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RAP, a member of the Ras superfamily of small GTPases and its putative GTPase activating proteins and guanine nucleotide exchange factors in raw 264.7 macrophages

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**RAP, A MEMBER OF THE RAS SUPERFAMILY OF SMALL GTPASES
AND ITS PUTATIVE GTPASE ACTIVATING PROTEINS AND GUANINE
NUCLEOTIDE EXCHANGE FACTORS IN RAW 264.7 MACROPHAGES**

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By

Monika Zofia Tucholska

B.Sc. Ryerson University, 2005

A thesis

Presented to Ryerson University

in partial fulfillment of the

requirements for the degree of

Master

in the Program of

Molecular Science

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RAP, A MEMBER OF THE RAS SUPERFAMILY OF SMALL GTPASES AND ITS PUTATIVE GTPASE ACTIVATING PROTEINS AND GUANINE NUCLEOTIDE EXCHANGE FACTORS IN RAW 264.7 MACROPHAGES.

Monika Zofia Tucholska

M.Sc. candidate in Molecular Science, Ryerson University, 2008

ABSTRACT

The Fc γ receptor is a cell surface protein essential in the immune response that binds IgG-opsonized particles resulting in phagocytosis. Phagocytosis is a process used to remove pathogens and confine them in a vacuole that will enable their breakdown. The members of the Ras superfamily of small G proteins have been identified in samples where the activated Fc γ receptor complex was captured and analyzed using tandem mass spectrometry. The protein Rap, belonging to the Ras superfamily, guanosine triphosphatases (GTPase) activating proteins (GAPs), which promote the dissociation of GTP, and guanine nucleotide exchange factors (GEFs), that permits the exchange of GDP for GTP, were detected by SEQUEST in RAW 264.7 macrophages and further analyzed using various methods. In this study, Raps, RasGAPs, and RapGEFs, were observed by tandem mass spectrometry and sequence correlation analysis. The selected isoforms were confirmed by Western blots, live cell confocal microscopy with fluorescent fusion constructs and antibody staining to verify the localization of Ras proteins, specifically Rap1, p120RasGAP and C3G, a RapGEF, to the activated Fc receptor.

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LIST OF SELECTED ABBREVIATIONS

ADP	adenosine diphosphate
AF6	afadin
Arf6	ADP ribosylation factor 6
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
CNBD	cyclic nucleotide binding domain
DAG	diacyl glycerol
DIC	differential interference contrast
EEA1	early endosome autoantigen 1
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
ESI	electrospray ionization
FcγR	receptor that binds the constant (Fc) portion of IgG
FYVE	Fabp-YTOB-Vac1p-EEA1
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GPI	glycosylphosphatidylinositol
GRP	guanyl nucleotide-releasing protein
GRF	guanyl nucleotide-releasing factor
GTP	guanosine triphosphate
HA	influenza virus hemagglutinin
IHOP	information hyperlinked over proteins
IgG	Immunoglobulin
ITAM	immunoreceptor tyrosine based activating motif
ITIM	immunoreceptor tyrosine based inhibitory motif
LAMP	lysosomal associated membrane protein
LARC	live-cell affinity receptor chromatography
LBPA	lysobisphosphatidic acid
LC	liquid chromatography
M6PR	mannose-6-phosphate receptor
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MS/MS	tandem mass spectrometry
NAPDH	nicotinamide adenine dinucleotide phosphate
OSBP	oxysterol-binding protein
ORP1L	OSBP-related protein-1L
OX-LDL	oxidized low-density lipoprotein
PAK	Pak protein kinase
PBS	phosphate -buffered saline
PH	pleckstrin homology binding domain
PI	phosphatidylinositol
PI3K	phosphatidylinositol 3-kinase

PI(3)P	phosphatidylinositol 3-phosphate
PKC	protein kinase C
PLC	phospholipase C
RFP	red fluorescent protein
RILP	Rab-interacting lysosomal protein
SPA-1	signal-induced proliferation associated gene-1
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH2	Src homology-2 binding domain
SH3	Src homology-3 binding domain
SHP2	SH2 domain-containing tyrosine phosphatase
SOS	Son of sevenless
SRBC	sheep red blood cells
SYK	spleen tyrosine kinase
TEC	Tec protein tyrosine kinase
TGN	trans-Golgi network
TLR	Toll-like receptor
VPS34	vacuolar protein sorting-associated protein 34
VPS/HOPS	vacuole protein sorting/homotypic fusion and vacuole protein sorting

INTRODUCTION

Cell surface proteins such as the receptor complexes are recognized as potential targets for many immune system associated diseases (Davis, 2007). As members of the immunoglobulin superfamily (Fridman, 1991), Fc γ receptors have shown great potential as immunotherapy targets because of their ability to regulate the immune response through the interaction with IgG antibodies enabling the stimulation of pro or anti-inflammatory responses (Nimmerjahn, *et al.*, 2008). These membrane proteins play a vital role in the regulation of the immune response in mammalian cells via complex cascades of signaling networks. The identification of these signaling proteins and the roles that they play specifically in the uptake of foreign matter has been studied extensively but not all signaling molecules and their functions have been identified (Greenberg, *et al.*, 2002). Further investigation of the pathway proteins may provide great insight towards understanding the immune response and the activating and inactivating molecules that may have potential as therapeutic targets in the treatment of many human associated diseases.

White blood cells such as neutrophils and macrophages are capable of removing pathogens and confining them in a vacuole that will enable their breakdown. This process is known as phagocytosis. Macrophages provide a working model of the phagocytic pathway due to their ability to engulf IgG-opsonized particles such as latex beads. This model provides insight about the proteins and their functions within the Fc γ receptor complex as well as the cellular process that are involved in immunity and the signaling events that trigger the inflammation response (Greenberg *et al.*, 2002). This study will investigate selected members of the Ras superfamily and their associated regulatory proteins in RAW 264.7 macrophages at the site of the activated Fc γ receptor complex.

Immune Response

The antigen nonspecific innate immune system and the antigen specific adaptive immune system are the two related components of the vertebrate immune system that function to protect against invading organisms (Hoffmann, *et al.*, 1999). The adaptive immune system is comprised of memory cells such as the autoreactive T lymphocytes and autoantibody producing B lymphocytes among others, which possess a variety of cell surface receptors that enable recognition of pathogens. The engagement of these adaptive receptors requires the recognition of a specific antigen belonging to a pathogen that has previously infected the body, thus resulting in a highly specific and amplified response (Medzhitov, *et al.*, 1997). In the absence of prior exposure to a pathogen the adaptive response may take days or weeks to trigger an immune response, thus putting the victim at high risk of harm or even death.

In contrast, the innate immune system corresponds to the immune response that does not require previous exposure to a pathogen in order to elicit an antimicrobial effect and it is the primary line of defense against microbial infections (Greenberg *et al.*, 2002). The innate immune system is composed of anatomical barriers, the complement system, secreted antimicrobial peptides and particularly phagocytosis and its ancillary reactions. Phagocytosis by macrophages initiates the innate immune system via the activation of specific phagocytic receptors that aid in the discrimination between pathogens and self. These receptors engage in a specific recognition mechanism that enables identification of conserved motifs on pathogens that are not found in higher eukaryotes (Aderem, *et al.*, 1999). Cellular receptors that are involved in the recognition mechanism are mannose receptors, that recognize pathogen associated residues such as mannans and integrins, as well as scavenger receptors that recognize modified lipoproteins including low density lipoprotein (LDL), resulting in the formation of foam cells that have been identified in

atherosclerotic plaques (Brown, *et al.*, 1983). Toll-like receptors (TLR) interact with pathogen-associated molecular patterns leading to inflammatory responses as well as development of antigen-specific adaptive immunity (Takeda, *et al.*, 2003). The production of specific antibodies by the adaptive immune system or the naive antibody repertoire, provide high affinity IgG that permit efficient interaction with the family of Fc γ receptors. Thus antibodies that recognize epitopes on infectious agents represent the intersection between the adaptive immune system and the innate immune system via the Fc receptor pathway that is stimulated by their Fc domains (Aderem *et al.*, 1999).

Fc receptors

The family of Fc receptors for IgG or Fc γ receptors, simultaneously trigger various activating and inhibitory signaling pathways that generate an immune response, as well as function in the process of antigen presentation, regulation of B-cell activation, plasma-cell survival and immune-complex-mediated maturation of dendritic cells (Bolland, *et al.*, 1999). Fc γ receptors fall into approximately three categories divided according to their affinity for IgG, the structure of the Fc fragment and the activating or inhibitory signaling pathway they induce through direct association with their immunoreceptor tyrosine based activating motifs (ITAM) and immunoreceptor tyrosine based inhibitory motifs (ITIM) localized on the cytosolic α -chain (Fig. 1). In order of highest to lowest affinity for IgG they are: Fc γ R1 cluster of differentiation (CD) 64, Fc γ RIIA/B/C (CD32), and Fc γ RIIIA/B (CD16). The Fc γ R1 is the high-affinity receptor in both mouse and human, and all other receptors retain medium to low affinity for the Fc fragment of IgG. These classes of receptors have been recognized in mice and homologous genes for the family of Fc γ receptors have also been identified in humans and monkeys, thus implying

that not all but some genes and functions are conserved between species (Nimmerjahn, *et al.*, 2006). Although differences between human and mouse Fc γ receptors are evident, the mouse provides a valuable model of the mechanisms involved in Fc γ receptor signaling, which has been confirmed in human models (Davis, 2007; Nimmerjahn, *et al.*, 2005; Revetch, 2003).

The complexity of the Fc γ receptor family can be better illustrated by their interaction with the antibody Fc fragment and number of different IgG subclasses that can bind the receptor with different affinities and vary between species. In humans, for instance there are four different subclasses of IgG, namely IgG1-IgG4. In mice there are also four, IgG1, IgG2a, IgG2b and IgG3, that vary by their affinity and specificity to different Fc γ receptors (Dijstelbloem, *et al.*, 2001; Nimmerjahn *et al.*, 2005). Antibodies mediate effector functions and cellular immune responses by interacting with Fc γ receptors and stimulating pro and anti-inflammatory functions. The activation of cell surface receptors via antibodies varies extremely and has engaged much interest in the field of immunotherapy. For instance, enhancing or blocking antibody binding has been known to regulate the immune response and improve the therapeutic outcome in many human diseases (Nimmerjahn *et al.*, 2008).

Figure 1: The family of human Fcγ receptors and mouse homologs.

Extracellular regions are representative of IgG binding domains (square, triangle, circle), the ligand binding α-chain extends through the membrane and attaches to the cytosolic ITAMs, represented by rectangles and ITIMs by diamonds. The glycosylphosphatidylinositol (GPI) linked receptor is only present in human neutrophils and is not a mouse homolog (Nimmerjahn *et al.*, 2008).

Phagocytosis

Phagocytosis is the process by which foreign particles and apoptotic bodies are eliminated and it is a dynamic component of the innate immune system. Phagocytic leukocytes such as neutrophils and monocytes/macrophages are essential players in the immune system since they destroy invading pathogens (Rabinovitch, 1995). A remarkably complex, and biologically important phagocytic signaling pathway triggers the encapsulation of foreign particles and microbial pathogens by phagocytosis. Phagocytic leukocytes have two essential functions in the immune system, the first of which is in the microbial death pathway whereby the ingested particle is directed to the lysosomes and treated with hydrolytic enzymes, and the

resulting phagolysosome is targeted to the phagocyte oxidase complex. The second function is to utilize phagocytosis to direct antigens to peptide presenting molecules such as MHC I and II compartments (Greenberg *et al.*, 2002). It is these two functions that allow phagocytosis to serve a dual role in the innate immune response, as well as link the innate and acquired immune responses. Self or nonself antigen presentation on the surface of a host cell is of great importance for the initiation and generation of acquired immunity. MHC class II molecules take time in their transport to the cell surface from the trans-Golgi network (TGN), that sorts and delivers proteins to various cell destinations, in order to intersect with the endocytic pathway (Traub, *et al.*, 1997). In professional antigen-presenting cells, such as macrophages, the late endosomes and lysosomes become enriched in the MHC class II molecules and these specialized endo/lysosomes become known as the MHC class II compartments (MIICs). It is thought that antigen presentation is a result of the fission and fusion events involved in the maturation of the phagosome and that a portion of the phagosome containing a partially degraded antigen undergoes fission and fuses with a MIIC (Vieira, *et al.*, 2002).

Phagocytosis is triggered by a complex network of signaling proteins, which is mediated by the activation of cell surface receptors. Many different cell surface phagocytosis promoting receptors that mediate innate immune responses in mammalian cells have been described (Greenberg *et al.*, 2002). The cell surface receptors of the innate immune system must discriminate between various pathogens and its own cellular components. Cell surface receptors fall into two categories; those that bind pathogens or particles coated with a host-derived ligand, termed opsonic receptors, and those that bind ligands endogenous to the pathogen or external particle, termed non-opsonic receptors (Aderem *et al.*, 1999). Opsonic receptors are those that recognize particles coated with antibodies by the host complement system and are recognized by

the Fc receptors respectively (Underhill, *et al.*, 2002). The roles of the diverse cell surface receptors in phagocytosis are specific in signaling actin polymerization and particle internalization (Aderem *et al.*, 1999).

The functions of the innate immune system resulting from phagocytosis are mediated by the interaction of cell surface receptors with their cognate ligands. The response of phagocytes, such as macrophages to engulf particles, is triggered and regulated by multi-ligand receptors on the cell surface including the Ig superfamily members such as the Fc receptors. The family of Fc gamma receptors for IgG not only stimulate the innate response but also determine whether an immunogenic or tolerogenic response is initiated upon ligand recognition, therefore making them attractive targets for novel immunotherapy practices (Nimmerjahn *et al.*, 2008). Immunogenic reactions are those that efficiently mediate pro-inflammatory responses and lead to the removal of infected cells, whereas tolerogenic responses do not initiate the immune response and suppress inflammatory responses. The many ligands that are recognized as pathogens or foreign by the innate immune receptors and stimulate the activation of phagocytes via the Fc receptor include hydrophobic surfaces such as polystyrene particles, IgG, ox-LDL, C-reactive protein, other modified lipids and apoptotic cells (Febbraio, *et al.*, 2001). The activation of the Fc receptor by binding IgG-opsonized particles is a well studied process, however the events that proceed the triggering of the complex cascade of protein activation and inactivation processes are much less understood and not all the proteins involved in the Fc receptor pathway have been identified. Studies involved in the identification of specific proteins and their roles in the uptake of pathogens via the Fc receptor complex pathway may provide potential targets in the treatment of disease (Davis, 2007).

Many signaling pathways in cellular processes converge on and regulate phosphoinositide 3-kinase (PI3K), whose inositol lipid products are key mediators in trafficking of membranes along the endocytic pathway and are critical for phagosome maturation (Vieira *et al.*, 2002). The family of PI3Ks phosphorylate the free OH groups of the inositol ring producing phosphoinositol-3,4,5-triphosphate [PI(3,4,5)P₃]. The activated PI3K converts the plasma membrane lipid phosphoinositol-4,5-bisphosphate [PI(4,5)P₂] into PI(3,4,5)P₃. Three classes of PI3Ks have been derived which are based on their regulation and substrate preference. Class I PI3K is activated by a cell surface receptor tyrosine kinase, such as epidermal growth factor receptor (EGFR), or G-protein-coupled receptors to generate PIP₃. Once activated by their receptors, class I PI3K phosphorylates plasma membrane phosphoinositides that bind with and then activate serine-threonine kinase Akt (Baumgartner, *et al.*, 2000; Domin, *et al.*, 1997; Jones, *et al.*, 2005; Riley, *et al.*, 2006). Akt are protein kinases that regulate cell survival, metabolism, angiogenesis as well as various human diseases when deregulated (Manning, *et al.*, 2007). The class II PI3K is a p170 molecule and these proteins are found predominantly in the membrane of cells and are activated by insulin, epidermal growth factor (EGF), or platelet-derived growth factor. Class II enzymes as of yet, have not been identified in phagosome maturation or formation (Vieira *et al.*, 2002). Class III PI3Ks however play a key role in intracellular trafficking through the synthesis of PI(3)P and PIP₂ (Knight, *et al.*, 2006). These classes of PI3Ks have very significant and different mechanisms in endocytic trafficking. For instance, the activation of class I PI3K results in the inhibition of degradative processes of endocytosis, while class III PI3K is required for the formation of vesicles and their subsequent transport to the lysosomes (Deretic, 2006; Vieira *et al.*, 2002). Thus, class I and class III PI3Ks are important regulatory molecules in phagocyte maturation (Vieira *et al.*, 2002).

