ml, Sigma, St. Louis, MO, *E. coli* serotype 0127:B8), or both rm IFN- γ and LPS was added. At 0, 3, and 9 h after treatment, plates were taken from each treatment group and the cells were removed by scraping. The scraped cells and medium were collected in 50 mL sterile tubes and put on ice. The tubes were then centrifuged at 150 g at 4 ° C for 10 min. The supernatant was decanted and the pellet was washed with 10 mL of ice cold PBS. The tubes were recentrifuged as before. Washing and centrifugation steps were repeated with PBS two more times. The cell pellet was suspended in 1 mL TriPure reagent (Roche, Indianapolis, IN) and total RNA was purified using the manufacturer's protocol. Total RNA was measured by absorbance at 260 nm, 280 nm, and 230 nm to confirm purity and establish concentration of the RNA. Electrophoresis was used to confirm RNA integrity by analyzing 10 ug of total RNA per well in a 1% agarose formaldehyde gel. Ethidium bromide was used to stain the RNA and confirm ribosomal subunit RNA integrity (Nassir et al., 1996).

The effect of proliferation was measured on cells without the addition of either IFN- γ or LPS. As needed, cells were transferred to 100 mm culture plates and allowed to adhere and grow. The plates were taken at 0, 8, 12, 15, and 24 h later and the cells were harvested and total RNA prepared as above. In order to prepare cytosolic protein preparations, three additional plates were taken at 0, 10, 15, 20, 24, and 28 h later and the cells were harvested and washed as above. The final cell pellets were resuspended in 3 mL of ice cold S-12 buffer and rapidly frozen at -80 ° C for 10 min and then thawed. Next, the samples were subjected to sonication (pulsed 0.7 s per 1.0 s for 2 min at a

power setting of 4). Then, the samples were centrifuged at 40,000 g for 30 min. The soluble portion (cytosol) was removed and the pellets discarded. The protein concentrations of the cytosol fractions were measured using the Markwell method (Markwell et al., 1978). Finally, aliquots of the samples (100 ug) were loaded on a 10% SDS tris-tricine polyacrylimide gel, and after electrophoresis proteins were transferred to a PVDF membrane (0.1 um). The membrane was stained with amido black to determine protein distribution. The membrane was blocked in 5% non-fat milk in PBS for 1 h. Next, the membrane was incubated with a polyclonal anti-CRIP rabbit antibody (made against a peptide representing amino acid residues 64-77 of CRIP and coded UF 83) in 5% non-fat milk in PBS-T for 14 h at 4 ° C. Then, the membrane was washed in PBS-T three times for 10 min each, and incubated in anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) for 2 h at room temperature. Finally, the membrane was incubated with ECF western blotting reagent (Amersham Pharmacia Biotech, Arlington Heights, IL) for 3 min and scanned on a STORM 840 phosphorimager (Amersham Pharmacia, Sunnyvale, CA).

Real Time RT-PCR of CRIP and CRT RNA

Real Time RT-PCR using the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA) was performed in order to quantify 18S, CRIP, CRT, and TNF- α (tumor necrosis factor-alpha) mRNA levels. Reverse transcription first converted these RNAs into cDNA. Specific primers and fluorescence resonance energy transfer (FRET) probes (purchased from Biosource International, Camarillo, CA) designed to amplify and detect these cDNAs were selected with Primer Express Software (Applied Biosystems). The Real Time RT-PCR primers and probes were as follows:

CRIP forward 5'-GCGACAAGGAGGTGTATTTTCG-3'

reverse 5'-TTTCCACATTTCTCGCACTTC-3'

FRET probe 5'-FAM-TGACGTCACTAGGCAAGGACTGGCAT-BHQ1-3'

CRT forward 5'-GCCATCTATTTCAAAGAGCAGTTCT-3'

reverse 5'-CGGACTTATGTTTGGATTCTGA-3'

FRET probe 5'-FAM-ACGGAGATGCCTGGACCAACCG-BHQ1-3'

TNF- α forward 5'-TCTCAGGCCTTCCTACCTTCAG-3'

reverse 5'-CTGTGAGGAAGGCTGTGCATT-3'

FRET probe 5'-FAM-CCTTTCCAGACTCTTCCCTGAGG-BHQ1-3'

Normalization to determine relative abundances of these RNA's was with 18S ribosomal RNA using primers and a Taqman probe (purchased from Applied Biosystems) designed to amplify 18S cDNA.

Nondenaturing PAGE and Western Analysis

Nondenaturing PAGE was used to characterize proteins potentially bound to CRIP in vivo. RAW 264.7 cells were transferred to 100 mm culture plates and allowed to adhere and grow for 12 h (~60% confluency). The cells were harvested and washed as above. The final cell pellet was resuspended in 3 mL of ice cold low ionic strength S12 buffer (10 mM Tris, 70 mM NaCl, 5 mM MgSO₄ * 7H₂O, pH 8.0) and rapidly frozen at -80 ° C for 10 min and then thawed. Next, the sample was subjected to sonication (pulsed 0.7 s per 1.0 s for 2 min at power setting 4). Then, the sample was centrifuged at 40,000 g for 30 min. The soluble portion of the sample was removed and the pellet

discarded. The protein concentration was measured using the Markwell method (Markwell et al., 1978). Finally, aliquots of the sample (100 ug) were loaded and run on a nondenaturing 7.5% Tris-Glycine polyacrylimide gel and transferred to a PVDF membrane (0.1 um). The membrane was stained with amido black to determine how the proteins were distributed. Next, the membrane was cut into two halves. Both halves of the membrane were blocked in 5% non-fat milk in PBS for one h. One half of the membrane was incubated with polyclonal anti-CRIP rabbit antibody (UF 83), while the other half of the membrane was incubated with anti-CRT rabbit antibody (Santa Cruz Biotech). Both were in 5% non-fat milk in PBS-T and both incubations were for 14 h at 4 ° C. The membranes were washed in PBS-T three times for 10 min each, and then incubated in anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) for 2 h at room temperature. The membranes were then incubated with ECF western blotting reagent (Amersham Pharmacia Biotech) for 3 min and scanned on a STORM 840 phosphorimager (Amersham Pharmacia Biotech).

Immunocytochemistry

The RAW 264.7 cells were plated on polylysine-coated slides (Fisher) for 12 and 24 h in DMEM culture medium as mentioned earlier. Next, the cells were fixed for 12 min in 4% paraformaldehyde in PBS, washed 3 times for 5 min each with PBS, and then permeabilized by incubation with Triton X-100 (0.1%) in PBS for 30 min. The nonspecific sites were blocked by incubation with 4% BSA in PBS for 30 min. The cells were then incubated with a 1:100 dilution of polyclonal rabbit anti-CRIP antibody (UF 83) and a 1:200 dilution of polyclonal goat anti-CRT antibody (Santa Cruz Biotech) (in 0.1% Triton X-100 in PBS) for 1 h at room temperature. After washing 3 times for 5 min each with PBS, the cells were incubated with secondary antibodies (Texas red conjugated

anti-goat [Santa Cruz Biotech] and FITC conjugated anti-rabbit [Sigma]) at a 1:100 dilution for 30 min at room temperature. The cells were then washed 3 times for 5 min each with PBS, mounted with mounting medium, and visualized using a confocal microscope at the microscopy core lab of University of Florida's Brain Institute.

Statistical Analysis

Tests for significance were performed using the Student's t-test or factorial ANOVA with Graphpad Quickcalcs Software (San Diego, CA, Graphpad.com). Differences were considered statistically significant if the P value was < 0.05 .

CHAPTER 3 RESULTS

Identification of Possible CRIP Binding Partner Using Affinity Chromatography

Cytosolic extracts of mouse spleen, thymus, and intestine were incubated separately in the CRIP affinity purification column with the hope of identifying a protein binding partner for CRIP. Aliquots of the fraction eluted with 1 M NaCl were analyzed by 10% SDS-PAGE. Silver staining of the gels revealed a band of approximately 55 kD was present that bound to the column for each of the samples from the spleen, thymus, and intestine (Figure 3-1). The 55 kD band was amino acid sequenced at UF's Protein Core. The sequence obtained was DPAIYFKEQFLDGXA. A BLAST search revealed the sequence was identical to that of mouse calreticulin precursor. Westerns analysis of thymus and intestinal proteins eluted off the CRIP column was performed with a calreticulin (CRT) antibody (Santa Cruz Biotechnology) that revealed that the 55 kD band that bound to the column in these samples was also CRT (Figure 3-2).

A control column with no protein bound to it was also constructed to evaluate whether CRT would bind nonspecifically to the column. CRT did not bind to the control column (Figure 3-3), and therefore the interaction between CRIP and CRT may be specific.

Effect of RAW 264.7 Cell Proliferation on CRIP, CRT, and MT mRNA

CRIP, CRT, and MT mRNA levels were studied in RAW cells as a function of time in culture and extent of confluence. Compared to initial levels, CRIP mRNA levels significantly increased at 8 h ($P < 0.04$). Levels significantly decreased between 12 and 15

h ($P<0.03$) and 15 and 24 h ($P<0.001$) (Figure 3-4). CRT mRNA levels were significantly increased between 0 and 12 h ($P<0.004$) and 8 and 12 h ($P<0.04$). Levels significantly decreased between 15 and 24 h ($P<0.005$) (Figure 3-4). MT mRNA levels significantly increased at 8 h ($P<0.006$) compared to initial levels. Between 8 and 24 h, levels significantly decreased ($P<0.008$) (Figure 3-4). Expression of MT, CRIP, and CRT mRNA each followed a similar parabolic curve, peaking at different time points, 8, 12, and 15 h respectively (Figure 3-4). Percent confluence at the time points examined, 0, 8, 12, 15, and 24 h, after plating was approximately 30, 40, 60, 70, and 90%, respectively when judged by observation (Figure 3-4).

