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Specific Patterns of Cdc42 Activity Are Related to Distinct Elements of T Cell Polarization

Irina Tskvitaria-Fuller,* Abhinav Seth,† Neeta Mistry,‡ Hua Gu,§ Michael K. Rosen,†§ and Christoph Wülfling‡*‡¶

T cell polarization toward and within the cellular interface with an APC is critical for effective T cell activation. The Rho family GTPase Cdc42 is a central regulator of cellular polarization. Using live-cell imaging, we characterized the spatiotemporal patterns of Cdc42 activity and their physiological regulation. Using three independent means of experimental manipulation of Cdc42 activity, we established that Cdc42 is a critical regulator of T cell actin dynamics, TCR clustering, and cell cycle entry. Using quantification of three-dimensional data, we could relate distinct spatiotemporal patterns of Cdc42 activity to specific elements of T cell activation. This result suggests that Cdc42 activity in specific locations at specific times is most critical for its function in T cell activation. The Journal of Immunology, 2006, 177: 1708–1720.

Materials and Methods

Cells and reagents

In vitro-primed primary T cells from SC.C7 (13) and Cbl-b-deficient SC.C7 (14) TCR transgenic mice were used in all experiments and were generated as described previously (15). The use of these mice has been reviewed and approved by the University of Texas (UT) Southwestern Institutional Animal Care and Use Committee. As APCs, CH27 and I-Ek-GFP-transfected A20 B cell lymphoma cells (15) were used. Agonist peptide concentrations were adjusted by dilution into the null peptide moth cytochrome c (MCC) D93E/K99F/T102S (15). Costimulation blockade with Abs against ICAM-1 or B7-1/B7-2 was as described previously (16).

Imaging

The live-cell Cdc42 sensor consists of aas 230–289 of WASP, directly followed by enhanced GFP, and the last 14 aas of Cdc42 containing the CAAX box. A control sensor with a 100-fold reduced affinity for active Cdc42 contains three mutations, F244D, H246D, and H249D. I-Ek-GFP (15), actin-GFP, (16) and tubulin-GFP (16) were used as described. The Cdc42 sensor, actin-GFP, and tubulin-GFP were introduced into primary T cells by retroviral transduction using a Moloney murine leukemia virus-derived system as described (16). The microscopy system and image acquisition have been described in detail (16). Briefly, primary T cells and peptide-activated APCs were allowed to interact at 37°C on the microscope stage. Every 20 s, a differential interference contrast (DIC) bright field image and 21 GFP images spaced 1 μm in z covering the entire cell were acquired. Staining with WASP GTPase-binding domain (GBD) was

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performed according to an established protocol (8), and images were acquired on a Delovan deconvolution microscope with a ×60 magnification.

**Image analysis**

For all image analysis, we generated three-dimensional reconstructions of productive cell couples. Time points were 1 s, 3 s, 5 s, and 7 s after cell couple formation for TCR/MHC accumulation and every 20 s between 20 min before and 100 min after cell couple formation plus ≥5 s after cell couple formation for actin, MTOC, and Cdc42 sensor analysis. Time 0 was set to the first time point with a maximally spread T cell/APC interface. This result in a more reliable synchronization of single-cell data than using the often-ambiguous first membrane contact between T cell and APC that occurred 20–40 s before the maximal spreading of the interface. Analysis of MHC/TCR accumulation, early actin spreading, and MTOC reorientation was as percentage of cell couples fulfilling precisely defined fluorescence intensity and geometry criteria, as established (15, 16).

Cdc42 sensor accumulation and sustained actin accumulation were analyzed with accumulation and centrality indices, as described in Results. Only cells with Cdc42 sensor fluorescence intensity at the T cell/APC interface of ≥10% above the respective cell and fluorescence background were analyzed. In these cells, the percentage of total fluorescence translocated to the area of accumulation was determined in background-corrected top down projections of the three-dimensional data. We measured the total integrated fluorescence at the area of accumulation, subtracted the area of accumulation times the average cellular fluorescence intensity (to account for the amount of fluorescence that would have been in the area of accumulation in a hypothetical cell without a Cdc42 enrichment), and we expressed the difference as fraction of the total integrated fluorescence of the T cell. To determine the area of accumulation as a percentage of the interface area, measurements were taken in DIC and three-dimensional fluorescence images. Unambiguous determination of the interface area is not possible in either image. As the best approximation, we measured the interface diameter in the DIC images and the diameter of the area of accumulation in the three-dimensional fluorescence images. Next, we assumed comparable geometry in both dimensions of the interface, as verified for the area of accumulation in the fluorescence images and seen for the entire interface in experiments with supported lipid bilayers as APC substitutes. We could then calculate the size of the area of accumulation relative to the interface area as the ratio of the square of the diameter of the area of accumulation to the square of the interface diameter. Comparing the accumulation and centrality indices to more traditional means of analysis, percentage of cell couples with accumulation in any pattern and percentage of cell couples with accumulation in a central pattern, respectively, we found that they were closely related (data not shown). Comparing the more traditional quantification to the indices-based one in the correlation analysis (see of Fig. 10), the major conclusions re-emerged. However, in contrast to the indices-based quantification, in the analysis based on percentage of cell couples with a particular phenotype, stronger relations between patterns of Cdc42 accumulation and elements of T cell polarization, as expressed for example by the expression of the regeneration index, were not always the ones from the other spatiotemporal patterns of Cdc42 accumulation analysis.