The elimination of invading microorganisms and other foreign particles by macrophages and neutrophils can be distributed among three different stages relevant to the molecules that are active at each stage. The three stages of phagocytosis consist of the early phagosome, the late phagosome and the phagolysosome. Initially the internalization of a foreign particle into the plasma membrane derived vacuole, known as the phagosome, is not competent to kill and eliminate the ingested microorganism. This is because the phagosome is similar to the lipid bilayer plasmalemma and its contents resemble the extracellular milieu, thus only enabling internalization but not digestion (Scott, *et al.*, 2003; Vieira *et al.*, 2002). Following sealing, the phagosome undergoes a sophisticated series of rapid fission and fusion events that result in the modification and formation of vesicles and tubules, their limiting membrane and their contents. This process is known as phagosome maturation and bestows the vacuole with a host of degradative properties, which are central to its microbial function (Beron, *et al.*, 1995; Tjelle, *et al.*, 2000). Phagosome maturation is dependant on a remodeling sequence that resembles the progression of the endocytic pathway, whereby phagosomes fuse extensively with sorting and late endosomes and lysosomes, and are ultimately converted from plasmalemma-like organelles to a lysosome like hybrid organelle, the phagolysosome (Beron *et al.*, 1995; Pitt, *et al.*, 1992). This process is thought to involve various fusion events with components of the endocytic pathway, as well as the removal of components by vesicular fission. These sequential interactions have been termed the "kiss and run" hypothesis (Desjardins, 1995) and bring about tremendous changes to the phagosomal contents and membrane. It has been proposed that through this mechanism, endosomes or lysosomes and phagosomes all form transient hybrid organelles connected by fusion pores that allow selective exchange of some membrane and luminal components to occur, followed by fission. Regardless of the detailed mechanism involved in the

sequential acquisition of fusion determinants during phagosome maturation, there is an overall agreement that it is the orderly interaction with elements of the endocytic pathway that grants lytic ability to the phagosome.

Phagosome maturation and the endocytic pathway are interrelated. The maturation process that provides the phagosome with lytic activity depends critically on the interaction of the nascent vacuole with the endocytic pathway (Vieira *et al.*, 2002). The endocytic pathway is an organized continuum of organelles ranging from early endosomes to lysosomes. In endocytosis, after internalization of a receptor-ligand complex, the contents of an endocytic vesicle are targeted to a sorting endosome, also known as an early endosome. Sorting endosomes are responsible for organizing and re-routing the assortment of internalized molecules. The ligand and its receptor dissociate and the receptor is returned back to the cell periphery in recycling endosomes, while the ligand continues along the degradative stages of the endocytic pathway towards lysosomes. Sorting or early endosomes are recognized by the presence of Rab5 or early endosome antigen 1 (EEA1), or experimentally by applying short pulses of ligands or fluid-phase markers.

The early phagosome

Rab5, a member of the superfamily of small G-proteins is thought to control the earliest identified stages of phagosomal maturation (Duclos, *et al.*, 2000; Roberts, *et al.*, 2000). The recruitment of Rab5 to the endosomal membrane has been suggested to be modulated by Ras, through the mediation of guanine nucleotide exchange factor RIN1 (Barbieri, *et al.*, 1998; Hoffenberg, *et al.*, 2000; Li, *et al.*, 1997). When in its GTP bound form, Rab5 can interact with several effectors, such as Rabenosyn-5, Rabaptin-5, and Rabex-5. Rabaptin-5 is a cytosolic

protein that is recruited to endosomes by Rab5-GTP and inhibits GTP hydrolysis, and Rabex-5 is a 60 kDa cytosolic nucleotide exchange factor specific for Rab5 that exists in association with Rabaptin-5 (Hoffenberg *et al.*, 2000; Horiuchi, *et al.*, 1997; Lippe, *et al.*, 2001). A novel Rab5 effector, Rab5 FYVE-finger effector, Rabankyrin-5, was recently discovered to localize to the early endosomes and shown to stimulate fusion activity (Schnatwinkel, *et al.*, 2004).

Like other small GTPases, Rab5-GTP is capable of recruiting downstream effectors. One such effector is EEA1, which at each terminus contains a spatially separate Rab-5-binding domain, thereby allowing it to serve as a bridge between two early endosomes (Mu, *et al.*, 1995). EEA1 also contains a FYVE zinc finger domain that interacts with PI(3)P and is required for the endosomal targeting of EEA1 (Lawe, *et al.*, 2000; Patki, *et al.*, 1997; Stenmark, *et al.*, 1996). Membranes containing Rab5 and PI(3)P are permitted to be tethered or bridged to the membrane due to the formation of antiparallel dimers by EEA1 (Callaghan, *et al.*, 1999).

Another critical Rab5 effector is vacuolar protein sorting-associated protein 34 (hVPS34), a type III PI3K. Class I, II and III PI3K are all capable of PI(3)P synthesis *in vitro* and regulate endocytic traffic, however, it is the Class III kinase that is the biologically relevant source of PI(3)P and is the PI3K that participates in phagosome maturation (Leevers, *et al.*, 1999; Vanhaesebroeck, *et al.*, 1997). VPS34 exists in a complex associated with p150, a VPS15-like serine/threonine kinase subunit of the complex, which is thought to regulate the membrane association and activity of VPS34 (Murray, *et al.*, 2000; Murray, *et al.*, 2002). Besides EEA1, Rab5 also recruits the p150/VPS34 complex to generate PI(3)P, a lipid that is crucial in the subsequent maturation process. Nascent phagosomes acquire PI(3)P shortly after sealing and it localizes to the membrane of the phagosomes within 1-2 minutes into the sealing process and persists for approximately 10 minutes, disappearing subsequently. Downstream effectors of

PI(3)P contain specific binding domains for the lipid, such as the PX domain in the p40 subunit of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex or the FYVE domain found in EEA1 (Ellson, *et al.*, 2002; Gaullier, *et al.*, 1998; Patki, *et al.*, 1998). NADPH oxidase complex is a membrane-bound enzyme consisting of multiple subunits that generate highly-reactive superoxides in the phagosome by transferring electrons from NADPH across the membrane. Binding to PI(3)P through its FYVE domain is required for the interaction of EEA1 with the docking and fusion machinery of the endosomal membrane (Wurmser, *et al.*, 1999).

While the fusion events that characterize phagosome maturation are important, remodeling of the phagosome also requires a loss of membrane and luminal components through budding, or fission, of vesicles. This process is imperative for ensuring that the mature phagosome maintains a constant surface area despite ongoing fusion events. In the endocytic pathway, internalized cell surface receptors that are destined for degradation are targeted to the inner vesicles of a subset of late endosomes, while receptors that are destined to be recycled back to the plasma membrane remain on the endosomal limiting membrane. Specialized endosomes, termed multivesicular bodies, allow the cell to physically separate proteins intended for lysosomal destruction from those that are to be conserved by the cell.

The transition of the sorting endosome or early phagosome to the late endosome/phagosome is indicated by the absence of Rab5, EEA1, and PI(3)P from the phagosomal membrane, followed by the acquisition of Rab7, Rab9 and other molecular markers (Vieira *et al.*, 2002). The equilibrium between the active membrane bound Rab5-GTP and the inactive cytoplasmic Rab5-GDP may be regulated by GAPs. GAPs control the lifetime of the activated state of Rab proteins and are thought to promote Rab GTPase hydrolysis by properly aligning a catalytically important glutamine residue and inserting a so-called arginine finger

residue directly into the Rab active site to stabilize the transition site of the hydrolysis reaction (Pfeffer, 2005). Possible GAPs for Rab5 include p120RasGAP, the tumor suppressor tuberin which is a product of the TSC2 complex, and RN-tre (Haas, *et al.*, 2005; Lanzetti, *et al.*, 2000; Liu, *et al.*, 1998). Another GAP for Rab5 includes RabGAP-5. The over expression of this GAP results in a loss of EEA1 from endosomes and blocks endocytic trafficking, while the depletion of this GAP results in increased endosome size, more endosome associated EEA1, and disrupts the trafficking of lysosomal-associated membrane proteins (LAMPs), specifically LAMP-1 (Haas *et al.*, 2005; Pfeffer, 2005). Regulation of Rab5 by RabGAP-5 limits the amount of activated Rab5 and regulates trafficking through endosomes.

The late phagosome

The late endosome/phagosome is characterized by an increase in acidity and the presence of Rab7, Rab9, mannose 6-phosphate receptor (M6PR), syntaxin 7, LAMPs and lysobisphosphatidic acid (LBPA), which appear between 15 and 45 minutes after phagosome sealing (Vieira *et al.*, 2002). The increase in acidity of the late endosome causes the release of lysosomal enzymes from the M6PR, thus permitting the recycling of the receptor to the TGN (Nishi, *et al.*, 2002). In studies where the pH of the phagosome was artificially increased it was observed that phagosome maturation was arrested and Rab5 remained on the phagosome in its GTP-bound form (Huynh, *et al.*, 2007). The mechanism of this effect is still unknown, but it can be speculated that a low pH might function in recruiting RabGAP-5, which inactivates Rab5.

The small G-protein Rab7 functions in the transition between early and late phagosomes and/or phagolysosomes. A recent fundamental breakthrough in understanding the role of the Rab5 to Rab7 switch on endocytic organelles, referred to as Rab conversion, has given insight

into the mechanism of cargo progression between the early and late endosomes (Rink, *et al.*, 2005). This process occurs via a bulk change of a Rab5 organelle into a Rab7 late endosome. The loss of Rab5 and acquisition of Rab7 requires the class C vacuole protein sorting/homotypic fusion and vacuole protein sorting (VPS/HOPS) complex, which is a known Rab7 GEF and interacts with Rab5. Rab5 mutants defective in GTP hydrolysis result in endosomes that recruit Rab7 but fail to displace Rab5, since GTP hydrolysis is prevented. Therefore, it is likely that following class C VPS/HOPS-dependant recruitment of Rab7 onto Rab5-positive endosomes, the completion of conversion involves negative feedback between Rab7 and Rab5 domains, possibly through the action of a Rab5-GAP (Rink *et al.*, 2005). There are three effectors for Rab7: the Rab7-interacting lysosomal protein (RILP), oxysterol-binding protein (OSBP)-related protein-1L (ORP1L), and Rabring 7 (Cantalupo, *et al.*, 2001; Johansson, *et al.*, 2005; Jordens, *et al.*, 2001; Mizuno, *et al.*, 2003). Rabring7 functions in late endocytic trafficking and lysosome biogenesis downstream of Rab7. It has also been discovered that Rabring7 is a Rab7 target protein on the basis that it binds the GTP-bound form of Rab7 and is recruited by the GTP-bound form of Rab7 onto late endosomes/lysosomes (Mizuno *et al.*, 2003). ORP1L also localizes to the late endosome/lysosome and interacts physically with GTP-bound form of Rab7 (Johansson *et al.*, 2005). Likewise, GTP-bound Rab7 recruits RILP, and in turn RILP is capable of simultaneously binding microtubule motor dynein/dynactin on Rab7-positive organelles. These motors are responsible for the transport of Rab7-positive organelles toward the minus end of microtubules so they may be removed from the cell periphery and directed towards the perinuclear region (Colucci, *et al.*, 2005; Jordens *et al.*, 2001).

Another small G-protein that localizes to the late phagosome is Rab9. Rab9 is required for the transport from late endosomes to the TGN and for lysosome biogenesis (Pfeffer, *et al.*,

2004). Rab9 protein resides in a late endosome microdomain along with M6PR, which play a role in carrying newly synthesized lysosomal enzymes from the TGN to the endosomes, and the return to the Golgi (Ganley, *et al.*, 2004). The stability of Rab9 on the late endosomes requires interaction with the effector tail-interacting protein of 47 kDa (TIP47), which binds directly to Rab9 and enhances its affinity for M6PR cytoplasmic domains (Carroll, *et al.*, 2001).

The phagolysosome

The transition of the early phagosome into a mature, late phagosome, and eventually to the phagolysosome is mediated by Rab7, RILP, and LBPA (Vieira *et al.*, 2002). The elevated concentrations of hydrolytic proteases and extreme acidity (pH 4.5-5) are characteristic of the phagolysosomes and are required in order to degrade the remaining cargo. Lysosomes and phagolysosomes share many markers with the late endosomes/phagosomes, including LAMPs, which can be best identified in "pulse-chase" labeling experiments through the delivery of internalized probes to a terminal, lysosomal compartment in ensured.

Phagosome maturation is driven by a variety of cellular processes ranging from receptor biology, signal transduction, cytoskeletal remodeling, membrane trafficking and ion transport. All these biological processes follow an ordered sequence of events. From the number of various molecules described, there are still existing gaps in understanding the function of proteins in the entire phagocytic process that remain to be elucidated.

The activation of the Fc receptor complex with IgG-opsonized micro particles stimulates the recruitment and assembly of proteins that play specific roles in the internalization of foreign particles. Specific proteins associated with the activated Fc receptor complex on the surface of cells have been studied by various methods including fluorescently tagged antibodies or proteins

(Boros, *et al.*, 1991; Segal, *et al.*, 1984). Fc mediated phagocytosis is dependant on PI3K and Ras super family pathways but the exact class, isoforms and regulatory factors are not known (Scott, *et al.*, 2005; Vieira, *et al.*, 2001). The function of one protein in a multi protein pathway may be of great importance, but the identification and isolation of all proteins in a complex may be more useful. Although surface proteins have been affinity captured from the cell plasma membrane by biotinylation (Sostaric, *et al.*, 2006), the isolation of the whole super molecular complex and its associated pathway has been challenging and not entirely successful (Barnidge, *et al.*, 2003; Schuchardt, *et al.*, 2008; Wu, *et al.*, 2003). The assembly of a receptor complex with all its participating molecules occurs only in live cells where the cellular energy, membranes, electrical potentials and active cytoskeleton are all required (Yeung, *et al.*, 2006). Hence the Fc receptor complex was activated by a ligand opsonized microscopic beads and captured from the surface of live cells. The proteins of the internalized phagosome have been enumerated by tandem mass spectrometry (MS/MS) (Foster, *et al.*, 2006; Garin, *et al.*, 2001) and the proteins of the Fc receptor complex have been collected on the cell surface by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) (Jankowski, *et al.*, 2008).

Data generated by LC-ESI-MS/MS can be searched against a library of proteins to identify the peptides within the sample (McCormack, *et al.*, 1997). Utilizing a data analysis software designed to correlate acquired tandem mass spectra of peptides with amino acid sequences from protein and nucleotide databases can be done quickly and efficiently using SEQUEST (Yates, *et al.*, 1995). The list of peptides identified by the search algorithm SEQUEST can be analyzed based on cumulative unified scores, and fully tryptic sequences, if the samples were digested with trypsin, in order to eliminate false positive identification. To further investigate the true presence of the protein, confirmation by microscopy using the same

IgG-opsonized micro beads, fluorescently labeled antibodies, and protein constructs fused with GFP may be used (Jankowski *et al.*, 2008). In this study three proteins identified with high cumulative scores by LC-ESI-MS/MS, were analyzed by laser confocal microscopy in order to confirm their presence at the activated Fc receptor complex.