Effect of IFN- γ and LPS on CRIP, CRT, and TNF- α mRNA Expression and Nitric Oxide Production in RAW 264.7 Cells

In order to measure CRIP and CRT mRNA expression in activated RAW cells, the cells were allowed to grow to approximately 80% confluence prior to the addition of LPS and/or IFN- γ . Relative CRIP and CRT mRNA abundance was measured at 0, 3, and 9 h after treatment. CRIP mRNA levels significantly ($P<0.03$) decreased in all groups from 0 to 9 h (Figure 3-5A). At 9 h expression was not significantly different between the control and IFN- γ treated cells, but was significantly lower ($P<0.003$) in those treated with LPS +/- IFN- γ compared to the control. Similarly, by 9 h in culture expression of CRT mRNA was significantly ($P<0.04$) lowered in all cells except those treated with IFN- γ ($P<0.07$) (Figure 3-5B). Likewise, at 9 h expression was not significantly different between the control and IFN- γ treated cells, but was significantly lower ($P<0.02$) in those treated with LPS +/- IFN- γ compared to the control. The latter observations suggest a specific biological response induced by LPS +/- IFN- γ treatment of RAW 264.7 cells is responsible for the decrease in CRIP and CRT mRNA expression.

Treatment of these cells with LPS +/- IFN- γ has been reported to result in secretion of TNF- α , which induces nitric oxide production through an autocrine response (MeBmer et al., 1996). For that reason, TNF- α mRNA expression and nitric oxide production were monitored as positive controls at 0, 3, and 9 h in culture. Relative expression of TNF- α mRNA levels (Figure 3-6A) did not change significantly during the entire 9 h culture period in control and IFN- γ treated cells. However, TNF- α mRNA levels increased markedly ($P < .011$) from initial values in the LPS +/- IFN- γ treated cells at 3 h, but decreased markedly ($P < .031$) between 3 and 9 h. In order to measure nitric oxide production, samples of the media were taken at 0, 3, and 9 h. Between 0 and 3 h no nitric oxide was detected in the medium of any group (Figure 3-6B). Control and IFN- γ treated cells did not release any nitric oxide into the medium, but LPS +/- IFN- γ treated cells increased nitric oxide release significantly ($P < 0.0006$) by 9 h.

Effect of RAW 264.7 Cell Proliferation on CRIP Protein Levels

CRIP protein levels were studied in the cells as a function of time and confluence. Protein expression increased up to 10 h (approximately 60% confluency) and declined thereafter (Figure 3-7). CRIP protein levels followed the mRNA levels very closely.

Nondenaturing PAGE of RAW 264.7 Cell Cytosolic Extracts at 60% Confluency

Peak induction of CRIP mRNA levels occurred at 12 h after plating the cells. At that time the RAW cells were at approximately 60% confluency. Therefore, based upon the hypothesis that CRIP functions in cell proliferation, it was decided that at this time point CRIP would most likely be complexed with CRT, if it is a protein partner. In order to identify a CRIP:CRT complex, the cells were allowed to grow for 12 h and cytosolic fractions (supernatants after 40,000 g centrifugation) were obtained. Aliquots of these extracts were run on a nondenaturing 7.5% polyacrylimide gel and transferred to a PVDF

membrane. The membrane was cut in half. One half of the membrane was incubated with an anti-CRIP rabbit antibody, and the other half was incubated with an anti-CRT antibody. After Western analysis with both membranes, the banding pattern was analyzed by phosphorimaging. The CRIP and CRT antibodies each identified one primary band with other minor bands. These bands were in identical positions in both membranes probed with the CRIP and CRT antibodies (Figure 3-8). Therefore, one interpretation is that CRIP and CRT are possibly in complex with each other and with other proteins. Upon repetition of the nondenaturing gel only the major band in the CRIP and major band in the CRT Western analysis were detected. The minor bands that were detected in identical places as the major bands on opposing Westerns could not be reproduced.

Colocalization of CRIP and CRT

In studies using confocal microscopy and immunocytochemistry both CRIP and CRT were shown to colocalize in RAW 264.7 cells at 12 h after plating on slides and there was less colocalization at 24 h when CRIP expression had decreased (Figure 3-9). The immunofluorescence confirms the Western data. At 12 h CRIP expression is higher than at 24 h as measured by both Western and immunofluorescence.

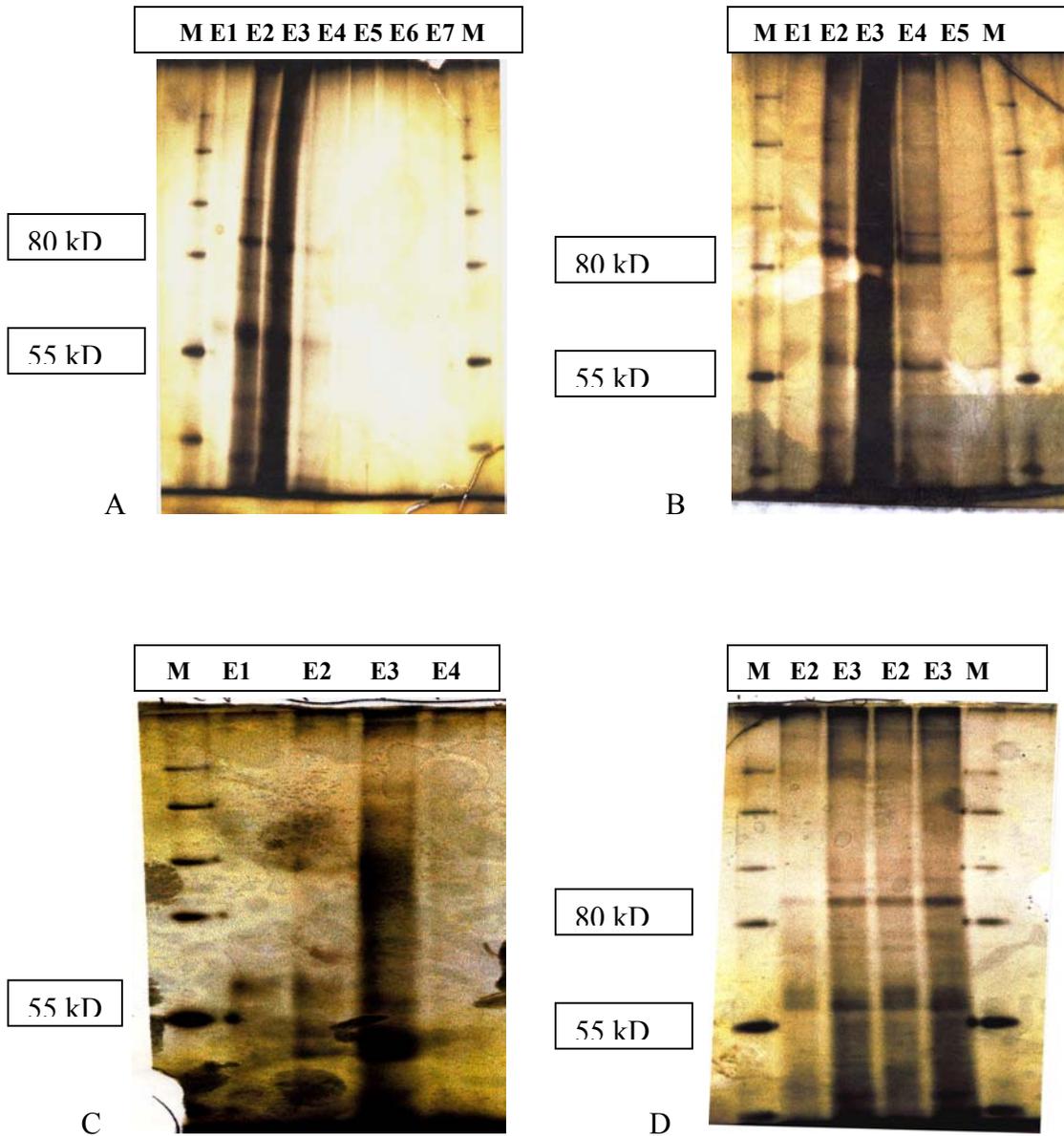


Figure 3-1 Silver stained 10% SDS tris-glycine polyacrylimide gels of proteins eluted from the CRIP affinity purification column. (A) Silver staining of thymus cytosolic proteins eluted off the column identified a 55 and 80 kD band. (B) Silver staining of spleen extracts eluted off the column also identified a 55 and 80 kD band. (C) Silver staining of intestinal extracts eluted off the column identified only a 55 kD band. (D) Salt was removed from the column eluate prior to electrophoresis. Note the improved apparent resolution of the 55 and 80 kD bands. M stands for marker and E# stands for elution number.

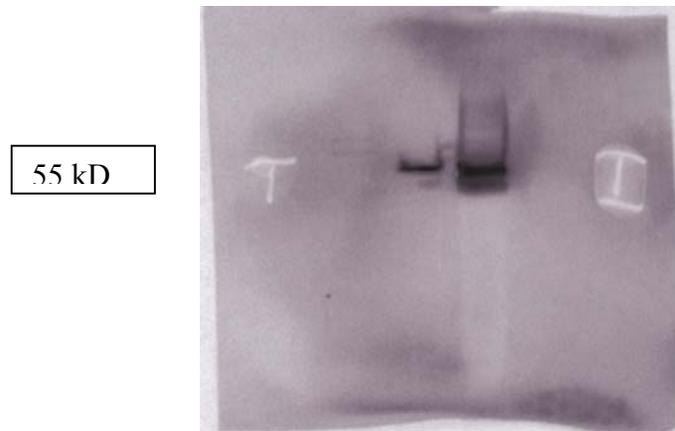


Figure 3-2 Western analysis of thymic (left) and intestinal (right) proteins eluted from the CRIP column performed with a calreticulin (CRT) antibody (Santa Cruz Biotechnology) revealed that the 55 kD band that bound to the column in these samples was CRT.

