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The Actin Cytoskeleton and Lymphocyte Activation

Minireview

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All stages in lymphocyte life and activation are associated with profound changes in cell morphology that depend on a functional tubulin and actomyosin cytoskeleton. Lymphocytes migrate through blood and lymph vessels, transgress vessel walls and extracellular matrix spaces, home into lymphoid organs, interact with antigen-presenting cells (APCs), and adhere to target cells. During lymphocyte maturation, developing T and B cell precursors are in continuous contact with stromal cells of the thymic or bone marrow microenvironments, and these physical contacts are crucial prerequisites for differentiation and selection. Since many signaling molecules are associated with cytoskeletal scaffolds, the cytoskeletal structure and scaffold geometries can directly regulate the molecular dynamics of signaling and biochemical responses. This minireview examines the emerging role of the actin cytoskeleton as an integral component of lymphocyte activation.

Caps and Supramolecular Activation Clusters

The notion that the actin cytoskeleton might be involved in intracellular signaling was first raised in the 1970s in studies showing that treatment of cells with cytochalasin, a fungal metabolite that inhibits actin reorganization, blocked formation of the Cap structure after IgM treatment of B cells (dePetris and Raff, 1973). The Cap is an asymmetric assembly of receptors and signaling molecules that forms on the surfaces of lymphocytes following stimulation (Figure 1A). Because the Cap includes many molecules that are involved in lymphocyte activation, it was thought that the Cap might be required for conveying signals to the cell interior. The significance of the Cap was called into question by reports that capping might be a nonspecific process related to membrane flow and cell motility. Moreover, whereas Cap formation requires 3–5 min, tyrosine phosphorylation characteristic of early activation events occurs within 10 s of antigen receptor engagement. Thus, the question seemed settled: the slow-forming Cap had no role in signaling.

Recent developments have cast new light on this problem. T cell activation depends on contact between T cell receptors (TCRs) and major histocompatility complex (MHC) ligands expressed on APCs. MHC molecules display short antigenic peptides to the polymorphic TCR. TCR-mediated stimulation leads to the organization of supramolecular activation clusters (SMACs) or "organized contacts" at the interfaces of physical contact between T cells and APCs (Monks et al., 1997). SMACs are highly organized focal interaction sites containing antigen receptors, coreceptors, and adhesion and signaling molecules. Recent experiments using three-dimensional immunofluorescence to visualize contact areas between T cells and APCs incubated with antigenic peptides indicate that a SMAC is organized into distinct spatial domains (Monks et al., 1998). The central area of a SMAC contains the TCR, CD3, $p56^{lck}$ and $p59^{fyn}$ kinases, and protein kinase C (PKC)θ, while the peripheral regions are enriched in the adhesion molecule LFA1 and the cytoskeletal protein talin (Figure 1B). Based on measurements of physical dimensions and molecular interactions, it has also been proposed that the coreceptors CD4 and CD8, the costimulatory molecule CD28, and the adhesion molecule CD2 may cosegregate with the central SMAC domain, whereas the phosphatase CD45 and adhesion molecule CD43 might be excluded from the central contact zone (Shaw and Dustin, 1997). The segregation of molecules in SMACs is mirrored in the orientation of molecules on the APC surface. MHC class II molecules and the LFA1-ligand ICAM1 congregate in the corresponding contact zone of the APC (Monks et al., 1998; Wülfing et al., 1998). When T cells were activated with altered peptides that did not trigger cytokine production or T cell proliferation, CD3 and talin formed molecular clusters but did not segregate into SMACs (Monks et al., 1998). Thus, segregation of receptors and signaling molecules within SMACs appears to be critical for the initiation of physiological responses leading to lymphocyte activation. Spatial segregation of activated receptors and signal transducing molecules might provide a unique environment promoting optimal signal transduction via inclusion and recruitment of activating signaling molecules and exclusion of potential negative regulators such as the CD45 tyrosine phosphatase.

