REGULATION OF RAP2 IN B LYMPHOCYTES

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ABSTRACT

Ras and members of the Ras-related family of small GTP-binding proteins are involved in many biological functions. These include regulation of cell growth, differentiation, and activation; actin cytoskeleton rearrangement, membrane trafficking, vesicle trafficking and nuclear transport. These GTP-binding proteins cycle between an inactive GDP-bound form or an active GTP-bound form which can bind downstream effectors.

Rap1A, Rap1B, Rap2A, and Rap2B proteins share approximately 50% sequence identity with Ras. In addition, Rap proteins share a similar effector region as Ras, but have different flanking amino acids. Despite the similarities, the functions of Rap1 and Rap2 have not been completely elucidated although recent studies suggest that both Rap proteins may be involved in cell migration and adhesion.

Previous work has shown that Rap1 is activated by many receptors. In our laboratory, we showed that Rap1 was activated by BCR cross-linking and CXCR4 stimulation. Hence, the first aim of this thesis was to determine if these receptors also activated Rap2. This is because Rap2 may or may not have the same function as Rap1 in B lymphocytes. The second objective was to uncover how BCR cross-linking and CXCR4 activate Rap proteins. A PLC inhibitor was used to elucidate the mechanism of this Rap activation signalling pathway. Finally, the third goal was to develop loss of function approaches for blocking Rap1 and Rap2 activation. Expression of the Rap-specific GAP proteins, RapGapII and Spa-1, was used to inhibit activation of Rap1 and Rap2 in B cell lines.

These experiments have resulted in three major findings. First I showed that anti-Ig antibodies, stromal cell-derived factor-1 (SDF-1), and phorbol 12,13-dibutyrate (PdBu) all activate Rap2 in B cells with activation kinetics similar to that for Rap1. Second, I showed that the BCR activates Rap2 via PLC-γ, and Rap2 activation can be inhibited by 1-[6-[[17β-3-

methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122). Finally, I showed that RapGapII and Spa-1 differentially inhibit the activation of Rap1 and Rap2.

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BCR	B cell antigen receptor
BLNK	B cell linker protein
DAG	Diacylglycerol
ECL	Enhanced chemiluminescence
EGF	Epithelial growth factor
ERK	Extracellular signal-regulated kinase
FDC	Follicular dendritic cell
GNEF	Guanine-nucleotide exchange factor
HRP	Horseradish peroxidase
ICAM	Intercellular adhesion molecule
IP_3	Inositol trisphosphate
LPA	Lysophosphatidic acid
LESTR	Leukocyte-derived seven-transmembrane domain receptor
mIg	Membrane immunoglobulin
NFAT	Nuclear factor of activated T cells
PDGF	Platelet-derived growth factor
PdBu	Phorbol 12,13-dibutyrate
PI3K	Phosphatidylinositol 3-kinase
$PI(4,5)P_2$	Phosphatidylinositol 4,5-biphosphate
$PI(3,4,5)P_3$	Phosphatidylinositol 3,4,5-trisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C

PTK	Protein tyrosine kinase
RasGRP	Ras guanyl nucleotide-releasing protein
Rlf	RalGDS-like factor
RPIP8	Rap2-interacting protein 8
SDF-1	Stromal cell-derived factor-1
SOS	Son of sevenless
TPA	12-O-tetradecanoylphorbol 13-acetate
U73122	1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione
U73343	1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione

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To my mother, Ticky Li, who suffers from myasthenia gravis.

CHAPTER ONE

INTRODUCTION

1.0 B Cells and their function

B lymphocytes play a vital role in the host immune system by protecting the body from foreign pathogens. The two primary roles of B lymphocytes are to present foreign antigens to T lymphocytes and to produce specific antibodies directed against foreign antigens (Peakman and Vergani, 1997). In short, B lymphocytes sample the microenvironment with their receptors. If a threat is present, a specific population of B lymphocytes is activated by the combination of antigen stimulation and T lymphocyte-derived signals. These activated B cells will proliferate and differentiate into antibody-producing plasma cells. Antibodies eliminate pathogens by three main mechanisms: neutralization, opsonization, and complement activation. Although each B cell recognizes only one or a few related antigens via their B cell antigen receptor (BCR), the diversity of antigen binding sites on the population of B cells is so abundant that the repertoire for antibody specificity within an individual exceeds 10^8 and presumably can recognize any pathogen one might encounter (Anderson, 1999).

How B lymphocytes develop, mature, become activated, and differentiate is therefore of much interest. As the following thesis is primarily focused on the effects of certain external cues being transmitted to the interior of the B cell, a brief discussion on signal transduction in B lymphocytes is explained below.

1.0.1 Signal transduction by key receptors in B lymphocytes

Receptors are important components of any cell, and many are located at the plasma membrane. When a surface receptor binds to a specific extracellular molecule, a signalling cascade is initiated. First the receptor is activated and then the signal is relayed to other

intracellular signalling molecules. The net result is that changes in cell functions occur (Campbell, 1996). These changes vary from gene expression to cytoskeletal rearrangements.

Many receptors regulate B cell development, activation, and tolerance. The following thesis will look at two important receptors that reside on the surface membrane of B lymphocytes: BCR and CXCR4.

Briefly, BCR signalling leads to activation or tolerance. The combination of antigen-induced BCR signalling and co-stimulatory signals provided by helper T lymphocytes causes B cells to proliferate and then differentiate into antibody-secreting plasma cells (Sharon, 1998). In contrast, for immature B cells, self-antigen-induced BCR signalling in the bone marrow often leads to B cell apoptosis or B cell anergy. Thus, tolerance to self-antigens in the body is maintained.

CXCR4 signalling is also important because it directs B cells and B cell progenitors to the proper location. The ligand for CXCR4, SDF-1, helps recruit naïve B cells into lymphoid organs where they can encounter foreign antigens (Okada et al., 2002). SDF-1, made by stromal cells in the bone marrow, retains B progenitors in the bone marrow where B cell maturation can take place (Janeway et al., 2001).

1.0.2 The role of the BCR in B cell activation

The BCR consists of an antigen-binding subunit (mIg) and a signalling subunit (CD79a/b). The membrane immunoglobulin (mIg) consists of four polypeptides – two heavy chains of either 50 kDa (IgG) to 72 kDa (IgM) in molecular weight and two 25 kDa light chains – that are held together by disulfide bonds (Gold and Matsuuchi, 1995). At the amino termini of both the heavy and light chains are the variable regions. These regions give each BCR its unique antigen binding specificity. Associated with the mIg in a non-covalent manner are two disulfide-bonded transmembrane polypeptides, Igα (CD79a) and Igβ (CD79b). These two polypeptides

are the main signalling components of the BCR complex because their carboxyl termini extend across the membrane and into the cytoplasm and provide the only significant cytoplasmic domains present in the BCR complex (Campbell, 1999; Kurosaki, 1997).

The BCR is important for two main reasons: It controls the development and maturation of B cell progenitors, and it controls the survival and activation of mature B lymphocytes (Gold and Matsuuchi, 1995; Janeway et al., 2001). During B cell development in the bone marrow, gene rearrangements are occurring on the heavy chain genes in an attempt to generate a pre-BCR. Signalling by the pre-BCR serves as an important checkpoint for B cell development and allows for further gene rearrangements of the light chain genes. Successful light chain gene arrangement leads to cell-surface expression of the BCR and then each B cell undergoes negative selection in order to eliminate those with BCR's that react strongly to self-antigens. It is unknown at the present time how B cells are able to sense a functional BCR and thereby receive additional signals for maturation.

After development, B cells are released from the bone marrow and they circulate within the body's lymphatic system and blood stream. It has been shown that continuous expression of the BCR and signalling via the BCR in B cells are required for continued survival in the periphery (Lam et al., 1997). Most importantly, upon recognizing pathogen-associated antigens, the B cell is activated. Again, BCR signalling occurs.

Hence, BCR signalling appears to control survival, activation, development, and death. Mutations in the BCR that lead to constitutive signalling or absence of signalling could cause immunodeficiency, autoimmunity, and even malignancy. This is why understanding how signal transduction works within B lymphocytes is important.

1.0.3 Signal transduction by the BCR

The initial BCR signalling events begin at the receptor when the BCR binds tightly with an antigen, which causes multiple antigen receptors to cluster (See Appendix I). Membrane Ig cross-linking or clustering is required for signal generation and is the initial step to B cell activation (Gold et al., 1992). This is because clustering allows receptor associated protein tyrosine kinases (PTK) of the Src family – Lyn, Fyn, Blk, and Lck – to come in close proximity to activate one another and phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAM; [D/E]xxYxx[L/I]xxxxxxxYxx[L/I]) of other CD79a/b chains (Campbell, 1999; DeFranco, 1997; Kurosaki, 1997). ITAMS that are approximately 26 amino acid in length (Kurosaki, 1997) and found on the cytoplasmic domains of the BCR receptor complex are phosphorylated by these PTKs. Phosphorylated ITAMs provide binding sites for proteins that possess Src homology 2 (SH2) domains, such as Syk – another PTK. Once Syk is recruited and binds to the phosphorylated ITAMs on the Igα and Igβ chains of the BCR complex, Syk, itself, gets phosphorylated and activated by the Src-family tyrosine kinases, and its activity is greatly enhanced (DeFranco, 1997). Another tyrosine kinase that gets activated after BCR engagement is the Tec-family kinase Bruton's tyrosine kinase (Btk).

Syk recruitment to the BCR is a crucial event because Syk activates a multitude of downstream signalling proteins which lead to the activation of three major signalling pathways: the phospholipase C-γ (PLC-γ) pathway, the phosphatidylinositol 3-kinase (PI3K) pathway, and the Ras pathway (Kurosaki, 1997). Adaptor proteins, such as B cell linker protein (BLNK), Cbl, Crk, Grb2, and Shc, are vital to the activation of these signal transduction pathways even though these proteins possess no intrinsic enzymatic function (Peterson et al., 1998). Their role is to mediate protein-protein interactions and to localize certain proteins to membranes.

Activation of the PLC-γ pathway begins with PLC-γ recruitment to the membrane via the binding of PLC-γ2's SH2 domain to BLNK, an adaptor protein that is phosphorylated by Syk

(Campbell, 1999). Once PLC-γ is brought to the plasma membrane, it is phosphorylated and activated by Btk. PLC-γ then cleaves the plasma membrane phospholipid phosphatidylinositol 4,5-biphosphate ($PI(4,5)P_2$) into inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Scharenberg and Kinet, 1998). IP_3 causes the release of Ca^{2+} ions from the endoplasmic reticulum while DAG activates multiple isoforms of the protein kinase C (PKC) family of serine/threonine kinases (Gold, 2000). The liberation of calcium activates a phosphatase known as calcineurin, and calcineurin ultimately dephosphorylates the transcription factor nuclear factor of activated T cells (NFAT). NFAT then translocates to the nucleus and stimulates transcription. PKC, on the other hand, activates the transcription factor, NF-κB, by initiating a pathway that leads to the degradation of the inhibitory subunit IκB (Gold, 2000). This permits the migration of NF-κB into the nucleus.

BCR engagement also activates PI3K (Gold et al., 1992), which consists of two subunits, the regulatory subunit p85 and the catalytic subunit p110 (Okkenhaug and Vanhaesebroeck, 2001). PI3K activation can occur by several means. First the CD19 co-receptor on B lymphocytes contain two YxxM motifs that are tyrosine phosphorylated after BCR engagement and provide the correct binding sites for the two SH2 domains on p85. The binding of p85 induces a conformational change within PI3K that increases its specific activity (Gold, 2000). The secondary method of PI3K activation is via adaptor proteins. For example, it has been shown that PI3K can bind via its SH2 domains to Cbl and Gab1 (Gold et al., 2000; Ingham et al., 1998). These adaptor proteins localize PI3K to its substrates, which are located in the plasma membrane. This is possible because adaptor proteins either contain PH domains which are capable of binding to certain membrane phospholipids, thus allowing for membrane recruitment, or they contain other protein interaction domains, for example PTB/SH2-like domains, that allow for interactions with membrane proteins.

The main function of PI3K is to phosphorylate $PI(4,5)P_2$ on the 3-position of the inositol ring, creating phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$) (Rameh and Cantley, 1999). $PI(4,5)P_2$ is the substrate of PLC-γ whereas $PI(3,4,5)P_3$ is involved in recruiting PH domain-containing signalling proteins to the membrane (Rameh and Cantley, 1999). Some of these PH domain-containing signalling proteins include PDK1, Akt kinases, and Btk.

The third signalling pathway to get activated after BCR cross-linking is the Ras pathway (Harwood and Cambier, 1993; Saxton et al., 1994; Su and Karin, 1996). Ras activation in B lymphocytes is most likely activated by the DAG → Ras guanyl nucleotide-releasing protein (RasGRP) pathway (Tognon et al., 1998) with minor contributions by the Shc/Grb/Son of Sevenless (SOS) pathway. It is believed that PLC-γ2-generated DAG recruits the RasGRP protein to the plasma membrane where Ras is located. RasGRP is a guanine-nucleotide exchange factor (GNEF) that activates Ras by triggering the displacement of GDP from Ras in order to generate an active GTP bound form of Ras. The other pathway begins with the membrane recruitment of the adaptor proteins Shc (Saxton et al., 1994) and Grb2. Shc and Grb2 mediate the recruitment of the GNEF, SOS, to the membrane where it activates membrane bound Ras. It has been suggested that the Shc/Grb2/SOS complex is recruited to the plasma membrane by Gab1 or BLNK (Fu et al., 1998; Ingham et al., 1998) after BCR ligation.

Active Ras binds to and activates the serine/threonine kinase, Raf1. This subsequently sets off a cascade of kinases (Kolch, 2000) in which Mek phosphorylates and activates the extracellular signal-regulated kinases (ERK). This leads to the phosphorylation of the transcription factor, Elk, and the induction of Fos expression. Without ERK activation, B cell proliferation does not occur (Richards et al., 2001).

1.0.4 The role of CXCR4 in B cell function

Chemokines are small chemoattractant proteins that stimulate the migration and activation of cells. In B cells, the chemokine SDF-1 retains B cell progenitors in the bone marrow microenvironment and allows for B cell maturation. SDF-1, SLC, and ELC recruit B cells into lymphoid organs while BLC recruits activated B cells into lymphoid follicles where they proliferate (Okada et al., 2002). Finally SDF-1 recruits plasma cells back to the bone marrow where they can survive for long periods of time (Hargreaves et al., 2001). In this thesis, I will focus on the signalling by the receptor for SDF-1, CXCR4.

Originally called leukocyte-derived seven-transmembrane domain receptor (LESTR), CXCR4 was isolated in 1994 by Loetscher et al. (Loetscher et al., 1994). At the time, much research was focused on chemotactic agonists, such as interleukin-8 (IL-8) and related cytokines, and how they could attract leukocytes by binding to seven-transmembrane domain, G protein-coupled receptors. CXCR4 was such a receptor. Today, CXCR4 is most often recognized as the entry cofactor for T cell line-trophic (T-trophic) Human Immunodeficiency Virus-1 (HIV-1) (Hori et al., 1998) and the receptor that can bind to the chemokine, stromal cell-derived factor-1 (SDF-1)

Briefly, CXCR4 is a seven-transmembrane domain receptor coupled to heterotrimeric G_i proteins. It was named after the type of chemokine to which it binds. Chemokines, such as SDF-1, are potent chemoattractants and are named according to the position of their first two conserved and closely paired cysteines (CXC, CX_3C, CC, or C) (Murdoch, 2000). As SDF-1 binds LESTR exclusively (Baggiolini, 1998) and is grouped under the superfamily of CXC chemokines, LESTR was redesignated as CXCR4.

The interaction between CXCR4 and SDF-1 is quite important for many reasons. First, CXCR4 is expressed by all B lymphocytes and monocytes, expressed by most T lymphocytes, and weakly expressed in natural killer cells (NK cells) (Hori et al., 1998). SDF-1, formerly known as pre-B-cell growth-stimulating factor (PBSF), is secreted by bone marrow stromal cells

(D'Apuzzo et al., 1997). CXCR4 knockout mice are deficient in B cell progenitors in the fetal liver and bone marrow (Ma et al., 1999). SDF-1 knockout mice also contain severely reduced B-cell progenitors in the fetal liver and bone marrow (Nagasawa et al., 1996) which suggests that both the ligand and the receptor have similar functions. Together, it has been shown that SDF-1 and CXCR4 are responsible for confining B cell precursors within the bone marrow environment and promoting their survival, which allows them to mature and differentiate (Baggiolini, 1998; Bleul et al., 1996; D'Apuzzo et al., 1997). SDF-1 and CXCR4 have also been implicated in recruiting mature B cells into lymphoid organs and plasma cells to the bone marrow (Hargreaves et al., 2001; Okada et al., 2002).

Because the CXCR4 receptor is responsible for a multitude of B cell functions (Murdoch, 2000), it is important to understand how these signals are conveyed upon activation of the receptor.

1.0.5 Signal transduction via CXCR4

Most chemokine receptors, like CXCR4, signal through heterotrimeric G proteins (Baird et al., 1999; Mohle et al., 2001; Ward and Westwick, 1998), which consist of three subunits: Gα, Gβ, and Gγ (See Appendix II). CXCR4 likely signals via $G\alpha_i$ since SDF-1-induced migration is blocked by pertussis toxin, a toxin which prevents the activation of $G\alpha_i$ (Thelen, 2001). When chemokine receptors are in their inactive state, the Gα subunit is GDP-bound and the complex is not associated with the receptor. Upon chemokine signalling, the trimeric G protein complex associates with the receptor, and the GDP bound to the Gα subunit is displaced by GTP. The heterotrimeric protein then dissociates into two components: GTP bound Gα and Gβγ (Thelen, 2001). The two subunits then go and activate other effector molecules such as PLC and PI3K.

