BIOLOGICAL EFFECTS OF VASOACTIVE INTESTINAL PEPTIDE/PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE RECEPTOR 1 (VPAC1) AND VPAC2 ON CHEMOTAXIS AND ITS REGULATION OF THE TUMOR SUPPRESSOR IKAROS IN LEUKEMIC T CELLS

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Title

Biological Effects of Vasoactive Intestinal Peptide/Pituitary Adenylate Cyclase Activating Polypeptide Receptor 1 (VPAC1) and VPAC2 on Chemotaxis and its Regulation of the Tumor Suppressor Ikaros in Leukemic T cells

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ABSTRACT

One in three children and young adults under the age of 20 diagnosed with cancer has some form of leukemia. Leukemia patients with mutations in the tumor suppressor transcription factor Ikaros (IK), an anti-leukemic factor that is critical for the development of blood cells; have a poor prognosis despite modern chemotherapy. There is, therefore, a critical need to understand the biology of IK. Research by our laboratory has identified a neurotransmitter, called vasoactive intestinal peptide (VIP), which blocks proliferation through one of its receptors (vasoactive intestinal peptide/pituitary adenylate cyclase activating polypeptide receptor 1(VPAC1)), but blocks apoptosis through a second inducible receptor (VPAC2), while both receptors have chemotactic properties in primary T cells. Some leukemia patients have reversed VIP receptor expression (low VPAC1; high VPAC2), and we hypothesizes that this contributes to a selective growth advantage for leukemic T cells. Understanding the biology by which VIP receptors regulate cellular growth (proliferation and apoptosis) and movement (chemotaxis) will be pivotal in establishing their signaling pathways as future drugs target candidates in the fight against leukemia. We hypothesized that VIP/VPAC1 signaling would alter the expression of IK protein and that VIP would direct cellular migration of both VPAC1 and VPAC2 expressing leukemic T cells. To test this hypothesis, we first asked whether VIP/VPAC1 signaling affects the expression and/or the phosphorylation profile of IK a transcription factor that regulates cellular growth and proliferation. The second question was whether VPAC1 and/or VPAC2 signaling differentially control leukemic cell movement. By one- and two-dimensional polyacrylamide gel electrophoresis followed by Western blot analysis,

we showed that VIP signaling suppresses IK expression and changes the isoelectric pools of IK protein in a human leukemia cell line. By using leukemia cells that only express VPAC1 or VPAC2 receptors; we demonstrated that VIP promoted cellular movement, but that this effect was controlled by different pathways elicited by VPAC1 versus VPAC2. Collectively, these data support the notion that the nervous system naturally contributes to normal blood cell function, but after leukemogenesis, the VIP signaling axis may exacerbate the leukemia phenotype.

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DEDICATION

I would like to dedicate this dissertation to my family. First and foremost, I would like to dedicate this to my parents, Ron and Beth, who were always there to support me in the decisions of life from high school, college, to graduate school. Their belief in me that I could accomplish anything that I set my heart and mind to was greatly appreciated. Dad, this work is truly dedicated to you, from the time I was a child you taught me to never give up and to continue to fight for what you believe in. You were an inspiration to me when you beat cancer the first time, and you were never dishearten when word that it had returned and was terminal. You demonstrated to me how one should live their life by doing what you LOVE and spending time with those you LOVE. I LOVE you and miss you Dad, not a day goes by where I do not think about you! Mom, you demonstrated to me what true LOVE and DEDICATION was by your continued willingness to help Dad in everything he did; you showed me what a TRUE friend was. Without your strength and support, I would not be where I am today.

Of course, I also want to dedicate this to the rest of my family Rich, Jen, Paityn, and Matt Vandersteen and Kraig, Deb, Alecia, Pam, and Bree Winzenburg who have been supportive of me in my academic career.

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ABBREVIATIONS

2DE..... Two-dimensional gel electrophoresis

aa.....Amino acid

AC.....Adenylate cyclase

AICD ..... Activation induced cell death

ATP.....Adenosine triphosphate

Bp ..... Base pair

BSA ..... Bovine serum albumin

BTC .....Basal transcriptional complex

BTM.....Basal transcriptional machinery

cAMP .....cyclic adenosine monophosphate

CD .....Cluster of differentiation

Cdk .....Cyclin dependent kinase

chFBS.....characterized fetal bovine serum

ChIP..... Chromatin immunoprecipitation

CK II..... Casein kinase II

CKI..... Cyclin kinase inhibitor

CM ..... Complete Media

CREB..... cAMP-dependent responsive element binding protein

CtBP .....C-terminal binding protein

Da ..... Dalton

DAG.....1,2-diacylglycerol

DBD .....DNA binding domain

DN .....Double negative

DNA .....Dexoyribose nucleic acid

DN-IK .....Dominant negative Ikaros

DP .....Double positive

DRB 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole

DTT .....Dithiothreitol

EDTA .....Ethylenediaminetetraacetic acid

EGTA ..... Ethyleneglycoltetraacetic acid

EMSA .....Electrophoretic mobility shift assay

EPAC .....Exchange protein directly activated by cAMP

ETP .....Early T cell progenitor

GDP ..... Guanine diphosphate

GPCR ..... G protein coupled receptor

GrB .....Granzyme B

GTP ..... Guanine triphosphate

HAT ..... Histone acetyltransferase

HBSS .....Hank's buffered saline solution

HDAC ..... Histone deacetylase

Hr ..... hour

HRC ..... Histone remodeling complex

HRP .....Horse radish peroxidase

i[cAMP] .....intercellular cAMP

IBMX 3-isobutyl-1-methyxanthine

IEF ..... Isoelectric focusing

IK ..... Ikaros

IK-DN ..... Ikaros dominant negative

IL ..... Interleukin

IMDM ..... Iscove's modified Dulbecco's medium

IP_3 ..... Inositol 1,4,5-triphosphate

Min ..... minute

M-MLV ..... Moloney-Murine Leukemia Virus

NaCl ..... Sodium chloride

NaOH ..... Sodium hydroxide

NF-DM ..... Non-fat dry milk

NP-40 ..... Nonidet P-40

NuRD ..... Nucleosome remodeling and histone deacetylase chromatin remodeling complex

PBMC ..... Peripheral blood mononuclear cells

PC-HC ..... Pericentromeric heterochromatin

PCI ..... Protease cocktail inhibitor

PHI ..... N-terminal histidine and C-terminal isoleucine amide

pI ..... Isoelectric point

PIP_2 ..... Phosphatidylinositol 4,5-bisphosphate

PKA ..... Protein kinas A

PKC ..... Protein kinase C

PLC ..... Phospholipase C

PMA.....Phorbol 12-myristate 13-acetate

PP.....Peyer's patches

PP1.....Protein phosphatase 1

PTM.....Posttranslational modification

PTx.....Pertussis toxin

qRT-PCR..... quantitate real time polymerase chain reaction

RAMP.....Receptor activity-modifying protein

RIPA.....Radioimmunoprecipitation assay

RT.....Reverse transcriptase

SDS.....Sodium dodecyl sulfate

SDS-PAGE.....Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SOC..... Super optimal broth

SP..... Single positive

SS.....Serum Starved

SUMO..... SUMOylation

SWI/SNF.....Switch/sucrose nonfermentable

TBS..... Tris buffered saline

TBS-T..... Tris buffered saline-tween

TCR.....T cell receptor

TdT.....Deoxynucleotideyl transferase

Th..... Helper T cell

TM..... Transmembrane

Treg.....Regulatory T cell

TSS.....Transciptional start site

VH..... Volt hour

VIP..... Vasoactive intestinal peptide

VPAC..... Vasoactive intestinal peptide/pituitary adenylate cyclase-activating polypeptide

CHAPTER 1. INTRODUCTION

General Introduction and Background

Leukemogenesis

One of three cancers diagnosed in children and young adults under the age of 20 is leukemia (American Cancer Society, 2012). Annually there are over 144,000 new cases diagnosed with a mortality rate of 53% (Leukemia and Lymphoma Society, 2011). Leukemia is the uncontrolled proliferation of blood cells (Hanahan & Weinberg, 2000). One etiology of leukemogenesis is the dysregulation of the tumor suppressive protein Ikaros (IK) (Marçais et al., 2010; Meleshko, Movchan, Belevtsev, & Savitskaja, 2008; Mullighan et al., 2009; Winandy, Wu, & Georgopoulos, 1995). IK is a master transcriptional regulator that is responsible for the regulation of genes needed for lymphopoiesis, T cell maturation, and regulation of the cell cycle (Georgopoulos, 2009; Georgopoulos et al., 1994; Molnár et al., 1996; Nakayama et al., 2000; Yoshida, Ng, Zuniga-Pflucker, & Georgopoulos, 2006). In a 2009 report, it was determined that 25% of all leukemic patients had altered IK protein expression, with 80% of these patients having a mutation in the IK gene (*Ikznf1*). These mutations altered IK's DNA binding domain or precludes its protein expression. The remaining 20% had an increase in smaller IK isoforms, which have been demonstrated to ablate DNA binding of full-length IK proteins (Nishii, Katayama, Miwa, et al., 2002; Nishii, Katayama, & Shiku, 2002). These patients were also identified to have a poor prognosis with less than a 20% survival rate despite aggressive, modern

chemotherapy treatments (Mullighan et al., 2009). Therefore, there is a critical need to understand the biology of IK and its regulation as a tumor suppressor.

The Ikaros Gene Family

Ikaros, the founding member of a zinc finger family of transcription factors, is critically important in the development and maturation of blood cells called hematopoiesis (Figure 1) (Cortes, Wong, Koipally, & Georgopoulos, 1999; Georgopoulos, 2002; John, Yoong, & Ward, 2009). Additional members of this zinc finger family include Aiolos, Helios, Eos, and Pegasus. IK, along with its other family members (Aiolos, Helios, and Eos), play key roles in the commitment of a pluripotent stem cell to differentiate into one of the three arms of hematopoiesis including erythroid (red blood cells), myeloid (neutrophils, eosinophils, and basophils), and lymphoid (T, B, and NK cells) cells (Hahm et al., 1998; Kelley et al., 1998; Morgan et al., 1997; Perdomo, Holmes, Chong, & Crossley, 2000) (Figure 1). Maturation of T cells is specified by altered expression of proteins, which direct lineage commitment, differentiation, and maturation into normal lymphocytes (John & Ward, 2011). Aiolos increases gene expression of the critical antiapoptotic factor Bcl-2 (Rebollo, Ayllón, Fleischer, Martínez, & Zaballos, 2001; J. H. Wang et al., 1998). Helios expression is significantly increased in T regulatory (Tregs) cells and is hypothesized to be a potential upstream regulator of the Foxp3 gene (Cai, Dierich, Oulad-Abdelghani, Chan, & Kastner, 2009; Hahm et al., 1998). Eos is myeloid lineage specific, while Pegasus expression is more widely distributed than other family members that are primarily limited to hematopoietic cells. It is unable to dimerize with other family members, and its

biological function is unknown (John & Ward, 2011; Perdomo et al., 2000). While IK is expressed in lymphoid and myeloid cells and is responsible for cell differentiation (Georgopoulos, 1997; Lo, Landau, & Smale, 1991; Payne et al., 2003; Payne, Nicolas, Zhu, Barsky, & Crooks, 2001).

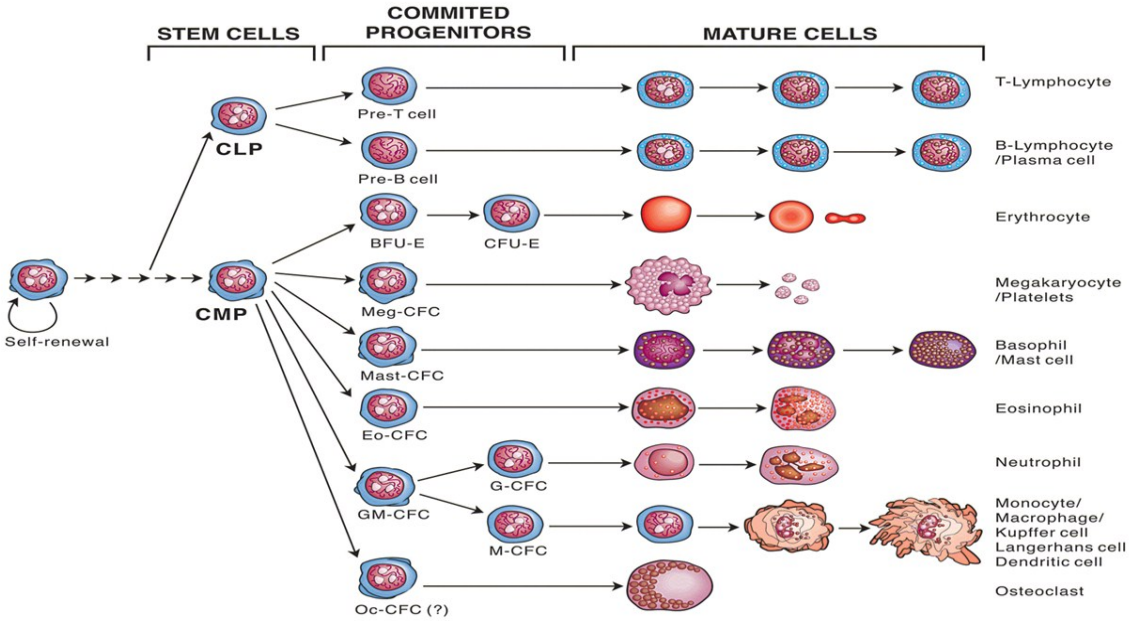


Figure 1. Maturation of blood cells through the process of hematopoiesis. Blood cells develop from a self-renewal pluripotent stem cell located in bone marrow. Cells differentiate into the three arms of the immune system of lymphocytes (T, B, and NK cells), erythroid (red blood cells), and myeloid (neutrophils, eosinophils, and basophils). Image adapted from www.bloodlines.stemcells.com/chapters.html.

IK was originally identified as a lymphocyte transcription factor called Lyf-1. Lyf-1 was identified to be bound to the terminal deoxynucleotideyl transferase (TdT), λ5, and VpreB promoters, suggesting that it had a role in transcriptional regulation (Lo et al., 1991). Biological roles of IK are hematopoiesis, immune function, tumor suppression, transcription, and chromatin remodeling (Kim et al., 1999; Koipally & Georgopoulos, 2000, 2002b; Koipally, Kim, et al., 1999; Koipally,

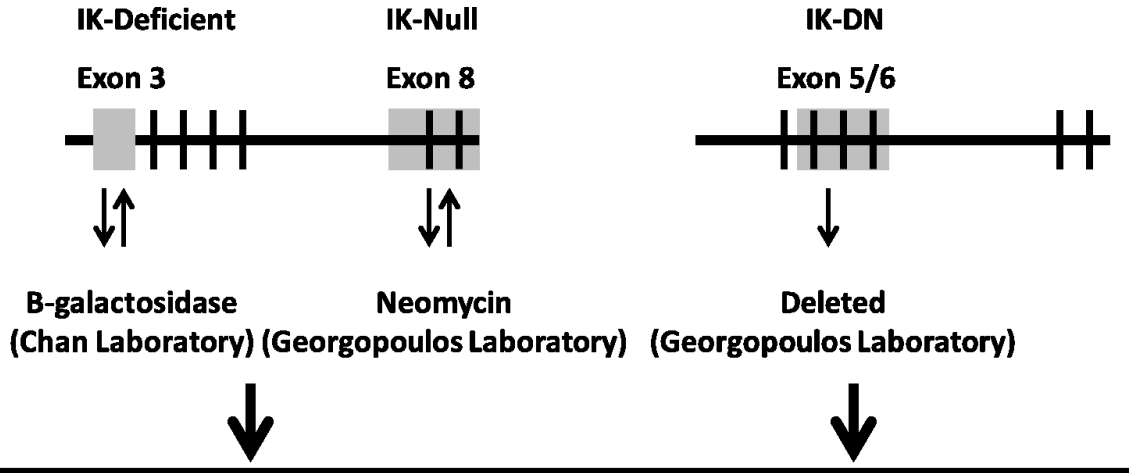
Renold, Kim, & Georgopoulos, 1999). IK contains two C-terminal hunchback-like zinc finger domains encoded within exon 8 that are utilized for protein-protein dimerization between IK and other family members (Sun, Liu, & Georgopoulos, 1996). Four krűppel-like zinc fingers are encoded within exons 4, 5, and 6 and have a high affinity for the core DNA sequence of GGGAA (Molnár & Georgopoulos, 1994). Although the IK gene contains eight exons, only seven are translated into a 519 amino acids (aa) full length IK protein (Hahm et al., 1994). Exon 3a, a variant exon, was later identified to be translated in humans, but absent in mice, yielding the longest known isoform to date, termed IK H (H for human) (Nakayama et al., 2000; Payne et al., 2001; Sun, Goodman, et al., 1999).

Ikaros Mouse Models Develop Leukemia

IK was identified to be present in mature T cells, as well as the earliest T cell progenitors, and therefore was hypothesized to play a role in the development of the T cell lineage. The presence of IK in early T cell progenitors was based on *in situ* hybridization analysis of mouse stem cells. These stem (progenitor) cells located in bone marrow would migrate to the thymus for T cell maturation. These same studies showed that IK expression remained high and was identified as being bound to the promoter of TdT gene (Georgopoulos, Moore, & Derfler, 1992; Hahm et al., 1994; Lo et al., 1991). The TdT gene encodes for a T cell specific endonuclease critical for T cell development. IK's role in T cell biology was tested with the generation of different transgenic mouse models (Figure 2).

A dominant negative (DN) mouse model was first created where exons four and five, which encodes three of the four DNA binding krűppel-like zinc fingers

were deleted from the IK gene to prevent IK from binding to DNA. $IK^{DN/DN}$ mice are unable to develop the lymphoid arm of the immune system resulting in the loss of T, B, and natural killer (NK) cells. While heterozygous mice ($IK^{+/DN}$) appeared to have normal immune cells at birth, but within 3-6 month of age tumors formed throughout the body from a thymocyte subpopulations through a loss of heterozygosity (loss of the wild type allele) (Georgopoulos et al., 1994).



	Fetal	Birth	Adult		Fetal	Birth	Adult
$IK^{-/-}$	No T, B, or NK cells	T cells rebound	Hyperproliferative & develop tumors	$IK^{DN/DN}$	No T, B, or NK cells		
$IK^{+/-}$	Normal cell development		Hyperproliferative & develop tumors	$IK^{+/DN}$	Normal cell development		3-6 months thymic tumors by loss of heterozygosity

Figure 2. Ikaros leukemia mouse models. IK-deficient mice were generated by replacing exon 3 with β-galactosidase. IK-null mice were generated by replacing exon 8 with neomycin. IK-DN mice were generated by deleting exons 5 and 6. All three strategies resulted in T cell leukemia (tumors). Additional phenotypes for each model are indicated.

Another transgenic model generated was the IK null mouse. In these mice, exon 8 was replaced with the neomycin gene. This alteration in the *Iknzf1* gene resulted in unstable protein due to the absence of its C-terminal dimerization

domain. Homozygous mice (IK $^{null/null}$) lacked all fetal T, B, and NK cells, but unlike the DN model, T cells recovered after birth. The recovery of T cells is due to Aiolos' compensation, whose expression is turned on upon birth (J. H. Wang et al., 1996). Moreover, the IK-DN and IK-null data confirmed that IK deficiency was critical for B and NK cell development more so than T cells development. Also, the fact that IK-DN mice showed no T cell rebound after birth suggest that IK binds other proteins (Aiolos), which lead to the eventual discovery of additional IK family members. When these homozygous mice matured into adults, they soon developed hyperproliferative T cells, which resulted in malignant transformation of T cells (leukemia) that caused tumors throughout the body. Mice that were heterozygous ($IK^{+/null}$) had normal fetal T, B, and NK cell development, but succumbed to leukemia upon adulthood due to loss of the wild type allele (Sun et al., 1996). Interestingly, heterozygous ($IK^{+/null}$) mice that underwent a thymectomy were "cured" of developing leukemia. This supported the notion that T cell leukemia occurring in the IK knockout mouse models was originating from a developing subpopulation within the thymus (Dumortier et al., 2006). Comparing the phenotype of these two models, it was determined that the DN mice had a more severe phenotype than the null model due to the lack of fetal and adult lymphocytes. This demonstrated that the DN isoform could prevent recovery of the T cells by sequestering Aiolos, which compensated for the loss of IK protein (Kelley et al., 1998; J. H. Wang et al., 1996). The most recent IK knockout model swapped β-galactosidase for exon 3 generating an IK deficient mouse model.

These mice had a similar phenotype as the IK null and also developed leukemia upon birth (Kirstetter, Thomas, Dierich, Kastner, & Chan, 2002).

T Cell Development

T cells are derived from a pluripotent stem cell originating in the bone marrow that commit towards the lymphoid lineage (Hansen & Zapata, 1998; McGrath, Koniski, Malik, & Palis, 2003). The earliest T cell progenitor migrates from the bone marrow to the thymus through a not well understood, pertussis toxin sensitive mechanism (Delgado et al., 1996; Leceta, Martínez, Delgado, Garrido, & Gomariz, 1996). One possible mechanism to explain the migration from bone marrow to the thymus is a chemotactic response of the stem cell to the neuropeptide, vasoactive intestinal peptide (VIP). VIP is secreted by VIPergic nerves that innervate the thymus and from thymocytes themselves (Delgado, Martinez, Leceta, & Gomariz, 1999). Such high VIP levels within the thymus could generate a concentration gradient that stem cells could utilize to migrate to the thymus (Bellinger, Lorton, Brouxhon, Felten, & Felten, 1996; Felten, Felten, Carlson, Olschowka, & Livnat, 1985). VIP is the ligand for vasoactive intestinal peptide receptors 1 (VPAC1) and 2 (Juarranz et al., 1999). VPAC1 and 2 are G protein coupled receptors (GPCRs) that are known to couple efficiently to the heterotrimeric $G_{\alpha s\beta\gamma}$ proteins, which upon ligand binding result in an activation of adenylate cyclase (AC). It is the $G_{\alpha s}$ protein that pertussis toxin ADP-ribosylates to “short-circuit” G proteins from interacting with GPCRs thereby blocking signal transduction (Paccani et al., 2008). To support this hypothesis, mature CD4 T

cells express high levels of VPAC1 and is chemotactic towards VIP (Johnston et al., 1994) suggesting that this may also be a mechanism for stem cells as well.

Maturation and differentiation of T cells occurs upon progenitor T cells entry into the thymus (Figure 3). Double negative 1 cells (DN1) are the earliest thymocyte progenitor (ETP) immature T cell isolated from the thymus and has high VPAC1 expression (Vomhof-DeKrey, Sandy, et al., 2011). At the DN1/ETP stage these cells lack expression of cluster of differentiation 4 and 8 (CD4 and CD8) surface markers and express an immature CD3 receptor (T cell receptor, TCR) and c-kit (CD117) a tyrosine kinase receptor. During maturation in the thymus, DN1 cells differentiate into DN2, DN3, DN4, double positive (DP; for CD4 and CD8 expression), single positive (SP) CD4 or CD8 T cells. In the DN3 stage, the TCR is rearranged by insertion of nucleic acids by TdT which allows for the grand diversity of antigens that the TCR is able to recognize (Lee et al., 1987), (Lo et al., 1991). Expression of TdT is controlled by IK protein and without IK these cells have an immature TCR and lead to maturation arrest (Mombaerts, Clarke, et al., 1992; Mombaerts, Iacomini, et al., 1992). Collectively, IK deficiency causes T cells to become less dependent on T cell signaling due to an immature TCR. It is for this reason that they develop into a hyperproliferative leukemic T cell.

IK has been implicated in the maturation of T cells in several ways. IK was first identified as a transcriptional repressor for TdT, which is essential for proper T cell receptor expression (Lo et al., 1991). Simultaneously, IK was classified as a “control enhancer element” for CD3δ (signaling component of the TCR) (Georgopoulos et al., 1992). The generation of the IK knockout mouse models

demonstrated a failure for lymphoid differentiation (Georgopoulos et al., 1994; Kirstetter et al., 2002; J. H. Wang et al., 1996). These studies identified IK as a critical regulator in the development of mature lymphocytes by regulating the expression of relevant gene targets, such as TdT and CD3δ.

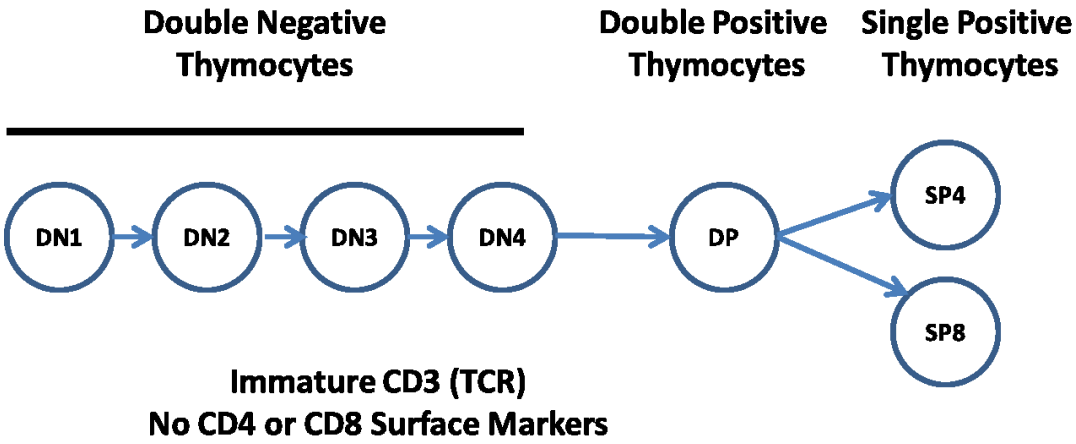


Figure 3. Maturation of T cells within the thymus. Stem cell progenitors originating from bone marrow, enter the thymus and are designated DN (double negative) 1 cells. T cell maturation proceeds through metabolically distinct subsets during which the T cell receptor (TCR) is rearranged (DN3) and co-expression of both CD4 and CD8 surface markers occurs (DP stage). Expression of either CD4 or CD8 is silenced to form either single positive lineage.

Further evidence for IK acting as a master regulator for T cell development was the isolation of a DN3 T cell from a $IK^{+/null}$ mouse, called JE131 cells (Kathrein, Lorenz, Innes, Griffiths, & Winandy, 2005). These cells are a rapidly proliferating immature T cell that is unable to differentiate into DN4 cells due to IK deficiency. Kathrein et al. reintroduced full length IK 1 into the JE131 cells and they successfully differentiated into DN4, DP, and SP cell with a skewing towards CD8 T cell lineage. This demonstrated that IK was a key regulator in the maturation of T cells, and possibly more important for CD8 T cells.

Ikaros Isoforms and their Role in Leukemia

Alternative splicing in eukaryotic cells is nature's mechanism to derive several protein isoforms from a single gene by alternately processing RNA. Transcribed RNA contains both introns and exons, called primary mRNA, which requires intron removal prior to protein translation. The process of removing introns from RNA, called splicing, is catalyzed via the protein/RNA spliceosome complex (Grabowski, Seiler, & Sharp, 1985). The spliceosome is responsible for excision of introns from the primary mRNA species and rejoining of adjacent exons. During this process, the spliceosome catalyzes two transesterification reactions resulting in removal, and the rejoining of adjacent exons together. Alternative splicing is a process that causes exons to be skipped, thus creating shorter splice variants encoding for different protein isoforms of the "same" gene (Figure 4).

Several different IK isoforms have been identified in T cells. To date, seventeen different isoforms have been reported (Iacobucci, Lonetti, Cilloni, et al., 2008; Javahery, Khachi, Lo, Zenzie-Gregory, & Smale, 1994; Meleshko et al., 2008; Payne et al., 2003; Payne et al., 2001). The two major isoforms expressed in primary T cells are IK 1 and IK 2 (Hahm et al., 1994). Both of these isoforms contain the DNA binding domain (DBD) consisting of three or four zinc fingers that facilitate high affinity DNA binding. These two isoforms are maintained in naïve T cells and are responsible for maintaining expression and preventing rapid cellular proliferation by setting the threshold for T cell activation (Avitahl et al., 1999; Ronni et al., 2007). Upon activation of human T cells, IK H isoform is upregulated. It is

hypothesized that each of these isoforms has a unique biological function. IK 1 co-localizes to pericentromeric heterochromatin (PC-HC), which coincides with genetic silencing while IK H does not have the same localization pattern suggesting it may have a different role in gene regulation (Li et al., 2011).

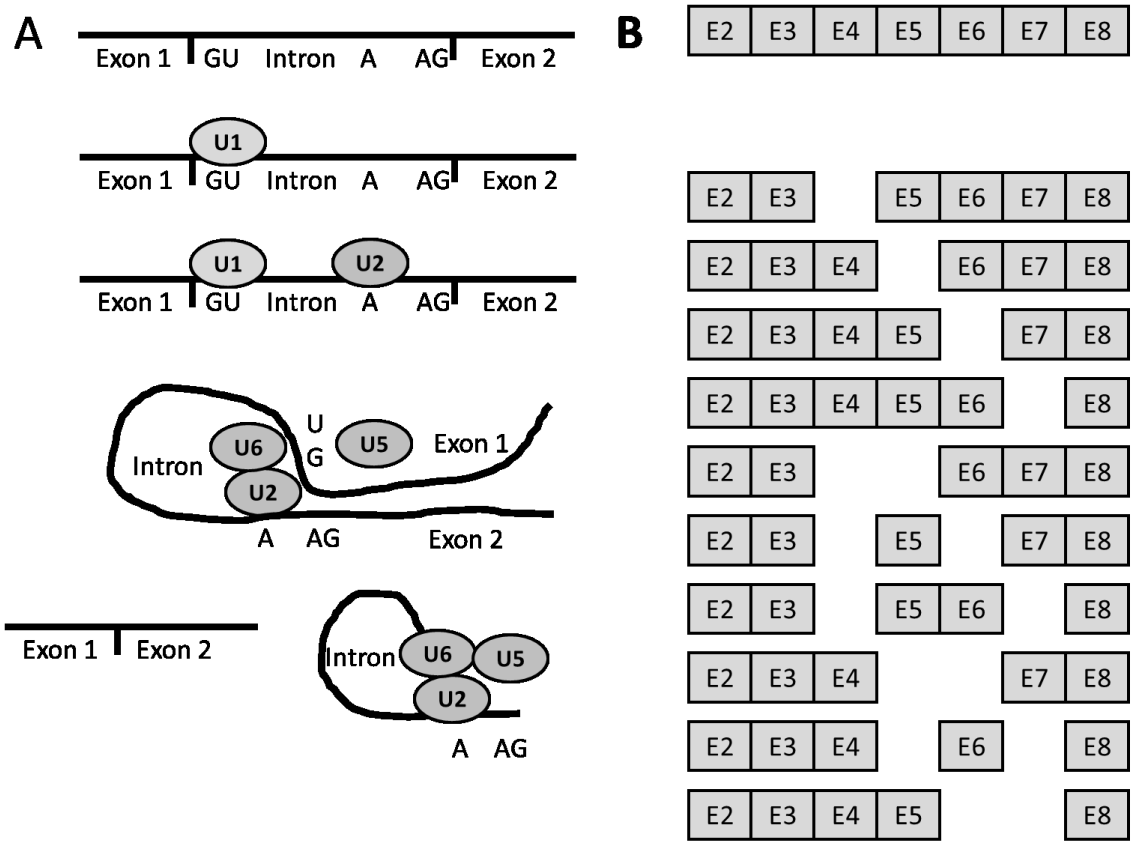


Figure 4. Alternative splicing mechanism schematic for mRNA. A. Splicing of primary RNA is catalyzed by the spliceosome consisting of proteins and RNA molecules to remove introns and rejoining exons. **B.** Alternative splicing caused by skipping exons can create several different mRNA species that encode for different protein products.

Additional IK isoforms to those discussed above have been identified. All known isoforms contains exons 2, 3, and 8, but exons 3a-7 can be alternatively spliced out generating additional IK isoforms (Figure 5). Note that exon 1 is part of the mRNA species, but it is not translated and its importance is unknown. Most of

IK isoforms lack the DBD or contain ≤ 2 zinc finger motifs and are classified as IK dominant negative (IK-DN) isoforms (Georgopoulos et al., 1994; Iacobucci, Lonetti, Cilloni, et al., 2008; Sun, Crotty, et al., 1999; Sun, Heerema, et al., 1999). The DN designation is because these isoforms are able to dimerize with DNA-binding isoforms rendering them inert. There have also been reports indicating a splicing event truncating exon 8. Taken together, the IK gene can produce up to 64 possible mRNA transcripts. Most of the DN isoforms lack the nuclear targeting sequence hypothesized to be encoded within exon 6, resulting in there sequestration in the cytoplasm (Cobb et al., 2000). IK isoforms unable to transverse the nuclear membrane are hypothesized to dimerize with larger forms to enter the nucleus (personal communication with Susan Winandy). In the nucleus, dimers containing DN isoforms inhibit DNA binding due to the lack of the critical zinc fingers needed to bind DNA.

Figure 5. Schematic representation of Ikaros protein. The diagram represents the eight transcribed exons. Exons 3A-7 can undergo alternative splicing resulting in numerous Ikaros isoforms. The black vertical bars represent either the DNA binding zinc fingers or the dimerization zinc fingers.

The function of IK-DN isoforms has been identified to inhibit DNA binding, but additional function (e.g. cytoplasmic functions) of these isoforms remains largely unknown. The shortest known isoform, IK 6 has increased expression in

leukemia cells lines. IK 6 has also been implicated in promoting the immortality of T cells by increasing expression of the antiapoptotic factor, termed Bcl-xl (Ezzat, Zhu, Loeper, Fischer, & Asa, 2006), and this and other Bcl-2 family members are known to prevent apoptosis (programed cell death) (Ezzat et al., 2006; Kano et al., 2008).

Regulation of Ikaros

Regulation of IK gene expression has largely remained an enigma along with the control of alternative splicing that form the vast variety of IK splice variants. However, its biochemical activities have been studied extensively in mice to understand their ability to bind DNA and regulate the cell cycle. IK undergoes tremendous posttranslational modifications (PTMs) at numerous residues throughout its primary sequence including phosphorylation, SUMOylation, and ubiqutination that regulate IK's ability to bind DNA and interact with chromatin remodeling factors (Dovat et al., 2002; Gómez-del Arco, Koipally, & Georgopoulos, 2005; Gómez-del Arco, Maki, & Georgopoulos, 2004).

IK's DNA binding affinity is regulated by phosphorylation of specific residues. A critical tyrosine (Y140) residue on the N-terminal region of the linker region between zinc fingers one and two is critical to maintain a dephosporylated state to ensure high DNA binding affinity. This Tyr residue is phosphorylated during the G_2/M transition of the cell cycle, which ablates the DNA-IK interaction (Dovat et al., 2002; Jantz & Berg, 2004). Additional regions within the primary aa sequence classified as p1, p2, and p3 are able to be phosphorylated and alter DNA binding affinity (Figure 6). The p1 site consists of a 21 aa sequence on the

C-terminus that contains five serine/threonine residues that when phosphorylated decrease IK's affinity for certain DNA sequences permitting G_1/S transition leading to cellular division. The p2 site is a single serine residue (S63) and p3 residues were undefined due to a weak phosphorylation pattern (Gómez-del Arco et al., 2004). Interestingly, a recent study by Li et al., revealed in Molt 4 leukemic T cells that there is a different phosphorylation pattern between IK H and 1. IK 1 becomes hyperphosphorylated and IK H is hypophosphorylated during $G_1 \rightarrow S$ progression, while both isoforms show similar phosphorylation patterns in G_2/M most likely due to the evolutionary conserved linker phosphorylation sites (Li et al., 2011).

The phosphorylation of IK is inversely correlated to its DNA binding affinity. In the naïve state, IK 1 is hypophosphorylated and has a high affinity for DNA, recruiting the biological machinery needed to either enhance or inhibit transcription. Upon T cell activation proliferation increases by 200 fold (Winandy et al., 1995) and cells transition from G_1 to S phase. TCR stimulation results in an increase in activity of the oncogene casein kinase II (CK II) (Filhol, Cochet, & Chambaz, 1990a, 1990b; Yu, Spector, Bae, & Marshak, 1991). CK II has increased activity in several different types of cancer (Ahmed, Gerber, & Cochet, 2002; Unger, Davis, Slaton, & Ahmed, 2004) and is the major kinase responsible for the phosphorylation of IK protein at the p1, p2, and p3 regions (Gómez-del Arco et al., 2004). As a result of phosphorylation, IK 1 is recruited to the pericentromeric heterochromatin (PC-HC) and silences genes (Brown et al., 1997; Cobb et al., 2000; Liberg, Smale, & Merkenschlager, 2003). When IK is in a

hyperphosphorylated state, it acts as a proto-oncogene by no longer being able to inhibit (and possibly promotes) cell cycle progression.

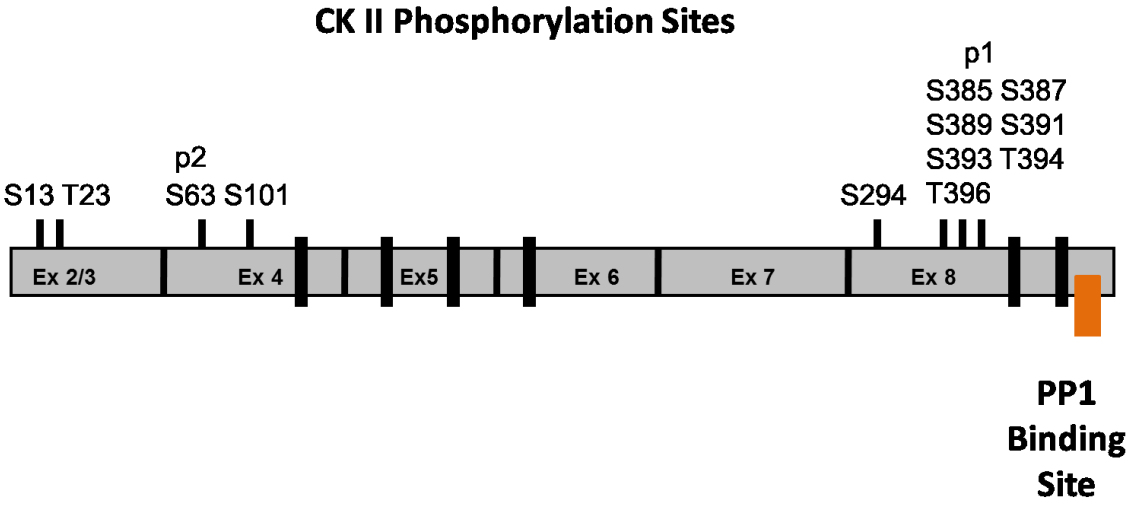


Figure 6. Casein kinase II phosphorylation sites and protein phosphatase 1 binding sites in Ikaros protein. Known phosphorylation sites within Ikaros' primary amino acid sequence by casein kinase II (CK II) are indicated above (p1 and p2). The protein phosphatase 1 (PP1) binding site located on the far C-terminus of the Ikaros protein as indicated by the vertical orange rectangle, which is responsible for dephosphorylating Ikaros. Vertical black bars represent the zinc fingers encoded in their respected exons (Ex).

Protein phosphatase 1 (PP1) maintains IK in a hypophosphorylated state *in vivo*. PP1 binds to IK near its C-terminal dimerization domain (Figure 6). When IK's PP1 binding site is mutated, loss of DNA binding occurs due to an increase in phosphorylation at the p1 site, and IK 1 fails to localize to PC-HC during S phase of the cell cycle. The dynamic phosphorylation pattern of IK is controlled therefore, by opposing enzymatic activities of PP1 and TCR dependent CK II. A shift in this phosphorylation equilibrium has pronounced effects on DNA-binding affinity, subnuclear localization, and gene expression (Gurel et al., 2008; Popescu et al., 2009). In a hyperphosphorylated state, IK protein undergoes ubiquitination ultimately resulting in protein degradation (Gómez-del Arco et al., 2004; Popescu

et al., 2009). Whether PP1 activity can rescue hyperphosphorylated IK protein from degradation is not known.

IK protein can be SUMOylated (SUMO), which is a small ubiquitin-like protein modification, at two lysine residues (K28 and K240). This modification alters IK's ability to regulate gene expression. SUMOylated IK protein still undergoes its dynamic redistribution to the PC-HC upon G_1/S transition, however, decreased gene expression is not observed. This PTM reduces IK's ability to interact with transcriptionally repressive complexes (NuRD, Sin3, and CtBP) (Gómez-del Arco et al., 2005).

Ikaros as an Epigenetic Factor

IK acts as a transcription factor by altering gene expression through the recruitment of histone remodeling complexes (HRC). The DBD of IK consists of four two-cysteine and two-histidine (C2H2) krűppel-like zinc fingers that are tetrahedrally coordinated to a zinc atom to form a ββα secondary structure that binds to the major groove of DNA (Figure 7) (Molnár et al., 1996; Rebollo & Schmitt, 2003). Ikaros and all its family members recognize the same core recognition sequence of GGGAA, and yet the same sequence is utilized by other proteins such as Notch and Elf-1 (Kathrein, Chari, & Winandy, 2008; Molnár & Georgopoulos, 1994).

IK family members are responsible for the recruitment of histone modifying enzymes that modify histone tails and regulate accessibility of the transcriptional machinery that is essential for mRNA synthesis. The HRCs that IK recruits are able to alter gene expression by remodeling chromatin structure to a more

compact state (silencing) or a more open state (permissive) (Figure 8) (Kim et al., 1999; Koipally & Georgopoulos, 2000; Koipally, Kim, et al., 1999; Liberg et al., 2003; O'Neill et al., 2000). Two well characterized PTMs that alter chromatin structure are acetylation and methylation of lysine residues on histone tails that protrude from each spherical nucleosome circumference, the repeating element of chromatin. Acetylation is an epigenetic marker that removes the positive charges on the histone tails and disrupts the electrostatic attraction to the polyanionic DNA double helix “spooled” around it. In addition, acetylated histone tails break the attractive forces adjacent nucleosomes. Collectively, these decreased interactions cause a more open chromatin landscape that is transcriptionally permissive as it allows more efficient accessibility of the basal transcriptional complex (BTC) to bind DNA. Acetylation of lysine residues is catalyzed by histone acetyltransferase (HAT) enzymes. HATs are part of the larger chromatin remodeling complex, called switch/sucrose nonfermentable (SWI/SNF) (Kim et al., 1999). IK can also interact with at least three repressive complexes, two of which contain histone deacetylase (HDAC) enzymes. They are part of the nuclear remodeling and deacetylase (NuRD) complex and Sin3 complex (Ahringer, 2000; Georgopoulos, 2002; Koipally & Georgopoulos, 2002a; Koipally, Heller, Seavitt, & Georgopoulos, 2002; O'Neill et al., 2000). A non-HDAC containing repressive complex that IK interacts with is C-terminal binding protein (CtBP) (Hu et al., 2007; Koipally & Georgopoulos, 2002b; Perdomo & Crossley, 2002).

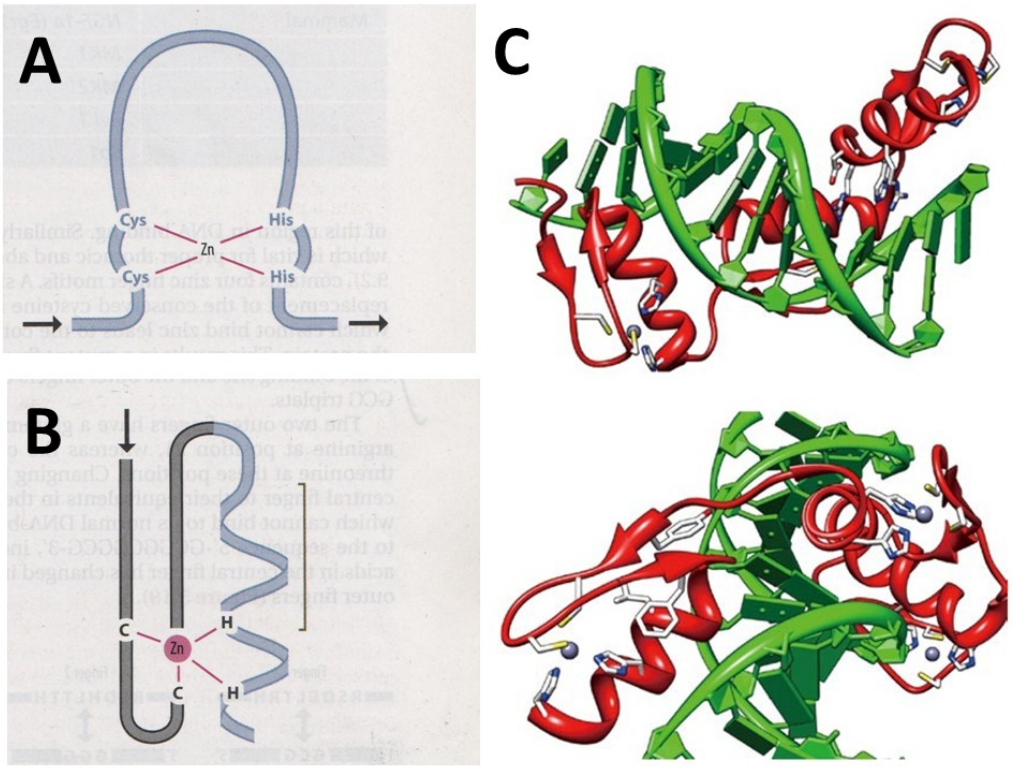


Figure 7. Representation of the krűppel-like zinc fingers and DNA interaction. A. Coordination of zinc in a C2H2 zinc finger. **B.** C2H2 zinc fingers are composed of two antiparallel lines (solid line) and an α-helix (curvy line) which binds to the major groove of DNA. **C.** Representation of three zinc fingers coordinated with the major groove of DNA. Figures are adapted from Gene regulations 6th Ed. and Marvin Payne.

