Rac1b, a Variant of Rac1, Interacts with Calmodulin

by

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ABSTRACT

Rac1b, splice isoform of Rac1, is a member of the Rho family of small GTP-binding proteins that has been found to be up-regulated in the cancers of breast, colon and the lung. Rac1b consists of an additional 19 amino acid insertion [VGETYGKDITSRGKDKPIA] close to the switch II domain, a region important for the interaction of Rac1 with various regulators and effectors. This insertion leads to the intracellular predominance of active GTP-bound form of Rac1b and also renders it ineffective to interact with Rho guanine nucleotide dissociation inhibitors (Rho GDI's).

Previously, a 14 amino acid region [AVKYLECSALTQRG] essential for calmodulin (CaM) binding has been established in Rac1. A similar region also exists in Rac1b. In the present work, we have determined that as for Rac1, Rac1b also interacts with calmodulin in a calcium dependent manner. We have also demonstrated that Rac1b binds to calmodulin directly. However, the putative CaM binding region in the two proteins differ as the commercially synthesized CaM binding peptide for Rac1 failed to compete with Rac1b for binding to calmodulin.

In addition, using the PAK-CRIB domain in pull down assays that interacts with the GTP-bound form of Rac1b, we have established that CaM plays an important role in the activation of Rac1b. Experiments using W7, the inhibitor for CaM, revealed that activation of Rac1b in the presence of W7 is reduced in response to growth factor agonists such as Heregulin β-1 and EGF. However, it was observed that the addition of W7 has no role in *in vitro* GDP/GTP binding to Rac1b. Molecular modeling and docking studies were also carried out to predict the possible sites in Rac1b that potentially can interact with CaM.

In summary, the results presented here demonstrate that CaM interacts with Rac1b in a calcium dependent manner. Additionally, CaM plays an important role in the activation of Rac1b thus indicating a role for CaM in cancer progression.

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Finally, I would like to express my deepest appreciation and gratitude to my loving family for their continuous support, patience and inspiration.

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LIST OF ABBREVIATIONS

BMP – Bone Morphogenic Proteins

Ca^{2+} - Calcium

CaM – Calmodulin

CNBr – Cyanogen bromide

DMEM – Dulbecco's modified Eagle's medium

DNA – Deoxyribonucleotide acid

EDTA – Ethylene diaminetetraacetic acid

EGF – Epidermal growth factor

EGTA – Ethylene glycol tetraacetic acid

FBS – Fetal bovine serum

FGF – Fibroblast growth factor

GAP – GTPase activating protein

GEF – Guanine nucleotide exchange factor

GDI – Guanine nucleotide exchange factor

GDP – Guanosine-5'-diphosphate

GM-CSF – Granulocyte macrophage colony stimulating factor

GST – Glutathione S-transferase

GPCR – G-protein coupled receptors

GSH – Glutathione

GTP – Guanosine-5'-triphosphate

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

kDa – KiloDalton

KCl – Potassium chloride

LB – Luria-Bertani

MAPK – Mitogen-activated protein kinase

$MgCl_2$ – Magnesium chloride

MMP-3 – Matrix Metalloproteinase-3

NADPH – Nicotinamide adenine dinucleotide phosphate

NF-κB – Nuclear factor kappa-light chain enhancer of activated B cells

PBS – Phosphate buffered saline

PDB – Protein data bank

PDGF – Platelet-derived growth factor

PI3K – Phosphoinositide 3-kinase

PMSF – Phenylmethylsulfonyl fluoride

PVDF – Polyvinyl difluoride

RIPA – Radioimmune precipitation buffer

ROS – Reactive oxygen species

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TGF-β – Transforming Growth Factor-β

TNF – Tumor necrosis factor

VEGF – Vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

1.1. Signal Transduction

Each cell in a multicellular organism continuously responds to a variety of signals containing vital information related to development, tissue repair, immunity and homeostasis. These signals can be soluble factors that are either generated locally (e.g. synaptic transmission) or distantly (e.g. hormones and growth factors). Stimulus can also be generated through ligands that are associated on the surface of other cells or from the extracellular matrix itself. Signal transduction occurs when an extracellular signalling molecule activates a cell surface receptor, triggering intracellular events such as production of second messengers which, in turn, activate other proteins and enzymes. These events are responsible for eliciting physiological cellular responses in the body. Errors in signal transduction can lead to structural alterations and loss of control mechanisms, resulting in a diseased state.

1.2. Receptors in Signal Transduction

Receptors involved in signal transduction are broadly classified into five major families. The first family of receptors includes the cytokine receptors that play a major role in regulating the function of the haematopoietic system and in coordinating immune responses. The largest family of cytokine receptors include the haematopoietin receptors that respond to factors such as interleukins, prolactin growth hormone and granulocyte macrophage colony stimulating factor

(GM-CSF) (Uings and Farrow 2000). Examples of other cytokine receptors include receptors for interferon and tumor necrosis factor (TNF). Receptor tyrosine kinases (RTKs) form the second family of receptors that act as receptors for many polypeptide growth factors such as epidermal growth factor (EGF) and platelet derived growth factor (PDGF), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF). These receptors are crucial for regulating important cellular processes including, proliferation and differentiation, migration, cell metabolism and cell cycle control. All RTKs have an extracellular ligand binding domain, a transmembrane helix and a cytoplasmic region that are generally activated through ligand-induced dimerization (Lemmon and Schlessinger (2010).

The third class of receptors include the Ligand Gated Ion Channels (LGICs) that act as barrier to the flux of ions across the cell membrane. LGICs mediate the rapid action of neurotransmitters and transduce the action of ligand binding into an electrical stimulus. There are three families of LGICs: first family is the cationic P2X (adenosine triphosphate, ATP) receptor, second family is the glutamate –activated cationic receptors which include the NMDA (N-methyl-D-aspartate) receptors, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic-acid) receptors and kainate receptors and, the third and the largest family is the Cys-loop receptor superfamily. The Cys-loop family consists of both anionic channels such as ϒ-amino butyric acid (GABA) and glycine receptors as well as cationic channels such as nicotinic acetylcholine receptors (nAChRs), serotonin (5-HT_3) and zinc activated ZAC receptors (Hogg, Buisson et al. 2005). Integrins, the transmembrane adhesion receptors, constitute the fourth family of receptors that function to mediate cell-cell and cell-extracellular matrix adhesion. Integrins also induce bidirectional signalling across the cell membrane to regulate cell proliferation, activation,

migration and homeostasis (Hynes 2002). Extracellular matrix (ECM) proteins such as collagen and fibronectin bind to these receptors that consist of two subunits, α and β, which heterodimerize to form different combinations with varying binding specificity and signalling properties (Zhang and Wang 2012).

The fifth class of receptors is called the G protein-Coupled Receptors (GPCR) that are characterized by an N-terminal extracellular domain, seven transmembrane helices separated by loops and an intracellular C-terminal domain. GPCRs are the largest family of membrane proteins that mediate most cellular responses to hormones and neurotransmitters, as well as being responsible for olfaction, vision and taste. GPCRs in vertebrates are commonly divided into five families on the basis of their sequence and structure: rhodopsin (family A), secretin (family B), glutamate (family C), adhesion and Frizzled/Taste (Rosenbaum, Rasmussen et al. 2009). Ligand binding to GPCRs on the extracellular side induces a conformational change in the receptor, leading to the activation of bound heterotrimeric G-proteins on the intracellular side. These activated heterotrimeric G-proteins trigger intracellular signaling cascades.

In addition to these, members of Transforming Growth Factor-β (TGF-β) superfamily constitute another important class of signal transducing receptors. The members of this family including TGF-β, activin, nodal and Bone Morphogenic Proteins (BMP's) regulate vital processes such as cell proliferation, differentiation, apoptosis, cell plasticity and migration (Huang and Chen 2012). Binding of TGF-β to its serine-threonine kinase receptors leads to the activation of Smad transcription factors resulting in gene transcription. TGF-β signaling can also activate other serine-threonine kinases including mitogen-activating protein kinases (MAPK),

phosphatidylinositol 3′-kinase, calcium-calmodulin dependent kinases, rho-associated protein kinases and others (Kamato, Burch et al. 2013). Disruption in the activity of these receptors has been implicated in various human diseases, including cancer.

1.3. G Proteins

Guanine nucleotide binding (G) proteins are a family of proteins involved in the transmission of signals generated through various stimuli acting on the exterior of a cell to its interior. These signal transducing proteins are involved in the regulation of many cellular processes, ranging from translation to exocytosis. G proteins generally function as a molecular switch and undergo two alternate conformations – an active GTP-bound conformation and an inactive GDP-bound conformation. The hydrolysis of GTP and the release of an inorganic phosphate (P_i) results in the relaxation of the protein into an inactive GDP-bound state. This structural change accompanied by the dissociation of P_i ensures that high affinity interactions with effector molecules are optimal in the triphosphate state (Gasper, Meyer et al. 2009). The superfamily of G proteins consists of several families including translational factors, heterotrimeric GTP-binding proteins, protooncogenic *ras* proteins, other signal recognition particle and its receptor as well as P-loop ATPases (Leipe, Wolf et al. 2002, Gasper, Meyer et al. 2009).

Signal transducing GTP-binding proteins are classified into two major groups. The first group is the high molecular weight or the heterotrimeric GTP-binding proteins (G proteins), consisting of three subunits α, β, and ϒ. The second group is the low molecular weight or the monomeric

GTP-binding proteins known as the Ras-like or small G-protiens. My research focus is on the small monomeric G-proteins.

1.4. Small GTP-binding Proteins

Small GTP-binding proteins are monomeric G-proteins that comprise over 150 members with molecular mass between 20-40 kDa (Konstantinopoulos, Karamouzis et al. 2007). The Ras superfamily of small G-proteins can structurally be classified into five subfamilies: Ras (Ras sarcoma oncoproteins), Rho (Ras homologous proteins), Rab (Ras-like proteins in brain), Sar1/Arf (ADP-ribosylation factor family proteins) and Ran (Ras-like nuclear proteins) (Wennerberg, Rossman et al. 2005, Konstantinopoulos, Karamouzis et al. 2007, Rojas, Fuentes et al. 2012). These proteins regulate diverse intracellular pathways including transmembrane signal transduction, cytoskeletal reorganization, gene expression, intracellular vesicle trafficking and nucleocytoplasmic transport.

1.5. MCF7 cells as the Model System

MCF7 is an epithelial cancer cell line, derived from the breast adenocarcinoma. It is an invasive breast ductal carcinoma cell line that was derived by pleural effusion from a breast cancer patient. It is widely used for *in vitro* breast cancer studies due to its retention of several characteristics particular to the mammary epithelium, including the ability to process estrogen. It contains both estrogen and progesterone receptors.

In this study, all the experiments have been performed using MCF7 as the cell model. Rac1b, an isoform of the small G-protein Rac1, is up-regulated in breast cancer (Schnelzer, Prechtel et al. 2000) which makes MCF7 cell line as the ideal model for studying the role of calmodulin in Rac1b function.

Chapter 2

LITERATURE REVIEW

2.1. The Ras Superfamily

RAS genes were initially discovered in the rodent genome as the viral genes with high oncogenic properties. This led to the study of these genes, their cellular counterparts, and the 21 kDa (p21) Ras proteins encoded by these genes (Cox and Der 2010). The Ras superfamily of small GTP-binding proteins (small GTPases) consists of a large group of highly conserved proteins that function as molecular switches by alternating between an inactive GDP-bound and an active GTP-bound state. The molecular switching between the inactive and active states is coupled with signal transduction to downstream effectors and is regulated by other set of proteins that comprise of the guanine nucleotide exchange factors (GEFs) and the GTPase activating proteins (GAPs) (Bos, Rehmann et al. 2007). Members of the Rho and the Rab families are also influenced by the guanine nucleotide dissociation inhibitors (GDIs) (Csepanyi-Komi, Levay et al. 2012). In addition, the small GTPases undergo post-translational modifications for protein-protein interactions and membrane localization (Konstantinopoulos, Karamouzis et al. 2007).

GEFs catalyze Ras activation by modifying the nucleotide binding site present between the switch I and switch II of the protein resulting in the replacement of GDP for GTP. The active GTP-bound Ras thus, binds and activates several downstream effectors and regulates important cellular functions. On the other hand, GAPs accelerate the slow intrinsic GTPase hydrolysis

activity by stabilizing glutamine residue 61 and insertion of an arginine residue in the active state to stabilize the transition state of GTP-hydrolysis reaction (Bos, Rehmann et al. 2007) (Figure 1).

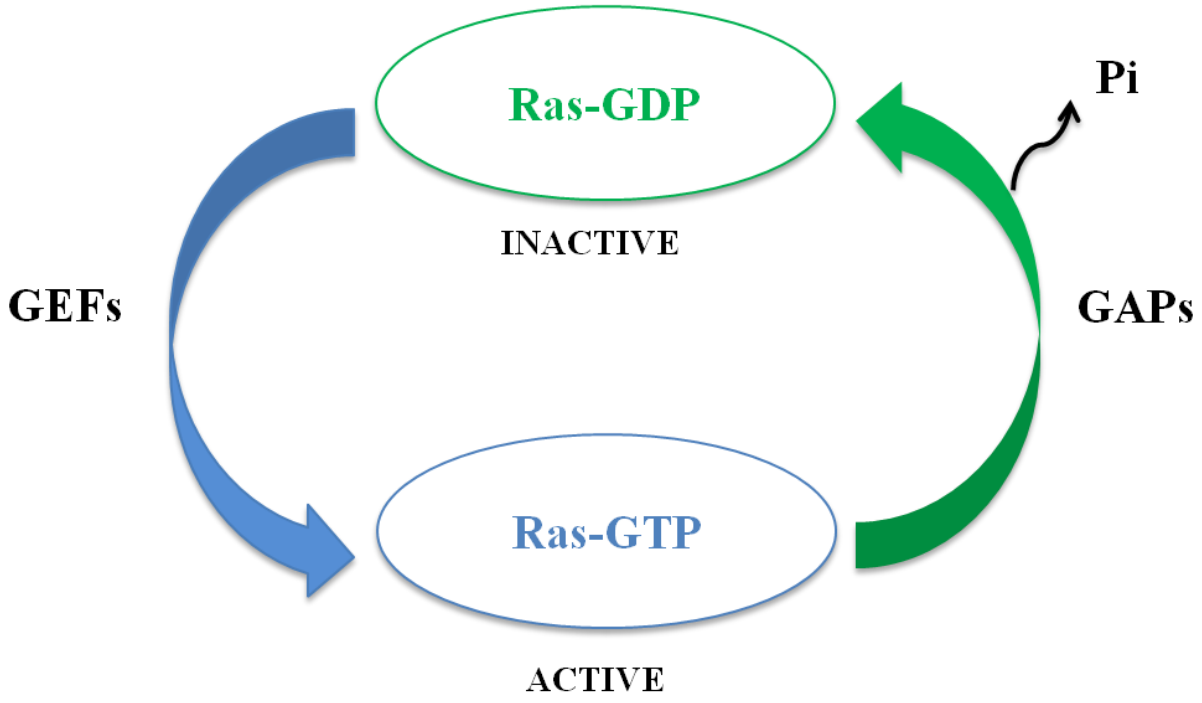

Figure 1: **Schematic diagram of the GTPase cycle of Ras proteins.** The cycling of the Ras proteins between the inactive GDP-bound and the active GTP-bound states is regulated by guanine nucleotide exchange factor (GEF) and the GTPase activating protein (GAP). GTP loading is catalyzed by GEFs whereas GAPs accelerate the hydrolysis of GTP to GDP.

