EXCHANGE PROTEIN ACTIVATED BY CAMP: DRUG TARGET AND BIOMARKER IN CHRONIC LYMPHOCYTIC LEUKEMIA

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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University of California, San Diego

2009
# TABLE OF CONTENTS

Signature Page........................................................................................................... iii

Table of Contents........................................................................................................ iv

List of Abbreviations.................................................................................................... vi

List of Figures............................................................................................................... vii

List of Tables............................................................................................................... x

Acknowledgements..................................................................................................... xi

Abstract....................................................................................................................... xii

1. Introduction........................................................................................................... 1
   1.1. Chronic lymphocytic leukemia................................................................. 1
   1.2. Apoptosis in CLL......................................................................................... 2
   1.3. cAMP: An important intracellular second messenger................................. 3
   1.4. cAMP and CLL......................................................................................... 5
   1.5. Downstream mediators of cAMP: PKA and Epac...................................... 6
   1.6. PKA and Epac in apoptosis of CLL-cells.................................................. 8
   1.7. Hypothesis and Goals................................................................................. 9
      1.7.1. Hypothesis One................................................................................ 9
      1.7.2. Hypothesis Two.............................................................................. 10

2. Materials and Methods....................................................................................... 11
   2.1. PBMC isolation.......................................................................................... 11
   2.2. SNP discovery............................................................................................. 11
      2.2.1. Cellular genomic DNA extraction...................................................... 11
      2.2.2. Epac-1 and Epac-2 polymerase chain reaction (PCR) primer design... 11
      2.2.3. PCR.................................................................................................... 12
      2.2.4. PCR product sequencing and sequence alignment............................... 13
   2.3. Real Time PCR............................................................................................ 14
   2.4. Flow Cytometry.......................................................................................... 16
   2.5. Statistical analyses....................................................................................... 17

3. Results: Single nucleotide polymorphisms...................................................... 18
   3.1. SNPs in the Epac-1 promoter region........................................................... 18
   3.2. SNPs in the Epac-2 promoter region........................................................... 25
4. Results: Expression of downstream mediators of cAMP

4.1. The mRNA expression of Epac-1 is increased whereas the mRNA expression of PKAI is decreased in patients with CLL

5. Results: Pro- and anti-apoptotic effects of PKA and Epac in CLL

5.1. Epac activators are anti-apoptotic whereas PKA activators are pro-apoptotic in CLL

5.2. Inhibition of PKA inhibits apoptosis whereas inhibition of Epac-dependent pathways is pro-apoptotic

5.3. Correlation of Epac expression with basal and drug-induced apoptosis

6. Discussion

7. Supplementary Figures

References
LIST OF ABBREVIATIONS

Epac = Exchange protein activated by cAMP

cAMP = 3’,5’ adenosine monophosphate

PKA = Protein kinase A

AKAP = A-kinase anchoring protein

CNG = cyclic-nucleotide gated channel

PDE = phosphodiesterase

GPCR = G-protein coupled receptor

CLL = Chronic Lymphocytic Leukemia

PBMC = Peripheral Blood mononuclear cells
LIST OF FIGURES

**Figure 1.1.** Schematic of the 3’5’-cyclic adenosine monophosphate (cAMP) signaling pathway……………………………………………………………………………………………………5

**Figure 1.2.** Schematic diagram of Epac-1 and Epac-2 protein domain homologies……..7

**Figure 1.3.** Protein domain diagram of Epac-2 before and after cAMP binding and Rap catalysis…………………………………………………………………………………………8

**Figure 3.1.** Map of novel and previously known SNPs in the 938-bp area immediately upstream of the Epac-1 exon 1 translation start site…………………21

**Figure 3.2.** Epac-1 SNP frequency in both normal PBMC and CLL…………………22

**Figure 3.3.** Epac-1 SNP distribution by frequency in normal, indolent-CLL, aggressive-CLL, and total CLL……………………………………………………………………………………………………23

**Figure 3.4.** Diagram of possible linkage disequilibrium between Epac-1 SNPs……24

**Figure 3.5.** Map of novel (black) and previously known (blue) SNPs in an 892-bp area immediately upstream of Epac-2 exon 1 translation start site…28

**Figure 3.6.** Epac-2 SNP frequency in normal and CLL…………………………………29

**Figure 3.7.** Epac-2 SNP distribution by frequency in normal, indolent-CLL, aggressive-CLL, and total CLL……………………………………………………………………………………30

**Figure 3.8.** Diagram of possible linkage disequilibrium between Epac-2 SNPs………31

**Figure 4.1.** Cycle thresholds normalized to 28S loading control for of MRP4, MRP5, Rlα, RIIβ, Epac-1, PDE3B, PDE7B in PBMC from CLL patients and normal PBMC as measured by real-time PCR……………………………………………………………………….35

**Figure 4.2.** Fold-change in the expression of MRP4, MRP5, Rlα, RIIβ, Epac-1, PDE3B, PDE7B in CLL compared to normal PBMC as measured by real-time PCR…36

**Figure 4.3.** Fold-change in the expression of MRP4, MRP5, Rlα, RIIβ, Epac-1, PDE3B, PDE7B in indolent-CLL compared to normal PBMC as measured by real-time PCR……………………………………………………………………………………37

**Figure 4.4.** Fold-change in the expression of MRP4, MRP5, Rlα, RIIβ, Epac-1, PDE3B, PDE7B in aggressive-CLL compared to normal PBMC as measured by real-time PCR……………………………………………………………………………………38
Figure 4.5. Comparison of fold-change in mRNA expression between patients with either indolent- or aggressive-CLL versus normal PBMC, as measured by real-time-PCR……………………………………………………………………………39

Figure 4.6. Ratio of R1α/R1β mRNA in normal PBMC, indolent-CLL, aggressive-CLL and total CLL……………………………………………………………………………40

Figure 5.1. Drug-induced apoptosis as measured by flow cytometry in CLL-cells in response to 50 µM 8Me, 50 µM N6, 50 µM 8Cl, 10 nM IR-284, or 100nM IR-284……………………………………………………………………………44

Figure 5.2. Drug-induced apoptosis as measured by flow cytometry in normal PBMC in response to 50 µM 8Me, 50 µM N6, or 100 nM IR-284………………………………44

Figure 5.3. Drug-induced apoptosis as measured by flow cytometry in CLL-cells in response to 100 nM IR-284 alone or with 5 µM PKI………………………………….45

Figure 5.4. Drug-induced apoptosis in CLL-cells as measured by flow cytometry in response to increasing doses of the Rap1-inhibitor GGTI-298 (100 nM, 1 µM, 10 µM)………………………………………………………………………………46

Figure 5.5. Drug-induced apoptosis in CLL-cells as measured by flow cytometry in response to increasing doses of GGTI-298 (100 nM, 1 µM, 10 µM), of 10 nM IR-284, or both combined……………………………………47

Figure 5.6. Drug-induced apoptosis in CLL-cells from two different patients as measured by flow cytometry in response to 1 µM GGTI-298, 10 nM IR-284 or both……………………………………………………………………48

Figure 5.7. Drug-induced apoptosis in CLL-cells as measured by flow cytometry in response to 50 µM 8Me, 10 µM GGTI-298 or both………………………………49

Figure 5.8. Correlation of Epac-1 mRNA fold change and basal apoptosis in CLL……50

Figure 5.9. Correlation of Epac-1 mRNA fold change and drug-induced (100 nM IR-284) apoptosis in CLL-cells…………………………………………………51

Figure 5.10. Correlation of R1β mRNA fold change and Epac-1 mRNA fold change………………………………………………………………………………52

Figure 5.11. Correlation of PDE7B mRNA fold change and Epac-1 mRNA fold change………………………………………………………………………………53

Figure 6.1. Schematic for the typical cAMP pathway, with the pro- and anti-apoptotic effects of Epac and PKA in CLL highlighted…………………………61
Figure S1. NCBI sequence of the first 1089 bp upstream of the Epac-1 exon 1 translation start site……………………………………………………………………………………………………62

Figure S2. NCBI sequence of the first 1191 bp upstream of the Epac-2 exon 1 translation start site………………………………………………………………………………………………63
LIST OF TABLES

Table 2.1. PCR protocol for Epac-1/2 promoter region PCR……………………………..13

Table 2.2. PCR primers for Epac-1 and Epac-2 1000-bp promoter……………………..13

Table 2.3. Real-time PCR protocol……………………………………………………………15

Table 2.4. RT-PCR primers for each gene studied…………………………………15

Table 3.1. Demographic data for normal and CLL subjects examined for Epac-1 promoter region SNPs………………………………………………………..20

Table 3.2. Epac-1 SNP distribution by frequency in normal PBMC, indolent-CLL, aggressive-CLL, and total CLL ……………………………… …………….……..20

Table 3.3. Epac-1 SNP distribution in normal PBMC and CLL, sorted by hetero- and homozygosity…………………………………………………………….21

Table 3.4. Demographic data for the normal and CLL subjects examined for Epac-2 promoter region SNPs………………………………………………26

Table 3.5. Epac-2 SNP distribution by frequency in normal, indolent-CLL, aggressive-CLL, and total CLL…………………………………………….27

Table 3.6. Epac-2 SNP distribution in normal and CLL, sorted by hetero- and homozygosity…………………………………………………………….27
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ABSTRACT OF THE THESIS

Exchange protein activated by cAMP: Drug target and biomarker in chronic lymphocytic leukemia