Ikaros' Role as a Transcriptional Activator

Evidence that IK transactivates gene expression has been vital in understanding its role in the immune system. IK binds to the regulatory element of the CD8α gene directing T cell lineage (Harker et al., 2002). IK was also identified as an enhancer of transcription for the CD3δ gene, which must be properly rearranged to form a signaling competent TCR (Georgopoulos et al., 1992). This was confirmed in the JE131 mouse cell line devoid of endogenous IK protein by showing that these cells lacked a rearranged T cell receptor. In addition, this study also identified that IK was a master regulator of the cell cycle by arresting

$G_1 \rightarrow S$ transition and inducing protein expression of $p27^{kip1}$. To confirm these results, the DN IK 7 isoform was reintroduced into JE131 cells and proliferation remained high with no measurable increase in the $p27^{kip1}$ protein (Kathrein et al., 2005).

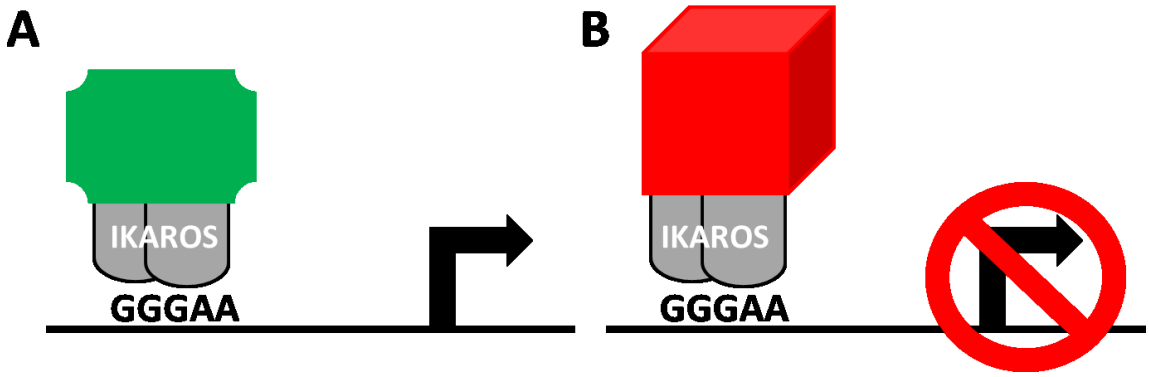


Figure 8. Ikaros acts as a transcriptional repressor or activator for genes. **A.** Ikaros (grey cylinders) binds the core GGGAA DNA sequence and recruits transcriptional activators (green rectangle), which increases the efficiency of the BTC assembly and the transcriptional rate of RNA synthesis. **B.** Ikaros binding to DNA recruits a suppressive complex (red box), which inhibits RNA synthesis efficiency of the BTC assembly.

Ikaros' Role as a Transcriptional Repressor

In addition to transactivation of gene expression, IK also suppresses transcription. Consequently, IK plays a duel role as a transcription factor based on which chromatin remodeling complex it recruits to gene targets. Transcriptional suppression mainly occurs via one of two mechanisms. The first mechanism is through the recruitment of histone modifying enzymes that deacetylate histone tails, restoring their positive charge and allowing for tighter DNA association. Such chromatin structure compaction reduces the efficiency of the basal transcription machinery (BTM) from assembling and results in transcriptional repression. The family of enzymes that accomplish this acetyl group removal are called HDAC

enzymes. Two HDAC containing complexes that IK is known to bind and recruit to promoters are the NuRD and Sin 3 complexes (Koipally & Georgopoulos, 2002a; Koipally et al., 2002; Koipally, Kim, et al., 1999; Koipally, Renold, et al., 1999; Sridharan & Smale, 2007). The second mechanism that IK can inhibit transcription is through an HDAC independent mechanism with the CtBP complex (Koipally & Georgopoulos, 2002b; Sridharan & Smale, 2007).

One gene that IK suppresses is vasoactive intestinal peptide/pituitary adenylate cyclase-activating polypeptide (VPAC) receptor 1 (*Vipr1*). Within the 10 kilobase pair (kbp) sequences flanking the promoter region (5 kbp upstream and downstream of the transitional start site (TSS)) there are seventeen core consensus sites for IK, some of which may play a major role in transcriptional regulation of VPAC1 (unpublished data; Ronni et al., 2007). First identified in 2002, recombinant IK 1 was transfected into a mouse fibroblast cell line (NIH 3T3), and VPAC1 mRNA transcript was suppressed by up to 90%. However, when IK 5 was expressed into these cells, there was no decrease in the expression of VPAC1 mRNA transcript (G. Dorsam & Goetzl, 2002). Together, this study demonstrated that IK-dependent down-regulation of VPAC1 was isoform-specific as the DN IK 5 failed to show an effect. It is this 2002 report that the Dorsam laboratory uses to warrant further investigation on IK-mediated regulation of VPAC1.

Continued research in this area resulted in Ronni et al, confirming that IK protein was bound to the promoter region of VPAC1 by an *in vivo* chromatin immunoprecipitation (ChIP) assay. These experiments indicated that IK was not

engaged at the VPAC1 promoter in naïve T cells, but upon activation, IK bound to its promoter region. Interestingly, VPAC1 is downregulated by 80% in activated T cells. It is enticing to speculate that the downregulation of VPAC1 in activated T cells might be the result of IK acting as a transcriptional inhibitor. Moreover, IK H expression is increased upon T cell activation, and could be reasonable candidate in mediating this silencing of VPAC1. However, ChIP assays specific for IK H were unable to detect IK at the promoter of VPAC1 during T cell activation (Ronni et al., 2007), ruling this isoform out and supporting either IK 1 and/or 2 as the inhibiting isoform. Our laboratory have extended these observations by utilizing HuT 78 cell line that expresses endogenous IK isoforms and showed similar data to primary T cells by suppressing of VPAC1 mRNA levels, in activated cells and correlated with enhanced IK engagement at the VPAC1 promoter by qPCR and ChIP analysis. Additional ChIP analyses revealed NuRD components (HDAC1 and Mi-2β) co-localizing with IK at the VPAC1 promoter suggesting the mode for suppression is through NuRD recruitment. In naïve HuT 78 cells, overexpression of IK 1 yielded no change, while IK 5 resulted in a 20-fold increase in VPAC1 mRNA, but curiously IK was not detected at the VPAC1 promoter (Benton et al., manuscript in preparation). In total, these data imply that IK engages the VPAC1 promoter only during T cell activation (when IK 1 is hyperphosphorylated and IK H is dephosphorylated), recruits NuRD and shuts down transcriptional expression through HDAC activity. IK also plays a repressive role in naïve T cells as well, either in the malignant transformation of HuT 78 cells or indirectly inducing a VPAC1 repressor (Pei, 1998)

Ikaros and VPAC2 Regulation

VPAC2 expression profile in T cells differs from VPAC1 and has been coined the inducible receptor due to its lack of expression in naïve T cells, but is upregulated upon activation. Similarly to VPAC1, VPAC2 contains fourteen IK consensus sequences that could potentially be utilized for gene regulation. This data suggest that VPAC2 may also be regulated by IK similarly to VPAC1. In support of this hypothesis, VPAC2 receptor is expressed very highly in developing wild type DN3 cells, but is undetectable in IK deficient JE131 DN 3 cells (Kathrein et al., 2005; Vomhof-DeKrey, Sandy, et al., 2011). These data demonstrated that IK may not only play a critical role in the expression of VPAC2, but also my act as a transcriptional activator.

Ikaros Regulates the Cell Cycle

T cell activation leads to cell cycle entry, where cells either undergo cell division (proliferation), or initiate program cell death (apoptosis). The cell cycle is divided into four phases termed: Gap 1 (G_1), DNA synthesis (S), Gap 2 (G_2), and mitosis (M). A fifth metabolically distinct cell cycle phase called G_0 also exists, but since T cells are generally not in this phase it will not be discussed further. The majority of cells are arresting in G_1 phase, which is the cell cycle phase where cells increase in size and prepare for $G_1 \rightarrow S$ phase transition and DNA replication. Upon external proliferative signals (e.g. TCR signaling), cells progress from G_1 phase into S phase passing a cellular “point of no return” committing the cell to divide or die. The S phase is when the cell undergoes genome duplication (DNA

replication) in preparation for cellular division. The G2 phase is a gap between synthesis of DNA and mitosis. In this state, cells continue to grow in size and ensure all cellular machinery needed for mitosis is synthesized. Also, the replicated genome is checked for errors and if necessary repaired by the mismatch mechanism. Cellular division occurs during the M phase resulting in the generation of two daughter cells released into the G_1 phase (Nourse et al., 1994; van den Heuvel, 2005).

External signals (e.g. TCR activation or IL-2) enhance the probability for cell cycle entry. In the absence of proliferative external signals, T cells remain in the G_1 phase by maintaining the expression of cell cycle arresting proteins like $p21^{kip1}$, and silencing cyclin proteins that are necessary components of cell cycle promoting cyclin dependent kinases (Cdks). Cdks are small serine/threonine protein kinases that are constitutively expressed in the cell while cyclins are upregulated during specific cell cycle phases (Figure 9). These proteins form Cyclin-Cdk complexes that regulate cell cycle progression. For example, the CyclinE-Cdk2 complex allows for progression from the G_1 to S phase. The $S \rightarrow G_2$ transition is regulated by the activation of Cyclin A and Cdk2. The G_2/M transition is regulated by Cyclin B-Cdk1 complex. Cellular division is completed after the release of two daughter cells into the $G_{0/1}$ phase. This is tightly controlled by Cyclin D and Cdk4/5. The regulation of Cyclins-Cdks activity is further regulated by Cdk inhibitors (CKI), including the Cit/Kip family of proteins that are responsible for preventing transition between cell cycle phases by inhibiting Cyclin-Cdk complexes from phosphorylating their repertoire of proteins necessary for cell

cycle progression (Reynisdóttir, Polyak, Iavarone, & Massagué, 1995; van den Heuvel, 2005). $p27^{kip1}$ is an inhibitor of CyclinE-Cdk2 complexes and arrest cells in the G_1 phase (Denicourt & Dowdy, 2004). Its protein expression is in early G_1 phase, but declines as cells progress toward the G_1/S transition point (Nourse et al., 1994; Reynisdóttir et al., 1995). $p27^{kip1}$ is regulated by phosphorylation. When phosphorylated, it dissociates from the CyclinE-Cdk2 complex and is exported to the cytoplasm for degradation. This dissociation of $p27^{kip1}$ releases active CyclinE-Cdk2 and sets in motion the transition from G_1 to S phase (Denicourt & Dowdy, 2004).

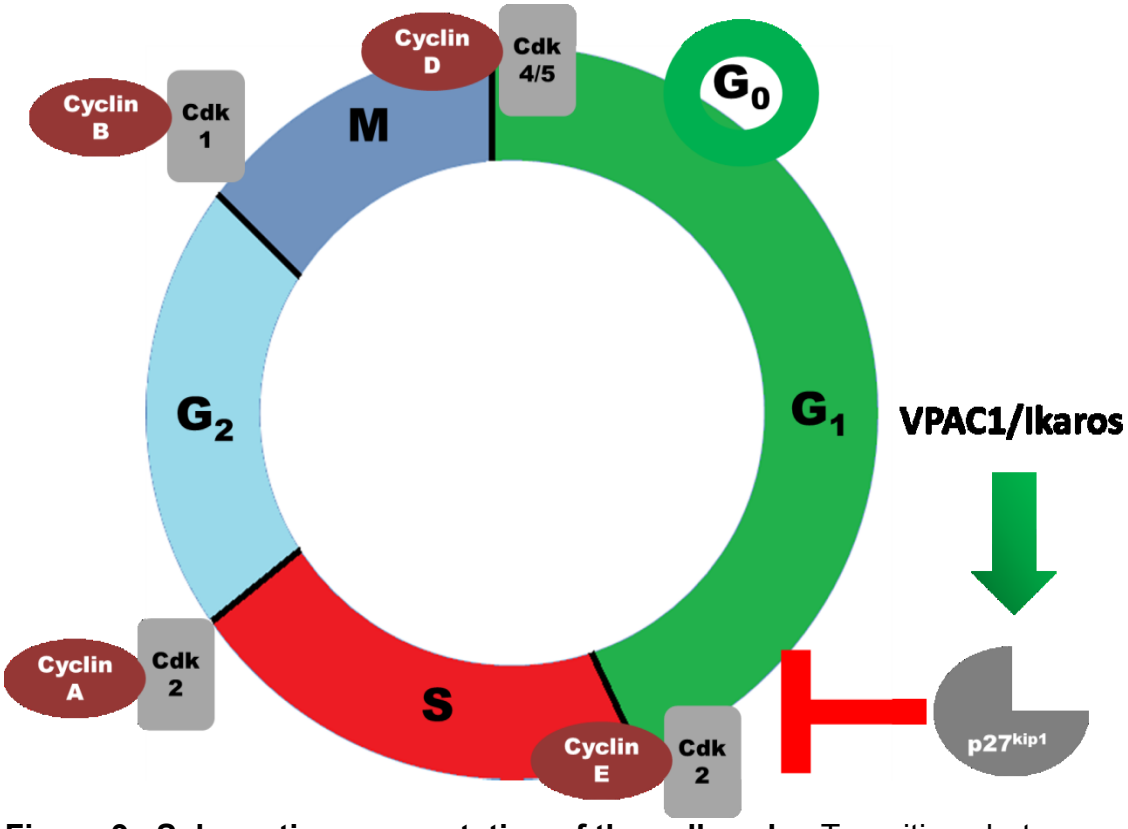


Figure 9. Schematic representation of the cell cycle. Transitions between phases of the cell cycle are regulated by Cyclin-Cdk complex as indicated. G_1/S transition is regulated by CyclinE-Cdk2 enzymatic activity, while $p27^{kip1}$ inhibits its activity. Regulation of $p27^{kip1}$ is regulated by both Ikaros and VPAC1 signaling.

The tumor suppressor IK is known to inhibit cell cycle progression. Kathrein et al. demonstrated that reintroduction of IK 1 into the JE131 murine DN3 thymocytes caused them to cease proliferating and arrest in G_1. This cell cycle arrest was accompanied by differentiation from the DN3 stage to the DP and CD8 single positive subset further supporting IK as an essential factor for T cell development. Ikaros 1 positively regulates the expression of $p27^{kip1}$, and in the absence of IK or presence of DN isoforms there is a lack of $p27^{kip1}$ protein synthesized (Kathrein et al., 2005). These data demonstrated that IK is a *bona fide* transcription factor that regulates cell cycle entry.

Vasoactive Intestinal Peptide Receptor 1 and the Cell Cycle

VPAC1 expression is dependent upon the activation status of T cells. In resting cells, VPAC1 expression is high and transmits signals that negatively regulate TCR signal transduction, and is therefore considered an antiproliferative receptor for T cells. However upon activation, VPAC1 expression is decreased up to 80% within 24 hours of TCR stimulation (Vomhof-DeKrey et al., 2008). Our research strongly supports the idea that TCR-induced downregulation of VPAC1 mRNA is due to the recruitment of the NuRD complex by IK (see above).

VPAC1 signaling regulates T cell activation by inhibiting interleukin-2 (IL-2) upregulation, which becomes elevated 200 fold during T cell activation (Delporte et al., 1995; Ganea & Sun, 1993; Martinez et al., 1999; Ottaway, 1987; Ottaway, Cheng, & Bjerknes, 1987; Ottaway, Lewis, & Asa, 1987; Sun & Ganea, 1993). VIP/VPAC1 signaling inhibits T cell activation and subsequent proliferation in a time dependent fashion. When T cells are pretreated, or exposed to VIP within 30

min of TCR activation, VIP/VPAC1 signaling is able to inhibit IL-2 production and suppress cellular proliferation (H. Y. Wang, Jiang, & Ganea, 2000). VIP/VPAC1 impedes the cell cycle entry of activated T cells by blocking the downregulation of $p27^{kip1}$ protein that in turn potently blocks G_1/S transition by inhibiting CyclinE-Cdk2 activity. VPAC1 signaling maintains $p27^{kip1}$ in a dephosphorylated state and thus inhibiting activity of G_1/S CyclinE-Cdk2 complex and preventing cell cycle entry (Pozo, Anderson, & Gonzalez-Rey, 2009). Thus, both VPAC1 and IK inhibit cell cycle progression by the same protein, $p27^{kip1}$, at different levels of regulation with IK acting on its transactivation, while VIP/VPAC1 signaling blocking is phosphorylation.

Expression of VPAC1 is altered in leukemic cells. In numerous mouse, rat, and human T cell leukemia/lymphoma cell lines with various phenotypic signatures, VPAC1 expression was suppressed or silenced as compared to levels found in mature primary T cells, CD34+ bone marrow progenitor cells, and human reference pool (G. P. Dorsam, Benton, Failing, & Batra, 2011). Many of these T cell lines also had a concomitant elevation in VPAC2 message (Delgado, Pozo, & Ganea, 2004; G. P. Dorsam et al., 2011). Unpublished data collected in the Dorsam laboratory from three human T cell leukemia patients validated that VPAC1 expression was low/absent, while two out of three patients had increased VPAC2 expression. These data clearly show that leukemic cells have a VIP receptor expression profile consistent to that of activated T cells. With low VPAC1 expression, leukemic T cells may more readily bypass cell cycle arrest normally maintained by VIP/VPAC1 signaling thereby providing a growth advantage.

Whether VIP receptor reversal in T cell leukemia is authentic aberrant expression or rather normal for the developmental stage the cell is arrested in is a question currently being pursued by our laboratory.

Cellular Regulation of Apoptosis by Ikaros

Apoptosis is a complex signaling mechanism that is designed to force cellular death under several environmental and/or cellular conditions. T cells undergo apoptosis during contraction phase of an immune response that takes place after pathogen clearance and is necessary for immune homeostasis. Cell suicide also takes place in the thymus during T cell maturation, where it is estimated that 98% of all developing thymocytes die (Yang & Ashwell, 1999). Finally, apoptosis is a critical function to prevent cell division if mutations, DNA lesions or other genetic abnormalities are unable to be corrected. Indeed, cancer cells bypass this process. In many hematological cancers, the Bcl-2 family of antiapoptotic proteins is overexpressed. Cancer cells utilize this defect to prevent cellular death as another growth advantage mechanism (e.g. a growth advantage). Two Bcl-2 family proteins, Bcl-2 and Bcl-xl, are factors that have been implicated in the protection from chemotherapy treatments (Sezaki et al., 2003).

Expression of IK-DN isoforms is increased in both T and B cell leukemia (Iacobucci, Lonetti, Cilloni, et al., 2008; Iacobucci, Lonetti, Messa, et al., 2008; Kano et al., 2008; Russell et al., 2008; Wojcik, Griffiths, Staggs, Hagman, & Winandy, 2007). As DN IK isoforms interact with DNA binding isoforms either in the nucleus or after *de novo* synthesis in the cytoplasm, they neutralize the DNA binding potential of the cellular IK pool thereby blocking its ability to differentially

regulate gene expression and/or “tether” distal chromosome regions together. It is these DNA binding activities that mediate IK’s tumor suppressor activities. However, it is unclear whether IK-DN isoforms have distinct functions separate from its dominant negative activities. The overexpression of DN isoforms has been implicated in the survival of leukemic cells (Sezaki et al., 2003). The increase in expression of IK 6 has been found in myeloid and lymphoid leukemia cells along with an increased expression of the antiapoptotic factor Bcl-xl protein. This elevated expression was a result of increased acetylation in the promoter of the Bcl-xl gene. Interestingly, knocking down expression of all IK isoforms by short interfering RNA (siRNA) was unable to mimic the increase of Bcl-xl protein expression, demonstrating that IK 6 was altering gene expression by binding to and preventing HDAC histone remodeling complexes (e.g. NuRD and Sin3) from being recruited to the Bcl-xl promoter (Ezzat et al., 2006; Kano et al., 2008). However, in order for this interpretation to be valid, siRNA knockdown of IK must have engaged a compensatory IK-dependent mechanism to recruit HDAC histone remodeling complexes to the Bcl-xl locus, but these studies have yet to be completed.

VIP and the Immune System

Vasoactive intestinal peptide (VIP) is a neuropeptide that has many effects on the immune system. VIP can be delivered to organs by innervating VIPergic nerves to the lungs, spleen, thymus, and gut where immune cells reside (Bellinger et al., 1996; Felten et al., 1985). Secondly, VIP is secreted by T cells, specifically CD4 Th2 effector cells (Delgado & Ganea, 2001). When VIP is secreted, it is able

to act in either an autocrine (on self) or paracrine (on neighboring cells) fashion. VIP binds to VPAC1 receptors resulting in an antiproliferative signaling cascade, which inhibits IL-2 due to elevated cyclic adenosine monophosphate (cAMP) levels within the cell. The inhibition of IL-2 by VIP occurs in both CD4 and CD8 T cells (H. Y. Wang et al., 2000). The decreased expression of IL-2 transcript was further identified to be controlled by a decrease in c-Jun expression in T cells and an upregulation of JunB protein (Tang, Welton, & Ganea, 1995; H. Y. Wang et al., 2000). Transcriptional regulation of IL-2 is controlled by several regulatory sites for AP-1, NFAT, NF-κB, Oct, and CD28RE. The AP-1 transcription regulating complex consists of a heterodimer containing c-Fos/c-Jun, which binds to the promoter and activates transcription. VIP signaling decreases c-Jun protein expression allowing for JunB to replace c-Jun in the AP-1 complex. This altered AP-1 complex is no longer able to be recruited to the promoter of IL-2 resulting in decreased expression (H. Y. Wang et al., 2000).

Additionally, VIP is able to control T cell biology in its ability to regulate cell differentiation. VIP acts on newly activated T cells and skew differentiation towards less proliferative Th17 and/or Treg effector cells (Figure 10). Such an effect would inhibit Th1 inflammatory effector cell generation. It is noteworthy to mention that such a skewing towards Tregs, for example, would be considered antiproliferative. Lastly, Th2 effector cells may result if VPAC2 levels become induced during T cell activation as supported by VPAC2 knockout and transgenic mouse models. VIP/VPAC2 signaling is known to induce cytokines that are responsible for dictating Th2 differentiation, such as increased levels of IL-4 and

IL-5 (Delgado, Leceta, Sun, Gomariz, & Ganea, 2000; Vassiliou, Jiang, Delgado, & Ganea, 2001). In the manner previously described, VIP inhibits the production of IL-2 and IFNγ which are critical factors that direct T cell differentiation towards a Th1 response. Additionally, VIP is able to induce a phenotype in T cells known as Tregs. These cells are able to limit an immune response and "regulate" their activation.

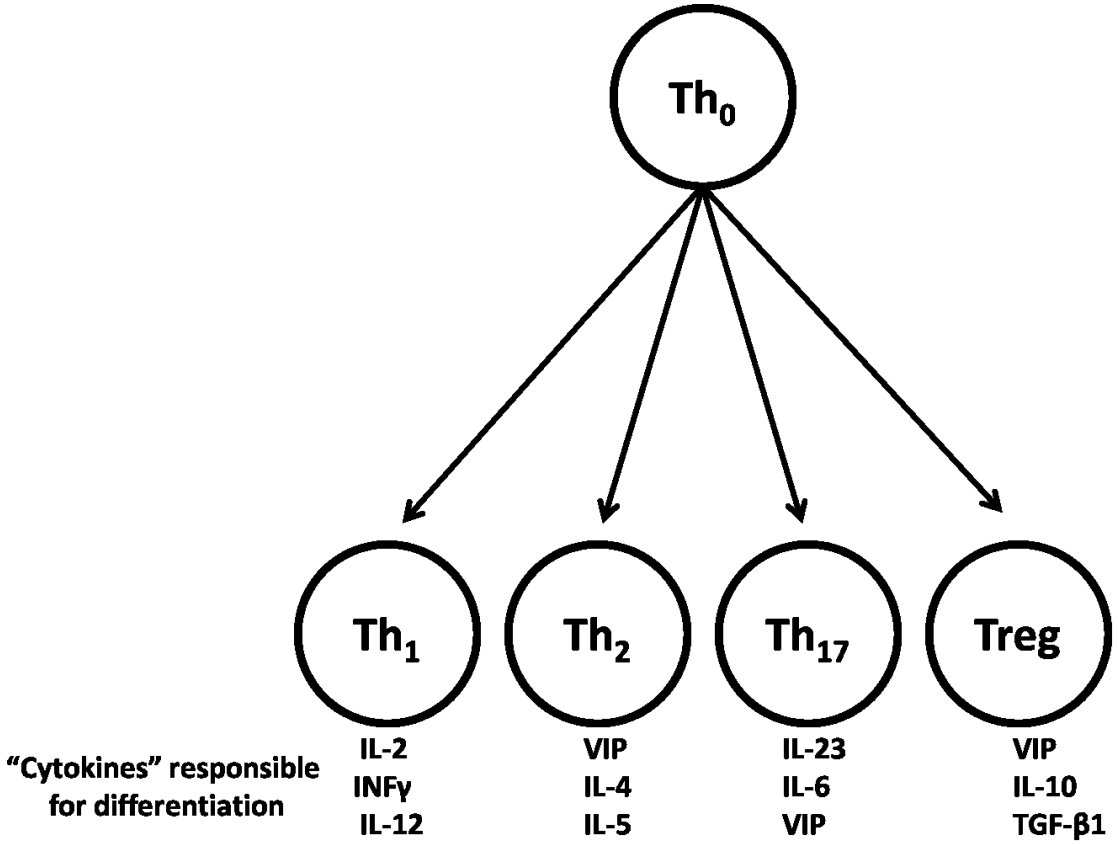


Figure 10. Differentiation of Th0 CD4 T cells into effector T cells. Naïve T cells upon activation (Th0) differentiate into one of four different effector cells. Effector cell differentiation occurs due to the presence of different cytokines that commit cells into Th1, Th2, Th17, or Treg cells.

VIP is able to regulate T lymphocyte apoptosis by regulation of cell surface proteins. Cytotoxic T cells kill other T cells by cellular death upon the interaction of Fas/FasL. The increased expression of FasL on T cells interacts with Fas on the

surface of cytotoxic T cells resulting in cellular apoptosis due to fragmentation of DNA. VIP decreases expression of FasL on CD4 T cells, thus averting cellular death (Delgado & Ganea, 2000a, 2000b, 2000c). Th1 cells are more susceptible to apoptosis by FasL than Th2 cells as VIP protects Th2 cells better than Th1 (Roberts et al., 2003; Zhang et al., 2003).

An additional mechanism for T cell death is the upregulation of granzyme B (GrB) (Delgado & Ganea, 2000a; Sharma, Delgado, & Ganea, 2006a, 2006b). In Th2 effector cells there is an increase in VPAC1 and 2 mRNA while in Th1 cells VPAC2 expression is similar to the naïve state. When using VPAC2 specific agonists, there was an increase in protection from activated induced cell death (AICD) in Th2 cells, but this was not observed on Th1 cells due to their lack of VPAC2 expression. This mechanism for the increased protection from AICD was determined to be AC→cAMP dependent, but protein kinas A (PKA) independent. It was determined that exchange protein directly activated by cAMP (EPAC) activation resulted in the ablation of AICD, demonstrating that VIP signaling through $G_{\alpha s}$→AC→cAMP→EPAC resulted in altered gene expression of GrB. In summary, VPAC2 specific agonist induce survival of Th2 cells, but not Th1 cells, by preventing the stimulation of GrB and the upregulation of FasL (Sharma et al., 2006a, 2006b).

VIP Induces Chemotaxis

The biological effect of chemotaxis for T lymphocytes towards VIP was first discovered by Ottaway et al (Ottaway, 1984). He identified that T cells were able to migrate to the Peyer's Patches (PP) within the gut demonstrating that VIP

directed cellular migration (chemotaxis). These data also showed that upon pretreatment with VIP, which lowered VIP receptor binding sites on the cell surface, before replacing T cells back into rats, they lacked the ability to migrate to the PP demonstrating that this phenomenon was due to the effects of VIP receptors present on the cell surface. Not known at the time, VIP treatment internalized the predominant VIP receptor expressed in T cells, VPAC1, and supporting their involvement in T cell migration to the gut (Ottaway, 1984; Ottaway, Cheng, et al., 1987; Ottaway & Greenberg, 1984). Additional studies have demonstrated that VIP can stimulate chemotaxis *in vitro* for both CD4 and CD8 T cells. These data confirmed that chemotaxis was controlled by VPAC receptors. Activated T cells were not as responsive to VIP in chemotaxis assays compared to naïve T cells. This difference was explained by the direct correlation between VIP receptor surface expression (Johnston et al., 1994). Interestingly, contradictory data has been presented demonstrating that VIP is able to inhibit chemotaxis in cells that express VPAC1. These data were collected from a transformed lymphoblastic T cell line (HuT 78 cells), and demonstrated that VIP was able to inhibit cellular migration towards TNFα and IL-4 (Xia, Gaufo, Wang, Sreedharan, & Goetzl, 1996). Similarly, Th1 cells also lack significant chemotactic response to VIP, which predominantly expresses VPAC1 at lower levels. While VPAC2 expressing cells (SupT1 cells) moved towards VIP in a concentration dependent fashion (Xia, Leppert, et al., 1996; Xia, Sreedharan, Dazin, Damsky, & Goetzl, 1996). In toto, these data demonstrated a discrepancy between wild type and leukemic T cells. Wild type cells are sensitive towards VIP mediated by

VPAC1 in naïve T cells and by VPAC2 in Th2 effector cells, but in contrast, VPAC1 expressing leukemic T cells have retarded chemotactic responses, and VPAC2 expressing leukemic cell have robust migration responses towards VIP.

VPAC Receptors on T Cells

The expression profile of VIP receptors has been investigated from the earliest of T cell progenitors (ETP/DN1) in the thymus to mature CD4/CD8 T cells. Expression mapping of both VPAC1 and 2 has been completed during the maturation process of T cells in the thymus by Vomhof-DeKrey et al. In total thymocytes, VPAC1 is the predominantly expressed receptor; however, there is an alteration in expression during specific phases of T cell maturation. In the DN1/ETP population VPAC1 was highly expressed (1000:1 ratio, VPAC1:VPAC2) while upon differentiation into DN2 cells VPAC1 expression plummets to nearly undetectable levels while VPAC2 increases (1:4 ratio). Further differentiation into the DN3 subset reveals VPAC2 levels increase while VPAC1 remained low (1:11 ratio). Interestingly, the DN4 stage showed restoration of VIP receptor ratios with VPAC1 expression 18-fold higher than VPAC2 (Vomhof-DeKrey, Sandy, et al., 2011).

Further maturation of T cells identifies that VPAC1 is the predominantly expressed receptor in mature T cells (CD4 and CD8). In murine T cells, VPAC1 is predominantly expressed receptor in both CD8 and CD4 T cells. Upon activation VPAC1 expression decreases in both cell types and VPAC2 has induced expression in CD4 T cells but not in CD8 T cells (Vomhof-DeKrey, Haring, & Dorsam, 2011). Similarly, CD4 T cells can be further subdivided into different

effector cells. These include Th1, Th2, Th17, and Tregs. VPAC1 is the predominantly expressed receptor in Th1 cells with VPAC2 levels similar to naïve T cells. While Th2 cells have and increased expression of both VPAC1 and 2 receptor expression (Lara-Marquez, O'Dorisio, O'Dorisio, Shah, & Karacay, 2001).

Purpose of this Research

The overall goal of this research was to investigate how VIP affects T cell biology. To this end, we first focused on whether VIP signaling could change the expression levels and/or phosphorylation profile of the master T cell regulator Ikaros. Second, we asked whether VIP signaling, through VPAC1 versus VPAC2, induced different chemotactic effects in T cells.

The purpose of the first project was to determine if VIP/VPAC1 signaling altered IK biology. Both VPAC1 and Ikaros are important for maintaining T cells in an unactivated state. VPAC1 suppresses T cell activation by inhibiting IL-2 production and inhibiting the G_1/S transition in the cell cycle by maintaining the CyclinE-Cdk2 inhibitor protein $p27^{kip1}$ in a dephosphorylated state. Additionally, IK protein sets the threshold for T cell activation by acting as a master regulator that controls the expression of $p27^{kip1}$ transcription. IK in the hypophosphorylated state is able to bind DNA and allow transcription or actively repress it. These critical functions of IK are dependent upon its phosphorylation state. Additionally, it has been reported that CK II is the major kinase responsible for the phosphorylation of IK, while additional kinase consensus sites were also identified. Therefore, we hypothesized that VIP signaling though the VPAC1 receptor would alter the expression of IK protein levels and its phosphorylation pattern. To test this

hypothesis, we utilized the leukemic T cell line HuT 78 cells and determined the expression of IK protein. IK protein levels and alterations in phosphorylation were determined by one- and two-dimensional Western blot analysis. We determined that VIP/VPAC1 signaling in naïve HuT 78 cells did not alter IK protein expression. However, in activated HuT 78 cells there was a decrease in nuclear IK protein. Additionally, we also determined that VIP/VPAC1 signaling in activated HuT 78 cells had an increase in the number of isoelectric IK species. These data supported that VIP/VPAC1 signaling in activated Hut 78 cells resulted in alterations in IK biology. VIP/VPAC1 signaling, presumed through $G_{\alpha s}$ activates PKA, results in increased IK phosphorylation and subsequently IK protein is degraded resulting in the decrease of IK protein observed in VIP treated activated HuT 78 cells

The goal of the second project was to determine if the biological role of VPAC1 and VPAC2 on chemotaxis was a result of different receptor function or signaling cascade. Due to the discrepancy of the chemotactic responses elicited by T cells where naïve T cells were chemotactic towards VIP while Th1 effector cells are unable to migrate towards VIP, even though both cells predominantly express VPAC1. Additionally, activated T cells have a retarded chemotaxis response while Th2 effector cells migrate similar to naïve T cells towards VIP. Lastly, VPAC2 expressing SupT1 cells show a migratory response to VIP. Therefore, we hypothesize that VPAC1 and 2 chemotactic responses would be through the same signaling cascade. To test this hypothesis, we utilized two leukemic T cell lines that predominantly express either VPAC1 or VPAC2. We

investigated the signaling cascade by measuring intercellular cAMP response upon ligand simulation and coupling to $G_{\alpha s}$. The biological function of chemotaxis was determined by quantitating the number of cells that migrated towards VIP in a modified Boyden chamber. We discovered that VPAC1 couples less efficiently to $G_{\alpha s}$ than VPAC2 resulting in different cAMP profiles elicited upon VIP treatment. Additionally, we discovered that both VPAC1 and VPAC2 expressing cells migrated towards VIP, but using different signaling cascades to achieve cellular migration. To this end, we were able to conclude that VPAC1 and VPAC2 have similar biological effects on T cells, but the method of interpreting external stimuli (VIP) is different between the two receptors.

These studies shed light on the potential regulation of the tumor suppressor IK and the signaling cascade responsible for T cell migration toward the neuropeptide VIP. These data suggest that inhibition of VIP signaling in activated leukemic T cells may allow for IK protein to suppress T cell activation by the recruitment of IK protein into the nucleus. Additionally, inhibition of both VPAC1 and 2 receptors in leukemia may prevent the migration of T cells to regions rich in VIP (e.g. lungs) where VIP/VPAC2 signaling increases cellular survival by the dysregulation of apoptosis.

CHAPTER 2. THE ROLE OF VIP SIGNALING ON THE EXPRESSION LEVELS AND PHOSPHORYLATION PROFILE OF THE TUMOR SUPPRESSOR IKAROS IN HUT 78 T CELLS

Introduction

Ikaros

Ikaros was discovered by two independent laboratories. The lymphoid specific transcription factor LyF-1 was discovered by the Smale group (UCLA) in 1991. LyF-1 was identified to bind to the TdT gene and suppress its transcriptional expression (Hahm et al. 1994, Lo, Landau and Smale 1991). TdT protein is responsible for the insertion of nucleotides into the coding joints of the T cell receptor (TCR, CD3) which, allows for the TCR diversity to recognize antigens (Morabito et al. 1987). At the same time IK was discovered by Katia Georgopoulos (Harvard University). IK was identified as a transcription factor that acted as a "control enhancer element" for the CD3δ gene (TCR) (Molnár and Georgopoulos 1994, Georgopoulos, Moore and Derfler 1992). The LyF-1 protein was identified to be encoded by the IK gene (*Ikzf*1) two years later (Hahm et al. 1994).

IK is a hematopoietic specific transcription factor found in most mature blood cells and in their most early progenitor bone marrow stem cells. IK mRNA expression was identified in embryonic pluripotent stem cells, thymus, and spleen in developing mouse embryos. *In situ* hybridization revealed that pluripotent stem cells express IK mRNA in cells that developed into the liver, but expression was lost at day 10.5 when stem cells differentiate and commit to a liver lineage. In

cells that would differentiate into the thymus and spleen (development of these organs begins at day 16), IK expression was increased and remained elevated throughout the development of the embryo. These data demonstrated that IK mRNA was expressed in pluripotent stem cells and in organs where T cells develop and reside.

Ikzf1 gene encodes a zinc finger protein. *Ikzf1* gene is located on chromosome 11 in human and chromosome 7 in mice (Iacobucci et al. 2009). The IK gene is transcribed into eight exons, seven of which, are translated into full-length IK 1 protein. IK 1 is a 519 aa protein that contains six zinc fingers (Hahm et al. 1994, Molnár and Georgopoulos 1994). Two C-terminal hunchback-like zinc fingers are encoded within exon eight and are responsible for IK dimerization. Additionally, there are four N-terminal krűppel-like zinc fingers that are encoded within exons four, five, and six. These four fingers constitute the DNA binding domain (DBD), which binds to the core sequence of GGGAA with high affinity (Figure 5) (Molnár and Georgopoulos 1994). Three of the four krűppel-like fingers are needed to bind to the major groove of DNA (Figure 7) (Molnár and Georgopoulos 1994, Hahm et al. 1994).

IK primary transcripts are alternatively spliced generating a multitude of protein isoforms. Five additional IK transcripts were initially identified along with IK 1 (Hahm et al. 1994, Molnár and Georgopoulos 1994). These transcripts were a result of alternative splicing of exons 4-7. IK 2 transcript lacks exon 4 and one of the DNA binding zinc fingers, but still has high DNA binding affinity. Additional isoforms identified were IK 3, 4, 5, and 6. Each of these isoforms share the first 53

amino acids which, are encoded by exons 2 and 3 and the last 236 aa encoded by exon 8. Isoforms 4-6 contain 0-2 of the krűppel-like zinc fingers and are unable to bind DNA.

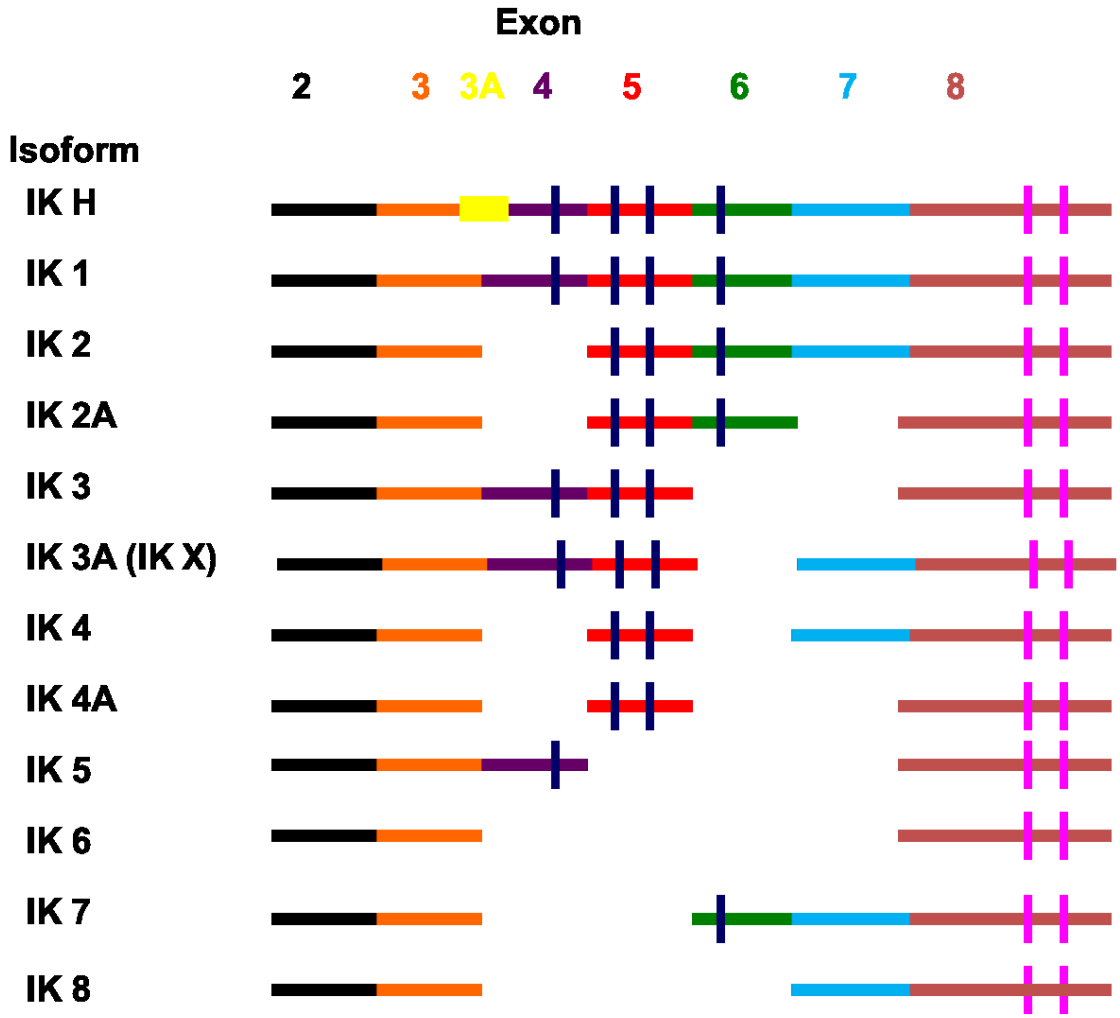


Figure 11. Alternatively spliced mRNA yields several Ikaros isoforms. Schematic representation of the predominant Ikaros isoforms found in mouse and human T cells. Exons 4-6 encode the DNA binding domain with the vertical bars, and the dimerization zinc fingers are located in exon 8. IK 5, 6, and 8 isoforms lack the needed DNA binding zinc fingers and are classified as dominant negative.

Alternative splicing of exons 4-7 for mouse IK made the theoretical number of possible IK isoforms equal to 64. Similar to mice, all known human IK isoforms contain exons 2, 3 and 8, where exons 4-7 are alternatively spliced. Unique to

humans, an additional 60 bp sequence was discovered in human IK transcripts and was termed exon 3A (Sun et al. 1999, Nakayama et al. 2000). IK transcripts containing exons 1-8 and 3A encodes IK H (H for human), the largest molecular weight IK isoform surpassing IK 1 due to the addition of exon 3A. Due to the high sequence homology between human and mice, it is reasonable to assume that IK H would also be expressed in mouse. However, to date its expression in mouse has not yet been reported, and therefore is most likely expressed at substantially lower levels if at all. The lack of detection in murine cells suggests that there is a species difference in IK biology. The additional exon 3A has been identified in two other isoforms called IK 5+ and IK 6+ (Iacobucci et al. 2008, Payne et al. 2003). The "+" nomenclature corresponds to the presence of exon 3A. Additionally, human IK contains an alternative splicing site that truncates 10 aa from the C-terminus of exon 7 (Payne et al. 2003). The additional exon and the additional splicing site increase the number if IK isoforms reported to 17 (Figure 11).

