



PKC-theta-mediated signal delivery from the TCR/CD28 surface receptors

Noah Isakov^{1*} and Amnon Altman²

¹ The Shraga Segal Department of Microbiology and Immunology, Faculty of Health Sciences and the Cancer Research Center, Ben-Gurion University of the Negev, Beer Sheva, Israel

² Division of Cell Biology, La Jolla Institute for Allergy and Immunology, La Jolla, CA, USA

Edited by:

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*Correspondence:

Noah Isakov, The Shraga Segal Department of Microbiology and Immunology, Faculty of Health Sciences and the Cancer Research Center, Ben-Gurion University of the Negev, P.O. Box 653, Beer Sheva 84105, Israel.
e-mail: noah@bgu.ac.il

Protein kinase C-theta (PKC θ) is a key enzyme in T lymphocytes, where it plays an important role in signal transduction downstream of the activated T cell antigen receptor (TCR) and the CD28 costimulatory receptor. Interest in PKC θ as a potential drug target has increased following recent findings that PKC θ is essential for harmful inflammatory responses mediated by Th2 (allergies) and Th17 (autoimmunity) cells as well as for graft-versus-host disease (GvHD) and allograft rejection, but is dispensable for beneficial responses such as antiviral immunity and graft-versus-leukemia (GvL) response. TCR/CD28 engagement triggers the translocation of the cytosolic PKC θ to the plasma membrane (PM), where it localizes at the center of the immunological synapse (IS), which forms at the contact site between an antigen-specific T cell and antigen-presenting cells (APC). However, the molecular basis for this unique localization, and whether it is required for its proper function have remained unresolved issues until recently. Our recent study resolved these questions by demonstrating that the unique V3 (hinge) domain of PKC θ and, more specifically, a proline-rich motif within this domain, is essential and sufficient for its localization at the IS, where it is anchored to the cytoplasmic tail of CD28 via an indirect mechanism involving Lck protein tyrosine kinase (PTK) as an intermediate. Importantly, the association of PKC θ with CD28 is essential not only for IS localization, but also for PKC θ -mediated activation of downstream signaling pathways, including the transcription factors NF- κ B and NF-AT, which are essential for productive T cell activation. Hence, interference with formation of the PKC θ -Lck-CD28 complex provides a promising basis for the design of novel, clinically useful allosteric PKC θ inhibitors. An additional recent study demonstrated that TCR triggering activates the germinal center kinase (GSK)-like kinase (GLK) and induces its association with the SLP-76 adaptor at the IS, where GLK phosphorylates the activation loop of PKC θ , converting it into an active enzyme. This recent progress, coupled with the need to study the biology of PKC θ in human T cells, is likely to facilitate the development of PKC θ -based therapeutic modalities for T cell-mediated diseases.

Keywords: protein kinase C-theta, PKC θ , CD28, Lck, signal transduction, costimulation

INTRODUCTION

Protein kinase C-theta (PKC θ) is a key regulator of signal transduction in activated T cells that is linked to multiple pathways downstream of the T cell antigen receptor (TCR; Isakov and Altman, 2002). Engagement of the TCR and the resulting formation of diacylglycerol (DAG) are sufficient for promoting PKC θ recruitment to cell membranes (Monks et al., 1997, 1998). However, localization of PKC θ to the immunological synapse (IS) is entirely dependent on the concomitant ligation of the CD28 coreceptor (Huang et al., 2002). Localization of PKC θ at the center of the IS is essential for activation of signaling pathways that promote T cell-dependent immune responses against distinct antigens and pathogens. While the recruitment of PKC θ to the IS of TCR/CD28 engaged T cells has been extensively studied, information on the molecular basis for this highly selective process has been relatively scarce until recently. The present manuscript provides background information on the molecules involved in this

process and describes in more detail the studies that clarified a new mechanism by which PKC θ is being recruited to the center of the IS and is essential for the induction of PKC θ -dependent activation signals.

THE PKC FAMILY

Protein kinase C was discovered by Nishizuka and colleagues, who demonstrated a new kinase that undergoes activation by limited proteolysis (Inoue et al., 1977), or by translocation to the plasma membrane (PM), where it associates with specific cofactors (Takai et al., 1979). The membrane-associated PKC-activating factor turned to be DAG (Kishimoto et al., 1980). DAG, together with inositol 1,4,5-trisphosphate (IP₃), are products of phospholipase C-mediated hydrolysis of the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP₂; Berridge and Irvine, 1984; Nishizuka, 1984). These two second messengers transduce signals from a plethora of activated receptors: the hydrophobic

DAG remains bound to the cell membrane where, in addition to PKC, it activates effector molecules such as RasGRP, a guanine nucleotide exchange factor (GEF) for Ras (Lorenzo et al., 2000), while the hydrophilic IP₃ diffuses through the cytosol and binds IP₃-receptors, which function as ligand-gated Ca²⁺ channels in the endoplasmic reticulum (ER), thereby triggering the release of free Ca²⁺ ions into the cytoplasm (Takai et al., 1979; Khan et al., 1992; Bourguignon et al., 1994). The utilization of phorbol esters, which mimic the activity of DAG, together with Ca²⁺ ionophores, demonstrated that PKC also plays an essential role in the induction of T lymphocyte proliferation (Truneh et al., 1985; Isakov and Altman, 1987) and reactivation of effector cytotoxic T cells (Isakov and Altman, 1985; Isakov et al., 1987).