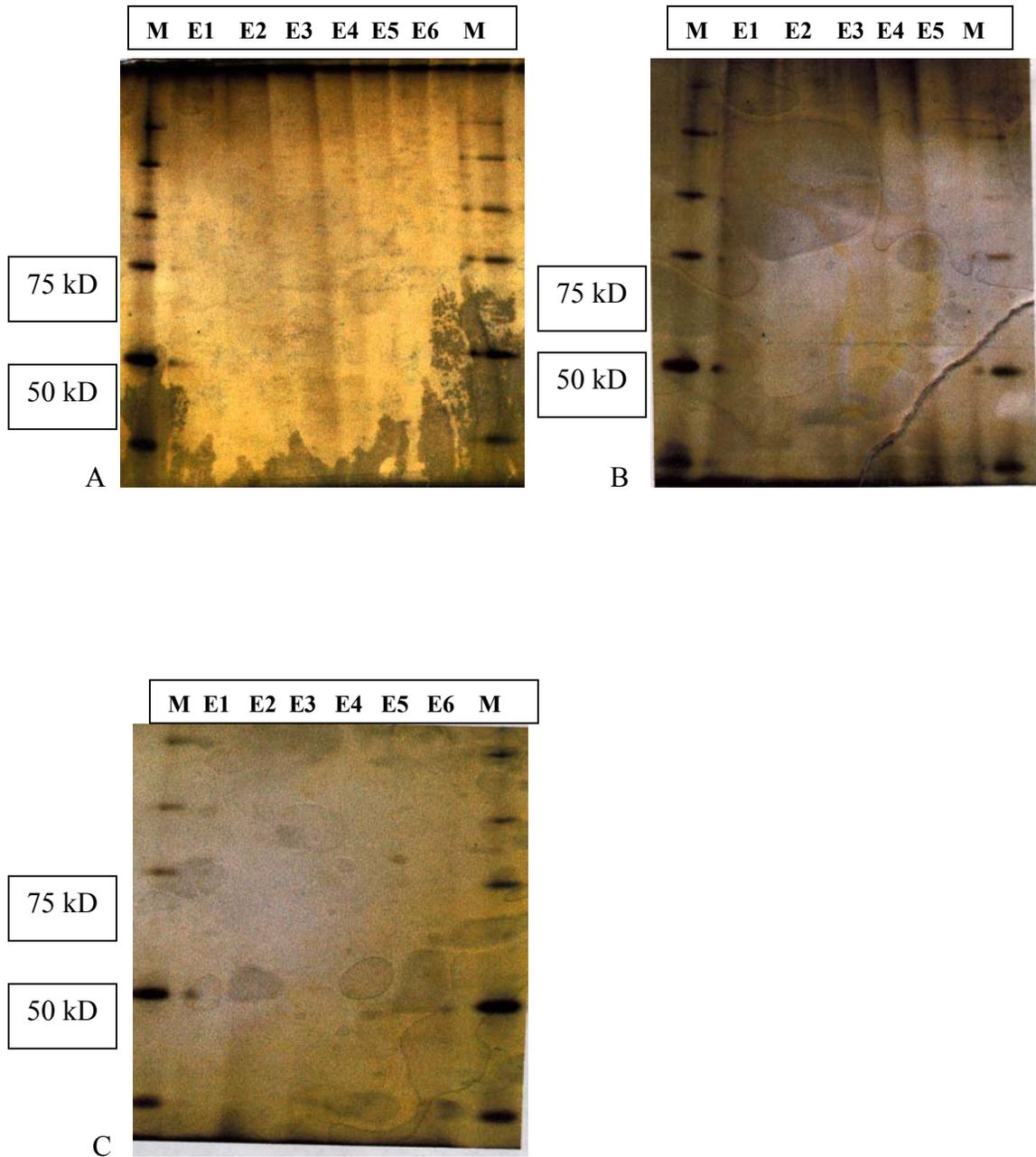


Figure 3-3 Silver stained 10% SDS tris-glycine polyacrylimide gels of proteins that eluted off of the control column. The molecular weight of the 50 and 75 kD marker bands are labeled. (A) Silver staining of thymus cytosolic proteins eluted off the column identified no bands (B) Silver staining of spleen extracts eluted off the column identified no bands (C) Silver staining of intestinal extracts eluted off the column identified no bands.

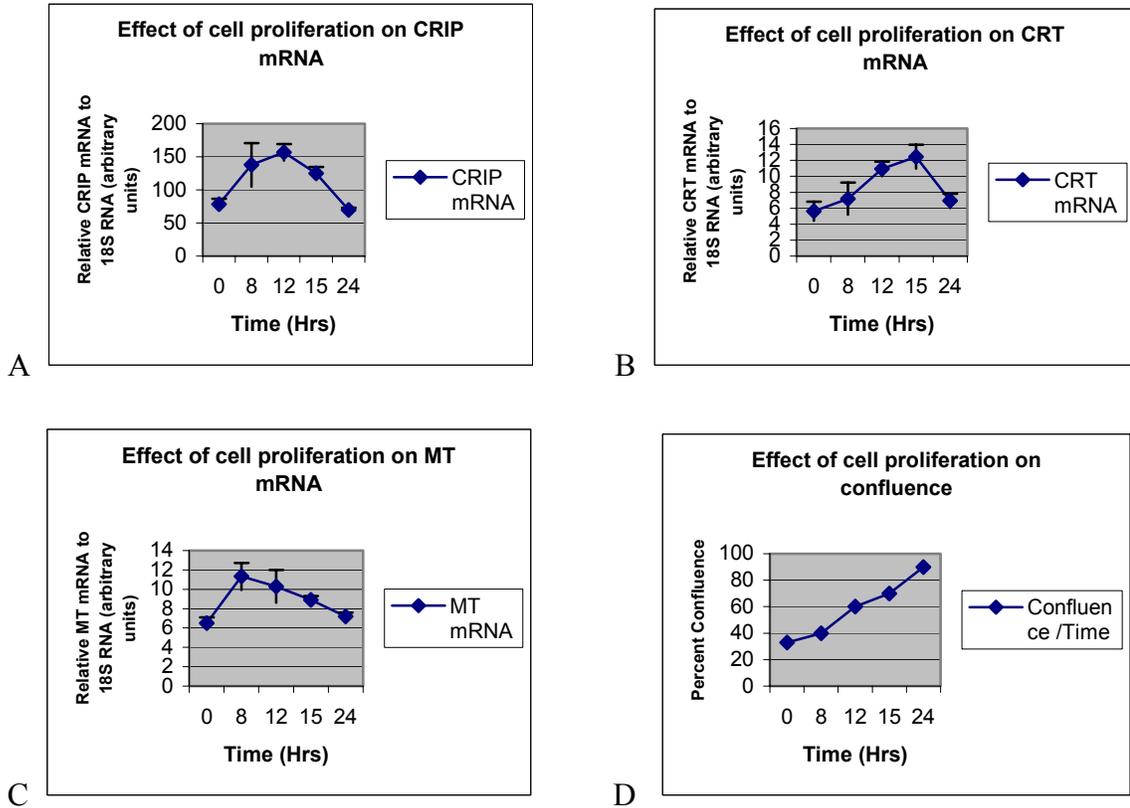


Figure 3-4 Effect of RAW 264.7 cell proliferation on CRIP (A), CRT (B), and MT (C) mRNA levels relative to 18S RNA as measured by real time RT PCR and approximate confluence (D) at 0, 8, 12, 15, and 24 h after plating cells at a 1:3 ratio.

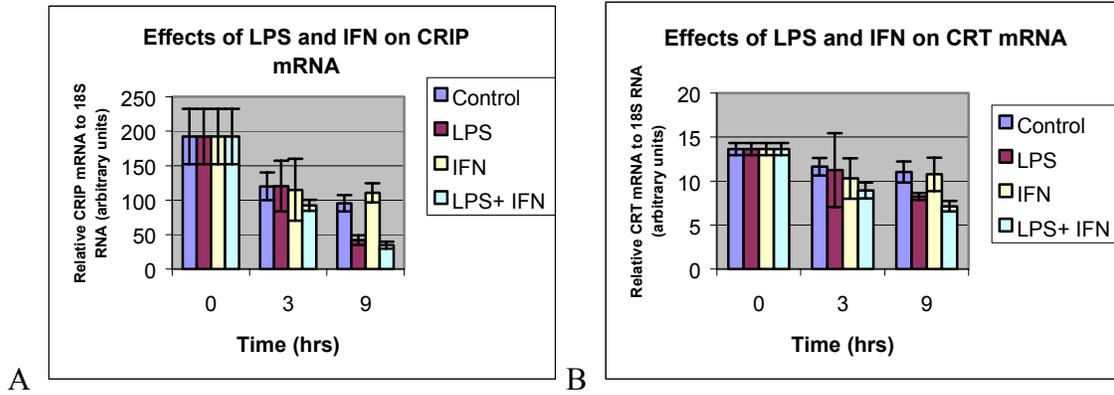


Figure 3-5 Effects of LPS and IFN- γ treatment of RAW 264.7 cells at 80% confluency on CRIP (A) and CRT (B) mRNA levels relative to 18S RNA as measured by real time RT PCR at 0, 3, and 9 h after treatment.

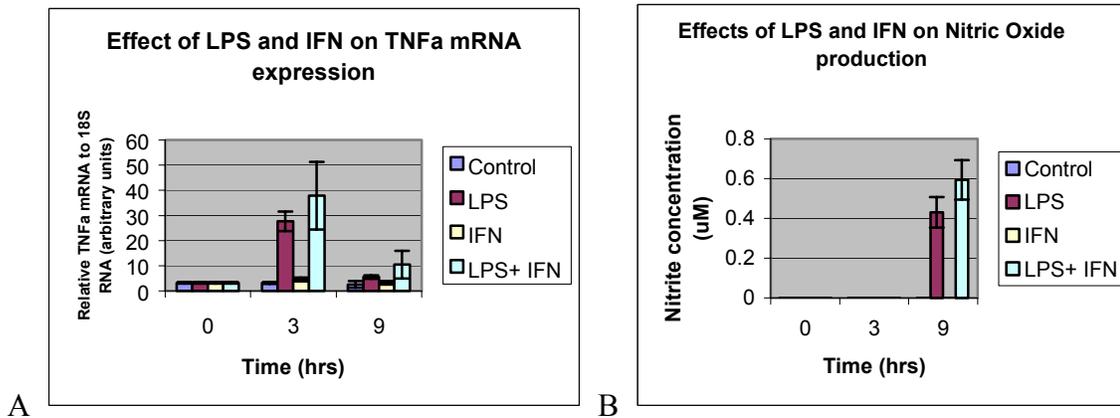
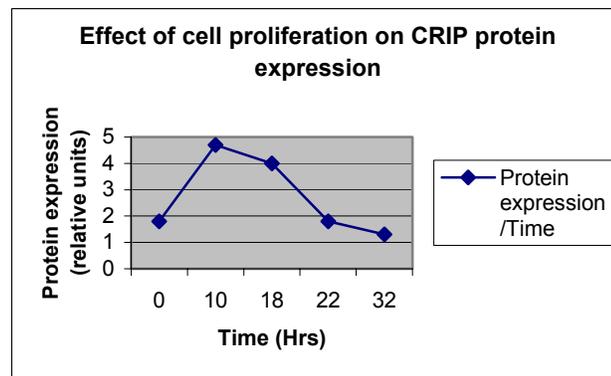
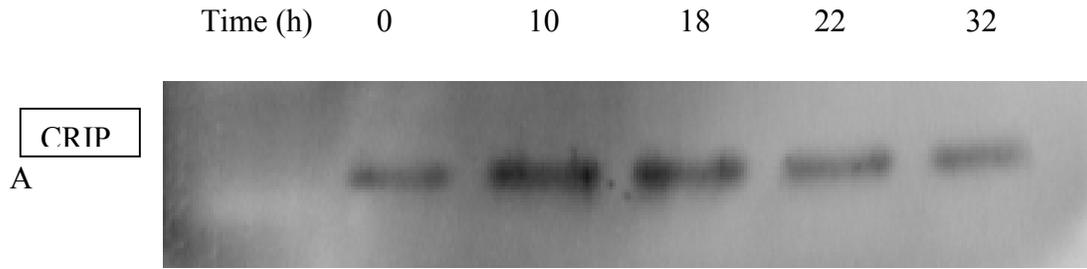


Figure 3-6 Effects of LPS and IFN- γ treatment of RAW 264.7 cells at 80% confluency on (A) TNF- α mRNA levels relative to 18S RNA as measured by real time RT PCR and (B) nitric oxide production as measured by addition of Greiss reagent to medium at 0, 3, and 9 h after treatment.



