UCSF
UC San Francisco Previously Published Works

Title
Cytoskeletal polarization of T cells is regulated by an immunoreceptor tyrosine-based activation motif-dependent mechanism.

Permalink
https://escholarship.org/uc/item/0bz9r07j

Journal
The Journal of cell biology, 140(4)

ISSN
0021-9525

Authors
Lowin-Kropf, B
Shapiro, VS
Weiss, A

Publication Date
1998-02-01

DOI
10.1083/jcb.140.4.861

Peer reviewed
Polarization of a T cell response towards a triggering antigen-presenting cell (APC) is thought to contribute to the specificity of the immune response. Upon encountering an APC, T cells rapidly undergo cytoskeletal polarization, which includes the formation of a tight collar of polymerized actin at the T cell–APC interface and the reorientation of the microtubule-organizing center (MTOC) towards the bound APC (Geiger et al., 1982; Ryser et al., 1982). Whereas F-actin accumulation at the cell–cell interface was suggested to stabilize and favor continuous T cell antigen receptor (TCR)–antigen interactions (Valitutti et al., 1995b), MTOC reorientation is thought to position the T cell secretory apparatus into close proximity with the APC. Indeed, several studies showed that repositioning of the MTOC was accompanied by the polarized concentration of cytokines and cytotoxic mediators at the T cell–APC/target interface (Kupfer and Dennert, 1984; Kupfer et al., 1991, 1994). Moreover, Poo et al. (1988) have demonstrated that limited TCR cross-linking in a cloned helper T cell line triggered the release of cytokines preferentially over the area of receptor cross-linking. Thus, the engagement of only a fraction of TCRs on a helper T cell results in the polarized delivery of cytokines towards the site of the stimulus.

Cytoskeletal polarization of a T cell is dependent on the presence of specific antigen (Kupfer and Singer, 1989), suggesting the involvement of a TCR-mediated signaling pathway. A variety of cellular responses, including gene activation, proliferation, and secretion of cytokines and cytotoxic mediators, have been reported to be initiated by these cytoskeletal changes did not depend on activation of additional coreceptors. Moreover, single subunits of the TCR complex, namely TCR-ζ and CD3ε, were equally effective in inducing cytoskeletal polarization. However, mutagenesis of the immunoreceptor tyrosine-based activation motifs (ITAMs), present three times in TCR-ζ and once in CD3ε, revealed that the induction of cytoskeletal rearrangements required the presence of at least one intact ITAM. In agreement with this result, lack of functional Lck, the protein tyrosine kinase responsible for ITAM phosphorylation, abolished both MTOC reorientation and polarized actin polymerization. Both inhibitor and transient overexpression studies demonstrated that MTOC reorientation could occur in the absence of Ras activation. Our results suggest that APC-induced T cell polarization is a TCR-mediated event that is coupled to the TCR by the same signaling motif as TCR-induced gene activation, but diverges in its distal signaling requirements.
TCR engagement. Interestingly, activation of Jurkat T cells with immobilized TCR-specific antibodies could induce actin polymerization and formation of F-actin-rich pseudopods, suggesting that TCR-mediated signaling pathways may indeed regulate cytoskeletal organization (Melamed et al., 1991). Furthermore, a recent report implies a role for the small GTPase CDC42 in APC-mediated T cell polarization (Stowers et al., 1995). Members of the Rho subfamily of small GTPases (Rho, Rac, CDC42) are evolutionary conserved regulators of the actin cytoskeleton in a variety of different cell systems (Hall, 1994). Consequently, TCR-mediated signals might activate small GTPases by an as yet unidentified mechanism. However, T cell–APC conjugate formation involves the clustering and engagement of various other T cell surface receptors besides TCR–antigen interactions, including the CD8/CD4 coreceptors and integrins (Kupfer and Singer, 1989; Kupfer et al., 1987). It is conceivable that triggering of these additional receptors is required for or at least contributes to APC-induced T cell polarization.