Protein kinase C enzymes transduce a myriad of signals from a large number of cell surface receptors that are coupled to phospholipase C and phospholipid hydrolysis. They regulate the function of effector molecules by phosphorylating specific serine and threonine residues. The PKC family includes 10 structurally and functionally related isoforms (for more details, see the first review by Pfeifhofer-Obermair et al., 2012), grouped into three subfamilies based on the composition of their regulatory domains and their respective cofactor requirements (Newton, 1995; Meller and Parker, 1998). The first subfamily includes conventional PKCs (cPKC; α , β I, β II, γ) that are regulated via two DAG-binding C1 domains organized in tandem near the cPKC amino terminus (Hurley et al., 1997; Johnson et al., 2000; Ho et al., 2001) and an adjacent Ca²⁺ and phospholipid-binding C2 domain (Nalefski and Falke, 1996; Johnson et al., 2000). The second group includes novel PKCs (nPKC; δ , ϵ , η , θ) that are DAG-dependent, but Ca²⁺ and phospholipid independent for their activity. The third group includes atypical PKCs (aPKC; ζ , λ/ι) that are DAG-, Ca²⁺-, and phospholipid-independent. While PKC enzymes are involved in metabolic processes in different cell types, many studies implicate PKC enzymes in signal transduction networks that convert environmental cues into cellular actions (Rosse et al., 2010). Six of the PKC isoforms, including PKC α , δ , ϵ , η , θ , and ζ are expressed at varying amounts in T cells (Meller et al., 1999). Immunological studies using different genetic models and pharmacological drugs indicated that distinct PKC isoforms are required for different aspects of the activation and effector functions of T cells. The results suggest that distinct PKC isoforms may serve as drug targets for different T cell mediated adaptive immune responses (Baier and Wagner, 2009).

PROTEIN KINASE C-THETA

Protein kinase C-theta is a Ca²⁺-independent nPKC isoform exhibiting a relatively selective pattern of tissue distribution, with predominant expression in T lymphocytes (Baier et al., 1993; Meller et al., 1999), platelets (Chang et al., 1993; Meller et al., 1998; Cohen et al., 2009), and skeletal muscle (Osada et al., 1992; Chang et al., 1993). It has a unique ability to translocate to the center of the IS of activated T cells (Monks et al., 1997, 1998) where its full activation requires the integration of TCR and CD28 costimulatory signals (Huang et al., 2002; Tseng et al., 2008; Yokosuka et al., 2008). Engagement of the TCR and the CD28 coreceptor initiates a series of PKC θ -dependent signaling

events leading to activation of transcription factors, including NF- κ B, AP-1, and NF-AT, which are critical for T cell activation, proliferation and differentiation (Baier-Bitterlich et al., 1996; Coudronniere et al., 2000; Dienz et al., 2000; Lin et al., 2000; Sun et al., 2000; Pfeifhofer et al., 2003). Under certain activation conditions, PKC θ can translocate to the nucleus where it directly associates with chromatin and is involved in the regulation of microRNAs and T cell-specific inducible gene expression program (Sutcliffe et al., 2011). The exact mechanism by which the membrane-bound PKC θ delivers signals to the nucleus has not been fully resolved but studies provided information on a number of effector molecules that operate along this pathway in activated T cells. These studies demonstrated that PKC θ -mediated regulation of NF- κ B activity involves the multisubunit inhibitor of κ B (I κ B) kinase (IKK) complex (Coudronniere et al., 2000; Dienz et al., 2000; Khoshnan et al., 2000; Lin et al., 2000; Bauer et al., 2001).

An important upstream effector in the NF- κ B signaling pathway is I κ B α , which binds NF- κ B in the cytoplasm of resting T cells and mask its nuclear localization signal (NLS), thereby preventing NF- κ B translocation to the nucleus (Mercurio et al., 1997; Regnier et al., 1997; Jacobs and Harrison, 1998). IKK-mediated phosphorylation of I κ B α signals the protein for degradation (Karin, 1999), exposes the NF- κ B NLS and promotes NF- κ B translocation to the nucleus and the induction of NF- κ B-mediated gene transcription. T cells from PKC θ -deficient (*Prkcd*^{-/-}) mice fail to respond to TCR stimulation with degradation of I κ B α (Sun et al., 2000), supporting the model whereby PKC θ regulates NF- κ B activity through its effect on IKK-I κ B α . Some of the effector molecules that link PKC θ to IKK have been identified and include the PKC θ substrate protein, caspase activation and recruitment domain (CARD) and membrane-associated guanylate kinase (MAGUK) domain-containing protein-1 (CARMA1). This scaffold protein is primarily expressed in lymphocytes (Bertin et al., 2001; Hara et al., 2003), where it links PKC θ to NF- κ B activation in T cells (Ruland et al., 2001, 2003; Ruefli-Brasse et al., 2003; Xue et al., 2003). Phosphorylation of CARMA1 by PKC θ in TCR/CD28-stimulated T cells, promotes CARMA1 association with the B-cell lymphoma/leukemia 10 (Bcl10) and mucosa-associated lymphoid tissue 1 (MALT1) proteins (Matsumoto et al., 2005; Sommer et al., 2005) leading to recruitment of the trimolecular complex to the IS (Gaide et al., 2002; Che et al., 2004; Hara et al., 2004) and activation of the IKK complex (McAllister-Lucas et al., 2001). Furthermore, overexpression of CARMA1, Bcl10, and MALT1 in T cells, followed by TCR/CD28 stimulation, resulted in the formation of a CARMA1-Bcl10-MALT1 trimolecular complex, where all three proteins were required for maximal activation of NF- κ B (McAllister-Lucas et al., 2001; Ruland et al., 2001). It should be noted that in some studies (Khoshnan et al., 2000), but not others (Lin et al., 2000), PKC θ was found to directly associate with members of the IKK complex, particularly IKK β , suggesting the potential existence of an additional linear route from PKC θ to NF- κ B. The transcription factor AP-1, similar to NF- κ B, is a primary physiological target of PKC θ (Baier-Bitterlich et al., 1996; Li et al., 2004), while regulation of the NF-AT transcription factor requires cooperation between PKC θ and calcineurin, a Ca²⁺-dependent serine/threonine phosphatase (Pfeifhofer et al., 2003).

