Formins Regulate the Actin-Related Protein 2/3 Complex-Independent Polarization of the Centrosome to the Immunological Synapse

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DOI 10.1016/j.immuni.2007.01.008

SUMMARY

T cell receptor (TCR)-mediated cytoskeletal reorganization is considered to be actin-related protein (Arp) 2/3 complex dependent. We therefore examined the requirement for Arp2/3- and formin-dependent F-actin nucleation during T cell activation. We demonstrated that without Arp2/3-mediated actin nucleation, stimulated T cells could not form an F-actin-rich lamellipod, but instead produced polarized filopodia-like structures. Moreover, the microtubule-organizing center (MTOC, or centrosome), which rapidly reorients to the immunological synapse through an unknown mechanism, polarized in the absence of Arp2/3. Conversely, the actin-nucleating formins, Diaphanous-1 (DIA1) and Formin-like-1 (FMNL1), did not affect TCR-stimulated F-actin-rich structures, but instead displayed unique patterns of centrosome colocalization and controlled TCR-mediated centrosome polarization. Depletion of FMNL1 or DIA1 in cytotoxic lymphocytes abrogated cell-mediated killing. Altogether, our results have identified Arp2/3 complex-independent cytoskeletal reorganization events in T lymphocytes and indicate that formins are essential cytoskeletal regulators of centrosome polarity in T cells.

INTRODUCTION

Within minutes of antigen-presenting cell (APC) recognition, T cells undergo cytoskeletal polarization, involving formation of an F-actin-rich lamellipodium (Bunnell et al., 2001) and microtubule-organizing center (MTOC) reorientation to the immune synapse (IS) (Kupfer et al., 1987). Although the mechanisms controlling polarization of these cytoskeletal elements remain unclear, this restructuring is essential for T cell function (Vicente-Manzanares and Sanchez-Madrid, 2004). In addition, the speed with which this cytoskeletal reorganization occurs makes the T cell-APC recognition process a unique model for studying the molecular mechanisms controlling cytoskeletal polarization.

TCR-mediated MTOC (or centrosome) reorientation has been suggested to be regulated by only a few signaling molecules (Ardouin et al., 2003; Combs et al., 2006; Kuhne et al., 2003; Martin-Cofreces et al., 2006; Serrador et al., 2004; Stowers et al., 1995). Functionally, MTOC polarization is thought to control the positioning of the secretory apparatus for directed release of lymphokines in T-helper cells (Kupfer et al., 1991) or cytotoxins in cytolytic cells (Kupfer and Dennert, 1984). Also, centrosome-plasma membrane contact in T cells is thought to be necessary for directed secretion and to be F-actin dependent (Stinchcombe et al., 2006). However, the putative actin regulators controlling this centrosome polarization remain to be identified.

Several studies making use of pharmacologic agents have demonstrated that F-actin reorganization is crucial for T cell activation (Campi et al., 2005; Holsinger et al., 1998; Valitutti et al., 1995). The actin-related protein (Arp) 2/3 complex, which is directly stimulated by activators such as Wiskott-Aldrich Syndrome protein (WASP) and WASP-family verprolin homologous (WAVE) proteins, nucleates F-actin into an expanding array of individual filaments branching off one another. This Arp2/3 complex-generated F-actin meshwork is proposed to produce sheet-like lamellipodia and spike-like filopodia. Even though filopodia are predicted to arise from the Arp2/3 complex-dependent dendritic network through selective bundling of Arp2/3 complex-generated filaments (Biaysheva et al., 2004), this idea remains controversial (Faix and Rottner, 2006). In fact, it is now suggested that the Arp2/3 complex is dispensable for filopodia formation (Steffen et al., 2006). Indeed, genetic evidence in yeast, Drosophila, and C. elegans reveals that the Arp2/3 complex is not the sole nucleator for all F-actin-containing structures (Evangelista et al., 2002; Hudson and Cooley, 2002; Severson et al., 2002; Tolliday et al., 2002). This suggests that F-actin nucleators are specialized, producing
specific architectural frameworks that coordinate distinct cellular functions.

Foramin family proteins can also nucleate F-actin, and unlike the Arp2/3 complex, formins nucleate linear F-actin filaments and are proposed to generate unbranched structures, such as actin cables, filopodia, and stress fibers (Faix and Grosse, 2006). Formins are conserved modular proteins sharing characteristic formin homology (FH) domains (Higgs, 2005). The FH1 domain interacts with the G-actin binding protein profilin, providing G-actin for filament assembly, while the adjacent FH2 domain nucleates F-actin. In yeast, formins participate in microtubule organization, spindle positioning, polarized cell growth, and contractile ring formation during cytokinesis (Faix and Grosse, 2006). However, the functions of mammalian formins are not well understood, with recent evidence suggesting roles in cell motility, adhesion, and microtubule capture and stabilization (Faix and Grosse, 2006; Kobielał et al., 2004; Wen et al., 2004). In fact, there appears to be a strong, yet ill-defined, link emerging between mammalian formins and the microtubule cytoskeleton.

