All roads lead to actin: the intimate relationship between TCR signaling and the cytoskeleton

Summary: Regardless of cell type, the regulation of the actin cytoskeleton is tightly linked to vital biological properties such as polarity, motility, cell–cell contact, exocytosis and proliferation. In the immune system, where rapid and efficient response to antigen-activated stimuli is crucial, an overwhelming amount of data implicate the actin cytoskeleton and its regulators as central to immune function. Increasingly, the cytoskeleton is considered an essential amplification step in T cell receptor (TCR)-, costimulatory-, and integrin-mediated signaling. Advances in genetic manipulation and confocal imaging have led to a keen appreciation of the importance of TCR signal integration by the actin cytoskeleton. This review outlines recent advances in elucidating the regulation of T cell function through the actin cytoskeleton. We also examine intriguing parallels between the immune system and other models of cytoskeletal regulation.

Introduction

T cells are highly dynamic and depend heavily on the cortical actin cytoskeleton for determining shape. The shape of the T cell throughout its life cycle gives some indication of its different functions. During circulation of T cells between the blood and lymphoid organs, the T cell must make the transition from a spherical resting cell, with antigen and adhesion receptors fairly evenly distributed on its surface, to a highly polarized cell containing a uropod (1–3). In the latter form, the T cell is migratory and drawn to chemotactic gradients as evidenced by the high levels of adhesive molecules and chemokine receptors selectively expressed on its leading edge. The trailing edge of the T cell uropod contains the T cell antigen receptor (TCR), large surface glycoproteins, and cellular organelles such as the endoplasmic reticulum (ER), Golgi apparatus, and microtubule-organizing center (MTOC).

Upon encountering an antigen-presenting cell (APC) in the lymph nodes or spleen, the T cell rapidly deforms itself against the target cell and reorients its cellular organelles to
the interface between the T cell and the APC. During the course of the encounter with APCs, several cytoskeletal structures may be observed in the T cell, including flat sheet-like ruffles (lamellipodia), thin microspikes (filopodia), and larger projections called pseudopodia (reviewed in 4). Once the T cell has successfully received antigenic stimulation, it undergoes clonal expansion and migrates out into the tissue in search of infected cells. When an infected cell is detected, the T cell must once again reorient its cellular machinery to the interface in anticipation of performing its effector functions. Here, stimulation results in the polarized secretion of cytolytic granules and/or cytokines in response to recognition of the infected cell.

None of the shape changes, movement, polarization, proliferation, or exocytic events just described are possible without the interaction between the TCR and the actin cytoskeleton. In fact, treatment with inhibitors of actin polymerization results in the abolition of all of these T cell functions (discussed later). Thus, appreciating the evidence for the function of the cytoskeleton in multiple T cell functions, how is the activation signal received through the TCR translated into cytoskeletal activity and then to T cell function? Here we review the molecular and biochemical means through which TCR signals regulate cytoskeletal reorganization. We also discuss recent relevant observations in the study of the relationship between T cell activation and the cytoskeleton.

**Links between the cytoskeleton and T cell function**

Initiation: from the TCR to the kinases

Engagement of the TCR by infected cells, or APCs, delivers signals to the T cell that result in activation and clearance of infection. The TCRζ chain dimers are the subunits of the TCR signaling complex responsible for recruiting intracellular tyrosine kinases that propagate T cell activation signals (reviewed in 5, 6). A striking example of the interaction of the TCR with the cytoskeleton is the physical linkage of a large proportion (30–40%) of the TCRζ chain population to the actin cytoskeleton (7, 8, reviewed in 9). Other T cell surface receptors such as CD2, CD4, CD8, CD11a/18, CD44, and class I major histocompatibility complex (MHC) receptors are also constitutively associated with the cytoskeleton (10). Upon stimulation, these receptors, as well as TCRζ, are recruited to the actin cytoskeleton and cluster or cap in the region of interface between the T cell and the APC. The observation of this redistribution of receptors on the T cell surface during APC engagement led to the description of the immunological synapse (11). Synapse formation and its role in signal transduction has been extensively and thoughtfully reviewed in the past few years (1, 3, 12–16). For this reason, we only briefly review it here and later touch upon more recent revelations in synapse/cytoskeleton relationships.

Although the phenomenon of ‘capping’ or clustering of TCRs was reported over a decade ago, these structures were largely observed as a result of antibody-induced activation of T cells. It was not until similar types of TCR aggregates were seen in T : APC interfaces that the concept of immunological synapse formation began to evolve. Kupfer and colleagues (17) were the first to report a reproducible pattern of redistribution for the TCR, CD2, and leukocyte function-associated antigen-1 (LFA-1) (CD11/18) upon successful T : APC conjugation. These supramolecular adhesion complexes (SMACs), or immune synapses, formed a pattern in which a central cluster (cSMAC) of TCR, CD2, and the costimulatory receptor, CD28, are surrounded by a concentric peripheral cluster (pSMAC) of CD11/18 adhesion molecules. Immune synapses have been demonstrated in helper and cytolytic T cells as well as in natural killer (NK) cells, suggesting that the synapse is an important regulator of the interaction between lymphocytes and APCs (14, 17–19).

The immune synapse may function to overcome the steric hindrance between the T cell and the APC. Because the TCR and other molecules in the cSMAC only extend 7–10 nm from the cell surface compared to the 40–45 nm length of the glycocalyx and other extended surface molecules on both the T cell and APC, there is a potential physical inhibition of TCR and antigen–MHC contact. The synapse may surmount this barrier by sorting larger molecules away from the cSMAC and into the pSMAC in order to allow close membrane juxtaposition between the T cell and the APC (reviewed in 12).

The ability to physically segregate receptors also yields the potential to regulate activation by altering the physical distribution of intracellular proteins linked to the receptors. TCR complexes found on the leading edges of T cells are exquisitely more sensitive to antigenic provocation than those on the distal or trailing edges (20, 21). Because neither the TCR nor any of the other receptors segregated into the cSMAC and pSMAC have any inherent enzymatic activity, the molecules recruited to them during the course of TCR engagement are central catalysts in T cell activation.

