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J Immunol 2012; 188:5421-5427; Prepublished online 23 April 2012;
doi: 10.4049/jimmunol.1200242
http://www.jimmunol.org/content/188/11/5421

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Stathmin Regulates Microtubule Dynamics and Microtubule Organizing Center Polarization in Activated T Cells

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Polarization of T cells involves reorientation of the microtubule organizing center (MTOC). Because activated ERK is localized at the immunological synapse, we investigated its role by showing that ERK activation is important for MTOC polarization. Suspecting that ERK phosphorylates a regulator of microtubules, we next focused on stathmin, a known ERK substrate. Our work indicates that during T cell activation, ERK is recruited to the synapse, allowing it to phosphorylate stathmin molecules near the immunological synapse. Supporting an important role of stathmin phosphorylation in T cell activation, we showed that T cell activation results in increased microtubule growth rate dependent on the presence of stathmin. The significance of this finding was demonstrated by results showing that CTLs from stathmin−/− mice displayed defective MTOC polarization and defective target cell cytolysis. These data implicate stathmin as a regulator of the microtubule network during T cell activation. The Journal of Immunology, 2012, 188: 5421–5427.

Interestingly, one of the most important effectors of DAG is Ras-GRP, the GTP exchange factor that functions to activate Ras and subsequently the ERK-MAPK pathway (13). A number of studies have shown that active ERK accumulates at the immune synapse (14, 15) and ERK activation is also thought to be important for MTOC polarization in T cells (16–18). Consistent with an important role for ERK in T cell polarization, cytolytic activity mediated by CTLs and NK cells is inhibited with ERK inhibition (16, 17). Additionally, NK cells lacking the ERK-MAPK scaffold KSR1, which is required for the localization of ERK to the immune synapse, also fail to polarize their granules and kill target cells poorly (14).

In this study, we investigated the potential role of ERK in MTOC reorientation. After confirming that ERK is required for MTOC polarization, we hypothesized that a specific substrate of ERK might be a regulator of the microtubule cytoskeleton. Because it is a known ERK substrate (19–23), we focused on the microtubule binding protein stathmin (OP18) as a possible link between ERK and the microtubule cytoskeleton. The stathmin family of proteins is highly conserved and functions by binding to free tubulin heterodimers in the cytoplasm and thereby regulates the concentration of free tubulin (24). Phosphorylation of stathmin by a number of serine-threonine kinases, including ERK, results in release of bound tubulin heterodimers and enhanced polymerization of the microtubule network.

Although stathmin was originally characterized as an oncoprotein overexpressed in T leukemia cells (25), little is known about its function in developing and mature T cells (26). Previous studies verify that it becomes phosphorylated after TCR stimulation, but the biological outcome in T cell activation is not known (27–29). Analysis of stathmin-deficient mice showed a reduction in thymocyte cellularity and peripheral T cell numbers, but additional immune cell analyses were not reported (30).

We found that stathmin is rapidly phosphorylated downstream of the TCR and that phosphorylated stathmin is localized to the immune synapse. Consistent with the importance of ERK localization at the synapse, T cells lacking the MAPK scaffold KSR1 showed defects in stathmin localization. This was important for MTOC polarization, as we found that microtubule growth rates were slowed in the absence of stathmin, resulting in a delay of MTOC reorientation.
reorientation and defects in CTL cytolysis. To our knowledge, these data are the first to implicate stathmin in the regulation of microtubule dynamics in activated T cells.

**Materials and Methods**

**Mice**

Stathmin knockout mice on a C57Bl6/J background have been previously described (30) and were a gift from Dr. Gleb Shumyatsky (Rutgers University). Stathmin+/− mice were crossed with OT-1 TCR (31) and AND TCR (32) transgenic mice. Age- and gender-matched stathmin wild-type or heterozygous littermate controls were used for all experiments. KSR1 knockout mice on a C57Bl6/J background were previously described (33) and were crossed to AND TCR transgenic mice. All mice were housed under specific pathogen-free conditions in the Washington University animal facilities in accordance with institutional guidelines.