In T cells, SDF-1 stimulation leads to the activation of PLC and PKC (Yonezawa et al., 2000). As a result of PLC activation, intracellular Ca^{2+} is increased, DAG is produced, and

ERK2 is activated. In other cell lines – human megakaryoblasts, human platelets, and hematopoietic progenitors – SDF-1 signalling activates Akt, focal adhesion components (RAFTK, Cas, Paxillin, FAK, Crk, CrkL), PI3K, ERK, SHP2, SHIP, Cbl, Fyn, and NF-κB (Chernock et al., 2001; Majka et al., 2000; Tilton et al., 2000; Wang et al., 2000). Akt activation promotes cell survival by inactivating several apoptosis-mediating proteins such as BAD, and caspase-9. Activation of focal adhesion components is important in cell migration because it is responsible for the formation of focal adhesions and the reorganization of actin cytoskeleton. Other proteins such as SHP2, Cbl, and Fyn are also involved in CXCR4 signalling and may act as key mediators of SDF-1-induced responses. Whether all of these proteins are also activated in B lymphocytes has not been confirmed. In this thesis, I will show that CXCR4 activates the Rap1 and Rap2 GTPases, which are members of the Ras family of GTPases.

1.1 Ras and Ras-like proteins

The Ras superfamily of proteins are small, 20-25kDa GTPases that regulate a wide variety of cellular functions. The superfamily is subdivided into several subfamilies based on the similarities that exist in their effector domain: Ras, Rho/Rac, Rab, Ran, Rad, and Arf (Geyer and Wittinghofer, 1997; Zwartkruis and Bos, 1999). Each subfamily seems to have distinct biological functions. For example, the Rab, Arf, and Arf-like GTPases are involved in the assembly, loading, targeting, budding and fusion of vesicles to their appropriate cellular compartments. The Rac/Rho subfamily controls cytoskeletal organization, mitogenesis, transformation, protein kinase cascades, phospholipid metabolism and transcriptional regulation. Lastly, Ran GTPases are necessary for nuclear transport (Macara et al., 1996; Rebhun et al., 2000). Hence Ras and Ras-like proteins control a multitude of functions in cells.

Rap proteins belong to this subfamily of Ras proteins, and Rap1 was first identified as a putative antagonist of Ras signalling (Kitayama et al., 1989). Therefore I will first discuss Ras and Ras signalling.

1.1.1 The importance of Ras and Ras-family members

Originally, Ras GTPases were intensely studied because 10-20% of human tumors contain mutated versions of these genes (Bos, 1998; Macara et al., 1996), and it was speculated that Ras proteins somehow contributed to the aberrant regulation of growth stimulatory signalling pathways in these cells (Campbell et al., 1998). Now it is known that Ras proteins are part of an important signalling pathway of protein kinases, as described before, that often link surface receptor signalling to gene expression. By being situated at the inner surface of the plasma membrane (Campbell et al., 1998) where most receptors are located, they serve as signal transducing switches that control cell growth, transformation, differentiation, and apoptosis (Bos, 2001; Kolch, 2000). Presently, more and more signalling pathways are being discovered that emanate from Ras.

Activated forms of Ras interact with many downstream molecules. Some of these include Raf1, A-Raf, RalGDS, PI3K, RalGDS-like factor (Rlf), Rgl, and Rin1 (Bos, 1998; Macara et al., 1996; Zwartkruis and Bos, 1999). Raf1 and A-Raf regulate the ERK signalling pathway. Rlf, RalGDS and Rgl are members of the RalGEF family and serve to connect Ras to the small GTPase Ral. PI3K, as mentioned before, is involved in generating $PI(3,4,5)P_3$, which regulates the activity of proteins involved in cell motility, cell cycle control, and cell differentiation. The function of Rin1 is unknown.

Other Ras subfamily members include R-Ras, TC21/R-Ras2, M-Ras/R-Ras3, Rap1A, Rap1B, Rap2A, Rap2B, RalA, RalB, Rit, Rin, Dex-Ras, Rheb, Rhes, NOEY2, κB-Ras1, κB-Ras2, Ha-Ras, K-Ras, and N-Ras (Rebhun et al., 2000). Not a lot is known about these Ras-like

proteins relative to what we know about Ras. However, their interactions with downstream effectors often hint at their possible role in cell function. These downstream effectors are however often shared, and cross-talk may occur.

1.1.2 GTPases

Proteins that belong to the GTPase superfamily are viewed as molecular switches that can be turned on by binding to GTP and turned off by hydrolysing GTP back to GDP. In this cycle, GTPases have three conformational states. The first is a GDP-bound inactive state. When the GDP is released, the GTPase is in its empty state. Under physiological conditions, the GTPase is now more likely to take up GTP in its empty guanine nucleotide binding site than GDP because of the higher concentration of GTP within the cytosol (Bourne et al., 1991). This overall reaction can be driven forward by the excess of GTP over GDP in the cytoplasm, by a greater affinity of the GTPase for GTP than GDP, or with the help of other regulatory molecules, which will be described below. Upon GTP binding, the GTPase is now in its active state until it reverts back to its inactive state when GTP is hydrolysed. There are many known classes of GTPases. All of them share a conserved fold and a general mechanism of action (Corbett and Alber, 2001).

1.1.3 GNEFs and GAPs

As described from above, there are regulatory proteins that can facilitate and catalyze the release of bound GDP from GTPases and promote its replacement by GTP. These proteins are known as GNEFs. Conversely, GAPs are regulatory proteins that accelerate GTP hydrolysis by GTPases.

GNEFs and GAPs for Ras may not exclusively regulate Ras; they may also regulate other Ras-like proteins as well. GNEFs that have been reported to catalyze GDP-GTP exchange on Ras are SOS, RasGRF, and RasGRP (Bos, 1998; Zwartkruis and Bos, 1999). Finally, GAPs that

have been reported to stimulate the intrinsic GTPase ability of Ras are RasGAP and neurofibromin (NF1) (Campbell et al., 1998; Zwartkruis and Bos, 1999).

1.2 Rap1 and Rap2

Presently, four Ras-related Rap proteins have been identified: Rap1A, Rap1B, Rap2A, and Rap2B (Reuther and Der, 2000). Rap1A, a 21 kDa protein, was discovered by Kitayama et al. in 1989 via its ability to apparently reverse the transformed phenotype of human fibroblasts expressing activated forms of Ras (Kitayama et al., 1989). Since Rap proteins are very similar in structure to Ras, it was suggested at the time that Rap might antagonize Ras-induced responses by competing with its effectors. Additionally, there was also the presumption that Rap proteins may be involved in growth control. However, over-expressed Rap2 does not share the same characteristic in Ras-transformed murine fibroblasts as Rap1A, nor does it interfere with Ras-induced transformation (Jimenez et al., 1991). Recently, experiments looking at the over-expression of Rap1 in other cell lines did not observe interference or antagonism within the Ras signalling pathway (Zwartkruis et al., 1998). The ability of over-expressed activated Rap1 to block the Ras → Erk pathway may just be an artefact generated by the high concentration of constitutively active Rap1. It is believed that under physiological conditions, even maximal levels of endogenous activated Rap1 could never sequester all the Ras effectors away from Ras.

Since many GTPases have important signalling functions, our laboratory has investigated the role of Rap in B cell signalling. A short description of Rap proteins is included in order for the reader to understand the molecular experiments explained later on in the thesis.

1.2.1 General characteristics of Rap1 and Rap2

Rap1A, Rap1B, Rap2A and Rap2B proteins share approximately 50% amino acid sequence identity with Ras (Janoueix-Lerosey et al., 1992; Jimenez et al., 1991) (See Appendix III). More specifically, Rap1A and Rap1B are 95% identical (Janoueix-Lerosey et al., 1998),

Rap2A and Rap2B are 90% identical (Nancy et al., 1999), and Rap1 and Rap2 proteins are 60% identical in terms of sequence identity (Beranger et al., 1991; Jimenez et al., 1991; van den Berghe et al., 1999). In terms of structural organization, Rap1 proteins have the exact same effector region as Ras, but have different flanking amino acids. Because the effector region confers the ability of Ras to interact with its unique set of effector molecules, it was believed that Rap1 could also interact with this same set of molecules. The effector domain in Rap2 differs from Rap1 by one amino acid substitution at position 39 – phenylalanine for serine. However, this amino acid substitution has no effect on Rap proteins since it has been shown experimentally that Rap1A altered in this way still displays the same set of properties (van den Berghe et al., 1999). As to cellular localization, it is known that Ras proteins are located at the plasma membrane whereas Rap proteins are concentrated near nuclear membranes (Bos, 2001).

Due to the structural similarities and a common effector region, one might expect that Rap and Ras proteins would have a shared set of regulatory proteins and effector molecules. For instance, a GNEF specific for both Rap1 and Ras is RasGRP2 (Clyde-Smith et al., 2000), and effector molecules that interact with both Ras and Rap are Raf, PI3K, and Rlf (Janoueix-Lerosey et al., 1998; Nancy et al., 1999; Traver et al., 2000). However, the similarities end here. More and more regulatory proteins and effector molecules are being discovered that are only specific for Rap proteins and not Ras, whereas some are specific for Rap and other Ras-like proteins. For example, PDZ-GEF1 is a GNEF specific for Rap1 and Rap2 but not Ras (de Rooij et al., 1999). Even amongst the Rap-associated proteins, some of these regulatory proteins and effector molecules have been characterized to interact with Rap1 but not Rap2 and vice versa. An example would be Rap2-interacting protein 8 (RPIP8), which interacts exclusively with Rap2 but not Rap1 or Ras (Janoueix-Lerosey et al., 1998). The other variable to consider is that some of these regulatory molecules and effector molecule interactions with Rap proteins only occur in certain cell types because of cell-type specific expression.

At present time, GNEFs that are known to activate both Rap1 and Rap2 are Epac1, Epac2 (de Rooij et al., 2000; de Rooij et al., 1998), and PDZ-GEF1 (de Rooij et al., 1999). Other GNEFs that have been shown to activate Rap1 are CalDAG-GEFI (Clyde-Smith et al., 2000), CalDAG-GEFIII (Yamashita et al., 2000), C3G (van den Berghe et al., 1999), smgGDS (Ichiba et al., 1997), and guanine nucleotide exchange factor for Rap1 (GFR) (Ichiba et al., 1999). It is not known whether these GNEFs also activate Rap2.

GAPs that can inactivate Rap1 and Rap2 are RapGapI (Janoueix-Lerosey et al., 1992), RapGapII (Mochizuki et al., 1999; McLeod, Li et al., JI, 2002) and Spa-1 (Hattori et al., 1995; Nur-E-Kamal, 1996; Tsukamoto et al., 1999; Wada et al., 1997). Another GAP that has been reported to regulate Rap1 is tuberin (Wienecke et al., 1995).

While the Rap proteins share a structural similarity with Ras, their effector regions are almost exactly the same. The ability of Rap proteins to compete with Ras effectors was inferred from this observation. For example, activated forms of Rap1 can bind to the Ras effector molecules: Raf, Rlf, and PI3K (Nancy et al., 1999; Traver et al., 2000; van den Berghe et al., 1999). However, Rap1 cannot bind to all of the subset of Ras effectors. In addition, Rap1 has its own subset of effectors that includes Ral-GEF (van den Berghe et al., 1999), RalGDS, and B-Raf. Rap2 can also bind RalGDS, but it has its own subset of effectors as well which includes RPIP8 (Janoueix-Lerosey et al., 1998) and RGS14 (Traver et al., 2000).

1.2.2 Known functions of Rap proteins

Despite the numerous studies looking at Rap protein effectors, regulatory proteins, and activators, the functions of Rap1 and Rap2 have not been fully elucidated. Much of what is known has come from the study of Rap1. Rap1 has been implicated in the control of platelet activation, T cell anergy, neuronal differentiation, cell migration, and cell adhesion.

Rap1 has been found to be expressed at high levels in human neutrophils and human platelets. Rap1 is activated by a large variety of stimulating agents in neutrophils: fMLP, PAF, GM-CSF, phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA), and IgG-coated particles (M'Rabet et al., 1998).

Stimulation of human platelets by von Willebrand factor (vWF) causes the activation of Rap1B (Franke et al., 2000; Torti et al., 1999). Zwartkruis et al. have also tested for the ability of Rap1 proteins to be activated in a variety of cell lines. In these studies, Zwartkruis et al. showed that Rap1 is activated by a variety of growth factor receptors including receptor tyrosine kinases such as the platelet-derived growth factor (PDGF) receptor and epithelial growth factor (EGF) receptor, and G protein-coupled receptors including the receptors for lysophosphatidic acid (LPA), thrombin, and endothelin (Zwartkruis et al., 1998).

York et al. showed that Rap1 was involved in nerve growth factor signalling, which is important for neuronal differentiation (York et al., 1998). More specifically, protein kinase A (PKA) activation is required for Rap1 activation and this leads to B-Raf activation, sustained activation of ERK, and neuronal differentiation via an ERK-dependent pathway (Vossler et al., 1997).

In T lymphocytes, activated Rap1 blocks IL-2 gene transcription (Boussiotis et al., 1997). This is consistent with the later observation that Rap1 is activated in anergic T cells (Carey et al., 2000) that have been stimulated by TCR engagement alone in the absence of co-stimulatory signals. If the co-stimulatory molecule, CD28, is engaged, Rap1 activation was blocked.

Increased Rap1 expression has been shown to alter cell morphology in *Dictyostelium discoideum* by altering cytoskeletal function (Rebstein et al., 1997). In contrast, Rap1 loss-of-function mutations in Drosophila disrupt cell migrations and cause abnormalities in cell shape during morphogenesis of cells in the eye disk (Asha et al., 1999).

Lately, multiple studies have shown that Rap proteins regulate cell adhesion. For example, activated Rap1 promotes cell adhesion in Hela cells, and inhibiting Rap activation via the expression of Spa-1 Rap-GAP opposes adhesion (Tsukamoto et al., 1999). Suga et al. have demonstrated that activated Rap1 leads to the activation of the β_2-integrin LFA-1, which binds intercellular adhesion molecule-1 (ICAM-1) (Suga et al., 2001). Activated Rap1 also increases T cell adhesion to the LFA-1-ligand ICAM. In addition, Rap activation regulates the avidity of the VLA-4 ($\alpha_4\beta_1$) integrin which binds vascular cell adhesion molecule-1 (VCAM-1) (Reedquist et al., 2000). In related studies, Rap1 was found to positively regulate integrin activation in T cells by promoting the clustering of LFA-1 and VLA-4 (Katagiri et al., 2000; Sebzda et al., 2002). The ability of Rap-GTP to promote integrin activation in T cells suggests that it could be important for T cell extravasation.

Despite the many studies on Rap1, not much is known about Rap2. It is clear that certain cellular properties of Rap2 are markedly different from Ras and Rap1. As mentioned before, overexpression of Rap2 does not oppose Ras-transformation of murine fibroblasts whereas Rap1 does. While activated Rap1 and Rap2 share common effectors, they also activate their own subset of effectors. All these facts suggest that there may be biological differences between Rap1 and Rap2.

1.2.3 Rap proteins in B lymphocytes

In previous experiments conducted in our laboratory, it was shown that BCR engagement activates Rap1, but that Rap1 does not modulate Ras signalling (McLeod and Gold, 2001; McLeod et al., 1998). The BCR activates Rap1 via PLC-γ2-dependent production of DAG. Rap1 activation is greatly reduced in PLC-γ2 deficient variant of the DT40 chicken B cell line. Recent experiments in our laboratory have also shown that Rap1 and /or Rap2 promote B cell adhesion and migration towards chemokines (Gold et al., 2000; McLeod, Li et al., JI, 2002).

1.3 WEHI-231, A20, and 2PK3 B cell lines as model systems

Three mouse monoclonal cell lines were employed to study Rap activation in B cells. B cell lines offer excellent models for the study of signal transduction and the biochemistry of signalling proteins. This is because B cell lines are easy to grow to large numbers and have been extensively characterized and widely used for signalling studies. The use of a mouse model is applicable since Rap proteins are conserved across species. For example, human and mouse Rap1 proteins are 88% identical in terms of amino acid sequence. Rap2 proteins between the two species are also 88% identical. Thus, results in these mouse cell lines can be used to infer similar cellular processes that take place in humans and other mammalian systems.

A brief description of three B cell lines used in the following study is described below.

1.3.1 WEHI-231 cells

WEHI-231 is a mouse tumor cell line that expresses surface IgM, but does not secrete immunoglobulin (Ig) (Lanier and Warner, 1981) unless stimulated with lipopolysaccharide (LPS). The special feature about these immature B cells is that when their BCRs are cross-linked with anti-Ig antibodies used as a surrogate for antigen, they will undergo growth arrest and apoptosis (Boyd et al., 1981). This characteristic of WEHI-231 makes them the favourite model system to study negative selection (King and Monroe, 2000) of self-reactive B cells as immature B lymphocytes going through development in the bone marrow undergo a similar process of growth arrest and cell death when their prototypic receptors bind strongly to self antigens.

Rap proteins are known to regulate B cell migration towards the chemokines SDF-1 (McLeod, Li, et al., JI, 2002), and this process is thought to be important for confining immature B cells with stromal cells within the bone marrow microenvironment. Therefore, WEHI-231 cells are also a good model for SDF-1 signalling studies.

1.3.2 A20 cells

The A20 B cell lymphoma line was first characterized by Kim et al. (Kim et al., 1979). These mature B cells express IgG and were derived from a BALB/c inbred mouse. As A20 B cells have been used previously for adhesion studies, they will be employed to study Rap proteins as Rap proteins are implicated in the activation of integrins.

1.3.3 2PK-3 cells

The 2PK-3 B cell lymphoma line was also derived from a BALB/c inbred mouse. 2PK-3s express IgG2 at the surface and are representative of mature B lymphocytes prior to the plasma cell stage of differentiation (Warner et al., 1979). 2PK-3 have been utilized in migration studies (Reif and Cyster, 2000) as they respond to SDF-1 and other chemokines. Hence, this cell line will also be used for the study of Rap proteins.

1.4 Thesis objectives

The role of Rap proteins in regulating cell function is not completely understood. Not much is known about their role in B cells. Importantly, a lot of work has been done on Rap1 but little is known about Rap2.

The first objective of this thesis was to characterize the activation of Rap2 proteins by BCR cross-linking and CXCR4 stimulation in B cells. Previous experiments have shown that anti-Ig antibodies and SDF-1 can activate Rap1 in B cells. The goal was to determine if there is coordinate regulation of Rap1 and Rap2 proteins. If there is, this suggests that maybe Rap1 and Rap2 possess the same upstream activators.

The second objective was to elucidate how BCR cross-linking activates Rap proteins. Phorbol 12,13-dibutyrate (PdBu) and a PLC inhibitor were used to test the hypothesis that Rap2 is activated by PLC-dependent production of DAG, as is the case for Rap1 in B cells.

