Minireview

T Cell Receptor Signaling: Beyond Complex Complexes*

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The adaptive phase of the immune response begins with engagement of CD4⁺ helper T cells of the T cell antigen receptor (TCR) by its ligand, a small foreign peptide bound to a cell surface protein of the class II major histocompatibility complex (peptide-MHC) expressed on an antigen-presenting cell. This engagement initiates a series of biochemical events that can differentially signal the naive T cell to: 1) enter into a pathway leading to generation of effector T cells with the onset of rapid proliferation and production of effector cytokines; 2) enter into a state of antigenic non-responsiveness known as anergy; or 3) die by apoptosis. The type of response elicited depends on multiple factors including the affinity of the interaction, the duration of the interaction, and the presence or absence of various costimulatory signaling inputs such as those provided by the CD4 coreceptor and the CD28 costimulatory receptor. In this review we provide an overview of the signaling events that are associated with the first of these outcomes: T cell activation. To present an overview of sufficiently broad scope, the depth of discussion of each aspect of TCR signaling is by necessity limited, and the reader is referred to the reviews cited throughout this text for consideration of these events in more detail.

TCR Structure

The TCR is composed of six different polypeptide chains. The specificity of ligand binding is dictated by the clonotypic TCRα and TCRβ chains, which arise from a process of genetic rearrangement that results in millions of receptor variants. These chains form a heterodimer that binds directly to peptide-MHC. Communication of TCRαβ engagement by peptide-MHC to the intracellular signaling machinery occurs via the TCR-associated CD3 chains, which are arranged into three dimers: γε, δε, and ζζ (1). Each CD3 chain contains immunoreceptor tyrosine-based activation motifs (ITAMs); one each in γ, δ, and ε and three in ζ. The eponymous features of these motifs are a pair of tyrosine residues separated by 9–11 amino acids. These tyrosines become rapidly phosphorylated by the Src-family kinase Lck following TCR stimulation; a required event for initiating TCR signaling (2, 3).

Tyrosine Kinase Cascade, Phosphorylation of Linker Proteins, and Assembly of Signalsome

Given the primacy of ITAM phosphorylation by Lck in TCR signaling, an especially important question to answer is how is ITAM tyrosine phosphorylation maintained below the signaling threshold prior to TCR engagement. Current data suggest that multiple mechanisms act in concert to block spontaneous TCR signaling. At the first level, there is a physical sequestration of Lck away from the TCR by virtue of differential partitioning of Lck and the TCR into lipid rafts (4, 5). Lipid rafts are heterogeneous lipid microdomains relatively enriched in sphingomyelin, glycosphingolipids, and cholesterol that spontaneously form in cell membranes as a consequence of the biochemical properties of the lipids that comprise the membrane. Lck (by virtue of it being myristoylated and palmitoylated) constitutively partitions to the lipid rafts, whereas the unstimulated TCR is largely excluded from this fraction. The CD8 ITAMs are also maintained in a subcritical state of tyrosine phosphorylation by tyrosine phosphatases, which have ready access to the TCR prior to TCR stimulation but more limited access following TCR stimulation (6, 7). In addition, prior to TCR engagement, Lck is maintained in an inactive state by the combined actions of Csk and PEP. Csk is a tyrosine kinase that phosphorylates the negative regulatory C-terminal tyrosine residue of Lck, and PEP is a hematopoietically restricted phosphatase that associates with Csk via the SH3 domain of Csk and dephosphorylates the activation loop tyrosine of Lck. Csk is co-localized to the lipid raft resident Lck via binding to tyrosine-phosphorylated Cbp/PAG, which is constitutively associated with the lipid rafts (8, 9).

Following stimulation, there is increased distribution of TCR to the lipid rafts and sequestration of negative regulatory tyrosine phosphatases away from the TCR (4, 5). Concurrently Lck becomes activated via dephosphorylation of the regulatory C-terminal tyrosine in response to increased exposure to CD45, a transmembrane phosphatase that dephosphorylates the negative regulatory site, and decreased exposure to Csk as Cbp/PAG is transiently dephosphorylated (9). In addition, because a portion of Lck is constitutively associated with the CD4 coreceptor, the peptide-MHC-induced co-localization of TCR with CD4 results in an increased local concentration of Lck around the TCR. The ITAMs of the CD3 chains subsequently become fully tyrosine-phosphorylated. Once fully phosphorylated, these motifs serve as binding sites for ZAP-70, which binds via its tandem SH2 domains (10). ZAP-70 is activated following ITAM recruitment via Lck-mediated tyrosine phosphorylation of the activation loop tyrosine (Tyr-493) of ZAP-70. Activated ZAP-70 autophosphorylates at tyrosines 292, 315, and 319. These sites serve to recruit various positive and negative signaling effectors to the TCR complex (10, 11). In addition to serving as a scaffold via self-phosphorylation, ZAP-70 also phosphorylates a restricted set of substrates following TCR stimulation. These include α-tubulin, Sam-68, Vav-1, VHR, Shc, Gab2, LAT, and SLP-76 (11). These latter two substrates in particular have been recognized to play a pivotal role in TCR signaling and are considered in more detail below.

