REGULATION OF Rac1 AND Cdc42 GTPases THROUGH DIRECT INTERACTION WITH CALMODULIN

By

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A thesis submitted to the Faculty of Graduate Studies
in partial fulfillment of the requirements for the
degree of

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ABSTRACT

Rac1 and Cdc42 are members of the Rho family of small GTPases and have been shown to induce lamellipodia and filopodia formation, respectively. This leads to changes in cytoskeleton organization, and as a consequence, affects cell migration. In the present work using calmodulin affinity binding assays and HeLa and platelet cell lysates, we demonstrate that endogenous Rac1 and Cdc42 interact with calmodulin (CaM) in both a Ca^{2+}-dependent and Ca^{2+}-independent fashion. We also have established that Rac1 and Cdc42 bind calmodulin directly. This novel interaction was further confirmed in cells using co-immunoprecipitation studies.

Using calmodulin database analysis to search for potential calmodulin binding sequences, we identified a novel CaM binding sequence [AVKYLECSALTQRG] within the C-terminus of Rac1 that can potentially interact with calmodulin. With an *in vitro* assay using a synthetic peptide representing the potential calmodulin binding region of Rac1, we have confirmed that the peptide binds calmodulin. A similar region exists in Cdc42.

Time course activation studies demonstrated that the optimal point of Rac1 and Cdc42 activation in human platelets was 60 sec. and 25 sec., respectively. Using the optimal activation point for each protein, the role of calmodulin in the function of Rac1 and Cdc42 was examined. Thrombin caused activation of Rac1 and Cdc42. The potent calmodulin antagonist, W7, abolished thrombin-mediated activation of Rac1 but enhanced the thrombin activation of Cdc42. Addition of W7 resulted in the activation of Cdc42 over basal levels and W7 did not inhibit thrombin mediated activation of Cdc42.

Immunocytochemistry analysis was carried out to study changes in Rac1, Cdc42 and CaM localization. In resting platelets Rac1, Cdc42 and calmodulin are distributed in both the cytoplasm and plasma membrane. Upon stimulation with thrombin, Rac1 and Cdc42 localized to plasma membrane and platelet aggregation occurred. The addition of W7 or W7 plus thrombin did not cause localization of Rac1, Cdc42 and calmodulin to plasma membrane compared to thrombin treatment alone. This further resulted in an inhibition to platelet aggregation.

In summary, the results presented here demonstrate calmodulin as a new interacting partner for Rac1 and Cdc42. This interaction plays a role in coordinating Rac1 and Cdc42 activation which may regulate cell migration and cytoskeleton remodeling.

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LIST OF ABBREVIATIONS

AEBSF - 4-[(2-aminoethyl)]-benzenesulfonyl fluoride

BSA - Bovine serum albumin

Ca^{2+} - Calcium

CaM - Calmodulin

CaMBP - CaM binding protein

CNBr - Cyanogen bromide

DMEM - Dulbecco's modified Eagle's medium

EDTA - Ethylene diaminetetraacetic acid

EGF - Epidermal growth factor

EGTA – Ethylene glycol-bis (2-aminoethyl ether)-N,N,N',N'-tetraacetic acid

ER - Endoplasmic reticulum

FBS - Fetal bovine serum

GAP - GTPase activating protein

GEF - Guanine nucleotide exchange factor

GDI - Guanine nucleotide dissociation inhibitor

GDP - Guanosine-5'-diphosphate

GST - Glutathione S-transferase

GPCR- G-protein coupled receptors

GSH - Glutathione

GTP - Guanosine-5'-triphosphate

IPTG - Isopropyl-1-thio-β-D-galactopyranoside

kb - Kilobase

kDa - KiloDalton

LB - Luria-Bertani

LMW-PTP - Low molecular weight protein tyrosine phosphatase

$MgCl_2$ - Magnesium chloride

MAPK - Mitogen-activated protein kinase

MEK - MAPK kinase

NaH_2PO_4 - Sodium dihydrogen phosphate

$NaHCO_3$ - Sodium bicarbonate

NaF - Sodium fluoride

PAR-1 - Protease-activated receptor-1

PBS - Phosphate buffered saline

PGDF - Platelet-derived growth factor

PIP_2 - Phosphatidylinositol bisphosphate

PKC - Protein kinase C

PLC - Phosphoinositide-specific phospholipase C

PMA - Phorbol 12-myristate 13-acetate

PMSF - Phenylmethylsulfonyl fluoride

PVDF - Polyvinyl difluoride

RIPA - Radioimmune precipitation buffer

SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis

CHAPTER 1

INTRODUCTION

1.1. Signal Transduction and Cell Surface Receptors

Each cell in a multicellular organism is constantly exposed to external signals that contain information for eliciting appropriate responses. External signals move from the outside to the inside of the cell through cell surface receptors. Cell surface receptors determine the fate and response as a consequence of a particular stimulus acting on a cell. Upon stimulation, the extracellular signal is transmitted across the cellular membrane initiating production of secondary messengers which, in turn, cause the activation of other proteins and enzymes. These subsequent reactions lead to alteration in cellular function and elicit physiological responses depending on the stimuli.

Receptors involved in signal transduction are classified into five general classes. The first class of receptors penetrates the plasma membrane and has intrinsic enzyme activity. This class has a single membrane spanning helix and extracellular ligand binding domain. Receptors in this class include, tyrosine kinases (e.g. PDGF, insulin, EGF and FGF receptors), tyrosine phosphatases (e.g. CD45 protein of T cells and macrophages), guanylyl cyclases (e.g. natriuretic peptide receptors) and serine/threonine kinases (e.g. activin and TGF-β receptors) (Uings and Farrow, 2000). Receptors with intrinsic tyrosine kinase activity are capable of autophosphorylation as well as phosphorylating other substrates. Upon binding of a growth factor to its receptor, it induces both receptor dimerization and an increase in the activity of the kinase. The degree of activation is

controlled by ligand binding in that it can cause downregulation of surface receptors and reduction in the signal transduction cascades (Uings and Farrow, 2000). Several families of receptors lack intrinsic enzyme activity, yet are coupled to intracellular tyrosine kinases through protein-protein interactions (Alberts *et al.*, 2002).

The second class of receptors includes the steroid receptors. These receptors, upon ligand binding, migrate to the nucleus where the ligand-receptor complex directly affects gene transcription (e.g. vitamin D) (Alberts *et al.*, 2002). The third class of receptors acts as a barrier to the flux of ions across the cell membrane. These include the ligand gated ion channel receptors that work by altering membrane permeability to specific ions (Uings and Farrow, 2000). Examples of this class are receptors in the central nervous system such as GABA, 5HT, glycine and nicotinic acetylcholinic (nACh) receptors. The GABA and glycine receptors are anion channels that allow the movement of chloride ions while the nACh receptor allows movement of monovalent cations such as sodium (Uings and Farrow, 2000). The fourth class of receptors include cytokine receptors that play an important role in the haematopoietic system and in coordinating immune responses. An example of cytokine receptors include tumor necrosis factor (TNF) and haematopoietin receptors which respond to cytokines such as interleukins (IL 1-5), prolactin growth hormone, and granulocyte macrophage colony stimulating factor (GM-CSF) (Uings and Farrow, 2000).

The fifth class of receptors is called G-Protein Coupled Receptors (GPCR). Receptors of the GPCR class interact with G-proteins and have a structure that is

characterized by seven transmembrane spanning domains. They activate signal transduction through the coupling of their cytoplasmic domains to a family of heterotrimeric GTP-binding proteins (G-proteins) (Uings and Farrow, 2000). Examples of receptors belonging to this class include the adrenergic receptors, odorant receptors, chemokine receptors and some hormone receptors (e.g. glucagon, angiotensin, vasopressin and bradykinin). They act on a wide variety of signal transduction pathways such as those involving adenylyl cyclase, tyrosine kinase cascades and phospholipases (Uings and Farrow, 2000). GTP-binding proteins therefore play an important role in cell signaling and signal transduction.

1.2. GTP-Binding Proteins

There are three known superfamilies of GTP-binding proteins (G-proteins) (Takai *et al.*, 1994; 2001). The first group includes initiation and elongation factors involved in protein synthesis. The second group consists of heterotrimeric G-proteins (e.g. G_s, G_i, G_q) which are made up of three subunits α, β, and γ (Takai *et al.*, 1994; 2001; Sidhu and Bhullar, 2005). These subunits regulate metabolic enzymes, ion channels, transporters and other cellular components that regulate many cellular processes (Takai *et al.*, 1994; 2001; Sidhu and Bhullar, 2005). Heterotrimeric G-protein coupled receptors contain seven membrane spanning regions that transmit extracellular signals (e.g. hormones, neurotransmitters, chemokines, autocrine and paracrine factors) to G-proteins on the inner surface of plasma membrane (Takai *et al.*, 1994; 2001). The third group of G-proteins includes small monomeric G-proteins known as Ras-like or small GTP-binding proteins (Takai *et al.*, 1994; 2001). My focus will be on this group of G-proteins.

1.3. Small GTP-Binding Proteins

The small G-proteins consist of a single polypeptide chain of 200-300 amino acids and have a molecular weight ranging from 20-40 kDa. (Takai *et al.*, 1994; 2001). There are approximately 150 known eukaryotic small G-proteins that have been characterized and divided into five families: Ras, Rho, Rab, Sar1/Arf and Ran (Paduch *et al* 2001). Small GTPases regulate diverse intracellular pathways including receptor-mediated signaling and gene expression (eg. Ras), nuclear transport (Ran), intracellular vesicle trafficking (Rab and Sar1/Arf proteins) and cytoskeletal dynamics (Rho, Rac, Cdc42) (Corbett and Alber, 2001; Takai *et al.*, 2001).

1.4. Platelets as the Model System

In most of our studies, we have used human platelets as our cell model. Platelets are anucleated blood cells that are discoid in shape and are derived from megakaryocytes (Italiano *et al.*, 2003). They play an important role in hemostasis and in preventing bleeding from damaged blood vessels. At the site of injury, platelets are activated upon adhering to exposed extracellular matrix proteins, collagen and laminins (Italiano *et al.*, 2003; Chang *et al.*, 2005). Subsequently, components of platelet storage granules (α and dense) including, von Willebrand factor (vWf), fibronectin, fibrinogen, thrombospondin and other molecules are released (Chang *et al.*, 2005). This causes the formation of a platelet adhesion plug as a result of platelet aggregation. The various signal transduction pathways in the platelet have been well defined with easily measurable end points. This makes platelets an ideal model for studying rapid morphological change in response to external stimuli.

CHAPTER 2

LITERATURE REVIEW

2.1. The Ras Superfamily

Ras p21 (Ras) protein was the first member of the Ras family of small GTPases to be discovered over 20 years ago in oncogenic viruses (Takai *et al.,* 2001). The Ras superfamily incorporates a large group of structurally and functionally conserved small GTP-binding proteins (small GTPases) that are analogous to an electric switch in which a GTP-bound state represents a switch in the "on" position while the GDP-bound state resembles the "off" position. Ras activation occurs when guanine nucleotide exchange factors (GEFs) contact switches I and II of Ras by forming a β-sheet. Switches I and II undergo a conformational change upon GTP- or GDP-binding. Ras binds with high affinity to effector proteins found in the cytoplasm when it is active (i.e. GTP-bound form) which leads to activation of downstream intracellular signaling pathways (Manser, 2002). The well known Ras effectors include Raf kinases, phosphatidylinositol-3-OH kinases and Ral-specific GEFs.

GTPase activating proteins (GAPs) speed up the slow intrinsic GTPase activity (Zerangue and Jan, 1998). GAPs stabilize an important catalytic glutamine residue in the switch II region of the GTPase and insert an arginine residue in the active site to stabilize the transition state of GTP hydrolysis reaction (Corbett and Alber, 2001). As a result, an antiparallel β-sheet is formed by both the GTPase and the GAP proteins. Hydrolysis of

GTP by GAPs leads to inactivation of the G-protein and dissociation from effectors (Manser, 2002).

2.2. Classifications, Structure and Function

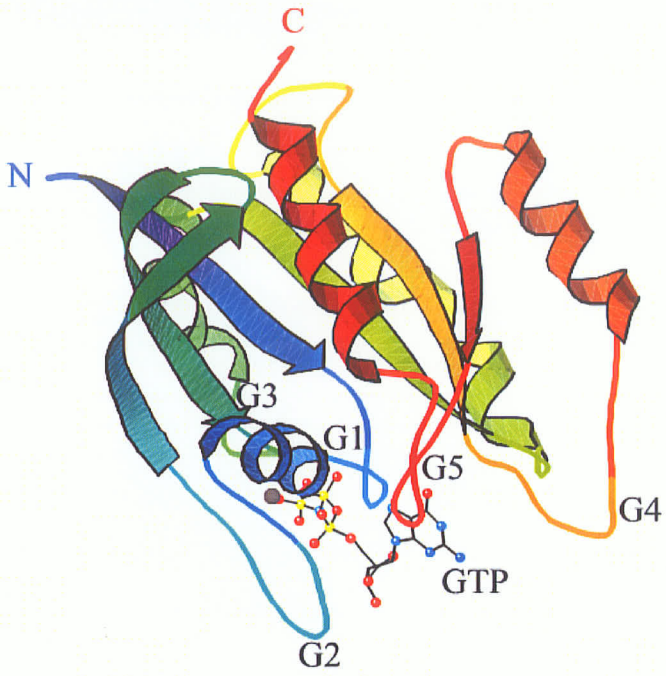

Figure 1: Structure of Ras GTPase. This structure (PDB id: 5P21) was generated using KiNG (Kinetic Image, Next Generation) software (http://kinemage.biochem.duke.edu).

The Ras superfamily of monomeric G-proteins contains more than 150 members that share a common structural design and molecular mechanism of action to those of Gα subunits of heterotrimeric G-proteins (Bourne *et al.*, 1991). Ras superfamily proteins are classified based on sequence similarity into four families: Ras-related, Rho, Rab/Ran, and Sar1/Arf (Colicelli, 2004).

The majority of the Ras proteins are bound to intracellular membranes and require a second signal for transport to the plasma membrane. They contain signal sequences at the C-terminal CAAX motif (A is aliphatic amino acid and X is methionine or serine) that allow them to bind to the plasma membrane. This motif signals three post-translational modifications: farnesylation, geranylgeranylation, AAX proteolysis, and carboxymethylation (Hancock *et al.*, 1989; Casey *et al.*, 1991). For N-Ras and H-Ras, the signal for membrane attachment also consists of one or two cysteines upstream of the CAAX motif (Hancock *et al.*, 1990). H-Ras, N-Ras, and K-Ras4A use palmitate fatty acid for membrane association whereas K-Ras-4B uses a lysine-rich polybasic sequence signal (Reuther and Der, 2000).

The Ras-related small G-proteins have similar conserved sequences and share a common structural core known as the G-domain. The structure of Ras consists of a hydrophobic core made of 6 β-sheet strands, connected by hydrophilic loops and α helices (Bourne *et al.*, 1991). The most highly conserved regions of the GTP-binding domains of G-protein family are the loop regions (G1-G5) but not the β-sheet or α-helices (**Figure 1**) (Paduch *et al.*, 2001; Sidhu and Bhullar, 2005). The G1 region (P-loop) is found between residues 10-17 in Ras. The hydrogens of the NH_2 groups from several residues and the ϵ-amino group of lysine 16 in this region form bonds with the α- and β-phosphates of GTP or GDP. The G2 region (switch I) is the effector-binding loop, found in Ras between residues 32-40 and is the site for the binding of effector molecules and GAP proteins (Paduch *et al.*, 2001; Sidhu and Bhullar, 2005). The G3 region or switch II region (residues 53-62 in Ras), is the most flexible element of the catalytic domain that is

found in all GTPases (Paduch *et al.*, 2001; Sidhu and Bhullar, 2005). The G4 region is made up of four hydrophobic or non-polar amino acids followed by a hydrophobic β strand and a hydrophilic loop (Bourne *et al.*, 1991; Sidhu and Bhullar, 2005). The last region in small GTPases is a conserved region called the G5 region (residues 144-146 of Ras) that is thought to have indirect interactions with guanine nucleotide (Bourne *et al.*, 1991; Sidhu and Bhullar, 2005).

2.3. The Rho (Ras homolog) Protein Family

In addition to their role in cytoskeleton reorganization, cell migration and adhesion, Rho proteins have been shown to regulate many cellular activities. These activities include regulation of cell polarity, endocytosis, vesicle trafficking, cell cycle progression, differentiation, oncogenesis and gene transcription (Burridge and Wennerberg, 2004). Based on primary sequence and known functions, there are 20 Rho proteins divided into 5 main groups: Rho-like, Rac-like, Cdc42-like, Rnd and RhoBTB subfamilies (**Table 1**). Rnd1 and Rnd2 are highly expressed in brain and have both been implicated in neurite outgrowth and branching (Burridge and Wennerberg, 2004). RhoBTB (Broad-Complex, Tramtrack, and Bric a brac) proteins are the most unusual members of the Rho family. The RhoBTB family was identified in the lower eukaryotes *Drosophila* and *Dictyostelium* (Rivero *et al.*, 2001). Presently, three RhoBTB proteins (RhoBTB1-3) have been identified in humans, mouse, vertebrates and insects but not in *Caenorhabditis elegans*, fungi or plants (Ramos *et al.*, 2002). They have large C-terminal extensions containing two BTB domains. In RhoBTB1 and RhoBTB2 (known as DBC2), the GTPase domain is Rho-like while the GTPase domain in RhoBTB3 is not and

therefore is not considered as a Rho protein. The function and signaling pathway of this group of proteins is unknown except that RhoBTB2 is downregulated in many breast cancers and its introduction into T47D breast cancer cells inhibits cell growth (Hamaguchi *et al.*, 2002; Burridge and Wennerberg, 2004). Recently, a couple of new Rho GTPases MIRO-1 and MIRO-2 have been discovered (Fransson *et al.,* 2003). However they have very little homology to the other Rho GTPases and lack the Rho specific insert loop in their GTPase domains (Burridge and Wennerberg, 2004).

Table 1

List of Rho family members[1]

Rho Family					
RhoA	Rac1	Cdc42	Rnd1	RhoBTB1	MIRO-1
RhoB	Rac2		Rnd2	RhoBTB2	MIRO-2
RhoC	Rac3		Rnd3	RhoBTB3	
RhoD	Rac4				
RhoF					
RhoG					
RhoH					
RhoJ					
RhoQ					
RhoU					
RhoV					
RhoT1					
RhoT2					

[1] (Fransson *et al.,* 2003; Colicelli, 2004)

2.4. Common Structural Features

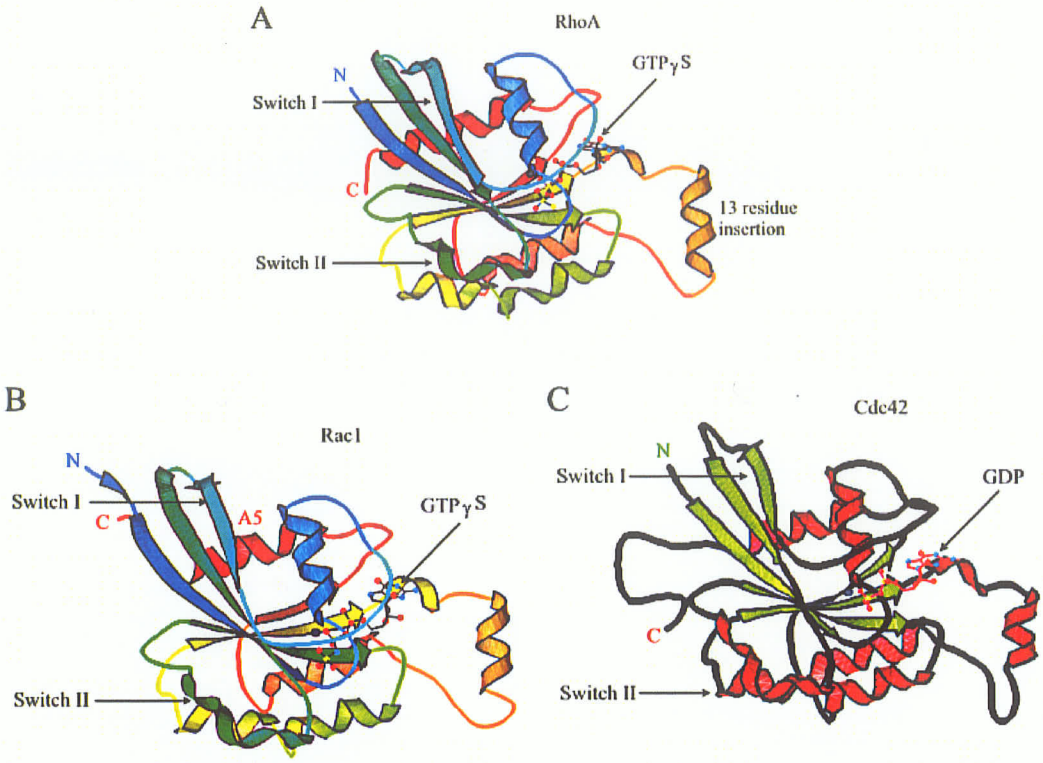

Figure 2: Structure of Rho GTPases. (A) RhoA in GTP bound form (PDB id: 1A2B), (B) Rac1-GTP (PDB id: 1MH1, (C) Cdc42-GDP (PDB id: 1AN0). These structures were created using KiNG (Kinetic Image, Next Generation) software (http://kinemage.biochem.duke.edu).