Lck, a tyrosine kinase vital to the initiation of signaling through its phosphorylation of TCRζ immunoreceptor tyrosine-based activation motifs (ITAMs), a portion of which undergoes activation-induced association with the cytoskeleton, is found in cSMACs (8, 22). For Lck this association is probably compounded by its interactions with the coreceptors
CD4 and CD8, both of which are linked to the cytoskeleton (10). A fraction of ZAP-70, another important proximal tyrosine kinase recruited to TCRζ upon stimulation, is constitutively associated with the actin cytoskeleton (7, 23). The compartmentalization of ZAP-70 to the plasma membrane may serve to reduce its recruitment time to TCRζ and its substrates upon activation (24). Also, sequestration of surface molecules and their associated intracellular proteins may increase signaling efficiency through the creation of pro-signaling molecular microdomains, sequestered away from down-regulatory signaling molecules (12, 25–27).

An example of this sequestration of down-regulatory molecules involves Csk, a negative regulator of Lck (28, 29). Upon TCR stimulation, Csk-binding protein (Cbp, also known as phosphoprotein associated with glycosphingolipid-enriched domains or PAG) is transiently dephosphorylated allowing the release of Csk from membrane rafts, thus relieving negative control of Lck. Two groups have recently reported that although Cbp is palmitoylated and thus membrane-associated, it may be recruited to regions of TCR signaling via two classes of cytoskeletal adapter proteins, the ezrin, radixin and moesin (ERM) family (discussed later) and the ERM-binding phosphoprotein of 50 kDa (EBP50) (30, 31). As we discuss, the ERM family of proteins are important for linkages between cell surface receptors and the cytoskeleton. Thus, a close relationship between ERMs, Cbp, and Lck implies tight coordination between cytoskeleton-mediated sequestration and TCR signaling.

The importance of immune synapse formation is underscored by the fact that failure to form mature synapses also results in the failure to activate mature T cells (type 2 helper T cells and immature thymocytes are the exception) (32, 33). When inhibitors of actin polymerization are employed, synapse formation, as well as T cell activation, is abolished (12, 27, 34–36). Many of the key regulators of actin polymerization are also segregated into the immune synapse. All these data point to a closely coupled relationship between immune synapse formation, TCR signaling, and actin cytoskeletal regulation.

Adapters from LAT to Nck
Linker for activation of T cells (LAT) is an adapter protein that couples the upstream signaling of Lck and ZAP-70 with downstream signaling events such as calcium flux, phosphatidylinositol (PI) turnover, and Ras activation (Fig. 1A) (reviewed in 6, 37, 38). LAT-deficient mice lack mature T cells; however, LAT-deficient mast cells, another immune cell type highly dependent on cytoskeletal function, have defects in granule exocytosis. This defect is due to an inability to phosphorylate src-homology 2 (SH2) domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) and activate phospholipase Cγ (PLCγ3), possible consequences of the loss of LAT as a nucleating site for these proteins (39). In turn, these molecules are required for signals necessary for cytoskeletal rearrangement (discussed later). Observations from LAT-deficient mast cells, as well as LAT-deficient transformed T cells, suggest that LAT also controls cytoskeletal organization in primary T cells (38).

Confocal imaging has revealed that TCR engagement is intimately linked to reorganization of the actin cytoskeleton (18, 40, 41). In order to improve resolution of the T cell interface, we stimulated T cells in slide chambers using poly-L-lysine and monoclonal antibody (mAb)-coated coverslips (40, C. L. Fuller, unpublished data). Imaging the contact points between the T cell and the coverslip by interference reflection microscopy (IRM) and fluorescence microscopy allowed high-resolution, time-resolved data concerning the nature of T cell activation to be obtained. Both transformed T cells and primary CD8+ cytotoxic T lymphocytes (CTLs) rapidly flatten and spread upon stimulation by mAb-coated coverslips (Fig. 2A). Actin-rich lamellae form a circumferential ring whose size approaches the diameter of the cell (~8–10 μm) and is approximately 1–2 μm thick (Fig. 2B). The formation of these rings is dependent on src-kinases, calcium, and LAT, as T cells treated with various inhibitors or transformed T cells lacking LAT do not exhibit maximal spreading or actin-ring formation (Fig. 3) (40). Because LAT is responsible for the recruitment of PLCγ3, SLP-76, and Vav, all of which are closely associated with regulation of actin remodeling, these results are not surprising.

To ensure that the spreading and ring structure were not artifacts of either transformed cells or the coverslip imaging method, primary influenza-specific CTLs were incubated with antigen (Ag)-pulsed target cells, and were fixed and stained for the helical actin polymer, filamentous actin (F-actin) (Fig. 4). Again, the formation of actin-dense rings was clearly observed when CTLs were stimulated in the presence of agonist peptide. In these images, even at the very earliest stages of T:APC contact, actin-rich structures were observed. These data confirm that stimulation of the actin cytoskeleton is one of the very earliest signals received by the T cell during activation.

Other adapter and scaffolding proteins, such as SLP-76 and the adhesion- and degranulation-promoting adapter protein (ADAP, previously known as FYB or SLAP-130), also play integral roles in the regulation of the actin cytoskeleton. Similar to LAT, SLP-76 is a scaffolding protein whose primary function is to bring signaling molecules into proximity of
one another (reviewed in 42–44). SLP-76 is bound by the adapter protein Gads, which in turn binds phosphorylated LAT (Fig. IA). Upon phosphorylation by ZAP-70, SLP-76 can be found in complexes with Vav, Nck, interleukin-2-induced tyrosine kinase (Itk), and ADAP in actin-rich lamellae (Fig. IB) (45). All of these proteins are likely to be key in SLP-76-mediated cytoskeletal regulation. As with LAT, thymocyte development is completely blocked in SLP-76-deficient mice, but defects in platelet morphology, integrin signaling, and actin remodeling seen in these mice would suggest a possible role for SLP-76 in integrin function and cytoskeletal regulation in mature T cells as well.