**Correlation analyses**

Correlation analyses were performed as described in Results. To be able to compare slopes of the regression curves of all four patterns of Cdc42 accumulation with one particular element of T cell activation, we multiplied each slope with the difference between the largest and smallest value of the corresponding quantitative measure of Cdc42 accumulation. The resulting numbers express how much the particular elements of T cell polarization change over the total observed range of changes of the Cdc42 pattern. The largest such product for an element of T cell activation was then set to one, and the ones from the other spatiotemporal patterns of Cdc42 activity were expressed as a fraction of this maximum.

The significance of differences between experimental conditions in analyses based on percentage of cell couples with a particular phenotype was calculated using a two-sample proportion z test. In analyses based on accumulation and centrality indices, single-cell (fractions of sensor translocation to the interface) and single-cell (fractions of sensor translocation to the interface) (area of accumulation as fraction of the interface area) were multiplied with the (fraction of cell couples above threshold) for each corresponding experimental condition, and the significant difference of such single-cell data for different experimental conditions was assessed using a Student’s t test.

**Results**

**Active Cdc42 accumulates at the T cell/APC interface.** To determine when and where Cdc42 is active during T cell activation, we designed a live-cell Cdc42 sensor, The GBD of WASP binds to GTP-bound Cdc42 with reasonable affinity ($K_{\text{d}} = 100$ nM) and high selectivity (19–21). To allow live cell detection of active Cdc42, we fused the GBD to GFP. To selectively monitor the vicinity of Cdc42, we added the Cdc42 CAAX box, which mediates prenylation. We then expressed the Cdc42 sensor in vitro-primed, primary T cells by retroviral transduction. As a T cell model, we used 5C.C7 T cells with CH27 B cell lymphoma APCs in the presence of 10 μM MCC, a “full stimulus.” Concomitant with interface formation, active Cdc42 accumulated at the T cell/APC interface (Fig. 1A) in 98% of the cell couples (n = 101). Enhanced accumulation at the center of the interface was observed in 65% of the cell couples. Interface accumulation of active Cdc42 dissipated over time. Only 25% of the cell couples showed accumulation of active Cdc42 5–15 min after interface formation in any pattern, only 3% at the center of the interface.

To quantify the amount and localization of Cdc42, we developed two new measures. To quantify the amount of active Cdc42 at the T cell/APC interface, we defined an “accumulation index.” For each cell couple, we first determined whether the fluorescence...
shown. In the top row of the Cdc42 sensor transduction with the Cdc42 sensor, and the presence of blocking B7 Abs (as a control of IL-2 production was quantified by intracellular cytokine staining. Consequently, CH27 APCs and 10^5 H9262 of a Cdc42 sensor-transduced 5C.C7 T cells with a CH27 B cell lymphoma APC in the presence of 10^5 H9262 translocated to the interface was determined from our three-dimension imaging data (Materials and Methods). The accumulation index was then defined as follows: accumulation index = fraction of cell couples above threshold × average fraction of sensor translocation interface. To measure how disperse Cdc42 activation was, we defined a centrality index. Again, cell couples were thresholded first. For cell couples above the threshold, we determined the area of accumulation as a percentage of the entire interface area (Materials and Methods). The only two patterns of Cdc42 sensor accumulation at the T cell/APC interface frequently observed were accumulation across the entire interface or accumulation at its center. A smaller area of accumulation thus represents more central accumulation. In addition, we took into account that more translocation of the Cdc42 sensor to a given area of accumulation constitutes a more intense accumulation. The centrality index was then defined as follows: centrality index = fraction of cell couples above threshold × average fraction of sensor translocation to the interface/area of accumulation as fraction of the interface area. These two indices generated similar but superior quantitative data compared with the widely used method to assess fluorescence accumulation, percentage of cell couples with accumulation above threshold in a particular pattern, as discussed in Materials and Methods. In the presence of a full T cell stimulus (CH27 APC with 10 μM MCC peptide), both indices reach maximal values within 20 min after the formation of a tight T cell/APC interface (Figs. 2 and 3). They slowly declined thereafter, the centrality index more rapidly than the accumulation index (Figs. 1 and 3). These data suggest that localized Cdc42 activation peaks during the initial establishment of a T cell/APC couple and that Cdc42 activity at the center of the T cell/APC interface is more prevalent during this peak of Cdc42 activation.