Rho GTPases share a common G-domain fold that is made up of a six-stranded β-sheet surrounded by α-helices. There are two segments: switch I and switch II between residues 28-44 and 62-69 respectively (Hakoshima *et al.*, 2003; Sidhu and Bhullar, 2005). This differentiates the RhoA-GTP and RhoA-GDP bound forms. These two segments are homologous to the Ras switch I (residues 32-38) and switch II (59-97), respectively (**Figure 2**). However, the only difference is that RhoA switch I is longer than Ras switch I and contains residues in the N-terminal half of the β-sheet (B2). Rho GTPases share a

main 13 residue insertion that is folded into an α-helical structure and has no significant impact on GDP or GTP conformations (**Figure 2**). Switches I and II of Rho GTPases in the GTP-bound conformation are stabilized by hydrogen bonds between the γ-phosphate and amide groups of both switches at threonine 37 (switch I) and glycine 62 (switch II). Removal of magnesium ions (Mg^{2+}) causes a large conformational alteration in both switches exposing the nucleotide binding site (Hakoshima *et al.,* 2003; Sidhu and Bhullar, 2005).

2.5. Rho GTPases: Rho, Rac, Cdc42

The Rho family of small GTP-binding proteins of the Ras superfamily, which includes Rac, Rho and Cdc42 proteins play distinct roles in actin assembly, regulation and motility. Rac1 and Cdc42 are involved in actin/myosin cytoskeleton rearrangement in many cells ultimately leading to changes in cell morphology (Nobes and Hall, 1995; Macara *et al.*, 1996; Hall, 1998). Rac1 was found to be involved in the induction of membrane ruffling, chemotaxis, and lamellipodia formation whereas Cdc42 is involved in filopodia formation (Nobes and Hall, 1999; Burridge and Wennerberg, 2004). There are three known members of the Rac family in mammals, all of which share a common structural arrangement and are encoded by distinct genes (Haeusler *et al.*, 2003). Rac1 is ubiquitously expressed and is the most extensively studied isoform. Rac2 is expressed in hematopietic cells and was suggested to function in regulating the oxidative burst in hematopoietic cells; however, it has also been shown that Rac1 is a major contributor to this process (Kozma *et al.*, 1997). Rac3 is expressed primarily in brain during development (McCarty *et al.*, 2005). The three amino acid sequences have 89-90%

sequence similarity (Bokoch *et al.*, 1995). There is only mammalian isoform of Cdc42 protein that is ubiquitously expressed (Bishop and Hall, 2000).

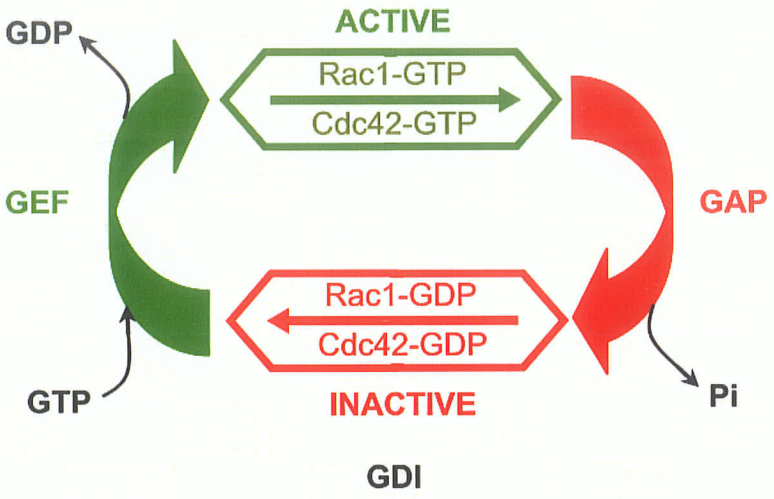

Figure 3: The GTPase cycle of Rac1 and Cdc42. Small GTPases Rac1 and Cdc42 are active when bound to GTP. GTP loading is catalyzed by a guanine nucleotide exchange factor (GEF). The GTPase activity of small G-proteins is increased by a GTPase activating protein (GAP) which accelerates the return of GTP-bound forms of Rac1 and Cdc42 to the GDP-bound forms. Guanine nucleotide dissociation inhibitors (GDI) block GTP for GDP exchange.

Like the rest of the Rho family of small GTP-binding protein, Rac1 and Cdc42 cycle between two states: the active GTP-bound and the inactive GDP-bound state that can subsequently interact with other effector proteins (**Figure 3**) (Hall, 1998; Manneville and Hall, 2002; Burridge and Wennerberg, 2004; McCarty *et al.*, 2005). The conformational differences between the GTP and GDP-bound forms are restricted to two surface loops called switch I and II (Cdc42/Rac1 amino acids 26-45 and 59-74 respectively) (Bishop and Hall, 2000). This enables effector proteins to discriminate

between the two forms (Bishop and Hall, 2000). The mechanism of hydrolysis of GTP to GDP is modified by several classes of helper proteins. Guanine nucleotide exchange factors (GEFs) of G-proteins catalyze the release of bound GDP and the capture of a GTP nucleotide activating the protein (Bourne *et al* 1990; Corbett and Alber, 2001). GTPase activating proteins (GAPs) promote the inactivation of the protein through hydrolysis of the bound GTP to GDP. Another example of regulatory proteins is the guanine nucleotide dissociation inhibitors (GDIs) that act by preventing nucleotide release (Corbett and Alber, 2001). This regulatory mechanism of activation and inactivation enables small GTP-binding proteins to transmit signals by acting as molecular switches.

Distinct actin structures are formed as a result of activation of Rho family members (Carpenter *et al.*, 1997). RhoA injection into cells mediates the formation of focal adhesions and stress fibers (Ridley and Hall, 1992) and ruffling in response to hepatocyte growth factor and phorbol esters (Nishiyama *et al.*, 1994). Injection of Rac1 into cells causes membrane ruffling and focal adhesion formation independent of RhoA (Ridley *et al.*, 1992). Cdc42 injection into cells causes microfilopodia formation and also leads to formation of Rac- and Rho-dependent ruffling and focal adhesion, respectively (Nobes and Hall, 1995; Kozma *et al.*, 1995). Membrane ruffles are closely related to lamellipodia and often used interchangeably. Lamellipodia are thin protrusive structures produced at the edge of migrating cells. Lamellipodia can evolve into ruffles when these membrane protrusions fail to adhere (Burridge and Wennerberg, 2004).

2.6. Rho, Rac1 and Cdc42 Effector Proteins

Table 2

Summary of effector proteins for small GTPases RhoA, Rac1 and Cdc42 [2]

RhoA	Rac1	Cdc42
PI3-kinase	PI3-kinase	PI3-kinase
PKN-RhoA(GTPγS)	PAK*	PAK-Cdc42(GMPPNP)
PI4,5-kinase	ACK	ACK-Cdc42(GMPPNP)
Phospholipase D	MLK2	MLK2
Rhophillin	MLK3	MLK3
Kinectin	IQGAP	IQGAP
Rhotekin	WASP	WASP-Cdc42(GMPPNP)
DGKζ	N-WASP	Arfaptin-Cdc42(GDP/GMPPNP)
PKN	p67phox-Rac1(GTP)	NADPH oxidase
MBS	S6-kinase	Par6-Cdc42(GMPPNP)
ROK/ROCK/Rho kinase	MRCK	PI4,5-kinase
Bni1	MSE5	DGK
Bnr1	Skm1	POSH
Pkc1	Gic1	POR1
P140mDia	Gic2	Sra-1
Fks1	Borg	S6-kinase
Fks2	Bni1	
	Ste20	
	Cla4	

Some of the known effectors of RhoA include protein kinase N (PKN), Rho-kinase and its homologs, rhophillin, rhotekin, citron, citron kinase, mDia and kinectin. These effector proteins bind RhoA at specific residues that distinguish RhoA from other GTPases such as Rac1 and Cdc42. These residues are lysine 27 in switch I, glutamic acid 47 in β-strand B2, glutamine 52 in β-strand B3 and glutamic acid 169 in α-helix A5 (Hakoshima *et al.,* 2003).

[2] (Bishop and Hall, 2000; Paduch *et al.*, 2001; Hakoshima *et al.,* 2003; Burridge and Wennerberg, 2004*)

The effectors of Rac1 and Cdc42 contain a common sequence motif (15 residues) known as the CRIB (Cdc42/Rac-interactive binding) motif (Hakoshima *et al.,* 2003). The presence of this motif shows that Rac1 and Cdc42 have very similar sequence identity. Effectors of Cdc42 are activated Cdc42-associated kinase (ACK), Wiskott-Aldrich syndrome protein (WASP), partition-defective protein Par6 and the Rac1/Cdc42 effector p21-activated kinase (PAK). Their CRIB motif binds Rac1/Cdc42 at β-sheet B2. These effectors also bind to switches I and II and α-helices A1 and A5. The p67phox is a subunit of phagocytotic NADPH oxidase and an effector of Rac1 that lacks the CRIB motif (Burbelo *et al.*, 1995; Hakoshima *et al.,* 2003). Another downstream effector of Rac1 which has been implicated in cytoskeletal reorganization and membrane ruffling is PAK. This serine/threonine kinase ($p65^{PAK}$) binds to both Cdc42 and Rac1 in a GTP-dependent manner *in vitro* (Manser *et al.*, 1994). It is activated by both Rac1 and Cdc42 and its expression can promote lamellipodia formation in addition to loss of stress fibers and focal adhesions (Sells *et al.*, 1997; Manser *et al.*, 1997). The table above summarizes all of the known effectors for RhoA, Rac1 and Cdc42 (**Table 2**).

In serum-starved quiescent cells, the expression of active Rac1 results in the formation of stress fibers due to downstream activation of Rho. Further addition of growth factors to stimulate Rac1 activity results in disassembly of stress fibers and focal adhesions (Sander *et al.,* 1999). These observations might be due to effects of PAK since it induces loss of stress fibers and focal adhesions by phosphorylating and inhibiting activity of myosin light chain and myosin II heavy chain kinases (Sander *et al.,* 1999; van Leeuwen *et al.*, 1999). Furthermore, Rac-mediated production of oxygen radicals causes

inhibition of the low molecular weight protein tyrosine phosphatase (LMW-PTP) which leads to increased phosphorylation and activation of p190RhoGAP. As a result, this causes inactivation of Rho (Nimnual *et al.*, 2003).

Fukuta and colleagues have recently found that an effector that binds Cdc42 and Rac1 known as IQGAP1 is associated with the protein CLIP170, which in turn binds to the growing ends of microtubules (Fukata *et al.*, 2002). Since Rac1 and Cdc42 are known to be activated at the leading edge of migrating cells, IQGAP1/CLIP170 complex is proposed to provide a signal that allows capturing and stabilization of the plus ends of microtubules (Manser, 2002). IQGAP1 and IQGAP2 proteins bind to active Cdc42 and Rac1 (Wessbach *et al.*, 1994; Brill *et al.*, 1996; Hart *et al.*, 1996; Kuroda *et al.*, 1996; McCallum *et al.*, 1996). IQGAP1 cross-links F-actin *in vitro*, an activity that is enhanced by Cdc42-GTP (Fukuta *et al.*, 1997). Calmodulin binds to IQGAP1 and inhibits its binding to actin and Cdc42. Therefore, it has been suggested that IQGAP1 oligomers may form after dissociation from calmodulin and upon binding to Cdc42 (Bishop and Hall, 2000).

2.7. Rho GTPases Localization

Similar to Ras proteins and G-proteins, Rho GTPases are synthesized as cytosolic proteins but have the ability to associate with membranes by virtue of a series of posttranslational modifications of the CAAX motif. This motif can undergo prenylation, AAX tripeptide proteolysis and carboxymethylation (Clarke, 1992). The capacity of Rho proteins to sequester in the cytosol is mediated by their interaction with RhoGDI. Rho proteins have been localized to the cytosol, plasma membranes including cholesterol rich microdomains, golgi, endosomes and nuclei (Michaelson *et al.*, 2001). Upon activation, they have been shown to translocate from cytosol to membranes. Rac1 and Cdc42 proteins are located on the plasma membrane and the cytosol. They tend to translocate between these two sites (Bohoch *et al.*, 1994; Boivin and Beliveau, 1995; Michaely *et al.*, 1999; Takai *et al.*, 2001). Due to presence of weak polybasic regions of Cdc42, this protein remains predominantly in the endomembrane; however, some is expressed in plasma membrane (Michaelson *et al.*, 2001). Cdc42 is also found in both cytosol and membrane of Golgi (Bohoch *et al.*, 1994; Michaelson *et al.*, 2001) and the ER and nuclear envelope (Michaelson *et al.*, 2001). Rac1 has been found to localize to the membrane of large pinocytotic vesicles which are induced by the expression of activated Rac1 (Robertson *et al.*, 1995). Fluorescently myc-tagged Rac1V12 shows expression on plasma membrane as well as internal membranes (Jou *et al.*, 2000). In addition, Rac1 translocates from cytosol to plasma membrane upon stimulation with agonists such as PDGF (Fleming *et al.*, 1996).

2.8. Rac1 and Cdc42 Agonists

Growth factors such as PDGF, EGF and insulin, activate Rac1 (Mertens *et al.*, 2003). PKC agonists such as PMA also activate Rac1 through PKC-dependent phosphorylation and activation of Tiam1 (Mertens *et al.*, 2003). The effects of several well known agonists such as thrombin, angiotensin II and collagen on Rac1 activation have been studied (Soulet *et al.*, 2001; Pelletier *et al.*, 2003). For example, thrombin and collagen activate Rac1 in platelets whereas in smooth muscle cells (SMC), Rac1 is activated by thrombin and angiotensin II (Pelletier *et al.*, 2003). In addition, growth factors such as epidermal growth factor and insulin as well as colony-stimulating factor-1 (CSF-1), steel locus factor (SLF), granulocyte-macrophage colony-stimulating factor (GM-CSF), fMetLeuPhe (fMLP), IL3 and IL-5 were also shown to induce activation of Rac1 (Grill *et al.*, 2002). Other known agonists of Rac1 include lysophosphatidic acid, sphingosine 1-phosphate and nitric oxide (Barber *et al.*, 2004; Hou and Ye, 2004). Cdc42 is activated by collagen and thrombin in human blood platelets (Azim *et al.*, 2000; Soulet *et al.*, 2001). EGF also stimulates activation of Cdc42 and Rac1 in Cos1 and A431 cells (Kurokawa *et al.*, 2004).

2.9. Pathways involved in Rac1 and Cdc42 Activation

Initial studies using Swiss 3T3 fibroblasts showed that Rac1 promotes polymerization of actin at the cell membrane producing lamellipodia and membrane ruffles (Ridley *et al.*, 1992). Similar observations have been noted in a wide variety of cell types including platelets using constitutively active and dominant negative Rac1 mutants (Hartwig *et al.*, 1995; Ridley, 2001; Burridge and Wennerberg, 2004).

Experiments carried out on mice deficient in Rac1 also produced similar observations (Gu *et al.*, 2003; Walmsley *et al.*, 2003). *In vivo* and *in vitro* studies using platelets show that Rac1 is required for platelet lamellipodia formation and for aggregate stability leading to thrombus formation (McCarty *et al.*, 2005). The regulation of Rac1 activation and lamellipodia formation in platelets enables them to withstand high shear forces. Moreover, Rac1 is also implicated in regulating pathways including oxidative bursts in neutrophils and in cellular growth pathways causing hypertrophy (Gregg *et al.*, 2003).

Rac1 activity is stimulated by G protein-coupled receptors (GPCRs) leading to activation of Janus kinase (Jaks) and signal transducers and activators of transcription (STATs) (Bokoch, 1995; Burstein *et al.*, 1998). Later, Rac1 activity was shown to be essential for the activation of the Jak/STAT signaling pathway (Pelletier *et al.*, 2003). The Jak/STAT signaling pathway is involved in development, differentiation, cell proliferation and survival, immune response, and oncogenesis (Pelletier *et al.*, 2003). Furthermore, reactive oxygen species (ROS) lead to activating phosphorylation of Jaks in a mechanism that has yet to be elucidated. A major source of ROS is the membrane bound NADPH oxidase complex that catalyzes the production of superoxide (Pelletier *et al.*, 2003). The activity of NADPH oxidase and ROS production is regulated by Rac1 and is involved in the activation of the Jak/STAT pathway (Pelletier *et al.*, 2003). The role of Cdc42 in the Jak/STAT signaling pathway is yet to be studied. Rac1 and Cdc42 activate c-Jun amino-terminal kinase (JNK) hence regulating the transcriptional activity of c-Jun (Coso *et al.*, 1995; Minden *et al.*, 1995). The inactivation of Cdc42 using gene-targeted mutations in mouse embryonic stem (ES) and mouse germ line cells show that Cdc42-

deficient ES cells exhibit normal proliferation and phosphorylation of mitogen- and stress-activated protein kinases such as Erk, JNK, and p38. On the other hand, Cdc42 deficiency causes very early embryonic lethality in mice and leads to irregular actin cytoskeletal organization in ES cells (Chen *et al.*, 2000). In fibroblastoid cell lines, the activation of Cdc42 is associated with activation of JNK, Erk, and p38 (Frost *et al.*, 1997; Bishop and Hall, 2000). In another study, the inhibition of Rac1 and Cdc42 in primary fibroblasts upregulates Akt and Erk activity via pathways involving PI(3)K, Akt, Raf and MEK but not Ras (Zugasti *et al.*, 2001). In human blood platelets, Rac1 is activated via GPCRs and phospholipase C (PLC) activation and calcium were essential for this activation (Soulet *et al.*, 2001). In contrast, Cdc42 activation in platelets is independent of PLC activation (Soulet *et al.*, 2001).

PI 3-kinase is involved in growth factor-induced membrane ruffling through studies conducted using platelet-derived growth factor (PDGF) receptor mutants and the PI 3-kinase inhibitor wortmannin (Wennstrom *et al.*, 1994; Kotani *et al.*, 1994). Rac1 and Cdc42 activate PI 4,5-kinase and PI 3-kinase (Tolias *et al.*, 1995). Rohatigi and colleagues have shown that PIP_2 promote actin polymerization in pathways downstream of Rac1 that are independent of Cdc42 (Rohatgi *et al.*, 2001).

