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Regulation of RasGRP1 protein in thymocyte development and peripheral T cells

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Author
Coakley, Kristen M.

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Regulation of RasGRP1 protein in thymocyte development and peripheral T cells

by

Kristen M. Coakley

DISSERTATION

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DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO
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by
Kristen M. Coakley
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Abstract

Lymphocytes express two Ras guanine exchange factors (RasGEFs), Sos and RasGRP1, which can act in concert to activate the Ras-MEK-ERK pathway downstream of antigen receptor signaling. RasGRP1 is capable of priming Sos and is required for optimal Sos activation. Sos is incapable of compensating for loss of RasGRP1 in lymphocytes as RasGRP1-deficient mice fail to develop single positive thymocytes. Conversely, overexpression of RasGRP1 in the thymus leads to the development of thymomas. Since RasGRP1 exists in this critical position between immunodeficiency and oncogenesis, it is important to understand the mechanisms underlying the regulation of RasGRP1 protein levels and activity.

My studies suggest that posttranslational modifications of RasGRP1 exist to limit RasGRP1 activity after antigen receptor stimulation. RasGRP1 appears to be monoubiquitinated and is phosphorylated in the basal state as well as upon TCR stimulation. Additionally, a hypomorphic allele of RasGRP1 (RasGRP1\textsuperscript{Anaef}) leads to relatively normal thymocyte development in the context of a wildtype TCR repertoire, but a modest impairment in positive selection is revealed when a transgenic TCR is used. Surprisingly, mice expressing this allele have appear to have phenotypically activated T cells, produce anti-nuclear antibodies and have hypercellular spleens by the age of 12 weeks. It appears that RasGRP1 may play a role in negatively regulating TCR signaling that was previously unrecognized. Thus, RasGRP1 must be properly regulated to prevent autoimmunity as well. Further work examining RasGRP1 protein function and regulation is required to understand the possibility and implications pharmacological targeting of this critical RasGEF in the disease state.
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CHAPTER 1

Introduction to T cell receptor (TCR) signaling during thymocyte development and peripheral T cell immune responses
Evolution of the adaptive immune system and T cell receptor generation

Since the beginning of life on Earth, there have been organisms that breach or usurp another organism’s defenses, profiting from their metabolism and body temperature, using their very being as a safe location for expansion and reproduction. As biologists, we have come to know them as parasites, viruses, and pathogenic bacteria. The evolutionary pressure to eliminate foreign invaders began early and persists to this day. Even some of the most basic of organisms have some sort of defensive mechanisms against invasion by foreign organisms (Cooper and Herrin 2006). Cells that developed in multicellular organisms devoted to this task were precursors to the modern day cell types that make up the “innate” immune system (Janeway and Medzhitov 2002). These cells use germline-encoded receptors capable of recognizing pathogen associated molecular patterns (PAMPs) or classes of antigens common to foreign bodies (Janeway and Medzhitov 2002).

However, as organisms coevolved with these pathogens, each developing mechanisms to evade the advances or defenses of the other, immune systems became more complex (Cooper and Herrin 2006; Lambrechts, Fellous et al. 2006). Evolution of the first sign of the “adaptive” immune system may have occurred as early as 500 million years ago with a chordate ancestor of both the jawless (agnathans) and jawed (gnathostomes) fishes as both groups appear to have evolved independent means of rearranging somatic genes to express unique cell surface receptors on two distinct subsets of immune cells (Cooper and Alder 2006). It is likely that mammalian mechanisms for rearranging somatic genes during lymphocyte development (using recombinase-activating gene, RAG) evolved
from that of the jawed fishes and were influenced by rounds of whole genome duplication and selective pressures from parasites (Flajnik and Kasahara 2010).

The adaptive immune system (beginning with cartilaginous fish and continuing with all living chordates: bony fish, amphibians, reptiles, birds and mammals) (Flajnik and Kasahara 2010) consists of two main types of hematopoietic cells: B cells and T cells. Both types of white blood cells originate from the common lymphoid progenitor cell in the bone marrow and begin development there. As maturation progresses, progenitor B cells remain in the bone marrow while progenitor T cells leave the bone marrow and travel through the blood stream to the thymus (Starr, Jameson et al. 2003; Zandi, Bryder et al. 2010). This multi-lobed organ, situated above the heart, contains many immune and endothelial cell types, all of which play a role in creating and maintaining the ideal environment for thymocyte development to occur (Rezzani, Bonomini et al. 2008; Dzhagalov and Phee 2011).

**Thymocyte development and life of a T cell**

Development of thymocytes into mature T cells proceeds in the thymus via a stepwise process with the ultimate “goal” being to produce a pool of T cells recognizing a broad range of peptide antigens that are also: 1) responsive to T cell receptor engagement by peptide loaded major histocompatibility complex (MHC) and 2) non-reactive to self antigen (Wang, Xiong et al. 2010).

The stages of thymocyte development can be easily tracked via their sequential expression of well-established cell surface markers (Fig. 1.1). Thymocytes begin development as CD4⁻ CD8⁻ double negative (DN) cells enter the thymus near the cortico-medullary junction (Starr, Jameson et al. 2003). Development of DN cells can be broken
Figure 1.1 – Thymocyte development in the thymus

Bone marrow progenitors (DN1: CD44+CD25−) enter the thymus from the blood stream (not pictured) at the cortico-medullary junction. These cells migrate outward toward the subcapsular epithelium and upregulate CD25 (DN2: CD44+CD25+). Upon reaching the subcapsular epithelium, they downregulate CD44 (DN3: CD44−CD25+). Successful pairing of TCRβ with an invariant pre-TCRα (β selection) results in signaling that downregulates CD25 expression (DN4: CD44−CD25−) and leads to the upregulation of both CD4 and CD8 (DP) and cell expansion. Upon production of a mature TCRβ/TCRα and successful recognition of MHC-peptide complex on medullary thymic epithelial cells (mTECs), CD4 or CD8 is downregulated (MHC I/II restriction dependent, respectively). These single positive T cells (CD4 SP and CD8 SP) exit the thymus via the lymphatics near the cortico-medullary junction (not pictured). This figure was modified from Starr et. al, Annual Reviews in Immunology, 2003.
Modified from Starr et al., Annual Reviews of Immunology 2003
down into 4 stages by the surface expression of additional cell surface markers, CD25 (also known as the IL-2 receptor) and CD44. The most immature cell type, DN1 cells (CD44⁺CD25⁻) begin migration toward the cortex and upregulate CD25 to become CD44⁺CD25⁺ DN2 cells (Ceredig and Rolink 2002). Upon reaching the subcapsular epithelium, these cells down regulate CD44 expression to become CD44⁻CD25⁺ DN3 cells (Ceredig and Rolink 2002). These cells are the first to rearrange and express a unique β chain coupled with a constant pre-TCR α chain (Palacios and Weiss 2007). Downregulation of CD25 occurs as thymocytes migrate back toward the medulla and the cells become CD44⁻CD25⁻ DN4 cells (Ceredig and Rolink 2002).

While the exact mechanism remains unclear, it is thought that interactions with cortical thymic epithelial cells (cTECs) and subsequent signaling from this pre-TCR complex allows the DN4 thymocyte to proceed to the next stage in development: CD4⁺ CD8⁺ double positive (DP) cells that rearrange and express a unique TCR α chain (Palacios and Weiss 2007). Successful rearrangement of an TCRα chain coupled to the existing TCRβ chain results in recognition of self-peptide-loaded MHC on cortical thymic epithelial cells (cTECs) (Starr, Jameson et al. 2003; Baldwin, Sandau et al. 2005). This process of positive selection ensures that thymocytes are capable of recognizing this protein complex on the surface of antigen presenting cells (APCs) and epithelial cells in the periphery during an immune reaction (reviewed in (Klein, Hinterberger et al. 2009). Remarkably, it is thought that the self-peptides loaded into the MHC complexes are generated via a thymus-specific proteosomal degradation process that allows for presentation of peptides different than those seen during negative selection in the medulla (Takahama, Nitta et al. 2010). At the same time or shortly thereafter, cells also undergo
negative selection. This process ensures that thymocytes that react too strongly to the MHC-self-peptide complex are deleted via apoptosis (reviewed in (Kroger, Flores et al. 2010). Medullary thymic epithelial cells (mTECs) express the transcription factor autoimmune regulator (AIRE) which allows for ectopic expression of normally tissue-specific self peptides found elsewhere in the body (e.g., insulin) (Anderson and Su 2011). It is hypothesized that cells that react too strongly at this stage are, or have the potential to become, self-reactive cells capable to responding to peripheral self-antigens and causing autoimmune disorders if allowed to proceed through development and into the periphery (Kroger, Flores et al. 2010). Differential self-peptide generation in different thymic epithelial subsets prevents peptide presentation redundancy and aids in the development of the most diverse TCR repertoire possible (Takahama, Nitta et al. 2010). Successful navigation through both of these checkpoints results in DP cells downregulating either their CD4 or CD8 surface expression and becoming mature CD4\(^+\)CD8\(^-\) or CD4\(^-\)CD8\(^+\) single positive (SP) T cells capable of exiting the thymus and patrolling the periphery (Starr, Jameson et al. 2003).

Once in the periphery, these T cells may meet the antigen their antigen receptor recognizes (e.g., loaded into an MHC molecule on an epithelial cell). This recognition will trigger a response that will allow the T cell to clonally expand, recruit other immune cells to the area and activate B cells to produce antibodies against that same intruder (Zhu and Paul 2010; Zhang and Bevan 2011; Zygmunt and Veldhoen 2011). Once the pathogen is cleared from the body, most of these expanded T cells are preferentially deleted via apoptosis, while some will remain in circulation as memory T cells capable of
faster and stronger recognition of the same pathogen (Youngblood, Davis et al. 2010; Pepper and Jenkins 2011).

**TCR Signaling**

All T cells express on their surface a T cell receptor (TCR) capable of recognizing a unique antigen sequence in the context of a major histocompatibility complex (MHC). T cells can be classified as either αβ T cells or γδ T cells based on the expression of specific components of surface T cell receptor (Kreslavsky, Gleimer et al. 2010). A mature, functional αβ TCR is a heterodimeric protein consisting of both a β chain and an α chain (Bonnet, Ferrier et al. 2009; Jouvin-Marche, Fuschiotti et al. 2009). The recognition pocket for peptide-loaded MHC is made up of portions of both chains and combinations of different α and β chains create additional layers of receptor diversity (Wucherpfennig, Gagnon et al. 2010).

The TCR is a transmembrane protein capable of transducing signal received from the extracellular domain to the intracellular space (Smith-Garvin, Koretzky et al. 2009). While is still unclear how this signal is actually transmitted to the intracellular space, it is thought that aggregation of TCRs at the surface along with conformational changes in the TCR itself or, more likely, in the associated CD3 chains and/or physical separation of the TCR complex from negative regulators of signaling (e.g. CD45) may work in concert to aid in this process (van der Merwe and Dushek 2011). What does seem clear is that TCR-associated CD3 contains the signaling ITAMs to which downstream proteins can bind and amplify the signaling cascade in a sensitive manner (Guy and Vignali 2009).
Recognition of its cognate peptide-MHC complex by TCR and coreceptor binding (such as CD4) leads to the activation of Src family kinases (Lck), which phosphorylate the SH2 domains on CD3 (Fig. 1.2a). These phosphorylated SH2 domains act as docking sites for proteins like Syk-family kinase member, Zap-70. Zap-70 phosphorylates Slp-76 and SH2 domains on the linker for activated T cells (LAT) (Au-Yeung, Deindl et al. 2009). The LAT scaffolding protein acts as a docking site for many signaling molecules capable of activating a multitude of signaling pathways including the Ras signaling pathway (Fuller and Zhang 2009). Phosphorylated PLCγ binds to phosphorylated Y136 of LAT and converts membrane component phosphotidylinositol bisphosphate (PIP2) to diacylglycerol (DAG) and inositol triphosphate (IP3) (Sommers, Lee et al. 2005). The generation of these second messengers is crucial for the activation of multiple signaling pathways (Kabouridis 2006; Oh-hora 2009) including calcium-calcineurin and Ras-Mek-Erk cascades described in further detail below.

**Calcium Signaling in T lymphocytes**

Activation of PLCγ leads to the production of the second messenger, IP3. This small molecule binds to IP3 receptors on the endoplasmic reticulum (ER) (Fig. 1.2b) (Lewis 2001). Binding of IP3 to its receptor leads to the release of ER calcium stores into the cytoplasm (Lewis 2001). A transmembrane calcium sensor in the ER membrane, stromal interaction molecule-1 (STIM-1) is activated by the depletion of ER calcium stores (Liou, Kim et al. 2005). STIM-1 interacts with the Orai1 subunit of calcium-release-activated calcium (CRAC) channels at the cell membrane (Hogan, Lewis et al. 2009). This interaction results in CRAC channel opening and calcium from outside the cell moves
Figure 1.2 – T cell signaling downstream of the T cell receptor (TCR)

(a) Upon recognition of its cognate peptide/MHC complex, combined with co-receptor binding (CD4 is shown), Src family kinase Lck phosphorylates ITAMs on CD3 and TCRζ chains. This leads to recruitment of the Syk family kinase Zap-70 which phosphorylates SLP76 (not shown) and the linker of activated T cells (LAT). The LAT adaptor protein acts as a docking station for many additional proteins (not all shown), including PLCγ. Upon binding to LAT, PLCγ converts PIP2 at the plasma membrane into the second messengers, inositol triphosphate (IP3) and diacylglycerol (DAG). Effects of these molecules are detailed in parts (b) and (c), respectively. (b) IP3 binds to IP3 receptors that release calcium from the endoplasmic reticulum. In response to the lower calcium levels in the ER, stromal interaction molecule-1 (STIM1) activates calcium-release-activated calcium (CRAC) channels at the cell membrane. The resulting influx of calcium activates multiple proteins including calmodulin and calcineurin. Activated calcineurin dephosphorylates the transcription factor, NFAT, which can then translocate into the nucleus to fulfill its effector function. (c) DAG directly recruits both PKCs and the RasGEF, RasGRP1. PKC phosphorylates RasGRP1 on T184. This increases RasGRP1’s RasGEF ability. The active RasGTP produced binds to the allosteric pocket of the other RasGEF, Sos. This binding increases Sos’s RasGEF activity 75 fold, generating a large pool of Ras capable of activating many pathways including the Raf-MEK-ERK phosphorylation cascade. Ultimately, the activation of Erk leads to the translocation of AP-1 to the nucleus. AP-1 pairs with NFAT to contribute to changes in gene transcription including the production of IL-2.
down the calcium concentration gradient into the cell (Oh-hora 2009). Calcium then binds to, and activates, several calcium-regulated proteins including PKCs and calmodulin (Feske 2007). Binding of calcium to calmodulin results in a conformational change crucial to its activation (Hogan, Chen et al. 2003). Calmodulin activates the phosphatase calcineurin, which dephosphorylates the transcription factor, NFAT (Gwack, Feske et al. 2007). This dephosphorylation event allows NFAT to migrate into the nucleus where it can bind to DNA and contribute to changes in gene transcription including the production of IL-2 (Hogan, Chen et al. 2003).

**Ras Activation in T lymphocytes**

Ras guanine exchange factors (RasGEFs) are responsible for Ras activation as Ras cannot exchange GDP for GTP without them (Bos, Rehmann et al. 2007). RasGEFs activate Ras by binding with their GEF pocket to inactive RasGDP and forcing the dissociation of the GDP molecule so that empty Ras can bind the abundant molecule, GTP, to form an active RasGTP molecule. RasGEFs are recruited via different mechanisms to the membrane where they can activate Ras (Fig. 1.2c). Interestingly, lymphocytes express two types of RasGEFs: Ras guanyl releasing factor (RasGRP) and the *son of sevenless* homologue (SOS) (Ebinu, Stang et al. 2000). Early TCR signaling events such as phosphorylation of the adapter LAT by Src and Syk family kinases is required for functional RasGEF localization (Weiss and Littman 1994) (Fig. 1.2a). SOS is recruited to phosphorylated LAT via binding with Grb2 (Roose and Weiss 2000). RasGRP1 is recruited via the second messenger diacylglycerol (DAG) produced by LAT-bound activated PLCγ (Roose and Weiss 2000) (Fig 1.2c). RasGRP1 converts the inactive form
of Ras (RasGDP) to the active form (RasGTP). RasGTP can bind to a second pocket for Ras in Sos, the allosteric pocket, this binding event increases Sos’ RasGEF activity (Margarit, Sondermann et al. 2003). This amplification of the Ras signal leads to the activation of many downstream effector pathways including the Ras-Raf-Mek-Erk cascade. Activation of this pathway causes the translocation of the transcription factor, AP-1 (Macian, Lopez-Rodriguez et al. 2001). AP-1 can pair with NFAT to regulate the expression of many genes (Macian, Lopez-Rodriguez et al. 2001).

**RasGRP1 as a key player in Ras activation in T cells**

RasGRP1 is a dominant RasGEF functioning (1) as a trigger for Ras signaling and (2) as a catalyst for SOS function in T lymphocytes (Roose, Mollenauer et al. 2007). The RasGRP1 knockout mouse experiences a block in T cell development at positive selection, indicating that expression of SOS cannot compensate for a loss of RasGRP1 expression (Dower, Stang et al. 2000). The dominance of RasGRP1 is also supported by cellular experiments that show that direct targeting of SOS to the membrane, previously shown to be sufficient for Ras activation (Brose, Betz et al. 2004), is dependent on intact RasGRP1 function (Roose, Mollenauer et al. 2005). This observation was not completely understood until the crystal structure of Sos was solved.

Elucidation of the crystal structure of Sos by the Kuriyan group showed that this protein not only contains a classical RasGEF pocket but also a second pocket capable of binding Ras (Margarit, Sondermann et al. 2003). This second pocket was termed an allosteric pocket because when it binds an active RasGTP molecule, an allosteric change occurs, increasing Sos RasGEF activity 75 fold (Margarit, Sondermann et al. 2003). This
discovery revealed the interesting conundrum that the product of Sos activity (RasGTP) was required for optimal activation of Sos. Previous work by Jeroen Roose has elucidated important crosstalk between these two RasGEFs in lymphocytes (Roose, Mollenauer et al. 2007).

Because lymphocytes express multiple RasGEFs that can communicate with one another to coordinate signaling, these cells are able to control the activation of Ras in a precise manner. Activation of RasGRP1 through direct recruitment to the PLCγ-induced DAG at the membrane, allows the cell to generate a small initial pool of RasGTP (Fig. 1.3a) (Roose, Mollenauer et al. 2007). Once generated, this RasGTP can bind to the allosteric pocket of Sos and increase its RasGEF activity (Margarit, Sondermann et al. 2003). Thus, SOS has the potential to be a powerful RasGEF but it requires priming by RasGTP, which can be provided by RasGRP1. This crosstalk allows the cell to turn a “linear” input of signal via the TCR engagement with MHC-peptide (one MHC-peptide complex interacts with one TCR at a time) into “digital” output via Ras-MEK-Erk pathway (generation of large amounts of RasGTP amplifies the effect into multiple pathways) (Fig. 1.3b). This interaction results in the creation of an on/off switch for the rapid and robust activation of T lymphocytes capable of differentiating between a “weak” and “strong” TCR engagement with relatively few, if any, cells acting at an “intermediate” state.

However, biology is not an exact science. Many of the events described in the life cycle of a T cell can go wrong. Recent research abounds with hypotheses and methods by which T cells can, in fact, contribute to pathogenesis of disease in addition to their roles
Figure 1.3 – Crosstalk between two RasGEFs in lymphocytes

(a) Example of proposed signaling events downstream of “weak” TCR signaling in the absence of Sos activation. In this scenario, the linear input given through the engagement of TCR results in analog increases in the Ras-Erk activation (bottom histograms).  