Here we examined the contributions of the Arp2/3 complex and formins to cytoskeletal regulation during T cell activation. We found that Arp2/3 complex-depleted cells, which could not form F-actin-rich lamellipodia, still polarized actin-based filopodia and were even capable of MTOPC polarization. In contrast, the formins, Diaphanos-1 (DIA1) and Formin-like-1 (FMNL1), did not regulate F-actin accumulation at the IS, but instead colocalized with the centrosome and controlled MTOPC polarization and cell-mediated killing. Thus, we found that the Arp2/3 complex and formins distinctly regulated the T cell cytoskeleton, and we identified the formins as essential regulators of centrosome polarity during T cell activation.

RESULTS

TCR Stimulation Leads to Distinct Arp2/3 Complex-Independent F-Actin Structures

In order to examine the role of the Arp2/3 complex in T cells, we generated short-hairpin RNA (shRNA) vectors to silence both Arp2 and Arp3. Transfection of these vectors into Jurkat T cells led to a substantial depletion of Arp2 and Arp3 (Figures 1A and 1B). Consistent with studies in nonhematopoietic cells (Steffen et al., 2006), suppression of either Arp2 or Arp3 with each individual targeting vector led to a decrease in expression of other Arp2/3 complex components (Figures 1A and 1B). Thus, the integrity of the Arp2/3 complex as a whole is dependent on the presence of either Arp2 or Arp3 in T cells.

Because the Arp2/3 complex is considered essential for TCR-mediated F-actin reorganization, we first examined the morphology of TCR-stimulated Arp2/3 complex-depleted GFP-actin-expressing Jurkat cells spreading after TCR ligation (Bunnell et al., 2001). GFP-actin Jurkat cells were transfected with shRNA vectors containing a separate mCherry transcriptional cassette (Nolz et al., 2006). Control mCherry-expressing cells spread onto anti-TCR-coated coverslips in an ordered fashion, forming a round, actin-rich lamellipod (Figures 1C; see Movie S1 in the Supplemental Data available with this article online). Spreading was maximal by 5 min after initial coverslip contact, with the central area corresponding to the cell body visually devoid of GFP-actin (Figure 1C). This was followed by a 15–20 min retraction phase, characterized by disassembly of the lamellipod and the return of GFP-actin to the cell body (Movie S1). Differential interference contrast (DIC) images indicated retrograde flow and ruffling of the extended lamellipod throughout the spreading process (Movie S1).

In contrast to control transfectants, neither shArp2- nor shArp3-transfected cells formed lamellipodia, but instead displayed a dramatically different phenotype, extending long, dynamic actin-rich filopodia in response to TCR stimulation (Figure 1C; Movies S2 and S3). These structures were not as long-lived as the lamellipod and were not uniformly produced, with cells displaying distinct morphologies (Figure 1C). Also, Arp2/3 complex-depleted cells retained substantial GFP-actin in their cell bodies, yet were seemingly capable of flattening against the activating coverslip (Movies S2 and S3). In contrast to Arp2-suppressed cells, shArp3-transfected cells transiently displayed periodic membrane bursts occurring between adjacent filopodial structures (Movie S3), which were most likely due to inefficient suppression of Arp3. Phalloidin staining of control and Arp2-suppressed T cells indicated that the GFP-actin enrichment seen in Figure 1C indeed correlated with F-actin polymerization (Figures 1D–1F), suggesting that Arp2/3 complex-independent actin reorganization was responsible for the microspike formation. In fact, shArp2-transfected cells no longer produced filopodia upon cytochalasin treatment (not shown), but did form them upon colchicine treatment (not shown), indicating an actin-dependent yet microtubule-independent mechanism for formation of these filopodia.

In order to more closely visualize the morphological differences between control and Arp2-suppressed cells, we utilized scanning electron microscopy (SEM). Unstimulated control cells did not undergo morphological alterations in response to the coverslip (Figure 1G), while anti-TCR-stimulated control cells extended flat sheet-like structures, which apparently displayed filopodia protruding at the leading edge (Figure 1H, left). After spreading completely, control cells were dramatically flattened against the activating coverslip, with apparent ruffling at the periphery (Figure 1H, right). Arp2-suppressed cells sent out filopodial appendages, which extended only from the area in contact with the coverslip (Figure 1I, left), SEM also confirmed that shArp2-transfected cells indeed flattened against the coverslip (Figure 1I, right). These data indicated that TCR-induced F-actin polymerization arises independently of the Arp2/3 complex, minimally in the form of microspikes.

Arp2/3 Complex-Independent Filopodia Polarize during APC Recognition

Because of the altered morphology of activated Arp2/3 complex-suppressed cells, we examined whether they
could respond to APCs with polarized F-actin. To analyze this physiological response, control and suppressed T cells were allowed to form conjugates with superantigen (SEE)-pulsed NALM6 or Raji B cells. As expected, control GFP+ cells flattened against the stimulating NALM6 cells, showing a robust band of polarized F-actin at the IS (Figure 2A). Interestingly, conjugates were found between GFP+ Arp2- and Arp3-suppressed cells and SEE-pulsed NALM6 cells, and upon analysis, these pairs frequently displayed polarized F-actin. However, similar to the filopodial structures observed in shArp2- and shArp3-suppressed cells that were activated on coverslips, Arp2- and Arp3-depleted cells produced disorganized F-actin rich projections over the B cell surface (Figure 2A). To determine whether costimulation altered this response by Arp2- and Arp3-depleted cells, we also used SEE-pulsed RAJI cells, which express B7, the costimulatory ligand that binds CD28 on T cells. We found that although control GFP+ cells flattened tightly against the RAJI cells, the Arp2- and Arp3-suppressed T cells responded with similar polarized F-actin-based protrusions (Figure 2A).