CHAPTER 3

CALMODULIN, CALCIUM AND G-PROTEINS

3.1. Calmodulin and Calcium

Ca^{2+} ions are important secondary messengers in a variety of cellular signaling pathways (Berridge *et al.*, 2000). For example, an increase in intracellular Ca^{2+} concentration is essential for muscle contraction. The intracellular calcium ion concentration is 10^{-7}M compared to extracellular and intraorganelle concentrations that may go as high as 10^{-3}M (Berridge *et al.*, 2000; Yamniuk and Vogel, 2004). Therefore, the cell is prepared for a rapid intracellular Ca^{2+} influx at any given time. The majority of the effects of Ca^{2+} in the cell are mediated through proteins that bind calcium. Calmodulin (CaM), a major calcium (Ca^{2+}) binding protein, is a highly conserved 16.7 kDa ubiquitous eukaryotic protein that binds to over 100 different targets in the cell, including serine/threonine kinases, in response to changes in intracellular Ca^{2+} levels (Yamniuk and Vogel, 2004). Calmodulin is highly acidic (pI approx 4.6) and has a unique structure consisting of two globular domains possessing two helix-loop-helix Ca^{2+}-binding motifs referred to as EF-hands, connected by a central linker (**Figure 4**) (Yamniuk and Vogel, 2004; Rashid *et al.*, 2004). The α-helical structure within these two domains opens upon calcium binding allowing the exposure of the hydrophobic binding sites, which then allows other target proteins to bind to calmodulin (Rashid *et al.*, 2004). As a result, the proteins bind a total of four Ca^{2+} ions. The carboxyl-terminal lobe binds Ca^{2+} with high affinity (K_d 10^{-7}M) whereas the amino-terminal binds with lower affinity (K_d 10^{-6}M) (Agell *et al.*, 2002). Calcium-free calmodulin (apo-CaM) is a tertiary

structure of CaM that is formed in the absence of Ca^{2+}. The distinct structural differences in calcium free-CaM (apo-CaM) compared to the calcium saturated form occur in the central linker region, which separates EF-hands I and II from EF-hands III and IV (**Figure 4**).

Figure 4: Schematic diagram of the protein sequence of human CaM. The four EF hand domains are separated by a long central linker α-helix

3.2. Calmodulin-Binding Motifs

Table 3

Summary of CaM-binding motifs[3]

Motifs	Common Characteristics
1-10 *	- Two bulky hydrophobic residues spaced by 8 amino acid residues [FILVW]xxxxxxxx[FILVW]. - Some have additional anchoring residues in the middle (1-5-10 or 1-4-10)
1-14 *	- Two bulky hydrophobic residues spaced by 12 amino acid residues - Additional anchoring residues are often also found in these motifs (1-5-8-14 or 1-8-14)
1-16 *	- Bulky hydrophobic residues separated by 1-14 amino acids - Atypical binding orientation
IQ	- [IVL]Qxxx[RK]xxxxx[RK] where X is any amino acid - Binds CaM in absence of Ca^{2+} - Have amphipathic character with a hydrophobic and basic face - Interacts with both apo-CaM and Ca^{2+}-CaM

[3] Bind CaM in presence of Ca^{2+} (Sidhu and Bhullar, 2005)

There are specific calcium-dependent binding sequences that classify target proteins as calmodulin-binding proteins. These typical CaM-binding sequences range between 16-30 amino acids and tend to form amphipathic α-helices with a hydrophobic and basic face (Sidhu and Bhullar, 2005). The table above summarizes the four groups that identify CaM-binding motifs. In addition, the CaM-binding motif that binds CaM largely in the absence of calcium is called the IQ motif (**Table 3**). This motif is recognized by the sequence ([IVL]Qxxx[RK]xxxxx[RK] where x is any amino acid (Bahler and Rhoads, 2002; Sidhu and Bhullar, 2005). IQ motifs also have amphipathic character with a hydrophobic and basic face. Therefore, some proteins containing these IQ motifs can bind to both Ca^{2+}-CaM and apo-CaM (Bahler and Rhoads, 2002; Sidhu and Bhullar, 2005).

3.3. Structural Conformational Rearrangements

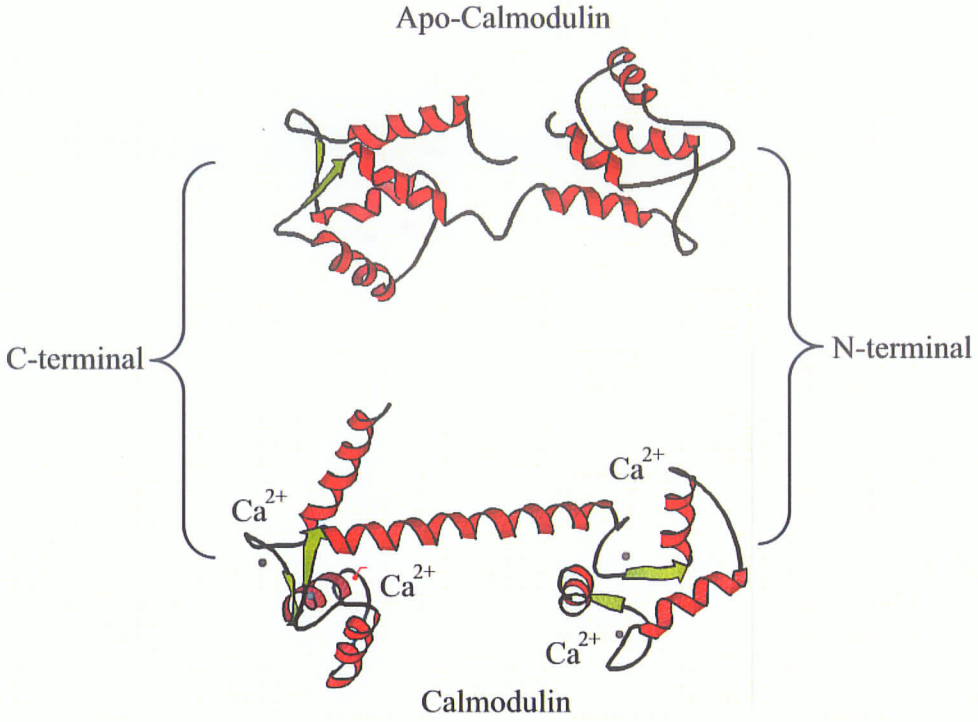

Figure 5: Structures of calcium-free calmodulin (Apo-calmodulin; PDB id: 1CFD) and calcium-bound calmodulin (PDB id: 1CLL). Calcium-bound calmodulin contains four calcium ions: two per EF domain pair. These structures were created using KiNG (Kinetic Image, Next Generation) software (http://kinemage.biochem.duke.edu).

The binding of each Ca^{2+} ion stabilizes the partially unstructured loop region of its corresponding EF-hand motif and causes an overall stabilization of the internal mobility of all of the helices in the protein. This causes a major reorientation of the partner helices within each EF-hand as they move from nearly antiparallel orientation in apo-CaM to almost perpendicular orientations in Ca^{2+}-CaM (Yamniuk and Vogel, 2004). During this helical rearrangement, the binding patches on the surface of each domain start to open. Each domain consists largely of hydrophobic amino acid side chains which have

been buried within the interior domain in apo-CaM. Therefore, each domain is often described as moving from a "closed" conformation in apo-CaM, to an "open" conformation in Ca^{2+}-CaM (**Figure 5**). The hydrophobic binding patches that open in both domains of CaM are rich in Met residues and are completely buried (i.e. solvent inaccessible) in apo-CaM. These residues become highly solvent accessible when calcium is bound.

3.4. Regulation of Calmodulin-Binding Proteins via Calmodulin

The binding of CaM to CaM-binding proteins (CaMBPs) modulates the function of these proteins and as a result affects many aspects of cell function. For example, CaM regulates cyclic nucleotide levels in the cell through the modulation of CaM-dependent guanylate cyclase, CaM-dependent adenylate cyclase, and CaM-dependent phosphodiesterase. Furthermore, CaM regulates phosphorylation of cellular proteins through the activation of a variety of kinases. These kinases include CaM kinase (CaMKs) I, II, III, IV, and V, phosphorylase kinase, elongation factor kinase, inositol (1,4,5)-tris-phosphate 3-kinase (IP_3-3K), and myosin light chain kinase (MLCK) (Agell *et al.*, 2002). CaM also mediates the dephosphorylation of cellular proteins through the activation of the CaM-dependent protein phosphatase 2B (calcineurin) which is activated by displacement of an autoinhibitor domain (AID) (Sidhu and Bhullar, 2005). The AID contains a CaM-binding site and upon binding of CaM to the target protein, the autoinhibitory domain undergoes conformational change allowing full enzyme activity (Sidhu and Bhullar, 2005). In addition, CaM also affects cytoskeleton rearrangements by

binding to microtubule associated proteins such as MAP2, Tau, α-spectrin, MLCK, and caldesmon (Agell *et al.*, 2002).

Transcriptions factors whose phosphorylation is regulated by CaM include CREB or NAFT4. Smad1 and Smad2 are CaMBPs that are involved in the regulation of TGFβ (Agell *et al.*, 2002). Another mechanism of CaM action is through the induction of channel dimers. Small conductance Ca^{2+}-CaM-activated potassium (SK) channels are Ca^{2+} gated membrane channels that bind constitutively to CaM. Increase in intracellular calcium induces a conformational change in the CaM-target complexes leading to the formation of CaM-SK channel dimers. Lastly, calcium and CaM have been shown to modulate activation of the Ras/Raf/MEK/ERK pathway (Agell *et al.*, 2002).

3.5. Role of Calmodulin in G-protein Regulation

Calmodulin binds to RalA and RalB and is necessary for thrombin-mediated activation in platelets (Clough *et al.*, 2002). CaM also binds to RalA in a Ca^{2+}-dependent fashion in erythrocytes (Wang *et al.*, 1997). In addition, CaM enhances loading of Ral with GTP (Wang *et al.*, 1997; Wang and Roufogalis, 1999) and is reported to have a role in the regulation of Ral (Park *et al.*, 1999). CaM has been suggested to be associated with RalA dissociation from synaptic vesicle membranes in a Ca^{2+}-dependent fashion that is independent of nucleotide status (Park *et al.*, 1999). A similar regulatory mechanism was observed for K-RasB (Sidhu *et al.*, 2003) and the Ras-related Rab3 small GTPase (Park *et al.*, 1999). Calmodulin also binds K-RasB and is inhibitory for its activation (Villalonga *et al.*, 2002).

3.6. Hypothesis

It has been shown that epidermal growth factor induced Ca^{2+} influx is mediated by Rac proteins (Peppelenbosch *et al.*, 1996) and that calmodulin antagonists block the activation of small GTPases Rac1 and Ras in neutrophils (Lian *et al.*, 2001). However, no information exists as to how calmodulin regulates Rac1 GTPase activity. In addition, it is not known if other members of the Rho family of small GTPases (e.g. Cdc42) are also regulated by CaM. The fact that CaM has been shown to interact with and regulate the activation of several small GTPases has led us to test the hypothesis that CaM interacts with and regulates the activity of Rac1 and Cdc42.

CHAPTER 4

RESEARCH OBJECTIVES AND EXPERIMENTAL APPROACH

Current evidence in the literature suggests that calmodulin antagonists block the activation of small GTPases Rac1 and Ras in neutrophils (Lian *et al.*, 2001). However, no information exists as to how calmodulin regulates Rac GTPase activity. In addition, it is not known if other members of the Rho family of small GTPases (e.g. Cdc42) are also regulated by calmodulin. The fact that CaM has been shown to interact with and regulate the activation of several small GTPases (Clough *et al.*, 2002; Villalonga *et al.*, 2002; Sidhu and Bhullar, 2001; Wang *et al.*, 1997) has led us to our current hypothesis.

The main hypothesis of the present work is that calmodulin interacts with Rac1 and Cdc42 and regulates their activity.

Specific Objectives

I. Investigate binding of calmodulin to Rac1 and Cdc42.

   a. To determine whether the binding of calmodulin to Rac1 and Cdc42 is direct by conducting *in vitro* binding experiments using purified recombinant proteins.

   b. To investigate whether calmodulin binding to Rac1 and Cdc42 is influenced by calcium concentrations within physiological range.

c. To confirm this novel interaction in cells using co-immunoprecipitation studies.

II. To identify the CaM binding region in Rac1.

a. To determine the CaM binding region in Rac1 and Cdc42 using sequence analysis.

b. To illustrate whether the proposed synthetic Rac1-CaM binding site can be used to inhibit binding of endogenous Rac1 to calmodulin.

III. To establish whether calmodulin binding regulates Rac1 and Cdc42 activation in platelet.

a. To establish the optimal time-point of Rac1 and Cdc42 activation in cells.

b. To illustrate effects of calmodulin inhibition on Rac1 and Cdc42 activation.

c. To examine the distribution of Rac1, Cdc42 and calmodulin in platelet and determine effects of calmodulin inhibition on the distribution and localization of Rac1, Cdc42 and calmodulin using fluorescent immunocytochemistry in conjunction with confocal microscopy.

CHAPTER 5

MATERIALS AND METHODS

5.1. MATERIALS

Sepharose 4B coupled Calmodulin (Cat# 17-0529-01), cyanogen bromide (CNBr)-activated Sepharose beads (Cat# 17-0981-01), ECL plus Western Blotting Detection System (Cat# RPN2132), Hybond PVDF transfer membrane (Cat# RPN303F) and Hyperfilm™ ECL (Cat# RPN3114K) were purchased from Amersham Biosciences. Bovine brain Calmodulin (Cat# 208694) and W7•HCl (Cat# KP31380) were purchased from EMD Biosciences Calbiochem. Thrombin (Cat# T-6884), glutathione agarose (Cat# G4510), GTPγS (Cat# G-8634), GDPβS (Cat# G-7637) and actin rabbit polyclonal antibody (Cat# A-5060) were purchased from Sigma. Dulbecco's Modified Eagle Medium (DMEM, Cat# 11995-065), penicillin-streptomycin (Cat# 15140-122), sodium bicarbonate solution (Cat# 25080-094), trypsin-EDTA (Cat# 25300-062), fetal bovine serum (FBS, Cat# 16140071) and ProLong® Gold antifade reagent (Cat# P36930) were obtained from Invitrogen. Triton X-100 (Cat# 161-0407), prestained SDS-PAGE low range molecular weight marker (Cat# 161-0305), BSA standard (Cat# 500-0007) and BioRad protein assay dye reagent (Cat# 500-0006) were purchased from BioRad Laboratories. Monoclonal Rac1 (Cat# 610651), monoclonal IQGAP1 (Cat# 610611) and monoclonal Cdc42 (Cat# C70820) antibodies were obtained from BD Transduction Laboratories. Calmodulin (Cat# 05-173) and IQGAP2 (Cat# 05-505) antibodies were obtained from Upstate Biotechnologies. Calmodulin rabbit polyclonal antibody (Cat# sc-5537), monoclonal Cdc42 immunoprecipitation-positive antibody (Cat# sc-8401), Texas

Red conjugated goat-anti rabbit (Cat# sc-2780), goat-anti mouse FITC conjugate (Cat# sc-2010) and protein A/G PLUS agarose beads (Cat# sc-2003) were purchased from Santa Cruz Biotechnology. Control mouse IgG_1 (Cat# NC-748-P1) was purchased from MediCorp Inc. The predicted Rac1 CaM-binding peptide (NH_2-AVKYLECSALTQRG-COOH) was custom synthesized by Sigma Genosys. All other reagents used were from Sigma except where indicated. GST-Rac1 and GST-Cdc42, and GST-Pak1 bacterial expression plasmids were kindly provided by Dr. R. Weinberg (Whitehead Institute for Biomedical Research, MIT, Cambridge, MA) and Dr. M. Hoshino (National Center of Neurology and Psychiatry, Tokyo, Japan) respectively.

5.2. METHODS

5.2.1. Isolation of GST Fusion Proteins

GST and the recombinant GST fusion proteins, GST-Rac1, GST-Cdc42 and GST-Pak1, were expressed in DH5-α *Escherichia coli* cells using 0.5 mM IPTG for induction as described previously (Jilkina and Bhullar, 1996). Briefly, 0.5 mM IPTG was added to an overnight grown bacterial culture and expression was allowed for 2 hrs at room temperature. The cells were collected by centrifugation at 6,000 x g for 20 min. NETT buffer, consisting of 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 1% Triton X-100, 1 mM PMSF plus 10 mg/ml lysozyme, was added to resuspend the bacterial cell pellet. To disrupt the cells, the mixture was subjected to ultrasonic cell disruption with 2-3 pluses of 30-45 sec (3 times x 20 sec at 10% of the output at control setting N#2) using an ultrasound disruptor, Model W-375. The bacterial cell lysate was centrifuged at 12,000 x g for 30 min to remove insoluble materials. Glycerol (20%) was added to the

supernatant and aliquots were frozen at -80°C until required for an experiment. The GST fusion proteins were purified after incubating the above bacterial supernatant with 250-750 µl of glutathione agarose beads (prepared in 1:1 volume of NT buffer {20 mM Tris-HCl, pH 8.0, 100 mM NaCl}) for 30 min at 4°C. The beads were washed three times with NETT (lacking lysozyme) and two times with NT buffer to remove unbound proteins. The purity of the final protein preparations was assessed using SDS-PAGE.

5.2.2. Cell Culture

HeLa cells were maintained in 100 mm plates or 75 cm^2 cell culture flasks using DMEM supplemented with 10% FBS (v/v), 1.5 g/L sodium bicarbonate ($NaHCO_3$) and 100 U penicillin/streptomycin at 37°C in 5% CO_2 and 95% air (v/v).

5.2.3. Preparation of Human Platelets

Blood was collected into acid citrate dextrose anti-coagulant (3.8 mM citric acid, 7.5 mM trisodium citrate, 125 mM dextrose; 3.6 mL anticoagulant/20 mL whole blood) by venipuncture of human volunteers who had denied taking medication known to interfere with platelet function within the previous 2 weeks. Platelet-rich plasma was obtained by centrifugation at 600 x g for 20 min at room temperature. The supernatant containing the purified platelets was centrifuged at 1,000 x g for 15 min at room temperature, and the resultant platelet pellet was resuspended in HEPES/Tyrode buffer (10 mM HEPES, pH 7.4, 137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH_2PO_4, 1.7 mM $MgCl_2$ and 5 mM glucose). The platelets were allowed to equilibrate for 30 min at 37°C prior to being used in an experiment. Platelet studies were approved by the Human

Research Ethics Board of the University of Manitoba and informed consent was obtained from all the volunteers.

5.2.4. GST-Pak1 pull out of Rac1-GTP, Cdc42-GTP and IQGAP1 from HeLa cell lysate

To confirm that GST-Pak1 interacts only with the GTP-bound form of Rac1 and Cdc42, *in vitro* loading of Rac1 and Cdc42 in cell lysates with GTPγS or GDPβS was performed (Benard *et al.*, 1999). HeLa cells at 80% confluence were washed twice with PBS and lysed in Buffer S (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1% NP-40, 2.5 mM $MgCl_2$, 10 mM NaF, 10% glycerol, 1 mM sodium orthovanadate $\{Na_3VO_4\}$, and a protease inhibitor cocktail consisting of 200 mM benzamidine, 2 μg/μl aprotinin, 83.4 mM AEBSF, 5 μg/μl leupeptin, and 0.025 mM pepstatin A), then rocked for 30 min at 4°C. Total cell lysate were centrifuged at 14,000 x g for 10 min at 4°C. To supernatant samples (~1 mg), 10 mM EGTA (final concentration) and guanine nucleotides (100 μM GTPγS or 100 μM GDPβS) were added and the mixture was incubated at 30°C for 15 min. At the end of the incubation, magnesium chloride ($MgCl_2$) was added to a final concentration of 60 mM to lock in nucleotides. The mixture was incubated with 100 μl GST-Pak1 bound to glutathione-agarose beads for 2 hrs while rocking at 4°C. Unbound proteins were removed by washing three times with binding buffer. 30 μl of Laemmli's sample buffer (Laemmli, 1970) was added to washed beads and heated at 100°C for 5 min. Eluted proteins were subjected to 12% SDS-PAGE, electrophoretically transferred to PVDF membranes, and Western blotting was performed with anti-Rac1 (1 μg/ml), anti-Cdc42 (1 μg/ml) or anti-IQGAP1 (1 μg/ml) antibodies and horse-radish peroxidase

(HRP)-conjugated secondary goat anti-mouse antibody (1:5000 dilution). The antigen-antibody complex was detected using enhanced chemiluminescence (ECL) reagents.