Among the many molecules found associated with SLP-76 is Vav, a well-studied cytoskeleton regulator and a guanine-nucleotide exchange factor (GEF) for small GTPases (reviewed in 46). Activated by Lck/Fyn upon receptor crosslinking, Vav, in turn, activates the GTPases Cdc42 and Rac through SLP-76-dependent interactions (Fig. IB,C) (47–49). Mice deficient for Vav expression are impaired in the phosphorylation of SLP-76, thus negating many of the scaffolding properties of SLP-76 including interaction with PLCγ and the association of TCRζ with the cytoskeleton (47, 50). The lack of interaction between PLCγ and SLP-76 in these mice is further compounded by the lack of Itk, and thus PLCγ, activation (50). Vav-deficient mice also exhibit defects in T cell development, calcium flux, proliferation, and cytokine production, as well as clustering of the TCR and lipid domains (47, 51–55).

The requirement for Vav in T cell SMAC formation and actin remodeling has been directly demonstrated (53). T cells from Vav-deficient mice are severely defective in synapse formation as well as in cytoskeletal rearrangement when encountering Ag-bearing targets. The role of Vav in bridging TCR and cytoskeletal signaling is exemplified by its constitutive association with TCRζ, which is itself highly associated with the actin cytoskeleton (56). Cytochalasin D treatment of wild-type T cells diminishes cytokine production and proliferation to

**Fig. 1. Overview of selected protein interactions relevant to TCR and cytoskeletal signaling.** (A) Proteins involved in the LAT signaling cluster. (B) The SLP-76–WASP complex. (C) Molecular model of WASP binding partners and actin nucleation. (D) Actin cytoskeleton–TCR signaling component interactions.
levels equivalent to those in Vav-deficient mice (52). These data highlight the importance of Vav in cytoskeleton- and TCR-dependent lymphocyte effector functions.

The Rho family GTPases consist of Rac, Cdc42, and Rho, small G proteins activated by GEFs, such as Vav, and localized to the cell membrane by prenylation. The Rho family has long been associated with actin remodeling of the cytoskeleton. As exemplified by actin structures such as stress fibers, lamellipodia, and filopodia, many changes in the shape of cells are attributed to regulation by these GTPases (reviewed in 4, 57). Rac and Cdc42 are thought to be responsible for the induction of new actin polymerization at leading edges, while Rho is more closely associated with reorganization of existing structures involved in retraction at the distal edge (58). In T cells, the presence of Cdc42 in cSMACs suggests the role of this GTPase in early TCR–cytoskeleton signaling (59). The common characteristic for Rho family members is that, in their activated form, they facilitate regulation of actin filament reorganization through effectors such as actin-related proteins 2/3 (Arp2/3) and Wiskott–Aldrich syndrome protein (WASP) family members (Fig. 1C).

ADAP is a multidomain adapter involved in the regulation of adhesion and degranulation, both actin-dependent functions, through its interactions with Fyn and SLP-76, as well as TCRζ (reviewed in 60, 61). ADAP, whose expression is limited to a few cell types including T lymphocytes, interacts with a host of

Fig. 2. Confocal microscopy examination of T lymphocyte cytoskeletal remodeling. (A) Confocal IRM imaging at the coverslip of live CTLs on coverslips coated with either poly-L-lysine, stimulatory mAbs or other activating reagents. Using a 633-nm laser focused on the coverslip, CTL spreading can be observed as a dark area where the T cell membrane is tightly apposed to the coverslip. (B) Actin ring formation is observed by fixing cells and staining with phalloidin-TR (actin) and CT-Green (cytoplasm) counterstain. Images are pseudocolored accordingly.
may play a similar role in the integration of TCR and cytoskeleton signaling. ADAP-deficient mice have defective T cell proliferation and cytokine production responses, stressing the relationship between these biological endpoints and cytoskeletal control (64, 65). Although ADAP colocalizes with F-actin at T cell interfaces, ADAP-deficient mice do not appear to have actin polymerization defects, making the exact role of ADAP somewhat obscure (63). These mice are unable to mount successful immune responses, pointing to the overall importance of ADAP in T cell-dependent immunity, although through an as yet undetermined mechanism.

Nck, an adapter protein recruited to SLP-76, has been shown to bind WASP and WASP-interacting protein (WIP), key components in actin polymerizing machinery (Fig. 1B) (66, 67). Along with WASP and ADAP, Nck colocalizes with SLP-76 to the immune synapse (66). Recruitment of WASP and its associated proteins may be the major function of Nck. Another possible role for Nck has been proposed. Gil and associates (68, 69) report that Nck directly binds CD3ε upon TCR engagement-induced exposure of proline-rich sequences within CD3ε. Recruitment of Nck to CD3ε was found to precede phosphorylation of ITAMs, one of the earliest TCR signaling events. Mutation of Nck to prevent its recruitment to CD3ε leads to disruption of T:APC conjugate formation, actin-ring formation, and cytokine production. These data provide evidence for alternatives to CD3ε ITAM phosphorylation as a means by which T cell activation is linked to the cytoskeleton.

Fig. 3. Pharmacological inhibition of cytoskeletal remodeling. CTLs pretreated with src-kinase inhibitor (PP2), actin polymerization inhibitor (cytochalasin D), or microtubule polymerization inhibitor (colchicine) were allowed to spread on prewarmed coverslips. Cells were then fixed and labeled with phalloidin-TR (actin) and CT-Green (cytoplasm) counterstain. Images are pseudocolored accordingly.