Analysis of the Fc receptor associated proteins by LC-ESI-MS/MS has identified many known and previously studied proteins of the phagocytic receptor (Botelho, *et al.*, 2000; Chung, *et al.*, 2000; Ellis, *et al.*, 1991). Many low-abundance regulatory protein isoforms relevant to the Fc receptor were also observed. The agreement between proteins identified by live-cell affinity receptor chromatography (LARC) with previously identified molecules of the Fc receptor that were studied by biochemical and genetic methods supports the discovery of novel proteins associated with the receptor complex. The recruitment of Src-protein kinase, spleen tyrosine kinase (SYK), phospholipase C (PLC), protein kinase C (PKC), PI3K, SHIP, Tec protein tyrosine kinase (TEC), Cdc42, Rap, Pak protein kinase (PAK), GAP, GEF, GRP, and Crk associated tyrosine kinase to the activated Fc receptor was reported by LC-ESI-MS/MS followed by biochemical analysis (Jankowski *et al.*, 2008). Furthermore, the recruitment of proteins specific to the Fc receptor and their functions can be further investigated by confocal microscopy using the same ligand coated beads as were used by LARC. The intensity of the fluorescence at the bead of an immunostained cell or a cell transfected with a fluorescently fused protein will provide an independent and quantitative representation of the presence and amount of that protein at the site of the activated Fc receptor complex.

The proteins identified by LARC represent a diverse collection of cell signaling proteins responsible for many cellular functions previously noted. The importance of this study lies in the use of the literature and information hyperlinked over proteins (IHOP) network analysis to

suggest which members of the Ras superfamily are likely to play a role in phagocytosis. Moreover there is need to infer which proteins are likely to regulate the Rap function in the Fc receptor pathway. The members of the Ras superfamily of small GTP binding proteins is evident in the data obtained from LARC and the controls but some of the activators and effectors of this group of proteins are only evident in the LARC data and not the controls, such as the inactivating protein for Ras, p120RasGAP, and the activating protein of Rap1, a GTP binding protein belonging to the superfamily of Ras proteins, Rap1GEF. This study will target the reliability of the mass spectrometric data obtained by LARC for Rap1, p120RasGAP and Rap1GEF, as well as verify the presence of these low abundance, signal transduction proteins at the site of the activated receptor complex.

Small GTP-binding proteins

Signal transduction within the living cell is mediated in part by the conversion and exchange of GTP to GDP through the action of GTPases. The monomeric group of proteins ranging between 20-40 kDa in molecular mass regulate a wide variety of cellular functions and signal the initiation and termination of various cellular processes by binding GTP molecules. The super-family of small GTP-binding proteins (G-proteins) is of great importance in eukaryotic cells and contains more than 100 members structurally classified into five sub-families: the Ras, Rho, Rab, Arf/Arl, Era-like, GTP translation factor and other families as listed by NCBI conserved domain database (Table 1). These members regulate gene expression, cytoskeletal reorganization, vesicle trafficking, nucleocytoplasmic transport and microtubule organization. (Takai, *et al.*, 2001).

Table I: The superfamily of small Ras-like G-proteins

Table depicting the members of each subfamily of Ras-like G-proteins including Ras, Rho, Rab, Arf/Arl, Era-like, GTP translation factor and others classified structurally.

<u>Ras</u>	<u>Rho</u>	<u>Rab</u>	<u>Arf/Arl</u>	<u>Era like</u>	<u>GTP translation factor</u>	<u>Others</u>	
Rap like	RhoA like	Rab28	Rab5 related	Sar1	YihA-EngB	EF1 alpha	Ran
Rap1	Rac1 like	Rab35	Rab6	Arf6	HflX	EF-Tu	G-alpha
Rap2	RhoBTB	Rab39	Rab7	Arf1/5 like	Obg	EF2	CDC-Septin
RSR1	Cdc42	Rab26	Rab18	Arl1	Ygr210	EF-G	GBP
H-N-K Ras	RhoG	Rab4	Rab19	Alr4/7	DRG	IF2-eIF5B	AlG1
RalA/B	Miro1	Rab30	Rab3	Arl5/8	NOG	eIF2-gamma	Toc34 like
ARHI	Rho2	Rab33A/B	Rab2	Arl2	YchF	SelB euk	BMS1
Rit-Rin-Ric	Wrch1	Rab9	Rab8/10/13 like	Arl3	EngA1	LepA	Miro2
RRP22	Rnd	Rab15	Rab11 like	ARLTS1	EngA2	TypA-BipA	Centaurin-gamma
Rhes like	Rnd3/RhoE/Rho8	Rab24	Rab1-Ypt1	Arl6	Era	CysN-ATPS	p47-IIGP-like
Ras2	Rnd1/Rho7	RJL	RabL4	ARD1	trmE	Snu114p	SR-beta
M-R Ras like	Rnd1/Rho6	Rab12	RabL3	Arl10 like	GTPBP1 like	TetM like	
RERG-RasL11	Rho4 like	Rab40	Rab23	Arfrp1		RF3	
RheB	Rop like	Rab14	Rab32/38	Arl211/Arl13 like		EF-G-bact	
Ras-dva	Rho3	Rab21	Rab36/34	Arl9/Arfrp2 like		SelB	
RGK	Tc10	RabL2	Rab20				
		RabA like	Rab27A				
			Spg1				

G-proteins are present in two interconvertible forms: GDP-bound inactive form and GTP-bound active form. Molecular switches stimulate the upstream dissociation of GDP from the GDP-bound form that is followed by the binding of GTP, leading to a conformational change enabling the interaction and activation of downstream effectors. The rate-limiting step of the GDP/GTP exchange is the dissociation of GDP from the inactive form, which leads to the activation of G-proteins but this process is extremely slow. In order to catalyze the dissociation of GDP, regulators are necessary to speed up the reaction and convert G-proteins to an active GTP-bound form. Activation of small G-proteins is achieved by upstream regulators and downstream effectors. These regulators and effectors are recruited to the protein of interest and regulate the amplitude and the kinetics of signaling. It is this process that triggers a downstream

signal transduction that regulates cellular functions such as cell growth, differentiation, and survival (Downward, 2003; Schlessinger, 2000).

Several small Ras superfamily GTPases have been known to control and coordinate the successive steps in phagocytic uptake by mammalian cells (Fig. 2). A group of GTPases belonging to the Rho family, Cdc42 and Rac, have been confirmed to localize to the site of particle binding during phagocytosis, where Cdc42 regulates pseudopod extension and Rac regulates phagosome closure (Caron, *et al.*, 1998; Castellano, *et al.*, 2001). GTP bound Cdc42, Rac1 and Rac2 have been implicated as distinct regulators that coordinate the rearrangement of actin and membranes during phagocytosis in macrophages via the downstream activation of the Arp 2/3 complex which functions to localize and orient actin polymerization to the site of particle binding (Hoppe, *et al.*, 2004). Rap1, another member of the Ras superfamily has been linked to signaling pathways that enable adhesion-like receptors to actively internalize particles via phagocytosis (Caron, 2003). Although Rap1 has shown to be an essential prerequisite for the internalization of a complement-opsonized particle the mechanism of its action is still unclear. Several Rab proteins have also been found on phagosomal membranes including Rab3, Rab4, Rab5, Rab7, Rab9, Rab10, Rab11 and Rab14 (Desjardins, *et al.*, 1994; Garin *et al.*, 2001). In order to achieve fully functional phagocytosis a mechanism enabling membrane replenishment must be present to compensate for the net loss of the plasma membrane. Adenosine diphosphate (ADP) ribosylation factor 6 (Arf6) and Rab11 are two GTPases essential in membrane delivery to the cell periphery. The function of Arf6 is essential for pseudopod extension around IgG-opsonized particles and recycling the phagosomes to the plasma membrane or the nascent phagosomes (Niedergang, *et al.*, 2003). Similarly Rab11 regulates phagosome recycling and is also required for optimal Fc receptor mediated phagocytosis (Cox, *et al.*, 2000). Other Rab

proteins are mostly involved in phagosome maturation, such as Rab5 that functions in early phagosome fusion and Rab7 that acts as a marker in the late phagosome (Roberts *et al.*, 2000; Vieira, *et al.*, 2003). Aside from the functions mentioned previously little is known about the remaining isoforms and their roles. Currently the numbers of proteins interacting with G-proteins that modulate activation and downstream signaling is increasing and the ongoing study to provide insight about their functions in the mechanism of phagocytosis continues.

Figure 2: Role of Small G-proteins in Phagocytosis.

This theoretical model illustrates the subsequent steps during phagocytosis upon binding of an IgG-opsonized microbead followed by Fc receptor activation and the GTPases that regulate the signaling cascades involved in adhesion dependant activation, actin polymerization and pseudopod extension, membrane delivery and phagosome maturation.

Ras proteins

As members of the G family of proteins, Ras proteins function as fundamental regulatory switches that control normal cell growth and differentiation by cycling between active and inactive conformations at the plasma membrane. Deregulation of the activated Ras family has drawn much attention to this specific group of G-proteins when its members were identified in signaling cascades involved in oncogenesis (Grewal, *et al.*, 2006; Hancock, 2003). Structurally, Ras proteins are 30-55% homologous to one another and all share consensus amino acid sequences responsible for specific interaction with GDP, GTP and GTPase, as well as a region interacting with downstream effectors (Bourne, *et al.*, 1991). The activation of Ras and its members is dependant on the ratio of bound GTP to GDP and since Ras proteins have a slow rate of GTP/GDP exchange and possess low GTPase activity these processes are regulated by GAPs and GEFs also known as guanine nucleotide exchange proteins (GEPs) (Cullen, *et al.*, 2002; Donovan, *et al.*, 2002; Quilliam, *et al.*, 2002). These regulators facilitate the exchange of GDP-bound Ras to GTP-bound Ras, thereby inactivating and activating Ras (Bollag, *et al.*, 1991; Downward, 1992b; Downward, 1992a; Hall, 1992). When in its GTP-bound form, Ras can activate effector proteins stimulating downstream pathways that control proliferation, differentiation and apoptosis. Thus, the activation of Ras is induced by a large variety of extracellular signals, most notably signals that activate cell surface receptors, whose activation targets many downstream pathways essential for cellular processes. However GEFs and GAPs represent a large and divergent protein family that is not yet fully understood and it is difficult to identify which family members are recruited to the Ras isoforms and trigger specific biological responses.

Regulation of Ras activity

The Ras superfamily cycles between two conformational forms induced by the binding of either GDP or GTP. GEFs induce the dissociation of GDP from Ras and allow for its association with GTP, which in turn is hydrolyzed to GDP through the intrinsic GTPase activity of GAPs. A number of specific GAPs and GEFs for Ras have been identified that are essential for the normal functioning of Ras. The GAPs associated with Ras are: p120GAP, neurofibromin, and GAP1, and the GEFs are: son of sevenless (SOS), Ras guanyl nucleotide-releasing protein (GRP), Ras guanyl nucleotide-releasing factor (GRF) (Zwartkruis, *et al.*, 1999). Little is known about how the GAPs and GEFs are regulated, but it has been proposed that GAPs can have additional functions as effectors of Ras signaling (Clark, *et al.*, 1997; Medema, *et al.*, 1992). Effectors of Ras are proteins that specifically bind to and are activated by the GTP-bound form of Ras and the activation of effectors usually results from the translocation from the cytosol to the plasma membrane.

Activation of plasma membrane receptors leads to the recruitment of GEFs, which stimulate the activation of Ras, subsequently activating its effectors that signal cellular pathways. For instance, the activation the receptor tyrosine kinase leads to the recruitment of GEFs that activate Ras and in turn activate its effector Raf-1, from which the phosphorylation cascade proceeds, translocating extracellular signal-regulated kinase (ERK) or mitogen-activated protein kinase (MAPK), which promotes gene transcription (Wittinghofer, 1998). The downstream function of the ERK/MAPK pathway renders the Ras family of GTPases proto-oncogenic, since mutations leading to continuous activation of Ras are among the most prevalent in human cancers. The Ras-Raf-MAP kinase pathways have been most extensively studied however, Ras also activates PI3K which has been implicated in mitogenic signaling and inhibition of apoptosis,

thus confirming its involvement in intracellular protein degradation process in cell growth control (Lee, *et al.*, 2002). Other known effectors for Ras are the p110 catalytic subunit of PI3K, Ral GDP dissociating stimulator (GDS), afadin (AF6), Byr2 protein kinase (homolog to mammalian MAP kinase), and as mentioned Raf-1 (Goldfinger, 2008). Ras functions as a molecular switch linking receptor kinase activation to downstream cytoplasmic or nuclear events through two key effectors: Raf and class I PI3K (Furuta, *et al.*, 2004; Rodriguez-Viciano, *et al.*, 1997). Class I PI3K associate with an adapter molecule of 85 kDa containing two Src homology-2 (SH2) domains. The function of the p85 subunit allows PI3K to migrate from the cytosol to the membrane where its p110 catalytic subunit can bind to Ras and activate PI3K (Baumgartner *et al.*, 2000).

p120RasGAP

The p120RasGAP is a key element of the Ras signaling pathway of small inner membrane-localized G-proteins that are known to regulate gene expression in cellular processes such as differentiation, migration, polarity, growth and death (Olson, *et al.*, 2000; Takashi Matozaki, 2000). p120RasGAP was the first GAP to be characterized at a molecular level and is active on Ha-Ras, Ki-Ras, N-Ras and R-Ras but not on Rho/Rac/Rab proteins (Table 1) (Boguski, *et al.*, 1993; Gibbs, *et al.*, 1988; Trahey, *et al.*, 1987). This negative regulator of Ras is a cytosolic protein that stimulates the intrinsic GTPase activity of Ras through its C-terminal GAP domain (Trahey, *et al.*, 1988; Vogel, *et al.*, 1988). The GAPs facilitate the switch between the active GTP-bound Ras to the inactive GDP-bound Ras, thus effectively switching the GTPase off and inactivating Ras (Bar-Sagi, *et al.*, 2000; Etienne-Manneville, *et al.*, 2002; Mackay, *et al.*, 1998). Ras proteins have been known to function irregularly in certain cancers,

leading to decreased apoptosis and increased invasion and metastasis (Adari, *et al.*, 1988; Downward, 2003). Other studies have addressed the importance of the GAPs for Ras in cell motility, whereby RasGAP is directly involved in cell movement and indirectly involved in complex formation to direct orientation and migration (Sarang V. Kulkarni, *et al.*, 2007). Therefore, it is important to focus on the specific roles of the p120 RasGAP and the selective pathways that these proteins regulate in cellular functions. Since RasGAP has been proposed to participate in cell signaling, identifying its partners is an essential step in understanding its regulatory function (Pronk, *et al.*, 1994).

The binding domain of the 120 kDa GAP for Ras plays an essential role in the recruitment of some proteins to the cell periphery (Bradley, *et al.*, 2006). These domains include two SH2 domains, an Src homology-3 (SH3) domain, and pleckstrin homology (PH) and protein kinase C2 homology domains in addition to the GAP domain. The orientation of the binding domains from the NH₂-terminal region contains two SH2 domains flanking an SH3 domain, a central region containing CalB and PH domains and the carboxy-terminal responsible for the catalysis that drives the conversion of Ras-GTP to Ras-GDP (Fig. 3) (Gawler, *et al.*, 1995; Pawson, 1995). These domains mediate the recruitment of RasGAP to the membranes and their receptors whereby it subsequently localizes to Ras.

Figure 3: The Human RasGap protein (Swiss-Prot, P20936).

RasGAP contains a total of 1047 amino acid residues with the amino terminal region consisting of the SH3 domain flanked by two SH2 domains, followed by a pleckstrin homology (PH) domain, a membrane targeting C2 domain, and a GTPase activating domain (GAP) at the carboxy-terminal.