5.2.5. GST-Pak1 pull out of Rac1-GTP, Cdc42-GTP and IQGAP2 from Human Platelet lysate

The *in vitro* loading of Rac1 and Cdc42 with GTPγS or GDPβS was also performed using platelets to confirm that GST-Pak1 interacts only with the Rac1-GTP and Cdc42-GTP forms. Platelets were isolated as mentioned above and were lysed using RIPA lysis buffer consisting of 50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.5% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 10 mM $MgCl_2$, 2.5 mM EGTA and a protease inhibitor cocktail consisting of 200 mM benzamidine, 2 μg/μl aprotinin, 83.4 mM AEBSF, 5 μg/μl leupeptin, and 0.025 mM pepstatin A. The platelet lysate (~2 mg) was centrifuged at 14,000 x g for 10 min at 4°C. The loading of Rac1 and Cdc42 with GTPγS or GDPβS was carried out as described for HeLa cells. The proteins bound to GST-Pak1 beads were eluted using Laemmli's sample buffer and subjected to 12% SDS-PAGE, electrophoretically transferred to PVDF membranes, and Western blotting was performed with anti-Rac1 (1 μg/ml), anti-Cdc42 (1 μg/ml) or anti-IQGAP2 (1 μg/ml) antibodies. After incubation with horse-radish peroxidase (HRP)-conjugated secondary goat anti-mouse antibody (1:5000 dilution), the antigen-antibody complex was visualized using ECL reagents.

5.2.6. CaM Sepharose Pull-down of Rac1 and Cdc42 from HeLa cell and Platelet lysate

HeLa cells were washed twice with phosphate-buffered saline and lysed in CaM binding buffer containing 20 mM HEPES, pH 7.4, 200 mM KCl, 1 mM $MgCl_2$, 0.55% Triton X-100 and a protease inhibitor cocktail consisting of 200 mM benzamidine, 2 $\mu g/\mu l$ aprotinin, 83.4 mM AEBSF, 5 $\mu g/\mu l$ leupeptin, and 0.025 mM pepstatin A for 30 min at 4°C. Platelets were isolated as previously described and lysed in CaM binding buffer containing protease inhibitors cocktail. The total cell lysates were centrifuged at 14,000 x g for 10 min at 4°C. After centrifugation, supernatant samples from HeLa (~1 mg) and platelets (~2 mg) were incubated with 100 μl of CaM Sepharose 4B beads (3:1 ratio of settled gel to buffer) that were previously equilibrated in CaM binding buffer. Treatment conditions included: buffer, buffer plus 5 mM or 10 mM EGTA, buffer containing 5 mM Ca^{2+}, buffer containing 5 mM or 10 mM EGTA plus variable Ca^{2+} concentrations (1 mM Ca^{2+}, 3 mM Ca^{2+} or 5 mM Ca^{2+}). Blank Sepharose 4B beads (100 μl) were used as control. The reaction mixture was incubated for 2 hrs at 4°C. Unbound proteins were removed by washing three times in CaM binding buffer. Laemmli's sample buffer was added to washed beads and heated at 100°C for 5 min. Eluted proteins were subjected to 12% SDS-PAGE, electrophoretically transferred to PVDF membranes, and Western blotting was performed with anti-Rac1 or anti-Cdc42 (1 $\mu g/ml$) antibodies and horse-radish peroxidase (HRP)-conjugated secondary goat anti-mouse antibody (1:5000 dilution). The antigen-antibody complex was detected using enhanced chemiluminescence (ECL) reagents.

5.2.7. GST-Rac1 and GST-Cdc42 Interaction with Pure Calmodulin

Purified GST or GST-Rac1 or GST-Cdc42 bound to GSH-agarose beads were washed with MOPS buffer consisting of 30 mM MOPS, pH 7.2, 1% NP-40 and 100 mM KCl. In addition to buffer alone, 5 mM or 10 mM EGTA, 5 mM Ca^{2+}, or 5 mM or 10 mM EGTA plus different Ca^{2+} concentrations (1 mM Ca^{2+}, 3 mM Ca^{2+}, 5 mM Ca^{2+}) plus 20 μg of pure CaM were added to tubes containing GST or GST-Rac1 or GST-Cdc42 (100 µl) and allowed to shake for 2 hrs at 4°C. After incubation, the beads were washed three times with MOPS buffer. Laemmli's sample buffer was added to washed beads and heated at 100°C for 5 min. Eluted proteins were subjected to 12% SDS-PAGE, electrophoretically transferred to PVDF membranes, and Western blotting was performed with anti calmodulin (1 μg/ml) antibody and horse-radish peroxidase (HRP)-conjugated secondary goat anti-mouse antibody (1:5000 dilution). The antigen-antibody complex was visualized using ECL.

5.2.8. Rac1 and Cdc42 Activation Assay

Platelets were purified as described previously and challenged with thrombin (1 U/ml) for different periods to determine optimal time-points for Rac1 and Cdc42 activation. Having determined the optimal time point for Rac1 and Cdc42 activation, platelets were stimulated for the appropriate time using the following conditions: no addition, W7 (150 µM), thrombin (1 U/ml), W7 (150 µM) plus thrombin (1 U/ml). Platelets were incubated for 5 min with W7 prior to addition of thrombin. Platelets were lysed using RIPA buffer (50 mM Tris, pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 2.5 mM EGTA, and a protease

inhibitor cocktail consisting of 200 mM benzamidine, 2 μg/μl aprotinin, 83.4 mM AEBSF, 5 μg/μl leupeptin, and 0.025 mM pepstatin A and 1 mM PMSF) and shaken for 30 min at 4°C. The cell lysate was centrifuged at 14,000 x g at 4°C. After centrifugation, the supernatant (~2 mg) was transferred into a separate tube and stored immediately on ice. The amount of activated Rac1 or Cdc42 in platelet lysate was determined using GST-Pak1. Thus, the centrifuged platelet lysate was incubated with GST-Pak1 coupled to GSH-agarose beads (100 μl) at 4°C for 2 hrs. After incubation, the beads were washed three times with cold Rac1-Cdc42 washing buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, 1% Triton X-100, 5 mM EGTA and a protease inhibitor cocktail consisting of 200 mM benzamidine, 2 μg/μl aprotinin, 83.4 mM AEBSF, 5 μg/μl leupeptin, and 0.025 mM pepstatin A and 1 mM PMSF). The final bead pellet was suspended in 30 μl of Laemmli's sample buffer and heated at 100°C for 5 min. Eluted proteins were separated using 12% SDS-PAGE, electrophoretically transferred to PVDF membranes, and Western blotting was performed with mouse anti-Rac1 or anti-Cdc42 monoclonal antibodies (1 μg/ml) and horse-radish peroxidase (HRP)-conjugated secondary goat anti-mouse antibody (1:5000 dilution). The antigen-antibody complex was detected using ECL.

5.2.9. Immunoprecipitation of Rac1, Cdc42 and CaM from HeLa cells and Platelets

HeLa cells were lysed with Triton lysis buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 10 mM EGTA, 1mM Na_2VO_4) containing a protease inhibitor cocktail tablet (Roche Diagnostics). The mixtures were put on ice immediately and sonicated. The lysates were clarified by centrifugation (14,000 x g for 10 min), and the supernatants (~1 mg) were

pre-cleared by adding 30 µl of protein A/G Plus-agarose beads for 2 hrs. After centrifugation, the supernatant was incubated with 4 µg/ml of control mouse IgG_1 or 4.0 µg/ml of mouse anti-CaM monoclonal or 4.0 µg/ml of mouse anti-Cdc42 monoclonal or 2 µg/ml of control mouse IgG_1 or 2.0/ml µg of mouse anti-Rac1 monoclonal antibodies, with gentle rocking at 4 °C overnight. Protein A/G Plus-agarose beads (30 µl) were added to the mixture and rocked at 4 °C for 1 hr. Similar steps were performed using platelet cell lysate with the exception of incubating pre-cleared supernatant (~2 mg) with 4 µg/ml of control mouse IgG_1 prior to incubation with 4.0 µg/ml of mouse anti-Rac1 monoclonal or 4.0 µg/ml of mouse anti-Cdc42 monoclonal antibodies for 3 hrs. The antigen-antibody complex was precipitated for 1 hr by the addition of 30 µl A/G Plus-agarose beads.

The beads were collected by centrifugation (16,000 × *g* for 25 sec.) and washed four times in 1× Triton lysis buffer minus detergent (50 mM Tris, pH 7.2, 10 mM EGTA, 1mM Na_2VO_4). The beads were resuspended in 20 µl of Laemmli's sample buffer, heated for 5 min at 100°C, centrifuged at 14,000 x g for 1 min and the proteins were separated using 12% SDS-PAGE. Polypeptides were transferred to PVDF membranes and probed with mouse anti- Rac1 (1 µg/ml), anti-Cdc42 (1 µg/ml), or anti-CaM (1 µg/ml) monoclonal antibodies and horse-radish peroxidase (HRP)-conjugated secondary goat anti-mouse antibody (1:5000 dilution). The antigen-antibody complex was detected using ECL.

5.2.10. Coupling of Potential CaM-binding Peptide to Sepharose CNBr Beads

The potential CaM binding peptide from Rac1 was coupled to CNBr-activated Sepharose beads according to the manufacturer's instructions. Briefly, peptide (2.5 mg/ml) was dissolved in 1x PBS and mixed 0.5:1 v/v with 0.1-0.3 g of CNBr-activated Sepharose beads that had been washed in 1 mM HCl (15 times bead volume) and a final wash in PBS, and mixture gently rocked overnight at 4°C. The beads were pelleted, the supernatant decanted, 1.0 ml of 10 mM Tris-HCl, pH 7.5, added and the mixture rocked gently at 4°C for 3 hrs to block empty sites on the beads. The beads were pelleted and washed in 1.0 ml of 10 mM Tris-HCl, pH 8-9, followed by washing in 1.0 ml of 100 mM acetate buffer, pH 4.0. This washing process was repeated 5 times, after which the beads were recovered and stored in 20% ethanol at 4°C until needed for experiment.

5.2.11. In Vitro Binding Assays

CaM-binding peptide coupled to CNBr-activated Sepharose beads was incubated with 20 μg of pure calmodulin in Buffer B (50 mM Tris-HCl, pH7.5, 10% glycerol, 200 mM NaCl, 2 mM $MgCl_2$) and rocked gently for 2 hrs or 24 hrs at 4°C. In an alternative approach, HeLa (~1 mg) and platelet cell (~2 mg) lysates lysed in Buffer B were incubated with peptide coupled to beads while rocking gently for 2 hrs or 24 hrs at 4°C. The beads were collected by centrifugation and unbound proteins removed by washing three times in Buffer B. Laemmli's sample buffer (30 μl) was added to beads and heated at 100°C for 5 min. Eluted proteins were subjected to 12% SDS-PAGE, electrophoretically transferred to PVDF membranes, and Western blotting was performed with anti-CaM (1 μg/ml) antibodies and horse-radish peroxidase (HRP)-conjugated

secondary goat anti-mouse antibody (1:5000 dilution) and antigen-antibody complex was visualized using ECL.

5.2.12. In Vitro Competition Assay

To assess if the peptide inhibits Rac1 binding to CaM, CaM Sepharose beads (100 µl) were incubated with different concentrations of free peptide (0 µM, 250 µM, 500 µM, 750 µM, 1000 µM) and rocked gently for 2 hrs in CaM binding buffer (20 mM HEPES, pH 7.4, 200 mM KCl, 1 mM $MgCl_2$, 0.55% Triton X-100 and protease inhibitors cocktail consisting of 200 mM Benzamidine, 2 µg/µl Aprotinin, 83.4 mM AEBSF, 5 µg/µl leupeptin, and 0.025 mM Pepstatin A). The supernatant was decanted and beads were washed once with CaM binding buffer. HeLa cells and platelets were lysed in CaM binding buffer containing protease inhibitors cocktail. The cell lysates were centrifuged at 14,000 x g for 10 min. The supernatant from HeLa (~1 mg) and platelets (~2 mg) was added to washed beads and rocked gently for 2 hrs at 4°C. Blank Sepharose 4B beads (100 µl) were used as control. Unbound proteins were removed by washing three times in CaM binding buffer. Laemmli's sample buffer (30 µl) was added to washed beads and heated at 100°C for 5 min. Eluted proteins were subjected to 12% SDS-PAGE, electrophoretically transferred to PVDF membranes, and Western blotting was performed with anti-CaM (1 µg/ml) antibody and horse-radish peroxidase (HRP)-conjugated secondary goat anti-mouse antibody (1:5000 dilution). The antigen-antibody complex was detected using enhanced chemiluminescence (ECL) reagents.

5.2.13. Fluorescent Immunocytochemistry

To localize Rac1, Cdc42 and CaM *in vivo*, we used a fluorescent immunocytochemistry technique in conjunction with confocal microscopy. Platelets were prepared according to the previously mentioned protocol. Platelets were left untreated or treated with thrombin alone (1 U/ml), the calmodulin inhibitor W7 alone (150 μM) for 5 min, with W7 (5 min) plus thrombin. The reaction was stopped by the addition of cold 4% paraformaldehyde (PFA) in PBS + 0.1% Triton X-100. A few drops of treated and control platelets were placed on Fisherbrand Superfrost Plus microscope slides (Fisher Scientific) and allowed to fix for 30 min. Fixed cells were washed three times with PBS at room temperature. Prior to treatment with antibodies, fixed platelets were blocked by incubating in cold PBS + 1% BSA for 30 min. The fixed platelets were incubated with primary monoclonal mouse anti-Rac1 (1:100 dilution), polyclonal rabbit anti-CaM (1:50-100 dilution) or monoclonal mouse anti-Cdc42 (1:50 dilution) antibodies for 1 hr in a humidified chamber. After incubation, platelets were washed three times with PBS + 1% BSA prior to incubation for 1 hr with secondary FITC (Green Fluorescent Dye) (1:200 dilution) for Rac1 or Cdc42 and Texas Red (Red Fluorescent Dye) (1:200 dilution) for CaM in humidified chamber. Prior to mounting coverslips on cells, ProLong Antifade Gold mounting medium was applied. Slides were sealed with clear nail polish and an Olympus 1X70 inverted confocal microscope with Normarski DIC optics was used to evaluate staining. Images were analyzed using FluoView software.

5.2.14. Protein Determination

Total protein concentrations in cell lysates were determined by incubating lysate samples with BioRad dye reagent (BioRad laboratories). The absorbance at 595 (A_{595}) of the stained mixture was measured and compared to a standard curve of known protein BSA concentrations.

5.2.15. Statistical Analysis

Where required the autoradiograph was scanned and bands quantified using Bio-Rad "Quantity One" program. The output was normalized and descriptive statistical analysis and t-tests were performed.

CHAPTER 6

RESULTS

Calmodulin has been shown to interact with, and regulate the activity of several small GTPases (Wang *et al.*, 1997; Sidhu and Bhullar, 2001; Villalonga *et al.*, 2002; Clough *et al.*, 2002). In the present study we have investigated if CaM interacts with Rac1 and Cdc42 both *in vitro*, and in cells, and studied the role of CaM in Rac1 and Cdc42 function.

6.1. Endogenous Rac1 and Cdc42 Interact with CaM-Sepharose

To determine if Rac1/Cdc42 and CaM interact, and assess the Ca^{2+} dependence of such, CaM-Sepharose beads pull down assays were used. Results from HeLa and platelet cell lysates demonstrated that endogenous Rac1 (**Figure 6**) and Cdc42 (**Figure 7**) interact with CaM. There was no binding of endogenous Rac1 and Cdc42 to blank Sepharose beads (**Figures 6 & 7**). The interaction between Rac1 and CaM showed some Ca^{2+}-dependency since addition of 5 mM EGTA (**Figure 6A**) almost eliminated binding and addition of increasing concentration of Ca^{2+} enhanced the binding between Rac1 and CaM (**Figure 6A**). Increasing the concentration of EGTA to 10 mM did not completely abolish the binding of Rac1 to CaM (**Figures 6 B & C**). A similar pattern of results was observed for the binding of Cdc42 to CaM (**Figure 7**). The addition of 5 mM EGTA to the binding buffer did not completely abolish the interaction between Cdc42 and CaM even though an increasing concentration of Ca^{2+} enhanced the binding of Cdc42 to CaM (**Figure 7A**). Using a higher concentration of EGTA (10 mM) did not completely inhibit the binding of Cdc42 to CaM (**Figures 7 B & C**).

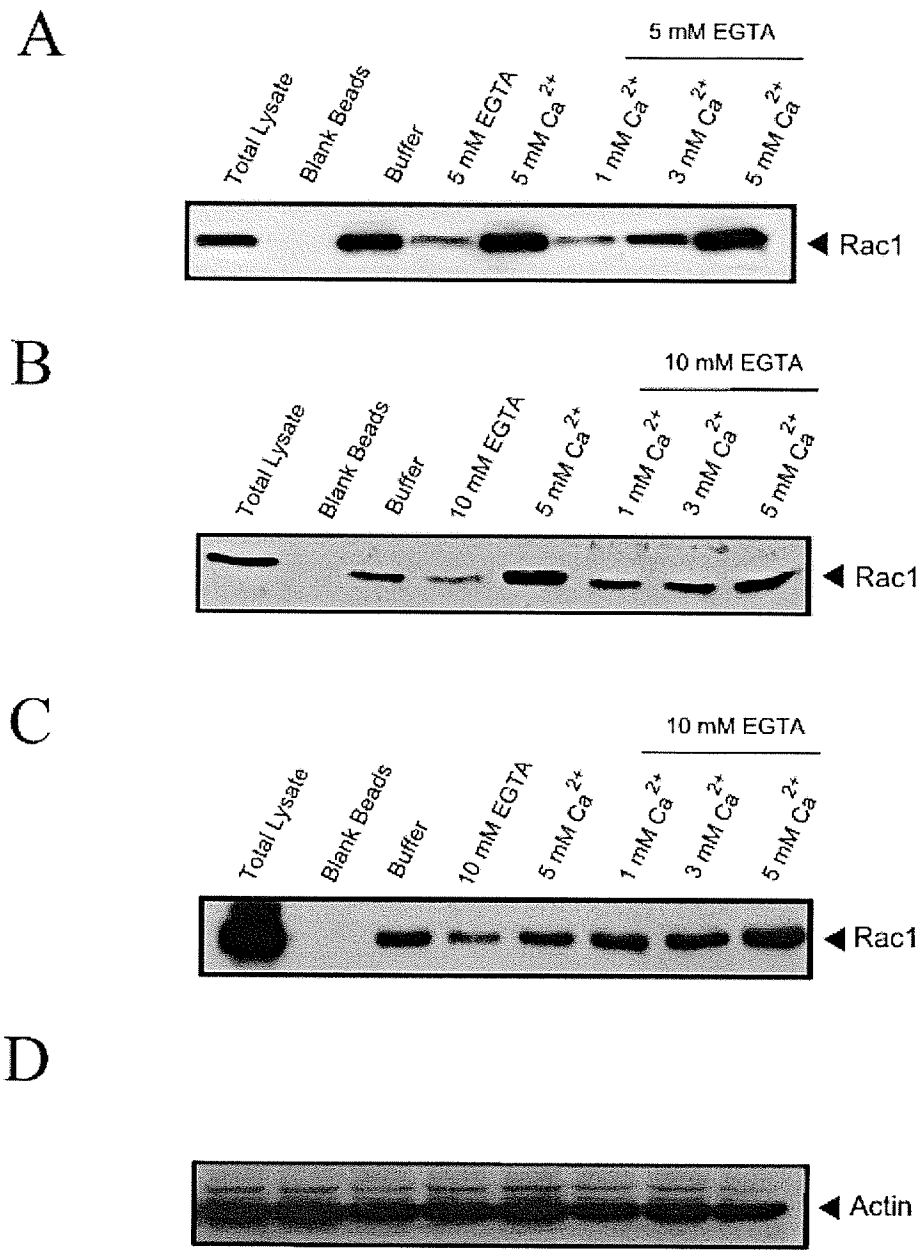

Figure 6: Endogenous Rac1 interacts with CaM-Sepharose. To determine Rac1 and CaM interaction and determine the Ca^{2+} dependence of such, CaM-Sepharose 4B beads (100 μl) were incubated for 2 hrs at 4°C with HeLa and platelet cell lysates. (A) HeLa cell lysates were incubated with beads in buffer alone, in the presence of 5 mM EGTA, in the presence of 5 mM Ca^{2+}, and in the presence of 5 mM EGTA + differing Ca^{2+} concentrations (1 mM Ca^{2+}, 3 mM Ca^{2+}, 5 mM Ca^{2+}). (B) HeLa cell lysates were incubated with the same conditions as in (A) except 10 mM EGTA was used. (C) Platelet cell lysate was exposed to the same conditions as in (B). Equal amount of protein in the starting platelet lysates was confirmed in (D) by probing for actin. This was also confirmed in HeLa cells. Sepharose 4B beads (100 μl) were used as control. After washing the beads, bound proteins were eluted using Laemmli's sample buffer, subjected to 12% SDS-PAGE, transferred to PVDF membranes, and probed with anti-Rac1 antibody. The antigen-antibody complex was visualized using ECL. The experiments were repeated a minimum of three times and gave identical results.