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Chronic lymphocytic leukemia (CLL), the most common adult leukemia in the Western world, is associated with the accumulation of B-lymphocytes due to decreased apoptosis. The second messenger 3’5’-cyclic adenosine monophosphate (cAMP) promotes apoptosis by unknown mechanisms in peripheral blood mononuclear cells (PBMC) isolated from patients with CLL. The actions of cAMP are mediated by protein kinase A (PKA) and Exchange protein activated by cAMP-1 (Epac-1). Using real-time PCR we find that compared to PBMC from healthy subjects, CLL-cells have elevated Epac-1 and decreased PKA regulatory subunit RIIβ mRNA expression. Studies using traditional PCR reveal that this increase in Epac-1 mRNA expression does not result from single nucleotide polymorphisms (SNPs) in the promoter region of Epac-1. Use of
fluorescent-activated cell sorting (FACS) (to assess annexin 5 staining, a marker for apoptosis) revealed that 48 hr treatment with an Epac-selective analog (8-pCPT-2Me-cAMP [8Me], 50 µM) inhibited apoptosis of CLL-cells but that a PKA-selective analog (N⁶- Phenyladenosine-cAMP [N6], 50 µM) induced apoptosis. Inhibition of Rap-1, the downstream mediator of Epac-1, with N-4-[2(R)-Amino-3-mercaptopropyl]amino-2-naphthylbenzoyl-(L)-leucine methyl ester [GGTI-298] (100 nM - 10 µM, 48h) increased apoptosis of CLL-cells and prevented the anti-apoptotic effect of Epac activation. These results reveal that CLL is associated with increased Epac-1 expression and that unlike PKA activation, Epac-1 activation (in a Rap-1 dependent manner) enhances the survival of the malignant B-cells. Approaches that decrease the expression and function of Epac-1 and increase the expression and function of PKA thus may be beneficial in treating CLL by increasing the pro-apoptotic effects of cAMP.
1. Introduction

1.1. Chronic Lymphocytic Leukemia

B-cell chronic lymphocytic leukemia (CLL) is the most common adult leukemia in the Western world.\(^1\) In the United States CLL has an incidence of 3.5 per 100,000 per year and primarily affects men over the age of 50: CLL occurs in men twice as often as in women.\(^2,3\) CLL is characterized by the arrest of malignant B-cells in the G\(_0\) phase of the cell cycle that then fail to undergo apoptosis.\(^2\) The median survival for CLL is roughly 10 years, but its progression is highly variable such that patients can survive from months to years after diagnosis.\(^2\) Rai et al. and Binet et al. developed clinical staging systems for CLL that classify patients into early (Rai 0, Binet A), intermediate (Rai I/II, Binet B) or advanced stages (Rai III/IV, Binet C), for which survival is 10, 5-7, and 1-3 years, respectively.\(^3,4,5\) Since treatment is not effective at early stages of CLL, which occurs in 70-80% of patients, predictive biological markers are needed to help determine when treatment is required.\(^2,3\)

Although a number of prognostic markers for CLL have been identified, each alone cannot consistently and accurately determine disease progression. High levels of serological markers, such thymidine kinase levels, \(\beta\) 2-microglobulin, and soluble CD23 are used to identify the stages of CLL.\(^6,7\) Unfortunately, the cut-off levels for these markers are highly variable in different laboratories performing the test, thus limiting their clinical utility.\(^6\) Irregular chromosomal structures or cytogenetic abnormalities in genomic DNA have also been correlated with risk for disease progression: those with del(13q) have a low risk for disease progression, whereas patients with del(17p),
del(11q), or trisomy 12 are at a high risk.\textsuperscript{6,8} Mutations of the variable region of the immunoglobulin heavy chains (IgVH) determine CLL progression: patients with leukemic IgVH genes that are unmutated (i.e., > 98% similar to a similar germ-line gene) are more likely than patients with mutated IgVH genes to have the advanced form of CLL.\textsuperscript{6,9} The presence of a 70-kD zeta-associated protein (ZAP-70) in CLL-cells is predictive of unmutated IgVH and also independently predictive of CLL progression.\textsuperscript{6,10} While each marker correlates to some degree with the progression of CLL, they are all indirect and do not provide insight into the mechanism of the disease. Further research is required that will define markers that more closely mirror disease progression, such as those that are directly involved with the reduced apoptosis of the malignant B-cells that characterize CLL.

1.2. Apoptosis in CLL

Apoptosis can be initiated by either the intrinsic or extrinsic pathways.\textsuperscript{11} A variety of factors, including hypoxia, cytotoxic drugs and radiation initiate the intrinsic death pathway by disrupting the mitochondrial membrane.\textsuperscript{12,13} This membrane disruption results in release of cytochrome c from the mitochondrial intermembrane space (IMS) and produces an inhibition of mitochondrial function. Cytochrome c combines with cytosolic Apaf-1 and pro-caspase 9 within a multiprotein complex called the apoptosome to activate caspase 9. Caspase 9 then activates the “effector caspase,” caspase 3. SMAC (second mitochondrial-derived activator of caspases) is also released from the mitochondrial IMS and inhibits the caspase 3-suppressing inhibitor of apoptosis proteins (IAPs). Capase 3 triggers a cascade of caspase activation and this cascade leads to cell
death.\textsuperscript{14,15} This process includes the deactivation of enzymes that inhibit apoptosis, destruction of cell structures, and activation of certain enzymes via regulatory subunit cleavage.\textsuperscript{16}. The extrinsic and intrinsic death pathways differ in that the former is activated by a membrane-bound death receptor (e.g., Fas), and does not trigger the disruption of the mitochondrial membrane. Cell-surface death receptors initiate apoptosis by activating caspase 8 within the death-inducing signaling complex (DISC), which in turn activates the caspase cascade and produces cell death.\textsuperscript{11,12,13}

In CLL, the apoptosis of B-cells is blunted, perhaps as a result of the overexpression of anti-apoptotic proteins, such as Bcl-2, and inhibition of pro-apoptotic proteins like p53 and ATM.\textsuperscript{13,17}

1.3. cAMP: An important intracellular second messenger

3',5' cyclic adenosine monophosphate (cAMP) is a second messenger that is activated by ligand (hormone or neurotransmitter) binding to cell surface 7-transmembrane receptors, G-protein-coupled receptors (GPCRs); increasing cAMP induces apoptosis in a number of malignant-cells.\textsuperscript{18,19} Activation of GPCRs promotes a conformational change that leads to coupling to intracellular heterotrimeric G-proteins, which are comprised of a guanosine diphosphate (GDP)-bound G\textsubscript{\alpha}-subunit and a G\textsubscript{\beta\gamma} complex. Receptor activation triggers the exchange of guanosine triphosphate (GTP) for GDP on the G\textsubscript{\alpha}-subunit, thus facilitating dissociation from G\textsubscript{\beta\gamma} and from the receptor as well. G\textsubscript{\alpha} is a G-protein that activates membrane-bound adenylyl cyclase (AC), which catalyzes the formation of cAMP. cAMP acts via a number of downstream mediators such as protein kinase A (the most well characterized), Exchange protein directly
activated by cAMP (Epac) and membrane-bound cyclic-nucleotide gated channels (CNGs, see Fig. 1.1). A balance between formation by AC and hydrolysis by cyclic nucleotide phosphodiesterases (PDEs) controls the intracellular level of cAMP and the duration of its signaling. Recent data indicate that the intracellular levels of cAMP can also be controlled by the presence of multidrug resistance proteins 4 and 5 (MRP4 and 5), which facilitate the efflux and lowering cellular levels of cAMP.

Recent research has revealed that the regulation of cAMP formation and hydrolysis is much more complex than previously thought. For example, 10 families of AC and 11 families of PDE (PDE4, PDE7 and PDE8 are selective for the hydrolysis of cAMP) exist, each differing in regulatory characteristics, and cellular and subcellular distribution to tightly control the intracellular levels of cyclic nucleotides. Furthermore, early measurements identified whole-cell cAMP concentrations in most tissues at \( \sim 10^{-7} \) M. With a PKA binding affinity for cAMP measured \textit{in vitro} to be around \( 10^{7}-10^{8} \) M, and binding affinities measured \textit{in vitro} for Epac and CNGs to be \( \sim 10^{-6} \) M, such a level of cAMP would imply that PKA would be constantly active, while Epac and CNGs would never be activated. This however is not the case as cAMP is not distributed evenly throughout the cytosol but is concentrated with cAMP-signal transduction pathways proteins in specific cellular compartments. PKA, Epac, PDEs, ACs and other proteins involved in the cAMP-pathway are coupled together in subcellular compartments by A-kinase anchoring proteins (AKAPs, see Figure 1.1). AKAPs allow for effective transduction of cAMP signaling through its downstream effectors.
Figure 1.1. Schematic of the 3’5’-cyclic adenosine monophosphate (cAMP) signaling pathway showing the $G_\alpha$ subunit of a G-protein coupled receptor (GPCR) activating adenylyl cyclase (AC), which in turn converts ATP into cAMP. This cAMP subsequently activates activating Exchange protein activated by cAMP (Epac), Protein kinase A (PKA), and cyclic nucleotide gated channels (CNGs) cAMP effluxes from the cell via multidrug resistance proteins 4 and 5 (MRP4 and 5), and is broken down into 5’AMP by phosphodiesterases (PDEs). Several proteins in the schematic are also anchored to membranes (not shown) by A-kinase anchoring proteins (AKAPs).

1.4. cAMP and CLL

Levels of cAMP and activity of PKA are lower in CLL-cells than in PBMC from normal subjects, suggesting a disease-related defect in this pathway.\textsuperscript{27,28} CLL-cells have increased mRNA and protein expression of PDE7B (cAMP-specific PDE) and total PDE7 activity, which, in part, likely contribute to the low intracellular concentration of cAMP.\textsuperscript{29} Although PDE7B is the only PDE significantly upregulated in CLL-cells compared to normal cells, PDE4B is the most highly expressed PDE in both CLL and normal cells, identifying PDE7B and PDE4B as possible therapeutic targets for the disease.\textsuperscript{30} PDE7, PDE4 and dual PDE4/7 inhibitors kill CLL-cells but do not kill normal
B-cells. Together these data suggest that PDE inhibition could be a novel means to treat CLL by increasing cAMP and selectively killing malignant B-cells.