TCR and cytoskeleton signaling proteins including, Fyn, SLP-76, Vav, Nck, Arp2/3, WASP, and Evl, and localizes to the immune synapse (Fig. 1A,B) (60–63). Thus, as LAT is believed to be the linker between TCR proximal and distal events, ADAP

Fig. 4. Antigen-presenting cell-induced CTL cytoskeletal remodeling. Fibroblast antigen-presenting cells bearing the appropriate MHC were grown in poly-L-lysine-coated slide chambers and pulsed with influenza peptide. Excess peptide was then removed by washing before addition of CT-Green-labeled live CTLs. T cells were allowed to spread and were then fixed and stained with phalloidin-TR (actin). CTLs can be distinguished from stress fiber-containing fibroblasts by the green cytoplasmic label. The leftmost image is a z-slice of a CTL (red and green, upper cell) engaging an APC. The same Z-stack was reconstructed in 3D using imaging software (rightmost image). The image is a top-down view of the CTL atop the actin-streaked fibroblast. Note the prominent ring of polymerized actin (red) at the interface between CTL and APC.
Actin nucleation: ARP2/3 and WASP

The Arp2/3 complex, first described in yeast, is the primary member of the actin nucleating machinery (reviewed in 70–72). The evolutionarily conserved Arp2/3 complex is a heteromeric complex that consists of seven subunits: two actin-related proteins (Arps) and five novel proteins (72). The Arp2/3 complex is the only known mammalian nucleator of actin, although recent studies in yeast suggest there may be more (see formins, discussed later). Arp2/3 allows for the polarized growth (pointed/slow-growing end to barbed/fast-growing end) and branching of F-actin. Because of these unique abilities, all other actin regulators interact with actin in conjunction with the Arp2/3 complex to stimulate actin nucleation. Stinchcombe and colleagues (73) have found Arp2/3 in cSMACs in cytolytic T cells, suggesting the involvement of actin polymerization in driving T cell activation.

The mammalian WASP family proteins [WASP, N-WASP, and WASP-family verprolin-homologous proteins (WAVES)] activate the Arp2/3 complex and are thus key to nucleation of new actin filaments in mammalian cells (reviewed in 58, 70, 74–76). The significance of WASP was recognized when mutations in WASP were defined as the causative agent in the human Wiskott–Aldrich syndrome (77, 78). T cells from WAS patients have abnormal microvilli, cell surface markers, polarization, and motility. Through interactions with SLP-76 via Nck, PIP2, GTPases, profilin, Arp2/3, and actin monomers, WASP family members are crucial in translating G-protein activation to actin polymerization (Fig. 1B,C). Because of the direct relationship of WASP family members with numerous TCR signaling proteins and actin polymerization, the nature of those interactions warrants closer examination. WASP interactions have not always been directly demonstrated in T cells. Rather, theories as to the function of WASP in T cells have been drawn from studies using yeast 2-hybrid technology and from studies defining interactions in other cell types such as fibroblasts, NK cells, and B cells.

WASP family members can perhaps best be understood through examination of their structural domains (Fig. 1C). WASP and N-WASP contain one or two N-terminal domains, respectively, termed WASP homology domain I or Ena/VASP homology domain I (WH1/EVH1) (reviewed in 79). WH1 domains mediate interactions with other signaling proteins through binding of proline-rich motifs. When the WH1 domain is overexpressed in mice, TCR stimulation is defective, yet actin polymerization is normal (80). This finding would suggest that binding partners of the WH1 domain may be sequestered away from their normal interactions and functions downstream of the TCR. Although the ability of the WH1 domain to interact with a great number of signaling proteins might be implied, to date, data from fibroblast models suggest that WIP is the only known WASP WH1 binding partner (74, 81).

WIP, which is composed of an actin-binding domain and several proline-rich regions, is thought to be an important mediator of the activity of WASP, perhaps through its interaction with Nck. WIP knockout mice have normal T lymphocyte development, but F-actin structures and T-APC interaction are greatly perturbed. As a result, synapse formation and thus cytoskeleton-dependent effector functions, such as polarization, proliferation and cytokine production, are detrimentally altered (82). WIP and WASP are also important in nuclear factor of activated T cell (NFAT)-dependent interleukin (IL)-2 transcription (83, 84). Disruption of WIP–WASP interactions in T cells completely abrogates NFAT-mediated activation of transcription, thus possibly explaining the loss of cytokine production and proliferation in WIP-deficient mice.

The basic and GBD/CRIB domains of N-WASP interact with phosphatidylinositol (4,5)-bisphosphate (PIP2) and Cdc42 (58, 70, 74, 75). While the exact PIP2 binding site on WASP is still unknown, it is believed to be the region between the basic and GBD domains (Fig. 1C) (76). The GBD domain has recently also been found to be important in WASP regulation (85). In its resting state, WASP masks its GBD, PIP2, SH3, and Arp2/3 binding sites, by intramolecular binding between the GBD and C-terminal coflin homology (CH) domain (part of the VCA domain, discussed later), preventing actin nucleation. Cdc42 competes with GBD for CH binding, thus relieving WASP autoinhibition (86). The GBD domain also plays a role in binding to PIP2, thus allowing localization of WASP to the membrane for activation. In T cells, the localization of WASP to the cSMAC has also been demonstrated to be GBD domain-dependent (59). Other reports show data proposing that the recruitment of the TCR to actin-rich patches in the cSMAC is WASP dependent, another example of the coupling of TCR signaling to actin regulation (87).

The proline-rich domain of WASP interacts with a multitude of SH3 domain-containing proteins: Grb2, Fyn, Itk, Nck, PLCγ1, profilin, and the p85 subunit of PI3K (66, 88–91). In T cell colocalization studies, the proline-rich domain of WASP was required for its cSMAC recruitment; however, Cdc42 was not (59). This finding stresses the importance of the SH3 domain proteins associated with WASP, especially Nck, to recruit it to the synapse. Only one protein, WASP interacting SH3 protein (WISH), is currently known to activate WASP/Arp2/3 independent of GTPases (92). The stoichiometry and temporal aspects of binding for these proteins with
WASP are not clearly understood, but collective data suggest that WASP serves as a bridge between the TCR and the actin cytoskeleton signaling machinery.