IK isoforms that lack three of the four krűppel-like zinc fingers are unable to bind DNA and are classified as DN-IK isoforms. The hunchback-like dimerization zinc fingers allows for IK protein to bind as hetero- or homodimers. Homodimers of IK H, 1, or 2 contain the needed three or four zinc fingers in the DBD that bind the major groove of DNA. IK 5, 6, and 8 homo-and heterodimers (even with isoforms containing 3 or more zinc fingers) lack more than two zinc fingers in the DBD and are unable to bind DNA thus acting in a dominant negative fashion (Figure 12) (Li et al. 2011a, Payne et al. 2011, Sun, Liu and Georgopoulos 1996, Winandy, Wu and Georgopoulos 1995, Molnár and Georgopoulos 1994).

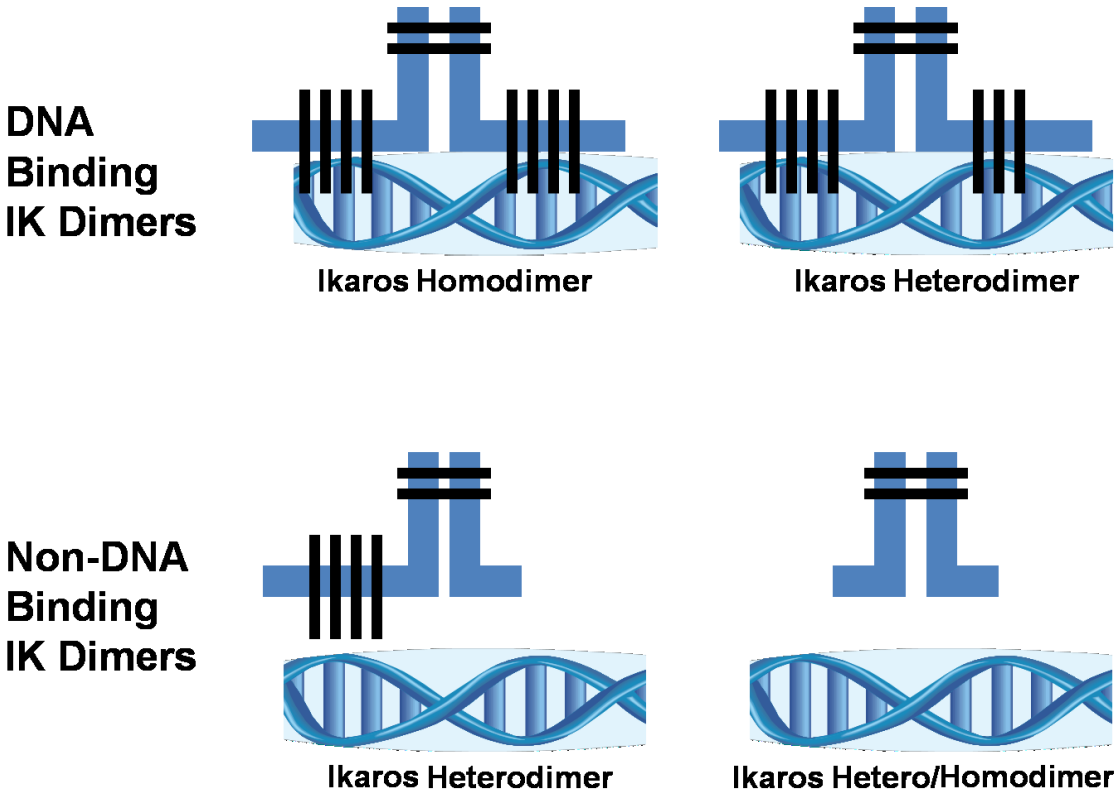


Figure 12. Ikaros forms DNA and non-DNA binding dimers. Ikaros protein forms dimers with their C-terminal zinc fingers. Three or four DNA binding zinc fingers are needed per monomer to bind DNA. IK dimers that lack this number are unable to bind DNA.

Larger IK isoforms reside in the nucleus, while shorter forms are sequestered in the cytoplasm. It is hypothesized that IK contains a nuclear targeting sequence encoded by exon 6. However, no such sequence has been functionally confirmed. Overexpression of larger IK isoforms (1 and 2) has identified that they translocate into the nucleus, while shorter isoforms reside in the cytoplasm. Each of the shorter isoforms lack exon 6, and supports the hypothesis that it encodes a nuclear targeting sequence (Cobb et al. 2000). Interestingly, even though IK 3 and 3A both contain the required three or more zinc fingers for binding DNA, they lack exon 6, suggesting that these two isoform would be unable to enter the nucleus without a compensatory mechanism. These two isoforms

have been identified in the nucleus, and the exact mechanism regarding this movement remains an enigma (Payne et al. 2003, Payne et al. 2001).

Evidence suggests that IK isoforms have distinct functions. For example, the largest human IK H isoform is upregulated upon T cell activation and has different DNA binding affinity than IK 1. This has been demonstrated by its lack of recruitment to PC-HC in cycling cells and its lack of engagement to the VPAC1 promoter as assessed by ChIP assays (Gurel et al. 2008, Ronni et al. 2007). IK 3A has been only identified in myeloid cells isolated from umbilical cord blood, but is “lost” upon birth. IK 3A has not been identified in other cell types to date, suggesting that it has a specific role in myeloid differentiation (Payne et al. 2003, Payne et al. 2001). The IK 6 isoform has been implemented in the immortality of both B and T cells by mediating the regulation the expression of the antiapoptotic factor basal cell lymphoma-extra long (Bcl-xl). This was an important observation showing that DN isoforms were capable of elevating antiapoptotic gene expression thereby acting in a proto-oncogene manner (growth promoting), and the opposite function of their DNA binding counterparts. Because the induction of Bcl-xl was associated with hyperacetylation of histones near its promoter, the mechanism by which IK 6 overexpression works could be through the binding of and sequestration of HDAC containing complexes that are unable to engage the Bcl-xl promoter (Ezzat et al. 2006, Kano et al. 2008).

Dysregulation of the IK genes lead to the loss of T, B, and NK cells. Three transgenic mouse models have been generated to demonstrate that IK is a master regulator in the development of T, B, and NK cells. Two transgenic mice models

generated a reduction in IK protein. These were the IK null and IK deficient models (Figure 2). The IK null mice were generated be replacing exon 8 with the neomycin gene. These mice therefore lacked the dimerization domain and the protein is quickly degraded. Mice that are homozygous ($IK^{null/null}$) lack fetal mature T, B, and NK cells, but upon birth T cells rebound. The rebound of T cells was a result of the increased expression of Aiolos upon birth. Mice that are heterozygous ($IK^{+/null}$) have normal T cell development (Wang et al. 1996, Kelley et al. 1998, Sun et al. 1996). Similar results were obtained in the IK deficient model where exon 3 was swapped for the β-galactosidase gene (Kirstetter et al. 2002). The third mouse model was the IK dominant negative strain, where exons 4-7 were deleted. The $IK^{+/DN}$ mice expressed IK protein that lacked a DBD. These mice also lacked fetal and interestingly adult T, B, and NK cells (Winandy et al. 1995, Georgopoulos et al. 1994). This demonstrated that DN-IK isoforms were able to completely ablate normal developmental activity for DNA binding IK isoforms resulting in the complete block of the lymphoid arm of hematopoiesis.

Heterozygous IK transgenic mice resulted in the formation of T cell leukemia after 3-6 months of age (Figure 2). Homo- and heterozygous IK null or deficient mice developed rapid proliferation of immature T cells and ultimately develop thymic tumors. IK $^{DN/DN}$ mice resulted in a lethal phenotype, while the IK $^{+/DN}$ mice developed thymic tumors within 3-6 months of birth (Wang et al. 1996, Georgopoulos et al. 1994). These studies demonstrated that either a lack of IK or overexpression of DN isoforms resulted in T cell leukemia. Interestingly, due to the lack of B cell development in any of the mouse models, it was not clear the

contribution that IK played in B cell leukemia (Kirstetter et al. 2002, Mullighan et al. 2008, Nera et al. 2006).

Reintroduction of IK protein in IK null JE131 T cells allowed for differentiation and maturation of CD8 T cells. The Winandy laboratory isolated a rapidly proliferating immature DN3 thymocyte from the IK null mouse, clonally expanded the isolated cell to generate the JE131 cell line. IK 1 was reintroduced into the JE131 cells resulted in the further differentiation into DN4, DP, and SP8 T cells. IK 1 resulted in the skewing of mature T cells towards mature CD8 T cells. However, IK 7 reintroduction into JE131 cells failed to differentiate and remained as a DN3 immature thymocyte (Kathrein et al. 2005, Urban and Winandy 2004). These data demonstrated that reintroduction of full-length IK protein, but not DN-IK isoforms, could rescue T cell differentiation and maturation in IK deficient thymocytes when IK protein is absent.

Table 1. Ikaros gene targets and their function.

Gene Name	Biological Function
Terminal deoxynucleotidyl Transferase	Catalyzes the addition of nucleotides in the hyper variable region of the TCR
Cluster of differentiation 8 (CD8)	Co-receptor associated with the TCR
Myc	Oncogenic transcription factor
Cluster of differentiation 3	Couples antigen recognition on T cells to cell signaling
Cluster of differentiation 2	Interacts with lymphocyte expressed molecules to mediate cell adhesion
Recombination activating gene (Rag-1)	Involved in the rearrangement and recombination of immunoglobulin and TCR genes during VDJ recombination
Interleukin 10 (IL-10)	Anti-inflammatory cytokine
Vasoactive intestinal peptide/pituitary adenylate cyclase-activating polypeptide receptor-1 (VPAC1)	Anti-inflammatory/anti-proliferative GPCR that influences immune response against pathogens

IK is a tumor suppressor and regulates cell cycle entry. The dysregulation of the cell cycle results in the rapidly proliferating leukemia T cell phenotype (Hanahan and Weinberg 2000). JE131 thymic cells overexpressed IK 1 and have a reduction in their cellular proliferation rates. It was observed that 80% of JE131 cells were arrested in the G_1 phase of the cell cycle. The decreased proliferation rate was consistent with the increased expression of the $p27^{kip1}$ protein, a Cdk inhibitor (Kathrein et al. 2005). The $p27^{kip1}$ protein is a G_1 cell cycle arresting protein that binds to the CyclinE-Cdk2 protein complex and inhibits its activation. CyclinE-Cdk2 activity is needed for the G_1/S cell cycle progression, and inhibition of this complex prevents cell cycle entry (Figure 9) (Denicourt and Dowdy 2004, van den Heuvel 2005). This resulted in designating IK as a *bona fide* T cell tumor suppressor.

IK's role as a transcription factor occurs through interactions with chromatin remodeling complexes. IK localizes to the PC-HC in cycling cells, which is the site of transcriptionally silenced genes. It interacts with a 2MDa complex known as nucleosome remodeling and deacetylase chromatin complex, which is a chromatin remodeling complex that suppresses gene expression. NuRD consist of several different proteins, such as histone deacetylase 1 and 2 (HDAC 1 and 2), and the ATPase enzymes Mi-2α and Mi-2β (Brown et al. 1997, Kim et al. 1999, Sridharan and Smale 2007). IK has also been co-purified with components of the Sin3 (HDACs and RbAp48) and C-terminal Binding Protein (CtBP) complex (HDACs, znF127, and CtBP1), which are two additional transcriptional repressor complex (Sridharan and Smale 2007). Validation of IK's capacity to regulate gene

expression was confirmed by its ability to suppress gene expression when fused to a Gal4 DBD and transfected into cells containing an artificial readout gene (e.g. luciferase) under the promoter control consisting of tandem Gal4 binding sites. Mutation or deletion of the GGGAA sequence in the promoter of Gal4 prevented IK from binding as determined by electrophoretic mobility shift assays (EMSAs) and expression of Gal 4 was restored. This demonstrated that IK was a repressor when bound to the Gal 4 promoter (Koipally et al. 1999). Besides interacting with repressive complexes, IK has also been co-purified with the switch/sucrose nonfermentable complex (SWI/SNF), which is a transcriptional activator (Sridharan and Smale 2007). Also in developing T cells, a tyrosine kinase receptor called flk-2 had reduced expression along with c-kit in mice that lacked the IK protein. IK has also been demonstrated to up regulate CD8a in the IK null mice when reintroduced (Harker et al. 2002, Nichogiannopoulou et al. 1999). Additional genes that are under tight transcriptional regulation by IK are found in Table 1.

Phosphorylation of IK protein regulates its biological function and interaction with DNA. IK is bound to DNA in the G_1 and S phases of the cell cycle, but affinity is completely lost when cells enter the M phase due to phosphorylation within the DBD (Gurel et al. 2008, Dovat et al. 2002, Gómez-del Arco, Maki and Georgopoulos 2004). During mitosis, IK 1's DNA binding affinity is regulated by a critical tyrosine (Y140) residue that caps the α-helix in the linker region of the DBD (Figure 13). When Y140 is phosphorylated during the G_2/M transition IK's DNA binding affinity is completely ablated. IK H and 1 isoforms contain the Y140 residue and upon entry into the M phase IK protein is found to be diffuse and

unlocalized with DNA (Dovat et al. 2002, Li et al. 2011b). High affinity binding to DNA can also be attenuated by phosphorylation at additional non-DBD regions. In a hypophosphorylated state, IK is able to bind DNA with high affinity (G_1 phase) in euchromatin, but excluded from PC-HC. In a hyperphosphorylated state (S phase) where phosphates are added to regions other than at its linker sites in the DBD, DNA binding characteristics change and show decrease affinity towards DNA consisting of single IK binding sites (euchromatin), but high affinity for DNA consisting of double IK binding sites (PC-HC) (Li et al. 2011b).

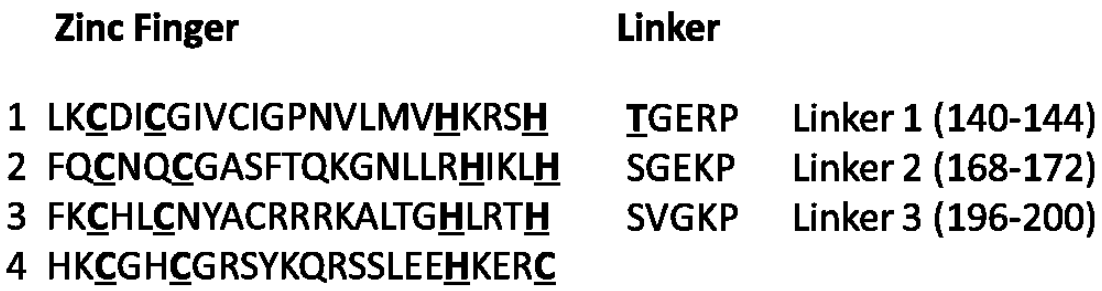


Figure 13. Critical phosphorylation sites in the DBD linker region that inhibits DNA binding affinity. The single letter, amino acid sequence abbreviations for the four DNA binding zinc fingers are shown. The cysteine (C) and histidine (H) residues that tetrahedrally coordinate with the zinc ion are bolded and underlined. The evolutionary conserved tyrosine (T) residue in the first linker between zinc finger 1 and 2 is critical to maintain DNA binding. Upon G_2/M transition the T140 is phosphorylated and DNA binding affinity is ablated.

Three additional regions of IK protein are known to be phosphorylated in addition to the DBD linker regions. These regions are the p1, p2, and p3 sites (Figure 6). The p1 site is located on the C-terminus of the IK protein consisting of a 21 aa sequence containing five serine/threonine residues that upon phosphorylation decreases IK's affinity for gene targets containing a single DNA binding site. When these residues were mutated to an alanine residue (hypophosphorylated) or glutamic acid residue (hyperphosphorylated) IK protein

had high affinity for DNA (Gómez-del Arco et al. 2004). These data suggest that the P1 site is not responsible for attenuating IK-DNA binding. Similar results have been identified where aspartic acid mutations do not mimic the phosphorylated state of a protein (Huang and Erikson 1994, McDowell et al. 2001, Dulhanty and Riordan 1994, Germann et al. 1996). The p2 region is a single serine residue (S63) located near the first DNA binding zinc finger, and p3 was undefined and only possesses few phosphorylation sites (Gómez-del Arco et al. 2004). Both IK H and 1 contain the aforementioned phosphorylation sites, but have opposite phosphorylation pattern during the cell cycle. IK 1 is hyperphosphorylated upon G_1/S transition and co-localizes to the PC-HC, while IK H becomes hypophophorylated and is co-localized to both the PC-HC and other regions of DNA (Li et al. 2011b, Ronni et al. 2007).

Casein kinase II is the major regulatory enzyme responsible for the phosphorylation of IK, including p1 (Gómez-del Arco et al. 2004). Several putative kinase recognition sites for protein kinases such as PKA, PKC, Cdk, and CK II have been identified within IK's amino acid sequence. During T cell activation brought on by TCR signaling, there is a simultaneous increase in the activity of CK II and IK hyperphosphorylation. This correlation led to the discovery that CK II was indeed responsible for the phosphorylation of the p1, p2, and p3 residues of IK (Figure 6). Inhibition of CK II by its specific inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) reduced the phosphorylation of IK at p1, p2, and p3 sites. Additionally, the inhibition of CK II by DRB prevented the progression from G_1 to S in the cell cycle, demonstrating that IK is a tumor

suppressor in the hypophosphorylated state. When CK II was overexpressed, IK protein became hyperphosphorylated and the cells rapidly proliferated (Gómez-del Arco et al. 2004, Dovat et al. 2011, Song et al. 2011). Additional kinases potentially phosphorylate IK, including PKA, but no reports have been published.

Protein phosphatase 1 (PP1) physically interacts with IK thereby antagonizing its phosphorylation by kinases like CK II. PP1 interacts with the RVXF motif located on the far C-terminus of IK (Figure 6). Mutations occurring at this region blocks PP1 from binding to IK and becomes unable to dephosphorylate IK protein shifting the equilibrium towards a hyperphosphorylated state. Such a shift in phosphorylation due to the loss of PP1 binding results in a reduction of DNA binding affinity for IK normally observed in activated, proliferating T cells (Figure 14). The dynamic phosphorylation state of IK is important to maintain in order for the cell to regulate cell cycle arrest versus cell cycle entry. Interestingly, some of the hyperphosphorylated IK protein becomes ubiquitinated and targeted for protein degradation by the 26S proteasome (Dovat et al. 2011, Song et al. 2011, Popescu et al. 2009). In conclusion, it may be the binding of PP1 to IK that prevents a pool of IK avoid degradation during T cell activation and transcriptionally acts on gene targets within the unique environment of the PC-HC.

Thus far, phosphorylation of IK has been identified as the predominant PTM. However, there are additional PTMs that regulate IK biology. As described above, a second PTM that results from the hyperphosphorylation of IK is ubiquitination by E3 ligase that “tags” IK for degradation through the 26S proteasome pathway (Figure 14) (Xie and Varshavsky 2000). A third PTM for IK

protein is SUMOylation (SUMO), a small ubiquitin like protein, at two lysine residues (K28 and K240). This modification significantly alters IK's ability to act as a tumor suppressor. SUMO modified IK protein still undergoes its dynamic redistribution to PC-HC upon G_1/S transition, however, genes normally downregulated by IK are not observed. This PTM reduces IK's ability to interact with repressor complexes (Gómez-del Arco, Koipally and Georgopoulos 2005).

Figure 14. Posttranslational modifications and consequences for Ikaros protein. Ikaros phosphorylation and SUMOylation result in altered biological effects within the cell. IK phosphorylation state is regulated by opposing enzymes CK II and PP1. In the hyperphosphorylated state IK allows for cell cycle progression and IK protein can be ubiquitinated and tagged for protein degradation. SUMOylation of IK protein alters its interaction with HDAC containing complexes.

IK is a transcriptional repressor of the *vipr1* gene (Dorsam and Goetzl 2002). The overexpression of IK 1 in NIH 3T3 cells has been demonstrated to downregulate the expression of VPAC1at the mRNA and protein levels, while IK 5 was unable to decrease VPAC1 expression. EMSAs have demonstrated that IK is able to bind to the promoter of the VPAC1 gene using either GST recombinant IK, overexpressed IK in NIH 3T3 cells or from activated primary T cells (Dorsam and

Goetzl 2002, Li et al. 2011b, Ronni et al. 2007). Similarly, *in vivo* ChIP assays have validated that IK 1 is bound to the VPAC1 promoter after T cell activation that correlated with VPAC1 mRNA downregulation (Ronni et al. 2007). Unpublished data in the Dorsam laboratory has also validated that IK 1/2 is engaged at the VPAC1 promoter in PMA/ionomycin activated HuT 78 cells along with co-engagement of NuRD components, Mi-2β and HDAC1 (Figure 15) (Benton et al.; manuscript in preparation). IK H has not been detected in proximity to the VPAC1 loci, and since it has been demonstrated to act as a transcriptional activator, than perhaps IK 1/2 dimers (homo and hetero) are principally responsible for VPAC1 downregulation (Ronni et al. 2007). The occupancy of IK 1/2, Mi-2β, and HDAC1 complex correlates with the downregulation of VPAC1 expression upon T cell activation. VPAC1 mRNA is downregulated 80% during T cell activation in human and murine T cells (Vomhof-DeKrey and Dorsam 2008, Vomhof-DeKrey et al. 2008, Johnston et al. 1994). VPAC1 protein is also decreased upon T cell activation, presumably due to the decrease in mRNA available to be translated into protein. However, a change in the stability of VPAC1 mRNA during T cell activation cannot be rule out.

Vasoactive Intestinal Peptide Receptor 1

VPAC1 is a GPCR that signals through PKA. VIP is the endogenous ligand that binds to VPAC1 and initiates signaling upon a conformational change in its transmembrane (TM) domains. One conformational change results in TM 3 and 6 separating from each other, and TM 6 rotates revealing critical residues needed for signaling proteins to binding. The exposure of these residues allows for an

important class of signaling molecules to bind, called heterotrimeric $G_{\alpha s\beta\gamma}$ proteins. Receptor interaction with the G protein trimer results in the exchange of GDP for GTP. This exchange leads to the dissociation of the βγ-dimer from the αs-subunit. The αs-subunit translocates along the cytoplasmic leaflet of the plasma membrane, through its palmitoylation modification, and activates adenylate cyclase by docking to a $G_{\alpha s}$ binding site. This causes an increase in adenylate cyclase activity and generates the secondary messenger cAMP from ATP. Two cAMP molecules bind to each of the regulatory subunits of PKA, which releases both catalytic subunits (Figure 16).

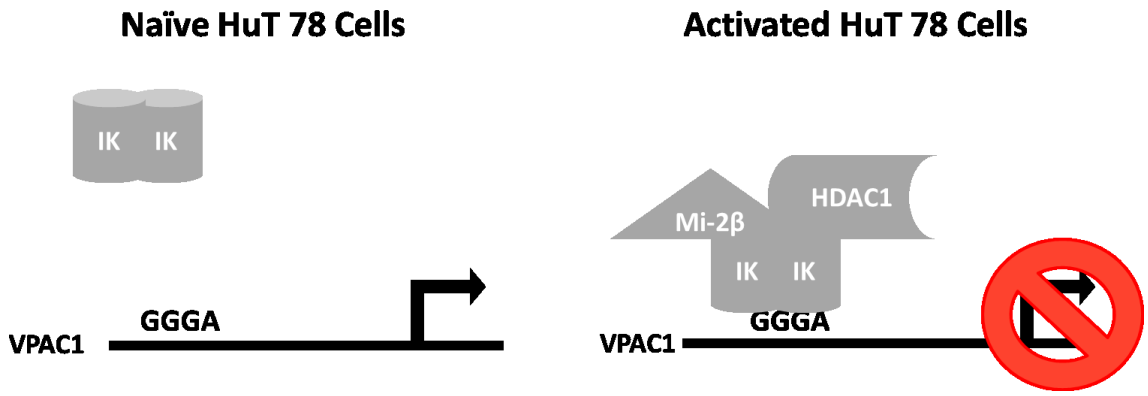


Figure 15. Engagement of IK in the VPAC1 promoter suppresses its transcription. The *vipr1* gene is presented with IK binding sites demarked by GGGA. In naïve HuT 78 cells (left), IK is not engaged at the VPAC1 promoter and *vipr1* is expressed. In activated T cells (right) IK, Mi-2β, and HDAC1 are all found spanning the transcriptional start site of the VPAC1 promoter. Mi-2β and HDAC1 are part of the 2MDa NuRD complex that inhibits transcription through its HDAC and ATPase activity

VPAC1 signaling suppresses T cell activation and its subsequent proliferation by inhibiting IL-2 expression. Additionally, IL-2 activates the CyclinE-Cdk2 complex and is impeded by VIP/VPAC1 signaling (Nourse et al. 1994). The expression of IL-2 is regulated by the transcriptional complex AP-1, which consists of c-Fos and c-Jun (Tang, Welton and Ganea 1995). The cAMP elicited from

VIP/VPAC1 signaling inhibits the expression of c-Jun in T cells and increases the expression of JunB. In activated T cells, c-Fos/c-Jun AP1 complex binds to the promoter of the IL-2 and recruits the transcriptional machinery needed for gene expression. However, due to the suppression of c-Jun and the increase in JunB protein resulting from VIP/VPAC1 signaling, the AP-1 complex replaces the c-Jun with JunB. The c-Fos/JunB AP-1 complex is unable to be recruited to the IL-2 promoter resulting in the lack of IL-2 expression (Wang, Jiang and Ganea 2000). The suppression of IL-2 maintains cells in a non-proliferating state as IL-2 is the major chemokine released to activate T cells.

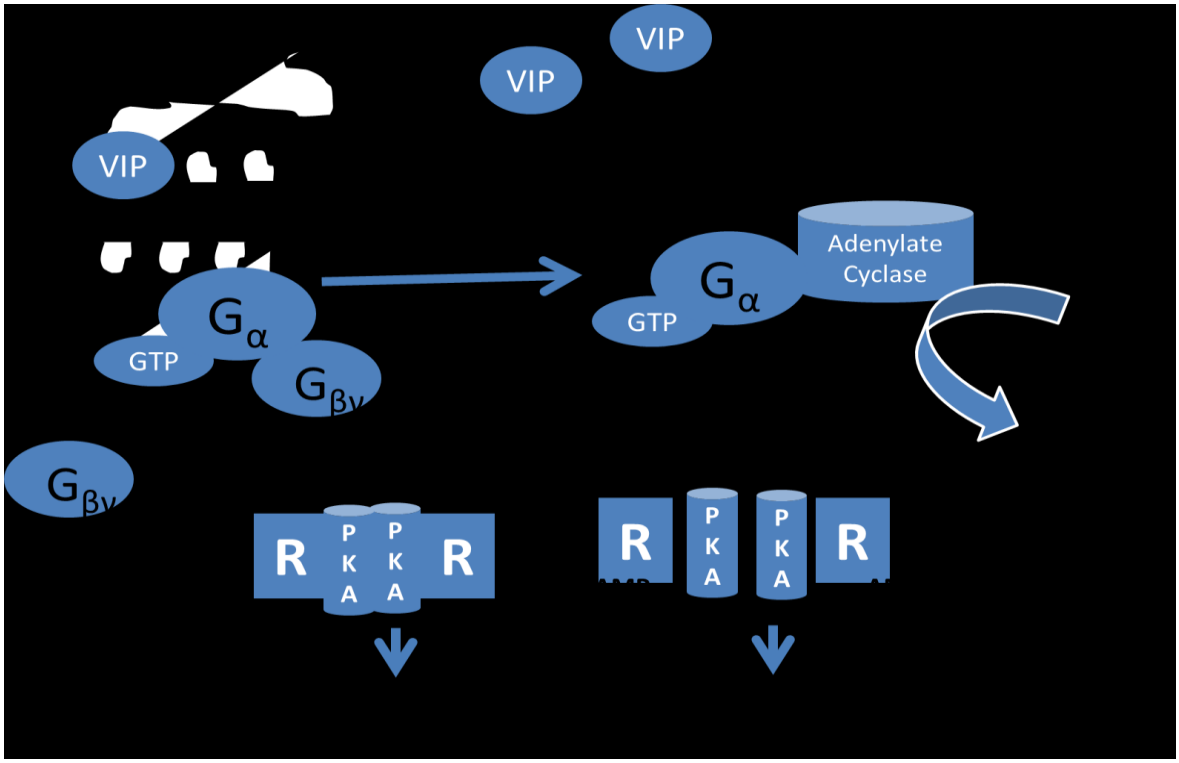


Figure 16. The $G_{\alpha s}$ signaling pathway elicited by VIP/VPAC1 signaling. VIP binds to VPAC1 and initiates the $G_{\alpha s}$ signaling cascade. Upon exchange of GDP with GTP, the α_s-subunit dissociates from the heterotrimeric G-protein complex allowing αs-subunit to activate adenylate cyclase. ATP is converted to cAMP and releases the regulatory subunits necessary to activate PKA. Activated PKA dimers phosphorylate a number of targets thereby "reshuffling" their biological activity and transiently change many cellular functions, including gene expression and chemotaxis.

VIP/VPAC1 signaling additionally suppresses cell cycle entry. In addition to the suppression of IL-2 to impede T cell activation and entry into the cell cycle, VPAC1 also regulates the cell cycle inhibitory protein $p27^{kip1}$. Cell cycle progression is regulated by Cyclin-Cdk complexes, and these complexes are key regulators needed for the transition between phases of the cell cycle. The $p27^{kip1}$ protein binds to the CyclinE-Cdk2 complex and inhibits its activity (Njaine et al. 2010, Denicourt and Dowdy 2004, Nourse et al. 1994). The CyclinE-Cdk2 complex allows for the progression between the G_1 and S phase. In the hypophosphorylated state $p27^{kip1}$ is bound to Cdk2 and inactivates the CyclinE-Cdk2 complex. When $p27^{kip1}$ is phosphorylated it dissociates from Cdk2 and translocates into the cytoplasm where it is degraded. VPAC1 is able to maintain $p27^{kip1}$ in a dephosporylated state thus preventing cell cycle entry (Pozo, Anderson and Gonzalez-Rey 2009). Additionally IL-2 activates the CyclinE-Cdk2 complex and is impeded by VIP/VPAC1 signaling (Nourse et al. 1994).

VPAC1 is highly expressed in T cell. In murine CD4 T cells, VPAC1 is highly expressed at both the mRNA and protein levels and expression is decreased by 80% at the mRNA level and protein upon activation (Vomhof-DeKrey and Dorsam 2008, Vomhof-DeKrey et al. 2008, Delgado et al. 1996, Johnson et al. 1996, Leceta et al. 1996). Upon TCR stimulation, the antiproliferative properties elicited by VPAC1 signaling have a short window of opportunity to impede T cell activation. If activated T cells are treated with VIP within 30 min post activation, VIP/VPAC1 signaling is able to suppress the extent

of T cell activation. After 30 min VIP treatment does not impede cell cycle entry and VPAC1 expression decreases (Wang et al. 2000).

The focus of this research was to determine if VIP/VPAC1 signaling alters IK biology by modulating its expression and/or phosphorylation profile. The antiproliferative properties elicited from VIP/VPAC1 signaling and that Ikaros sets the threshold for T cell activation suggested a potential link between the two proteins. We identified putative PKA phosphorylation sites within the primary amino acid sequence of IK and prompted us to propose this hypothesize. Specifically, we hypothesized that VIP/VPAC1 signaling would increase IK protein expression and/or block its activated phosphorylation profile by a PKA-dependent mechanism. This hypothesis was tested by the utilization of the VPAC1 expressing HuT 78 T cell line to determine whether VIP treatment altered IK protein expressed by Western blots and whether there was a change in the isoelectric species of IK by two-dimensional electrophoresis/Western blots. We concluded that VIP/VPAC1 signaling does not alter the expression of IK in unactivated Hut 78 cells. However, we showed that VIP/VPAC1 signaling suppressed nuclear IK protein increase observed upon T cell activation by one-dimensional Western blots. Additionally, we were able to conclude that VIP altered the isoelectric pattern of immunoreactive IK species in activated Hut 78 cells in both the cytoplasm and the nucleus. These data were the first to determine that VIP/VPAC1 signaling could modify the tumor suppressor IK protein and represents the intriguing regulatory axis of the nervous system impinging the immune system.

Materials and Methods

Reagents

HuT 78 and NIH-3T3 cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's modified Eagle's medium, Iscove's modified Dulbecco's medium, penicillin, streptomycin, L-glutamine, phosphate buffered saline without Ca^{2+} or Mg^{2+}, characterized fetal bovine serum, and trypsin were purchased from Cellgro (Manassas, VA). Opti-MEM, Lypofectamine 2000, pcDNA 3.1 His A tag expression vector were obtained from Invitrogen (Carlsbad, CA). Sodium chloride, potassium chloride, ethylenediaminetetraacetic acid (EDTA), ethyleneglycoltetraacetic acid (EGTA), tris, sodium hydroxide, sodium dodecyl sulfate, sodium deoxycholate, HEPES, nonidet P-40, glycerol, 2-mercaptoethanol, ammonium persulfate, methanol, acetic acid, commassie blue, and Ponceau S were bought from Sigma-Aldrich (St. Louis, MO). Vasoactive intestinal peptide was purchased from American Peptides (Sunnyvale, CA). Protease cocktail inhibitor set III and Super Signal West femto chemiluminescent reagent were obtained from Thermo Fischer Scientific (Waltham, MA). Tween 20, ECL Plus, and 2-D clean up kit were purchased from GE Healthcare (Piscataway, NJ). Silverstain Plus kit, DC Protein assay, tetramethylethylenediamine (TEMED), nitrocellulose, and 30% Acrylamide/Bis solution (37.5:1) were bought from Bio-Rad (Hercules, CA). Rabbit anti-human Lamin A/C, rabbit anti-human Actin, rabbit anti-human Actin-HRP, and goat anti-rabbit HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-mouse Ikaros-CTS antibody and Ikaros H cDNA was a kind gift from Dr. Dovat (University of Wisconsin, Madison, Madison, WI). Qiashredders, RNeasy mini kits, and RNase-free DNase I was

obtained from Qiagen (Valencia, CA). Deoxynucleotides, reverse transcriptase, Taq DNA polymerase were procured from Promega (Madison, WI). Phenol/chlorophorm/isoamyl alcohol (25:24:1) was purchased from Research Organics (Cleveland, OH) Gene specific primers were obtained from Integrated DNA technologies (Coralville, IA). Phusion HF DNA polymerase and BAM HI were purchased from New England BioLabs (Ipswitch, MA). Rabbit anti- mouse SP1 was purchased from EMD-Millipore (Billerica, MA). pCMV-Tag2B expression vector was bought from Stratagene (Santa Clara, CA) and the cDNA for mouse Ikaros 1, 2, and 5 were a kind gift from Dr. Georgopolous (Harvard University, Cambridge, MA).

Tissue Culture

HuT 78 cells were cultured in 77% Iscove's modified Dulbecco's medium (IMDM), supplemented with 20% characterized fetal bovine serum (chFBS), 100 units/mL penicillin (1%), 100 µg/mL streptomycin (1%), and 4 mM L-glutamine (1%) (HuT 78 completed media). Cells were collected, centrifuged at 300 x g for 5 min, and seeded at 2-3 x 10^5 cells/mL three times a week (every 48-72 hr) with fresh complete media. Cells were incubated at 37°C in 5% CO_2. NIH-3T3 cells were propagated in 89% Dulbecco's modified Eagles medium, 10% defined FBS, 100 U/mL penicillin (1%), and 100 ug/mL streptomycin (1%). Cells were seeded at 2 x 10^5 cells/T-25 and media was exchanged 3 times a week. Cells were incubated at 37°C in 10% CO_2.

Radioimmunoprecipitation Assay

Isolation of IK protein was completed using radioimmunoprecipitation assay buffer (RIPA) to isolate total cellular protein. Cells were collected and washed in PBS three times prior to lysing. Cell pellets (1×10^7) were resuspened in 1 mL RIPA buffer (150 mM sodium chloride (NaCl), 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM HEPES [pH 7.4] and 1x Protease Cocktail Inhibitor set III (PCI)), incubated for 30 min at 4°C, by vortexing every 10 min. Samples were centrifuged at 8,000 x g for 15 min at 4°C and supernatant was collected and either frozen at -80°C or protein was quantitated by a DC protein assay prior to further analysis.

Nuclear Protein Isolation

Cytoplasmic and nuclear proteins were isolated based on a modified Schreiber protein isolation method (Schreiber et al. 1989). Cells (1×10^7) were collected and washed three times in PBS. Cell pellets were gently resuspened in 1 mL ice-cold Buffer A (10 mM HEPES [pH 7.9], 10 mM potassium chloride (KCl), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethyleneglycoltetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), and 1x PCI) and allowed to swell on ice for 15 min. NP-40 detergent (0.063% v/v) was utilized to permeabilize plasma membranes, briefly vortexed, and centrifuged (8,000 x g) for 30 seconds at 4°C. Supernatant (cytoplasmic protein fraction) was removed, cell pellets were washed in Buffer A, and supernatant was completely removed. Nuclei were resuspended in 200-350 µL of ice-cold Buffer C (20 mM HEPES [pH 7.9] 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1X PCI), vortexed, and incubated for 30 min

on ice. Samples were centrifuged at 8,000 x g for 5 min at 4°C. Supernatant (nuclear protein fraction) was removed and protein concentration was determined by DC Protein Assay or snap frozen in an ethanol-dry ice bath and stored at -80°C until used.

Time Course of Naïve and Activated VIP Treated Cells

Hut 78 cells were artificially treated to mimic T cell activation with phorbol 12-myristate 13-acetate (PMA) and ionomycin. This strategy was employed due to a dysfunctional zeta receptor (CD3ζ). Cells were seeded at 5 x 10^5 cells/mL for 24 hr prior to activation to synchronize cell cycle. Cells were collected, counted, and resuspended at 1 x 10^6 cells/mL in complete media. Cells were activated with 10 ng/mL PMA and 1 μM ionomycin or untreated to maintain an unactivated phonotype. Cells were incubated +/- VIP (10^{-8} M) for 0, 4, 8, or 24 hr. Cells were collected, washed in PBS, and lysed as previously described.

DC Protein Assay

Protein concentration was determined using Lowery based DC Protein Assay as described by manufacturer (Bio-Rad, 2008). Briefly, Reagent A and S were combined in a 50:1 ratio for solution A'. In a 96 well plate, BSA protein standard (2 mg/mL) or unknown sample, 20 μL reagent A' and 200 μL Reagent B were combined for final volume of 230 μL. Samples were incubated for 15 min and absorbance was measured at 750 nm by a Thermo Scientific Multiscan Spectrum Microplate Spectrophotometer (Waltham, MA).

SDS-PAGE and Immunoblots

Separation of proteins based on molecular weight was completed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a Bio-Rad mini-PROTEAN tetra cell apparatus (Hercules, CA). Proteins were applied to a discontinuous separating (resolving) and stacking gel (see table 2 for gel percentages). Protein samples (10-50 µg) were denatured with 5x loading dye (200 mM Tris [pH 8.8], 25% v/v glycerol, 0.2% w/v SDS, 0.02% w/v bromophenol blue, and 5% v/v 2-mercaptoethanol), and incubated at 100°C for 10 min for soluble proteins. Duplicate gels were loaded and electrophoresed with Laemmli running buffer (25 mM Tris base, 192 mM glycine, and 0.1% SDS, pH of solution is not adjusted) at 200 V for 1 hr.

Table 2. Separating and stacking gels used for SDS-PAGE.

Reagent	5% Stacking	12% Resolving
Acrylimide-Bis (30%)	3.4 mL	4 mL
4X Resolving Buffer (1.5 M Tris [pH 8.8], 10% w/v SDS)	-	2.6 mL
4X Stacking Buffer (1.5 M Tris [pH 8.8], 10% w/v SDS)	0.680 mL	-
10% w/v Ammonium Persulfate	50 ul	100 µL
TEMED	5 µL	5 µL
Water	3.4 mL	3.3 mL

SDS-PAGE gels were transferred to a nitrocellulose membrane using the Laemmli transfer buffer (25 mM Tris-base, 192 mM glycine, 20% methanol, and 0.1% SDS, pH is not adjusted) at 4°C for 140 volt hr (VH). Transfer efficiency to nitrocellulose was determined by Coomassie staining of the gels and Ponceau staining of the nitrocellulose membrane. Membranes were stained for 5-10 min with Ponceau stain (0.1% w/v Ponceau S in 5% acetic acid), followed by several washes with water to remove excess stain unbound to the negatively charged

proteins. For complete removal of Ponceau S, membranes were destained in 50 mM sodium hydroxide (NaOH) with gentle agitation for 1 min followed by 2, five min washes in water and a 10 min wash in TBS-T (100 mM Tris [pH 7.6], 150 mM NaCl, and 0.1% Tween-20). Gels were incubated with Coomassie stain (20% acetic acid, 50% methanol, and 0.05% w/v Coomassie blue) for 45 min and destained with 10% acetic acid until signals were clearly visual over background. Membranes were incubated in blocking buffer (2.5% non-fat dry milk in TBS-T [NF-DM TBS-T]) with gentle agitation for 30 min and subsequently washed 3 times for 5 min in TBS-T. Membranes were incubated with primary antibody in 2.5% NF-DM TBS-T (1:5,000 dilution for IK-CTS, 1:10,000 SP1, Lamin A/C, and Actin-HRP) for 1 hr at RT or overnight at 4°C. Unbound primary antibody was removed by washing the membrane 3 times for 10 min each with TBS-T and shaking. Secondary antibody (1:10,000 goat anti-rabbit HRP) was incubated for 30 min in 2.5% NF-DM TBS-T. Immunoblots were washed 6 times with TBS-T for 10 min to minimize background signals. Protein of interest was visualized by Super Signal West femto Chemileuminescent substrate and imaged with a SynGene Chemi Genius (Cabridge, England) or by ECL Plus and imaged using a GE Healthcare Storm Imager 865 (Piscataway, NJ). Validation of equal loading of protein was determined by Actin, Lamin A/C, or SP1 probing after stripping of nitrocellulose membranes and reprobing. Membranes were incubated for 30 min at 50°C in stripping solution (25 mM Tris [pH 7.8], 1% SDS, and 0.25% v/v 2-mercaptoethanol) followed by washing in TBS-T for 1 hr exchanging wash buffer 6 times. To determine complete stripping of primary and secondary antibodies,

membranes were developed to ensure minimal background before reprobing. Membranes were rinsed in TBS-T for 10 min and reprobed as described above.

Two-Dimensional Gel Electrophoresis

Ikaros isoelectric species were identified by separating proteins by 2-dimensional gel electrophoresis (2DE). Isolated nuclear protein was purified to remove salts and concentrate sample with a 2-D clean up kit from GE Healthcare. Briefly, nuclear protein (100 µg) was aliquoted into a 1.5 mL tube and 300 µL of Precipitant solution was added, vortexed, and incubated on ice for 5 min. Co-Precipitant solution (300 µL) was added, vortexted, and centrifuged (16,000 x g) for 15 min at 4°C. Supernatant was decanted and 40 µL of Co-precipitant solution was added directly to the protein pellet, and incubated for 5 min on ice. Samples were centrifuged for 5 min at 16,000 x g at 4°C. Supernatant was carefully removed by aspiration with a drawn-out Pasture pipette. The protein pellet was dislodged with 25 µL water, 1 mL wash buffer, and 5 µL wash additive. Samples were vortexed for 30 seconds and incubated at -20°C for 30 min (could be stored up to 1 week). Samples were centrifuged for 5 min, supernatant was completely removed, and air dried for 5 min. Reconstitution of protein pellet was completed in 125 µL Destreak Rehydration Solution and 0.60% v/v IPG 3-11NL buffer. Samples were loaded into a strip holder between the electrodes followed by addition of the isoelectric focusing (IEF) strip (3-11 nonlinear, 7 cm strips). Strips were covered in 100 µL of covering solution (mineral oil) to minimize evaporation of sample. Samples were electrophoresed using the following parameters for a GE Healthcare IPG Phor III (Piscataway, NJ): Step 1-100 V 15 hr, 2. Step 200 V

200 VH, 3. Gradient 500 V 300 VH, 4. Gradient 1000 V 600 VH, 5. Gradient 2000 V 1800 VH, 6. Gradient 3000 V 3000 VH, 7. Gradient 5000 V 7000 VH, 8. Step 5000 V 1250 VH. Upon completion of IEF, strips were incubated in SDS equilibration buffer (6 M urea, 75 mM Tris [pH 8.8], 29.3%v/v glycerol, 2% w/v SDS, 0.002% w/v bromophenol blue, and 10mM 2-mercaptoethanol) for 15 min twice. Proteins were separated using a 12% resolving gel, IEF strip was loaded into the well and covered with sealing solution (0.5% w/v agarose M, 0.002% w/v bromophenol blue) and electrophoresed at 60 V until dye front was electrophoresed off the gel. Gels were either silver stained or transferred to nitrocellulose for immunoblots.