B

Figure 3-7 (A) Western analysis of RAW cell cytosolic CRIP protein levels up to 32 h after plating. (B) Densitometric measurement of CRIP protein increased from initial levels up till 10 h. At 22 h CRIP protein expression was back at initial levels.

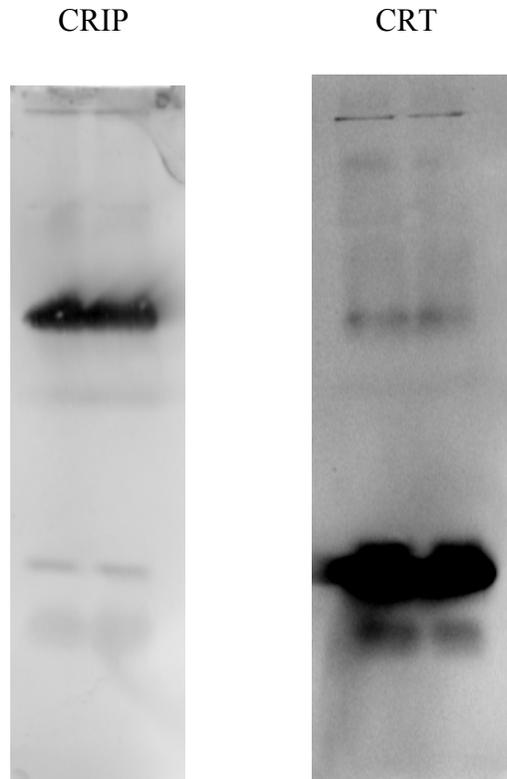


Figure 3-8 Western analysis of RAW 264.7 cell cytosol protein extracts analyzed in a nondenaturing 7.5% polyacrylide gel and transferred to PVDF membrane (0.1 um) for analysis with CRIP and CRT antibodies.

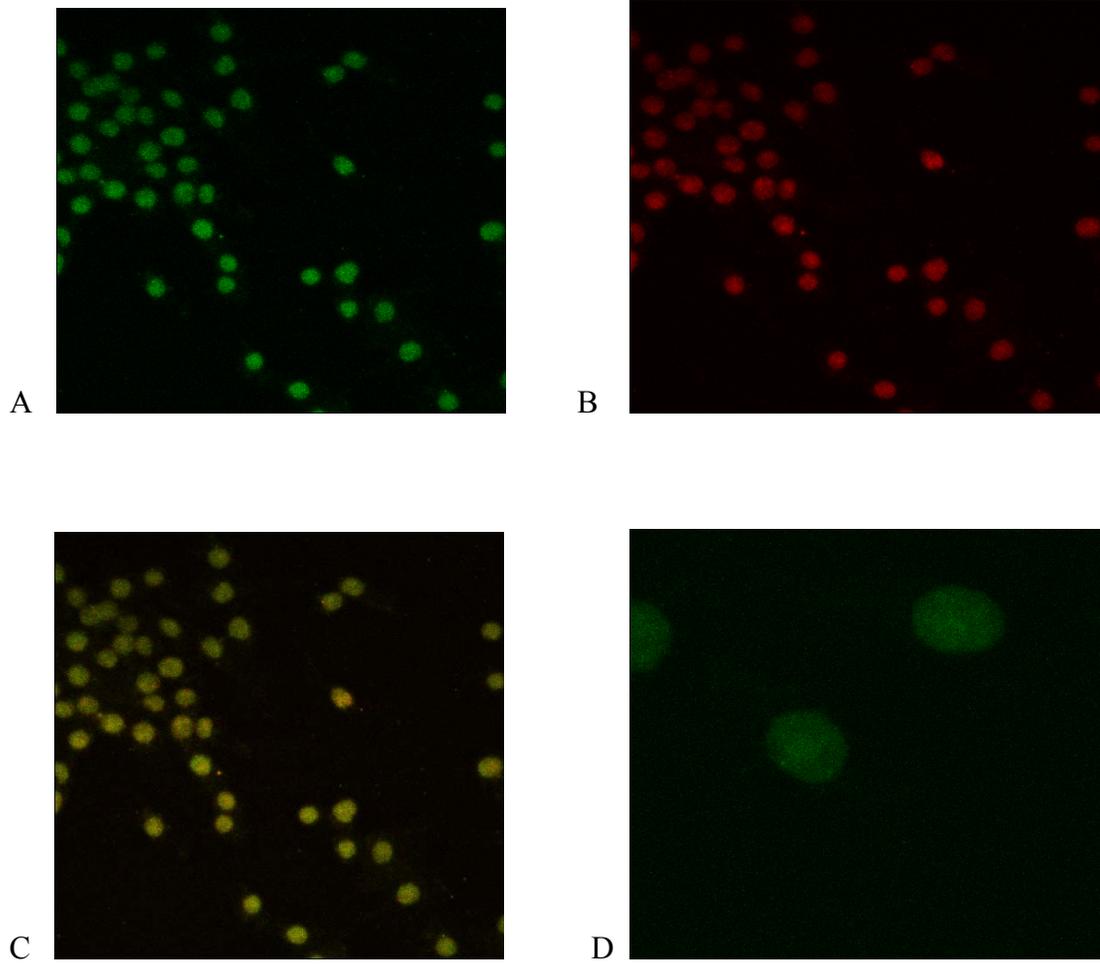


Figure 3-9 RAW 264.7 cells were incubated with polyclonal rabbit anti-CRIP (UF 83) and anti-rabbit conjugated FITC antibodies (Sigma) and polyclonal goat anti-CRT (Santa Cruz Biotech) and anti-goat conjugated Texas Red antibodies (Santa Cruz Biotech) 12 and 24 h after plating on slides images were obtained using confocal microscopy. (A) CRIP (green) localization in cells at 12 h at 900 X (B) CRT (red) localization in cells at 12 h at 900 X (C) CRIP and CRT (yellow) colocalization in cells at 12 h at 900 X (D) CRIP (green) localization in cells at 12 h at 3000 X.

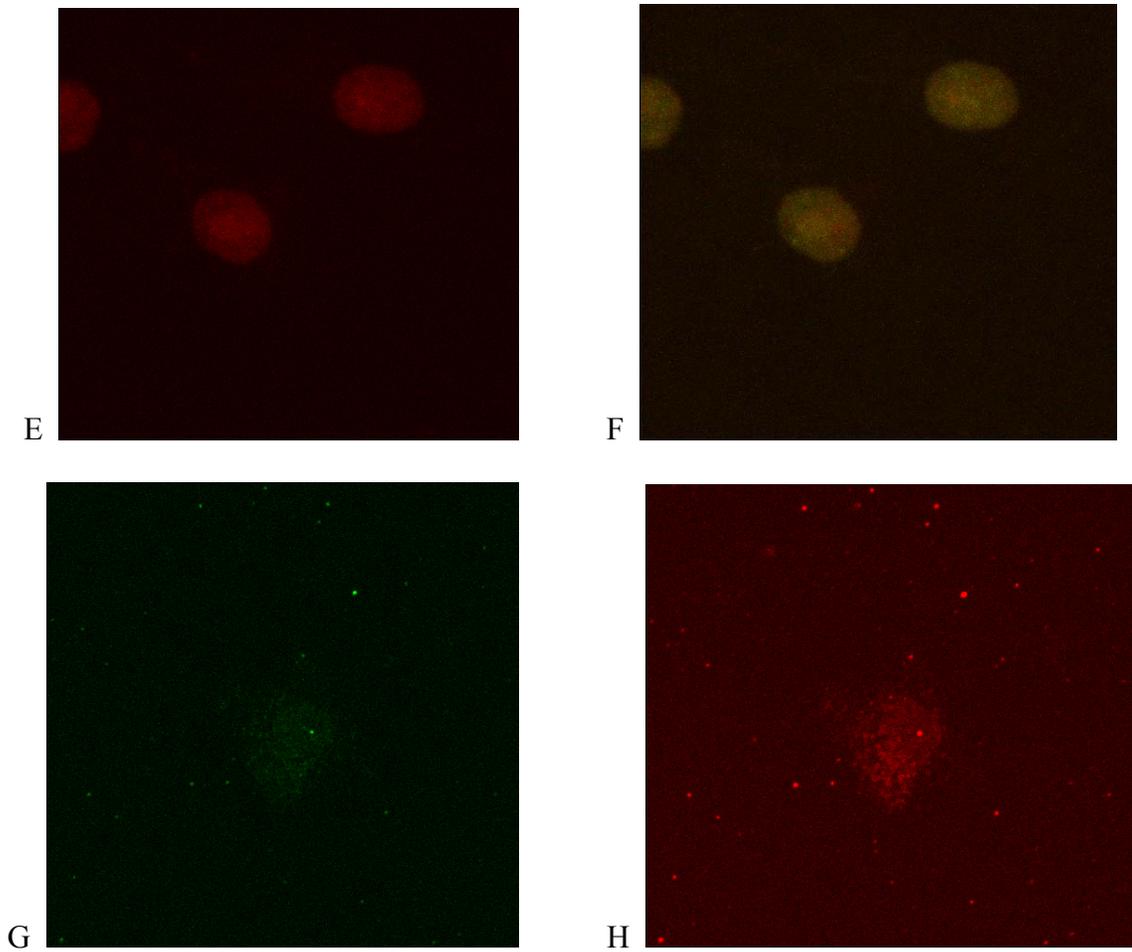


Figure 3-9—Continued. (E) CRT (red) localization in cells at 12 h at 3000 X (F) CRIP and CRT (yellow) colocalization in cells at 12 h at 3000X (G) CRIP (green) localization in cells at 24 h at 1800 X (H) CRT (red) localization in cells at 24 h at 1800 X.