(b) Example of proposed signaling events downstream of “strong” TCR signaling which results in the recruitment and activation of a second RasGEF, Sos. Binding of active RasGTP to Sos increases RasGEF activity and greatly amplifies the signal. In this scenario, the linear input received through the TCR is translated into digital increases (Off/On) in the Ras-Erk activation (bottom histograms). Figure courtesy of Jeroen P. Roose.
Figure courtesy of Jeroen P. Roose
in the resolution of others (Goverman 2011; Otsuki, Hayashi et al. 2011; Roep and Peakman 2011). Rapid expansion of T cells during an immune response is remarkably similar to the rapid expansion of cells during leukemia/lymphoma. What controls this expansion and prevents it from going out of control? Humans are constantly reacting against pathogens, expanding and contracting their T cell pools, but they develop T cell lymphomas only rarely (Graux 2011). Conversely, genetic mutations exist that perturb the normal development of the immune system and lead to immune deficiencies and impair immune responses to pathogens (Kersseboom, Brooks et al. 2011; Rezaei, Hedayat et al. 2011). Remarkably, there are even those mutations that lead to initial lymphopenia followed by aberrant expansion of T cells and subsequent autoimmune syndromes (Liston, Enders et al. 2008). In order to understand these sorts of biomedical phenomena, it is necessary to develop a deeper understanding of the T cell and the underlying mechanisms that allow for the controlled development and expansion of this cell type.
CHAPTER 2

Posttranslational modification and degradation of RasGRP1 protein
in T lymphocytes
Summary

T cell acute lymphoblastic leukemia/lymphoma (TALL/L) involves the abnormal proliferation of cells from the lymphocyte compartment. Patients with TALL/L often show elevated levels of the active form of the Ras oncoprotein, RasGTP, in peripheral blood and bone marrow cells. However, relatively few of these TALL/L patients have the typical somatic activating mutations in Ras itself that would explain the accumulation of RasGTP. This finding suggests that many TALL/L patients may have abnormal expression or activity of signaling proteins upstream of RasGTP. Correspondingly, transgenic mice overexpressing wild type RasGRP1 using an exogenous promoter develop thymomas and skin carcinomas. Thus, non-cancerous cells must have a mechanism to limit RasGRP1 function.

Surprisingly, the negative regulatory factors that turn off this strong, RasGRP1-driven proliferative signal and prevent uncontrolled proliferation like that seen in TALL/L patients are unknown. Decreases in RasGRP1 protein level after T cell receptor (TCR) stimulation clearly precedes more delayed RasGRP1 transcript degradation. Differential binding of antibodies specific for phosphorylation consensus sites and ubiquitin suggest posttranslational modification of RasGRP1. RasGRP1 is a relatively stable protein in the basal state but its half-life decreases dramatically after crosslinking of T cell receptors. This decrease in half-life seems dependent on signals in addition to those mediated by diacylglycerol, as calcium seems to play a synergistic role. Thus it appears that posttranslational modifications of RasGRP1 play a role in negative regulation of this dominant RasGEF.
Introduction

Isoforms of Ras (K-, N-, and H-Ras) and the activating mutations that can occur in these proteins are subject to much study in the oncology (Bos 1989; Omerovic, Laude et al. 2007) and developmental (Schubbert, Shannon et al. 2007) fields. Somatic, activating mutations in Ras proteins generally obliterate the intrinsic GTPase activity of Ras and result in an accumulation of RasGTP within the cell. Different cancer types display varying frequencies of Ras activating mutations. For example, nearly 90% of pancreatic cancers contain an activating mutation in the K-Ras protein (Friday and Adjei 2005). This is in stark contrast to the mere 10% of T cell acute lymphoblastic leukemia/lymphomas (TALL/L) that have been shown to contain a K-Ras or N-Ras activating mutation (Ahuja, Foti et al. 1990; Yokota, Nakao et al. 1998; Wiemels, Zhang et al. 2005). Interestingly, 50% of patients with TALL/L are seen with elevated levels of RasGTP in peripheral blood and bone marrow cells (von Lintig, Huvar et al. 2000). These numbers suggest that up to 40% of patients diagnosed with TALL/L may have mutations in or aberrant expression of signaling components that lead to enhanced GTP-loading of Ras or decreased hydrolysis of RasGTP to RasGDP.

T cells and Ras Signaling

As described in Chapter 1, cells utilize intracellular signal transduction pathways to transmit extracellular signals to the nucleus in order to influence gene expression and elicit changes in cell biology. Specifically, T lymphocytes recognize pathogenic invasion through the T cell receptor (TCR) and are required to rapidly divide in response to the invasion. Ras GTPases are crucial in this process. Membrane-bound Ras cycles between
inactive (GDP-bound) and active (GTP-bound) states and functions as a switch. The Ras cycle is controlled by 1) Ras guanine nucleotide exchange factors (RasGEFs) that displace bound GDP to allow for GTP binding and by 2) RasGTPase activating proteins (RasGAPs) that aid in the hydrolysis of GTP to GDP (Genot and Cantrell 2000). Once in the active state, RasGTP binds to and recruits effectors to transmit signals to multiple effector pathways, including the RAF-MEK-ERK pathway, which results in transcription of genes, e.g. those that drive cell division (Katz and McCormick 1997; Campbell, Khosravi-Far et al. 1998). Lymphocytes, in particular, are known to induce Ras activation in a robust and sensitive manner following antigen receptor engagement (Roose, Mollenauer et al. 2007). To accomplish this in a controlled manner, T cells express two Ras guanine nucleotide exchange factors (RasGEFs), SOS and RasGRP1 as well as many negative regulators including the Ras-GTPase activating proteins (RasGAPs), p120 and CAPRI (Lapinski, Qiao et al.; Lockyer, Kupzig et al. 2001; Vigil, Cherfils et al. 2010). Previous work has demonstrated that RasGRP1 and SOS work in a coordinated positive feedback loop that leads to the accumulation of RasGTP and eventually induces T cell proliferation (Roose, Mollenauer et al. 2007) (this is also reviewed in Chapter 1).

**Transcriptional and Posttranslational Regulation of RasGRP1**

Expression and activity of proteins can be controlled on many levels within the cell. Regulation of transcription factors can control whether or not a gene is translated (Alberts, Johnson et al. 2002). Regulation of mRNA transcript can control whether or not a particular mRNA is transcribed into protein (Alberts, Johnson et al. 2002). And finally, posttranslational modifications, compartmentalization and/or degradation can
control protein activity (Alberts, Johnson et al. 2002). What is currently known about RasGRP1 RNA and protein regulation is described in detail below.

**Regulation via control of RasGRP1 mRNA**

While this was not the focus of my work, it is important to also give a brief overview about what is known about transcriptional regulation of RasGRP1 as this control does contribute to the regulation of RasGRP1 activity. Messenger RNA level can be controlled through suppression of transcription factor activity and/or through degradation of existing mRNA, often through microRNA binding to the 3’UTR. Interestingly, upon TCR stimulation of primary T cells for 72 hours, RasGRP1 mRNA transcript levels are significantly reduced (Diehn, Alizadeh et al. 2002). Unpublished data from the Roose lab shows that, in both Jurkat cells and lymphoma cell lines from the Matthias Wabl screen, RasGRP1 transcript levels are reduced after 24 hours of either PMA (a chemical diacylglycerol mimetic) or TCR stimulation. Both stimuli induce the same level of transcript degradation, suggesting that RasGRP1 mRNA levels are indeed negatively regulated in T cells. Since the diacylglycerol (DAG) signal is sufficient (as evidenced by the results following the addition of PMA alone), this mRNA regulation appears to be calcium independent. As far as we know, no careful time course analysis of mRNA expression after stimulation of T cells has been performed.

The transcription factor responsible for RasGRP1 transcription is unknown, but support of 3’UTR regulation of RasGRP1 mRNA comes from the retroviral insertional mutagenesis screen performed by Matthias Wabl’s lab (unpublished, personal communication). One lymphoma contained retroviral insertion in the 3’UTR of *rasGRP1*
(Fig. 2.1a). The 3’ UTR of rasGRP1 contains a putative binding site for the microRNA Mir21 that may be affected by this retroviral insertion and further work in the Roose lab will seek to determine if this is the cause of the lymphoma.

The mRNA for rasGRP1 is also thought to contain an alternate “START” ATG (methionine) sequence (Fig. 2.1a). Use of this site results in a protein product that is approximately 4kD smaller (86kD) than the full-length (90kD) RasGRP1 protein (Poon and Stone 2009). It is currently unknown if specific cell contexts cause the mRNA to be preferentially read starting at the alternate START ATG site. Observation in the Roose lab has seen equal expression of both forms of RasGRP1 protein in T lymphocytes and the protein appears as a doublet on a Western blot when certain antibodies are used. In support of the existence of isoform preference in T cells under specific circumstances, recent studies of lupus patients have uncovered preferential expression of an 86kD version of RasGRP1 in affected individuals that is thought to occur through alternative splicing of mRNA, specifically the excision of exon 11 (Yasuda, Stevens et al. 2007).

**Regulation via control of RasGRP1 protein localization**

Proteins can be regulated via a variety of mechanisms including recruitment/sequestration and posttranslational modification. As previously mentioned, RasGRP1 is directly recruited to patches of diacylglycerol in membranes. This recruitment occurs via RasGRP1’s C terminal C1 domain (Fig. 2.1b) (Ebinu, Stang et al. 2000). The exact location of this recruitment remains unclear. Work performed by Mark Philips’ group using DT40 B cells suggests that the majority of RasGRP1 activity occurs at the Golgi
Figure 2.1 – RasGRP1 regulatory domains

(a) Diagram of the rasgrp1 locus. RNA splices between exons are shown in grey. rasgrp1 contains one alternate start ATG in the first exon (red). The 3’ untranslated region (UTR) contains a putative binding site for the regulatory microRNA, MiR21 (blue). Locations and frequencies of retroviral insertion sites uncovered during an insertional mutagenesis screen performed by the Mathias Wabl lab are shown (black arrows). (b) Diagram of RasGRP1 protein. Together the Ras Exchange Motif (REM, yellow) and Cdc25 domain (purple) form the catalytic domain. The Cdc25 motif contains two PKC phosphorylation sites (T184, blue; S198, pink). The EF hands (orange) are thought to be involved in binding of calcium and protein localization. The C1 domain (green) binds DAG contained in cell membranes. At the C-terminus, there is a pair of alpha helices that are predicted to form a coiled-coil domain (red). This domain may enable RasGRP1 to form dimers or bind additional proteins that contain similar domains.
and ER and is not seen at the plasma membrane (Bivona, Perez De Castro et al. 2003; Perez de Castro, Bivona et al. 2004). A major caveat to this localization work (and that presented in (Tazmini, Beaulieu et al. 2009)) is that this RasGRP1 activity is only seen in DT40 B cells when Ras is simultaneously overexpressed, thus large amounts of localization at the Golgi could be due to the large amount of plasmid-encoded protein being produced. As the plasma membrane seems to be the primary site of TCR signaling activity in the majority of T cells (Sykulev 2010), it seems likely RasGRP1 activity would occur here as well. Indeed, recent work by Ignatio Rubio was able to document Ras activity at the plasma membranes of T cells using a system that did not require Ras overexpression (Rubio, Grund et al. 2010).

RasGRP1 also contains 2 EF hands (Fig. 2.1b) (Ebinu, Bottorff et al. 1998). In other proteins, these motifs are generally thought to induce conformational change following calcium binding. Early in vitro work demonstrated that purified RasGRP1 protein was capable of binding Ca\(^{++}\) ions (Ebinu, Bottorff et al. 1998). Binding of Ca to EF hand 2 enhanced the binding of calcium to EF hand 1 (Ebinu, Bottorff et al. 1998). Work in the Kay lab found that EF hand 1, but not EF hand 2, was required for localization to the Golgi to occur (Tazmini, Beaulieu et al. 2009) although this work was also performed in DT40 cells in the presence of Ras overexpression. My own work on the function of EF hands in RasGRP1 protein activity will be discussed in Chapter 3.

**Regulation via control of RasGRP1 protein-protein interaction**

RasGRP1 has been shown to interact with multiple proteins in vivo. Specifically, activation of RasGRP1 (and the entire Ras activation pathway) seems to require the
RhoGEF, Vav. This interaction was first shown in DT40 B cells and was shown to require PLC\(\gamma\) with F-actin playing an important supporting role (Caloca, Zugaza et al. 2003). Further work with Vav\(^{-}\) thymocytes showed that Vav’s effect on RasGRP1 activity, and Ras activation in general, seemed to be related to defective LAT phosphorylation and subsequent reduction in PLC\(\gamma\) recruitment (Reynolds, de Bettignies et al. 2004).

Work performed in Jurkat cells using overexpression of the adaptor protein, SKAP55, showed an augmentation of TCR signaling after stimulation when the protein was expressed at 0.5-2.0 times the endogenous protein amount (Kosco, Cerignoli et al. 2008). The augmentation resulted in an increase in stimulus-induced P-Erk production and increases in reporter gene expression (luciferase under the AP-1 transcription factor or interleukin 2 promoter) were observed (Kosco, Cerignoli et al. 2008). In contrast, larger amounts of SKAP55 overexpression led to inhibition of Ras-Erk signaling that could be rescued using constitutively active forms of Ras (Kosco, Cerignoli et al. 2008). The authors hypothesized that excessive amounts of SKAP55 in the cell would disrupt the formation of a normal protein complex important in Ras signaling. The search for a protein binding partner that affected Ras signaling in T cells led to the discovery that RasGRP1 directly binds the C-terminus SKAP55 (Kosco, Cerignoli et al. 2008). A second group has confirmed that the C-terminus of SKAP55 can bind RasGRP1 directly and argue that this interaction may serve to limit RasGRP1 activity downstream of LFA-1 mediated integrin signaling (Schneider, Wang et al. 2008). In light of the strict dosage requirements seen in the first study, and the possible differences in function downstream
of different transmembrane molecules, the inhibitory/aumentation effects of SKAP55 binding to RasGRP1 may be difficult to fully elucidate.

Lastly, collaboration between the Roose Lab and the Kuriyan group at UC Berkeley has revealed that RasGRP1 may contain a C-terminal coiled-coil domain (Fig. 2.1b). This domain appears conserved across species but it not present in other RasGRP family members (unpublished). Work is ongoing to determine if RasGRP1 forms dimeric complexes with itself or other proteins. Formation of RasGRP1 homodimers would also explain the previously documented phenomenon of weakened RasGRP1 alleles acting in a dominant negative fashion when reconstituted into a RasGRP1 deficient cell line (ΔDAG-JPRM-441) that still contains 10% wildtype RasGRP1 protein (Roose, Mollenauer et al. 2005).

**Regulation via control of RasGRP1 protein activity**

Posttranslational modifications, such as phosphorylation, can act to enhance or inhibit protein activity. RasGRP1 is phosphorylated on threonine 184 (T184) (Fig. 2.1b). This phosphorylation event increases RasGRP1 GEF activity (Zheng, Liu et al. 2005; Stone 2011). This phosphorylation event is reduced in the presence of a DAG-responsive novel PKC inhibitor, rottlerin (Roose, Mollenauer et al. 2005). While it is not clear which PKC phosphorylates RasGRP1 in T cells, it is suggested that another phosphorylation event on RasGRP1 occurs in B cells via PKCδ (Limnander, Depeille et al. 2010).

In addition to the activity-enhancing phosphorylation of T184, RasGRP1 appears to be phosphorylated and/or modified in other ways on additional residues. Firstly, RasGRP1 migrates as a higher molecular weight species on an SDS PAGE gel in stimulated versus
unstimulated Jurkat cells (Jereon Roose, unpublished). Mutation of T184 to alanine (T184A) prevents phosphorylation of T184 and reduces the activity of RasGRP1. However, the RasGRP1-T184A species runs as a higher molecular weight species than the wildtype (wt) version on a reduced SDS PAGE gel, suggesting additional modifications on RasGRP1 that are not on T184 (Jeroen Roose, unpublished). Mutation of another predicted PKC phosphorylation site to alanine, S198A, does not change mobility but results in increased phosphorylation of RasGRP1 detected with a phospho-serine (PKC substrate) antibody (Jeroen Roose, unpublished). However, both wt and S198A RasGRP1 species demonstrate a similar level of phosphorylation when a third phospho-specific antibody, phospho-Ser/Thr (Akt substrate) is used. Therefore, while it is clear that RasGRP1 is phosphorylated and these posttranslational modifications can affect protein activity, there has been no formal, comprehensive study of RasGRP1 phosphorylation sites and the mechanisms by which they control RasGRP1 function and stability.

**RasGRP1 and oncogenesis**

Since RasGRP1 seems poised at the beginning of a positive feedback loop leading to the activation of Ras (and subsequent cell proliferation), it is not surprising that transgenic RasGRP1 mouse models are prone to developing tumors. Mice overexpressing RasGRP1 using the keratin 5 promoter in epidermal cells developed spontaneous skin papillomas (Oki-Idouchi and Lorenzo 2007) which were more likely to progress into larger carcinomas (compared to wild type mice) when treated with carcinogenic agents (Luke, Oki-Idouchi et al. 2007). Development of cutaneous squamous cell carcinomas in
response to skin wounding in these mice was also RasGRP1 transgene dosage dependent (Diez, Garrido et al. 2009). Mice with a transgenic overexpression of RasGRP1 in thymocytes develop thymic lymphomas independent of TCR signaling (Klinger, Guilbault et al. 2005). Consistent with this finding, a recent T cell retroviral insertional mutagenesis screen performed by Matthias Wabl indicates that an insertion in RasGRP1 (Fig. 2.1a) is present in 237 of 3000 T cell lymphomas analyzed, making it the fourth most common insertion site uncovered in this in vivo mouse screen (Hartzell In Revision). Interestingly, only one insertion was found in another RasGRP family member, RasGRP4. No insertions were found in SOS1, SOS2, RasGRP2 or RasGRP3.

Given that T lymphocytes potently activate Ras through RasGRP1 during both thymic development and peripheral immune responses but generally do not become cancerous, these cells must have a mechanism in place to limit RasGRP1 activity. Conversely, aberrant, or loss, of RasGRP1 regulation resulting in elevated or prolonged activity could very well lead to a lymphoproliferative phenotype. Remarkably, little work has been done to examine how T cells turn off this robust proliferative signal and thereby prevent aberrantly dividing cells akin to those seen in TALL/L patients.
Results

**Differential binding of antibodies suggests additional posttranslational modifications on RasGRP1**

To confirm the previously described differences in binding of phospho-antibodies against common consensus sites, I repeated immunoprecipitations of RasGRP1 from Jurkat cells stimulated with TCR crosslinking antibodies (Fig. 2.2a). Binding of phosphorylated PKC consensus site (p-S/T) antibody increased dramatically after 5 minutes of stimulus. This is likely representative of the known PKC-mediated phosphorylation event on T184 in RasGRP1. However, I cannot be certain that multiple phosphorylated PKC consensus sites are being recognized. In contrast, binding of Akt consensus site (p-S) antibody was elevated in the basal site, slightly increased at the 5 minute time point and back to basal levels by 10 minutes (Fig. 2.2a). This binding pattern is suggestive of additional phosphorylation sites that may exist on RasGRP1 at the basal state and that this event appears to preferential occur on one RasGRP1 isoform (presumably the 90kD, although aligning of different blots was not conclusive).

Interestingly, it appears that RasGRP1 may also be ubiquitinated. Ubiquitin can use its own lysines (K) 48 or 63 to form either multimeric ubiquitin chains or single ubiquitins attached to lysines present on the target protein (Dikic, Wakatsuki et al. 2009). Although exceptions continue to be discovered, it is thought that utilization of K48 to form chains leads to the proteosome-mediated degradation of the target protein (Dikic, Wakatsuki et al. 2009). Alternatively, usage of K63 is thought occur singly and to act as a subcellular localization signal (Chen and Sun 2009). An antibody recognizing both types of
Figure 2.2 – Evidence of posttranslational modifications to RasGRP1 protein

(a) Jurkat cells were stimulated using TCR crosslinking antibodies for the times indicated. RasGRP1 protein was immunoprecipitated (antibody clone E80) and resulting purified protein was probed with antibodies recognizing phosphorylated PKC consensus site (p-S/T), phosphorylated Akt consensus site (p-T), pan-ubiquitin (K48 and K63 linkages), K63 linkage ubiquitin only and total RasGRP1 protein (antibody clone E160).

(b) Scansite predictions for PKC serine/threonine phosphorylation sites in RasGRP1 protein with confidence scores (rightmost columns). Sites conserved between human and mouse are shown with conserved sequence in bold. Most sites fall within the catalytic region, with one present in the second EF hand. Scansite also predicted the known phosphorylation event (T184) in the catalytic region with similar confidence (Score human/Score mouse columns).
a

Jurkat
IP: E80
(RasGRP1)

+αTCR
Time (min):
0  5  10  30

WB:
PKC consensus
Akt consensus
pan ubiquitin
K63 linkage ubiquitin
E160 (RasGRP1)

b

Scansite
Predicted serine and threonine phosphorylation sites:

<table>
<thead>
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<th>Score human</th>
<th>Score mouse</th>
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ubiquitin attachment shows increased binding to RasGRP1 after 5 minutes of stimulation (Fig. 2.2a). This pattern mimics that seen using a ubiquitin antibody that specifically recognizes K63 linkage. Therefore, ubiquitinated RasGRP1 may be trafficked to another compartment after stimulation through the T cell receptor. Interestingly, it appears that both forms of RasGRP1 protein (86kD and 90kD) appear to be ubiquitinated.

To predict which sites may be phosphorylated in RasGRP1, we utilized Scansite, a computer software program capable of recognizing kinase consensus sites. Scansite identified 11 conserved serines in RasGRP1 that fit a consensus motif for PKC kinases (Fig. 2.2b). These sites received high confidence scores (greater than 0.9) and were highly conserved between mouse and human RasGRP1 amino acid sequence. Attempts at direct identification of phosphorylated/ubiquitinated residues via Mass Spec will be discussed in the Discussion Section of this chapter.

**RasGRP1 protein is relatively stable in the absence of stimulus**

Posttranslational modifications (phosphorylation/ubiquitination, etc.) can lead to protein instability or turnover (Okiyoneda, Apaja et al.). Since regulation of RasGRP1 mRNA is incompletely understood and cells are capable of constantly producing new protein, it is not sufficient to use normal western blot technique to investigate basal protein turnover. Instead, it is necessary to radiolabel individual proteins in order to follow these same proteins over time (Bonifacino 2001). To examine the basal stability of RasGRP1 protein (protein half-life), I metabolically labeled Jurkat cells or 1156O mouse lymphoma cells using Translabel reagent (a mixture of $^{35}$S-cysteine and $^{35}$S-methionine). After 20 minutes, cells were diluted using culture medium supplemented with unlabeled cysteine.
and methionine (5mM each). Most RasGRP1 protein made during the Translabel incubation period incorporate at least some radioactive methionine/cysteines. An aliquot of cells was lysed at the indicated time points (Fig. 2.3a). RasGRP1 was immunoprecipitated from the lysates and run on a gel. After drying, the gel was exposed to a phosphoimaging screen for 18 hours. Since the same radiolabeled protein was still present after four hours of incubation with unlabeled amino acids, RasGRP1 appears to be a relatively stable protein with little turnover in the basal state. The loss of RasGRP1 after the 20 hours is likely due to the observed massive cell death after a long incubation in the acidic amino acid supplemented culture media.

To examine RasGRP1 turnover without the use of radioisotopes, I used an inhibitor of protein biosynthesis, cyclohexamide (Fig. 2.3b and 2.3c). Incubation with this inhibitor for 30 minutes (the 0 minutes time point) did not decrease the total amount of RasGRP1 protein in the cell. Taking additional time points out to 2 hours did not see a decrease in the amount of protein regardless of treatment (Fig 2.3b). This supports the idea that in the basal cell state, RasGRP1 protein is turned over at a slow rate.

**Stimulus decreases the half-life of RasGRP1 protein**

To further examine this apparent degradation of RasGRP1 protein, cells labeled with Translabel were washed and stimulated using TCR crosslinking antibodies or the diacylglycerol mimetic, PMA. As before, RasGRP1 was immunoprecipitated from lysates and the gel was exposed to a phosphoimaging screen for 18 hours (Fig. 2.4). After TCR stimulation, radiolabeled RasGRP1 protein was rapidly degraded by 20 minutes. In contrast, radiolabeled RasGRP1 protein in cells stimulated using PMA or
Figure 2.3 – RasGRP1 protein is relatively stable in cells at the basal state

(a) Jurkat cells (human) and 1156O cells (mouse, overexpressing RasGRP1 protein) were metabolically labeled with $^{35}$S cysteine and methionine for 20 minutes. Radioactive label was diluted out with media containing 5mM unlabeled cysteine and methionine. Post-washout, cells were lysed at times indicated, lysates were cleared and RasGRP1 protein was immunoprecipitated. Dried gel was exposed on a phosphoimager screen for 18 hours. (b) Wildtype thymocytes were pre-incubated with cyclohexamide (inhibitor of biosynthesis) for 30 minutes or left untreated (PBS). Lysates were taken at time points indicated. Note the presence of a similar amount of protein for both treatments. (c) 1156O cells were pre-incubated with cyclohexamide (inhibitor of biosynthesis) for 30 minutes. Note the presence of a similar amount of protein in the 0 time point lane for both treatments. Cells were stimulated with antiTCR crosslinking antibodies.
a

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<th>20'</th>
<th>2h</th>
<th>4h</th>
<th>20h</th>
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Jurkat

1156O

stimulus: none

b

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<tr>
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<th>30</th>
<th>60</th>
<th>120</th>
<th>0</th>
<th>5</th>
<th>30</th>
<th>60</th>
<th>120</th>
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</table>

WB: RasGRP1

cells: thymocytes
stimulus: none

+cyclohexamide


c

<table>
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<tr>
<th>Time (min):</th>
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<th>5</th>
<th>30</th>
<th>60</th>
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</table>

WB: RasGRP1

cells: 1156O
stimulus: αTCR/αCD4

+cyclohexamide
Figure 2.4 – RasGRP1 protein is actively degraded after TCR stimulus

1156O mouse lymphoma cells were metabolically labeled with $\text{S}^{35}$ cysteine/methionine for 20 minutes. Cells were washed 3 times with label-free medium and rested for 30 minutes. Cells were stimulated with PMA, TCR crosslinking antibody, or warm PBS and lysed at times indicated. Lysates were cleared and RasGRP1 protein was immunoprecipitated. Dried gel was exposed on a phosphoimaging screen for 18 hours.
<table>
<thead>
<tr>
<th>Time of stimulus (min):</th>
<th>0</th>
<th>2</th>
<th>5</th>
<th>20</th>
<th>60</th>
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</thead>
<tbody>
<tr>
<td>αTCR/αCD4</td>
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<tr>
<td>PMA</td>
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<tr>
<td>PBS (no stimulus)</td>
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mock stimulated with PBS was not degraded until 60 minutes after stimulation. What is most important to note is the increase in the rate of degradation in the TCR stimulated samples relative to the PMA treated and mock stimulation samples. These differences lead to the hypothesis that stimulus through the TCR leads to rapid, active degradation of RasGRP1 protein. Intriguingly, the addition of TCR crosslinking antibodies (additional time points in Fig. 2.3c) resulted in the rapid disappearance of the RasGRP1 protein band although the rate was not increased in the presence of cyclohexamide. This decrease in protein could be due to either active protein degradation or simple inhibition of mRNA levels/translation combined with passive protein turnover. Given that RasGRP1 protein levels appear stable at the basal state, it is likely that this observation is due to active protein degradation.

**Decrease in RasGRP1 protein after stimulus is degradation**

Using the same RasGRP1 overexpressing lymphoma cell line (1156O), I observed that RasGRP1 protein is degraded in response to TCR stimulation (“DAG+ calcium”) but not to in response to the DAG-mimetic PMA (“DAG” only) (Fig. 2.5a), even though both treatments are known to result in T184 phosphorylation (Roose, Mollenauer et al. 2005). Thus, either phosphorylation at position T184 is insufficient as a degradation signature or factors dependent on “DAG+calcium” TCR signaling are, in part, responsible for degradation of RasGRP1 protein. RasGRP1 protein and transcript levels are elevated in this lymphoma cell line but the cells appear to retain the ability to degrade RasGRP1 specifically in response to TCR stimulation.
It is possible that RasGRP1 overexpression in this cell line could result in “rewiring” of signaling networks and that this observed degradation was an anomaly specific to the cell line rather than a normal mode of protein regulation. Therefore, I sought to confirm this observation in additional cell types. A time course using a second cell system, human leukemic T cell line Jurkat cells, stimulated with TCR crosslinking antibody (C305) showed a similar reduction in protein level over time (Fig. 2.5b). Significantly, when non-leukemic wildtype thymocytes from a 5 week-old mice were stimulated using TCR crosslinking antibody, the same degradation response was observed (Fig. 2.5c). This result is especially striking as not all cells in the thymus express a fully functional TCR and therefore receive less stimulus. Thus it appears that signaling components downstream of the TCR result in degradation of RasGRP1 protein in cell lines and primary cells.

While these results seem to point at the active degradation of RasGRP1 protein after TCR stimulus, it still remained possible that RasGRP1 was instead recruited to an insoluble intracellular compartment. This was especially possible given the earlier observation that RasGRP1 may be K63 ubiquitinated (a subcellular localization signal). To test this theory, I stimulated 1156O lymphoma cells using TCR crosslinking antibody. Cells were then lysed using either NP40 lysis buffer (as per usual) or using more stringent SDS lysis buffer capable of solubilizing all cell components (Fig. 2.5d). Probing these lysates for RasGRP1 protein revealed similar rates and levels of degradation in both treatments. This result shows that RasGRP1 is likely degraded in response to TCR stimulation and is not sequestered in a different subcellular compartment.
Figure 2.5 – RasGRP1 is actively degraded after TCR stimulus in a variety of cells

(a) 1156O cells were stimulated using either PMA or TCR/CD4 crosslinking antibodies. Cells were lysed using Nonidet P 40 (NP40) lysis buffer at times indicated. Lysates were probed for the presence of RasGRP1 protein. Grb2 is included as a loading control. (b) Jurkat cells were stimulated using TCR crosslinking antibody. Cells were lysed using NP40 lysis buffer at times indicated. Lysates were probed for the presence of RasGRP1 protein. Grb2 is included as a loading control. (c) Wild type thymocytes were stimulated using TCR crosslinking antibodies. Cells were lysed using NP40 at times indicated. Lysates were probed for the presence of RasGRP1 protein. Alpha tubulin is included as a loading control. (d) 1156O cells were stimulated using TCR crosslinking antibodies. Cells were lysed using either NP40 lysis buffer or SDS boil at times indicated. Lysates were probed for the presence of RasGRP1 protein. Alpha tubulin is included as a loading control.
a  
1156O  
Stimulus:  
Time (min):  
<table>
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<tr>
<th></th>
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<th>αTCR + αCD4</th>
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WB: RasGRP1  

WB: Grb2  

b  
Jurkat  
+ αTCR:  
Time (min):  
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<th>90</th>
<th>120</th>
<th>150</th>
<th>180</th>
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WB: RasGRP1  

WB: Grb2  

c  
thymocytes  
+ αTCR:  
Time (min):  
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<th>60</th>
<th>120</th>
</tr>
</thead>
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WB: RasGRP1  

WB: α tubulin  

d  
1156O  
αTCR + αCD4  
Time (min):  
<table>
<thead>
<tr>
<th></th>
<th>NP40 lysis</th>
<th>SDS lysis</th>
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RasGRP1 degradation response requires additional signals (calcium)

In an attempt to determine possible downstream signaling components involved in the degradation response, I stimulated lymphoma cells in the presence of various inhibitors of components downstream of the TCR: Src family kinases, PLC\(\gamma\), and MEK kinase (Fig. 2.6). While RasGRP1 protein levels decreased in cells incubated in PBS alone or MEK inhibitor (U1026), protein levels remained constant in cells preincubated with Src kinase inhibitor (PP2) and PLC\(\gamma\) inhibitor (U71322). Additionally, use of a calcium chelator (BAPTA) to inhibit the activity of classical calcium-responsive PKC kinases also slowed RasGRP1 degradation. These results suggest that RasGRP1 is posttranscriptionally regulated by proteins that act downstream of PLC\(\gamma\) but upstream of MEK kinases. It is also likely that these components require calcium themselves (such as PKCs) or the activation of other calcium-regulated proteins (such as calmodulin or calcineurin).

Two-dimensional gels reveal that RasGRP1 may be modified on additional sites to T184

In order to fully investigate the posttranslational modifications present on RasGRP1 protein, it would be ideal to have an idea of how heterogenous RasGRP1 phosphorylation may be. The best method for this would be two-dimensional electrophoresis. In this method, cells are lysed in a non-ionic buffer (like NP40) and the protein of interest is immunoprecipitated or otherwise enriched (e.g. column filtration). This enriched sample must then be thoroughly cleaned to remove any ionic materials (e.g. salts). The sample is then loaded into a immobilized pH gradient gel. When an electric current is run through this gel (isoelectric focusing), the proteins will move through the gradient until they reach
Figure 2.6 – RasGRP1 degradation rates differ in the presence of inhibitors of signaling pathway components

1156O cells were preincubated for 20 minutes with inhibitors of Src family kinases (PP2), PLCγ (U71322), MEK kinase (U0126) or an intracellular calcium chelator (BAPTA). PBS indicates cells that were rested in PBS only (no inhibitor) but were also stimulated using the same TCR crosslinking antibodies. Cells were lysed using NP40 lysis buffer at times indicated after TCR crosslinking and lysates probed for the presence of RasGRP1 protein by Western blot. Degradation of RasGRP1 protein was inhibited or delayed with inhibitors of Src family kinases, PLCγ, or an intracellular calcium chelator (BAPTA).
### Experiment 1

**1156O + αTCR + αCD4**

Preincubated with:

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<th>PLCγ inhibitor</th>
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**WB: RasGRP1**

![Western Blot Image]

### Experiment 2

**1156O + αTCR + αCD4**

Preincubated with:

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<thead>
<tr>
<th></th>
<th>PBS</th>
<th>MEK inhibitor</th>
<th>Calcium chelator</th>
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**WB: RasGRP1**

![Western Blot Image]
the pH at which their overall charge is neutral (isoelectric point). For example, RasGRP1’s predicted isoelectric point is near pH7. If RasGRP1 is phosphorylated once, this adds additional negative charge to the overall molecule. This means that in the fixed pH gradient, the modified protein would move more toward the acidic end (lower pHs) because this is the point at which it is now isoelectrically neutral. Phosphorylation adds a negative charge and reduces the pKa of the protein. As such, the protein will focus to a more acidic portion of the gel. After isoelectric focusing, the entire pH gel strip is loaded onto a regular SDS PAGE gel and when the electric current is run through the gel again, all protein separate by molecular weight. Thus, if RasGRP1 had only one phosphorylation site (T184), we would see two spots with a molecular weight of 90kD on a 2D gel after stimulation with PMA: one spot toward the acidic side of the gel (with one phosphorylation event at T184) and one (presumably smaller spot) toward the basic side (unphosphorylated RasGRP1).

In order to examine how many posttranslationally-modified forms of RasGRP1 protein might exist, I optimized two-dimensional gel electrophoresis for RasGRP1 protein (more about this process can be found in the Chapter 4 and Materials and Methods section of this chapter). I immunoprecipitated RasGRP1 from 1156O mouse lymphoma cells that were left unstimulated or stimulated with either PMA or TCR crosslinking antibodies. I then ran 2D gels using pH strips with a fixed range of pH3-pH10 (Fig 2.7a-c). Finally gels were silver stained to visualize all proteins present.

Unexpectedly, the largest variety of posttranslational modifications seems to be at the basal state (Fig. 2.2a). In this state is appears that RasGRP1 has 3 forms. This may be representative of forms with 0, 1, and 2 phosphorylation events (as they appear to move
toward the pH 3 side of the gel). However, because of the way isoelectric focusing occurs, it cannot be ruled out that RasGRP1 has instead 1, 2 and 3 phosphorylation events in the basal state (etc.). After 5 minutes of stimulation with PMA, it appears that there is a large, relatively homogeneous, shift to the right (toward pH 3) indicating that additional posttranslational events are occurring (**Fig. 2.7b**). A similar shift occurs after 10 minutes of stimulation with TCR crosslinking antibodies (**Fig. 2.7c**). The shift is not as homogeneous here and may be due to either slower kinetics of modification after TCR stimulation in comparison to that seen with PMA or it may represent the removal of modifications to return to the basal state. A time course for RasGRP1 modification was not completed.

Finally, it is also interesting to note the presence of additional proteins on the gels. All three conditions reveal the presence of a protein that is very close in size to what is presumed to be RasGRP1 that does not appear to change in response to stimulus (circled, **Fig. 2.7a-c**). It would be interesting to find out if this protein is 1) the other isoform of RasGRP1 or 2) an additional co-precipitating protein (or non-specific species recognized by the IP antibody). Other co-precipitating proteins are also clearly visible in all gels although the most abundant protein on the gel is the heavy chain of the antibody used to immunoprecipitate RasGRP1. Additional work will need to be done to determine what these additional proteins are and what roles, if any, they play in RasGRP1 activity/regulation.
Figure 2.7 – Two-dimensional gel analysis of RasGRP1 protein reveals complexity of posttranslational modifications

1156O cells were left unstimulated or stimulated using PMA or TCR crosslinking antibodies. RasGRP1 protein was immunoprecipitated and applied to a 2 dimensional gel with a pH range of 3-10. All gels were imaged using silver staining. (a) 2D gel of unstimulated 1156O. Note three spots indicated by arrowheads (right). Circled is a common spot that may be the long form of RasGRP1 or a co-precipitating protein. (b) 2D gel of 1156O stimulated for 5 minutes with PMA. Note the shift to the right, indicating posttranslational modifications that occur (arrow) to make the protein “more negative”. The full size gel also shows a variety of spots at different sizes that may be co-precipitating proteins. (c) 2D gel of 1156O stimulated for 10 minutes with anti-TCR/anti-CD4 crosslinking antibodies. Note the shift to the right, indicating posttranslational modifications that occur (arrow) to make the protein “more negative”. There appear to be 2 spots (marked by arrowheads).
Discussion

I have provided substantial evidence that RasGRP1 is posttranslationally regulated. Differential binding of antibodies recognizing phosphorylated consensus motifs suggest that in addition to the known activating phosphorylation event on T184, there appears to be a basal level of phosphorylation on RasGRP1. It is possible that this basal phosphorylation acts in an inhibitory manner and must either be removed by a phosphatase or countered by phosphorylation at T184 after stimulation through the T cell receptor. The 2D gel data support the theory of basal phosphorylation as three separate spots appear in the basal state. These spots condense in the direction consistent with phosphorylation after stimulation, but it seems that at least some of the RasGRP1 present in the cell at the basal state is in some way posttranslationally modified.

Unfortunately, it is not possible to know which posttranslational modification/phosphorylation sites are being used in each of the spots seen on the 2D gel. For example, one spot may represent a pool of RasGRP1 protein that is modified (e.g., phosphorylated) on one amino acid residue. Another, to the right, represents RasGRP1 that is modified on a total of two amino acids residues. This could mean the same amino acid as the spot to the left plus an additional amino acid or two completely novel amino acids. The best way to investigate this question would be to successfully extract each spot from the gel and send the gel pieces for Mass Spectrometry. Gel spot extraction and mass spectrometry was attempted using both 2D and 1D gels of immunoprecipitated RasGRP1, but was not successful. Results were largely full of keratin and no attempts resulted in the visualization of any RasGRP1 peptides, let alone those that were
phosphorylated. Personal communication with other labs including Jim Stone and
members of the Art Weiss lab has revealed that others have also tried Mass Spec with
RasGRP1 protein and this work has rarely, if ever, resulted in any RasGRP1 peptides
being recognized by the detector. For some unknown reason, it appears that RasGRP1
peptides generated during the digestion and laser excitation processes are not prone to
“flying” in the Mass Spec machine. Significant advances this technology or additional
biochemical knowledge of RasGRP1 protein will be necessary before Mass Spec will be
a useful technique to study RasGRP1 phospho-peptides.

The binding of K63 linked ubiquitin antibody to RasGRP1 after TCR stimulation is also
an interesting result. As stated before, ubiquitins that use K63 as a linkage point are
thought to be more involved in either subcellular localization signals or may act as
signaling/activation events themselves. These ubiquitins can also coordinate binding of
other proteins in complexes. Additional work with RasGRP1 to confirm the presence of
ubiquitin on RasGRP1 itself (and not a co-IP protein of similar molecular weight) as well
as the overall number of ubiquitins in the chain will be important in determining a
molecular mechanism, be it signaling or localization.

Much of this project grew from the initial observation that RasGRP1 seems to be
degraded after TCR stimulation. As a potent activator of Ras, a known oncogene, it
seems logical that it would be of utmost importance to the T cell participating in an
immune response to be able to quickly and accurately eliminate this signaling trigger.
One effective way to remove this sort of signaling event would be to completely degrade
that protein acting as the initiation point of the signaling cascade. As seen, RasGRP1 appears to be a relatively stable protein in the basal state and initiation of signaling is required for degradation to occur. Furthermore, degradation appears specifically in response to the full complement of components downstream of the TCR, i.e., introduction of DAG (PMA) at the cell membrane is not sufficient to cause degradation and additional factors activated by TCR crosslinking are required. This is in contrast to other proteins known to be recruited to the membrane via DAG analogues, like protein kinase C (PKC), which after activation are degraded in a ubiquitin-dependent manner when cells are treated with either PMA or bryostatin (Stone, Stang et al. 2004). Thus it appears that RasGRP1 protein degradation requires an additional signal besides DAG. This, in and of itself, appears to be a sort of regulation, preventing unnecessary degradation of RasGRP1 after encounters that continuously trigger low level basal signaling events.

In the future it would be interesting to determine the exact mechanism of degradation. Initial experiments performed using proteosome inhibitor, MG132 did not prevent the degradation of RasGRP1. It could be that degradation does not occur via the proteosome but instead occurs via the endocytic pathway. Other researchers have noticed TCR signaling occur on intracellular vesicles within the cell that later fuse with the endosomal pathway (Purbhoo, Liu et al. 2010). Studies looking at immune synapses also note the funneling of signaling molecules into the center of the point of contact (cSMAC) that later move away from TIRF imaging plane of view (into the cell) (Varma, Campi et al. 2006). It remains possible that RasGRP1 is transported away from the plasma membrane along with the large TCR signaling complex and degraded in an intracellular vesicle. As
such, further study using endosomal pathway inhibitors may prove useful in determining this mode of degradation.

These results, together with the published observation that transgenic or retroviral overexpression of RasGRP1 contributes to uncontrolled cellular proliferation, lead me to the hypothesis that normal cells must have a specific intrinsic method to regulate RasGRP1 protein levels over time in response to stimuli. Although these data do not provide an exact mechanism, I am confident that further work in the Roose lab will continue to examine posttranslational modifications on RasGRP1 and how these modifications contribute to or help to prevent the overproliferation of lymphoid cells like that seen in TALL/L.
Materials and Methods

Cell Stimulations:
Cells were stimulated using 20ng/mL PMA (Calbiochem). To mimic TCR engagement, cells were pre-labeled using anti-CD3 primary antibody (10µg/mL, UCSF Monoclonal Antibody Core, clone 2C11). Crosslinking was achieved using goat anti-hamster antibody (10µg/mL, Jackson ImmunoResearch). Rabbit anti-human RasGRP1 (clone E80 and clone E160) was produced by Epitomics, Inc. (Burlingame, CA, USA). When indicated, cells were pre-incubated with the following inhibitors: cyclohexamide, src family kinase inhibitor (PP2, Calbiochem), PLCγ inhibitor (U73122, Calbiochem), MEK inhibitor (U0126, Cell Signaling) and calcium chelator (BAPTA-AM, Invitrogen).

Immunoprecipitation:
Pre-cleared lysates were incubated with Sepharose beads (GE Healthcare) pre-labeled with RasGRP1 antibody (E80) at 4°C for 1 hour. Beads were spun down and protein removed using 2x sample buffer containing betamercaptoethanol. Protein was run on western blotting gels as described above.

Western Blotting:
Cells were lysed using Nonidet-P40 lysis buffer (1%) supplemented with protease and phosphatase inhibitors. Lysates were run on 10% acrylamide Bis-Tris gels and transferred onto PDVF filter (Millipore, Immobilon-P). Blots were probed for RasGRP1 (in house, E80 and E160), Alpha tubulin (Sigma) or Grb2 (Santa Cruz), PKC consensus
sequence, Akt consensus sequence, pan-ubiquitin and K63 linkage ubiquitin (all Cell Signaling). Signal from primary antibodies detected using HRP conjugated secondary antibodies: Sheep anti-mouse HRP (GE Healthcare) and goat anti-rabbit HRP (SouthernBiotech). Blots were developed using Pierce ECL Western Blotting Substrate (ThermoScientific) and images recorded using a chemiluminescence imager (Fuji, LAS-4000).

**Metabolic labeling at basal state:**

Cells (Jurkat or 1156O; 120 x 10^6) were rested in cysteine/methionine-free media (Sigma) supplemented with 10% dialyzed fetal bovine serum (HyClone) for 30 minutes at 37°C. Cells were then pulsed for 10 minutes with 1mCi of ^35^S Translabel No-Thaw Reagent (MP Biomedicals) added to the media. Cells were then diluted in warm chase media (RPMI supplemented with 10% FBS, 5mM unlabeled cysteine, and 5mM unlabeled methionine, Acros Organics). Aliquots were taken at time points indicated and lysed in 1% NP40 lysis buffer as per usual. Prior to immunoprecipitation, lysates were precleared by incubating with Pansorbin (Calbiochem) and unlabeled sepharose beads (GE Healthcare) 2x 1 hour for each reagent. Immunoprecipitates were then run on gels as described above.

**Metabolic labeling followed by stimulation:**

Cells (Jurkat or 1156O; 120 x 10^6) were rested in cysteine/methionine-free media (Sigma) supplemented with 10% dialyzed fetal bovine serum (HyClone) for 30 minutes at 37°C. Cells were then pulsed for 10 minutes with 1mCi of ^35^S Translabel No-Thaw...
Reagent (MP Biomedicals) added to the media. Cells were then washed twice in warm chase media (RPMI supplemented with 10% FBS, 5mM unlabeled cysteine, and 5mM unlabeled methionine, Acros Organics). Cells were labeled on ice with 10ug/mL primary antibody (2c11 and anti-CD4, both UCSF Monoclonal Antibody Core). Cells were then stimulated using either 10ug/mL secondary mix (goat anti-hamster and goat anti-rat, Jackson ImmunoResearch) or 20ng/mL PMA (Calbiochem) for the times indicated. Cells were lysed in 1% NP40 lysis buffer as per usual. Prior to immunoprecipitation, lysates were precleared by incubating with Pansorbin (Calbiochem) and unlabeled sepharose beads (GE Healthcare) 2x 1 hour for each reagent. Immunoprecipitates were then run on gels as described above.

**Hot gel imaging:**

After electrophoresis, gels were dried using a gel dryer (BioRad) at 80°C for 2 hours. Dried gels were then exposed to a phosphorimaging screen (GE Healthcare) overnight. Imaging screen was scanned using a Typhoon phosphorimager (GE Healthcare).

**2D gel electrophoresis:**

Cells were lysed in 1% NP40 lysis buffer and immunoprecipitated for RasGRP1 as described above. Protein was removed from sepharose beads using sample rehydration buffer (Rehydration Buffer 2, Invitrogen) + 100mM DTT (Sigma). Ionic contaminants were removed from immunoprecipitates using 2D Clean-up Kit (Amersham Biosciences) and following manufacturer’s instructions. The following day, protein was rehydrated using 150ul rehydration buffer + 100mM DTT. Protein was loaded onto dried fixed pH
gradient strips (Invitrogen Zoom IPG Runner) and gels were allowed to rehydrate overnight at room temperature. Gels were loaded into isoelectric focusing cassettes (Invitrogen) and protein was focused over the course of 3 hours (limits: 0.1mAmps and 0.1 watts per strip; Program: 175 volts for 15 minutes, 175-2000 volts 45 minutes, 2000 volts for 60 minutes). Best results were achieved if paper electrode wicks were changed after the first 7 minutes (Harper, Mozdzanowski et al. 2001; Chevallet, Tastet et al. 2004; Görg, Klaus et al. 2007). After focusing, gels were equilibrated for 15 minutes (tumbling with 10mL of Equilibration buffer +0.05g DTT) and alkylated for 15 minutes (tumbling with 10mL of Equilibration buffer + 0.045g iodoacetamide). 

**Equilibration buffer:** 15% glycerol, 370mM Tris pH 8.8, 1% SDS, 6M Urea. Strips were then loaded onto a second dimension gel (IPG well, Invitrogen) and run for 1.5 hours at 200 volts.

**Silver Staining:**

Gels were marked using a glass pipet. Gels were fixed for 30 minutes and an additional 18 hours using fixation buffer (30% ethanol, 10% glacial acetic acid) to allow for removal of 2D gel carrier ampholytes (Invitrogen). Gels were then washed 4 times in ddH$_2$O to rehydrate. Gels were sensitized using 8mM sodium thiosulfate (Sigma) followed by two washes in ddH$_2$O (all steps 1 minute). Gels were then placed in 12mM silver nitrate (Fisher) for 20 minutes. Gels were developed using developing buffer (10g galactose, 100mL 0.5M boric acid, 150 mL sodium hydroxide, 50ul 0.5M sodium thiosulfate, 250mL dH$_2$O) until spots were clearly seen (10-30 minutes). Gels were transferred into Stop buffer (20g Tris, 10mL glacial acetic acid, 490mL ddH$_2$O) for 30
minutes and ddH₂O for one hour (Chevallet, Luche et al. 2006; Chevallet, Luche et al. 2008). Digital images of gels were recorded using a Fuji LAS-4000 in bright field.
CHAPTER 3

An Autoimmune Mutation in the Calcium-Binding Domain of Rasgrf1 Reveals Unique Compensation during Thymocyte Selection
Summary

Activation of Ras and the downstream ERK kinases by the Ras guanine nucleotide exchange factor Rasgrp1 is essential for T-cell differentiation, but Rasgrp1 regulation is incompletely understood. Here we characterize a mouse point mutation, Rasgrp1\textsuperscript{Anaef}, in Rasgrp1’s second EF hand that profoundly impairs activation of Rasgrp1 and Ras-ERK signals in response to strong TCR stimuli yet has remarkably little effect on thymocyte positive selection. Surprisingly, Rasgrp1\textsuperscript{Anaef} thymocytes efficiently compensate for this defective allele, and indeed Rasgrp1-dependent induction of CD44 was enhanced by the EF-hand mutation. Consequently, peripheral T cells with elevated CD44 expression expand in Rasgrp1\textsuperscript{Anaef} mice. The concurrent production of anti-nuclear autoantibodies provides an exciting novel model to study the relationship between Rasgrp1, thymic selection, and human autoimmune diseases.
Introduction

Thymocyte development and Ras-MEK-Erk signaling

Developing thymocytes undergo positive selection to generate a population of T lymphocytes with a broad spectrum of antigen-specific T cell receptors (TCR). Concurrently, lymphocytes carrying high affinity, potentially self-reactive, TCRs are eliminated (negative selection) to avoid the development of autoimmunity. During these selection processes, differences in incoming signals from the TCR are converted to discriminating signal output (Starr, Jameson et al. 2003). It was recognized early on that the small GTPase Ras plays a role in thymocyte selection (Swan, Alberola-Ila et al. 1995). The canonical RasGTP-RAF-MEK-ERK pathway is the best-studied Ras effector pathway downstream of TCR engagement and the impaired positive selection of ERK-1 and -2 doubly deficient thymocytes underscores the importance of this pathway during development (Fischer, Katayama et al. 2005).

RasGEF activity in thymocytes

There are three Ras guanine exchange factor (RasGEF) families that can activate Ras: SOS, RasGRP, and RasGRF. T - and B-lymphocytes express SOS1, SOS2, RasGRP1, and RasGRP3 (reviewed in (Stone 2011). Following TCR engagement, Son of Sevenless (SOS)-1 and -2 are recruited to the plasma membrane via a Grb2-phospho-LAT interaction. Simultaneously, the second messenger diacylglycerol (DAG), generated via PLCγ, directly recruits Ras guanine nucleotide releasing protein (RasGRP1) to the plasma membrane (Ebinu, Bottorff et al. 1998). While RasGRP1 and SOS1 can synergize
to induce high-level Ras activation (Roose, Mollenauer et al. 2007), RasGRP1 also serves a non-redundant role in priming SOS1 via RasGRP1-produced RasGTP (Das, Ho et al. 2009). In agreement with a non-redundant function, thymocyte development is severely impaired in Rasgrp1-deficient mice and not compensated for by other RasGEFs (Dower, Stang et al. 2000). These mice exhibit a strong defect in positive selection and impaired ERK phosphorylation in thymocytes (Priatel, Teh et al. 2002). Older Rasgrp1-deficient mice develop splenomegaly and demonstrate autoantibodies in the serum (Layer, Lin et al. 2003; Coughlin, Stang et al. 2005), although these phenotypic characteristics appear milder in Rasgrp1-deficient mice on a pure C57BL/6 background (Chen, Priatel et al. 2008).

**RasGRP1 regulation in thymocytes**

Although RasGRP1 plays a critical role in the activation of Ras, relatively little is known about its regulation in normal T lymphocytes or the in vivo importance of such regulation. In addition to membrane-recruitment via its DAG-binding C1 domain (Ebinu, Bottorff et al. 1998; Zheng, Liu et al. 2005), RasGRP1’s GEF activity is enhanced by the inducible phosphorylation of threonine 184 (Roose, Mollenauer et al. 2005; Zheng, Liu et al. 2005). TCR-induced activation of phospholipase C γ (PLC γ) not only results in production of DAG but also of inositol 1,4,5-trisphosphate (IP3), which binds to IP3 receptors on the endoplasmic reticulum to activate the calcium pathway (Feske 2007). Interestingly, RasGRP1 also contains a pair of EF hands, motifs that often come in pairs that can bind calcium to induce a conformational change (Gifford, Walsh et al. 2007). Previously, the second EF hand (EF2) of RasGRP1 was reported to bind calcium in vitro.
In chicken DT40 B cells, the EF\textsubscript{1} domain enables the recruitment function of a PT domain (plasma membrane targeting domain) (Tazmini, Beaulieu et al. 2009). Using GFP-tagged RasGRP1 expression, this C-terminal PT domain has been defined to cooperate with the C1 domain to recruit RasGRP1 to the membrane. Notably, PT domain’s contribution is substantial in BCR-stimulated B cell lines, very modest in T cell lines, and negligible in fibroblasts (Beaulieu, Zahedi et al. 2007). Thus, RasGRP1’s EF hands likely play a regulatory role but this role might differ from cell to cell type. To date, no \textit{in vivo} function for RasGRP1’s EF\textsubscript{1} or EF\textsubscript{2} hands has been described in T lymphocytes.

**Mouse models with hypomorphic signaling proteins may provide insights to human disease**

Recent analyses of various mouse models carrying hypomorphic alleles of signaling molecules ZAP70 and LAT have revealed that single amino acid polymorphisms can cause severe, paradoxical immune phenotypes. In contrast to the absolute blocks in thymocyte development in ZAP-70- or LAT-deficient mice (Negishi, Motoyama et al. 1995; Zhang, Sommers et al. 1999), these hypomorphic alleles result in only a relative developmental impairment combined with aberrant immune function of the resultant peripheral T cells. For example, an SKG allele of the kinase ZAP-70 has reduced binding-affinity for phospho-TCRζ and leads to autoimmune arthritis in mice (Sakaguchi, Takahashi et al. 2003). Point mutations in ZAP70’s catalytic domain that reduce kinase activity to intermediate levels result in autoantibody expression and hyper-IgE production, whereas more severe decreases in kinase activity result in T cell
deficiency (Siggs, Miosge et al. 2007). Mutation of the single tyrosine in LAT
(LAT\textsuperscript{Y136F}) results in hyperproliferative lymphocytes of a TH2 type (Aguado, Richelme et al. 2002; Sommers, Park et al. 2002).

**Genetic variants in human disease**

A major form of human genetic variation comprises single nucleotide variants that cause amino acid substitutions (missense variants; SNVs) or modify the level of gene expression rather than knocking out protein expression: most people inherit ~12,000 missense gene variants (Project 2010). Therefore mouse models that analyze the consequences of missense variants may serve as useful models to better understand complex human immune diseases. Common tag SNVs near \textit{RASGRP1} are associated with susceptibility to autoimmune (Type 1) diabetes and to thyroid autoantibodies in Graves disease (Qu, Grant et al. 2009; Plagnol, Howson et al. 2011), while two unstudied \textit{RASGRP1} missense SNVs are currently known (rs62621817, rs75648785). A fruitful approach for identifying missense gene variants that dysregulate immune function has been through \textit{N}-ethy\textit{N}-nitrosourea (ENU) mutagenesis (Nelms and Goodnow 2001; Jenne, Enders et al. 2009).

Here we describe the analysis of a novel ENU mutant mouse with a missense variant, \textit{Rasgrp1\textsuperscript{Anaef}}, that reveals a new regulatory function of Rasgrp1’s second EF hand. Examination of thymocyte selection in \textit{Rasgrp1\textsuperscript{Anaef}} mice unmasks striking compensation for this hypomorphic allele, resulting in aberrant accumulation of peripheral CD4\textsuperscript{+} T cells with upregulated CD44 expression and formation of anti-nuclear antibodies.
Results

Identification of a \textit{Rasgrp1}\textsuperscript{Anaef} mouse strain with a mutated EF hand in Rasgrp1

As part of a mouse genome-wide screen for immune phenotypes induced by ENU mutagenesis (Nelms and Goodnow 2001; Jenne, Enders et al. 2009), we identified a variant C57BL/6 pedigree with ~25% of offspring age 8-16 weeks displaying decreased percentages of CD4$^+$ T cells in peripheral blood (Fig. 3.1a), elevated CD44 expression on CD4$^+$ cells (Fig. 3.1b, c), and a high occurrence of homogeneous antinuclear antibodies (Fig. 3.1d, e). The CD4/CD44 trait was used to map the mutation in an F2 intercross to an interval between 114 and 121.2 Mb on chromosome 2 (Figure A.1a). Sequencing of the exons of \textit{Rasgrp1}, the only candidate gene within this interval with a known immune function, identified an A to G point missense mutation in codon 519 within exon 13 (Fig. 3.1f). The mutant codon encodes a neutral amino acid glycine (G) instead of arginine (R), a large polar molecule with a positive charge (Rasgrp1 R519G; Fig. 3.1f). R519 is located in Rasgrp1’s second EF Hand (EF$^2$) (Fig. 3.1g, h), reported to bind calcium \textit{in vitro} (Ebinu, Bottorff et al. 1998). While there is no crystal structure for RasGRP1 to date, sequence analysis indicates R519 is positioned in the calcium-binding loop and is flanked by two putative $\alpha$ helices. The sequence of the calcium-binding loop in Rasgrp1 closely resembles those found in the calcium-binding calmodulin (CaM) and calcium and integrin binding protein (CIB) with conserved residues at the “X”, “Y”, and “Z” positions, which are critical for positioning of the calcium ion (Fig. 3.1g) (Grabarek 2006; Gifford, Walsh et al. 2007). The charged R519 residue in Rasgrp1 is identical in CIB, and conserved in Rasgrp3. The ENU-generated allele was named “Rasgrp1\textsuperscript{Anaef}” to
Figure 3.1 – An ENU mouse mutant with anti-nuclear antibodies and CD44hi phenotype maps to Rasgrp1

(a,b) Representative results of flow cytometric analysis of blood from 6-12 week old (B6.Anae x CBA/J) F2 mice used to map the Anaef mutation. Mice displaying decreased CD4 cells and increased CD44 expression on CD4 cells in repeated bleeds were designated “affected” individuals (red squares) compared to “unaffected” littermates (black circles). (c) CD44 surface expression on CD4+ peripheral blood cells from representative homozygous mutant and wild-type mice. (d, e) Analysis of antinuclear antibodies (ANA) by indirect immunofluorescence on HEP-2 cells for a B6xB10.Rasgrp1Anaef mouse and wild-type littermate. Plasma from Rasgrp1Anaef mice show homogeneous staining of the nucleus of interphase cells and positive chromatin bars in dividing cells (marked with arrow). Magnification 20x. (e) Quantitation of positive ANA results for wild-type and Rasgrp1Anaef C57Bl/6xC57Bl/10 siblings tested at 15 weeks of age. (f) Sequence trace of Rasgrp1Anaef exon 13 aligned to wildtype Rasgrp1 sequence. (g) Model of a typical EF hand with conserved residues in the calcium (Ca)-binding loop flanked by the N- and C-terminal α-helices. Comparison of CaM (calmodulin, top), CIB (calcium and integrin binding protein, middle), Rasgrp3, and Rasgrp1 EF hand sequences with conserved residues highlighted. (Acidic, red; Basic, blue; Calcium-binding residues, highlighted in grey; ENU-mutated residue, underlined).

(h) Linear schematic of Rasgrp1 protein domains, phosphorylation site threonine 184, and position of R519G mutation in the second EF hand. (i) Rasgrp1 protein expression in Rasgrp1Anaef/Anaef thymocytes, compared to Rasgrp1 expression in Rasgrp1Anaef/WT thymocytes and wildtype thymocytes. α tubulin expression functions as loading control.
a) Affected
• Unaffected

b) % CD4 + CD4

20 40 60 80
% B220

% CD4
0 0 10 20 30

c) CD4+ cells peripheral blood

% Max

10^3 10^4 10^5
CD4

Anaef
Mean gate = 19.4%
SD = 3.8%

WT
Mean gate = 8.1%
SD = 2.8%
P < 0.0001

d) WT

Anaef

Number of mice

20 40

e) ANA negative
• ANA positive

f) AnaEF:
Wild type:

G L I S

AnaEF:
Wild type:

G D E I T

G D E I T

R S 19G

DAG-binding

h) 1 58 110 183 440 797

REM Cdc25 G1

P_T184

Ca2+-binding loop

position: 1 3 5 7 9 12

CaM EF1:
DKKEEK TI TTKK

CIB EF3:
DKKEEK TL NRDE

Rasgrp3 EF2:
DKKEEK LI SKDE

Rasgrp1 EF2:
DKDREG LI SRDE

i) m/m m/+ +/+ WB:

60 87 100

% relative expression

α tubulin
reflect the combination of antinuclear antibody (ANA) production and the amino acid substitution in the EF hand. Genotyping of this mutation in multiple generations of C57BL/6 offspring (Fig. A.1b, A.1c) demonstrated a correlation between inheritance of the Rasgrp1<sup>Anaef</sup> allele and the immunological abnormalities described above and below, with heterozygous (<sup>Anaef</sup>/wt) mice demonstrating an intermediate immune phenotype to homozygotes (data not shown). The R519G substitution caused an approximate 40% decrease in Rasgrp1 protein levels in homozygous Rasgrp1<sup>Anaef</sup> thymocytes (Fig. 3.1i). Since heterozygous Rasgrp1<sup>-/-wildtype</sup> thymocytes express half the Rasgrp1 dosage but do not display an abnormal immune phenotype (Dower, Stang et al. 2000), we hypothesized that the R519G substitution in Rasgrp1 has a different impact than Rasgrp1 deficiency.

**RasGRP1<sup>Anaef</sup> demonstrates impaired activation of RasGRP1 and the Ras-ERK pathway**

Past studies have focused on the requirement of the second messenger DAG for recruitment of RasGRP1 to the membrane via its C1 domain (Ebinu, Bottorff et al. 1998; Roose, Mollenauer et al. 2005) and for the activating phosphorylation event on T184 in RasGRP1(Roose, Mollenauer et al. 2005; Zheng, Liu et al. 2005). These studies established that PMA, a synthetic analog of DAG, is an effective activator of the RasGRP1-Ras-ERK pathway. To investigate the biochemical effect of the Rasgrp1<sup>Anaef</sup> allele, we stably reconstituted a RasGRP1-deficient Jurkat cell line (JPRM441) that expresses ~10% of residual, wildtype RasGRP1 protein (Roose, Mollenauer et al. 2005; Zheng, Liu et al. 2005) with full-length RasGRP1 containing the R519G mutation (Fig. 3.2a). Stimulation of these clonal cell lines with PMA followed by a RasGTP pull-down
Figure 3.2 – Rasgrp1<sup>Anaef</sup> is a hypomorphic allele with impaired activation of Rasgrp1, Ras, and the downstream ERK kinases.

(a) RasGRP-1 deficient Jurkat cells (JPRM441) were stably reconstituted with a construct expressing R519G-RasGRP1 (Rasgrp1<sup>Anaef</sup> allele; clones #67, 68, 69). Protein lysates from an equivalent number of cells were probed for expression of RasGRP1 and α tubulin (loading control). Percent expression of RasGRP1 in each cell line (relative to the parental wildtype Jurkat) is shown. RasGRP1<sup>Anaef</sup> protein levels in clones #68 and #69 were similar to those found in the parental Jurkat cell line and to expression of wildtype RasGRP1 in a reconstituted JPRM441 clone 208 that we previously characterized (Roose, Mollenauer et al. 2005) and were utilized in subsequent assays. (b) Jurkat, JPRM441, and JPRM441 cells reconstituted with Anaef- or WT RasGRP1 were left unstimulated or stimulated with 25 ng/ml PMA and RasGTP levels were determined through a RasGTP pull down assay. (c,d) The indicated cell lines were stimulated for 0, 1, 5, or 10 minutes (see key) with a high (25ng/ml, b) or medium dose (5ng/ml, c) of PMA, stained for intracellular phosphorylated –ERK-1 and -2 (P-ERK), and analyzed by flow cytometry. Results for JPRM441-RasGRP1<sup>Anaef</sup> clones #68 are shown, similar results were obtained with clone #69. (e) The indicated stable cell lines were stimulated with a low dose (2ng/mL) of PMA or a combination of PMA and ionomycin (1µM) and stained for intracellular P-ERK. (f) Jurkat, JPRM441, and JPRM441 cells reconstituted with Anaef- or WT RasGRP1 were stimulated with PMA (5 ng/mL) and analyzed for RasGRP1 phosphorylation on threonine 184 (P-T184-RasGRP1). Figure 2b is a representative example of two independent experiments. All other panels are representative images of at least 3 experiments.
assay demonstrated that Rasgrp1\textsuperscript{Anaef} is a hypomorphic allele resulting in impaired GTP-loading of Ras (Fig. 3.2b).

We subsequently examined the effects of RasGRP1\textsuperscript{Anaef} on the patterns of ERK phosphorylation, downstream of RasGTP. Stimulation of cells expressing RasGRP1\textsuperscript{Anaef} with PMA revealed a defective induction of phospho-ERK (P-ERK) that was most notable with the lower dose of PMA (PMA MED; 5ng/mL) and contrasted the effective induction of P-ERK signals in Jurkat and JPRM441-WT-RasGRP1 cells (Fig. 3.2c and 3.2d). In fact, P-ERK responses in JPRM441-RasGRP1\textsuperscript{Anaef} cells were more impaired than in the parental JPRM441 cells, indicating a dominant negative effect, which was also observed at the level of Ras activation (Fig. 3.2b). We previously reported a dominant negative effect for ΔDAG-RasGRP1, a form of RasGRP1 lacking the DAG-binding C1 domain (ΔDAG), possibly the result of competition with the residual expression of RasGRP1 in JPRM441 cells (Roose, Mollenauer et al. 2005) (Fig. 3.2d).

EF hands are known to bind calcium and EF\textsubscript{2} in the Rasgrp1 protein binds calcium \textit{in vitro} (Ebinu, Bottorff et al. 1998), but PMA does not trigger calcium fluxes. While total intracellular calcium concentrations are low in resting cells, there is an enrichment of calcium ions near the negatively charged polar headgroups of phospholipids in the plasma membrane (Hille 2001). To test if increasing intracellular calcium would synergize with PMA stimulation and produce a more robust response through the RasGRP1-Ras-ERK pathway we utilized the calcium ionophore ionomycin. Ionomycin, combined with a low dose of PMA in Jurkat and JPRM441-WT-RasGRP1 cells, resulted in more rapid kinetics of P-ERK induction compared to a low dose of PMA stimulation alone, whereas ionomycin alone had only minimal effects on P-ERK (Fig.
3.2e and A.2a). As before, RasGRP1<sup>Anaef</sup> demonstrated a dominant negative effect with the synergistic stimuli.

Since DAG-induced phosphorylation of RasGRP1 on threonine 184 (T184) increases its RasGEF activity (Roose, Mollenauer et al. 2005; Zheng, Liu et al. 2005), we examined how the Anaef mutation in EF<sub>2</sub> might impact this phosphorylation event. To this end we first generated a monoclonal antibody specific for P-T<sub>184</sub>-RasGRP1 (Fig. A.2b). Stimulation of our panel of cell lines with PMA demonstrated that the Anaef mutation in RasGRP1 strongly impairs the activating phosphorylation event on T184 (Fig. 3.2f).

Thymocytes from Rasgrp1<sup>Anaef</sup> mice display severe defects in Rasgrp1 and ERK activation

To examine the global biochemical effect of RasGRP1<sup>Anaef</sup> expression in primary cells, we first stimulated thymocytes from wildtype or Rasgrp1<sup>Anaef</sup> mice with anti-CD3 crosslinking antibodies and probed lysates for tyrosine-phosphorylated proteins. Both thymocyte populations demonstrated similar induction of total phospho-tyrosine patterns and similar activating phosphorylation of Lck and Zap-70 that lie upstream of Rasgrp1 (Fig. 3.3a). TCR-induced elevation of intracellular calcium was indistinguishable from wildtype in Rasgrp1<sup>Anaef</sup> CD<sup>4+</sup>SP and CD<sup>8+</sup>SP thymocytes, but there was a consistently lower peak calcium response in mutant CD<sup>4+</sup>CD<sup>8+</sup> double positive (DP) cells (Fig. 3.3b). Additionally, there was normal CD3-induced tyrosine phosphorylation of PLC<sub>γ1</sub> (Fig 3.3c), indicating that PLC<sub>γ1</sub> activation and IP3 generation following in vitro stimulation by CD3 crosslinking is largely intact in Rasgrp1<sup>Anaef</sup> thymocytes. By contrast, TCR-
Figure 3.3 – Severely impaired Rasgrp1 and ERK activation in \(\text{Rasgrp1}^{\text{Anaef}}\) thymocytes

(a) Thymocytes from \(\text{Rasgrp1}^{\text{Anaef}}\) and age-matched WT mice were stimulated with anti-CD3 antibody (clone 2C11, 10\(\mu\)g/mL) and whole cell lysates were analyzed for induction of tyrosine phosphorylated proteins (4G10). The same lysates were analyzed for specific phospho-Lck and phospho-Zap70 residues. \(\alpha\) tubulin is a loading control. Surface TCR expression levels on \(\text{Rasgrp1}^{\text{Anaef}}\) thymocytes were indistinguishable from wildtype (data not shown). (b) Thymocytes were loaded with the Indo-1 AM calcium dye, labeled with anti-CD3 antibody (10\(\mu\)g/mL), and stimulated with goat anti-hamster secondary crosslinking antibody (30\(\mu\)g/mL). Calcium flux following stimulus addition was measured over 6 minutes. Histograms plot the ratio of \(\text{Ca}^{2+}\) bound Indo-1 AM to \(\text{Ca}^{2+}\) unbound dye as calcium traces over time, gated on CD4\(^+\), CD8\(^+\) single positive or CD4\(^+\)CD8\(^+\) double positive cells for an individual experimental mouse. Histograms for thymocytes from B6.\(\text{Rasgrp1}^{\text{Anaef}}\) or age-matched WT B6 mice (both CD45.2; red: \(\text{Rasgrp1}^{\text{Anaef}}\), blue: WT) are shown overlaid with those from WT B6.SJL-CD45.1 thymocytes (black) that were mixed in at a 1:1 ratio in the same stimulus tube and distinguished by staining with anti-CD45.1 antibody. (c) Phosphorylation of PLC\(\gamma\)1, Rasgrp1, and ERK in total thymocytes from \(\text{Rasgrp1}^{\text{Anaef}}\) and age-matched WT mice that were stimulated as in figure 3a. Loading controls are in Fig. A.2c. (d, e) Thymocytes from \(\text{Rasgrp1}^{\text{Anaef}}\) and age-matched WT mice were stimulated with PMA (30ng/ml in 3d) or with anti-CD3 antibody (5\(\mu\)g/mL in 3e) and crosslinked using secondary antibody (Goat anti-hamster, 20\(\mu\)g/mL) for the indicated time points, fixed, and stained for intracellular p-ERK. Gating on CD4\(^+\)CD8\(^+\) (double positive) and CD4\(^+\) or CD8\(^+\) single
positive thymocytes is shown and numbers indicate the percentages of cells below or above the arbitrarily set reference point in the histograms. All data panels are representative of 3 independent experiments.
induced phosphorylation of T₁₈₄-Rasgrp1 was drastically impaired in Rasgrp¹Anaef thymocytes and ERK phosphorylation was reduced (Fig. 3.3c, A.2c).

We subsequently examined the patterns of P-ERK induction by intracellular flow cytometric staining and electronic gating on thymocyte subsets following in vitro stimulation. As in the cell lines, Rasgrp¹Anaef CD4⁺CD8⁺ double positive (DP), CD4⁺ single positive (SP) and CD8⁺ SP thymocytes were much less responsive to PMA stimulation in terms of P-ERK induction, compared to age-matched wildtype thymocytes (Fig. 3.3d). Stimulation of wildtype DP thymocytes with a sub-maximally stimulating concentration of anti-CD3 antibody resulted in low-levels of ERK phosphorylation that is typically observed for wildtype DP thymocytes (Starr, Jameson et al. 2003; Gallo, Winslow et al. 2007), but strongly induced P-ERK in CD4⁺ SP and CD8⁺ SP cells. By contrast, corresponding P-ERK responses were greatly reduced in Rasgrp¹Anaef DP, CD4⁺ SP and CD8⁺ SP (Fig. 3.3e). Thus expression of Rasgrp¹Anaef in thymocytes severely impacts Rasgrp1 phosphorylation and ERK activation in DP and SP thymocytes, which is not compensated for by other RasGEFs under these in vitro stimulation conditions.

Unanticipated, efficient thymic production and peripheral maintenance of Rasgrp¹Anaef T cells with a diverse TCR repertoire

Given the marked defects in in vitro CD3-induced ERK activation in Rasgrp¹Anaef thymocytes, we expected to observe a dramatic defect in thymocyte development. Surprisingly, development of mature thymocyte subsets was relatively normal in Rasgrp¹Anaef mice when compared to age-matched wildtype controls, and contrasted with the dramatic decrease in CD4⁺ SP and CD8⁺ SP thymocytes in Rasgrp¹⁻/⁻ knockout mice.
(Fig. 3.4a and b). Transitions in early thymocyte development through CD25/CD44 subsets of CD4+CD8- double negative (DN) Rasgrp1AnaeF thymocytes occurred normally (Fig. A.2d) and the Rasgrp1AnaeF allele caused no deficit in the transition from the DN to CD4+8+ DP stage in irradiated mice competitively reconstituted with CD45.2+ Rasgrp1AnaeF and CD45.1+ wildtype bone marrow (Fig. A.2e). Likewise the Rasgrp1AnaeF mutation did not decrease the subset of DP cells that have begun to be positively selected into SP cells, marked by induction of CD69 and increased cell surface TCR (Swat, Dessing et al. 1993; Punt, Suzuki et al. 1996; Azzam, Grinberg et al. 1998) (Fig. 3.4a, lower panels). In competitive bone marrow chimeras reconstituted with a mixture of B6 marrow homozygous for the Anaef mutation and B6.SJL-CD45.1 congenic marrow with wildtype RasGRP1, the Rasgrp1AnaeF thymocytes exhibited no competitive disadvantage as they matured from DP to SP cells (Fig 3.4c).

Consistent with normal thymic development, the percentages and absolute number of CD4+ and CD8+ T cells in the spleen or lymph node were also very similar between 5 week-old Rasgrp1AnaeF and wildtype mice (Fig. 3.4d and A.3a). However, by 12 weeks, Rasgrp1AnaeF mice developed increased splenic cellularity (Fig. A.3b) with elevated numbers of splenic CD4+ T cells (Fig. 3.4d). To test whether this might reflect failure of thymic negative selection we measured the frequency of Vβ11+ SP thymocytes that recognize self-superantigen encoded by endogenous mouse mammary tumor virus, since these are negatively selected in conjunction with I-E MHC molecules in H2k haplotype mice but not in mice with the I-E negative H2b haplotype (Starr, Jameson et al. 2003). Deletion of Vβ11+ SP thymocytes occurred equally efficiently in Rasgrp1AnaeF mice and
Figure 3.4 – Surprisingly normal development of \textit{Rasgrp1}\textsuperscript{Anaef} thymocytes with a wildtype TCR repertoire

\textit{(a)} Developmental thymocyte subsets from RasGRP1\textsuperscript{Anaef}, RasGRP1\textsuperscript{-/-} and wildtype mice. Total thymocytes were stained for surface expression of CD4 and CD8. Expression of CD69 versus TCR\(\beta\) expression was electronically gated for on CD4\(^+\)CD8\(^+\) double positive thymocytes. Numbers represent percentages of cell populations in the quadrants or in drawn gate. Plots are representative of 3 mice per genotype. \textit{(b)} Absolute cell numbers of DP, CD4\(^+\) SP and CD8\(^+\) SP thymocytes were enumerated and plotted. Student’s t tests P value symbols: ** \(p < 0.005\) , * \(p < 0.05\), n.s. = not significant. (n = 3 mice per group; representative of two independent experiments). \textit{(c)} Lethally irradiated CD45.1\(^+\) B6 recipient mice were reconstituted with CD45.1\(^+\) WT bone marrow cells mixed with bone marrow cells from CD45.2\(^+\) \textit{Rasgrp1}\textsuperscript{Anaef} or CD45.2\(^+\) WT donors. The percentage of CD45.2\(^+\) cells amongst the indicated thymocyte subsets was determined in 2 separate experiments 8-10 weeks after reconstitution. \textit{(d)} Total numbers splenic CD4\(^+\) and CD8\(^+\) cells from 5 week-old versus 12 week-old \textit{Rasgrp1}\textsuperscript{Anaef} mice and age matched B6 wildtype controls. (n= 4 mice) * \(p < 0.05\) \textit{(e)} Thymocytes from \textit{Rasgrp1}\textsuperscript{Anaef} or WT non-transgenic mice on the B10.BR (H-2\(^k\)) or B6 (H-2\(^b\)) backgrounds were stained for CD4, CD8 and V\(\beta\)11 surface expression and the percent V\(\beta\)11\(^+\) cells amongst CD4 SP and CD8 SP populations were enumerated and plotted. \textit{(f)} Splenocytes from \textit{Rasgrp1}\textsuperscript{Anaef} and WT mice were stained for CD4, CD25 and FoxP3 expression. Percentages of CD4\(^+\) FoxP3\(^+\) cells are shown (left). Total numbers of \textit{Rasgrp1}\textsuperscript{Anaef} and WT CD4\(^+\) FoxP3\(^+\) cells are shown on the right.
wild-type controls (Fig. 3.4e). This result contrasts to the compensatory increased frequency of self-superantigen recognizing Vβ11+ thymocytes observed in mice carrying Zap70 alleles that are incrementally impaired (Siggs, Miosge et al. 2007). Since hypomorphic Zap70 alleles also impair thymic regulatory T cell (Treg) formation (Siggs, Miosge et al. 2007), we analyzed the frequency and number of Foxp3+ CD4 cells and found that these were not diminished by the Rasgrp1Anaef mutation, but instead were increased (Fig. 3.4f). Collectively, these data indicate that the severe biochemical defect observed with the Rasgrp1Anaef allele in vitro (Fig. 3.3) is efficiently compensated in vivo (Fig. 3.4).

Analysis of Rasgrp1Anaef TCR transgenic mice reveals mildly decreased positive selection but intact negative selection

One mechanism of compensation for defective thymocyte signaling molecules is through selection of TCRs with higher affinities for MHC/peptide (Starr, Jameson et al. 2003). To test this possibility we bred the Rasgrp1Anaef mutation into three different TCR-transgenic backgrounds. Bone marrow chimeras were prepared by reconstituting H2k mice with wildtype or Rasgrp1Anaef bone marrow bearing the MHC II-restricted 3A9 TCR transgene. In non-transgenic recipients, which provide an environment permissive to positive selection, there was no effect of the Rasgrp1Anaef allele on the number of DP cells (Fig. 3.5a, 3.5b) but there was a 6-fold reduction in the number of CD4+ SP Foxp3- thymocytes expressing the transgenic 3A9 αβ TCR (TCR3A9) (Fig. 3.5a, 3.5c). This 6-fold decrease in positive selection contrasts with the 50-fold decrease in TCR3A9 SP cells caused by the mildest of the Zap70 alleles (Siggs, Miosge et al. 2007). A modest, cell
Figure 3.5 – Modestly impaired positive selection but intact negative selection of Rasgrp1<sup>Anaef</sup> thymocytes expressing the TCR<sup>3A9</sup>

(a) Thymocyte selection was examined on the TCR<sup>3A9</sup>-transgenic backgrounds, which is positively selected on MHC II and negatively selected in mice expressing hen egg lysozyme (HEL) in the thymus. Lethally irradiated recipient mice with or without Aire-dependent thymic expression of HEL under the insulin promoter (Akkaraju, Ho et al. 1997; Liston, Lesage et al. 2003) were reconstituted with Rasgrp1<sup>Anaef</sup> or WT bone marrow (both on the 3A9 TCR background). Thymocytes were stained for CD4, CD8, Foxp3 and the “clonotypic” antibody (1G12) specific for the 3A9 αβ TCR (denoted TCR<sup>3A9</sup> on the figure). Representative plots (left) and total cell counts (right) of (b) DP cells, (c) CD4SP Foxp3<sup>−</sup> TCR<sup>3A9+</sup> cells and (e, f) CD4SP Foxp3<sup>+</sup> TCR<sup>3A9+</sup> cells are shown (n= 4, 5, 8 and 8 mice per group, as listed from left to right on the figure). Percentages of Foxp3<sup>+</sup>/Foxp3<sup>−</sup> CD4<sup>+</sup> SP cells emerging in the presence of HEL were calculated and plotted in (f). Numbers indicate the percentage of cells inside the gate or quadrants. Student’s t tests P value symbols: ** p = 0.001 to 0.01; * p = 0.01 to 0.05. n.s. = no statistical difference. (d) Lethally irradiated CD45.2<sup>+</sup> B10.BR mice (lacking HEL expression) were reconstituted with a mixture of 3A9 TCR transgenic bone marrow cells from CD45.2<sup>+</sup> Rasgrp1<sup>Anaef</sup> and CD45.1<sup>+</sup> WT donors. The percentage of CD45.2<sup>+</sup> cells amongst the indicated thymocyte subsets was determined 8-10 weeks after reconstitution. Paired t tests P value symbol: * = 0.02.
autonomous defect in positive selection was confirmed by analyzing competition between TCR^{3A9} thymocytes with wild-type and mutant Rasgrpl in mixed bone marrow chimeras (Fig. 3.5d). In the mixed chimeras, the contribution of the Rasgrpl^{Anaef} cells to the CD4^{+} SP population was decreased ~50% relative to their contribution to the DP population of the same animals.

In a negatively selecting environment, namely in recipients expressing the TCR^{3A9} cognate ligand hen egg lysozyme (HEL) in AIRE\(^{+}\) medullary thymic epithelial cells, there was a 6-fold decrease in the number of Rasgrpl^{Anaef} CD4^{+} SP Foxp3\(^{-}\) TCR^{3A9+} thymocytes compared to the numbers in a positively selecting environment (Figs. 3.5a and 3.5c). This was comparable to the magnitude of negative selection observed in TCR^{3A9+} thymocytes with wild-type RasGRP1, and is consistent with the evidence of normal negative selection of V\(\beta\)11\(^{+}\) cells above. The number of Foxp3\(^{+}\) CD4^{+} SP TCR^{3A9+} thymocytes formed during negative selection was not significantly decreased by the Rasgrpl^{Anaef} allele, whereas it was decreased during positive selection (Figs. 3.5a and 3.5e). As a result, among all CD4^{+} SP TCR^{3A9+} thymocytes with high avidity for self antigen (HEL) an increased proportion expressed Foxp3 (Fig 3.5f). Taken together, we conclude that the Rasgrpl^{Anaef} allele causes a qualitative defect on selection of thymocytes: decreasing positive selection modestly, increasing the proportion of high-avidity Foxp3\(^{+}\) Tregs, but leaving negative selection of intact.

The Rasgrpl^{Anaef} allele also resulted in an approximately 50% decrease in positive selection of CD8^{+} SP thymocytes in female mice expressing the MHC I-restricted HY
TCR (Fig. 3.6a, b) and of CD4+ SP thymocytes in mice expressing the MHC II-restricted OTII TCR, transgenic for TCRα and β chains (Fig. 3.6c, d). All positively selected CD4+ SP Rasgrp1Anaef thymocytes expressed the OTII TCR (Vβ5+ and Vα2+) and continued to do so as CD4+ T cells in the spleen (data not shown and Fig. A.4a). Rasgrp1Anaef HY+ male mice showed depletion of HY-TCR bearing DP thymocytes similar to wildtype controls, consistent with the previous finding that negative selection is intact in Rasgrp1−/− HY+ males (Dower, Stang et al. 2000; Priatel, Teh et al. 2002) (Fig. A.4b, c). Consistent with the 50% decrease in positive-selection into CD4+ SP cells, Rasgrp1Anaef OTII+ DP thymocytes showed an ~50% reduction in the percentage of TCRβhiCD69hi cells compared to wild-type OTII counterparts (Fig. 3.6e). Analyses of competitive mixed chimeras confirmed a partial cell-autonomous deficit within OTII− Rasgrp1Anaef thymocytes to mature from the DP to the CD4+ SP stage during positive selection (Fig. 3.6f), resulting in a lower contribution of Rasgrp1Anaef CD4+ T cells in spleen (Fig. A.4d). In contrast to the modest decrease in positive selection, both DP and CD4+ SP thymocytes from OTII+ Rasgrp1Anaef mice displayed a strong defect in P-ERK induction following in vitro TCR stimulation (Fig. 3.6g and h) or PMA stimulation (Fig. A.4e, A.4f).

Evidence for increased tonic signals in Rasgrp1Anaef double positive thymocytes

To investigate how relatively efficient positive selection of Rasgrp1Anaef DP thymocytes might occur in the context of a diverse TCR repertoire, we directly compared DP and SP thymocytes from wildtype, Rasgrp1−/−, and Rasgrp1Anaef mice for expression of cell surface markers that are induced by TCR signaling. Expression on DP and SP thymocytes
Figure 3.6 – Mildly impaired positive selection but severely impaired TCR-induced ERK phosphorylation in Rasgrp1^Anaef thymocytes expressing the OTII or HY TCR

(a,b) Thymocytes from Rasgrp1^Anaef or age-matched WT female mice with a restricted HY TCR were stained for CD4 and CD8 surface expression. Representative plots and total cell counts are shown (n=4 mice). * p < 0.05. (c,d) Thymocytes from Rasgrp1^Anaef or age-matched WT mice with a restricted OTII TCR were stained for CD4 and CD8 surface expression. Representative plots and total cell counts are shown (n=6 mice). Student’s t tests P value symbols: ** p < 0.005 , * p < 0.05. The OTII TCR is positively selected on MHC II and HY TCR is positively selected on MHC I in female mice and negatively selected in male mice expressing male antigen. (e) Surface expression of TCRβ and CD69 on double positive thymocytes in Anaef OTII^+ mice compared to age-matched WT OTII^+ controls. Dot plots and histograms are representative of 4 mice. Numbers indicate percentage of double positive thymocytes within the TCRβ^hiCD69^hi gate. (f) Competitive bone marrow reconstitution with Rasgrp1^Anaef OTII^+ and WT OTII^+ (B6) bone marrow into F1 mice from C57BL/6 x C57BL/6.SJL-C57.1 cross and thymuses analyzed 12 weeks after transplantation. The percentage of CD45.2^+ Rasgrp1^Anaef cells contributing to the CD4^+CD8^− (DN), CD4^+CD8^+ (DP), and CD4^+ (SP) thymocyte subsets was determined and plotted for three mice. * p < 0.05. (g, h) Thymocytes from Rasgrp1^Anaef OTII^+ or WT OTII^+ mice were stimulated with anti-CD3 antibody (5µg/mL) for the indicated time points and stained for intracellular p-ERK. P-ERK levels for CD4^+CD8^+ DP and for CD4^+ SP cells are shown. Representative examples of 3 independent experiments.
of the homing and adhesion receptor, CD44, depends on signaling by Rasgrp1 and mTOR. mTOR signaling is greatly reduced in Rasgrp1−/− knockout mice (Gorentla, Wan et al. 2011) and CD44 expression on DP and SP thymocytes and peripheral T cells is greatly decreased in mTOR-deficient mice (Gorentla, Wan et al. 2011) and in thymocytes from Rasgrp1-deficient mice (Priatel, Teh et al. 2002; Priatel, Chen et al. 2007) and Fig. 3.7f, g). By contrast, CD44 expression was increased on DP and SP thymocytes and peripheral naïve T cells from Rasgrp1Anaef mice (Fig. 3.7a, b). Staining for L-selectin (CD62L) to gate peripheral CD4 cells into naïve and activated/memory T cells showed that there was a homogeneous shift to higher CD44 on the naïve peripheral T cells similar to the shift observed in thymocytes (Fig. 3.7c). Analysis of mixed bone marrow chimeras containing wild-type and mutant T cells showed that the paradoxically elevated CD44 on Rasgrp1Anaef T cells reflected a direct, cell-autonomous effect of the mis-sense mutation (Fig. 3.7d, A.5a) and was not secondary to homeostatic processes that might occur when all of the T cells carry the Rasgrp1 defect. A substantial proportion of the paradoxical CD44 expression remained when the repertoire was fixed to the TCR^3A9 (Fig. 3.7e).

CD69 upregulation on DP and SP thymocytes is a measurement of Ras signals accumulated over time (D'Ambrosio, Cantrell et al. 1994), while CD5 measures tonic signaling by the pre-TCR and TCR (Swat, Dessing et al. 1993; Punt, Suzuki et al. 1996; Azzam, Grinberg et al. 1998). CD69 was diminished on DP and SP thymocytes from Rasgrp1−/− mice, while CD5 was not significantly altered in the knockout (Fig. 3.7g). By contrast, Rasgrp1Anaef DP thymocytes exhibited a small, but statistically significant,
homogeneous increase in CD69 and CD5 expression (Fig. 3.7g, A.5b). Like CD44, the elevated CD5 expression persisted in CD4$^+$ SP Rasgrp1$^{Anaef}$ thymocytes while the CD69 levels were comparable to wild-type (Fig. 3.7f, 3.7g). In contrast, the few Rasgrp1$^{-/-}$ thymocytes that develop into CD4$^+$ or CD8$^+$ SP (Fig. 3.4a) had decreased CD69 and CD44 but normal or slightly elevated levels of CD5 (Fig. 3.7f, 3.7g, A.5) (Priatel, Teh et al. 2002; Priatel, Chen et al. 2007). Thus Rasgrp1$^{Anaef}$ thymocytes demonstrate signs of increased tonic signals at the pre-selection DP stage and during SP cell differentiation that may compensate for defective TCR-Rasgrp1$^{Anaef}$ signals, resulting in effective maturation of Rasgrp1$^{Anaef}$ SP with persisting elevated CD44 expression post-selection.
Figure 3.7 – CD69, CD5, and CD44 expression levels indicate increased tonic signaling in Rasgrp1<sup>Anaef</sup> thymocytes.

(a-c) Analysis of CD44 expression on thymocyte subsets and CD4<sup>+</sup> splenocytes from wildtype and Rasgrp1<sup>Anaef</sup> mice. (b) The mean fluorescence of CD44 staining for each genotype is depicted with each dot representing an individual mouse, * p < 0.05 (c) Expression analysis for CD44 in CD62L<sup>Hi</sup> and CD62L<sup>Lo</sup> CD4<sup>+</sup> splenocytes. (d,e) Competitive bone marrow reconstitution with Rasgrp1<sup>Anaef</sup> and WT (B6) bone marrow on a diverse repertoire (d) or restricted by the TCR3A9 (e). Splenocytes are analyzed by FACS staining for CD44 expression in a CD4<sup>+</sup> or CD4<sup>+</sup> TCR<sup>3A9+</sup> gate. Each dot represents an individual mouse. CD44 MFI values in CD4<sup>+</sup> cells coming from the different CD45.1 and CD45.2 donors are graphed. ** p < 0.005 , * p < 0.05. Similar elevated MFI’s for CD44 were observed on Rasgrp1<sup>Anaef</sup> CD4<sup>+</sup> splenocytes that were gated for CD4<sup>+</sup>CD45<sup>+</sup>/LOW (Fig. S5a). (f, g) Contrasting CD69, CD5, and CD44 expression levels between thymocyte subsets of Rasgrp1<sup>Anaef</sup> and Rasgrp1<sup>−/−</sup> mice. Representative histograms are shown in panels 7f and S5b and S5c. Mean fluorescence intensities (MFIs) of CD69, CD5 and CD44 expression on DP, CD4<sup>+</sup> SP and CD8<sup>+</sup> SP thymocytes of wildtype (WT), Rasgrp1<sup>−/−</sup>, or Rasgrp1<sup>Anaef</sup> mice were determined. MFIs were normalized for expression in wildtype mice (set at 1.0) and plotted as difference compared to wildtype expression levels with statistical analysis. CD8<sup>+</sup> SP thymocytes are depicted in figures S5c and S5d. (n = 3 mice per group, representative of 2 independent experiments). ** p < 0.005 , * p < 0.05.
Discussion

Here we have characterized a novel mouse model \textit{Rasgrp1}^{Anaef} and provide the first \textit{in vivo} evidence that EF hands play a role in RasGRP1 GEF function. The Rasgrp1^{Anaef} mutation in EF$_2$ results in severely impaired phosphorylation of T$_{184}$ in RasGRP1, an activating modification induced by DAG signals. To date no RasGRP1 structure is known and the exact molecular mechanism of RasGRP1 activation via its EF$_2$ domain requires future investigation. Given that EF$_2$ binds to calcium \textit{in vitro} (Ebinu, Bottorff et al. 1998) and that calcium binding to EF hands often induces a conformational change (Gifford, Walsh et al. 2007), we hypothesize the \textit{Anaef} mutation might decrease efficient calcium binding to EF$_2$ and result in a loss of a conformational change that normally facilitates RasGRP1 phosphorylation on threonine 184. It is interesting to note that phosphorylation of RasGRP1 on T$_{184}$ increases its RasGEF activity but does not appear to be an absolute requirement for RasGRP1 activity (Roose, Mollenauer et al. 2005; Zheng, Liu et al. 2005). It is therefore probable that some function of RasGRP1^{Anaef} is preserved.

Given the profound biochemical defect of \textit{Rasgrp1}^{Anaef} in thymocytes, we were struck by the almost normal thymocyte development and selection on a diverse TCR repertoire. These results are in sharp contrast to the severely impaired thymocyte development in \textit{Rasgrp1}^{-/-} mice (Dower, Stang et al. 2000), due to a strong defect in positive selection (Priatel, Teh et al. 2002). Positive selection can occur over a large range of ligand affinities until a threshold in signals defines a switch in thymocyte cell fate, from positive to negative selection (Daniels, Teixeiro et al. 2006). The range of positively selecting
ligands allows for a level of flexibility during thymocyte selection but can also allow for unintentional selection of TCRs with high affinity for MHC/peptide as a mechanism to compensate for defective intracellular molecules (Starr, Jameson et al. 2003). Normally, any higher affinity T cells that have been positively selected on thymic cortical epithelium would be negatively selected in the thymic medulla. Decreased kinase activity of Zap-70 results in linked deficits in positive and negative selection, so that high affinity Vβ11+ thymocytes that recognize an endogenous retroviral self-superantigen escape deletion (Siggs, Miosge et al. 2007). In contrast, we observed normal deletion of Vβ11+ thymocytes and high affinity TCR3A9 thymocytes in the Rasgrp1Anaef mice, indicating that compensation in the formation of Rasgrp1Anaef SP thymocytes is not through selection of a repertoire with high-affinity TCRs that would normally have been deleted. The comparison between Rasgrp1Anaef thymocytes with a polyclonal TCR repertoire, where there was no decrease in SP thymocyte formation even under competitive repopulation conditions, and Rasgrp1Anaef thymocytes with three different fixed TCRs where there was a decrease in the efficiency of forming SP cells, indicates that part of the compensation must involve selection of different TCRs. However, part of the compensation for the profound TCR signaling defect must occur within the context of a fixed TCR because there was only a modest cell autonomous decrease in TCR3A9 or TCROTII CD4+ SP cell formation caused by the Anaef mutation. This modest effect on positive selection contrasts with the near complete block in TCR3A9 positive selection caused by a much more subtle deficit in TCR signaling caused by the mildest Zap70 hypomorphic mutations (Siggs, Miosge et al. 2007).
A key to the process of compensating for the EF-hand mutation comes from the opposite effects of the Anaef and knockout RasGRP1 mutations on CD44 induction in DP and SP thymocytes, which is increased by the Anaef mutation but greatly decreased by the RasGRP1 knockout. Expression of CD44 on T cells is induced by the mammalian target of rapamycin (mTOR) – S6 kinase signaling pathway, as it is greatly reduced in animals with reduced mTOR (Zhang, Readinger et al. 2011) (CCG and A. Enders, unpublished data). Absence of RasGRP1 protein results abolishes TCR-induced mTOR activation (Gorentla, Wan et al. 2011) and greatly decreases CD44 expression (Priatel, Teh et al. 2002; Priatel, Chen et al. 2007). The opposite effect of the Anaef mutation indicates that this aspect of RasGRP1’s function is not only retained but is enhanced by the EF-hand mutation. Enhancement of RasGRP1-induced CD44 is not explained by selection for higher affinity TCRs because it is observed on all DP thymocytes (not just the CD69+ subset) and on T cells bearing a fixed TCR3A9.

The small, but statistically significant, increase in CD69 and CD5 expression in a homogenous pattern on CD4+CD8+ DP Rasgrp1Anaef T cells is indicative of an increase in tonic, pre-selection signals in the entire population of DP cells. Interestingly, similarly modest but uniform shifts in CD5 expression have been observed for DP thymocytes with impaired function of negative regulators of TCR signaling. Examples are a wedge-mutant of the phosphatase CD45 (Hermiston, Zikherman et al. 2009) or deletion of the kinase Csk (Zikherman, Jenne et al. 2010) that both result in loss of negative regulation of Src family kinases. Similarly increases in CD5 expression are seen in mice deficient for the Src-like adapter molecule (SLAP) (Sosinowski, Killeen et al. 2001) and the E3 ubiquitin
ligase c-Cbl (Naramura, Kole et al. 1998) that regulate levels of TCR expression. Based on our results and the abovementioned studies, we postulate that Rasgrp1 also has an unexpected negative regulatory role. It is possible that RasGRP1, through its EF-hand, engages a negative feedback loop that dampens low level tonic or positive selection signals from the TCR. In this view, the EF-hand mutation in Rasgrp1<sup>Anaef</sup> impairs the negative feedback loop in addition to impairing the role of RasGRP1 in supporting high ERK phosphorylation when TCRs are strongly stimulated. A “one step forward, one step backward” defect of this nature could increase tonic and low level TCR-induced ERK and mTOR signaling sufficiently to support positive selection and higher CD44 expression, while not allowing the high ERK phosphorylation that occurs when TCRs are strongly crosslinked. How RasGRP1 integrates differences in TCR signal strength, and whether this is governed in part by the EF-hand domain, are important problems for further research. The concept of Rasgrp1<sup>Anaef</sup> as a faulty off-on switch would fit with our data and focuses attention on the need for in-depth studies of RasGRP1 dynamics to understand thymocyte selection.

Our new Rasgrp1<sup>Anaef</sup> mouse model adds to an emerging category of mouse models with point mutation versions of TCR signaling proteins, along with Zap70<sup>skg</sup> (Sakaguchi, Takahashi et al. 2003), Zap70<sup>murdock</sup> and Zap70<sup>mrdless</sup> hypomorphic alleles (Siggs, Miosge et al. 2007), LAT<sup>Y136F</sup> mice (Aguado, Richelme et al. 2002; Sommers, Park et al. 2002), and Card11<sup>unmodulated</sup> mice (Jun, Wilson et al. 2003), that demonstrate a partial deficit in T cell signaling that precipitates autoimmunity or allergy. Given the huge number of missense variants in each person (Project 2010), patients with autoimmune diseases are more
likely to have point mutations in various genes than complete loss of gene expression. We detected anti-nuclear antibodies at an early age (15 weeks) in RasgrplAnaef mice on a C57BL/6 background. In the case of complete Rasgrpl deficiency, some T cells develop and become self-reactive in the periphery and aged Rasgrpl−/− mice demonstrate splenomegaly and the production of anti-nuclear antibodies (Layer, Lin et al. 2003; Coughlin, Stang et al. 2005). Interestingly, the immune pathology is milder in Rasgrpl−/− mice on a pure C57BL/6 background (Chen, Priatel et al. 2008) and disappears when mice are additionally deficient for Rasgrp-3 (Coughlin, Stang et al. 2005), the Rasgrp isoform most highly expressed in B cells (Stone 2011). Splice variants of RasGRP1 have been documented for patients with SLE (Yasuda, Stevens et al. 2007) and abnormal microRNA-driven downregulation of RasGRP1 expression may play a role in aberrant DNA methylation in Lupus CD4+ T cells (Pan, Zhu et al. 2010). In addition, SNPs in RasGRP1 have been described in genome-wide association studies of autoimmune diabetes and thyroid disease (Qu, Grant et al. 2009; Plagnol, Howson et al. 2011). We propose that RasgrplAnaef mice may provide a useful model system for further studies to help elucidate how RASGRP1 variants contribute to autoimmune disease, and to help target future efforts to modulate this pathway pharmacologically.
Materials and Methods

Mice

Mice were housed in pathogen-free conditions and experiments approved by either the Australian University Animal Ethics and Experimentation Committee (Goodnow group) or the Institutional Animal Care and Use Committee of the University of California, San Francisco (Roose group, AN084051-01). C57BL/6, C57BL/6.SJL-CD45.1, RAG 1/2\(+\), B10Br, B10Br.CD45.1, B10Br TCR\(^{3\alpha9}\), B10Br.insHEL and B10Br TCR\(^{3\alpha9}\).insHEL mice were obtained from ANU Bioscience Services. C57BL/6-TgN(OT-II.2a) and C57BL/6-HY_TCRtg were obtained from the Arthur Weiss Lab.

ENU mutagenesis

The RasGRP1\(^{Anaef}\) strain was established through Ethylnitrosourea (ENU)-mediated mutagenesis of B6 mice at the Australian National University as previously described (Randall, Lambe et al. 2009). Once identified, the RasGRP1\(^{Anaef}\) strain was backcrossed onto B6 for three additional generations.

Genetic mapping of the Anaef mutation.

Affected RasGRP1\(^{Anaef}\) mice were crossed onto the CBA/J background to generate heterozygous F1 mice. F1 mice were intercrossed to yield mice homozygous for the Anaef mutation and carrying a mix of C57BL/6 and background CBA/J single nucleotide polymorphisms (SNPs). Genomic DNA isolated from both affected and unaffected mice was used as template for SNP mapping at the Genomics Institute of the Novartis Research Foundation (San Diego, CA). SNP markers were spaced approximately every
3-5 Mbp throughout the genome. Once a defined interval was established, the RasGRP1 encoding gene was sequenced from genomic DNA from both affected Anaef and WT mice. All exons were amplified by PCR with primers spanning the whole exon and the flanking intronic regions. A single point mutation was identified in exon 13.

**Genotyping**

Roose lab Anaef mice were genotyped using MS-PCR. Primers were combined in a single reaction with Taq, Taq buffer and dNTPs (all New England BioLabs). Goodnow lab Anaef mice were genotyped by APF Genomics Services. Genotyping followed the manufacturer’s instructions for Amplifluor PCR (SNP FAM/JOE; Millipore).

**Transfections and stable cell lines**

Transfections and creation of stable cell lines was performed as previously described in Roose et al., 2005 (Roose, Mollenauer et al. 2005).

**Antinuclear antibody testing**

Diluted mouse plasma was applied to HEp-2 slides (Inova, USA). AlexaFluor488-conjugated goat anti-mouse IgG (Invitrogen) was added and slides mounted with Dako fluorescence mounting medium. Photos were taken using an Olympus IX71 microscope and WIB filter with 20x lens and exposure time of 1/25 seconds.
Cell Surface Flow Cytometry Stainings:

Thymocytes and splenocytes from RasGRP1^{Anael} mice and age-matched wild type controls were stained for: CD44, CD4, CD45.1, CD 45.2, CD5, CD69, TCRb, CD25, OTII TCR, HY TCR (all BD Pharmingen), CD8, (UCSF Monoclonal Antibody Core, clone YTS169.4). Flow cytometry was performed on a FACSort (Becton Dickinson). 3A9^{+} TCR cells were detected using a custom 1G12 monoclonal antibody, a biotinylated secondary (A85.1) and streptavidin Qdot605 (Invitrogen) tertiary marker. For intracellular Foxp3, cells were fixed and permeabilized using a Foxp3 staining kit (eBioscience), then labeled with anti-Foxp3 (FJK-16s). For Vβ deletion assay, 30x10^6 cells from each H2k/H2b mouse were stained using biotinylated Vbeta5.1/2 and Vbeta11 (both BD Pharmingen) and and streptavidin Qdot605 (Invitrogen) tertiary marker.

Cell Stimulations:

Cells were stimulated using 25ng/mL (HIGH), 5ng/mL (MED), or 2ng/mL (LOW) PMA (Calbiochem) with/without ionomycin (10μM, Sigma). To mimic TCR engagement, cells were pre-labeled using anti-CD3 primary antibody (10μg/mL, UCSF Monoclonal Antibody Core, clone 2C11). Crosslinking was achieved using goat anti-hamster antibody (10μg/mL, Jackson ImmunoResearch). Overnight stimulations of CD4^{+} T cells were performed using plate-bound anti-CD3 antibody. Plates were pre-incubated with 0.2μg/mL, 0.4μg/mL and 1.6μg/mL antibody overnight and cells applied at 37°C for an additional 16 hour incubation before CD69 expression was assessed by flow cytometry.
**Calcium Flux:**

Thymocytes from age-matched wild type and RasGRP1\textsuperscript{Anaef} mice were mixed 1:1 with congenically marked wild type thymocytes. Cell mixtures were labeled with Indo-1 AM (Invitrogen) as per manufacturer’s instructions and anti-CD3 antibody and stained for CD4, CD8 and CD45.1/2 (all BD Biosciences). Cells were warmed to 37°C and baseline measurements collected for 30 seconds. Crosslinking was achieved using goat anti-hamster antibody (30µg/mL, Jackson ImmunoResearch). Measurements were taken using an LSRII Flow Cytometer (BD Biosciences) for a total of 6 minutes at 37°C.

**Intracellular Flow Cytometry Stainings:**

Procedure was performed as described in Das et al., 2009 (Das, Ho et al. 2009). Cells were fixed using Cytofix Cell Fixative (BD Biosciences). Cells were permeabilized using 90% Methanol. Primary staining for phospho-Erk occurred using rabbit anti-mouse p-Erk antibody (Cell Signaling, #4377S) followed by staining with goat anti-Rabbit PE (Jackson ImmunoResearch).

**Ras Pulldown**

Activation of Ras was analyzed using a RasGTP pulldown assay (Upstate) as previously described (Roose, Mollenauer et al. 2005).

**Western Blotting:**

Cells were lysed using Nonidet-P40 lysis buffer (1%) supplemented with protease and phosphatase inhibitors. Lysates were run on 10% acrylamide Bis-Tris gels and
transferred onto PDVF filter (Millipore, Immobilon-P). Blots were probed for RasGRP1 (Santa Cruz, M199), Alpha tubulin (Sigma), phospho-tyrosine (In house antibody prep, clone 4G10), phospho-Zap70 (Y493; Cell Signaling), phospho-PLCγ (Y783; Cell Signaling) and phospho-Lck (Y416; Cell Signaling). Rabbit anti- human RasGRP1 (clone E80) was produced by Epitomics, Inc. (Burlingame, CA, USA). Mouse anti-RasGRP1 p-T184 (clone JR-pT184RG1-4G7) was produced by AnaSpec (Fremont, CA, USA). Signal from primary antibodies detected using HRP conjugated secondary antibodies: Sheep anti-mouse HRP (GE Healthcare) and goat anti-rabbit HRP (SouthernBiotech). Blots were developed using Pierce ECL Western Blotting Substrate (ThermoScientific) and images recorded using a chemiluminescence imager (Fuji, LAS-4000).

**Bone Marrow Chimeras**

Bone marrow was collected and depleted of T cells and NK cells by magnetic labelling using biotinylated anti-TCRβ and anti-NK1.1 and streptavidin microbeads before passage through a MACS LD column (Miltenyi Biotec). 2x10^6 cells were transferred into lethally irradiated recipients. In 100% bone marrow chimeras, marrow was transferred into HEL transgenic and non-transgenic mice. In mixed bone marrow chimeras, a 50:50 mix of congenically marked mutant or wild type bone marrow was transferred into non-transgenic recipients.
CHAPTER 4

Insights into RasGRP1 regulation and beyond
In the previous chapters, I have shown how changes in the way RasGRP1 protein is posttranslationally modified or changes in the primary amino acid sequence of RasGRP1 can affect its regulation and by extension, T cell function or development. While much has already been covered, I would like to highlight a few future experiments relating to both projects that I feel would further our knowledge of RasGRP1 regulation and T cell mediated disease.

**Future experimental directions for RasGRP1 posttranslational modification projects**

As with all projects in this stage of completion, the results leave something to be desired. While these experiments suggest that posttranslational modification of RasGRP1 will prove biologically interesting, much of the work remains to be done. Accordingly, experiments have already begun to elucidate the mechanisms and function of ubiquitination of RasGRP1. Using a denature/renature protocol (i.e., using an antibody to IP RasGRP1, denaturing the proteins in the sample to disrupt protein bonding pairs, allowing the proteins to refold and re-IPing for RasGRP1) Evan Markegard in the Roose lab has determined that RasGRP1 may be monoubiquitinated. This type of ubiquitin linkage suggests a role for ubiquitin that is not as a proteosomal degradation signal but may instead serve as an intracellular sorting signal or ubiquitin binding domain (UBD)-containing protein recognition site (Grabbe and Dikic 2009). UBD-containing proteins continue to be discovered (acting in addition to known immune regulatory A20 and c-Cbl proteins) and the protein structure of ubiquitin can provide a multitude of binding surfaces that may make ubiquitin signaling more diverse and flexible than the more
characterized phosphorylation cascades (Dikic, Wakatsuki et al. 2009). It is interesting to consider that RasGRP1 could use this ubiquitination event to translate signal downstream (perhaps feeding into the unexpected negative regulatory role for RasGRP1 suggested using Anaef mice discussed in Chapter 3). Furthermore, many of these UBD-binding proteins are implicated in the endocytosis pathway. Either function could ultimately serve to terminate RasGRP1 activity at the membrane without involvement of the proteosomal pathway.

Work by Steve Bunnell and Larry Samelson’s groups has focused on the formation of clusters containing LAT, ZAP-70 and SLP-76 at the plasma membrane after TCR engagement (Bunnell, Singer et al. 2006; Sylvain, Nguyen et al. 2011). Under normal conditions, it appears that these clusters move toward the center of the point of engagement and are eventually endocytosed into the cell (Bunnell, Singer et al. 2006). Strikingly, while these clusters form normally in the absence of PLCγ, T cells are not activated (Bunnell, Singer et al. 2006). The authors suggest a “critical factor” is missing, it seems possible this factor is RasGRP1. Since these clusters are commonly endocytosed, it remains possible that RasGRP1 is normally transported along with these other complexes into the endocytic pathway of the cell. It would be interesting to develop a tagged version of RasGRP1 and use similar microscopy techniques to visualize this movement and determine if ubiquitination of RasGRP1 plays an active role in this process.
Conversely, further work is needed to determine if RasGRP1 can use an ubiquitin to bind other proteins at the membrane and if this binding plays a role in anchoring RasGRP1 to the site of activity or otherwise enhances activity. If so, RasGRP1 may one day serve as a target for drugs like the peptidomimetic inhibitors recently developed against inhibitors of apoptosis (IAPs) and B cell lymphoma 2 (Bcl2) (Dikic et al, Nat Rev Immunol 2009). Disruption in this way might then contribute to the treatment of TALL/L with high levels of RasGRP1 activity.

Of course, phosphorylation sites remain of interest and the availability of reagents makes probing this possibility relatively simple. Previous work by Andre Limnander while in the Weiss lab uncovered a role for phosphorylation of RasGRP1 at serine 332 in the P-Erk induction downstream of store-operated Ca\(^{++}\) entry (SOCE) in B cells (Limnander, Depeille et al. 2010). Further experiments using both the ablation (S332A) mutant and phosphomimetic (S332D) mutant tranfected into RasGRP1 deficient Jurkat could determine if this phosphorylation event occurs in T cells, if this event is biologically relevant, and if it contributes to RasGRP1 degradation. Generation of an antibody against this phosphorylated residue in RasGRP1 could also be useful to examining this phenomenon in primary cells.
Areas for needed technical improvements in RasGRP1 posttranslational modification projects

The area I believe still hold the most potential for a willing researcher involves the use of 2D gels to further investigate these “spots” as the Roose lab so lovingly calls them. Firstly, the amount of RasGRP1 protein on the 2D gels can likely be increased by some method and the resolution improved. Unfortunately, 2D electrophoresis requires specific conditions that all change depending on the properties of the protein you want to observe, the number of strips you are trying to resolve at a given time and how well you were able to remove ionic contaminants from your sample. I spent a good deal of time trying my best to optimize conditions for 2D gel analysis of RasGRP1. I am certain that I have not yet perfected it. If I were to be continuing with the project, I would first optimize the “enrichment” step, namely, the immunoprecipitation of RasGRP1. As the immobilized pH gradient gels are limited in the total amount of protein that can be loaded, it is wasteful to take up that space with unwanted protein, e.g., the IP antibody. Furthermore, if the data regarding SKAP55 turns out to be particularly relevant, its size at 55kD is likely to be obscured by this large band of Ig protein (Kosco, Cerignoli et al. 2008). Therefore, I would highly recommend performing the IP step using antibody that is chemically conjugated to the beads so that any protein that proceeds into the cleaning step is RasGRP1 or something that binds RasGRP1 in vitro.

Since the isoelectric point of RasGRP1 is predicted to be around 7, it should also be possible to increase the resolution of the 2D gels by using immobilized pH gels with a
narrower range of pH, e.g., pH 4-7. Changing the pH of the strip will necessitate another round of optimization but will likely be worth the effort. Lastly, what I regret most in placing this project aside to pursue the more immediately fruitful thymocyte development project is that I was never able to use any inhibitors during my stimulations nor did I transfer these 2D gels to Western blot membranes for probing with HRP-conjugated antibodies (against RasGRP1, RasGRP1 p-T184 or others). Continued use of 2D gels combined with these efforts are likely to provide some very intriguing data.

A major caveat to most of the work described in Chapter 2 is that the best data come from a mouse cell line known to overexpress RasGRP1 protein, 1156O. At the time that I joined the Roose lab, a reliable antibody recognizing the human form of RasGRP1 was not available. As such, even though I would perform experiments using the more widely recognized human cell line for biochemical analysis, Jurkat, I was not able to consistently obtain meaningful results. Since that time, the Roose lab has generated and tested a monoclonal antibody capable of specifically recognizing human RasGRP1 protein (E80). Future work with this project will surely use Jurkat cells and all experimental conditions will need to be optimized for this (dosage of drugs, rest times before stimulation, etc.)

Differences in total half-life between the pulse-chase RasGRP1 radiolabel experiment and the previous basal stability experiment are likely due to differences in experimental set up (see Materials and Methods). The washing step, as opposed to the dilution of cells in media 1) takes more time and 2) introduces large fluctuations in temperature. In addition, these cells needed to be rested and labeled using primary antibody prior to
stimulus. All of these factors increased the time between the end of metabolic labeling and the actual moment of lysis. A repeat of the basal state analysis using the same technique for preparing the pulse-chase samples might shed light on this question.

**Future Directions RasGRP1*Anaef* mice**

While it is clear that there are positive selection defects in these mice, it remains a bit of a puzzle as to how these thymocytes are able to make it through the thymus and into the periphery with such a low level of P-Erk induction. My experiments show that both the double positive and single positive thymocytes in *Anaef* mice displayed severe defects in P-Erk production after stimulation. Previous experiments with RasGRP1/-/- mice with forced expression of a “strong” TCR, 2C, found that while double positive thymocytes were defective in P-Erk induction, the single positive thymocytes were normal in their P-Erk induction (Priatel, Teh et al. 2002). The authors state that this is likely due to the use of an alternate pathway to upregulate P-Erk in those cells that managed to be positively selected, likely aided by this high affinity TCR. Intriguingly, RasGRP1 *Anaef* mice do not appear to select for higher affinity TCRs and produce these “normal” single positives capable of high P-Erk induction (Chapter 3). Their single positive thymocyte remain hypo-responsive even once they become naïve T cells in the periphery. Instead, it appears that they have “just enough” P-Erk signal to be positively selected, but while this threshold is met to create T cells, it comes at a cost (namely, the production of autoantibodies at a later time). Our data using selection markers suggests an increase in tonic, pre-selection signals as the result of a loss of negative regulation via RasGRP1 and increased mTOR signaling. It would be most interesting to look at thymocyte
development both pre- and post-selection using an allelic series of RasGRP1: RasGRP1\(^{+/+}\), RasGRP1\(^{+/−}\), RasGRP1\(^{+\text{Anaef}}\), RasGRP1\(^{\text{Anaef}/\text{Anaef}}\), RasGRP1\(^{\text{Anaef}−}\), and RasGRP1\(^{−/−}\) to further probe where this threshold lies.

During positive and negative selection, thymocytes also receive signals from other cell surface receptors such as integrins and the co-stimulatory molecule, CD28 (Starr, Jameson et al. 2003). In addition, surface receptors are often internalized and recycled after stimulation (Bunnell, Singer et al. 2006). In the experiments using TCR crosslinking antibodies on RasGRP1\(^{\text{Anaef}}\) thymocytes it was assumed that this type of stimulus would result in the same cellular response as stimulation of thymocytes using selecting peptide-MHC on the surface of thymic epithelial cells. To examine the possibility that thymocytes from RasGRP1\(^{\text{Anaef}}\) mice may experience signal differently when stimulated using peptide-MHC, experiments using CD4\(^+\)T cells co-cultured with antigen-loaded antigen presenting cells (followed by FACs or Western blot for P-Erk induction) could be performed. However, this experiment is also limited by the fact that developing thymocytes and mature T cells can react differently to the same peptide (Ignatowicz, Kappler et al. 1996). Breeding of the RasGRP1\(^{\text{Anaef}}\) mice into the OT I TCR background and using fetal thymic organ culture to test a degenerate series of selecting peptides and tetramers (like those used by Ed Palmer’s group (Daniels, Teixeiro et al. 2006)) would be a useful start to addressing this question in thymocytes. If RasGRP1\(^{\text{Anaef}}\) thymocytes are positively and negatively selected using the same range of peptide affinities as wildtype thymocytes, it would suggest that there are indeed differential effects of TCR
crosslinking and peptide-MHC binding that were not revealed during the previous experiments.

While RasGRP1 Anaef mice were selected based, in part, on their production of anti-nuclear antibodies, it remains unclear what contributions T cells play in the phenotype. Intriguingly, older RasGRP1/− also develop autoantibodies but this is prevented in mice doubly deficient in RasGRP1 and RasGRP3 (Layer, Lin et al. 2003; Coughlin, Stang et al. 2005). As B cells also express some RasGRP1 protein along with RasGRP3, it is likely that this phenotype requires both immune cell types. It is also possible that additional cell types contribute as well. In addition, there appears to be an expansion of both B cells and T cells over time, suggesting that T cells are at least somewhat involved in what appears to be a minor autoimmune response. Studies examining RasGRP1 in systemic lupus erythematosus (SLE) have documented splice variants not present in healthy individuals and aberrant micro-RNA control of RasGRP1 may also play a role in aberrant DNA methylation in these patients (Pan, Zhu et al.; Yasuda, Stevens et al. 2007). Taken together with the mouse data using RasGRP3 knockouts, it seems as though RasGRP1 and RasGRP3 may interact or participate in a balance of signal that has not been previously appreciated. Carefully controlled cell transfer experiments (co-injection of RasGRPAnaef and wildtype B and T cells) might be able to address this question. As RasGRP1 Anaef mice exist on the B6/C57 background that is normally resistant to the development of overt autoimmunity, it might also be worth while to backcross these mice onto more susceptible backgrounds (Perry, Sang et al. 2010).
There is also anecdotal evidence that RasGRP1\textsuperscript{Anaef} mice develop enlarged lymph nodes and large amounts of scar tissue in response to minor skin irritations as they age. Although it was never quantified and did not seem to lead to early morbidity, it seems as though this situation (in which multiple immune cell types are interacting) was aberrantly controlled and could be interesting in following up on. The additional use of infection models that are TH1-mediated versus TH2-mediated would provide further insight to peripheral T cell function in these mice, particularly given the TH2 skewing phenotype of the Lat\textsuperscript{Y136F} mice (Aguado, Richelme et al. 2002; Sommers, Lee et al. 2005). Since these mice cannot recruit PLC\textgreek{y} to the membrane to produce DAG, it is likely that many of the defects seen in this mouse are directly related to the lack of RasGRP1 function. Followup in the RasGRP1\textsuperscript{Anaef} mice should examine whether a similar skewing phenotype exists in the periphery of these mice and if this affects their cytokine production and immune responses to infections.

On a similar note, the phenotype of CD4\textsuperscript{hi} CD4\textsuperscript{+} T cells that appears to be mostly TCR-independent remains to be examined (Chapter 3). Our studies suggest that there is an undiscovered negative regulatory role played by RasGRP1. CD44 expression is known to be mediated by mTOR-S6 signaling in T cells (Zhang, Readinger et al. 2011). Knockout of RasGRP1 eliminates mTOR activation downstream of the TCR (Gorentla, Wan et al. 2011) and decreased surface CD44 expression (Priatel, Teh et al. 2002; Priatel, Chen et al. 2007). This study using the RasGRP1Anaef mice displays an opposite effect, with CD44 expression increased on all T cells regardless of developmental stage or TCR transgene expression. This expression was not explained by the selection of high-affinity...
TCRs and led us to the idea that it is possible that *Anaef* mutation contributes to a “gain of function” in regard to its signaling toward mTOR. The mTOR pathway affects many other genes as well as CD44 and this increased signaling likely has broader implications on T cell biology that have been thus far underappreciated in these mice. As such, a study using pre-selection double positive thymocytes (wildtype, RasGRP1 knockout and *Anaef*) and microarray analysis should reveal genes that show decreased expression in the knockout scenario but are increased in *Anaef* cells. These genes are likely to be candidates that contribute not only to the CD44\textsuperscript{hi} phenotype but also the selection compensation mechanisms and, possibly, later B cell autoimmune involvement.

Of course, all of this work would be of use in informing researchers if a RasGRP1 inhibiting drug is indeed a viable biological option. As RasGTP has proven to be a difficult pharmacologic target, the search continues for a way to shut down aberrant Ras signaling in the disease state. Work in cancer cells seems to indicate that the targeting of RasGRP1 may be a viable option in some cases where RasGRP1 transcript is high and causing increased RasGTP levels (Hartzell In Revision). Further elucidation of posttranslational modifications would inform the development of that drug by providing points of interest that could be exploited by a small molecule inhibitor. However, my studies using the crippled RasGRP1 allele (RasGRP1\textsuperscript{*Anaef*}) that later seems to contribute to autoimmune disease suggest that chronic, but incomplete, blockage of RasGRP1 activity in such patients may lead to later complications and may not be helpful at all in treating T cell mediated autoimmune disease. If such a therapeutic route is explored, it will need to find the appropriate balance and weigh the costs and benefits.
APPENDIX
Supplemental Figures to Chapter 3
Figure A.1 – Mapping of the Rasgrp1 mutation in ENU mutant mice with anti-nuclear antibodies and CD44\textsuperscript{hi} phenotype and genotyping

(a) SNP analysis of ENU founder mice crossed with CBA/J mice. Phenotypes of affected mice tracked with a genomic region between 114-121.2 on chromosome 2. The Rasgrp1 gene lies in this interval.

(b) Genotyping of Rasgrp1\textsuperscript{Anaef/Anaef}, Rasgrp1\textsuperscript{Anaef/WT}, and wildtype mice in the Roose lab was performed using MS-PCR as previously described (Bottema and Sommer 1993; Rust, Funke et al. 1993). Products are wt: 202bp and R519G: 223bp.

(c) Genotyping of Rasgrp1\textsuperscript{Anaef/Anaef}, Rasgrp1\textsuperscript{Anaef/WT}, and wildtype mice using Amplifluor PCR in the Goodnow Lab.
**a**

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Region of Interest: 114-121.2 Mb

**Unaffected Littermates**

**Affected AnaEF**

**b**

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Program Cycle:

30'' 94°C
30'' 65.3°C
30'' 72°C
x 30 cycles

Primer Sequences:

R519G Forward -
5'AGGCTGCTCTCTTTCTCAC3'

R519G Reverse wild type -
5'TCATGAAGTAGGCCGTATCTCGTCGCT3'

R519G Reverse mutant -
5'TGGAATAGATGGAGCTGCCCTATGAAAGTGCCGTATCTCGATCC3'

**c**

Red Inputs (RasGRP1_Anaef allele)

Green Inputs (WT (+) allele)

Program Cycle:

10'' 95°C
20'' 60°C
40'' 72°C
x 35 cycles

Primer Sequences:

886004-1 -
5'GAAGGTGACCGATTTAGTATGCTGTAGCCGTATCTCGTCTCT3'

886004-2 -
5'GAAGGTGACCGATTTAGTATGCTGTAGCCGTATCTCGTCTCC3'

886004-4 -
5'CCTGACCTCAATGACCTGAAT3'
Figure A.2 – Biochemical aspects and early thymocyte development for Rasgr1Anaef.

(a) Ionomycin-induced P-ERK responses. The indicated stable cell lines were stimulated with ionomycin (1μM) for the indicated times and stained for intracellular p-ERK. Representative histograms are shown.

(b) Characterization of JR-pT184RG1-2G9 monoclonal antibody. Whole cell lysates (WCL) of RasGRP1 immunopercipitations with m133 monoclonal antibody of the indicated cell samples were subjected to western blot analysis with JR-pT184RG1-2G9 antibody. Note the induction of phosphorylated RasGRP1 in PMA-stimulated Jurkat cells and the reduction of this phosphor-RasGRP1 species in JPRM441 cells. Backblotting for total RasGRP1 levels was performed using a JR-E80-2 monoclonal antibody that recognizes human RasGRP1.

(c) Loading controls for the data presented in figure 3.3c. Levels of α-tubulin expression indicate equal protein loading of the WT and Rasgr1Anaef thymocyte samples. Our experience with the JR-pT184RG1-2G9 antibody has taught us that subsequent re-blotting for total mouse Rasgrp1 is problematic. In figure A.2c, the identical blot analyzed for P-T184-Rasgrp1 was stripped and re-blotted for total Rasgrp1 levels, indicating a seeming equal level of Rasgrp1 for WT and Rasgr1Anaef samples. However, when the same samples were run on a separate gel and directly blotted for Rasgrp1 we observed the 40% reduction in Rasgrp1 expression in Rasgr1Anaef samples as in figure 3.1i (not shown). Thus, the phospho-T184-Rasgrp1 blotting affects the subsequent total
Rasgrp1 blotting. We have observed a similar effect in the past for P-ERK and subsequent total ERK blotting of the same blot (Roose, Mollenauer et al. 2007).

(d,e) Normal transition of Rasgrp1Anaef thymocytes from the CD4−CD8− double negative to CD4+CD8+ double positive stage.

(d) Thymocytes from 5 week-old Rasgrp1Anaef mice and age-matched WT mice were stained for surface expression of CD4 and CD8. Electronic gating on CD4−CD8− double negative thymocytes combined with CD25 and CD44 staining revealed normal developmental progression in both mouse models. Representative dot plots are shown (n=5 mice). Numbers in the quadrants correspond to relative percentages in cell populations.

(e) Lethally irradiated B6 WT mice were injected with bone marrow from Rasgrp1AnaEF or WT mice (B6) mixed with that from B6-SJL-CD45.1 WT mice. Recipients were sacrificed after 12 weeks and stained for CD4, CD8, CD45.1 and CD45.2. Panels show total cell numbers expressing CD45.1 on gated populations in the thymus. Note that there was an approximately four-fold lower number for Rasgrp1AnaEF CD4−8− double negative (DN) thymocytes but that this ratio remained roughly the same for CD4+8+ double positive (DP) cells, indicating that development of Rasgrp1AnaEF thymocytes, transitioning from the DN to DP stage is comparable to wild type.
a. Ionomycin Alone
Jurkat
JPRM441 (RasGRP1 deficient)
JPRM441 + WT RasGRP1
JPRM441 + R519G RasGRP1

% max fluorescence

P-ERK
0 minutes 5 minutes 30 minutes

KEY
1 minute

b. Blotting:
RasGRP1 IP
m133 antibody

RasGRP1 (JR-E80-2)
phospho-T184-RasGRP1
(JR-pT184RG1-2G9)

WCL
phospho-T184-RasGRP1
(JR-pT184RG1-2G9)

WT
Anaef
TCR stim (min.)
P-T184-Rasgrp1
Rasgrp1
P-PLCγ1
P-ERK
α-tubulin
d. CD4^+^CD8^+^ Double negative thymocytes

CD5^+^CD25^+^ CD25^+^CD25^+^

WT
Anaef

3.4x
3.8x

Competitive BM reconstitution

Double Negative

Double Positive

B6-CD45.2-Anaef
B6-CD45.2-WT
B6-SJL-CD45.1-WT
Figure A.3 – Analysis of splenic and lymph node T cells and total splenocytes in Rasgrp1Anaef mice.

(a) Analysis of CD4 and CD8 T cell populations in the spleen and lymph node from 5 week-old Rasgrp1Anaef mice and age-matched WT mice through surface expression of CD4 and CD8 markers. Numbers indicate the percentage of cells inside the drawn gate.

(b) Total splenic cell counts from 5 week-old (left) and 12 week-old (right) RasGRP1Anaef (white) and wildtype (black) mice. Differences in total splenic counts of RasGRP1Anaef mice were statistically significant at 12 weeks of age, n=4 mice per genotype; * p<0.05.
Figure A.4 – Analysis of OTII- and HY-TCR transgenic thymocytes and splenocytes from Rasgrp1<sup>Anaef</sup> mice.

(a) Analysis of Vα2 and Vβ5 expression from the transgenic OTII-TCR in CD4<sup>+</sup> splenocytes from Rasgrp1<sup>Anaef</sup> or WT mice.

(b) Thymocytes from Rasgrp1<sup>Anaef</sup> or age-matched WT male mice with a restricted HY TCR were stained for CD4 and CD8 surface expression. Representative plots (left) and total CD4<sup>+</sup>CD8<sup>+</sup> double positive cell counts (right) are shown (n=10 mice) and indicate no statistical (n.s.) difference.

(c) Expression analysis of the transgenic HY-TCR in CD4<sup>+</sup>CD8<sup>+</sup> thymocytes of male Rasgrp1<sup>Anaef</sup> or WT mice indicates equal expression for the two genotypes.

(d) Competitive bone marrow reconstitution with Rasgrp1<sup>Anaef</sup> OTII<sup>+</sup> and WT OTII<sup>+</sup> (B6) bone marrow into F1 mice from C57BL/6 x C57BL/6.SJL-CD45.1 cross. Thymuses were analyzed 12 weeks after transplantation and presented in figure 3.6f. The percentage of CD45.2<sup>+</sup> Rasgrp1<sup>Anaef</sup> cells contributing to CD4<sup>+</sup> splenocytes and splenic neutrophils was determined in three mice and plotted. Note the modest decrease for Rasgrp1<sup>Anaef</sup> CD4<sup>+</sup> splenocytes, similar to the decrease in CD4<sup>+</sup> SP thymocytes (figure 3.6f), whereas splenic neutrophils show no significant difference.
(d, e) Thymocytes from Rasgrp1<sup>Anaef</sup> OTII<sup>+</sup> or WT OTII<sup>+</sup> mice were stimulated with PMA (20ng/mL) for the indicated times and stained for intracellular p-ERK. Gating on CD4<sup>+</sup>CD8<sup>+</sup> (double positive) and CD4<sup>+</sup> (single positive) thymocytes is shown.
a) CD4^+ splenocytes

b) Anaef HY^+ μ^+ WT HY^+ μ^+ CD4^+CD8^+ thymocytes

b) Anaef HY^+ μ^+ WT HY^+ μ^+ CD4^+CD8^+ thymocytes

c) Anaef HY^+ μ^+ WT HY^+ μ^+ HY TCR

d) Spleen OTII^+ TCR

% CD45.2^+ Neutrophils

% CD4^+ cells

* n.s.

e) DP, OTII^+ Anaef WT

f) CD4 SP, OTII^+ Anaef WT

p-ERK
Figure A.5 – Expression analysis of CD44, CD69, and CD5 expression in OTII- and HY-TCR transgenic thymocytes and splenocytes from Rasgrp1<sup>Anaef</sup> mice.

(a) Data accompanying figure 3.7d. MFI’s for CD44 were determined on Rasgrp1<sup>Anaef</sup> CD4<sup>+</sup> splenocytes that were gated for CD4<sup>+</sup>/LOW. Competitive bone marrow reconstitution with Rasgrp1<sup>Anaef</sup> and WT (B6) bone marrow on a diverse repertoire. Splenocytes are analyzed by FACS staining for CD44 expression in a CD4<sup>+</sup> gate. Each dot represents an individual mouse. CD44 MFI values in CD4<sup>+</sup> cells coming from the different CD45.1 and CD45.2 donors are graphed. P values are indicated.

(b-d) Contrasting CD69, CD5, and CD44 expression levels between thymocyte subsets of Rasgrp1<sup>Anaef</sup> and Rasgrp1<sup>−/−</sup> mice. Data accompanying figures 3.7f and 3.7g. Mean fluorescence intensities (MFIs) of CD69, CD5 and CD44 expression on DP, CD4<sup>+</sup> SP and CD8<sup>+</sup> SP thymocytes of wildtype (WT), Rasgrp1<sup>−/−</sup>, or Rasgrp1<sup>Anaef</sup> mice were determined. MFIs were normalized for expression in wildtype mice (set at 1.0) and plotted as difference compared to wildtype expression levels with statistical analysis. (n = 3 mice per group, representative of 2 independent experiments). ** p < 0.005 , * p < 0.05.
**a** Competitive BM reconstitution

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**b** CD4+CD8+ Double Positive

### WT

- CD44
- CD69
- CD5

### WT

- CD44
- CD69
- CD5

**c** CD8+ Single Positive

- Anaef
- Rasgrp1-/-

### WT

- CD44
- CD69
- CD5

### WT

- CD44
- CD69
- CD5

**d** MFI of expression normalized to MFI in WT

- CD44
- CD69
- CD5

### Anaef

- CD44
- CD69
- CD5

### Rasgrp1-/-

- CD44
- CD69
- CD5
References


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