2.2. Structure, Classification and Function of Ras GTPase

The Ras superfamily of proteins is classified into five major subfamilies that include the Ras, Rho, Rab, Arf and Ran families. These proteins function as regulators of important biological processes including transmembrane signal transduction (Ras), cytoskeletal reorganization (Rho), gene expression (Ras, Rho), intracellular vesicle trafficking (Rab), microtubule organization (Ran) and nucleocytoplasmic transport (Ran) (Konstantinopoulos, Karamouzis et al. 2007). Despite their functional diversity, these proteins exhibit high sequence similarity and a conserved ~ 20kDa G-domain that binds and hydrolyzes the nucleotide. The G-domain fold consists of six-stranded β sheet and five α helices and five highly conserved polypeptide loops (G1-G5) (Figure 2). The G1 loop (P-loop) connecting the β1 strand to the α1 helix is responsible for the binding of the α- and β-phosphate groups whereas the G2 loop connecting the α1 helix to the β2 strand, contains a conserved Thr residue for Mg^{2+} binding. The G3 loop provides residues for Mg^{2+} and ϒ-phosphate binding and the G4 and G5 loops recognize the guanine base (Paduch, Jelen et al. 2001).

A majority of Ras family proteins are subjected to lipid modifications to promote membrane localization and regulate protein interactions. The most common post-translational modification occurs at the carboxyl (C-terminal) end, where linkage of the 15 carbon atom farnesyl or the 20 carbon atom geranylgeranyl group occurs via thioester bond to a cysteine residue (Csepanyi-Komi, Levay et al. 2012). In Ras and Rho GTPases, prenylation occurs at the C-terminal CAAX box where the prenylated cysteine is followed by two aliphatic amino acids (A) and a non-specific amino acid (X). Prenylation is followed by the cleavage of tripeptide AAX motif and methylation of the C-terminal cysteine (de la Vega, Burrows et al. 2011). In Ral, in addition to

the membrane localization of protein these post translational modifications have also been shown to be important for calmodulin binding (Sidhu, Elsaraj et al. 2005).

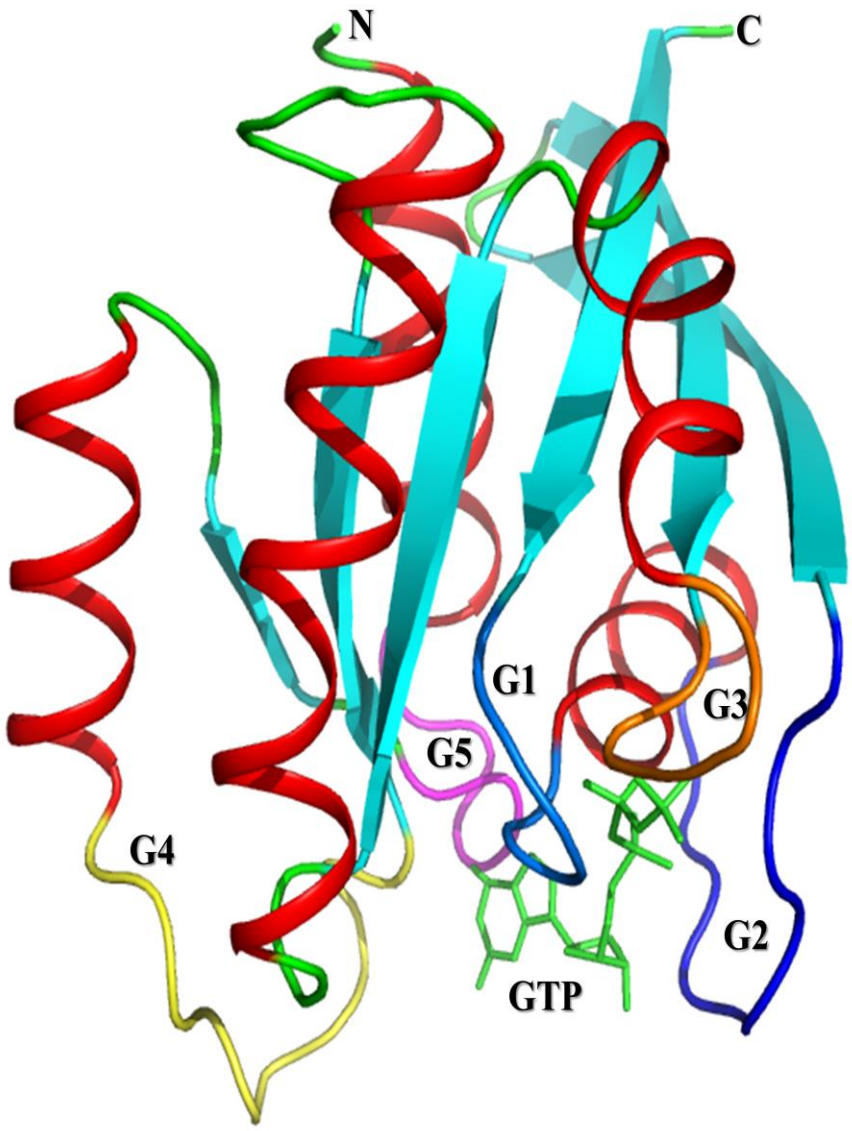

Figure 2: **Representation of the crystal structure of Ras GTPase.** This structure of Ras GTPase (PDB ID: 5P21) depicting the G-domain was created using the PyMOL software.

2.3. The Rho Protein Family

The Rho (Ras homologous) subfamily of proteins consists of more than 20 proteins in humans that show high conservation among their G1 to G5 loops. Rho proteins regulate important cellular functions including vesicle transport, microtubule dynamics, cell cycle progression and gene expression (Heasman and Ridley 2008). The Rho GTPases can be classified into eight subfamilies based on their amino acid sequence similarities. The members of the Rac, Cdc42, Rho and Rif families are classified as the classical Rho GTPases while others are the atypical Rho GTPases, that are predominantly GTP-bound (Heasman and Ridley 2008). Rho, Rac and Cdc42 proteins are the most widely studied members of the Rho GTPase family and regulate the assembly of actin-myosin filaments, lamellipodia and filopodia formation respectively (Bishop and Hall 2000). Cdc42 regulates endocytosis, transport between the endoplasmic reticulum and Golgi apparatus, post-Golgi transport and exocytosis whereas RhoA regulates endocytosis through its downstream target RhoA kinase (Chi, Wang et al. 2013).

Table 1: Rho GTPase family[1]

Classical Rho GTPases				Atypical Rho GTPases			
Rac1	Cdc42	RhoA	RIF	CHP	RhoH	RhoBTB1	RND1
Rac2	TC10	RhoB	RhoD	WRCH1		RhoBTB2	RND2
Rac3	TCL	RhoC					RND3

[1] Heasman S.J. *et al* 2008

Rho GTPases share a common G-domain fold, that consists of a six-stranded β-sheet surrounded by α-helices. The major structural differences between the GDP- and the GTP-bound forms of human RhoA are confined to switch I (residues 28-44) and switch II (residues 62-69) that correspond to residues 32-38 (switch I) and residues 59-97 (switch II) in the small GTPase, Ras. Hydrogen bonds between the ϒ-phosphate group and amide groups of Thr 37 (switch I) and Gly (switch II) stablilize the conformations of these switches in the GTP-bound form of RhoA. Removal of magnesium ions (Mg^{2+}) causes conformational changes in the switch I region of RhoA resulting in the opening of the nucleotide-binding site (Hakoshima, Shimizu et al. 2003).

2.4. Rho GTPases: Rac1 and Rac1b

2.4.1. Rac1

Rac1 is a member of the Rho family of small GTP-binding proteins that plays an important role in cytoskeletal remodelling and cell growth. On activation, Rac1 can interact with several downstream effectors to trigger various cellular responses including NADPH oxidase activation, secretory processes, phagocytosis of apoptotic cells, epithelial cell polarization, formation of cortical actin-containing membrane ruffles and lamellipodia, and induction of gene expression (Kumar, Rajendran et al. 2013). 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. The three Rac isoforms have different expression patterns, despite their high sequence similarity (80 – 90%). Rac1 is the best studied member of this family and is ubiquitously expressed, whereas Rac2 expression is mostly restricted to cells of haematopoietic origin and Rac3 is most abundant in the brain (Heasman and Ridley 2008).

Similar to the rest of the Rho family of small GTP-binding proteins, Rac1 cycles between the active and the inactive states and this cycling is controlled by GEFs and GAPs. In addition, Rho-GDP dissociation inhibitors (Rho-GDIs) can regulate Rac1 activity by sequestering the protein in the cytoplasm (Matos, Collard et al. 2003). Rac1 consists of three functional regions that include switch I (Figure 3). Switch I is the effector region that interacts with downstream effectors such as IQGAP1 and the proteins of NADPH complex whereas switch II is the region where interactions with the GEFs occur for the activation of Rac1. The insert region, a characteristic of Rho GTPases, is located between the β-strand 5 and the α-helix 4 (Kumar, Rajendran et al. 2013) and is essential for mitogenesis, apoptosis and regulating interactions with the downstream effectors.

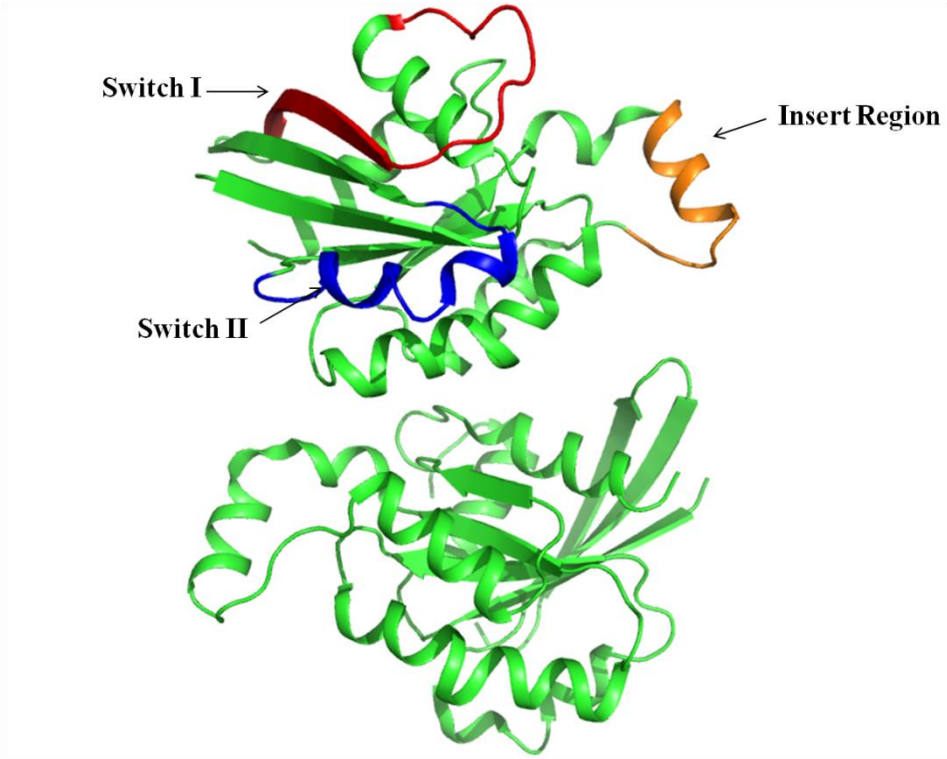

Figure 3: **Representation of Switch I (residues 26-45), Switch II (residues 59-74) and the Insert region (residues 124-135) in Rac1**. (Kumar, Rajendran et al. 2013). This structure of Rac1 (PDB ID: 3TH5) was created using the PyMOL software.

2.4.2. Rac1b

Rac1b, a splice isoform of Rac1, was first identified in human skin and the epithelial tissue of intestinal tract (Orlichenko, Geyer et al. 2010). Rac1b is found to be up-regulated in malignant colorectal cancer (Jordan, Brazao et al. 1999), breast cancer (Schnelzer, Prechtel et al. 2000) and lung cancer (Zhou, Licciulli et al. 2013). It contains a 19-amino acid insertion, not found in Rac1 resulting from the inclusion of exon 3b, containing 57 nucleotides between codons 75 and 76 (Singh, Karnoub et al. 2004) (Figure 4). This insertion in Rac1b is immediately behind the switch II region (residues 60-76) that greatly reduces its intrinsic GTPase activity and thus Rac1b is found preferentially in a GTP-bound active form in the cells (Figure 5).

The switch I and II regions in small GTPases in addition to being important regions for the alternation between the inactive and active forms of these proteins, also act as contact points for various effectors such as GEFs and GAPs. The Rac1b insertion is located between the loop L5-sequence ^{71}SYPQT^{75} that marks the end of the switch II region and the sequence ^{77}VFLICFS^{83} that constitutes the β4-strand and coincides with the beginning of exon 4 (Jordan, Brazao et al. 1999). Unlike Rac1, it does not interact with Rho-GDI and thus, most Rac1b remains in the active form bound to the plasma membrane (Matos, Collard et al. 2003) and allowing its interaction with effectors

Rac1

mqaikcvvvg dgavgktcll isyttnafpg eyiptvfdny sanvmvdgkp vnlglwdtag qedydrlrpl sypqtdvfli cfslvspasf envrakwype vrhhcpntpi ilvgtkldlr ddkdtieklk ekkltpityp qglamakeig avkylecsal tqrglktvfd eairavlcpp pvkkrkrkcl ll

Rac1b

mqaikcvvvg dgavgktcll isyttnafpg eyiptvfdny sanvmvdgkp vnlglwdtag qedydrlrpl sypqtvgety gkditsrgkd kpiadvflic fslvspasfe nvrakwypev rhhcpntpii lvgtkldlrd dkdtieklke kkltpitypq glamakeiga vkylecsalt qrglktvfde airavlcppp vkkrkrkcll l

Figure 4: **Amino acid sequences of small GTPases Rac1 and Rac1b.** Rac1 and Rac1b consist of 192 and 211 amino acids, respectively. The region in green represents the additional 19 amino acid insertion in Rac1b. The regions in red represent the previously identified CaM-binding region in Rac1 (Elsaraj and Bhullar 2008).