The third objective was to develop loss of function approaches to study the roles of Rap1 and Rap2 in B cells. As stated before, many GAPs regulate Rap proteins. Two Rap-GAPs were expressed in B cell lines in order to inhibit Rap activation and to determine if both suppress Rap1 and Rap2 or if they had differential effects on Rap proteins. This would allow us to explore the roles of Rap1 and Rap2 in B cells.

Note: Some of the work in this thesis (SDF-1-induced activation of Rap2) has been published in Sarah J. McLeod, Anson H. Y. Li, Rosaline L. Lee, Anita E. Burgess, and Michael R. Gold, *The Rap GTPases Regulate B Cell Migration Toward the Chemokine Stromal Cell-Derived Factor-1 (CXC Chemokine Ligand 12): Potential Role for Rap2 in Promoting B Cell Migration*, Journal of Immunology, 2002, In Press.

CHAPTER TWO

MATERIALS AND METHODS

2.0 Antibodies and other reagents

Goat anti-mouse IgM (μ chain specific) and rabbit anti-mouse IgG (Fcγ fragment specific) antibodies used for BCR ligation were purchased from Jackson ImmunoResearch Laboratories (Mississauga, Ontario, Canada). Mouse SDF-1α used to stimulate the CXCR4 receptor was obtained from R & D Systems (Minneapolis, MN), and the phorbol ester, PdBu, was procured from Sigma (Oakville, Ontario, Canada). The PLC inhibitor, 1-[6-[[17β-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122), and its negative control and structural analogue, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73343), were obtained from Biomol (Plymouth Meeting, PA). Glutathione Sepharaose 4B beads used for immunoprecipitation studies were acquired from Amersham Biosciences (Baie d'Urfe, Quebec, Canada). The anti-Rap1 rabbit polyclonal IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and the anti-Rap2 mouse monoclonal IgG_{2a} was obtained from BD Transduction Laboratories (Mississauga, Ontario, Canada). The secondary reagents used for chemiluminescence, protein A-horseradish peroxidase (HRP) and goat anti-mouse-HRP, were acquired from Bio-Rad (Hercules, CA). The western blotting detection reagent, ECL, was purchased from Amersham Biosciences.

2.1 B cell lines

2.1.1 WEHI-231, A20, 2PK-3 cells

The WEHI-231, A20, and 2PK-3 B cell lymphomas have been described previously (Kim et al., 1979; Lanier and Warner, 1981; Warner et al., 1979). All B cell lines can be obtained from American Type Culture Collection (Rockville, MD).

2.1.2 pMSCV RapGapII-expressing B cell lines

The cDNA for FLAG-tagged RapGapII (Mochizuki et al., 1999) in the pMSCV retroviral vector was a gift from Dr. Michiyuki Matsuda (Osaka University, Japan). The empty pMSCV vector or the pMSCV RapGapII vector was transfected into BOSC23 packaging cell lines and the resulting virus particles were used to infect WEHI-231 and 2PK-3 cells as described (Krebs et al., 1999). After selection in puromycin, stable bulk populations of cells were maintained in the presence of puromycin. A20 cells were transfected by electroporation. Individual puromycin-resistant clones were selected, expanded, and maintained in medium containing puromycin. Transfection of the WEHI-231 and 2PK-3 B cells was done by Sarah McLeod from our laboratory whereas the A20 transformants were prepared by May Dang-Lawson from our laboratory. FLAG-tagged RapGapII expression was assessed by immunoblotting with anti-FLAG M2 monoclonal antibodies (Sigma) and with anti-RapGapII antibodies (Mochizuki et al., 1999).

2.1.3 pLXSN Spa-1-expressing B cell lines

The cDNA for FLAG-tagged Spa-1 (Hattori et al., 1995) in the pLXSN retroviral vector was a gift from Dr N. Minato (Kyoto University, Japan). This vector or the empty pLXSN vector was transfected into BOSC23 cells and used to generate retroviruses for infection of WEHI-231 cells. Again electroporation was used for A20 cells. Selection was achieved in medium containing G418. FLAG-tagged Spa-1 expression was assessed by immunoblotting with anti-FLAG M2 monoclonal antibodies and with anti-Spa-1 antibodies, which was prepared by Blazej Szczygielski from our laboratory.

2.1.4 pMX-PIE Spa-1-expressing B cell lines

The pMX-PIE vector was a gift from Dr. A. Mui (University of British Columbia, British Columbia, Canada). The cDNA for FLAG-tagged Spa-1 was subcloned into the vector by Sarah McLeod and used for retroviral-mediated gene transfer into 2PK-3 B cells.

2.2 Cell culture

The WEHI-231, A20, and 2PK-3 murine B cell lines were grown in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT), 2 mM L-glutamine, 100 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 50 units per ml of penicillin-G, and 50 μg per ml of streptomycin (Pen/Strep, Gibco). To reduce the basal signalling background in WEHI-231 cells, these cells were grown overnight in medium containing 1% heat-inactivated fetal calf serum before performing the Rap activation assays.

2.3 Rap activation assay

Cells were resuspended to 2.5 x 10^7 per ml in modified Hepes-buffered saline (Saxton et al., 1994), and stimulated with 100 μg per ml anti-mouse Ig antibodies, 100 ng per ml (12.5 nM) recombinant murine SDF-1, or 100 nM PdBu. In studies using the PLC inhibitor, U73122, and its structural analogue, U73343 (Bleasdale et al., 1990; Smith et al., 1990), cells were pre-treated with these compounds at a final concentration of 20 μM for 20 minutes prior to stimulation.

The cell suspension was solubilized in an equal volume of Rap lysis buffer (50 mM Tris-HCl pH 7.4, 10% glycerol, 1% IGEPAL, 200 mM NaCl, 2 mM $MgCl_2$, 1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg per ml leupeptin, 1 μg per ml aprotinin) to terminate the reaction and placed on ice for 10 minutes. Detergent-insoluble material was removed by centrifugation at 14,400 RPM for 15 minutes at 4°C, and cell lysates were used for the Rap activation assay as described below.

The assay uses a GST-RalGDS fusion protein to selectively precipitate the active GTP-bound forms of Rap1 and Rap2. The preparation of GST-RalGDS has been described before (McLeod et al., 1998). Fifteen microlitres of the fusion protein was used per sample and mixed with 20 µl of glutathione Sepharose 4B beads in the cold for one hour. The beads were subsequently washed twice with Rap lysis buffer before being mixed with the cell lysates in the cold for another hour. The beads were then washed three times with Rap lysis buffer to wash away unbound proteins. Bound proteins were then eluted with SDS-PAGE sample buffer containing 100 mM dithiothreitol.

2.4 Protein analysis

2.4.1 SDS-PAGE and western blotting

Eluted proteins were separated using 1.5 mm-thick SDS-PAGE mini-gels and transferred to a nitrocellulose filter (Schleicher & Schuell, Keene, NH). Molecular mass standards were visualized by staining with Ponçeau S (Sigma). The filters were blocked with 5% milk powder in TBST (10 mM Tris-HCl, pH 8, 150 mM NaCl)/0.1% Tween 20 at room temperature for one hour. Mouse anti-Rap2 antibodies were diluted to 0.1 µg per ml in 5% milk powder in TBST, added to the filters and incubated overnight at 4°C. The filters were washed with TBST for 30 minutes at room temperature and then incubated with goat anti-mouse-HRP antibodies diluted at 1:2000 in 5% milk powder in TBST for 30 minutes at room temperature. The filters were washed once again with TBST for 30 minutes at room temperature and visualized by enhanced chemiluminescence detection. Filters were subsequently reprobed by first removing the bound antibodies on the filter by washing the filter with several changes of stripping solution (10 mM Tris-HCl, pH 8, 150 mM NaCl, pH adjusted to 2.0) for one hour. The filters were again blocked with 5% milk powder in TBST at room temperature for one hour. Rabbit anti-Rap1 antibodies were diluted to 0.5 µg per ml in 5% milk powder in TBST and added to the filters and incubated

overnight at 4°C. The filters were washed with TBST for 15 minutes at room temperature and then incubated with Protein A-HRP diluted at 1:2500 in 5% milk powder in TBST for 45 minutes at room temperature. The filters were washed with TBST for 15 minutes at room temperature and finally visualized by enhanced chemiluminescence (ECL) detection.

2.4.2 Quantitation and statistical analysis

Immunoreactive bands corresponding to activated Rap proteins were visualized by ECL. The X-ray films were scanned and saved as TIFF files. Densitometry was performed using ImageQuant (Molecular Dynamics, Sunnyvale, CA) software to analyze the intensity of the bands. Each experiment was repeated at least twice.

CHAPTER THREE

ACTIVATION OF THE RAP2 PROTEINS IN B LYMPHOCYTES

3.0 Rationale and summary

Previous work in our laboratory showed that Rap1 is activated when B lymphocytes are stimulated via the BCR or CXCR4. The aim of this chapter was to determine whether these receptors also activated Rap2 and to determine if they did so via a similar signalling pathway.

McLeod et al. have shown that Rap1 is activated in B lymphocytes after BCR ligation (McLeod and Gold, 2001) via PLC-γ2-dependent production of the second messenger DAG (McLeod et al., 1998). This was demonstrated using a PLC-γ2-deficient variant of the DT40 chicken B cell line. BCR-induced activation of Rap1 in PLC-γ2-deficient DT40 cells was significantly reduced when compared to the wild-type control. Addition of PdBu, a phorbol ester that mimics the action of DAG, into these cells caused Rap1 activation in these B cell lines (McLeod et al., 1998), suggesting that DAG mediates Rap activation. The other $PI(4,5)P_2$-derived second messenger does not activate Rap in B cells (McLeod et al., 1998).

Many chemokine receptors are coupled to heterotrimeric G_i proteins and signal via PLC-β activation, and this ultimately leads to an increase in intracellular DAG (Thelen, 2001). Our laboratory showed that SDF-1-induced signalling via CXCR4, a chemokine receptor found on B lymphocytes, also activates Rap1 (McLeod, Li, et al., JI, 2002). This work indicated that activation of the Rap GTPases is important for B cell migration in response to SDF-1 binding to CXCR4 (McLeod, Li, et al., JI, 2002).

Since Rap1 and Rap2 are closely related and regulated by similar GNEFs and GAPs, I hypothesized that BCR cross-linking and CXCR4 stimulation would also activate Rap2 via a PLC-γ2 → DAG pathway. In this chapter, I show that anti-Ig, SDF-1, and PdBu all activated

Rap2 in B cells with kinetics similar to that for Rap1 activation. I also show that the BCR activated Rap2 via PLC-γ2 and that Rap2 activation could be inhibited by PLC inhibitor, U73122, but not its structural analogue, U73343.

3.1 Activation of Rap2 by anti-Ig antibodies

In order to characterize Rap2 activation in B lymphocytes, I stimulated WEHI-231 and A20 cells with anti-Ig antibodies in order to cross-link the BCR. A 60 minute Rap activation time course was done to look at the kinetics of Rap2 activation, Rap activation was assessed using GST-RalGDS to pull down the activated GTP-bound forms of Rap proteins. After SDS-PAGE and transfer to nitrocellulose, filters were immunoblotted for Rap2 and then reprobed for Rap1 in order to detect the activated Rap1 and Rap2.

Figure 3.1 and 3.2 show that Rap2 was activated in WEHI-231 cells and A20 cells after BCR engagement. Rap2 activation was evident after two minutes of stimulation, was maximal at 10 to 30 minutes, and was still elevated even after one hour. Rap1 was also activated with similar kinetics.

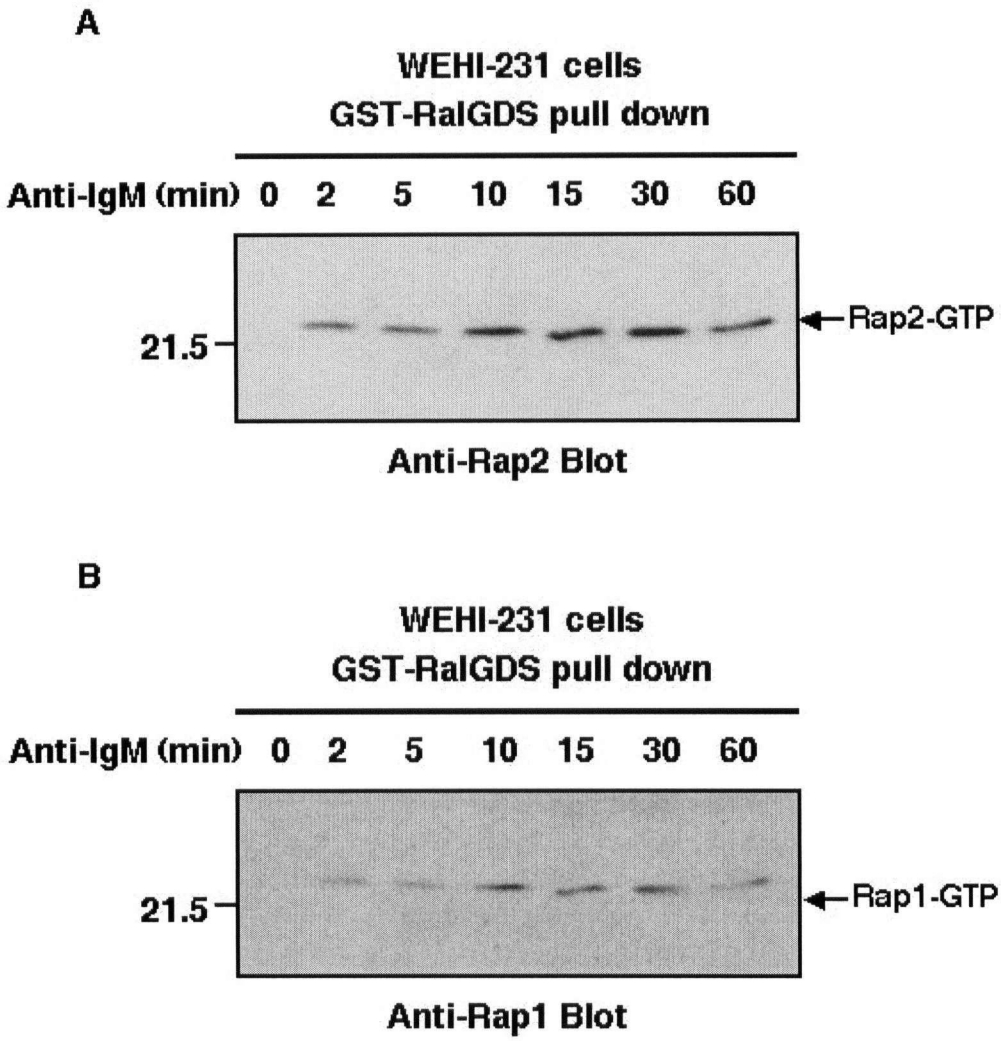

Figure 3.1. Anti-IgM activates Rap1 and Rap2 in WEHI-231 cells. WEHI-231 cells were stimulated with 100 μg/ml anti-IgM antibodies for the indicated times. A GST-RalGDS fusion protein was used to selectively precipitate the activated GTP-bound forms of the Rap proteins. Western blotting was used to detect the activated forms of (A) Rap2 and (B) Rap1. Molecular mass markers (in kDa) are shown to the left. Similar results were obtained in two independent experiments.

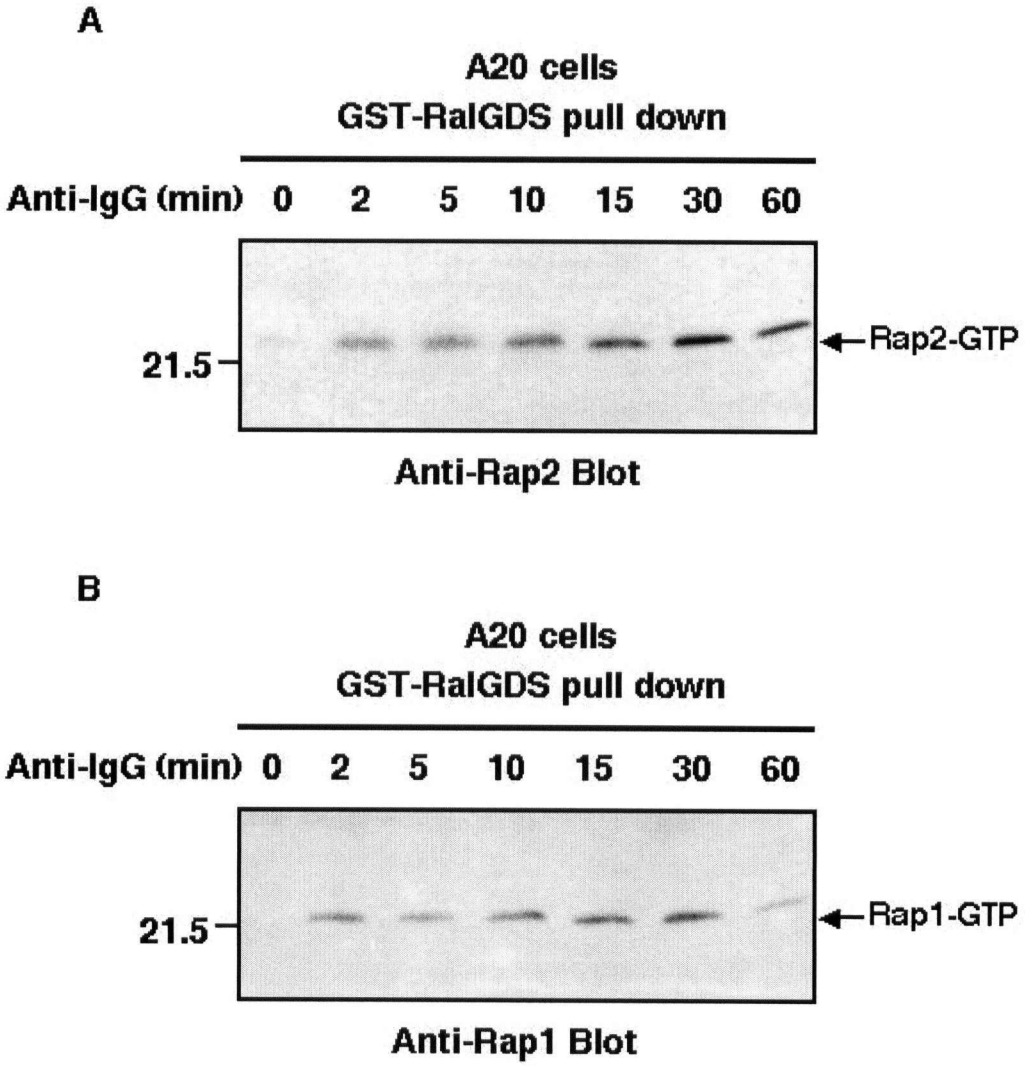

Figure 3.2. Anti-IgG activates Rap1 and Rap2 in A20 cells. A20 cells were stimulated with 100 μg/ml anti-IgG antibodies for the indicated times. A GST-RalGDS fusion protein was used to selectively precipitate the activated GTP-bound forms of the Rap proteins. Western blotting was used to detect the activated forms of (A) Rap2 and (B) Rap1. Molecular mass markers (in kDa) are shown to the left. Similar results were obtained in two independent experiments.