The TCR is coupled to intracellular signaling pathways by two noncovalently associated signal-transducing complexes, namely the CD3 (γ, δ, and ε) and the TCR-ζ chains. Signaling through these complexes requires a conserved sequence motif (YxxLx(6–8)YxxL), termed the immunoreceptor tyrosine-based activation motif (ITAM) (Irving and Weiss, 1991; Romeo and Seed, 1991). The CD3 and TCR-ζ chains each contain one and three ITAMs, respectively. Upon TCR engagement, the tyrosine residues in the ITAMs are rapidly phosphorylated by the Src-family protein tyrosine kinase (PTK) Lck (van Oers et al., 1996) and serve as docking sites for src-homology-domain 2 (SH2) containing signaling molecules such as the PTKs of the Syk/zeta-associated protein 70 (ZAP-70) family. After recruitment, Syk/ZAP-70 is activated by phosphorylation and contributes to the initiation of downstream signaling events such as calcium mobilization and activation of the Ras pathway. Single CD3 and TCR-ζ chains can independently activate a variety of qualitatively similar proximal and distal signaling events (Irving and Weiss, 1991; Letourneau and Klauser, 1991; Romeo and Seed, 1991; Shinkai et al., 1995), suggesting that the presence of multiple ITAMs in the TCR mainly serves the purpose of signal amplification. However, amino acids surrounding the ITAMs differ in their ability to induce apoptosis in a murine T cell line (Combadiere et al., 1996). Furthermore, the third TCR-ζ ITAM and the CD3ε ITAM have been implicated in coupling the TCR to the actin cytoskeleton in an activation-dependent manner (Rozdzial et al., 1995). Thus, individual subunits might couple the TCR to effector molecules involved in the regulation of cytoskeletal changes.

To investigate the role of TCR-mediated signaling pathways in APC-induced T cell polarization, we set up a conjugation assay using Jurkat T cells or mutants derived from it as effector cells, with antibody-coated, cell-sized latex beads as APCs. We show here that TCR cross-linking alone is sufficient to induce MTOC polarization and actin polymerization at the T cell–bead interface. Cytoskeletal reorientation required the presence of at least one intact ITAM. In support of this result, no cytoskeletal changes could be observed in the Lck-deficient Jurkat mutant JCaM1.6. Furthermore, our studies demonstrate that MTOC reorientation occurs in the absence of Ras activation. Our results imply that APC-induced T cell polarization is a TCR-mediated event that is coupled to the receptor by the same signaling motif as TCR-mediated gene activation, but diverges in its distal signaling requirements, since Ras is not required. The assay described in this study might be a useful tool to characterize signaling molecules involved in T cell polarization.

Materials and Methods

Cells and Reagents

The Jurkat T cell subclone E6, JCaM1.6 (Goldsmith et al., 1988) and large T antigen (TAG) Jurkat cells (kindly provided by Dr. G. Crabtree, Stanford University, Palo Alto, CA; Clipstone and Crabtree, 1992) were maintained in RPMI 1640 medium supplemented with 5% fetal calf serum (Intergen Co., Purchase, NY), 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Jurkat-derived stable clones were cultured in complete medium containing 10% FCS and 2 mg/ml G418 (GIBCO BRL, Gaithersburg, MD). JCaM1.6/Lck cells (Straus and Weiss, 1992) were maintained in complete medium containing 10% FCS and 250 μg/ml hygromycin (GIBCO BRL). Poly-L-lysine was obtained from Sigma Chemical Co. (St. Louis, MO). The MAPK inhibitor PD 098059 and Wortmannin were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA). Polystyrene latex microspheres (diameter 6 μm) were purchased from Polysciences Inc. (Warrington, PA). Antibodies were absorbed to the beads as previously described (Mescher, 1992). Briefly, 5–10 μg of purified antibody were mixed with 107 polystyrene beads in a final volume of 1 ml PBS, and incubated for 90 min at room temperature with constant tumbling. Beads were then blocked in 1.5 ml of PBS/1% BSA for 30 min. After three washes in PBS, latex beads were resuspended in PBS and stored at 4°C. Efficient antibody absorption was verified by flow cytometry.

Antibodies

Antibodies used for stimulation and immunofluorescence microscopy are as follows: the mAb C305 (IgM) specifically recognizes the Jurkat Ti β chain (Weiss and Stobo, 1984). Leu 4 (IgG1) is directed against the human CD3ε chain. RBC4 (IgM) recognizes the transferrin receptor. The mAb 9.1 (IgG3) is specific for human CD2 (Yang et al., 1986). Mouse mAb OKT8 recognizes an extracellular epitope of human CD8 and was acquired from American Type Culture Collection (Rockville, MD). The mAb 7G7B6 is directed against murine CD25 (Tac) and was obtained from American Type Culture Collection. A mouse mAb to human CD11a (IgG1, SPV-L7) was purchased from Zymed Laboratories, Inc. (S. San Francisco, CA). A rat mAb to α-tubulin (YOL1/34) was obtained from Harlan Sera-Laboratories (Crawley, UK) and was detected with an FITC-conjugated, affinity-purified donkey anti-rat (Fab′)2 antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). F-actin was detected with rhodamin-phalloidin (Molecular Probes, Inc., Menlo Park, CA). Antiphosphotyrosine mAb 4G10 was purchased from Upstate BioTechnology Inc. (Lake Placid, NY). An mAb against human Ha-Ras was purchased from Transduction Laboratories (Lexington, KY).

Plasmids

The dominant-negative ZAP-70 constructs SH2 (N-ε) and SH2 (N-ε) were described elsewhere (Qian et al., 1996). The N17Ras plasmid was a kind gift from D. Cantrell (ICRF, London, UK).