Of further interest, MRP1 is expressed at higher levels in some cancers compared to normal cells. If high expression levels of MRP4 and 5 occur in CLL, these may, in addition to increased PDE7B activity, contribute to the lower intracellular cAMP observed in CLL-cells.

1.5. Downstream mediators of cAMP: PKA and Epac

As outlined above, cAMP can activate a number of downstream effectors. In B-cells, cAMP activates both PKA and Epac. A key downstream mediator of PKA is the cAMP response element binding (CREB) protein, although PKA can also regulate extracellular signal-regulated kinases (ERKs) and nuclear factor of activated T-cells (NFAT). PKA is a heterotetramer consisting of two regulatory (R) and two catalytic (C) subunits. Activation of PKA involves binding of cAMP to the R subunits’ cAMP binding domain and dissociation of the R and C subunits. There are several isoforms of the regulatory (RIα, RIβ, RIIα, RIIβ) and catalytic (Cα, Cβ, Cγ) subunits, but variation only in the R subunits significantly effects the function of PKA. RI and RII isoforms are differentially expressed in different tissues and subcellular compartments via specific AKAPs. The variable and differential expression of RI and RII allows PKA to be ubiquitously expressed but selectively regulated. A difference in the RIα/RIIβ expression ratio has been observed between melanomas and melanocytes. The functional significance of this altered ratio is unknown but it may serve as a diagnostic marker.
Similar to PKA, Epac is also ubiquitously expressed. The main downstream mediator of Epac is the small GTPase Rap, which in turn regulates numerous downstream effectors. The two forms of Epac, Epac-1 and Epac-2, facilitate guanine exchange of Rap-1 and Rap-2, respectively. Both Epacs are multi-domain proteins that have an N-terminal auto-inhibitory regulatory region and a C-terminal catalytic region (Figure 1.2).

**Figure 1.2.** Schematic diagram of Epac-1 and Epac-2 protein domain homologies.

It is thought that the Epac protein “folds over” upon itself such that the regulatory region covers the catalytic region, unless cAMP binds to the regulatory region’s cyclic nucleotide binding domain (CBD). Upon cAMP binding, the “ionic latch” connecting the regulatory region and the catalytic region is disrupted, such that the catalytic CDC25-homology domain (CDC25HD) is open for RapGDP to bind and be converted to RapGTP (Figure 1.3). Both variants share a similar sequence homology, with the exception of an extra CBD of unknown function in Epac-2.
Figure 1.3. Protein domain diagram of Epac-2 before and after cAMP binding and Rap catalysis.

Epac-1 is widely expressed in many tissues but Epac-2 is selectively expressed at high levels in the brain and adrenal glands. Epac-1 has been identified as playing a role in numerous cellular processes, including gene transcription, cell adhesion, cell-cell junction formation, exocytosis, and cardiomyocyte gap junction formation, whereas Epac-2 has been associated with glucose-dependent insulin release.13,37

1.6. PKA and Epac in apoptosis of CLL-cells

The role of the two major cAMP downstream-mediators, PKA and Epac, in apoptosis of CLL-cells has not been fully elucidated. In a number of malignant cells, cAMP can induce apoptosis via a PKA-dependent pathway, for example in S49 cells (mouse T-cell lymphoma cells).18,19 In S49 cells cAMP/PKA upregulates the expression of pro-apoptotic proteins such as the BH3 family member Bim, and downregulates the production of anti-apoptotic proteins such as survivin. The resultant shift towards the production of pro-apoptotic proteins and action leads to the depolarization of the mitochondrial membrane and the release of the mitochondrial proteins cytochrome c and SMAC.19,14 In CLL, a similar cAMP-dependent mechanism for killing has been observed.27,30,32 Inhibition of PDE4, PDE7, and PDE4/7 kills CLL-cells via a
cAMP/PKA-dependent pathway that leads to mitochondrial depolarization, release of cytochrome c and a decrease in expression of the anti-apoptotic protein survivin.\textsuperscript{27,30}

Whereas PKA is pro-apoptotic, Epac activation has been shown to be anti-apoptotic in CLL.\textsuperscript{32} A recent study in a small number of patients, found that levels of the Epac-1 mRNA transcript are higher in CLL-cells than in normal PBMC.\textsuperscript{32} The addition of an Epac activator blunted basal apoptosis in these CLL-cells, suggesting that Epac-1 is anti-apoptotic: the mechanism by which Epac inhibits apoptosis is still unclear.\textsuperscript{32} Enhancing the PKA pathway and blunting the Epac pathway in CLL would be predicted to increase the apoptotic effect of drugs that raise cellular cAMP levels.

1.7. Hypothesis and Goals

1.7.1. Hypothesis One

As noted above, previous research in a small number of patients has identified higher levels of the Epac-1 mRNA transcript in CLL-cells compared to normal PBMC.\textsuperscript{11} Since changes in the DNA sequence of a gene’s promoter region could affect mRNA transcription, I sought to test the hypothesis that single nucleotide polymorphisms (SNPs) exist in the promoter of Epac-1 that might explain the increased expression of Epac-1 mRNA observed in patients with CLL.
1.7.2. Hypothesis Two

A second hypothesis is that increased Epac expression, which we sought to confirm in a larger population of CLL patients than was tested previously, would correlate with disease state and be of functional significance. Confirmation of higher expression levels of Epac in CLL could identify it as a potential target for the treatment of the disease, such that decreasing the expression of Epac may enhance the therapeutic (pro-apoptotic) potential of agents that act by increasing intracellular cAMP.
2. Materials and Methods

2.1 PBMC isolation

Blood was collected from healthy donors and CLL patients (to isolate peripheral blood mononuclear cells [PBMC and CLL-CELLS or CLL-cells, respectively] following informed consent, in agreement with institutional guidelines. All use of patient data and samples followed or exceeded the guidelines of the Health Insurance Portability and Accountability Act. The diagnosis of CLL was made by assessment of blood cell morphology and immunophenotyping. The patients’ median age was 61 yr (range, 44-75 yr), with a median WBC of 142 x 10^6 cells/ml (range, 15 to 460 x 10^6 cells/ml). PBMC were isolated by density-gradient centrifugation using Ficoll-Paque (Amersham Biosciences), washed, suspended in fetal-calf serum containing 10% DMSO then stored in liquid N₂ for subsequent use.

2.2. SNP discovery

2.2.1. Cellular genomic DNA extraction

Each 1 ml cell sample (both CLL and normal) was thawed and diluted in 2 ml of RPMI 1640 media (with added L-glutamine and phenol red, Sigma-Aldrich, St, Louis, MO). A 10 µl portion of cell/media solution was mixed with 90 µl 0.5 x 0.4% Trypan blue (Invitrogen, Carlsbad, CA), and 10 µl of this solution was pipetted onto a Bright-Line Hemacytometer (Reichert, Depew, NY) for cell counting. The original cells were resuspended in RPMI 1640 and pelleted at 1200 rpm for 5 min at 25 °C. Media was
aspirated and the cells resuspended to 5 million cells/ml; 1 ml was pelleted for storage of cells at –80 °C. Genomic DNA from pelleted cells was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

2.2.2. Epac-1 and Epac-2 polymerase chain reaction (PCR) primer design

Primers for human Epac-1 were designed for the 1000-base pair (bp) promoter region upstream of Exon 1 using the NCBI Entrez search engine (http://www.ncbi.nlm.nih.gov/sites/entrez, Bethesda, MD). The sequence was then pasted into the Primer3 online primer-designing program (http://frodo.wi.mit.edu/, MIT, Cambridge, MA) using standard settings. Several primer pairs were chosen from the program’s suggestions: Epac-2 primers were designed in the same manner. All primers were obtained from Integrated DNA Technologies (San Diego, CA) and diluted with diethyl pyrocarbonate- (DEPC) treated water to a stock concentration of 200 µM.

2.2.3. PCR

Each PCR reaction was comprised of 8 ng of patient DNA with 10 µM primer pairs and ZymoTaq PCR Premix (Zymo Research, Orange, CA) according to the manufacturer’s instructions. PCR was performed using a RoboCycler Gradient 96 thermocycler (Stratagene, La Jolla, CA) and the PCR protocol and primers shown in Table 2.1 and Table 2.2.
After completion of the PCR reaction, remaining primers or dNTPs were removed by adding ExoSAP-IT (USB Corporation, Cleveland, OH) according to the manufacturer’s instructions.