3.2 Activation of Rap2 by SDF-1

Because B lymphocytes migrate towards SDF-1 and Rap proteins are involved in adhesion in T cells and migration in *Drosophila*, we speculated that Rap proteins would also play a role in B cell migration. Our laboratory had previously shown that SDF-1 activates Rap1 in WEHI-231 and 2PK-3 cells (McLeod, Li, et al., JI, 2002). To determine whether SDF-1 also activates Rap2 GTPases, we stimulated WEHI-231 and 2PK-3 cells with SDF-1 and assayed Rap activation as in previous experiments. These experiments showed that Rap1 and Rap2 were activated in WEHI-231 and 2PK-3 cells after SDF-1 stimulation. Both Rap1 and Rap2 were activated within two minutes and remained activated for 30 minutes. The kinetics of Rap1 and Rap2 activation were similar. Maximal levels of Rap activation occurred between 10 to 30 minutes although there was some variability as to when this was achieved. It is possible that Rap proteins are continuously cycling between the activated and inactivated states during this time where a majority of the Rap proteins are activated. This would account for the lack of consistency in identifying maximal levels of Rap activation at a specific moment in time. Interestingly in 2PK-3 cells, the activated Rap2 appeared as a doublet. It is unknown what this doublet represents. It could be that these two proteins are Rap2A and Rap2B.

In summary, Figures 3.3 and 3.4 show that Rap2 is activated by SDF-1. The kinetics of Rap2 activation were similar to BCR-induced activation of WEHI-231 and A20 cells. This suggested that Rap1 and Rap2 were activated co-ordinately.

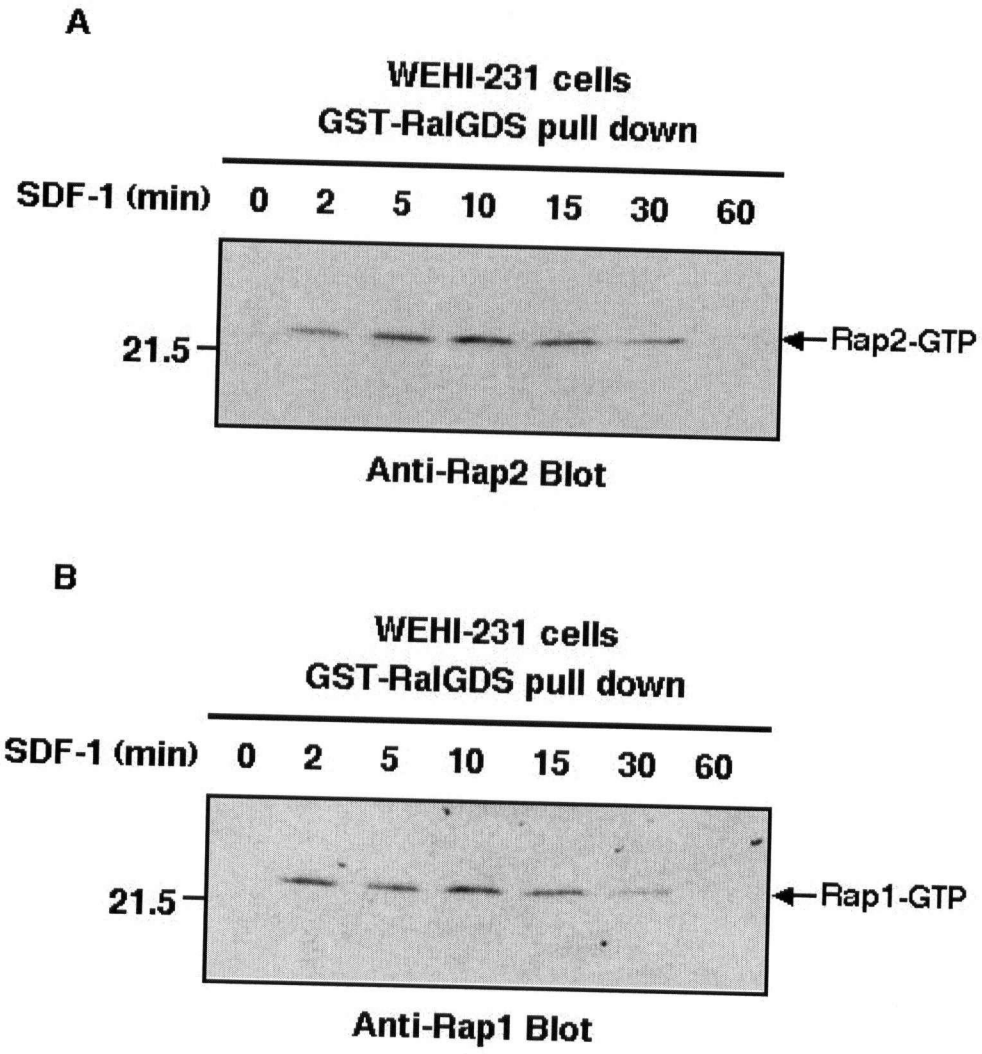

Figure 3.3. SDF-1 activates Rap1 and Rap2 in WEHI-231 cells. WEHI-231 cells were stimulated with 100 ng/ml (12.5 nM) SDF-1 for the indicated times. A GST-RalGDS fusion protein was used to selectively precipitate the activated GTP-bound forms of the Rap proteins. Western blotting was used to detect for the activated forms of (A) Rap2 and (B) Rap1. Molecular mass markers (in kDa) are shown to the left. Similar results were obtained in two independent experiments.

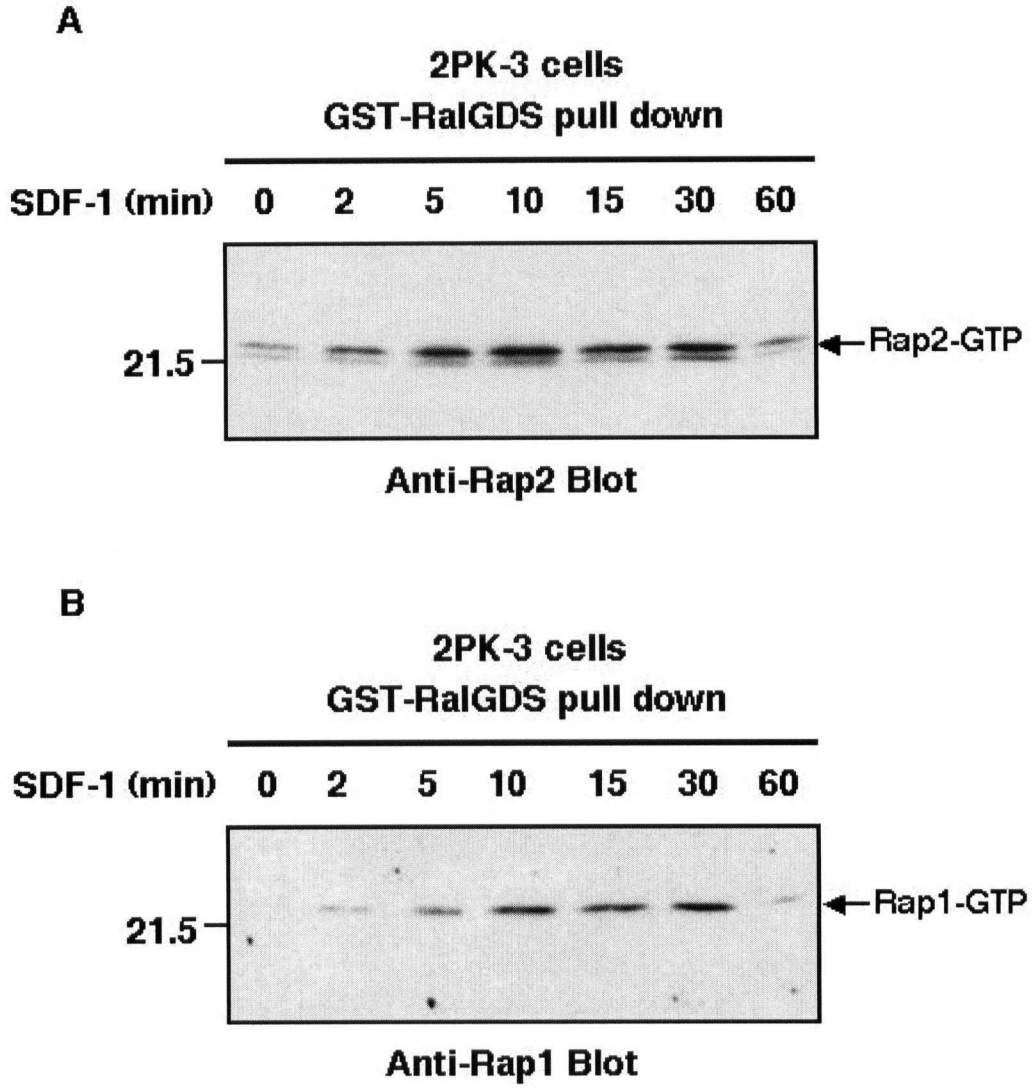

Figure 3.4. SDF-1 activates Rap1 and Rap2 in 2PK-3 cells. 2PK-3 cells were stimulated with 100 ng/ml (12.5 nM) SDF-1 for the indicated times. A GST-RalGDS fusion protein was used to selectively precipitate the activated GTP-bound forms of the Rap proteins. Western blotting was used to detect for the activated forms of (A) Rap2 and (B) Rap1. Molecular mass markers (in kDa) are shown to the left. Similar results were obtained in two independent experiments.

3.3 Mechanism of BCR-induced Rap2 activation

We showed previously that BCR-induced Rap1 activation was via a BCR → PLC-γ2 → DAG → Rap1 pathway. Since Rap1 and Rap2 are regulated by similar GEFs and GAPs (de Rooij et al., 2000; Hattori et al., 1995; Ichiba et al., 1997; Mochizuki et al., 1999; Tsukamoto et al., 1999), we hypothesized that Rap2 would be activated by the same pathway. We performed two tests to examine this. First, Rap2 should be activated by phorbol esters which mimic DAG. Second, Rap2 activation should be blocked by PLC inhibitors.

3.3.1 Activation of Rap2 by phorbol esters

First, I tested whether the DAG mimic PdBu could activate Rap2 in WEHI-231 cells. A 60 minute Rap activation assay was done as in previous experiments. Figure 3.5 shows that Rap1 and Rap2 were activated in WEHI-231 cells after PdBu stimulation. The kinetics were similar to that for anti-Ig- and SDF-1-induced Rap2 activation, with Rap2 being activated from two to 60 minutes. Reprobing of the filter showed that PdBu activated Rap1 with similar activation kinetics. Figure 3.5 (A) shows that Rap2 activation at 60 minutes dropped back down to basal levels whereas in Figure 3.5 (B), Rap1 activation at 60 minutes is still quite strong. Again, not much is known about Rap activation beyond the initial 15 minutes. There are insufficient time points to suggest that PdBu-induced Rap2 activation has stopped at 60 minutes. Preliminary experiments in our lab suggest that Rap proteins cycle between their active and inactive states in waves after one hour of stimulation. What is clear is that Rap2 can be activated by PdBu and the initial activation kinetics are very similar to Rap1. This suggested that Rap2 might also be activated via a PLC → DAG pathway.

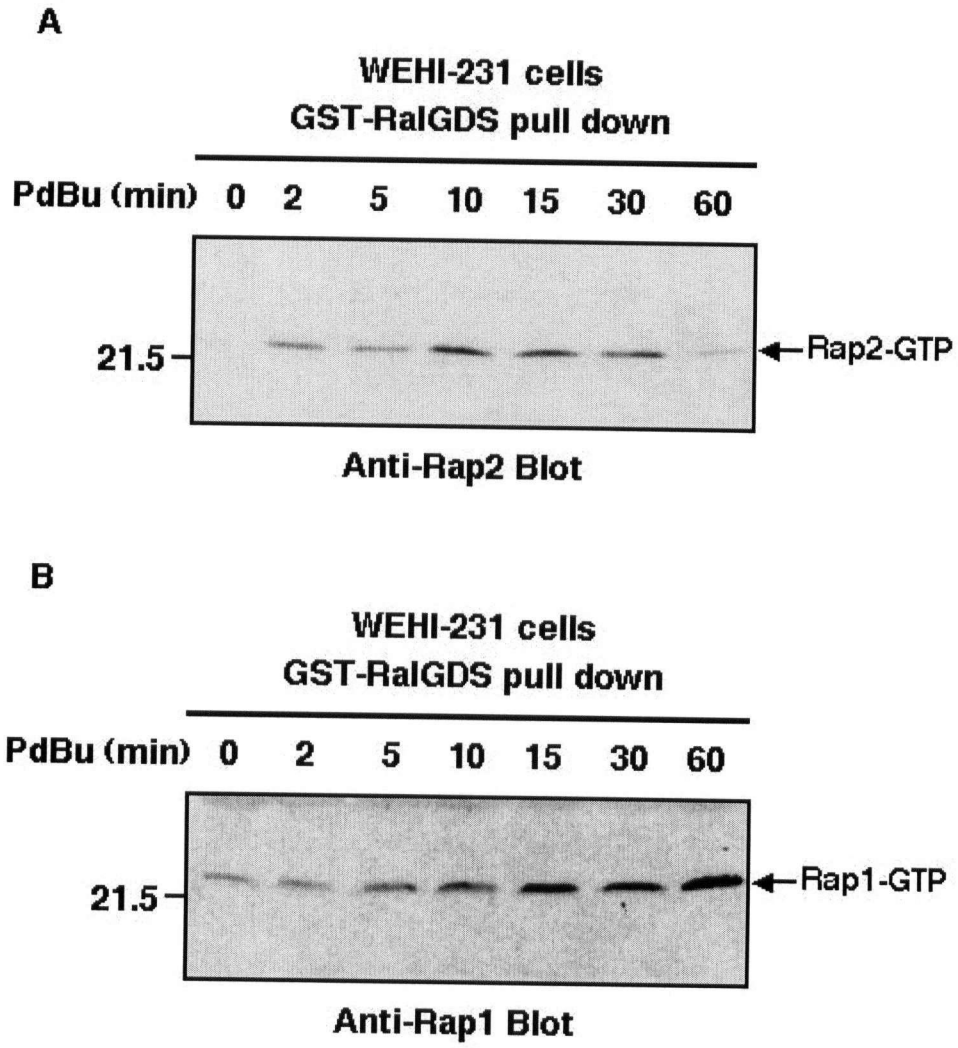

Figure 3.5. Rap1 and Rap2 are activated by the DAG mimic PdBu in WEHI-231 cells. WEHI-231 cells were stimulated with 100 nM PdBu, a phorbol ester that mimics the effect of DAG, for the indicated times. A GST-RalGDS fusion protein was used to selectively precipitate the activated GTP-bound forms of the Rap proteins. Western blotting was used to detect the activated forms of (A) Rap2 and (B) Rap1. Molecular mass markers (in kDa) are shown to the left. Similar results were obtained in two independent experiments.

3.3.2 Inhibition of Rap2 activation by the PLC inhibitor U73122

Based on the above experiment, a loss of function approach was used to test if indeed the PLC → DAG pathway leads to the activation of Rap2. The PLC inhibitor, U73122, was used to block DAG production. A structural analogue of U73122, U73343, was used as a negative control.

WEHI-231 cells were either pre-treated with U73122 or U73343 or left untreated and then stimulated with anti-Ig antibodies for 10 minutes. Filters were immunoblotted for Rap2 and then reprobed for Rap1. In order to quantitate the relative levels of Rap1 and Rap2 activation or inhibition in these cells, densitometry studies were performed using ImageQuant software. The intensity of each band that appeared on the filter is indicative of the amount of Rap activation at a given time. Obtaining a pixel volume for each U73122 band from the U73122 treatment and dividing this value with the pixel volume of its U73343 treatment control on the blot would give us the relative levels of Rap activation at each time point.

Figure 3.6 shows that Rap2 activation was partially inhibited by U73122. In contrast, Rap1 activation was almost completely inhibited when WEHI-231 cells were pre-treated with U73122. In both cases, Rap1 and Rap2 activation was still observed in U73343 pre-treated cells and was comparable to untreated cells. In addition, dimethyl sulfoxide (DMSO), the solvent used to dissolve U73122 and U73343, did not activate Rap1 or Rap2 and did not block Rap activation. Figure 3.7 shows that Rap1 activation was inhibited 60-70% whereas Rap2 activation was inhibited 40-50% in U73122 pre-treated cells.

In conclusion, PLC inhibitor, U73122, partially inhibited Rap1 and Rap2 activation in WEHI-231 cells. The partial inhibition seen could be a result of insufficient amounts of inhibitor used to block DAG production or that the inhibitor is not very efficient at blocking PLC in WEHI-231 cells. To test whether PLC activation was completely inhibited, one could measure IP_3 production, or one could look at other DAG-dependent events like the activation of the

Ras → ERK pathway and immunoblot for phosphorylated forms of ERK. An alternative way to assess the role of PLC-γ2 in Rap2 activation would be to use PLC-γ2 deficient DT40 cells, as it had been done for Rap1.