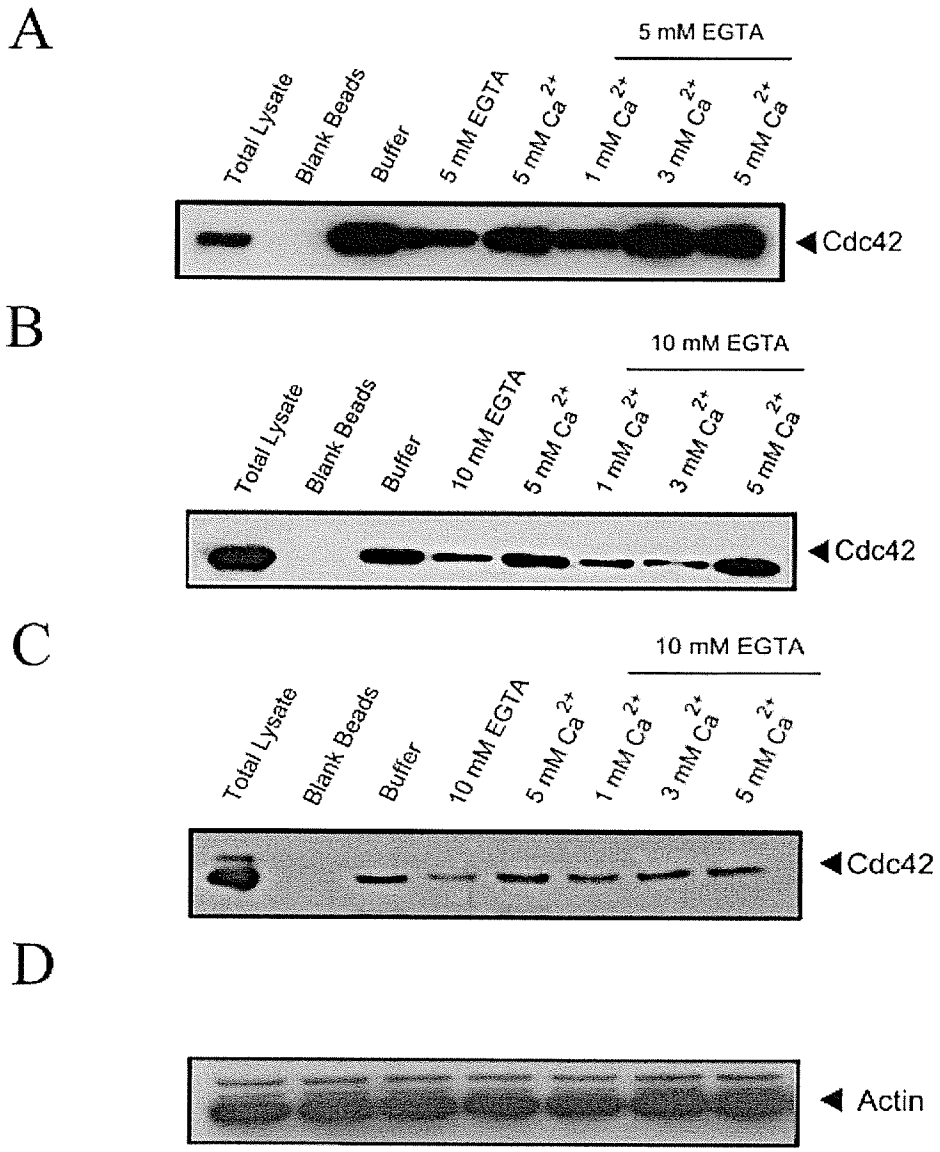

Figure 7: Endogenous Cdc42 interacts with CaM-Sepharose. To determine Cdc42 and CaM interaction and determine the Ca^{2+} dependence of such, CaM-Sepharose 4B beads (100 μl) were incubated for 2 hrs at 4°C with HeLa and platelet cell lysates. (A) HeLa cell lysates were incubated with beads in buffer alone, in the presence of 5 mM EGTA, in the presence of 5 mM Ca^{2+}, and in the presence of 5 mM EGTA + differing Ca^{2+} concentrations (1 mM Ca^{2+}, 3 mM Ca^{2+}, 5 mM Ca^{2+}). (B) HeLa cell lysates were incubated with the same conditions as in (A) except 10 mM EGTA was used. (C) Platelet cell lysate was exposed to the same conditions as in (B). Equal amount of protein in the starting platelet lysates was confirmed in (D) by probing for actin. This was also confirmed in HeLa cells. Sepharose 4B beads (100 μl) were used as control. After washing the beads, bound proteins were eluted using Laemmli's sample buffer, subjected to 12% SDS-PAGE, transferred to PVDF membranes, and probed with anti-Cdc42 antibody. The antigen-antibody complex was visualized using ECL. The experiments were repeated a minimum of three times and gave identical results.

6.2. Rac1 and Cdc42 bind to Pure CaM

To determine if Rac1 and Cdc42 bind directly to CaM, we used GST-Rac1 and GST-Cdc42 fusion proteins and pure bovine brain CaM in *in vitro* binding assays. The expression of both GST-Rac1 and GST-Cdc42 in *Escherichia coli* was assessed prior to performing experiments (**Figure 8**). The pure recombinant proteins demonstrated the expected molecular mass on SDS-PAGE analysis.

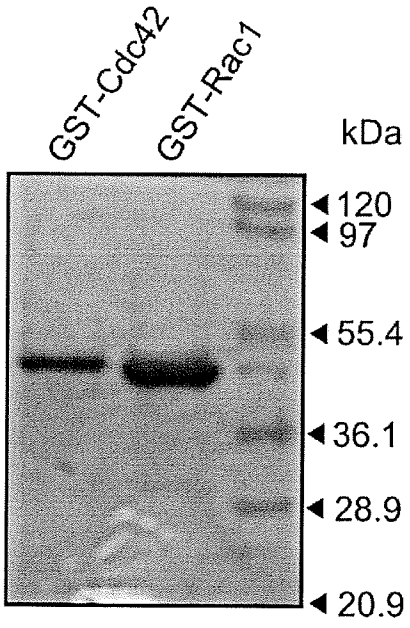

Figure 8: Purification of recombinant GST-Rac1 and GST-Cdc42 fusion proteins. Proteins were purified from *E. coli* after stimulation with IPTG and using GSH-agarose beads. The purified proteins were analyzed using 12% SDS-PAGE followed by Coommasie blue staining.

The recombinant proteins were then used to assess if Rac1 and Cdc42 interact directly with CaM. The results demonstrated that pure calmodulin indeed binds specifically to Rac1 (**Figure 9**) and Cdc42 (**Figure 10**). The CaM binding again demonstrated some Ca^{2+}-dependency for both Rac1 and Cdc42. There was no binding of CaM to GST (**Figures 9 & 10**). The increase in EGTA concentration from 5 mM to 10 mM did not abolish the binding and an increase in Ca^{2+} concentration enhanced the binding of calmodulin to both Rac1 (**Figure 9B**) and Cdc42 (**Figure 10B**). These results confirmed that CaM directly interacts with Rac1 and Cdc42.

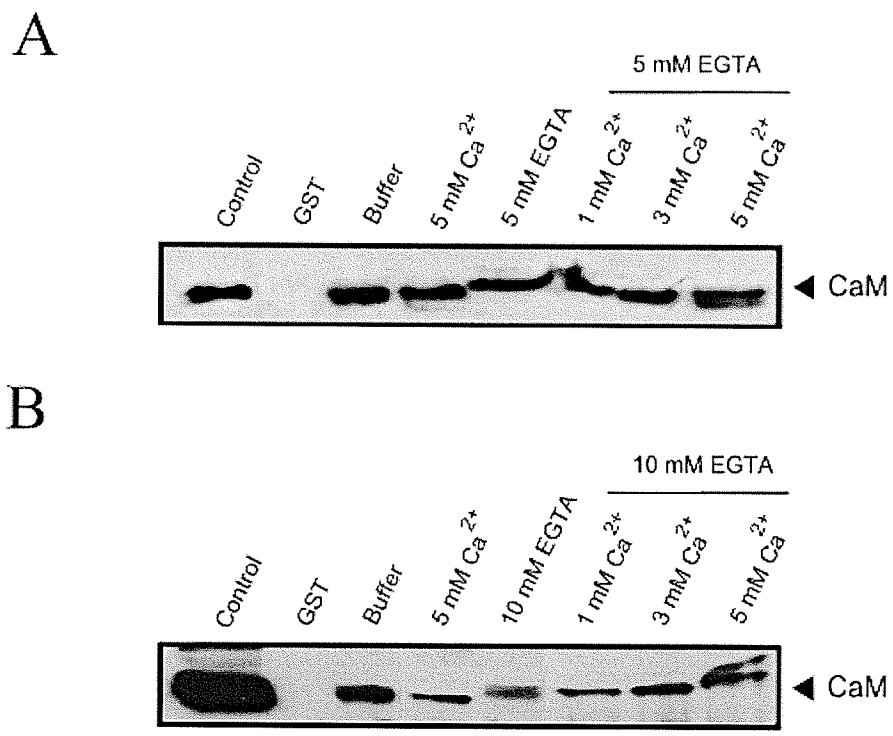

Figure 9: GST-Rac1 binds to purified bovine brain CaM. GST-Rac1 beads were incubated with purified CaM (20 μg) in MOPS buffer (30 mM MOPS pH 7.2, 1% NP-40, and 100 mM KCl) and allowed to shake for 2 hrs at 4°C. Purified bovine brain CaM (0.5 μg) is shown above in the control lane. GST beads were used as negative control. The incubation conditions included GST-Rac1 beads with (A) buffer alone, buffer + 5 mM EGTA, buffer + 5 mM Ca^{2+}, buffer with 5 mM EGTA plus varying Ca^{2+} concentrations (1 mM Ca^{2+}, 3 mM Ca^{2+}, 5 mM Ca^{2+}). In (B) same conditions as in (A) were used except 10 mM EGTA concentration was used. After incubation beads were washed and bound proteins were eluted using Laemmli's sample buffer and separated using 12% SDS-PAGE, transferred to PVDF membranes and probed with anti-CaM monoclonal antibody. The antigen-antibody complex was visualized using ECL. The experiment was repeated a minimum of three times and gave identical results.

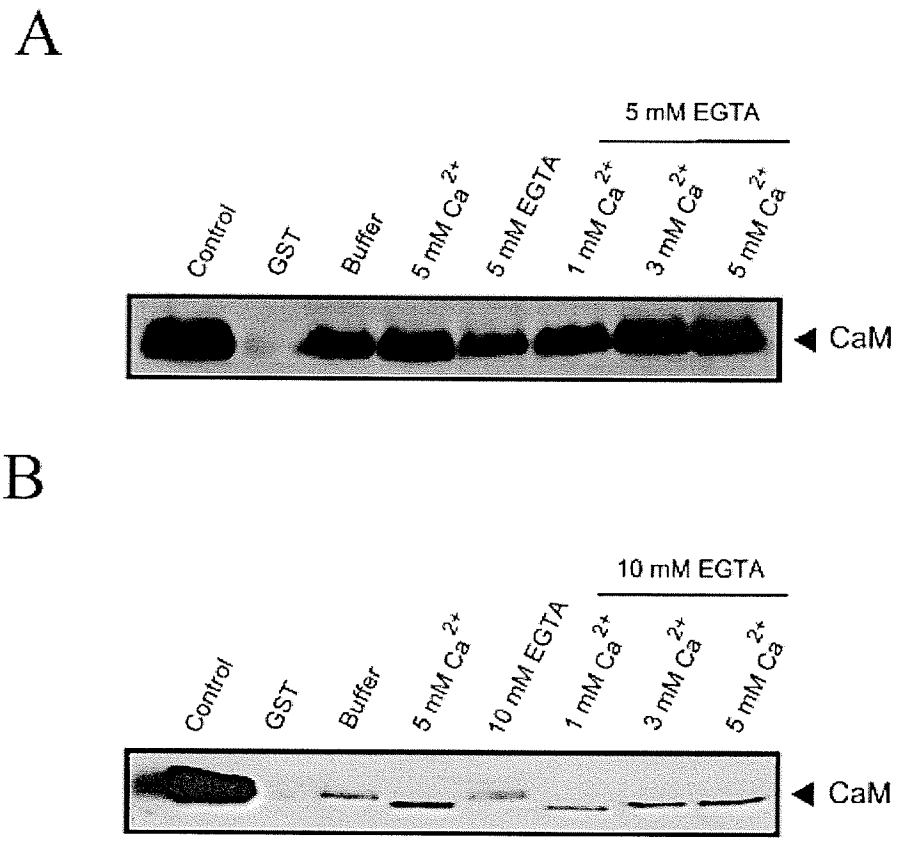

Figure 10: GST-Cdc42 binds to purified bovine brain CaM. GST-Cdc42 beads were incubated with purified CaM (20 μg) in MOPS buffer (30 mM MOPS pH 7.2, 1% NP-40, and 100 mM KCl) and allowed to shake for 2 hrs at 4°C. Purified bovine brain CaM (0.5 μg) is shown above in the control lane. GST beads were used as negative control. The incubation conditions included GST-Cdc42 beads with (A) buffer alone, buffer + 5 mM EGTA, buffer + 5 mM Ca^{2+}, buffer with 5 mM EGTA plus varying Ca^{2+} concentrations (1 mM Ca^{2+}, 3 mM Ca^{2+}, 5 mM Ca^{2+}). In (B) same conditions as in (A) were used except 10 mM EGTA concentration was used. After incubation beads were washed and bound proteins were eluted using Laemmli's sample buffer and separated using 12% SDS-PAGE, transferred to PVDF membranes and probed with anti-CaM monoclonal antibody. The antigen-antibody complex was visualized using ECL. The experiment was repeated a minimum of three times and gave identical results.

6.3. Calmodulin Database Search Analysis for Potential CaM-binding Sites

Since we established that calmodulin directly binds to Rac1, we next screened the Rac1 sequence for potential CaM-binding domains using the CaM target database (http://calcium.oci.utoronto.ca/).

```
Rac1            MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPVNLGLWDTAG 60
Cdc42           MQTIKCVVVGDGAVGKTCLLISYTTNKFPSEYVPTVFDNYAVTVMIGGEPYTLGLFDTAG 60
                **:**********************  **.**:*******:..**:.*:*  .***:****

Rac1            QEDYDRLRPLSYPQTDVFLICFSLVSPASFENVRAKWYPEVRHHCPNTPIILVGTKLDLR 120
Cdc42           QEDYDRLRPLSYPQTDVFLVCFSVVSPSSFENVKEKWVPEITHHCPKTPFLLVGTQIDLR 120
                ******************:***:***:*****:  **  **:  ****:**::****::***

Rac1            DDKDTIEKLKEKKLTPITYPQGLAMAKEIGAVKYLECSALTQRGLKTVFDEAIRAVLCPP 180
Cdc42           DDPSTIEKLAKNKQKPITPETAEKLARDLKAVKYVECSALTQKGLKNVFDEAILAALEPP 180
                **  .***** ::* .***    .  :*::: ****:*******:***.****** *.* **

Rac1            PVKKRKRKCLLL 192
Cdc42           EPKK-SRRCVLL 191
                  **  .*:*:**
```

Figure 11: CLUSTAL W multiple sequence alignment results for human Rac1 (Accession # AAM21111) and human Cdc42 (Accession # AAT70721). The proposed calmodulin binding sequences in Rac1 and Cdc42 are underlined. (CLUSTAL W 1.82 multiple sequence alignment)

The database revealed a region (amino acids 151-164) with CaM-binding potential located in the C-terminal of Rac1 (**Figure 11**). Calmodulin binding to target proteins can be calcium-dependent or -independent (Yamniuk and Vogel *et al.*, 2004). The typical calmodulin binding regions vary in length between 16-30 amino acids. They also have tendency to form amphipathic α- helices with a hydrophobic and a basic face (James *et al.*, 1995; Rhoads and Friedberg, 1997). The proposed 14 amino acid CaM-binding sequence is composed of 57.14% hydrophobic and 42.86% hydrophilic residues (**Table 4**).

Table 4

Properties of the putative Calmodulin binding region in Rac1
(http://calcium.oci.utoronto.ca/)

AVKYLECSALTQRG	
Average Residue Weight	108.63
Average Hydropathy	-0.05
Average Hydrophobic Moment	0.299
Average Propensity for Alpha-Helix Formation	1.046
Percent Hydrophobic Residue*	57.14
Percent Hydrophilic Residue**	42.86
Net Charge	+1
Number of Basic Residues	2
Number of Acidic Residues	1
* Hydrophobic Residues are ACFGHILMVWY	
** Hydrophilic Residues are DEKNPQRST	

This region of Rac1 has an alanine and a glycine residue at each end, which are weak hydrophobic amino acids and are characteristic of calmodulin binding motifs. It is not a calmodulin binding IQ motif since it does not comply with the IQ motif sequence [IVL]Qxxx[RK]xxxxx[RK] where X is any amino acid. A similar CaM-binding site in Cdc42 [AVKYVECSALTQK] that is composed of 53.85% hydrophobic and 46.15% hydrophilic residues has been identified (**Table 5**).

Table 5

Properties of the proposed Calmodulin binding region in Cdc42
(http://calcium.oci.utoronto.ca/)

AVKYVECSALTQK	
Average Residue Weight:	109.364
Average Hydropathy	0.054
Average Hydrophobic Moment	0.331
Average Propensity for Alpha-Helix Formation	1.085
Percent Hydrophobic Residue*	53.85
Percent Hydrophilic Residue**	46.15
Net Charge	+1
Number of Basic Residues	2
Number of Acidic Residues	1
* Hydrophobic Residues are ACFGHILMVWY	
** Hydrophilic Residues are DEKNPQRST	

6.4. Rac1 CaM-binding Peptide Binds Endogenous and Pure CaM

To determine whether the peptide synthesized [AVKYLECSALTQRG] binds CaM, we coupled it to CNBr-activated Sepharose beads. We used a direct approach in which the coupled peptide was added to HeLa and platelet cell lysates to examine binding to endogenous CaM.

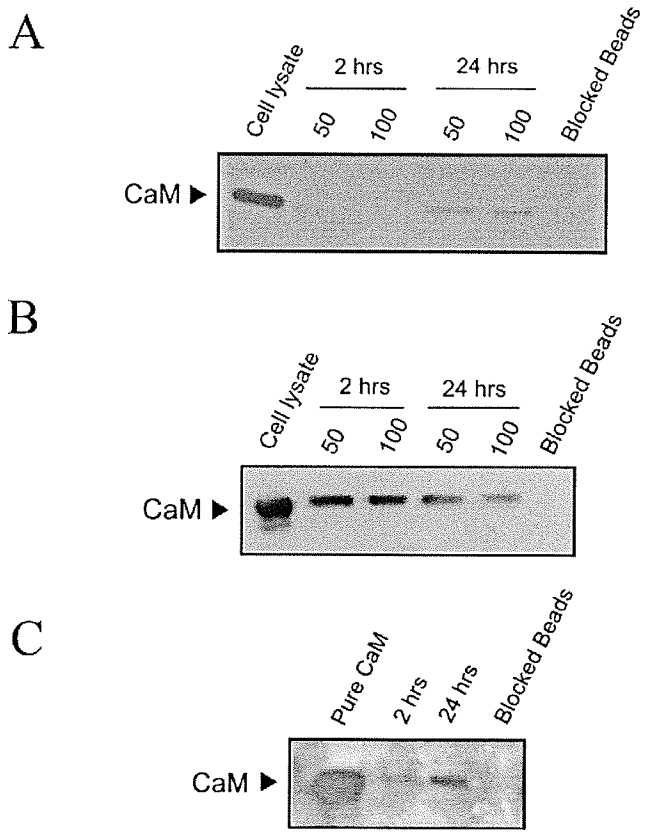

Figure 12: Coupled CaM-binding peptide binds endogenous and pure CaM from cell lysates. Binding of peptide (AVKYLECSALTQRG) coupled to CNBr activated Sepharose beads to CaM from HeLa (A), platelet (B) cell lysates and to pure CaM (C) over a 2 hrs or a 24 hrs period. Proteins were separated by SDS-PAGE and Western blotting was performed using antibodies against CaM.