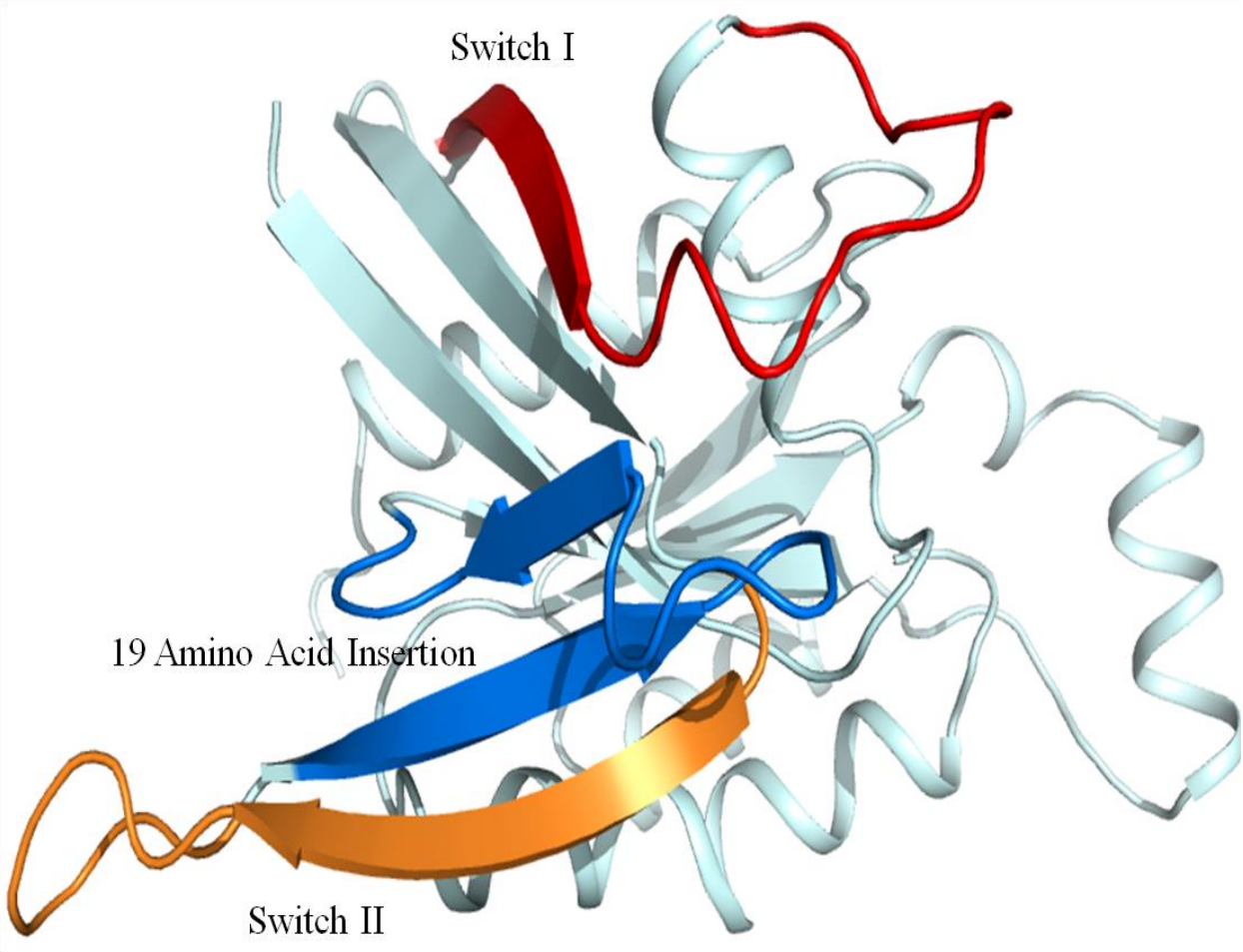

Figure 5: **Representation of the switch I, switch II regions and the 19 amino acid insert regions in Rac1b**. This structure of Rac1b (PDB ID: 1RYF) was created using the PyMOL software.

Previous studies have identified biochemical and signaling properties of Rac1b that are distinct from or overlap with Rac1. Both proteins are identical with respect to the C-terminal geranyl-geranylation motif CLLL that confers plasma membrane anchorage as well as the hydrophobic and polybasic motif that mediates interaction with lipid products of PI-3 kinase (Jordan, Brazao et al. 1999). Similar to Rac1, Rac1b can stimulate NFκB, Akt and ROS production (Nimnual, Taylor et al. 2010). When assessed in cultured cells, Rac1b has been found to have many of the properties of GTPase-defective Rac1 mutants. Exogenously expressed Rac1b promotes density- and anchorage- independent cell growth of NIH 3T3 cells, cell survival and cell cycle progression in mouse fibroblasts, and dishevelled-3-mediated detachment of colorectal cancer cells (Orlichenko, Geyer et al. 2010). However, Rac1b also shows distinct activities from Rac1. Rac1b displays a faster GEF-independent GDP/GTP exchange rate, an impaired GTPase activity and an inability to bind Rho GDI (Fiegen, Haeusler et al. 2004). Unlike Rac1, Rac1b does not promote cadherin-dependent disassembly of adherens junctions in keratinocytes, it does not induce cytoskeletal rearrangements in colorectal cancer cells as effectively as GTPase defective mutants of Rac1 and it is not as effective as Rac1 for activation of RelB (Orlichenko, Geyer et al. 2010). Rac1b is also defective in activation of two major downstream effectors of Rac1, p21-activated kinase (PAK) and c-Jun kinase (Jnk) (Matos, Collard et al. 2003). Rac1b has been shown to mediate an MMP-3-epithelial to mesenchymal transition in cultured cells, through the induction of reactive oxygen species (Radisky, Levy et al. 2005). Additionally, Rac1b has been identified to negatively regulate the activity of Rac1. The expression of Rac1b in HeLa cells interferes with the activation of Rac1 by PDGF, reduces the membrane-bound Rac1 and promotes an increase in Rho activity (Nimnual, Taylor et al. 2010).

2.5. Activation of Rac1b

The high GTP-GDP cycling rate, the inability to interact with Rho-GDI, results in the high activation state of Rac1b and its preferential binding to the plasma membrane. Due to its high activation status, even low amounts of endogenous Rac1b can contribute to the Rac-mediated downstream signaling mechanisms through selective interaction with Rac effector proteins. For example, Tiam1, an activator of Rac1 equally binds to and activates both Rac1 and Rac1b whereas, Bcr-GAP accelerates the intrinsic GTPase activity of both proteins (Matos, Collard et al. 2003). Rac1b does not activate full length PAK but binds to the PAK-CRIB domain in the active GTP-bound state. PAK-CRIB is the p-21 activated kinase 1 protein (PAK) that includes the highly conserved CRIB (Cdc-42/Rac-interactive binding) motif (Matos, Collard et al. 2003) that is used for pulling out the active form of Rac1.

The receptor for Epidermal Growth Factor (EGF) and related ligands (EGFR) is a widely expressed protein that plays an important role in regulating diverse biological responses, such as proliferation, differentiation, cellular motility and survival. The ErbB family of receptors, is a member of the receptor tyrosine kinase superfamily that consists of EGFR (HER1/ErbB1), HER/neu2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4) (Kasza 2013). These receptors are involved in the regulation of various cellular functions including cellular proliferation, differentiation, apoptosis, cell polarity as well as cell motility. Rac can be activated through GEFs, growth factors (EGF, platelet derived growth factor, hepatocyte growth factor) and G protein-coupled receptor ligands (sphingosine-1-phosphate, bombesin) (Wertheimer, Gutierrez-Uzquiza et al. 2012). Heregulins are a group of EGF-like ligands for the ErbB3 and ErbB4 receptors that are often expressed in breast cancer tissues (Dunn, Sinha et al. 2004, Kim, Han et al. 2012). Heregulins activate PI3K-Akt, Erk mitogen activated protein kinase (MAPK) in breast

cancer cells and also promote changes in cytoskeletal reorganization. In addition, MAPK and Akt have been shown to phosphorylate the estrogen receptor (ER), thereby resembling the effects of estrogenic activity (Nagashima, Suzuki et al. 2008). In breast cancer cells, heregulin β-1 (HRG) causes a strong activation of Rac mediated through ErbB2, ErbB3 and also by transactivation of EGFR (Yang, Liu et al. 2006). Thus, EGF and Hergulin β-1 were used in this study to understand the role of calmodulin in the regulation and activation of Rac1b, in the ER positive breast cancer line, MCF7.

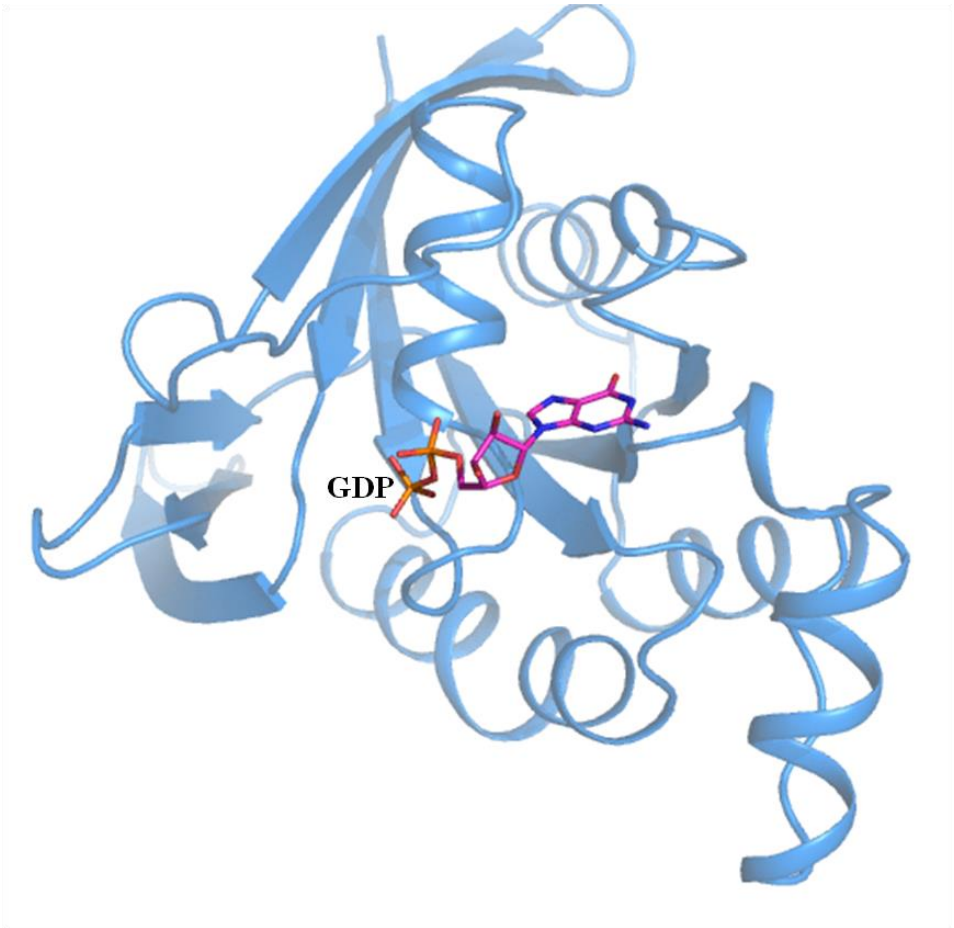

Figure 6: **Representation of the crystal structure of the inactive GDP-bound form of Rac1b** (PDB ID: 1RYF). The GDP region in the complex is represented in the form of sticks. This structure was created using the PyMOL software.

2.6. Calcium and Calcium Binding Proteins

Ca^{2+} ions are ubiquitous second messengers for a variety of cellular signaling pathways. By mass, Ca^{2+} represents the most abundant mineral in the human body. The intracellular Ca^{2+} ion concentration is maintained within the narrow range of 10^{-7} to 10^{-8} M, despite its much higher extracellular concentration of about 10^{-3} M (Zhou, Xue et al. 2013). This major gradient difference across the plasma membrane enables the large influx of Ca^{2+} ions in response to signaling events. In eukaryotic cells, Ca^{2+} functions as a universal and versatile signal with the ability to alter protein conformations and local electrostatic fields over a wide range of affinities (nM to mM). Ca^{2+} ions participate in the regulation of various biological processes, including muscle contraction, cell motility, hormonal secretion, neurotransmitter release, memory formation, endocytosis and exocytosis through proteins that bind calcium. By modulating the activity of these calcium binding proteins, Ca^{2+} ions are thus able to impact nearly every aspect of cellular life.

2.6.1. Calmodulin

Calmodulin (Calcium Modulated Protein, CaM), is a highly conserved 16.7 kDa ubiquitous calcium sensor protein, found in all eukaryotic organisms. CaM mediates a wide range of cellular processes including cell division and differentiation, gene transcription, DNA synthesis, membrane fusion and muscle contraction (Yanez, Gil-Longo et al. 2012). It is a highly acidic protein (pI approx 4.6) that binds to at least 300 different target proteins thereby regulating many intracellular events. These target proteins can be classified into Ca^{2+} dependent, Ca^{2+} independent, and Ca^{2+} inhibited proteins (Yamniuk and Vogel 2004, Xu, Chelikani et al. 2012).

Some target proteins recognize its Ca^{2+} -bound form (holo CaM), whereas others are modulated by its Ca^{2+} -free form (apo CaM) (Kortvely and Gulya 2004). The transition from apo- to Ca^{2+} -calmodulin involves significant structural changes in the protein. CaM as a cellular Ca^{2+} sensor regulates its target proteins through three main mechanisms: relief of auto-inhibition, active site remodeling or dimerization of target domains (Hoeflich and Ikura 2002, Macdougall, Wachten et al. 2009).

2.6.1.1 Structural Features of Calmodulin

Calmodulin structure has an overall length of 65 Å and consists of two globular domains, seven α-helices, four Ca^{2+} -binding EF domains (helix-loop-helix) and two short double-stranded antiparallel β-sheets between the pairs of adjacent Ca^{2+} -binding loops. The structure of calmodulin can be divided into four homologous segments with the first (amino terminal, residues 8 to 40) domain resembling most to the third (residues 81 to 113) and the second domain (residues 44 to 76) resembling most to the fourth (carboxy terminal, residues 117 to 148) (Rashid, Khurshid et al. 2004). Each of these four domains consists of two α-helices and a Ca^{2+} binding EF hand domain. The fourth and the fifth α-helices combine to form a long flexible "central helix" (residues 74-82) that reorients when calmodulin binds to other target proteins.