We next formed conjugates between control or shArp2-transfected T cells and SEE-pulsed Raji cells, and we imaged them by SEM (representative conjugates are shown in Figures 2B and 2C). In many cases, control cells maintained strong contacts with APCs, with the lamellipod extending over the APC surface (Figure 2B). In contrast, Arp2-depleted T cells did not produce lamellipodia or display impressive morphological alterations. Actually, these cells showed less contact with APCs, extending “finger-like” projections throughout the cell-cell contact zone (Figure 2C). These structures were also apparent in confocal images with shArp2- and shArp3-suppressed cells (Figure 2A). These data further support the notion that
polarized F-actin reorganization at the IS is not exclusively regulated by the Arp2/3 complex.

TCR-Mediated MTOC Polarization Is Arp2/3 Complex Independent

Since Arp2/3 complex-depleted T cells retained the ability to polarize F-actin structures, we next examined MTOC reorientation toward APCs, a hallmark of T cell polarization. We found that TCR-mediated MTOC repositioning is Arp2/3 complex independent, with shArp2-GFP- and shArp3-GFP-transfected cells efficiently polarizing their MTOC and microtubule system to face APCs (Figures 3A and 3B). Thus, while depletion of the Arp2/3 complex regulates activated T cell morphology, it does not control the formation or polarization of all F-actin-based structures or MTOC reorientation toward the IS.

Arp2/3 Complex-Dependent F-Actin Regulates Integrin Activation and TCR Internalization

TCR-mediated j2-integrin activation is considered actin dependent and is essential for T cell-APC conjugation (Kinashi, 2005). To examine whether j2-integrin activation directly requires Arp2/3 complex-mediated F-actin, we analyzed conjugate formation in cells suppressed for Arp2 and Arp3. We found that shArp2- and shArp3-transfected cells formed conjugates with SEE-pulsed B cells less frequently than did control transfectants (Figure 3C) and that single Arp2 or Arp3 suppression yielded similar results (not shown). Because j2-integrin-mediated conjugation was Arp2/3 complex dependent, we next investigated whether TCR-stimulated shArp2- or shArp3-transfected cells were capable of binding fibronectin, a j1-integrin-dependent event. As shown in Figure 3D, GFP-expressing Arp2- and Arp3-suppressed cells also demonstrated diminished TCR-mediated fibronectin adhesion.

Additionally, the Arp2/3 complex may participate in receptor internalization (Engqvist-Goldstein and Drubin, 2003). As shown in Figure 3E, basal TCR surface expression was unaffected without Arp2 or Arp3. However, Arp2- and Arp3-depleted cells displayed substantial TCR internalization defects (Figure 3F), even in spite of the fact that microtubules, which are suggested to regulate TCR internalization (Barr et al., 2006), still polarize in the absence of the Arp2/3 complex. We next analyzed the ability

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Figure 2. Arp2/3 Complex-Independent Filopodia Polarize during APC Recognition

(A) Jurkat cells were transfected with control, shArp2-GFP, or shArp3-GFP vectors (green). Each population was conjugated with SEE-pulsed/CMAC-stained NALM6 or Raji cells (blue) and stained with phalloidin (red).

(B and C) Control-transfected (B) or shArp2-transfected (C) Jurkat cells were conjugated with SEE-pulsed Raji cells and analyzed by SEM.
Figure 3. Arp2/3 Complex-Dependent F-Actin Polymerization Does Not Control MTOC Polarity, but Regulates Integrins and TCR Internalization

(A and B) Jurkat cells were transfected with control, shArp2-GFP, or shArp3-GFP vectors (green) (A). Each population was conjugated with unpulsed or SEE-pulsed/CMAC-stained Raji cells (blue), labeled with anti-α-Tubulin (red), and scored for MTOC polarization (B). Arrows indicate MTOC position.

(C) Jurkat cells were transfected with control or with both shArp2-GFP and shArp3-GFP vectors (green), and incubated with unpulsed or SEE-pulsed/PKH26-stained NALM6 B cells (red). Conjugation efficiency was determined by flow cytometry.

(D) Jurkat cells were transfected as in (A) and analyzed for fibronectin binding. The percent adhesion (GFP-negative, low, and high) in each sample was determined by flow cytometry.

(E) Jurkat cells were transfected as in (A) and stained on ice with anti-CD3-PE, and cell-surface expression of the TCR was analyzed on GFP+ cells.