The results demonstrate that there was no binding observed when blocked CNBr Sepharose beads (100 µl) were used as control in HeLa (**Figure 12A**) and platelet

(**Figure 12B**) cell lysates. But more binding was observed when coupled peptide was used. More binding was observed in samples incubated for 24 hrs (**Figure 12A**). A similar approach was taken using platelet cell lysates; however, there was more binding observed over 2 hours compared to 24 hours (**Figure 12B**). Again, there was no binding observed in the blocked CNBr beads lane. The opposite approach in which coupled peptide incubated with pure calmodulin was also used to establish whether the potential CaM-binding peptide will bind to pure calmodulin. Results demonstrated the Rac1-CaM binding peptide interacts with pure CaM (**Figure 12C**). There was no binding observed with blocked beads.

6.5. Rac1-CaM binding Peptide Competes with Rac1 for Binding to CaM-Sepharose

To determine whether the proposed Rac1-CaM binding peptide on Rac1 competes for binding to calmodulin, we used CaM-Sepharose beads as a tool.

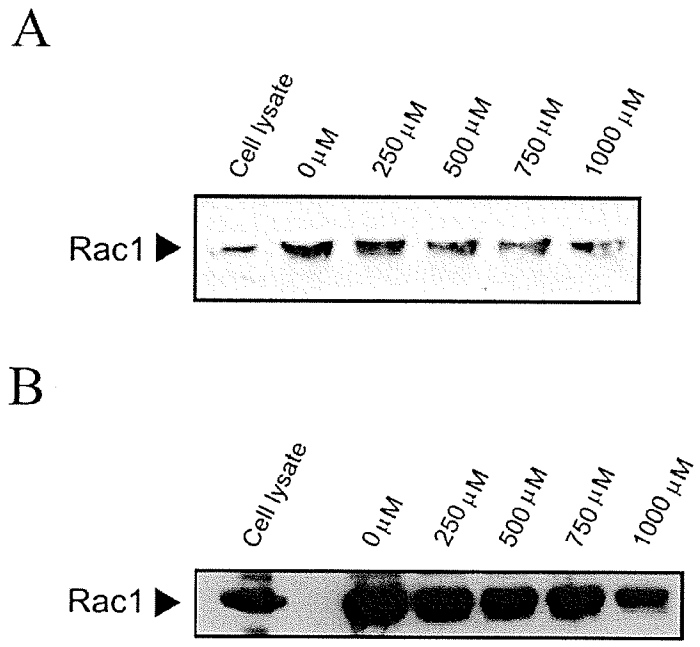

Figure 13: Free peptide competes with endogenous Rac1 for binding to CaM-Sepharose beads. CaM-Sepharose beads were used to establish whether Rac1 binding to these beads could be inhibited with the addition of CaM-binding peptide to HeLa (A) and platelet cell lysates (B). CaM-Sepharose beads were incubated with 0, 250, 500, 750, and 1000 μM of peptide for 2 hrs followed by addition of cell lysates for 2 hrs at 4 °C. After washing, proteins bound to beads were separated by SDS-PAGE and Western blotting was performed using antibodies against Rac1.

CaM-Sepharose beads were incubated with different concentrations of free peptide for two hours prior to incubation with HeLa (**Figure 13A**) and platelet (**Figure 13B**) cell lysates. Results demonstrated a decrease in Rac1 binding to CaM-Sepharose beads as the concentration of free CaM-binding peptide is increased (**Figure 13**). This indicates that the peptide is competing for binding of endogenous Rac1 to CaM-Sepharose beads.

6.6. Rac1 and Cdc42 are Associated with CaM in cells

Co-immunoprecipitation experiments were performed to establish whether endogenous Rac1 and Cdc42 are associated with Calmodulin in HeLa and platelets.

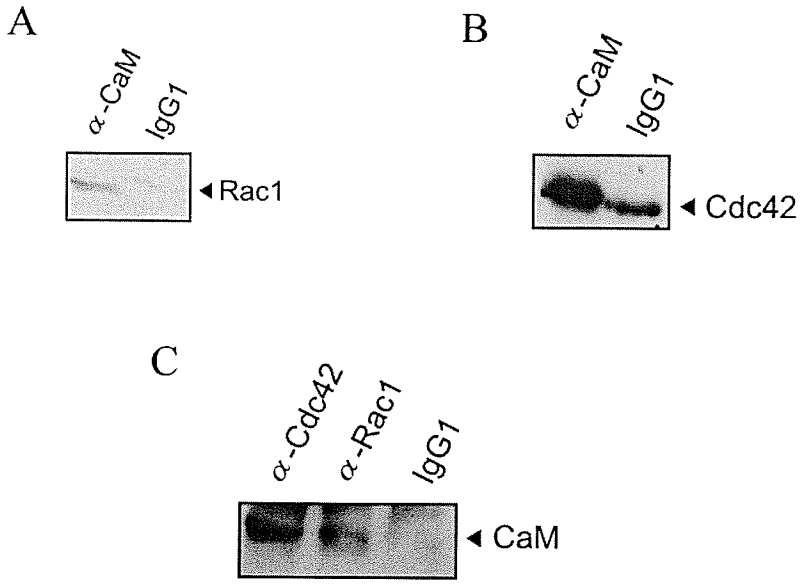

Figure 14: Rac1, Cdc42 and CaM coprecipitate from HeLa cell lysate. HeLa cells were lysed using Triton X-100 buffer and prepared for Co-IP as described under "Materials and Methods". Briefly, lysates were incubated with either anti-CaM antibody (A & B) or anti-Rac1 monoclonal or anti-Cdc42 monoclonal (C) antibodies or with mouse IgG_1 antibody. The antigen-antibody complex was isolated using protein A/G Plus-Agarose beads. The immunoprecipitated proteins were separated by 12% SDS-PAGE and probed with anti-Rac1 (A) or anti-Cdc42 (B) or anti-CaM (C) antibodies. These experiments were repeated at least three times and gave similar results.

Rac1 and Cdc42 coprecipitated from total HeLa (**Figures 14 A & B**) and platelets (**Figures 15 A & B**) with anti-CaM antibody but not with mouse IgG_1. In the reverse experiments, CaM coprecipitated from total platelet (**Figure 15C**) and HeLa (**Figure 14C**) extracts with anti-Rac1 and anti-Cdc42. The co-immunoprecipitation results provide further evidence of the interaction of Rac1 and Cdc42 with calmodulin in cells.

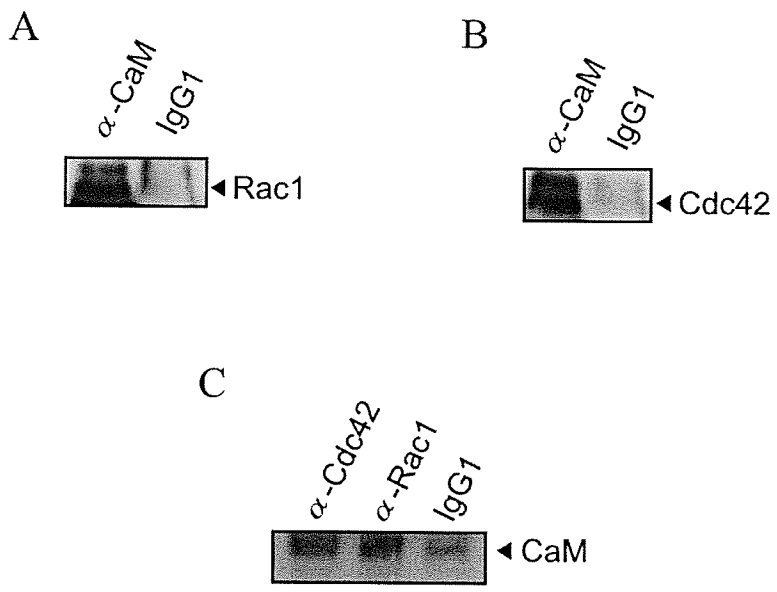

Figure 15: Rac1, Cdc42 and CaM coprecipitate from platelets. Platelets were lysed using Triton X-100 buffer and prepared for Co-IP as described under "Materials and Methods". Briefly, lysates were incubated with either anti-CaM antibody (A & B) or anti-Rac1 monoclonal or anti-Cdc42 monoclonal (C) antibodies or with mouse IgG_1 antibody. The antigen-antibody complex was isolated using protein A/G Plus-Agarose beads. The immunoprecipitated proteins were separated by 12% SDS-PAGE and probed with anti-Rac1 (A) or anti-Cdc42 (B) or anti-CaM (C) antibodies. These experiments were repeated at least three times and gave similar results.

6.7. Role of CaM in Rac1 and Cdc42 Function

Previously, this laboratory has shown that CaM is required for the activation of RalA and RalB in human platelets (Clough *et al.*, 2002). Thus, to assess a potential role of CaM in the activation of Rac1 and Cdc42, we used human platelets as the model cell system. We used the p-21 activated kinase (Pak) to determine the level of Rac1-GTP and Cdc42-GTP in cells. The p-21 activated kinase (Pak) is a downstream target of Rac1 and Cdc42 that interacts with the GTP-bound form of both Rac1 and Cdc42 (Manser *et al.*,

1994; Soulet *et al.*, 2001; Vidal *et al.*, 2002). This feature of Pak has been exploited to determine the level of active Rac1 and Cdc42 in cells (Bagrodia and Cerione, 1999; Soulet *et al.*, 2001; Vidal *et al.*, 2002). Thus, initially we have used GST-Pak1 (expressing the Rac1 and Cdc42 binding domain Pak1 coupled to the GST protein) fusion protein to confirm that it only interacts with the Rac1-GTP and Cdc42-GTP forms in HeLa cells and platelets. Expression and purification of recombinant GST-Pak1 in *E. coli* was assessed using SDS-PAGE (**Figure 16**).

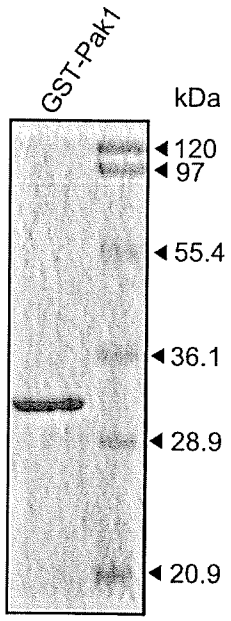

Figure 16: Purification of recombinant GST-Pak1 fusion protein. GST-Pak1 was purified from *E. coli* after stimulation with IPTG and using GSH-Agarose beads. Purified protein was analyzed using 12% SDS-PAGE followed by Coommasie blue staining.

Next we investigated if GST-Pak1 used in our studies bound only to the GTP-bound form of Rac1 and Cdc42 in both platelets and HeLa cells. Cell lysates were

incubated with GTPγS or GDPβS prior to incubation with GST-Pak1. The results demonstrated that in HeLa cells (**Figures 17 A & B**) and platelets (**Figures 17 C & D**), GST-Pak1 interacts only with Rac1 and Cdc42 that was loaded with GTPγS and not with the GDPβS loaded form of Rac1 or Cdc42.

IQGAP1 and IQGAP2 are actin binding proteins that bind to Rac1 and Cdc42. IQGAP1 was found to bind directly to activated forms of Cdc42 and Rac1 but not to active or inactive Ras or Rho, and it inhibits the GTPase activity of Cdc42 maintaining it in its active state (Hart *et al.*, 1996; Kuroda *et al.*, 1996; McCallum *et al.*, 1996). Little is known about the mechanism of IQGAP2 interaction with Rac1 and Cdc42. However, it was reported that IQGAP2 binds to calmodulin but neither the full-length nor the truncated IQGAP2 stimulated the activity Rac1 and Cdc42 (Brill *et al.*, 1996). A direct evidence for calmodulin binding by IQGAP1 has also been presented (Hart *et al.*, 1996; McCallum *et al.*, 1998). Therefore, it was crucial to provide evidence that binding of calmodulin to Rac1 and Cdc42 is independent of IQGAP influence in our particular cell models.

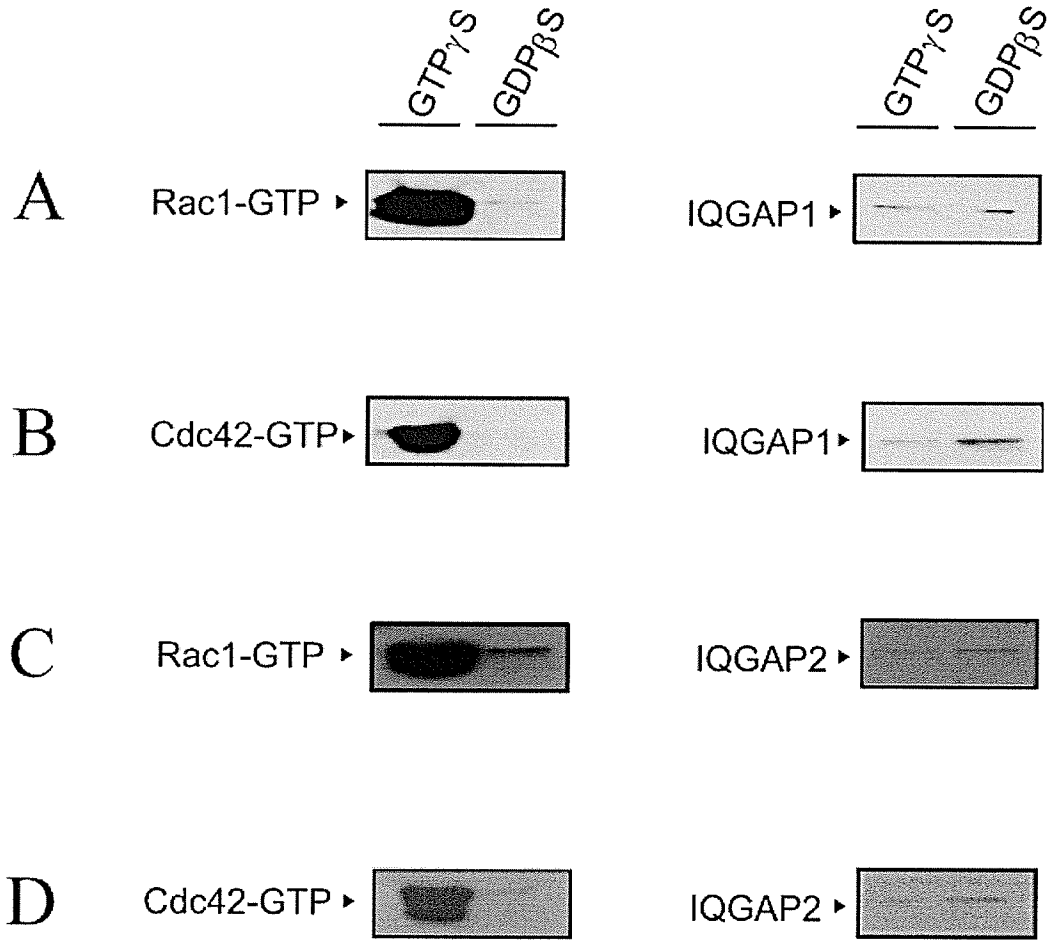

Figure 17: *In vitro* loading of Rac1 and Cdc42 in HeLa and platelets and levels of IQGAP1 and IQGAP2. HeLa and platelet cell lysates were prepared as described under "Materials and Methods". 100 μl of GST-Pak1 bound to glutathione-agarose beads was added to HeLa or platelet cell lysates that were previously incubated with guanine nucleotides (100 μM GTPγS or 100 μM GDPβS). Proteins were separated using SDS-PAGE and presence of Rac1 and Cdc42 analyzed by Western blotting. Parts (A & B) represent Rac1, Cdc42 and IQGAP1 in HeLa cells whereas Parts (C & D) represent Rac1, Cdc42 and IQGAP2 in platelets. The blots show a representative autoradiograph from experiments repeated three times with consistent results.

To investigate the levels of IQGAP1 and IQGAP2 present in cells when Rac1 and Cdc42 are active, we used the fusion protein GST-Pak1 as a tool that is known to bind to the active form of Rac1 and Cdc42. In the HeLa cell line, we examined the levels of IQGAP1 when Rac1 and Cdc42 were loaded with GTPγS or GDPβS. Results show that the levels of IQGAP1 associated with Rac1 and Cdc42 are not elevated irrespective of the guanine nucleotide status **(Figures 17 A & B)**. In contrast, we report that there was no IQGAP1 present in human platelets; however, IQGAP2 was present in platelets and its levels were not increased when Rac1 and Cdc42 were loaded with the guanine nucleotides GTPγS (**Figures 17 C & D**).

6.8. Determination of Optimal Time-point Rac1 and Cdc42 Activation

To determine if CaM is involved in activation of Rac1 and Cdc42, we initially established the optimal time-point for Rac1 and Cdc42 activation using the known agonist, thrombin.

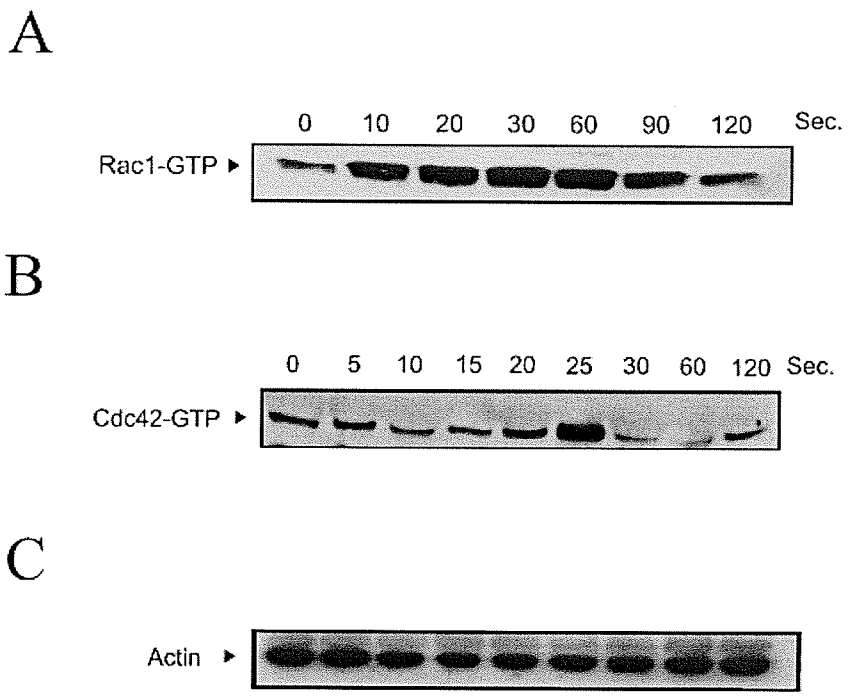

Figure 18: Time-course for optimal point of activation for Rac1 and Cdc42. Freshly drawn human platelets were prepared as described under "Materials and Methods", divided into 500 μl aliquots and incubated for 30 min at 37°C prior to thrombin treatment (1U/ml) for indicated times. After thrombin treatment, the platelets were lysed in RIPA buffer and incubated with 100 μl of GST-Pak1 beads for 2 hrs at 4°C to recover GTP-bound Rac1 or GTP-bound Cdc42. The beads were washed three times with Rac1 washing buffer. Bound proteins were eluted using Laemmli's sample buffer and Rac1-GTP or GTP-Cdc42 were detected using SDS-PAGE and Western blotting using a monoclonal antibody against Rac1 or Cdc42. Part (A) in the above figure shows the time-point at which Rac1 is maximally activated. Part (B) in the above figure shows the time-point at which Cdc42 is maximally activated. Part (C) represents confirmation of equal amount of protein in the starting platelet lysates by probing for actin. Sepharose 4B beads (100 μl) were used as control. The blots show a representative autoradiograph from experiments repeated at least three times with consistent results.