Silver Stain

Detection of proteins was completed using Silver Stain Plus as indicated by the manufacturer (Bio-Rad, 2008). Briefly, membranes were incubated in fixative (50% methanol, 10% acetic acid, and 10% fixative enhancer concentrate) for 20 min. Gels were rinsed 2 times for 10 min each wash in deionized (DI) water. Gels incubated for 20 min in staining solution (10% Silver complex solution, 10% reduction moderator solution, and 10% image development reagent) followed by incubation of the gel in development accelerator solution. Upon visualization of the protein, development was inhibited by incubating in a 5% acetic acid solution for 15 min followed by two sequential rinses in DI water for 5 min and imaged with a SynGene Chemi Genius (Cabridge, England).

Site Directed Mutagenesis

Deletions of exons 4-7 and 4-6 from full length IK 1 plasmid DNA were introduced by synthesizing complementary DNA catalyzed by the high fidelity Phusion HF DNA polymerase (1 Unit) (a kind gift from Dr. Stuart Haring) with1X Phusion HF buffer, 1.5 mM $MgCl_2$, 400 µM dNTPs (each), 1 µM primers (Table 3), 0.1 ng pCMV-Tag 2B-IK 1 plasmid with a final volume of 20 µL. Reaction conditions consisted of an initial denaturation step of 95°C 3 min, followed by 30 cycles of amplification of 95°C 1 min, 55°C 1 min, 72°C 1 min/kbp of plasmid (7 min), and a final extension of 72°C for 5 min. PCR product was digested with *Dpn*I restriction enzyme (a kind gift from Dr. Stuart Haring) for 2 hours at 37°C (Figure 17).

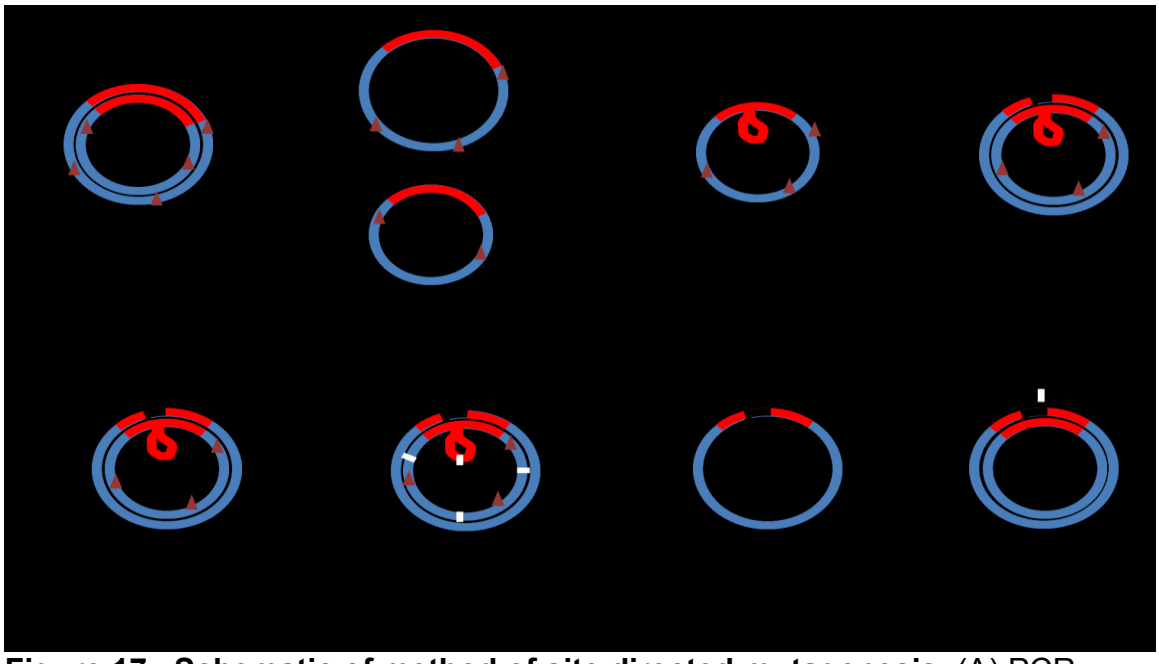


Figure 17. Schematic of method of site directed mutagenesis. (A) PCR amplification to generate pCMV-Tag2B IK 6 and IK 8 using site directed mutagenesis. PCR amplification was completed by initial denaturation of plasmids, mutational primers bind and PCR amplifies looping out the undesired DNA segment. (B) Upon completion of PCR, plasmids were *DnpI* digested to cleave methylated DNA, followed by second strand synthesis for IK 6 and 8 plasmids.

Alkaline Lysis Minipreps

LB medium was inoculated with a transformed bacterial colony and grown overnight at 37°C with vigorous agitation. Cultures were collected and centrifuged (16,000 x g), supernatants were decanted, cell pellet was lysed in GTE solution (50 mM glucose, 25 mM Tris [pH 8], 10 mM EDTA), and incubated at room temperature for 5 min. Two-volumes of NaOH/SDS solution (0.2N NaOH and 1% w/v SDS) was combined with lysed cells, and incubated on ice for 5 min. To the solution, 0.75 volumes potassium acetate solution (5M acetic acid and KOH pellets to pH 4.8) was added, vortexed, and incubated on ice for 5 min. Samples were centrifuged at 16,000 x g for 3 min, and the supernatant was collected. Two-volumes of 95% ethanol was combined with the supernatant and incubated for 2 min at room temperature. Samples were centrifuged for 1 min at 16,000 x g, room temperature. The supernatant was removed and the pellet was washed with 70% ethanol. The plasmid pellet was resuspended in TE buffer (10 mM Tris [pH 7.5] and 1 mM EDTA) and frozen at -20°C.

Flow Cytometry Analysis

One million cells were resuspended in 100 µL of binding buffer (PBS and 1% w/v BSA) and incubated with human CD25-PE (1:100 dilution) for 30 min at 4°C in the dark. Cells were washed two times with 2 mL PBS and centrifuged at 500 x g for 5 min followed by decanting off supernatant. Cells were resuspened in 300 µl PBS and analyzed on an Accuri C6 flow cytometer (Ann Arbor, MI).

RNA Isolation, cDNA Synthesis, and Traditional PCR

RNA isolation was completed using a Qiagen mini RNeasy kit, protocol used was a modified version from Qiagen's April 2006 handbook. Briefly, tissue culture cells were collected, centrifuged, and all supernatants were removed by aspiration. Cell pellets were lysed in 350 µL RLT buffer (RLT buffer supplemented with 1% v/v β-mercaptoethanol), vortexed, and transferred to a QIAshredder spin column. Samples were centrifuged at 8,000 x g for 2 min to sheer genomic DNA. QIAshredder columns were discarded, and 1 equivalent volume of 70% ethanol was added to the flow through and mixed well by pipetting up and down. Cellular lysates were transferred to an RNaesy spin column, centrifuged at 8,000 x g for 15 seconds to allow RNA bind to the column. Columns were washed with 350 µL RW1 buffer before an on-column DNase I treatment. DNase I (10 µL DNase I and 70 µL RDD buffer) was directly added to the column and incubated at 37°C for 45 min. Digested DNA was eluted off the column with a second 350 µL RW1 buffer wash, centrifuged for 15 seconds at 8,000 x g and flow-through discarded. Columns were washed with 500 µL RPE buffer, centrifuged for 2 min, and repeated. The collection tube was changed, centrifuged for an additional minute to remove all residual ethanol, and columns were placed in capped 1.5 mL centrifuge tubes. RNA was eluted with 50 µL RNase-free water (37°C) into a 1.5 mL tube where a second DNase I treatment was conducted. Equivalent volumes of DNase I/RDD buffer and RNA were incubated at 37°C for 45 min. RNA was further purified by the addition of 1 volume phenol/chloroform/isoamyl alcohol (25:24:1) to the DNase treated RNA (Moore and Dowhan, 2002). Samples were

vortexed for 2 min to create an emulsion and centrifuged at 8,000 x g for 10 min at room temperature to separate the organic phase from the RNA-containing aqueous (top) layer. RNA was collected by careful transfer to a new tube, and 3 M sodium acetate (pH 5.2) (1/10 volume) was added and vortexed briefly. To facilitate precipitation of RNA, 900 µl of ice cold absolute ethanol was added, briefly vortexed, and incubated at -80°C for $\geq$ 30 min. RNA samples were centrifuged at 12,000 x g for 30 min at 0°C. Ethanol was carefully aspirated not to disrupt RNA pellet and washed with 1 mL 70% ethanol to solubilize salts and centrifuged for an additional 10 min at 0°C. Ethanol was carefully removed as above and allowed to air dry for 5 min to ensure complete removal of ethanol. RNA pellets were resuspended in 50 uL of nuclease free water (37°C), and incubated for 10 minutes at 70°C. Total RNA (0.5-4 µg) was utilized for cDNA synthesis or stored at -80°C until needed.

RNA concentration and purity was determined by spectroscopy by measuring at 260 and 280 nm on a Thermo Scientific Nanodrop 2000C spectrophotometer (Waltham, MA). Reactions containing reverse transcriptase (RT+) and omitting reverse transcriptase (RT-) were performed to synthesize cDNA using 4 µg of total RNA. Prior to RT addition, both reactions designated RT+ and RT- had 1 µg random hexamers (500 ng/µL) added to a final volume of 30 µl, and samples were incubated at 69°C for 7 min followed by a quick chill on ice to minimize formation of secondary structures. To RT+ cDNA reactions, a master mix was added with final volumes of 1X Moloney Murine Leukemia Virus (M-MLV) buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl, 1 mM $MgCl_2$, and 50 mM

DTT), 4 mM dNTPs, 50 units RNasin, 400 units M-MLV RT, and RNase free water for a final volume of reaction at 50 µl. RT- cDNA reactions used the above master mix with nuclease free water replacing RT. Samples were mixed by flicking the tubes and briefly centrifuging to collect all liquid at the bottom of the tube. Samples were incubated for 1 hr at 37°C, and stored at -20°C or used immediately for traditional PCR.

cDNA was amplified by 500 nM of IK primers (Table 3), 500 nM dNTPs, and 1 Unit of Taq DNA polymerase with 1.5 mM $MgCl_2$. Reaction conditions began with an initial denaturation step of 95°C for 5 min, followed by 40 cycles of amplification of 95°C for 1 min, 62°C for 1 min, 72°C for 1 min and a final extension of 72°C for 10 min.

Table 3. Primer sets for traditional PCR for IK and site directed mutagenesis.

Primer Name	Primer	Accession Number
Ikaros Ex 2	5′-GCC TGT CCC TGA GGA CCT GTC-3′	NM_001025597
Ikaros Ex 3	5′-AGT AAT GTT AAA GTA GAG ACT CAG-3′	NM_001025597
Ikaros Ex 7	5′-GAT GGC TTG GTC CAT CAC GTG GGA-3′	NM_001025597
Ikaros 6 Mutant	5'- CAG CAG AACTCC AAG AGT GAT CGA GGC ATG GGA GAC AAGTGC CTG TCA GAC ATG CCC TAT -3'	
Ikaros 8 Mutant	5'- CAG CAG AACTCC AAG AGT GAT CGA GGC ATG CCA GTA ATT AAG GAA GAA ACT AAC CAC AAC -3'	

Lipofectamine 2000 Transfections

Adherent NIH 3T3 cells were seeded at 5 x 10^5 cells per T-25 tissue culture flask 24 hr prior to transfection. After 24 hr, cells were approximately 80% confluent and utilized for transient transfections. DNA master mix of 10 µg of plasmid DNA (pCMV, pCMV-IK 1, pCMV-IK 2, pCMV-IK 5, pCMV-IK 6, pCMV-IK 8) added to 625 µL Opti-MEM media was prepared. A second solution containing 25 µL Lipofectamine 2000 added to 625 µL Opti-MEM. Both solutions were

incubated for 5 min at room temperature followed by mixing 1:1 (650 µL each) and incubated at room temperature for 20 min. Media in the cell culture flask was removed and cells were washed with PBS. Fresh Opti-MEM (5 mL) was added to the culture and 1.25 mL of DNA:Lipofectamine master mix was added to each flask. Four hours post transfection, media was removed, cells were washed 2 times in PBS and complete media was added to transfection flask. Cells were collected 20 hr post-transfection by washing cells with PBS followed by removal of cells with Trypsin. Collected cells were washed in PBS and frozen as dry pelleted cells.

Genomic DNA Profile Analysis

Genomic profile analysis was completed by North Dakota State University DNA Forensic Laboratory. One million Hut 78 cells were collected, resuspended in PBS and delivered to the Forensic Laboratory. STR analysis was completed using the AB's AmpFℓSTR ® Identifiler ™.

Results

Since IK undergoes alternative splicing, the first question we asked was which IK isoforms are expressed in HuT 78 cells. To this end, total RNA and protein were isolated from HuT 78 cells for RT-PCR and Western blot analysis. IK mRNA species were amplified using primer sequences located in exon 4 and 8. Two RT-PCR products were observed matching the expected sizes for IK 1 (774 bp) and IK 3A (650 bp). Two additional species were detected at low levels. The smaller of the two is a candidate for IK 5 (519 bp) and the larger species of 1270 bp is bigger than any known IK isoform and may represent preprocessed (primary)

RNA. Additional IK isoforms might be present, since not all IK isoforms reported to date possess exon 4. To analyze IK protein, Western blot analysis demonstrated that IK 1 and 2 were the predominant isoforms expressed (Figure 18), as they comigrate with FLAG-Tag 2B IK 1 and 2 recombinant protein isolated from NIH 3T3 IK transfectants (note that FLAG-Tagged protein migrates slightly slower than wild type protein). These data demonstrated that HuT 78 cells predominately express IK 1 and IK 3A at the mRNA level, while IK 1 and IK 2 were the major isoforms detected at the protein level.

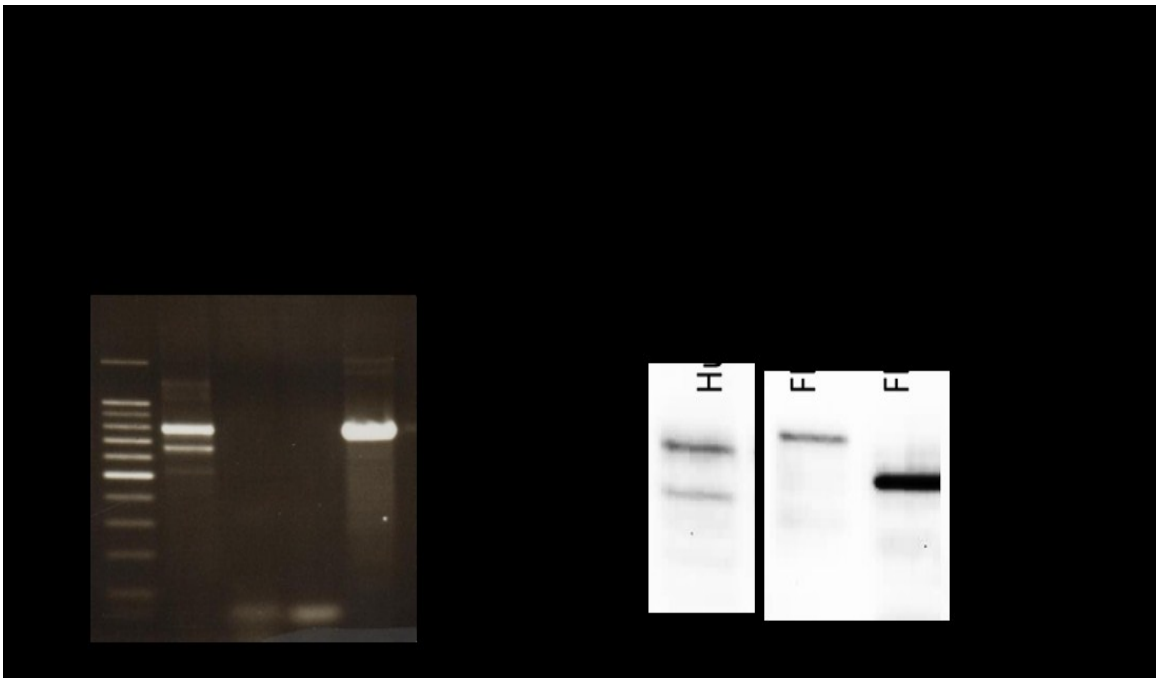


Figure 18. Ikaros 1 and 2 are the predominantly expressed isoforms in HuT 78 cells. Hut 78 cells were lysed and total cellular RNA and proteins were isolated as described in materials and methods. (A) RT-PCR amplification using primers specific for exons 4 and 8. Reactions were separated on 2% agarose gels and visualized by ethidium bromide staining. Digital documentation was performed on a SynGene instrument. (B) Western blot analysis using an indirect detection method of a rabbit anti-mouse IK primary antibody followed by a goat anti-rabbit-HRP secondary. Westerns were visualized by WESTfemto chemiluminescent substrate and digital documentation was performed on a SynGene instrument.

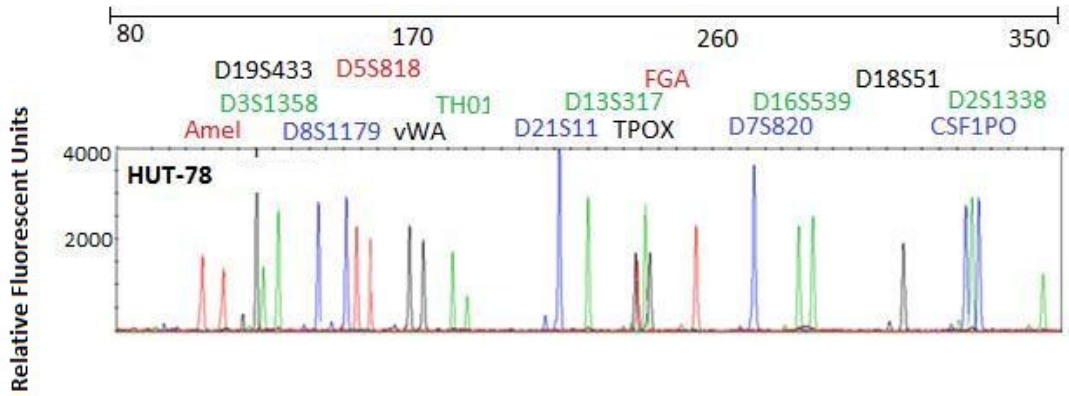


Figure 19. Genetic profile of Hut 78 cells. Hut 78 cell's short tandom repeats were analysed to validate genotype of cell line used after several passages. Electrophorogram of STR analysis of 15 loci and the amel as determined by AB's AmpFℓSTR ® Identifililer ™.

Table 4. Genetic profile for HuT 78 cells.

Gene Name	ATCC*	NDSU**
D3S1358		8 8
THO1	8 9	8 9
D21S22		8 9
D18S51		14 14
PENTA E		30 30
D5S818	11 12	11 12
D13S317	8 12	8 12
D7S820	8 11	8 11
D16S539	11 12	11 12
CSF1PO	11 12	11 12
PENTA D		8 8
Amelogenin	X Y	X Y
vWA	14 15	14 15
D8S1179		12 14
TPOX	8 9	8 9
FGA		21 25

*STR analysis completed by ATCC on 8 genes and amelogenin.
** STR analysis completed by NDSU Forensic Laboratory for 15 genes and amelogenin.

Malignat cells are inherently unstable and can accumulate additional genetic abnormalities. Therefore, the genetic profile of HuT 78 cells was measured after several passages of culture. STR analysis was completed on HuT

78 cells after several passages and compared to ATCC STR analysis. This analysis showed an identical match compared to ATCC for the 8 loci and the Amel genes. Additional STR loci were also identified by the NDSU Forensic Laboratory (Figure 19 and Table 4). HuT 78 cells did not have any detectable genetic mutations in their genome at these identified loci, and we concluded that this T cell line was indeed stable for our subsequent research. Importantly, with the rampent HeLa cell contamination of over 60% of the cell lines worldwide, our analysis confirmed a single, homogenous cell line (Lucey, Nelson-Rees and Hutchins 2009).

In the naïve state, we hypothesized that IK expression would be altered upon VIP treatment of Hut 78 cells. To determine the effect of VIP treatment of Hut 78 cells on nuclear IK protein, samples were collected and lysed in the presence or absence of VIP. Nuclear protein was separated by SDS-PAGE and IK protein expression was detected by Western blot analysis. There was not a significant change in the expression of nuclear IK protein in the naïve HuT 78 cells over a 24 hr time period when normalized to the nuclear Lamin A/C protein (Figure 20). There was a fluctuation in nuclear IK protein upon VIP treatment, but was not statistically significant. These data demonstrated that VIP treatment to naïve Hut 78 cells did not significantly alter the nuclear expression of IK protein.

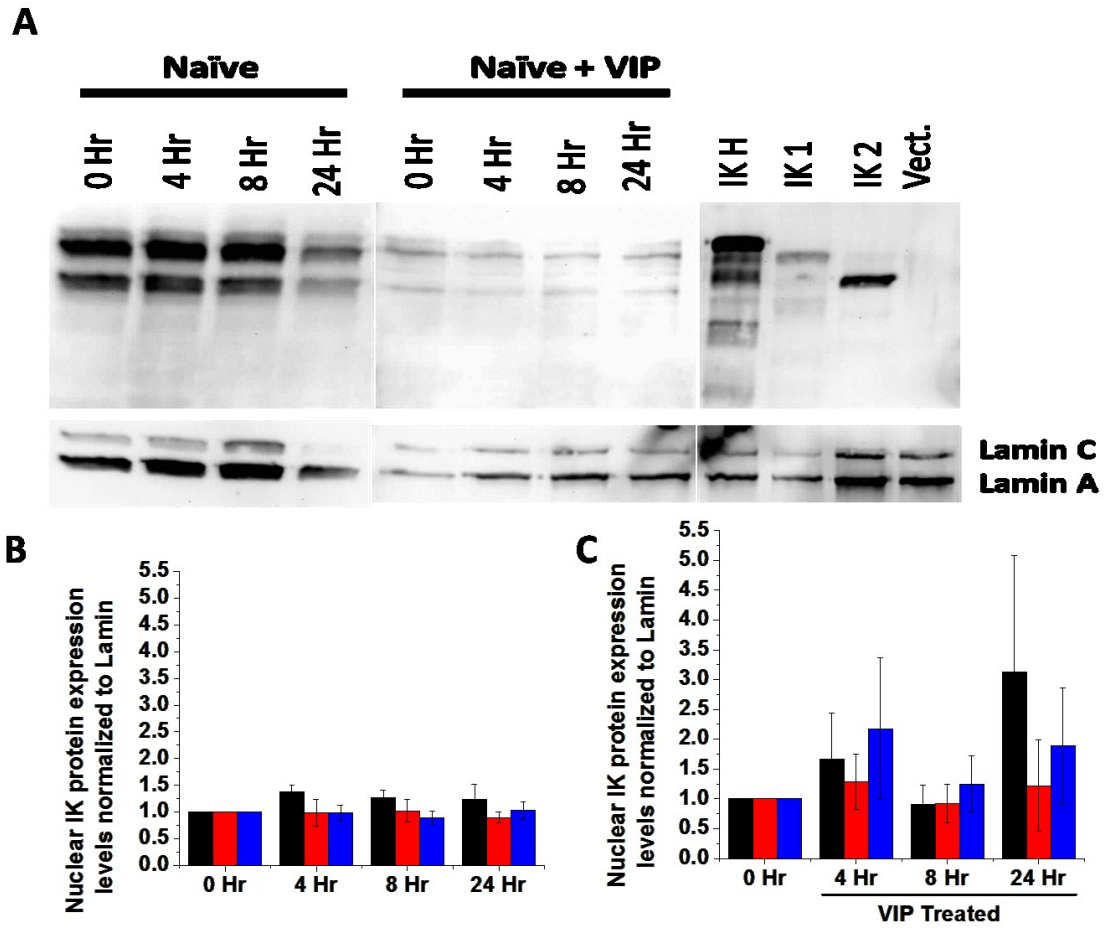
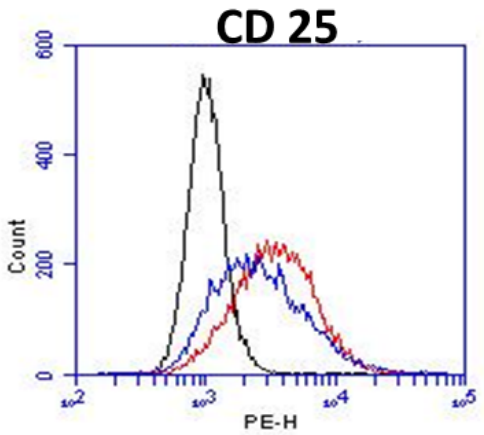


Figure 20. VIP does not alter nuclear IK protein expression in naïve HuT 78 cells. Naïve HuT 78 cells were treated +/- VIP (10^{-8} M) and incubated for 0, 4, 8, or 24 hr. Cells were collected, lysed, and nuclear protein was separated by SDS-PAGE. Proteins were transferred to nitrocellulose for immunoblots analysis (materials and methods). (A) Nuclear protein isolated from HuT 78 cells +/- VIP treated was probed for IK protein. Membranes were reprobed for Lamin A/C as normalizing controls. Densitometry of IK H (black), 1 (red) and 2 (blue) protein expression normalized to Lamin A/C for naïve (B) and VIP treated (C) HuT 78 cells. All experiments are n=3.

The validation of HuT 78 cells becoming activated with PMA/ionomycin and biological activity of VIP was completed by measurement of the T cell activation surface marker CD25. Cells were activated with PMA/ionomycin in the presence or absence of VIP for 24 hr and expression of CD25 was determined by flow cytometry and compared to unactivated HuT 78 cells. Upon PMA/ionomycin

treatment, there was an increase in CD25 surface protein expression in 75% of HuT 78 cells compared to naïve cells. VIP treated activated HuT 78 cells had a decrease in detectable CD25 protein expression by one-third (Figure 21). These data demonstrated that PMA/ionomycin treated cells were activated by the increase in CD25 expression compared to naïve HuT 78 cells. VIP inhibited CD25 upregulation observed in PMA/ionomycin treated HuT 78 cells and is consistent with its well-established anti-inflammatory effects.



Color	Status	% Positive
Black	Naïve	2.0%
Red	Activated	75.2%
Blue	Act + VIP 10^{-8}M	57.1%

Figure 21. VIP treatment of activated HuT 78 cells limited CD25 surface protein expression. HuT 78 cells were activated with PMA/ionomycin and treated +/- VIP (10-8 M) for 24 hrs. Activation of HuT 78 cells monitored by the expression of the early activation marker CD25 (IL-2 receptor) on naïve and activated HuT 78 +/- VIP treatment. Positive cells (%) for CD25 are indicated on the right panel.

Due to the suppression of CD25 expression in activated HuT 78 cells and that anti-proliferative response due to VIP/VPAC1 signaling, the potential effects of VIP on activated HuT 78 cells was investigated. HuT 78 cells were activated with PMA/ionomycin in the presence or absence of VIP and nuclear IK protein expression levels were determined by 1D Western blot analysis. PMA/ionomycin activated HuT 78 cells had an increase in nuclear IK 1 protein irrespective of the analysis time point, while little alteration in IK H and IK 2 was observed when

normalized to Lamin A/C nuclear protein (Figure 22). When HuT 78 cells were activated and co-treated with VIP (10^{-8} M) there was an observed decrease in nuclear IK protein for all three isoforms present with the greatest effect on IK 1 and IK 2.

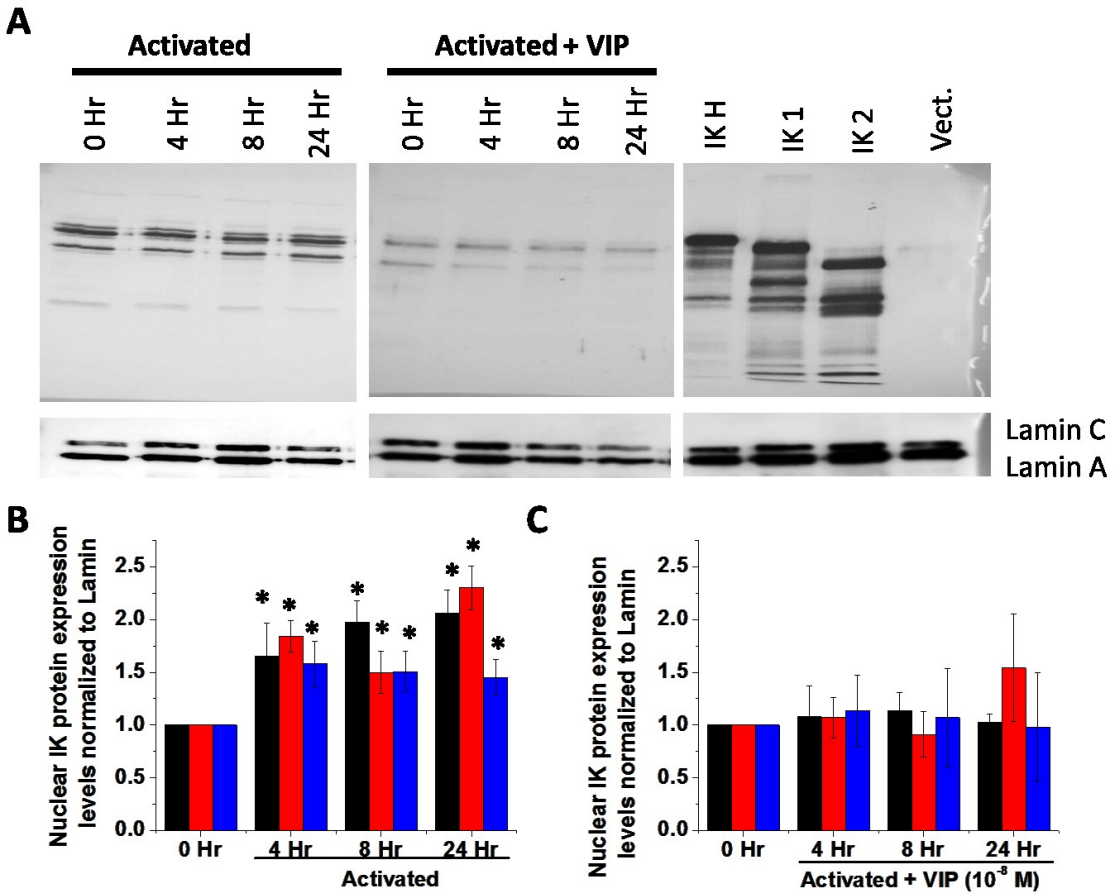


Figure 22. VIP treatment of activated HuT 78 cells blocked nuclear IK protein upregulation. Hut 78 cells were activated with PMA/ionomycin and treated +/- VIP (10^{-8} M) for 24 hr. Cells were collected, lysed, and separated by SDS-PAGE (see materials and methods). (A) Immunoblots of activated Hut 78 cells +/- VIP nuclear protein lysate probed against IK and Lamin A/C. Densitometry of IK H (black), 1 (red), and 2 (blue) in activated HuT 78 cells in the (B) absence and (C) presence of VIP. IK protein was normalized to Lamin A/C for loading control. All experiments are n=3, *p>0.05 and ** p>0.01.

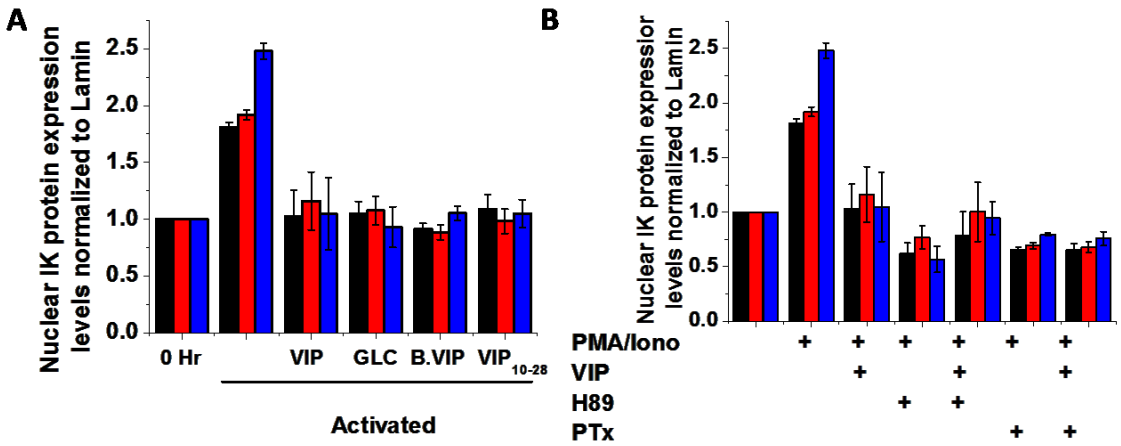


Figure 23. Increased Ikaros expression in activated HuT 78 cells appears to be non-specific. Activated HuT 78 cells treated with VIP, glucagon (irrelevant peptide), inactive (boiled) VIP, VIP_{10-28} (a partial antagonist) for 8 hr (A) or treated in the presence of H89 (PKA inhibitor) or pertussis toxin (PTx) (inhibition of AC) (B). Samples were lysed, nuclear IK protein was separated by SDS-PAGE, and probed against anti-IK CTS antibody. Densitometry ratios between IK H (black), 1 (red), and 2 (blue) signal divided by Lamin A/C were used to plot bar graphs. All experiments are n=3.

To determine if the suppression of nuclear IK protein was VIP specific in activated HuT 78 cells, cells were treated with additional peptides. Activated HuT 78 cells were treated with inactive (boiled) VIP, VIP_{10-28} (partial antagonist), glucagon (irrelevant peptide), or VIP in the presence of pertussis toxin (PTx) or H89. After 8 hr cells were collected, lysed, and nuclear protein was separated by SDS-PAGE. IK protein was detected by Western blots and normalized to Lamin A/C. Increased protein expression was detected upon activation as previously observed, however, irrespective of the treatment with inactive VIP, VIP_{10-28}, or glucagon there was no change in the detected IK protein from the unactivated sample (Figure 23). Activated HuT 78 cells treated with both VIP and the PKA inhibitor H89 has a slight increase in nuclear IK protein levels compared to H89 treated cells alone. Ptx treatment of activated cells lacked an increase in nuclear

IK protein and VIP treatment had no effect on IK expression levels. These experiments demonstrated that the increase in IK protein upon activation could be blunted by PTx, as expected since PTx is a known inhibitor of T cell activation. These data would have additionally suggested that the decrease in nuclear IK protein was not specific to VIP. However, due to the increased expression of Lamin A/C in activated murine CD4 T cells these data were deemed inconclusive.

In a large microarray study on murine primary CD4 T cells, VIP was determined to upregulate Lamin A/C gene expression in PMA/ionomycin activated cells. This could have caused the inconclusive results collected in figures 20 and 22. For this reason, a different nuclear normalizing protein was used and the analysis repeated. To this end, HuT 78 cells were activated with PMA/ionomycin and treated +/- VIP and IK protein was measured by immunoblot analysis and normalized to SP1. This analysis showed that only the IK 1 isoform was upregulated by PMA/ionomycin, and VIP co-treatment blocked this increase and lowered IK 2 in a time dependent fashion peaking at 24 hr (60% for both isoforms) (Figure 24). Interestingly, IK H was not affected by VIP signaling. These data demonstrated that VIP differentially decreased some, but not all IK isoforms. Moreover, these data support the idea that VIP signaling downregulates IK isoforms that are hyperphosphorylated (IK 1 and IK 2) (Ronni et al. 2007, Song et al. 2011), while having little effect on the isoform that is hypophosphorylated (IK H) during T cell activation.

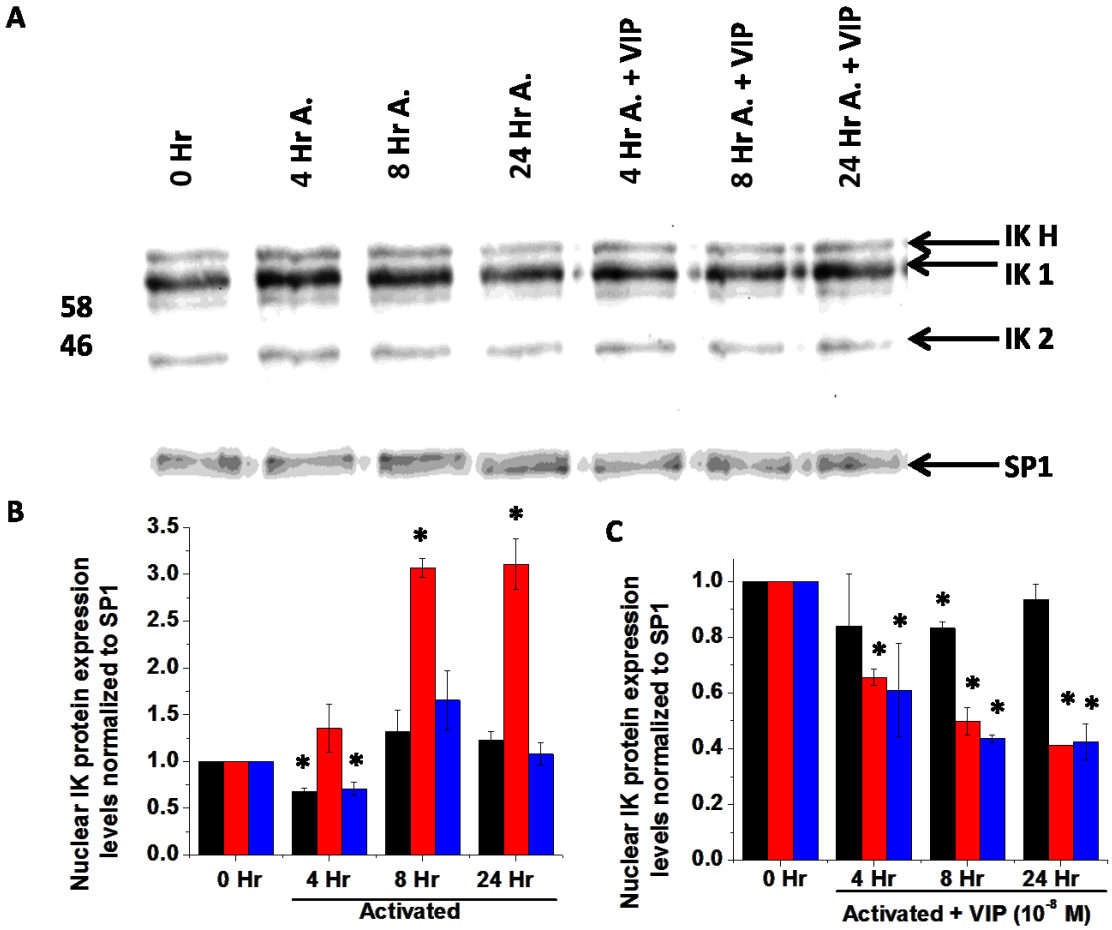


Figure 24. VIP decreases the expression of IK 1 and IK 2, but not IK H in activated HuT 78 cells. HuT 78 cells were activated with PMA/ionomycin +/- VIP, lysed, and nuclear protein was seperated by SDS-PAGE (materials and methods). Immunoblot analysis of IK and SP1 protein (A). Densitometry of IK H (black), 1 (red), and 2 (blue) protein expression normalized to nuclear protein SP1 for activated (B) and activated plus VIP (C). All experiments are n=3, *p>0.05 and ** p>0.01.

The alteration of nuclear IK protein expression due to VIP signaling through VIP/VPAC1 signaling cascade and the activation of PKA resulted in the inquire if IK contains putative PKA phosphorylation sites. The primary aa sequence of IK 1 was imputed into a PKA phosphorylation site predictor program by Mendel IMP Bioinformatics (mendel.imp.ac.at/pts1). Residues with a probability score of >0.30 were deemed as potential PKA phosphorylation sites are reported in Table 5.

Residues not identified to date to be phosphorylated are shown in figure 25. The identification of putative PKA phosphorylation sites in IK further supported the hypothesis that VIP/VPAC1 signaling through PKA would alter IK's phosphorylation status.

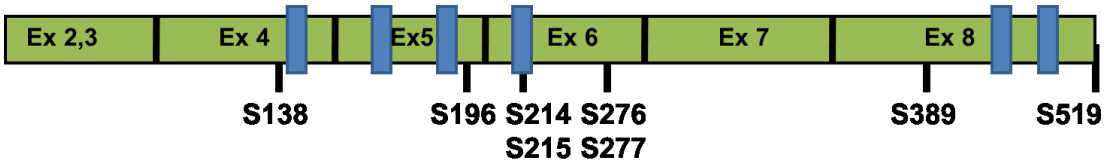


Figure 25. Putative PKA phosphorylation sites identified in Ikaros 1. Putative PKA phosphorylation residues within the primary IK 1 aa sequence. Analysis was performed by Mendel IMP Bioinformatics (mendel.imp.ac.at/pts1), scores >0.30 were considered as potential PKA phosphorylation residues.

Table 5. Putative PKA phosphorylation sites within Ikaros.

Residue*	Score**	Residue*	Score**
S276	0.65	S377	0.44
S277	0.63	S326	0.41
S398	0.58	S427	0.40
S196	0.57	S258	0.36
S442	0.50	S389	0.36
S138	0.49	S453	0.36
S215	0.47	S229	0.34
S409	0.46	S338	0.31
S519	0.45	S345	0.31

* Serine residues that are putative PKA phosphorylation sites
** Score (probability) for putative PKA phosphorylation sites as determined by Mendel bioinformatics.

We hypothesized that VIP signaling would activate PKA and complete with CK II to alter the phosphorylation state of IK protein maintaining its anti-leukemic activity. HuT 78 cells were activated with PMA/ionomycin treatment +/- VIP, collected after 4 hr and lysed. Samples were subjected to two-dimensional gel electrophoresis (2DE) followed by Western blot analysis. Three specific isoelectric species (high, intermediate, and low molecular weight (MW)) decreased upon T

cell activation (4 hr), but remained in the presence of VIP (Figure 26). Moreover, all three species were acidic (high MW) or the most acidic within a constellation of IK immunoreactive species (intermediate and low MW). These data demonstrated that acidic immunoreactive IK species normally lost during T cell activation were maintained by VIP treatment of activated cells, further supporting VIP's immunosuppressive effects by inhibiting T cell activation. Whether this is a direct (PKA) or indirect (immunosuppressive) effect by VIP is currently unknown.

Table 6. Calculated molecular weight and isoelectric point for Ikaros isoforms.

Ikaros Isoform	Molecular Weight (KDa)	Isoelectric Point (pI)
IK H	59.8	6.60
IK 1	57.5	6.12
IK 2	48.3	6.22
IK 3	47.6	5.75
IK 3A (IK X)	52.0	6.06
IK 4	43.1	6.12
IK 5	41.2	5.11
IK 6	32.0	5.01
IK 7	42.7	5.86
IK 8	36.9	5.15

We hypothesized that nuclear immunoreactive IK species are also altered in activated HuT 78 cells. To test this idea, we isolated nuclear protein from activated +/- VIP treated HuT 78 cells. Samples were separated by 2DE and immunoreactive IK protein detected by immunoblots. The 2DE separation of nuclear protein revealed fewer immunoreactive IK protein species in comparison to whole cell lysate (Figure 26). Two intermediate isoforms were detected in control cells (0 hr) (Figure 27). Upon activation there was a decrease in the number of isoelectric species (top intermediate species) and the detection of a new lower molecular weight species not observed in control. In contrast, activated

HuT 78 cells treated with VIP inhibited the appearance of the lower molecular weight species and the bottom intermediate IK immunoreactive species compared to activated cells in the absence of VIP. Lastly, there was a faint detection of a higher species in control (0 hr) that disappeared in activated in the absence but not presence of VIP treatment (4 hr). Collectively, IK immunoreactive species undergo a dramatic redistribution during T cell activation (PMA/ionomycin) by (1) losing the high MW species hypothesized to be IK H through dephosphorylation (Li et al. 2011b), (2) focusing into fewer isoelectric pools for the larger of the intermediate species thought to be IK 1 and (3) the appearance of several smaller species that may be a IK-DN isoform. These changes in isoelectric IK immunoreactive species would decrease the DNA binding potential of IK with the appearance of a DN IK immunoreactive species and skew the ratio of DNA binding IK isoforms to favor IK 1/2 versus IK H. This latter effect may shift the transcriptional regulation to overall repressive as IK H has been shown by Dovat's group to be a transcriptional activator. In the presence of VIP activated cells show further differences distinct to being activated without VIP and control. VIP blocks the appearance of the lower IK immunoreactive species and the disappearance of the higher IK immunoreactive species. In contrast, VIP dramatically lowers the detectable presence of the lower intermediate IK immunoreactive species, suggesting that this neuropeptide may shift the ratio of nuclear IK proteins to favor IK H and IK 1 but not IK 2.