The two globular Ca^{2+}-binding domains (the C- and N- terminal domains), each containing the two EF hand motifs and separated by the helical central linker region combine to form a dumbbell-like shape (Rashid, Khurshid et al. 2004). Upon binding of calcium, conformational changes occur resulting in the opening of the central helical structure in between the two domains (Figure 7). As a result, the methionine-rich hydrophobic binding sites are exposed,

which then allows target proteins to interact with calmodulin (Rashid, Khurshid et al. 2004, Yamniuk and Vogel 2004). Each globular domain consists of two such sites, with the sites in C-terminal domain having higher affinity for Ca^{2+} than the N-terminal sites (Chin and Means 2000).

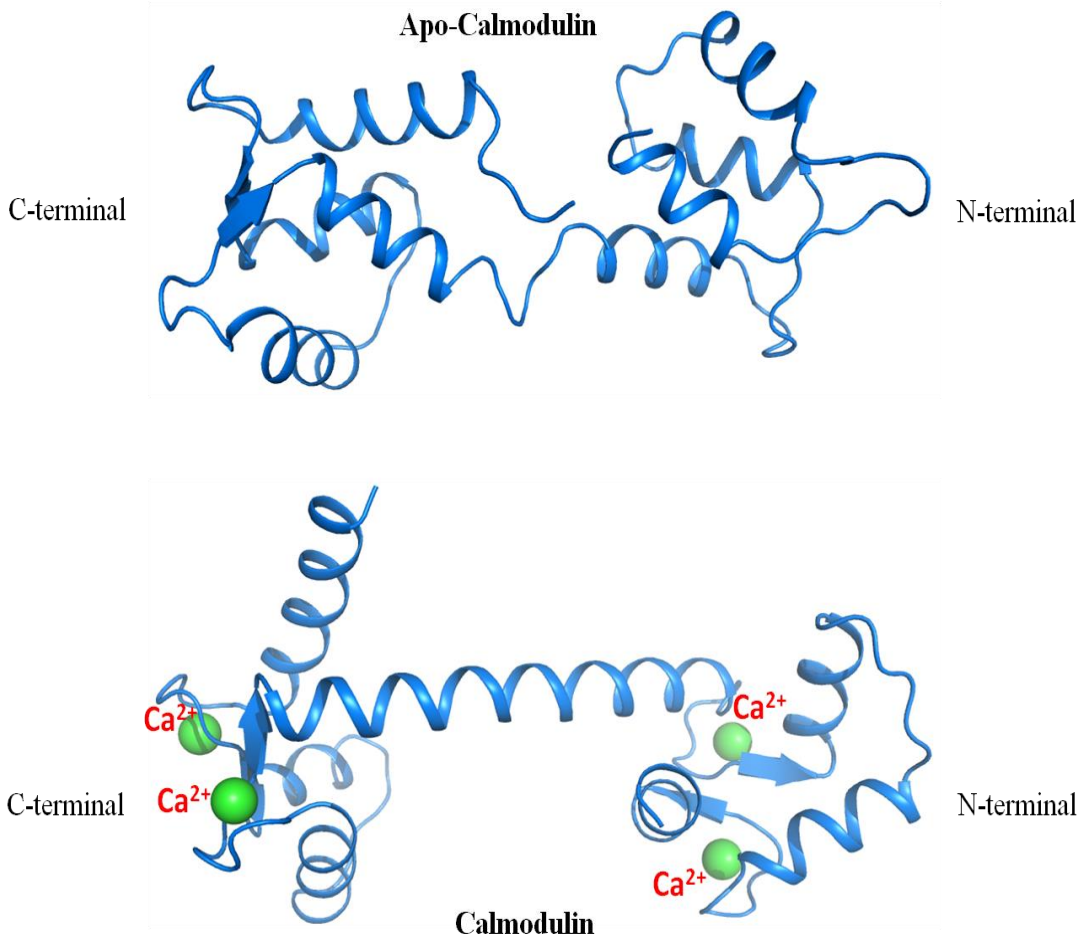

Figure 7: **Representation of the structures of calcium-free calmodulin (Apo-Calmodulin; PDB ID: 1CFD) and calcium-bound calmodulin (Holo-Calmodulin; PDB ID: 1CLL).** The calcium-bound calmodulin contains four calcium ions, represented in green spheres. These structures were created using the PyMOL software.

2.6.1.2. Calmodulin-Binding Motifs

Calmodulin is a highly conserved protein however, Ca^{2+}/CaM-binding domains of its target proteins exhibit low sequence homology. Based on the position of conserved hydrophobic residues in its target, CaM binding domains have been classified into distinct recognition motifs.

Table 2: Calmodulin-binding Motifs[2]

Motifs	Common Characteristics
1 - 10	• The two key bulky hydrophobic residues are spaced 8 residues apart (FILVW)xxxxxxxx(FILVW). • Some have additional anchoring residues in the middle (1-5-10). • Binds CaM in the presence of Ca^{2+}
1 - 14	• The two key bulky hydrophobic residues are spaced 12 residues apart (FILVW)xxxxxxxxxxxx(FILVW). • Some have additional anchoring residues in the middle (1-5-8-14 or 1-8-14). • Binds CaM in the presence of Ca^{2+}
1 - 16	• The key bulky hydrophobic residues are spaced 14 residues apart (FILVW)xxxxxxxxxxxxxx(FILVW). • Atypical binding orientation. • Binds CaM in the presence of Ca^{2+}
IQ	• (FILV)Qxxx(RK)Gxxx(RK)xx(FILVWY) where x is any amino acid. • Binds CaM in the absence of Ca^{2+} • Amphipathic character with a hydrophobic and basic faces. • Interacts with both apo-CaM and Ca^{2+}-CaM.
Others	• Sequences in this group do not belong to any of the above motifs. • Includes 1-12 motif in which the hydrophobic residues are separated by 10 residues and the basic motif which contains several lysine or arginine residues in the calmodulin binding sequence. • Binds CaM in the presence of Ca^{2+}

[2] http://calcium.uhnres.utoronto.ca/ctdb/ctdb/browse.html

These CaM-binding sequences usually vary between 15-30 amino acids in length and have a tendency to form amphipathic α-helices with a hydrophobic and basic face (Yap, Kim et al. 2000, Sidhu, Clough et al. 2005). The motif that mainly binds CaM in the absence of calcium is called IQ motif. IQ motif regions are 20-25 residues long, have an amphipathic character with hydrophobic and basic face, and thus can interact with both apo-CaM and Ca^{2+}/CaM (Bahler and Rhoads 2002, Sidhu, Clough et al. 2005). Examples of IQ motif-containing proteins that bind CaM in a Ca^{2+}-dependent manner include utrophin, Ras GRF1 (Ras guanine nucleotide-releasing factor), Nina C myosins, calcium vector target protein and others (Bahler and Rhoads 2002).

2.6.1.3. Role of Calmodulin in G-protein Regulation

Calmodulin has been shown to interact with and regulate the activity of several small G proteins. For example, calmodulin binds and regulates the thrombin-mediated activation of RalA, RalB and Rac1 in platelets as well as the activation of Rac1 in CHRF-288-11 cells (Clough, Sidhu et al. 2002, Elsaraj and Bhullar 2008, Xu and Bhullar 2011). However, in platelets the binding of CaM to Cdc42 has been shown to have an inhibitory role indicating that CaM in platelets acts to maintain Cdc42 in the inactive state (Elsaraj and Bhullar 2008). A similar inhibitory mechanism for CaM has been observed in the case of K-RasB (Villalonga, Lopez-Alcala et al. 2001, Sidhu, Clough et al. 2003). CaM is involved in the dissociation of RalA from synaptic vesicles in a Ca^{2+}-dependent manner and also the dissociation of K-RasB from platelets and MCF7 cell membrane (Park, Lee et al. 1999, Sidhu, Clough et al. 2003). CaM also binds to Rab3A and Rab3B, plays a role in its dissociation from the membrane and also regulates its function (Sidhu, Clough et al. 2003). In addition, studies have also demonstrated the role of calmodulin in

neuronal signaling pathways through its interaction with the endogenous small GTPase Rin (Hoshino and Nakamura 2003).

2.7 Hypothesis

Calmodulin interacts and regulates the activity of several small GTPases. Previous studies have shown that calmodulin binds and regulates the activation of Rac1 in a Ca^{2+}-dependent manner, and that this activation is inhibited in the presence of calmodulin antagonists. However, no information exists if calmodulin also binds and regulates the activity of Rac1 isoform, Rac1b. Since, Rac1b has similar sequence to Rac1 with an additional 19 amino acid insertion and exhibits the previously established CaM-binding region in Rac1 has led us to test the hypothesis that CaM interacts with and regulates the activity of Rac1b.

Chapter 3

RESEARCH OBJECTIVES AND EXPERIMENTAL APPROACH

Previous studies in the literature have shown that Calmodulin (CaM) can interact with and regulate the activity of several small GTPases (Clough *et al*., 2002; Villalonga *et al*., 2002; Sidhu and Bhullar, 2001; Wang *et al*., 1997). It has also been established that the members of the Rho family of small GTPases including Rac1 are regulated by calmodulin. The interaction between Rac1 and CaM has been shown to be direct. Using CaM database analysis and *in vitro* peptide competition assays a 14 amino acid region crucial for CaM binding has been established in Rac1(Elsaraj and Bhullar, 2008). Rac1b, a splice isoform of Rac1 has the same amino acid region. However, no information exists if CaM also plays an important role in the regulation of Rac1b activity and led to our current hypothesis.

The hypothesis of the current work is that calmodulin interacts with and regulates the activation of Rac1b.

Specific Objectives

I. Investigate binding of calmodulin to Rac1b.

   a. To determine whether the binding of calmodulin to Rac1b is direct by conducting *in vitro* binding experiments using purified recombinant proteins.
   b. To investigate whether calmodulin binding to Rac1b is calcium dependent.

c. To establish whether the binding of Rac1b is specific to calmodulin using calgranulin B, another Ca^{2+} binding protein.

II. To identify the possible CaM interacting residues in Rac1b.

a. To determine if the CaM binding region is the same in Rac1 and Rac1b.
b. To illustrate whether the established Rac1-CaM binding site can be used to inhibit binding of endogenous Rac1b to calmodulin.

III. To establish whether calmodulin binding regulates Rac1b activation in MCF7 cells.

a. To illustrate effects of calmodulin inhibition on Rac1b activation.
b. To study the role of CaM in *in vitro* GDP/GTP binding to Rac1b.

Chapter 4

MATERIALS AND METHODS

3.1. MATERIALS

Sepharose 4B coupled Calmodulin (Cat# 17-0529-01), cyanogen bromide (CNBr)-activated Sepharose beads (Cat# RPN2132), Hybond PVDF transfer membrane (Cat# RPN303F) and Hyperfilm^{TM} ECL (Cat# RPN 2232) were purchased from Amersham Biosciences. Trypsin, fetal bovine serum (FBS), calmodulin from bovine testes (Cat# P1431), glutathione agarose (Cat# G4510), GTP and GDP (Cat# G8634 and G7637) and anti-rabbit polyclonal antibody (Cat# A-5060) were purchased from Sigma-Aldrich. W7-HCl (Cat# 681629), W5-HCl (Cat# 681625), monoclonal anti-calmodulin antibody (Cat# 05-173) and polyclonal Rac1b antibody (Cat# 09-271) were purchased from EMD Millipore. Dulbecco's Modified Eagle Medium (DMEM, Cat# 11995), penicillin-streptomycin (Cat# 15140) were purchased from Invitrogen. Triton X-100 (Cat# 161-0404), prestained SDS-PAGE low range molecular weight markers (Cat# 1610305) were purchased from BioRad Laboratories. 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. Restriction enzymes and molecular biology reagents were from New England Biolabs, Invitrogen.

3.2. METHODS

3.2.1. Preparation of LB (Luria-Bertani)-ampicillin plates

Petri dishes for growth of *E. coli* were prepared by dissolving 20 g of LB-agar in 500 ml of distilled water. The media was autoclaved for 45 min. Following autoclaving, the media was allowed to cool for approximately 30 min at RT with constant stirring at a slow speed on stirrer/hot plate (Corning PC-320). 500 µl of 100 mg/ml stock solution of ampicillin (final concentration 0.1 mg/ml) was added, the LB-Agar was poured into sterile petri dishes (20-25 ml/plate), and allowed to solidify for 4-5 hours at RT. The plates were stored at 4°C until use.

3.2.2. Transformation of competent *E. coli*

For transformation with plasmids, a vial of frozen competent *E. coli* cells was thawed on ice. 50 µl of the cells were transferred to a 1.5 ml eppendorf tube and 0.1 µg (1-5 µl) of the plasmid was added to the tube. The mixture was incubated on ice for 30 min. The cells were further incubated at 42°C for 30 seconds, cooled on ice for 2 min and then 500 µl of SOC media was added to the cells. The tube was shaken gently for 1 hour at 37°C. A momentary spin was given and 100 µl of the mixture was spread on the LB plates and colonies were allowed to form overnight at 37°C. For plasmid miniprep, few selected colonies were inoculated in 5 ml of LB media containing 5 µl of 100 mg/ml ampicillin stock solution and allowed to grow overnight at 37°C with vigorous shaking.

DNA fragments to be ligated were excised from agarose gel and purified using the gel purification kit from Qiagen according to manufacturer's instructions. The purified DNA was mixed in the ratio of 1:2 of vector to insert in 10 µl that also contained 1 unit of T4 DNA ligase mix (Takara Bio. Inc.). Ligation mixtures were incubated for 30 min at 16°C.

3.2.3. *Cloning of Rac1b in pGEX-4T2*

The cDNA for Rac1b was purchased from Origene (RC224262). Rac1b gene in pCMV6 vector was amplified by PCR with the forward (5' GGATCCCCAGGAATTCCCATGCAGGCCATCAAGTGT 3') and reverse primers (5' GCGGCCGCTCGAGTCGACTCACAACAGCAGTTTTCT 3') using the Platinum *Taq* DNA Polymerase High Fidelity kit according to the manufacturer's instructions. The amplication product was analyzed by 1 % agarose gel electrophoresis.