(F) Jurkat cells were transfected as in (A), stained on ice with anti-CD3-PE, crosslinked for the indicated times at 37°C, incubated in cold stripping buffer (removing surface antibodies), and analyzed by flow cytometry for TCR internalization in GFP+ cells.

Bars in (B)–(D) and (F) represent mean ± SD from three independent experiments.
of shArp2- and shArp3-transfected cells to signal efficiently after TCR ligation. So far, the role of the actin cytoskeleton in regulating calcium-related signaling events and mitogen-activated protein kinases (MAPKs) in lymphocytes has been controversial (Hao and August, 2005; Holsinger et al., 1998; Rivas et al., 2004; Valitutti et al., 1995). Interestingly, our studies indicate that Arp2- and Arp3-suppressed Jurkat cells display normal TCR-mediated PLC-γ1 phosphorylation and Ca²⁺ mobilization (Figures S1A and S1B). Also, we found prolonged ERK phosphorylation in the absence of Arp2 or Arp3 (Figure S1A), which may be a direct result of diminished TCR internalization. Thus, although the Arp2/3 complex was not required for MTOC and F-actin polarization or certain TCR-mediated signaling events, integrin activation and TCR internalization were diminished. This indicates that F-actin generated independently of the Arp2/3 complex may have distinct roles from Arp2/3 complex-nucleated F-actin during T cell activation.

Formins Display Distinct Cytoskeletal Colocalization in T Cells

To identify potential Arp2/3 complex-independent mechanisms for F-actin polarization in T cells, we examined formins because of their suggested role in filopodia formation. Formin expression in T cells has not been analyzed, although lymphocytes have been demonstrated to express FMNL1 (Favaro et al., 2006). Indeed, we found FMNL1 mRNA and protein to be expressed in both Jurkat and peripheral blood human CD4⁺ (hCD4⁺) T cells (Figures 4A and 4B), with primary T cells expressing more protein than Jurkat cells (Figure 4C). In contrast, while mRNA for the other formin-like family members (FMNL2 and FMNL3) could be detected, very little protein was immunoprecipitated from Jurkat or hCD4⁺ T cell lysates (Figures 4B and 4D). However, we detected FMNL2 and FMNL3 protein in nonimmune cell types (Figures 4B and 4D). Additionally, of the Diaphanous family members (DIA1, DIA2, and DIA3), DIA1 appeared to be the predominant isoform expressed in T cells (Figures 4A and 4E).

We next analyzed the localization of these formins by immunofluorescence. We found fringe-like localization of FMNL1 at the leading edge of the lamellipod in spreading T cells (Figure S2B). This FMNL1 accumulation was also apparent at the edge of F-actin structures, which formed during APC recognition (Figure S5A), along with a distinct, “ring-like” localization of FMNL1 within the T cell body (Figure S5B). Upon further analysis, we found that this
FMNL1 ring reoriented along with the MTOC to face stimulating APCs at later time points (Figure 5C). Moreover, a similar FMNL1 ring polarized with the MTOC in hCD4+ T cells (Figures 5D and 5E). Interestingly, we identified that the centrosome was positioned precisely within the center of this FMNL1 ring structure (Figure 5F).

In contrast to FMNL1, DIA1 was enriched in a fine line surrounding the lamellipod of spreading T cells (Figure S2A). In conjugates, DIA1 did not display remarkable accumulation with F-actin, although it was within F-actin-rich areas (Figure 5G). However, like FMNL1, DIA1 displayed a distinct point of localization within the T cell body (Figures 5G and 5H), and upon closer analysis appeared to be in a “starburst” pattern overlaying the MTOC and microtubules (Figure 5I). Additionally, DIA1 co-localized with the MTOC in primary hCD4+ T cells (Figures 5J and 5K) and was also found surrounding the centrosome (Figure 5L). Moreover, DIA1 localized throughout the Arp2/3 complex-independent filopodia, whereas FMNL1 was frequently enriched at the tips (Figure S2).
Formins Regulate T Cell Polarity

A

B

C

D

E

Vector shDIA1

Time (hrs) 72 24 48 72

anti-DIA1

anti-ZAP70

Vector shFMNL1a shFMNL1b

Time (hrs) 72 24 48 72

anti-FMN1

anti-ZAP70

-SEE

+SEE

Transfected T cells in conjugates (%)

Vector shFMNL1 shDIA1

Conjugates with polarized F-actin (%)

Vector -SEE Vector +SEE shFMNL1 +SEE shDIA1 +SEE

F-Actin GFP Merge

Vector -SEE

Vector +SEE

shFMNL1 +SEE

shDIA1 +SEE
contrast to DIA1, DIA2 was not found to localize with F-actin-rich T cell lamellipodia or filopodia (not shown), but did similarly surround the centrosome (Figure S3).