To establish specificity of the Cdc42 sensor, we generated a control sensor with 100-fold reduced affinity for Cdc42/GTP (A. Seth and M. K. Rosen, unpublished data) by making three-point mutations in the WASP GBD, F244D, H246D, and H249D. In T cells before APC contact, the vast majority of the control sensor was localized in the vicinity of the MTOC (Fig. 2A), as is the majority of endogenous Cdc42 (8). This contrasts with the predominant plasma membrane localization of the wild-type sensor. This disparate localization of the wild-type and control sensors before T cell activation suggests that wild-type sensor localization is dominated by binding of its GBD to a constitutive pool of active Cdc42 at the cell membrane. In response to a full stimulus (CH27 APCs with 10 μM MCC peptide), the control sensor accumulated at the T cell/APC interface only slightly above cellular background (Fig. 2A). The accumulation and centrality indices at individual time points analyzed (Fig. 2B) were <95 and 22% of those of the wild-type sensor (p < 0.001), respectively. In no case was control sensor interface accumulation more intense than the internal accumulation in the vicinity of the MTOC. Such minimal control sensor accumulation is consistent with the residual affinity of the mutated WASP GBD for active Cdc42. The distinct behaviors of the wild-type and control sensors before and after T cell/APC couple formation thus indicate that the former reports specifically on active Cdc42. As a further control, we used a purified GBD-GFP fusion protein to stain fixed cell couples. Active Cdc42 accumulated in the same patterns as seen with the live-cell biosensor (Fig. 1B), confirming our live cell data and previous data (8, 25). Furthermore, we tested whether the Cdc42 sensor interfered with T cell activation at the expression level achieved by retroviral transduction. As assayed by IL-2 secretion, it did not (Fig. 1C). In summary, we designed a live-cell imaging sensor for Cdc42 that specifically reports on active Cdc42 without interfering with T cell activation upon its retroviral expression in primary T cells. We found that active Cdc42 accumulates at the T cell/APC interface.
preferentially immediately after its formation and preferentially at its center.

**Experimental strategy**

Our working hypothesis for the following investigation of the role of Cdc42 in T cell activation is that specific spatial and temporal patterns of Cdc42 activity control distinct elements of T cell activation. As an ideal test for our hypothesis, we would block one specific spatial pattern of Cdc42 activity at a specific time while leaving all other spatiotemporal patterns undisturbed and assess the consequences for T cell activation. For example, one might desire to ablate Cdc42 activity at the center of the T cell/APC interface immediately after cell couple formation but somehow enable Cdc42 to remain active at the periphery of the interface during that time and in all locations thereafter. However, such precise perturbations are currently not feasible. As an alternative, we sought to test our hypothesis by making multiple perturbations of Cdc42 activity. Some would affect some spatiotemporal patterns of Cdc42 activity more than others. We can then apply the same perturbations to various elements of T cell activation. A collection of such perturbations could then be used to discover significant correlations between changes in specific spatiotemporal patterns of Cdc42 activity and distinct elements of T cell activation, a first step to causality.

In the next three sections, we will introduce the perturbations of Cdc42 activity and quantify their effects on the spatiotemporal patterns of Cdc42 activity (Figs. 3–5). In the subsequent four sections, we will quantify how central elements of T cell activation are affected by these perturbations (Figs. 6–9). This will both define the role of Cdc42 in regulating T cell polarization/activation and allow the discovery of quantitative relations between spatiotemporal patterns of Cdc42 activity and elements of T cell activation, as discussed in the last section (Fig. 10).

For our analysis of Cdc42 activity, we will distinguish two spatial and two temporal patterns. We will address whether Cdc42 is active over the entire T cell/APC interface or selectively at its center. The clustering of various receptors and signaling intermediates at the center of the T cell/APC interface has identified it as a region of special interest. We will analyze Cdc42 activity within the first and the second minute after tight cell couple formation.
Within the first minute, distinct and dramatic morphologically changes occur. T cell actin spreads toward the edges of the interface (16), the uropod retracts (26), and a deep invagination forms at the center of the interface (K. Singleton, N. Parvaze, K. R. Dama, K. S. Chen, P. Jennings, B. Purtic, M. D. Sjaastad, C. Gilpin, M. M. Davis, and C. Wüsting, submitted for publication).

FIGURE 3. Interface accumulation of active Cdc42 is regulated by the TCR and CD28. A, Cdc42 sensor-transduced 5C.C7 T cells were activated by CH27 APCs in the presence of MCC agonist peptide. Accumulation (on the left) and centrality indices (on the right), as indicated, are given at the noted time points relative to the formation of a tight T cell/APC interface. The conditions were 10 μM MCC agonist peptide (full stimulus), 10 μM MCC with 10 μg/ml anti-B7-1 and anti-B7-2 Abs (anti-B7, B7 blockade), a 1/100 dilution of MCC into the null peptide MCC D93E/K99T/T102S (10 μM total concentration) (0.1 μM MCC, low agonist peptide), a combination of 0.1 μM MCC, anti-B7, and 10 μg/ml an ICAM Ab (limiting), and 10 μM MCC with 1/3 μM WASP GBD or with 100/350 nM Cdc42dn. Twenty-six to 107 cell couples (on average 48) from at least three independent experiments were analyzed per condition. Only 12 cell couples could be analyzed under limiting conditions, because T cell activation was rare. Cdc42 sensor data with a full T cell stimulus are the same as in Fig. 2B. B, The data from A are pooled so that the 0, 20, and 40 s time points are listed as 1 min, and the 60, 80, and 100 s time points are listed as 2 min. p values (Student’s t test) vs 10 μM MCC are given at the right of each panel. n.s. indicates p > 0.05.