3.2.3.1. Restriction Digestion and Ligation

The PCR product and the pGEX-4T2 vector were digested with restriction enzymes EcoRI and SalI as follows: 2 µg PCR product or pGEX-4T2, 2 µl BSA (10 x), 2 µl NEB 3 buffer, 2 µl ECoRI, 2 µl SalI, and reaction volume made upto 20 µl with water in a tube. The contents were mixed and incubated for 2 hour at 4°C. The linearized plasmid and gene were purified by 1 % agarose gel electrophoresis using Qiagen gel extraction kit and eluted in a total volume of 20 µl water. The Rac1b gene was then ligated into the plasmid pGEX- 4T2 using the Takara ligation

kit. The ligation mixture was used for transformation of competent *E. coli* DH5α as described earlier.

Isolated colonies from the previous step were picked and used to inoculate 5 ml of LB media containing ampicillin. After growth at 37°C for 16-18 hours, DNA was purified from the cultures using Qiagen DNA purification miniprep kit. The DNA was eluted in 20 µl sterile water and restriction digestion was done using EcoRI and SalI enzymes. The samples were analyzed by gel electrophoresis on a 1 % agarose gel. The isolates showing the correct banding pattern were sequence confirmed (Robarts Research Institute, London, ON).

3.2.4. *Isolation of GST Fusion Proteins*

GST and the recombinant GST fusion proteins, GST-Pak1, GST-Rac1 and GST-Rac1b, were expressed in DH5α *Escherichia coli* cells using 0.5mM IPTG for induction as previously described (Jilkina and Bhullar, 1996). Briefly, 0.5mM IPTG was added to an overnight grown 200 ml bacterial culture and allowed to shake for 2 hours at room temperature. The cells were centrifuged 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. The bacterial culture was left at room temperature for 15 min. To disrupt the cells, the mixture was subjected to ultrasonic cell disruption with 2-3 pulses of 30-45 sec (3 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 material. 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, consisting of 20 mM Tris-HCl, pH 8.0, 100 mM NaCl) for 30 min at 4°C. The beads were washed three times with NT buffer to remove unbound proteins. The purity of the final protein preparations was assessed using SDS-PAGE.

3.2.5. *Cell Culture*

MCF7 cells were maintained in 100 mm plates using DMEM supplemented with 10% FBS (v/v) and 100 U penicillin/streptomycin at 37°C in 5% CO_2 and 95% air (v/v).

3.2.6. *CaM Sepharose Pull-down of Rac1b from MCF7 cell lysate*

MCF7 cells were washed 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 2 µg/µl aprotonin, 5 µg/µl leupeptin, and 1 mM PMSF. MCF7 cells were lysed in CaM binding buffer containing protease inhibitors cocktail. The total cell lysate was centrifuged at 14,000 x g for 10 min at 4°C. After centrifugation, supernatant samples from MCF7 cell lysate were incubated with 80 µl of CaM Sepharose 4B beads that were previously equilibrated in CaM binding buffer. Treatment conditions included: buffer, buffer containing 10 mM EGTA, buffer containing 5 mM Ca^{2+} , buffer containing 10 mM EGTA plus variable Ca^{2+} concentrations (1 mM Ca^{2+} or 3 mM Ca^{2+} or 5 mM Ca^{2+}). Blank Sepharose 4B beads (80 µl) were used as control. The reaction was incubated for 2 hours 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-Rac1b (1 µg/ml) antibody and horse-radish peroxidase (HRP)-conjugated secondary goat anti-rabbit antibody (1:10,000 dilution). The antigen-antibody complex was detected using enhanced chemiluminescence (ECL) reagents.

3.2.7. *GST-Rac1b Interaction with Pure Calmodulin*

Purified GST or GST-Rac1b 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, 10 mM EGTA, 5 mM Ca^{2+}, 10 mM EGTA plus different Ca^{2+} concentrations (1 mM Ca^{2+} or 3 mM Ca^{2+} or 5 mM Ca^{2+}) plus 20 µg of pure CaM were added to tubes containing GST or GST-Rac1b (100 µl) and allowed to shake for 2 hours 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-CaM (1 µg/ml) antibody and horse-radish peroxidase (HRP)-conjugated secondary goat anti-mouse antibody (1:10,000 dilution). The antigen-antibody complex was visualized using ECL reagents.

3.2.8. Rac1b Activation Assay

MCF7 cells were serum starved for 48 hours followed by washing with phosphate buffered saline and trypsinization. The cells were centrifuged and the cell pellet was re-suspended in 1 ml of PBS. The cells were stimulated using the following conditions: no addition, W7 (150 µM, 30 min), EGF (50 ng/ml, 30 min) or Heregulin β1 (50 ng/ml, 10 min), W7 + EGF or Heregulin β1, W5 (150 µM, 30 min) and W5 + EGF or Heregulin β1. After incubation, MCF cells were lysed using RIPA buffer (50 mM Tris-HCl 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 2 µg/µl aprotonin, 1 µg/µl leupeptin and 1 mM PMSF). The cell lysate was centrifuged for 10 min at 14,000 x g at 4°C. After centrifugation, the supernatant was transferred into a separate tube and stored immediately on ice. The amount of activated Rac1b in MCF7 cell lysate was determined using GST-Pak1. Thus, the centrifuged MCF7 cell lysate was incubated with GST-Pak1 coupled to GSH-agarose beads (75 µl) at 4°C for 2 hours. After incubation, the beads were washed three times with cold Rac1b 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 2 µg/µl aprotonin, 1 µg/µl leupeptin 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 membrane, and Western blotting was performed with rabbit anti-Rac1b polyclonal antibody (1 µg/ml) and horse-radish peroxidase (HRP)-conjugated secondary goat anti-rabbit antibody (1:10,000 dilution). The antigen-antibody complex was detected using ECL.

3.2.9. In vitro GDP/GTP – Rac1b binding assay

To assess the role of calmodulin in the *in vitro* binding of Rac1b to GDP or GTP, *in vitro* loading of Rac1b in MCF7 cell lysate with GTPϒS or GDPβS was performed. MCF7 cells at 80% confluence were washed twice with PBS and lysed in Buffer S (1% NP-40, 2.5 mM MgCl2, 10 mM NaF, 10% glycerol, 1 mM sodium orthovanadate (Na3VO4), and a protease inhibitor cocktail consisting of 2 µg/µl aprotonin, 1 µg/µl leupeptin and 1 mM PMSF). The lysate was rocked for 30 min at 4°C followed by centrifugation at 14,000 x g for 10 min at +4°C. The supernatant samples were incubated with or without 50 µM W7-HCl for 15 min, prior to the addition of 10 mM EGTA (final concentration) and guanine nucleotides (100 µM GTPϒS or 100 µM GDPβS). The mixture was incubated at 30°C for 15 min. At the end of incubation, $MgCl_2$ was added to a final concentration of 60 mM to lock in the nucleotides. The mixture was incubated with 100 µl GST-Pak1 bound to glutathione-agarose beads for 2 hours while rocking at 4°C. Unbound proteins were removed by washing three times with binding buffer. 30 µl of 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-Rac1b (1µg/ml) antibody and horse-radish peroxidase (HRP)-conjugated secondary goat anti-rabbit antibody (1:10,000 dilution). The antigen-antibody complex was detected using ECL reagents.

3.2.10. In Vitro Competition Assay

To assess if the Rac1 CaM-binding peptide inhibits Rac1b 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 hours in CaM binding buffer. The supernatant was decanted and beads were washed once with CaM binding buffer. MCF7 cells were lysed in CaM binding buffer containing protease inhibitor cocktail and the cell lysate was centrifuged for 10 min at 14,000 x g at 4°C. The supernatant from MCF7 cell lysate was added to washed beads and rocked gently for 2 hours at 4°C. Unbound proteins were removed by washing three times in CaM binding buffer. 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 momoclonal mouse anti-Rac1 or polyclonal rabbit anti-Rac1b antibodies. The antigen-antibody complex was detected using ECL.

3.2.11. Analyzing the specificity of small G proteins binding to Calmodulin

To determine the specificity of small G proteins binding with calmodulin, Calgranulin B, another Ca^{2+} binding protein was used to determine its binding. MCF7 cells were lysed in MOPS buffer. The lysate was centrifuged for 10 min at 14,000 x g at 4°C. Equal amount of centrifuged lysate was incubated with either GST, GST-Rac1 or GST-Rac1b coupled to GSH-agarose beads at 4°C for 2 hours. After incubation, the beads were washed three times with MOPS buffer. 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 13% SDS-PAGE, electrophoretically transferred to PVDF

membranes, and Western Blotting was performed with rabbit anti-calgranulin B (H-90) polyclonal antibody (1:1000 dilution) and horse-radish peroxidase (HRP)-conjugated secondary goat anti-rabbit antibody (1:10,000 dilution). HL-60 whole cell lysate was used as the positive control. The antigen-antibody complex was detected using ECL.

3.2.12. *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 was performed with One-way ANOVA using Tukeys *post hoc* test.

3.2.13. Molecular modeling

The full length Rac1b model is not available so in order to build the whole protein, Rac1b template PDB ID: 1RYF was used, using SWISS MODEL. Up to five template structures per batch were superimposed using an iterative least squares algorithm. A structural alignment was generated after removing incompatible templates. To generate the core of the model, the backbone atom positions of the template structure were averaged. The templates were thereby weighted by their sequence similarity to the target sequence, while significantly deviating atom positions were excluded. The reconstruction of the model side chains was based on the weighted positions of corresponding residues in the template structures. Starting with conserved residues, the model side chains were built by iso-sterically replacing template structure side chains. To refine the protein structure geometry, which have been introduced by the modeling algorithm

was regularized in the last modeling step by steepest descent energy minimization using the GROMOS96 force field. To assess the interaction between the theoretical model of Rac1b and CaM, ZDock server was used to analyze binding. CaM (PDB ID: 1CFD) was docked with Rac1b using Z DOCK server (http://zdock.bu.edu/). The docking calculations were carried out using Fast fourier transform based protein docking method using ZDock. This involves searches of all possible binding modes in the translational and rotational space between two proteins and evaluates each by an energy scoring function. The poses with the best energy scores were chosen for further analysis. The model was visualized using PyMol.

Chapter 5

RESULTS

The important role that Calmodulin plays in regulating the activity of several small GTPases has previously been established (Agell, Bachs et al. 2002, Elsaraj and Bhullar 2008, Xu, Chelikani et al. 2012). In the present study we have investigated the role of CaM in the regulation and activation of small GTPase, Rac1b, in MCF7 cells.

4.1. Endogenous Rac1b interacts with CaM-Sepharose

To determine the interaction between Rac1b from MCF7 cell lysate and CaM, and to assess their Ca^{2+} dependence, CaM Sepharose pull down assays were used. Results showed that endogenous Rac1b from MCF7 cells interacts and binds with CaM Sepharose beads (Figure 8A). No interaction was observed between Rac1b and blank Sepharose beads (Figure 8A). The interaction between Rac1b and CaM showed Ca^{2+} dependency since the addition of 5 mM Ca^{2+} enhanced the binding between the two proteins whereas the addition of 10 mM EGTA significantly inhibited this binding (Figure 8B). The lysates in each sample contained equal amounts of total protein as determined by the anti-Rac1b antibody (Figure 8C). Quantification of the data confirmed these results and established the Ca^{2+} dependency for the interaction between Rac1b and CaM (Figure 9).

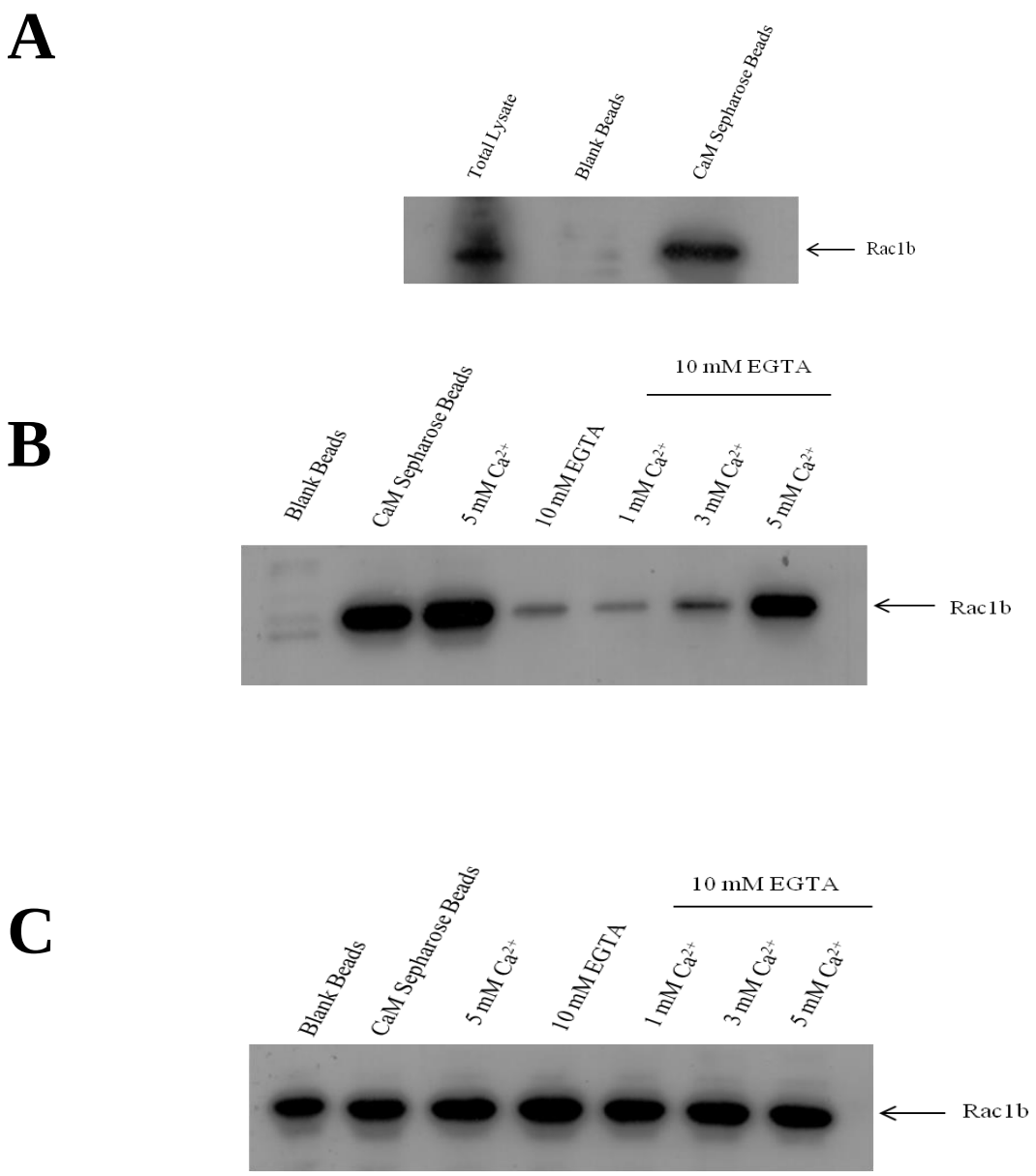

Figure 8: **Endogenous Rac1b interacts with CaM Sepharose**. (A) To assess the interaction between Rac1b and CaM and blank Sepharose and CaM-Sepharose 4B beads (80 µl) were incubated for 2 hrs at 4°C with MCF7 cell lysate. (B) To determine the Ca^{2+} dependence of this interaction, MCF7 cell lysate was incubated with Sepharose beads, CaM-Sepharose 4B beads and with CaM-Sepharose 4B beads in the presence of 5 mM Ca^{2+}, 10 mM EGTA, and in the presence of 10 mM EGTA + different concentrations of Ca^{2+} (1 mM Ca^{2+}, 3 mM Ca^{2+}, 5 mM Ca^{2+}). (C) Equal amount of protein in each experimental sample was confirmed by probing the starting cell lysate for Rac1b. The figures above are a representative of three separate experiments.