**FMNL1 and DIA1 Do Not Control TCR-Induced F-Actin Accumulation at the IS**

Because FMNL1 and DIA1 displayed unique cytoskeletal localization patterns and potentially regulate Arp2/3 complex-independent F-actin polymerization, we generated shRNA-targeting vectors against FMNL1 and DIA1 (Figures 6A and 6B). Use of these suppression vectors abrogated centrosomal staining by both anti-FMNL1 and anti-DIA1, demonstrating the specificity of the antibody staining (Figure S4). We next examined whether suppression of these formins affected Arp2/3 complex-independent filopodia formation. Like control, shFMNL1a-mCherry-, shFMNL1b-mCherry-, and shDIA1-mCherry-transfected cells spread normally (Figures S5A–SSD). Likewise, GFP-actin cells cotransfected with shArp2-mCherry and shFMNL1 or shDIA1 vectors (individually or combined) produced microspikes upon activation (Figures S5E–S5I). Also, shFMNL1- and shDIA1-transfected cells efficiently formed conjugates and displayed normal F-actin accumulation at the IS (Figures 6C–6E). Moreover, FMNL1- and DIA1-depleted T cells showed normal PLCγ1, ERK, and calcium responses after TCR stimulation (Figures S1C and S1D). Thus, although these formins localized in the lamellipod of spreading cells and to the filopodia of Arp2- or Arp3-suppressed cells, their depletion did not affect the formation of these F-actin structures or signaling. This suggested that FMNL1 and DIA1 function distinctly, because they do not impact Arp2/3 complex-regulated cellular processes, such as lamellipod formation and integrin activation.

As a means to further analyze whether formins produce Arp2/3 complex-independent filopodia, we studied the role of lymphocyte-expressed ENA and VASP proteins (EVL; ENA/VASP-like and VASP; vasodilator-stimulated phosphoprotein). This family of proteins is proposed to be necessary for formin-dependent filopodia formation through their bundling of formin-mediated F-actin (Schröenbeck et al., 2006). In spreading cells, EVL was accumulated at the very leading edge and colocalized with F-actin within the lamellipod, whereas VASP primarily localized only to the edge of the lamellae (Figure S6). In addition, EVL was within Arp2/3 complex-independent filopodia, while VASP localized to the tips (Figure S6). Interestingly, shEVL-mCherry-, shVASP-mCherry-, as well as doubly shEVL-mCherry- and shVASP-mCherry-transfected cells spread normally (Figures S7A–S7D). Likewise, GFP-actin cells cotransfected with shArp2-mCherry and shEVL or shVASP vectors (individually or both) efficiently formed filopodia (Figures S7E–S7I). Thus, Arp2/3 complex-independent filopodia formation in T cells is also EVL and VASP independent.

**FMNL1 and DIA1 Regulate MTOC Polarization**

Recent evidence implicates F-actin reorganization in establishing T cell centrosomal polarity (Stinchcombe et al., 2006). Given that formins colocalize with the T cell centrosome, we examined their role in TCR-mediated MTOC polarization. In contrast to Arp2/3 complex depletion, which did not affect MTOC reorientation (Figures 3A and 3B), loss of FMNL1 or DIA1 did reduce MTOC polarization (Figures 7A and 7B). Thus, although FMNL1 and DIA1 did not affect the formation of F-actin-based structures at the IS, they do regulate centrosome polarization in T cells.

Centrosome polarization is essential for the directed release of granules during cytotoxic T cell (CTL)-mediated killing, so we were next interested in the ability of siFMNL1- and siDIA1-transfected primary hCD8+ T cells to kill target cells. Like hCD4+ T cells, hCD8+ T cells expressed FMNL1 and DIA1, which colocalized with the MTOC (Figure S8). However, we found that primary human T cells (CD4+ and CD8+) also highly expressed a smaller 80 kDa variant of DIA1 (Figure S8C), which along with the larger variant was specifically depleted with siDIA1 (Figure 7C). Interestingly, CTL clones did not express DIA2 (Figure S8C). In a redirected cytotoxicity assay, we found decreased target cell lysis by FMNL1- and DIA1-depleted CTLs, while combined suppression of these formins substantially reduced CTL-mediated killing (Figure 7C). This suggested that the diminished centrosomal polarization in the absence of FMNL1 and DIA1 negatively affects CTL function.

So far, regulation of TCR-mediated MTOC polarization has been shown to involve FYN, ZAP70, LAT, SLP76, and VAV1 (Ardouin et al., 2003; Kuhne et al., 2003; Martin-Cofreces et al., 2006). These components are known to be required for numerous signaling pathways downstream of the TCR including the activation of rho family GTPases. Importantly, regulation of the formin proteins is primarily thought to occur through interactions with these small GTPases, with DIA1 being regulated by RHOA-C (Faix and Grosse, 2006) and FMNL1 predicted to be RAC1 regulated (Yayoshi-Yamamoto et al., 2000). However, we found that in addition to binding GTP-bound RAC1 (Figure 7D), FMNL1 also directly and specifically interacted with GTP-RHOA (Figure 7E), but not GTP-CDC42 (Figure 7F). It is surprising that DIA1 and FMNL1 are potentially regulated through interactions with RHO and RAC.
yet MTOC polarization in T cells has been suggested to be CDC42 dependent (Stowers et al., 1995). However, this CDC42 dependency for MTOC polarity was shown utilizing overexpression of dominant-negative CDC42, which may have pleiotropic effects on signaling. Thus, we specifically depleted RAC1 and CDC42 with shRNA and analyzed TCR-mediated MTOC polarization. Interestingly, RAC1 suppression, but not CDC42 suppression, resulted in diminished MTOC polarization in Jurkat cells (Figure 7G). This suggested that RAC1, possibly through its effects on FMNL1, is directing TCR-mediated movement of the MTOC to the IS.