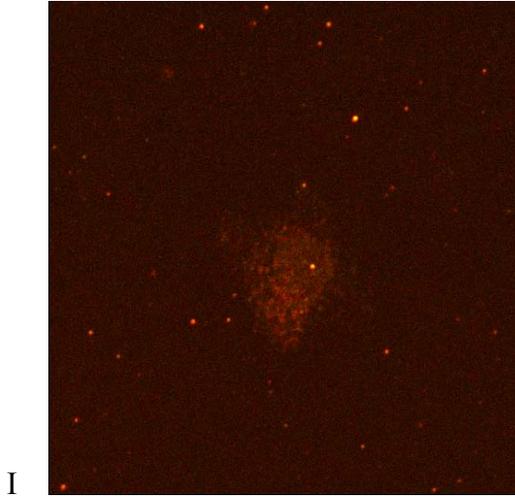


Figure 3-9—Continued. (I) CRIP and CRT (yellow) colocalization in cells at 24 h at 1800 X.

CHAPTER 4 DISCUSSION

Proteins with LIM domains can act as adaptors for the formation of multiprotein complexes. Many of these proteins are involved in cell growth and differentiation (Bach, 2000; Dawid et al., 1998). In this study we show that CRIP binds to the protein CRT, and that mRNA expression of both proteins is upregulated during cell spreading and proliferation and downregulated by cell growth arrest in RAW 264.7 cells. In addition, mRNA expression of both proteins is further downregulated by treatment of these cells with LPS +/- IFN- γ . Therefore, the similar expression of both CRIP and CRT mRNA on a temporal basis and evidence that they interact in vitro and colocalize suggest they act as protein partners for a function related to proliferation.

Ever since it was first identified, CRT has been studied as a chaperoning protein in the endoplasmic reticulum (Ostwald & MacLennan, 1974). Recently, CRT was identified in other areas of the cell, and has also been shown to both inhibit cell proliferation by binding nuclear steroid hormone receptors and to be necessary for cell spreading because of its ability to bind integrins in the cell membrane and regulate integrin-mediated cell adhesion and calcium signaling (Coppolino & Dedhar, 1998).

Several in vitro studies have shown that CRT is able to bind to the KXFFKR sequence in between the two zinc fingers of all known nuclear steroid hormone receptors (Dedhar, 1994). In vitro binding of CRT to several steroid hormone receptors has also been shown to inhibit the ability of these receptors to bind to their DNA elements and upregulate transcription of the specific genes (Burns, 1994; Dedhar et al., 1994; Shago et

al., 1997; St. Arnaud et al., 1995; Winrow et al., 1995). Dedhar et al. (1994) co-transfected expression vectors with full length CRT and androgen receptor genes along with a chloramphenicol acetyltransferase (CAT) reporter gene driven by mouse mammary tumor virus (MMTV) long terminal repeat (LTR) containing androgen response elements into Vero fibroblast cells. This resulted in a dose-dependent CRT vector related inhibition of CAT activity induced by the androgen receptor. On the other hand, Winrow et al. (1995) showed that although purified CRT inhibits the binding of PPAR/RXR alpha heterodimers and other nuclear hormone receptors, to peroxisome proliferators-responsive DNA elements in vitro, overexpression of CRT in transiently transfected cultured cells had no effect on transactivation mediated by PPAR/RXR. Then again, Shago et al. (1997) and St. Arnaud et al. (1995) showed that over-expression of CRT can inhibit retinoic acid-sensitive neuronal differentiation and vitamin D-induced mineralization in osteoblasts. It may be possible that an interaction between CRIP and CRT prevents CRT from inhibiting the steroid hormone receptors from binding to DNA. During RAW 264.7 cell proliferation CRIP mRNA expression is upregulated before CRT (Figure 3-1 and 3-2). CRIP is upregulated by glucocorticoid hormones. Glucocorticoid response elements have been identified in the CRIP promoter and other steroid response elements may also be present (Levenson et al., 1993). Therefore, increased CRIP production before CRT during cell proliferation and spreading could allow the formation of a CRIP:CRT complex, which may prevent CRT from inhibiting growth by binding to nuclear steroid hormone receptors.

Additionally, CRT has been shown to co-localize and co-purify with integrins within cell adhesion complexes, and to bind to the KXGFFKR sequence in the integrin

alpha-subunit cytoplasmic domains (Coppolino & Dedhar, 1998). Furthermore, several studies have shown blocking CRT function by downregulating CRT mRNA or treating with anti-CRT antibodies inhibits adhesion and spreading of cells on an extra cellular matrix (ECM) (Coppolino et al., 1995; Leung-Hagesteijn et al., 1994). It is thought that the binding of CRT to the alpha subunit of integrins stabilizes integrins in an activated confirmation to allow binding to the ECM and spreading of cells (Coppolino & Dedhar, 1998).

Several LIM proteins have been identified within adhesion complexes. Wixler et al. (2000) reported that the four and one half LIM domain protein DRAL/FHL2 interacts with several alpha and beta integrin subunits, and localized DRAHL/FHL2 to cell adhesion complexes. Y. Tu et al. (1999) demonstrated that the five LIM-only domain protein PINCH directly interacts with integrin linked kinase (ILK), is recruited to integrin-rich sites in spreading cells, and acts as a bridge stabilizing the interaction between ILK and Nck-2.

LIM proteins also interact with the cysteine-rich zinc finger domains of PKC. Zhou et al. (1999) found that the three LIM domains of cypher 1 bind several isoforms of protein kinase C (PKC), which allows the protein to be phosphorylated by PKC. They suggest that cypher1 may function as an adaptor in striated muscle to couple PKC-mediated signaling, via its LIM domains, to the cytoskeleton (-actinin-2) through its PDZ domain. Kuroda et al. (1996) reported that the LIM 2 domain of LIMK1 is crucial for binding between LIMK1 and PKC gamma. The interaction between LIM proteins and PKC may relate to an interaction between CRIP, PKC, and CRT. CRT contains a recognizable site for phosphorylation by PKC (Michalak et al., 1992). Also, the receptor

for activated PKC was shown to interact with integrin beta subunit cytoplasmic tails (Liliental et al., 1998). It is possible that CRIP that is bound to CRT may facilitate the phosphorylation of CRT by PKC because of an interaction between PKC and CRIP's LIM domain (Figure 4-1). Other CRIP binding partners were bound by the CRIP affinity purification column. Unfortunately, we were unable to amino acid sequence their terminal ends for identification. Also, the nondenaturing gel detected CRIP and CRT together in bands of different molecular weights. This suggests that CRIP and CRT form complexes that include other proteins as well. Immunoprecipitation of complexed proteins using CRIP and/or CRT antibodies should be carried out to study CRIP's interaction with proteins in vivo. One of the other partners that bound to the CRIP affinity purification column had a molecular weight of around 80 kD, which is the same molecular mass as several isoforms of PKC (Chang et al., 2002). Phosphorylation of CRT may be required to stabilize its interaction with integrin alpha subunits as well as to stabilize integrins in an active conformation and allow cell adhesion and spreading to take place. Stabilizing the interaction between CRT and integrin alpha subunits may also be necessary to allow calcium influx that is essential for integrin mediated cell adhesion. In addition, CRIP may function as an adaptor protein that allows CRT to be phosphorylated by PKC which may cause translocation of the CRIP:CRT:PKC complex to the cell membrane where it binds integrins and stabilizes their function (Figure 4-1).

Within the endoplasmic reticulum CRT has been shown to be a chaperone necessary for the secretion of interferon-gamma and the synthesis of MHC I complexes (Kosuge et al., 2000 and Sadasivan et al., 1995 and 1996). Overexpression of CRIP in transgenic mice leads to increased secretion of Th2 (IL-6 and IL-10) cytokines and

decreased secretion of Th1 cytokines (IFN- γ and IL-2) after challenge of mice with LPS or mouse splenocytes with PHA (Lanningham-Foster et al., 2002). It is possible that overexpression of CRIP leads to low levels of CRT in the endoplasmic reticulum (Figure 4-1). This would inhibit the secretion of IFN- γ during an immune response, which would in turn lead to less CD4⁺ T cells differentiating into Th1 cells that primarily secrete IL-2 and IFN- γ . Therefore, less IL-2 and IFN- γ would be secreted during an immune response because of a decreased number of CD4⁺ T cells differentiating into Th1 cells. Also, as a consequence of less secretion of the Th1 cytokine, IFN- γ , more CD4⁺ T cells would develop into Th2 cells that primarily secrete IL-6 and IL-10. Neither IL-6 nor IL-10 have been shown to rely on the chaperone CRT for proper folding, and therefore, would still be secreted. Consequently, an increased concentration of Th2 cytokines would be detected. Furthermore, MHC I is an important antigen presenting protein that is required to activate a CD8⁺ T cell response. The CD8⁺ T cell response is important for clearing viral infections. When challenged with influenza virus, the ability of transgenic mice that overexpress CRIP to clear the virus from their lungs was severely compromised (Lanningham-Foster et al., 2002). CD8⁺ T cells also produce IFN- γ and IL-2 when activated. Consequently, decreased concentrations of CRT in the endoplasmic reticulum could also inhibit the formation of MHC I complexes on the surface of the cell membrane, which would lead to less activation of CD8⁺ T cells and production of IFN- γ and IL-2.