When phosphorylated, both LAT and SLP-76 act as linker/adapter proteins, which serve as nucleation points for the construction of higher order multimolecular signaling complexes (Fig. 1), often referred to as the signalsome (8, 12–14). Acting in concert, these linker/adapter proteins regulate the activation of PLCγ1 and the subsequent hydrolysis of phosphatidylinositol (IP)₁,₄,₅-P₃ to generate diacylglycerol (DAG) and inositol-1,4,5-P₃ (IP₃), second messengers in protein kinase C (PKC) and Ras activation (via DAG) and calcium mobilization (via IP₃). They also play an important role in the activation of another TCR-activated protein-tyrosine kinase, Itk (also known as Emt and Tsk). Itk serves as an integrator of signals arising from the signalsome and from phosphoinositide 3-kinase (PI3K; see below), because signals from both pathways are required for its activation (15, 16). Itk has been implicated in 1) PLCγ1 activation, 2) regulation of TCR-stimulated actin cytoskeleton reorganization via its involvement in regulating the Vav-1/Cdc42/WASP pathway (see below), and 3) in so-called “inside-out” signaling, which results in activation of β₁ integrin adhesion factors (15, 16). The Lat and SLP-76 signaling complexes also support the activation of multiple other signaling proteins, including mitogen-activated protein kinases (p38, Jnk, and Erk) and small molecular weight G proteins (Ras, Rnu, Rho, and Cdc42). A key feature of LAT is that it is a transmembrane adapter

* This minireview will be reprinted in the 2004 Minireview Compendium, which will be available in January, 2005.

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The abbreviations used are: TCR, T cell antigen receptor; MHC, major histocompatibility complex; ITAM, immunoreceptor tyrosine-based activation motif; PLC, phospholipase C; PI, phosphatidylinositol; DAG, diacylglycerol; IP₃, inositol-1,4,5-P₃; PKC, protein kinase C; PI3K, phosphoinositide 3-kinase; IκB, immunological synapse; SMAC, supramolecular activation complex; IL, interleukin; TNF, tumor necrosis factor; IκK, IκB kinase.

DOI 10.1074/jbc.R400012200

This paper is available on line at http://www.jbc.org/28827
Fig. 1. T cell receptor signaling events leading to activation of transcription factors. This figure presents an overview of some of the key signaling events linking the binding of peptide-MHC to the T cell antigen receptor (TCR/CD3) and the CD4 costimulatory receptor. The existence of key phosphorylation events is indicated on some of the signaling proteins by small gray circles labeled P; however, not all proteins undergoing TCR-stimulated phosphorylation are labeled. Ub designates ubiquitination. The nature of the different interactions is described in the accompanying text. For greater graphical clarity some important signaling events are not depicted; most notably the small molecular weight linkers Gads and Grb2 are excluded from the LAT/SLP-76 signalosome complex. Also not shown are the signals contributed from the CD28 costimulatory receptor toward the activation of NFAT (1-PI3K positive feedback loop).

protein that is constitutively targeted to the lipid rafts by virtue of palmitoylation of two juxtamembrane cysteine residues (8, 13, 14). It has no protein interaction domains other than the multiple tyrosine residues that are phosphorylated by ZAP-70 following TCR signaling. When phosphorylated, these tyrosines serve as binding sites for specific SH2 domain-containing proteins. There are a total of 8 tyrosine residues in LAT that are conserved between humans, mice, rats, and bovines (14). The C-terminal 5 tyrosine residues play critical roles in the ability of LAT to bind to PLCγ1 and the linker proteins Gads and Grb2. SLP-76 associates with LAT via Gads. SLP-76 can also directly bind to PLCγ1, indicating the existence of higher order interactions between these and possibly other signaling proteins in the signalosome. In addition to the proteins already mentioned, phosphorylated LAT also binds to PI3K, Grap, 3BP2, Shb, SOS, c-Cbl, Vav, and Itk, localizing these molecules in close proximity and a defined orientation to one another within the lipid raft domain of the plasma membrane. Jurkat T cells deficient for LAT expression exhibit severe signaling defects to Ca2+ mobilization, mitogen-activated protein kinase activation, and NFAT activation (8, 13, 14).