Thrombin was previously shown to activate Rac1 and Cdc42 in human platelets (Soulet *et al.*, 2001; Vidal *et al.*, 2002). We used GST-Pak1 fusion protein to pull down

the active form of Rac1 and Cdc42 from platelets at different time-points. The optimal time-points for Rac1 and Cdc42 was determined to be 60 sec. and 25 sec., respectively (**Figure 18**). Soluet's group has previously shown that the optimum activation time-point for Rac1 is 30 sec. even though a 60 sec. stimulation time for Rac1 was used throughout their work (Soluet *et al.*, 2001). Time-course experiments performed on platelets have shown that Cdc42 is maximally activated at 20 sec (Vidal *et al.*, 2002). Our results are consistent with these findings.

6.9. Calmodulin Activates Rac1 and Inhibits Cdc42 Activation in Platelets

After the determination of the optimal time-point for Rac1 and Cdc42 activation, we investigated the role of calmodulin on Rac1 and Cdc42 activation in platelets. Thrombin caused a two and half fold increase in the activation of Rac1 in human platelets (**Figure 19**). This thrombin induced activation was abolished in the presence of the CaM inhibitor W7•HCl (**Figure 19**). In case of Cdc42, thrombin caused a 1.5-fold increase in Cdc42 activation in human platelets (**Figure 20**). This result is consistent with that reported in (Vidal *et al.*, 2002). Addition of W7•HCl alone to platelets resulted in activation of Cdc42 (**Figure 20**). Incubation of platelets with W7•HCl prior to addition of thrombin did not inhibit Cdc42 activation (**Figure 20**). Thus, these results suggest that CaM has opposing effects in the activation process of Rac1 and Cdc42. In case of Rac1, CaM is necessary for the activation. However, CaM is inhibitory to Cdc42 activation since addition of W7•HCl resulted in activation of Cdc42 and W7•HCl did not inhibit thrombin dependent activation of Cdc42.

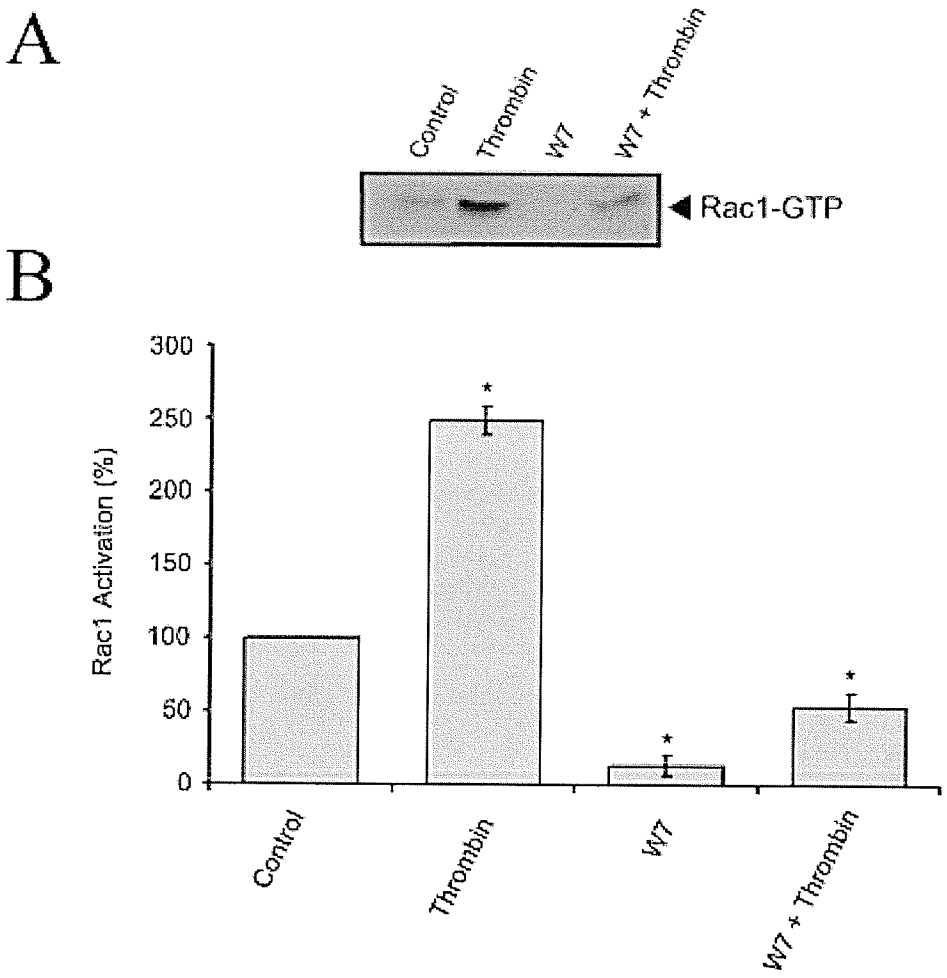

Figure 19: Calmodulin is required for thrombin induced activation of Rac1. Freshly drawn human platelets were prepared as described under "Materials and Methods", divided into 500 μl aliquots and incubated for 30 min at 37°C prior to thrombin treatment (1U/ml). Five minutes before thrombin treatment, the indicated aliquots were treated with 150 μM of calmodulin inhibitor W7. After thrombin treatment for 1 min, the platelets were lysed in RIPA buffer and incubated with 100 μl of GST-Pak1 beads for 2 hrs at 4°C to recover GTP-bound Rac1. The beads were washed three times with Rac1 washing buffer. Bound proteins were eluted using Laemmli's sample buffer and Rac1-GTP detected using SDS-PAGE and Western blotting using a monoclonal antibody against Rac1. The experiment was repeated a minimum of three times using blood from three different donors. Rac1-GTP levels were significantly ($p < 0.05$) different from those in control platelets upon the addition of *thrombin, *W7, and *W7 plus thrombin. Part (A) shows levels of Rac1-GTP determined using Western blot analysis. In part (B), values for Rac1-GTP in various samples were quantified from three separate experiments.

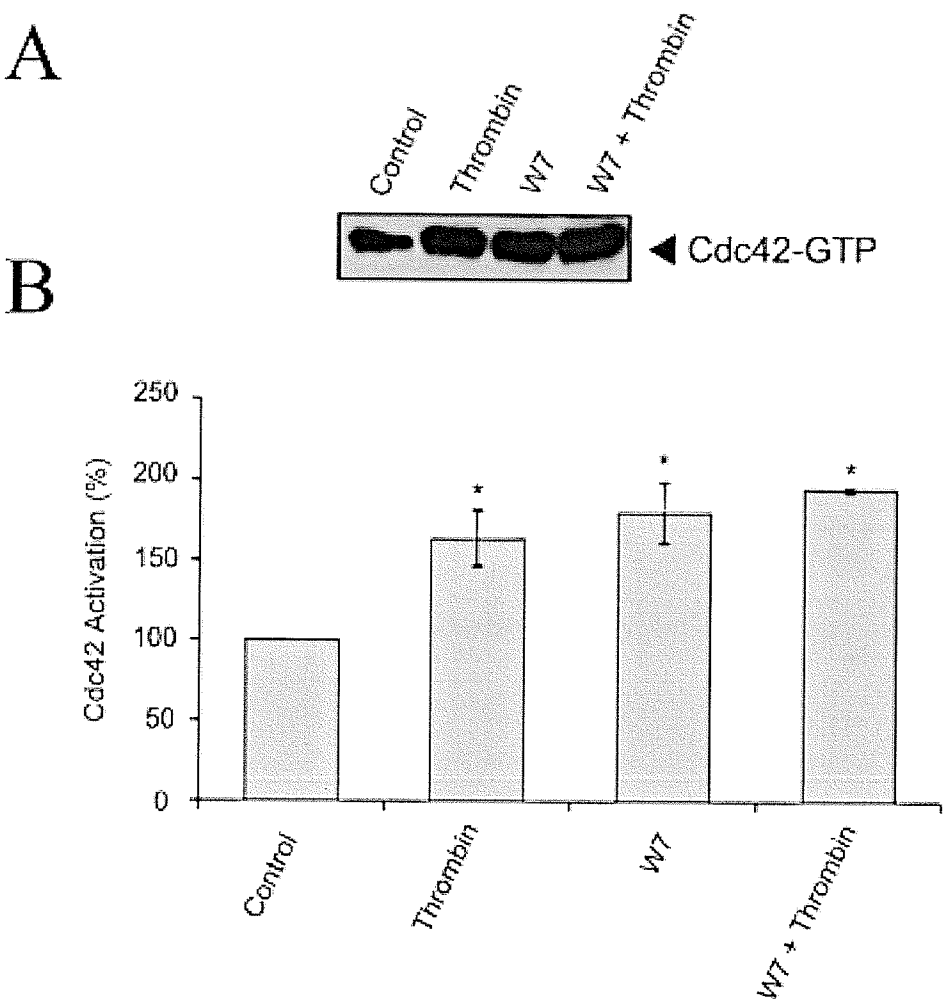

Figure 20: Calmodulin inhibits Cdc42 GTPase Activity. Freshly drawn human platelets were prepared as described under "Experimental Procedures", divided into 500 μl aliquots and incubated for 30 min at 37°C prior to thrombin treatment (1U/ml). Five minutes before thrombin treatment, the indicated aliquots were treated with 150 μM of calmodulin inhibitor W7. After thrombin treatment for 25 sec., the platelets were lysed in RIPA buffer and incubated with 100 μl of GST-Pak1 beads for 2 hrs at 4°C to recover GTP-bound Cdc42. The beads were washed three times with Cdc42 washing buffer. Bound proteins were eluted using Laemmli's sample buffer and Cdc42-GTP detected using SDS-PAGE and Western blotting using a monoclonal antibody against Cdc42. The experiment was repeated a minimum of three times using blood from three different donors. Cdc42-GTP levels were significantly ($p < 0.05$) different from those in control platelets upon the addition of *thrombin, *W7, and *W7 plus thrombin. Part (A) shows levels of Cdc42-GTP determined using Western blot analysis. In part (B), values for Cdc42-GTP in various samples were quantified from three separate experiments.

6.10. Rac1 and Cdc42 localize to Plasma Membrane upon Activation

Fluorescent immunocytochemistry studies were performed to investigate Rac1, Cdc42 and calmodulin distribution and localization in platelets. In control samples, Rac1, Cdc42 and calmodulin were distributed equally between the cytoplasm and plasma membrane (**Figures 21 C-E & 22 C-E**). The merged images of Rac1 and calmodulin (**Figure 21D**) or Cdc42 and calmodulin (**Figure 22D**) indicate that these proteins are localized throughout the cell. The addition of thrombin resulted in localization of Rac1, Cdc42 and Calmodulin to plasma membrane as well as platelet aggregation (**Figures 21 F-H & 22 F-H**). The addition of W7 to platelets resulted in more Rac1 and CaM or Cdc42 and CaM to be in the cytoplasm compared to thrombin treatment alone (**Figures 21 I-K & 22 I-K**). The addition of W7 plus thrombin **(Figures 21 L-N & 22 L-N)** caused some localization of Rac1, Cdc42 and CaM to membrane, formation of membrane protrusions and inhibited thrombin induced platelet aggregation.

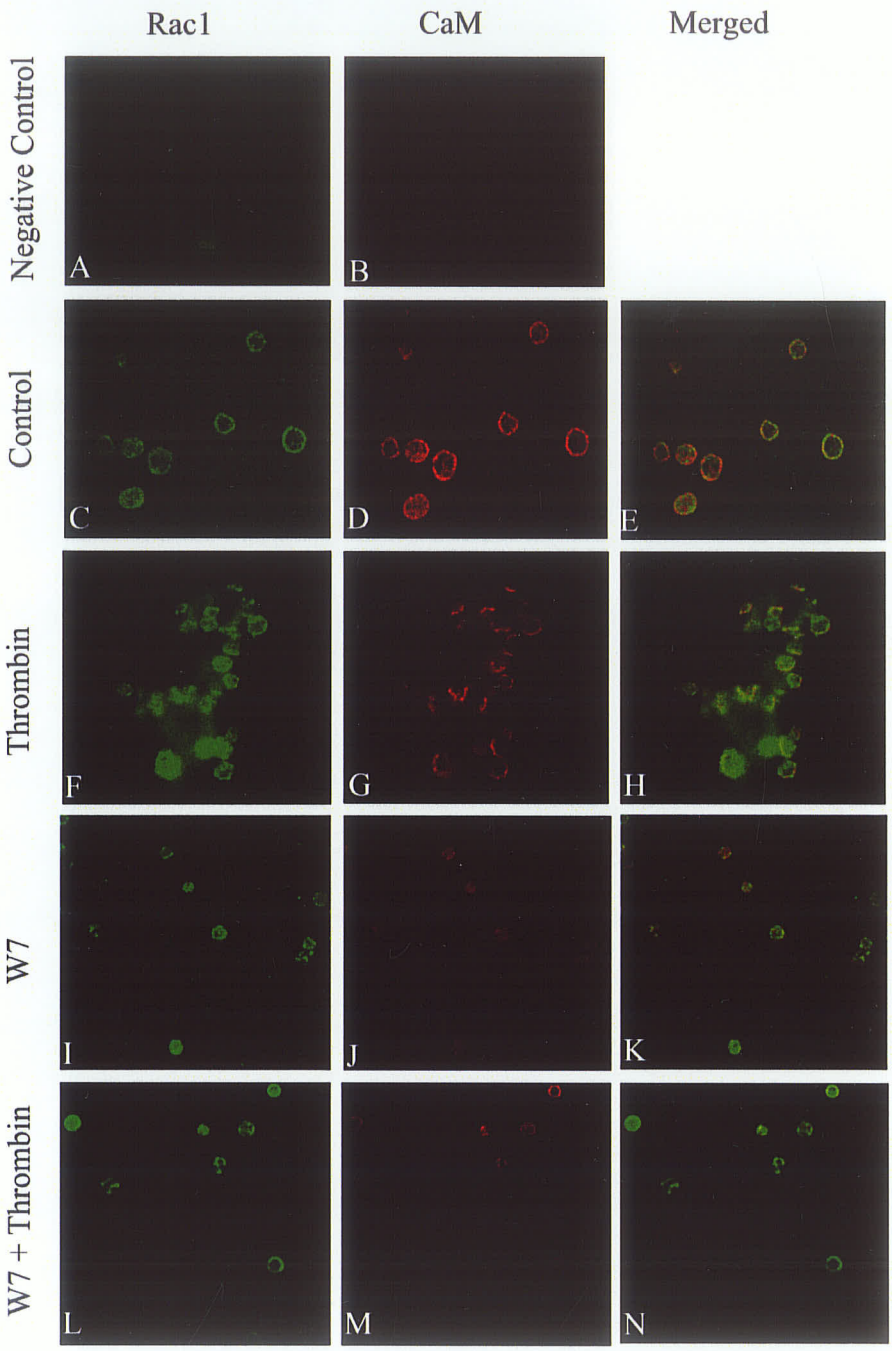

Figure 21: Fluorescent immunocytochemistry analysis of Rac1 and calmodulin localization. Freshly drawn human platelets were prepared as previously described and treated with thrombin alone (1 U/ml), the calmodulin inhibitor W7 alone (150 μM) for 5 min or with W7 (5 min) plus thrombin. The reaction was stopped with the addition of cold 4% PFA in PBS + 0.1% Triton X-100 for 30 min. Fixed cells were washed with PBS at room temperature. Cells were incubated with primary monoclonal mouse an anti-Rac1 (1:100 dilution) or polyclonal rabbit anti-CaM (1:100 dilution) antibodies for 1 hour in humidified chamber. Cells were washed three times with PBS + 1% BSA prior to incubation for 1 hr with secondary FITC (1:200 dilution) for Cdc42 and Texas Red (1:200 dilution) for CaM in humidified chamber. ProLong Antifade Gold mounting medium was applied to coverslips and the slides were viewed by confocal microscopy. Images were viewed under magnification of 100x oil emersion lens and analyzed using FluoView software.

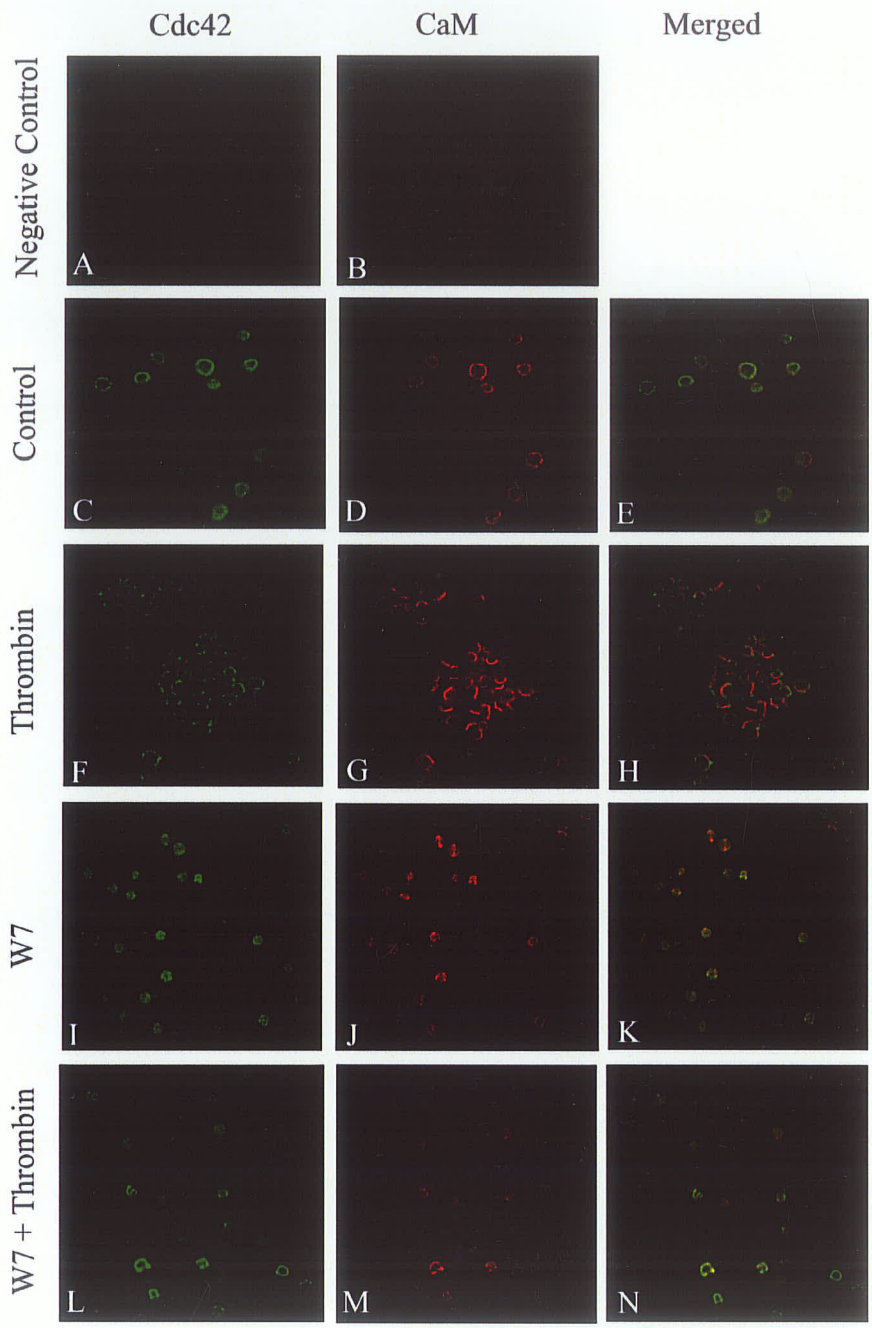

Figure 22: Fluorescent immunocytochemistry analysis of Cdc42 and calmodulin localization. Freshly drawn human platelets were prepared as previously described and treated with thrombin alone (1 U/ml), the calmodulin inhibitor W7 alone (150 μM) for 5 min or with W7 (5 min) plus thrombin. The reaction was stopped with the addition of cold 4% PFA in PBS + 0.1% Triton X-100 for 30 min. Fixed cells were washed with PBS at room temperature. Cells were incubated with primary monoclonal mouse an anti-Cdc42 (1:50 dilution) or polyclonal rabbit anti-CaM (1:50 dilution) antibodies for 1 hour in humidified chamber. Cells were washed three times with PBS + 1% BSA prior to incubation for 1 hr with secondary FITC (1:200 dilution) for Cdc42 and Texas Red (1:200 dilution) for CaM in humidified chamber. ProLong Antifade Gold mounting medium was applied to coverslips and the slides were viewed by confocal microscopy. Images were viewed under magnification of 100x oil emersion lens and analyzed using FluoView software.