Chimera Construction and Clones

Stable clones expressing the TT-ε chimeras were kindly provided by Dr.
Creation of the CD8-z
The outside primers and fragment substitution were the same as for the
before plating out in medium containing 2 mg/ml of G418 (GIBCO BRL).
Ras. After 36 h, dead cells were eliminated by centrifugation over a Ficoll
plasmid. All constructs were subcloned into pcDNA3 (Invitrogen
constructed with EcoRV and BamHI, and substituted for the wild-type CD8-
created by PCR mutagenesis from the CD8-
the following primers were used: 5' primer (within Taq): 5' ACAGATCCTCAGGTAGCAGTG 3'; 3' primer (within CD3ε): 5' TCGGATATCTTTCCTTGGAGCCCCT 3'. Wild-type TT-T76 and CD8-
expression vector (Irving and Weiss, 1991). The mu-
plasmid. The CD8-
were generated as described elsewhere (Irving and Weiss, 1991). Con-
expression vector (Irving and Weiss, 1991). The mu-
the following primers were used: 5' CAGGATTCCTAAGCAGCTC 3', and 3' TTCTGTTGGAGAATTGCTC 5'. To
the following primers were used: 5' GAGGGATCGATGT-
the following primers were used: 5' GCCCTGTTCATAGGAAGCTCA 3', and 3' TCCTCTCCGAGGATCATCTT 5'. To
the following primers were used: 5' AGGGCTCTCAGTTGAGATGG 3', and 3' ACCGGCCCTGAGAAGTAC 5'.

Conjugate Formation and Immunofluorescence Microscopy
Jurkat T cells or derived clones and mutants were mixed with antibody-
coated latex beads at a 1:1 ratio. After centrifugation for 5 min at 100 g, the
cell–bead mixture was incubated for an additional 5–25 min at 37°C. Conjugates were then resuspended, plated onto poly-l-lysine-treated
slides, and fixed for 30 min in 3.4% paraformaldehyde at room tempera-
ture. Fixed cells were permeabilized for 4 min in PBS/0.1% Triton X-100
and blocked for 10 min in PBS/0.2% BSA. Cells were labeled for 30 min
with antibody diluted in PBS/0.2% BSA, followed by two washes of 10 min
each. After secondary antibody labeling, cells were incubated for 10 min
in 1% ovalbumin-phalloidin. After three final washes, slides were
mounted. Samples were either viewed under a laser scanning microscope
(LSM 410; Carl Zeiss, Inc., Thornwood, NY) (1st experiment) or a con-
ventional Microphot-FXA microscope (all other experiments). The
MTOC was scored as reoriented if it was located in close proximity to
the T cell plasma membrane between the T cell nucleus and the bead contact
area. At least 100 conjugates were scored in each experiment.

Transient Transfection Conjugate Formation Assay
Cell (10^6) were transiently cotransfected by electroporation, as described
above, with 30 µg of the indicated TT chimera and 40 µg of a vector con-
taining no insert or expressing dominant-negative mutants of ZAP-70 and
Ras. After 26 h, dead cells were eliminated by centrifugation over a Ficol

Results

Anti-TCR-coated Latex Beads Induce MTOC Reorientation and Polarized Actin Polymerization in Jurkat T Cells

Previous T cell polarization studies performed in a B cell–T cell system demonstrated the dependence of cytoskeletal rearrangements during conjugate formation on the presence of antigen, implying the participation of the TCR in these events (Kupfer and Dennert, 1984). The involvement of accessory molecules including integrins and coreceptors such as CD4/CD8 made it difficult to evaluate to what extent TCR-linked pathways were involved and sufficient for T cell cytoskeletal polarization. To address this question, we set up an in vitro assay using Jurkat T cells as effectors, and antibody-coated, cell-sized latex microspheres as APCs. Previous studies have demonstrated that such artificial APCs, either coated with TCR-specific antibodies or antigen–major histocompatibility complexes (MHC), could induce early and late signaling events, such as tyrosine phosphorylation and exocytosis, in T cells (Anel et al., 1994; Mescher, 1992). In contrast to soluble or plate-
bound TCR-specific antibodies, the use of latex beads allowed us to study signaling events in response to a focal stimulus. In a first set of experiments, we investigated whether triggering of the TCR alone was sufficient to induce reorientation of the microtubule cytoskeleton and polarized actin polymerization. Latex beads coated with the CD3ε-specific antibody Leu4 were mixed with Jurkat