All three PKC θ -regulated transcription factors have corresponding binding sites on the IL-2 gene promoter, and their binding to the IL-2 gene is essential for optimal IL-2 response (Isakov and Altman, 2002).

While PKC θ -mediated regulation of NF- κ B activity in TCR/CD28-stimulated T cells has been studied in great detail, PKC θ is also involved in the regulation of additional cellular functions, and physically associates with additional binding partners. Besides CARMA1, PKC θ physically associate with 14-3-3 τ (Meller et al., 1996), Cbl (Liu et al., 1999), Fyn (Ron et al., 1999), Lck (Liu et al., 2000), AKT (Bauer et al., 2001), moesin (Pietromonaco et al., 1998), PICOT (Witte et al., 2000), and the HIV nef protein (Smith et al., 1996). Some of these molecules (i.e., Lck) phosphorylate PKC θ and may affect its activity and/or subcellular distribution, while others, which serve as substrates for PKC θ (i.e., Cbl, 14-3-3 τ and moesin) may regulate cellular functions, such as cytoskeletal reorganization.

DIFFERENTIAL REQUIREMENTS FOR PKC θ BY DISTINCT T CELL SUBPOPULATIONS

Initial characterization of PKC θ -deficient T cells suggested the involvement of PKC θ in cellular responses leading to T cell activation, proliferation, and cytokine production (Sun et al., 2000; Pfeifhofer et al., 2003; Anderson et al., 2006). Subsequent *in vitro* and *in vivo* investigations and the analysis of *Prkccq*^{-/-} mice in different disease models demonstrated differential requirements for PKC θ by distinct T cell subpopulations and during the induction of selected types of immune responses. Thus, PKC θ was found to be essential for the induction of Th2-type immune responses to allergens or helminth infection (Marsland et al., 2004; Salek-Ardakani et al., 2004) and the induction of Th17-mediated experimental autoimmune encephalomyelitis (EAE) that serves as a model of multiple sclerosis (Salek-Ardakani et al., 2005; Anderson et al., 2006; Tan et al., 2006; Marsland et al., 2007; Kwon et al., 2012), and other experimental autoimmune diseases (Anderson et al., 2006; Healy et al., 2006; Marsland et al., 2007; Chuang et al., 2011). In contrast, Th1-dependent mouse resistance to *Leishmania major* infection was intact in *Prkccq*^{-/-} mice (Marsland et al., 2004; Ohayon et al., 2007), and PKC θ was dispensable for CTL-mediated protective antiviral responses, most likely reflecting compensation by innate immunity signals (Berg-Brown et al., 2004; Giannoni et al., 2005; Marsland et al., 2005, 2007; Valenzuela et al., 2009). Consistent with the *in vivo* findings, *in vitro* induction of CD4+ T cell polarization by optimal T cell-antigen-presenting cell (APC) coculture conditions, demonstrated a requirement for PKC θ during Th2 and Th17 cell development, and only moderate effect of PKC θ on Th1 cell development (Marsland et al., 2004; Salek-Ardakani et al., 2004, 2005). Additional studies performed in *Prkccq*^{-/-} mice demonstrated the requirement for PKC θ in the induction of graft-versus-host (GvH) and alloreactive T cell-mediated immune responses (Valenzuela et al., 2009). In contrast, PKC θ -deficient T cells retained the ability to induce graft-versus-leukemia (GvL) responses in allogeneic bone marrow (BM) transplanted mice (Valenzuela et al., 2009).

Protein kinase C-theta also contributes to allograft rejection, as shown by Manicassamy et al. (2008) using an adoptive transfer

model. In these studies, *Rag*^{-/-} mice reconstituted with *Prkccq*^{-/-} T cells were unable to reject cardiac allografts, in contrast to the acute allograft rejection observed in the wild-type T cell reconstituted *Rag*^{-/-} mice. However, this was due to lack of PKC θ -regulated expression of anti-apoptotic molecules, such as Bcl-x_L, which led to apoptosis of the effector T cells; transgenic expression of Bcl-x_L in *Prkccq*^{-/-} T cells restored their ability to reject the cardiac allografts. The rejection of cardiac allograft by *Prkccq*^{-/-} mice was only slightly delayed (Manicassamy et al., 2008; Gruber et al., 2009), suggesting compensation by other PKC isoforms. Indeed, mice lacking both PKC θ and PKC α , demonstrated a significantly delayed rejection of cardiac allografts (Gruber et al., 2009).

The overall positive role of PKC θ in the activation of effector T cells (T_{eff}) and the promotion of adaptive immune responses raise questions about the nature of its function in regulatory T cells (T_{reg}) that suppress T_{eff} functions. This issue has recently been partially resolved by Zanin-Zhorov et al. (2010) who found that PKC θ mediates negative feedback on T_{reg} functions. Furthermore, activation of T_{reg} resulted in sequestration of PKC θ away from the IS, and inhibition of PKC θ activity (using the C20 compound) increased the suppressive activity of T_{reg} (Zanin-Zhorov et al., 2010, 2011). *In vivo* studies demonstrated that T_{reg} development in the thymus of *Prkccq*^{-/-} mice is impaired leading to reduced numbers of T_{reg} cells in the periphery (Schmidt-Suppryan et al., 2004; Zanin-Zhorov et al., 2010, 2011), although activity of these mature PKC θ -deficient T_{reg} cells was intact (Gupta et al., 2008).