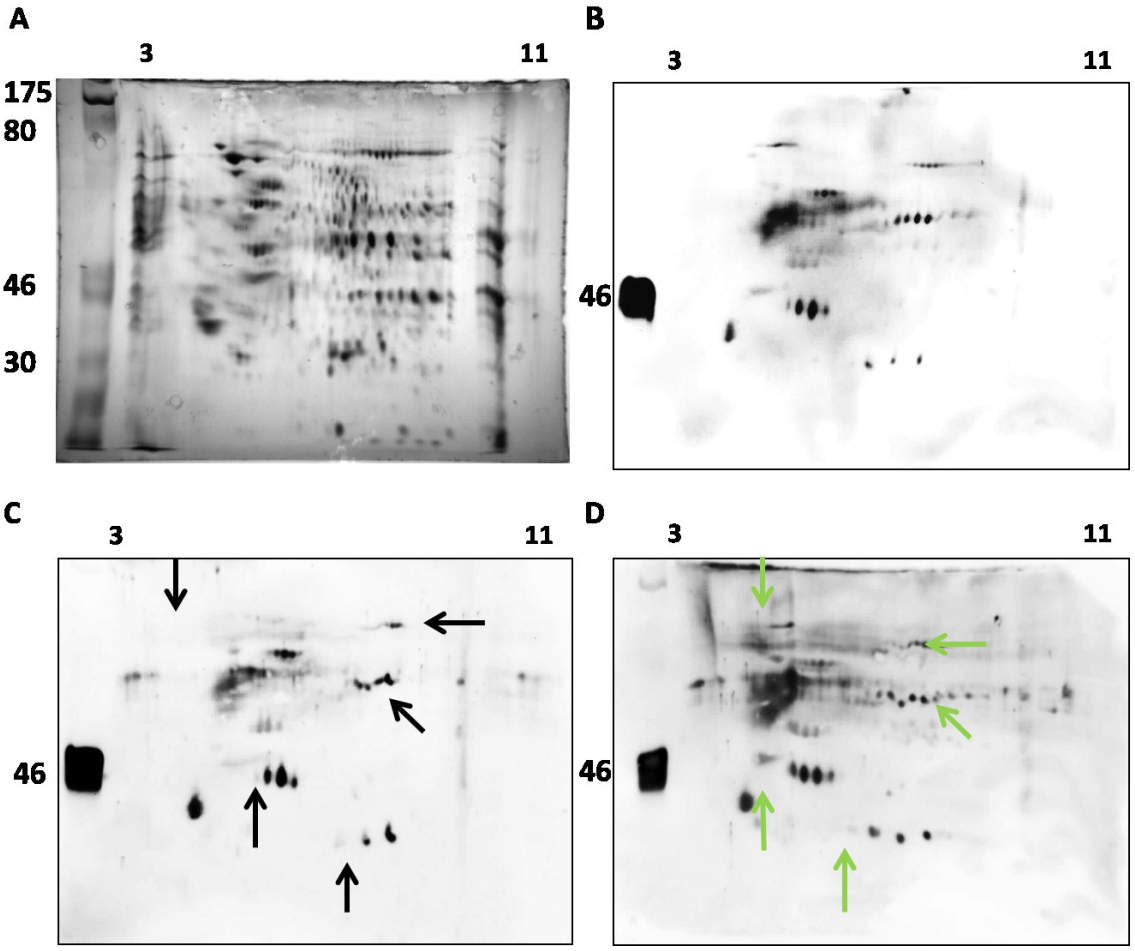


Figure 26. Acidic immunoreactive Ikaros species differentially modulated by T cell activation and VIP signaling. Total cell lysate was separated by isoelectric focusing of proteins on a 3-11 nonlinear pH gradient followed by separation by molecular weight (Materials and Methods). (A) Silver stained gel of control HuT 78 cell lysate. Western blot analysis for immunoreactive IK species in HuT 78 cells activated for 0 hr (B), 4 hr in the absence (C), or presence (D) of VIP. Black arrows represent immunoreactive IK species lost upon activation, while green arrows are alterations in IK between activated and VIP treated cells.

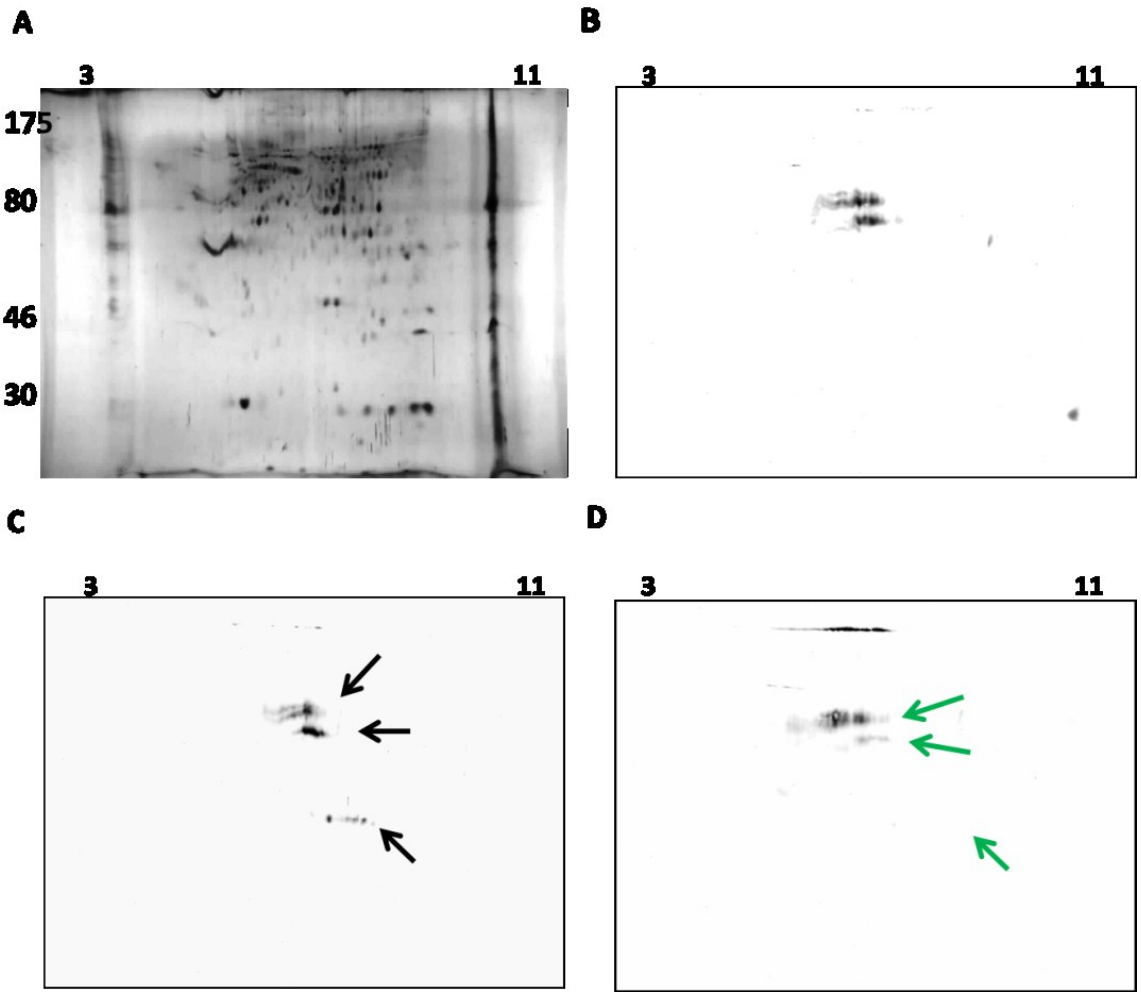


Figure 27. VIP treated activated HuT 78 cells has a unique nuclear protein expression pattern and ablates a lower molecular weight species. Nuclear proteins were separated by 2DE and IK protein was detected by IB (Materials and Methods). HuT 78 cells were activated for 4 hr +/- VIP and compared to no activation (0 hr). Nuclear cell lysate were separated and immunoreactive IK protein detected. Silver stained 2DE of 0 Hr sample (A). B-D separated 2DE gels were IB at 0 hr (B), and activated for 4 hr in the absence (C) and presence (D) of VIP. Black arrows depicts altered isoelectric species between 0 and 4 hr activated samples and green arrows represent differences in isoelectric species between activated and VIP treated samples.

Site directed mutagenesis was used to generate two additional IK isoforms for positive controls for RT-PCR and Western blots. IK 6 and IK 8 isoforms were created to identify the lower molecular weight species identified in the activated Hut 78 cells. Site directed mutagenesis was completed with pCMV FLAG-Tag 2B

IK 1 plasmid to generate IK 6 and IK 8 plasmids. Primers specifically designed to remove exons 4-7 (IK 6) and exons 4-6 (IK 8) from the IK 1 cDNA construct. Restriction digest was completed on IK 1, 6, and 8 pCMV-Tag2B plasmids with *Bam HI* cleaving once in the multiple cloning region and once within the IK sequence (Figure 28). A 1000 bp amplicon was detected for IK 1, while a 900 bp sequence was identified for IK 8, and a 760 bp amplicon was identified for IK 6 plasmid. These amplicons were the appropriate sizes for each of the created IK plasmids and will be validated by sequencing.

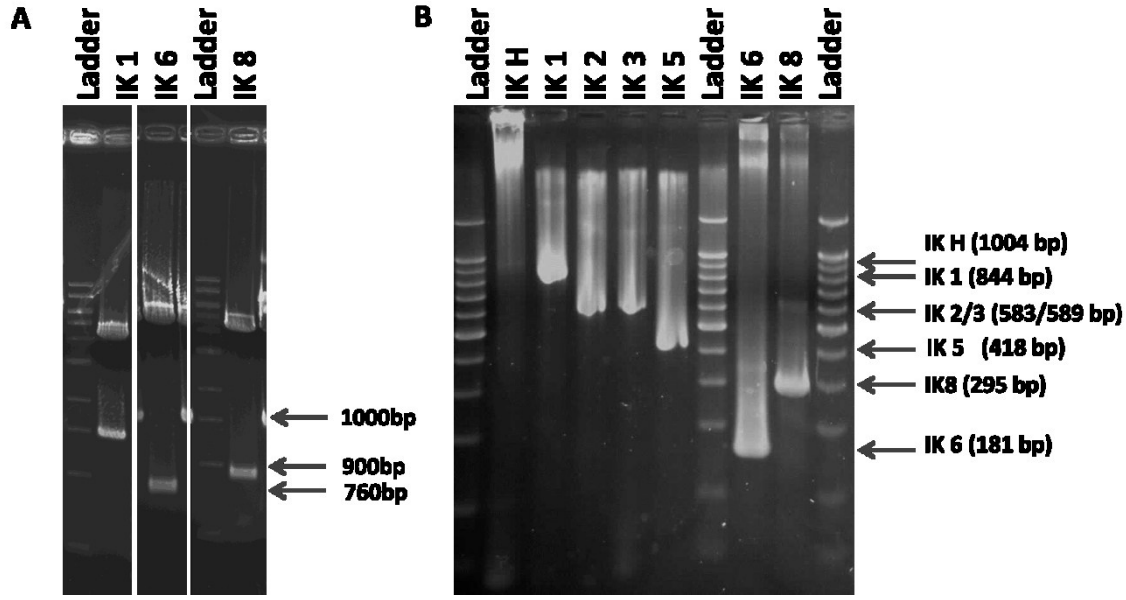


Figure 28. Ikaros 6 and 8 plasmid constructs created by site directed mutagenesis. Site directed mutagenesis was used to synthesize IK 6 and 8 plasmids from the pCMV-Tag 2B IK 1 construct (materials and methods). (A) *Bam HI* restriction digest of mutated plasmids generated fragments of the approximate size of IK 6 and 8. (B) PCR amplification of all IK plasmid constructs to determine IK mRNA species in HuT 78 cells.

Discussion

This study yielded several interesting and unknown findings of the T cell biology of Ikaros. We were able to conclude that VIP signaling in the naïve HuT

78 cells was unable to alter IK protein expression in the nucleus. We observed that VIP suppressed IK protein in activated HuT 78 cells. Additionally, we determined that VIP was altered IK protein expression in activated HuT 78 cells when normalized to Lamin A/C, a decrease in IK protein was observed when normalized to SP1. Lastly, we were able to conclude that VIP altered the isoelectric species of IK protein in activated HuT 78 cells.

HuT 78 cells express both DNA binding and dominant negative IK isoforms. IBs for IK protein revealed that IK 1 and 2 isoforms are expressed in HuT 78 cells as the predominant expressed IK isoforms. These are also two of the three major isoforms found in healthy human T cells (Ronni et al. 2007, Hahm et al. 1994). A lower molecular weight isoform was detected in the activated cells by 2D Western blots. Additional evidence that HuT 78 cells expressed DN isoforms was demonstrated in the RT-PCR using IK primers located within exons 4 and 8, two additional species with a smaller amplicon representing IK 5 and IK 3A. It would be intriguing if Hut 78 cells expressed IK 3A since this isoform has only been identified to only be expressed in the myeloid cell lineage (Payne et al. 2003, Payne et al. 2001). These data would suggest that IK 3A is not myeloid specific or that in leukemic T cells alternative splicing of the primary IK transcript is dysregulated and allows for the expression of this isoform.

We hypothesized that VIP/VPAC1 signaling would alter the expression levels of IK protein in naïve HuT 78 cells. VIP/VPAC1 canonical signaling through PKA could potentially phosphorylate IK (Figure 25) and result in a hyperphosphorylated IK protein, which would undergo ubiquitination and

degradation (Popescu et al. 2009, Gómez-del Arco et al. 2004). However, we were unable to detect a change in the IK protein upon VIP treatment in naïve HuT 78 cells when normalized to Lamin A/C nuclear protein. These results suggest that VIP signaling in the naïve HuT 78 cells does not regulate IK's protein expression this may be due to the naïve status of the cells.

Activated Hut 78 cells treated with VIP suppressed IK protein expression. Nuclear lysate from activated HuT 78 cells revealed that IK H, 1, and 2 proteins increased upon T cell activation. In active primary CD4 T cells there is an increase in IK H and 1 protein expression while IK 2 protein expression decreased (Ronni et al. 2007). These data demonstrated that leukemic HuT 78 cells acted similar to normal T lymphocytes for IK H and 1 while IK 2 protein expression does not mirror primary T cells. Next we investigated if VIP/VPAC1 signaling in activated HuT 78 cells would alter IK protein expression. VIP treated cells resulted in all three IK isoforms having similar levels to unactivated cells. These data suggest that IK protein expression is suppressed by VIP. This data was the first to implicate VIP/VPAC1 as a regulator of IK. The suppression of IK was unexpected as VPAC1 is an antiproliferative receptor and was hypothesized to increase IK protein to maintain HuT 78 cells in the naïve/unactivated state as IK is known to regulate T cell cycle entry.

Lamin A/C is transcriptionally regulated by VIP in PMA/ionomycin activated murine CD4 T cells. A microarray study was completed using murine CD4 T cells that predominantly express VPAC1 (similar to HuT 78 cells VIP receptor profile) were PMA/ionomycin activated and treated with VIP. After 5 hr of activation,

microarray analysis was completed and Lamin A gene expression was increased by 1.3 fold while unaffected in the naïve VIP treated cells. The increase in Lamin A/C expression could potentially mask an increase in nuclear IK protein upon VIP treatment in activated HuT 78 cells, if activated VIP treated HuT 78 cells responded similar to the primary murine CD4 T cells in the microarray study. It is unknown if the increase in Lamin A/C translates between species (mouse to human), but without further investigation it is warranted to hypothesize that Lamin A/C expression may be altered in HuT 78 cells too. The increased expression of IK in the activated state, but suppression of IK protein for activated Hut 78 cells treated with VIP may be a consequence of normalizing to Lamin. Therefore, activated cells treated with VIP will be normalized to SP1 nuclear protein.

IK protein levels are decreased in activated HuT 78 cells treated with VIP. Activated HuT 78 cells resulted in the increase expression of IK protein mirroring the results obtained when normalized to Lamin A/C. VIP treatment of activated Hut 78 cells resulted in a reduction of detectable IK protein in the nucleus when normalized to SP1. VIP/VPAC1 signaling resulted in decreased nuclear IK protein expression, a possible reason for this decrease is that when IK becomes hyperphosphorylated it is degraded as previously reported (Song et al. 2011, Popescu et al. 2009, Gómez-del Arco et al. 2004). The observed decrease of IK protein upon treatment of activated HuT 78 cells with VIP for both Lamin A/C and SP1 demonstrate that IK protein is regulated by VIP/VPAC1. Interestingly the suppression of IK when normalized to SP1 was greater than when normalized to Lamin. These data would suggest that VIP/VPAC1 signaling decreases Lamin

A/C protein expression in HuT 78 cells unlike primary murine CD4 T cells where an increase in transcript was detected.

Increased IK protein in activated HuT 78 cells is PTx sensitive. The increase in nuclear IK protein in activated HuT 78 cells demonstrates that IK protein responds similar to activated CD4 T cells (Ronni et al. 2007). Interestingly, the increase of IK was blunted upon PTx treatment in the activated HuT 78 cells. The activation method utilized in these experiments was PKC and Ca^{2+} dependent and $G_{\alpha s}$ independent and therefore inhibition of AC was unexpectently observed. These data do support that T cell activation is PTx sensitive and by inhibition of activation in HuT 78 cells (Paccani et al. 2008). IK protein should mirror the naïve VIP treated IK results.

Activation of HuT 78 cells was completed using a well-established method with PM and ionomycin (Boulougouris et al. 1998, Chatila et al. 1989). PMA activates PKC while ionomycin opens Ca^{2+} channels, which mimics T cell activation similar to TCR simulation. Upon activation of HuT 78 cells there was an increase in the surface protein expression of the IL-2 receptor (CD25). Interestingly, a decrease in surface protein expression was observed when activated Hut 78 cells were co-treated with VIP. VIP is known to have antiproliferative effects by inhibiting the production and secretion of IL-2 by a cAMP dependent mechanism (Wang et al. 2000) and VIP is known to induce a $CD4^{+}CD25^{+}$ Treg population (Delgado et al. 2005, Chen et al. 2005). However, suppression of CD25 by VIP has not been demonstrated to the best of our knowledge. These data would demonstrate that VIP not only inhibits cytokine

production (IL-2 and INFγ) needed for T cell activation, but also regulates the receptor expression for these cytokines. Therefore, VIP may downregulate CD25 to prevent activation if IL-2 is secreted by other cells.

IK protein in the cell is maintained in several different isoelectric species. Total cellular protein isolated from HuT 78 cells separated by isoelectric focusing, SDS-PAGE, and probed using an IK-CTS antibody revealed that there are numerous isoelectric pools of IK for each isoform. 2DE Western blots identified at least six different isoforms present in the naïve HuT 78 cells. Each of the immunoreactive IK isoforms detected ranged from 3-7 isoelectric species. Numerous phosphorylation sites have been characterized for IK, which could result in this unique pattern (Gómez-del Arco et al. 2004, Song et al. 2011, Popescu et al. 2009, Gurel et al. 2008, Ronni et al. 2007). Previous 2DE have been completed by digestion of IK 1 protein and separating peptide fragments to determine different phosphorylation sites (Song et al. 2011, Dovat et al. 2002, Gómez-del Arco et al. 2004). Unlike previous experiments that have focused on IK 1 protein, these experiments show all endogenous IK isoforms present in the Hut 78 cells and their unique isoelectric pattern. Any PTM that alters the isoelectric point of IK (Table 6) will result in a different pool of IK protein; these small changes may not be detectable by 1D Western blots. Therefore, small changes such as phosphorylation can be resolved at a higher resolution due to a change in the overall charge of IK and can be identified.

Activation of HuT 78 cells resulted in a decrease in the number of isoelectric species of immunoreactive IK. Also, activation increased the relative

intensities of specific IK pools, these data coincide with the increase in phosphorylation of IK upon T cell activation as previously reported (Gómez-del Arco et al. 2004, Dovat et al. 2011, Li et al. 2011b, Song et al. 2011, Dovat et al. 2002). These data show that within 4 hr of T cell activation IK protein is posttranslationally regulated (presumably by phosphorylation) and results in the decrease of identifiable isoelectric species. These data support the hypothesis that depending upon the isoelectric state of IK, it is able to interact with different chromatin remodeling complexes that are either suppressors or activators. This may be how IK is able to act as a repressor and activator by its discrimination for specific binding partners and its DNA affinity. The understanding of which isoelectric species of IK is interacting with each chromatin remodeling complex will increase our understanding of how IK is able to act as a rheostat for gene regulation.

VIP increases the number of immunoreactive nuclear IK species in activated HuT 78 cells. VIP treatment of activated HuT 78 cells result in a unique isoelectric pattern from 0 and 4 hr activated cells. The redistribution of IK pools decreases the intensity of IK species, and would correlate with the decrease of IK protein observed at the 1D level. The increase of isoelectric species of IK demonstrates that VIP/VPAC1 signaling not only decreases nuclear protein expression, but also includes posttranslational modifications of IK protein by increasing the number of isoelectric species. These data are the first to demonstrate that VIP is able to alter IK protein through posttranslational modifications as detected by the increase in immunoreactive IK species. It is

currently hypothesized that the PTM of IK protein that alters the isoelectric pattern is due to phosphorylation by PKA. Several residues have been identified to be putative phosphorylation sites for PKA (Table 5, Figure 25). This idea is further supported in that VIP/VPAC1 (the predominant VIP receptor expressed on HuT 78 cells) signaling results in an increase in i[cAMP] which, is an activator for PKA (O'Dorisio et al. 1981)

Several immunoreactive IK species are confined to the cytoplasm. Separation of cytoplasmic and nuclear proteins result in the reduction of IK isoforms detected. In total cell lysate, there were at least 6 different isoforms detected while in the nuclear fraction there were only two in the 0 hr sample. These data demonstrate that the cytoplasm is rich in IK protein. The numerous species could potentially be a result of IK protein being synthesized as protein translation occurs in the cytoplasm. Additionally, the lack of lower molecular weight IK isoforms supports the hypothesis that these cells lack the nuclear targeting sequence and are unable to enter the nucleus without dimerizing with a larger isoform. Lastly, the question that remains is whether the IK protein in the cytoplasm is important and do they have a cytoplasmic function?

Nuclear IK protein undergoes an alteration in isoelectric species upon activation and VIP treatment. At 0 hr, there are two IK isoforms detected with 3-5 different isoelectric species. Upon activation these two isoform of IK have a reduction in the number of immunoreactive IK pools, which coincides with the observation in the total cell lysate Western blots. Interesting, there is an additional lower MW species detected in the 4 hr activated nuclear lysate not observed at 0

hr. This data would suggest that upon activation of HuT 78 cells smaller isoforms of IK are translocated into the nucleus. These smaller isoforms upon translocation into the nucleus would impede IK's engagement with DNA due to the lack of the zinc fingers needed to bind DNA (Li et al. 2011a). Another possible scenario for the detection of a lower molecular weight species is that hyperphosphorylated IK protein is being degraded and we are detecting IK breakdown products (Dovat et al. 2011, Song et al. 2011, Popescu et al. 2009, Gómez-del Arco et al. 2004). The degradation of IK protein is a possibility, we have observed a breakdown product when overexpressing IK in NIH 3T3 cells that yield lower molecular weight immunoreactive IK (Figure 18, 20, and 22, positive control lanes for IK 1 and 2). The possibility that this is the cause of the smaller fragments is unlikely since only three isoelectric species were detected and all have a similar molecular weight.

VIP treatment of activated Hut 78 cells alters the isoelectric species of IK protein from activated cells alone. Immunoreactive IK species detected in activated HuT 78 cells treated with VIP have an altered expression profile compared to naïve and activated HuT 78 cells. This altered expression demonstrated that VIP was able to regulate the isoelectric profile of IK protein. There is a decrease in intensity of the lower of the two higher molecular weight species, presumed to be IK 2 isoform. In addition to the decreased expression, there is an increase in number of higher molecular weight isoelectric species of IK. This would suggest that VIP is able to regulate both of these isoforms. Interesting, there is a complete loss of the lower molecular weight species that was detected in the activated HuT 78 nuclear cell lysate. This loss of the lower molecular weight

species suggests that VIP prevents the translocation of lower molecular weight species into the nucleus. If the lower molecular weight species was a degradation product of IK upon activation it would demonstrate that IK protein is protected from degradation by VIP.

The determination of whether the lower molecular weight species in the activated HuT 78 cell lysate is a breakdown product could be determined by use of a protease inhibitor to prevent the degradation of IK protein. If the lower molecular weight species was a breakdown product, these signals should be ablated upon protease inhibition. Additionally, increasing the resolution of the 2DE by decreasing the pH range from 3-11 (nonlinear) to a more neutral range of pH 5-8 and to use larger isoelectric focusing strips from 7 cm to 13 cm to increase separation and resolution. Identification of the lower molecular weight species could be determined by spot picking and sequencing by mass spectroscopy.

If the lower molecular weight species was a DN isoform of IK, this could be supported by overexpressing DN isoforms, separated by 2DE and probing for IK. However, PTMs to IK proteins in non-T cells may be mute if the PTMs are unique to lymphocytes and not in other cell types (i.e. NIH 3T3 cells). Utilization of positive controls for additional IK isoforms expressed in HuT 78 cells could aid in the identity of the lower molecular species. To generate additional IK-DN isoforms, site directed mutagenesis was completed from the pCMV Tag 2B IK 1 plasmid construct. Site directed mutagenesis removed exons 4-7 for IK 6 and exons 4-6 for IK 8 generating two additional positive controls to be utilized.

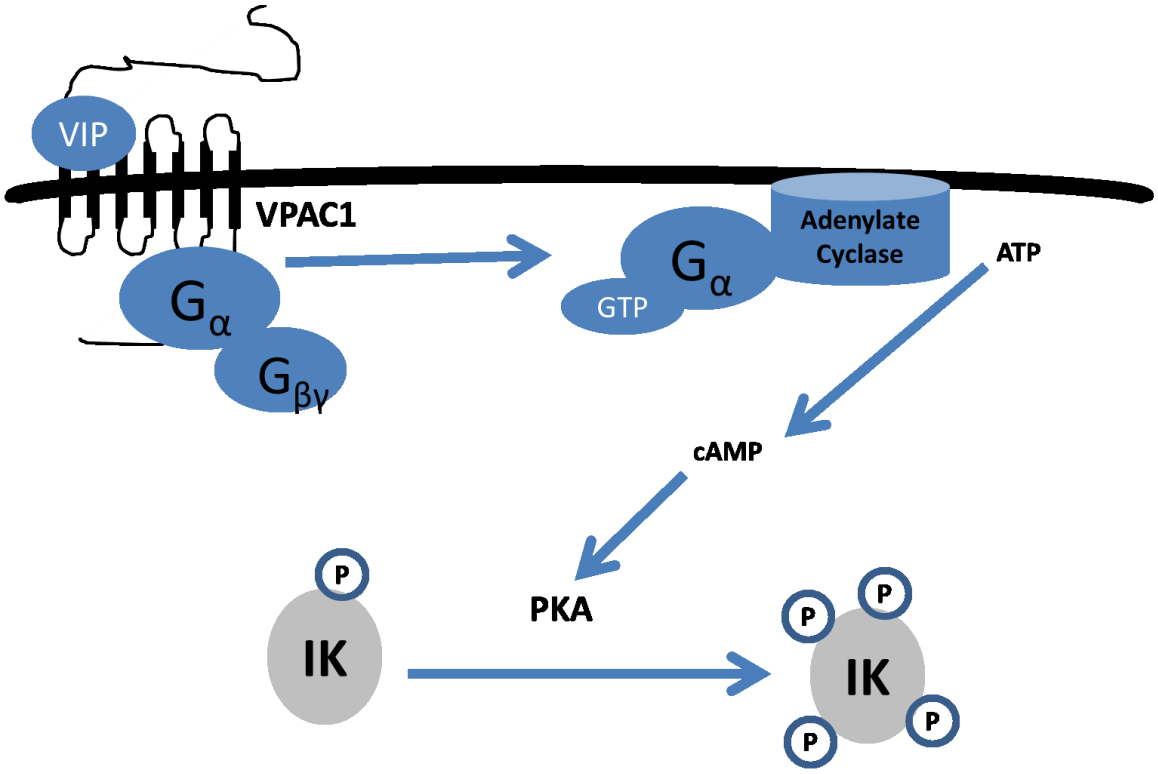


Figure 29. Working hypothesis for VIP/VPAC1 signaling to alter Ikaros biology. VIP binding to the VPAC1 results in the activation of the Gαs signaling cascade. IK protein is maintained in a hypophosphorylated state, but PKA increases the number of phosphorylated residues in return decreasing IK protein stability and altering its isoelectric points.

These data demonstrated that VIP signaling in naive Hut 78 cells does not alter nuclear IK protein expression determined by one-dimensional Western blot analysis. However, activated HuT 78 cells treated with VIP resulted in a decrease of nuclear IK protein compared to the activated cells. Additionally, the number of IK isoelectric species identified is altered in HuT 78 cells between a naïve and activated cell state. Our data supports that the reduction in the number of isoelectric species of IK would result from IK protein having a similar phosphorylation pattern upon activation. These data also demonstrated that IK's isoelectric state can be regulated by VIP signaling through VPAC1 in HuT 78 cells

(Figure 29). The increase in the number of immunoreactive IK species detected in total and nuclear cellular proteins, demonstrated that VIP signaling altered both cytoplasmic and nuclear IK protein. Additionally, VIP appears to impede the detection of a lower molecular weight species suggesting that VIP may regulate the translocation of IK-DN species into the nucleus. Lastly, these data are the first to demonstrate that VIP/VPAC1 signaling decreased the expression of the IL-2 receptor (CD25) in activated HuT 78 cells.

These data suggest that in activated T cells IK undergoes posttranslational modifications that allows for the rearrangement of IK protein localization and alters its biological function. The decrease in IK protein detected upon T cell activation would allow for T cells to enter into the cell cycle due to the loss of IK protein that sets the threshold for T cell activation by lowering the amount of IK protein in the nucleus this would allow for a lower threshold to be reached to allow for cell cycle entry. Additionally, the regulation of IK protein by the antiproliferative receptor VPAC1 signaling upon VIP binding would demonstrate that in leukemia VPAC1 does not retain its antiproliferative properties and that cancer cells may be able to highjack VPAC1 to aid in its ability to survive. These data potentially suggest that in activated leukemic T cells that VIP has the potential to be a “growth factor” allowing for a rapid proliferation.

CHAPTER 3. HUMAN T CELLS EXPRESSING VASOACTIVE INTESTINAL PEPTIDE RECEPTORS 1 AND 2 MEDIATE CELLULAR MIGRATION THROUGH ALTERNATIVE SIGNALING PATHWAYS

Introduction

VIP Receptor Expression in T Cells

VPAC1 and 2 are GPCRs that are ubiquitously expressed in all cell types. VPAC expression was first detected in immune cells in the early 1980's (Guerrero et al. 1981, O'Dorisio et al. 1980, O'Dorisio et al. 1981). Peripheral blood mononuclear cells (PBMCs) isolated from whole human blood were identified to have a single class of receptors as assessed by ^{125}I-VIP and Scatchard analysis. This study calculated 1,700 VIP receptors/cell with a K_D of 0.47 nM (Payan et al. 1984). Subsequent examination of highly purified T cells (>95% purity) validated even higher cell surface expression density of VIP receptors. CD4 T cells bound greater amounts of ^{125}I-VIP than CD8 T cells. This ^{125}I-VIP binding assay revealed approximately 8,400 binding sites/CD4 T cell, while activated CD4 T cells had a decrease by ~40% (5,100 receptors) after 6-8 hr of activation (Figure 30) (Johnston et al. 1994). For example, the VIP receptor responsible for binding VIP on CD4 T cells was most likely VPAC1 as it is expressed 500 times greater than VPAC2 at the mRNA level (Dorsam et al. 2010). Upon CD3/CD28 activation of CD4 T cells, VPAC1 mRNA expression levels decreased 80% (Vomhof-DeKrey and Dorsam 2008, Vomhof-DeKrey et al. 2008). Also, additional human immune cells express VPAC receptors as radioactively labeled ^{125}I-VIP was able to identify specific binding sites on B and NK cells at a lower level (Calvo et al. 1986). This is

most likely why there is a discrepancy between the number of VIP receptors in PBMCs and purified CD4 T cells.

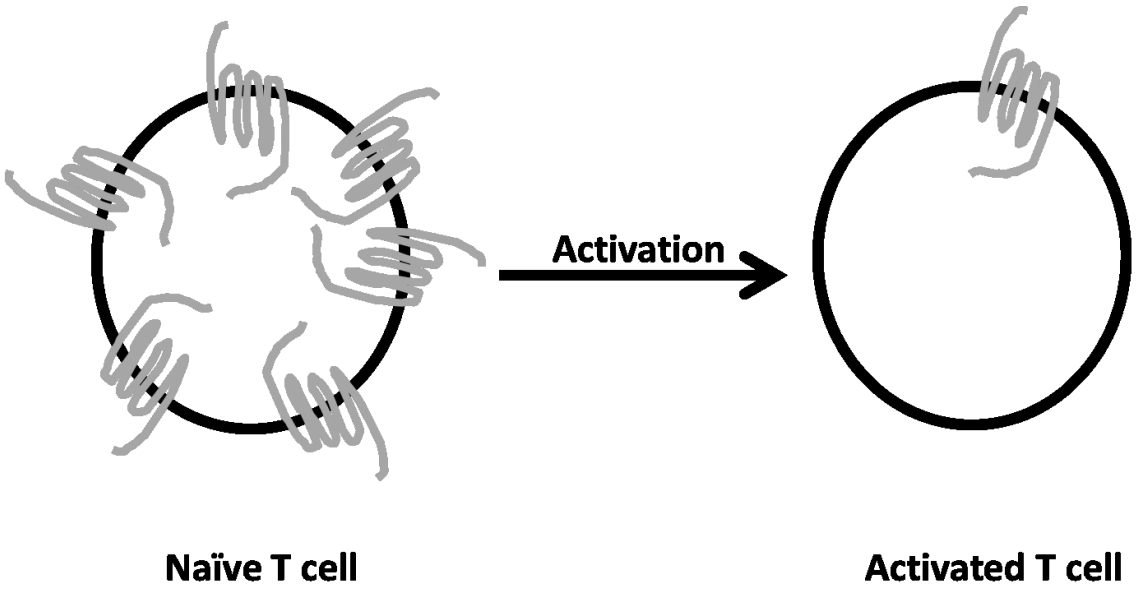


Figure 30. Expression profile of VPAC1 on T lymphocytes. In naïve T cells, VPAC1 expression is maintained at a high level. Upon activation of T cells VPAC1 protein is removed from the plasma membrane at 80-90% reduction in surface protein from naïve cells.

The expression profile of VPAC receptors on T cells is activation status dependent. In the naïve state, when cells are in the G_1 phase, VPAC1 is the predominantly expressed receptor (Hermann et al. 2012, Dorsam et al. 2010, Benton et al. 2009). Upon activation, VPAC1 expression at both the mRNA and protein levels decreased after 24 hr by qRT-PCR and antibody staining/flow cytometry (Hermann et al. 2012, Vomhof-DeKrey et al. 2008). These data agree with the study reported by Johnston et al. (Johnston et al. 1994). VPAC2 is expressed at low levels in the naïve state, but is upregulated in activated mouse T cells, which resulted in this receptor to be coined the "inducible." This was first observed by Delgado et al. (Delgado et al. 1996b).

The expression of VIP receptors was unknown until the 1990's after VPAC1 and 2 receptors were isolated and cloned. The VPAC1 gene (*vipr1*) was cloned in 1992 and the VPAC2 gene (*vipr2*) was cloned soon after (Ishihara et al. 1992, Svoboda et al. 1994, Lutz et al. 1993). In many leukemic cancers there is an increase in the number of VIP specific binding sites compared to healthy cells (Finch, Sreedharan and Goetzl 1989, Beed et al. 1983, Cheng et al. 1993, Robichon et al. 1993). The reason for this was because both VPAC1 and 2 receptors bind VIP with similar affinity (Couvineau, Amiranoff and Laburthe 1986, Ciccarelli et al. 1995) and researchers were unable to distinguish between the two receptors and the receptors were not cloned until the 1990's. In healthy naïve T cells ^{125}I-VIP binding sites identified were predominantly the VPAC1 receptor. However, in leukemic cells the predominantly expressed VIP receptor is VPAC2. Therefore, the increase in VIP specific binding sites was a result of increased expression of VPAC2 and not VPAC1. VPAC2 is the predominantly expressed receptor at the mRNA level in mouse, rat, and human T cell lines with little or no VPAC1 mRNA detected (Dorsam et al. 2011, Delgado et al. 2004b). Similarly, patients with T and B cell leukemia had a decrease in VPAC1 and an increase in VPAC2 mRNA expression (unpublished data). The decrease in VPAC1 expression may result in the leukemic cells having a hyperproliferative phenotype as VPAC1 suppresses T cell activation and proliferation.

The Neuropeptide called Vasoactive Intestinal Peptide

Vasoactive intestinal peptide (VIP) was first isolated by Said and Mutt in 1970 from the small intestine of swine. This peptide was originally shown to result

in vasodilatation regulation and reduction of arterial blood pressure (Said and Mutt 1970a, Said and Mutt 1970b). The VIP gene (*vip*) maps to human chromosome 6 (Tsukada et al. 1985). The VIP gene transcribes a six exon mRNA species. The mRNA coding for human VIP is translated into an 170 aa precursor peptide, preproVIP(Itoh et al. 1983, Nishizawa et al. 1985). This peptide undergoes posttranslational processing to yield two functional neuropeptides (Figure 31), the 28 aa VIP and a 27 aa peptide with N-terminal histidine and C-terminal isoleucine amide (PHI) (Fahrenkrug 2010).

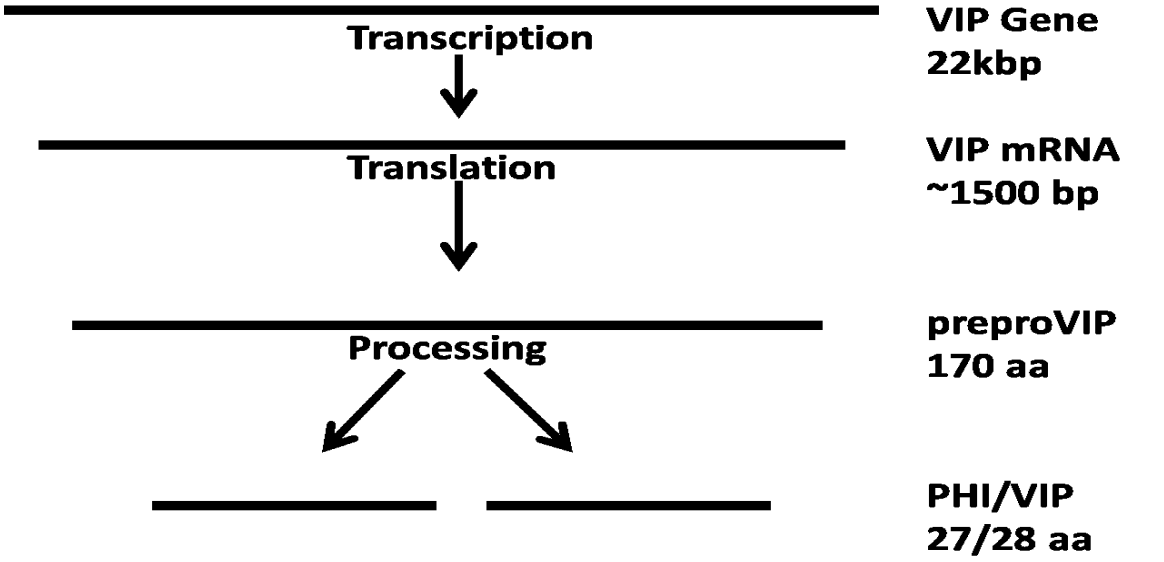


Figure 31. The VIP gene encodes two neuropeptides. VIP gene encodes for both vasoactive intestinal peptide and N-terminal histidine and C-terminal isoleucine amide (PHI). The 170 aa preproVIP is processed to release both a 27 and 28 aa neuropeptides.

VIP is a highly conserved peptide across species as it has an identical aa sequence in human, bovine, rat, goat, rabbit, swine, and canine (Bunnett et al. 1984, Dimaline et al. 1984, Gossen et al. 1990, Eng et al. 1986, Said and Mutt 1970a). Slight diversity occurs in both guinea pig and chicken with a deviation of

four amino acids (Eng et al. 1986, McFarlin et al. 1995). The evolutionarily conserved nature of VIP indicates important biological function including smooth muscle relaxation in intestinal and respiratory tissue, and stimulation of electrolyte secretion in the gut (Barbezat and Grossman 1971, Piper, Said and Vane 1970). Later studies identified that VIP affects thymus biology by altering cytokine production, apoptosis, differentiation, and mobility (Ernström, Gafvelin and Mutt 1995, Xin et al. 1997, Xin and Sriram 1998, Delgado et al. 1996a, Pankhaniya et al. 1998).

Activated CD4 T cells (Th0) can differentiate into four subsets of effector cells called Th1, Th2, Th17, and Tregs (Figure 10). Type 1 helper T cells (Th1) stimulate phagocyte-mediated defense by ingestion and killing of microbes. These cells produce inflammatory cytokines such as interferon-γ (INF-γ), IL-2, and tumor necrosis factor (TNF) and activate macrophages to target microbes. Th2 cells stimulate IgE and eosinophil/mast cell immune response. These cells produce IL-4 and IL-5 cytokines. IL-4 cytokines stimulate production of IgE antibodies from B cells, while IL-5 activates eosinophils (Abbas and Lichtman 2009). Additionally, release of IL-4 and IL-5 cytokines from Th2 cells result in the inhibition of IFNγ and IL-2 production and further the differentiation of activated Th0 cells into Th2 effectors (Delgado et al. 1999c, Delgado et al. 1999d, Delgado et al. 1999a, Delgado et al. 1999e). Th17 cells defend against some bacterial and fungal infections and inflammatory disorders such as multiple sclerosis, inflammatory bowel disease, and rheumatoid arthritis. Regulatory T cells (Tregs) block the activation of lymphocyte specific self-antigens (Abbas and Lichtman 2009).

VIP plays a major role in the differentiation of naïve CD4 helper T cells. T cell differentiation is regulated by the local cytokine environment. VIP signaling favors the differentiation of Th0 cells into Th2 cells by upregulating the expression and secretion of the Th2-promoting IL-4 and IL-5 cytokines, and simultaneously inhibits Th1-promoting IFNγ and IL-2 production (Delgado et al. 1998, Delgado and Ganea 1999, Delgado et al. 1999a, Delgado et al. 1999e, Delgado and Ganea 2001c). This has been verified in vivo through the use of transgenic mice overexpressing human VPAC2 on CD4 T cells that showed increased expression of Th2 promoting cytokines (Voice et al. 2001). In contrast, VPAC2 knockout mice demonstrated the opposite phenotype by skewing towards Th1 promoting cytokines expression (Goetzl et al. 2001). These two mouse models clearly demonstrate a role VIP/VPAC2 receptor signaling in directing differentiation towards Th2 cells.

Vasoactive Intestinal Peptide Receptor 1

The expression profile of VIP receptors has been established to be ubiquitous. VIP receptors have been identified in the liver, lungs, adipose tissue, intestine, and in lymphocytes (Laburthe et al. 1979, Prieto, Laburthe and Rosselin 1979, Desbuquois 1974, Bataille, Freychet and Rosselin 1974). The identification of VIP was in 1970 (Said and Mutt 1970a). It was not until 1993 that the human VPAC1 receptor was identified (Sreedharan et al. 1993).