**Cell culture and Abs**

EL-4, RMA-S, CH27, and Jurkat cells were maintained in RPMI 1640 supplemented with 10% FBS and 2 mM glutamine. T cells isolated from OT-1 or AND TCR transgenic mice T cells were cultured in IMDM supplemented with 10% FBS, 2 mM glutamine, nonessential amino acids, sodium bicarbonate, penicillin, and streptomycin. TCR stimulation was performed with anti-mouse CD3 (2C11) and anti-mouse CD28 (37.51) or anti-human CD3 (OKT3). Anti-stathmin (O01383), anti-α-tubulin (FITC conjugated), and anti-p-ERK Abs were from Sigma-Aldrich. Anti-stathmin p-S24 Ab (ab47398) was from Abcam. ERK2 and PKCθ Abs were from Santa Cruz Biotechnology. PLCγ1 and p-PLCγ1 Abs were from Cell Signaling Technology. Anti-mouse CD107a-PE (1D4B) and anti-mouse CD68-FITC (53-6.7) were from BD Biosciences. All secondary Abs were made in mouse. Anti-stathmin inhibitors (e.g., UO126, Rp-cAMP, and cytochalasin D) were allowed for 1 h before stimulation, then plated onto anti-CD3–coated coverslips to induce MTOC polarization. Cells were also treated with UO126 for 1 h and then plated onto anti-CD3–coated coverslips with 1 μM calcium release very slow. Lysis was then performed and plated in 96-well U-bottom plates (10,000 cells/well) with CTLs at varying E:T cell ratios and cultured for 4–6 h at 37˚C. Supernatants were then collected and read on a MicroBeta counter (PerkinElmer). Specific lysis was calculated as follows: [(average sample cpm − average spontaneous cpm)/(average maximum cpm − average spontaneous cpm)] × 100.

**Degranulation assay**

Whole spleens from stathmin wild-type (littermate controls) or knockout OT-1 TCR transgenic mice were stimulated with the addition of 1 μM SIN1FEK peptide for 5 d to generate CTLs. A standard [3H]release assay was performed. Briefly, [3H]-labeled target cells pulsed with SIN1FEK were plated in 96-well U-bottom plates (10,000 cells/well) with CTLs at varying E:T cell ratios and cultured for 4–6 h at 37˚C. Supernatants were then collected and read on a FACSCaliber (BD Biosciences).

**Microtubule growth rate measurements**

Whole spleens from stathmin wild-type (littermate controls) or knockout mice were activated with anti-CD3 for 18–20 h and transformed with retrovirus expressing EB3-GFP. At day 4 following transformation, T cells were purified and plated on glass-bottom dishes coated with anti-CD3 or CD45 Abs. Live images were taken using the Olympus FV1000 confocal microscope after cells were settled for 15–45 min on the dishes. Images of individual cells were obtained every 2 s for 3–5 min. Microtubule growth rate measurements were generated using the Ttrack plugin for ImageJ. Statistical analysis was obtained from >50 microtubule tracks from >10 cells each from three separate experiments.

**Results**

**Inhibition of ERK decreases efficiency of MTOC polarization**

ERK was previously shown to be important for cytolysis by CD8 T cells (16), suggesting that it may play a role in T cell polarity. To address this more directly, we tested whether the MTOC can polarize in activated T cells when ERK is inhibited by measuring MTOC polarization in the presence or absence of the MEK inhibitor U0126. Because MEK activation is required for ERK activation, MEK inhibition blocks ERK activation. Mouse T cells were treated with U0126 for 1 h and then plated onto anti-CD3–coated coverslips to induce MTOC polarization. Cells were also treated with inhibitors to block actin polymerization (cytochalasin d) and cAMP (Rp-cAMP) as positive and negative controls, respectively. After staining with anti-tubulin to visualize the MTOC, the percentage of cells whose MTOC was polarized was measured by confocal microscopy. Cells were scored as polarized when the MTOC was clearly visible in the center of the cell at a focal plane adjacent to the glass coverslip.
We noted a 40–50% decrease in MTOC polarization in the cells treated with MEK inhibitor (Fig. 1B), confirming that ERK activity is important for MTOC polarization in T cells.

**Stathmin is rapidly phosphorylated downstream of the TCR in an ERK-dependent manner**

Recently, it was shown that ERK is recruited to the immunological synapse (14, 15). This suggests that the polarized recruitment of ERK may function to phosphorylate proteins at the synapse that may be important for MTOC polarization. We focused on stathmin because it is a well-known ERK substrate and functions to modulate microtubule dynamics. Therefore, we examined the relationship between ERK and stathmin in the regulation of microtubule dynamics in activated T cells.

We first wanted to confirm that stathmin is phosphorylated by ERK following TCR engagement. To test this, Jurkat T cells were treated with PMA (Fig. 2A) or anti-CD3 (Fig. 2B) for various times. Immunoblotting of cell lysates with an Ab that recognizes phosphorylated stathmin showed that stathmin is phosphorylated within 15 min of stimulation and remains phosphorylated for 2 h (Fig. 2A, 2B). This was ERK-specific, as treatment with the MEK inhibitor UO126 largely abrogated stathmin phosphorylation (Fig. 2A, 2B). This finding was confirmed in primary T cells stimulated with peptide-loaded APCs (Fig. 2C and data not shown). Taken together, these data demonstrate that stathmin is phosphorylated in an ERK-dependent manner downstream of the TCR.