THE IMMUNOLOGICAL SYNAPSE

Adaptive immune responses are dependent on the effective communication between antigen-specific T cells and APCs. At the very early phase of the activation response, T cells interact via their TCR with cognate peptide-MHC complexes on the surface of APCs and both cell types respond by redistributing their receptors/ligands to the contact area that rearranges as a platform for effective signaling (Dustin and Zhu, 2006). The IS, representing the interface between a T cell and an APC, is formed by specific protein microclustering (Yokosuka et al., 2005) and their segregation into one of two separate regions: a central core [central supramolecular activation clusters (cSMAC)], which contains the TCR and costimulatory receptors, and a peripheral region [peripheral supramolecular activation clusters (pSMAC)], which contains adhesion molecules, such as LFA-1 (Dustin, 2009). T cell surface receptor engagement triggers signaling cascades that result in the recruitment of multiple membrane-anchored and cytoplasmic effector molecules, including kinases, adaptor proteins, and cytoskeletal components, to the IS (Dustin et al., 2010). One of the most prominent proteins to be recruited to the IS of antigen-responding T cells is PKC θ , which localizes at the cSMAC (Monks et al., 1997, 1998). Additional high-resolution imaging analysis by TIRF microscopy demonstrated that PKC θ colocalizes with CD28, and demonstrated that the cSMAC is divided into two structurally and functionally distinct compartments: a central TCR^{high} compartment, where signaling is terminated (Vardhana et al., 2010) and TCR-associated signaling complexes are internalized and degraded, and an outer TCR^{low} “ring” where PKC θ and CD28 colocalize (Yokosuka et al., 2008).

CD28

CD28 is a type 1 transmembrane glycoprotein that is constitutively expressed as a disulfide-linked homodimer on all CD4+ and CD8+ murine T cells and majority of CD4+ and CD8+ human peripheral blood T cells (Gross et al., 1990; Vallejo, 2005). The human CD28 precursor protein is 220 amino acids long (218 in mouse) and the mature protein possesses 202 amino acids (218 in mouse) due to cleavage of an amino-terminal leader sequence (18 and 19 amino acids in the human and mouse CD28, respectively). In addition, CD28 possesses a cytoplasmic tail of 41 amino acids (38 in mouse) that is critical for signal transduction and coreceptor-induced cell stimulation. Physiological activation of CD28 is mediated by one of two natural ligands expressed on the surface of APCs, CD80, and CD86, which directly associate with a conserved motif [MYPPPY (single amino-acid letter code)] in the extracellular region of CD28 (Kariv et al., 1996; Truneh et al., 1996). Engagement of CD28 provides costimulatory signals that complement or synergize with those provided by the TCR, leading to optimal activation of T cells (Thompson et al., 1989; Harding et al., 1992). CD28 engagement increases IL-2 production (Thompson et al., 1989; Jain et al., 1995; Reichert et al., 2001) and IL-2 receptor expression (Shahinian et al., 1993), and provides survival signals by upregulating the anti-apoptotic protein, Bcl-X_L (Boise et al., 1993). In addition, CD28 synergizes with the TCR in providing potent signals for activation of c-Jun kinase (JNK), p38 MAP kinase, and IKK pathways (Su et al., 1994; Harhaj and Sun, 1998), and activation of the NF- κ B (Michel et al., 2000; Diehn et al., 2002) AP-1 (Rincon and Flavell, 1994) and NF-AT transcription factors (Michel et al., 2000; Diehn et al., 2002).

The positive role of CD28 in T cell activation was demonstrated in CD28-deficient (*Cd28*^{-/-}) T cells, in which TCR engagement in the absence of CD28 costimulation resulted in anergy and/or tolerance induction upon rechallenge with the same antigen (Appleman and Boussiotis, 2003). T cell proliferation and Th2-type cytokine secretion were also severely impaired in *Cd28*^{-/-} mice or wild-type mice treated with CD28 antagonists (Green et al., 1994; Lucas et al., 1995; Rulifson et al., 1997; Schweitzer et al., 1997; Gudmundsdottir et al., 1999). Furthermore, lack of CD28-mediated costimulation led to reduced immune responses against infectious pathogens (Shahinian et al., 1993; King et al., 1996; Mittrucker et al., 2001; Compton and Farrell, 2002) and allografts (Salomon and Bluestone, 2001) and impaired GvH disease (Via et al., 1996), contact hypersensitivity (Kondo et al., 1996), and asthma (Krinzman et al., 1996).

T cell receptor engagement in the absence of CD28 costimulation induces an unbalanced signaling response in which TCR-mediated Ca²⁺ influx predominates. This leads to activation of calcineurin which dephosphorylates NF-AT leading to its nuclear translocation and induction of a limited set of anergy-associated genes resulting in T cell anergy (Macian et al., 2004). CD28, in contrast to the TCR, does not induce a Ca²⁺ response (Lyakh et al., 1997). Instead, CD28-coupled costimulatory signals induce the activation of NF- κ B and AP-1, and concomitant AP-1 association with NF-AT, conditions that promote IL-2 production and rescue of the T cells from a state of anergy (Macian et al., 2004).