The VPAC1 cDNA was cloned from human colon adenocarcinoma HT29 cell line resulting in a 52 kDa protein (Sreedharan et al. 1993). The VPAC1 gene (*Vipr1*) is a 55 kbp sequence located on human chromosome 3 (Cardoso et al.

2007, Lutz et al. 1993, Sreedharan et al. 1995). *Vipr1* gene is transcribed into a 2.2 kbp mRNA sequence that encodes a 13 exon mRNA species that yields the 457 aa full-length VPAC1 protein (Sreedharan et al. 1993, Sreedharan et al. 1995). VPAC1 is a seven transmembrane heptahelical serpentine receptor that has an extended extracellular N-terminal tail (>120 aa) and a shorter intracellular C-terminal tail. VPAC1 has high affinity for the neuropeptide VIP with a K_d ~1nM (Sreedharan et al. 1993).

VPAC1 is a G protein coupled receptor that can bind to several G-proteins to initiate a signaling cascade (Figure 32). VIP/VPAC1 signaling has been demonstrated by our laboratory and others to efficiently couple to the $G_{\alpha s\beta\gamma}$ protein. VIP binds to the VPAC1 protein resulting in the conformation change and allowing for the exposure of critical residues located within the third intercellular loop and the C-terminal tail. G proteins bind to these exposed residues and become activated upon exchange of the bound GDP for GTP. The αs-subunit dissociates and activates AC. Adenylate cyclase enzymatically converts ATP to cAMP, which activates PKA (Calvo et al. 1986, Calvo, Guerrero and Goberna 1989, Couvineau et al. 1990, Kermode et al. 1992). Additionally, VPAC1 is able to couple to the $G_{\alpha i}$ and $G_{\alpha q}$ proteins (Van Rampelbergh et al. 1997, McCulloch et al. 2000)

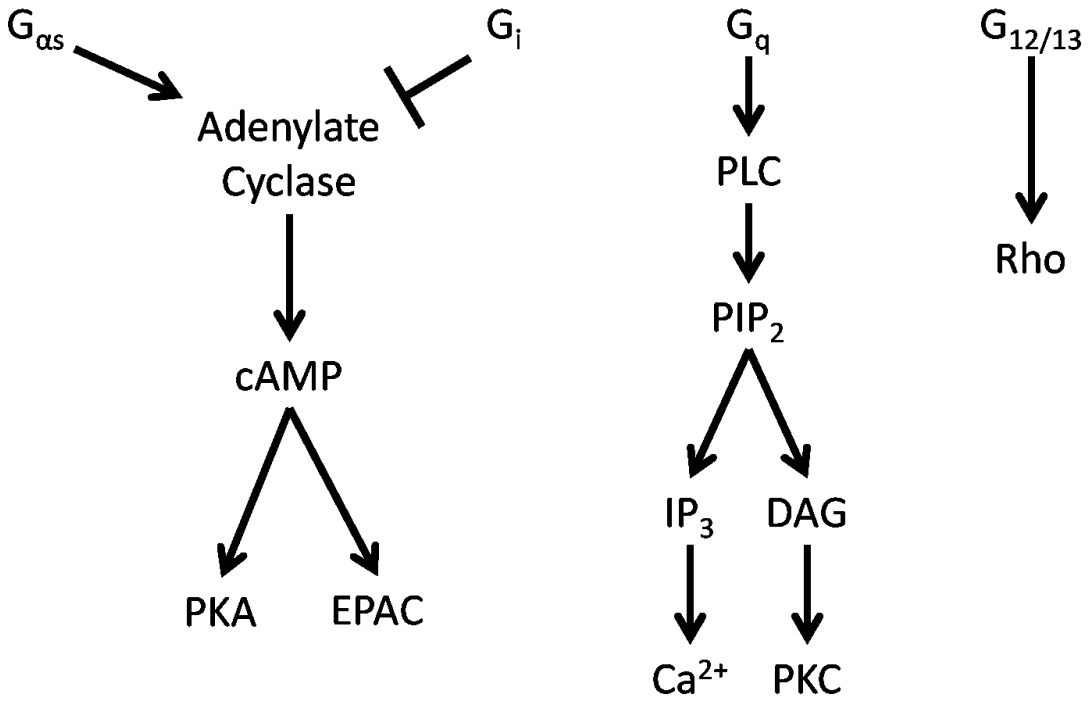


Figure 32. GPCR signaling cascades elicited upon ligand binding. VPAC1 and 2 are able to couple to any of the four major G proteins and initiate signal transduction. Signaling cascade of activated α-subunit is responsible for the initiation of signaling depicted here.

VIP signaling has antiproliferative action on newly activated T cells mediated by the predominant VIP receptor, VPAC1. VIP impedes T cell activation by inhibiting critical cytokines that are expressed upon TCR stimulation to enhance T cell activation. One of these cytokines that VIP suppresses is IL-2 (Ganea and Sun 1993, Sun and Ganea 1993, Gomariz et al. 2001). VIP suppresses IL-2 expression by regulation of two transcription factors that bind and promote transcriptional expression. VIP/VPAC1 couples to $G_{\alpha s}$ resulting in an increase of i[cAMP] and activation of PKA , which phosphorylates nuclear factor of activated T cells (NF-AT) protein (Delgado and Ganea 2001b). Phosphorylated NF-AT protein maintains cytoplasmic localization and preventing NF-AT from binding to the IL-2 promoter and initiating transcription (Hermann-Kleiter et al. 2006). Several

additional transcription factors are regulated by VIP signaling including NF-κB, IRF-1, MAPK, and CRE (Delgado, Varela and Gonzalez-Rey 2008), which result in the suppression of the pro-inflammatory cytokines such as TNFα, IL-12, and iNOS (Leceta et al. 1996). NF-κB is a transcriptional activator for the pro-inflammatory IL-12. VIP/VPAC1 signaling impedes the phosphorylation of NF-κB's regulatory subunit and sequestering an inactive NF-κB in the cytoplasm (Le Beau et al. 1992, Leceta et al. 2000, Ganea and Delgado 2003). VIP/VPAC1 signaling also increases the anti-inflammatory cytokines, such as IL-10, further suppressing the immune response. The induction of IL-10 is mediated by the increase in cAMP-dependent CREB binding protein (Delgado et al. 1999c).

Another immunosuppressive mechanism that VPAC1 is known to inhibit in T cells is the G_1/S cell cycle transition. The antiproliferative properties of VPAC1 results from the regulation of the cyclin dependent kinase inhibitor $p27^{kip1}$ by impeding protein degradation, as well as, maintaining nuclear localization (Anderson and Gonzalez-Rey 2010, Kathrein et al. 2005). T cells are unable to transition from G_1 to S phase of the cell cycle with high levels of $p27^{kip1}$ as it associates with and inactivates a G_1/S Cyclin-Cdk nuclear complex. For normal cell cycle progression, $p27^{kip1}$ protein is phosphorylated resulting in the dissociation of the $p27^{kip1}$-CyclinE-Cdk2 complex. This complex is required to phosphorylate many proteins required for $G_1 \rightarrow S$ progression. VIP/VPAC1 signaling acts in opposing cell proliferation by maintaining a tight association of $p27^{kip1}$-CyclinE-Cdk2 (Reynisdóttir et al. 1995, van den Heuvel 2005).

More recently, a 5TM VPAC1 receptor has been identified, which has similar affinity for VIP (Bokaei et al. 2006). The 5TM splice variant lacks the sixth and seventh transmembrane and the third intercellular and extracellular loop. Due to the lack of the third intercellular loop, the 5TM VPAC1 receptor is unable to couple to G proteins. Overexpression of the 5TM VPAC1 receptor demonstrated a lack of an i[cAMP] response upon VIP treatment, while overexpressed 7TM VPAC1 elicited a robust i[cAMP] response. This data supported the inability of the 5TM VPAC1 receptor to couple to G proteins. It was hypothesized that the 5TM VPAC1 receptor signals through a tyrosine kinase. Additionally, four VPAC1 splice variants have been discovered in our laboratory (Hermann et al, manuscript in preparation). These splice variants also were unable to couple to G proteins and did not elicit a i[cAMP] response upon exogenous VIP treatment. Shorter splice variants have yet to be cloned and we are unaware of additional possible exons that are removed.

Vasoactive Intestinal Peptide Receptor 2

VPAC2 is another GPCR that binds VIP with high affinity with a unique expression profile in the immune system. VPAC2 is expressed at low levels (if at all) on naïve CD4 splenocytes (Metwali et al. 2000), absent in murine CD8 splenocytes (Vomhof-DeKrey et al. 2011a), and low in T cells isolated from peripheral blood (Lara-Marquez et al. 2001). In contrast to the downregulation of VPAC1 in activated T cells, VPAC2 mRNA and surface protein expression increases under certain environmental conditions (e.g. Th2 versus Th1

chemokines). Therefore, VPAC2 is considered the inducible VIP receptor in T cells (Delgado et al. 1996b).

The VPAC2 gene (*vipr2*) was first cloned in 1994, and is encoded by a 117 kbp DNA sequence that located on chromosome 11 (Svoboda et al. 1994). *Vipr2* transcribes a 457 aa, 7TM receptor that contains 13 exons (Cardoso et al. 2007). VPAC2 was demonstrated to be a functional receptor that couples to $G_{\alpha s}$ and elicits a robust i[cAMP] response upon VIP treatment (Svoboda et al. 1994).

VPAC2 has been identified to play a key role skewing T cell differentiation into Th2 effector cells. In the absence of VPAC2, there was a lack of Th2 effector T cells as evidence by the Th1 promoting cytokines, whereas VPAC2 transgenic mice showed the opposite phenotype resulting in an increase in the Th2 promoting cytokines (Voice et al. 2003). These data demonstrate that upon activation of T cells VIP/VPAC2 contributes to tailoring of the immune system to clear infection by differentiating towards a humoral Th2 immune response distinct from a cell-specific Th1 response. VIP/VPAC2 signaling induces the expression of JunB and c-Maf, which are essential transcription factors needed to initiate IL-4 cytokine transcription (Voice et al. 2003, Wang et al. 2000). Additionally, VIP signaling inhibits the expression of the Th1 cytokine IL-2 and inhibiting Th1 differentiation (Ganea and Sun 1993, Sun and Ganea 1993).

In addition to regulating the immune response and differentiating T cells, VIP signaling through VPAC2 also prevents apoptosis. VPAC2 signaling increases cell survival due to its regulation of anti-apoptotic proteins. For example, VIP/VPAC2 induces the anti-apoptotic factor Bcl-2 protein expression

(Gutiérrez-Cañas et al. 2003, Onoue et al. 2004, Sahin et al. 2006). Bcl-2 transcription is regulated by the cAMP responsive element binding protein (CREB), which binds to the Bcl-2 promoter and initiates transcription upon VIP/VPAC2 signaling (Xiang et al. 2006, Mehrhof et al. 2001, Wilson, Mochon and Boxer 1996). Additionally, VPAC2 signaling in a PKA-dependent mechanism is responsible for the phosphorylation of the pro-apoptotic protein BAD resulting in its inactivation and enhanced survival response (Sastry, Karpova and Kulik 2006a, Sastry et al. 2006b). Therefore, VPAC2 expression may be upregulated in activated T cells to prevent the cell from undergoing apoptosis during the cell cycle and an immune response.

VPAC1 and VPAC2 Signaling

GPCRs utilize three major signaling cascades to elicit a cellular response from extracellular stimuli. These cascades are propagated by different heterotrimeric G protein complexes consisting of $G_{\alpha s}$ (stimulatory), $G_{\alpha i}$ (inhibitory), and $G_{\alpha q}$ monomers that VPAC1 and 2 couple to resulting in a diverse of biological responses (McCulloch et al. 2000). Some of these responses include phagocytosis and proliferation (Hamm 2001, Karnik et al. 2003, Kristiansen 2004). Each of these biological responses result from a conformational change in the VPAC receptor whereby its TM3 and TM6 shift upon ligand binding, which produces the conformational change and efficient coupling of G protein complexes. Heterotrimeric G protein complexes consist of an α-, β-, γ-subunit, and the inactive G protein complexes are bound with a GDP. Upon binding of the heterotrimeric G protein complex to the intracellular tail of the VIP receptor, GDP is

exchanged with GTP allowing for the dissociation of the α-monomer from the βγ-dimer. $G_{\alpha s}$ monomers "stimulate" AC activity elevating the production of i[cAMP] (Figure 32). The increase in localized cellular cAMP activates downstream effector enzymes including PKA or EPAC. Activation of PKA results from the binding of two cAMP molecules to two regulatory subunits bound to the two PKA catalytic subunits forming an inactive PKA tetramer (Figure16). Binding of cAMP releases the regulatory subunits from the catalytic subunits allowing for the phosphorylation of PKA target proteins.

The remaining two signaling pathways VIP receptors are known to interact with are $G_{\alpha s}$ and $G_{\alpha q}$. The inhibitory pathway of AC occurs upon VPAC receptors coupling with the $G_{\alpha i}$ pathway. The Gαi trimeric proteins dissociate and the α-subunit binds and inhibits AC activity. The third pathway is $G_{\alpha q}$, which utilizes phospholipase C (PLC) to cleave phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 increases the intracellular Ca^{2+} concentration and DAG activates protein kinase C (PKC).

There are several additional signaling mechanisms that VPAC receptors are known to initiate. VPAC receptors are known to interact with small G-protein ADP-ribosylation factor (McCulloch et al. 2001) or receptor activity-modifying protein 2 (RAMP 2), which initiates a signaling cascade that is G protein independent (Sexton et al. 2006, Christopoulos et al. 2003). In addition to these signaling mechanism, VPAC1 also contains a PDZ domain on its C-terminus, which allows for additional signaling. The two major functions of PDZ domains are to anchor integral membrane proteins (receptors, transporters, channels, and

adhesion proteins) and bind with other PDZ domains creating a network scaffolding that creates higher order oligomers (Day and Kobilka 2006). Gee et al. recently demonstrated that the PDZ domain of VPAC1 interacts with the synaptic scaffolding molecule (S-SCAM also known as MAGI-2). This interaction identified VPAC1's utilization of the PDZ domain to alter signaling. S-SCAM binding inhibits VPAC1 internalization and cAMP generation (Gee et al. 2009a, Gee et al. 2009b). These results suggest that the ability for VPAC1 to couple with $G_{\alpha s}$ is dependent on whether its PDZ domain is engaged with S-SCAM. This mechanism can contribute to the "cell-context" signaling phenomena observed for these receptors.

Vasoactive Intestinal Peptide Transcriptome

Recently, a large microarray study was completed resulting in the generation of the largest known transcriptome regulated by VIP signaling in murine CD4 T cells. CD4 T cells were either maintained in the naïve state or activated using phorbol 12-myristate 13-acetate (PMA) and ionomycin to mimic T cell activation and treated +/- VIP (10^{-7} M). As a result of this study, it was identified that VIP signaling (through VPAC1 in the naïve state) uniquely altered expression of 248 genes and in the activated state 206 genes. Of the genes identified, 60 were altered in both the naïve and activated state. Based on analysis of the activated CD4 T cell microarray data, VIP was identified to modulate a unique gene set involved in cellular movement (Table 7). VIP signaling was presumed to be through VPAC1 as a result of the limited activation time of 5 hr, this would most likely not be enough time to upregulate VPAC2 surface expression and VIP would be degraded before VIP/VPAC2 signaling could be initiated. These data

demonstrated a possible mechanism that is responsible for the antiproliferative effects of VPAC1 and the chemotactic response T cells have towards VIP.

Table 7. VIP regulated genes in naïve and activated CD4 T cells.

Symbol	Gene Name	Naïve Fold Change	Activated Fold Change
Cxcl10	Chemokine (C-X-C motif) ligand 10 (IP-10)		1.8
Ccr8	Chemokine (C-C motif) receptor 8		1.8
Il5	Interleukin 5 (Il5)	1.7	
Egfr	Epidermal growth factor receptor (Egfr)	1.4	
Adam15	Disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 15 (Adamts15)	1.3	
Grb7	Growth factor receptor bound protein 7 (Grb7)	1.3	
Pak1	p21 (CDKN1A)-activated kinase 1 (Pak1)	1.5	
Snail1	Snail homolog 1 (Drosophila) (Snai1)	1.5	
Qrich	Glutamine rich 2 (Qrich2)	1.4	
App	Amyloid beta (A4) precursor protein (App)	1.3	

Chemotactic Responses to VIP

VIP is a known chemoattractant for the homing of naïve T cells to Peyer's patches in the gut. Downregulation of VIP receptors decreases migration and supported VIP as a chemoattractant (Ottaway 1984, Ottaway and Greenberg 1984). This migration phenomena is not limited to T cells as it is a chemoattractant with other cell types that express high levels of VPAC1 (Xia et al. 1996a). Naïve CD4 T cells have a greater chemotactic response towards VIP than activated CD4 T cells, and was suggested by the authors of the study to be due to the decrease in the number of VIP specific binding sites on activated T cells (Johnston et al. 1994). For example, Th1 cells lack a chemotactic response, while

VIP is a chemoattractant for Th2 cells (Delgado, Gonzalez-Rey and Ganea 2004a). Interestingly, Th1 cells and leukemic T cells that predominantly express VPAC1 lack of cellular migration towards VIP (Schratzberger et al. 1998). While Th2 cells express both VPAC1 and 2 and SupT1 cells that predominantly express VPAC2 were chemotactic towards VIP (Xia et al. 1996c). Due to this vast difference of results that VPAC1 could be chemotactic towards VIP in naïve T cells and Th2 effector cells, but not in Th1 or cell lines that express VPAC1, we investigated the role of VIP as a chemoattractant for VPAC1.

We set forth to understand the signaling mechanism that was responsible for the cellular migration towards VIP in cells that predominantly expressed VPAC1 or VPAC2. We hypothesized that VPAC 1 and 2 receptors would utilize the same signaling cascade but would have different biological effects on chemotaxis.

Summary of Results

The major goal of this project was to determine if VPAC1 and VPAC2 have the same biological function (chemotaxis) utilizing the same signaling pathway. It has been reported that VPAC1 and VPAC2 expressing cells both are chemotactic towards VIP. However, most studies that have demonstrated chemotaxis utilize cells that express both VPAC1 and VPAC2. Therefore, we decided to utilize two cell lines that predominantly express either VPAC1 or VPAC2. We hypothesized that VPAC1 and VPAC2 are chemotactic towards VIP employing the same signaling cascade initiated upon VIP binding. We tested this hypothesis by using HuT 78 cells that only express VPAC1 and Molt 4 cells that predominantly express

VPAC2. We validated receptor expression by TaqMan qRT-PCR and flow cytometry. Additionally, we investigated the signaling cascade elicited upon VIP binding by measuring i[cAMP] elicited and coupling to $G_{\alpha s}$. Lastly, we demonstrated that VIP receptors were chemotactic towards VIP. We discovered that quantitating mRNA expression of VPAC receptors does not correlate to VIP surface expression. Additionally, we also discovered that Hut 78 cells resulted in a unimodal i[cAMP] response with maximal response at 10^{-10} M VIP, while Molt 4 cells had a robust i[cAMP] response that was VIP concentration dependent. To this end, we determined that VPAC1 does not couple to $G_{\alpha s}$ while VPAC2 does supporting the cAMP response. Lastly, we determined that VPAC2's chemotactic response was adenylate cyclase and PKA dependent. Surprisingly, chemotaxis for VPAC1 expressing Hut 78 cells was adenylate cyclase, PKA, and PKC dependent. These data demonstrated that VIP receptors have the same biological response of chemotaxis, but utilize two different signaling cascades for directed cellular migration.

Materials and Methods

Reagents

HuT 78, Molt 4, SupT1, and NIH-3T3 cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's modified Eagle's medium, Iscove's modified Dulbecco's medium, RPMI 1640, penicillin, streptomycin, L-glutamine, phosphate buffered saline without Ca^{2+} or Mg^{2+}, characterized fetal bovine serum, and trypsin were purchased from Cellgro (Manassas, VA). Sodium chloride, potassium chloride, ethylenediaminetetraacetic

acid (EDTA), ethyleneglycoltetraacetic acid (EGTA), Tris, sodium hydroxide, sodium dodecyl sulfate, sodium deoxycholate, glycerol, 2-mercaptoethanol, ammonium persulfate, methanol, acetic acid, Commassie blue, and Ponceau S were bought from Sigma-Aldrich (St. Louis, MO). Vasoactive intestinal peptide, VIP_{10-28}, and Glucagon was purchased from American Peptides (Sunnyvale, CA). Protease cocktail inhibitor set III and Super Signal West femto chemiluminescent substrate were obtained from Thermo Fischer Scientific (Waltham, MA). Tween 20, ECL Plus, and Ficoll-Paque were purchased from GE Healthcare (Piscataway, NJ). DC Protein assay, tetramethylethylenediamine (TEMED), nitrocellulose, and acrylamide-bis were bought from Bio-Rad (Hercules, CA). Qiashredders, RNeasy mini kits, and RNase-free DNase I was obtained from Qiagen (Valencia, CA). Deoxynucleotides, reverse transcriptase, Taq DNA polymerase were procured from Promega (Madison, WI). Phenol/chlorophorm/isoamyl alcohol (25:24:1) was purchased from Research Organics (Cleveland, OH) Gene specific primers were obtained from Integrated DNA technologies (Coralville, IA). QCM™ Fluorimetric cell migration assay was purchased from EMD-Millipore (Billerica, MA). Naïve CD4/CD45RA T cell isolation kit was purchased from Miltenyi Biotech (Cambridge, MA). FAM-labeled VIP and FAM-labeled Scrambled peptide were obtained from Anaspec (Fremont, CA). Red blood cell lysis buffer, CD4-APC, and CD45RA-FITC antibodies were procured from Ebiosciences (San Diego, CA). cAMP ELISA kit was purchased from Cayman Chemicals (Ann Arbor, MI). Gαs assay kit was obtained from NewEast Biosciences (King of Prussia, PA). Whole human blood was procured from Bioreclaimation (Westbury, NY)

Tissue Culture

HuT 78 cells were cultured in 77% IMDM, supplemented with 20% chFBS, 100 units/mL penicillin (1%), 100 µg/mL streptomycin (1%), and 4 mM L-glutamine (1%) (HuT 78 completed media). Molt 4 and Sup T1 cells were propagated in 89% RPMI 1640, 10% chFBS, 100 units/mL penicillin, and 100 µg/mL streptomycin (SupT1/Molt 4 complete media). Cells were collected, centrifuged at 300 x g for 5 min, and seeded at 2-3 x 10^5 cells/mL three times a week (every 48-72 hr) with fresh complete media. Cells were incubated at 37°C in 5% CO_2.

RNA Isolation, cDNA Synthesis, and pRT-PCR

RNA isolation protocol was a modified version from Qiagen's April 2006 handbook. Briefly, tissue culture cells were collected, centrifuged, and all supernatants were removed by aspiration. Cell pellets were lysed in 350 µL RLT buffer (RLT buffer supplemented with 1% v/v β-mercaptoethanol), vortexed, and transferred to a QIAshredder spin column. Samples were centrifuged at 8,000 x g for 2 minutes to sheer genomic DNA. QIAshredder columns were discarded, and 1 equivalent volume of 70% ethanol was added to the flow through and mixed well by pipeting up and down. Cellular lysates were transferred to an RNaesy spin column, centrifuged at 8,000 x g for 15 seconds to allow RNA bind to the column. Columns bound with predominantly RNA and DNA were washed with 350 µL RW1 buffer before an on-column DNase I treatment. DNase I (10 µL DNase I and 70 µL RDD buffer) was directly added to the column and incubated at 37°C for 45 min. Digested DNA (free nucleotides) were eluted off the column with a second 350 µL RW1 buffer wash, centrifuged for 15 seconds at 8,000 x g and flow-through

discarded. Columns were washed with 500 µL RPE buffer, centrifuged for 2 min, and repeated. The collection tube was changed, centrifuged for an additional minute to remove all residual ethanol, and columns were placed in capped 1.5 mL centrifuge tubes. RNA was eluted with 50 µL RNase-free water (37°C) into a 1.5 mL tube where a second DNase I treatment was conducted. Equivalent volumes of DNase I/RDD buffer and RNA were incubated at 37°C for 45 min. RNA was further purified by the addition of 1 volume phenol/chloroform/isoamyl alcohol (25:24:1) to the DNase treated RNA (Moore and Dowhan 2002). Samples were vortexed for 2 min to create an emulsion and centrifuged at 8,000 x g for 10 min at room temperature to separate the organic phase from the RNA-containing aqueous (top) layer. RNA was collected by careful transfer to a new tube, and 3 M sodium acetate (pH 5.2) (1/10 volume) was added and vortexed briefly. To facilitate precipitation of RNA, 900 µl of ice cold absolute ethanol was added, briefly vortexed, and incubated at -80°C for $\geq$ 30 min. RNA samples were centrifuged at 12,000 x g for 30 min at 0°C. Ethanol was carefully aspirated not to disrupt RNA pellet and washed with 1 mL 70% ethanol to solubilize salts and centrifuged for an additional 10 min at 0°C. Ethanol was carefully removed as above and allowed to air dry for 5 min to ensure complete removal of ethanol. RNA pellets were resuspended in 50 uL of nuclease free water (37°C), and incubated for 10 minutes at 70°C. Total RNA (0.5-4 µg) was utilized for cDNA synthesis or stored at -80°C until needed.

RNA concentration and purity was determined by optical density measured at 260 and 280 nm on a Nanodrop 2000C Spectrophotometer (Thermo Scientific,

Waltham, MA). Reactions containing reverse transcriptase (RT+) and omitting reverse transcriptase (RT-) were performed to synthesize cDNA using 4 µg of total RNA. Prior to RT addition, both reactions designated RT+ and RT- had 500 ng random hexamers added to a final volume of 30 µl, and samples were incubated at 69°C for 7 min followed by a quick chill on ice to minimize formation of secondary structures. To RT+ cDNA reactions, a master mix was added with final volumes of 1X Moloney Murine Leukemia Virus (M-MLV) buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl, 1 mM $MgCl_2$, and 50 mM DTT), 4 mM dNTPs, 50 units RNasin, 400 units M-MLV RT, and RNase free water for a final volume of reaction at 50 µl. RT- cDNA reactions used the above master mix with nuclease free water replacing RT. Samples were mixed by flicking the tubes and briefly centrifuging to collect all liquid at the bottom of the tube. Samples were incubated for 1 hr at 37°C, and stored at -20°C or used immediately for qRT-PCR.

Table 8. Primer-probe sequences utilized in qRT-PCR.

Gene	Forward	Reverse	Probe	Accession Number
hVPAC1	5'-ACA AGG CAG CGA GTT TGG AT-3'	5'-GTG CAG TGG AGC TTC CTG AAC-3'	5'-/56-FAM/CCA CCC TTC TGG TCG CCA CA/36-TAMSp/-3'	NM_004624
hVPAC2	5'-CGT GAA CAG CAT TCA CCC AGA AT-3'	5'-GCT GAC GGT CTC TCC CAC AT-3'	5'-56-FAM/ACG GTG CCC TCC CCA AAA GT/36-TAMSp/-3'	NM_003382
hHPRT	5' -GGC AGT ATA ATC CAA AGA TGG TCA A-3'	5'-GTC TGG CTT ATA TCC AAC ACT TCG T-3'	5'-/56-FAM/CAA GCT TGC TG TGA AAA GGA CCC C/36-TAMSp/-3'	NM_000194

Real time quantitative PCR (qRT-PCR) was utilized to determine VPAC1 and VPAC2 expression. Samples were normalized with HPRT as the housekeeping gene. cDNA synthesized from HuT 78, Molt 4, or human reference pool (HRP) was utilized in qRT-PCR reactions based on a series of dilutions in nuclease free water that empirically determined the linear range for all amplicons

(1:2 dilutions for VPAC1, 1:4 dilutions for VPAC2 and HPRT). qRT-PCR reactions consisted of 10 µL of diluted cDNA, 1X Taqman buffer, 375 nM forward and reverse primers, 250 nM probe to a final volume of 25 µL. Real time qPCR was completed using an Applied Biosystems (ABI) 7500 fast qPCR themocycler. Primers and probes utilized in detection of VPAC1 and 2 are identified in Table 8. The delta-delta Ct method was utilized for calculating relevant VPAC1 and 2 expression compared to the constitutively expressed HPRT. HRP was utilized as a comparison that represents an average expression of 10 different tissues. TaqMan primer-probe method was utilized instead of SYBR green due to low expression of VPAC1 receptors in T cell lines used.

Flow Cytometry

Identification of VIP binding sites on HuT 78, Molt 4, HT 29, and CHO-K1 cells was completed by FAM-labeled VIP with detection by flow cytometry. One million cells were resuspended in 100 µL of binding buffer (1x PBS and 1% w/v BSA) and incubated with 10^{-5}-10^{-7} M VIP-FAM or scrambled peptide-FAM for 30 min at 4°C in the dark to inhibit internalization of peptides. Cells were washed two times by adding 1-2 mL PBS and centrifuged at 500 x g for 5 min followed by decanting off supernatant. Cells were resuspened in 300 µl PBS and analysed on an Accuri C6 flow cytometer (Anarbor, MI). Specific VIP binding (VIP-Scramble) was determined for each sample.

T Cell Isolation

Naïve CD4 T cells were isolated from whole human blood obtained from Bioreclamations Inc (Westbury, NY). Lymphocytes were isolated using a density

gradient by layering blood on top of the Ficoll-Paque. Blood samples were centrifuged at 1500 x g for 30 min at RT. The mononuclear cell layer was removed and washed in Hank's buffered salt solution (HBSS) for 20 min and centrifuged at 500 x g at RT. Removal of red blood cells were completed using Red Blood Cell Lysis buffer, cells were resuspended, incubated with gentle agitation, and centrifuged at 500 x g for 5 min at RT. Cell count was determined with 0.2% Trypan blue and counted using a hemocytometer. Naïve CD4 CD45 RA cells were isolated from the lymphocyte population. Depletion of memory CD4 T cells and non-CD4 T cells a cocktail of CD45RO, CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD56, CD123, anti-TCRγ/δ, anti-HLA-DR, and CD235A biotinylated antibodies were used. Cells were incubated for 10 min, centrifuged at 300 x g for 10 min, washed with PBS, and incubated with secondary anti-biotin antibody for 15 min. Cells were washed, resuspended in 500 ul Blue buffer (PBS [pH] and BSA), and negatively selected using a Miltenyi Automax (Cambridge, MA). CD4 T cell purity was determined by labeling cells with CD4-APC and CD45RA-FITC labeled antibodies and measured by flow cytometry. Briefly, total lymphocytes, purified CD4 T cells, and lymphocytes depleted of CD4 T cells (2 x 10^5 cells) were resuspended in 100 µL binding buffer and incubated with 1 µg CD4-APC and 1 µg CD45RA-FITC antibodies at 4°C for 30 min. Cells were washed twice with PBS and resuspended in 300 µL of PBS. Cells were analyzed as previously described with the Accuri C6.

cAMP ELISA

To determine the maximum biological activity upon ligand binding to VPAC receptors, intracellular cAMP (i[cAMP]) was measured. T cell lines were incubated in either complete or reduced serum media for 24 hr prior to assay at 5 x 10^5 cells/mL at 37°C, 5% CO_2. Cells were washed 2 times in PBS and resuspended at 1 x 10^7 cells/mL in HBSS supplemented with 0.1% BSA and 0.75 mM 3-isobutyl-1-methyxanthine (IBMX), followed by a 45 min incubation to inhibit phosphodiesterase activity. Cells were treated with varying concentrations of VIP_{1-28} (10^{-14}-10^{-6} M), VIP_{10-28} (10^{-8} M), vehicle control, or forskolin (80 µM) for 30 min at 37°C, 5% CO_2. Cells were collected and lysed in 200 µL of 0.1 M HCl by incubating with vigorous shaking for 20 min at RT. Cell lysates were centrifuged for 10 min at 1000 x g, and diluted with equal volume of EIA buffer (1 M phosphate solution, 1% BSA, 4 M NaCl, 10 mM EDTA and 0.1% sodium azide). Intercellular cAMP levels were measured using a competitive cAMP EIA ELISA.

$G_{\alpha s}$ Assay

Complete and serum starved T cell lines were cultured at 5 x 10^5 cells/mL for 24 hr prior to assay. Cells were collected and washed 3 times in PBS, and reseeded at 1 x 10^7 cells/mL in HBSS. Samples were treated with either VIP or water for 15 min at 37°C, 5% CO_2. Cells were washed to remove ligand, lysed in 1 mL of lysis buffer (50 mM tris [pH 7.4], 150 mM NaCl, 1mM EDTA, 1%Triton X-100, and 1x PIC) for 30 min on ice, centrifuged, and supernatant was collected. Positive (GTP) and negative (GDP) controls were completed using 800 µg of HT29 cell lysate treated with either GDP or non-hydrolysable GTPγS and incubated for

90 min with gentle agitation. For IPs, 800 µg of total protein was incubated for 1 hr with protein A magnetic beads and 1 µg of the GTP-$G_{\alpha s}$ antibody added to a final volume of 1 mL. Immunoprecipitations were washed 3 times in lysis buffer followed by incubating in 20 uL of 2x SDS-PAGE sample buffer. Samples were loaded on a 5% stacking and 15% separating gel and electrophoresed.

Microarray Analysis

All microarray experiments and statistical analyses were performed by the Marshall University Microarray Facility. To determine regulation of gene expression, HuT 78 and Molt 4 cells were subjected to VIP treatment for 2, 6, and 24 hr. Cells were collected, washed in PBS, and lysed in RLT buffer as previously described to isolate RNA. Isolated RNA was then sent to Marshall University for microarray analyses.

Chemotaxis

To determine the biological ability for VIP to act as a chemoattractant, cell migration assays were performed. Cell migration was determined by the number of cells that migrated from the upper to lower chamber in the modified Boyden chamber system. HuT 78 and Molt 4 cells were serum starved for 24 hr prior to assay, collected, and washed with PBS. Molt 4 cells required a basement matrix for migration, 3.5 µg/cm^2 mouse collagen IV in 0.5 N HCl was used to coat the porous membrane for 1 hr, at 37°C. Chambers were washed three times with PBS to remove residual HCl. HuT 78 and Molt 4 cells were either resuspended at 1 x 10^6 (24 well plate) or 7.5 x 10^5 (96 well plate) cells/mL in reduced serum media and aliquoted into the upper chamber. The lower chamber consisted of reduced

serum media and the desired chemoattractant concentrations (Table 9). Cells were incubated for 4 hr at 37°C, 5% CO_2 to allow for chemotaxis. Cells were then lysed with 4x cell lysis buffer containing CyQUANT® GRDye (1:75 dilution) and assayed in duplicate as recommended by the manufacturer. All inhibitors utilized in this study (Table 9) were incubated with cells 30 min prior to assay. Chemotactic response was measured by SPECTRA max Gemini EM fluorometer with excitation of 480 nm and detecting at 520 nm; statistics were completed using paired statistical analysis by Origin 8.0.

Table 9. Chemoattractants and inhibitors utilized in chemotaxis assays.

Chemoattractant	Reference	Inhibitor	Reference
TNF-α	(Xia et al. 1996a)	H89	(Davies et al. 2000)
IL-4	(Xia et al. 1996a)	PKI	(Davies et al. 2000)
Serum	(Johnston et al. 1994)	SQ22536	(Barcova et al. 1999)
VIP	(Xia et al. 1996c)	Bisindolylmaleimide	(Martiny-Baron et al. 1993)
GRO-α	(Xia et al. 1996a)	Calphostin C	(Martiny-Baron et al. 1993)

Results

In an attempt to study the unique cellular signaling effects of VIP elicited by VPAC1 versus VPAC2, it was essential to first identify T cell lines that predominantly express only one of these receptors. To this end, VPAC receptor expression was measured at the mRNA and protein levels. Total RNA isolated from HuT 78 and Molt 4 cells propagated in either complete (CM) or reduced serum (SS) media, and quantitative RT-PCR (qRT-PCR) was used to measure the transcriptional expression profile for each receptor. Initially, SYBR green was

used to identify VPAC1 transcripts, but due to low expression levels TaqMan primer-probes were used that resulted in greater sensitivity (data not shown) (Figure 33A). Based on TaqMan qRT-PCR, HuT 78 cells exclusively expressed detectable VPAC1 mRNA levels (Figure 33B), albeit at ~1% compared to HRP control. Importantly, there was no detection of VPAC2 message in either media condition. In contrast, Molt 4 cells predominantly expressed VPAC2 mRNA levels 340-fold greater compared to VPAC1 levels. Serum starving Molt 4 cells decreased VPAC2 expression by approximately one-half. Molt 4 cells expressed similar low levels of VPAC1 mRNA compared to HuT 78 cells in both complete and reduced serum conditions. These data confirmed that HuT 78 and Molt 4 cells predominantly express only one of the VPAC receptors at the mRNA level.

Even though the qRT-PCR data confirmed predominant VPAC1 expression in HuT 78 cells, and VPAC2 expression in Molt 4 cells, the tremendous difference in expression magnitude (340-fold) between VPAC2 and VPAC1 was a concern. Therefore, VIP specific binding was measured as an indirect measurement of receptor protein. To measure surface VPAC receptor expression, FAM-labeled VIP ligand was incubated with HuT 78 and Molt 4 cells cultured in complete media and measured by flow cytometry (Figure 33C). A 42 aa scrambled peptide conjugated to FAM was used to determine non-specific binding. Interestingly, HuT 78 cells showed 2-fold more specific VIP binding sites compared to Molt 4 cells. The mean fluorescent values from this analysis are presented in Table 10. These results clearly demonstrate a lack of agreement between qPCR and ligand binding measurements. Nonetheless, these data and that of qRT-PCR support equivalent

expression of VIP binding sites on HuT 78 and Molt 4 cell lines, presumably due to VPAC1 and VPAC2 surface protein expression.

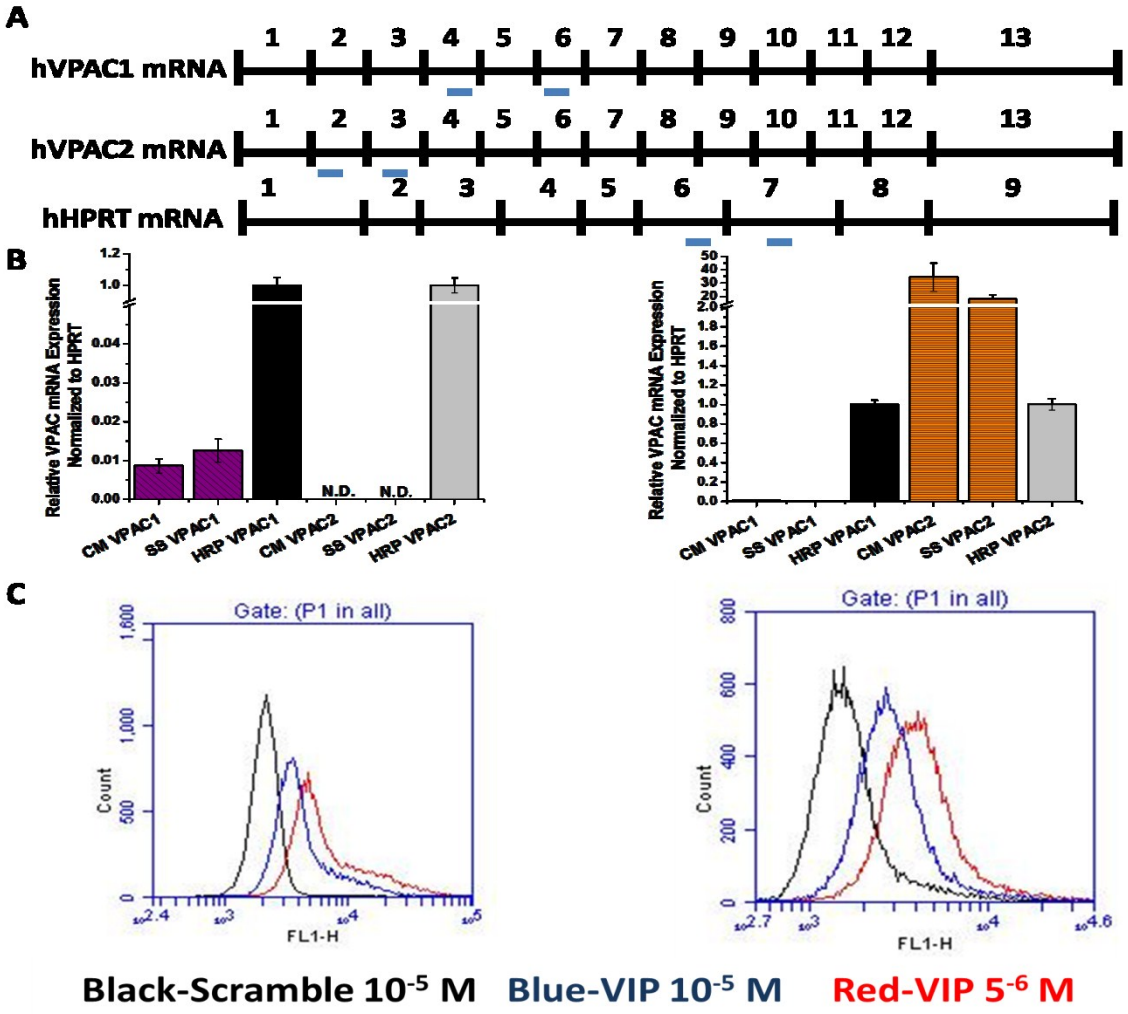


Figure 33. HuT 78 cells exclusively express VPAC1, while Molt 4 cells predominantly express VPAC2. RNA isolated from HuT 78, Molt 4 cells (cultured in complete [CM] or reduced serum [SS] media), and human reference pool (HRP) total RNA (materials and methods) was utilized for qPCR measurement for VPAC1 and VPAC2 normalized to HPRT. (A) Primer sets utilized for qRT-PCR for each amplicon as indicated. (B) VPAC1 and 2 expression was normalized to HPRT for HuT 78 (left panel) and Molt 4 (right panel). (C) Measurement of VIP specific binding sites on HuT 78 (left panel) and Molt 4 (Right panel) were identified using FAM-labeled VIP and analyzed using an Acuri C6 Flow Cytometer. VIP specific binding sites were calculated based on a normalization of fluorescence over non-specific binding using a FAM-labeled scrambled peptide. These experiments were preformed 3 independent times.

Table 10. Mean fluorescents values of FAM-labeled VIP verses scramble.

Sample (HuT 78)	Mean FL1*		Sample (Molt 4)	Mean FL1*
Scramble 10^{-5} M	2253.7		Scramble 10^{-5} M	1943.2
VIP 10^{-5} M	9747.5		VIP 10^{-5} M	5227.0
VIP 5^{-6} M	5272.5		VIP 5^{-6} M	3427.3
VIP 10^{-6} M	2201.4		VIP 10^{-6} M	1900.5

*FL1-FAM fluorophore detected

The next question we asked was whether VIP treatment of HuT 78 and Molt 4 cells would elicit different signaling responses due to their unique VIP receptor expression profile. Since the cloning of VPAC1 and 2 receptors in the 1990's, it has become well-established that both receptors are coupled to the cAMP/PKA pathway through $G_{\alpha s}$. Consequently, this signaling pathway was investigated by measuring i[cAMP]. This experiment was accomplished by adding endogenous VIP ligand, a partial antagonist VIP_{10-28}, and a known AC activator, forskolin. HuT 78 and Molt 4 cells were cultured in both complete and reduced serum media. The purpose for reduced serum media was to lower the basal metabolic rates thereby enhancing VIP signaling. When Hut 78 cells were pre-incubated for 24 hr prior to i[cAMP] assay in complete media , VIP treatment failed to significantly alter i[cAMP] levels (Figure 34A). In stark contrast, serum starved HuT 78 cells showed a unimodal increase in i[cAMP] peaking at 10^{-10} M VIP (Figure 34B). VPAC2 expressing Molt 4 and SupT1 (another VPAC2 expressing T cell) cells showed a similar VIP concentration dependent response in i[cAMP] in complete media (Figure 34C and E). However, in serum starved media, there was a marked enhancement in relative i[cAMP] elicited by VIP treatment for both Molt 4 and SupT1, but the highest [VIP] 10^{-6} M a sharp reduction of i[cAMP] in SupT1 cells.