Figure 1. Anti-TCR-coated latex beads induce MTOC reorienta-
tion and polarized actin polymerization in Jurkat T cells. Jurkat T
cells were mixed at a 1:2 ratio with anti–CD3ε-coated latex beads (left) or poly-L-lysine-coated latex beads (right). After 30 min
at 37°C, conjugates were stained with the antitubulin antibody
body YOL1/34 (a) and rhodamine-phalloidin to visualize F-actin
(b). The position of cell-bound latex beads is indicated by an as-
terisk, the position of the MTOC by an arrowhead. Bar, 5 µm.
T cells at a 1:2 ratio and incubated for 30 min at 37°C. Formed conjugates were fixed and stained for tubulin and F-actin. The position of the MTOC within the T cells was analyzed microscopically. Only MTOCs directly underlying the bound latex bead were scored positive. In the majority of all conjugates (73 ± 2%), the T cell MTOC was facing the latex bead contact area (Fig. 1 a). Polymerized actin was concentrated in a dense collar at the bead attachment site. F-actin-rich pseudopods protruded from the T cell surface, and in many cases surrounded the bead (Fig. 1 b). Similar results were obtained at shorter incubation times (10–20 min) and with latex beads coated with the Jurkat CD11a/LFA-1, the transferrin receptor, and CD2. Conjugates were stained with antitubulin antibody. Each column represents the average of at least four individual experiments in which >100 conjugates were scored for MTOC reorientation. MTOCs positioned between the bead contact site and the T cell nucleus, in close proximity to the T cell plasma membrane, were scored as positive for reorientation.

To determine whether cytoskeletal polarization was specifically associated with TCR cross-linking, antibodies directed against a variety of T cell surface molecules (CD11a/LFA-1, transferrin receptor [TfR] and CD2) and poly-l-lysine were bound to latex beads and used in the conjugate formation assay. All surface molecules analyzed were expressed at comparable levels in Jurkat T cells and similar numbers of bead–cell conjugates were formed (data not shown). As summarized in Fig. 2, TCR/CD3-specific beads displayed MTOC polarization in ∼80% of all scored conjugates, whereas beads coated with either anti–CD11a, anti–TfR, anti–CD2, or poly-l-lysine were randomly positioned (31 ± 5, 26 ± 2, 39 ± 4, and 26 ± 6% of scored conjugates, respectively), demonstrating that cytoskeletal polarization is specifically associated with TCR/CD3 cross-linking.

Figure 2. MTOC reorientation in Jurkat cells is specifically associated with TCR/CD3 cross-linking. Jurkat T cells were mixed at a 1:2 ratio with latex beads coated either with poly-l-lysine or antibodies against CD11a/LFA-1, the transferrin receptor, and CD2. Conjugates were stained with antitubulin antibody. Each column represents the average of at least four individual experiments in which >100 conjugates were scored for MTOC reorientation. MTOCs positioned between the bead contact site and the T cell nucleus, in close proximity to the T cell plasma membrane, were scored as positive for reorientation.

Figure 3. Chimera surface expression in stable Jurkat-derived clones expressing chimeras between Tac and the cytoplasmic tail of TCR-ζ (TT-ζ), CD3ε (TT-ε), or a truncated version of CD3ε (TT-εT). Stable clones were analyzed for chimera surface expression by FACS® analysis using an FITC-labeled Tac-specific antibody. The solid line represents untransfected Jurkat T cells.

Clustering of Chimeras Containing the Cytoplasmic Domain of CD3ε or TCR-ζ Can Induce MTOC Reorientation and Actin Polymerization

Several studies support the view that the presence of multiple ITAMs in the TCR might serve the purpose of signal amplification (Irving et al., 1993; Shinkai et al., 1995). However, single TCR subunits can associate with distinct SH2-containing signaling molecules and show qualitative differences in the induction of certain cellular responses (Combadiere et al., 1996; Osman et al., 1996). This suggests that distinct TCR subunits could couple the receptor to qualitatively different signaling pathways. To evaluate the ability of single TCR chains to induce cytoskeletal changes, we analyzed Jurkat T cells stably expressing a chimera between the extracellular and transmembrane domain of CD25 (Tac) with either the cytoplasmic tail of TCR-ζ (TT-ζ), CD3ε (TT-ε), or a truncated form of CD3ε (TT-εT) containing only the first three amino acids of the cytoplasmic tail. An ITAM motif is present once in the CD3ε chain and three times in the TCR-ζ chain. The ITAMs in both chains differ in their nonconsensus sequences and were shown to bind signaling molecules with different affinities (Osman et al., 1996). As demonstrated previously (Letourneur and Klausner, 1991; Shinkai et al., 1995), TT chimeras are expressed as monomeric molecules and do not associate with endogenous CD3 subunits. Clones with similar surface expression of the chimeric proteins (Fig. 3) were chosen and mixed with anti–Tac-coated latex beads for 10 min. The percentage of conjugate formation was comparable (data not shown). As shown in Fig. 4 A, clustering of the cytoplasmic tail of either TCR-ζ or CD3ε was sufficient to induce MTOC reorientation. The extent of MTOC reorientation triggered by either chimera was comparable and was similar to levels induced by cross-linking of the complete TCR-CD3 complex. Furthermore, both chimeras could induce actin polymerization (Fig. 4, B and C). In contrast, no cytoskeletal polarization was de-
tected towards beads bound to the truncated TT-eT chimera. These results suggest that both the ζ and ε chains can trigger qualitatively similar signals, which are normally generated by the TCR and induce T cell polarization. Furthermore, the presence of one ITAM motif was sufficient to induce cytoskeletal rearrangements, and the number of ITAMS had no obvious quantitative effect on the induction of cytoskeletal polarization. However, in transient expression experiments where chimera surface expression was lower, the TT-ζ chimera induced MTOC reorientation less efficiently than TT-ε (see below and data not shown). This result supports the view that multiple ITAMs can act as signal amplifiers under suboptimal activation conditions.