FIGURE 4. A biochemical determination of Cdc42 activity matches the imaging data. Cdc42 GTP was precipitated from 5C.C7 T cell extracts with the PAK GBD and quantified by Western blotting. In A, a representative blot is shown. Numbers below each band indicate experimental conditions, as listed on the right. Numbers above each band indicate measured band intensity. In B, the quantization of all experiments is given as the average amount of precipitated Cdc42 GTP ± SD relative to a full stimulus. The T cell activation conditions were as follows: 10 μg/ml anti-TCR plus 10 μg/ml anti-CD28 (full stimulus), 10 μg/ml anti-TCR only, 0.1 μg/ml anti-TCR plus 10 μg/ml anti-CD28 (low anti-TCR), 10 μg/ml anti-TCR plus 10 μg/ml anti-CD28 plus 100 nM Cdc42dn, and Cbl-b-deficient 5C.C7 T cells and littermate control wild-type 5C.C7 T cells with 10 μg/ml anti-TCR plus 10 μg/ml anti-CD28. Independent experiments (≥2) were performed per condition.
frequency of cell couple formation was also diminished (I. Tskvitaria-Fuller and C. Wülfing, data not shown), these data should be compared cautiously to the blockade of individual receptors. To avoid such complications, our subsequent investigations are limited to conditions of undisturbed cell couple formation.

To further validate the live-cell Cdc42 sensor and to corroborate regulation of Cdc42 activity by TCR and CD28, we examined the amount of active Cdc42 biochemically. We precipitated active Cdc42 from cell extracts with the GBD of the p21-activated kinase and quantified its amounts by Western blotting (Fig. 4). T cell activation by APC resulted in a prohibitive background of Cdc42 activity in the cell extracts. T cells were therefore activated with Abs. We used 10 μg/ml anti-CD3 plus 10 μg/ml anti-CD28 as a full stimulus, 10 μg/ml anti-CD3 in the absence of anti-CD28 to assess the role of CD28, and a 100-fold reduced concentration of anti-CD3 Abs, i.e., 0.1 μg/ml, in combination with 10 μg/ml anti-CD28 to assess TCR signaling. Similar to the live-cell sensor data, Cdc42 activation depended on both the TCR and CD28 (Fig. 4B). Absence of CD28 engagement reduced the amount of precipitated Cdc42 to 51 ± 25% of a full stimulus, a reduced TCR signal to 32 ± 22% (Fig. 4B).

**Perturbing Cdc42: Cbl-b suppresses sustained Cdc42 activation**

Cbl-b is a negative regulator of the guanine nucleotide exchange factor (GEF) Vav (27, 28), an activator of Cdc42. To investigate the role of Cbl-b in the activation of Cdc42, we transduced primary T cells derived from Cbl-b-deficient, 5C.C7 TCR transgenic mice (14) with the Cdc42 sensor and activated them with a full stimulus (CH27 APCs plus 10 μM MCC peptide). In comparison to littermate controls, over the first minute after cell couple formation, the average Cdc42 centrality index was increased by 28% (p < 0.01). Over the second minute, both centrality and accumulation indices were enhanced by 97 and 59%, respectively (p < 0.001) (Fig. 5). At longer times (>5 min) after cell couple formation, the Cdc42 centrality and accumulation indices were increased even more by 150 and 168% (p < 0.01), respectively. For reasons that could not be elucidated, 5C.C7 T cells that were obtained as littermate controls for the Cbl-b-deficient T cells showed less Cdc42 accumulation than those that were used for all other studies (compare Figs. 3A and 5). Interestingly, biochemical Cdc42.GTP pull-down data showed only a moderate increase (22% at 5 min after T cell activation) in overall Cdc42 activity in Cbl-b-deficient T cells (Fig. 4B). The more dramatic enhancement of local Cdc42 activity, as measured with the Cdc42 biosensor, vs global Cdc42 activity, as measured biochemically, suggests that Cbl-b predominantly suppresses the localized activation of Cdc42 at the T cell/APC interface. Cbl-b thus is a negative regulator of sustained activation of Cdc42 at the T cell/APC interface.