The binding domains of p120 RasGAP have been closely studied and many interacting proteins have been identified for each domain. The SH2 domains bind phosphorylated tyrosine residues and in RasGAP have been noted to interact with p56, p62^{dok}, and p190 RhoGAP (Di Cristofano, *et al.*, 1998; Ellis, *et al.*, 1990). Other important molecules such as an SH2 domain-containing tyrosine phosphatase (SHP2) has been shown to down-regulate phosphorylation and disengage RasGAP, thus sustaining Ras activation by the interaction of Gab1 with the RasGAP SH2 domains (Montagner, *et al.*, 2005). The SH3 domains are conserved in many cellular signaling proteins and mediate protein interactions via specific proline-containing peptides. The complex formation of p190 RhoGAP with p120 RasGAP upon tyrosine phosphorylation results in a conformational change that exposes the target binding surface of the RasGAP SH3 domain allowing for additional protein interactions (Hu, *et al.*, 1997). Furthermore, one of the RasGAP SH3 domain binding protein is G3BP, a 68 kDa cytosolic protein containing two RNA-binding domains that signify that this interaction may modulate translational efficiency of mRNA and is essential for proper growth and development (Parker, *et al.*, 1996; Zekri, *et al.*, 2005). The carboxy-terminal catalytic domain interacts with and enhances the GTPase activity of proto-oncogenic p21 Ras and acts as a direct negative regulator of p21 Ras signaling by accelerating Ras GTPase activity (Adari *et al.*, 1988; Zhang, *et al.*, 1990).

Rap proteins

Another group of small GTPases belonging to the Ras family of proteins are Raps, which are regulated by an array of specific GEFs and GAPs. Rap1 has been noted to regulate various cellular processes including cell proliferation, differentiation, cytoskeletal rearrangement, MAPK signaling, cadherin-mediated cell junction, as well as integrin-mediated cell adhesion (Bos, 2005;

Bos, *et al.*, 2001; Stork, 2003). There are five isoforms of Rap: Rap1A, Rap1B, Rap2A, Rap2B, and recently discovered Rap2C, (Paganini, *et al.*, 2006) with which Rap1A and Rap1B share more than 90% sequence homology and show no distinct variation in cellular functions and for the purpose of consistency will be referred to as Rap1 from now on. Recently Rap1 was shown to be present at the site of the activated phagocytic receptor in RAW 264.7 macrophages (Jankowski *et al.*, 2008). Rap1 is a monomeric, Ras-like small GTPase regulator that functions in cellular processes by coupling signals generated within the cellular membranes to intracellular effectors. The signal transduction of Rap1 is common to that of Ras due to the similarities of the effector domain region between Rap and Ras. The activation of Rap1 is mediated by distinct GEFs that increase the concentration of GTP-bound Rap1 to elicit a cellular response. The localization of Rap1 has been monitored and shown to recruit to perinuclear structures (Yanhong Liao, *et al.*, 2001), TGN (Caron, 2003; Johannes L. Bos, *et al.*, 2001), lysosomal vesicles (Johannes L. Bos *et al.*, 2001), endosomes (Caron, 2003; Chengbiao Wu, *et al.*, 2001; Randall D. York, *et al.*, 2000), and most interestingly, the plasma membrane (Xianlong Gao, *et al.*, 2001; Yusuke Ohba, *et al.*, 2003). For some cellular responses, the choice of cell-type-specific Rap1 exchangers may both activate Rap1 and direct it to a specific target. As is the case with many GTP binding proteins, the roles of Rap and its associated proteins in cellular functions are complex and not yet fully understood. However, evidence has suggested that Rap exerts biological activities in cellular growth and differentiation as well as in phagocyte specific pathways eluting to the fact that Rap may play a very specific role in certain cells (Bokoch, 1993).

There are various specific inactivating GAPs associated with the signaling pathways of Rap. The GAPs specific for Rap are Rap1GAP (Rubinfeld, *et al.*, 1991), the tuberous sclerosis

complex 2 product tuberin (Wienecke, *et al.*, 1995), and SPA-1 (signal-induced proliferation associated gene-1) (Hattori, *et al.*, 1995). Specific RapGAP activities have been detected in the cytosol and the plasma membrane of different cells (Kikuchi, *et al.*, 1989; Ueda, *et al.*, 1989).

Numerous GEFs have been identified as activators of Rap1, including C3G (a homolog to Rap1GEF), cyclic adenosine monophosphate (cAMP) GEFs, Ca^{2+} and/or diacyl glycerol (DAG) activated GEFs (CalDAG-GEFs) and PDZ-GEFs (Bos *et al.*, 2001; Ehrhardt, *et al.*, 2002). Rap1 has been proven to be vital for various morphogenic processes in migration and cell shape and mutations of Rap1 impair embryo development in some organisms (Asha, *et al.*, 1999; Baena-Lopez, *et al.*, 2005). Mutations of the Rap1 effectors have also been shown to cause significant effects in signaling pathways. C3G binds through the proline-rich domain (PRD) to the SH3 domain of Crk, an adaptor protein involved in tyrosine kinase-dependant activation of Rap1 occurring on membrane receptors and adaptor proteins (Gotoh, *et al.*, 1995). cAMP-GEFs contain two cyclic nucleotide binding domains (CNBDs) one of which, cAMP binds and activates thereby stimulating Rap-dependant signaling (Bos *et al.*, 2001; de Rooij, *et al.*, 1998). CalDAG-GEFs are regulated by distinct calcium and DAG binding domains and were identified as a critical regulator in inside-out integrin activation in human T lymphocytes, neutrophils, and platelets (Kawasaki, *et al.*, 1998; Pasvolsky, *et al.*, 2007). PDZ-GEFs are recruited to membrane receptors and proteins via cAMP-binding domains and a PDZ domain that is specific for Rap1 and Rap2, a close relative of Rap1 (de Rooij, *et al.*, 1999).

The function of GEFs and GAPs is cyclical and complimentary, where GEFs promote the conversion of GDP-bound or inactive form of small G protein to GTP-bound or active forms and GAPs enhance the hydrolysis of bound GTP to GDP. Ras and Rap1 proteins have both been shown to recruit to the plasma and endosomal membranes (Ehrhardt *et al.*, 2002; Hancock,

2003). Ras and Rap1 share extensive homology within the regions involved in effector binding and both bind to the effectors Raf-1, B-Raf, RalGDS and AF-6 (Bos *et al.*, 2001). Rap1 antagonizes the actions of Ras in a variety of cellular systems, but it also enhances Ras action in others (Stork, 2003). The similarity between Ras and Rap are evident and the individually distinct cellular effects that they exert may be due to the consequence of their localization. Ras residing in the plasma membrane and Rap in the perinuclear membrane compartments may result in differential signaling (Mochizuki, *et al.*, 2001). These two signaling molecules have been of interest in recent studies due to their involvement in a wide range of cellular processes, however, due to the extreme diversity of signal transduction their actions still lack full consensus.

The direct association of Rap with the phagocytic receptors can be demonstrated by molecules common to both the protein and the process. Recent findings suggest that Rap signaling positively contributes to cadherin-mediated adhesion (Price, *et al.*, 2004). The route of entry by pathogens through the host cell phagocytic pathway is mediated by adhesion molecules that bind E-catherin (Mengaud, *et al.*, 1996). E-catherin is a transmembrane adhesion molecule important in the formation and maintenance of cell contacts in solid tissue. In this light, it is possible that Rap may function to regulate cell-surface adhesion receptors, such as the phagocytic receptor. It has also been shown that Ras regulates the activation of PI3K, the key regulatory element of endocytic trafficking. Although the main GTPases that drive the phagocytic pathway belong to the Rab subfamily of small G-proteins, there are many functions within phagocytosis that are still not yet explained and provide the rationale that Ras and its associated members may also play unknown roles in phagocytosis.

RATIONALE

Considerable evidence is available from the genetic and biochemical analysis of the innate immune system and the signaling agents associated with the Fc receptor that might be potential therapeutic targets for the prevention of immune associated diseases. Over the last two decades substantial progress in the understanding of signal transduction at a molecular level has resulted in the identification of signaling cascades involved in innate immunity. The Fc receptor signaling pathway and actin assembly resulting in pseudopod extension and remodeling of the cell membrane has been extensively studied and is best understood in comparison to other cell surface receptors (Aderem *et al.*, 1999; Cox, *et al.*, 1999). The uptake of IgG coated particles by phagocytic cells is well established, thus making the Fc receptor an ideal surface receptor to use as an experimental model (Kasai, *et al.*, 1982). The effect of many proteins at the cell surface as activators or inhibitors leads to complex downstream signaling pathways that are still not well understood. The use of liquid chromatography coupled to tandem mass spectrometry is a practical method that can be used to identify most regulatory proteins of the Fc receptor pathway. However the sensitivity of these instruments is still not at its ideal level and extensive sample preparation is required, thus further investigation of these proteins via biochemical analysis is necessary.

p120RasGAP may regulate some Ras-dependant cellular processes and has been shown to be important in growth and development through its association with key regulatory proteins. The determination of p120 RasGAP at the site of the activated receptor in RAW 264.7 macrophages will further expand our understanding of this complex protein and its regulatory functions that have been shown to promote downstream signaling aside from inactivation of Ras (Clark *et al.*, 1997; Wittinghofer, 1998). GAPs and GEFs may direct the immediate response to

stimuli by triggering necessary associations in order for signaling proteins to carry out cellular functions. Understanding the regulatory roles of GAPs and GEFs may provide insight on various human immune diseases.

METHODS AND MATERIALS

Reagents

Monoclonal mouse anti-Ras-GAP was obtained from BD Transduction Laboratories (San Jose, CA). Monoclonal rabbit anti-Rap1A/Rap1B was obtained from Cell Signaling Technologies, Inc. (Danvers, MA), monoclonal rabbit anti-C3G was obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Cy2-, Cy3-, and Cy5- conjugated anti-rabbit and anti-mouse secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). The 2 micron polystyrene beads were obtained from Bangs Laboratories (Fishers, IN). Human IgG and all other reagents were obtained from Sigma-Aldrich unless otherwise stated.

Nucleic acids

The following plasmids encoding p120 RasGAP and Rap1A were used in these experiments. Construct for p232-mRFP-N1, was generously provided by William D. Bradley (Department of Molecular Biophysics and Biochemistry, Yale University, New Haven) (William D. Bradley, et al., 2006). The p232-mRFP-N1 construct consisted of the SH2-SH3-SH2 (232 kDa) domains of p120 RasGAP were cloned into the pmRFP-N1 vector (Clontech's pEYFP-N1 vector with EYFP excised and mRFP from Roger Tsein cloned into the AgeI and NotI sites). The mRFP sequence is 3' to the 232 sequence, resulting in a carboxyl-terminal tag constructed by Tony Koleske and Bill Bradley. Primer information is not known, however, template was likely pRcHA-p120RasGAP from Jeff Settleman. 232 was cloned into the pmRFP-N1 vector using XhoI and AgeI. This 232 fragment of p120RasGAP binds to the RasGAP binding region of p190RhoGAP disrupting its ability to bind p120RasGAP. This event will disrupt the

translocation of p190RhoGAP to the cell periphery and prevents its ability to inhibit RhoA in vivo (Bradley *et al.*, 2006).

Constructs for EGFP-Rap1A (in pFLAG-CMV-2) were generously donated by Dr. Tohru Kataoka (Department of Molecular and Cellular Biology, Kobe University, Japan). A BamH I/BamH I fragments encoding wildtype Rap1A was subcloned into the BamH I site of the mammalian expression vector pFLAG-CMV-2 and a Hind III/Hind III fragment encoding EGFP was inserted into the Hind III site of pFLAG-CMV-2 (Xianlong Gao *et al.*, 2001).

Cell culture

The macrophage RAW 264.7 cell line was obtained from ATCC (American Type Culture Collection, VA, USA). Macrophage cells were cultured in Dulbecco's modified Earl's alpha-MEM with 5% heat-inactivated fetal bovine serum in 25 cm² culture flasks at 37 °C, with 5% CO₂ (Jankowski *et al.*, 2008).

Experimental Procedures

Live-cell affinity receptor chromatography (LARC) coupled to LC-MS/MS

The Fc receptor complex was captured by LARC as described by Jankowski *et al.*, 2008 (Jankowski *et al.*, 2008). IgG-coated polystyrene beads were presented to the surface of live RAW 264.7 macrophage cells for 30 min on ice. Unbound beads were washed away and the assembly of the Fc receptor complex was activated by warming the cells for a maximum of 10 min at 37 °C. The internalization of the beads was quenched with ice cold buffer, thus keeping the complex intact and preventing internalization. The cells were disrupted using a French Press

(FA-078A, Thermo Electron, Waltham, MA, USA) and the beads were isolated by sucrose gradient centrifugation (Beckman, USA) (Jankowski *et al.*, 2008). The protein fraction obtained from the extractions was digested with trypsin and analyzed by LC-MS/MS in at least three replicates alongside controls as described by Jankowski *et al.*, (2008). The MS/MS data files generated by the LC-ESI-ion trap were searched against a non-redundant library of proteins using the SEQUEST algorithm, with scoring conditions and settings recommended by the manufacturer (Chelius, *et al.*, 2002; Yates, 1998).

Confirmation of proteins identified by SEQUEST

Manual inspection of proteins generated by search algorithms is required in order to eliminate false positives. Lists of peptides identified from the MS/MS data by SEQUEST were manually inspected for proteins of interest and correlated to the full length FASTA protein sequences from the NCBI website. The sequences for Rap, p120 Ras Gap and Rap GEF 1 were correlated by BLAST to screen for homologous proteins or peptides that were identified by SEQUEST. Peptide sequences generated from the MS/MS data were aligned with the FASTA protein sequences and accepted as true if at least three different peptides from a single protein identified by SEQUEST were present in the FASTA sequence.

Confirmation by confocal microscopy

In order to confirm that the proteins identified by LARC following LC-MS/MS were truly associated with the Fc receptor complex, additional experimentation using Western Blots and confocal microscopy coupled with antibody staining or fluorescently fused constructs of

endogenous proteins were used. Image analysis of both live and fixed RAW 264.7 macrophage cells was done by using Zeiss LSM 510 laser scanning confocal microscope with a 100x oil immersion objective.

Western blot of RAW 264.7 cell lysates

Confirmation of the presence of RasGAP and Rap1A/Rap1B, and C3G was done on RAW cell lysates using Western blotting technique. Cell lysates were prepared by scraping cells that were grown to 80-90% confluency in a 75 cm² culture flask in cell culture conditions. Approximately 5 ml of 2xSDS-PAGE sample buffer containing approximately 5mM PMSF, 5mM AEBSF, 5mM EDTA salt and eukaryotic anti-protease cocktail (Sigma-Aldrich) was added to the scraped cells and immediately heated for 10min at 100°C. Approximately 30 µl of cell lysate alongside a molecular weight marker was run on discontinuous polyacrylamide gels with 4% monomer stacking gels with Tris pH 6.8 and 7% monomer separating gels with tris pH 8.8 in tricine anode and cathode buffers (Schagger, *et al.*, 1987). Proteins from SDS-PAGE were transferred to Immobilon-P Transfer Membrane, 0.45µm pore size (Millipore, Bedford, MA) (Burnette, 1981). The membrane was stained with Coomassie stain with no acetic acid, de-stained with 50% methanol, dried, and marked with a pencil indicating the location of the molecular weight marker bands. The membrane was soaked in 100% methanol until completely de-stained and blocked with PBST (PBS + 0.1% Tween) 5% skim milk, and 1% goat serum for 30 min. After blocking, membranes were washed 3 times with PBST prior to incubating with 1:2000 primary antibody for RasGAP, Rap1A/Rap1B, or C3G, in PBST for 30 min, and washed 3 times again. The membrane was incubated with 1:20,000 HRP conjugated secondary antibody

in PBST for 30 min, washed extensively with PBST, and developed with ECL Western blotting substrate solution (Pierce, Rockford, IL).