The forces driving the formation of SMACs and Caps require actin polymerization in the T cell but not in the APC (Wülfing et al., 1998). Thus, a signaling mechanism must first activate actin polymerization resulting in the assembly of the Cap or SMAC. But what is the mechanism underlying molecular segregation? Engagement of CD2 initiates a process of protein segregation, receptor clustering, and polarization of the T cell cytoskeleton (Dustin et al., 1998). A novel SH3 domain–containing adaptor molecule, CD2-associated protein (CD2AP), can mediate this cytoskeletal polarization. Since the binding of CD2AP to CD2 depends on T cell activation, it has been suggested that TCR activation mobilizes CD2AP, which engages CD2, resulting in receptor clustering, the building of intracellular scaffolds, and T cell polarization. However, mice lacking CD2 have no apparent defects in T cell activation and lymphocyte development, suggesting either that a surface receptor

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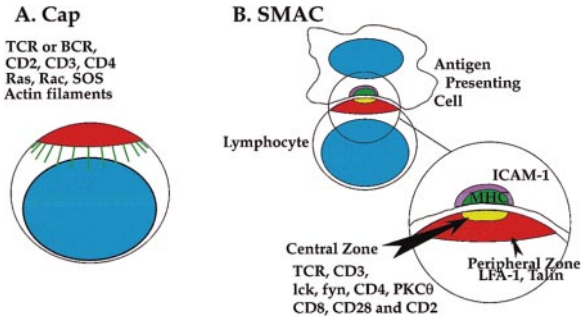

Figure 1. Comparison of the Structure of Caps (A) and SMACs (B)

Only a few of the many receptors and signaling molecules shown to be within the Cap are listed. Caps and SMACs are probably not equivalent. The fundametal property of SMACs is the specific organization of proteins at the contact site. A similar organization has not been shown for Caps.

other than CD2 can mediate receptor clustering, and/or that CD2AP can associate with other adhesion molecules.

Molecular Forces Driving Actin Reorganization

The connection between signaling and Cap formation has been revealed in recent studies of genetically engineered mouse mutants. The assembly of the Cap is defective in T and B cells lacking the guanine nucleotide exchange factor VAV (Fischer et al., 1998; Holsinger et al., 1998). VAV links TCR stimulation to activation of the Rho-family kinases Rac1, CDC42, and RhoA. VAV-deficient T cells and thymocytes exhibit defects in antigen receptor–induced actin polymerization and recruitment of actin to the TCRζ chain (Fischer et al., 1998; Kong et al., 1998). Cap formation is also impaired by a deficiency of the CDC42-associated Wiscott Aldrich syndrome protein (WASP) (Snapper et al., 1998). In addition to defects in antigen receptor capping, human Wiscott Aldrich syndrome is characterized by immunodeficiency, thrombocytopenia, and lymphoid malignancies. Transfection of a gene encoding a dominant-negative Rac1 mutant also results in a failure of Cap assembly in lymphocytes (Holsinger et al., 1998). These experiments indicate that VAV, Rac1, and WASP link antigen receptor engagement to cytoskeletal reorganization, receptor clustering, and Cap formation.

The GTPases Rac1, CDC42, and Rho function as molecular switches in cells and orchestrate receptor-mediated cellular responses such as cytoskeletal changes and DNA synthesis (Lamarche et al., 1996). Target molecules for Rac1, CDC42, and RhoA differ widely in their functions and cellular distribution and include such molecules as phosphatidylinositol 4-phosphate 5-kinase (PIP5K), WASP, myosin light chain (MLC) phosphatase, and the p21-activated kinase (PAK). PIP5K phosphorylates PIP, resulting in the production and local accumulation of phosphatidylinositol 4,5-bisphosphate (PIP_2) (Chong et al., 1994). High concentrations of PIP2 can dissociate actin-binding proteins such as profilin and gelsolin and promote interactions between actin and the cytoskeletal proteins vinculin and talin, which could be one mechanism for local actin polymerization and anchoring of the actin cytoskeleton to the cell membrane. Recent evidence indicates that in B cells stimulation of the CD19 coreceptor leads to the recruitment of VAV, which then regulates PIP5K activation (O'Rourke et al., 1998). Thus, regulation of actin polymerization and Cap formation by VAV might be achieved in part by the Rho- or Rac1-mediated activation of PIP5K. However, VAV also directly associates with talin and vinculin (Fischer et al., 1998). VAV might scaffold a signaling pathway promoting actin polymerization and anchor actin nucleation molecules required for the formation of Caps and SMACs (Figure 2).