**Phosphorylated stathmin is enriched at the immune synapse of primary CTLs**

To determine whether phosphorylated stathmin localizes to the immunological synapse, Abs to p-stathmin were used to stain TCR transgenic primary T cells conjugated with peptide-coated APCs. This demonstrated a clear accumulation of p-stathmin at the immune synapse in ~72% of conjugates (Figs. 2C, 3). Interestingly, no specific enrichment was seen using an Ab that recognizes all stathmin molecules (Fig. 2D). This suggests that the recruitment of ERK to the synapse allows for local phosphorylation of stathmin molecules. Because stathmin is a cytoplasmic molecule, phosphorylation is likely to be dynamic, resulting in the continuous release of microtubule subunits (34). This suggests that polarized stathmin phosphorylation might be important in the regulation of microtubule dynamics at the immune synapse during T cell activation.

**Synapse localization of phosphorylated stathmin is disrupted in KSR1−/− T cells**

We previously showed that the scaffold molecule KSR1 facilitates the localization of p-ERK at the immunological synapse (14). We could therefore use KSR1-deficient cells to confirm whether the localization of p-ERK at the immunological synapse was involved in polarized stathmin phosphorylation. Cell conjugates generated using wild-type or knockout KSR1 TCR transgenic T cells and peptide-pulsed APCs were stained with p-stathmin Abs. As expected, p-stathmin was localized at the immune synapse of wild-type cells, but p-stathmin polarization was significantly decreased.
in KSR1-deficient T cells (Fig. 3). This supports the idea that KSR1-dependent recruitment of p-ERK to the immune synapse allows for polarized phosphorylation of stathmin at the immune synapse.

To confirm that stathmin was involved in MTOC polarization, we assessed MTOC polarization in T cells from wild-type and stathmin-deficient mice to anti-CD3–coated coverslips. We observed an ~50% decrease in MTOC polarization in stathmin knockout T cells compared with wild-type T cells at multiple time points (Fig. 4A). To extend these findings to a more physiologic assay, we assessed MTOC polarization in APC conjugates with wild-type or stathmin knockout T cells (Fig. 4B). Consistent with our data from anti-CD3–coated coverslips, we observed a significant decrease in MTOC polarization after 5 min in stathmin knockout T cells compared with wild-type T cells. These data support a role for stathmin in regulating MTOC reorientation during T cell activation.

To determine whether the defect in MTOC polarization in stathmin-deficient T cells was physiologically significant, we performed a number of assays of T cell function. Because MTOC polarization is required for efficient cytolysis by CD8 T cells, we first tested whether stathmin knockout CTLs were defective in cytolysis. Indeed, CTLs generated from stathmin-deficient animals demonstrated a defect in specific lysis of peptide-loaded targets using a conventional [51Cr]-release assay (Fig. 4C). To determine whether stathmin knockout T cells were defective in degranulation, we also measured surface LAMP-1 staining during a 45-min incubation with target cells (Fig. 4D, 4E). We found that the magnitude of degranulation as measured by LAMP-1 staining was lower in stathmin-deficient cells compared with wild-type cells.

**FIGURE 3.** KSR1 is required for immune synapse localization of phosphorylated stathmin. (A) CD4+ T cells from KSR1 wild-type or knockout AND TCR mice were stimulated with CH27 B cells loaded with 1 μg moth cytochrome c peptide for 5 d until rested. T cells were purified and conjugated with CH27 cells loaded with peptide. Conjugates were stained with p-S24 stathmin Ab and analyzed by confocal microscopy. (B) Graph represents three experiments of >20 conjugates each. Scale bar, 10 μm. *p < 0.001 (two-tailed unpaired t test).