WASP has the ability to interact with actin monomers and Arp2/3 through the evolutionarily conserved C-terminal WASP homology domain 2 (WH2) and verprolin, coflin, and acidic region (VCA) domains (93, reviewed in 94). Actin binding to the WH2 domain of WASP is believed to enhance the affinity of WASP for the Arp2/3 complex, thus providing a positive feedback loop for actin polymerization (74). In mice engineered to lack the VCA domain of WASP, thymocyte development is blocked at an immature stage, a property not seen in WASP-deficient mice. This blockage suggests, along with data on synapse formation (discussed later), that cytoskeletal cues are fundamental to not only mature T cell function but thymocyte development as well.

WASP may also play a role in the down-regulation of activation signals via internalization of activated TCRs (95). With the help of the newly described endocytic adapter, intersectin 2, WASP may cooperate with Cdc42 to target surface TCRs to clathrin-coated endocytic vesicles. Along with data on distal polarization and sequestration (see ERMs, later), this is yet another example of the cytoskeleton playing a role in the dissipation as well as the activation of TCR signaling via spatial instead of enzymatic means.

The process of actin reorganization
The cortical actin-binding protein, cortactin, is not a member of the WASP family, but it can activate the Arp2/3 complex to a much lesser extent. Cortactin has acidic, proline-rich, and SH3 domains like the WASP, but lacks WH1, WH2, or WHD domains (72, 96). Instead of WH2 domains, cortactin utilizes its repeat domain, consisting of a helix–turn–helix structure, to directly bind F-actin. The N-terminal acidic domain (NTA) of cortactin mediates Arp2/3 binding. Cortactin is thought to synergize with N-WASP to activate Arp2/3, and recent findings support this hypothesis. Either alone or with the aid of N-WASP, cortactin is able to activate the Arp2/3 complex and stabilize actin networks (97). Through its VCA- and NTA-binding domains, cortactin is able to bridge the Arp2/3 complex and F-actin without inhibiting simultaneous N-WASP interactions. In T cells, the cortactin homolog Lck-binding protein 1 (LckBP1) or HS1, is heavy phosphorylated upon TCR stimulation and believed to be tightly regulated via Lck interactions (98–100). As with EBP50, this positions an important cytoskeletal regulator in close proximity to upstream TCR signaling events, again suggesting parallel progression along both signaling pathways.

Ena/VASP family members localize to the leading edge of fibroblast lamellipodia and promote F-actin elongation by competing with capping proteins for access to fast-growing ends (101). The Ena/VASP family has also been found to play a role in the extension of pseudopodia, another lymphocyte function that is highly dependent on actin reorganization. Subcellular localization of Ena/VASP proteins is regulated by binding the WH1 domain of proteins such as ADAP (63). In macrophage and transformed T cell pseudopodia, Ena/VASP family members colocalize with SLP76, ADAP, Nck, WASP, and profilin (63, 102). The prevention of ADAP binding to these proteins abrogates TCR-induced actin remodeling. Evi, an Ena/VASP family member, is found in regions of dynamic actin remodeling such as adhesions, lamellae and microspikes formed in the leading edge of T lymphocytes as a result of TCR stimulation (Fig. 1A) (103). Evi is aided by profilin and ADAP in nucleating actin polymerization while under the negative regulation of cAMP-dependent kinase (63, 103).

Regulation of F-actin elongation in all cells is thought to occur through three mechanisms: regulation of capping proteins, severing of existing F-actin, and de novo nucleation (see Arp2/3 discussed earlier) (104). Gelsolin, profilin, and coflin add to the complex picture of cytoskeletal regulation occurring at the level of actin monomers and filaments. Elongation is achieved through the addition of actin-ATP monomers to the fast-growing end of the growing actin filament (reviewed in 105). One mechanism for the initiation of rapid monomer addition to the growing end is for slow-growing ends to be severed, thus freeing actin-ADP to be activated and funneled to fast-growing ends (105). Gelsolin is one of the proteins responsible for severing and capping of F-actin slow-growing ends in response to calcium flux and changes in pH in T cells and many other cell types (106, 107). Gelsolin capping serves to stabilize F-actin as well as prevent elongation at the slow-growing end (Fig. 1D). Once gelsolin-capped F-actin is uncapped through Rac-mediated mechanisms, new fast-growing ends are created where polymerization can begin again (107). In response to intracellular calcium flux, gelsolin is believed to act as a regulator of Rac-mediated membrane ruffling (108). As gelsolin is also regulated by PI(3)K, it is easy to see how it might be regulated in leading edges of lamellae by PI(3)K, which is recruited by WASP/WAVE family members. The recycling of actin monomers from the pointed end to growing end of F-actin may also explain the speed with which actin structures are formed.

The process of actin reorganization requires the rapid transport of actin monomers to fast-growing ends by actin-binding proteins such as profilin and coflin (Fig. 1C). Profilin promotes
fast-growing end elongation by regulating pools of monomeric actin-ATP, chiefly through induction of G-actin ADP–ATP exchange (70). This control mechanism prevents spontaneous actin monomer polymerization. Profilin acts as a shuttle between ADP- and ATP-bound pools of G-actin and the actin nucleating point induced by WASP/WAVE and Arp2/3. Profilin is also essential for Cdc42-mediated filopodia formation (109).

Cofilin is found complexed with actin-ADP monomers at sites of lamellae advancement in neuronal and T cells (110, 111). Cofilin, also known as actin-depolymerizing factor (ADF), regulates F-actin filaments through severing and/or increasing the rate at which actin-ADP is lost from slow-growing or pointed ends (depolymerization), inhibiting actin polymerization (107). Cofilin activation in T cells results from stimulation through coreceptors CD2, CD4, CD8, and CD28, but not through TCR-mediated stimulation alone (111). Costimulation through these T cell coreceptors mediates cofilin dephosphorylation by the serine/threonine phosphatases PP1 and PP2A (112). Dephosphorylated cofilin then destabilizes TCR-induced actin filaments by increasing slow-growing end depolymerization. The activity of cofilin is negatively regulated by phosphorylation mediated by the Rac effector LIM-kinase (LIM-K), which is itself regulated by PAK and Rho-associated kinase (ROCK) in fibroblast and endothelial cells (discussed later) (113–117). Upon serine phosphorylation by LIM-K, cofilin is inactivated and loses its actin-ADP binding ability. Cofilin phosphorylation thus increases the pool of free actin-ADP, favoring actin monomer binding by profilin and thus ADP/ATP exchange and fast-growing end polymerization.