**Table 2.1.** PCR protocol for Epac-1/2 promoter region PCR.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>15 minutes</td>
</tr>
<tr>
<td>2</td>
<td>94</td>
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<tr>
<td>3</td>
<td>59</td>
<td>30 seconds</td>
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<tr>
<td>4</td>
<td>72</td>
<td>1 minute, 15 seconds</td>
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<tr>
<td>5</td>
<td>72</td>
<td>10 minutes</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>Hold until sample removal.</td>
</tr>
</tbody>
</table>

**Go to Step 2 34 more times.**

**Table 2.2.** PCR primers for Epac-1 and Epac-2 1000-bp promoter.

<table>
<thead>
<tr>
<th></th>
<th>Forward primer (5’→3’)</th>
<th>Reverse primer (5’→3’)</th>
<th>T&lt;sub&gt;m&lt;/sub&gt; (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epac-1</td>
<td>TGAGCCTCGGTAAGC</td>
<td>CCGTGCAGGCTCTAGCA</td>
<td>59</td>
</tr>
<tr>
<td>Epac-2</td>
<td>AAGCAATGCTATGTCCCTTCC</td>
<td>GCAGCGACCATGTTGAGC</td>
<td>61</td>
</tr>
</tbody>
</table>

**2.2.4. PCR Product Sequencing & Sequence Alignment**

PCR products were sequenced by Genewiz (La Jolla, CA) and the sequences analyzed using the computer program FinchTV (http://www.geospiza.com/Products/finchtv.shtml, Geospiza, Seattle, WA). To identify SNPs in Epac-1 and Epac-2 promoters regions of the patient samples, gene sequences were compared to NCBI’s standard sequence via the computer program CLUSTALW (Kyoto University, Kyoto, Japan) hosted by the San Diego Supercomputer Center’s Biology Workbench (http://workbench.sdsc.edu/, San Diego, CA).
2.3. Real Time PCR

RNA was extracted from 5 million CLL-cells and normal PBMC using an extraction kit RNeasy (Qiagen) according to the manufacturer’s instructions. cDNA was synthesized using Superscript III Reverse Transcriptase kit (Invitrogen), as per the manufacturer’s instructions. Real-time PCR was performed using 8 ng cDNA, 0.5 µM forward and reverse primers, and qPCR Mastermix Plus for Sybr Green I (Eurogentec, San Diego, CA) and the Opticon 2 RT-PCR machine (MJ Research, Waltham, MA). The RT-PCR program and RT-PCR primers used are shown in Table 2.3 and Table 2.4, respectively. Primer efficiency was calculated for each primer set before use and samples were compared using the relative cycle threshold (Ct) method, normalizing to 28S rRNA.
Table 2.3. Real-time PCR protocol.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>2 minutes</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>10 minutes</td>
</tr>
<tr>
<td>3</td>
<td>95</td>
<td>15 seconds</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>30 seconds</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>1 minute</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>Plate read.</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>Go to Step 3 34 more times.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Construct melting curves for samples by heating the plate from 60°C to 95°C. Read plate every 0.2°C, holding the temperature for 1 second.</td>
</tr>
</tbody>
</table>

Table 2.4. RT-PCR primers for each gene studied. T_m=60°C for each primer.

<table>
<thead>
<tr>
<th></th>
<th>Forward primer (5’→ 3’)</th>
<th>Reverse primer (5’→ 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRP4</td>
<td>TGGTGCAGAAGGGGACTTAC</td>
<td>GCTCTCCAGAGCACCACATCTT</td>
</tr>
<tr>
<td>MRP5</td>
<td>AGTGCTCTGAAGCCCATCC</td>
<td>CCAGAGAAGAAAGCCACGAA</td>
</tr>
<tr>
<td>R1α</td>
<td>GGGAAGCACAAGACTGAGAAAGC</td>
<td>ACAGCAGCTGACCCCTCTAA</td>
</tr>
<tr>
<td>R1β</td>
<td>GTCAAAGATGGGGAGCATGT</td>
<td>AGCACCAGGAGGATAGCAG</td>
</tr>
<tr>
<td>Epac-1</td>
<td>ACTTTATCCCCAACTTGATG</td>
<td>TCCTCCAGAAATCTCTCAGAC</td>
</tr>
<tr>
<td>PDE3B</td>
<td>TGATGCTGTGGTTTGCTAC</td>
<td>AAAAAATATTCGCGTCTGCG</td>
</tr>
<tr>
<td>PDE7B</td>
<td>TATACCAAGCCCGCTCTGC</td>
<td>TTCCATTTGTCAAACGATCA</td>
</tr>
<tr>
<td>28S</td>
<td>GCCTAGCAGCCGACTTAGAA</td>
<td>AAATCACATCGCGTCAACAC</td>
</tr>
</tbody>
</table>
2.4. Flow Cytometry

1 million CLL-cells or normal PBMC/ml RPMI 1640 were plated in a 12-well cell culture plate (Greiner Bio-One, Monroe, NC) and incubated for 48 hr at 37 °C in air plus 5% CO₂ with vehicle, the Epac-specific activator [8-(4-Chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate, 8Me, 50 µM, Biolog, Bremen, Germany], a PKARII-activator [N⁶-Phenyladenosine- 3′, 5′- cyclic monophosphate, N⁶, 50 µM, Biolog, Bremen, Germany], a PKARI-activator [8-Chloroadenosine-3′,5′-cyclic monophosphate, 8Cl, 50 µM, Biolog, Bremen, Germany], a dual PDE4/7 inhibitor [IR-284, (4-(3-chloro-4-methoxyphenyl)-2-(1-morpholine-4-carbonyl) piperadin-4-yl)-4a, 5,8,8a-tetrahydrophthalazin-1(2H)-one, CSD Cancer Center Medicinal Chemistry Core], or a the Rap-1 inhibitor [GGTI-298, N-[[4-(2-(R)-Amino-3-mercaptopropyl)amino]-2-naphthylbenzoyl]leucine methyl ester trifluoroacetate salt, Sigma Aldrich, St. Louis, MO].

After 48 hr incubation the cells were pelleted by centrifugation at 1200rpm for 5 min at 4°C, washed in 1 ml of cold PBS with 1% FBS, washed in 1ml Hank’s Balanced Salt Solution (HBSS) (Invitrogen), resuspended in 100 µl of the HBSS, and treated with 1 µl of annexin V/fluorescein isothiocyanate (FITC) (Becton Dickinson Biosciences, San Jose, CA). After 15 min incubation at 37 °C, the cells were treated with another 200 µl of HBSS and 10 µl of 30 µg/ml propidium iodide. Each 300 µl cell sample was then analyzed by flow cytometry using FACScan (Becton Dickinson Biosciences, San Jose, CA).
2.5. Statistical analyses

Values are expressed as mean ± SEM. Statistical significance in a comparison between two groups was based on an unpaired Student t test or paired t test for comparing combined treatments. Major and minor SNP allele frequencies were compared between diseased and normal using Fisher’s exact test. Groups were correlated using Spearman’s test. A value of $P < 0.05$ was considered statistically significant.
3. Results: Single nucleotide polymorphisms

Transcription of DNA to mRNA is normally regulated by a variety of influences that include transcription factors, RNA interference, and enhancers. Transcription factors often regulate transcription by binding to the noncoding promoter region upstream of a gene’s coding region. Therefore, changes to the DNA sequence of a gene’s promoter region could affect mRNA transcription. Variations between the genomes of individuals are common and are termed polymorphisms if they occur in at least 1% of a population. The most common type of polymorphism is a mutation of one base to another, termed a single nucleotide polymorphism (SNP). SNPs in coding regions can either be silent or can alter the encoded protein by changing one of its component peptides or by stopping translation. SNPs in noncoding regions, such as the promoter, can also produce effects on the production and function of a protein, but the mechanism by which they produce such effects is less clear.

3.1. SNPs in the Epac-1 promoter region

To identify SNPs in the promoter region of Epac-1 we initially amplified 1000 bp of the promoter (see primers in Table 2.4) from normal and CLL patients, sequenced those fragments and performed multiple sequence alignments. We identified 7 SNPs, including 3 that are novel, in the 1000-bp region immediately upstream of the Epac-1 exon 1 translation start site (Fig. 3.1). Previously known SNPs in this region (in the National Center for Biotechnology Information’s (NCBI) SNP database (dbSNP)) are marked in blue and with dbSNP’s rs# identifier in Figure 3.1. The position of each SNP (relative to the translation start site), their positive strand nucleotide changes, and their
frequencies (%) were as follows: -249 T>C (3.2%), -265 C>G (11.4%), -655 T>C (44.3%), -705 C>T (10.8%), -741 A>C (4.4%), -792 G>A (11.4%), and -938 G>A (27.8%, Figure 3.1). All SNPs except -265 C>G were found at frequencies that differ significantly from Hardy-Weinberg equilibrium (Fisher’s exact test, P>0.001). The entire positive strand genomic DNA sequence examined is depicted in Figure S1.

The demographic data for the CLL and normal subjects analyzed for Epac-1 promoter region SNPs are shown in Table 3.1. All examined normal subjects and CLL patients are Caucasian. The sample sizes for normal subjects and CLL patients and for CLL disease states are similar, but sample sizes between the sexes are not equal since CLL is more common in men than woman.

The SNPs -265 C>G, -655 T>C, -705 C>T, -792 G>A, and -938 G>A occurred at frequencies of at least 10% (Figure 3.2) in both normal and CLL patients. The -655 T>C and -938 G>A SNPs were found most frequently in both populations.

The frequency of each of the 7 SNPs in normal subjects (n=35), indolent-CLL (n=24), aggressive-CLL (n=20), and total CLL (n=44) are shown in Figure 3.3 and Table 3.2. The -938 G>A SNP was significantly more frequent (P<0.05) in cells from patients with aggressive-CLL than in normal subjects. Based on analysis with Fisher’s exact test, all other SNPs occurred at similar frequencies in normal subjects and CLL patients, and in patients with different CLL disease states. Therefore, the -938 G>A SNP may be potentially useful as a marker for aggressive-CLL.

We found no strong evidence for linkage disequilibrium (LD) of six of the seven discovered Epac-1 SNPs (Figure 3.4, black denotes strong evidence for LD, white denotes strong evidence for recombination, and gray shows that evidence is
uninformative) as determined by the Haploview software (Broad Institute, Cambridge, MA).

Table 3.3 summarizes the heterozygosity and homozygosity of Epac-1 promoter-region SNPs found in normal subjects (n=35), patients with indolent-CLL (n=24), aggressive-CLL (n=20), and in the total patient population with CLL (n=44).

Table 3.1. Demographic data for normal subjects and CLL patients examined for Epac-1 promoter region SNPs.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Total (number/%total)</th>
<th>Indolent CLL</th>
<th>Aggressive CLL</th>
<th>Total CLL</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Both (total)</td>
<td>79 (100%)</td>
<td>24 (30%)</td>
<td>20 (25%)</td>
<td>44 (56%)</td>
<td>35 (44%)</td>
</tr>
<tr>
<td>Male</td>
<td>58 (73%)</td>
<td>16 (20%)</td>
<td>15 (19%)</td>
<td>31 (39%)</td>
<td>27 (34%)</td>
</tr>
<tr>
<td>Female</td>
<td>21 (27%)</td>
<td>8 (10%)</td>
<td>5 (6%)</td>
<td>13 (17%)</td>
<td>8 (10%)</td>
</tr>
</tbody>
</table>

Table 3.2. Epac-1 SNP distribution by frequency in normal subjects (n=35), indolent-CLL (n=24), aggressive-CLL (n=20), and total CLL (n=44).