**FIGURE 4.** Stathmin−/− T cells have impaired MTOC polarization and are defective in cytolysis. (A) Stathmin knockout or littermate control T cells were purified, rested for 30 min, and plated on anti-CD3–coated glass coverslips for the indicated times. Cells were fixed, permeabilized, and stained with anti-αβ-tubulin and imaged using confocal microscopy. Data are represented as means ± SEM and >50 cells per condition were scored in three experiments. All knockout values are significantly different from wild-type. (B) Rested T cells from stathmin knockout OT-1 or littermate OT-1 controls were conjugated with peptide-pulsed CFSE-labeled RMA-S cells onto glass coverslips for 5 min at room temperature. The cells were then fixed, permeabilized, stained with Hoechst and anti-αβ-tubulin, and analyzed using confocal microscopy. Data are represented as means ± SEM and >50 cells per condition were scored in three experiments. Images represent cells scored as polarized or not polarized. Scale bars, 10 μm. (C) Cytolysis by stathmin knockout or littermate control CTLs was assayed using a standard [51Cr]-release assay. Cells were plated at indicated E:T ratios and incubated for 4–6 h. Supernatants were counted on a MicroBeta counter and specific lysis was calculated. Differences in specific lysis between stathmin wild-type and knockout CTLs are significant at all E:T ratios. Graph is representative of two experiments. (D and E) Degranulation was assayed by LAMP-1 staining during 45 min of OT-1+ stathmin knockout or control T cell incubation with peptide-loaded RMA-S cells. Graphs show means ± SEM and are representative of four experiments. *p < 0.001 (unpaired t test).
(Fig. 4E). The basal level of LAMP-1 staining was, however, higher in stathmin-deficient cells, suggesting constitutive non-specific degradation in these cells (Fig. 4D). These data support the idea that stathmin is involved in MTOC polarization and cytolysis by CD8+ T cells. Surprisingly, stathmin knockout T cells performed normally in other assays of T cell function, including proliferation and migration (Fig. 5A, 5B). Stathmin knockout T cells also produced similar levels of IFN-γ compared with wild-type T cells (Fig. 5C).

**T cells from stathmin−/− mice have impaired PKC0 polarization and microtubule dynamics**

Our data suggest that ERK phosphorylates stathmin at the immune synapse following T cell activation and that this leads to the dynamic restructuring of the microtubule network facilitating MTOC polarization. It is possible, however, that defects in TCR proximal signaling, rather than defects in microtubule dynamics, contribute to the MTOC polarization defect we observed in stathmin-deficient cells. To rule out this possibility, we first tested whether conjugate formation was affected in the absence of stathmin. No difference between wild-type and stathmin-deficient T cells was seen (data not shown). Additionally, activation of ERK and PLCγ was normal in stathmin-deficient T cells (Fig. 6A). Lastly, stathmin-deficient T cells also fluxed calcium at wild-type levels (Fig. 6B). This suggests that proximal TCR signaling is intact in stathmin knockout T cells.

Because PKC0 polarization was previously shown to precede MTOC polarization in T cells (12), we also assessed PKC0 polarization in stathmin knockout T cells. T cell conjugates were generated and stained using Abs to PKC0. We found that there was a defect in PKC0 polarization in stathmin-deficient T cells as compared with wild-type cells (Fig. 6C). Although PLCγ is known to control PKC0 recruitment to the immunological synapse (12), we did not detect defects in PLCγ activation in stathmin-deficient T cells (Fig. 6A). Therefore, it seems unlikely that a defect in DAG production is the cause of the PKC0 polarization defect, although we did not directly measure DAG accumulation at the synapse and therefore cannot completely rule out this possibility. Our data suggest, however, that PKC0 polarization may depend on an intact microtubule network. Consistent with this, we found that nocodazole-mediated disruption of microtubules in T cells blocked PKC0 polarization (data not shown).

Because defects in early TCR signaling are probably not responsible for the MTOC polarization defects in stathmin-deficient T cells, we directly tested whether stathmin plays a role in microtubule dynamics by measuring such dynamics in T cells stimulated with anti-CD3 or a control Ab (CD45). To label microtubules, we transduced cells with a GFP fusion protein of EB3, a protein that binds to the ends of growing microtubules (35). Using real-time confocal microscopy, we found that T cell activation strongly enhanced microtubule growth rate with microtubules in activated cells moving at a growth rate of ~50% greater than cells plated on the control Ab (Fig. 6D). This enhancement appeared to require stathmin, as the microtubule growth rate in stathmin-deficient T cells was much slower compared with wild-type T cells (Fig. 6D). These data support a role for phosphorylated stathmin in regulating microtubule growth in activated T cells, MTOC polarization, and cytolysis by CD8+ T cells.

**Discussion**

In this study, we showed that the localization of ERK to the immunological synapse plays a role in T cell polarization. By using T cells deficient in KSR1, a scaffolding molecule required for ERK localization at the synapse, we were able to correlate the loss of polarized ERK localization with defective polarized stathmin phosphorylation. This supports the idea that the retention of activated ERK at the synapse allows it to phosphorylate specific substrates at the synapse in a polarized fashion. We suspect that the localized phosphorylation of ERK substrates may play a role in T cell polarization.