Role of Actin-Dependent Structures in T Cell Activation

Several groups have reported that actin polymerization is necessary for promoting the correct orientation and contacts between lymphocytes and APCs, a process that depends on CDC42 (Stowers et al., 1995). This requirement reflects the well-characterized role of actin polymerization in cell motility, and not a requirement for actin polymerization in signaling. The possibility that Rac1, VAV, and Caps are necessary for signal transduction at some point distal to the initial cell–cell contact has been raised by an analysis of the signaling pathways disrupted in the absence of VAV. Consistent with the time course of capping, there is little or no defect in the tyrosine phosphorylation of major substrates downstream of the TCR in the absence of VAV or the Cap. Activation of JNK/SAPK, p38 kinases, MAPK, and NF-κB also appears normal. However, despite earlier reports it is now clear that Ca^{2+} signaling is defective in VAV-deficient lymphocytes (Turner et al., 1997; Fischer et al., 1998; Holsinger et al., 1998). Ca^{2+} mobilization from intracellular stores and the activation of PKC isoforms depend on hydrolysis of PIP2 by PLC-γ1. A shortage in PIP2 generation due to defective activation of PIP5K could account for the Ca^{2+} signaling defect in VAV-deficient cells. PIP2 turnover occurs very rapidly in the membrane, such that the activation of PIP5K is the rate-limiting step for replenishment of PIP2 levels. However, inhibition of actin polymerization in thymocytes and human T cell lines also reduces Ca^{2+} flux during activation by specific peptide/MHC complexes on APCs (Valitutti et al., 1995; Kong et al., 1998). These data imply that sustained Ca^{2+} flux depends on reorganization of the actin cytoskeleton and PIP2 production.

What is the function of receptor clustering? In VAV-deficient T cells, although the TCRs do not cluster in response to antigenic stimulation, the principal signaling pathways can still operate. However, the level of signal transduction achieved is not sufficient to produce a complete T cell response. In the absence of VAV, early thymocyte development at the pre-TCR stage, positive and negative thymocyte selection, peptide/MHC- and antigen receptor–mediated thymocyte apoptosis, antigen receptor–induced cell cycle progression, and the activation of immune response genes such as the IL-2 gene are impaired (Turner et al., 1997; Fischer et al., 1998; Holsinger et al., 1998; Kong et al., 1998). Similarly, deletion of the *WASP* gene severely impairs T cell proliferation and cytokine production (Snapper et al., 1998). Moreover, inhibition of actin polymerization by Cytochalasin D mimics defects in Vav- or WASP-deficient T cells or T cell lines overexpressing dominant-negative Rac1. Cytochalasin D inhibits IL-2 production, proliferation, TCR capping, and peptide/MHC-mediated thymocyte

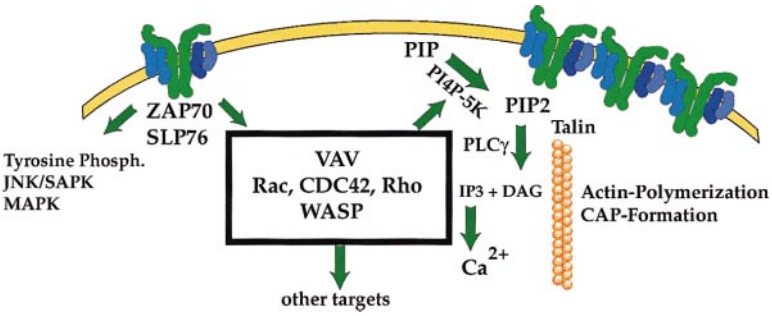

Figure 2. Plausible Biochemical Sequence of Events Linking TCR Engagement to the Formation of the Cap or SMAC