<table>
<thead>
<tr>
<th>SNP</th>
<th>Normal</th>
<th>Indolent CLL</th>
<th>Aggressive CLL</th>
<th>Total CLL</th>
</tr>
</thead>
<tbody>
<tr>
<td>-249 T&gt;C</td>
<td>2.9</td>
<td>4.2</td>
<td>2.5</td>
<td>3.4</td>
</tr>
<tr>
<td>-265 C&gt;G</td>
<td>15.7</td>
<td>6.3</td>
<td>10.0</td>
<td>8.0</td>
</tr>
<tr>
<td>-655 T&gt;C</td>
<td>35.7</td>
<td>52.1</td>
<td>50.0</td>
<td>51.1</td>
</tr>
<tr>
<td>-705 A&gt;C</td>
<td>7.1</td>
<td>14.6</td>
<td>12.5</td>
<td>13.6</td>
</tr>
<tr>
<td>-741 A&gt;C</td>
<td>2.9</td>
<td>4.2</td>
<td>7.5</td>
<td>5.7</td>
</tr>
<tr>
<td>-792 G&gt;A</td>
<td>11.4</td>
<td>12.5</td>
<td>10.0</td>
<td>11.4</td>
</tr>
<tr>
<td>-938 G&gt;A</td>
<td>21.4</td>
<td>29.2</td>
<td>37.5</td>
<td>33.0</td>
</tr>
</tbody>
</table>
Table 3.3. Epac-1 SNP distribution in normal (n=35) and CLL (n=44), sorted by heterozygosity (Hetero) and homozygosity (Homo).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-249 T&gt;C</td>
<td>3.2</td>
<td>0.0</td>
<td>3.4</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>-265 C&gt;G</td>
<td>3.8</td>
<td>7.6</td>
<td>3.4</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>-655 T&gt;C</td>
<td>10.0</td>
<td>25.7</td>
<td>30.7</td>
<td>20.5</td>
</tr>
<tr>
<td></td>
<td>-705 A&gt;C</td>
<td>8.6</td>
<td>2.9</td>
<td>6.8</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>-741 A&gt;C</td>
<td>2.9</td>
<td>0.0</td>
<td>5.7</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>-792 G&gt;A</td>
<td>11.4</td>
<td>0.0</td>
<td>5.7</td>
<td>5.7</td>
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<tr>
<td></td>
<td>-938 G&gt;A</td>
<td>19.3</td>
<td>13.6</td>
<td>14.3</td>
<td>8.6</td>
</tr>
</tbody>
</table>

Figure 3.1. Novel (black) and previously known (blue) SNPs in the 938-bp area immediately upstream of the Epac-1 exon 1 translation start site. Known SNPs are also marked with the NCBI SNP database (dbSNP) rs identifiers.
Figure 3.2. Epac-1 SNP frequency in normal and CLL (n=79).
Figure 3.3. Epac-1 SNP distribution by frequency in normal (n=35), indolent-CLL (n=24), aggressive-CLL (n=20), and total CLL (n=44). Significance as measured by Fisher’s exact test is depicted by a single asterisk for P<0.05.
Figure 3.4. Diagram of possible linkage disequilibrium (LD) between Epac-1 SNPs. Intersecting squares between SNPs are color-coded for strong evidence for linkage disequilibrium (black), uninformative evidence (light gray), or strong evidence for recombination (white) as predicted by the Haploview software.
3.2. SNPs in the Epac-2 promoter region

To identify SNPs in the promoter of Epac-2 we amplified 1000 bp of the promoter (see primers in Table 2.4) from normal and CLL patients, sequenced those fragments, and performed multiple sequence alignments. We identified 8 SNPs in the 1000-bp promoter region immediately upstream of the Epac-2 exon 1 translation start site (Figure 3.5). The positions of the SNPs (relative to the translation start site), their positive strand nucleotide changes and frequencies (Figure 3.6) were as follows: -109 C>A (4.6%), -242/-243 GCGG (4-bp insert, 12.3%), -222 C>A (0.8%), -357 C>A (10.8%), -418 C>T (5.4%), -419 C>T (5.4%), -776 G>T (13.8%), and -892 A>G (10.8%). All SNPs were found at frequencies that differ significantly from Hardy-Weinberg equilibrium (Fisher’s exact test, P>0.001). Figure 3.5 shows that 6 of the 8 discovered SNPs are novel. SNPs already in dbSNP are marked in blue and noted by their rs# identifiers. The entire positive strand genomic DNA sequence is depicted in Figure S2.

Table 3.4 shows the demographic information for the normal subjects and CLL patients examined for Epac-2 promoter region SNPs. All examined normal subjects and CLL patients are Caucasian. More patients with CLL and more males were examined compared to the number of non-CLL subjects and females.

Figure 3.7 shows the absolute frequency of each of the 8 SNPs in normal PBMC (n=8), indolent-CLL (n=31), aggressive-CLL (n=26), and total CLL (n=57); the frequency of each SNP is listed in Table 3.5. Of the 8 SNPs identified, 4 (-242/243 GCGG, -357 A>C, -776 G>T, -892 A>G) had a frequency of >10% in both normal subjects and patients with CLL (n=65, Figure 3.7). As shown in Figure 3.7, the -892 A>G SNP was significantly more frequent (P<0.05) in normal subjects than in patients with
aggressive-CLL. All other SNPs were of similar frequencies in normal subjects and CLL patients and in patients with different CLL disease states, as determined by Fisher’s exact test.

There is strong evidence that -418 C>T and -419 C>T are in LD with each other (Figure 3.8, black denotes strong evidence for LD, white denotes strong evidence for recombination, and gray shows no strong evidence for either) as determined by the software. No strong evidence was found for LD among the other 5 Epac-2 SNPs.

Table 3.6 summarizes the heterozygosity and homozygosity of Epac-2 promoter-region SNPs found in normal subjects (n=8) and patients with indolent-CLL (n=31), aggressive-CLL (n=26), and in the total CLL patient population (n=57).

**Table 3.4.** Demographic data for the normal subjects and CLL patients examined for Epac-2 promoter region SNPs.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Total (number/%total)</th>
<th>Indolent CLL</th>
<th>Aggressive CLL</th>
<th>Total CLL</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Both (total)</td>
<td>65 (100%)</td>
<td>31 (48%)</td>
<td>26 (40%)</td>
<td>57 (88%)</td>
<td>8 (12%)</td>
</tr>
<tr>
<td>Male</td>
<td>45 (69%)</td>
<td>23 (35%)</td>
<td>18 (28%)</td>
<td>41 (63%)</td>
<td>4 (6%)</td>
</tr>
<tr>
<td>Female</td>
<td>20 (31%)</td>
<td>8 (12%)</td>
<td>8 (12%)</td>
<td>16 (25%)</td>
<td>4 (6%)</td>
</tr>
</tbody>
</table>
Table 3.5. Epac-2 SNP distribution by frequency in normal (n=22), indolent-CLL (n=62), aggressive-CLL (n=52), and total CLL (n=114).

<table>
<thead>
<tr>
<th>SNP</th>
<th>Normal</th>
<th>Indolent CLL</th>
<th>Aggressive CLL</th>
<th>Total CLL</th>
</tr>
</thead>
<tbody>
<tr>
<td>-108 C&gt;A</td>
<td>0.0</td>
<td>9.8</td>
<td>0.0</td>
<td>5.3</td>
</tr>
<tr>
<td>-222 C&gt;A</td>
<td>0.0</td>
<td>1.6</td>
<td>0.0</td>
<td>0.9</td>
</tr>
<tr>
<td>-242/-243 GCGG</td>
<td>18.8</td>
<td>12.9</td>
<td>9.6</td>
<td>11.4</td>
</tr>
<tr>
<td>-357 A&gt;C</td>
<td>6.3</td>
<td>9.8</td>
<td>7.7</td>
<td>11.4</td>
</tr>
<tr>
<td>-418 C&gt;T</td>
<td>6.3</td>
<td>4.8</td>
<td>5.8</td>
<td>5.3</td>
</tr>
<tr>
<td>-419 C&gt;T</td>
<td>6.3</td>
<td>4.8</td>
<td>5.8</td>
<td>5.3</td>
</tr>
<tr>
<td>-776 G&gt;T</td>
<td>25.0</td>
<td>14.5</td>
<td>9.6</td>
<td>12.3</td>
</tr>
<tr>
<td>-892 G&gt;T</td>
<td>25.0</td>
<td>12.9</td>
<td>3.8</td>
<td>8.8</td>
</tr>
</tbody>
</table>