Cell staining

Cells were scraped and grown on 2.5 cm glass cover slips in six-well plates containing 1-2 mL media. Cells were allowed to grow 24-48 hrs until ~30% confluence was reached following transfection or immuno staining. Sheep red blood cells (SRBC) or 2 micron polystyrene beads were opsonized with rabbit anti-SRBC or human IgG at room temperature, for 30min and washed three times with 1xPBS as described previously (Marshall J.G., *et al.*, 2001). Cells grown on glass cover slips were incubated with opsonized particles for 5-7 min at 37 °C. Cover slips with cells were put on ice and washed with ice cold PBS and fixed for 30 min with 4% paraformaldehyde in 1xPBS. The fixed cells were gently washed 3 times with 1xPBS and then 3 times with 1xPBS and 5% glycine. After this step cells were either left overnight in 1xPBS with 0.1% MgCl at 4°C or were permeablized with 0.1% Triton in 1xPBS (or PBST) for 30 min before blocking. Cells were blocked with 5% skim milk and 1% donkey serum in 1xPBST for 1 hour. After blocking the cover slips were washed 3 times with 1xPBST. On the cleaned cover of the 6 well plate, 24 µl of PBST was spotted and incubated with 1 µl primary antibody, the cover slips were then placed face down on the 25µl spot, avoiding air bubbles, and incubated at room temperature for 30 min. Then the cover slips were placed back in the wells and washed 3 times with 1xPBST. Cells were incubated with the cy2-conjugated secondary antibody at 1:20000 dilution in 1xPBS for 30 min at room temperature. The cover slips were washed 3-5 times with 1xPBS and fixed onto microscope slides with DAKO (Dako, Denmark) until dry and analyzed using a laser confocal microscope.

Competent Escherichia coli (E.coli)

The GFP and RFP fused constructs were re-dissolved in sterile TE buffer (10mM Tris-HCl pH 7.6-8, 1mM EDTA). Inducing competence in the *E.coli* strain DH5 Alpha (ATCC) was carried out as described by Hanahan (Hanahan, 1983). LB broth was inoculated with *E.coli* strain DH5 Alpha stock and incubated overnight at 37°C. Approximately 1ml of the overnight culture was transferred into 5 mL LB broth and grown at 37°C, shaking at 200 rpm for 2 hrs. From this culture 1 ml was drawn out and added to 100 ml pre-warmed LB broth and allowed to grow until an optical density of 0.3 to 0.45 was reached (approximately 2hrs). This culture was chilled on ice and centrifuged at 3500 rpm / 8000 g for 5 min at 4°C. Supernatant was discarded and the pellet was resuspended in 2/5 original volume Tfb I buffer at pH 5.8 (30 mM potassium acetate, 100 mM RbCl₂, 50 mM MgCl, 15% glycerol, 10 mM mM CaCl₂ · 2H₂O) and chilled on ice for 5 min. The contents were centrifuged at 3500rpm/6000g for 5 min at 4°C and the pellet was resuspended in 1/25 original volume Tfb II buffer pH 6.5 (10 mM MOPS, 75 mM CaCl₂ · 2H₂O, 10 mM RbCl₂, 15% glycerol) and chilled on ice for 15 min. Competent cells were stored in 100 µl aliquots at -80°C.

Cell transformation

Competent *E.coli* cells were thawed on ice and 50-100 µl was incubated with approximately 1mg/ml DNA and left on ice for 30 min. The cells were heat shocked at 42°C for 90 seconds, and chilled on ice for 1-2 min. The cell suspension was added to 1 ml LB broth and incubated at 37°C, shaking at 200 rpm for 1 hr minimum. The cell culture was plated on LB agar plates alone, and containing 50 µg/ml kanomycin or 100 µg/ml ampicillin and grown overnight at 37°C. Colonies that grew on plates indicating resistance were picked off and grown in 2ml LB

containing the specific antibiotic for 6 hrs. Further, 5 ml LB containing antibiotic was inoculated with 100 µl of the 6 hr culture and grown overnight in the same conditions. The cells were later harvested and the plasmid was extracted using Q1Afilter Plasmid Maxi kit (QIAGEN, Valencia, Calif.).

DNA Extraction

Transformed *E.coli* cells were grown in 100 – 250 ml LB media containing specific antibiotic for 6-8 hrs, and harvested as described in the manufacturers protocol for Q1Afilter Plasmid Maxi kit (QIAGEN, Valencia, Calif.). The concentration of DNA in TE buffer was determined by UV absorption at 260 nm.

Cell Transfection

The RAW 264.7 macrophage cells were transfected using FuGENE 6 transfection reagent (Roche Diagnostics, USA) according to manufacturers directions or electroporated as described in the Nucleofection protocol (Amaxa, Cologne, Germany) using the setting file #D-032. The cells were normally grown in 25 cm² culture flasks at 37 °C, 5% CO₂ for 24 to 48 hrs until 90% confluency was reached. Cells were scraped, pelletized and resuspended in amaxa nucleafector solution as provided by the manufacturer, combined with DNA and electroporated. Following electroporation transfected cells were quickly diluted in pre-warmed media and incubated in cell culture conditions overnight followed by immunostaining or analysis by live cell confocal microscopy. The RAW 264.7 macrophages were transfected with p232-mRFP-N1 and EGFP-Rap1A (in pFLAG-CMV-2) alongside a pmaxGFP control (provided by manufacturer).

RESULTS

Analysis of proteins associated with the activate Fc receptor complex may provide insight on the regulation of the innate immune response. In this experiment, RAW 264.7 macrophages were exposed to latex beads coated with an Fc receptor activating ligand and the proteins within the assembled complex were identified and analyzed using tandem mass spectrometry. Mass spectrometry data was further confirmed by visualizing phagocytic macrophages that were immunostained or transfected with fluorescently fused constructs via confocal microscopy

Correlation of LC-MS/MS data to protein sequences

Analysis of data obtained by live cell affinity receptor chromatography followed by tandem mass spectrometry revealed a number of potential molecules related to the Ras superfamily of proteins. Interestingly, many Ras and Rap associated GEFs and GAPs were detected. Rap1 and its variants were detected with high scores, some above 75,000, mostly with three or more different tryptic peptides (Table II). Rap1A was detected with the highest score at 77 410 with 4 different peptides, the second highest score was seen for cAMP dependant Rap1 at 3433, with 8 different peptides, third highest score for another Rap isoform was apparent at 2983 representing 5 different peptide sequences. Ras isoforms were not apparent in the data collected from the LARC samples.

Approximately the same number of GEFs associated with Rap and Ras were also detected with relatively high scores in the range of 100 to above 14000 (Table III). PDZ GEF, a known GEF for Rap was detected with the highest score at 14,092, with 21 different tryptic peptides. The second highest score was for Rap1GEF at 5837 and 14 different peptides. Aside from the predominant presence of Rap GEFs, a poor score was obtained for RasGEF at 375 and

only one tryptic peptide was identified.

On the contrary, Ras family associated GAPs were detected with much more significant scores than Rap associated GAPs (Table IV). p120RasGAP, a known deactivator of Ras, was identified in three different isoforms with scores of 2323, 9596, and 1908. The isoforms of p120RasGAP were detected with at least 5 different peptides, all ending in a tryptic cleavage. Rap associated GAPs were detected with much less intense scores and not as many peptides. Rap1 GAP was detected with efficiency scores of 2856, 1730 and 2378 with at least three different peptide sequences. The Ras family of proteins was detected with high scores for Rap1 and its variants from the LARC experiment, thus providing evidence that the mass spectrometry data is in fact reliable. Also the data collected for the p120RasGAP suggests that this molecule may be present at the activated Fc receptor and the data associated with RapGEF also supports this notion.

To further validate and minimize false positive identifications by SEQUEST only proteins with at least three different peptide sequences were considered to be true positives (Cargile, *et al.*, 2004). Multiple peptides identified by LC-MS/MS were manually correlated to the full length FASTA protein sequences of Rap1/Rap2, p120RasGAP and Rap GEF1 (Fig. 4). Rap1A was identified by 4 different tryptic peptides that correlated to the full length proteins sequence, from which two sequences were overlapping (Fig4, Panel A). Ras p21 protein activator of p120RasGAP was distinctly identified by six different tryptic sequences that correlated to the full length FASTA protein (Fig4, Panel B). And finally, 5 tryptic peptides identified by tandem mass spectrometry were in exact alignment with the native protein sequence for RapGEF1 (Fig.4, Panel C). The identification of these proteins by mass spectrometry seems reliable since the proteins were identified by at least three different peptide

sequences essentially confirming the presence of Rap, p120RasGAP and RapGEF at the site of the IgG receptor complex captured by LARC to be true.

Furthermore good quality MS/MS spectra was obtained for Rap1, p120 RasGAP and Rap1GEF (Fig. 5) with good mass accuracy, typically within m/z 0.2, and resolution of approximately 12, 000 that permitted a clear delineation of 2+, and 3+ charge states. LC-MS/MS spectra for Rap1 from the LARC experiment is shown for the 2+ peptide LVVLGSGGVGK (MH+ 986.19), 3+ peptide SKINVNEIFYDLVR (MH+ 1710.96), and 2+ peptide INVNEIFYDLVR (MH+ 1495.71) with correlating b and y ions. The spectra fragmentation for three selected peptides for p120RasGAP is indicated for the 3+ peptide EPVPMQDQGQVLNDTVDGK (MH+ 2071.26), the 2+ peptide YIYGCLQKSVQHK (MH+ 1567.84), and the 3+ peptide PSDNTPGDYSLYFRTNENIQRFK (MH+ 2763.96). Moreover, mass spectra of the selected fragmentation patterns for Rap1GEF peptides are indicated by fully tryptic 2+ peptide MKPPKIPFMPLLLK (MH+1654.20), 3+ peptide CNEVQLWVATEILLCSQLGK (MH+ 2248.65), and 3+ peptide VLHLVSQWISLYK (MH+ 1586.90). Peptides identified for these three proteins show accurate b and y ion series alignment, all tryptic peptides and well-resolved peaks.

Table II: Detection of Ras superfamily member Rap1 and related proteins in RAW 264.7 macrophages.

Proteins sample collected by LARC method were analyzed by LC-MS/MS and identified using SEQUEST algorithm. This table indicates various isoforms of Ras and Rap1 identified with different peptided sequences, protonated parent ion (MH+), charge, score, total score, and accession number.

Reference	Sequence	MH+	Charge	Score	Score XC	Accession Delta Cn
rap2 interacting protein x [Mus musculus]	QEMELAMK	980.19	2	294.76	2698.46	21312036.0
	QEMELAMKMLEKDVICEK	2056.48	3	768.11	1.38	0.0
	VKEESSYLLESNRK	1682.86	3	1220.81	1.77	0.1
	QQLDDLRLALKHELAFK	1926.21	3	266.19	2.05	0.1
	IITLQEEMER	1262.46	2	148.59	1.07	0.0
RAS, guanyl releasing protein 2; RAP 1A protein-specific guanine nucleo	MASTLDLDK	994.15	2	1307.57	1307.57	6755290.0
					1.69	0.1
unnamed protein product [Mus musculus] for Rap1 8	HTLDGAACLLNSNKYSPSR				40.48	12861034.0
	VSIK	2475.81	2	40.48	0.74	0.0
RAP1A, member of RAS oncogene family; RAS-related protein RAP1A [Homo s	LVVLGSGGVGK	986.19	2	2855.44	77410.60	4506413.0
	LVVLGSGGVGK	986.19	2	5884.80	1.65	0.3
	LVVLGSGGVGK	986.19	2	3058.54	2.63	0.4
	LVVLGSGGVGK	986.19	2	4575.36	2.25	0.2
				14301.1	2.51	0.3
	SKINVNEIFYDLVR	1710.96	3	9	3.90	0.4
	INVNEIFYDLVR	1495.71	2	6	3.75	0.4
	INVNEIFYDLVR	1495.71	2	6	4.13	0.5
	LVVLGSGGVGK	986.19	2	2104.65	2.08	0.2
	LVVLGSGGVGK	986.19	2	1658.87	2.13	0.2
	VVGKEQQQLAR	1299.46	2	168.46	1.16	0.0
				14301.1		
	SKINVNEIFYDLVR	1710.96	3	9	3.90	0.4
					1429.22	28522596.0
	GSRAVPLVLVGTKADLDA					
similar to RAP2A, member of RAS oncogene family; K-REV; RAP2, member o	ER	2068.36	3	943.65	2.03	0.0
	IPANALETWPAERFIG	1786.02	3	181.75	1.19	0.0
	CEPSVEELFSK	1268.42	2	122.32	0.70	0.0
	TMVDHVFTQVVREMEALA					
	PPGQEAPR	2910.32	3	181.50	1.15	0.1
RAP2B, member of RAS oncogene family [Mus musculus]	VVVLGSGGVGKSALTVQF				531.93	13386338.0
	VTGSFIEK	2581.00	3	531.93	1.35	0.1
RAP1B, member of RAS oncogene family; K-REV; RAS-related protein RAP1B					400.04	7661678.0
	VKDTDDVPMILVGNK	1644.92	3	400.04	1.32	0.0
Rap2ip protein [Mus musculus] - mouse musculus]					398.29	28279470.0

RGSE_MOUSE Regulator of G-protein signaling 14 (RGS14) (RAP1/RAP2 interacting	RELEGVILELQEQLPDPPP PR	2523.87	3	398.29	1.39	0.1		
					2708.76	3914636.0		
	VVRISAKPTK	1099.35	3	99.99	1.14	0.0		
	KSLGSGESESESR	1353.38	2	580.78	1.37	0.1		
	ACERFQQIPASDTK	1594.78	2	150.21	0.81	0.1		
	REMTGGAMNSALRR	1550.79	2	447.27	1.02	0.1		
	PGKYCCVYLPDGTASLALA							
	RPGLTIR	2737.24	2	188.86	1.04	0.0		
	HLGVPNGR	849.96	2	418.19	1.27	0.1		
	QTCDieGLVELLNrvQSSG							
	AHDQRGLLR	3109.47	2	198.00	0.98	0.1		
	SPLYQECLLAEAEGR	1679.88	2	384.64	1.35	0.1		
	QTCDieGLVELLNrvQSSG							
	AHDQRGLLR	3109.47	2	198.00	0.98	0.1		
	PGEKQPMDLTPVSSVASQ							
	TLVLDTPPDAK	3152.52	2	42.81	0.83	0.0		
RAP1, GTP-GDP dissociation stimulator 1; cDNA sequence BC011279 [Mus m					2110.87	21704072.0		
	LLSALIRHSK	1138.39	3	580.47	1.50	0.1		
	LLSALIRHSK	1138.39	3	274.88	1.31	0.0		
	EDDVAELKTASDLMVLLL							
	LGDESMQKLFEGGK	3496.99	2	360.51	1.13	0.1		
	SEMPVQFKLLGTLRMLID							
	AQAEAAEQLGK	3286.86	3	501.17	1.70	0.1		
	NAKLVERLVEWCEAK	1789.09	3	280.58	1.34	0.0		
	SEMPVQFKLLGTLRMLID							
	AQAEAAEQLGK	3286.86	2	113.27	1.06	0.1		
Glutamate receptor, ionotropic, AMPA3 (alpha 3); cAMP-dependent Rap1 g					3433.69	8393313.0		
	DIQEFRIIEEMDR	1851.08	3	677.05	1.55	0.1		
	INTILEQVVILGKHSR	1821.16	3	439.49	1.49	0.0		
	YGARDPETKIWNMGVDEL							
	VYGR	2512.83	3	739.74	1.13	0.0		
	GFSILQAIMEAAVQNNWQ							
	VTAR	2448.79	2	55.15	1.06	0.0		
	NAPLKYSALTHDAILVIAE							
	AFR	2515.89	3	404.80	1.23	0.1		
	NTVQEHSFAFRFAVQLYNT							
	NQNTTEK	2942.15	2	69.11	0.87	0.1		
	GSALGTPVNLAVLK	1340.59	3	730.50	1.36	0.0		
	GAILSLGYYK	1198.44	2	66.48	0.90	0.0		
	LSIVGDGKYGARDPETK	1807.00	2	251.36	1.16	0.1		
Similar to TRF2-interacting telomeric RAP1 protein [Mus musculus]					2983.75	20071926.0		
	ADGYPIWSRQDDLDLQKD							
	DEDAR	2722.82	3	623.69	2.21	0.0		
	DDEDARDALVK	1247.30	3	964.42	1.64	0.0		
	ADGYPIWSR	1065.16	3	179.24	1.10	0.1		
	SSLTEHSWQSLKDRYLK	2079.30	3	431.95	1.32	0.0		
	ENDEAVKKMLVEATR	1733.97	2	784.45	1.99	0.0		
similar to V-SERA 4 [Mus musculus] or for Rap1 4					186.59	38077718.0		
	FQSAEDGSEGNFKGPRLDN TFLFQR	2862.06	3	186.59	1.27	0.0		
complement component C2 [Mus musculus] rotein 1 (Rap					1500.81	192435.0		
	RKQGISEFYADDIALLK	1968.24	0	240.43	1.34	0.0		
	QGISEFYADDIALLK	1683.88	3	628.58	1.41	0.2		
	IFSFEVNTVAITFASQPKT IMSILSER	3243.81	3	631.80	1.54	0.1		