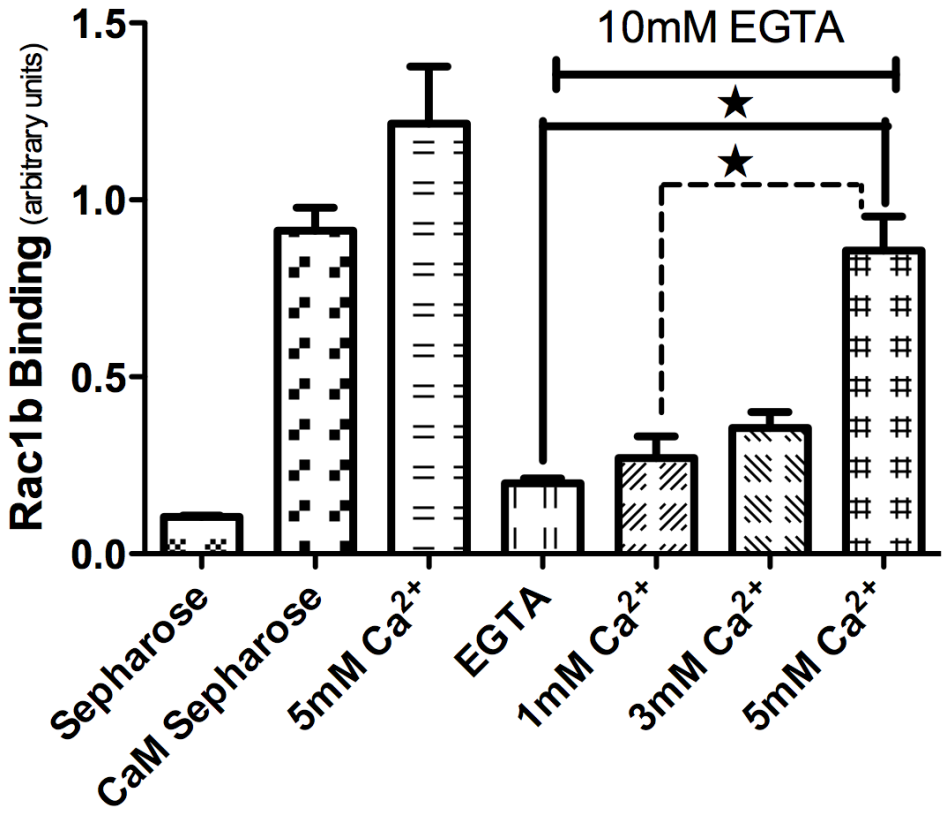

Figure 9: **Quantification results for the interaction of endogenous Rac1b with CaM Sepharose**. The experiment was repeated for a minimum of three times and quantification was done. Sepharose beads were used as a negative control. The units on Y-axis are represented in terms of band density. * p value < 0.05 is considered to be significant.

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4.2 Purification of recombinant GST-Rac1b fusion protein

GST-Rac1b fusion protein was used to determine the binding between Rac1b and pure bovine testes CaM. The expression of GST-Rac1b in *Escherichia coli* was assessed prior to performing the experiment (Figure 10). The pure recombinant protein demonstrated the expected molecular mass on SDS-PAGE analysis.

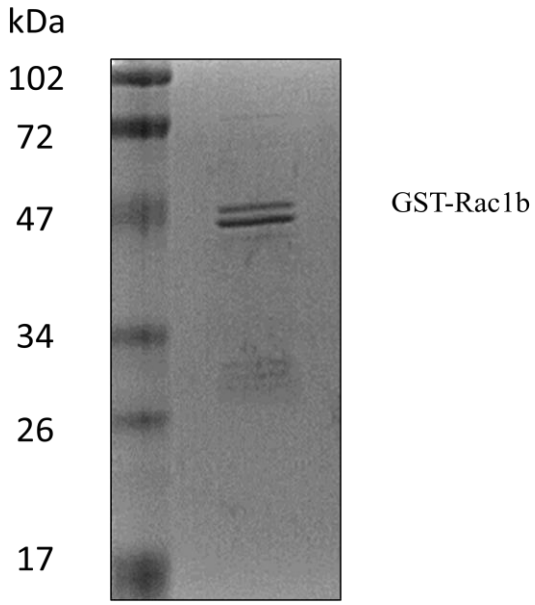

Figure 10: **Purification of recombinant GST-Rac1b fusion protein**. After purification from *E. coli* and stimulation using IPTG, Rac1b bound to GSH-agarose beads was analyzed on a 12% SDS-PAGE followed by staining with Coommasie blue.

4.3 Rac1b binds to Pure CaM

To determine if Rac1b binds directly to CaM, GST-Rac1b fusion protein and pure bovine testes CaM were used in *in vitro* binding assays. The experiments performed using GST-Rac1b showed that Rac1b binds to pure CaM in a Ca^{2+} dependent manner. The binding between GST-Rac1b and purified CaM was enhanced on the addition of 5 mM Ca^{2+} whereas a decrease in the binding was observed upon the addition of 10 mM EGTA to the buffer (Figure 11). Quantification of the data confirmed the observed results (Figure 11).

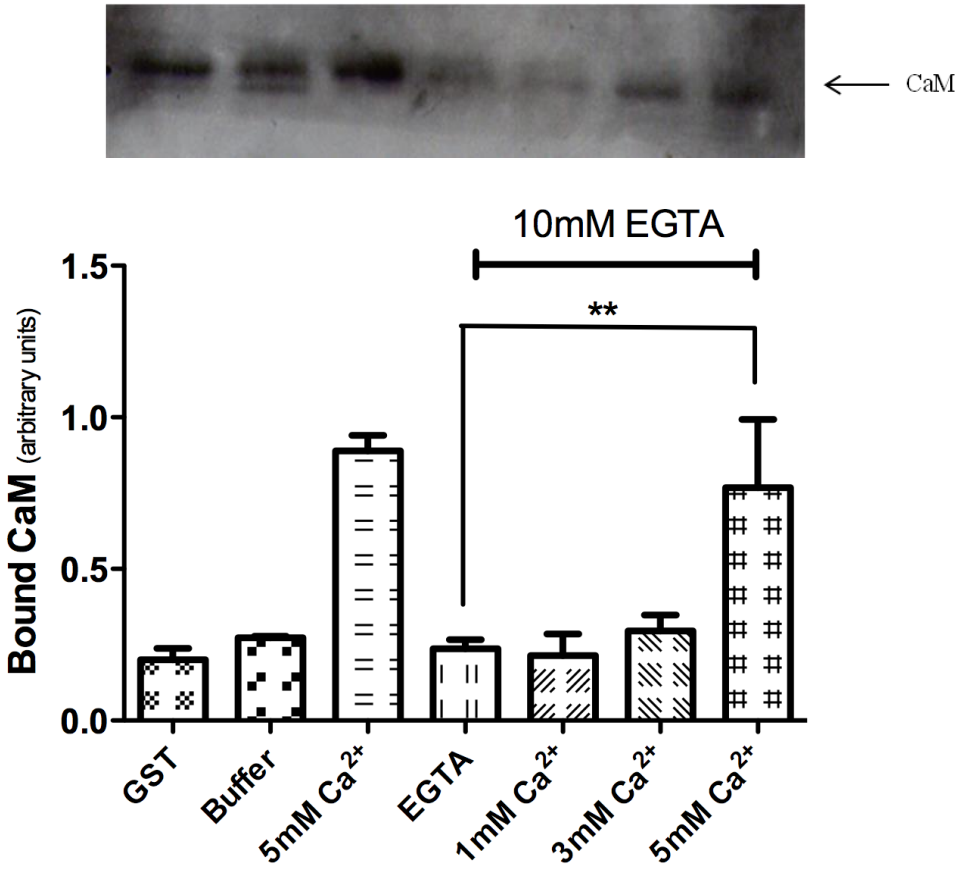

Figure 11: **GST-Rac1b binds to purified CaM**. To assess the interaction between Rac1b and pure CaM, GST-Rac1b beads and purified bovine testes CaM (20 µg) in MOPS buffer were incubated and allowed to shake for 2 hrs at 4°C. To determine the Ca^{2+} dependence of this interaction, the reaction mixture was incubated in different conditions including buffer alone, in the presence of 5 mM Ca^{2+}, 10 mM EGTA, and in the presence of 10 mM EGTA + different concentrations of Ca^{2+} (1 mM Ca^{2+}, 3 mM Ca^{2+}, 5 mM Ca^{2+}). GST beads were used as negative control. The experiment was repeated for a minimum of three times and quantification was done from different set of experiments. The units on Y-axis are represented in terms of band intensity. ** p value < 0.01 is considered to be significant.

4.4. Calmodulin activates Rac1b in MCF7 Cells.

The activation assays for Rac1b were done using Pak1, a type of p21-activated kinases (PAKs). PAKs are serine/threonine protein kinases that function as important mediators of Rac1 and Cdc42 GTPase function and are also involved in the pathways for Ras-driven tumorigenesis (Ong, Jubb et al. 2011). The p21-activated kinase (Pak1) is a downstream target of Rac1 and Cdc42 that interacts specifically with their GTP-bound forms. Previous studies from our lab have established a direct interaction between Rac1 and calmodulin. The role of CaM in the activation of Rac1 was studied using the recombinant GST-Pak1 fusion protein and W7, an inhibitor for calmodulin (Elsaraj and Bhullar 2008). In addition, the PAK-CRIB domain used for the pull down assays has been shown to bind equally with Rac1 and Rac1b (Matos, Collard et al. 2003). Thus, recombinant GST-Pak1 fusion protein was used to detect the active GTP-bound form of Rac1b. Expression and purification of recombinant GST-Pak1 in *E. coli* using SDS-PAGE was done prior to performing the experiment (Figure 12).

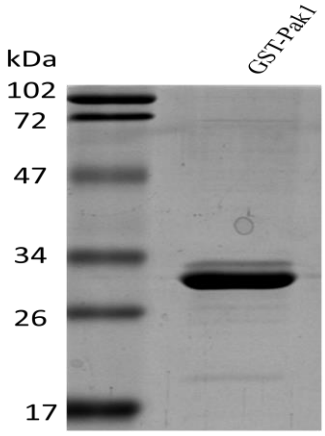

Figure 12: **Purification of recombinant GST-Pak1 fusion protein**. After purification from *E. coli* as described in the Materials and Methods, Pak1 bound to GSH-agarose beads was analyzed on a 12% SDS-PAGE followed by staining with Coommasie blue.

4.*3.1. Heregulin β-1 induced calmodulin activation of Rac1b.*

Heregulins are EGF-like ligands that act on the ErbB family of receptors and have previously shown to activate Rac proteins (Dunn, Sinha et al. 2004, Yang, Liu et al. 2006).

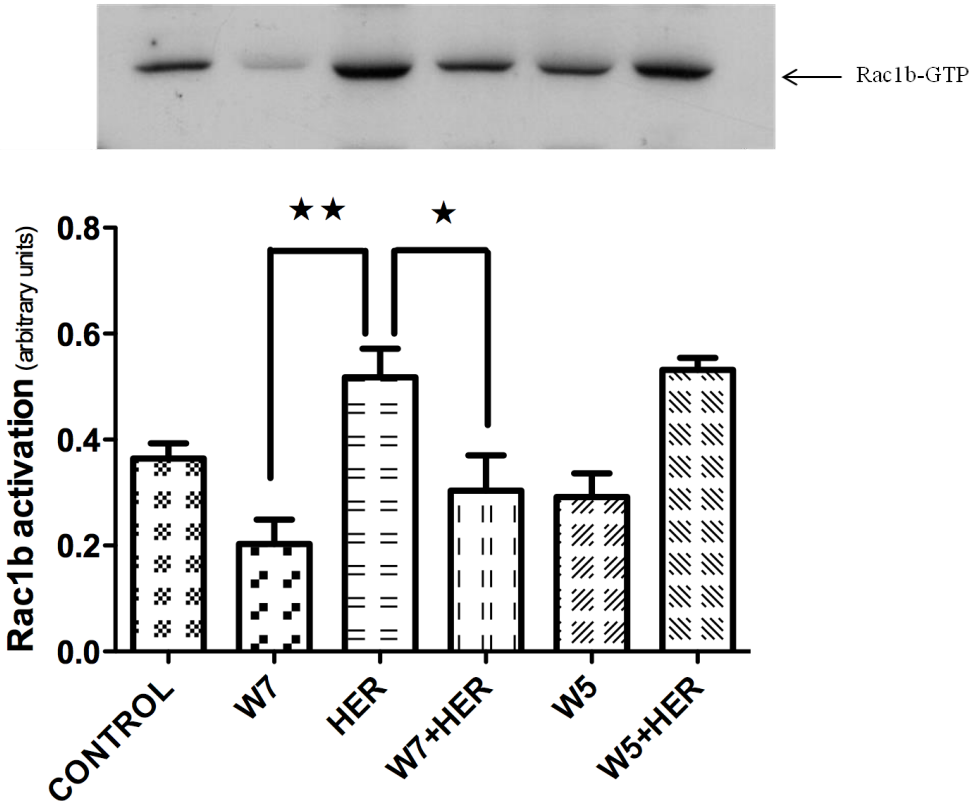

Figure 13: **Heregulin β-1 induced activation of Rac1b in MCF7 cells**. Serum starved MCF7 cells were prepared as described in "Materials and Methods" and stimulated with 50 ng/ml Heregulin β-1 for 10 min with or without prior incubation with 150 µM W5 or W7 for 30 min. After treatment, the cells were lysed in RIPA buffer and incubated with 75 µl of GST-Pak1 beads for 2 hrs at 4°C to recover the GTP-bound from of Rac1b. The beads were washed three times with Rac1b washing buffer and the bound proteins were eluted using Laemmli's sample buffer. Rac1b-GTP was detected using SDS-PAGE and Western Blotting using the polyclonal anti-Rac1b antibody. The experiment was repeated a minimum of three times and the Rac1b-GTP values were quantified from separate experiments. The units on Y-axis are represented in terms of band density. * p value < 0.05 and ** p value < 0.01 are considered to be significant.