SIGNALING DOWNSTREAM OF CD28

CD28 delivers signals in activated T cells via its cytoplasmic tail, which has no intrinsic catalytic activity, but possesses several protein-protein interaction motifs that enable it to associate with enzymes and other effector molecules (Boise et al., 1993; see **Figure 1**). In resting T cells, non-phosphorylated CD28 associates with the serine/threonine protein phosphatase protein 2A (PP2A), which dissociates from CD28 upon activation induced-phosphorylation of CD28 (Chuang et al., 2000). CD28 triggering by its ligands leads to phosphorylation of tyrosine residues (Raab et al., 1995; Teng et al., 1996; King et al., 1997) in the cytoplasmic tail of CD28, creating new docking sites for different effector molecules that initiate the activation of signaling cascades, and define the costimulatory functions of CD28 (Raab et al., 1995; Andres et al., 2004; Dodson et al., 2009).

The first motif in the human CD28 cytoplasmic tail, juxtaposed to the PM, contains a Y¹⁷³MNM sequence that undergoes tyrosine phosphorylation following the engagement of CD28 and serves as a binding site for the SH2 domain of p85, the regulatory subunit of the lipid kinase, phosphatidylinositol 3-kinase (PI3K; August and Dupont, 1994; Pages et al., 1994; Prasad et al., 1994; Truitt et al., 1994). The methionine residue at the +3 position confers specificity for p85 binding (Takeda et al., 2008), while the asparagine at the +2 position confers additional specificity for the SH2 domain of Grb2 and GADS (Songyang et al., 1993; Raab et al., 1995; Sanchez-Lockhart et al., 2004; Schneider et al., 1995; Harada et al., 2001). The relative concentration of PI3K, Grb2, and GADS at the vicinity of CD28 cytoplasmic tail, and the relative affinity of their SH2 domain for the phospho-Tyr¹⁷³-containing

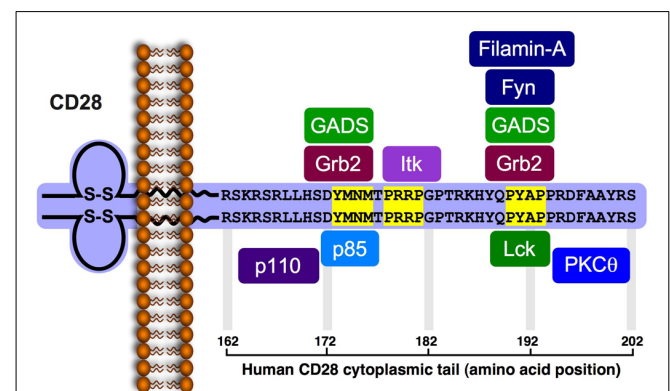


FIGURE 1 | Signaling motifs in the cytoplasmic tail of the human CD28 and binding partners.

The human *CD28* encodes a 220 amino acid-long protein (218 in the mouse) that includes a leader sequence of 18 residues (19 residues in the mouse). The mature protein (202 residues) possesses a 41 amino acid-long cytoplasmic tail that includes three potential protein-protein interaction motifs (highlighted in yellow). The phospho-Tyr¹⁷³ within the YNMN motif serves as a docking site for the SH2-containing proteins, p85, Grb2 and GADS. The P¹⁷⁸RRP motif can interact with the SH3 domain of Itk. The P¹⁹⁰YAP motif can interact with the SH3 domain of Grb2, GADS and Lck, as well as with filamin-A. Phosphorylation of Tyr¹⁹¹ within the PYAP motif creates a docking site for the Lck SH2 domain and enables PKC θ to interact via its V3 domain with the Lck SH3. Studies indicate that Tyr¹⁹¹ is important for CD28 and PKC θ localization to the cSMAC, and that the PYAP motif contributes to T cell activation and cytokine expression.

motif likely determine which of the three potential binding partners interacts with the activated CD28 and, hence, the resulting functional outcome. A second, nearby motif possesses the P¹⁷⁸RRP sequence, and serves as a binding site for the SH3 domain of IL-2-inducible T cell kinase (Itk; Marengere et al., 1997; Garcon et al., 2004). CD28-mediated activation of Itk is dependent on Lck (Gibson et al., 1996), but the actual role of Itk in CD28-induced costimulation is still controversial (Liao et al., 1997; Gibson et al., 1998; Yang and Olive, 1999; Li and Berg, 2005). A third, more distal, P¹⁹⁰YAP motif serves as a potential docking site for several different effector molecules. These include filamin-A, an actin binding protein and a scaffold for lipid raft formation, which utilizes repeat 10 (amino acids 1158–1246) for interaction with CD28 (Tavano et al., 2006), Grb2 and GADS adaptor proteins, which bind the P¹⁹⁰YAP motif via their SH3 domain (Okkenhaug and Rottapel, 1998; Ellis et al., 2000), and the Lck and Fyn protein tyrosine kinases (PTKs; Hutchcroft and Bierer, 1994; zur Hausen et al., 1997; Holdorf et al., 1999; Tavano et al., 2004). Both Lck and Fyn were implicated in the early phase of the CD28 signaling pathway (August et al., 1994) and coexpression studies demonstrated that the two PTKs could phosphorylate CD28, primarily on Tyr¹⁷³ at the Y¹⁷³MNM motif, thereby increasing the binding of p85- and Grb2-SH2 to CD28 (Raab et al., 1995). Lck and Fyn were also found to coimmunoprecipitate with CD28 from activated T cells (Hutchcroft and Bierer, 1994), where Lck interacted with the P¹⁹⁰YAP motif via its SH3 domain (Holdorf et al., 1999; Tavano et al., 2004), and Fyn interacted with the same motif using its SH2 domain (zur Hausen et al., 1997), although other studies indicated no interaction between CD28 and Fyn (Marengere et al., 1997). While presence of the two proline residues in the P¹⁹⁰YAP motif predicts interaction with SH3-containing proteins, binding studies demonstrated that the Lck-SH3 domain interacts with relatively low affinity ($K_d > 1 \mu\text{M}$) with peptides that contain the P¹⁹⁰YAP motif and correspond to residues 188–202 of human CD28, or 186–196 of murine CD28, respectively (Hofinger and Sticht, 2005).