C530044N13Rik protein [Mus musculus] or for Rap1	VRSAMEAADVFHRAR	1716.95	3	856.40			1310.07	19343815.0
	PKFFVLCGDLVHAMPOTP WR	2272.72	2	453.67			2.27	0.0
Rap2 interacting protein; Rap2 interacting protein 8 [Mus musculus]							1233.98	7949125.0
	PEGASNSKLYR	1222.33	2	539.85			1.02	0.2
	RHSFMSTEPLSAEASLSSDS QR	2424.59	3	694.13			1.89	0.0
RIKEN cDNA 8030491N06 [Mus musculus] for Rap1 o							1089.98	38087813.0
	GTRLTIMMSMRVMVR	1783.29	0	282.58			1.23	0.0
	FHGKASDAVR	1088.20	2	439.78			1.24	0.0
	IALEWVAIMYGLGALLEV							
	WVAFQOK	2850.41	3	206.41			1.40	0.0
	WLASLEKCLRFTIVNLLQSC VATR	2778.38	2	161.21			1.34	0.0
Unnamed protein product [Mus musculus] for Rap1 o							883.39	26350257.0
	HSPVPRPASRSSSPGAK	1718.90	0	372.77			1.30	0.1
	RNSAPESILLVPPTWPK	1906.22	3	510.62			1.17	0.1
Unnamed protein product [Mus musculus] 1 (Rap							397.54	26326413.0
	RLETIQDLRALSVLMVSISS LISPCFQER	3306.89	2	397.54			1.27	0.1
Unnamed protein product [Mus musculus] for Rap1 &							102.60	12857665.0
	NDWETTIEHFHVETLADN AIIVYQTHK	3302.60	2	102.60			0.82	0.0
FGF-14C (FHF-4C) [Mus musculus] meric RAP1 protein							453.10	6714538.0
	AGVTPSKSTSASAIMNGGK	1764.98	2	453.10			1.29	0.0
similar to Ras-related protein RAP-1A (C21KG) (KREV-1 protein) (GTP-bi							426.29	20875149.0
	KTPVGKEEA	959.08	2	426.29			1.06	0.1
similar to Rap2 binding protein 9 [Homo sapiens] [Mus musculus]							276.90	28501205.0
	GGGGGGGGGKK	731.78	2	276.90			1.37	0.0

Table III: Detection of Rap and Ras GEFs in RAW 264.7 macrophages

Protein sample collected from LARC were analyzed by LC-MS/MS and identified using SEQUEST algorithm. This table indicates various isoforms of Ras and Rap GEFs identified with different peptided sequences, protonated parent ion (MH+), charge, score, total score, and accession number.

Reference	Sequence	MH+	Charge	Score	XC	Accession Delta Cn
GFR_MOUSE Guanine nucleotide exchange factor for Rap1 &				5837.4	1	32171383.0
	MFLKTIYR	1072.35	2	514.81	1.31	0.1
	VLHLVSQWISLYK	1586.90	3	759.98	1.61	0.0
	VQLVKKFIK	1103.43	2	417.28	1.17	0.1
	VASCGPAPTSKSAENDAR	1690.78	2	372.39	1.12	0.1
	LQERDDIGRIELVHK	1822.06	3	154.93	1.12	0.0
	LQERDDIGRIELVHK	1822.06	3	154.93	1.12	0.0
	TEDSEESSDEILAR	1581.58	2	111.05	0.84	0.0
	KLFSELESLTDPSLNHK	1959.19	2	168.55	0.85	0.1
	LQERDDIGRIELVHK	1822.06	3	270.12	1.16	0.0
	HCRTNQFGSDVSPK	1576.72	2	226.06	1.04	0.0
	AQQNLNSFFAIVMGLNTASVSRL					
	SQTWEK	3242.66	2	132.06	0.85	0.0
	LTSAVQRELAIAVIALK	1684.02	0	297.15	1.41	0.0
	ALRNIIILQAADLVKDR	1923.29	3	417.10	1.33	0.1
	MFLKTIYR	1072.35	2	459.14	1.34	0.1
	AQQNLNSFFAIVMGLNTASVSRL					
	SQTWEK	3242.66	2	132.06	0.85	0.0
	VASCGPAPTSKSAENDARYVVVS					
	GTPEK	2750.98	3	649.72	1.48	0.2
	MKPPKIPFMPLLLK	1654.20	2	275.80	1	0.12
	CNEVQLWVATEILLCSQLGK	2248.65	3	324.30	2	0.12
Rap1 guanine-nucleotide-exchange factor directly activated by cAMP; ex				2658.9	2	21450061.0
	QDFNRIIK	1034.19	3	233.62	1.06	0.1
	NRYTVMSGTPEKILELLEAMR	2566.04	3	1165.51	2.30	0.2
	TMRLEEHGKVVLVLER	1910.28	2	291.62	1.00	0.1
	VSLEQATTEHVKAGK	1735.92	2	205.88	0.93	0.1
	TDEELDLIFEELLHIK	1958.20	3	762.28	1.59	0.2
PDZ domain containing guanine nucleotide exchange factor (GEF) 1 [Mus]				14092.	29	38076580.0
	DLPPFGINSPQALKK	1625.89	2	335.05	1.24	0.0
	SSIVSNSSFDSPVSLHDER	2163.29	2	312.61	1.35	0.0
	NMQKVEEEGEIVMVKEHR	2186.50	3	186.59	1.18	0.0
	SSIVSNSSFDSPVSLHDERRQR	2603.79	3	422.00	1.45	0.0
	SSIVSNSSFDSPVSLHDER	2163.29	2	259.52	1.06	0.1
	EAPLPFILLGGSEKGFIFVDSVDS					
	CSK	2914.32	3	590.68	1.79	0.1

	ESLEQAQSR	1048.09	2	388.01	1.10	0.2
	SSIVSNSSFDSVPVSLHDER	2163.29	3	421.10	1.56	0.0
	SSIVSNSSFDSVPVSLHDER	2163.29	2	267.15	1.21	0.1
	PTEAPAPGQTPPAAAAASRPGSK	2060.26	3	905.15	1.62	0.1
	SSIVSNSSFDSVPVSLHDER	2163.29	2	169.18	1.28	0.0
	SSIVSNSSFDSVPVSLHDER	2163.29	2	578.88	1.63	0.1
	SSIVSNSSFDSVPVSLHDER	2163.29	2	353.76	1.20	0.1
	SSIVSNSSFDSVPVSLHDER	2163.29	2	298.21	1.47	0.1
	AEILCMGNSFGVSPTMDKEYMK					
	GVMR	2896.44	3	388.02	1.81	0.0
	SSIVSNSSFDSVPVSLHDER	2163.29	2	233.58	1.53	0.0
	SSIVSNSSFDSVPVSLHDER	2163.29	2	300.04	1.35	0.0
	SSIVSNSSFDSVPVSLHDER	2163.29	2	141.37	1.00	0.0
	NMAKYRNVLSGQNLQPPVIPLFP					
	VIK	2938.53	2	70.47	0.73	0.0
	IALHCR	712.89	2	292.85	1.09	0.0
	DCLEKDPIDR	1204.34	2	155.55	1.13	0.0
	YRNVLSGQNLQPPVIPLFPVIK	2493.98	3	580.99	1.59	0.1
	ELCAVMVFVAVVER	1466.80	2	413.39	1.21	0.1
	LPLVIKLPENQEETEPSCLDEK	2525.86	2	106.59	1.09	0.0
	KLEWFNDPSLRDK	1762.00	2	1050.40	1.81	0.2
	DLTFLHEGNDISK	1376.45	0	159.99	1.05	0.1
	EYMKGVMR TK	1243.53	3	319.61	1.58	0.1
	IALHCRECK	1073.32	2	186.71	0.85	0.1
	EVVIQAIR	928.11	2	910.62	1.64	0.1
	DIVRDCLEK	1091.27	2	340.85	1.30	0.0
	VDGLVNFELKR	1290.49	2	471.81	1.25	0.1
	DITNPNDNR	1059.07	2	463.96	1.35	0.1
	TTWEKLPNK	1117.28	2	1330.57	1.83	0.1
	KSKANTVGGR	1018.15	2	384.38	1.07	0.0
	SSIVSNSSFDSVPVSLHDER	2163.29	2	302.66	1.09	0.1
EPC2_MOUSE cAMP-regulated guanine nucleotide exchange factor II (cAMP-GEFII) RapGEF 4					1298.5	
					6	32171510.0
	QISEDAPQK	1215.34	2	295.18	0.97	0.1
	.LGSGELIIVK	1086.31	3	564.48	1.05	0.1
	LEPLIPFMPLLIKDMTFTHEGNK	2783.35	2	130.05	1.56	0.1
	NHQDVRSYVRQLNVIDNQK	2355.56	3	194.93	1.06	0.0
	MDYALNNK	969.10	1	113.91	1.07	0.0
RasGEF domain family, member 1B; GPI-gamma 4; likely ortholog of mouse					1341.7	
					1	22749129.0
	SLTAHSSREK	1116.21	0	140.33	1.34	0.0
	KNVQQMMQCLIR	1492.86	3	344.85	1.76	0.0
	KNVQQMMQCLIR	1492.86	2	775.61	1.65	0.0
	FDILEHQMDPSSNFYNYRTALR	2718.99	2	80.92	0.77	0.1
RalGEF-like protein 3, mouse homolog [Homo sapiens]					992.77	42661965.0
	DAPAGSPASPQGPSTK	1719.84	2	116.58	0.83	0.0
	ATVAQFNTVTGCVLGSVLGAPGL	2700.11	2	313.37	0.94	0.1

Pdzhgef2 protein [Mus musculus] usculus] ulus] ctor	AAPQR					
	DAPAGSPASPQGPSTKLPLSL	2909.24	3	423.88	1.49	0.1
	DLPSPR					
	PGAAGASPTVR	984.09	2	118.51	0.87	0.1
	SRDAPAGSPASPQGPSTK	1963.10	1	20.43	0.77	0.0
RAB guanine nucleotide exchange factor (GEF) 1; Ras negative regulator				966.56		18044313.0
	KHTEDTISVASSLHSSPPASPQNSP					
	RK	2860.09	3	453.31	1.93	0.1
	KGYTLTPSSKCDNLSSSHSEISSR	2700.88	3	513.25	1.49	0.1
cAMP-GEFII [Mus musculus]				2791.7		9910316.0
				7		
	HIFNAIKITK	1185.44	2	233.07	1.17	0.1
	LDAQSLNLSQEDFDRYMSGQTSP					
	RK	2888.12	2	55.88	0.82	0.0
	NLDLLSQLNER	1315.46	2	284.36	1.15	0.0
	RIRALHWVTPQMLCVPVNEEIP					
	VSDMVVK	3491.15	3	415.78	1.64	0.1
	YMSGQTSPRKQESSEWPPEACLG					
	VK	2797.12	3	631.35	1.82	0.1
	LDAQSLNLSQEDFDR	1751.83	2	376.81	1.09	0.1
	HIFNAIKITK	1185.44	0	421.71	1.00	0.1
	TGQEVYKQTKMFLEAMPYK	2293.69	2	372.82	1.01	0.2
RasGEF domain family, member 1B; GPI-gamma 4 [Mus musculus]				106.63		17061829.0
	MLLLSQMGPDAMR	1600.96	2	106.63	0.91	0.0
Pdzhgef2 protein [Mus musculus] Mus musculus] UM-BI				375.77		31044455.0
	LAALSQYEEVLAKLSSTATDR	2267.52	3	375.77	1.47	0.0
				2200.6		19344048.0
				0		
	SLSQGSTNSNMLDVQGAHKK	2160.36	3	1397.89	1.80	0.3
	LRGTWEKLPSK	1315.55	2	326.62	1.18	0.0
	HLQDLQDLFDPSR	1584.72	2	476.09	1.44	0.0

Table IV: Detection of all GAPs by LARC in RAW 264.7 macrophages.

Protein samples obtained from the LARC method were analyzed by LC-MS/MS and identified using SEQUEST algorithm. This table indicates various isoforms of Ras and Rap GAPs identified with different peptided sequences, protonated parent ion (MH+), charge, score, total score, and accession number.

Reference	Sequence	MH+	Charge	Score	Score	Accession
File, Scan(s)					XC	Delta Cn
RAS p21 protein activator 1; p120-rasGAP; GTPase-activating protein [M					23233.97	21703900.0
	YIYGCLQKSVQHK	1567.84	2	800.10	2.01	0.1
	YIYGCLQKSVQHK	1567.84	2	497.47	1.97	0.1
	YIYGCLQKSVQHK	1567.84	2	82.41	0.85	0.0
	YIYGCLQKSVQHK	1567.84	2	1123.28	1.97	0.1
	YIYGCLQKSVQHK	1567.84	2	641.15	2.05	0.1
	YIYGCLQKSVQHK	1567.84	2	1484.67	2.27	0.2
	YIYGCLQKSVQHK	1567.84	2	961.17	1.70	0.2
	QVSSLVLHIEEAHKLPVK	2028.39	3	260.53	1.22	0.0
	YIYGCLQKSVQHK	1567.84	2	221.94	1.26	0.0
	YIYGCLQKSVQHK	1567.84	2	588.85	1.77	0.0
	YIYGCLQKSVQHK	1567.84	2	594.58	1.71	0.1
	YIYGCLQKSVQHK	1567.84	2	344.35	1.20	0.1
	YIYGCLQKSVQHK	1567.84	2	557.51	1.52	0.1
	SNKHRMIMFLDELGNVPE					
	LPDTTEHSR	3168.55	2	703.51	1.00	0.3
	KSKDPDILFMR	1350.61	2	67.02	0.89	0.0
	PSDNTPGDYSLYFRTNENI					
	QRFK	2763.96	3	908.25	1.66	0.2
	EPVPMQDQGQVLNDTV					
	GK	2071.26	3	784.62	1.65	0.0
	EPVPMQDQGQVLNDTV					
	GK	2071.26	3	784.62	1.65	0.0
	YIYGCLQKSVQHK	1567.84	2	338.13	1.51	0.0
	YIYGCLQKSVQHK	1567.84	2	2321.53	2.41	0.2
	YYNSIGDIIDHYRK	1757.93	2	92.39	0.81	0.0
	GEKLLYPVAPPEPVEDR	1910.16	2	202.65	1.08	0.0
	WKNLYFILEGSDAQLIYFE					
	SEKR	2851.21	2	96.76	0.71	0.0
	SKDPDILFMR	1222.44	2	481.54	0.97	0.1
	YIYGCLQKSVQHK	1567.84	2	796.10	1.87	0.1
	YIYGCLQKSVQHK	1567.84	2	818.84	1.92	0.1
	YIYGCLQKSVQHK	1567.84	2	552.76	1.72	0.0
	ATKPKGLIDLVSVCVYVV					
	HDSLFGFR	2706.16	3	289.38	1.63	0.1
	TKDAFYKNIVK	1327.55	3	878.88	1.99	0.0
	MCGDYIIGRR	1291.49	2	170.12	0.98	0.0
	YIYGCLQKSVQHK	1567.84	2	332.58	1.41	0.0
	YIYGCLQKSVQHK	1567.84	2	625.96	1.78	0.1
	YIYGCLQKSVQHK	1567.84	2	378.09	1.41	0.0
	YIYGCLQKSVQHK	1567.84	2	970.42	1.70	0.2
	YIYGCLQKSVQHK	1567.84	2	548.10	1.48	0.1