Unlike LAT, SLP-76 is a cytosolic protein (12). The structure of SLP-76 includes an acidic N-terminal region, which includes three sites of tyrosine phosphorylation (Tyr-113, Tyr-128, and Tyr-145). When phosphorylated, these sites can bind the SH2 domains of Vav-1, Nck, and Itk. The acidic region is followed by an extended proline-rich region, which binds the SH3 domains of Gads, Itk, and PLCγ1. The C-terminal portion of SLP-76 is comprised of a single SH2 domain, which binds primarily to ADAP, which has been reported to play a key role in inside-out signaling to integrins (17). Jurkat T cells that lack SLP-76 are defective in their ability to activate: 1) PLCγ1 and consequently Ca2+ mobilization and NFAT activation; 2) the Ras/Raf/Mek/Erk pathway; 3) NFκB; and 4) inside-out signaling to integrins (12, 17, 18).

Cytoskeletal Reorganization and Formation of Immunological Synapse

A new layer of complexity in the process of TCR signaling has come to light in recent years in the form of the immunological synapse (IS), which has also been referred to as the supramolecular activation complex (SMAC). This is a dynamic yet highly ordered structure that forms at the site of T cell contact with an antigen-presenting cell (19). The mature IS is characterized by a central region (c-SMAC) that is enriched in clustered TCR and PKCθ, surrounded by a peripheral ring (pSMAC) of adhesion factors such as LFA-1 and a distal ring (dSMAC) containing proteins such as the tyrosine phosphatases CD148 and CD45. The IS, although not required for initiating TCR signaling, is required for sustained signaling, IL-2 production, and proliferation (19). It also has the ability to act as a positive or negative servo, either amplifying weak TCR signals or attenuating strong signals (20).

Clustering of lipid rafts at the contact site and formation of the IS is an active process that requires several upstream signaling events to occur. Most important of these are the signals that lead to reorganization of the actin cytoskeleton. The proximal catalyst for this is the recruitment and activation of the Arp2/3 complex, which catalyzes the formation of new nucleation sites for actin polymerization. A key upstream regulator of Arp2/3 is WASP, which is rapidly recruited to lipid rafts following TCR/CD28 costimulation (13, 21). WASP is constitutively present at high stoichiometry in a complex with WIP and CrkL. WIP-bound WASP is refractory to activation. Upon TCR stimulation, this complex is recruited to the TCR (and consequently to the lipid rafts and the antigen-presenting cell contact site) via the binding of the CrkL SH2 domain to tyrosine-phosphorylated ZAP-70 (22, 23). Co-localization of this complex with activated PKCθ at the lipid rafts results in the phosphorylation of WIP and disruption of the WIP-WASP association, thereby facilitating activation of WASP by Cdc42 (22). In this model, WIP serves both to keep WASP basally inactive and to facilitate WASP activation upon TCR stimulation. WASP can also
be recruited to the lipid rafts via SH3 domain-mediated binding of Nck to a proline-rich region of WASP (24). In this case, the raft recruitment and activation of WASP are coordinated by SLP-76, which functions as a targeting scaffold, bringing WASP into close proximity with Cdc42 that has been activated by SLP-76-bound Vav-1 and also targeting WASP to the rafts via SLP-76-Gads-mediated binding to Lat. Itk also plays a critical role in this process, apparently at the level of supporting Vav-1 recruitment to the plasma membrane (25, 26).

**Activation of Transcription Factors**

Changes in gene expression represent the culmination of the TCR signaling pathway and are required for the T cell to gain full proliferative competence and the ability to produce effector cytokines. Three transcription factors in particular have been found to play a key role in TCR-stimulated changes in gene expression; these are NFκB, NFAT, and AP-1.

NFκB—NFκB is the collective name given to the dimeric transcription factors of the Rel family (27). Activation of NFκB is primarily controlled via the nuclear cytoplasmic partitioning of NFκB. In the absence of an activating signal, NFκB is retained in the cytoplasm by tight binding to an inhibitory IκB protein. Numerous stimuli including TNFα, IL-1, and TCR/CD28 costimulation activate an IκB kinase (IKK) complex containing two kinase subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ (also known as NEMO). The IKK complex phosphorylates IκB and targets it for ubiquitination and proteolysis via the 26 S proteasome complex. The degradation of IκB unmasks the nuclear localization sequence of NFκB, allowing translocation to the nucleus, where NFκB regulates the activity of its target genes (28).