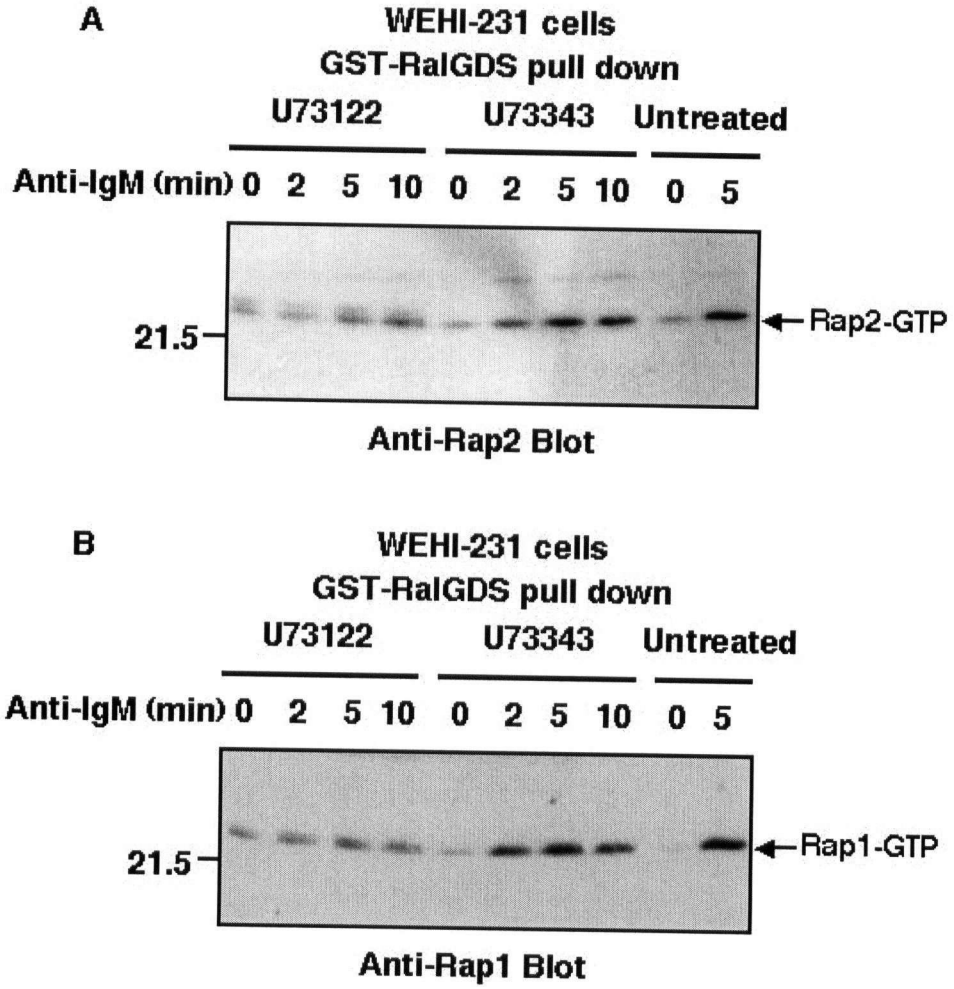

Figure 3.6. The PLC inhibitor U73122 partially inhibits BCR-induced Rap1 and Rap2 activation in WEHI-231 cells. WEHI-231 cells were pre-treated with 20 μM U73122 or 20 μM of U73343 for 20 minutes or left untreated for 20 minutes. The cells were then stimulated with 100 μg/ml anti-IgM antibodies for the indicated times. A GST-RalGDS fusion protein was used to selectively precipitate the activated GTP-bound forms of the Rap proteins. Western blotting was used to detect the activated forms of (A) Rap2 and (B) Rap1. Molecular mass markers (in kDa) are shown to the left. Similar results were obtained in two independent experiments.

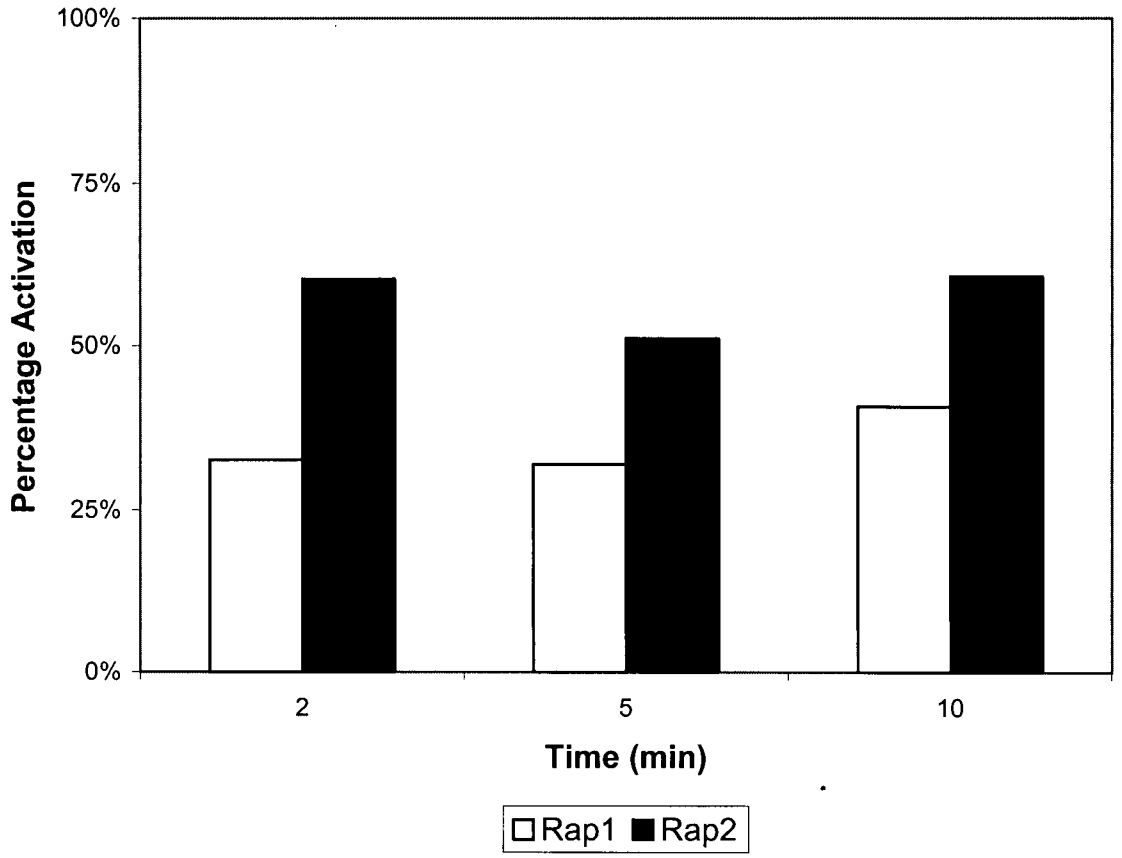

Figure 3.7. Relative levels of Rap1 and Rap2 activation in U73122 pre-treated WEHI-231 cells. Densitometry was performed on Figure 3.6 using ImageQuant. Relative levels of Rap1 and Rap2 activation in U73122 pre-treated WEHI-231 cells were calculated by taking the pixel volume for each time point, a representative of the amount of Rap activation, and dividing it by the pixel volume of its corresponding U73343 value. This ratio was expressed as a percentage.

3.4 Conclusion

This chapter has shown that anti-Ig and SDF-1 can stimulate the activation of Rap2. This is the first report of receptor-induced activation of Rap2. The activation kinetics of Rap2 activation were similar to Rap1. These studies suggest that Rap1 and Rap2 activation are co-ordinately regulated in B lymphocytes. This is not surprising since Rap1 and Rap2 share a common subset of GNEFs and GAPs.

The mechanism of Rap2 activation was also examined in this chapter. The ability of Rap2 to be activated by a PLC → DAG pathway was tested. PdBu addition stimulated Rap2 activation in WEHI-231 cells. The use of PLC inhibitor, U73122, in BCR-induced WEHI-231 showed that Rap2 activation is at least partly dependent on PLC activity. Parallel experiments conducted in the laboratory using U73122 in SDF-1-induced 2PK-3 cells showed that SDF-1-induced Rap2 activation was also PLC dependent. These two experiments therefore show that BCR-induced and SDF-1-induced activation of Rap2 is at least partly dependent on PLC. Since the efficacy of U73122 was not assessed, it is possible that these results underestimate the role of PLC in Rap2 activation. A more direct test of the role of PLC-γ2 in BCR-induced Rap2 activation would be to use the PLC-γ2-deficient DT40 cells.

CHAPTER FOUR

DEVELOPING LOSS OF FUNCTION APPROACH TO STUDY THE ROLES OF THE RAP1 AND RAP2 PROTEINS IN B LYMPHOCYTES

4.1 Rationale and summary

To study the roles of Rap1 and Rap2 in B cells, we wanted to use a loss of function approach to inhibit the activation of the endogenous Rap1 and Rap2 in B cells. To do this, we expressed two different Rap-GAPs in B cells. RapGapII and Spa-1 are two GAPs that accelerate the rate of GTP hydrolysis in Rap1 and Rap2 converting them to an inactive GDP-bound form (Ohba et al., 2000). Thus, RapGapII or Spa-1 was expressed individually in B cell lines and a Rap activation assay was performed to look for Rap1 and Rap2 inhibition when using the aforementioned stimuli from the previous chapter. I found that RapGapII and Spa-1 differentially inhibited Rap1 versus Rap2 activation. Both RapGapII and Spa-1 completely inhibited Rap1 activation. RapGapII inhibited Rap2 substantially but not completely whereas Spa-1 only caused modest inhibition of Rap2.

4.2 The effect of RapGapII expression on Rap1 and Rap2 activation

Preliminary experiments in our laboratory had shown that Rap1 activation was completely inhibited in RapGapII expressing and Spa-1 over-expressing cell lines (S. McLeod et al., unpublished results). I examined whether Rap2 activation was inhibited by these GTPases. First, WEHI-231 and A20 cells transfected with FLAG-tagged RapGapII or the empty vector, pMSCV were stimulated with anti-Ig antibodies and Rap activation was assayed over a 15 minute time course. Filters were immunoblotted for Rap2 and then reprobed for Rap1. The effects of Rap activation in cells expressing RapGapII were compared with vector control cells.

I found that Rap1 activation was completely inhibited in RapGapII-expressing WEHI-231 and A20 cells. This was a good control to test that the cells were indeed expressing

RapGapII because it had been shown before that Rap1 activation was inhibited in these cell lines. Figure 4.1 and 4.2 also show that RapGapII partially inhibited Rap2 activation in WEHI-231 and A20 cells. This inhibitory effect of Rap2 was more pronounced at later time intervals than at the beginning of the time course. Therefore, this difference in Rap inhibition showed that RapGapII differentially inhibited Rap1 and Rap2 activation in BCR-induced B cells, being more effective at inhibiting Rap1 than Rap2.

After looking at BCR-induced Rap2 activation in RapGapII-expressing WEHI-231 and A20 cells, we decided to look at SDF-1-induced Rap2 activation in RapGapII-expressing WEHI and 2PK-3 cells. WEHI-231 and 2PK-3 cells transfected with FLAG-tagged RapGapII or the empty vector, pMSCV, were stimulated with SDF-1. Figure 4.3 and 4.4 show that RapGapII expression completely inhibited Rap1 activation but only partially inhibited Rap2 activation, similar to what was seen for BCR stimulation.

To conclude the studies using RapGapII, PdBu was used to stimulate WEHI-231 cells transfected with FLAG-tagged RapGapII or the empty vector, pMSCV, to see if the same partial inhibitory effect on Rap2 activation was consistent. Figure 4.5 shows that RapGapII blocked Rap1 activation but failed to block Rap2 activation completely. In all experiments, the inhibitory effect of Rap2 activation by RapGapII is much stronger later in the time course.

In summary, RapGapII completely inhibited BCR-induced, SDF-1-induced, and PdBu-induced Rap1 activation but only partially inhibited Rap2 activation. This indicates that RapGapII is a more effective inhibitor of Rap1 than Rap2. Nevertheless, RapGapII did cause substantial inhibition of Rap2 activation.

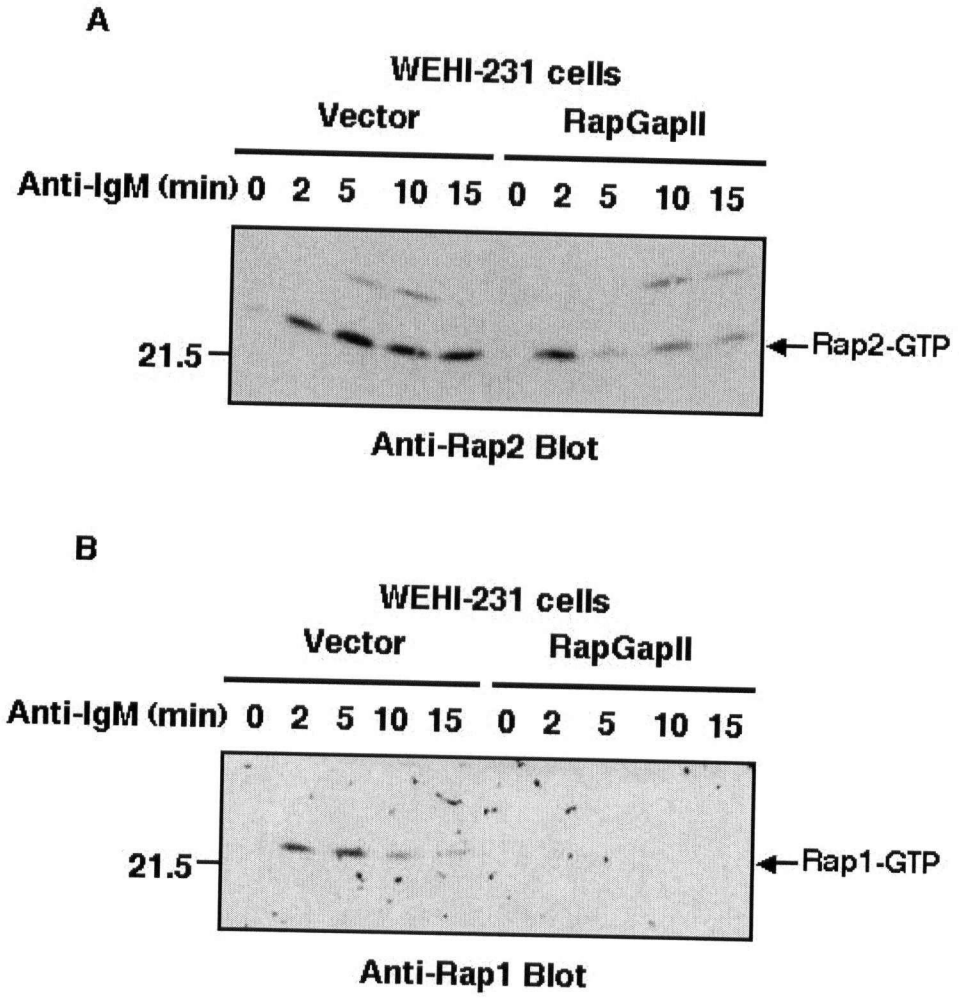

Figure 4.1. Effects of RapGapII expression on anti-IgM-induced Rap1 and Rap2 activation in WEHI-231 cells. Stable populations of WEHI-231 cells expressing the empty vector pMSCV or pMSCV-containing cDNA encoding FLAG-tagged RapGapII were stimulated with 100 μg/ml anti-IgM antibodies for the indicated times. A GST-RalGDS fusion protein was used to selectively precipitate the activated GTP-bound forms of the Rap proteins. Western blotting was used to detect the activated forms of (A) Rap2 and (B) Rap1. Molecular mass markers (in kDa) are shown to the left. Similar results were obtained in two independent experiments.

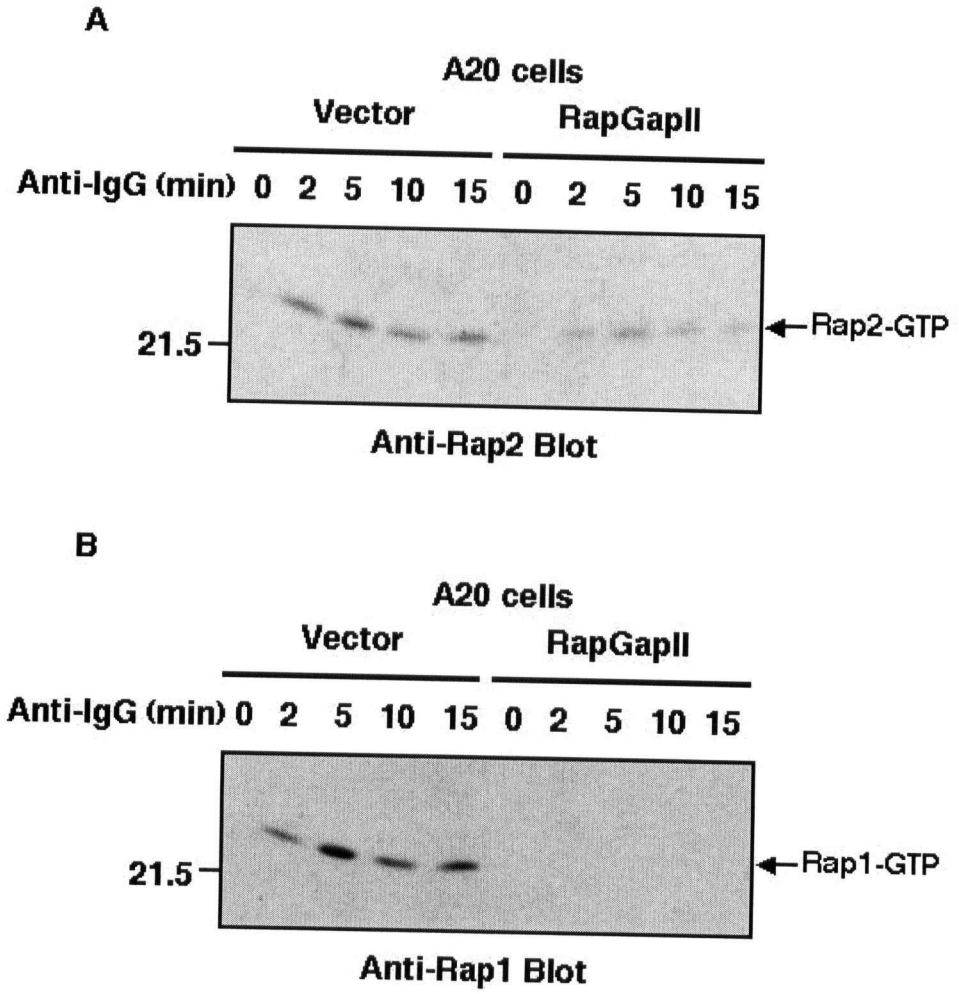

Figure 4.2. Effects of RapGapII expression on anti-IgG-induced Rap1 and Rap2 activation in A20 cells. Stable populations of A20 cells expressing the empty vector pMSCV or pMSCV-containing cDNA encoding FLAG-tagged RapGapII were stimulated with 100 μg/ml anti-IgG antibodies for the indicated times. A GST-RalGDS fusion protein was used to selectively precipitate the activated GTP-bound forms of the Rap proteins. Western blotting was used to detect the activated forms of (A) Rap2 and (B) Rap1. Molecular mass markers (in kDa) are shown to the left. Similar results were obtained in two independent experiments.