Other studies demonstrated that Tyr¹⁹¹ within the P¹⁹⁰YAP motif is one of two major phosphorylation sites in CD28-stimulated Jurkat T cells, and the only tyrosine residue within the CD28 cytoplasmic tail that is essential for delivery of costimulatory signals leading to CD69 expression and synthesis and secretion of IL-2 (Sadra et al., 1999). The latter findings raise the possibility that CD28 engagement-induced phosphorylation of Tyr¹⁹¹ creates a new and transient binding site for SH2-containing proteins, possibly Lck, since CD28 and Lck were shown to colocalize at the cSMAC (Tavano et al., 2004; Kong et al., 2011). Binding studies provided further support for this hypothesis by showing that a CD28-derived peptide that possesses phospho-Tyr¹⁹¹ interacts with the Lck-SH2 domain with a relatively high affinity ($K_d = 2.13 \mu\text{M}$; Hofinger and Sticht, 2005), at the range of other SH2-ligand interactions (Bauer et al., 2004). This binding affinity is about three orders of magnitude stronger than that for the Lck-SH3 domain. High affinity binding of Lck-SH2 to P¹⁹⁰pYAP occurs despite the difference between this sequence and the phospho-YEEI sequence predicted to be the preferred binding site of the Lck-SH2 domain (Songyang

et al., 1993). More recent studies indicated that PKC θ can also interact with the cytoplasmic tail of CD28, and that this interaction involves Lck as an intermediate molecule, as discussed below.

CD28 AND THE IS

Upon binding of its ligand, B7, CD28, similar to the engaged TCR, accumulates at the cSMAC of the IS although the two receptors initiates distinct but complementary signaling pathways. The transient recruitment of CD28 to the immature IS of TCR engaged T cells is very rapid and occurs within seconds of the onset of the calcium signal (Andres et al., 2004). Engagement of the TCR in *Cd28*^{-/-} T cells results in altered, diffuse pattern of distribution of PKC θ and LFA-1 at the IS, suggesting an essential role for CD28 in the initiation and stabilization of the mature IS (Huang et al., 2002; Sanchez-Lockhart et al., 2004). Furthermore, *in vivo* blocking of CD28 impairs the activity of effector molecules, including PKC θ (Jang et al., 2008), and inhibits T cell-dependent immune responses (Linsley and Nadler, 2009). CD28 engagement promotes a cytoskeleton-dependent recruitment of cell surface receptors (Wulfiging and Davis, 1998) and signaling molecules-containing lipid rafts that support building the IS and contribute to signal transduction from IS-residing receptors (Dustin and Shaw, 1999; Viola et al., 1999).

More recent studies demonstrated that in activated T cells, CD28 is recruited coordinately with the TCR to form microclusters at the cSMAC (Yokosuka et al., 2008). Upon progression of this initial step, the CD28 and TCR segregate to two spatially distinct subregions within the cSMAC, a central TCR^{high} subregion, where signaling is terminated and TCR-associated signaling complexes are internalized and degraded, and an outer TCR^{low} annular form that contain CD28 clusters, as well as PKC θ . CD28 and PKC θ were physically associated, as shown by PKC θ coimmunoprecipitation with CD28 from a lysate of PMA-stimulated T cells (Yokosuka et al., 2008).

PKC θ -CD28 INTERACTION AND RECRUITMENT OF PKC θ TO THE IS

T cell receptor engagement polarizes PKC θ and induce its recruitment to the IS, a response that is greatly augmented by CD28 ligation (Huang et al., 2002; Tseng et al., 2008; Yokosuka et al., 2008). Although the recruitment of PKC θ to the center of the IS (cSMAC) of is well documented, information on the molecular basis for this highly selective localization has been relatively scarce. Early studies have shown that PKC θ recruitment to the IS is indirectly dependent on the PI3K interaction motif within the CD28 cytosolic tail (Harada et al., 2001). Thus, mutation of Met¹⁷³ within the mouse YMN motif, which binds PI3K upon its tyrosine phosphorylation, resulted in decreased ability of CD28 to direct PKC θ recruitment to the cSMAC, and inhibited PKC θ -dependent activation of NF- κ B to and the *Il2* gene (Sanchez-Lockhart et al., 2004).