	YIYGCLQKSVQHK	1567.84	2	1214.02	1.71	0.2
	YIYGCLQKSVQHK	1567.84	2	719.68	1.63	0.1
RAS p21 protein activator 3; GAPIII activator 3; Ras GTPase-activating				9596.83	7110713.0	
	VVPFISAIASAEVKRTQDP NTIFR	2661.05	3	1340.36	2.04	0.2
	YADAVKNFLDLISSGR	1857.06	2	399.51	1.69	0.1
	SFQNYIRQQSEISTHSI IFECQGLPIVNGQCDPYAT VTLAGPFR	2039.19	3	1095.12	1.74	0.2
	FGMKNFKK	2911.35	2	122.61	1.09	0.1
	PTIEEICQSHK	1000.24	2	343.04	0.98	0.0
	PTIEEICQSHKSCEIDPVK	1285.45	2	231.73	1.27	0.1
	SIEQPILLKEGFMIKR	2157.46	3	1511.49	1.70	0.2
	ILRHSSSYEAWYFLQPR	1903.32	2	234.28	1.87	0.1
	ILRHSSSYEAWYFLQPR	2154.42	3	566.81	1.71	0.0
	DLKLSADVEPVASAAH ILGEVCRDK	2154.42	3	1019.11	2.12	0.1
	YADAVKNFLDLISSGR	2838.23	0	220.66	1.13	0.1
	VDLWNASNLKFGDEFLG ELR	1857.06	2	1409.59	1.29	0.3
	LNVVYTEDHVSSEYYSP LRDILLK	2324.58	3	405.81	1.60	0.1
	CIDETMKLAGMHYLVHT LKPTIEEICQSHK	3002.37	2	58.30	0.73	0.0
		3471.10	3	638.44	1.32	0.1
RAS p21 protein activator 2 [Mus musculus]				1908.75	16716601.0	
	YGSKENPIVGKIS	1392.58	2	130.34	1.17	0.0
	TLTLISKTIQIIGNWGCQSR KFLDEISSTETKESSTSE PVHLK	2233.62	3	1090.51	1.57	0.2
	LVPFITAVAELDLK	2650.88	3	263.90	1.32	0.0
	LEIRIDLWNNENLVQDVF LGEIK	1529.85	2	288.90	1.00	0.1
		2772.15	3	135.11	0.96	0.0
similar to Ras GTPase-activating protein SynGAP (Synaptic Ras-GTPase-a				4524.82	38082120.0	
	QQQVEKDSQIKSIHR	1858.09	3	677.09	1.56	0.1
	LGPLPRLNDISTALR	1750.08	3	690.47	1.75	0.1
	LWIEARELPPK	1465.77	2	351.88	1.16	0.1
	CFACRSAAER	1114.29	2	259.17	1.15	0.0
	ALYESEENCEVDPIK	1739.88	3	790.43	1.60	0.0
	EDIADRLISASFLR	1719.96	3	370.05	1.78	0.0
	VIQNLANSFK	1134.31	2	226.79	1.19	0.1
	LKEYSKSMDES	1473.64	2	536.08	1.28	0.1
	EDIADRLISASFLR	1719.96	2	324.20	1.70	0.0
	SVPGGKQYSMEAAPAF R	1965.22	1	65.61	0.82	0.0
	LGPLPRLNDISTALR	1750.08	3	233.05	1.40	0.0
RSG4_MOUSE RasGAP-activating-like protein 1				6880.34	13959574.0	
	EAPGTTSPRLVELWDWD MVGK	2388.69	3	862.91	1.81	0.1
	EVARTNDPNTLFR	1533.67	2	109.17	0.85	0.0

	GRCLRCHVR	1100.35	2	215.91	1.00	0.0
	LFLGRGLAGPFLDYLTR	1910.25	2	463.72	1.36	0.1
	TNDPNTLFRSNLASK DISGTSDFARVFWGNHS LETSTIKK	1765.91	2	379.44	1.22	0.0
	PPNGWFRLLPFPR	2895.18	2	57.44	0.82	0.0
	LFLGRGLAGPFLDYLTR	1597.89	3	883.32	1.12	0.2
	NDFLGMVEFTPQTLQKQP PNGWFR	1910.25	2	463.72	1.36	0.1
	LLPFPRAEDSGSLGALR DFLDQLVDVDEDEEAGGP ACALVQPSTIVR	2852.22	3	297.37	1.23	0.1
	QDLATKLVK	1857.10	3	504.78	1.23	0.2
		3203.48	3	103.49	1.10	0.0
		1016.22	2	2539.07	1.97	0.1
ras-GTPase-activating protein SH3-domain binding protein; GAP SH3 bind				19550.78	7305075.0	
	DFFQNFNGNVVELR	1585.75	2	3569.02	2.10	0.3
	VPASQRPRESK	1196.34	0	115.15	0.73	0.1
	LPNFGFVVFDDSEPVQK	1939.16	2	5467.31	2.75	0.4
	DFFQNFNGNVVELR	1585.75	2	10065.25	3.07	0.5
	LPNFGFVVFDDSEPVQK	1939.16	1	163.80	0.72	0.1
	PESKPSQIPPQR	1479.62	2	170.26	0.80	0.1
Cherp protein [Mus musculus] ; p120- rasGAP; GTPase				447.28	21595046.0	
	WDQYKGVGVALDDPYEN YR	2289.45	3	347.24	1.27	0.0
	LHLIYLINDVLHHWALTR GRSLPHSQR	3247.75	3	100.04	1.07	0.0
Rap1, GTPase-activating protein 1 [Mus musculus]				2856.48	38078906.0	
	QQVQGARMRK	1202.42	2	588.40	1.16	0.0
	NTDLFEMIEKMQGSR	1800.05	3	442.27	1.22	0.1
	WYTGTTPGR	1035.14	3	838.67	1.89	0.1
	WYTGTTPGR	1035.14	2	987.14	1.04	0.2
similar to Rap1 GTPase-activating protein 1 (Rap1GAP) [Mus musculus]				1730.96	38091476.0	
	IPLAGLSKLPVQIAK HFLGKDHLNFYCTGSSLG NLILSIK	1733.13	3	314.17	1.63	0.0
	RHFLGKDHLNFYCTGSSL GNLILSIK	2779.21	3	223.11	1.46	0.0
	HFLGKDHLNFYCTGSSLG NLILSIK	2935.40	3	173.05	1.20	0.0
	LPFTDGDGTQQR	2779.21	3	862.85	1.85	0.1
		1278.35	2	157.78	0.85	0.1
Rap1gal protein [Mus musculus] rogenase 12 protein				2378.12	30354392.0	
	LIVTFDEHVISNNFK	1777.01	3	628.56	1.76	0.1
	YDVIGDQEHLR	1345.44	2	512.50	1.11	0.1
	RSSAIGIENIQEVQEK GQLPSEVAPSCPPSQASHG HPTQTSASRGTLR	1958.17	3	683.50	1.77	0.1
	LPYTEGDAQQLQRK	3243.52	2	350.66	1.03	0.2
		1647.81	2	202.90	1.19	0.0

similar to Ras GTPase-activating protein
SynGAP (Synaptic Ras-GTPase-a

LKEYSKSMDESR	1473.64	2	536.08	536.08	38082120.0
			1.28		0.1

Figure 4. Correlation of peptides identified by LC-MS/MS and searched with SEQUEST to the full length FASTA protein sequence.

Panel A illustrates the four different peptides identified for Rap1A; Panel B illustrates the six different tryptic peptides identified correlating to the full protein sequence for RAS p21 protein activator 1. Panel C illustrates the five different tryptic peptides identified with MS/MS data correlating to the full protein sequence for Rap guanine nucleotide-exchange factor 5 for Rap1. The accession numbers for the FASTA sequences are indicated as listed by NCBI, and the identified peptides from the list of proteins generated by SEQUEST are indicated within the text in bold and underlined lettering, overlapping sequences are in red.

- A. gil4506413|ref|NP_002875.1| RAP1A, member of RAS oncogene family [Homo sapiens]

MREYK**LVVLGSGGVGKS**SALTVQFVQGIFVEKYDPTIEDSYRKQVEVDCQQCMLEILD~~DTAGTEQFTAMRDLY~~
MKNGQGFAVYSITAQSTFNDLQDLREQILRVKDTEDVPMILVGNKCDLEDER**VVGKEOGONLAR**QWCNC
AFLESSAK**SKINVNEIFYDLVR**QINRKTPVEKKKPKKKSCLL

- B. gil21703900|ref|NP_663427.1| RAS p21 protein activator 1 [Mus musculus]

MCGDYYIGGRRFSSLSDLIGYYSHVSCLLK**GEKLLYPVAPPEPVED**RRRVRAILPYTKVPDTEISFLKGDM
FIVHNELEDGWMWVTNLRTDEQGLIVEDLVEEVGREEDPHEGKIWFHGKISKQEAYNLLMTVGQVCSFLVRP
SDNTPGDYSLYFRTNENIQRFKICPTPNQFMGGRYYNSIGDIIDHYRKEQIVEGYLLK**EPVPMODGOVL**
NDTVDGKEIYNTIRRK**TKDAFYKNIVK**GYLLKKGKGR**WKNLYFILEGSDAOLIFYESEKR**ATKPKGLID
LSVCSVYVVDHSLFGRPNCFQIVVQHFSEEHYIFYFAGETPEQAEDWMKGLQAFCSLRKSSPGTSNKRRLQVS
SLVLHIEEAHKLPVKHFTNPYCNILNSVQVAKTHAREGQNPVWSEEFVDDLPDINRFEITLSNKTKKSKDP
DILFMRCQLSRLQKGHATDEWFLSSHIPLKGIEPGSLRVARYSMEKIMPEEEYSEFKELILQKELHVYALS
HVCQDRTLLASILLKIFLHEKLESLLLCTLNDRISMEDEATTFRATTLASTLMEQYMKATATQFVHHALK
DSILKIMESKQSCELSPSKLEKNEDVNTNLAHLLSILSELVEKIFMASEILPPTLR**YIYGCLOKSVOHK**WPTNNT
MRTRVVSQGFVFLRLICPAILNPRMFNIISDPSPIAARTLTLVAKSVQNLANLVEFGAKEPYMEGVNPFKSNKH
RMIMFLDELGNVPELPDTTEHSRTDLSRDLAALHEICVAHSDDELRTLSNERGVQQHVLKLLAITELLQKQKQ
QYTKTNDIR

- C. gil32171383|spl|Q8C0Q9.2|RPGF5_MOUSE Rap guanine nucleotide exchange factor 5 (Guanine nucleotide exchange factor for Rap1) (M-Ras-regulated Rap GEF) (MR-GEF)

MTTGDCQTLSSRRISNPYLEHSPSQIYGENSSCAGRALRNIIILQAADLVKDRVNLKGFYRRSCVGSSELVDWLE
HCPFVQCRSMAIGVWQLLDMGIMSSVDQHLVYFDNYVFYQSSDECSYLYCEFEREEEWQKGVKLLLELV
HLIPARAGICDLHQKTEDSEESSDEILARLTSVQRELAAVIALKARKSAIEQDDENADKHVTVTEANNGPDP
QAGVMCK**LOERDDIGRIELVHK**LARENCQFLQTEKKESEKLEQQDDEVMTMVQVKEQQQSVLVLKK**YASCG**
PAPTSQSAENDARYVVVSGTPEKILEHLLNDLHLAEVQHKETETLLDDFLLTYTFMTTDDLCQALLRHYS
KKYQGEENSVDPCRKRK**VLHLVSOWISLYK**DWLHEDEHSMFLKTIYRNVLDVVEYPILEKELKEFQKIL
GVYRRHTVDEYSPQKKNKALFHQFSLKENWLQHRGTVAETEEIFCHVYITEHSYISVKAKVSSTAQEILKVVA
EKLQRAEEDLALVAITFSGEKHEFQPNDLAISKSLEASGRIYVYRKDLADTLNPLAENEESQQRSRILGMNT
WDLALELMSFDWLSLFSIHEQELIYFTFSRQNGENTVNLSLLQRCNEVQLWVATEILLCSQLGKR**VOLVK**
KEIKIAAHCKAQONLSFFAIVMGLNTASVSRLSOTWEKIPGKFKKLFSELESITDPSLNHKA YRDAFKKM
KPPKIPFMPLLLKDVTFIHEGNKTFLDNLVNFELHMIADTVRTL RHCRTNQFGSDVSPKEQQELKSYVNHLY
VIDSQALFELSHRLEPRA

Figure 5. Fragmentation evidence for selected peptide sequences.

Fragmentation spectra was obtained from LC-MS/MS using the SEQUEST algorithm illustrating the peptide sequences from Rap1A, p120RasGAP and Rap1 GEF.

Rap1A:

P120RasGAP:

Rap1 GEF:

Confirmation of proteins by Western blot

Western blots were done to confirm the presence of Rap1, RasGAP and C3G, a Rap1GEF, in RAW 264.7 macrophage cell lysates. A strong band was detected at 21 kDa for the blot incubated with Rap1A/Rap1B rabbit monoclonal antibody signifying the presence of this protein within the macrophage cell lysate in its full and not cleaved form (Fig. 6). Rap1 (26B4) Rabbit monoclonal antibody detects endogenous levels of total Rap1A and Rap1B protein, which were both identified by tandem mass spectrometry. The bands observed for the blot incubated with mouse anti-RasGAP were apparent at approximately 102 kDa and 75 kDa with quite strong bands and a very light band was present at 120 kDa signifying that the 120 kDa protein may have been spliced by proteases (Fig. 6). The detection of C3G, a 121 kDa protein, was done with a rabbit monoclonal anti-C3G antibody. C3G, a GEF for Rap, was detected with a strong band at approximately 43 kDa and a second, much more faint band was detected at approximately 58 kDa. This yet again signifies that C3G might have been cleaved by proteases during the lysis of RAW 264.7 macrophages.

Figure 6. Western blot against p120RasGAP and Rap1A /Rap1B in RAW macrophage cell lysates.

Cells were boiled in SDS-PAGE sample buffer in the presence of AEBSF, PMSF and eukaryotic anti-protease cocktail before probing with 1 μ l mouse anti-p120RasGAP, 1 μ l rabbit anti-Rap1A/Rap1B and 1 μ l rabbit anti-C3G and detection with goat anti-mouse and goat anti-rabbit HRP conjugated secondary antibody and detection by ECL.

Confirmation by Immunostaining with Confocal Microscopy

The localization of p120RasGAP, Rap1A/Rap1B, and C3G to the cell periphery near the bound IgG-opsonized particle was observed by staining fixed cells with mouse monoclonal anti-RasGAP, rabbit monoclonal anti-Rap1A/Rap2A and rabbit anti-C3G monoclonal antibodies and detecting with secondary antibody conjugated to cyan 3 via laser confocal microscopy. The confocal image representing antibody staining for RasGAP shows definite cup formation around the four IgG-opsonized beads bound to the cell membrane, and not for the bead that has been internalized (Fig. 7). The localization of Rap1A/Rap1B to the cell periphery is also indicated by an increase in fluorescence at the bead that is bound to the Fc receptor and not for the beads that have already been internalized (Fig. 7). C3G antibody staining was done to predict the presence of GEFs for Rap due to the lack of reagents for other GEFs. As an indicator, C3G shows strong accumulation near the bound beads (Fig. 7). Thus antibody staining showed increased fluorescence at the bead suggesting the recruitment of RasGAP and Rap1A/Rap1B to the site on the plasma membrane as well as a potential activator or isoform of C3G, a GEF for Rap, where the receptor complex was activated by the binding of the ligand coated beads (Fig. 7).