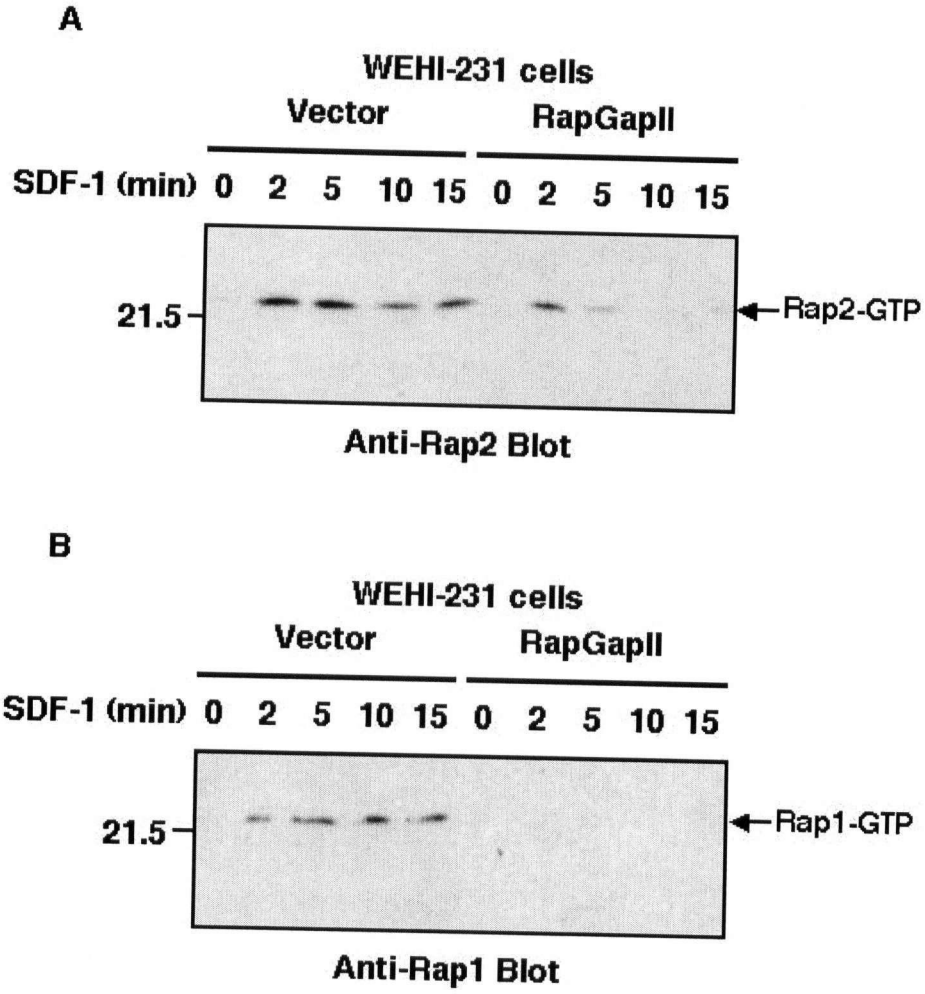

Figure 4.3. Effects of RapGapII expression on SDF-1-induced Rap1 and Rap2 activation in WEHI-231 cells. Stable populations of WEHI-231 cells expressing the empty vector pMSCV or pMSCV-containing cDNA encoding FLAG-tagged RapGapII were stimulated with 100 ng/ml (12.5 nM) SDF-1 for the indicated times. A GST-RalGDS fusion protein was used to selectively precipitate the activated GTP-bound forms of the Rap proteins. Western blotting was used to detect the activated forms of (A) Rap2 and (B) Rap1. Molecular mass markers (in kDa) are shown to the left. Similar results were obtained in two independent experiments.

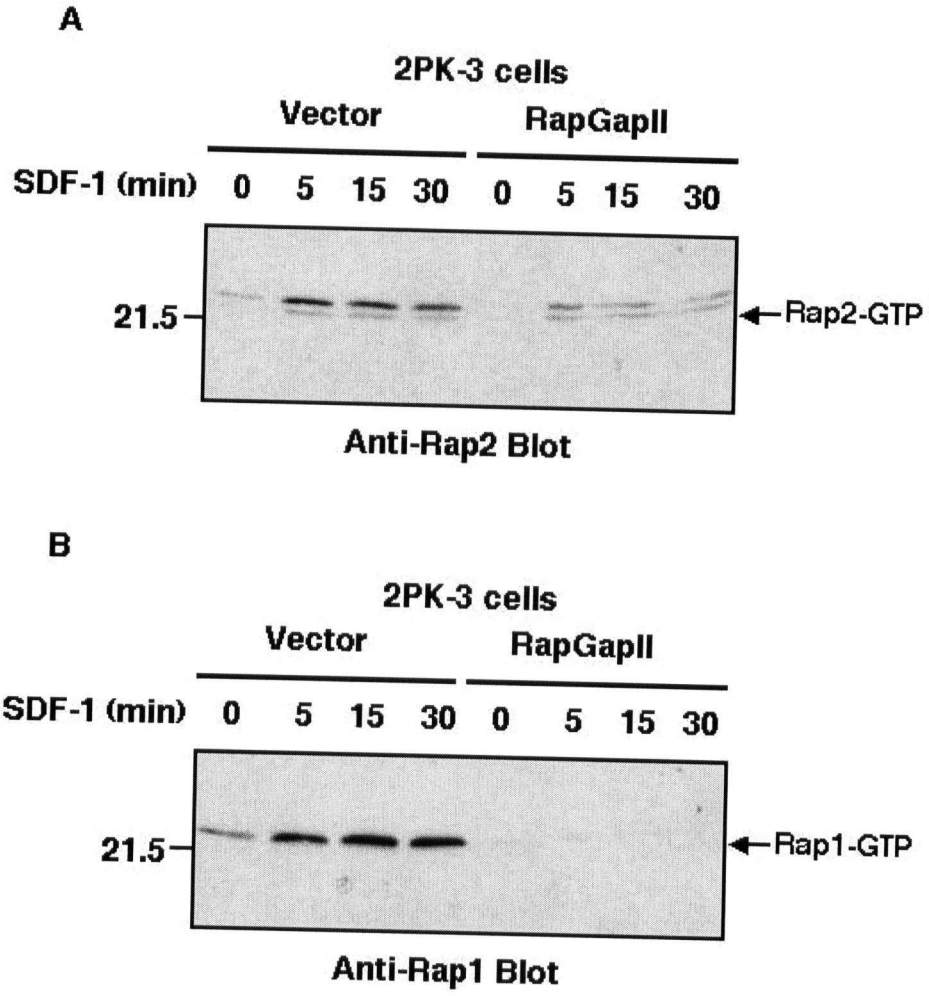

Figure 4.4. Effects of RapGapII expression on SDF-1-induced Rap1 and Rap2 activation in 2PK-3 cells. Stable populations of 2PK-3 cells expressing the empty vector pMSCV or pMSCV-containing cDNA encoding FLAG-tagged RapGapII were stimulated with 100 ng/ml (12.5 nM) SDF-1 for the indicated times. A GST-RalGDS fusion protein was used to selectively precipitate the activated GTP-bound forms of the Rap proteins. Western blotting was used to detect the activated forms of (A) Rap2 and (B) Rap1. Molecular mass markers (in kDa) are shown to the left. Similar results were obtained in four independent experiments.

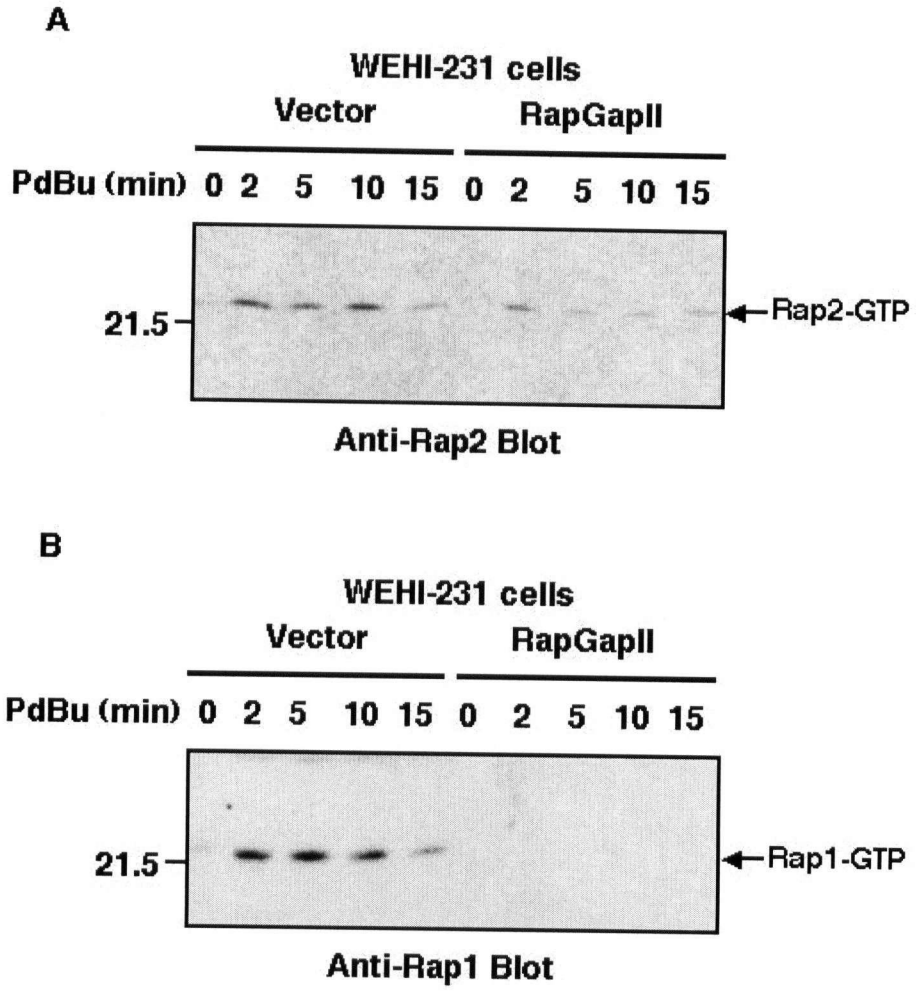

Figure 4.5. Effects of RapGapII expression on PdBu-induced Rap1 and Rap2 activation in WEHI-231 cells. Stable populations of WEHI-231 cells expressing the empty vector pMSCV or pMSCV-containing cDNA encoding FLAG-tagged RapGapII were stimulated with 100 nM PdBu for the indicated times. A GST-RalGDS fusion protein was used to selectively precipitate the activated GTP-bound forms of the Rap proteins. Western blotting was used to detect the activated forms of (A) Rap2 and (B) Rap1. Molecular mass markers (in kDa) are shown to the left. Similar results were obtained in two independent experiments.

4.3 The effect of Spa-1 expression on Rap1 and Rap2 activation

The second GAP to be utilized for the loss of function study was Spa-1. Similar to RapGapII, previous work had shown that Rap1 activation was completely inhibited in Spa-1 over-expressing cell lines (S. McLeod et al., unpublished results). The experiments in section 4.2 have already shown that Rap1 activation was more efficiently inhibited by RapGapII than was Rap2 activation.

WEHI-231 and A20 cells transfected with FLAG-tagged Spa-1 or the empty pLXSN vector were stimulated with anti-Ig antibodies. Figures 4.6 and 4.7 show that Spa-1 inhibited Rap1 activation completely, as was shown previously. In contrast, Spa-1, like RapGapII, only partially inhibited anti-Ig-induced Rap2 activation in WEHI-231 cells. There was very little inhibition in anti-Ig-induced Rap2 activation in A20 cells when compared to WEHI-231 cells. This could be a result of different amounts of Rap2 being activated in different cell lines and Spa-1's inability to inhibit Rap2 activation efficiently.

The ability of Spa-1 to inhibit Rap2 activation following CXCR4 stimulation with SDF-1 was investigated next. 2PK-3 cells transfected with FLAG-tagged Spa-1 or the empty pMX-PIE vector were stimulated with SDF-1. The use of the pMX-PIE vector, which encodes EGFP after an internal ribosomal empty site, provided an advantage for migration studies being conducted in the laboratory. Figure 4.8 showed that Spa-1 only partially inhibited SDF-1-induced Rap2 activation whereas Spa-1 completely blocked SDF-1-induced Rap1 activation.

Finally, PdBu was used to stimulate WEHI-231 cells transfected with FLAG-tagged Spa-1 or the empty pLXSN vector to see if Spa-1 had any inhibitory effect on PdBu-induced Rap2 activation. Figure 4.9 again shows that Spa-1 partially inhibited Rap2 activation while completely inhibiting Rap1 activation in WEHI-231 cells.

Thus, these experiments have shown that activation of Rap1 is completely blocked by RapGapII and Spa-1. In contrast, Rap2 is less effectively inhibited by RapGapII and Spa-1.

RapGapII caused significant but not complete inhibition of Rap2 activation. In contrast, Spa-1 caused only modest inhibition of Rap2.

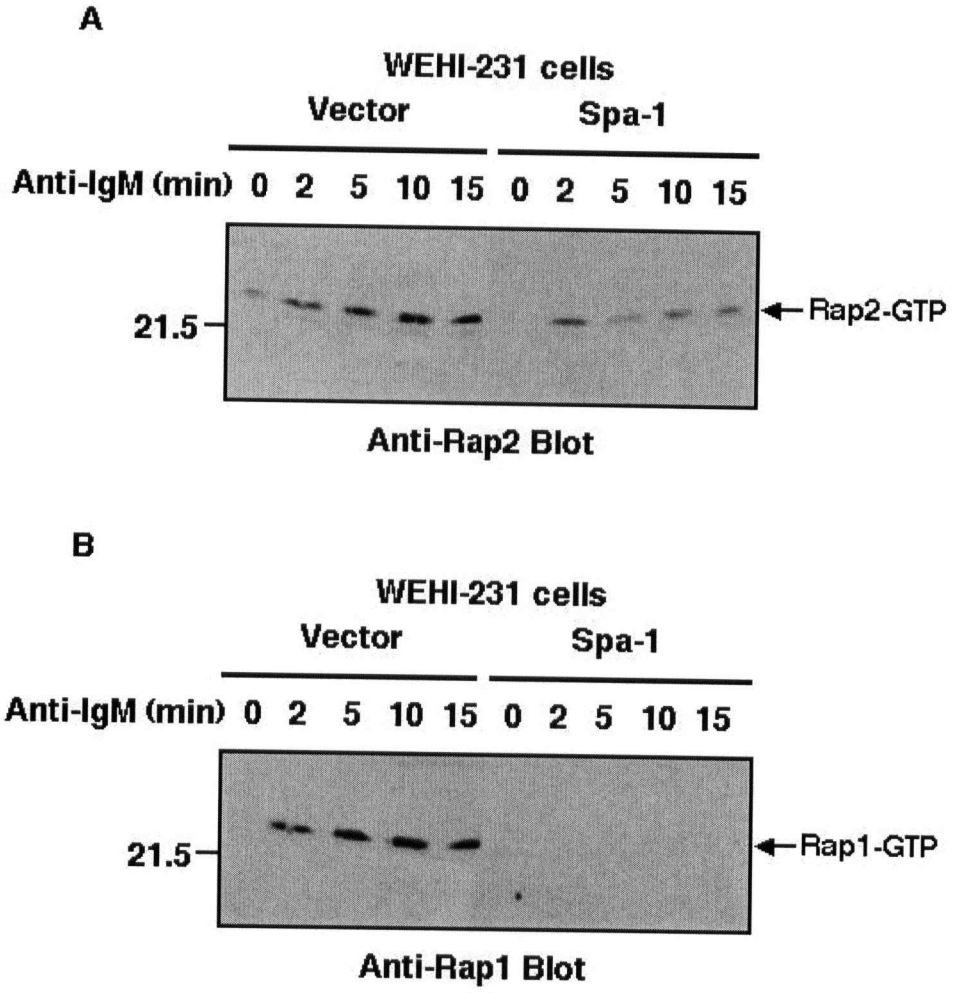

Figure 4.6. Effects of Spa-1 expression on anti-IgM-induced Rap1 and Rap2 activation in WEHI-231 cells. Stable populations of WEHI-231 cells expressing the empty vector pLXSN or pLXSN-containing cDNA encoding FLAG-tagged Spa1 were stimulated with 100 μg/ml anti-IgM antibodies for the indicated times. A GST-RalGDS fusion protein was used to selectively precipitate the activated GTP-bound forms of the Rap proteins. Western blotting was used to detect the activated forms of (A) Rap2 and (B) Rap1. Molecular mass markers (in kDa) are shown to the left. Similar results were obtained in two independent experiments.