Table 3.6. Epac-2 SNP distribution in normal subjects (n=8) and CLL patients (n=57), sorted by heterozygosity (Hetero) and homozygosity (Homo).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>-108 C&gt;A</td>
<td>0.0</td>
<td>0.0</td>
<td>3.5</td>
<td>1.8</td>
</tr>
<tr>
<td>-222 C&gt;A</td>
<td>0.0</td>
<td>0.0</td>
<td>0.9</td>
<td>0.0</td>
</tr>
<tr>
<td>-242/-243 GCGG</td>
<td>18.8</td>
<td>0.0</td>
<td>11.4</td>
<td>0.0</td>
</tr>
<tr>
<td>-357 A&gt;C</td>
<td>6.3</td>
<td>0.0</td>
<td>4.4</td>
<td>7.0</td>
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<td>-418 C&gt;T</td>
<td>6.3</td>
<td>0.0</td>
<td>1.8</td>
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<tr>
<td>-419 C&gt;T</td>
<td>6.3</td>
<td>0.0</td>
<td>1.8</td>
<td>3.5</td>
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<tr>
<td>-776 G&gt;T</td>
<td>0.0</td>
<td>12.5</td>
<td>8.8</td>
<td>3.5</td>
</tr>
<tr>
<td>-892 G&gt;T</td>
<td>0.0</td>
<td>25.0</td>
<td>8.8</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Figure 3.5. Novel (black) and previously known (blue) SNPs in an 892-bp area immediately upstream of Epac-2 exon 1 translation start site. Known SNPs are also marked with the NCBI SNP database (dbSNP) rs identifiers.
Figure 3.6. Epac-2 SNP frequency in normal subjects and patients with CLL (n=65).
Figure 3.7. Epac-2 SNP distribution by frequency in normal subjects (n=22) and patients with indolent-CLL (n=62), aggressive-CLL (n=52), and in the total CLL population (n=114). Significance (P<0.05), as measured by Fisher’s exact test, is depicted by a single asterisk.
Figure 3.8. Diagram of possible linkage disequilibrium between Epac-2 SNPs. Intersecting squares between SNPs are color-coded for strong evidence for linkage disequilibrium (dark gray), uninformative evidence (light gray), or strong evidence for recombination (white) as predicted by the Haploview software.
4. Results: Expression and function of downstream mediators of cAMP

A previous study with a small sample size reported that CLL-cells had elevated Epac-1 mRNA levels compared to those of normal PBMC. We sought to assess this possibility in a larger sample size and also to examine the mRNA levels of other downstream mediators of cAMP in CLL and see if they might correlate with disease state and apoptosis. We also examined the RIA/RIIB ratio in CLL and normal PBMC to determine if, as in melanomas, this ratio might be a biomarker for cancer.

4.1. The mRNA expression of Epac-1 is increased whereas the mRNA expression of PKARIIβ is decreased in patients with CLL.

We examined the mRNA expression of Epac-1, PKA RIA and RIIβ subunits and MRP4 and MRP5 (cAMP efflux channels) in CLL and normal PBMC. Figure 4.1 demonstrates, by comparing normalized Ct values (lower values equal higher expression), that PKARIA is among the highest expressed mRNAs in normal PBMC and CLL-cells. Using the ΔΔCt method we compared mRNA expression using real-time PCR of each of these genes in CLL-cells vs. normal PBMC. In addition to mRNA for Epac, PKA and the MRPs we also investigated the expression of PDE3B and PDE7B as positive controls, since previous data show that their expression is down-regulated and up-regulated, respectively, in CLL. Figures 4.2 shows that CLL is associated with a prominent increase in mRNA expression for Epac-1 (n=38, 218.2±38.1-fold, P<0.001, two very high outliers were excluded: ones that were 2964- and 3624-fold increased) and PDE7B (n=56, 20.08±2.3-fold, P<0.01). By contrast, we found that patients with CLL had a
significant decrease in mRNA expression of RIIβ (n=22, -39.5±9.3-fold, P<0.001) and PDE3B (n=33, -57.05±7.0-fold P<0.01). No change in the mRNA expression of RIα, MRP4, or MRP5 was observed in CLL patients compared to normal subjects.

As outlined above, it would be clinically beneficial to distinguish between patients who are in the indolent and aggressive stages of CLL. We tested whether expression of one of our genes of interest might separate patients in the two stages of CLL. Patients with indolent-CLL (Figure 4.3) had mRNA expression of Epac-1 (n=17, 208.9±38.8-fold, P<0.001, two very high outliers were excluded, as noted above) and PDE7B (n=22, 14.6±2.4-fold, P<0.05) that were significantly increased compared to expression in PBMC from normal subjects; only PDE3B (n=17, -66.7±11.4-fold,) was decreased in expression compared to that of normal PBMC. In aggressive-CLL (Figure 4.4) the mRNA expression of Epac-1 (n=19, 226.4±64.4-fold, P<0.001) and PDE7B (n=31, 23.1±3.5-fold, P<0.01) were increased whereas mRNA expression of RIIβ (n=10, -62.5±15.5-fold, P<0.001) and PDE3B (n=27, -55.5±7.4-fold, P<0.01) were decreased compared to the values observed for normal PBMC. Furthermore, in patients with aggressive-CLL, unlike those with indolent-CLL, mRNA expression of RIα (n=14, -3.5±0.5-fold, P<0.05) and MRP4 (n=14, -4.9±1.0-fold, P<0.01) were significantly decreased compared to the values in normal PBMC.

As shown in Figure 4.5, we observed no significant difference in mRNA expression of MRP4, MRP5, PKARI, PDE7B, or PDE3B between patients with indolent-CLL or aggressive-CLL. However the mRNA expression of PKARII was significantly higher in patients with aggressive-CLL than those with indolent-CLL (P<0.05). These data suggest that changes in RIIβ expression may distinguish the stages of CLL.
Previous data indicate that the ratio of RIα/RIIβ protein is higher in melanomas than in melanocytes, therefore we investigated this ratio in patients with CLL. Figure 4.6 shows that compared to normal PBMC, CLL-cells have a higher ratio of expression of RIα mRNA to RIIβ mRNA (n=23, 211±52.2-fold, P<0.01) with similar ratios in patients with indolent (n=13, 201±66.4-fold, P<0.05) or aggressive (n=10, 224±87.4-fold, P<0.05) CLL (normal PBMC, n=12, 8.1±1.5-fold). The results shown in Figures 4.2, 4.3, and 4.4 suggest that the increase in this ratio is the result of the decreased RIIβ expression with CLL rather than an increase in expression of RIα.
Figure 4.1. Cycle thresholds (lower value denotes higher expression) normalized to 28S for MRP4 (n=33), MRP5 (n=31), R1α (n=33), R1β (n=22), Epac-1 (n=38, Ct values for two outliers were excluded: 14.9 and 14.6), PDE3B (n=33), PDE7B (n=56) in from CLL-cells and normal PBMC (n=12 for Epac-1, n=14 for all others), as measured by real-time PCR. Significance determined by Student’s t-test is depicted by (*) for P<0.05, (**) for P<0.01, and (***) for P<0.001 compared to normal.
**Figure 4.2.** Fold-change in the expression of MRP4 (n=33), MRP5 (n=31), R1α (n=33), R1β (n=22), Epac-1 (n=38, two very high outliers were excluded: 2964- and 3624-fold), PDE3B (n=33), PDE7B (n=56) in CLL-cells compared to normal PBMC, as measured by real-time PCR. Significance determined by Student’s t-test is depicted by (*) for P<0.05, (**) for P<0.01, and (***) for P<0.001 compared to normal.
Figure 4.3. Fold-change in the mRNA expression of MRP4 (n=19), MRP5 (n=18), RIα (n=19), RIIβ (n=12), Epac-1 (n=17), PDE3B (n=17), PDE7B (n=21) mRNA in patients with indolent-CLL compared to PBMC from normal subjects, as measured by real-time PCR. Significance determined by Student’s t-test is depicted by (**) for P<0.01, and (***)) for P<0.001 compared to normal.
Figure 4.4. Fold-change in mRNA expression of MRP4 (n=14), MRP5 (n=13), RIα (n=14), RIIβ (n=10), Epac-1 (n=19, two very high outliers were excluded: 2964- and 3624-fold), PDE3B (n=27), PDE7B (n=31) in PBMC from patients with aggressive-CLL compared to PBMC from normal subjects, as measured by real-time PCR. Significance determined by Student’s t-test is depicted by (**) for P<0.01, and (***) for P<0.001 compared to normal.
Figure 4.5. Comparison of fold-change in mRNA expression between patients with indolent-CLL or aggressive-CLL versus normal PBMC, as measured by real-time-PCR. The sample sizes (n) for each mRNA transcript are the same as in Figures 2.2 and 2.3. Significance determined by Student’s t-test is depicted by (*) for P<0.05, and compared results for patients with indolent-CLL vs. aggressive-CLL.
Figure 4.6. Ratio of RIα/RIIβ mRNA in normal PBMC (n=12), and patients with indolent-CLL (n=13), aggressive-CLL (n=10) and the total CLL population (n=23). Significance determined by Student’s t-test is depicted by (*) for P<0.05, (**) for P<0.01 compared to normal PBMC.
5. Results: Pro- and anti-apoptotic effects of PKA and Epac in CLL

5.1. Epac activators are anti-apoptotic whereas PKA activators are pro-apoptotic in CLL

As noted above, Epac activators reportedly attenuate apoptosis in a small number of patients with CLL and PDE4/7 inhibitors induce apoptosis in CLL.\textsuperscript{27,30,32} We assessed a larger sample size and investigated the effects of activation and inhibition of Epac, PKA regulatory subunits, and of a protein downstream of Epac (Rap) on apoptosis of CLL-cells.