DISCUSSION

In this study, we found that TCR-stimulated Arp2/3 complex-depleted cells still polarized F-actin in the form of filopodial protrusions and were capable of MTOC polarization. In contrast, DIA1 and FMNL1 did not regulate F-actin polarization but instead controlled MTOC polarity. Thus, the Arp2/3 complex does not exclusively govern TCR-mediated cytoskeletal reorganization.

Upon stimulation, Arp2- and Arp3-suppressed T cells form atypical filopodia. However, these structures probably normally arise during activation but are obscured by global actin reorganization events within the Arp2/3 complex-driven meshwork. Indeed, Arp3-suppressed T cells displayed remnants of lamellipod formation, which transiently originated and spread between Arp2/3 complex-independent spikes. Also, SEM images revealed apparent filopodial tips protruding from the lamellipod edge in spreading cells. In fact, microspikes have been described buried within the lamellipodial meshwork in fibroblasts (Small, 1981). Thus, Arp2/3 complex-independent filopodia formation might concomitantly direct Arp2/3 complex-mediated lamellipod formation. In this regard, they are not functionally redundant between different formin family members. It is also interesting that depletion of EVL and VASP, which do not appear to participate in actin dynamics (lamellipodia or filopodia) that lead to F-actin polarization at the IS. It remains possible that Arp2/3 complex-independent filopodial formation results from another T cell-expressed formin or that there is functional redundancy at the IS.

FMNL1 and DIA1 localize within F-actin-rich structures, suggesting their involvement in F-actin polarization. Nevertheless, these formins do not appear to participate in actin dynamics (lamellipodia or filopodia) that lead to F-actin polarization at the IS. It remains possible that Arp2/3 complex-independent filopodial formation results from another T cell-expressed formin or that there is functional redundancy between different formin family members. It is also interesting that depletion of EVL and VASP, which are proposed to be required for the formation of formin-mediated filopodia (Schirenbeck et al., 2006), did not affect the formation of filopodia in the absence of Arp2 or Arp3. Thus, either formins do not require an association with these actin-bundling proteins to promote the formation of filopodia in T cells, or formins are not the major regulator of microspike formation in Arp2/3 complex-suppressed cells. It is possible that other actin nucleators, such as SPIR proteins, could regulate these spikes.

Interestingly, although there may be functional compensation between formins in the generation of filopodia in the absence of Arp2 or Arp3, loss of FMNL1 or DIA1 individually affects MTOC reorientation, suggesting that, at least in this regard, they are not functionally redundant. In fact, it is clear that these formins display strikingly different patterns of centrosome colocalization, which may provide the basis for why they each independently regulate MTOC polarization. It is already thought that formins are essential for actin-dependent processes leading to polarity of the yeast MTOC (Faix and Grosse, 2006). Thus, microtubule regulation may be a general property of this protein family in

Figure 7. FMNL1 and DIA1 Control TCR-Mediated MTOC Polarization

(A and B) Jurkat cells (A) were transfected with shRNA-GFP vectors as indicated and conjugated with unpulsed or SEE-pulsed CMAC-stained Raji B cells (blue). The conjugates were stained with anti-αTubulin (red) and scored for MTOC polarization (B). Arrows indicate MTOC position.

(C) hCD8+ T cells were transfected with siRNA against FMNL1 and/or DIA1 and then used in a redirected cytotoxicity assay. Data were analyzed by the Student’s t test, *p < 0.05 and **p < 0.0002.

(D–F) Purified FLAG-tagged RAC1 (D), CDC42 (E), or RHOA (F) was loaded with GDP or GTP-γS and then analyzed for binding to GST control or GST-FMNL1 GBBD (GTPase binding domain). GST-PAK-GBBD was used as a positive control for binding to GTP-loaded RAC1 and CDC42, whereas RHOTEKIN-GBBD was used for RHOA.

(G) Jurkat cells were transfected with the indicated shRNA-GFP vectors and then analyzed for MTOC polarization as in (A) and (B). Bars in (B), (C), and (G) represent mean ± SD from three independent experiments.

Immunity 26, 177–190, February 2007 ©2007 Elsevier Inc. 187
eukaryotic cells, with a formin network controlling the actin-dependent processes that allow microtubule positioning. In fact, recent studies established the importance of formins in microtubule stabilization and suggest their role in migration through possible effects on microtubule polarization (Eng et al., 2006; Yamana et al., 2006). Therefore, even though the polarization of recognizable actin structures is unaffected by loss of FMNL1 and DIA1, we propose that these proteins each control distinct actin processes that are not as readily observed but are necessary for centrosome positioning toward the IS.