Glucocorticoids are anti-inflammatory steroid hormones that have been shown to inhibit the production of Th1 cytokines, like IFN- γ , and stimulate the production of Th2 cytokines, like IL-10, during an immune response (Elenkov et al., 2002). CRIP has been

shown to be induced by treatment with dexamethasone (DEX), a potent synthetic glucocorticoid-like hormone, because of glucocorticoid response elements in its promoter (Levenson et al., 1993). Initial studies in RAW 264.7 cells have shown that CRIP mRNA levels are upregulated by treatment with DEX (data not shown). Part of the mechanism by which glucocorticoids may downregulate secretion of IFN- γ may be by upregulating CRIP expression. CRIP could then bind to CRT and prevent CRT from entering the endoplasmic reticulum (ER). Less CRT in the ER would prevent proper folding of IFN- γ and decrease the secretion of IFN- γ by the cell.

MeBmer et al. (1996) reported that nitric oxide (NO) released endogenously via iNOS induction triggered by treating RAW 264.7 cells with 10 μ g/mL LPS and 100 U/mL IFN- γ led to apoptotic cell death in 50% of the stimulated cells after 24 h. Furthermore, Oyadomari et al. (2001) reported that treating mouse MIN6 pancreatic beta cells with an NO donor caused apoptosis to occur in these cells in similar time course as in RAW 264.7 cells. Treatment of MIN6 cells with an NO donor led to depletion of endoplasmic reticulum calcium stores which induced the endoplasmic reticulum stress protein, CCAAT/enhancer-binding protein-homologous protein (CHOP), which plays an important role cell growth arrest and apoptosis in these cells. In addition, NO has been shown to activate guanylate cyclase, leading to increased intracellular cGMP concentrations, and the ensuing activation of cGMP-dependent protein kinase (PKG) in pancreatic beta cells (Loweth et al., 1997). In this study, treatment of RAW 264.7 cells with LPS +/- IFN- γ led to NO production after only 9 h. Both CRIP and CRT mRNA expression in cells treated with LPS +/- IFN- γ were only significantly decreased from the control after 9 h, when NO was produced. Taking into consideration the effects of NO on

cell calcium, cGMP, and apoptotic cell death, it is possible that both CRIP and CRT mRNA expression are downregulated by some signal cascade that is activated when cells produce NO.

Given that both CRIP and CRT mRNA expression are upregulated during cell spreading and CRT has been shown to be essential for cell adhesion and spreading, it is possible that downregulation of CRIP and CRT mRNA expression is related to the concurrent apoptosis. Conran et al. (2001) showed that eosinophil cell surface integrin expression and function were altered by treatment of cells with an NO donor. The NO donor significantly inhibited ability of the eosinophils to bind to serum and fibronectin. If the CRIP:CRT complex is important in stabilizing integrins and allowing cell adhesion, downregulation of CRT and CRIP mRNA expression would inhibit cell adhesion by disrupting integrin function. Blocking cell adhesion in the LIM 1863 colon carcinoma cell line by generating an anti-integrin antibody that blocked integrins from being able to trigger cell adhesion has been reported to lead to rapid apoptosis (Bates et al., 1994). Therefore, if the CRIP:CRT complex is important in targeting CRT to integrins, or stabilizing integrins in a confirmation that triggers adhesion to an ECM, downregulation of CRT and CRIP mRNA expression would inhibit cell adhesion and lead to apoptosis.

Okadaic acid, an inhibitor of intracellular protein serine/threonine phosphatases, has been reported to modulate integrin affinity (Hedman et al., 1992). Coppolino et al. (1999) have recently reported that incubation of PC-3 and Jurkat T cells with okadaic acid inhibited integrin adhesion to collagen. They also reported that okadaic acid inhibited CRT binding to integrins. Kwon et al. (2001) reported that treatment of U937 cells with okadaic acid led to apoptosis in these cells. They also reported that this

apoptotic response was inhibited by treatment of these cells with phorbol myristate acetate, an activator of PKC. Therefore, it is possible that protein serine/threonine phosphatases are responsible for activation of PKC. Activated PKC may then phosphorylate CRT by interacting with the CRIP:CRT complex and this would lead to binding of CRT and integrins. Binding of CRT and integrins would then stimulate cell adhesion which would lead to proliferation. Conversely, lack of activation of PKC would lead to lack of an interaction between CRT and integrins, lack of cell adhesion, and apoptosis.

It is also interesting to note that both CRT and CRIP have not been identified in any prokaryote genomes that have been sequenced to date (Clarke et al., 1998 and Michalak et al., 1999). If these proteins are binding partners that act together to stimulate adhesion to the ECM and cell proliferation, this interaction would represent a eukaryotic-specific function.

In conclusion, both CRT and CRIP mRNA expression are upregulated during cell spreading and proliferation and downregulated by conditions that lead to apoptosis in RAW 264.7 cells. Both CRIP and CRT have been shown to associate *in vitro* and colocalize *in vivo*. The response of CRT and CRIP mRNA expression during RAW 264.7 cell spreading and proliferation suggests that the CRIP:CRT complex is necessary to trigger cell adhesion and spreading. Additionally, the fact that overexpression of CRIP in transgenic mice decreases secretion of Th1 cytokines suggests that the CRIP:CRT complex may also prevent CRT from being transported to the endoplasmic reticulum, where it has important chaperoning functions, especially in regulating a Th1 immune response, in cells.

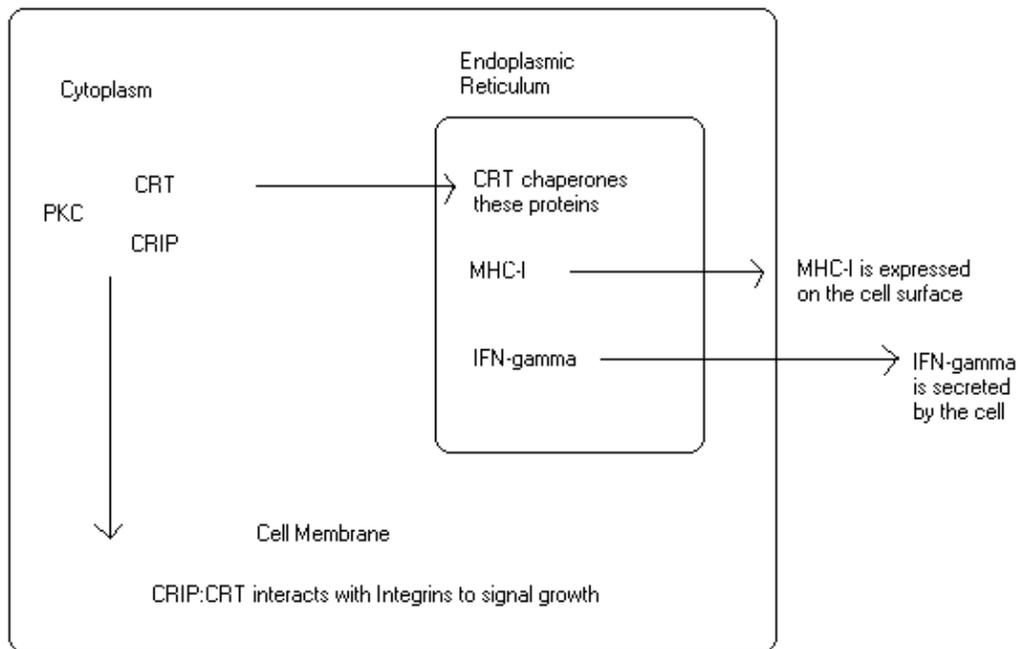


Figure 4-1 Model of potential CRIP interaction with CRT and PKC which would cause translocation of CRT to integrins at the cell membrane and block translocation of CRT into the endoplasmic reticulum.

LIST OF REFERENCES

- Apostolova M D, Cherian M G. Delay of M-phase onset by aphidicolin can retain the nuclear localization of zinc and metallothionein in 3T3L1 fibroblasts. *J Cell Physiol* (2000) 183:247-253.
- Atar D, Backx P H, Appel M M, Gao W D, Marban E. Excitation-transcription coupling mediated by zinc influx through voltage-dependent calcium channels. *J Biol Chem* (1995) 270:2473-2477.
- Bach I. The LIM domain: Regulation by association. *Mechanisms of Development* (2000) 91:5-17.
- Bates R C, Buret A, Van Helden D F, Horton M A, Burns G F. Apoptosis induced by inhibition of intercellular contact. *J Cell Biol* (1994) 125:403-415.
- Berg J M, Shi Y. The galvanization of biology: A growing appreciation for the roles of zinc. *Science* (1996) 271:1081-1085.
- Beyersmann D, Haase H. Functions of zinc in signaling, proliferation, and differentiation of mammalian cells. *BioMetals* (2001) 14:331-341.
- Borrello M G, Mercalli E, Perego C, Degl'Innocenti D, Ghizzoni S, Arighi E, Eroini B, Rizzetti M G, Pierotti M A. Differential interaction of Enigma protein with the two RET isoforms. *Biochem Biophys Res Commun* (2002) 296:515-22.
- Burns K. Modulation of gene expression by calreticulin binding to the glucocorticoid receptor. *Nature* (1994) 367:476-480.
- Chai F, Troung-Tran A Q, Evdokiou A, Young G P, Zalewski P D. Intracellular zinc depletion induces caspase activation and p21 Waf1/Cip1 cleavage in human epithelial cell lines. *J Infect Diseases* (2000) 182:S85-S92.
- Chang P, Tucker M A, Hicks P H, Prince C W. Novel protein kinase C isoforms and mitogen-activated kinase kinase mediate phorbol ester-induced osteopontin expression. *IJBCB* (2002) 34:1142-1151.
- Chesters J K, Petrie L, Vint H. Specificity and timing of the Zn²⁺ requirement for DNA synthesis by 3T3 cells. *Exp Cell Res* (1989) 184:499-508.