Remarkably, TCR stimulation uses a completely different pathway for activating IKK than other stimuli (e.g. TNFα and IL-1), and many of the early signaling proteins that have been described above are required including Lek, ZAP-70, SLP-76, PLCγ1, and Vav-1 (29). A key step in NFκB activation is the activation of PKCθ and its translocation to lipid rafts and the IS. PKCθ is a member of the “novel” class (DAG-responsive, Ca2+/-independent) of PKCs, is selectively expressed primarily in T cells, and has the distinction of being the only PKC isozyme that is known to translocate to the lipid rafts (30). The translocation of raft/IS recruitment remains undetermined but appears to involve CARMA-1 (see below). The target of PKCθ in activating IKK also remains unknown. It does not directly phosphorylate IKK and may act through calmodulin-dependent kinase II (29, 30).

A critical upstream element in raft recruitment and activation of PKCθ is Vav-1-mediated activation of Rho family G proteins and reorganization of the actin cytoskeleton (30). Raft localization of PKCθ also requires Lek, PI3K, PDK1, SLP-76, PLCγ1, and CARMA-1 (29). Notably, the requirement for PLCγ1 is independent of its enzymatic activity, because neither pharmacological inhibition nor expression of a dominant-negative allele of PLCγ1 inhibits PKCθ raft recruitment (31, 32). PLCγ1 is likely acting as a scaffolding protein in this pathway, possibly in a multimolecular complex with SLP-76 and Vav-1. PKCα but not PKCβ, both “conventional” PKC isotypes (responsive to DAG and Ca2+), also plays an important but as yet undefined role upstream of PKCθ in TCR/CD28 costimulated but not TNFα-stimulated NFκB activation (33).

Another pathway that affects PKCθ activation is PI3K. PI3K phosphorylates the D3 position on the inositol ring of PI-4,5-P3 to generate PI-3,4,5-P3. This lipid serves as a selective binding site in the plasma membrane for certain pleckstrin homology domain-containing proteins. The serine/threonine kinase Akt is one of these proteins (as is Itk). Akt is activated in response to PI3K activation, and Akt and PKCθ physically and functionally interact to synergetically activate NFκB (34, 35). PI-3,4,5-P3 may also affect PKCθ via activation of Vav-1 although activation of Vav-1 by PI-3,4,5-P3 is controversial (36, 37). More recently, Vav-1 has been implicated as an upstream regulator of PI3K, so Vav-1 may function in a positive feedback loop for PI3K activation. Interestingly, the placement of Vav-1 upstream of PI3K may help to explain why Vav-1 is required for PKCθ recruitment to lipid rafts in T cells from Vav-1−/- mice but is dispensable for recruitment in Vav-negative Jurkat T cells (37, 38). A notable difference between these two model systems is that Jurkat T cells have constitutively high levels of PI-3,4,5-P3, because they fail to express the phosphatases that catalyze PI-3,4,5-P3 (39, 40). Thus Jurkat T cells have substantial resting levels of PI-3,4,5-P3 in the absence of Vav-1-mediated activation of PI3K.

Most recently a series of genetic studies have established critical roles for a number of novel proteins in TCR/CD28-costimulated NFκB activation. These include: 1) CARMA-1 (also known as CARD11 and Bimp3), which is a lymphocyte-specific scaffold molecule containing a caspase-recruitment domain (CARD) and a membrane-associated guanylate kinase-like domain; 2) Bcl10, which is a CARD-containing serine/threonine kinase; and 3) MALT1/paracaspase, which is composed of an N-terminal death domain followed by two immunoglobulin-like domains and a C-terminal caspase-like domain (41–43). CARMA-1 is constitutively associated with lipid rafts. Upon TCR stimulation, CARMA-1 acts as a scaffold/adaptor protein recruiting Bcl10, PKCθ, and IKK to the lipid rafts/IS and placing them in juxtaposition to one another and to the TCR (44–47).