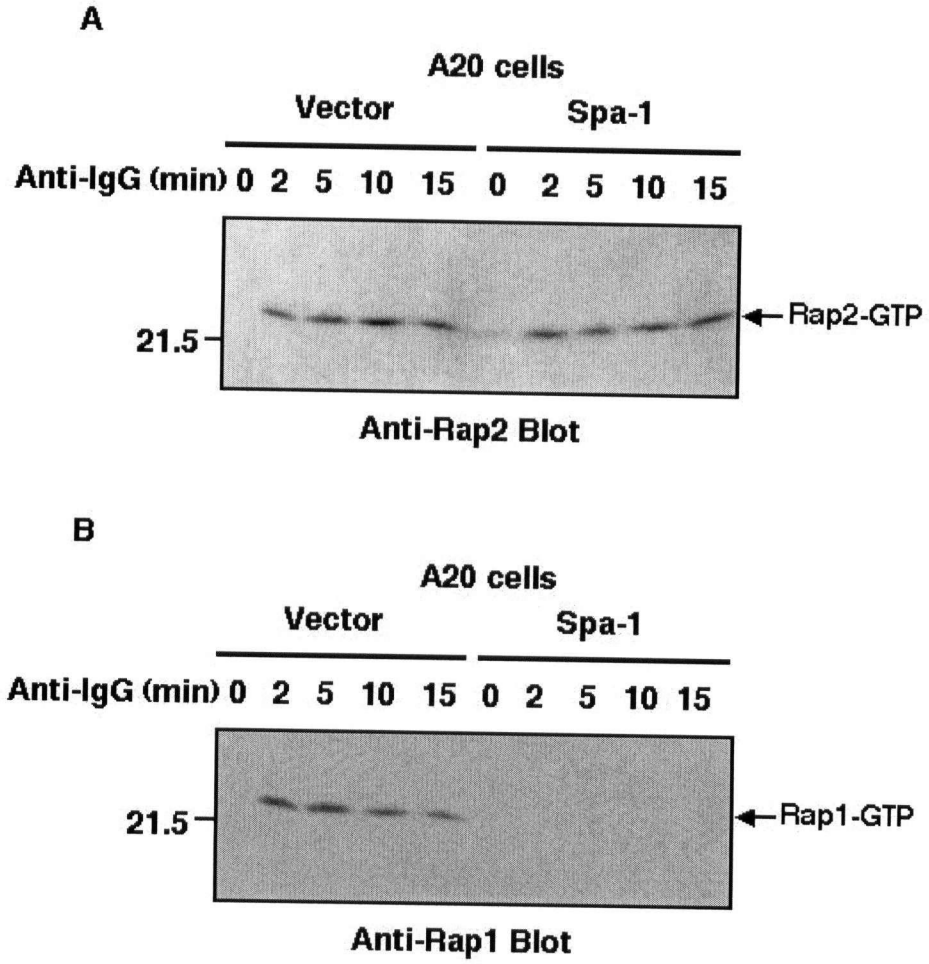

Figure 4.7. Effects of Spa-1 expression on anti-IgG-induced Rap1 and Rap2 activation in A20 cells. Stable populations of A20 cells expressing the empty vector pLXSN or pLXSN-containing cDNA encoding FLAG-tagged Spa1 were stimulated with 100 μg/ml anti-IgG antibodies for the indicated times. A GST-RalGDS fusion protein was used to selectively precipitate the activated GTP-bound forms of the Rap proteins. Western blotting was used to detect the activated forms of (A) Rap2 and (B) Rap1. Molecular mass markers (in kDa) are shown to the left. Similar results were obtained in two independent experiments.

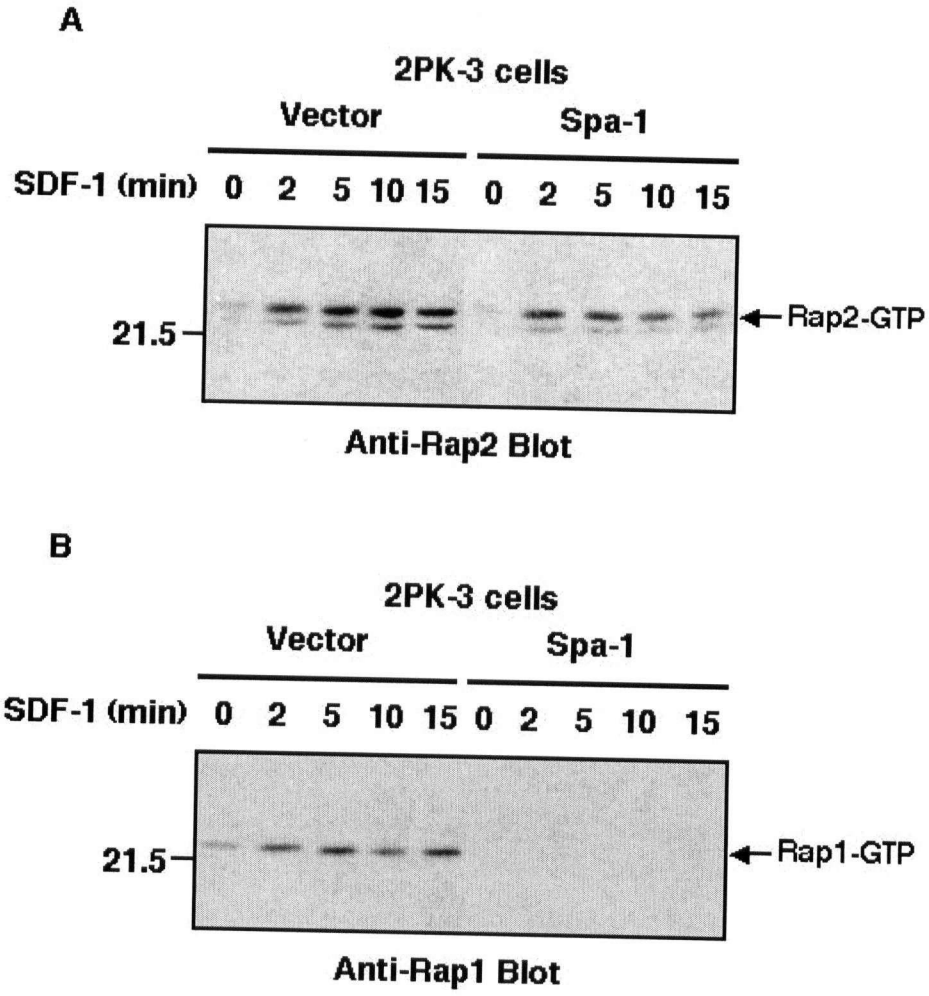

Figure 4.8. Effects of Spa-1 expression on SDF-1-induced Rap1 and Rap2 activation in 2PK-3 cells. Stable populations of 2PK-3 cells expressing the empty vector pMX-PIE or pMX-PIE-containing cDNA encoding FLAG-tagged Spa1 were stimulated with 100 ng/ml (12.5 nM) SDF-1 for the indicated times. A GST-RalGDS fusion protein was used to selectively precipitate the activated GTP-bound forms of the Rap proteins. Western blotting was used to detect the activated forms of (A) Rap2 and (B) Rap1. Molecular mass markers (in kDa) are shown to the left. Similar results were obtained in five independent experiments.

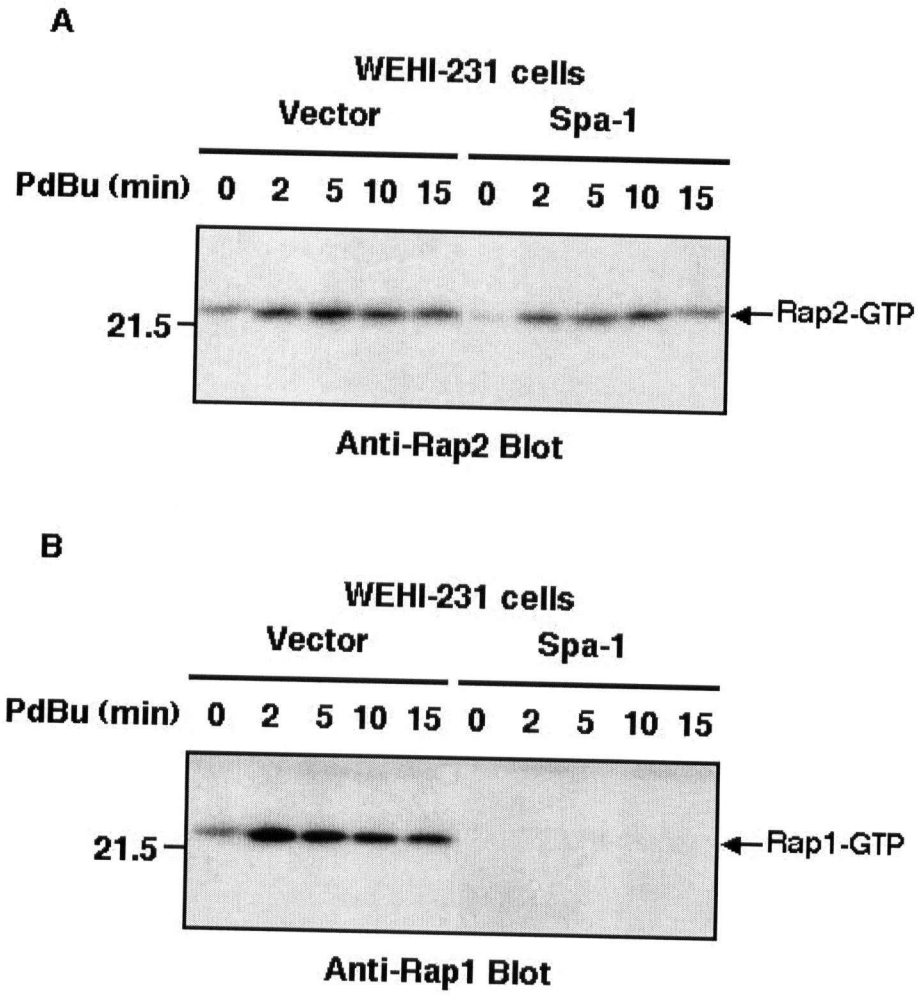

Figure. 4.9. Effects of Spa-1 expression on PdBu-induced Rap1 and Rap2 activation in WEHI-231 cells. Stable populations of WEHI-231 cells expressing the empty vector pLXSN or pLXSN-containing cDNA encoding FLAG-tagged Spa1 were stimulated with 100 nM PdBu for the indicated times. A GST-RalGDS fusion protein was used to selectively precipitate the activated GTP-bound forms of the Rap proteins. Western blotting was used to detect the activated forms of (A) Rap2 and (B) Rap1. Molecular mass markers (in kDa) are shown to the left. Similar results were obtained in two independent experiments.

4.4 Conclusion

This chapter has shown that both RapGapII and Spa-1 completely inhibit Rap1 activation in B cells when stimulated with anti-Ig antibodies, SDF-1, and PdBu. RapGapII inhibits Rap2 substantially but not completely whereas Spa-1 only causes modest inhibition of Rap2. One possible explanation for this partial inhibitory effect is that perhaps at lower dosages of stimuli, better inhibition would be seen because less Rap2 proteins would become activated. A dose response would need to be conducted to test this assumption.

Figures 4.1 to 4.9 also showed that it was easier to inhibit Rap1 than Rap2. There are several explanations for this. First, there could be more activated forms of Rap2 than Rap1 in B cells. Second, Ohba et al. have alluded to the fact that Rap2 has a low sensitivity to Rap-GAPs (Ohba et al., 2000), and this may account for the high levels of activated Rap2 observed in anti-Ig-induced, SDF-1-induced, and PdBu-induced RapGapII- or Spa-1-expressing B cell lines.

Parallel studies conducted in the laboratory have shown that RapGapII significantly inhibits B cell adhesion and migration. The inability of RapGapII to completely inhibit Rap2 activation could account for the small population of adherent and migratory B cells. Spa-1 also does not inhibit migration or adhesion very much. This could be because there is still sufficient Rap2 activation in these cells. Therefore, more studies are required to quantitate the difference in inhibition of Rap2 by RapGapII and Spa-1.

CHAPTER FIVE

DISCUSSION

5.1 Summary

One of the primary aims of my thesis was to see if Rap2 was activated by the same stimuli as Rap1. This was shown in Chapter 3 when we observed Rap2 activation in B cells that were stimulated with anti-Ig antibodies or SDF-1. The other goal of this thesis was to investigate how BCR engagement and CXCR4 signalling activated Rap2. We saw that addition of a DAG mimic, PdBu, induced Rap2 activation whereas the use of a PLC inhibitor, U73122, significantly inhibited Rap2 activation. This implied that Rap2 was activated at least in part, through a PLC → DAG pathway. The last aim of my thesis was to develop loss of function approaches for blocking Rap1 and Rap2 activation. We observed that RapGapII and Spa-1 both inhibited Rap1 activation. RapGapII caused significant inhibition of Rap2 activation while Spa-1 was less effective at inhibiting Rap2 activation. The significance of these findings will be discussed below.

5.2 Coordinate activation of Rap1 and Rap2 in B cells

While Rap1 has been shown to be activated by many receptors, little was known about Rap2 activation. My work is the first report of receptor-induced activation of Rap2. More specifically, I showed for the first time that Rap2 is activated by both the BCR and CXCR4 in B cells. Previously, Rap2 was only shown to be activated by PMA and by cell adhesion.

Initially, our laboratory looked at Rap2 because we wanted to know if it was redundant in function with Rap1 in B lymphocytes. This is because the amino acid sequence identity between Rap2 and Rap1 is quite high, 60% (Janoueix-Lerosey et al., 1998; Nancy et al., 1999; Traver et al., 2000), and both Rap proteins possess a virtually identical effector region. On top of this,

many of the GNEFs and GAPs that regulate Rap1 can interact with Rap2 (Ohba et al., 2000). However, there exists the possibility that Rap2 may have overlapping functions with Rap1 or alternatively, Rap2 may have a completely different function from Rap1. This is because while Rap2 shares most of the effector proteins with Rap1 (Ohba et al., 2000), both Rap proteins possess their own distinct subset of effector molecules that they can bind to. I showed that Rap2 was activated by both the BCR and CXCR4 and that the activation kinetics between Rap2 and Rap1 were quite similar. Hence, this observation raised the possibility that Rap2 could be co-ordinately regulated by the same regulatory proteins as Rap1.

Many receptors, when stimulated, are capable of activating Rap1 (Bos, 1998). This is because Rap-GNEFs are capable of coupling almost any receptor signalling pathway to Rap1. Consistent with this idea, I showed that Rap2, like Rap1, is activated at least in part, through a PLC → DAG pathway in B cells. Although many of these studies have not looked at Rap2 activation, I speculate that many other receptors that activate Rap1 will activate Rap2 as well.

5.3 Activation of Rap2 via the PLC pathway

Previous experiments had shown that BCR activated Rap1 via a BCR → PLC-γ2 → DAG → Rap1 pathway. We hypothesized that Rap2 would also be activated by the same pathway.

My results indicate that BCR activates Rap2 via PLC-dependent production of DAG. This is because the phorbol ester, PdBu, activated Rap2 whereas the PLC inhibitor, U73122, blocked Rap2 activation. Since ionomycin, a calcium ionophore, did not activate Rap2 (S. McLeod, unpublished results), it supports the idea that DAG is the PLC-derived second messenger that activates Rap2.

If Rap1 and Rap2 activation are downstream of DAG, the interesting question raised is which Rap-GNEFs and Rap-GAPs are regulated by DAG in B cells. Known Rap-GNEFs that

are regulated by DAG are CalDAG-GEFI (Kawasaki et al., 1998), RasGRP2 (Clyde-Smith et al., 2000), and CalDAG-GEFIII (Yamashita et al., 2000). However, most of these GNEFs are also activated by intracellular Ca^{2+} increases whereas addition of ionomycin in B cells did not stimulate Rap1 (McLeod et al., 1998) or Rap2 activation (S. McLeod, unpublished results). It is also not known whether these GNEFs are expressed in B cells. Thus, there may be another DAG-regulated GNEF in B cells that does not respond to Ca^{2+}. As for Rap-GAPs, Spa-1, RapGapI, RapGapII, Tuberin GAP^{IP4BP}, and E6tP1 are known to stimulate GTPase Rap1 activity (McLeod and Gold, 2001). However, only Spa-1, RapGapI, and RapGapII are known to regulate Rap2 and only Spa-1 is known to be expressed in B cells (McLeod and Gold, 2001). Clearly more studies need to be done. In order to investigate which GNEFs and GAPs couple the BCR and CXCR4 to Rap1/2 activation, loss of function studies can be utilized. Generating specific GNEF/GAP-deficient B cell lines and then looking for Rap1 and Rap2 activation will be quite useful in seeing which GNEFs and GAPs regulate Rap1 and Rap2. A complementation study can be used to follow-up on this experiment in which the GNEF and GAP that was missing is replaced. Another approach would be to over-express certain GNEFs and GAPs in these B cell lines and to see if they have any effect on Rap1 and Rap2 activation. GNEFs and GAPs that do not affect Rap1 and Rap2 in B cells will have no visible effects.

5.4 Differential regulation of Rap1 and Rap2 by RapGapII and Spa-1

We knew previously that RapGapII inhibited Rap1 activation. Since Rap2 could have redundant functions as Rap1, we needed to know whether RapGapII could also inhibit Rap2. Because using one approach could give us artefacts, it was important to use a second loss-of-function approach to study Rap1 and Rap2 inhibition. Hence, we over-expressed Spa-1, another Rap-specific GAP (McLeod and Gold, 2001).

Our results showed that Spa-1 effectively inhibited Rap1 but not Rap2. In contrast, RapGapII caused significant inhibition in Rap1 and Rap2 activation. This is supported by experiments conducted by Ohba et al. in NIH 3T3 cells. They showed that Rap2 functions as a slowly responding molecular switch compared to Rap1 because Rap2 appears to have a low sensitivity to GAP proteins resulting in a high GTP/GDP ratio on Rap2 (Ohba et al., 2000).

Since RapGapII and Spa-1 differentially inhibit Rap1 versus Rap2 activation, this can be used to identify functions that can be carried out by Rap2 in the absence of Rap1 activation. This is discussed in the following section.

5.5 The role of Rap proteins in cell migration and cell adhesion

When Rap1 (then called Krev-1) was first cloned, it was believed to have the ability to revert Ras transformed cells (Kitayama et al., 1989). Because the 21 kDa protein, Rap1, shared a 50% amino acid sequence identity with Ras, it was believed that the function of Rap1 was to suppress Ras signalling by interacting with Ras effectors via competitive inhibition. This theory was further strengthened when it was revealed that Rap1 and Ras had the same effector region (Kitayama et al., 1989). However, this theory began to lose support when it was demonstrated that Rap2, which also shared a virtually identical effector region as Ras, neither exhibited growth-promoting nor growth-inhibitory effects in several independent cell lines (Jimenez et al., 1991). Later, Zwartkruis et al. showed that Rap1 activation did not inhibit Ras effector signalling when Rap1 signalling was activated by stimulating several growth factor receptors such as PDGF, EGF, and the G protein-coupled receptors for LPA, thrombin and endothelin. It is believed that Ras inhibition by Rap1 was an artefact due to over-expressing activated Rap1.