**Perturbing Cdc42: direct manipulation of Cdc42 activity**

To causally determine roles of Cdc42 in T cell polarization and activation, we needed to manipulate its activity directly. We had to take into account that Cdc42 is essential for cell survival (10, 11). Manipulation of Cdc42 activity thus should be short-term and as moderate as minimally required. Furthermore, to account for limitations in the specificity of individual reagents, multiple reagents with independent mechanisms of action were desirable. Here we used three reagents. To interfere with Cdc42 function, we used the dominant-negative (dn) N17 mutant of Cdc42 and the WASP GBD. As specificity controls for Cdc42dn, we used N17 Rac1 and N19 RhoA (17). To enhance Cdc42 function, we used the V12 ca mutant (17). Cdc42dn and the control reagents Rac1dn and RhoAdn function by blocking access of endogenous GTPases to GEFs (29). Because it is likely that Cdc42 and Rac1 share GEFs,
some overlap between the effects of Cdc42dn and Rac1dn is expected. We found only moderate overlap, as described in another manuscript (I. Tskvitaria-Fuller, N. Mistry, and C. Wulfing, manuscript in preparation). The WASP GBD uses a mechanism of action that is distinct from Cdc42dn. It competes with endogenous effectors for access to active Cdc42. Cdc42ca provides increased levels of Cdc42 activity, because it is locked in the GTP-bound state. The localization of this activity is independent of the localization of Cdc42 GEFs. It is likely the same as the overall Cdc42 distribution. Cdc42ca thus likely provides increased Cdc42 activity more globally throughout the cell than activation of endogenous Cdc42 by receptor engagement.

As used, none of these reagents affected the frequency of cell couple formation and interface diameters (Fig. 6). All three reagents were introduced into cells by protein transduction, where addition of a 10-aa peptide from the HIV tat protein confers membrane-permeability (12). Fusion proteins were produced in E. coli. They were taken up by 100% of T cells within ≤30 min (data not shown). Taking advantage of the quantitative nature of protein transduction, we performed dose responses to determine the minimal reagent concentrations to achieve a saturated blockade of Cdc42 activity, as described in detail in another manuscript (I. Tskvitaria-Fuller, N, Mistry, and C. Wulfing, manuscript in preparation). Briefly, 100 nM and 350 nM Cdc42dn yielded indistinguishable phenotypes in blocking Cdc42 activation, as assayed by interface accumulation of the Cdc42 sensor. To allow a larger data set in the description of the effect of Cdc42dn on Cdc42 activation, these data were therefore pooled. For the analysis of the effect of Cdc42dn on various elements of T cell polarization, only the minimal concentration that showed saturated interference with Cdc42 sensor localization, i.e., 100 nM, was used to maximize specificity. Similarly, 1 and 3 μM WASP GBD yielded indistinguishable interference with Cdc42 sensor accumulation, allowing data pooling to describe the effect of WASP GBD on Cdc42 activation. However, because 3 μM was more potent than 1 μM WASP GBD in other assays, the higher concentration was used for the analysis of the effect of WASP GBD on various elements of T cell polarization.

To assess the spatiotemporal patterns of Cdc42 activity upon direct manipulation of its activity, we determined them in the presence of Cdc42dn and WASP GBD. In the presence of the full stimulus (CH27 APC plus 10 μM MCC peptide), both inhibitors blocked sensor recruitment to T cell/APC interface. 100/350 nM Cdc42dn reduced the accumulation index over the first and second minute after cell couple formation by 16% (p = 0.01) and 15%...
(p = 0.01), the centrality index by 24% (p = 0.02) and 17% (not significant), respectively (Fig. 3B). A total of ½ μM WASP GBD did not affect the accumulation index. It reduced the centrality index by 29% (p < 0.001) and 22% (p < 0.05) (Fig. 3B). Direct interference with Cdc42 activity using Cdc42dn and WASP GBD thus left substantial residual Cdc42 activity at the interface. However, so did B7 blockade and a low agonist peptide concentration (Fig. 3). In fact, the magnitude of the Cdc42dn/WASP GBD effects was comparable to those of reducing TCR ligand density or blocking CD28 engagement (Fig. 3). To explain this shared residual Cdc42 activity, we suggest that a substantial minimal level of Cdc42 activity is required to allow efficient cell couple formation. Lower levels of Cdc42 activity would not be detectable in T cell/APC couples, because they interfered with cell couple formation. This suggestion is supported by the observation that both limiting T cell stimulation (data not shown) and increased doses of Cdc42dn (Fig. 6A) interfered with cell couple formation. The observation that Cdc42 activity at the interface was reduced to comparable levels upon addition of Cdc42dn/WASP GBD and upon physiological reduction of the T cell stimulus establishes that Cdc42dn and WASP GBD reduce Cdc42 activity to close to the minimal level compatible with efficient cell couple formation. We call such interference “physiologically saturating” and will apply this expression to the magnitude of the effects of Cdc42dn/WASP GBD on various elements of T cell polarization/activation below. To corroborate that direct interference with Cdc42 activation by 100 nM Cdc42dn was comparable to reduced TCR or CD28 engagement, we performed biochemical Cdc42.GTP pull-down assays. Adding 100 nM Cdc42dn to a full stimulus reduced Cdc42 GTP loading to 54 ± 28%, comparable to lack of CD28 engagement (51 ± 25%) or a reduced TCR stimulus (32 ± 21%) (Fig. 4B).