To confirm that the secondary staining was specific for the primary antibodies, control cells were incubated with IgG-opsonized beads, fixed and stained only with the secondary antibody conjugated to cy3 anti-mouse and anti-rabbit and imaged using the same settings as for the cells stained with both the primary and secondary antibodies. The control stained with only donkey anti-mouse cy3 secondary antibody revealed no fluorescence at the bead (Fig.7) signifying that the signal obtained for RasGAP is true, and there is no non-specific binding. The control stained only with donkey anti-rabbit cy3 secondary antibody revealed fluorescence specifically at the bead and nowhere else in the cell (Fig. 7). This implies that the anti-rabbit

secondary antibody reacts with the human IgG, which the beads were coated with and that the signal obtained for Rap1A (Fig.7) is a summed fluorescence of the localization of Rap1A to the bead and the fluorescence from the bead alone. The anti-rabbit cy3 antibody staining seems to be significantly present on the bead itself within the cy3 donkey anti-rabbit (Cy3DantiR) secondary antibody control, however with the cells that were stained with Rap1A/Rap1B and C3G the distribution of fluorescence seems to be present within the body of the cell and predominantly around the bound beads instead of the entire surface of the bead. Rap1A/Rap1B stained cells seem to fluoresce throughout the cytosol and form a haze around the bound bead but do not display a specific increase in fluorescence within the internalized beads. The same can be seen for C3G staining, where the whole body of the cell fluoresces as well as the plasma membrane around the bound particles and not in particular the whole bead in comparison to the cy3 anti-rabbit controls. Although non-specific binding was observed for the anti-rabbit secondary controls, the signal seems to be the strongest only at the bead and not throughout the cell body.

Figure 7. Immunofluorescent staining of RAW 264.7 macrophages.

Cells were grown on glass coverslips in alphaMEM overnight and then exposed to IgG-opsonized beads for 10min, washed, fixed and stained with 1 μ l antibody followed by cy2 conjugated secondary antibody. Control images illustrating cells stained only with secondary donkey anti-mouse (DantiM) and donkey anti-rabbit (DantiR) cy3 show non specific binding. Panels illustrate the localization of mouse anti-RasGAP and rabbit anti-Rap1A/Rap1B and rabbit anti-C3G to the cell periphery near the binding site of the IgG-opsonized bead (indicated by arrows). Images show the rhodamine (red) light channel, the white light channel or differential interference contrast (DIC) image.

Confirmation by confocal microscopy with transfected fusion proteins

The accumulation of p120RasGAP and Rap1 in the stimulated phagocytes was studied by transient transfection of fusion proteins consisting of essential binding domains linked to GFP or RFP. Cells transfected with the EGFP-Rap1A wildtype construct and the p232-mRFP-N1 dominant negative construct were visualized by live-cell confocal microscopy to study the localization of these proteins to the site on the plasma membrane where the receptor was activated by a ligand-coated bead. In the cells transfected with the Rap1A linked to EGFP it is evident from the fluorescence intensity that Rap1A localizes through the cytoplasm and the plasma membrane but is not present in the nucleus (Fig. 8). The line scan corresponding to the distribution of Rap1A within the cell indicates a uniform distribution of fluorescence throughout the body of the cell where there is no bead bound to the surface of the cell. The fluorescence drops significantly at the nucleus and spikes again in the cytosol of cell near the nucleus and slightly less at the bead. Although the fluorescence intensity at the phagocytic cup is comparable to that of the entire cell, the cup formation is evident and Rap1A, a cytosolic protein, localizes to the membrane, thus possibly participating in the uptake of the ligand-coated particle.

Figure 8. Quantitation of the distribution of EGFP-Rap1A in transfected RAW 264.7 macrophage cells.

RAW 264.7 cells were transfected with EGFP-Rap1A construct and exposed to IgG-opsonized SRBCs to initiate phagocytosis, during which confocal images were acquired every 30 seconds. The image below represents two cells transfected with EGFP-Rap1A in the presence of SRBCs, below is a pixel intensity graph corresponding to the image above. To quantify the accumulation of EGFP-Rap1A a line was drawn through the image and fluorescence intensity of individual pixels was measured at the site of phagocytosis in comparison to that of the entire cell.

The distribution of RasGAP in the stimulated phagocyte was studied by transient transfection of a fusion protein consisting of the SH2-SH3-SH2 binding domain of p120RasGAP linked to RFP. The fluorescence distribution of the line scan shows intensity saturation at the phagocytic cup, then drops down at the nucleus and spikes again within the body of the cell (Fig. 9). The distribution of p232-mRFP-N1 indicated that the binding partner for the binding domain of p120RasGAP is present in the cytosol and is recruited from the cytosol to the cell periphery upon activation of the Fc receptor complex. Interestingly, the distribution of the wildtype Rap and the binding region of p120RasGAP appears to be quite similar.

The effect of the dominant negative construct that binds to the RasGAP binding region may have an effect on the number of particles taken up by the cell in comparison to the control cells. The cell transfected with p232-mRFP-N1 engulfs more IgG-opsonized beads than the neighboring cells that show no sign of transfection (Fig. 10). Thus it is possible that the dominant negative construct may have an effect on phagocytosis and disrupts the regulatory function of RasGAP at the site of the activated receptor and by binding RasGAP interferes with other signaling proteins necessary for regulation of phagocytosis.

These preliminary studies of Ras proteins may provide sufficient data for further investigation into the functions of GAPs and GEFs and the binding partners they might interact with.

Figure 9. Quantitation of the distribution of the dominant negative fragment for p120RasGAP in RAW 264.7 macrophages.

Macrophage cells were transfected with p232-mRFP-N1, consisting of the binding region of p120RasGAP. RAW 264.7 cells were transfected with p232-mRFP-N1 construct and exposed to IgG-opsonized SRBCs to initiate phagocytosis, during which confocal images were acquired every 30 seconds. The image below represents two cells transfected with p232-mRFP-N1 in the presence of SRBCs, below which is a pixel intensity graph corresponding to the above image. To quantify the accumulation of EGFP-Rap1A, a line was drawn through the image and fluorescence intensity of individual pixels was measured at the site of phagocytosis in comparison to that of the entire cell.

Figure 10. Expression of p120RasGAP RFP dominant negative construct in RAW 264.7 macrophage cells.

Live cells were transfected by electroporation using amaxa with p232-mRFP-N1 construct, exposed to 2 μ m polystyrene beads and imaged under laser confocal microscopy over a 30 min time duration. This illustration shows a transfected cell (red) engulfing more IgG-opsonized beads than its neighboring non-transfected cells. Images show the rhodamine (red) light channel, the white light channel, and combined red and white light channels.

DISCUSSION

In this study the member of Ras superfamily of GTP binding proteins, Rap and some regulatory proteins were identified by tandem mass spectrometry and confirmed by detection with antibodies, immunostaining, and transient transfections with GFP fusions of protein binding domains coupled to laser confocal microscopy. The mass spectrometry data for Rap, GAPs and GEFs shows significant scores, with at least three different peptides identified for each protein, as well as tryptically cleaved peptides. Data meeting all these aforementioned criteria has shown to eliminate false positives 95% of the time (Cargile *et al.*, 2004). Here we conclude that based on cumulative scores the identification of Rap, GAPs and GEFs was accurate.

Ras superfamily members are noted to be involved in vesicle transport and fusion. For instance, Rap proteins are consistent with the vesicular supply of plasma membrane during early events of phagocytosis (Pizon, *et al.*, 1994; Seastone, *et al.*, 1999). The presence of Ras superfamily member Rap was observed in RAW 264.7 macrophages by LC-MS/MS. LC-MS/MS was able to identify various isoforms of Rap including Rap1A, Rap1B, Rap2A, Rap2B, Rap1/Rap2 and various other isoforms. Some isoforms of Rap were seen with higher relative scores and identified by a number of different tryptic peptides. A FASTA alignment for Rap1A indicated that the four peptides that were identified by tandem mass spectrometry were in fact aligned with the protein sequence listed by NCBI. Fragmentation spectra for the three selected peptides identified for Rap1A shows low noise and well resolved peaks thus signifying that the detection by SEQUEST and the spectra are reliable. ←

The analysis of collected samples by the LARC method has shown evidence for the presence of Rap activating GEFs. Among the different GEFs identified for Rap, there was evidence that Ras GEFs were also present in the samples. RapGEF1 and PDZ GEF were

identified with a greater number of peptides than other GEFs associated with Ras or Rap. The FASTA alignment of the RapGEF1 correlated five peptide sequences to the full length protein sequence, thus suggesting that spectrometric analysis generated reliable results. Mass spectra for peptides specifically associated with RapGEF1 shows well resolved peaks and low noise levels for the three peptides identified by LARC. It can be concluded that LARC was able to reveal some of the relevant isoforms of the Rap associated GEFs.

Analysis of the captured activated receptor complex by LARC from the surface of live cells revealed the presence of GAP variants for Ras. A distinct presence of p120RasGAP was observed with relatively higher scores and greater number of peptides compared to other identified GAPs. This implies that RasGAPs are present within the activated receptor complex captured by LARC. The alignment of 6 different peptide sequences to the full length FASTA protein sequence confirmed that the sequences generated by tandem mass spectrometry were in agreement with the native sequence of the p120RasGAP protein. Good fragmentation patterns with high relative abundance and low noise were obtained for peptides correlated with the native sequence of p120RasGAP. Therefore, p120RasGAP and associated GAP isoforms were detected with high accuracy by the LARC method suggesting their localization to the activated receptor before it is internalized.

Biochemical techniques supporting the association of Ras superfamily member Rap and its associated GEFs and GAPs indicate that these molecules are present in RAW 264.7 macrophages. Western blotting supported the presence of Rap1/Rap2 in RAW 264.7 macrophages, although the associated p120RasGAP and the RapGEF C3G were detected with less confidence possibly due to cleavage of these proteins within the whole cell lysate or due to degradation in strong detergents used in SDS-PAGE and sample buffer. Proteins bearing an

immunological relation to those observed by tandem mass spectrometry were confirmed by western blot. Therefore, it can be concluded that Rap, p120RasGAP and RapGEF are present in 264.7 macrophages.

Immunostaining of RAW 264.7 macrophages in the presence of an IgG-opsonized beads was used to further address the association of Rap, RasGAP and C3G. RasGAP was detected at the site of the activated receptor near the bead. The presence of Rap1A/Rap1B and C3G near the nascent phagosome was also seen in immunostained macrophages. Thus it can be concluded that RasGAP, Rap1A/Rap1B and C3G were observed at the site of the activated receptor near the bound particle in RAW 264.7 macrophages. Similarly, cells transfected with Rap1 and p120RasGAP show distribution of fluorescence at the cup formation in live macrophages. Utilizing antibodies and fluorescent fusion proteins to detect Ras protein Rap, p120RasGAP, and RapGEF has indicated that these proteins localize to the activated receptor and may play a role in phagocytosis. Thus it can be concluded that Rap, RasGAP and RapGEF play a role at the activated Fc receptor complex however, further experimentation utilizing knock outs or silencing RNA is required to investigate further into the functions of these proteins and study the effects of suppression of these molecules during phagocytosis.

The distribution of the Rap1 and p120RasGAP within live macrophages is very comparable and may indicate that these two proteins act together in the phagocytic pathway. Another point supporting this argument may be the presence of a GAP for Ras within the mass spectrometry data, but a lack of Ras itself. Based on these preliminary results it can be hypothesized that the equal distribution of the binding domain of p120RasGAP and Rap may suggest that RasGAP may play a role in the inactivation of Rap or be its downstream effector. The support provided by mass spectrometry data for the presence of Rap GEFs at the activated

receptor contributes to support the theory that Rap is activated and may interact with p120RasGAP. I conclude that p120Ras GAP and Rap1 showed localization to the activated Fc receptor complex by live cell confocal microscopy and therefore are associated with the receptor complex. Further investigation into the signaling pathway of these Ras proteins may provide sufficient evidence for this argument.

There is much support for the association of Rap with integrin mediated cell adhesion implying that Rap is associated with the stage of the phagocytic pathway that has to do with the rearrangement of the cytoskeleton at the surface of the cell in the formation of the phagocytic cup (Pizon *et al.*, 1994; Seastone *et al.*, 1999). However the association of GAPs and GEFs that trigger pathways of Rap are not well understood and their functions are not yet known. Rabs are currently the GTPases that drive the early stages of phagocytosis, but not all their interactions and functions are known (Vieira *et al.*, 2002). Belonging to the same family of small G-proteins, Rabs and Ras both act as molecular switches that mediate signaling of cellular functions. This study shows the association of the Ras superfamily member Rap and its associated GAPs and GEFs. Therefore, it is possible that the Ras family of proteins and its associated regulatory molecules may play a significant role in the phagocytic pathway.

Ras and Rap are highly homologous and it may be possible that they share common effectors. It has been proposed that Rap functions as an antagonist by trapping Ras effectors in an inactive complex and if overexpressed, it can revert to the morphological phenotype of K-Ras (Kitayama, *et al.*, 1989). Although this has been documented, it is not the case for all scenarios, and Rap is not always found to be capable of reverting to the transformed phenotype characteristic of Ras transformed cells (Sato, *et al.*, 1994). The overexpression of Rap can interfere in Ras effector signaling, but it has been shown that it is unlikely that Rap activation

solely serves to repress Ras signaling. Here, we propose that this might be the case. The detection of the p120RasGAP and RapGEF isoforms by tandem mass spectrometry may suggest that these two molecules regulate Rap, however further studies to investigate this argument are still required.

CONCLUSION

Mass spectrometry was able to accurately identify Ras superfamily member Rap, its associated GEF proteins and GAPs. The LARC method has shown evidence to capture receptor associated proteins by mass spectrometry and further confirm their localization to the Fc receptor by confocal microscopy (Jankowski *et al.*, 2008) with high accuracy, thus suggesting that the Ras inactivator p120GAP may in fact be present at the activated complex. Additionally, antibody staining has shown that Rap, p120RasGAP and C3G are recruited to the cell membrane at the site of the activated receptor near the bound bead. Expression of fluorescent fusion proteins of the p120RasGAP binding domain and Rap was observed within the cytosol and near the phagocytic cup in RAW 264.7 macrophages. Ras family of proteins, especially Rap and their associated GAPs and GEFs, may play a role in phagocytosis and further in triggering an innate immune response, although further evidence is required to establish the mechanisms involved.

FUTURE OBJECTIVES

Future directions that may provide more support for the arguments made in this study could focus on establishing evidence for the function that p120RasGAP and RapGEF may play in phagocytosis. The ability to use mass spectrometry and antibodies for the detection of specific proteins near the sight of the activated Fc receptor is sufficient for gaining a foundation in the investigation of molecular signaling within a cellular pathway. However, further study is required to identify the various isoforms and track their activity throughout the entire process of internalizing ligated particles. The use of GFP fused constructs of the critical binding domains of Ras, Rap, p120RasGAP and RapGEF are all required to track the activation of Rap and the deactivation of Ras simultaneously. Also the use of dominant negative constructs that have the ability to interfere with the native binding of these interacting molecules is also necessary in order to investigate the physical effect on phagocytosis. Another method that can be used to completely investigate the role of these molecules is by using silencing RNA. Silencing RNA has the ability to silence or significantly reduce the expression of a specific protein within a cellular network thus enabling the study of the proteins function in phagocytosis. For instance, reduced phagocytosis upon silencing of a specific protein will render that protein as an essential signaling molecule in the uptake of pathogens. Confocal microscopy enables the visualization of the effects that dominant negatives or silencing RNA may have on the role of Ras, Rap, p120RasGAP, and RapGEF in the uptake of opsonized particles. Phagocytosis assays to investigate the potential of phagocytosis in cells that have been transfected with silencing RNA or dominant negatives can provide a statistical analysis of the effects on the uptake of ligated particles as well (Steinberg, *et al.*, 2007). In this light further experimentation is required to identify the roles of these molecules in the Fc receptor complex although, mass spectrometry

data and immunostaining has provided evidence that these molecules are present during phagocytosis.

Inactivation of Ras by various drugs can also provide potential methods for analyzing the contribution of Ras and its related proteins to the activated Fc receptor. Manumycin A is a Ras specific inhibitor that was shown to inactivate class I PI3K (Li, et al., 2007). Further investigation of the actions of this inhibitor could lend further insight on the role of Ras protein in the activation of the Fc receptor.

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