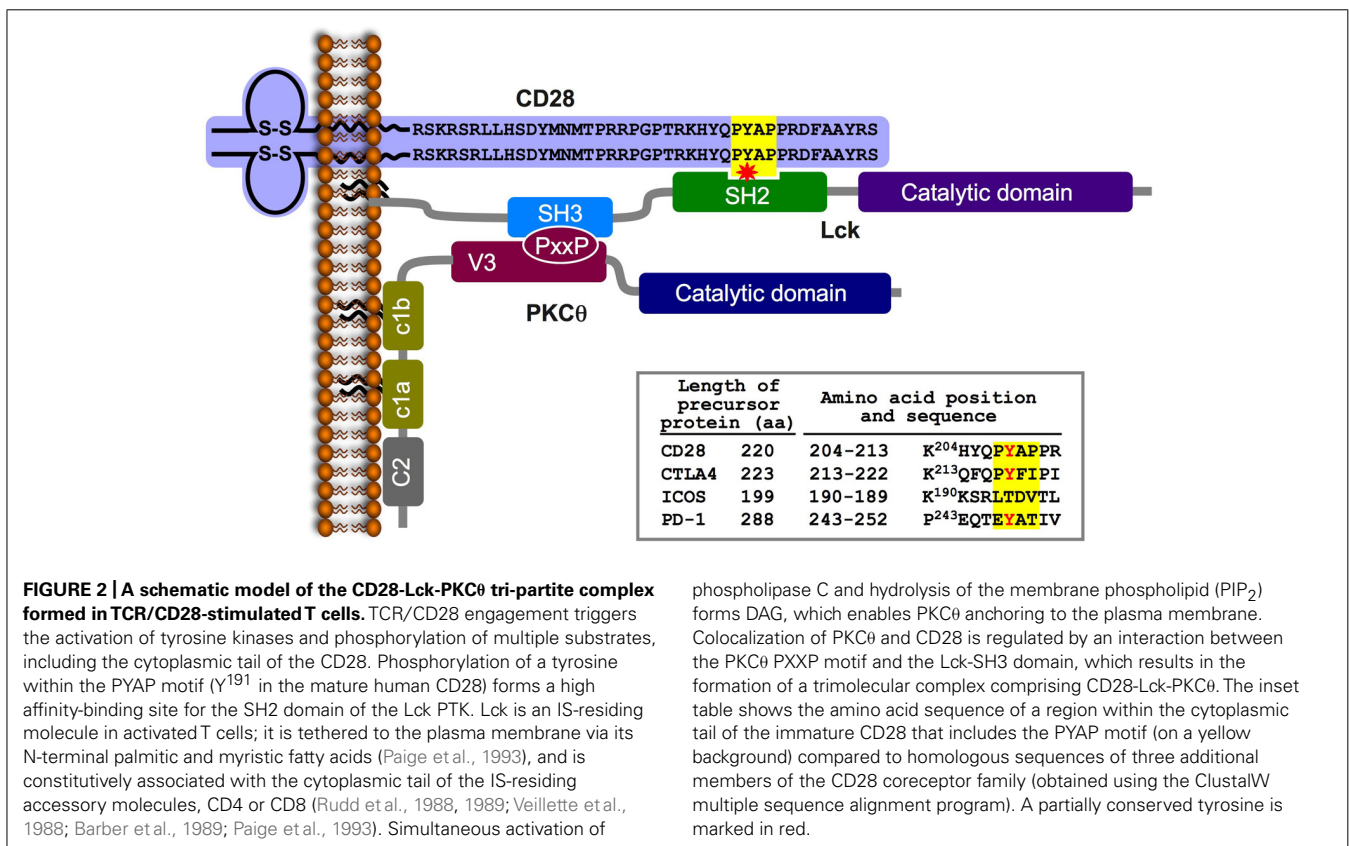
Following the recently reported PKC θ -CD28 association in PMA-stimulated T cells (Yokosuka et al., 2008), we conducted a detailed structure-function analysis of this association in TCR-stimulated T cells (Kong et al., 2011). We demonstrated that PKC θ physically associated with the cytoplasmic tail of CD28

following TCR/CD28 costimulation. Taking advantage of the fact that PKC δ , the closest relative of PKC θ , does not translocate to the IS after T cell-APC interaction (Monks et al., 1997), we compared the amino acid sequence analysis of PKC θ and PKC δ and found that they diverged significantly only in their V3 (hinge) domain, corresponding to amino acids ~291–378 of human PKC θ , suggesting a potential role for this region in targeting PKC θ to the IS. Indeed, a V3-deletion mutant of PKC θ (PKC θ - Δ V3) or an exchange mutant of PKC θ , in which the native V3 domain was replaced by the PKC δ V3 domain, did not coimmunoprecipitate with CD28, and failed to translocate to the IS (Kong et al., 2011) and to activate PKC θ -dependent reporter genes such as the CD28 response element (RE/AP). Conversely, the isolated V3 domain of PKC θ localized in the center of the IS and associated with CD28. Moreover, T cells recovered from mouse BM chimeras on a *Prkca*^{-/-} background reconstituted with the same PKC θ mutants failed to proliferate and produce IL-2 in response to CD3/CD28 costimulation, and their ability to upregulate CD69 or CD25 expression was reduced. Given the critical role of the V3 domain in directing the CD28 association and IS localization of PKC θ , we argued that this domain will function as a dominant negative mutant by disrupting the activation-dependent association between endogenous CD28 and PKC θ . As expected, ectopic expression of the isolated PKC θ V3 domain blocked the recruitment of endogenous PKC θ to CD28 and the IS, and severely inhibited PKC θ -dependent functions, including CD25 and CD69 upregulation, T cell proliferation and IL-2

production, and Th2 and Th17 (but not Th1) differentiation and inflammation.

Fine mapping of the PKC θ V3 domain identified an evolutionarily conserved proline-rich (PR) motif (ARPPCLPTP; corresponding to amino acid residues 328–336 of human PKC θ) within the PKC θ -V3 domain, which was required for PKC θ -CD28 association, PKC θ localization to the IS, and induction of PKC θ -mediated functions. Insertion of this motif into the V3 domain of PKC δ enabled this altered PKC δ form to translocate to the IS and activate PKC θ -dependent signal. The two internal proline residues in this motif (Pro-331 and -334) were particularly critical in this regard (Kong et al., 2011).