We began our studies by trying to identify a specific ERK substrate at the immunological synapse. Given the critical role of MTOC polarization in T cell polarity, we searched for an ERK substrate that might be a regulator of the microtubule cytoskeleton. This led us to examine stathmin, a known ERK substrate and microtubule regulator (19, 21, 22, 24). Stathmin functions by binding to tubulin αβ heterodimers, sequestering them, and preventing them from being assembled into microtubules (36). Because microtubule assembly is critically dependent on the concentration of free tubulin heterodimers (37), the localized release of tubulin is likely to play an important role in localized assembly and growth of microtubules. Consistent with this hypothesis, we showed that microtubule growth was strongly enhanced by T cell activation in a mechanism that was ERK- and stathmin-dependent. To our knowledge, our studies are the first to show that T cell activation enhances the growth rate of microtubule movement. It seems reasonable to speculate that an increased rate of microtubule growth at the immunological synapse may play a role in repositioning of the MTOC.
Previously, it was shown that mice lacking stathmin demonstrate decreased thymocyte and peripheral cell numbers, suggesting an important role for stathmin in T lymphocyte function; however, no other T lymphocyte abnormality was reported (30). In this study, we found that T cells lacking stathmin showed decreased PKC\(\alpha\) polarization, delayed MTOC polarization, decreased microtubule growth rates, and moderate defects in CTL killing, as well as decreased Ag-induced degranulation due to increased basal degranulation. Surprisingly, we did not detect any other defects in T cell function, including proliferation, migration, conjugate formation (data not shown), immune synapse formation (data not shown), and cytokine release. Although this might suggest that there are compensatory pathways activated in the absence of stathmin, we were unable to detect mRNA expression of other stathmin family proteins (RB3, SCLIP, and SGC10) in either wild-type or knockout mice (data not shown), suggesting that there is not a compensatory increase in expression of other stathmin family members.

Given the importance of MTOC reorientation in directing T cell polarity and polarized intracellular trafficking and secretion (4, 7), it is surprising that the role of microtubules during T cell activation is relatively unexplored. This may be due to previous studies that showed that TCR signaling, proliferation, and cytokysis are largely unaffected by treatment with drugs that inhibit microtubules (38–40). Given that these inhibitors profoundly inhibit T cell polarization, our results suggest that role of microtubules in T cell activation be re-examined in more physiological situations.

We found, for example, that nocodazole treatment blocked MTOC polarization and also inhibited PLC\(\gamma\) (data not shown). This could also explain some of the inconsistencies in the literature regarding the role of microtubules (41, 42). Nevertheless, our results suggest that microtubule regulation is an important aspect of T cell activation.

Our work also implicates the ERK-MAPK pathway as playing an important role in regulating cell polarity. One function of the immunological synapse may be to help the assembly of a signaling apparatus that directs the reorientation of the cell. It is interesting to speculate that the effects of DAG on cell polarization (10) may be related to the ability of DAG to activate the Ras-MAPK signaling pathway via the activation of Ras-GRP (13). Because DAG is constrained to the plasma membrane and can be quickly inactivated by lipid kinases (43), Ras activation occurs in a localized fashion in the immunological synapse. Previously, we showed that the recruitment of the scaffold KSR1 to the immunological synapse functions to tether and hold activated ERK at the synapse (14). In the absence of KSR1, we speculated that the effects of DAG on cell polarization (10) may be related to the ability of DAG to activate the Ras-MAPK signaling pathway via the activation of Ras-GRP (13). Because DAG is constrained to the plasma membrane and can be quickly inactivated by lipid kinases (43), Ras activation occurs in a localized fashion in the immunological synapse. Previously, we showed that the recruitment of the scaffold KSR1 to the immunological synapse functions to tether and hold activated ERK at the synapse (14). In the absence of KSR1, we speculated that activated ERK would diffuse away from the synapse and into the cytoplasm. Our work suggests that holding ERK at the synapse may function to allow it to phosphorylate specific substrates involved in cell polarization, such as stathmin. Given that ERK can phosphorylate a wide variety of different proteins, it seems likely that stathmin might not be the only protein phosphorylated by ERK involved in cell polarity. The challenge for the immediate future will be to identify these other substrates.
Acknowledgments

We thank Dr. U.K. Schubart and Dr. G.P. Shumyatsky for providing stathmin 1−/− mice.

Disclosures

The authors have no financial conflicts of interest.

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