This abrupt decrease in i[cAMP] is most likely due to homologous desensitization (Bohn et al. 2004, Gainetdinov et al. 2004), brought on by the tremendous response of 30-fold increase in i[cAMP]. The partial antagonist elicited an increase i[cAMP], but at reduced levels as expected. Collectively, the data clearly revealed differential cAMP profiles elicited by VIP in T cell lines expressing VPAC1 or VPAC2. In addition, the sensitivity to VIP by VPAC1 expressing HuT 78 cells was 1000-fold greater (10^{-10} M) than VPAC2 expressing Molt 4 and SupT1 cells (10^{-6} M VIP). Whereas the maximal i[cAMP] generated by VPAC2 expressing Molt 4 and SupT1 cells was an order of magnitude higher compared to VPAC1 expressing HuT 78 cells.

To determine how the i[cAMP] response elicited upon VIP treatment from HuT 78, Molt 4, and SupT1 cells compared to naïve human CD4 T cells, we isolated CD4+CD44RA+ T cells from whole human blood. Naïve CD4/CD45RA T cells, well-established as high expressers for VPAC2 expression, were purified to 90% (Figure 35C) by magnetic bead technology. Repeating VIP treatment with purified T cells showed a lack of an increase in i[cAMP] levels, while the antagonist VIP_{10-28} elicited a detectable cAMP response over water control (Figure 35D). Lymphocytes depleted of CD4 T cells also lacked an increase in i[cAMP] due to both VIP and VIP_{10-28} treatment. In all samples, an increase in i[cAMP] was detected after treatment of cells with forskolin demonstrating functional AC activity. These data demonstrated that primary CD4 T cells respond to VIP in a similar fashion to HuT 78 cells cultured in complete media. Unfortunately, serum starving primary T cells causes massive cell death and this condition was not

possible to study. Nonetheless, it is important to emphasize a failure for VPAC1 Gαs coupling and cAMP signaling in primary T cells. Perhaps, the general acceptance for VIP receptor coupling to $G_{\alpha s}$ is based on artificial overexpression systems using Chinese Hamster Ovary (CHO-K1) cells rather than primary cells. Also, Molt 4 cells have an activated Th2 phenotype (O'Dorisio et al. 1981, Beed et al. 1983) and activated T cells (Anderson and Gonzalez-Rey 2010) show a robust VIP induced cAMP response and could provide an explanation for differential signaling due to either VIP receptor type or activated phenotype.

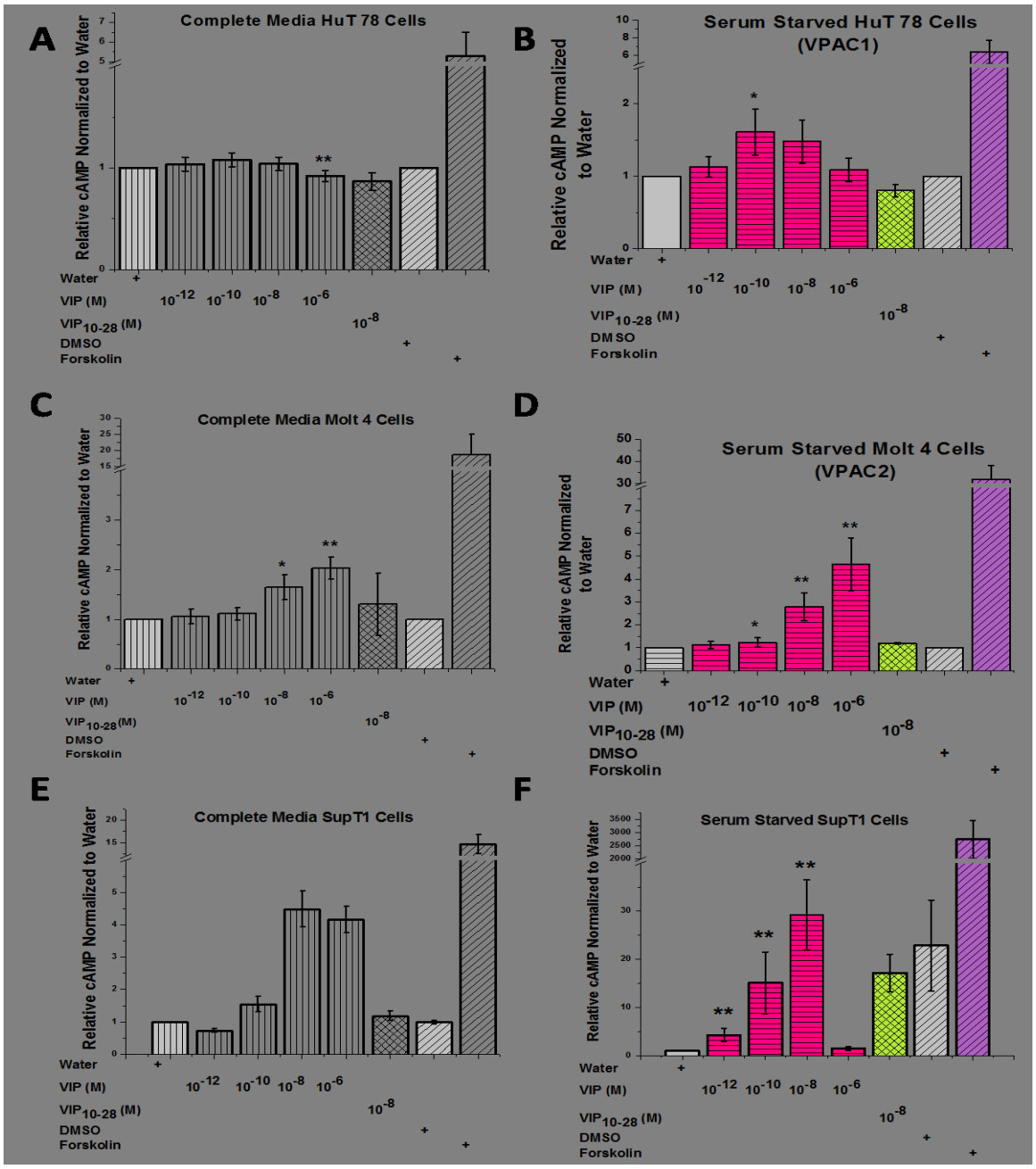


Figure 34. T cell line expression either VPAC1 or VPAC2 receptors elicit different i[cAMP] responses upon stimulation with VIP. HuT 78 (A and B), Molt 4 (C and D), and Sup T1 (E and F) were assayed for i[cAMP] elicited upon treatment with varying concentrations of VIP, forskolin (80 μM), or VIP_{10-28} as indicated. Cells were either cultured in complete (CM) or reduced serum (SS) media for 24 hours followed by IBMX treatment for 45 min. Forskolin was utilized as a positive control for activation of AC, VIP_{10-28} an antagonist for VPAC receptors, while DMSO and water were vehicle controls. cAMP was detected using a competitive ELISA (Cayman Chemical, Anarbor MI), and results are mean +/- SEM and n=5-15. *$p<0.05$ and ** $p<0.01$

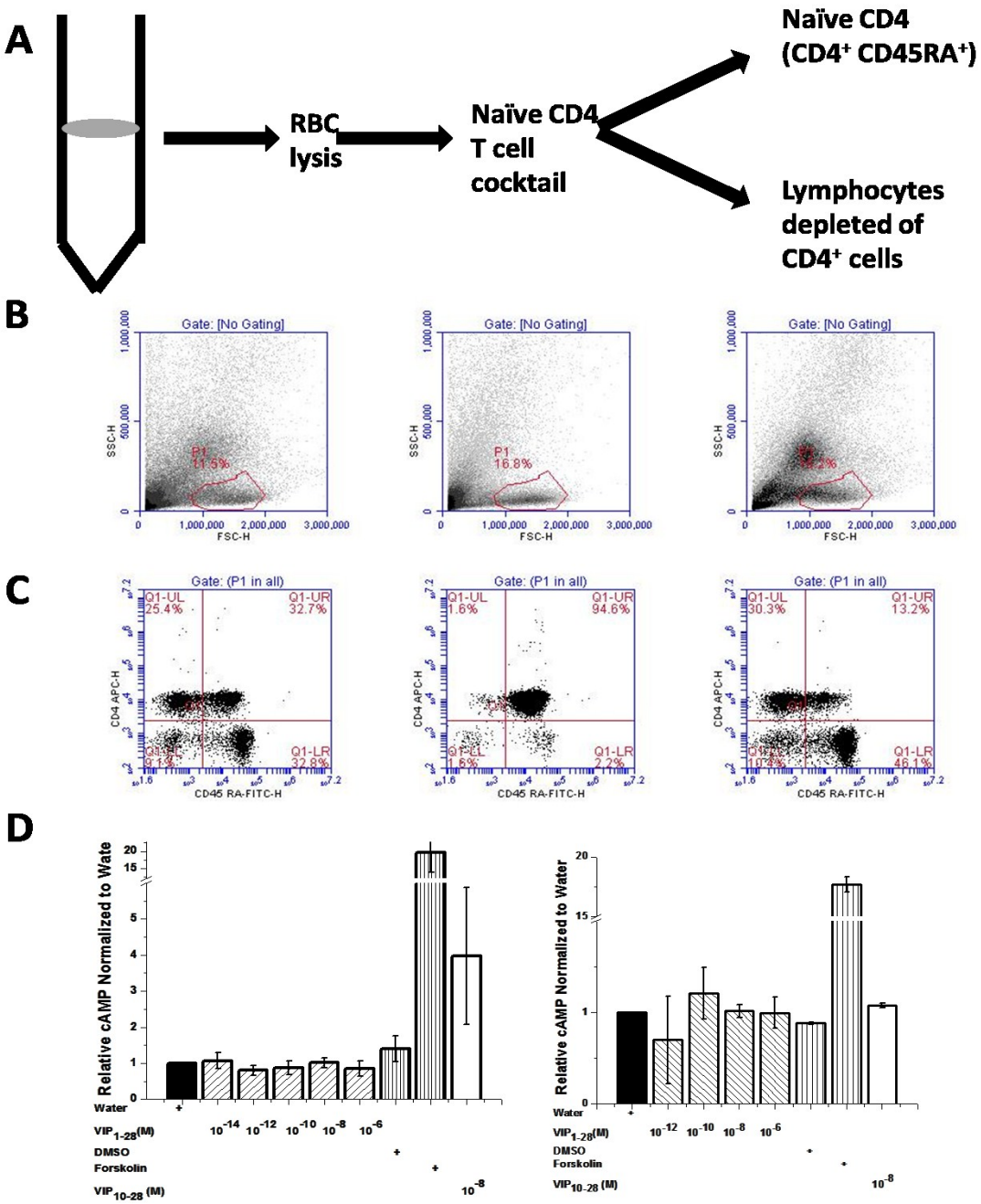


Figure 35. CD4$^+$ CD45RA$^+$ T cells lack a cAMP response upon VIP treatment. Naïve CD4+CD45RA+ T cells were isolated from whole human blood (A) using a ficoll gradient followed by naïve CD4 T cell depletion using mangetic bead technology (materials and methods). Cells were stained with CD4-APC and CD45RA-FITC antibodies and analyzed pre- and post-purification by flow cytometry measuring forward and side scatter(B) and fluorescence for CD4 and CD45RA. Purifed naïve CD4 (D) and depleted lymphocytes were utilized in a competitive ELISA to quantitate the i[cAMP] levels due to VIP_{1-28}, a known antagonist VIP_{10-28} , or forskolin, a potent AC activator. All experiments are n=3.

The increase in i[cAMP] by forskolin was 3-fold greater in Molt 4 cells compared to HuT 78 cells in complete media, and 6-fold higher with serum starving. This suggested to us that basal adenylate cyclase activity , which forskolin activates, is higher in Hut 78 cells and could explain the reduced sensitivity to forskolin. In an attempt to gain support for this question, basal cAMP levels were measured in both cell lines cultured in complete and serum starving conditions. As hypothesized, HuT 78 cells endogenously generated ~5-fold higher levels of i[cAMP] compared to Molt 4 cells (Figure 36) irrespecitve of culturing conditions. Also, SupT1 and naïve CD4/CD45RA T cells have similar levels of endogenous cAMP to Molt 4 cells (data not shown). These data may explain the modest i[cAMP] spikes by forskolin and VIP in HuT 78 cells compared to Molt4 cells.

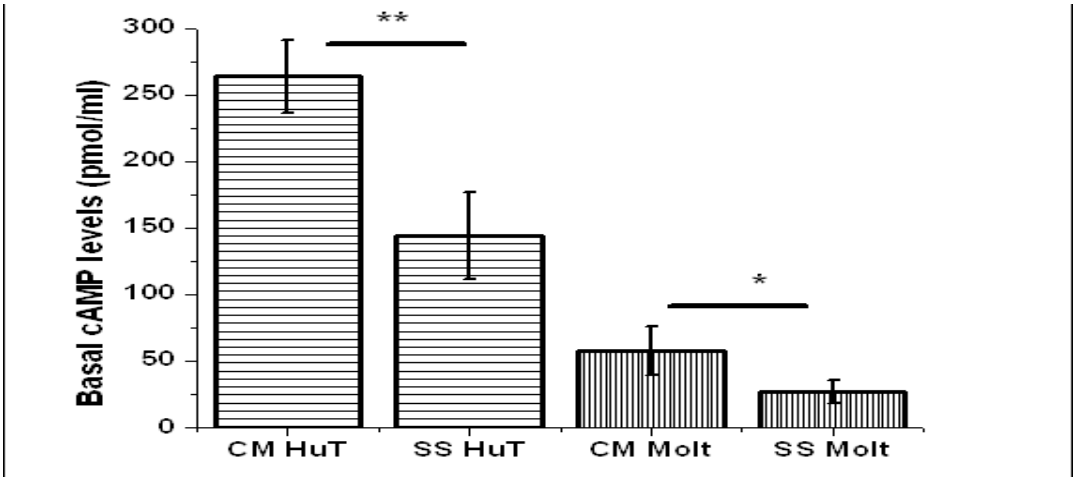


Figure 36. HuT 78 cells have higher basal endogenous levels of i[cAMP] compared to other T cell lymphocytes. Endgoenous cAMP levels were determined by a competitive ELISA (see materials and methods). HuT 78 and Molt 4 cells were cultured in either complete (CM) or reduced serum (SS) media were assayed 30 min post VIP treatment for i[cAMP]. Results are mean +/- SEM and n=5-15. *p<0.05 and ** p<0.01

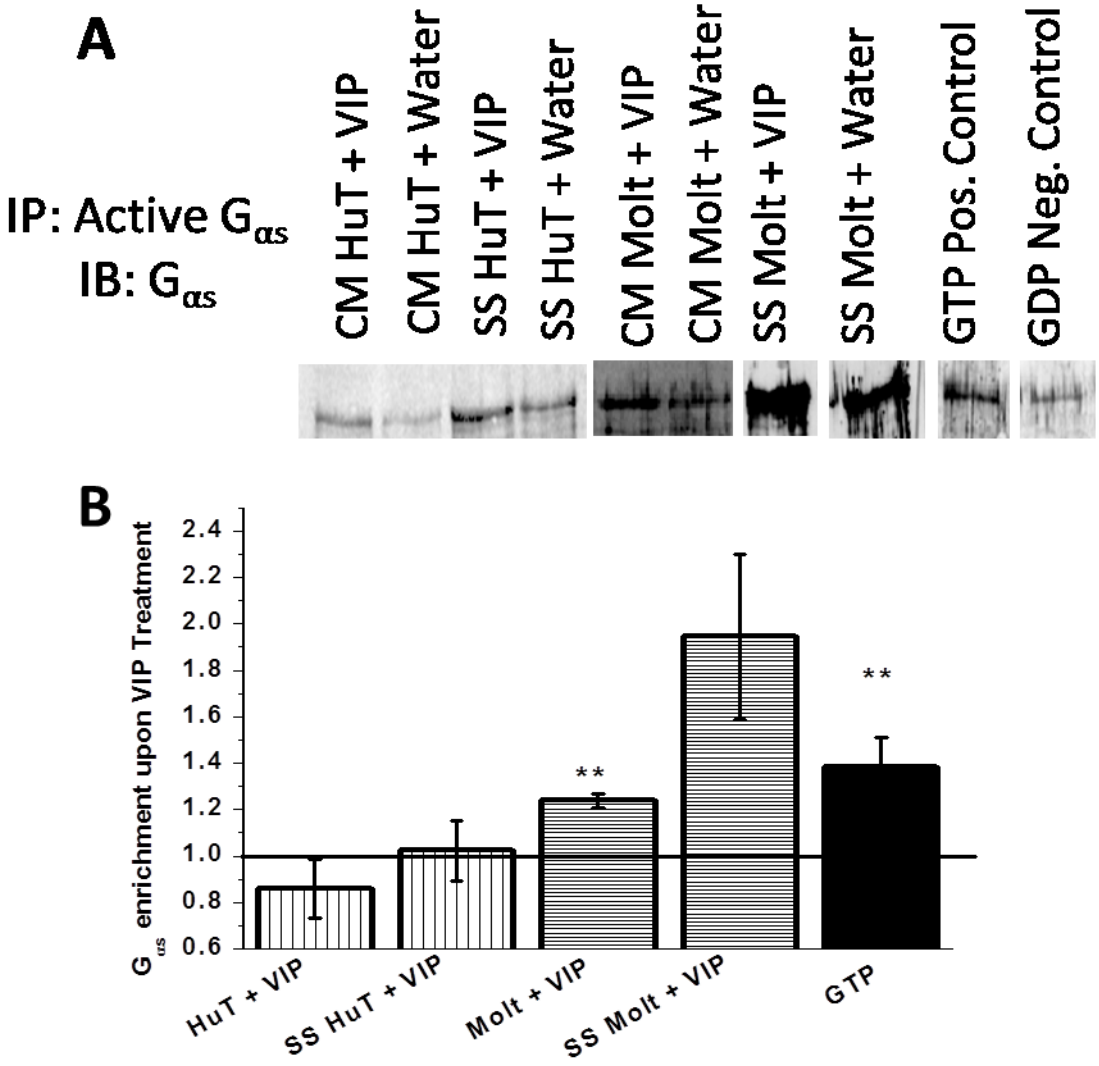


Figure 37. VPAC2, but not VPAC1 couples to $G_{\alpha s}$ to elicit a cAMP response. Cells were treated +/- VIP, lysed, and immunoprecipitated for the active GTP-$G_{\alpha s}$ complex (see materials and methods). (A) Samples were subjected to SDS-PAGE and IB for $G_{\alpha s}$. (B) Densitometry of active $G_{\alpha s}$ was determined using the Rolling-Ball method (GE Healthcare STORM 685) with enrichment determined over control (water). All experiments are n=3, *p>0.05 and ** p>0.01.

Canonical signaling of both VPAC receptors is accomplished by efficient coupling with $G_{\alpha s}$ that results in AC activation and a spike in i[cAMP] (Figure 16) , called “cAMP clouds.” Due to the radically different cAMP response obtained between HuT 78 and Molt 4 cells, upon exogenous VIP treatment we determined

the extent of receptor coupling efficiency to $G_{\alpha s}$. Immunoprecipitations were performed to pull down the active form of $G_{\alpha s}$ upon VIP treatment of both T cell lines. HuT 78 and Molt 4 cells were incubated in CM or SS media and treated with VIP (10^{-8} M) or water (Figure 37). Molt 4 cells irrespective of the culture media has an increase of active $G_{\alpha s}$ complex precipitated upon VIP treatment compared to water (Figure 37). Surprisingly, HuT 78 cells failed to show a significant exchange of GDP upon VIP treatment despite culture conditions in complete or serum free media. HT 29 cells were utilized for both positive and negative controls by saturating the system with either a non-hydrolyzable GTPγS or GDP resulting in an expected 40% increase in the positive control based on company standards. These data demonstrate that VIP induces strong GTP exchange in Molt 4 cells, but not HuT 78 cells, consistent with its more potent elevation of i[cAMP] levels. It would therefore follow that VPAC2, unlike VPAC1, efficiently couples with Gαs, and VPAC1 signaling induces a weak i[cAMP] increase through a non-canonical $G_{\alpha s}$ independent mechanism.

VIP/VPAC signaling has demonstrated previously to alter hundreds of genes in murine primary CD4 T cells. However, this study only evaluated T cells predominantly expressing VPAC1, as VPAC2 expression is only found on Th2 activated cells. To determine changes in gene expression due to VPAC1 (HuT 78) or VPAC2 (Molt 4) signaling upon VIP stimulation in whole human genome microarray studies were conducted (Agilent platform, Marshall University). Both HuT 78 and Molt 4 cells were incubated +/- VIP (10^{-8} M) for 2, 6, and 24 hr, lysed, and total RNA isolated (Figure 38A). i[cAMP] was measured for each sample to

ensure a biological response resulting from VIP. Microarray experiments were completed at Marshall University, initial results showed no differential regulation of genes. VIP signaling is drastically different in human malignant T cells compared to primary murine T cells, which we conclude may be due to the leukemic transformation that usurps GPCR function (Dorsam and Gutkind 2007). Additionally, differences in gene regulation response might be species specific and VIP gene regulation may not translate between mouse and human.

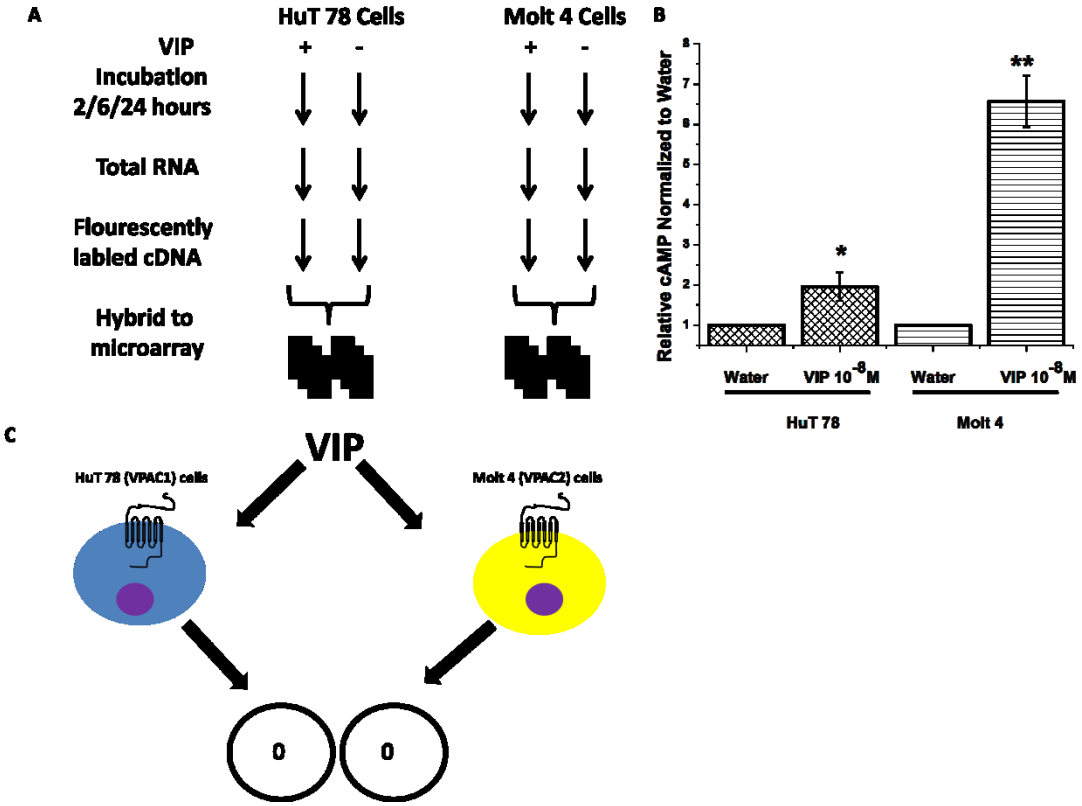


Figure 38. VPAC1 and VPAC2 fail to alter gene expression in leukemic T cells. (A) Serum starved HuT 78 and Molt 4 cells were treated for 2, 6, and 24 hours with +/- VIP (10^{-8} M). Total RNA was isolated, cDNA was synthesized and fluorescently labeled and hybrized to microarrays (see materials and methods). (B) Cells were serum starved for 24 hr and treated with VIP or vehicle control for 30 min and analyzed for i[cAMP]. Data is presented as mean +/- SEM for pooled data. (C) Differential regulation of gene expression between HuT 78 and Molt 4 cells. Microarrays were completed in sextuplets, * p>0.05 and ** p>0.01.

Table 11. Altered gene expression in HuT 78 cells due to VIP/VPAC1 signaling.

Gene Name	Set A (time)	Set B (time)	Accession Number
DTNA	3.26 (2 hr)	0.32 (24 hr)	NM_001390
RBM22	1.59 (24 hr)	0.73 (2 hr)	NM_018047
C10orf79	0.12 (2 hr)	5.66 (6 hr)	NM_05145
FAM5C	0.16 (2 hr)	2.88 (2 hr)	NM_199051
GK	0.12 (2 hr)	4.16 (6 hr)	NM_203391
C19orf30	0.14 (2 hr)	2.95 (6 hr)	NR_027148
THC2709441	0.31 (2 hr)	2.63 (6 hr)	THC2709441

Table 12. VPAC2 signaling alters gene expression in Molt 4 cells.

Gene Name	Set A (Time)	Set B (Time)	Accession Number
ADIPOQ	5.81 (24 hr)	1.44 (6 hr)	NM_004797

Due to the lack of identifiable gene targets, experiments were then regrouped by cAMP response. HuT 78 group A consisted of microarray experiments 1, 3, and 5 and group B consisted of experiments 2, 4, and 6. This data analysis allowed for the identification of seven genes that were differentially expressed in these two subsets (Table 11). While similar analyses for Molt 4 cells were completed resulting in only one gene that was altered (Table 12). No genes were identified using this method that overlapped between VPAC1 and 2 signaling further supporting different signaling from these two receptors. Validation of these gene changes by qRT-PCR is a major future goal. These data demonstrate a lack of gene targets due to VIP in leukemic T cells compared to normal mouse T cells.

The observation that VIP signaling in HuT 78 and Molt 4 cells was unable to alter gene expression changes, despite increase in i[cAMP] and confirmed Gαs GTP exchange in Molt 4 cells, created uncertainty regarding their cellular function in leukemia. One major cellular function reported by others (Ottaway 1984, Ottaway and Greenberg 1984) and us (Dorsam et al. 2010) is that VIP induces

cellular migration in primary murine T lymphocytes. Since mammals are "hardwired" with peripheral VIPergic scenery nerves innervating immune organs, an important consequence for VIP's chemotactic activity is regulating lymphocyte trafficking to these cellular compartments. Consequently we used HuT 78 and Molt 4 cells to determine whether VIP receptors are functionally active, and if so regulated chemotaxis differentially based on their discordant signaling parameters. To this end, chemotactic responses to VIP were measured. Molt 4 cells were serum starved to maximize chemotactic migration and VIP was used as a chemoattractant in the lower compartment of a Boyden chamber system. The two known chemoattractants (Serum and TNFα) resulted in an increase of chemotaxis over no chemoattractant control (Figure 39A). VIP was chemotactic in a concentration dependent mechanism for Molt 4 cells, with maximal response found at 10^{-6} M VIP (Figure 39B). Molt 4 cells were unresponsive to chemotaxis when both VIP and TNFα were both utilized in the lower chamber (Figure 39C). Molt 4 cells treated 5 min prior to assay lacked chemotaxis towards VIP (Figure 39C), supporting a rapid internalization of the VPAC2 receptor as shown by others (Johnston et al. 1994). These Molt 4 data demonstrated that VIP is a chemoattractant in a concentration dependent fashion, and suggest that signaling transmitted by VPAC2 mediated these effects.

Based on the data presented so far, VIP induces chemotaxis of Molt 4 cells through a VPAC→$G_{\alpha s}$→cAMP dependent pathway (Figures 34, 37, and 39). PKA is an enzyme bound by regulatory subunits as an inactive complex, there are multiple binding sites for the secondary messenger, cAMP, and that when bound

dissociates both regulatory subunits from the PKA catalytic dimer. Now activated, PKA phosphorylates serine/threonine residues on a multitude of cytoplasmic and nuclear proteins changing their biological activity and temporarily altering the cellular phenotype. To determine chemotactic responses toward VIP was PKA dependent, we tested a highly specific adenylate cyclase inhibitor (SQ22356) and two structurally different PKA inhibitors (H89 and PKI) (Figure 39D, E, and F). All three inhibitors did not show any difference to basal chemotaxis compared to vehicle control, but each completely ablated VIP induced chemotaxis as asserted by cAMP ELISA. These data support that Molt 4 cells are chemotactically sensitive to VIP is through the activation of AC and PKA.

VIP elicited peak i[cAMP] levels at 10^{-10} M VIP in HuT 78 cells, but lacked a response at 10^{-6} M VIP , which was the optimal VIP concentration for both cAMP and chemotaxis in Molt 4 cells. We hypothesized that 10^{-10} M VIP would also result in a maximum chemotactic response. Therefore, serum starved HuT 78 cells were incubated with varying levels of VIP (Figure 40) and assessed for chemotaxis. Unexpectedly, an increase in chemotaxis for HuT 78 cells was also observed in a VIP concentration dependent fashion with VIP yielding a maximal response at 10^{-6} M. VIP pretreatment of HuT 78 cells blunted the cellular migration to the lower chamber, and supported that chemotaxis towards VIP was dependent upon VPAC1 receptor expression. These data supported that VIP/VPAC1 transmit intercellular signaling that induces chemotaxis in HuT 78 cells through a i[cAMP] independent

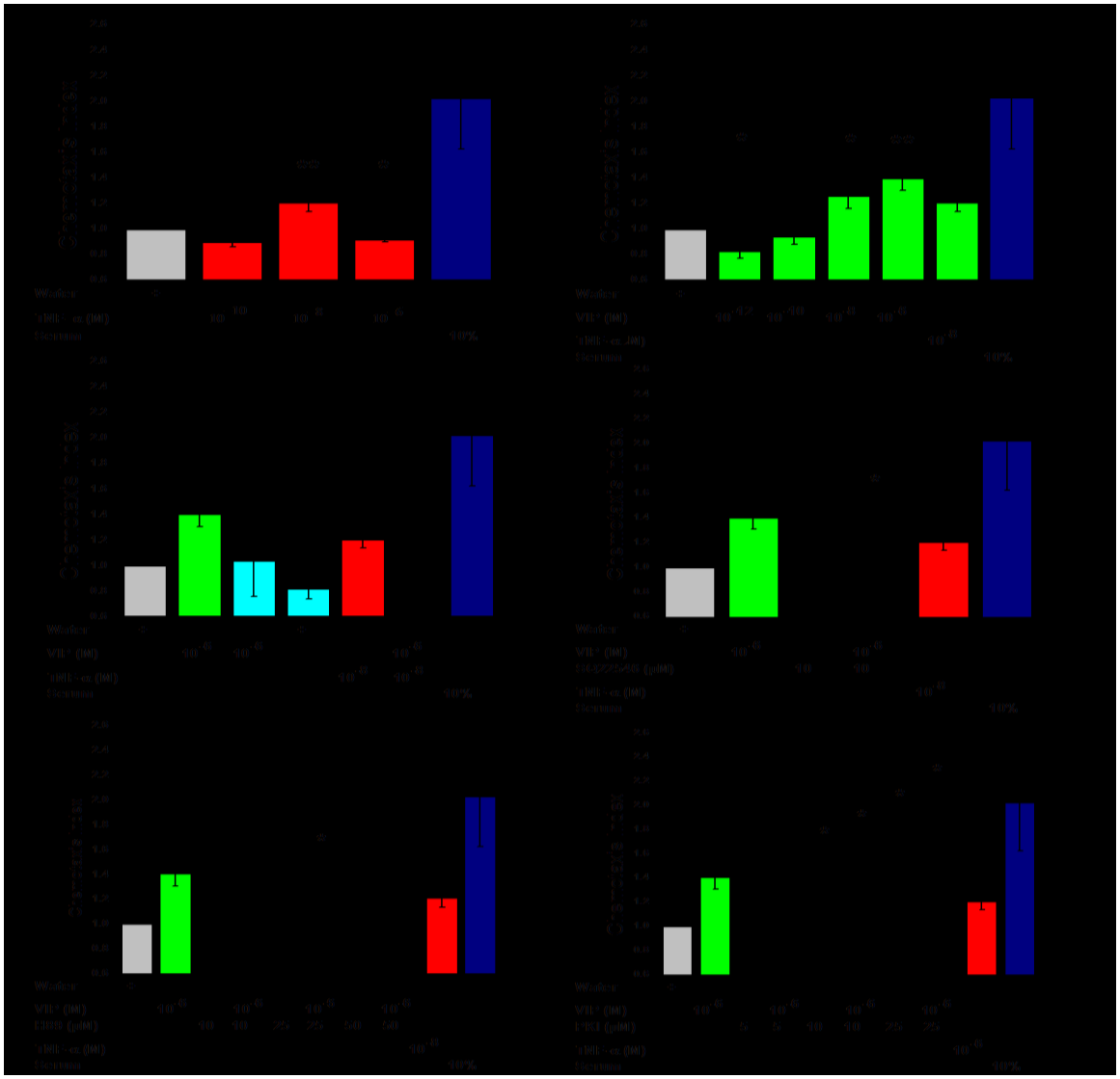


Figure 39. VPAC2 induced chemotaxis towards VIP is dependent upon PKA activity. Molt 4 cells were serum starved for 24 hr prior to the start of this assay (see materials and methods). Molt 4 cells were aliquoted in the upper chamber with varying concentraitons of TNF-α (A), or VIP (B) in the lower chamber. Cells were incubated for 4 rs at 37°C, cells that migrated to the lower chamber were lysed and quanitated with an interclating DNA dye (A). Cellular controls of VIP and TNFα additive response for chemotaxis along with pretreatment with VIP (light blue) initiating signaling prior to assay (C). Cell permeable inhibitor for AC, SQ22353 , (D) and two PKA inhibitiors, H89 (E) and PKI (F) were incubated for 30 min prior to assay measured as in A and compared to vehicle control. n=3-12, * p>0.05 and **p>0.01.

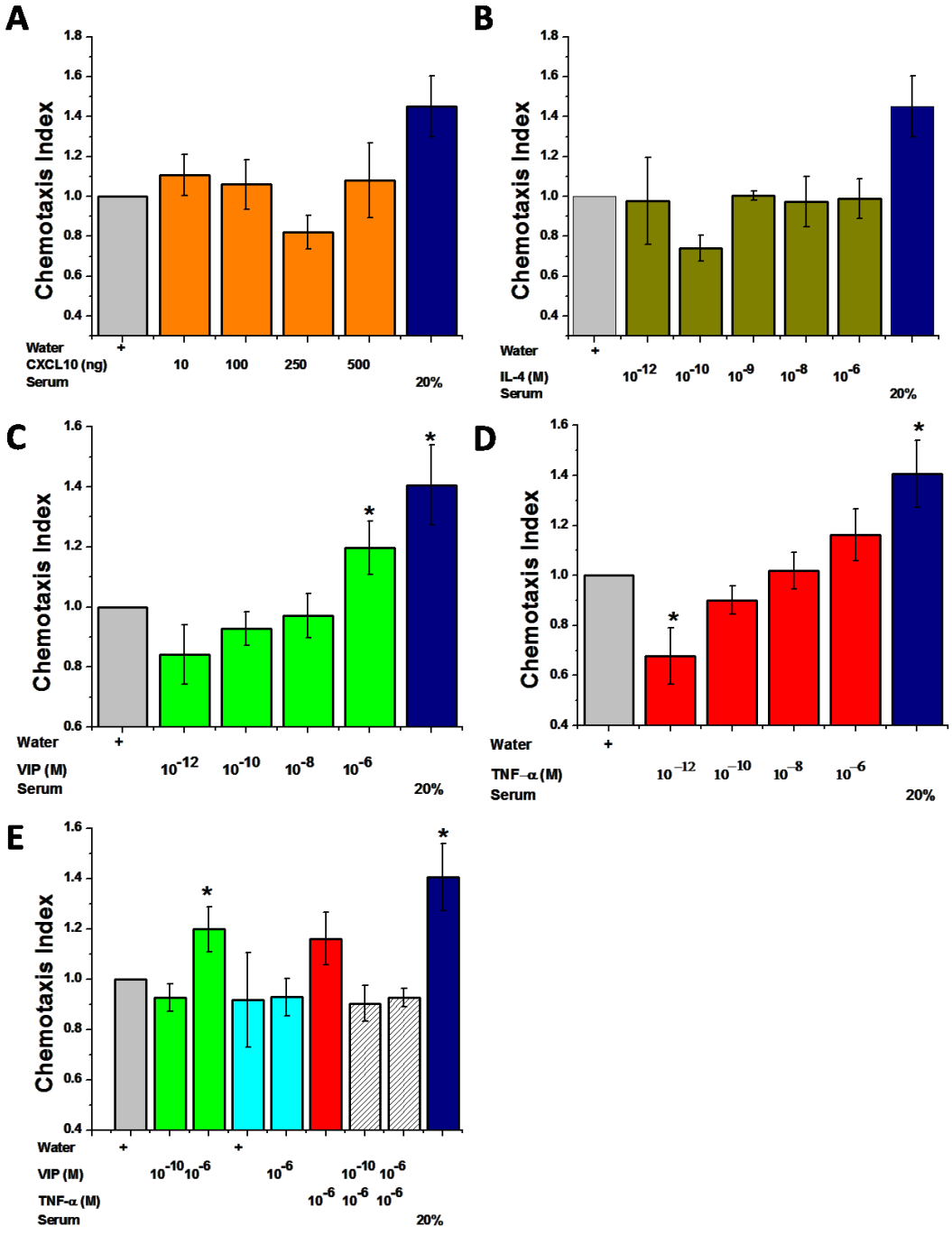


Figure 40. Differential chemotactic influence by VIP concentration on VPAC1 expressing HuT 78 cells. Serum starved HuT 78 cells chemotactic response to CXCL10 (A), IL-4 (B), VIP (C), or TNF-α (D). Cellular migration controls of pretreatment with VIP 5 min prior to assay (light blue bars) with either water or VIP as chemoattracant and an additive effect of VIP and TNFα were measured (E). All experiments are n=3, *p>0.05 and ** p>0.01.

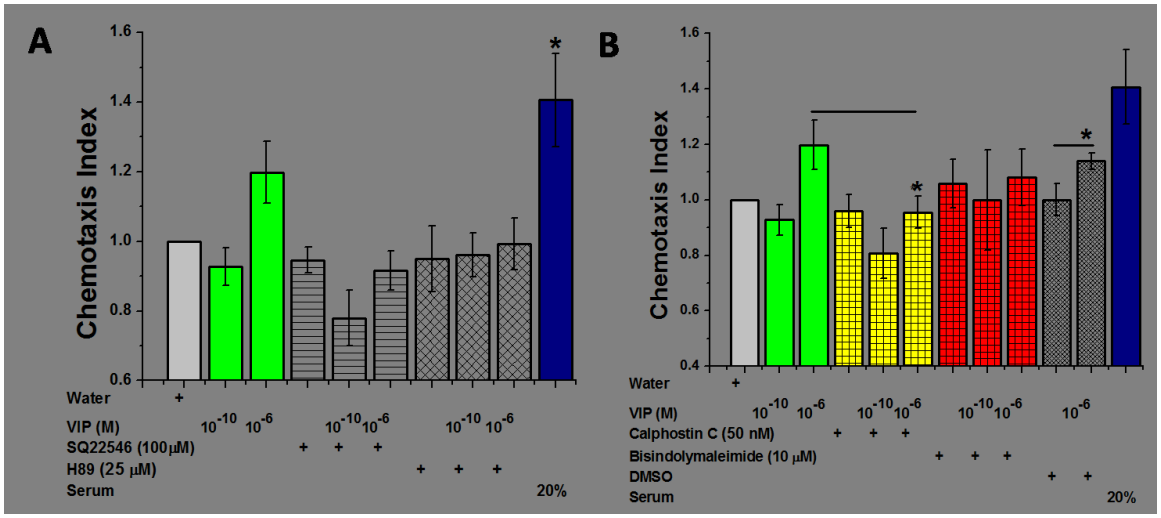


Figure 41. VIP chemotactic response is PKA and PKC mediated. VPAC1 signaling cascade responsible for chemotaxis was detemined using inhibitors towards AC, PKA, and PKC (materials and methods). HuT 78 cells were treated with SQ22536, H89 (A), bisindolymaleimide and calphostin C (B) 30 miniutes prior to the chemotaxis assay. All experiments are n=3, *p>0.05 and ** p>0.01.

To test whether VIP was chemotactic on HuT 78 cells through a i[cAMP] independent manner, we carried out pharmacological inhibitor studies. Due to the lack of i[cAMP] elicited, we first investigated the $G_{\alpha q}$ signaling pathway. Hut 78 cells were pretreated with two PKC specific inhibitors for either the regulatory (bisindolylmaleimide) or catalytic (calphostin C) subunits and the VIP chemotactic response was measured. Cells treated with calphostin C had a complete loss of chemotaxis towards VIP (Figure 41). As expected there was a reduction in cellular migration when Hut 78 cells were treated with bisindolylmaleimide, but to a lesser extent than with calphostin C. Additionally, we investigated if inhibition of AC and PKA would result in cellular migration towards VIP at 10^{-10} M. Surprisingly when HuT 78 cells were pretreated with either SQ22536 or H89 there was an inhibition of chemotaxis towards VIP demonstrating that PKA also plays a role in cellular

migration. These data support that the chemotactic response by VIP/VPAC1 is AC, PKA, and PKC dependent.

Discussion

In this study, we provide a better understanding for the signal transduction pathways elicited by VIP treatment in leukemic T cells. HuT 78 cells that exclusively express VPAC1 bound VIP and increase i[cAMP] modestly in a unimodal manner with the exogenous addition of 10^{-10} M VIP. However, Molt 4 cells, that predominantly express VPAC2, bound VIP protein, and increased i[cAMP] robustly in a concentration dependent manner. Curiously, only Molt 4 cells demonstrated detectable $G_{\alpha s}$-GDP→$G_{\alpha s}$-GTP exchange, even though both cell lines were chemotactically sensitive to VIP. Importantly both cell lines showed an inability to differentially regulate gene expression, unlike primary mouse T cells. Lastly, pharmacological inhibitors studies confirmed a $G_{\alpha s}$ → AC → PKA dependent pathway regulating Molt 4 cell chemotaxis to VIP. Whereas, $G_{\alpha s}$ →AC → PKA and $G_{\alpha q}$ → PKC are supported to regulate HuT 78 cell chemotaxis towards VIP. Taken together, VIP signaling in leukemic T cells , which induces similar cellular function (e.g. chemotaxis) is nonetheless cell context dependent, due to differences in (1) VIP receptor profile, (2) VIP ligand response, (3) magnitude of elicited i[cAMP], (4) $G_{\alpha s}$ coupling efficiency, and (5) pharmacological inhibitor sensitivity.

Quantifying mRNA expression for VPAC receptors in HuT 78 and Molt 4 cells was found in this study to be a poor indicator of the magnitude of surface VIP specific binding sites. In a direct comparison by qRT-PCR, Molt 4 cells expressed

350-fold greater VPAC2 versus VPAC1 mRNA levels in HuT 78 cells. However, VIP surface receptor expression revealed slightly higher VIP specific-binding in HuT 78 cells compared to Molt 4 cells (~2-fold). The qRT-PCR data would have predicted a greater VIP-specific binding in Molt 4 cells versus Hut 78 cells. The fact that there were an "similar" number of VIP binding sites on both cell lines supports the conclusion that there is a lack of a correlation between mRNA and protein expression for VPAC1 in HuT 78 cells. However, HuT 78 cells may be an exception as the HT29 cell line, which is reported to have an equivalent mRNA copy number of VPAC1 as Molt 4 cells have for VPAC2, showed a closer agreement in VIP-specific binding. Our data supports these findings as HT29 cells showed a similar VIP-binding to Molt 4 cells, but VPAC1 mRNA levels were equivalent to VPAC2 mRNA expression (data not shown). These data demonstrate that mRNA and surface protein do correlate between HT29 and Molt 4 cells but indicate massive discrepancies in the HuT 78 cells.