Apoptosis in CLL and normal PBMC was measured after 48 hr incubation with vehicle, 50 \( \mu \text{M} \) 8Me (an Epac activator), 50 \( \mu \text{M} \) N6 (a PKAII activator), 50 \( \mu \text{M} \) 8Cl (a PKAI activator), or 10 nM IR-284 (a PDE4/7 inhibitor) by FACS using annexin V-FITC and propidium iodide staining. Figure 5.1 shows that 8Me \((n=23, -4.7\%\pm1.35\%, P<0.05)\) inhibited the basal apoptosis of CLL-cells, suggesting that Epac is anti-apoptotic in these cells. In contrast, N6 \((n=33, 10.9\%\pm1.4, P<0.05)\), a PKARI\(\beta\) analog, increased apoptosis of CLL, implying that PKA is pro-apoptotic in CLL. However, 8Cl \((n=7, 1.8\%\pm3.3\%)\), a PKARI\(\alpha\) analog did not increase apoptosis of CLL. The PDE inhibitor, IR-284 \((10 \text{ nM}; n=9, 10.2\%\pm3.5\% \text{ and } 100 \text{ nM}; n=15, 18.6\%\pm2.6\%, \text{ both } P<0.05)\) increased apoptosis of CLL-cells, as previously reported.\textsuperscript{28} Together these data imply that the pro-apoptotic effects of the PDE4/7 inhibitors occur by raising cAMP that acts via PKARI\(\beta\) activation and that inhibition of Epac (which is anti-apoptotic) may enhance the apoptotic effect of PDE inhibition. Importantly, neither N6 \((n=5, -1.1\%\pm3.0\%)\), 8Me \((n=5, -1.5\%\pm2.3\%)\), nor IR-284 \((n=3, 7.5\%\pm4.3\%)\) significantly altered apoptosis of normal PBMC (Figure 5.2).
5.2 Inhibition of PKA inhibits apoptosis whereas inhibition of Epac-dependent pathways is pro-apoptotic

The PKA-selective inhibitor PKI (5 µM, n=6, 10.9%±1.5%) significantly (P<0.05) attenuated IR-284- (100 nM, n=6, 17.5%±4.6%) induced apoptosis (Figure 5.3), suggesting that increased cAMP promoted by the PDE4/7 inhibitor acts via PKA to promote the killing of CLL-cells. Because no Epac-selective inhibitors are available, we sought to assess the role of Epac in apoptosis by inhibiting its downstream mediator Rap-1 (with GGTTI-298). Figure 5.4 shows that GGTTI-298 significantly (P<0.01) increased apoptosis of CLL-cells at 10 µM (n=5, 26.0%±3.3%) compared to vehicle but this effect was not observed at lower concentrations of GGTTI-298. Co-administration of GGTTI-298 did not significantly increase the pro-apoptotic effect of 10 nM IR-284 in pooled data from studies with four CLL patients (n=4) (Figure 5.5). However, when analyzed individually two of these four patients showed a synergistic effect of 1 µM GGTTI-298 on the pro-apoptotic effect of IR-284. Although the numbers of patients studied is quite small, these data suggest that some CLL patients may benefit from inhibition of Epac-dependent pathways to enhance the pro-apoptotic response to cAMP/PKA (Figure 5.6).

Moreover, Figure 5.7 shows that 10 µM GGTTI-298 (n=5, 26.0%±3.3%) prevented the anti-apoptotic effect of 50 µM 8Me (n=5, 29.4%±4.2%), demonstrating that the anti-apoptotic effect of Epac activation is, at least in part, mediated through Rap-1.
5.3 Correlation of Epac expression with basal and drug-induced apoptosis

To test whether high levels of Epac may contribute to low basal apoptosis and blunt cAMP-promoted apoptosis in CLL, the basal level of apoptosis in CLL-cells was plotted against the fold-change in Epac-1 mRNA expression but the results showed no significant correlation (according to Spearman’s test, Figure 5.8). In addition, we found no significant correlation between the fold-change in PKARIIβ expression in patients with CLL and basal apoptosis (data not shown) or between PKARIIβ mRNA fold-change and Epac-1 fold-change in patients with CLL. (Figure 5.10, two Epac-1 outliers [341- and 279-fold] and four RIIβ outliers [-131-, –200-, –383-, and -459-fold] were excluded). Similarly, no significant correlation was found between drug-induced apoptosis (100 nM IR-284) in CLL and the fold-change in Epac-1 mRNA expression (Figure 5.9).

Of note, the fold-change in Epac-1 mRNA and PDE7B mRNA in patients with CLL correlate very highly (Figure 5.11, P<0.001, r=0.5395, Spearman’s test). This correlation suggests that there are distinct mRNA expression features of CLL subpopulations but more subjects would be required in order to obtain more definitive evidence as to whether expression of Epac-1 mRNA and/or PDE7B mRNA relate to particular clinical features and disease progression in CLL.
Figure 5.1. Drug-induced apoptosis (measured by flow cytometry) in CLL-cells in response to 50 µM 8Me (n=23), 50 µM N6 (n=33), 50 µM 8Cl (n=7), 10nM IR-284 (n=9), or 100 nM IR-284 (n=15). Significance as determined by Student’s t-test with (*) P<0.05 compared to vehicle.

Figure 5.2. Drug-induced apoptosis (measured by flow cytometry) in normal PBMC in response to 50 µM 8Me (n=5), 50 µM N6 (n=5), or 100 nM IR-284 (n=3).
Figure 5.3. Drug-induced apoptosis (measured by flow cytometry) in CLL-cells in response to 100 nM IR-284 alone (n=6) or together with 5µM PKI (n=6). Significance as determined by Student’s t-test with (**) P<0.01 compared to IR-284 alone.
Figure 5.4. Drug-induced apoptosis in CLL-cells (measured by flow cytometry) in response to 3 doses of the Rap1-inhibitor GGTI-298 (100 nM [n=4], 1µM [n=4], 10 µM [n=5]). Significance as determined by Student’s t-test with (**) P<0.01, compared to vehicle.
Figure 5.5. Drug-induced apoptosis in CLL-cells (measured by flow cytometry) in response to increasing doses of GGTI-298 (100 nM (n=4), 1 µM (n=4), 10 µM (n=5)), of 10 nM IR-284 (n=9), or both combined (n=4, n=4, n=6, in order of increasing GGTI-298 doses). Statistical significance as determined by Student’s t-test with (*) P<0.05, and (**) P<0.01, compared to vehicle.
Figure 5.6. Drug-induced apoptosis in CLL-cells from two different patients, as measured by flow cytometry, in response to 1 µM GGTI-298, 10 nM IR-284 or both.
Figure 5.7. Drug-induced apoptosis in CLL-cells (measured by flow cytometry) in response to 50 μM 8Me (n=23), 10 μM GGTI-298 (n=5) or both (n=5). Drug-induced apoptosis under different treatments are not significantly different as determined by Student’s t-test (P>0.05).
Figure 5.8. Correlation of Epac-1 mRNA fold-change and basal apoptosis in patients with CLL (n=11). Correlation is not significant (P>0.05), according to the Spearman test for correlation.
Figure 5.9. Correlation of Epac-1 mRNA, expressed as fold-change compared to normal, and drug-induced (100 nM IR-284) apoptosis in CLL-cells (n=10). Correlation is not significant (P>0.05) according to the Spearman test for correlation.
Figure 5.10. Correlation of the fold-changes in mRNA expression for RIIβ mRNA and Epac-1 (n=28). Two Epac-1 outliers (341- and 279-fold) and 4 RIIβ outliers (-131-., –200-., –383-., and -459-fold) were excluded. Correlation is not significant (P>0.05) according to the Spearman test for correlation.
Figure 5.11. Correlation of the fold-changes in PDE7B mRNA and Epac-1 mRNA (compared to normal PBMC) (n=43). Correlation is significant (P<0.001, ***, r=0.5395) as determined by the Spearman test for correlation.
6. Discussion

The current studies showed that the mRNA expression of Epac-1 is significantly increased in CLL-cells compared to normal-PBMC, results that are similar to previous published data (Figure 4.1).\textsuperscript{32} Initial studies aimed to investigate if the high expression of Epac-1 with CLL could be explained by SNPs in its promoter region. Transcription factors rely on binding to consensus DNA sequences in the promoter of genes; therefore SNPs in these sequences can disrupt their ability to bind to DNA. To our knowledge, this is the first study that has assessed for SNPs in the promoter rather than the coding region of the Epac-1 gene.\textsuperscript{39} Of the 7 SNPs discovered in the Epac-1 promoter region (Figure 3.3), only the SNP -938 G>A has a frequency that is significantly different in patients with CLL than in normal PBMC. However, no transcription factors are known to bind to the sequence containing this SNP (as predicted by Transcription Element Search Software [TESS]).\textsuperscript{40} Therefore, although our results identify a -938 G>A SNP and six other SNPs in the promoter of Epac-1, they do not explain the high Epac-1 mRNA expression that occurs in CLL-cells.

In linkage disequilibrium (LD) single nucleotides nonrandomly associate together during chromosome recombination. In SNP association studies it is important to determine if a “silent” SNP (such as -938 G>A) associates with a SNP that causes a change in phenotype. The -938 G>A SNP, along with the other 5 SNPs, are not in LD with each other (Figure 3.4), suggesting that no “silent” SNPs act together with 938 G>A SNP to alter phenotype.

Throughout our SNP discovery we aimed to uncover a new biomarker for CLL, since previous studies have shown that SNPs in certain genes can predict the
susceptibility to CLL and be associated with the outcome, for example, polymorphisms in the CD38 gene predict the risk of developing CLL.\textsuperscript{41,42} The -938 G>A SNP is found more significantly in patients with aggressive-CLL and perhaps may prove useful in predicting progression of the disease. Further analyses in more subjects are required to validate the -938 G>A SNP in the Epac-1 promoter as a biomarker in CLL.

Since Epac-2 mRNA expression is not correlated with CLL, SNP discovery in Epac-2 was used as a negative control. Interestingly, though, we found a 4-bp insertion between -242 and -243 in the promoter of Epac-2 and that this insertion was expressed heterozygously and found at frequencies of 10-20\% in normal PBMC and CLL. It is possible that this 4-bp insertion alters promoter activity. Future studies will aim to subclone this fragment to verify its existence and function.