Subcellular localization studies, while interesting, have not fully elucidated the functions of Rap proteins either. We know that Rap2 proteins undergo post-translational modifications that allow them to interact with membranes (Beranger et al., 1991). However, Rap proteins seem

to be associated with different membranes than Ras, which is located at the plasma membrane (Pizon et al., 1994). This brings into question whether Rap proteins could interact with Ras effectors at all since Ras effectors are localized at the plasma membrane and Rap proteins are confined to distinct areas of the cell such as the peri-nuclear membrane region (Bos, 2001) and the endoplasmic reticulum (Beranger et al., 1991). Recent studies using fluorescent resonance energy transfer have shown that activation of Rap1 begins outside the nucleus and spreads outwards towards the plasma membrane over time (Bos, 2001). The ability of Rap proteins to translocate to discrete areas of the cell was also confirmed in human platelets. Franke et al. showed that platelet aggregation correlated with the translocation of Rap1 proteins to detergent insoluble cytoskeletal fractions (Franke et al., 2000). This led us to believe that perhaps Rap proteins interacted with cytoskeletal components in B lymphocytes and somehow influenced adhesion and migration. Studies of Rap translocation to the cytoskeleton in B cell lines were inconclusive however (unpublished results).

More and more investigations are showing that Rap1 is involved in cell adhesion and cell migration. For example, Tsukamoto et al. showed that transiently expressing a Rap1 GAP, Spa-1, in HeLa cells induced rounding up of cells whereas transiently expressing the Rap-GNEF, C3G, induced extensive cell spreading (Tsukamoto et al., 1999). In another study in T cells, Reedquist et al. showed that activated Rap1 promotes VLA-4 and LFA-1-dependent adhesion (Reedquist et al., 2000). They speculate that Rap1 may coordinate adhesion-dependent signals during T cell migration or extravasation through epithelial cells. Finally Arai et al. demonstrated that CrkL-mediated cell adhesion in hematopoietic cells can be inhibited by a dominant negative mutant of Rap1, Rap1A-17N (Arai et al., 2001). These reports made us curious as to whether Rap1 and Rap2 could also mediate cell adhesion and migration in B lymphocytes.

Recently, our laboratory has shown that expressing RapGapII blocked B cell adhesion via LFA-1 and VLA-4 (S. McLeod, unpublished results). Also, treating B cells with SDF-1 induces

Rap activation, and we have shown that SDF-1-induced migration requires activation of the Rap GTPases (McLeod, Li, et al., JI, 2002). Expressing RapGapII in these cells blocked Rap activation and reduced SDF-1-induced migration significantly whereas expressing a constitutively active form for Rap2 – Rap2V12 – increased SDF-1-induced B cell migration. In other studies in the laboratory, expressing Rap2V12 in A20 cells promotes morphological changes (S. McLeod, unpublished results). The cells seemed to adhere to surfaces better than control cells. Taken together, these experiments indicate that Rap proteins clearly play a role in B cell adhesion and migration.

If Rap proteins are important for B cell adhesion and migration, the next question to ask is exactly how Rap proteins regulate these processes. Increase in cell adhesion depends on the activation of the LFA-1 and VLA-4 integrins in B cells. Sarah McLeod has shown that Rap activation is important for activation of both LFA-1 and VLA-4 by anti-Ig antibodies and by phorbol esters (S. McLeod, unpublished results). While the mechanism for integrin activation is not known, we do know that integrin activation requires integrin clustering which is dependent on their release from actin cytoskeleton. Maybe, activated Rap proteins regulate this process.

There are several situations during B cell development and activation in which adhesion and migration processes are required. First, B cell progenitor binding to bone marrow stromal cells is necessary for the production of mature B cells (Melchers and Rolink, 1999). Second, the ability for B cells to migrate across the endothelial cells in blood vessels and enter the lymphoid organs requires integrins (Melchers and Rolink, 1999). Third, antigen presentation between B and T cells requires cell-cell adhesion, which is mediated by integrins (Janeway et al., 2001). Finally, B cell survival in the periphery is dependent on cell-cell interactions with follicular dendritic cells (FDC). This process requires the B cells to adhere to the FDCs in order to receive survival signals. Therefore, it would be interesting to see if Rap proteins play a role in these processes.

5.6 Further work

Many questions still remain unanswered regarding the regulation and function of Rap proteins in B lymphocytes. I have shown that Rap1 and Rap2 can be activated by a variety of stimulants: anti-Ig antibodies, SDF-1, and PdBu. We also know that the activation kinetics of Rap1 are very similar to Rap2 suggesting coordinate regulation by the same upstream activators. The other finding of this thesis is that Rap2, like Rap1, seems to be activated via a PLC → DAG pathway. However, we do not know which GNEF or GAP regulates Rap activation/inhibition. Loss of function experiments creating B cell lines that are deficient in one specific GNEF or GAP will have to be generated in order to observe which GNEFs and GAPs couple the BCR and CXCR4 to Rap activation.

While over-expressing GAPs to inhibit Rap function is an alternative method to replace the loss of function effect of Rap proteins, it could nevertheless introduce artefacts in our studies as these GAPs might not completely inhibit Rap in certain specific situations, and they could interfere with other signalling pathways in B lymphocytes. Experiments conducted in our laboratory have shown that anti-Ig, SDF-1, and PdBu stimulation in several B cell lines expressing RapGapII does not interfere with ERK, p38, JNK, and Rac activation (R. Lee, S. McLeod, S. Christian et al., unpublished results). A better approach would be to create a conditional Rap1/2 knockout mutant. However, this would require multiple knockouts of Rap1A, Rap1B, Rap2A, and Rap2B. An alternative is to couple loss of function experiments with gain of function experiments, like over-expressing Rap2V12, a constitutively active form of Rap2.

We know that Spa-1 is found in B cells (S. McLeod, M. Gold, unpublished results). Results from Chapter 4 of the thesis also showed that Spa-1 was more active on Rap1 than Rap2. Since Spa-1 is ineffective at inhibiting Rap2, this suggests the existence of another GAP that

could regulate Rap2 activation in B lymphocytes. A yeast 2-hybrid analysis of proteins which interact with Rap2 could uncover other Rap-GAPs in B lymphocytes.

The best way to address whether Rap1 and Rap2 have the same or different functions in B lymphocytes would be to over-express Rap1V12 and Rap2V12 in B cell lines. This gain of function approach would enable us to see the phenotypic changes that occur and allow us to infer what functions are regulated by Rap1 or Rap2 in B cells. Unfortunately, we have been unable to express Rap1V12 into B cells. Constitutively active Rap1 seems to be toxic in B lymphocytes. What is required is a way to induce or regulate Rap1V12 expression. Clearly, more studies are required.

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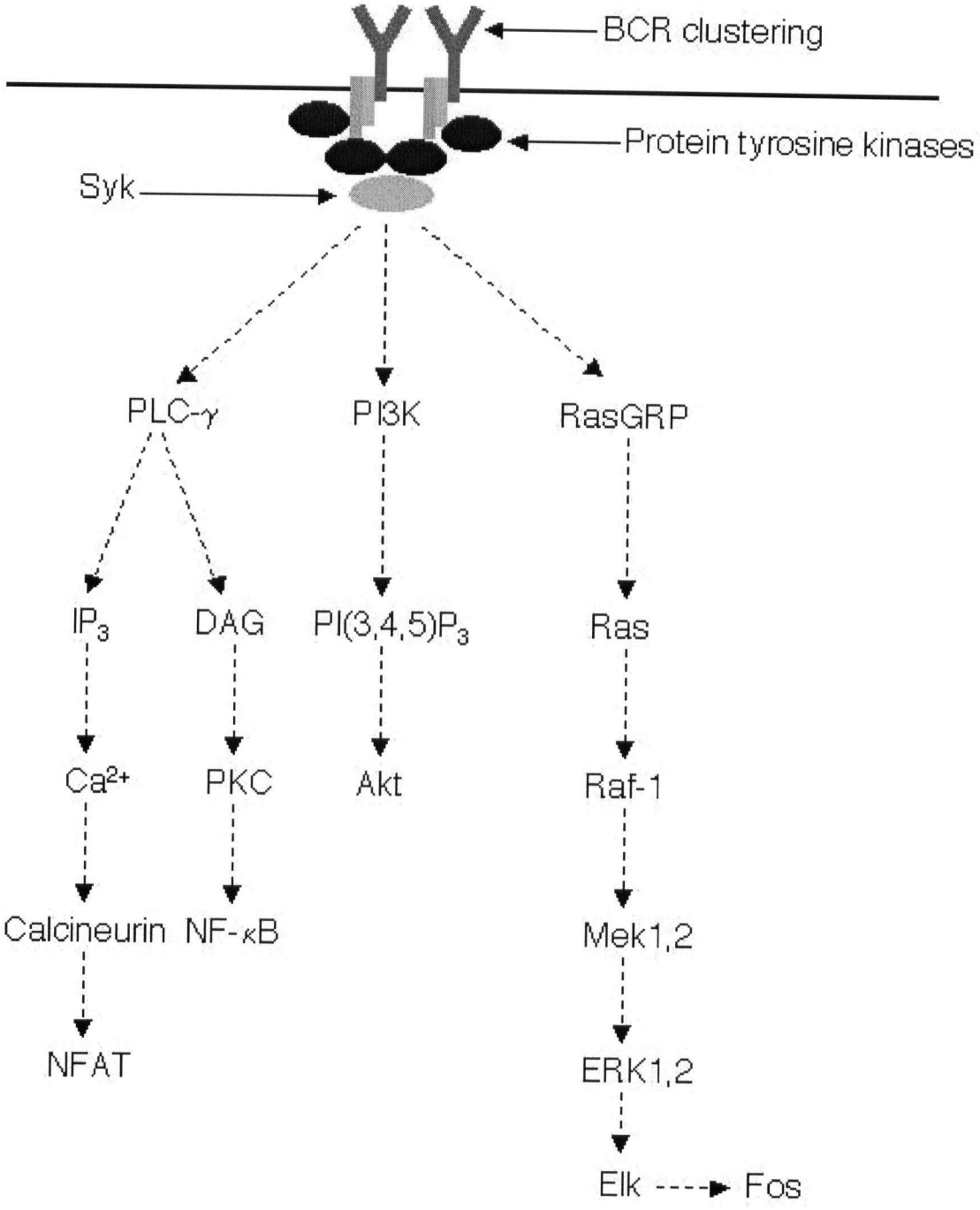
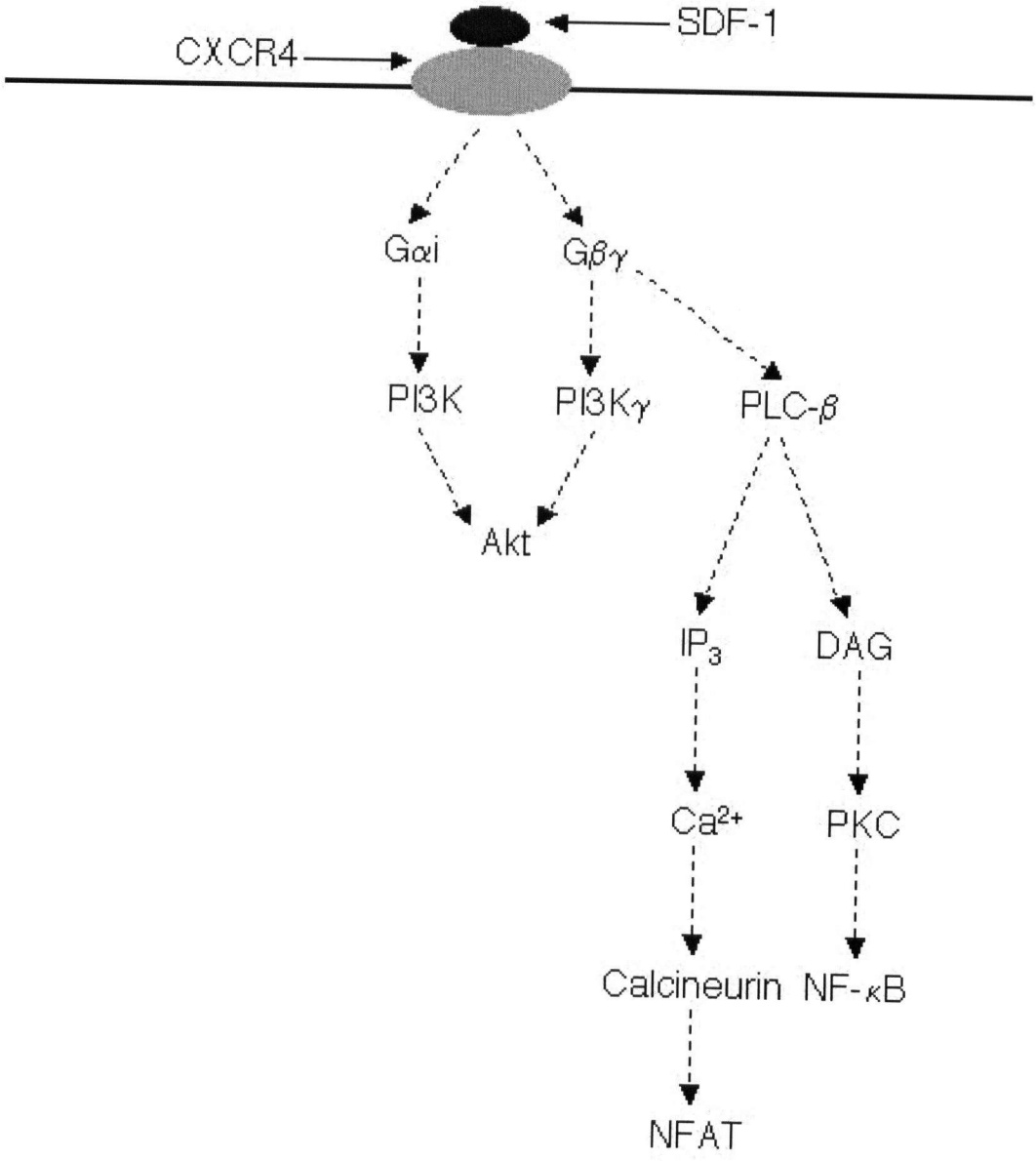
APPENDIX I

Overview of BCR signalling pathway



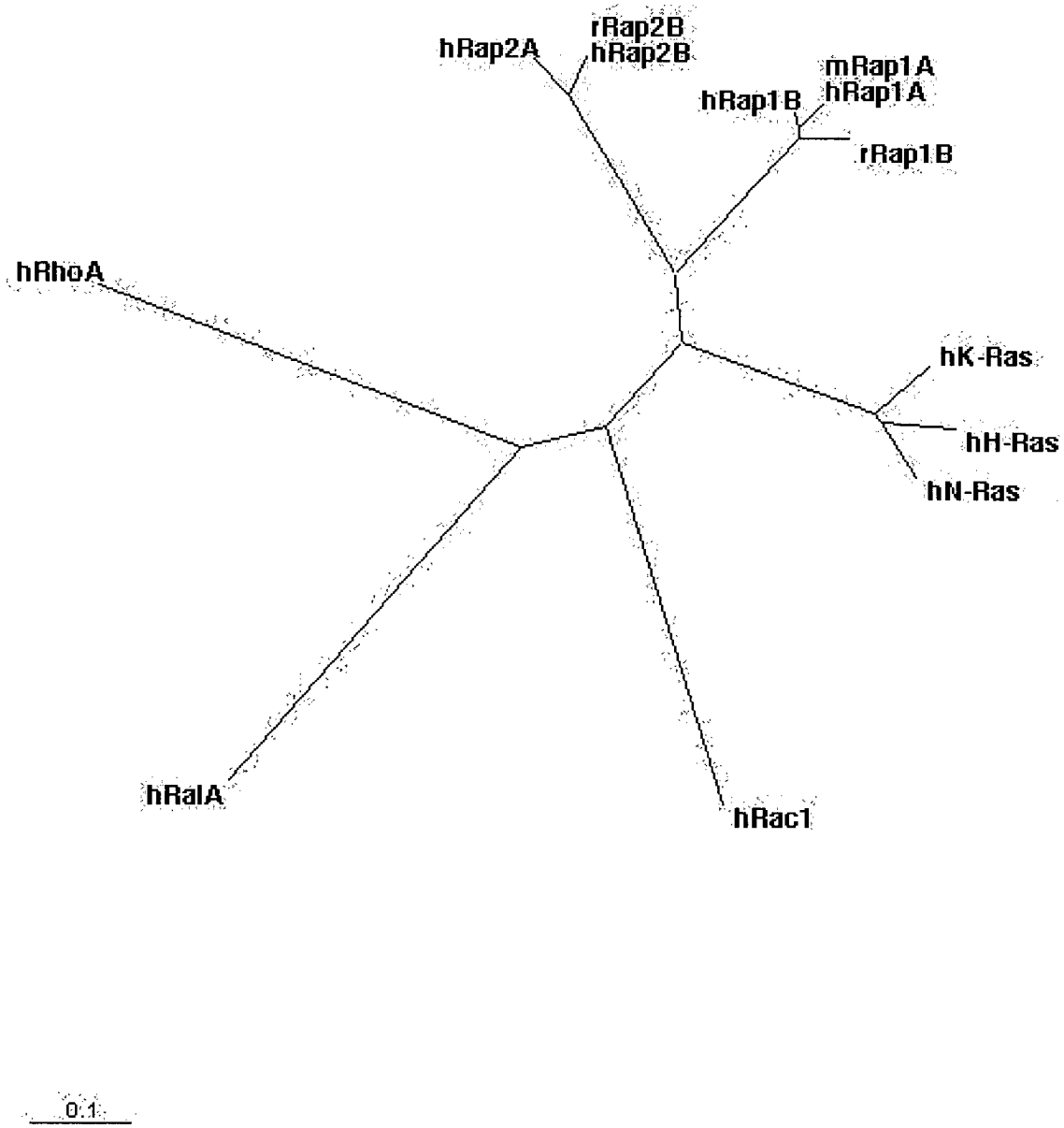
APPENDIX II

Overview of CXCR4 signalling pathway



APPENDIX III

Phylogenic tree of Rap and Ras family GTPases



Note: The following phylogenic tree was generated by aligning the amino acid sequences using ClustalW and drawn using TreeViewPPC. The letters before each gene denotes the species from which it came, where 'h' is human, 'm' is mouse, and 'r' is rat.