In summary, we have established three independent, quantitative reagents to manipulate Cdc42 activity in live primary T cells, Cdc42dn, WASP GBD, and Cdc42ca. Because of their distinct mechanisms of action, effects seen with a majority of the three reagents can confidently be assigned to Cdc42. In addition, we have quantified the effects of the inhibitory reagents on the accumulation of the Cdc42 sensor at the T cell/APC interface and compared these effects to those of a reduced TCR signal and costimulation blockade. This comparison established concentrations of Cdc42dn and WASP GBD that are physiologically saturating with respect to interference with Cdc42 activity. Using these concentrations, we will determine the role of Cdc42 in T cell polarization and activation in the next three sections. In addition, we have now completed the analysis of the spatiotemporal patterns of Cdc42 activity under various perturbations, as required for the final analysis of relations between patterns of Cdc42 activity and elements of T cell activation.

Cdc42-dependent elements of T cell activation: sustained actin accumulation

Actin dynamics mediate T cell polarization. Sustained actin accumulation at the T cell/APC interface (≥5 s after interface formation) is an important part of these dynamics (16). We assayed it using actin-GFP (16). For a more rigorous quantitative analysis of sustained actin accumulation, we defined an actin accumulation index analogous to the Cdc42 sensor accumulation index. We first assessed the sensitivity of sustained actin accumulation to direct interference with Cdc42 activity. In the presence of a full T cell stimulus (CH27 APC with 10 μM MCC peptide), 100 nM Cdc42dn significantly reduced the actin accumulation index by 54% (p < 0.001), and 3 μM WASP GBD reduced it by 32% (p < 0.001) (Fig. 7). The extent of these reductions was half to two-thirds of those seen with the low agonist peptide concentration (0.1 μM MCC) (72% reduction; p < 0.001) or B7 blockade (74% reduction; p < 0.001). The comparable reductions in sustained actin accumulation upon addition of Cdc42dn/WASP GBD vs reduced TCR or CD28 engagement establish near physiological saturation of the effects of Cdc42dn/WASP GBD. This suggests that Cdc42 is a critical regulator of sustained actin accumulation. One hundred nanomolar Rac1dn (29% reduction; p < 0.01) or 100 nM RhoAdn (4% reduction, not significant) showed significantly smaller reductions of the actin accumulation index than 100 nM Cdc42dn (p < 0.05 vs 100 nM Cdc42dn), establishing some specificity among these three Rho GTPases.

Next, we investigated whether additional Cdc42 activity could enhance sustained actin accumulation. Addition of 1 μM Cdc42ca to a full T cell stimulus did not increase the actin accumulation index but significantly decreased it by 27% (p = 0.001) (Fig. 7). To account for this reduction, we suggest that Cdc42-mediated enhancement of actin accumulation was already maximal at the full T cell stimulus. Further active Cdc42, in particular dispersed
throughout the cell as for Cdc42ca, would then compete for Cdc42 effectors, reducing localized actin accumulation at the interface. However, in contrast to the inhibitory effect at a full T cell stimulus, Cdc42ca completely and in a dose-dependent manner restored sustained actin accumulation that was depressed in the presence of suboptimal stimuli (reduced TCR or CD28 engagement) (Fig. 7). To gain insight into how Cdc42 regulates sustained actin accumulation (16). Both Cdc42dn and WASP GBD blocked entry of 5C.C7 T cells into the cell cycle, as determined by Hoechst staining (Fig. 8B). In the presence of a full T cell stimulus (CH27 APC with 10 μM MCC peptide), 100 nM Cdc42dn reduced the percentage of T cells in the cell cycle to 51 ± 11% of buffer only, 3 μM WASP GBD to 16 ± 3%, comparable to a B7 blockade (reduction to 21 ± 10%). In parallel, 3 μM WASP GBD reduced the percentage of T cells secreting IL-2 to 71 ± 5% of buffer only, comparable with a B7 blockade (59 ± 20% of control). Next, we asked whether additional active Cdc42 could enhance IL-2 secretion. Addition of 1 μM Cdc42ca did not change IL-2 secretion in the presence of a full T cell stimulus and could not compensate for reduced IL-2 secretion during B7 blockade (data not shown). This suggests a more limited involvement of Cdc42 in the regulation of IL-2 secretion as opposed to sustained actin accumulation. In summary, Cdc42 was required for cell cycle entry and contributed to IL-2 production.