**MTOC Reorientation and Actin Polymerization Require the Presence of an Intact ITAM**

ITAM phosphorylation is essential for signaling through the TCR in many cellular responses. Phosphorylated ITAMs serve as docking sites for downstream signaling molecules such as ZAP-70 (Osman et al., 1996). Furthermore, phosphorylated ITAMs can mediate the activation-induced association of TCR components with the actin cytoskeleton. In particular, the third ITAM of the TCR-ζ chain has been reported to be crucial for F-actin interaction (Rozdzial et al., 1995). To investigate the role of ITAM phosphorylation and F-actin binding in coupling the TCR to T cell polarization, we analyzed stable clones expressing a chimera between the extracellular/transmembrane domain of human CD8 and either the entire cytoplasmic tail of TCR-ζ (CD8-ζ), the cytoplasmic tail of TCR-ζ truncated after the second ITAM (CD8-ζT76), or the CD8-ζT76 chimera in which all four ITAM tyrosines were mutated to phenylalanine (CD8-ζ4F) (Fig. 5 A). Clones with comparable surface expression were used (Fig. 6). As shown before for the TT-ζ expressing clone, cross-linking of the CD8-ζ chimera efficiently induced MTOC reorientation and actin polymerization at the bead attachment site (Fig. 5, B and C). No obvious qualitative difference was observed for the CD8-ζT76 chimera, which lacks the third ITAM, suggested to mediate F-actin binding. Focal actin polymerization was as frequently detected...
with CD8-ζT76 as in the CD8-ζ chimera (Fig. 5 C). As judged by immunofluorescence microscopy, no significant difference in the extent of F-actin accumulation was observed (Fig. 5 D). Our data suggest that the observed clustering of F-actin at the site of TCR engagement occurs in the absence of the third ζ chain ITAM. In contrast, analysis of CD8-ζ4F demonstrated the strict dependence of both MTOC reorientation and actin polymerization on the tyrosine phosphorylation of the ITAM motif. Mixing of clone CD8-ζ4F with anti-CD8-coated latex beads still yielded conjugates, but MTOC positioning was random (38 ± 7%) (Fig. 5 B). The frequency and extent of actin polymerization was also dramatically reduced (Fig. 5, C and D). These results indicate that ITAM phosphorylation is required for TCR-induced cytoskeletal polarization.

To corroborate this finding, we analyzed cytoskeletal rearrangements in the mutant Jurkat cell line JCaM1.6, which lacks functional Lck. Src family kinases and in particular Lck have been shown to be responsible for ITAM phosphorylation in T cells (Iwashima et al., 1994; van Oers et al., 1996). Consistent with the failure to phosphorylate ζ and CD3 chains, TCR-ligation of JCaM1.6 fails to induce tyrosine phosphorylation, calcium influx, or CD69 expression (Straus and Weiss, 1992). Analyzed in our conjugate formation assay, JCaM1.6 failed to polarize towards CD3-specific latex beads. MTOC positioning was random (30 ± 4% of scored conjugates) and no actin polymerization was detected (Fig. 7, A and B). Reconstitution of JCaM1.6 with wild-type murine Lck (JCaM1.6/Lck) restored TCR-induced cytoskeletal changes comparable with that observed in the parental Jurkat cell line (Fig. 7, A and B). Similar results were obtained with the mutant Jurkat cell line J45.01, which lacks functional CD45 (data not shown). CD45 is a transmembrane tyrosine phosphatase required for the activation of Src family kinases (Weiss and Littman, 1994). Taken together, these results support the view that ITAM phosphorylation and subsequent recruitment of downstream signaling molecules to the TCR are a prerequisite for cytoskeletal alterations.