Complete activation of Vav requires a combination of two signals (Han et al., 1998): (1) binding of PI3K-generated phospholipids to VAV's plekstrin homology domain; (2) tyrosine phosphorylation of VAV mediated by the Src-family kinases $p56^{lck}$/$p59^{fyn}$, or the nonreceptor tyrosine kinase ZAP70. ZAP70 is recruited to the TCRζ chain and can bind VAV either directly or through the adaptor protein SLP76 (see accompanying minireview by C. E. Rudd in this issue of *Cell*). At this point the data on the pathways leading to SMAC formation are more speculative than those leading to the Cap.

apoptosis but does not impair known signaling pathways with the exception of Ca^{2+} mobilization (Holsinger et al., 1998; Kong et al., 1998). Thus, Vav-, Rac1-, and WASP-regulated cytoskeletal reorganization and receptor clustering are required for T cell maturation and the induction of physiological T cell responses.

Receptor clustering appears to be important for assembling signaling molecules at focal sites, an event that may promote sustained signaling necessary for lymphocyte activation. The induction of physiological responses may require that distinct signaling pathways be activated in a temporally and spatially coordinated fashion. Oscillations of Ca^{2+} flux within a focal site regulated by receptor clustering might be a mechanism by which multiple signaling pathways could be coordinated. Without SMACs or Caps, individual signaling pathways may fire, but the synergy is lost. Alternatively, the formation of SMACs or Caps may lead to the activation of a unique signaling cascade. The possibility that actin-dependent events activate a novel signaling pathway is suggested by findings that the downstream effects of Rac1 and CDC42 can be dissociated (Lamarche et al., 1996). Thus, Rac1 and CDC42 mutants have been identified that fail to activate PAK and JNK/SAPK but still lead to cytoskeletal changes and cell cycle progression. The pathways downstream of these Rac1/CDC42 alleles leading to proliferation and actin changes remain to be discovered.

Activation of PKC can bypass the functional defects in T cells deficient for VAV, Rac1, or WASP function (Fischer et al., 1998; Holsinger et al., 1998; Snapper et al., 1998). Moreover, PKC activation restores the susceptibility to apoptosis of *vav*$^{-/-}$ thymocytes, and inhibition of a PKC isoform inhibits TCR-mediated thymocyte apoptosis. Of the several PKC isoforms, VAV associates only with the Ca^{2+}-independent PKCθ molecule (Kong et al., 1998). PKCθ is highly expressed in the hematopoietic system, particularly in T cells, and cooperates with calcineurin to induce transcription of the T cell growth factor IL-2 (Werlen et al., 1998). This observation is reminiscent of the coordinated transactivation of the IL-2 gene by calcineurin and Vav/Rac1 (Wu et al., 1996; Holsinger et al., 1998). In addition, PKCθ translocates to the central areas of the SMACs, whereas PKCα, β1, δ, ε, and ζ remain in other regions (Monks et al., 1997, 1998). Thus, PKCθ is a candidate for an effector kinase that links Cap and SMAC formation to downstream signaling pathways.

Overexpression of VAV activates NF-AT-dependent transcription (Wu et al., 1996), suggesting that NF-AT is a nuclear terminus of Cap- and actin-dependent signaling. Since NF-AT transcription complexes control IL-2 expression, a biochemical pathway can be proposed that relays TCR engagement to VAV activation, actin polymerization, NF-AT activation, and expression of immune response genes (Figure 3). In VAV- and Rac1-mutant as well as Cytochalasin D–treated T cells, NFATc1 translocates from the cytoplasm to the nucleus following TCR engagement, but the NFAT transcriptional complex is inactive (Holsinger et al., 1998). Thus, Vav- and Cap-dependent signaling activates either a cascade that induces the expression of an as yet unknown nuclear subunit of NF-AT complexes, or a unique pathway that mediates direct NF-AT activation. The idea that there may be a novel signal transduction pathway emanating from Caps and SMACs is not far fetched. The most recent gene sequencing efforts indicate that there may be as many as 4,000 kinase and 10,000 transcription factor genes in the human genome. Thus, the vast majority of intracellular signaling pathways has yet to be discovered.