Cdc42-dependent elements of T cell activation: early actin accumulation

Within the first minute of T cell/APC couple formation, actin spreads to the edges of the interface (16). The concomitant clearing of actin at the center of the interface is related to a specific T cell invagination event at the center at the T cell/APC interface that we speculate serves to reset the T cell signaling machinery (K. Singleton, N. Parvez, K. R. Dama, K. S. Chen, P. Jennings, B. Pertic, M. D. Sjastad, C. Gilpin, M. M. Davis, and C. Wülfing, submitted for publication). We therefore assessed the sensitivity of early actin spreading to direct interference with Cdc42 activity. In the presence of a full T cell stimulus (CH27 APC with 10 μM MCC peptide), 100 nM Cdc42dn significantly (p = 0.001) reduced the percentage of cell couples showing early actin spreading from 74 to 36% (Fig. 9A). A total of 3 μM WASP GBD reduced the percentage moderately to 49% of cell couples. One hundred nanomolar Rac1dn and 100 nM RhoAdn allowed early actin spreading to occur in 64 and 63% of the cell couples, respectively (Fig. 9A), thus interfering significantly less than 100 nM Cdc42dn (p < 0.05 vs 100 nM Cdc42dn). These data establish Cdc42 as a specific regulator of early actin spreading. The reductions in early actin spreading by Cdc42dn/WASP GBD are comparable to those achieved by reductions in TCR signal strength (47% of cell couples with early actin spreading at 0.1 μM MCC; Fig. 9B). This physiological saturation of the effect of direct interference with Cdc42 suggests that Cdc42 is a critical regulator of early actin spreading.

Another critical element of T cell polarization is the clustering of receptors, among them the TCR and CD28, at the center of the T cell/APC interface. In 5C.C7 T cells such clustering is required for efficient IL-2 secretion (18). We therefore assessed the sensitivity of central TCR accumulation to interference with Cdc42 activity. In the presence of a full T cell stimulus (A20/EH-GFP APC with10 μM MCC peptide), 100 nM Cdc42dn and 3 μM WASP GBD significantly (p < 0.005) interfered with central TCR clustering (Fig. 8A). Only 28 and 22% of treated T cell/APC couples, respectively, clustered the TCR at the center of the T cell/APC interface, as opposed to 68% upon addition of buffer only. Similar reductions in central TCR accumulation were observed upon reduced TCR or CD28 engagement (low agonist peptide or B7 blockade) (15). This physiological saturation of the effect of direct interference with Cdc42 suggests that Cdc42 is a critical regulator of central TCR clustering.

Cdc42-dependent elements of T cell activation: cell cycle entry and IL-2 production

Next, we investigated the role of Cdc42 in T cell cycle entry. T cell cycle entry has previously been shown to depend on sustained actin accumulation (16). Both Cdc42dn and WASP GBD blocked entry of 5C.C7 T cells into the cell cycle, as determined by Hoechst staining (Fig. 8B). In the presence of a full T cell stimulus (CH27 APC with 10 μM MCC peptide), 100 nM Cdc42dn reduced the percentage of T cells in the cell cycle to 51 ± 11% of buffer only, 3 μM WASP GBD to 16 ± 3%, comparable to a B7 blockade (reduction to 21 ± 10%). In parallel, 3 μM WASP GBD reduced the percentage of T cells secreting IL-2 to 71 ± 5% of buffer only, comparable with a B7 blockade (59 ± 20% of control). Next, we asked whether additional active Cdc42 could enhance IL-2 secretion. Addition of 1 μM Cdc42ca did not change IL-2 secretion in the presence of a full T cell stimulus and could not compensate for reduced IL-2 secretion during B7 blockade (data not shown). This suggests a more limited involvement of Cdc42 in the regulation of IL-2 secretion as opposed to sustained actin accumulation. In summary, Cdc42 was required for cell cycle entry and contributed to IL-2 production.

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Next, we asked whether additional active Cdc42 could enhance early actin spreading. Similar to the regulation of sustained actin dynamics, addition of 1 μM Cdc42ca to a full T cell stimulus reduced the percentage of T cell/APC couples with early actin spreading to 60% (p = 0.01) (Fig. 9A). However, in contrast to the effect on sustained actin accumulation, Cdc42ca could not restore diminished early actin spreading in the presence of reduced TCR engagement. At 0.1 μM MCC, 47% of the cell couples displayed early actin spreading, in the additional presence of 100 nM to 1 μM Cdc42 30–40% (Fig. 9B). In summary, Cdc42 was required for early actin spreading. However, in contrast to the regulation of sustained actin accumulation, a ca mutant of Cdc42 could not compensate for a reduced TCR signal.

Cdc42-dependent elements of T cell activation: cell couple formation and MTOC reorientation

We have established that Cdc42 is a central regulator of T cell actin dynamics. Reagents to interfere with Cdc42 activity thus might impede T cell couple formation. However, in the presence of a full T cell stimulus (CH27 APC with 10 μM MCC peptide), 100 nM Cdc42dn or 3 μM WASP GBD did not affect the frequency of T cell/APC couple formation, nor interface diameters (Fig. 6). Only upon addition of 1 μM Cdc42dn, the percentage of T cells in contact with an APC that form a tight cell couple was significantly (p < 0.05) reduced from 61% with buffer only to 27% (Fig. 6A).