- Chesters J K, Petrie L, Travis A J. A requirement for Zn²⁺ for the induction of thymidine kinase but not ornithine decarboxylase in 3T3 cells stimulated from quiescence. *Biochem J* (1990) 272:525-527.
- Clarke N D, Berg J M. Zinc fingers in *Caenorhabditis elegans*: Finding families and probing pathways. *Science* (1998) 282:2018-2022.
- Conran N, Ferreira H H, Lorand-Metze I, Thomazzi S M, Antunes E, De Nucci G. Nitric oxide regulates human eosinophil adhesion mechanisms in vitro by changing integrin expression and activity on the eosinophil cell surface. *Br J Pharmacol* (2001) 134:632-638.
- Coppolino M, Leung-Hagesteijn C, Dedhar S, Wilkins J. Inducible interaction of integrin alpha2 beta1 with calreticulin. *J Biol Chem* (1995) 270:23132-23138.
- Coppolino M, Woodside M J, Demaurex N, Grinstein S, St. Arnaud R, Dedhar S. Calreticulin is essential for integrin-mediated calcium signaling and cell adhesion. *Nature* (1997) 386:843-847.
- Coppolino M G, Dedhar S. Molecules in focus: Calreticulin. *The International Journal of Biochemistry and Cell Biology* (1998) 30:553-558.
- Coppolino M G, Dedhar S. Ligand-specific, transient interaction between integrins and calreticulin during cell adhesion to extracellular matrix proteins is dependent upon phosphorylation/dephosphorylation events. *Biochem J* (1999) 340:41-50.
- Cossack Z T. Decline in somatomedin-C, insulin-like growth factor I with experimentally induced zinc deficiency in human subjects. *Clin Nutr* (1991) 10:284-291.
- Coyle P, Zalewski P D, Philcox J C, Forbes I J, Ward A D, Lincoln S F, Mahadevan I, Rofe A M. Measurement of zinc in hepatocytes by using a fluorescent probe, Zinquin: Relationship to metallothionein and intracellular zinc. *Biochem J* (1994) 303:781-786.
- Csermely P, Szamel M, Resch K, Somogyi J. Zinc can increase the affinity of phorbol ester receptor in T lymphocytes. *Biochem Biophys Res Comm* (1988a) 154:578-583.
- Csermely P, Szamel M, Resch K, Somogyi J. Zinc can increase the activity of protein kinase C and contributes to its binding to the plasma membrane in T lymphocytes. *J Biol Chem* (1988b) 263:6487-6490.
- Cui L, Takagi Y, Wasa M, Sando K, Khan J, Okada A. Nitric oxide synthase inhibitor attenuates intestinal damage induced by zinc deficiency in rats. *J Nutr* (1999) 129:792-798.

- Cui L, Wasa M, Takagi Y, Sando K, Khan J, Okada A. Nitric oxide synthase inhibitor attenuates inflammatory lesions in the skin of zinc-deficient rats. *Nutrition* (2000) 16:34-41.
- Dawid I B, Breen J J, Toyama R. LIM domains: multiple roles as adapters and functional modifiers in protein interactions. *Trends Genet* (1998) 14:156-162.
- Dedhar S. Novel functions for calreticulin: interaction with integrins and modulation of gene expression. *Trends Biochem Sci* (1994) 19:269-271.
- Dedhar S, Rennie P S, Shago M, Hagesteijn C Y, Yang H, Filmus J, Hawley R G, Bruchovsky N, Sheng H, Matusik R J. Inhibition of nuclear hormone receptor activity by calreticulin. *Nature* (1994) 367:480-483.
- Dreno B. Oligoelements et peau. *Dermatologie Pratique* (1996) 182:1-3.
- Duvall E, Wyllie A H. Death and the cell. *Immunol Today* (1986) 7:115-119.
- Elenkov I J, Chrousos G P. Stress hormones, proinflammatory and antiinflammatory cytokines, and autoimmunity. *Ann NY Acad Sci* (2002) 966:290-303.
- Erlandsen S L, Parsons J A, Taylor T D. Ultrastructural immunocytochemical localization of lysozyme in Paneth cells of man. *J Histochem Cytochem* (1974) 22:401-413.
- Fernandes P R, Samuelson D A, Clark W R, Cousins R J. Immunohistochemical localization of cysteine-rich intestinal protein in rat small intestine. *FASEB J* (1995) 9:A738.
- Forbes I J, Zalewski P D, Giannakis C, Petkoff H S, Cowled P A. Interaction between protein kinase C and regulatory ligand is enhanced by a chelatable pool of cellular zinc. *Biochim Biophys Acta* (1990) 1053:113-117.
- Grummt F, Weinmann-Dorsch C, Schneider-Schaulies J, Lux A. Zinc a messenger of mitogenic induction: Effects on diadenosine tetraphosphate and DNA synthesis. *Exp Cell Res* (1986) 163:191-200.
- Haase H, Beyersmann D. Uptake and intracellular distribution of labile and total Zn(II) in C6 rat glioma cells investigated with fluorescent probes and atomic absorption. *Biometals* (1999) 12:247-254.
- Hallquist N A, Khoo C, Cousins R J. Lipopolysaccharide regulates cysteine-rich intestinal protein, a zinc-finger protein, in immune cells and plasma. *J Leuk Biol* (1996) 59:172-177.
- Hanada K, Tamai K, Sawamura D, Hashimoto I, Muramatsu T. Dynamic changes in intracellular location of metallothionein in rat keratinocytes after ultraviolet-B radiation. *J Invest Dermatol* (1998) 110:98-100.

- Hansson A. Extracellular zinc ions induce mitogen-activated protein kinase activity and protein tyrosine phosphorylation in bombesin-sensitive Swiss 3T3 fibroblasts. *Arch Biochem Biophys* (1996) 328:233-238.
- Harper B D, Beckerle M C, Pomies P. Fine mapping of the alpha-actinin binding site within cysteine-rich protein. *Biochem J* (2000) 350:269-274.
- Hedman H, Lundgren E. Regulation of LFA-1 avidity in human B cells. Requirements for dephosphorylation events for high avidity ICAM-1 binding. *J Immunol* (1992) 149:2295-2299.
- Hobert O, Moerman D G, Clark K A, Beckerle M C, Ruvkun G. A conserved LIM protein that affects muscular adherens junction integrity and mechanosensory function in *Caenorhabditis elegans*. *J Cell Biol* (1999) 144:45-57.
- Huang J S, Mukherjee J J, Chung T, Crilly K S, Kiss Z. Extracellular calcium stimulates DNA synthesis in synergism with zinc, insulin, and insulin-like growth factor I in fibroblasts. *Eur J Biochem* (1999) 266:943-951.
- Hubbard S R, Bishop W R, Kirschmeier P, George S J, Cramer S P, Hendrickson W A. Identification and characterization of zinc binding sites in protein kinase C. *Science* (1991) 254:1776-1779.
- Khanna N C, Tokuda M, Waisman D M. Conformational changes induced by binding of divalent cations to calregulin. *J Biol Chem* (1986) 261:8883-8887.
- Khoo C, Cousins R J. Purification and properties of rat cysteine-rich intestinal protein. *Biochem J* (1993) 299:445-450.
- Khoo C, Hallquist N A, Samuelson D A, Cousins R J. Differential expression of cysteine-rich intestinal protein in liver and intestine in CCl₄-induced inflammation. *American Physiological Society* (1996) G613-G617.
- Kosuge T, Toyoshima S. Increased degradation of newly synthesized interferon-gamma (IFN-gamma) in anti CD3-stimulated lymphocytes treated with glycoprotein processing inhibitors. *Biol Pharm Bull* (2000) 23:545-548.
- Kuo S M, Kondo Y, DeFellipo J M, Einstorff M S, Bahuson R R, Lazo J S. Subcellular localization of metallothionein IIA in human bladder tumor cells using a novel epitope-specific antiserum. *Toxic Appl Pharmacol* (1994) 125:104-110.
- Kuroda S, Tokunaga C, Kiyohara Y, Higuchi O, Konishi H, Mizuno K, Gill G N, Kikkawa U. Protein-protein interaction of zinc finger LIM domains with protein kinase C. *J Biol Chem* (1996) 271:31029-31032.
- Kwon T K. Phorbol myristate acetate inhibits okadaic acid-induced apoptosis and downregulation of X-linked inhibitor of apoptosis in U937 cells. *Biochem Biophys Res Commun* (2001) 287:135-141.

- Langmade S J, Ravindra E, Daniels P J, Andrews G K. The transcription factor MTF-1 mediates metal regulation of the mouse ZnT1 gene. *J Biol Chem* (2000) 275:34803-34809.
- Lanningham-Foster L, Green C L, Langkamp-Henken B, Davis B A, Nguyen K T, Bender B S, Cousins R J. Overexpression of CRIP in transgenic mice alters cytokine patterns and the immune response. *Am J Physiol Endocrinol Metab* (2002) 282:E1197-E1203.
- Leung-Hagesteijn C Y, Milankov K, Michalak M, Wilkins J, Dedhar S. Cell attachment to extracellular matrix substrates is inhibited upon downregulation of expression of calreticulin, an intracellular integrin alpha-subunit-binding protein. *J Cell Sci* (1994) 107:589-600.
- Levenson C W, Shay N F, Lee-Ambrose L M, Cousins R J. Regulation of cysteine-rich intestinal protein by dexamethasone in the neonatal rat. *Proc Natl Acad Sci, USA* (1993) 90:712-716.
- Levenson C W, Shay N F, Cousins R J. Cloning and initial characterization of the promoter region of the rat cysteine-rich intestinal protein gene. *Biochem J* (1994) 303:731-736.
- Li Z, Stafford W F, Bouvier M. The metal ion binding properties of calreticulin modulate its conformational flexibility and thermal stability. *Biochemistry* (2001) 40:11193-11201.
- Lichtlen P, Wang Y, Besler T, Georgiev O, Certa U, Sack R, Schaffner W. Target gene search for the metal-responsive transcription factor MTF-1. *Nucleic Acids Research* (2001) 29:1514-1523.
- Liliental J, Chang D D. Rack1, a receptor for activated protein kinase C, interacts with integrin beta subunit. *J Biol Chem* (1998) 273:2379-2383.
- Loweth A C, Williams G T, Scarpello J H, Morgan N G. Evidence for the involvement of cGMP and protein kinase G in nitric oxide-induced apoptosis in the pancreatic B-cell line, HIT-T15. *FEBS Lett* (1997) 400:285-288.
- Markwell M A, Haas S M, Bieber L L, Tolbert N E. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* (1978) 87:206-210.
- McNall A D, Etherton T D, Fosmire G J. The impaired growth induced by zinc deficiency in rats is associated with decreased expression of the hepatic insulin-like growth factor I and growth hormone receptor genes. *J Nutr* (1995) 125:874-879.
- MeBmer U K, Reimer D M, Reed J C, Brune B. Nitric oxide induced poly(ADP-ribose) polymerase cleavage in RAW 264.7 macrophage apoptosis is blocked by Bcl-2. *FEBS Lett* (1996) 384:162-166.