Bcl10, in turn, acts by targeting IKK for lysine 63-linked ubiquitination on lysine 399 (48). Bcl10-induced IKKγ ubiquitination and subsequent NFκB activation requires the Bcl10-associated proteins, MALT1/paracaspase, and the ubiquitin-conjugating enzyme UBC13. Unlike lysine 48-linked polyubiquitination, which generally targets proteins for degradation in the proteasome (as is the case with IκB), lysine 63-linked ubiquitination can often regulate protein degradation or intermolecular interactions (49). Notably K939R IKKγ was defective in reconstituting NFκB activation in an IKKγ-deficient Jurkat T cell line (48). The precise mechanism by which IKKγ ubiquitination activates NFκB activation is unknown but presumably involves changes in the structure of IKK that lead to increased IKK activity toward IκB. Bcl10 is also found in complex with IKKγ, IKKα, and IKKβ following stimulation and may play a role in recruiting or stabilizing association of the IKK complex to the lipid rafts and the immunological synapse via the association of Bcl10 with CARMA-1.

That there is yet further complexity to the signaling pathway leading to TCR/CD28-costimulated NFκB activation is suggested by a number of recent studies, including the observation that the cytosolic adaptor protein She is required for nuclear transport of c-Rel (50). In addition, another CARD-containing serine/threonine kinase, Rip2 (also known as RICK, CARDIAK, CCK, and Ripk2) has also been implicated in TCR/CD28-costimulated NFκB activation (51–53). There are also additional kinases that clearly play a role in NFκB activation. These include calmodulin-dependent kinase II (MEK1, MEKK2, MLK3), COT/Tpl-2, and NIK (29).

**NFAT**—In comparison with NFκB, the pathway leading to activation of NFAT is much simpler (54, 55). The rate-limiting step in NFAT activation is the removal of key phosphate groups from the NFAT-sequestering protein (56–58). These include calmodulin-dependent kinase II (MEK1, MEKK2, MLK3), COT/Tpl-2, and NIK (29).

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NFκB—In comparison with NFκB, the pathway leading to activation of NFAT is much simpler (54, 55). The rate-limiting step in NFAT activation is the removal of key phosphate groups from the N terminus of the NFAT protein. Phosphorylation of these residues masks the nuclear localization sequence on NFAT, and when the phosphates are removed NFAT can translocate to the nucleus and regulate the expression of various genes. The dephosphorylation of NFAT is specifically carried out by the Ca2+/-calmodulin-regulated phosphatase, calcineurin. Consequently, TCR-stimulated activation of PLCγ1, with the subsequent production of IP3 and increase in intracellular Ca2+, is a critical component of NFAT activation.

Vav-1 is also recognized as playing a key role in NFAT activation (30). One of the importance of Vav-1 in TCR-stimulated NFAT activity is manifest at multiple steps. In mouse T cells, loss of Vav-1 is associated with greatly impaired Ca2+ influx as a consequence of impaired activation of Itk, Tec, and PLCγ1. These effects are at least in part because of defective PI3K activation in these cells. Keeping in mind the previously discussed defect in PI-3,4,5-P3 metabolism that is characteristic of Jurkat T cells, the important effector role that PI3K plays in Vav-1 signaling may explain why the Vav-1-negative Jurkat T cell line fails to recapitulate the phenotype of the Vav-1−/- mouse T cells (37, 38). However, despite high basal PI-3,4,5-P3, Vav-1-negative Jurkat T cells still show defective NFAT activation, which has been attributed to defective opening of calcium release-activated channels (57) and defective Jnk activation (38).

The NFAT activation/deactivation cycle is completed by the rephosphorylation of NFAT, which causes NFAT to repartition into
the cytosol. This reaction can be carried out by several different serine/threonine kinases including CK1, CK2, Jnk, Erk, and p38; however, it is the GSK3 serine/threonine kinase that appears to play the dominant role in inactivating NFAT in T cells (54, 55). The prominent role of GSK3 in NFAT deactivation also provides an additional pathway by which PI3K can lead to increased or sustained NFAT activation. GSK3 is basally highly activated and is inactivated when phosphorylated upon an N-terminal serine residue. Akt is particularly effective at phosphorylating and inactivating GSK3 and is probably a good thing.

**APA**—Like NFκB activation, the activation of AP-1 requires PKCα activation, and PKCα−/− mice fail to activate AP-1 in response to TCR stimulation (30, 58). The AP-1 transcription factor is not a simple matter to activate a resting T cell. Given how dangerous players interact with one another. However, we are still far from comprehending a few of the lines and some of the ways in which the TCR engagement (11). A great many (maybe even most) of the response to TCR stimulation (30, 58). The AP-1 transcription factor is probably a good thing. Given how dangerous players interact with one another. However, we are still far from comprehending a few of the lines and some of the ways in which the TCR engagement (11). A great many (maybe even most) of the response to TCR stimulation (30, 58). The AP-1 transcription factor is probably a good thing.