In trying to more precisely define the nature of the inducible PKC θ -Lck complex, we focused on the potential contribution of Lck kinase. This possibility was considered in view of previous studies demonstrating a functional relationship between CD28, PKC θ , and Lck. First, in stimulated T cells, Lck can be recruited to the tyrosine-phosphorylated distal PR motif (P¹⁹⁰Y*AP) in the cytoplasmic tail of CD28 via its SH2 and SH3 domains, respectively (Miller et al., 2009; see **Figure 2**). This motif directs the colocalization of PKC θ and CD28 to the cSMAC (Yokosuka et al., 2008) and is apparently involved in additional biological functions, including the stabilization of IL-2 mRNA, reorganization of lipid rafts, and sustained autophosphorylation and activation of Lck at the IS (Holdorf et al., 2002; Sanchez-Lockhart et al., 2004; Dodson et al., 2009). Second, Lck phosphorylates and associates with PKC θ , and mutation of the major Lck phosphorylation site on PKC θ (Tyr⁹⁰)



inhibited PKC θ -dependent activation events in stimulated T cells (Liu et al., 2000).

Our further analysis confirmed the physical and functional CD28-Lck-PKC θ link by demonstrating that Lck function as an intermediate to recruit PKC θ to CD28 upon T cell stimulation. The Lck-SH3 domain interacted with the PR motif in the PKC θ V3 domain, while the Lck SH2 domain interacted with phospho-Tyr¹⁹¹ in the P¹⁹⁰YAP motif in the CD28 cytoplasmic tail. Taken together, the above findings demonstrate a unique signaling mode of CD28 and establish the molecular basis for the specialized localization and function of PKC θ in antigen-stimulated T cells.

THE GLK-PKC θ LINK

Recent studies demonstrated that recruitment of PKC θ to the cSMAC in activated T cells is essential but not sufficient for the full activation of PKC θ and its downstream target molecules. These studies further showed that the germinal center kinase (GSK)-like kinase (GLK) also translocates to the IS of TCR-engaged T cells where it phosphorylates the activation loop of PKC θ , converting it into an active enzyme (Chuang et al., 2011). Of interest, however, despite the importance of PKC θ in the thymic development of natural regulatory T cells (nTregs; Schmidt-Supprian et al., 2004), GLK-deficient mice displayed normal nTreg development (Chuang et al., 2011). These results emphasize the important role of post-transcriptional regulation of PKC θ that occurs at several steps and involve different checkpoints at distinct sites within the activated T cell.

CONCLUSIONS AND FUTURE PERSPECTIVES

Identification and characterization of the molecular mechanism by which PKC θ associates with CD28 and colocalizes with it at the cSMAC has provided important information relevant to the mechanism by which CD28 and PKC θ contribute to signal transduction in TCR/CD28-engaged T cells. These findings also raise new questions relevant to the mechanism of interaction of CD28 and PKC θ and their specific role in the induction of distinct T cell-mediated immune responses. One obvious question relates to the mechanism by which PKC θ is sequestered away from the IS of activated T_{reg} cells. It would be interesting to determine whether a CD28-Lck-PKC θ tri-partite complex (Kong et al., 2011) occurs in T_{reg} cells, and determine the mechanism that enables PKC θ recruitment away from the T_{reg}-APC contact area. A possible explanation for this process was provided by Yokosuka et al. (2010) showing that CTLA-4 competes with CD28 in recruitment to the cSMAC. In addition, it is not known whether PKC θ is involved in a second signal delivery during the costimulation of $\gamma\delta$ T cells (Ribot et al., 2011).

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Despite the extensive amount of studies on the biology of PKC θ in mouse T cells, very little is known about its regulation and function in human T cells. This is a substantial gap that would need to be filled if PKC θ is destined to fulfill its promise as a clinically relevant drug target (Altman and Kong, 2012). As discussed earlier, the dependence of T cell-mediated deleterious autoimmune/inflammatory responses, including GvHD, on PKC θ , but its dispensable role in beneficial responses (antiviral immunity and GvL response) make it an attractive clinical drug target with potentially advantage over global immunosuppressive drugs such as calcineurin inhibitors (e.g., cyclosporine A), which have pronounced toxic side effects. Indeed, there has been considerable interest among pharmaceutical companies in developing small molecule selective PKC θ catalytic activity inhibitors, and AEB071, the most advanced of these compounds, which inhibits other PKC family members in addition to PKC θ , is currently in early clinical trials (Evenou et al., 2009).

Nevertheless, small molecule inhibitors of protein kinases often have toxic side effects because of their lack of absolute specificity, which reflects the relatively high conservation of catalytic domains within the protein kinase family, and even more so within the PKC family. Furthermore, since catalytic kinase inhibitors in current clinical use are ATP competitors, they need to be used at relatively high and potentially toxic concentrations in order to effectively compete with ATP, whose intracellular concentration is \sim 1 mM. As a result, there has recently been considerable interest and progress in developing allosteric kinase inhibitors, which bind to sites other than the catalytic site in kinases and, thus, are likely to be much more selective and less toxic (Lamba and Ghosh, 2012). Our recent study (Kong et al., 2011) demonstrates a new potential approach for attenuating PKC θ -dependent functions utilizing allosteric compounds based on the critical PR motif in the V3 domain of PKC θ that will block its Lck-mediated association with CD28 and recruitment to the IS, which is obligatory for its downstream signaling functions. This new approach could serve as a basis for the development of new therapeutic agents that would selectively suppress undesired T cell-mediated inflammation and autoimmunity or prevent graft rejection, while preserving desired immunity, such as antiviral responses.

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