Recently, it was suggested that the centrosome must contact the plasma membrane at the IS to allow T cell secretion (Stinchcombe et al., 2006). In this study, they proposed that the CDC42-binding actin-regulatory protein IQGAP1, which interacts with microtubule plus-end complexes, may provide the force for positioning the centrosome by linking actin-dependent processes to microtubule ends. In support of this, we found that shRNA-depletion of IQGAP1 affects MTOC polarization (T.S.G. and D.D.B., unpublished observation), suggesting that these plus-end complexes are, in fact, essential for centrosome polarity in T cells. It is of interest that formin family proteins may also bind microtubule plus-end complexes (Faix and Grosse, 2006), and therefore might be required for their movement. Thus, it is likely that formin-dependent and formin-independent processes acting along microtubules and directly at the MTOC will participate in TCR-mediated centrosome polarity and cell-mediated cytotoxicity.

Surprisingly, although dominant-negative expression studies have indicated the importance of CDC42 in MTOC polarization during T cell activation (Stowers et al., 1995), we find that depletion of RAC1, but not CDC42, abrogates this process in T cells. While it is possible that the residual expression of CDC42 after suppression is sufficient to drive MTOC reorientation, this finding is interesting because DIA1 is RHO regulated, FMNL1 interacts with RHOA and RAC1, and IQGAP1 binds RAC1 (in addition to CDC42). However, we must note that in one report, FMNL1 was suggested to be CDC42 regulated (Seth et al., 2006). In this study they could not detect GTP-RAC1 or GTP-CDC42 binding to FMNL1, so they instead analyzed relief of FMNL1 autoinhibition by using a large molar excess of CDC42 and truncated FMNL1 domains. Thus, this may not reflect a physiological mechanism of FMNL1 regulation. Further studies are needed to define the signaling pathways linking formin-mediated actin regulation to microtubule dynamics in T cells. In particular, the generation of reagents to specifically target and detect different members of the very homologous rho family (RHOA-C) GTP-binding proteins will help in dissecting the pathways linking formins to the TCR.

In contrast to MTOC polarization, β2 integrin activation appears to be specifically Arp2/3 complex dependent and does not rely on FMNL1 or DIA1. This is interesting because WAVE2 controls integrin activation in T cells (Nolz et al., 2006), and we observe a similar β2 integrin defect between Arp2/3 complex- and WAVE2-suppressed cells, suggesting that WAVE2 regulation of β2 integrins is Arp2/3 complex dependent. However, the β1 integrin defect of WAVE2-depleted cells seems more severe than that seen with loss of either Arp2 or Arp3 (not shown) (Nolz et al., 2006). Interactions between the WAVE2 complex and formins may be important in this regard. In fact, it was recently demonstrated that formins might predominately regulate β3 integrins (Butler et al., 2006), suggesting that integrin subsets might also be separately regulated through distinctly formed actin frameworks. Further dissection of the mechanisms by which distinct pools of F-actin are regulated to control both polarity and integrin function is necessary to fully understand these subtle complexities.

Altogether, we demonstrate that the Arp2/3 complex is not the sole regulator of TCR-mediated cytoskeletal dynamics, and we find that formins control the dynamic centrosomal polarization necessary for T cell function and cellular cytotoxicity. Through the continued characterization of the distinct regulatory mechanisms that collaboratively control cytoskeletal restructuring, we can begin to functionally dissect the specific contributions of the diverse F-actin architectural frameworks and their roles in establishing cell polarity.

**EXPERIMENTAL PROCEDURES**

**Reagents and Plasmids**

Reagents were from Sigma unless otherwise specified. Anti-CD3 (OKT3) was from the Mayo Pharmacy and anti-ZAP70, anti-PLCγ1, and anti-WAVE2 have been previously described (Gomez et al., 2005; Nolz et al., 2006). Anti-Arp3 and anti-CDC42 (BD Transduction Laboratories), anti-Arp2 (clone H-84; Santa Cruz), and anti-ARPC2 (Bethyl Laboratories) were used. We obtained monoclonal anti-FLAG, anti–α-Tubulin, and anti–γ-Tubulin from Sigma. We also used antibodies against ERK1/2, pERK1/2 (T202/Y204), and pPLCγ1 (Y783) from Cell Signaling Technology. Antisera against FMNL1, FMNL2, and FMNL3 were generated by immunizing rabbits with KHL-conjugated synthetic peptides (Cocalico Biologicals; Table S1). Antibodies against EVL(AA261-340) and VASP(AA231-325) were generated similarly, but with GST-fusion proteins. Anti-CD3ε:PE was from BD Immunocytometry Systems. The pFRT-H1p, pCMS3, eGFP:H1p, and pCMS3:mCherry:H1p shRNA vectors have been described (Gomez et al., 2005; Nolz et al., 2006). RT-PCR was performed as described (Nolz et al., 2006). See Table S1 for RT-PCR primers and shRNA targeting sequences.