- Michalak M, Milner R E, Burns K, Opas M. Calreticulin. *Biochem J* (1992) 285:681-692.
- Michalak M, Corbett E F, Mesaeri N, Nakamura K, Opas M. Calreticulin: one protein, one gene, many functions. *Biochem J* (1999) 344:281-292.
- Nartey N O, Cherian M G, Banerjee D. Immunohistochemical localization of metallothionein in human thyroid tumors. *Am J Pathol* (1987) 129:177-182.
- Nassir F, Blanchard R K, Mazur A, Cousins R J, Davidson N O. Apolipoprotein B mRNA editing is preserved in the intestine and liver of zinc-deficient rats. *J Nutr.* (1996) 126:860-864.
- Nguyen T O, Capra J D, Sontheimer R D. Calreticulin is transcriptionally upregulated by heat shock, calcium, and heavy metals. *Mol Immunol* (1996) 33:379-386.
- Nix D A, Beckerle M C. Nuclear-cytoplasmic shuttling of focal contact protein, zyxin: a potential mechanism for communication between sites of cell adhesion and the nucleus. *J Cell Biol* (1997) 138:1139-1147.
- Nix D A, Fradelizi J, Bockholt S, Menichi B, Louyard D, Friederich E, Beckerle M C. Targeting of zyxin to sites of actin membrane interaction and to the nucleus. *J Biol Chem* (2001) 276:34759-67.
- Ostwald T J, MacLennan D H. Isolation of a high affinity calcium-binding protein from sarcoplasmic reticulum. *J Biol Chem* (1974) 249:974-979.
- Ouellette A J, Miller S I, Henschen A H, Selsted M E. Purification and primary structure of murine cryptdin-1, a Paneth cell defensin. *FEBS Lett* (1992) 304:146-148.
- Oyadomari S, Takeda K, Takiguchi M, Gotoh T, Matsumoto M, Wada I, Akira S, Araki E, Mori M. Nitric oxide-induced apoptosis in pancreatic beta cells is mediated by the endoplasmic reticulum stress pathway. *Proc Natl Acad Sci USA.* (2001) 98:10845-50.
- Parker P, Coussens L, Totty N, Rhee L, Young S, Chen E, Stabel S, Waterfield M D, Ullrich A. The complete primary structure of protein kinase C- the major phorbol ester receptor. *Science* (1986) 233:853-859.
- Ramsamooj P, Notario V, Dritschilo A. Enhanced expression of calreticulin in the nucleus of radioresistant squamous carcinoma cells in response to ionizing radiation. *Cancer Res* (1995) 55:3016-3021.
- Rearden A. A new LIM protein containing an autoepitope homologous to "senescent cell antigen." *Biochem Biophys Res Commun* (1994) 1201:1124-1131.

- Roderick H L, Campbell A K, Llewellyn D H. Nuclear localization of calreticulin in vivo is enhanced by its interaction with glucocorticoid receptors. *FEBS Lett* (1997) 405:181-185.
- Rogers J M, Taubeneck M W, Daston G P, Sulik K K, Zucker R M, Elstein K H, Jankowski M A, Keen C L. Zinc deficiency causes apoptosis but not cell cycle alterations in organogenesis-stage rat embryos: effect of varying duration of deficiency. *Teratology* (1995) 52:149-159.
- Roth H P, Kirchgessner M. Influence of alimentary zinc deficiency on the concentration of growth hormone (GH), insulin-like growth factor I (IGF-1) and insulin in serum of force-fed rats. *Horm Metab Res* (1994) 26:404-408.
- Sadasivan B, Cariappa A, Waneck G L, Cresswell P. *Cold Spring Harbor Symp Quant Biol* (1995) 60:267-275.
- Sadasivan B, Lehner P J, Ortmann B, Spies T, Creswell P. Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP. *Immunity* (1996) 5:103-114.
- Sadler I, Crawford A W, Michelsen J W, Beckerle M C. Zyxin and cCRP: two interactive LIM domain proteins associated with the cytoskeleton. *J Cell Biol* (1992) 119: 1573-1587.
- St. Arnaud R, Prud'homme J, Leung-Hagesteijn C, Dedhar S. Constitutive expression of calreticulin in osteoblasts inhibits mineralization. *J Cell Biol* (1995) 131:1351-1359.
- Samet J M, Silbajoris R, Wu W, Graves L M. Tyrosine phosphatases as targets in metal-induced signaling in human airway epithelial cells. *Am J Resp Cell Mol Biol* (1999) 21:357-364.
- Shago M, Flock G, Leung-Hagesteijn C, Grinstein M, Grinstein S, Giguere V, Dedhar S. Modulation of the retinoic acid and retinoid X receptor signaling pathways in P19 embryonal carcinoma cells by calreticulin. *Exp Cell Res* (1997) 230:50-60.
- Sjaastad M D, Lewis R S, Nelson W J. Mechanisms of integrin-mediated calcium signaling in MDCK cells: Regulation of adhesion by IP3 and store-independent calcium influx. *Mol Biol Cell* (1996) 7:1025-1041.
- Smirnova I V, Bittel D C, Ravindra R, Jiang H, Andrews G K. Zinc and cadmium can promote rapid nuclear translocation of metal response element-binding transcription factor-1. *J Biol Chem* (2000) 275:9377-9384.
- Takemoto H, Yoshimori T, Yamamoto A, Miyata Y, Yahara I, Inoue K, Tashiro Y. *Arch Biochem Biophys* (1992) 296:129-136.

- Tenaud I, Leroy S, Chebassier N, Dreno B. Zinc, copper, and manganese enhanced keratinocyte migration through a functional modulation of keratinocyte integrins. *Exp Dermatol* (2000) 9:407-416.
- Truong-Tran A Q, Carter J, Ruffin R E, Zalewski P D. The role of zinc in caspase activation and apoptotic cell death. *Biometals* (2001) 14:315-330.
- Tsujikawa K, Imai T, Kakutani M, Kayamori Y, Mimura T, Otak N, Kimura M, Fukuyama R, Shimizu N. Localization of metallothionein in nuclei of growing primary cultured adult rat hepatocytes. *FEBS Lett* (1991) 283:239-242.
- Tsujikawa K, Suzuki N, Sagawa K, Itoh M, Sugiyama T, Kohama Y, Otaki N, Kimura M, Mimura T. Induction and subcellular localization of metallothionein in regenerating rat liver. *Eur J Cell Biol* (1994) 63:240-246.
- Tu Y, Li F, Goicoechea S, Wu C. The LIM-only protein PINCH directly interacts with integrin-linked kinase and is recruited to integrin-rich sites in spreading cells. *Mol Cell Biol* (1999) 19:2425-2434.
- Turner C E, Glenney J R, Burridge K. Paxillin: a new vinculin-binding protein present in focal adhesions. *J Cell Biol* (1990) 111:1059-1068.
- Voet D, Voet J. *Biochemistry Second Edition*. New York: John Wiley & Sons, Inc. (1995) 377.
- Winrow C J, Miyata K S, Marcus S L, Burns K, Michalak M, Capone J P, Rachubinski R A. Calreticulin modulates the in vitro DNA binding but not the in vivo transcriptional activation by peroxisome proliferator-activated receptor/retinoid X receptor X receptor heterodimers. *Mol Cell Endocrinol* (1995) 111:175-179.
- Wixler V, Geerts D, Laplantine E, Westhoff D, Smyth N, Aumailley M, Sonnenberg A, Paulsson M. The LIM-only protein DRAL/FHL2 binds to the cytoplasmic domain of several alpha and beta integrin chains and is recruited to adhesion complexes. *J Biol Chem* (2000) 275:33669-33678.
- Woo E S, Lazo J S. Nucleocytoplasmic functionality of metallothionein. *Cancer Res* (1997) 57:4236-4241.
- Wu W, Graves L M, Jaspers I, Devlin R B, Reed W, Samet J M. Activation of the EGF receptor signaling pathway in human airway epithelial cells exposed to metals. *Am J Physiol* (1999) 277:L924-L931.
- Yoshimori T, Semba T, Takemoto H, Akagi S, Yamamoto A, Tashiro Y. Protein disulfide-isomerase in rat exocrine pancreatic cells is exported from the endoplasmic reticulum despite possessing the retention signal. *J Biol Chem* (1990) 265:15984-15990.

- Zalewski P D, Fobes I J, Giannakis C, Cowled P A, Betts W H. Synergy between zinc and phorbol ester in translocation of protein kinase C to cytoskeleton. *FEBS Lett* (1990) 273:131-134.
- Zalewski P D, Forbes I J. Intracellular zinc and the regulation of apoptosis. In: Lavin M., Watters D. eds. *Programmed Cell Death: The Cellular and Molecular Biology of Apoptosis*. Melbourne: Harwood Academic Publishers (2001): 73-86.
- Zhou Q, Ruiz-Lozano P, Martone M E, Chen J. Cypher, a striated muscle-restricted PDZ and LIM domain-containing protein, binds to alpha-actinin-2 and protein kinase C. *J Biol Chem* (1999) 274:19807-19813.
- Zhu Q, Zelinka P, White T, Tanzer M L. Calreticulin-integrin bidirectional signaling complex. *Biochem Biophys Res Commun* (1997) 232:354-358.

BIOGRAPHICAL SKETCH

John David Nicewonger II was born to John and Barbara Nicewonger in Fort Lauderdale, Florida on November 18, 1976. John was raised in south Florida. He graduated among the top ten in his class from Spanish River High School in Boca Raton in May, 1995. The following fall he began studies at the University of Florida. In May, 1999, he graduated with honors from the University of Florida earning a B. S. in microbiology and cell science.

In January, 2000, John decided to apply for a master's program in the Food Science and Human Nutrition Department at the University of Florida. He was accepted and awarded the Davis Graduate Nutrition Enhancement Award of \$5000 per year. In August of 2000 he began an assistantship under Dr. R. J. Cousins. While working toward finishing his project, John developed a passion for the study of immunology. He is planning on finishing his thesis in December, 2002, and will be applying to the University of Pittsburgh to begin doctorate studies in immunology and pathology.