As mentioned, LIM-K activity is regulated by phosphorylation by PAK1 and ROCK in T cells as well as in fibroblasts and neuronal cells (112–114). PAK1 is a member of a family of serine-threonine protein kinases activated through direct interaction with Cdc42 and/or Rac (reviewed in 115, 116). In the resting state, PAK1 is autoinhibited. Cdc42/Rac interaction with the PAK1 p21-binding domain frees the PAK1 catalytic domain in a manner analogous to the way WASP is activated by GTPases. In T cells, Nck was initially thought to recruit PAK to the plasma membrane through LAT, Vav1, SLP-76 and Nck (118). However, Ku and colleagues (119) later reported that neither LAT, SLP-76, nor Nck are required for PAK1 activation. Instead, PAK1 activation required ZAP-70, Rac1, a Rho GEF called PAK1-interacting protein (PIX), and PIX-interacting protein of 95 kDa (p95PKL).

ROCK is yet another effector of the GTPase, Rho. The kinase activity of ROCK regulates the myosin light chain (MLC) and myosin binding subunit (MBS) (117). ROCK is induced by GTP-bound Rho and arachidonic acid to change from an autoinhibited conformation to an active one. Activation of ROCK in T cells leads to stress fiber formation, membrane ruffling, motility, and filopodia formation, all structures closely associated with the Rho family GTPases (117, 120). The substrates responsible for these cytoskeletal reorganization events are the ERM family, MLC, adducin, and, as mentioned earlier, LIM-K (reviewed in 117).

 Ezrin and moesin, members of the ERM family of cytoskeletal adapter proteins expressed in T cells, localize to the synapse at the onset of TCR signaling (121, 122). ERM proteins colocalize in F-actin-rich areas with WASP and vinculin and are regulated by phosphatidylinositol (PIs) and ROCK (120). The N-terminal FERM (four.1, ezrin, radixin, moesin) domain of ERM molecules is responsible for membrane localization upon threonine phosphorylation or PIP2 binding (reviewed in 123). The C-terminus of these molecules contains the F-actin binding domain of ERMs. Reminiscent of WASP, PAK, and ROCK regulation, the binding of the N-terminal FERM and C-terminal domains of ERM is autoinhibitory. ROCK activates ERMs by threonine phosphorylation, allowing the release of intramolecular suppression (117).

Along with WASP, ERMs are thought to be key in coupling the rearrangement of the cytoskeleton to the plasma membrane and cell surface receptors, principally through their interactions with PIs (124). Among the ERM-associated cell surface receptors in T cells are CD43, CD44, CD95 and intercellular adhesion molecules (ICAM)-1, -2, and -3 (123). ERMs are also believed to be responsible for coupling the actin cytoskeleton and the MTOC (125). These linkages may be important for cellular functions such as polarization, directed migration, vesicle transport (granule and cytokine), and proliferation (126–129). These processes are closely coupled to ERM protein function as blockage of ERM function results in the loss of cytoskeletal structures such as microvilli and lamellipodia, deficient cytokine release and loss of T cell adhesions (130). New data regarding EBPs suggest the potential of ERMs to be regulators of vital early signal transduction events, specifically the activation of Lck (30, 31). As ERMs are present at the very early stages of synapse formation, they could facilitate movement of negative src-kinase regulators away from pro-signaling microdomains.

The interaction of moesin with CD43, a large surface mucin that is excluded from the T:APC synapse, is the prototype for many proposed ERM–surface receptor pairs (Fig.1D). CD43 and moesin preferentially localize to the distal uropod, or antipodal membrane of migrating T cells (25, 121, 131). CD43 relocalization to the distal pole of the T cell is mediated
by the TCR-inducible binding of moesin to CD43 in activated T cells (121, 130). The sequestration of CD43 away from the synapse is not a general characteristic of all large surface glycoproteins; CD45 is not similarly actively excluded (25). Overexpression of ERMs, ezrin in particular, disrupts synapse formation (122). Whether this is due to an inability to remove ezrin to distal poles remains unclear. ERMs have recently been demonstrated to be vital to the recruitment of lipid rafts to the synapse (31). These lipid rafts contain key TCR activation signaling proteins such as LAT. The regulation of signaling by ERMs through the creation of biochemically polarized microdomains in the membrane is a model for cytoskeleton-enhanced signaling through sequestration of synapse activators and inhibitors, both steric and enzymatic (132). It seems likely that other synapse formation events are similarly controlled.

The cytoskeleton in action: microtubule and actin–cytoskeletal cooperation

The microtubule filament network is closely associated with actin–cytoskeletal organization. Our data along with many others suggest a role for MTOC involvement in actin cytoskeletal function, as inhibitors of either the microtubule or actin cytoskeletal networks has consequences for the other network (Fig.3) (40, reviewed in 125). While a review of MTOC regulation is outside the scope of this review, recent data in T cells, specifically cytotoxic T lymphocytes, have revived interest in examining MTOC–actin coordination.

CTLs respond within minutes of encountering antigen-bearing target cells by the rapid release of perforin- and granzyme-containing cytolitic granules (reviewed in 133). Perhaps because of the rapid nature of the CTL response, they have served as models in the study of the T cell cytoskeleton for decades. Studies of CTL: APC interactions find that both cytokine and cytolitic granule secretion are polar and may be regulated by the same signals that mediate immune synapse formation (18, 73, 128, 134–136). Here, using the CTL effector function of granule exocytosis as an example, we discuss several recent connections between MTOC–actin cytoskeletal regulators and T cell effector function.