We also found that the -418 C>T and -419 C>T SNPs in Epac-2 are in LD (Figure 3.8), which is not surprising since they are only separated by one base pair. Of the 8 SNPs found in the Epac-2 promoter region only the frequency of -892 G>T occurred at a significantly different frequency in CLL and normal PBMC (the SNP was less frequent in CLL). According to TESS this SNP may alter the binding of the activator T-cell transcription factor 1 (TCF1).\textsuperscript{43} However, this transcription factor has only been found in T-cells and epithelial cells, but not in the brain or adrenal glands where Epac-2 is highly expressed.\textsuperscript{36,44,45} It therefore seems unlikely that this SNP affects Epac-2 transcription. Further studies to investigate the functional impact of the -892 G>T SNP on Epac-2 promoter activity, would require construction of luciferase reporter plasmids with a 5’-flanking region and the wild-type or SNP-containing gene.
Previous work in CLL has shown a correlation between abnormal levels of pro-apoptotic proteins (Bcl-2, Bax, p53) and PDEs but the current results provide the first comprehensive analysis of downstream mediators of cAMP in relation to CLL.\textsuperscript{46,47} We find that PKARI\textsubscript{α}, PKARII\textsubscript{β}, Epac-1, Epac-2, MRP4 and MRP5 are all expressed in both normal PBMC and CLL-cells. Among those entities, PKARI\textsubscript{α} is the highest expressed, however its expression does not significantly change with the disease. By contrast, mRNA expression of Epac-1 is prominently increased and that of PKARII\textsubscript{β} is significantly decreased in CLL versus normal PBMC (Figure 4.2, by \textasciitilde200-fold and \textasciitilde40-fold, respectively). Normal PBMCs are predominantly T-cells but CLL represents an enrichment in B-cells, which potentially may, at least in part, explain differences in the expression of the cAMP mediators. However, further data obtained in the lab (data not shown) show a similar difference in expression in Epac-1 and PKARII\textsubscript{β} in CLL vs. normal B-cells, thus implicating the malignancy in the expression pattern of those two downstream mediators in CLL. These data support the hypothesis that an imbalance of those effectors of cAMP contribute to the decreased apoptosis of B-cells in CLL.

The current studies also sought to determine if the genes of interest might be biomarkers for CLL and thus compared their expression in patients with indolent- and aggressive-CLL. In addition to the changes in Epac-1 and PKARII\textsubscript{β} noted above as indicators of CLL, MRP4 and PKARI were significantly decreased in aggressive-CLL. However, there was no significance difference in the expression of Epac-1, Epac-2, PKARI, MRP4 or MRP5 in patients with indolent-CLL vs. aggressive-CLL, thus suggesting that expression of those genes is unlikely to be useful as a prognostic
biomarker for the disease. Further analyses is required to determine if increased Epac expression associates with other clinical features of CLL or with drug responses. Of interest, PKARIIβ expression is significantly lower in aggressive-CLL than in indolent-CLL (Figure 4.5). These data support the hypothesis that PKARIIβ may be a novel biomarker in CLL but more subjects need to be investigated to determine if a specific fold-change of PKARIIβ can distinguish indolent- and aggressive-CLL.

Previous data has shown that certain cancers are associated with an altered ratio of RIα/RIIβ expression. For example, Mantovani et al., showed that a high RIα/RIIβ ratio commonly occurs in melanomas and that pharmacological and genetic manipulations to restore this unbalanced ratio lead to decreased proliferation in human melanoma cells. Akin to those findings are the results shown here that the ratio of RIα/RIIβ expression is higher in CLL compared to normal PBMC (Figure 4.6). In CLL it can be hypothesized that restoring the expression of PKARIIβ may be beneficial by increasing the apoptotic effects of drugs that raise cAMP (since other results shown here suggest that cAMP-induced apoptosis in CLL appears to be via PKARIIβ, Figure 4.2).

Of interest, the fold-change in mRNA expression of Epac-1 and PDE7B in CLL (compared to normal PBMC) are significantly correlated (Figure 5.10). This finding suggests that both genes may be transcriptionally regulated by a common mechanism that relates to disease progression, that the regulation of one gene may depend on the other (for example, that the upregulation of PDE7B and the subsequent decrease in cAMP leads the transcriptional regulation of Epac-1) or alternatively, that the expression pattern of PDE7B and Epac in CLL-cells is characteristic of the clonal expansion of a subset of
B-cells. Investigating the expression of both Epac-1 and PDE7B may prove useful for understanding the pathophysiology and identifying therapeutic targets in CLL.

It is important to point out that although the mRNA expression data shows several significant differences between normal PBMC and CLL and among different patients with CLL, mRNA expression does not always correlate with that of protein and thus, it will be necessary to define protein levels of Epac-1, PKA, and other downstream effectors in CLL and normal PBMC: preliminary data (not shown) shows an ∼14 fold-increase in Epac-1 protein levels in CLL compared to normal (n=16, P<0.01). In addition, activity of these downstream mediators should be measured such as via CREB phosphorylation for PKA and Rap-1 activation for Epac-1 to help confirm the functional significance of the changes in mRNA expression.33,47

The current studies show that Epac activation decreased the basal apoptosis of CLL-cells while PKA activation increased apoptosis of such cells (Figure 5.1). Since the PKARIα-selective analog (8Cl) did not significantly kill the CLL-cells, whereas the PKARIβ analog induced apoptosis, cAMP-promoted killing via PKA may be attributed to PKARIβ. Previous studies have shown that PKARIβ, not PKARIα, kills acute lymphoblastic leukemia cells, thus providing further evidence for its role in cAMP-promoted killing of malignant cells.49 However, the lack of apoptosis in CLL by the PKARIα analog (Figure 5.1) does not correlate with the high expression of PKARIα compared to PKARIβ in CLL-cells. This discrepancy may be attributable to the fact that PKARIβ analog N6 is ∼4 times more lipophilic than 8Cl (the PKARIα analog).50
Therefore, 8Cl may not as readily cross the plasma membrane compared to N6 and caution must be taken in interpreting the data.

Previous data show that a dual PDE4/7 inhibitor (IR-284) kills CLL-cells in a cAMP-dependent manner.\(^ {27} \) The current studies suggest that the pro-apoptotic effect of the PDE4/7 inhibitor occurs, at least in part, via PKA (likely PKAII\(\beta\), according to the results discussed above), since in addition to the pro-apoptotic effects of a PKA-selective analog in CLL, PKI blunts the IR-284-promoted killing (Figure 5.3). To investigate the role of Epac in the pro-apoptotic effect of IR-284 we inhibited a proximal downstream mediator of Epac, Rap; since no Epac inhibitors are commercially available, inhibition of Rap should mimic the effects of Epac inhibition. Treating CLL PBMC with the Rap inhibitor GGTI-298 increased basal apoptosis, and prevented the anti-apoptotic effect of the Epac activator, which supports data shown in WEHI-231 (immature B) cells that Rap-1 is a key downstream mediator of Epac-1 (Figures 5.4, 5.5, 5.7).\(^ {51} \) Furthermore, and of therapeutic relevance, Rap inhibition enhanced the pro-apoptotic effect of IR-284 in a small number of CLL patients (although pooled data did not show such an effect due to inter-individual differences, Figure 5.5 vs. 5.6). Together with the increase in Epac-1 expression in CLL, these findings suggest that inhibition of Epac-dependent pathways may prove useful for enhancing the pro-apoptotic effects of IR-284 or other drugs that increase cAMP levels and activate PKA.

The mechanism by which Epac activation protects cells from apoptosis in a Rap-1-dependent manner is unclear. Rap-1 has a large number of downstream targets that include extracellular signal regulated kinases 1 and 2 (Erk1/2), phosphatidylinositol-3-kinase (PI3K)-dependent protein kinase B/AKT and protein kinase C. In human
melanoma cells Epac activation via 8Me has been shown to stimulate AKT. Furthermore, a number of publications have shown that CLL is associated with prolonged activation of PI3K/AKT that leads to resistance to apoptosis in CLL. Increased expression of Epac in CLL-cells may contribute to sustained AKT activation and thus further support the potential of using Epac as a therapeutic target in CLL.

Pooled analysis of samples from numerous patients showed that expression of Epac-1 or PKAIIβ did not correlate with basal or drug-induced apoptosis (Figures 5.8 and 5.9). When working with clinical samples it is often difficult and misleading to pool cells from patients because individual patients do not necessarily exhibit identical genetic and biochemical features of a particular disease. In fact, patients with the highest Epac-1 expression (in Figure 5.8) are those that have the lowest basal apoptosis. This suggests that the increased levels of Epac-1 in those patients may help prevent apoptosis of the patient’s malignant B-cells.

In summary, the current studies show that in normal and CLL the ~1000-bp regions upstream of the Epac-1 and Epac-2 exon 1 translation start sites contain 7 SNPs and 8 SNPs, respectively. One SNP in each of the Epac-1 and Epac-2 promoter regions is found at significantly different frequencies in CLL-cells and normal PBMC. The SNP in the Epac-1 promoter does not affect transcription factor binding but it may be useful as a biomarker for CLL. In addition, Epac-1 mRNA expression is much higher in CLL-cells compared to normal PBMC whereas the mRNA expression of PKAIIβ is significantly lower; Epac activators decrease apoptosis in CLL-cells, whereas PKA activation increases apoptosis. Together these findings suggest that there is a defect in the pro- and anti-apoptotic pathways of cAMP in CLL and that these defects may explain, at least
in part, the increased accumulation of B-cells with the disease (Figure 6.1). In conclusion, data shown in this Masters thesis suggest a novel strategy for the treatment of CLL: decreasing the expression and function of Epac-1, while increasing that of PKAII\(\beta\) so as to increase the pro-apoptotic effects of drugs that act through raising cAMP and activate PKA or inhibit Epac effectors.

**Figure 6.1.** Schematic for the alterations in the cAMP pathway that occurs in CLL (increased Epac-1, and PDE7B, and decreased PKAII [denoted by the dashed arrows] and the pro- and anti-apoptotic effects of Epac and PKA in CLL are highlighted)
Figure S1. NCBI sequence of the first 1089 bp upstream of the Epac-1 exon 1 translation start site (marked by the green box). SNPs are marked in red.
Figure S2. NCBI sequence of the first 1191 bp upstream of the Epac-2 exon 1 translation start site (marked by a green box). SNPs are marked in red.
References:


