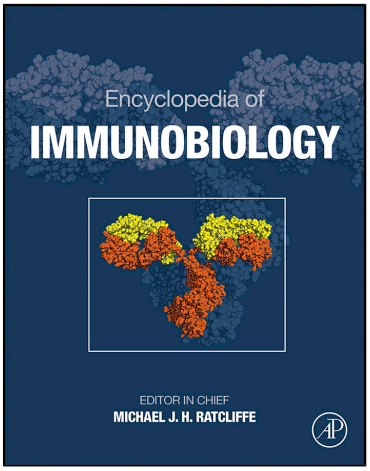

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Kinase and Phosphatase Effector Pathways in T Cells

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Abstract

Multiple interconnected effector pathways mediate the activation of T cells following recognition of cognate antigen. These kinase and phosphatase pathways link proximal T cell receptor (TCR) signaling to changes in gene expression and cell physiology. A key step in the TCR signaling cascade is the generation of the second messenger molecules inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 triggers calcium signaling, leading to activation of the transcription factor NFAT (nuclear factor of activated T cells), while DAG activates PKCθ and RasGRP1, which couple to the transcription factors NFκB (nuclear factor kappa B) and AP-1 (activator protein 1), respectively. The coordinated binding of these three transcription factors, NFAT, NFκB, and AP-1, to the promoter of the T cell survival cytokine interleukin-2 is a critical step in T cell activation. In this article, we cover the kinase and phosphatase pathways downstream of second messenger generation and discuss the role of these effectors in T cell development, activation, and differentiation.

Introduction

When a T cell interacts with an antigen-presenting cell (APC) bearing cognate antigen, a number of signaling and cell biological events occur that lead to full activation of the T cell. The site of contact between the T cell and the APC is known as the immunological synapse (IS), and a number of studies have shown that signaling molecules are recruited to or excluded from the IS in a coordinated, regulated manner. This spatial reorganization facilitates transduction of signals from the T cell receptor (TCR) and costimulatory molecules in a multistep fashion to eventually instruct transcription factors in the nucleus to induce gene expression. Receptor signals trigger the activation of proximal tyrosine kinases and the phosphorylation and assembly of adaptor complexes so that distal kinase and phosphatase effector pathways can be subsequently engaged to activate a number of important transcription factors. These transcription factors include nuclear factor of activated T cells (NFAT), nuclear factor kappa B (NFκB), and activator protein 1 (AP-1), which are of particular importance for T cells because of their role in inducing transcription of the T cell survival cytokine interleukin-2 (IL-2). Namely, there are binding sites for all three transcription factors in the *IL-2* promoter, and their coordinated binding is required for *IL-2* gene expression (Smith-Garvin et al., 2009; Navarro and Cantrell, 2014).

TCR and costimulatory signals, such as CD28, also act in concert with specific cytokine receptor signals to establish differentiation of naïve $CD4^+$ T cells into appropriate effector T cell subsets. Depending on the type of pathogen and local cytokine environment, naïve $CD4^+$ T cells can, for instance, differentiate into T-helper type 1 (Th1) cells that are important for clearing bacterial and viral infections or into T-helper type 2 (Th2) cells that are important for clearing helminth infections but can be pathogenic in asthma. Th17 cells are proinflammatory, while iTregs express the master transcription factor FoxP3 and are important for suppressing other T cells (O'Shea and Paul, 2010). A key function of these differentiated cells is to produce cytokines such as IFNγ (for Th1) and IL-4 (for Th2).

In this article we will focus on the different serine/threonine kinase and phosphatase effector pathways that lie downstream of the proximal tyrosine kinases and assembled adaptor complexes and are triggered by second messenger molecules. We will discuss the role of these kinase pathways in $CD4^+$ T cells.

Second Messenger Molecules: Connecting Proximal TCR Signaling to Effector Kinase and Phosphatase Pathways

Many molecular events have been defined to occur when the TCR is ligated by cognate antigen and the IS is formed. In short, the initiation of proximal TCR signaling and the formation of the LAT (linker for activation of T cells) signalosome ultimately lead to the binding and activation of the enzyme phospholipase C gamma 1 (PLCγ1) (**Figure 1**). The importance of PLCγ1 in a number of thymocyte and T cell processes was demonstrated using mice where *PLCγ1* is deleted exclusively in T cells (Fu et al., 2010). Deletion of *PLCγ1* results in reduced numbers of thymocytes due to defects in positive and negative selection, and very few T cells populate the secondary lymphoid organs. Those T cells that do make it to lymphoid organs have defects in proliferation and IL-2 production, and the mice develop an autoimmune, inflammatory disease. With respect to biochemical signals, *PLCγ1*-deficient T cells are impaired in their ability to activate the transcription factors NFAT, NFκB, and AP-1. Of note, PLCγ2 plays a similarly critical role in B lymphocytes (Wang et al., 2000), but we focus on T cells here.

How does PLCγ1 connect to activation of NFAT, NFκB, and AP-1? PLCγ1 hydrolyzes the phospholipid PIP2 (phosphatidylinositol 4,5-bisphosphate) at the plasma membrane (PM), generating two important second messenger molecules: the hydrophilic inositol trisphosphate (IP3), which diffuses into the cytosol (Huang and Sauer, 2010), and the hydrophobic diacylglycerol (DAG), which embeds in the PM (and other endomembranes) (Almena and Merida, 2011; **Figure 1**). IP3

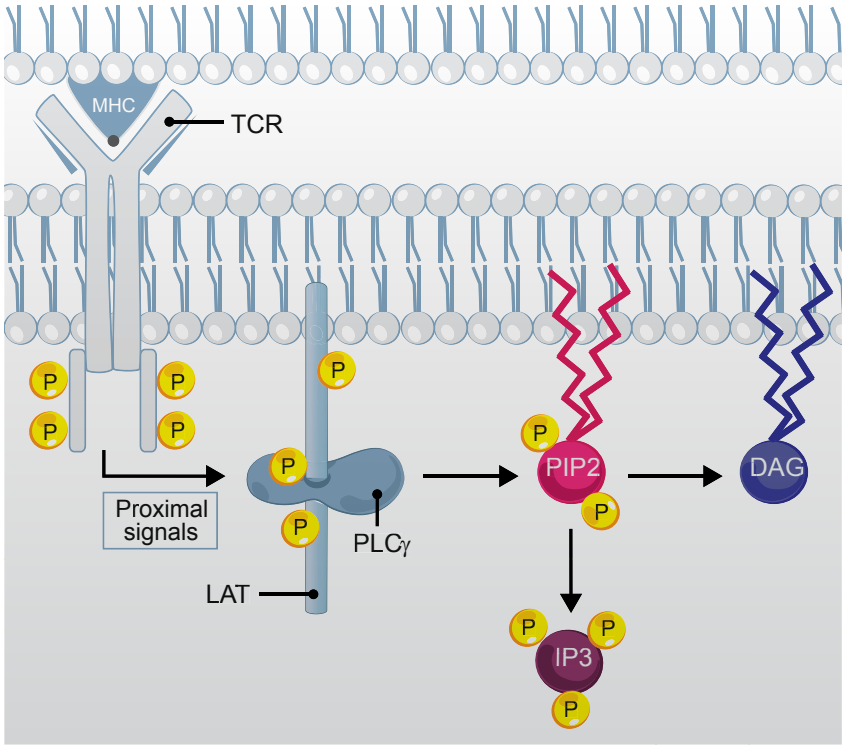

Figure 1 T cell receptor (TCR) stimulation and proximal signaling events result in the recruitment of PLCγ1 and the generation of second messenger molecules IP3 and DAG. Upon ligation of the TCR by cognate antigen bound to MHC, a number of proximal TCR signaling events (not depicted) are initiated that result in the formation of the LAT signalosome. PLCγ1 can bind to LAT, where it is activated by Tec kinases. Active PLCγ1 hydrolyzes the plasma membrane–associated phospholipid PIP2, leading to the production of two important second messengers: IP3 and DAG. IP3 is a soluble, hydrophilic molecule and diffuses into the cytosol. DAG is a hydrophobic molecule and embeds in the plasma membrane. It is recruited to the IS (immunological synapse), formed at the site of T cell: APC contact. Figure created by Anna Hupalowska.

activates calcium (Ca^{2+}) signaling and the transcription factor NFAT, and DAG activates effector proteins such as the protein kinase C family (PKCs) and the Ras guanine nucleotide releasing protein (RasGRP) family members, which can couple to the NFκB and AP-1 pathways, respectively (**Figure 2**). In the next sections, we will discuss kinase and phosphatase signaling pathways downstream of IP3 and DAG and highlight their role in thymocyte development and T cell activation and function.

Calcium Fluxes and Calcium-Trigger Signals in T Cells

Calcium is a critical second messenger and mediates a number of important biochemical and cell biological functions in T cells and other cells (Huang and Sauer, 2010; Feske, 2007; Hogan et al., 2010). Resting T cells maintain cytoplasmic stores of Ca^{2+} at ~100 nM concentration (Feske, 2007; Hogan et al., 2010), and upon receptor ligation, activated T cells flux Ca^{2+} and the intracellular concentration rises to ~1 μM. This is due to (1) release of Ca^{2+} that is stored in the endoplasmic reticulum (ER) into the cytosol as well as (2) influx of Ca^{2+} from the extracellular space to the inside of the cell (Feske, 2007; Hogan et al., 2010; Feske et al., 2015). There are a number of cellular proteins whose activation is controlled by binding to calcium, and many of these harbor a protein domain called the EF hand. EF hands typically come in pairs and have a helix-loop-helix structure. Each EF hand can bind one calcium ion, and Ca^{2+} binding typically induces a conformational change in the protein that facilitates subsequent signal transduction (Gifford et al., 2007). In the next sections, we will highlight the Ca^{2+} signaling pathway downstream of TCR-IP3 signals that culminates in NFAT activation, point out EF hand–containing proteins therein, and discuss the role of these Ca^{2+} effectors in T cell biology.

Release of Calcium Stores from the ER – Role of the IP3 Receptor

Generation of the second messenger IP3 from PIP2 at the PM is the first step in activating Ca^{2+} signaling. The soluble, hydrophilic IP3 molecule diffuses throughout the cytosol and binds to IP3 receptors (IP3Rs), which are channels located on the ER. IP3Rs also bind calcium, and it is thought that the combined binding of IP3 and Ca^{2+} leads to a conformational change, activating and opening the IP3R. This is followed by a rapid efflux of Ca^{2+} ions from the ER lumen into the cytosol (Taylor et al., 2009; **Figure 3**). Three genes (*ITPR1*, *2*, and *3*) encode the three IP3R homologs, which are ubiquitously expressed (Taylor et al., 2009). Experiments utilizing a chicken B cell line deficient for all *ITPR* genes clearly demonstrated that IP3 receptors

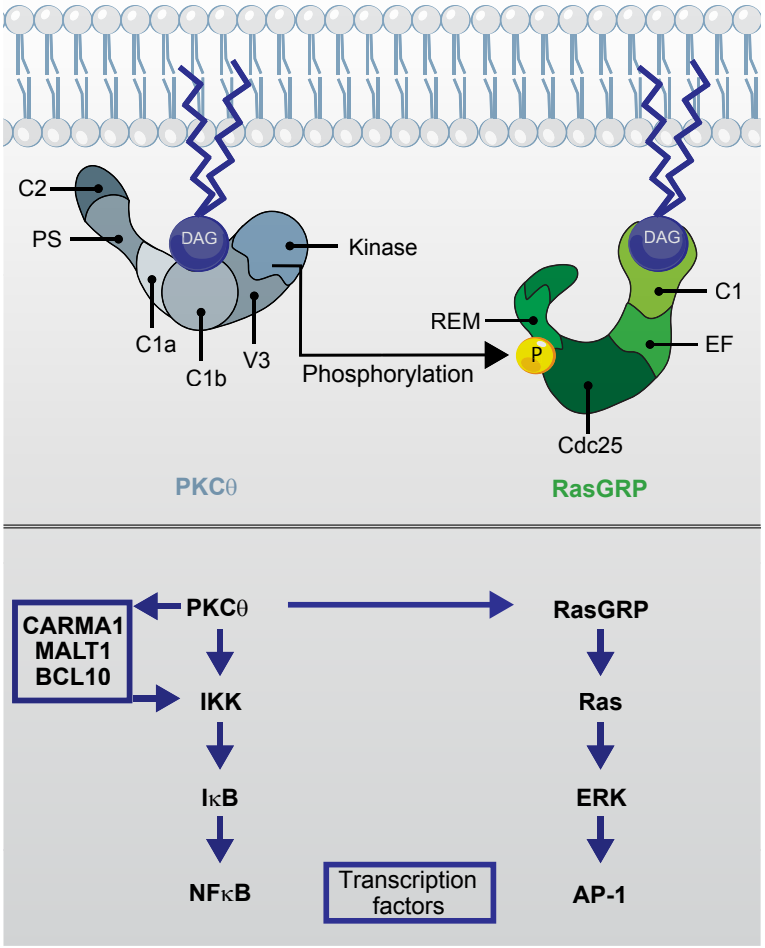

Figure 2 Diacylglycerol (DAG) allows for the recruitment of PKCθ and RasGRP1 via their DAG-binding C1 domains. Depiction of PKCθ and RasGRP1 with their protein domains and highlighting their role in propagating downstream signaling. Both proteins harbor typical C1 domains, which have a conformation that is compatible with binding to DAG. This C1-DAG interaction promotes recruitment of these effectors to the plasma membrane. Membrane-bound PKCθ activates the NFκB signaling pathway. DAG-bound RasGRP1 can interact with Ras and activate Ras-ERK-AP-1 signaling. PKCθ can also phosphorylate RasGRP1 at a threonine residue in the catalytic domain, though the role of this phosphorylation event is still unclear. Figure created by Anna Hupalowska.

are critical for calcium flux downstream of B cell receptor activation (Sugawara et al., 1997). A mouse model in which all three *ITPR* genes are deleted in T cells revealed a role for IP3R signaling in thymocyte development at the double-negative (DN) to double-positive (DP) transition. Interestingly, these knockout mice also develop thymic tumors that resemble T cell acute lymphoblastic leukemia/lymphoma (Ouyang et al., 2014). These studies demonstrate an important role for IP3Rs and release of stored calcium in controlling lymphocyte development and activation.

Calcium Entry from Outside the Cell – STIM and ORAI

Release of stored calcium from the ER downstream of TCR → IP3 signals results in the opening of CRAC (calcium release–activated calcium) channels at the PM. Open CRAC channels allow for influx of calcium from the outside of the cell into the cytosol. This influx is known as SOCE, or store-operated calcium entry. Several tools aided researchers in studying SOCE and calcium signaling in T cells, such as thapsigargin, an agent that leads to depletion of ER calcium stores, and ionomycin, a calcium ionophore (Hogan et al., 2010). It was known since the 1970s that activated T cells take up extracellular calcium, but the molecular players that physically form the channel the Ca^{2+} ions enter through were not identified until the mid-2000s. Seminal papers describe their discovery: these studies involved RNAi screens in *Drosophila* and HeLa cells, and the analysis of patients with primary immunodeficiency and CRAC channel activation (Hogan et al., 2010; Feske, 2007; Feske et al., 2015). These players are now known to be STIM (stromal interaction molecule) and ORAI (ORAI calcium release-activated calcium channel modulator). We will provide a brief summary of how STIM and ORAI function in T cells, and for further details on this topic we point the readers to these excellent reviews (Hogan et al., 2010; Feske et al., 2015).

STIM1 is a single-pass transmembrane protein that is predominantly localized to the ER. Lymphocytes and other

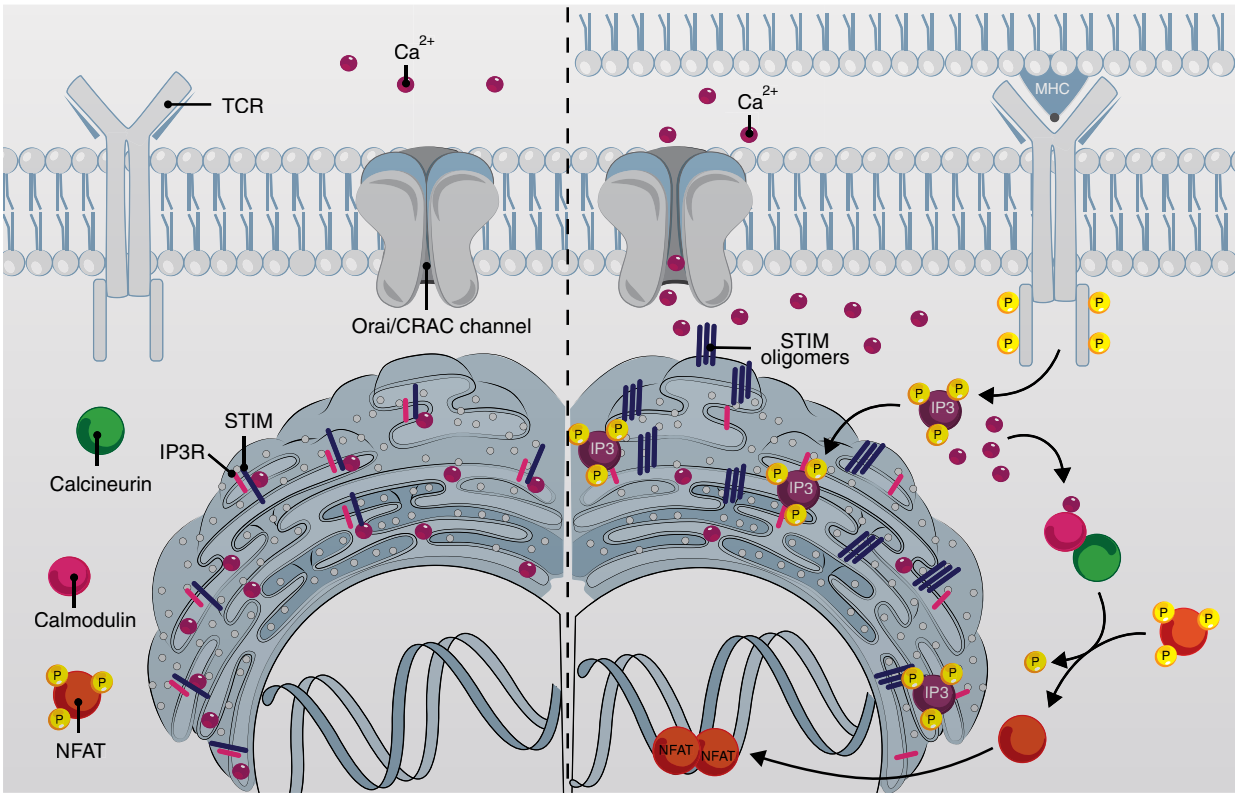

Figure 3 Generation of calcium signals in T cells resulting in NFAT transcriptional responses. Overview of calcium regulation in resting (left side) and receptor-activated (right side) T cells. In the basal state (left), there is little free calcium in the cytoplasm and instead it is stored in the endoplasmic reticulum (ER). Some of this calcium associates with the EF hands of STIM1 molecules, which span the ER membrane. Ca^{2+}-associated STIM molecules are inactive and do not form higher-order oligomers. The calcium-responsive transcription factor NFAT is sequestered in the cytoplasm via constitutive phosphorylation. Upon TCR activation (right), generated IP3 binds IP3 receptors (IP3Rs) on the ER membrane. These receptors open and release stored calcium into the cytosol. Ca^{2+} uncouples from STIM, and STIM oligomers form near the PM, where they interact with ORAI. ORAI molecules span the PM and are the pore-forming unit of the CRAC channel. STIM activates ORAI, opening the CRAC channel, allowing for the import of Ca^{2+} from outside the cell into the cytosol. One important consequence of this calcium flux is activation of NFAT. Calcium binds the EF hands of calmodulin, which activates calcineurin, a phosphatase. Activated calcineurin can dephosphorylate NFAT, and NFAT translocates to the nucleus where it activates its target genes. Note that some of the molecular details are not included in the figure. Figure created by Anna Hupalowska.

cells also express another STIM family member, STIM2, although mouse models with deficiencies in *STIM1* or *STIM2* reveal that STIM1 appears to be more important for Ca^{2+} signaling in T cells (Oh-hora et al., 2008). The portion of the STIM1 protein that resides in the ER lumen contains a pair of EF hands (Stathopulos et al., 2008). STIM1 is a calcium-sensing protein: in resting lymphocytes, the EF hands of STIM1 bind Ca^{2+} which is present in high concentration in the ER. Upon TCR ligation and depletion of ER Ca^{2+} stores, Ca^{2+} dissociates from STIM1, inducing a conformational change in the protein (Stathopulos et al., 2008) that allows STIM1 to oligomerize. Total internal reflection microscopy and electron microscopy studies demonstrated that STIM oligomers cluster at individual spots, called puncta. These puncta are found at sites where the ER and the PM are closely apposed, and calcium entry follows their formation (Wu, 2006). STIM1 recruits ORAI1 to these puncta by binding to the C-terminus of ORAI1 (Park et al., 2009), and STIM1 activates ORAI1 via an additional interaction (possibly the ORAI N-terminus) (Hogan et al., 2010; **Figure 3**). The *ORAI1* gene encodes the protein that forms the CRAC channel (specifically, the pore subunit of the CRAC channel), which is very selective for Ca^{2+} (Prakriya et al., 2006). The pore is not active and does not allow Ca^{2+} entry, however, until it complexes with STIM1. Of note, there are two *ORAI1* homologs, *ORAI2* and *ORAI3*, although their role in lymphocytes is still unclear (Feske et al., 2015).

Analysis of human patients with primary immunodeficiency and of genetic mouse models revealed critical physiological functions for STIM and ORAI. Several families were identified that had severe combined immunodeficiency accompanied by impaired CRAC channel activity (these disorders are also referred to as 'CRAC channelopathies'). These families were found to have mutations in *STIM1* (Fuchs et al., 2012; Picard et al., 2009) and *ORAI1* (Feske et al., 2006) that either abolish protein expression or lead to loss-of-function variants. Patients with *STIM1*- and *ORAI1*-mutated immunodeficiencies have similar clinical phenotypes that include recurrent infections (especially at a young age), autoimmunity later in life, muscular hypotonia, and ectodermal dysplasia (Feske et al., 2015). Interestingly, lymphocyte development is normal in

these patients, but their peripheral T cells are functionally impaired: these T cells do not flux Ca^{2+}, do not proliferate well in response to stimulation, and do not activate NFAT. These T cells also have impaired production of NFAT-responsive cytokines such as IL-2 (Hogan et al., 2010; Feske et al., 2015). Reconstituting the patient cells with WT *ORAI1* restores CRAC channel function.

Mouse models with gene deletions in STIM and ORAI (*STIM1*$^{-/-}$, *STIM2*$^{-/-}$, and *ORAI1*$^{-/-}$) or with a patient variant (*ORAI1*^{R93W}) knocked into the endogenous *ORAI1* locus were subsequently generated (Oh-hora et al., 2008; Gwack et al., 2008; Vig et al., 2007). These mice phenotypically resemble the human patients, in that thymocyte development is intact, yet peripheral $CD4^+$ T cells display profound defects in SOCE, NFAT activation, and NFAT-dependent cytokine production. These mice have been powerful tools for aiding our understanding of the roles STIM and ORAI play in a number of T cell effector functions (Oh-hora et al., 2008; Ma et al., 2010; Gwack et al., 2008; McCarl et al., 2010; Feske et al., 2015; Hogan et al., 2010).

A Calcium–Calmodulin–Calcineurin Phosphatase Pathway Regulates NFAT Activation

A key downstream consequence of calcium signaling is activation of the transcription factor family NFAT. The biochemical details of this pathway have been well studied and involve the sequential activation of the Ca^{2+} sensor calmodulin and the phosphatase calcineurin. Calmodulin contains four canonical EF hands, and these become fully occupied (each binding one Ca^{2+} ion) when the cytosolic concentration rises following SOCE. Ca^{2+}-bound calmodulin undergoes a conformational change, exposing a site necessary for interaction with its binding partners, including calcineurin (Babu et al., 1988; James et al., 1995). Calcineurin is a heterodimeric phosphatase comprised of a catalytic subunit (A) and a regulatory subunit (B), which heterodimerize, but the dimer is not fully active until it binds calmodulin (**Figure 3**). Calcineurin A consists of an N-terminal catalytic domain, a calcineurin B binding region, a calmodulin binding segment, and an autoinhibitory region. Calcineurin B also contains four EF hands, which have roles in both calcium sensing and in conferring stability on the heterodimer (Li et al., 2011). Calmodulin binding leads to a conformational change in calcineurin by displacing the autoinhibitory domain (Marshall et al., 2015). The importance of calcineurin in T cell activation is underscored by the fact that two clinically important immunosuppressive agents, cyclosporine A (CsA) and tacrolimus (also called FK506), were shown to be inhibitors of calcineurin (Liu et al., 1991). These drugs are extensively used in the clinic to treat autoimmunity and to improve survival following transplantation.

Upon activation, calcineurin docks onto NFAT in order to dephosphorylate and activate the transcription factor. NFAT proteins contain a transactivation domain, a regulatory domain, a DNA-binding domain, and a C-terminal domain. Within the regulatory domain lie several serine-rich motifs, and these are heavily phosphorylated in resting T cells by several kinases (Hogan et al., 2003; Macian, 2005; Müller and Rao, 2010). When these serines are phosphorylated, NFAT is in such a conformation that its nuclear localization signal (NLS) is masked (Macian, 2005). Upon antigen receptor stimulation and Ca^{2+} signaling, active calcineurin dephosphorylates NFAT, exposing the NLS. This permits the nuclear translocation of NFAT and induction of its target genes (**Figure 3**). Thus, NFAT serves as a molecular link between Ca^{2+} signaling and gene expression. NFAT typically partners with other transcription factors via protein–protein interactions to regulate specific gene expression patterns. AP-1 (downstream of Ras-ERK signaling, discussed below) is a common partner of NFAT in activated T cells (Jain et al., 1992); as such, T cells integrate kinase pathways downstream of IP3 and DAG for full activation. Upon termination of cell activation, NFAT can be rephosphorylated in the nucleus by export kinases to promote its export from the nucleus into the cytosol (Macian, 2005; Müller and Rao, 2010).

There are five NFAT family members (NFAT1-5), but we discuss NFAT1, 2, and 4 (which we collectively refer to as 'NFAT') here, as NFAT3 is not expressed in T cells and, unlike NFATs 1–4, NFAT5 is not regulated by Ca^{2+} (Macian, 2005). The various NFAT proteins seem to have redundant functions in T cells, as individual gene knockout mice have a mild impact on the immune system. Data from the study of mice deficient in multiple NFAT family members have revealed roles for NFAT in thymocyte positive selection, T cell differentiation, and anergy. For example, *NFAT1*$^{-/-}$ *NFAT2*$^{-/-}$ mice have profound defects in the production of IL-2 and other cytokines, and *NFAT1*$^{-/-}$ *NFAT4*$^{-/-}$ mice have hyperactive T cells and increased Th2 responses (Macian, 2005). For a thorough discussion of the role of NFAT in these effector functions, we point readers to several excellent reviews (Hogan et al., 2003; Macian, 2005; Müller and Rao, 2010).

As described here, signaling downstream of the TCR can lead to activation of NFAT through generation of the second messenger IP3 and Ca^{2+} signaling. However, to fully activate T cells and induce IL-2 production, NFAT needs to bind in a coordinated fashion to the *IL-2* promoter along with the transcription factors NFκB and AP-1, which are triggered downstream of DAG. The role of DAG and its downstream effector pathways in T cells will be discussed next.

DAG and C1 Domains

DAG is a glycerol molecule joined to two long chain fatty acids through ester linkages; it is a hydrophobic molecule and thus embeds in cellular membranes such as the PM (Carrasco and Merida, 2007; Krishna and Zhong, 2013). DAG is an important lipid for lymphocytes and other cell types both because it can be metabolized and used as a source of fatty acids, which are important for growth and development (Carrasco and Merida, 2007), and because it can act as a second messenger for activation of signaling pathways such as NFκB and AP-1 (**Figure 2**). How exactly does DAG generation downstream of the TCR trigger NFκB and AP-1 signaling pathways, ultimately leading to T cell activation? This is primarily achieved through the recruitment and allosteric activation of effector proteins that harbor a 'typical C1 domain,' which can bind to DAG. C1 domains have a conserved 50 amino acid sequence and a well-characterized structure; they contain a hydrophobic

base and a groove that form a cuplike structure to bind DAG. Binding of the C1 structure to DAG achieves membrane recruitment of proteins with such a domain (Carrasco and Merida, 2004). Examples of signaling proteins with C1 domains include members of the PKC family, protein kinase D (PKD) (Spitaler et al., 2006), RasGRPs, chimaerin family proteins, and munc13 proteins (Krishna and Zhong, 2013); PKCs (particularly PKCθ) and RasGRP1 will be discussed in greater detail subsequently (**Figure 2**).

Direct biochemical measurement of DAG levels in cells, and how receptor stimulation alters these levels, is challenging. Elegant microscopy studies used the isolated C1 domains from various proteins as tools to investigate DAG in cells (Carrasco and Merida, 2004; Spitaler et al., 2006; Quann et al., 2009). These tools, referred to as C1 domain probes, typically consist of the isolated C1 domain fused to green fluorescent protein (GFP). The C1 binds DAG and the associated GFP reports the subcellular location of DAG and potentially the localization of the protein whose C1 domain was used (though other protein domains can contribute to localization as well). These tools revealed that DAG and DAG effector proteins accumulate at the PM and within the IS in an organized way. For more detailed reading on subsequent studies and approaches, we refer the reader to these additional studies (Sato et al., 2006; Kunkel and Newton, 2010; Almena and Merida, 2011).

Generation and Turnover of DAG – Regulation Is Critical

In the 1980s it became clear that DAG is a potent T cell stimulant. Evidence for this came from experiments showing that phorbol esters (synthetic DAG mimetics) such as phorbol 12-myristate 13-acetate (PMA) are efficient T cell activators (Smith-Garvin et al., 2009). Cells cannot metabolize PMA, so upon administration it embeds in the PM and fully activates AP-1 and NFκB signaling to drive T cell effector functions, such as cytokine production (Weiss et al., 1984). For certain cell responses, PMA stimulation entirely bypasses the need for TCR stimulation or proximal TCR signaling components such as the tyrosine kinase ZAP70 (zeta chain–associated protein kinase 70) or the adaptor molecule LAT. Because of this, in the 1990s PMA became a useful tool to help delineate the TCR signaling pathway. Occurring in parallel in the 1980s, cancer researchers uncovered that 12-O-tetradecanoylphorbol-13-acetate (TPA), a phorbol ester similar to PMA, could be used to promote tumor formation in various cancer settings, particularly in mouse models of skin cancer where TPA is painted on the skin (Takigawa et al., 1983). These studies inspired the hunt for DAG/PMA-responsive effector molecules, which we will discuss later.

The abovementioned studies indirectly implied that DAG levels must be strictly regulated to ensure signaling occurs in the appropriate context. DAG is present at low levels in resting (unstimulated) lymphocytes; it can be generated by *de novo* synthesis or can be produced from cellular intermediates by the activity of several enzymes such as sphingomyelin synthase, phospholipase D, and phospholipase C (PLC). DAG is rapidly generated in response to TCR ligation. The PLC family member PLCγ1 is particularly important for DAG generation from PIP2 in TCR-activated T cells (**Figure 1**). It is of interest to note that PIP2 comprises less than 1% of the PM (Spitaler et al., 2006; Huang and Sauer, 2010), yet its hydrolysis has profound effects on the activation status of lymphocytes. This is achieved because newly generated DAG is not equally distributed but forms a gradient and accumulates at the IS. The localized clustering of DAG-responsive signaling molecules at the IS is important for the robust activation of downstream pathways and is functionally important for T cell polarization, activation, development, and differentiation (Smith-Garvin et al., 2009; Quann et al., 2011).

Conversely, DAG can be phosphorylated by a class of proteins called diacylglycerol kinases (DGKs), producing phosphatidic acid, and this can serve as a mechanism to dampen or turn off DAG signaling. Mouse models with T cell–specific deletions of *DGKα* and *DGKζ* (the DGK family members expressed in T cells), which effectively constitutively activate DAG signaling due to the inability to 'turn off' DAG, reveal a crucial role for these kinases in controlling thymocyte maturation and cell activation (Guo et al., 2008). $DGK\alpha^{-/-}$ $DGK\zeta^{-/-}$ mice have reduced numbers of single-positive (SP) thymocytes concomitant with elevated levels of Ras-GTP and phosphorylated ERK (Guo et al., 2008). It is still unclear whether this reduction is due to defects in positive selection or enhanced negative selection of thymocytes (Joshi and Koretzky, 2013). Furthermore, older compound heterozygous $DGK\alpha^{+/-}$ $DGK\zeta^{+/-}$ mice that are engineered to carry a transgenic TCR on thymocytes develop thymic lymphomas. These cells exhibit elevated Ras-ERK signaling which likely drives the cellular transformation. These genetic approaches highlight the importance of regulated, controlled DAG generation and turnover to ensure proper thymocyte development and T cell proliferation (Krishna and Zhong, 2013).

PKC Family – Classification and Structural Features

The PKC family of serine/threonine kinases harbor two tandem C1 domains (C1a and C1b) and thus are capable of binding DAG. This family consists of at least nine isoforms (α, β, γ, δ, ε, η, θ, ζ, ι), eight of which (all but γ) are expressed in T lymphocytes. The isoforms are further categorized into three groups: (1) the conventional PKCs (α, β, γ) require both DAG and Ca^{2+} for activation; (2) the novel PKCs (δ, ε, η, θ) require DAG but not Ca^{2+} for activation, and; (3) the atypical PKCs (ζ, ι) are activated via protein–protein interactions independently of both DAG and Ca^{2+} (Melowic et al., 2007; Quann et al., 2009; Pfeifhofer et al., 2003).

All PKCs contain C1 domains, but PKCθ is the dominant isoform to get recruited to the IS upon T cell contact with an APC (Monks et al., 1997). This IS localization occurs in an antigen-specific manner and is dependent on PLCγ1-derived DAG, as treatment of cells with a PLCγ1 inhibitor or with calphostin c (which competes for DAG binding) blocks PKCθ translocation to the PM (Diaz-Flores et al., 2003; Carrasco and Merida, 2004). Thus, DAG plays a critical role in the regulation of PKCθ translocation. Additional studies revealed that various structural domains of PKCθ in addition to the C1 domain are important for controlling its activation and membrane localization. PKCθ has a C2

domain at its N-terminus, followed by a pseudosubstrate (PS) domain, tandem C1 domains (C1a and C1b), a V3 (hinge region), and C-terminal kinase domains (**Figure 2**). In resting cells, PKCθ exists in a 'closed/inactive' conformation in the cytosol; the PS domain forms an intramolecular interaction with the kinase (catalytic) domain, such that substrate cannot bind to PKCθ (Wang et al., 2012). The C2 domain is also thought to be important for this autoinhibition (Melowic et al., 2007). This inhibition is relieved upon T cell activation: DAG binds to PKCθ, leading to PM translocation and inducing an allosteric change in the protein such that the activation loop can be phosphorylated and the kinase domain can be accessible by substrate. The membrane-associated protein is now in an 'open/active' conformation and can bind and phosphorylate its substrates (Wang et al., 2012).

Specifically, it is the C1b domain that allows for PM targeting in response to PMA or TCR/CD28 stimulation (Carrasco and Merida, 2004; Melowic et al., 2007; **Figure 2**). Interestingly, in *in vitro* experiments the full-length PKCθ protein remains at the T cell–APC contact site for a longer period of time than the isolated C1 domains of PKCθ do, suggesting that other domains of PKCθ are also important for stable recruitment of PKCθ to the IS (Carrasco and Merida, 2004). Mechanistically, it was shown that the V3 (hinge) region of PKCθ, which contains a proline-rich element, is important for IS localization and activation of downstream signaling pathways (Kong et al., 2011). In the current model, PKCθ is recruited to the IS in a transient, low-affinity manner via its C1b domain, and then binding of the V3 region to CD28 stabilizes PKCθ at the IS, allowing for signal propagation (Yokosuka et al., 2008; Kong et al., 2011).

PKCθ, NFκB, and AP-1 Pathways and T Cell Function

To understand the role of PKCθ *in vivo*, two groups independently generated *PKCθ* knockout mice using different gene-targeting strategies and both reported a striking defect in T cell proliferation and production of the T cell survival cytokine IL-2 in response to TCR/CD28 stimulation (Sun et al., 2000; Pfeifhofer et al., 2003). These mice revealed an important role for PKCθ in T cell proliferation and IL-2 production; *PKCθ* knockout T cells demonstrated decreased activation of the transcription factors AP-1 and NFκB in response to CD3/CD28 stimulation, which fits with the notion that these transcription factors need to bind to the promoter region of the *IL-2* gene to induce IL-2 expression. Indeed, the proliferation defect could be rescued by administering IL-2 to *PKCθ* knockout T cells exogenously. These findings were corroborated by two studies using a Jurkat T cell lymphoma cell line; both groups found that PKCθ could activate NFκB and AP-1 luciferase reporters in response to CD3/CD28 stimulation, and this effect could be blocked with rottlerin, a PKCθ inhibitor (Coudronniere et al., 2000) or with antisense oligos to PKCθ (Lin et al., 2000). More specifically, these groups found that PKCθ activity was important for activating IKKβ and for inducing degradation of IκB. One of these groups (Lin et al., 2000) also revealed a critical role for CD28 costimulation in PKCθ-dependent NFκB activation, which agrees with the biochemical connection between CD28 and the V3 region of PKCθ to stabilize this kinase at the IS (Lin et al., 2000; Kong et al., 2011).

Biochemically, the classical NFκB pathway has been well characterized in T lymphocytes (Thome et al., 2010; Hayden and Ghosh, 2012). Briefly, in the basal state homo- or heterodimers of the transcription factor NFκB are sequestered in the cytosol due to their association with the inhibitory subunit IκB. Upon TCR stimulation, a signaling cascade leads to the activation of the IKK complex (which consists of the regulatory protein NEMO and the kinases IKKα and IKKβ). Active IKKβ can then phosphorylate IκB family members (such as IκBα) on conserved serine residues. Phosphorylated IκBα is then ubiquitinated and degraded, releasing NFκB and exposing its NLS. The liberated NFκB dimers can then translocate to the nucleus, bind to target genes, and initiate NFκB-driven transcription. Of note, it was not immediately clear how PKCθ couples to AP-1 activation. In a subsequent section we will discuss how it was uncovered that PKCθ phosphorylates RasGRP1, which in turn activates the Ras-ERK-AP-1 pathway (Roose et al., 2005; **Figure 2**).

As discussed elsewhere in the Encyclopedia of Immunobiology, the molecular players upstream of the IKK complex can vary depending on the type of stimulation. In antigen receptor–stimulated lymphocytes, there is a pathway linking PKCθ to IKK/NFκB activation via the scaffold protein CARMA1 (also called CARD11). CARMA1 exists in a closed conformation in the cytosol. When CARMA1 becomes activated, it forms a complex (the CBM complex) with the scaffold protein MALT1 and the adaptor BCL10 (which are constitutively associated). Active PKCθ (and possibly other kinases) phosphorylates CARMA1 at specific serine residues, triggering a conformational change (to a more open form) that allows for CARMA1 to bind MALT1/BCL10 (via interactions of the CARD domains in CARMA1 and BCL10). Complexed MALT1 and BCL10 subsequently become ubiquitinated, which leads to the recruitment and activation of IKK (Oeckinghaus et al., 2007). Other studies have shown that PKCθ may also have a role in recruiting IKK to CBM (Lee et al., 2005). Thus, a TCR-PKCθ-CBM pathway exists that leads to NFκB activation in T cells. For a more in-depth discussion of NFκB signaling, we point the readers to two excellent reviews (Thome et al., 2010; Hayden and Ghosh, 2012).

A number of studies have documented important roles for PKCθ in various aspects of T cell–mediated immunity. In the appropriate cytokine milieu, activated T cells can differentiate into a number of effector subsets (including Th1, Th2, Th17, and iTreg) that mediate different functions (O'Shea and Paul, 2010). PKCθ has an important role in Th2 cells, as *PKCθ* knockout mice cannot efficiently clear *Nippostrongylus brasiliensis* worm infections due to impaired Th2 cell activity, and *PKCθ*-deficient mice do not develop allergic airway disease in challenge assays (Marsland, 2004; Salek-Ardakani et al., 2004). More subtle effects on Th17 and iTreg cells were observed (Salek-Ardakani et al., 2005; Tan et al., 2006), whereas PKCθ appears to be dispensable for Th1 cells. These studies reveal an important yet complex role for PKCθ signals in helper T cell differentiation and function, and at this point it is not understood why Th2 cells are most affected when PKCθ is absent.

A DAG-Induced Ras-ERK Signaling Pathway in T Cells

As mentioned earlier, DAG as a signaling molecule caught the attention of both cancer biologists and immunologists in the 1980s. A seminal paper from Julian Downward and Doreen Cantrell described the increased levels of active Ras-GTP when lymphocytes were stimulated with phorbol ester (Downward et al., 1990); this was the first time that signal-induced Ras activation was observed in noncancerous cells. Ras is a small GTPase that cycles between an inactive, GDP-bound state and an active, GTP-bound state (**Figure 4**). Ras is turned on when it is bound to GTP, as GTP-bound (but not GDP-bound) Ras is capable of binding downstream effector proteins such as RAF, PI3K (phosphoinositide 3-kinase), and RalGDS, which then can become activated and propagate downstream signaling (Castellano and Downward, 2010, 2011; Ahearn et al., 2012). Ras signaling can be turned off by hydrolysis of GTP into GDP. The nucleotide-bound states of Ras are regulated by two classes of proteins, RasGAPs (GTPase-activating proteins) and RasGEFs (guanine nucleotide exchange factors) (Jun et al., 2013a). Ras by itself has limited GTPase activity, and RasGAP proteins turn off Ras signaling by enhancing the GTPase activity of Ras, leading to GTP hydrolysis and Ras adopting a conformation such that it is unable to bind downstream effectors. Conversely, GEFs activate Ras by binding it and inducing a conformational change such that GDP is released; empty Ras subsequently associates with GTP, which is available at higher levels than GDP in the cytoplasm (Vetter and Wittinghofer, 2001; Bos et al., 2007). Lymphocytes express three families of GEFs: RasGRPs, SOS (son of sevenless), and RasGRFs (Ras guanine nucleotide releasing factors). However, RasGRFs appear to have a very limited role in Ras-ERK activation in T cells (Jun et al., 2013a; Ksionda et al., 2013), so in this article we will focus on the role of RasGRPs and SOS. Both of these RasGEFs activate Ras by inducing a conformational change that causes the release of otherwise tightly bound nucleotide (Boriack-Sjodin et al., 1998).

Briefly, the classical MAP kinase pathway downstream of Ras activation proceeds as follows: Ras-GTP binds the kinase RAF, and active RAF phosphorylates MEK (mitogen-activated protein kinase kinase), which in turn phosphorylates ERK1/2 (extracellular signal regulated kinase 1/2) on tyrosine and threonine residues (Ahearn et al., 2012). ERK1/2 are serine/threonine kinases and are able to activate a number of cytosolic proteins and transcription factors through phosphorylation, including the transcription factor ELK1, which in turn leads to activation of AP-1 (**Figure 2**). AP-1 consists of a heterodimer of the Jun/Fos transcription factors, and it (along with NFAT and NFκB, discussed above) contributes to IL-2 gene induction (Smith-Garvin et al., 2009).

The molecular components of the Ras-MAP kinase pathway have been shown to be important for T cells in studies using genetic mouse models. Generation of CD4 or CD8 SP thymocytes, the most mature stages of developing T cells in the thymus, is reduced when mice express

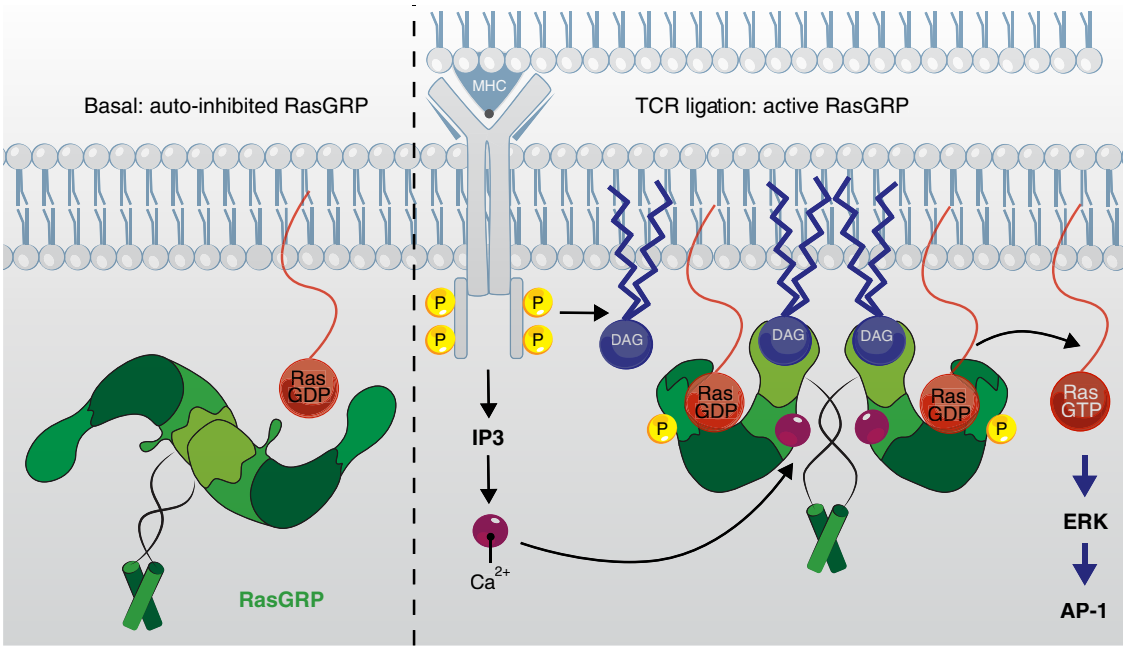

Figure 4 Autoinhibition of RasGRP1 and activation by DAG and calcium. In the basal state (left), RasGRP1 molecules exist in the cytosol as autoinhibited dimers. Dimerization is achieved via the C-terminal coiled-coil domain as well as by a C1-EF hand interface. This puts the protein in a closed conformation and buries the C1 domain of RasGRP1, so it is unable to bind DAG and be translocated to the plasma membrane, where it interacts with Ras. Autoinhibition is further achieved by the presence of a linker that blocks the Ras-binding domain of RasGRP1. When T cells are activated via the TCR (right), autoinhibition is relieved and RasGRP1 becomes activated. Calcium binds to the EF1 hand of RasGRP1, promoting a more open conformation and exposing the C1 domain. Open RasGRP1 molecules can translocate to the PM via C1-DAG binding, and at the PM they can bind to and activate Ras. Figure created by Anna Hupalowska.

a dominant negative form of Ras (S17N) transgenically under the control of the Lck promoter (which turns on early in thymocyte development). This inability to progress from the $CD4^+$ $CD8^+$ DP to the SP stage was shown to be due to impaired positive selection of these developing T cells (Swan et al., 1995). Consistent with a role for the Ras-RAF-MEK-ERK pathway, mice expressing catalytically inactive MEK1 in thymocytes as well as mice with thymocytes doubly deficient for *ERK1* and *ERK2* also have impaired positive selection and reduced numbers of SP cells (Alberola-Ila et al., 1995; Fischer et al., 2005).

The RasGRP Family of Ras Exchange Factors and Their Role in Ras-ERK Signaling

As discussed, it was known since 1990 that phorbol esters such as DAG can activate Ras; however, a direct molecular link remained elusive. For a substantial period, PKCs were thought to provide this link between DAG and Ras activation. However, thymocyte development (a process that, as discussed, requires intact Ras signaling) is normal in *PKCθ*-deficient mice (Sun et al., 2000). In 1998, the RasGEF RasGRP1 was identified by Jim Stone's group through a fibroblast transformation screen, surprisingly late in the cloning era (Ebinu, 1998). Since the sequence of RasGRP1 revealed both a Ras activation domain as well as a C1 domain, the link between DAG and Ras was quickly made.

Stone's group cloned *RasGRP1* from a cDNA library from the brain, indicating that *RasGRP1* is expressed in that tissue. Soon after, Robert Kay and colleagues also identified *RasGRP1* in a transformation screen, this time using a cDNA library from a T cell hybridoma line (Tognon et al., 1998). Subsequently, RasGRP1 was shown to be very highly expressed in thymocytes and T cells (Ebinu et al., 2000), and thus a large body of research has focused on RasGRP1 and its role in Ras activation in T cells. However it is of note that in addition to developing and mature T cells, *RasGRP1* is expressed in other hematopoietic cells such as B cells (Coughlin et al., 2005), mast cells (Liu et al., 2007), neutrophils (la Luz Sierra, 2010), and natural killer cells (Lee et al., 2009). RasGRP1 is also expressed in non-hematopoietic cells, such as cells in several regions of the brain (Kawasaki et al., 1998), in keratinocytes of the skin (Rambaratsingh et al., 2003), and in intestinal epithelial cells (Depeille et al., 2015).

RasGRP1 is a member of the RasGRP family of RasGEFs. This family is comprised of four members: RasGRP1, 2, 3, and 4. The four molecules all share several protein domains, including the catalytic module, consisting of an REM (Ras exchange motif) and a CDC25 domain, which binds Ras, C1 domains, and a pair of EF hands (**Figures 2** and **4**). However, there are variations in these domains across the different RasGRPs, and the molecules thus seem to have distinct biochemical function and regulation. For further details on the different RasGRP proteins we refer you to an article (Ksionda et al., 2013). Here we exclusively focus on RasGRP1 because of its evident role in thymocytes and T cells.

RasGRP1's C1 domain has high homology to the C1 of PKCs and has high affinity for phorbol esters (Lorenzo et al., 2000). The earliest studies of RasGRP1 showed that the full-length protein, but not a construct lacking the DAG-binding C1 domain, could be detected in membrane fractions after subcellular fractionation (Ebinu, 1998). Later, it was shown using probes consisting of an isolated RasGRP1 C1 domain fused to GFP that RasGRP1 is found in the cytoplasm (often perinuclear) in resting Jurkat cells and translocates to the PM upon stimulation with TCR/CD28 or PMA (Carrasco and Merida, 2004). The C1 domain was also shown to be important for RasGRP1's signaling capabilities, as cells harboring a RasGRP1 ΔC1 construct display reduced Ras-GTP loading, less phosphorylation of ERK (Ebinu, 1998), and reduced cellular transformation (Tognon et al., 1998).

Classical EF hands (such as the pair found in calmodulin, discussed above) come in pairs, and each hand typically binds one calcium ion. Early studies showed that RasGRP1 was capable of binding calcium (Ebinu, 1998), but it was noted that the EF hands of RasGRP1 are unique in that the region between the two EF hands is shorter than what is typically observed. Later, it was found that RasGRP1 only binds one Ca^{2+} ion, via the first EF hand (EF1) (Iwig et al., 2013; **Figure 4**).

The presence of the DAG-binding C1 domain and the EF hands in RasGRP1 suggested that the protein is regulated by both of these second messengers. Our lab and the Kuriyan lab recently solved the crystal structure of RasGRP1, which led to a model for how RasGRP1 is autoinhibited in the basal state and activated upon receptor stimulation. The catalytic domain of RasGRP1 is intrinsically active (Iwig et al., 2013), so autoinhibition is critical to prevent spurious RasGRP1-Ras signaling. The C1 and EF hands play crucial regulatory roles here. The crystal structure revealed that RasGRP1 autoinhibition in the basal state is achieved via a dimer interface that prevents DAG binding and via a linker domain that blocks the Ras-binding site (Iwig et al., 2013). The autoinhibited dimer interface forms such that the C1 domain of each RasGRP1 monomer contacts the EF2 and CDC25 domains of the other, thereby burying the C1 domains (and presumably preventing its ability to bind DAG and translocate to the PM). The C-termini of two RasGRP1 molecules also form a coiled coil, providing an additional dimerization interface (**Figure 4**). The C-terminal tail domain of RasGRP1 appears to be functionally important as well, as mice where endogenous *RasGRP1* is replaced with a version that lacks the tail domain have impaired thymocyte development, T cell proliferation, and autoimmunity. This tail-deficient RasGRP1 is also impaired in PMA-induced PM translocation (Fuller et al., 2012). As noted, autoinhibition is further achieved by the presence of a CDC25-EF linker, which lies in the Ras-binding site of RasGRP1.

How and in what order of events RasGRP1 is activated is unknown, but DAG, calcium, and RasGRP1 phosphorylation play a role (Roose et al., 2005; Iwig et al., 2013; Ksionda et al., 2013). NMR scattering studies imply that calcium binding to EF1 induces a conformational change from an autoinhibited dimer to an active dimer (Iwig et al., 2013; **Figure 4**). RasGRP1 is inducibly phosphorylated by PKCθ following receptor stimulation (Roose et al., 2005; Zheng et al., 2005); however, the biochemical and physiological relevance of this phosphorylation is still not well understood. Notably, this PKCθ-RasGRP1 connection may explain why PKCθ-deficient cells fail to activate the transcription factor AP-1 (Sun et al., 2000; Pfeifhofer et al., 2003; **Figure 2**).

Strict Regulation of RasGRP1-Kinase Signals and Consequences of Perturbed Regulation

RasGRP1-deficient mice were generated by Jim Stone's group in 2000 and revealed an important physiological role for RasGRP1 in thymocyte development (Dower et al., 2000). Strikingly, thymocytes from *RasGRP1* knockout mice are developmentally arrested at the DP stage, leading to greatly reduced absolute numbers and percentages of $CD4^+$ and $CD8^+$ SP thymocytes. In line with the fact that so few thymocytes make it through development, *RasGRP1*-deficient mice also have very few mature T cells populating their spleen and lymph nodes. Biochemically, $RasGRP1^{-/-}$ thymocytes have impaired RasGRP1-Ras-ERK signaling, demonstrated by the inability to phosphorylate ERK in response to TCR or phorbol ester stimulation. *RasGRP1*-deficient Jurkat T cells similarly are impaired to induce P-ERK or upregulate the activation marker CD69 (Roose et al., 2005). Subsequent studies in which $RasGRP1^{-/-}$ mice were crossed to TCR transgenic mice revealed that RasGRP1 is required for thymocyte positive selection but, interestingly, RasGRP1 is not required for negative selection (Priatel et al., 2002).

Conversely, dysregulated RasGRP1 activity *in vivo* can lead to disease. RasGRP1 overexpression overwhelms basal autoinhibition and leads to T cell leukemia (Klinger et al,. 2004; Oki et al., 2011; Hartzell et al., 2013). $RasGRP1^{Anaef}$, a mouse missense variant with a point-mutated EF2, weakens autoinhibition, and follicular helper-like T cells (Tfh-like cells) aberrantly accumulate as a result of increased basal $RasGRP1^{Anaef}$-mTOR (mammalian/mechanistic target of rapamycin) signaling (Daley et al., 2013). These cells aberrantly stimulate B cells to produce autoantibodies, driving a lupus-like disease. It is of interest to note that *RasGRP1* variants have been implicated in human autoimmune disease as well: splice variants in *RasGRP1* have been reported in systemic lupus erythematosus (Yasuda et al., 2007), and single nucleotide variants near *RasGRP1* have been identified in genome-wide association studies of type 1 diabetes and Graves' disease (Qu et al., 2009; Plagnol et al., 2011).

A Second Ras-ERK Pathway that Generates Digital Effector Kinase Signals

The SOS1 and SOS2 RasGEFs are ubiquitously expressed in all cell types, including lymphoid cells. In T lymphocytes that are stimulated through the TCR, these RasGEFs are recruited to the membrane via the adaptor molecule Grb2, which binds to phosphorylated tyrosines in LAT (Jun et al., 2013a). Like RasGRP1, SOS1 has a catalytic domain consisting of REM and CDC25 modules, which can bind and activate Ras. The identification of the DAG-RasGRP1-Ras pathway in T lymphocytes immediately brought up a conundrum as to why these cells would have two pathways, via SOS and via RasGRP, to activate Ras following stimulation of the same receptor (the TCR). The fact that *RasGRP1*-deficient mice display such a profound defect in thymocyte positive selection implies that SOS cannot compensate for RasGRP1 deficiency (Dower et al., 2000). Vice versa, SOS RasGEFs play a role at the TCRβ selection checkpoint, earlier in thymocyte development, and defects at this checkpoint observed in *SOS1*-deficient mice are not compensated for by RasGRP1 (Kortum et al., 2011).

Biochemical and computational studies on these two families of RasGEFs, aided by structural work, revealed that RasGRP and SOS can signal to Ras in distinct patterns. RasGRP1 activates Ras (and the downstream ERK kinase) in an analog manner: there is a linear correlation between signal input to RasGRP1 and Ras-ERK signal output (Roose et al., 2007; Das et al., 2009; Jun et al., 2013a; Ksionda et al., 2013). By contrast, SOS1 can signal to Ras-ERK in a digital or bimodal manner: when the input signal to SOS1 crosses a threshold, a jump in the intensity of the Ras-ERK signal occurs (Roose et al., 2007; Das et al., 2009; Prasad et al., 2009; Jun et al., 2013b,a). The molecular basis for the digital Ras-ERK signal lies in a positive feedback loop on SOS1 (**Figure 5**). Crystal structures of SOS1 with Ras demonstrated that there is a second binding site for Ras on SOS1, distal of the catalytic pocket. Occupancy of this allosteric pocket by Ras-GTP increases the GEF activity of SOS1 *in vitro* (Margarit et al., 2003; Sondermann et al., 2004; **Figure 5**). Since SOS1 itself generates Ras-GTP, this phenomenon creates a positive feedback loop that was demonstrated to exist in several cell types, including lymphocytes (Boykevisch et al., 2006; Roose et al., 2007; Das et al., 2009; Jun et al., 2013a; Jeng et al., 2012; Depeille et al., 2015). RasGRP1 also facilitates engagement of this positive feedback loop over SOS by generating some initial Ras-GTP to prime SOS1 (Roose et al., 2007).

Thus, lymphocytes have a choice of signaling to Ras-ERK in an analog (only RasGRP) or digital (RasGRP and SOS) manner, but the true *in vivo* implication of analog versus digital Ras-ERK signals still needs to be elucidated. The phenotypes of the different genetic mouse models clearly indicated that these RasGEFs play distinct roles during thymocyte development (Dower et al., 2000; Kortum et al., 2011, 2012). While this could be the consequence of altered analog or digital Ras-ERK signaling, it is also possible that the defects in thymocyte development are caused by impaired signals through other (non-Ras) pathways that depend on either RasGRP1 or on SOS1.

Concluding Remarks

Here we have provided an overview of the several major effector kinase and phosphatase pathways that connect proximal TCR signaling to NFAT, NFκB, and activator AP-1 transcription factors, and we discussed the role these pathways play in T cells. We want to emphasize that we did not attempt to cover all effector pathways but focused on the more established ones. For example, in many cancer settings, PI3K is an effector molecule of Ras-GTP (Castellano and Downward, 2010, 2011). In T cells, CD28 costimulation is thought to enhance PI3K activation, but the signaling pathways triggered by CD28 are poorly understood and the exact connection between CD28 and PI3K is controversial as well (Boomer and Green, 2010). PI3K is a lipid kinase that converts PIP2 into PIP3 (phosphatidylinositol 3,4,5-trisphosphate) at the membrane resulting in the recruitment of plextrin homology (PH)-domain containing proteins such as the kinase AKT. The PH domain binds to PIP3. One of the pathways that AKT can activate involves mTOR, which regulates metabolic processes in cells and is covered elsewhere in the *Encyclopedia of Immunobiology*.

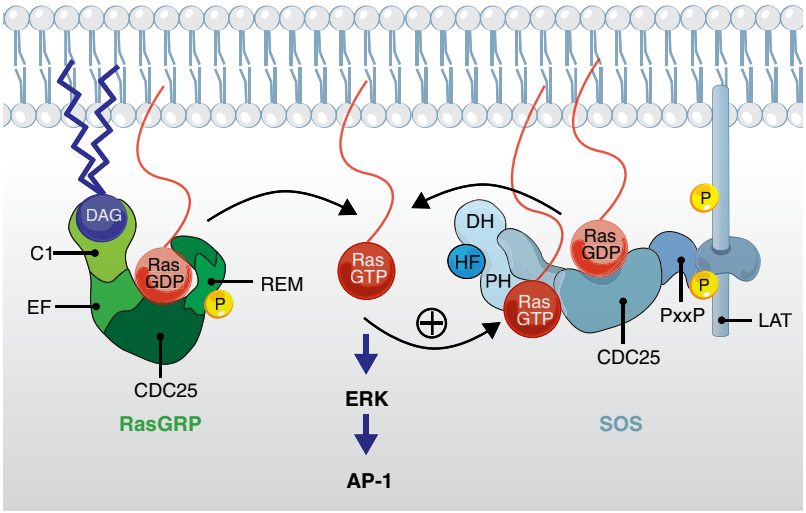

Figure 5 Coexpression of RasGRP1 and SOS1 in lymphocytes allows for choices in analog or digital Ras-ERK signaling. The catalytic module of RasGRP1, the REM-CDC25 domains, can bind Ras and activate it, producing Ras-GTP. There is a linear relationship between the amount of signal input and the amount of Ras-ERK output. In contrast, SOS1 can bind Ras at two distinct sites, one in its catalytic domain (also comprised of REM-CDC25 domains) and another at an allosteric site distal to the catalytic module. The allosteric site binds Ras-GTP, and when Ras-GTP is bound there it greatly enhances the GEF activity of SOS. SOS1 itself can initially generate Ras-GTP that then positively feeds back on SOS1 by binding to the allosteric site. Additionally, RasGRP1 can provide this initial burst of Ras-GTP that binds to the allosteric site of SOS1. This positive feedback loop means that SOS1-Ras signaling occurs in a digital fashion: once a certain threshold of signal input is crossed, a jump in the intensity of the Ras-ERK-AP-1 output occurs. Figure created by Anna Hupalowska.

Lastly, it is intuitive to focus on the catalytic activity of enzymes such as kinases, phosphatases, and RasGEFs, but it is important to keep in mind that these enzymes can also fulfill noncatalytic, adaptor functions and couple to signaling proteins in an unanticipated manner. For example, we have identified a P38 kinase pathway that relies on the presence but not the catalytic function of the RasGEF SOS1 (Jun et al., 2013b). Similarly, the *RasGRP1* variant *RasGRP1*^{Anaef} signals to mTOR at an increased intensity resulting in increased expression of the mTOR target gene CD44 (Daley et al., 2013). However, Ras target genes, such as CD69 and TCRβ, are expressed at normal levels in *RasGRP1*^{Anaef} T cells (Daley et al., 2013), arguing that there is also a noncanonical signaling pathway downstream of the RasGEF RasGRP1 to mTOR, revealed by the *RasGRP1*^{Anaef} variant.

See also: **Autoimmunity:** Animal Models of Autoimmunity; Systemic Lupus Erythematosus. **Development of T Cells and Innate Lymphoid Cells:** TCR Affinity and Signaling during Thymocyte Selection. **Signal Transduction:** Adapter Molecules in Immune Receptor Signaling; Cellular Metabolism Controls Lymphocyte Activation and Differentiation; Immunological Synapses; TCR Signaling: Proximal Signaling; Ubiquitin Signaling to NF-κB. **T Cell Activation:** Th1 Cells; The Differentiation and Function of Th2 and Th9 Cells; Treg Cells.

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