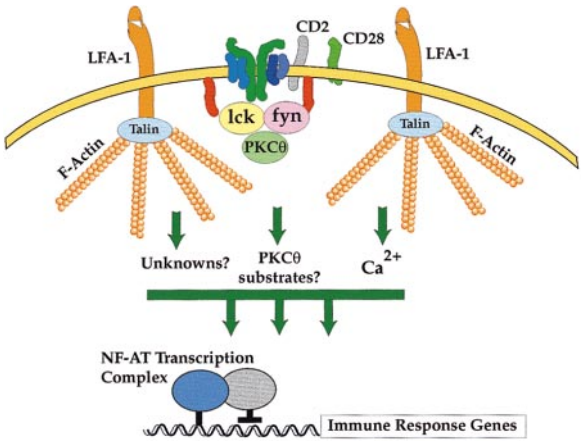

Figure 3. Signaling Pathways Leading from the Cap to Transcription of Immune Response Genes

See text for details.

The Mechanism of Actin-Dependent Receptor Clustering

The studies reviewed above provide compelling evidence that clustering of signaling molecules, possibly in specific architectures, is an essential step in lymphocyte activation. Antigen receptor activation may lead to the organization of focal actin-scaffolded signaling "highways" whose function is to sustain TCR signaling and coordinate downstream signaling events such that complete activation is achieved and late events such as proliferation and cytokine secretion can occur. Receptor clustering could favor sustained signaling in three ways: (1) by increasing the likelihood of contacts between the TCR and the MHC-bound ligand; the increased concentration of TCR molecules in a high-density zone would allow low-affinity receptors to initiate and maintain signals; (2) by increasing the concentration of cytosolic signaling molecules and second messengers at regionally organized focal points in the proximity of TCRs; and (3) by excluding negative regulatory molecules such as phosphatases from the zone of antigen receptor signaling.

T cells display ~30,000–40,000 TCR on their surface, but only 50–100 peptide/MHC complexes on APCs are required for T cell activation. Moreover, it appears that survival of naive peripheral CD4 and CD8 T cells depends on continuous recognition of self-peptide/MHC complexes by the TCR (Tanchot et al., 1997). Why are these T cells not constantly activated? Ligand-induced formation of Caps and SMACs may introduce an additional level of regulation of lymphocyte effector functions. Thus, TCR-mediated MAPK, SAPK/JNK, or NF-κB induction in the absence of Caps and SMACs might regulate cell survival. However, formation of higher order clusters appears necessary to induce immune responses such as proliferation and expression of regulatory cytokines. In this scenario, recruitment and activation of a Cap and SMAC–dependent signaling cascade may be the crucial and limiting step required for the induction of lymphocyte effector functions. Moreover, Cap and SMAC formation and cluster-dependent signaling might be important molecular mechanisms that regulate immunological tolerance and lymphocyte unresponsiveness.

A role for an actin-dependent clustering of signaling molecules is in line with fundamental regulatory mechanisms in signaling that might be stated in chemical terms as "effective molarity," encompassing concepts of both proximity and orientation. The widespread use of dimerization domains, localization domains, and protein–protein interaction domains as well as scaffolding proteins may reflect the importance of effective molarity as a regulatory mechanism in intracellular signaling. Most steps in intracellular signaling can be regulated by induced proximity in specific architectures, and actin-dependent clustering of signaling molecules could well serve the purpose of controlling effective molarity. Future understanding is likely to depend on the identification of the biochemical pathway(s) that are defective in the absence of Caps and SMACs and novel strategies to test the function of the molecules involved in these signaling clusters.

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