Overexpression of a Dominant-Negative ZAP-70 Mutant Impairs MTOC Reorientation

A crucial step in TCR-mediated signaling is the recruitment of ZAP-70 to phosphorylated ITAMs after TCR engagement. Recruitment leads to tyrosine phosphorylation and activation of ZAP-70, which, in turn, contributes to the activation of both early and late signaling cascades. Several studies suggest that ZAP-70 plays in important role in TCR-mediated signaling. For example, lack of functional ZAP-70 in humans and mice results in severe developmental and functional defects in the T cell compartment (Arpaia et al., 1994; Chan et al., 1994; Elder et al., 1994). Furthermore, introduction of a dominant-negative mutant form of ZAP-70 into T cells suppresses TCR-mediated tyrosine phosphorylation of several signaling molecules and activation of NF-AT, a nuclear factor essential for inducible interleukin 2 gene expression (Qian et al., 1996). To assess the role of the ZAP-70–ITAM interaction in TCR-mediated MTOC reorientation, we transiently cotransfected Jurkat cells with the activating chimera TTε and the dominant-negative ZAP-70 mutant SH2 (N+C) (Qian et al., 1996). SH2 (N+C) is thought to inhibit TCR

Figure 6. CD8 chimera surface expression in stable Jurkat-derived clones. Stable clones were analyzed for chimera surface expression by FACS® analysis using an FITC-labeled CD8-specific antibody. The solid line represents untransfected Jurkat T cells.

Figure 7. MTOC reorientation and actin polymerization are abolished in the Lck-deficient cell line JCaM1.6. Jurkat T cells and the signaling mutant JCaM1.6 were bound to anti-CD3ε beads and scored as described in Fig. 2 for (A) MTOC reorientation and (B and C) actin polymerization. C shows actin polymerization in Jurkat T cells (a), the Lck-deficient mutant JCaM1.6 (b), and JCaM1.6/Lck reconstituted with murine Lck (c). Arrows point to cell-bound beads. Averages of seven (A) and three (B) experiments are shown. Bar, 5 μm.
signal by blocking phosphorylated ITAMs (Qian et al., 1996). Transfected cells were mixed with anti–Tac latex beads 36 h later, and conjugates were formed for 10 min at 37°C. Similar to our studies in stable clones, engagement of TT-ε-induced MTOC reorganization, although to a lesser extent (67 ± 1% of the conjugates). As expected, engagement of transiently expressed TT-ε failed to induce MTOC reorientation (20 ± 6%) and served as a negative control. Coexpression of dominant-negative ZAP-70 markedly reduced MTOC reorientation by ~50–60%, whereas expression of a mutant SH2 (N + C) form (SH2 (N + C)), (Qian et al., 1996), unable to bind to ITAMs, had only minimal effects (Fig. 8). Expression of both mutants did not affect TT-ε surface expression (data not shown). Moreover, comparable results were obtained in Jurkat TAg cells (data not shown). These results demonstrate that engagement of transiently expressed activating chimeras can induce MTOC reorientation. Moreover, a dominant-negative mutant of ZAP-70 encompassing the two SH2 domains impairs both TCR-mediated MTOC reorientation (shown here) and NF-AT gene activation (Qian et al., 1996). This suggests that both responses are mediated by the same proximal signaling events requiring molecules that interact with phosphorylated ITAMs.

**MTOC Reorientation Occurs in the Absence of Ras Activation**

Two major signaling pathways, namely the Ras and the PLC-γ1 pathway, are activated after TCR engagement. Activation of the small GTPase Ras by the TCR induces the extracellular signal-regulated kinase (ERK 1,2) cascade involving several cytoplasmic serine/threonine kinases. ERKs phosphorylate transcription factors of the AP-1 family and are necessary for TCR-mediated NF-AT gene activation and T cell development (Alberola-Ila et al., 1995; Izquierdo-Pastor et al., 1995; Swan et al., 1995). In certain cell types, ERKs are associated with microtubules (Morishima-Kawashima and Kosik, 1996; Reszka et al., 1995) and phosphorylate microtubule-associated proteins (MAPs). MAPs stimulate the polymerization of new and preexisting microtubules. Interestingly, these microtubule-promoting properties are lost after in vitro phosphorylation by ERKs (Hoshi et al., 1992). Thus, TCR-induced ERK activation could influence microtubule dynamics. To address this question, we took advantage of the recently described compound PD 098059, a selective inhibitor of the ERK-activating enzyme MAPK/ERK kinase (Dudley et al., 1995). To characterize the effect of PD 098059 on TCR-induced ERK activation, Jurkat T cells were preincubated for 30 min with increasing concentrations of PD 098059 and were stimulated for 10 min by the addition of C305-coated latex beads. Cells were then lysed and analyzed by tyrosine phosphorylation by immunoblotting. As shown in Fig. 9 A, incubation with PD 098059 resulted in a dose-dependent inhibition of ERK phosphorylation in response to TCR engagement, whereas the phosphorylation status of various other proteins remained unchanged. The identity and quantity of ERK detected was confirmed by stripping and reblotting the same membrane with a phospho-ERK–specific antibody (data not shown). This experiment demonstrates that PD 098059 indeed specifically inhibits ERK phosphorylation. However, the inhibitor had no significant effect on MTOC reorientation (Fig. 9 B), even at concentrations that substantially reduced ERK phosphorylation. This suggests that ERKs are not involved in the regulation of cytoskeletal events associated with MTOC reorientation.