**Cell Culture, Transfection, Immunoprecipitation, GST Pull-Down, and Immunoblot Analysis**

Jurkat-E6, hCD4+, RAJI, NALM6, P815, and GFP-actin-Jurkat-E6 cells were transfected by electroporation at 295V with 3.5 μg of plasmid DNA. Anti-ZAP70, anti-PLCγ1, and anti-RAC1 (clone 23A8; Upstate Biotechnology) were used in this study. In addition, polyclonal antibodies against DIA1 and DIA2 (Bethyl Laboratories) were used. We obtained monoclonal anti-FLAG, anti–α-Tubulin, and anti–γ-Tubulin from Sigma. We also used antibodies against ERK1/2, pERK1/2 (T202/Y204), and pPLCγ1 (Y783) from Cell Signaling Technology. Antisera against FMNL1, FMNL2, and FMNL3 were generated by immunizing rabbits with KHL-conjugated synthetic peptides (Cocalico Biologicals; Table S1). Antibodies against EVL(AA261-340) and VASP(AA231-325) were generated similarly, but with GST-fusion proteins. Anti-CD3ε:PE was from BD Immunocytometry Systems. The pFRT-H1p, pCMS3, eGFP:H1p, and pCMS3:mCherry:H1p shRNA vectors have been described (Gomez et al., 2005; Nolz et al., 2006). RT-PCR was performed as described (Nolz et al., 2006). See Table S1 for RT-PCR primers and shRNA targeting sequences.
protein (25 μg) was bound to GSH-agarose (30 μl) followed by one wash. FLAG/His-tagged GTPases were purified from insect cells by the FastBac system and Probound resin (Invitrogen) and loaded with GDP or GTP*S. For loading, 25 mM of purified GTPase was incubated with 50 μM GDP or GTP*S in GTPase Loading Buffer (20 mM Tris [pH 7.5], 50 mM NaCl, 1 mM EDTA, and 0.1% Triton) for 10 min at 37°C, and then MgCl₂ was added to a final concentration of 2.5 mM. This GTPase-containing solution was then added to the GST-fusion protein bound agarose, rotated for 25 min at 4°C, and then washed two times.

Cytotoxicity Assays
The redirected 51Cr-release assays were performed as previously described (Billaudeau et al., 2000). CD8⁺ T cells were used 48 hr after transfection with siRNA and were able to kill P815 cells only in the presence of OKT3 (1 ng/ml). In all cases, spontaneous release did not exceed 10% of maximum lysis. Lytic units were calculated based on 20% cytotoxicity (Billaudeau et al., 2000).

Immunofluorescence and Live Cell Imaging
Immunofluorescence of fixed Jurkat- or hCD4⁺-containing conjugates was performed as described (Gomez et al., 2005). For quantification, 50–100 conjugates were chosen randomly, and an individual blinded to the experiment scored conjugates consisting of one GFP⁺ T cell and one blue CMAC-stained B cell. Conjugates showing distinct labeling at the cell-cell contact site were scored positive. MT0C polarization studies were performed similarly, except conjugates were allowed to form for 30 min and scored positive if the MTOC (based on β-Tubulin staining) was against the cell-cell interface. For immunofluorescence of spreading cells, Jurkat cells were settled for 5 min onto poly-L-lysine-coated coverslips (Gomez et al., 2006), which were preincubated with or without anti-CD3 (20 μg/ml in PBS) overnight at 4°C. For live cell imaging, Jurkat cells were imaged spreading onto anti-TCR-coated coverslips as described (Nolz et al., 2006).

Scanning Electron Microscopy
For scanning electron microscopy (SEM), cells were prepared on glass coverslips as above (Gomez et al., 2005) but were placed in fixative (4% formaldehyde and 1% glutaraldehyde in sodium phosphate buffer [pH 7.3]) at 4°C for up to 36 hr. The coverslips were treated with a series of solution exchanges, including two phosphate buffer washes followed by sequential 60% ethanol, 70% ethanol, 80% ethanol, 95% ethanol, and 100% ethanol washes. Coverslips were placed into a critical point dryer while in 100% ethanol for drying, sputter-coated with gold-palladium, and imaged on a Hitachi H-4700 SEM.

TCR Expression and Internalization
Jurkat cells were transfected with the indicated GFP-shRNA vectors, and basal TCR expression and TCR internalization were analyzed by flow cytometry gating on GFP⁺ cells as described (Gomez et al., 2005).

Adhesion Assays, Conjugate Analysis, and Calcium Mobilization
Adhesion assays were performed as described (Nolz et al., 2006). Conjugate assays were performed as described (Gomez et al., 2005; Nolz et al., 2006). Calcium mobilization studies were done as previously described (Gomez et al., 2005).

Supplemental Data
Supplemental Data include nine figures, one table, and three movies and can be found with this article online at http://www.immunity.com/cgi/content/full/26/2/177/DC1/.

ACKNOWLEDGMENTS
We would like to thank R.A. Schoon and C.J. Dick for their help with the cytotoxicity assays. This work was supported by the Mayo Foundation, NIH grant R01-AI065474 to D.B., NIH grant F31-AI068624 to T.S.G., NIH grant R01-CA47752 to P.J.L., and NIH grants R01-AI038474 and R01-AI031126 to Y.S. The authors declare that they have no competing financial interests.

Received: October 6, 2006
Revised: December 20, 2006
Accepted: January 8, 2007
Published online: February 15, 2007

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