The lack of correlation between mRNA and protein levels in HuT 78 and Molt 4 cells could be a result of several factors. First, low mRNA expression of VPAC1 in HuT 78 suggests that VPAC1 mRNA could be unstable and quickly degraded. Second, VPAC1 mRNA could be a target of a microRNA that degrades the mRNA template, which would result in decrease protein expression. Recently, microRNA 525-5p has been identified to target the 3' UTR of VPAC1 resulting in a decrease of VPAC1 mRNA and protein (Cocco et al. 2010). However, this mechanism is highly unlikely due to the fact that targeting of the 3'UTR of VPAC1 mRNA should result in degradation of RNA prior to protein translation. Third,

protein stability of VPAC1 may have a longer half-life than VPAC2. Fourth, splice variants of VPAC1 may not be detected by our qRT-PCR primer-probe method. Indeed, a 5TM VPAC1 splice variant was identified in HuT 78 cells (Bokaei et al. 2006), and evidence for four additional splice variants have been discovered by our laboratory, as these isoforms could lack the exon sequence used in qRT-PCR detection. Due to the high mRNA levels detected in the Molt4 cells by pRT-PCR, while they are reported to have significantly lower surface protein expression (19,000 receptors/cell) there are potentially several possibilities for this discrepancy. First, the half-life of VPAC2 mRNA may be significantly longer than VPAC1. Second, posttranscriptional and translational regulation may result in a lack of VPAC2 protein expressed. Lastly, VPAC2 may have a short half-life in T cells as it is primarily expressed in activated cells while they progress through the cell cycle.

The hypothesis that VIP would elicit biological activity in a concentration dependent manner through a $G_{\alpha s}$-dependent signaling cascade was based on previously published data. In an overexpression system for VPAC1 and VPAC2 in CHO-K1 cells, VIP treatment elicited an i[cAMP] response in a concentration dependent manner, this data is supported by data collected in our laboratory. Also, HuT 78 cells reportedly have a 2-fold increase in i[cAMP] upon VIP binding in a concentration dependent fashion, but unfortunately these published results were not presented in graphical form and only discussed in the text. However, H9 cells, a derivative of HuT 78 cells, lacked an i[cAMP] response upon VIP treatment and actually suppressed i[cAMP] levels (Goursaud et al. 2005). In HuT 78 cells,

our data did not support either of the reported studies, as we showed a unimodal peak at 10^{-10} M VIP. These discrepancies are possibly a result of culture conditions (type of media (RPMI 1640 versus IMDM and 20%, 10%, and 0.5% serum conditions) and cellular background.

There are several additional possible reasons for the discrepancy in elicited i[cAMP] in Hut 78 cells. Desensitization of the VPAC1 receptors may result in lack of cAMP as GPCR's typically undergo desensitization at higher concentrations of ligand binding. Additionally, VPAC1 is known to internalize quickly upon ligand binding, internalization of VPAC1 receptor results in uncoupling from $G_{\alpha s}$ and binding to an arrestin protein, resulting in the activation of a different signaling cascade. VPAC1 receptors have the potential to couple to a different G protein molecule such as $G_{\alpha i}$ or $G_{\alpha q}$ as previously reported. These alternative signaling pathways would result in the absence of i[cAMP] generated due to other secondary messengers and could explain why we were unable to measure i[cAMP] elicited in HuT 78 cells. VPAC1 also contains a PDZ domain, which is a scaffolding protein that is able to bind PKC and PLC (Ranganathan and Ross 1997). Recent research has identified the formation of hetero- and homo-oligomers of different isoforms as in the case with oxytocin receptor and result in the alteration in G protein signaling (Devost and Zingg 2004, Hasbi et al. 2004). The 5TM VPAC1 receptor is unable to couple to G proteins due to its lack of the third intercellular loop and is hypothesized to signal through a tyrosine kinase (Bokaei et al. 2006). Lastly, VPAC1 receptor has been demonstrated to be pre-coupled to $G_{\alpha s}$ resulting in a continual activation (Martin Shreeve 2002) resulting in

higher basal cAMP levels that could explain our observation of HuT 78 cells having significantly higher i[cAMP] compared to Molt 4 cells. The high basal levels along with the lack of active $G_{\alpha s}$-GTP complex immunoprecipitated suggested to us that this may be a "cell specific" phenomenon occurring in the HuT 78 cells, which results in the lack of cAMP elicited upon VIP binding.

Both cell lines that predominantly express VPAC2 (Molt 4 and Sup T1) elicited an i[cAMP] response that was concentration dependent for VIP except for at the highest VIP concentration in serum starved SupT1 cells. These data support the reported increase in i[cAMP] upon overexpression of VPAC2 and in previously reported cAMP response for Molt 4 and SupT 1 cells. Reduction of serum in culture media resulted in an increase of secondary messenger (cAMP) over complete media cultured cells. Serum reduction limits exposure to several growth factors, thus slowing metabolism and lowering basal cAMP levels. In Molt 4 cells, these data demonstrated a maximal cAMP response upon saturation of VPAC2 with VIP. The K_d for VPAC2/VIP binding is approximately 1 nM, while at these concentrations there was minimal cAMP response. These data suggest that VPAC2 elicits a cAMP response upon complete saturation of receptors, with no apparent internalization and desensitization in Molt 4 cells.

A possible reason for the lack of response detected in HuT 78 cells may be due to the excessively high cAMP levels present. One explanation could be that HuT 78 cells were cultured in 20% chFBS media, with other cells propagated in only 10% serum. This elevated level of serum may have aided in the high endogenous cAMP levels detected, and "short-circuited" VIP signaling. This

notion is supported by the fact that HuT 78 cells were sensitive to VIP by eliciting i[cAMP] only after serum removal. Alternatively, VPAC1 may not increase cAMP if the VPAC1 receptor is pre-coupled to $G_{\alpha s}$ and is chronically signaling. Indeed, pre-coupling of VPAC1 has been identified to occur in murine brain, kidney, and lung cells (Martin Shreeve 2002) giving credence that this phenomenon may occur in other cell types too. If VPAC1 is pre-coupled to $G_{\alpha s}$, it would result in increased basal i[cAMP] levels and ligand binding may not be able to increase i[cAMP]. A less feasible possibility would be that HuT 78 cells have reached its maximum cAMP production, but forskolin increased i[cAMP] suggesting that this is not a reasonable explanation. In toto, these data suggest that the endogenously high levels of i[cAMP] in HuT 78 cells may contribute to ineffective VIP signaling and cAMP response.

Our results support that VPAC1 may not couple to $G_{\alpha s}$ and the cAMP elicited is through a non-canonical pathway. HuT 78 cells were assayed for active $G_{\alpha s}$-GTP upon VIP treatment or water and lacked an increase in active $G_{\alpha s}$-GTP complex. This raised the question, if $G_{\alpha s}$ is not activated in HuT 78 cells treated with VIP, what is responsible for the increase in cAMP? We speculate that an alternative method of activating AC is occurring. One possible mechanism could be that VIP/VPAC1 signaling is coupled to G_i, where the α-subunit inhibits AC activity, this is supported by our decrease in i[cAMP] observed at 10^{-12} M VIP. Furthermore, the βγ-subunits have the ability to activate specific isoforms of AC (Defer, Best-Belpomme and Hanoune 2000).

Previously, we have published the largest collection of VIP differentially regulated genes that represent its transcriptome in naïve and activated murine CD4 T cells (Dorsam et al. 2010). In that study, we identified that the VIP transcriptome resulted in the increase of several genes that participate in apoptosis and chemotaxis. However, that study was limited to study primary cells that predominantly expressed only VPAC1 with low levels of VPAC2 (<1%). Therefore, we completed a similar whole genome study using Molt 4 and Hut 78 cells in attempt to determine the gene regulation changes controlled by VPAC1 and 2. Interestingly, microarray results were unable to identify a single gene target that was differentially regulated. Reanalysis of this data set by grouping experiments based on the agreement of i[cAMP] elicited, yielding two sets (group A higher cAMP, group B lower cAMP). This method of analysis ignored the difference in time of VIP treatment and the magnitude of the change in expression for each set. This analysis determined that there were seven genes differentially upregulated in HuT 78 cells, but were curiously were not involved in chemotaxis or apoptosis. Fascinatingly, Lamin that was oppositely regulated in set A versus set B. We originally utilized the Lamin gene as a normalizing control for nuclear protein in Western blots and changed this practice based on these results. Similar analyses were completed for Molt 4 cells with sets A and B where only one gene was found to be regulated by VIP. These data demonstrated that leukemic T cells have radically limited sensitivity to VIP with respect to gene expression compared to wild type mouse CD4 T cells and supports the hypothesis that cancer cells

usurp cellular proteins to gain a selective advantage in cellular growth (Dorsam and Gutkind 2007).

Several studies have identified VIP is a chemoattractant for lymphocytes (Ottaway 1984, Johnston et al. 1994). However identity of the VIP receptor was not determined, thus making it impossible to know, which receptor was responsible for the directed cellular movement. Our study was designed to determine, which VIP receptor was responsible for chemotaxis as HuT 78 cells exclusively express VPAC1 receptors and Molt 4 cells predominantly express VPAC2. The utilization of these two cell lines would allow for us to determine, which VIP receptor was responsible for VIP chemotaxis. We hypothesized that VPAC2 expressing Molt 4 cells would migrate towards VIP. Sup T1 cells that predominantly express VPAC2 are chemotactic towards VIP in a concentration dependent system (Xia et al. 1996c) while these cells do express low levels of VPAC1. Data collected for Molt 4 cells resulted in a similar chemotactic response to VIP as SupT1 cells. These data support the idea that leukemic T cells predominantly expressing VPAC2 receptors move towards VIP in a concentration dependent manner.

The directed cellular migration of leukemic T cells to organs that contain innervating VIPergic nerves may result in death. The lungs contain high levels of VIP to suppress an immune response by naïve T cells due to the large number of antigens taken in while breathing. Additionally, this high level of VIP may also recruit leukemic T cells to the lungs. The recruitment of leukemic T cells to the lungs can result in the lungs filling with mucosal fluids and T cells, this increase

results in the lungs filling with fluid and can ultimately lead to “drowning.” Therefore, one possible mechanism to prevent the migration of leukemic T cells to the lungs is to inhibit its cellular migration.

Based upon the increase in i[cAMP] and the efficient coupling of $G_{\alpha s}$, it was hypothesized that VIP/VPAC2 signaling through $G_{\alpha s}$→AC→cAMP→PKA was responsible for the chemotactic response. The use of pharmacological inhibitors for AC resulted in a loss of chemotaxis for Molt 4 cells, similarly when PKA’s activity was inhibited by either PKI or H89 no cellular migration occurred (Figure 42). The lack of chemotaxis toward VIP in Molt 4 cells supports the hypothesis for this experiment that PKA activity controls directed cellular movement towards VIP of VPAC2 expressing T cells. The utilization of VPAC2 expressing cells to migrate towards VIP may have a duel function. Besides the demonstrated chemotactic response to VIP, VPAC2 may be critical in the survival of cancer cells by evading apoptosis. It is reasonable to argue that these cells migrate towards VIP to utilize the ligand for its antiapoptotic effect on the cell due to VPAC2’s ability to control Bcl-2 proteins.

Discrepancies in VPAC1 expressing cells chemotactic response toward VIP have undermined the possible role VIP plays in chemotaxis. Naïve murine CD4 T cells migrated to the PP in the gut and upon activated there was a decrease in the cellular migration towards VIP rich regions. In contrast, activated Th2 cells are chemotactic towards VIP while Th1 cells do not migrate to VIP. Additionally, HuT 78 cells were reported to lack a chemotactic response towards VIP, and inhibited IL-4 and TNFα chemotaxis. In the present study, we found that HuT 78 cells had a

chemotactic response to VIP in a concentration dependent fashion , which is opposite of the observation made by Xia et al. (Xia et al. 1996a). These differences may be a result of the Boyden chambers used. One such example could be the pore size used in this experiment. We utilized an 8 μm pore while other study used a 12 μm pore. Hut 78 cells have an average diameter size of 14.5 μm and a range of 13-16 μm, and therefore the larger pore size of 12 μm may have resulted in greater random diffusion. In summary, our results agree with previous reports regarding i[cAMP] elicited by VIP in HuT 78 cells. However, the optimal ligand concentration and the effects on chemotaxis did not agree based on potential experimental strategies employed.

Interestingly, the chemotactic response of Hut 78 cells towards VIP in a concentration dependent fashion was similar to Molt 4 cells. We originally hypothesized that HuT 78 cells would elicit a chemotactic response that parallels it i[cAMP] response, as in the case with the Molt 4 cells. Curiously, our data indicated a concentration dependent chemotactic response by HuT 78 cells to VIP. These results supports that both VPAC1 and VPAC2 induces chemotaxis but through different signaling mechanisms. This hypothesis was tested using several pharmacological inhibitors. Unexpectedly, inhibition against AC and PKA ($G_{\alpha s}$ pathway) activity resulted in a complete loss of chemotaxis towards VIP at 10^{-6} M with little effect at 10^{-10} M VIP implying that the $G_{\alpha s}$ pathway that activated AC and PKA is needed for HuT 78 cells to migrate towards VIP. These data demonstrated PKA activity is needed for VIP chemotaxis even though there is no i[cAMP] elicited upon VIP binding.

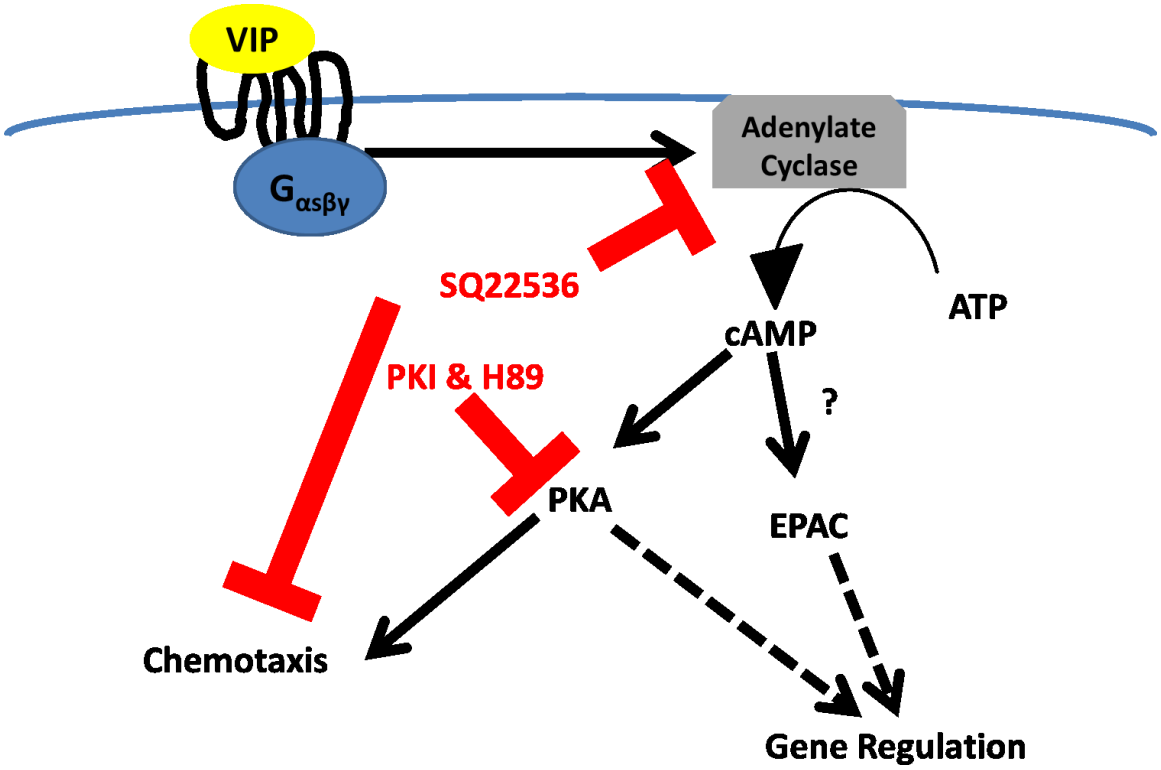


Figure 42. Signaling cascade that results in VPAC2 chemotaxis. Exogenous VIP binds to PVAC2 allowing for the dissociation of the αs-subunit, which activates adenylate cyclase. This results in the conversion of ATP to cAMP, which can activated PKA or EPAC. Inhibition of adenylate cyclase and PKA resulted in inhibition of chemotaxis. Dashed lines are possible signaling results, but due to lack of data this was not confirmed.

Due to HuT 78 cells chemotactic response at 10^{-6} M VIP and the lack of i[cAMP] elicited, we hypothesized that VIP chemotactic response was due to VPAC1 signaling through the $G_{\alpha q}$ pathway. HuT 78 cells were treated with pharmacological inhibitors towards PKC. Upon treatment with an inhibitor for either the regulatory (calphostin C) or catalytic (bisindolylmaleimide) domain for PKC ($G_{\alpha q}$ pathway) there was a loss of cellular migration at 10^{-6} M VIP with little effect at 10^{-10} M VIP. In total, these data demonstrated that PKC also plays a role in the chemotactic response to VIP for HuT 78 cells, and both the $G_{\alpha q}$ and $G_{\alpha s}$ pathways are needed to induce chemotaxis as blocking one or the other with

inhibitors blocks chemotaxis (Figure 43). It is not understood why we were unable to measure $G_{\alpha s}$ (IP and cAMP) pathway involvement, while inhibition against their signaling effectors did show involvement.

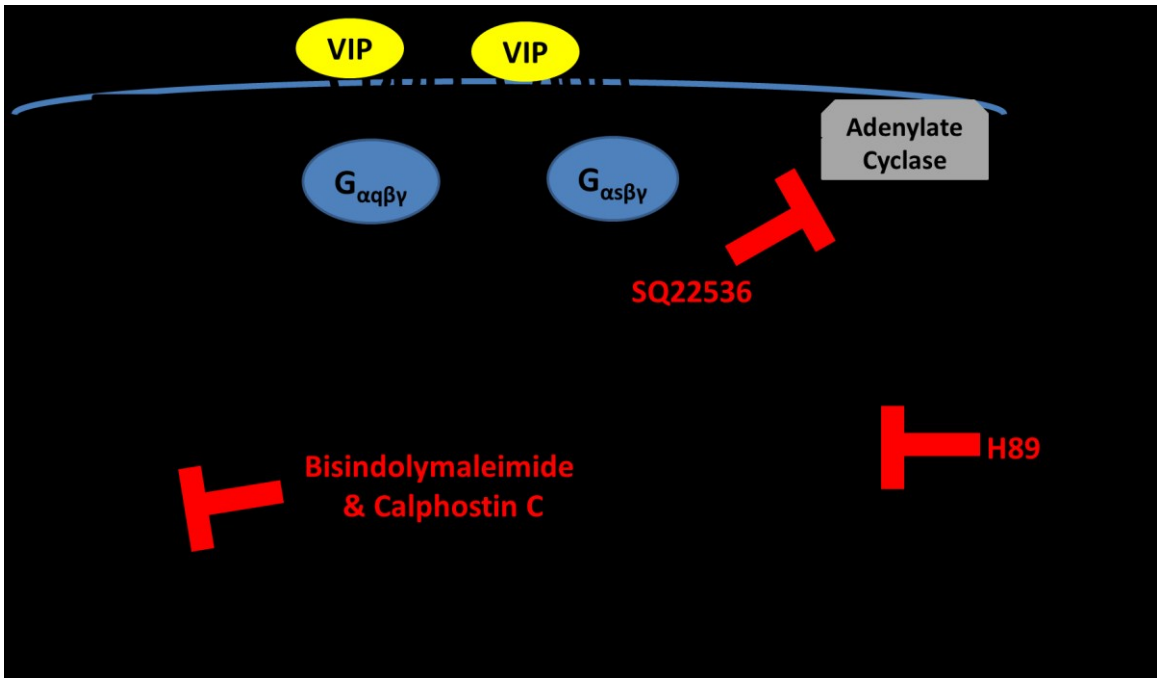


Figure 43. Signaling mechanism utilized by VPAC1 receptors in Hut 78 cells in response to VIP. VIP signaling resulted in chemotaxis, but was ablated when AC, PKA, and PKC activity was inhibited. These data demonstrated that VPAC1 receptors could couple to more than one G protein ($G_{\alpha s}$ and $G_{\alpha q}$). VIP possibly regulates gene regulation but very weakly.

The chemotactic response of VIP on VPAC1 and VPAC2 expressing cells and the signaling cascade responsible for the directed cellular movement was elicited in this study. VPAC2 clearly couples more efficiently to $G_{\alpha s}$ in leukemic T cells than VPAC1 observed by the i[cAMP] response and immunoprecipitated $G_{\alpha s}$-GTP complex. These data also demonstrated that VPAC1 is chemotactic in HuT 78 cells, but appears to be directed by two equally important mechanisms that utilize both PKA and PKC for cellular migration unlike VPAC2 that exclusively utilizes PKA. Understanding a chemotactic response for VPAC1 and 2 is critical in

understanding a possible mechanism to explain the cellular migration of T cells to the site of an immune response or to organs in the body where VIPergic nerves innervate and release VIP. We propose that recruitment of activated T cells expressing high VPAC2 and low VPAC1 by VIP may result in increased survival due to the inhibition of apoptosis by VPAC2 signaling. After the pathogen has been cleared, VIP receptor expression would switch with high VPAC1 and low VPAC2 promoting apoptosis and removal of these T cells no longer needed. This mechanism has developed to allow a single neuropeptide to have multiple functions within the same cell by regulating its temporal receptor expression.

CHAPTER 4. OVERALL DISUCSSION

Discussion, Implications, and Future Directions

We hypothesized that VIP signaling through VPAC1 in a $G_{\alpha s}$ dependent signaling cascade would alter the biology of IK in both naïve and activated T cells. To test this hypothesis, we utilized HuT 78 cells, which solely expressed VPAC1, to determine how VIP/VPAC1 signaling would alter IK protein expression and its posttranslational modifications by one- and two-dimensional gel electrophoresis and Western blot analyses. These experiments yielded previously unknown data about the regulation of the tumor suppressor IK. First, nuclear IK protein levels in naïve HuT 78 cells in the presence or absence of VIP was unaltered. Second, PMA/ionomycin activated HuT 78 cells had an increase in nuclear IK protein expression levels. VIP treated activated HuT 78 cells suppressed the increase of nuclear IK protein and decreased the surface protein expression of CD25. Lastly, VIP was determined to alter the isoelectric species of IK protein. These data demonstrated that IK protein is regulated by the neuropeptide VIP in activated HuT 78 cells.

We originally hypothesized that IK protein would have increased expression in HuT 78 cells upon VIP/VPAC1 signaling. This was hypothesized because VIP signaling through VPAC1 is known to have antiproliferative properties by maintaining $p27^{kip1}$ in a dephosphorylated state and the suppression of the activation chemokine IL-2. Additionally, IK is known to set the threshold for T cell activation, this is completed by regulation of the $p27^{kip1}$ gene expression. We set forth to determine if there was a change in IK protein in naïve HuT 78 cells when

treated with VIP. There was no change detected in IK protein over 24 hr between naïve cells treated with or without VIP. This data did not support our original hypothesis as we would have expected an increase in nuclear IK protein. However, upon activation with PMA/ionomycin there was an observed increase in nuclear IK protein levels. These data would suggest that IK protein is being recruited to the nucleus upon activation. The increase in IK protein could be a result of the cell's attempt to maintain a naïve state, since IK sets the threshold for T cell activation. Our hypothesis, if true would result in an increase of IK protein due to VIP treatment. Interestingly, a decrease in IK protein was detected in the activated state and undermined our hypothesis. However, IK protein is known to be maintained in a hypophosphorylated state in resting T cells and upon activation IK protein becomes hyperphosphorylated by CK II. Hyperphosphorylation of IK could result in decreased nuclear IK protein due to ubiquitination and protein degradation. The decrease of nuclear IK protein could have been a result of VIP/VPAC1 signaling through the $G_{\alpha s}$ signaling cascade, which would activate PKA. IK contains several putative consensus sequences for PKA phosphorylation. The decrease of IK protein expression could have resulted from both CK II and PKA phosphorylating IK and resulting in IK protein being "tagged" for degradation.

Due to the decrease in nuclear IK protein in activated VIP treated Hut 78 cells and the number of PKA consensus sites; we hypothesized that VIP/VPAC1 signaling in activated HuT 78 cells would alter the isoelectric species of IK. To determine if VIP/VPAC1 signaling altered the isoelectric states of IK, we separated total and nuclear IK protein by two-dimensional Western blots. We concluded that

there were several isoelectric immunoreactive IK species for each IK isoform. As expected, there was a decrease in the number of isoelectric species after four hours of activation. Phosphorylation of IK would alter the number of isoelectric species of IK, this data supports that upon T cell activation IK protein is hyperphosphorylated at the p1, p2, and p3 regions resulting in the alteration of isoelectric species detected (Gómez-del Arco, Maki and Georgopoulos 2004). Interestingly, the when activated HuT 78 cells were treated with VIP there was an increase in the number of IK isoelectric species detected. This supported the hypothesis that VIP/VPAC1 signaling through PKA could alter the isoelectric species of IK. Additionally, these data revealed that VIP signaling resulted in a different phosphorylation pattern in activated T cells. This data supported that other kinases (we propose PKA) are able to phosphorylate IK protein.

Unexpectedly, in two-dimensional Western blot analyses of activated HuT 78 cells a lower molecular weight immunoreactive IK species was detected. This lower molecular weight species is presumed to be a dominant negative IK isoform. The IK-DN isoform was not detected in the unactivated (0 hr) sample, and would suggest that upon activation of HuT 78 cells DN IK isoforms are translocated into the nucleus. Due to the lack of a nuclear targeting sequence in the DN isoforms, we hypothesized that the DN isoforms dimerizes with IK H, 1, or 2 which are able to transverse the nuclear membrane. The increase in detectable IK protein that was observed in the one-dimensional Western blots would support this hypothesis and explain the increase in nuclear IK 1 protein could dimerize and translocated the DN isoforms into the nucleus. Interestingly, 2D Western blots were unable to

detect the lower molecular weight species of IK in activated cells treated with VIP. The loss of the lower molecular weight species with VIP treated also supports the hypothesis that the DN isoforms are being recruited to the nucleus by dimerizing with larger isoforms as there was a decrease in the detected nuclear IK protein. Therefore, this suggested that if IK protein is not being translocated to the nucleus, the DN isoforms are unable to enter and impede IK-DNA interactions.

The working model for this data is that activation of HuT 78 cells results in the hyperphosphorylation of IK protein to allow for cell cycle progression by CK II and that activated HuT 78 cells attempt to suppress cell cycle entry by the recruitment of additional IK protein into the nucleus. Cell cycle progression from G_1 to S is controlled by the CyclinE-Cdk2 complex and is inhibited by the CDKI $p27^{kip1}$ protein. The transcriptional regulation of $p27^{kip1}$ is controlled by the expression of IK 1 (Kathrein et al. 2005). In activated cells, IK becomes hyperphosphorylated and has a decrease in DNA binding affinity, this decrease in affinity would displace IK protein from the $p27^{kip1}$ promoter and suppresses transcription as IK is an activator for this gene (Molnár and Georgopoulos 1994). The activation of HuT 78 cells results in an increase of IK protein in the nucleus, where IK 1/DN dimers are translocated from the cytoplasm into the nucleus. These DN containing IK dimers are unable to engage the $p27^{kip1}$ promoter due to the lack of the needed DNA binding zinc fingers. Therefore, cell cycle entry occurs. While VIP treatment of activated HuT 78 cells results in a hyperphosporylated state and IK protein is degraded allowing for cell cycle entry and prevents the translocation of IK-DN isoforms from the cytoplasm into the nucleus. The lack of

DN isoforms in the nucleus upon reentry into the G_1 phase allows for hypophosphorylated IK to reengage the $p27^{kip1}$ promoter allowing for transcription. Additionally, PKA may be phosphorylating cytoplasmic IK protein which could result in a hyperphosphorylated state and IK protein being tagged for degradation (Figure 44).

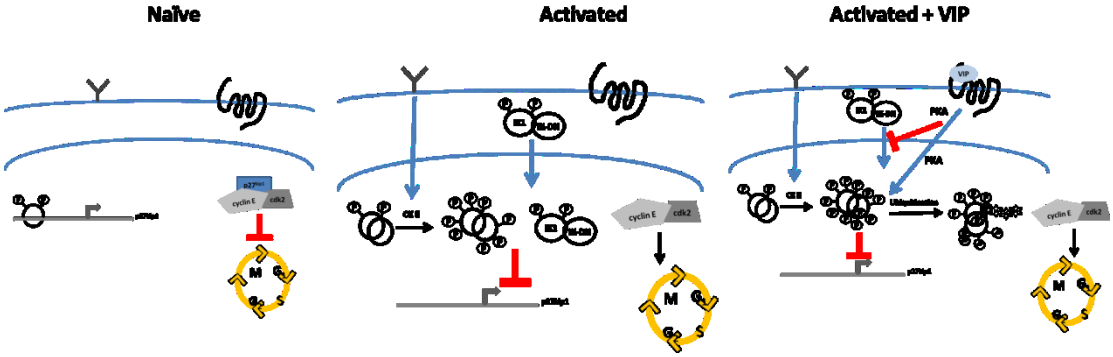


Figure 44. Hypothetical model of cell cycle progression in naïve and activated HuT 78 cells +/- VIP. In the naïve state, IK is engaged to the $p27^{kip1}$ promoter allowing for transcription. In the activated state, IK becomes hyperphosphorylated, dissociates form the $p27^{kip1}$ promoter, and additional IK is translocated into the nucleus. In VIP treated activated HuT 78 cells, PKA is activated resulting in additional phosphorylation of IK resulting in degradation and this also impedes DN isoforms from entering the nucleus.

Future experiments are needed to test this hypothesis. First, to determine if IK is engaged to the $p27^{kip1}$ promoter in naïve and activated HuT 78 cells can be assessed by ChIP analysis. It would be speculated that IK is engaged in the $p27^{kip1}$ promoter in the naïve but not in the activated state. Second, identification of the lower molecular weight species of IK in the activated but not in the activated/VIP treated cells. Isolation of the lower molecular weight IK species could be completed by isolation of this species (spot picking from a 2D SDS-PAGE) and protein sequencing by mass spectroscopy. Third, determine what posttranslational modification resulted in the alteration of the number of

immunoreactive isoelectric IK species. To determine if the PTM is phosphorylation, activated +/- VIP HuT 78 cells could be cultured with ^{32}PγATP. In activated HuT 78 cells, CK II will phosphorylate IK with ^{32}P labeled ATP. If there is additional phosphorylation detected in the VIP treated cells these data would strongly suggest that VIP signaling phosphorylates IK protein. Additionally, we could inhibit CKII activity with DRB and identify if there is an alteration in the isoelectric pattern of IK due to VIP treatment in activated HuT 78 cells. Lastly, to determine if the decrease in IK protein in activated HuT 78 cells treated with VIP was a result of protein degradation. Immunoprecipitation assays could be completed using a ubiquitin specific antibody on activated and activated/VIP treated cell lysate. Immunoprecipitated ubiquitin protein would be separated by SDS-PAGE, transferred to nitrocellulose, and probed for IK protein. If there is an increase in ubiquitinated IK protein detected between the activated +/- VIP treated cells this would support that VIP signaling increases IK protein degradation. Additionally, inhibition of the E3 ligase (responsible for ubiquitin tagging of proteins) in the VIP treated activated HuT 78 cells should increase the amount of nuclear IK protein present demonstrating that VIP signaling is increasing IK protein degradation.

We hypothesized that VPAC1 and 2 signaling have different biological effects on T cells. To test this hypothesis, we utilized two leukemic T cell lines that predominantly express either VPAC1 (HuT 78 cells) or VPAC2 (Molt 4 cells) to determine the biological effects on chemotaxis. We confirmed that VPAC 1 was the only VIP receptor expressed in HuT 78 cells and that VPAC2 was

predominantly expressed in Molt 4 cells. Interestingly, the mRNA expression levels of VPAC receptors did not correlate with the surface protein expression measured by FAM-labeled VIP. Additionally, we determined that VPAC1 couples less efficiently to $G_{\alpha s}$ than VPAC2 by immunoprecipitation for the active $G_{\alpha s}$-GTP complex and i[cAMP] elicited upon ligand binding. Lastly, we determined that both VPAC1 and VPAC2 expressing cells were chemotactic towards VIP using different signaling cascades. VPAC1 utilizes both PKA and PKC to initiate chemotaxis while VPAC2 utilizes PKA for directed cellular migration towards VIP.

VIP receptor expression was validated to ensure that HuT 78 and Molt 4 cells express a single VIP receptor. We determined that HuT 78 cells solely express VPAC1 at the mRNA level as previously reported (Xia et al. 1996a). Additionally, we confirmed that Molt 4 cells predominantly express VPAC2. We also determined VIP surface protein receptor expression on each cell line. Remarkably, HuT 78 cells express low VPAC1 mRNA levels while Molt 4 cells express high VPAC2 mRNA levels when normalized to a human reference RNA pool. However, this was the opposite of what was observed when we measured the surface protein expression. This data suggest that quantitating VPAC receptor expression by mRNA is not a good method for extrapolating to protein expression levels. One possible reason for the discrepancy is that VPAC receptors are known to be alternatively spliced (Bokaei et al. 2006, Grinninger et al. 2004). Two isoforms are known for both VPAC1 and VPAC2, the full-length 7TM and a shorter 5TM version. Our method of detection (qRT-PCR) does measure both splice variants. However, there may be additional splice variants that we are unable to

be detected. Our laboratory has potentially identified four additional VPAC1 splice variants, however, cloning of these splice variants has not been completed and the regions we amplify by qRT-PCR may not detect these isoforms.

The biological activity of VIP was determined for each receptor by measurement of intercellular cAMP produced upon ligand/receptor binding. HuT 78 cells lacked a i[cAMP] response when cultured in complete media, but when cells were cultured for 24 hr prior to assay in serum free media there was an increase in i[cAMP] at 10^{-10} M VIP. This lack of i[cAMP] could be explained by the expression of the 5TM VPAC1 receptor. The 5TM receptor lacks the third intercellular loop, which is needed for efficient coupling to G proteins. HuT 78 cells are reported to have 75,000 receptors/cell (Xia et al. 1996a) and to express both 5TM and 7TM VPAC1 mRNA (Bokaei et al. 2006). The overexpression of the 7TM VPAC1 receptor in CHO-K1 cells or endogenous VPAC1 expressing HT-29 cells both elicit a i[cAMP] response that is concentration dependent (Lara-Marquez et al. 2001, Alleaume et al. 2003, Langer, Gaspard and Robberecht 2006). It is hypothesized that the 5TM receptor signals through a tyrosine kinase, because when the 5TM VPAC1 receptor was overexpressed in CHO-K1 cells there was no cAMP response upon VIP treatment (Bokaei et al. 2006). HuT 78 cells express both 5 and 7 TM VPAC1 receptors (as reported by Bokaei et al.) is supported by the lack of active $G_{\alpha s}$ that was detected upon VIP treatment. The Molt 4 (VPAC2) cells elicited a i[cAMP] response in a VIP concentration dependent fashion along with an increase in active $G_{\alpha s}$ immunoprecipitated upon VIP treatment. These data support that VPAC2 couples more efficiently than VPAC1 to $G_{\alpha s}$ if the 7TM

receptor is the only VPAC1 receptor expressed in Hut 78 cells or that the Molt 4 cells do not express the 5TM splice variant and all surface receptors are able to couple with G proteins.

Originally we hypothesized that VIP would elicit different biological functions using the same signaling cascade through VPAC1 and VPAC2 receptors. Our endpoint analysis was chemotaxis, as it was reported that Sup T1 cells, which highly express VPAC2 were chemotactic towards VIP and that VIP inhibited chemotaxis in HuT 78 cells (Finch, Sreedharan and Goetzl 1989, Xia et al. 1996b, Xia et al. 1996a). However, we discovered that both VPAC1 and 2 expressing cells had a chemotactic response to VIP. As a result we modified our hypothesis that VPAC1 and 2 utilize different signaling cascades to result in the same biological function of chemotaxis. This was due to the lack of i[cAMP] elicited and inefficient coupling to $G_{\alpha s}$, while Molt 4 cells had a robust i[cAMP] response and efficient coupling to $G_{\alpha s}$. The Molt 4 cells chemotactic response towards VIP was PKA dependent while chemotaxis for HuT 78 cells was PKA and PKC dependent. This data supports that VPAC1 and 2 have the same biological effect on chemotaxis, but utilized different signaling cascades to obtain this biological function.

These data demonstrate that VPAC1 and 2 results in the same biological function (chemotaxis), but the consequence is through two different signaling cascades. Naïve T cell are known to be chemotactic towards VIP and highly express VPAC1 receptors with little/no VPAC2 receptor expression. This expression profile nearly mimics the expression of VPAC receptors on HuT 78

cells. The signaling cascade that resulted in chemotaxis was not previously established, it was presumed to be though the $G_{\alpha s}$ signaling cascade. This data, due to the lack of i[cAMP] response and coupling to active $G_{\alpha s}$, supports that VPAC1 receptor couples to a different G protein, presumably $G_{\alpha q}$ due to PKC's involvement in chemotaxis. We now know that upon activation there is a decrease in the number of VPAC1 receptors expressed on the surface of the T cell, and that VPAC2 surface expression increases. Therefore, it would be warranted to hypothesized that VPAC2 has a less chemotactic response to VIP as it is reported that activated T cells have a weaker chemotactic response towards VIP (Johnston et al. 1994). However, the chemotactic response of SupT1 cells, which predominantly express VPAC2 and low levels of VPAC1 demonstrates that these cells significantly migrate towards VIP. Interestingly, when we measured chemotaxis, we concluded that there was a greater chemotactic response for VPAC2 than VPAC1 expressing cells (Figure 45).

The alteration in VPAC receptors in leukemia may increase in cell survival. In naïve T cells, VPAC1 is the predominantly expressed receptor, while in leukemic T cells VPAC1 expression is suppressed and VPAC2 is the predominantly expressed receptor (unpublished data; Dorsam et al. 2011). It is unknown if leukemia causes the decrease in VPAC1 expression or if the downregulation of VPAC1 results in leukemia, but there is a correlation between the decreased expression of VPAC1 and leukemic T cells. One possible reason for this downregulation is that VPAC1 signaling maintains $p27^{kip1}$ protein in a dephosphorylated state, which prevents the G_1/S cell cycle transition. Therefore, it

would be important for a leukemic cell to decrease the expression of VPAC1 to allow for cell cycle progression. The increase in VPAC2 expression can easily be explained because VPAC2 signaling results in the increase in expression of the antiapoptotic Bcl-2 protein. Consequently, leukemic cells alter VIP receptor expression to maximize cell survival.

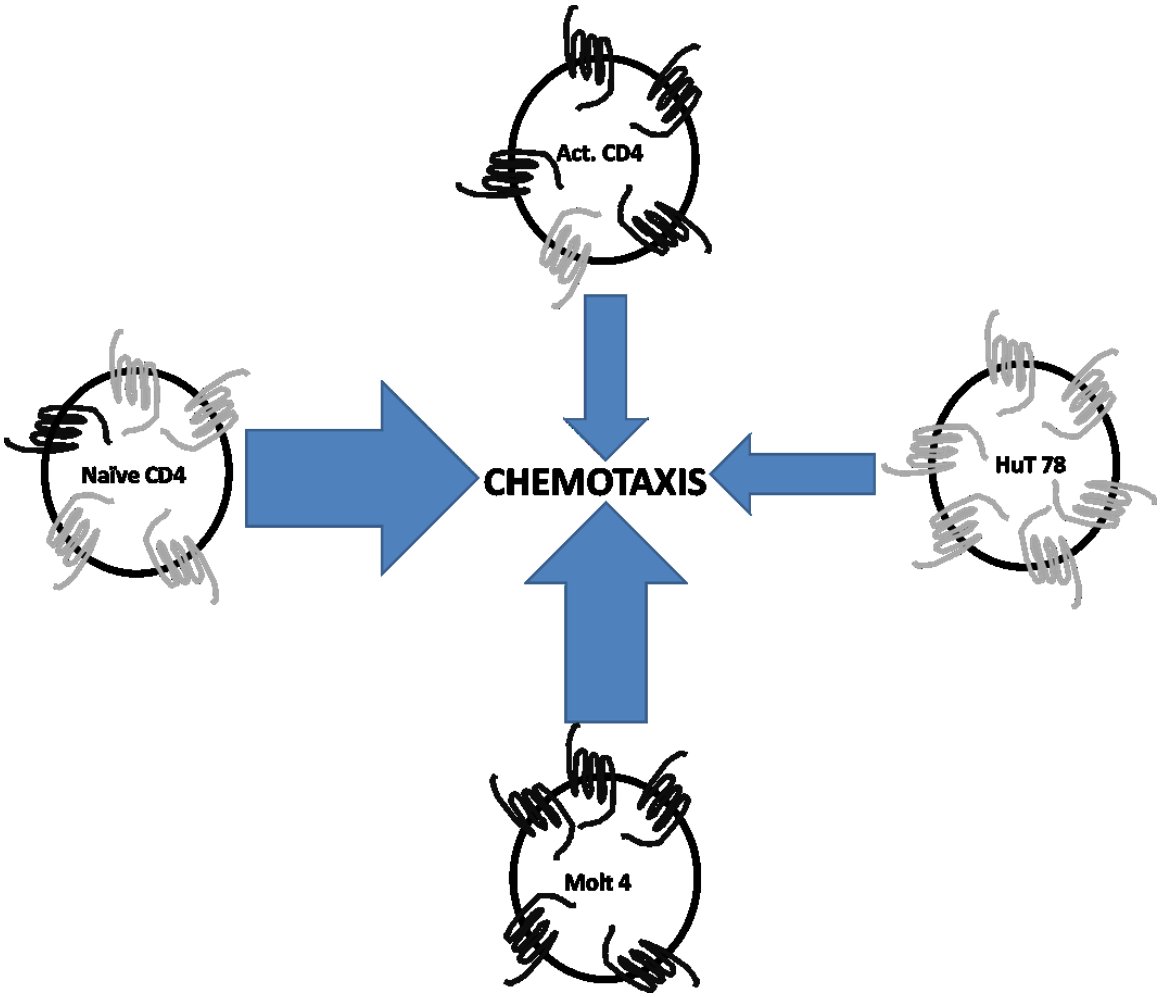


Figure 45. Hypothetical model for magnitude of VIP directed chemotaxis. VPAC1 (light gray) and VPAC2 (Black) receptors expression is depicted on naïve CD4, activated CD4, HuT 78, and Molt 4 cells. Size of arrow represents magnitude of chemotactic response due to VIP.

Chemotaxis towards VIP may result in increased leukemic T cell survival. Since VPAC1 and 2 expressing cells are chemotactic to VIP, and VIP/VPAC2

signaling increases cell survival by regulation of antiapoptotic factors. These two consequences of VIP signaling support the hypothesis that leukemic T cells dysregulate VIP receptors in order to support cell survival.

VPAC1 and 2 were previously thought to have different biological functions in the cell; however, we have demonstrated that VPAC1 and 2 result in chemotaxis towards VIP using different signaling cascades. It is still questionable if the biological effect of VIP signaling that results in chemotaxis is receptor specific or cell context dependent. To test this inquire, knocking down VPAC1 and VPAC2 in both HuT 78 and Molt 4 cell lines by targeting the 3' UTR of the mRNA to suppress VPAC receptor protein and repeat the chemotaxis assays. This will validate that chemotaxis is a VIP receptor mediated phenomena. Additionally, if the receptor expression on HuT 78 and Molt 4 cells could be switched that HuT 78 cells solely express VPAC2 and Molt 4 cells only express VPAC1 receptors to answer the cell context dependent question for signaling. The reevaluate of chemotaxis and signaling cascade elicited will allow us to answer if VIP/VPAC signaling is cell context dependent. These data would allow us to understand if the lack of i[cAMP] in HuT 78 cells was due to the expression of the 5TM VPAC1 receptor. Additionally, if Molt 4 cells had a lack of coupling to $G_{\alpha s}$ and i[cAMP] this would demonstrate that VPAC2 couples more efficiently than VPAC1 to $G_{\alpha s}$.

These data have demonstrated that VIP signaling alters T cell biology. First, we were able to conclude that VIP/VPAC1 signaling alters the expression of nuclear IK protein and alters the isoelectric species of IK in activated HuT 78 cells. Second, we demonstrated that VPAC1 and 2 receptors have similar biological

function (chemotaxis) but the signaling cascade utilized by each receptor is different. These data were the first to demonstrate that VIP signaling is able to regulate the tumor suppressor IK and that VPAC1 and 2 have similar biological cellular migration but utilize different signaling cascades. These allow for the possibility to inhibit chemotaxis for leukemic T cells and suppress a potential survival mechanism of cancer cells.

CHAPTER 5. REFERENCES

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