The orchestrated movement of the MTOC from the distal pole to the leading edge of the CTL is one of the key indicators of successful CTL activation. The importance of actin : MTOC interactions is demonstrated by the actin cytoskeleton dependence of this movement as well as the polarized cytokine and granule release that follows (126, 127). Injection of dominant negative Cdc42 mutants prevents actin polymerization as well as MTOC polarization (137). Disruption of the actin cytoskeleton by inhibitors or dominant negative Rac prevents MTOC movement and release of both cytokines and perforin granules (137–139). Conversely, disruption of the MTOC abrogates cytokine and granule release while also causing changes in the quality and duration of T cell actin remodeling (138, 140). This finding suggests that while not the central component of cytoskeletal organization, the MTOC is closely involved with key cytoskeletal players in directing CTL effector functions.

Lck, Vav, and ZAP-70 activation are important for the induction of cytoskeletal, cytokine and proliferative responses, and have been shown to constitutively associate with tubulin, the structural component of the MTOC, as well as with the actin cytoskeleton (23, 141, 142). Nocadazole, a potent microtubule inhibitor, prevented the phosphorylation of the TCRζ and ZAP-70 by Lck (142). Both ZAP-70 and the MTOC have been demonstrated to localize and remain in the synapse during activation (5, 141, 142). In fact, Kuhn and Poenie (143) report that the MTOC in CTLs is in fact physically linked to the pSMAC. These authors show that in fluorescent tubulin and LFA-1-stained CTLs, the pSMAC corresponds to points where the MTOC reaches the plasma membrane. They further show that the MTOC loops through and is anchored to the pSMAC. In this model, the reorientation of the MTOC from the trailing edge to the CTL: APC interface could be a mechanism for ‘sweeping’ together pSMAC components. In this scenario, it is easy to imagine a moesin: CD43-like regulation that might tie some of the pSMAC components to the closely membrane juxtaposed MTOC, strongly supporting data that the MTOC augments cytoskeletal function in synapse formation and thus signal transduction.

Cytolytic granules are known to travel to T : APC interfaces via microtubules and myosin motors (138, 144–146). Myosin motor proteins have been shown to help regulate synapse formation (34). In cells where myosin motor proteins were inhibited, calcium flux, adhesion molecule redistribution, and synapse formation were also disrupted. Two groups have recently discovered that one of the activators of the actin-based myosin motor Va, the small GTPase Rab27a, may play a role in CTL granule cytolysis (73, 129, 147). Originally studied in regards to a melanocyte defect, Rab27a-deficient mice have severely reduced lytic function due to an inability of lytic granules to dock and migrate to T : APC interfaces.

Striking data supporting the intimate role of TCR signaling, synapse formation, and cytolytic granule exocytosis have been reported recently (73). Stinchcombe and colleagues (73) report that Lck- and granzyme-containing lytic granules occupy discrete adjoining regions within the cSMAC. Amazingly, the signaling and effector domains of the cSMAC defined
by these two entities do not appear to overlap, suggesting that a highly ordered mechanism is involved in maintaining these distinct domains. It seems probable that the sequestration of these domains involves cytoskeletal regulation and underscores the need for integration of MTOC–actin cytoskeleton signals.

**Perspectives**

All cell types owe a great deal of their functionality to their actin cytoskeleton. T lymphocytes, melanocytes (see Rab27a, earlier), neurons, yeast, Listeria, and Dictyostelium may seem to have little in common on the surface, yet genetic manipulation, fluorescent fusion proteins, and confocal microscopy suggest that upon closer examination, the general principles governing actin regulation are likely to be universal. Here we look at several nonimmunological model systems to which molecular immunologists might look to find research that has led to advances in understanding cytoskeletal regulation.

**Lessons from bacteria, yeast, and amoeba**

Bacterial and viral pathogens such as Listeria, Shigella, and vaccinia have been closely studied because of their ability to regulate actin in order to allow their replication and dissemination in their host cells (reviewed in 148, 149). Using host cell actin machinery such as WASP, WIP, Nck, Arp2/3, gelsolin, and profilin, along with novel bacteria proteins such as VASP, ActA, IcsA, and intimin, bacteria and viruses are able to form intracellular actin tails and extracellular projections called ‘pedestals’, reminiscent of microspikes and pseudopodia in T cells.

Bacteria such as *Escherichia coli* must also tightly regulate actin in order to adhere to mucosal host cells. Enteropathogenic *E. coli* induce the formation of actin pedestals to which they adhere through the injection of bacterial actin nucleating proteins into the host cell cytoplasm (150). The translocated intimin receptor (Tir) is a newly described bacterial protein believed to induce actin nucleation through its recruitment of Nck (150, 151). Host cell proteins WASP and Arp2/3 are also required for pedestal formation (151). Whether Tir is a WIP homolog or a novel protein with yet undiscovered human homologs remains to be seen.

Formins purified from yeast led researchers to the discovery of a novel Arp2/3-independent mechanism of actin nucleation (152). Formins, in conjunction with profilin, were found to promote the formation of actin filaments *in vitro*. As many actin-related regulatory mechanisms have been evolutionarily conserved, the possibility for new mammalian actin nucleators independent of Arp2/3 formally exists.

Studies in Dictyostelium and other eukaryotic cells also provide insights into the relationship between signaling and the cytoskeleton (2, 153–156). These studies suggest that polarity following chemotactic stimulus is a product of the same type of sequestration of receptors and signaling molecules on distal poles seen in lymphocytes.

**Neuronal proteins**

Cytoskeleton proteins such as N-WASP, WAVE, Evl and Mena are found in neuronal cells as well as T cells. It has been suggested that there may be multiple parallels between neural and immunological synapse formation and cytoskeletal regulation (103, 157–159). Similarly, studies of growth cone regulation have helped to elucidate the function of proteins found in both neurons and T lymphocytes (103, 110, 160–162).