4.3.2. EGF-induced calmodulin activation of Rac1b.

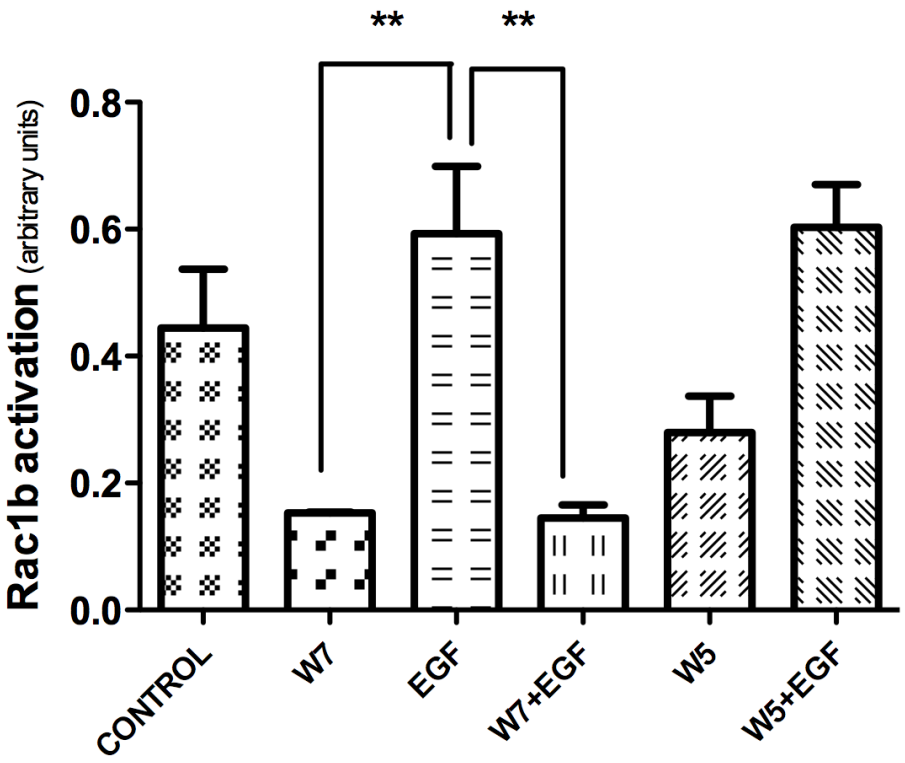

Figure 14: **EGF-induced activation of Rac1b in MCF7 cells**. Serum starved MCF7 cells were prepared as described in "Materials and Methods" and stimulated with 50 ng/ml EGF for 30 min with or without prior incubation with 150 µM W5 or W7 for 30 min. After treatment, the cells were lysed in RIPA buffer and incubated with 75 µl of GST-Pak1 beads for 2 hrs at 4°C to recover the GTP-bound from of Rac1b. The beads were washed three times with Rac1b washing buffer and the bound proteins were eluted using Laemmli's sample buffer. Rac1b-GTP was detected using SDS-PAGE and Western Blotting using the polyclonal anti-Rac1b antibody. The experiment was repeated a minimum of three times and the Rac1b-GTP values were quantified from separate experiments. The units on Y-axis are represented in terms of band density. ** p value < 0.01 is considered to be significant.

4.4 Role of W7 in in vitro GDP/GTP binding to Rac1b

W7, a potent calmodulin inhibitor was used to assess the role of calmodulin in *in vitro* Rac1b binding to GDP/GTP. GST-Pak1 beads were used to detect the binding of the active GTP-bound form of Rac1b with GTPϒS or GDPβS in the presence or absence of 50 μM W7. The results obtained showed no significant difference in the binding of Rac1b to the guanine nucleotides in the presence or absence of W7 suggesting that W7, has no effect in GDP/GTP binding to Rac1b in *in vitro* conditions (Figure 15).

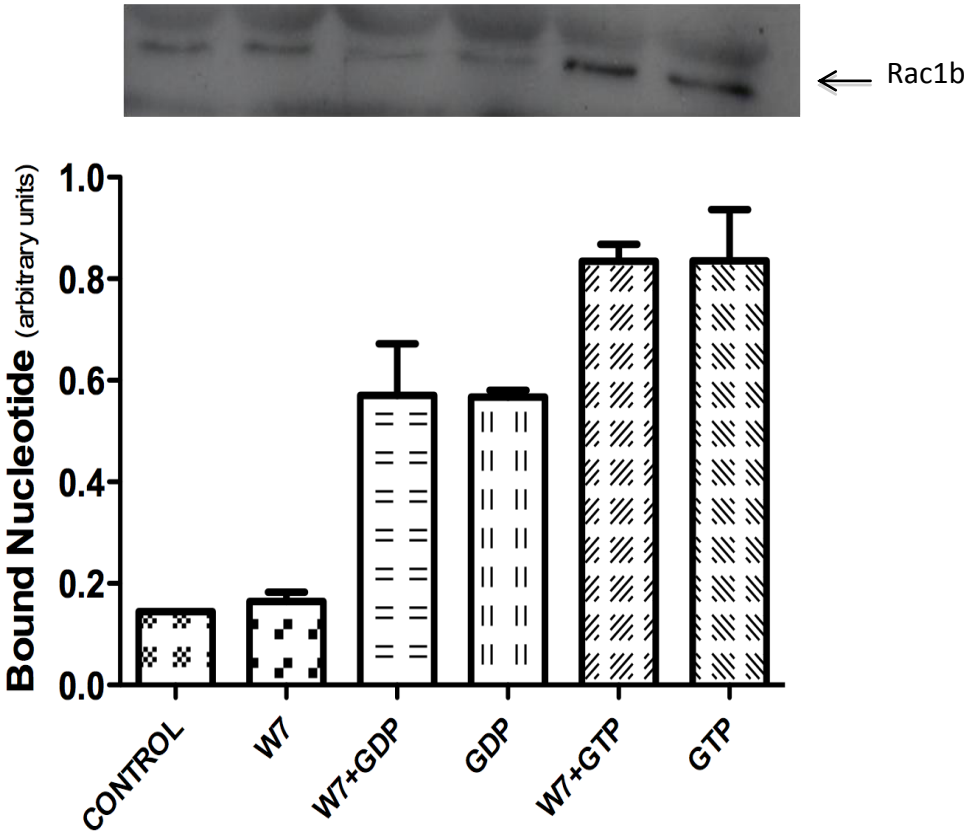

Figure 15: **Role of W7 in *in vitro* GDP/GTP binding to Rac1b**. MCF7 cell lysate was prepared as described under the "Materials and Methods" section. The lysate was incubated with or without 50 µM W7 for 15 min, prior to the addition of EGTA and guanine nucleotides (100 µM GTPϒS or 100 µM GDPβS). The mixture was incubated at 30°C for 15 min. At the end of incubation, $MgCl_2$ was added to a final concentration of 60 mM to lock in the nucleotides. The mixture was incubated with 100 µl GST-Pak1 beads for 2 hrs while rocking at 4°C. Unbound proteins were removed by washing beads three times. Bound proteins were eluted using Laemmli's sample buffer and subjected to 12% SDS-PAGE and Western Blotting was performed using the polyclonal anti-Rac1b antibody. The experiment was repeated for a minimum of three times and quantification was performed using the Bio-Rad "Quantity One" Program. The units on Y-axis are represented in terms of band density.

4.5 Analyzing the specificity of small G proteins binding to Calmodulin

To determine the specificity of small G protein binding to CaM, we tested the binding of Rho GTPases Rac1 and Rac1b with Calgranulin B. Calgranulin B (S100A-9 or MRP-14) is a member of the S-100 family of calcium binding proteins that exist as a homodimer or heterodimer with S100A-8 (Markowitz and Carson 2013) and is mainly involved in chronic inflammation and cancer. Calcium bound S100A-9 binds to arachidonic acid, cytoskeletal elements, Receptor for Advanced Glycation Endproducts (RAGE), Toll-like Receptor 4 (TLR4), the major fatty acid transporter CD36, matrix metalloproteinases (MMPs), fibronectin and heparin sulfate glycosaminoglycans (Bode, Luken et al. 2008, Markowitz and Carson 2013). GST-Rac1 and GST-Rac1b fusion proteins were used to assess the binding of Calgranulin B to Rac1 and Rac1b respectively. The results obtained showed almost no binding between Calgranulin B and Rac1 or Rac1b, indicating the specificity of Calmodulin for binding to the Rac1 and Rac1b GTPases (Figure 16). Binding was also not observed between CalgranulinB and Rac1 or Rac1b when additional calcium was added to the cell lysate. Expression and purification of recombinant GST, GST-Rac1 and GST-Rac1b in *E. coli* using SDS-PAGE was done prior to performing the experiment.

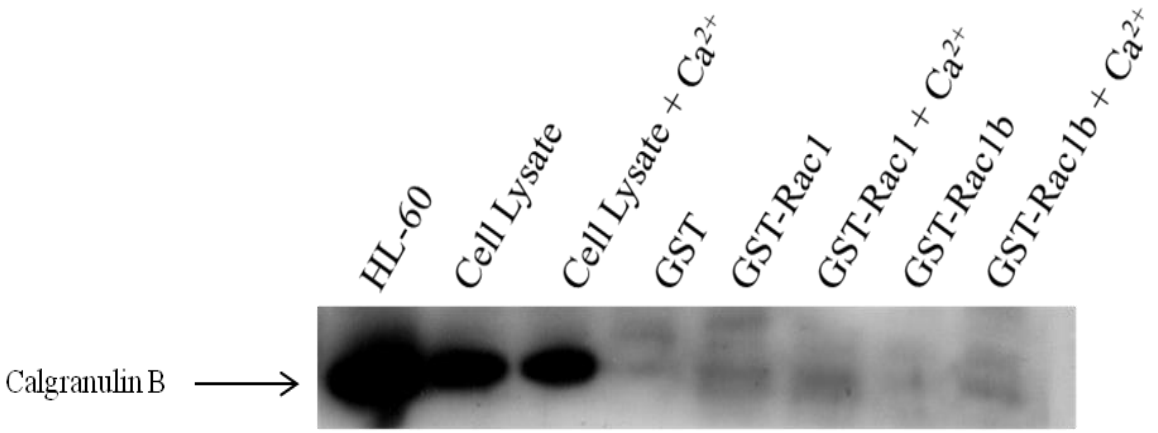

Figure 16: **S100A-9 interaction with Rac1 and Rac1b**. MCF7 cell lysate was prepared as described in the "Materials and Methods" section. Equal amounts of lysate was incubated with GST, GST-Rac1 and GST-Rac1b beads for 2 hr at 4°C. To determine the Ca^{2+} dependence of this interaction, 5 mM Ca^{2+} was added to the samples. After incubation, the beads were washed three times with MOPS buffer. Bound proteins were eluted using Laemmli's sample buffer and subjected to 13% SDS-PAGE. Western Blotting was performed with anti-calgranulinB (H-90) polyclonal antibody. HL-60 whole cell lysate was used as the positive control. The experiment was repeated for a minimum of three times with similar results obtained each time.

4.6 Rac1 CaM-binding peptide does not compete with Rac1b for binding to CaM-Sepahrose

Previous studies from our lab have determined a 14 amino acid CaM-binding region (amino acids 151-164) in the C-terminus of Rac1. The synthesized Rac1-CaM binding peptide [AVKYLECSALTQRG] has been shown to bind with both endogenous and pure CaM and also to compete with Rac1 for binding to CaM-Sepahrose (Elsaraj and Bhullar 2008). A similar amino acid sequence also exists in Rac1b. To determine if the CaM-binding regions in Rac1 and Rac1b are the same, we used the commercially synthesized Rac1-CaM binding peptide and CaM-Sepharose beads as a tool (Figure 17).

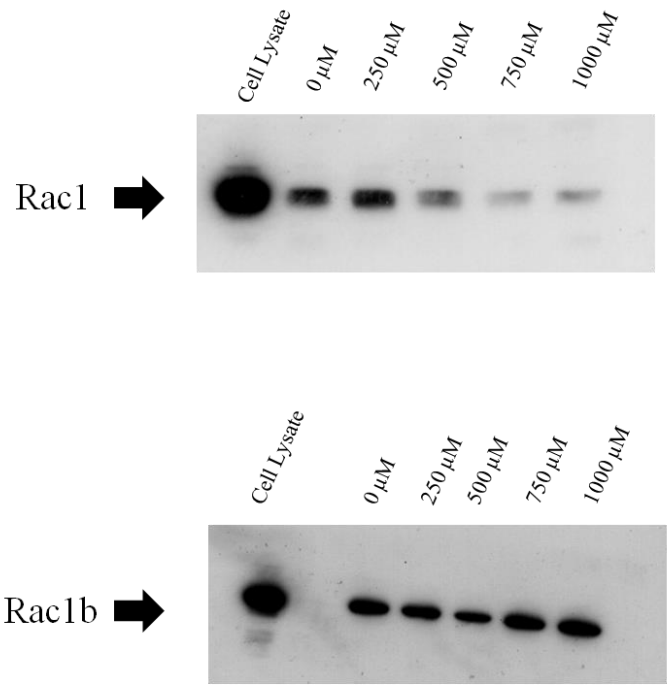

Figure 17: **Rac1 CaM-binding peptide interaction with endogenous Rac1 and endogenous Rac1b for binding to CaM Sepharose beads**. CaM Sepharose beads were incubated with 0, 250, 500, 750, and 1000 µM of peptide for 2hrs followed by addition of MCF7 cell lysate for 2 hrs at 4°C. After washing, proteins bound to the beads were separated by SDS-PAGE and Western Blotting was performed with anti-Rac1 and anti-Rac1b antibodies. The experiment was repeated for a minimum of three times with similar results obtained each time.

The results obtained demonstrate that the CaM-binding region in Rac1b is not the same as for Rac1 as higher concentrations of the synthesized free Rac1 CaM-binding peptide failed to inhibit the binding between CaM Sephaorse and Rac1b. The peptide competes with endogenous Rac1 for binding to CaM Sepharose beads but the same was not observed in the case of Rac1b (Figure 17).