**Discussion**

Upon binding to an APC, T cells undergo rapid and dra-
matic cytoskeletal changes that ensure the polarized delivery of an effector response. To date, T cell polarization has been mainly studied in a B cell–T cell system, where complex receptor–ligand interactions complicate the identification of the participating signaling pathways. In this report, we describe a simple bead-based conjugate formation assay to study this response. Jurkat T cells were chosen as model effector cells. Although this leukemic cell line may differ in many aspects from primary T cells, it has been a powerful tool in dissecting signaling pathways involved in T cell activation. Jurkat T cells can be easily manipulated by transient transfection approaches, and several TCR-signaling mutants have been generated and characterized. APCs have been replaced by antibody-coated latex beads. Since antibody-induced TCR cross-linking may induce stronger signals than TCR engagement by peptide–MHC complexes, our assay system may underscore the need for accessory molecules such as adhesion receptors or coreceptors. Furthermore, T cell activation with immobilized antibodies may not exactly reflect the role of receptor membrane motility and recycling in the generation of an intracellular signal. Nevertheless, the assay system should be useful to characterize the minimal requirements for polarized cytoskeletal rearrangements and to identify key-regulators of this mechanism. Our analysis provides evidence that TCR engagement alone is sufficient to induce MTOC reorientation and focal F-actin accumulation, suggesting that TCR-dependent signaling pathways can regulate cytoskeletal dynamics.

The induction of actin polymerization through antigen receptor activation has been previously observed in a variety of cell types including T cells, B cells, and macrophages (Greenberg et al., 1991; Melamed et al., 1991; Parsey and Lewis, 1993), but so far the underlying signaling pathways have been elusive. In contrast to cell activation by soluble factors, antigen-induced T cell activation is triggered by spatially constrained ligands; thus, TCR-induced cytoskeletal changes could influence the cellular response in several ways. First, cytoskeleton-dependent changes in cell shape and motility could increase the frequency of APC contacts, eventually resulting in the formation of stable conjugates (Negulescu et al., 1996). Second, actin polymerization could be required for the rapid reorganization and concentration of accessory molecules and receptors at the T cell–APC contact site, which would further increase conjugate stability and result in the formation of signaling

Figure 9. MTOC reorientation is mediated by Ras-independent pathways. (A) Jurkat T cells were incubated with the indicated concentrations of PD 098059 in DMSO for 30 min, followed by a 10-min stimulation with C305-coated latex beads (+). Untreated control cells were mixed with poly-I-lysine-coated beads (–). Cells were lysed and whole cell lysates were analyzed by immunoblotting with an antiphosphotyrosine antibody. (B) Jurkat T cells were incubated with the indicated concentrations of PD 098059 for 30 min. Subsequently, C305-coated latex beads were added at a 1:2 ratio and conjugates were incubated for 10 min. Conjugates were fixed and stained with antitubulin antibody. The average of four independent experiments is shown. (C) Tag Jurkat cells were cotransfected with TT-e and empty vector or N17Ras were analyzed for the presence of mutant Ras with a Ha-Ras-specific mAb.

Figure 10. PI-3 kinase is not required for TCR-mediated MTOC reorientation in Jurkat T cells. Jurkat T cells were incubated with the indicated concentrations of Wortmannin for 30 min. Subsequently, Leu4-coated latex beads were added at a 1:2 ratio and conjugates were incubated for 30 min. Conjugates were fixed and stained with antitubulin antibody. The average of four independent experiments is shown.
Furthermore, efficient signaling by peptide–MHC com-
gulates may need the contribution of adhesion molecules
ences or the different modes used to induce polarization.
Two systems could reflect either cell line–specific differ-
cations or the different modes used to induce polarization.