WASP-family verprolin homologous protein (WAVE) members of the WASP family share many similarities with WASP and N-WASP; however, one important difference is that WAVEs lack GBD domains (163). As a result, the ability of Rac to activate WAVE is not identical to the direct interaction of Cdc42 with WASP and N-WASP. Recent reports in neuronal cells and fibroblasts point to the insulin response substrate (IRSp53) as the mediator of Rac–WAVE interaction (164). IRSp53 is believed to bind Rac through its Rac-binding domain (RBD) and WAVE through the WAVE homology domain (WHD). In addition, although N-WASP and Cdc42 cooperation in actin polymerization has been clearly demonstrated, the role of WAVE/SCAR (suppressor of cAMP receptor mutation) and its Rho family activator Rac1 has only recently been elucidated (165, 166). WAVE/SCAR is negatively inhibited through its interactions with the Nck-associated protein (NAP). Whether the form of WAVE found in T cells, WAVE2, will have similar functions remains to be seen.

Semaphorins, transmembrane and metalloprotease–released proteins commonly found in neuronal growth cones, have recently generated intense interest. Proteins such as CD100 (Sema4D), CD232 (viral-encoded semaphorin receptor or A39R), and CD108 (murine semaphorin K1) are all highly expressed on T lymphocytes and may confer regulation of both motility and signaling. In the case of CD100, its cleavage from the surface of activated T cells allows binding to CD72 on B cells, releasing inhibitory pressure on antibody production (167).

The Eph family of receptor tyrosine kinases was first studied in the context of their ability to promote disassembly of the actin cytoskeleton in neuronal growth cones (reviewed in 168, 169). However, recent findings suggest that ephrins may be just as important in actin cytoskeleton assembly (170). Fibroblasts exhibited spreading and actin ring-type structures when
imaged during stimulation with ephrinA, in a manner strikingly similar to that described earlier for T cells. Some Eph RTKs have been shown to be upstream of Nck in neuronal cells, suggesting that Eph family members found on T lymphocytes may play similar roles (171). Interestingly, the EphB2 receptor has been linked to activation of PAK1 via PIX and PI-3K in mesodermal cells (172). Precisely what the role of the Eph family may be in T cell signaling, via TCR or other signaling pathways, remains to be seen.

Agrin and neuropilin-1 are among the most recently studied neuronal proteins shown to play a role in T cell immune function, most directly in the initiation of T: APC interaction. Agrin, a protein important to formation of neuromuscular junctions, has been implicated in the formation of T cell synapses (160, 173). In T cells, agrin appears to cause clustering of membrane lipid rafts to the site of T: APC interaction, paralleling its function in the nervous system. Neuropilin-1 aids in axon guidance in the nervous system, but has recently been studied in the context of T: dendritic cell interfaces (162, 174). Both dendritic cells and resting T cells express and translocate neuropilin-1 to their interface upon activation. Inhibition of neuropilin-1 prevents T cell activation, which can be demonstrated by the loss of proliferation following preincubation of dendritic and T cells with blocking antibodies. Closer examination of the parallels between neuronal and immune systems is warranted.

Concluding remarks

In just the last 2 years, the accumulated knowledge regarding TCR signaling has expanded tremendously. Advances in confocal microscopy such as two-photon in vivo imaging, fluorescence resonance energy transfer (FRET), and time-resolved imaging of fluorescent chimeras promise to help further unravel the kinetics and stoichiometry of TCR signaling. Bioinformatics allows for a much easier method for examining potential mammalian homologs of the vast number of well-documented cytoskeletal regulators in yeast and other model systems. The bridging of these disciplines promises to allow for more accurate and detailed analyses of TCR and cytoskeletal regulation, thus enabling a more lucid understanding of TCR and cytoskeletal regulation in vivo in the near future.

Abbreviations

Ab, antibody (mAb, monoclonal Ab)
ADAP, adhesion- and degranulation-promoting adapter protein
ADF, actin-depolymerizing factor
Ag, antigen
Arp2/3, actin-related proteins 2/3
CBP, Csk-binding protein
CRIB, Cdc42/Rac interactive binding domain (also called GBD)
EBP50, ERM-binding phosphoprotein of 50 kDa
ENa, enabled protein
ERM, ezrin, radixin and moesin
EVH1, Ena/VASP homology domain 1 (also called WH1)
EVL, Ena/VASP-like protein
F-actin, filamentous actin
FYB, Fyn-binding protein (now ADAP)
G-actin, globular actin
GBD, GTPase-binding domain (also called CRIB)
GEF, guanine-nucleotide exchange factor
IRSp53, insulin response substrate of 53 kDa
Itk, IL-2-induced tyrosine kinase
LAT, linker for activation of T cells
LckBP1, Lck-binding protein 1
MLC, myosin light chain
N-WASP, neuronal-Wiskott—Aldrich syndrome protein
NTA, amino terminal acidic domain
Nap125, Nck-associated protein of 125 kDa
p95PKL, PIX-interacting protein of 95 kDa
PAG, phosphoprotein associated with glycosphingolipid-enriched domains
PAK, p21-activated kinases
PH, pleckstrin homology domain
PI, phosphatidylinositol
PIP₂, phosphatidylinositol (4,5) biphosphate
PIX, PAK1-interacting protein
PRS, proline-rich sequence
RBD, Rac-binding domain
ROCK, Rho-associated kinase
RTK, receptor tyrosine kinase
SCAR, suppressor of cAMP receptor mutation
SKAP-55, Src-kinase associated phosphoprotein of 55 kDa
SLAP-130, SLP-76 associated protein of 130 kDa (now ADAP)
SLP-76, SH2 domain containing leukocyte phosphoprotein of 76 kDa
SMAC, supramolecular adhesion complexes, proximal (p), central (c), and distal (d)
VASP, vasodilator-stimulated phosphoprotein
VCA, verprolin—homology, coflin—homology, and acidic domain
WASP, Wiskott—Aldrich syndrome protein
WAVE, WASP-family verprolin homologous protein
WH1/2, WASP homology domain 1/2 (WH1 also called EVH1)
WIP, WAVE interacting protein
WISH, WASP interacting SH3 protein
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