4.7 Molecular Modelling

Rac1b model was constructed from the PDB ID: 1YRF using the SWISS MODEL program as described in the "Materials and Methods" section. To predict the possible interaction sites of Rac1b with CaM, protein-protein docking of Rac1b model and CaM crystal structure (PDB ID: 1CFD) was carried out using the ZDOCK server. Rac1b-CaM complex was analyzed using PyMOL and the possible interacting residues are listed below.

Rac1b residues	CaM residues
ASN 39	GLU 87
TYR 40	GLU 87
SER 41	GLU 83
SER 41	GLU 84
SER 41	GLU 87
ASN 43	LEU 112
ASN 43	GLU 84
LYS 49	GLU 11
ASN 52	GLU 84
ASN 52	ASP 80
ASN 52	GLU 84
LEU 53	GLU 84
GLY 54	GLU 84
TYR 72	ASP 50
PRO 199	ARG 37
PRO 200	ARG 37
VAL 195	GLY 40
LEU 196	GLY 40

Table 3: List of possible Rac1b and CaM interacting residues identified from molecular modelling and docking studies.

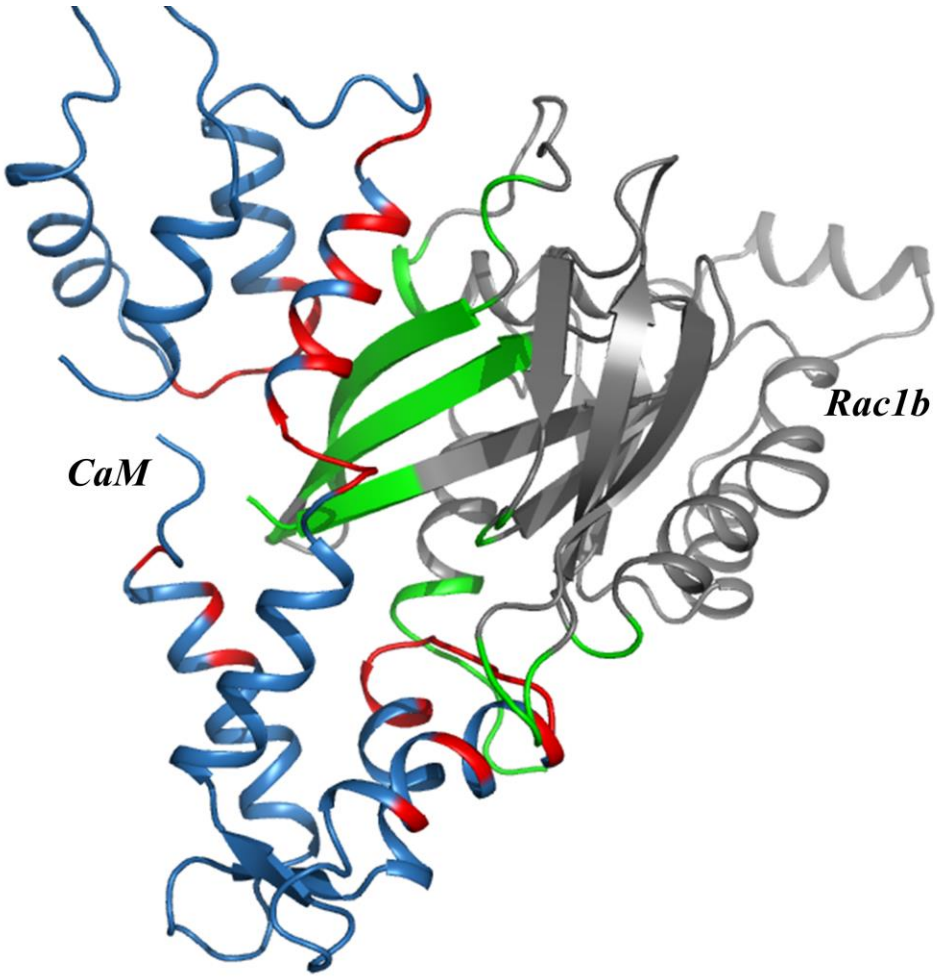

Figure 18: **Modelling of Rac1b-CaM docking complex**. Rac1b model is represented in grey color and CaM crystal structure is represented in blue. CaM interacting regions in Rac1b are depicted in green whereas, Rac1b interacting region in CaM are depicted in red color. This docking complex structure was generated using ZDOCK.

Chapter 6

DISCUSSION AND CONCLUSIONS

Apart from playing an important role in physiological cellular functions, the aberrant activity of Rac1 has also been implicated in tumorigenesis. Overexpression of Rac1 has been reported in cancers of the breast, lung and colon (Bosco, Mulloy et al. 2009). In breast cancer, overexpression of Rac1 in the early phase and its hyperactivation during the aggressive form of the disease has been indicated (Schnelzer, Prechtel et al. 2000). Hyperactivation of not just Rac1, but also an important Rac1 GEF, Tiam1, has also been found in the highly invasive breast tumors (Yang, Liu et al. 2006). Rac1b is a splice isoform of Rac1 that exists primarily in the active GTP-bound state in the cell. Expression of constitutively active mutants of Rac1 (Q61L Rac1 and Rac1 V12), its splice isoform Rac1b and various Rac GEF's (Tiam1 and Vav) have been shown to promote oncogenesis in fibroblasts (Bosco, Mulloy et al. 2009). Previous studies have also established that Matrix Metalloproteinase-3 (MMP-3) induces expression of Rac1b in mammary epithelial cells that lead to increased levels of cellular reactive oxygen species (ROS), expression of transcription factor SNAIL, epithelial mesenchymal transition, genomic instability and ultimately results in tissue structure disruption and malignant transformation (Lee, Chen et al. 2012).

In comparison to Rac1, Rac1b poorly activates p21-activated kinases (Pak's) and Jun N-terminal kinase but its expression in fibroblasts under serum starved conditions stimulates cell-cycle progression and survival (Matos and Jordan 2005). The additional 19 amino acid insertion close to the switch II region and its impaired interaction with Rho-GDI's favours the existence of the

GTP-bound form of Rac1b in the cells. On the other hand, similarly to Rac1, Rac1b can stimulate NFκB, Akt and ROS production (Nimnual, Taylor et al. 2010). In addition, Rac1b can also be activated through Rac1-GEF's via PI3K products (Tiam1) or via direct tyrosine phosphorylation (Vav) as well as inhibition of either of these pathways blocks the activation of both Rac1 and Rac1b (Matos and Jordan 2005).

In spite of its significant expression and role in certain tumors, the mechanism of regulation of Rac1b in cells is not yet fully understood. Thus, it is crucial to understand the mechanisms by which this small GTPase interacts and modulates the downstream signaling effects of its regulators and effector proteins.

Calmodulin is a ubiquitous calcium binding protein that in response to increased intracellular Ca^{2+} concentration undergoes conformational changes thus, interacting with other calcium binding proteins and regulating physiological functions. Calmodulin has previously been shown to interact with and regulate the activity of several small GTPases. It binds to RalA and RalB and is necessary for the thrombin induced activation of these GTPases in platelets (Clough, Sidhu et al. 2002). Similar regulatory mechanisms were observed for K-RasB and Rab3 (Park, Farnsworth et al. 1997, Sidhu and Bhullar 2001, Sidhu, Clough et al. 2003). A novel calcium dependent interaction has also been observed between calmodulin and small GTPases Rac1 and Cdc42 *in vitro* and in cells (Elsaraj and Bhullar 2008). The fact that CaM has previously been shown to interact and regulate the activation of Rac1 has led us to investigate if CaM interacts and regulates the activity of its splice isoform, Rac1b.

Our results demonstrate a novel interaction between calmodulin and the small GTPase Rac1b *in vitro* and in cells. The interaction shows calcium dependency since the addition of calcium chelator, EGTA reduced the binding between calmodulin and Rac1b whereas the addition of increasing concentrations of calcium enhanced this binding. Rac1b was also shown to bind directly to pure CaM in a similar manner. This suggests that similar to Rac1, Rac1b also has a Ca^{2+} -dependent CaM binding site. Additionally, to test specificity of the interaction between small GTPases and calmodulin, binding assays between Calgaranulin B and Rho GTPases, Rac1 and Rac1b were carried out. Calgranulins are members of the S-100 family of small calcium binding proteins with immunological functions (Bargagli, Olivieri et al. 2008). Studies have also detected Calgranulin A and Calgranulin B in breast and prostate cancers (Celis, Gromova et al. 2006, Gebhardt, Nemeth et al. 2006). Results obtained from these experiments demonstrated almost no binding between Calgranulin B and Rac1 or Rac1b, indicating high specificity of the interaction and role of CaM in regulation of Rac1 and Rac1b.

Previously, it has been established that small GTPases interact with CaM in both a Ca^{2+} dependent as well as in a Ca^{2+} independent manner. Studies have shown separate C-terminal and N-terminal CaM binding domains in small GTPases. To test if the same is true for Rac1b as well, we used the commercially synthesized CaM biding peptide for the previously established C-terminal CaM binding region in Rac1 (Elsaraj and Bhullar 2008). Despite the presence of identical amino acid sequences in both the proteins, the Rac1-CaM binding peptide failed to compete with Rac1b for binding to CaM, thereby suggesting a different CaM binding region in Rac1b. The addition of EGTA did not completely abolish the interaction between Rac1b and CaM further indicates that the interaction between CaM and Rac1b, can possibly be occurring in

both a Ca^{2+} -dependent as well as in a Ca^{2+} -independent manner. The 19 amino acid insertion, immediately behind the switch II region in Rac1b not only renders it ineffective to the action of Rho GDI's but also modifies the activity and regulation of Rac1b. Thus, it could be possible that despite the presence of identical CaM binding regions in the two proteins, the additional 19 amino acid insertion in Rac1b, modifies its CaM binding region making it distinct from Rac1.

The binding sites in CaM typically contain basic and hydrophobic amino acids. For instance, the lysine and arginine residues (K153 and R163) in Rac1-CaM binding domain have been shown to be essential for the interaction between the two proteins. In addition, the thrombin-induced activation of their mutants (K153A and K153A/R163A) was shown to be significantly reduced, demonstrating the essential role of lysine and arginine in Rac1-CaM interaction (Xu, Chelikani et al. 2012). In K-RasB, three regions including the hypervariable region, the α-helix between amino acids 151 and 166, and the Switch II regions have been established to play an important role in calmodulin binding (Abraham, Nolet et al. 2009). Within the hypervariable region of K-Ras4B, the hydrophobic farnesyl group and the positively charged amino acids bind specifically to the C-terminal domain of Ca^{2+} -loaded calmodulin, while the GTPϒS loaded catalytic domain of K-Ras4B may interact with the N-terminal domain of calmodulin (Abraham, Nolet et al. 2009). Recently, it has also been shown that calmodulin binding modulates the activity of K-RasB as well as inhibits its phosphorylation at Ser181 (Alvarez-Moya, Barcelo et al. 2011). Thus, in addition to non-polar hydrophobic amino acids, the role of serine in CaM-K-RasB interaction establishes an important role of hydrophilic or polar amino acids in CaM binding. In our study, the commercially synthesized Rac1-CaM binding peptide failed to compete with Rac1b for binding to CaM-Sepharose beads. Moreover, the possible Rac1b- CaM interacting

residues in our study show serine and other hydrophilic amino acids as key residues for CaM interaction and thus, possibly explaining the presence of a different CaM binding region in Rac1b.

W7, the calmodulin antagonist inhibits the activation of small GTPases such as RalA, RalB and Rac1. Activation assays from a previous study has demonstrated the inhibitory role of W7, during thrombin-induced activation of Rac1, but not for Cdc42 (Elsaraj and Bhullar 2008). Similar results for Rac1b were observed in this study, where the addition of growth factor agonists such as Epidermal growth factor and Heregulin β-1 enhanced the activation of Rac1b but in the presence of W7, this activation was inhibited. However, our study also shows that W7 does not affect the binding of GDP/GTP to Rac1b in *in vitro* conditions thus, suggesting that loading of GTP by Rac1b is not CaM dependent.

In conclusion, previous literature and the results from this study establish an important role for CaM in the regulation and activation of Rho GTPase Rac1, and its splice isoform Rac1b. The results in this study demonstrate the interaction between CaM and Rac1b, to be Ca^{2+} -dependent. This study has also established that the CaM binding regions in Rac1 and Rac1b differ. Using molecular modelling and docking studies, we have identified the possible interacting residues for Rac1b and CaM interaction. However, additional site-directed mutagenesis studies are required to identify the exact calmodulin binding domain in Rac1b. CaM binding to K-Ras4B activates Akt, a protein kinase that plays a role in cell survival and resistance to cancer therapy (Abraham, Nolet et al. 2009). It is possible that Rac1b also activates Akt in a similar fashion. These findings

suggest calmodulin to be a new interacting partner for Rac1b in its growth factor mediated activation thus, substantiating the potential role of CaM in the progression of human cancers.

Chapter 7

FUTURE DIRECTIONS

The research presented here has established calmodulin to be a novel interacting partner for Rac1b. Calmodulin is the ubiquitous protein responsible for mediating the effects of calcium ions on multiple signal transduction pathways. Since Rac1b has a major role in the initiation and advanced stages of various human cancers, it is essential that further studies be carried out to better understand the structural and functional aspects through which calmodulin plays a role in regulating the activity of Rac1b. The identification of the exact amino acid residues in Rac1b crucial for binding to calmodulin will be of interest to determine the mechanism by which the additional 19 amino acid insertion in Rac1b affects its regulation with calmodulin and other effectors.

In our study, the synthesized peptide for the CaM-binding region in Rac1 failed to compete with Rac1b to bind with calmodulin suggesting the presence of other calmodulin binding regions. It will be of interest to determine as to how the modification of the flexible switch regions in Rac1b impact its calmodulin binding sites. Previous studies have also shown the presence of calmodulin binding region in the N-terminus part of small GTPases (Clough, Sidhu et al. 2002). Thus, it is also possible that a CaM-binding region exists in the N-terminus of Rac1b. This could be tested by carrying out site-directed mutagenesis followed by *in vitro* binding assays.

The tumor promoting role of Rac1b has been established in cancers of the human breast, colon and the lung. Therefore, it will be of relevance to analyze the effect of calmodulin and calmodulin inhibitors on Rac1b in cancer. In addition, it will be of help to determine how this

novel interaction between CaM and Rac1b can affect downstream pathways that result in genomic instability, altered epithelial mesenchymal transition and malignant transformation.

This study has established a role for calmodulin in the regulation of Rac1b. Future studies for the identification of newer effectors that control the activity of small GTP-binding proteins are imperative to have a better understanding of signal transduction. These studies will aid in the elucidation of mechanisms that are involved in the deregulation of the physiological cellular function and development of disease.

Chapter 8

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