CHAPTER 7

DISCUSSION AND CONCLUSIONS

Rac1 and Cdc42 are small GTP-binding proteins that are involved in actin/myosin cytoskeleton reorganization leading to changes in cell shape (Nobes and Hall, 1995; Macara *et al.*, 1996; Hall, 1998). Rac1 is involved in membrane ruffling induction, chemotaxis, and lamellipodia formation whereas Cdc42 is involved in filopodia formation (Nobes and Hall, 1999; Burridge and Wennerberg, 2004). Human platelets contain approximately 2-3 μM Rac1 and 0.2-0.3 μM Cdc42, both of which are predominantly (>90%) found in the cytosol (Azim *et al.*, 2000; Schmidt *et al.*, 2003). In platelets, Rac1 is required for lamellipodia formation and for aggregate stability leading to thrombus formation (McCarty *et al.*, 2005). Filopodia formation and lamella spreading have been observed in platelets stimulated with thrombin through the G-coupled proteinase-activated receptor-1 (PAR1) (Vidal *et al.*, 2002). Thrombin-mediated activation of platelet Rac1 and Cdc42 occurs via PAR1 (Azim *et al.*, 2000; Soulet *et al.*, 2001; Vidal *et al.*, 2002).

The regulation of Rac1 and Cdc42 in cells is not fully understood, but the interaction of these GTPases with other proteins is important for their function. Thus, IQGAP1 and IQGAP2 are proteins that have recently been identified in human platelets (Schmidt *et al.*, 2003) and are known to bind to the active form (i.e. GTP-bound) of Rac1 and Cdc42 (Wessbach *et al.*, 1994; Kuroda *et al.*, 1996; Brill *et al.*, 1996; Hart *et al.*, 1996; McCuallum *et al.*, 1996). IQGAP1 binds directly to activated forms of Cdc42 and

Rac1 and it inhibits the GTPase activity of Cdc42 maintaining it in its active state (Hart *et al.*, 1996; Kuroda *et al.*, 1996; McCallum *et al.*, 1996). IQGAP1 and IQGAP2 also bind calmodulin (Brill *et al.*, 1996; Hart *et al.*, 1996) and this prevents IQGAP1 from interacting with Cdc42-GTP (Joyal *et al.*, 1997). No information exists on whether calmodulin binding to IQGAP1 affects the regulation of Rac1. In addition, the effects of calmodulin binding to IQGAP2 on the small GTPases Rac1 and Cdc42 has yet to be elucidated.

Calmodulin interacts with a variety of small GTPases (Wang *et al.*, 1997; Sidhu and Bhullar, 2001; Villalonga *et al.*, 2002; Clough *et al.*, 2002). Calmodulin binds to RalA and RalB and is necessary for thrombin-mediated activation of these GTPases in platelets (Clough *et al.*, 2002). It has also been shown that CaM can promote GTP-GDP exchange on RalA (Park *et al.*, 1999). CaM also causes dissociation of RalA from synaptic vesicle membranes in a Ca^{2+}-dependent fashion that is independent of the guanine nucleotide status of Ral (Park *et al.*, 1999). Similar regulatory mechanisms were observed for K-RasB (Sidhu *et al.*, 2003), the small GTPase Rab3A (Park *et al.*, 1997) and Rab3B (Sidhu and Bhullar, 2001).

In response to an increase in intracellular Ca^{2+} concentration, calmodulin undergoes a conformational change that allows it to interact with its target proteins and modify their function. Thus, calmodulin and calmodulin-binding proteins play an important role in intracellular Ca^{2+} signaling and in various physiological functions including glycogen metabolism, secretion, muscle contraction, actin/cytoskeletal

organization and cell division (Walsh, 1994). Epidermal growth factor-induced Ca^{2+} influx is mediated by Rac proteins (Peppelenbosch *et al.*, 1996) and calmodulin antagonists block the activation of small GTPases Rac and Ras in neutrophils (Lian *et al.*, 2001). However, no information exists as to how calmodulin regulates Rac GTPase activity. In addition, it is not known if other members of the Rho family of small GTPases (e.g. Cdc42) are also regulated by CaM. The fact that CaM has been shown to interact with and regulate the activation of several small GTPases (Wang *et al.*, 1997; Sidhu and Bhullar, 2001; Clough *et al.*, 2002; Villalonga *et al.*, 2002) has led us to investigate if CaM interacts and regulates the activity of Rac1 and Cdc42.

We have found a novel interaction between calmodulin and the small GTPases Rac1 and Cdc42 *in vitro* and in cells. The interaction exhibited calcium dependency since addition of EGTA reduced the binding of calmodulin to Rac1 and Cdc42. Rac1 and Cdc42 were also shown to bind directly to pure calmodulin in a similar fashion. The addition of higher concentrations of EGTA did not completely abolish the binding which suggests that the binding is partially calcium-independent. This suggests that Rac1 and Cdc42 potentially have two binding sites for CaM: one Ca^{2+}-dependent and a second site that is Ca^{2+}-independent. We have previously demonstrated that a similar scenario exists in case of RalA and RalB in that this GTPase also has an N-terminal Ca^{2+}-independent and a C-terminal Ca^{2+}-dependent binding site for CaM (Clough *et al.*, 2002). Whether Rac1 and Cdc42 have similar types of CaM binding sites in their N-terminal and C-terminal is not known. The interaction of calmodulin with Rac1 or Cdc42 was further confirmed in cells using co-immunoprecipitation. The results obtained from co-

immunoprecipitation provides evidence that calmodulin is associated with these GTPases in a complex in cells.

The interactions of CaM with its target proteins are predominantly hydrophobic and strong electrostatic interactions (Wang *et al.*, 1997). The proposed Rac1-CaM binding peptide identified by us is located within amino acids 151-164 in the C-terminal of Rac1. This region is composed mostly of hydrophobic amino acid residues and can serve as the CaM-binding region in Rac1. A similar region is present in Cdc42 but its ability to bind CaM has not been confirmed.

A recent study has demonstrated a role for Rac1 in the activation of the NADPH oxidase complex that is necessary for the downstream activation of tyrosine phosphorylation of Jaks and STAT-dependent transcription in smooth muscle cells (Pelletier *et al.*, 2003). The GPCR agonists AngII and thrombin have been shown to activate Rac1 in vascular smooth muscle cells and this was sufficient for enhancing the phosphorylation of Jaks and the transcriptional activity of STATs (Pelletier *et al.*, 2003). There is also evidence suggesting an important role for Cdc42 in cell migration (Fukata *et al.*, 2002). The inhibition of Cdc42 in primary fibroblasts and astrocytes undergoing migration leads to impaired migration and lamellipodia formation (Nobes and Hall, 1995; Hall, 1998; Manneville and Hall, 2002) However, the effects of Cdc42 in the regulation of certain pathways appears to be cell type specific. For example, in several fibroblastoid cell lines, Cdc42 activation has been linked with the activation of JNK, Erk and p38 (Frost *et al.*, 1997; Bishop and Hall, 2000). Conversely, the use of dominant negative

Cdc42 in primary fibroblasts resulted in upregulation of Akt and Erk activity (Zugasti *et al.*, 2001); and the loss of Cdc42 gene in ES cells had no effect on Erk, JNK, and p38 activation but prevented filopodia structure formation (Chen *et al.*, 2000). However, in another study (Czuchra *et al.*, 2005) reported that Cdc42-deficient fibroblastoid and ES cells maintained the ability to form filopodia and lamellipodia and that dominant negative Cdc42 did not inhibit filopodia and lamellipodia formation. This implies that filopodia formation may be regulated by GEFs that are distinct from those controlling cell polarization. CaM acts like a GEF for the Ral GTPase (Park *et al.*, 2001), so it is possible that CaM may play a similar role in controlling Rac1 and Cdc42 activation and thus regulate the Jak/STAT signaling pathway.

In platelets, Rac1 and Cdc42 activation occurs after stimulation of G-coupled PAR-1 by thrombin (Soulet *et al.*, 2001). The optimal time-points for Rac1 and Cdc42 activation by thrombin were re-examined in platelets and determined to be 60 sec. and 25 sec., respectively. These results are different from those of Soulet's group since they have shown that the optimal activation time-point for Rac1 was 30 sec. even though a 60 sec. time-point for Rac1 stimulation was used in their studies (Soulet *et al.*, 2001). Vidal *et al* found that Cdc42 was maximally activated at 20 sec (Vidal *et al.*, 2002). Our result is consistent with their work since our optimal time-point for Cdc42 activation in platelets was 25 sec.

The role of calmodulin in Rac1 and Cdc42 function was examined using platelet as our cell model system. Previously it was shown that the calmodulin inhibitor W7

inhibited the activation of Rac1 in neutrophils (Lian *et al.*, 2001). Similar results were obtained in our studies with platelets. The addition of thrombin after W7 did not reverse the inhibitory effects of W7 on Rac1 activation. This suggests a role for calmodulin in Rac1 activation. In contrast, the activity of Cdc42 was increased after the addition of W7 and a further increase was observed upon the addition of W7 plus thrombin. This suggests that calmodulin activates Rac1 but inhibits Cdc42 activation in platelets. Calmodulin binding to IQGAP1 prevents the later protein from binding to active Cdc42, which raised the question of whether the increase in Cdc42 activity in the absence of calmodulin is due to the association of IQGAP1 with Cdc42-GTP. In our studies, we could not detect IQGAP1 in human platelets but it was detected in HeLa cells. However, we did detect IQGAP2 in human platelet lysate. The role of calmodulin binding to IQGAP2 is unknown, but levels of IQGAP2 associated with Rac1 and Cdc42 were not increased upon loading of these GTPases with GTPγS. This suggests that activated Rac1 or Cdc42 are associated with IQGAP2 but that the observed activation is independent of IQGAPs association with these small GTPases.

Active forms of Cdc42 and Rac1 were shown to accumulate in response to thrombin, causing Rac1 to localize to the plasma membrane and Cdc42 to associate with the actin cytoskeleton (Azim *et al.*, 2000). This was also shown by immunocytochemical analysis in which Rac1 and Cdc42 translocated to platelet plasma membrane upon stimulation with thrombin (Vidal *et al.*, 2002). Here we show by immunocytochemical analysis that Rac1, Cdc42 and calmodulin are present in both the cytoplasm and the plasma membrane. The addition of thrombin caused localization of Rac1, Cdc42 and

calmodulin to plasma membrane and platelet aggregation. However, the effects of the calmodulin inhibitor W7 alone or W7 plus thrombin have not previously been investigated using an immunocytochemical approach. We have shown that the addition of W7 to platelets resulted in higher levels of Rac1, Cdc42 and calmodulin in the cytoplasm compared to control. The addition of W7 plus thrombin caused some localization to plasma membrane, in addition to formation of membrane protrusions, but this was much reduced compared to platelets treated with thrombin alone. Treatment with W7 or W7 plus thrombin did not lead to platelet aggregation. This suggests that calmodulin also plays a role in platelet aggregate formation possibly through its function in Rac1 and Cdc42 activation.

Rac1 and Cdc42 must be activated before actin assembly. Activated Cdc42 stimulates activation of Rac1 which in turn stimulates activation of Rho. Therefore, in the context of actin assembly, the primary effect of Cdc42 activation is induction of filopodia, whereas the secondary effect is induction of membrane ruffling and tertiary effects include Rho mediated stress fiber formation (Nobes and Hall, 1995; Kozma *et al.*, 1995; Bashour *et al.*, 1997). However, the steps between their activation and platelet actin remodeling are not fully understood (Vidal *et al.*, 2002). In platelets, the inhibition of Cdc42 and Rac1 blocks thrombin-induced filopodia extension and lamellae spreading, confirming that these events are dependent on Cdc42 and Rac1 (Vidal *et al.*, 2002). Stimulation of platelets by thrombin induced filopodia extensions and lamellae spreading with an increase in actin polymerization and the consequent change in platelet shape, leading to platelet aggregation. In addition, the use of the dominant-negative mutant

N17Rac1 prevented exposure of actin filaments prior to thrombin stimulation in platelets. However, when the constitutively active mutant V12Rac1 was introduced, it promoted the formation of actin filaments (Hartwig *et al.*, 1995).

Activation assay results indicate that the calmodulin inhibitor W7 prevents thrombin-induced Rac1 activation but has no effect on the activation of Cdc42 by thrombin. Immunocytochemistry results show an absence of platelet aggregation under these conditions. This finding supports previous work showing that Cdc42 and Rac1 activation is required for platelet aggregation (Azim *et al.*, 2000). In addition, our studies demonstrate that activation of Cdc42 alone is not sufficient for platelet aggregation. This suggests that both of these GTPases must be activated in concert in order to promote platelet aggregation. Furthermore, it is possible that W7 may have an effect on other proteins needed for actin polymerization (e.g. actin).

In conclusion, these studies have established that the calcium sensor protein, calmodulin, interacts with and regulates the activity of small GTPases Rac1 and Cdc42. This interaction was found to be partially Ca^{2+}-dependent. The binding of calmodulin to Rac1 increases its activation in cells while its binding to Cdc42 inhibits activation of this GTPase. Further, using a synthetic peptide with the sequence of a CaM-binding target region in Rac1, we have identified the site for Rac1-CaM interaction. We have also confirmed this novel interaction in cells using co-immunoprecipitation. Immunocytochemical analyses have revealed distribution and localization of Rac1, Cdc42 and calmodulin as well as the potential role for calmodulin in regulating platelet

aggregation. These findings have lead us to a proposed model (**Figure 23**) which highlights calmodulin as a new interacting partner for Rac1 and Cdc42 in the thrombin-mediated PAR-1 signaling pathway known to activate small GTPases Rac1 and Cdc42 leading to platelet aggregation.

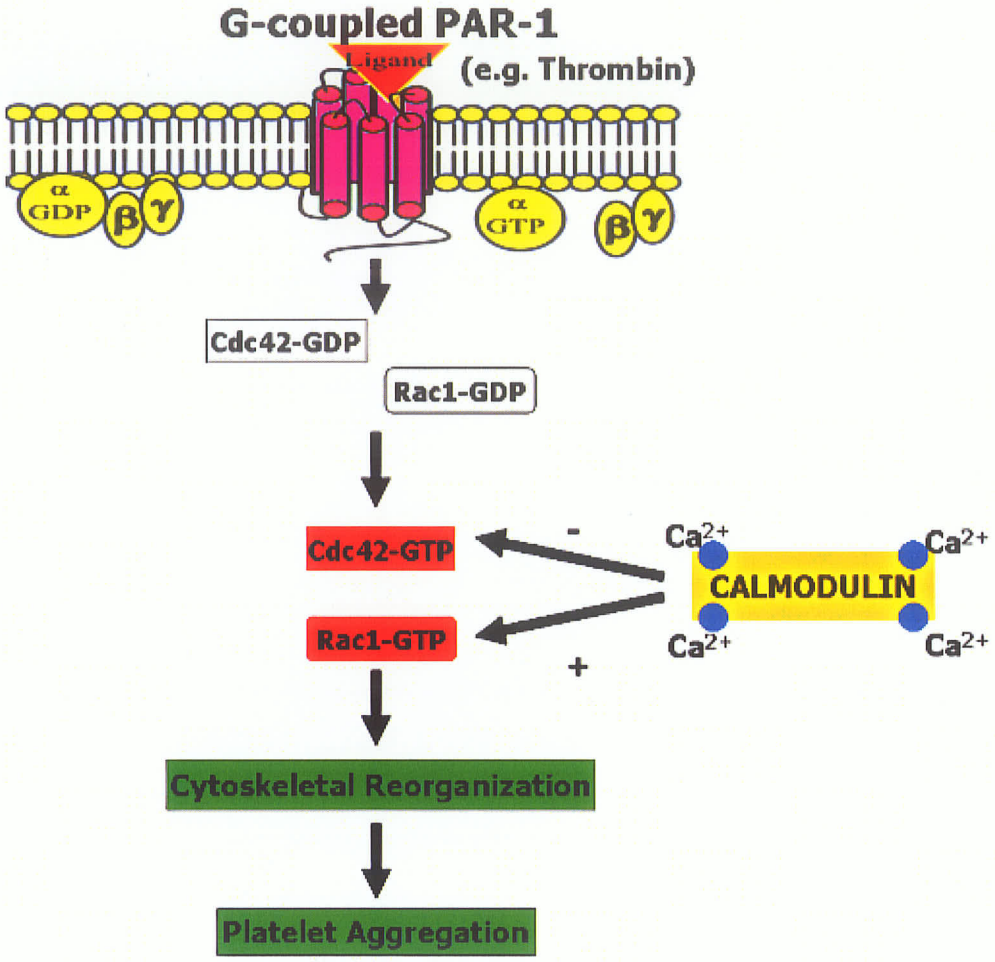

Figure 23: Proposed model for Rac1 and Cdc42 activation. Rac1 and Cdc42 are activated through G-protein coupled proteinase-activated receptor (PAR-1). Upon the binding of a ligand (an agonist) to its receptor on the cell surface, Rac1 and Cdc42 are activated. Calmodulin, a new interacting partner for Rac1 and Cdc42 binds to both proteins and regulates their activation. This may cause an alteration in cytoskeletal dynamics and platelet aggregation. The "+" indicates that CaM is required for activation and the "-" indicates that CaM inhibits the activation step.

CHAPTER 8

FUTURE DIRECTIONS

The research presented here has established calmodulin as a novel interacting partner for small GTPases Rac1 and Cdc42 that was shown to regulate their activity in cells. Further studies are still needed to characterize detailed structural and functional aspects of this regulation. The proposed Cdc42-CaM binding peptide can be synthesized and similar binding experiments can be performed to examine its potential for being a CaM-binding region. The *in vitro* binding of calmodulin to RalA has been previously shown to occur at the C-terminus (Wang *et al.* 1997; Wang and Roufogalis, 1999). Later, it was shown that calmodulin also binds to the N-terminus part of calmodulin (Clough *et al.*, 2002). Thus, it is possible that there may be another CaM-binding site on Rac1 that is located in the N-terminus. This could be investigated by using restriction enzymes, keeping the N-terminal residues and inserting them into a GST-fusion vector and carrying out *in vitro* binding assays. Similar experiments could be done with Cdc42 since there is also the potential of two CaM binding sites present as discussed earlier. It is also important to determine the exact amino acid residues in the Rac1-CaM binding peptide that make up the CaM binding domain. This could be achieved by *in vitro* mutagenesis techniques, followed by the binding assays described here. To confirm if these interactions occur *in vivo*, yeast two-hybrid system can be used.

It is of interest to try to reproduce results obtained from platelet studies using other cell lines to examine whether the proposed mechanisms are universal. In addition,

determining how this novel CaM interaction with Rac1 and Cdc42 impacts downstream pathways such as the Jak/STAT signaling pathway and cell functions such as development, differentiation, cell proliferation and survival, immune response, and oncogenesis would be of interest. The use of different Rac1 mutant mammalian vectors and their effect on downstream Jak/STAT pathway activation in presence or in absence of calmodulin would add additional data. This would provide a better understanding of mechanisms involving Rac1/Cdc42 activation and the precise role that calmodulin plays in signal pathway cascades leading to activation of cell functions.

To gain better understanding of pathways involving small GTPase signal transduction, it is fundamental to continue identifying new interacting partners. Those partners will provide us with better idea, on the role of small GTPases in regulating cell function. This research has examined a novel interaction and regulatory mechanism for Rac1 and Cdc42 activity involving calmodulin. This work opens the possibilities for continued research into several diverse areas involving small GTP binding proteins.

CHAPTER 9

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