Since the PI-3 kinase inhibitor Wortmannin had no ef-
fect on extracellular calcium showed similar effects in our con-
jugate assay (Stowers et al., 1995). This result suggests that
ItAM phosphorylation is a key event in T cell activation.
Our analysis of ITAM–regulated T Cell Polarization
ITAM phosphorylation is a key event in T cell activa-
tion. Our analysis of ITAM–chain chimeras and Jurkat mutants
demonstrates that MTOC reorientation and actin poly-
merization require the presence of at least one intact
ITAM and a functional ITAM-phosphorylating Src-family
kinase. Similarly, Cox et al. (1996) showed recently that
clustering of the ITAM-containing Fc receptor γ subunit
can trigger submembranous actin assembly in the chicken
B cell line DT40. Actin polymerization required ITAM
phosphorylation and the presence of the PTK Syk. In T
cells, the Syk/ZAP-70 family member ZAP-70 can bind to
phosphorylated ITAMs of all TCR signaling subunits with
very high affinity (Osman et al., 1996). Recruitment of
ZAP-70 to the receptor results in ZAP-70 phosphoryla-
tion and activation. Overexpression of a dominant-nega-
tive mutant of ZAP-70, which blocks phosphorylated
ITAMs, markedly reduced MTOC reorientation in our
studies. This result suggests that ZAP-70 or another phos-
pho-ITAM-binding molecule may be involved in the regu-
lation of microtubule dynamics. In fact, both ZAP-70 and
Syk can associate and efficiently phosphorylate α-tubulin
in vitro (Huby et al., 1995; Peters et al., 1996). Further-
more, two recent reports provide evidence that T cell ac-
tivation leads to the rapid tyrosine phosphorylation of
α-tubulin (Cardine et al., 1995; Ley et al., 1994). Impor-
tantly, phosphorylated α-tubulin was not incorporated
into microtubules, but remained in the soluble, unpoly-
merized tubulin pool. Syk was found to phosphorylate a
site in the COOH terminus of α-tubulin, which coincides
with a region involved in MAP binding (Peters et al., 1996).
Since MAPs are involved in regulating the polymer-
ization status of tubulin, it is possible that TCR-induced
activation of ZAP-70 leads to the phosphorylation of
α-tubulin and the subsequent dissociation of MAPs. This
in turn could destabilize existing microtubules and lower
the pool of tubulin monomers available for polymeriza-
tion. Such changes in microtubule dynamics would occur
locally at the site of TCR engagement and might result in
the repositioning of the MTOC. Although the model de-
scribed is very attractive, the possibility remains that dom-
inant-negative ZAP-70 impairs MTOC reorientation by
inhibiting the binding of other SH2-containing effector
molecules to ITAMs. Taking advantage of membrane-target-
ged ZAP-70/Syk chimeras, future experiments will as-

Protocols involving in the regulation of microtubule dy-
namics are often good substrates for ERKs and protein ki-
nase C (PKC) (Gelfand, 1991). Members of both kinase
families are known to be activated upon T cell activation.
Thus, apart from the aforementioned ZAP-70-mediated
mechanism, changes in the microtubule cytoskeleton could
be regulated by one of these pathways. Our experiments
show that MTÖC reorientation can occur in the absence
of Ras activation. Neither overexpression of dominant-
negative Ras, nor inhibition of MAPK/ERK significantly
affected MTOC reorientation. These results suggest that
Ras-independent pathways regulate microtubule rearran-
gements in response to TCR engagement. In earlier
studies, MTOC reorientation was shown to be dependent
on extracellular calcium (Kupfer et al., 1987). Incubation
of B cell–T cell couples in the presence of EGTA reduced
MTOC reorientation from >90% to 55 ± 5%. Chelation
of extracellular calcium showed similar effects in our con-
jaguate formation assay (data not shown). MTOC reorientation was significantly reduced, but not abolished (from 78% to 50 ± 8%). This suggests that MTOC reorientation may in part be regulated by a calcium-dependent pathway. Indeed, repositioning of the MTOC could be a complex event involving the shortening of microtubules and the subsequent anchoring of the MTOC at the site of TCR engagement. Thus, impairment of one of these events could result in partial MTOC reorientation. A more simple explanation relates to the fact that intracellular calcium stores are not depleted under our assay conditions. Thus, release of intracellular calcium may be sufficient to partially activate calcium-dependent effector molecules. Interestingly, Ca^2+/calmodulin-dependent kinase family members phosphorylate MAPs (Gelfand, 1991). Thus, several kinase families might participate in the regulation of MTOC reorientation.

The assay system described here should prove useful to further characterize the effector molecules involved in T cell cytoskeleton dynamics. Once identified, it will be interesting to assess the physiological role of these effectors in maintaining a polarized intercellular immune response.

The authors thank members of the Weiss laboratory for helpful discussions, and in particular Drs. D. Yablonski and N.S.C. van Oers for critical reading of the manuscript. We gratefully acknowledge the technical assistance of T. Laroche and the laser scanning microscope.

This work was supported in part by grants from the Boehringer Ingelheim Fonds (B. Lowin-Kropp) and the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation (DYG-1356 to V. Smith Shapiro).

Received for publication 16 December 1997 and in revised form 29 December 1997.

References