The reorientation of the MTOC toward the T cell/APC interface is a critical element of T cell polarization. Cdc42 has been suggested to be required for it (9). However, we found only small effects of Cdc42dn on MTOC reorientation only at high concentrations. Cdc42dn did not significantly (p > 0.05) disturb the eventual localization of the MTOC behind the center of the interface. With a score of 1 indicating MTOC localization at the interface, 3 at the opposite side of the T cell, the final average MTOC position in the presence of a full T cell stimulus (CH27 APC with 10 μM MCC peptide) and 100 nM, 350 nM, and 1 μM Cdc42dn was 1.18, 1.23, and 1.33, as opposed to 1.02 upon addition of buffer only. However, Cdc42dn slowed MTOC reorientation moderately. Although at a full T cell stimulus, 80% of the MTOC translocation was complete within 80 s of cell couple formation, this percentage dropped to 20% upon addition of ≥350 nM Cdc42dn (Fig. 6C). For comparison, even under limiting T cell activation conditions, 50% of the MTOC reorientation was complete after 80 s (16).

In summary, interference with Cdc42 activation only moderately impeded cell couple formation and MTOC reorientation only at high concentrations of Cdc42dn. This is in sharp contrast to the sensitivity of actin dynamics and central TCR accumulation to interference with Cdc42. These data suggest that actin dynamics and central TCR accumulation, but not MTOC reorientation and cell coupling, are primary targets of Cdc42. Our final analysis of the relations between specific spatiotemporal patterns of Cdc42 activity with distinct elements of T cell polarization will therefore not include cell couple formation and MTOC reorientation.

Specific patterns of Cdc42 activity relate to distinct elements of T cell polarization

In the preceding sections, we have, first, quantified spatiotemporal patterns of Cdc42 activity and, second, elements of T cell activation that are dependent on Cdc42 under the same set of perturbations. These data provide an opportunity to relate patterns of Cdc42 activity to elements of T cell activation. Specific regulation of a particular element of T cell activation by a distinct pattern of Cdc42 activity should be evident in a tight correlation through the perturbations. We used five T cell activation conditions with varying degrees of Cdc42 activity, a full stimulus, B7 blockade, low agonist peptide, Cdc42dn, and WASP GBD. We used four patterns of Cdc42 accumulation, accumulation and centrality indices over the first and second minute after cell couple formation (Fig. 3). We used five Cdc42-dependent elements of T cell activation, sustained actin accumulation (Fig. 7), central TCR clustering (Fig. 8A), T cell cycle entry (Fig. 8B), IL-2 production (text), and early actin spreading (Fig. 9). To examine relations between these patterns of Cdc42 accumulation and these elements of T cell activation, we pair-wise plotted each of the four measures of Cdc42 accumulation...
against each of the five elements of T cell activation conditions (an example is shown in Fig. 10A). These 20 plots generated two types of information. The slopes of the regression curves measure how strongly changes in a particular Cdc42 pattern are related to a distinct element of T cell activation (Fig. 10B). Large slopes, i.e., strong relations, indicate that the respective element of T cell activation is sensitive to the corresponding spatiotemporal pattern of Cdc42 activity. In addition, correlation coefficients with their SEs measure the reliability of a relation (Fig. 10C). For correlation coefficients $>0.5$, all SEs were $\leq 0.08$. Centrality and accumulation indices are abbreviated as "cen" and "acc," respectively. Because no significant relations exists between IL-2 production and any pattern of Cdc42 activity, slopes of the correlation graphs are meaningless and not included in B. The relative slope and the correlation coefficient for the relation between the Cdc42 centrality index at 1 min and sustained actin dynamics are not visible. They are 0.264 and 0.18, respectively.

This analysis established that specific patterns of Cdc42 activity were linked to distinct elements of T cell activation. Specifically, sustained actin accumulation was most closely related to the Cdc42 accumulation and centrality indices over the second minute after T cell/APC couple formation ($r^2 = 0.78 \pm 0.01$ for the accumulation index, $r^2 = 0.81 \pm 0.01$ for the centrality index). Early actin spreading was most closely related to the Cdc42 centrality index over the first minute after cell couple formation ($r^2 = 0.76 \pm 0.08$). Central TCR accumulation was consistently related to the Cdc42 centrality index at 1 min ($r^2 = 0.51 \pm 0.07$ for the centrality index at 2 min). T cell cycle entry was most closely related to the Cdc42 centrality index at 2 min ($r^2 = 0.58 \pm 0.02$). No pattern was strongly related to IL-2 secretion, consistent with the comparatively smaller effects of direct manipulation of Cdc42 activity. In general, changes in

![FIGURE 10. Specific patterns of Cdc42 accumulation are related to distinct elements of T cell activation. In A, an example of a correlation analysis of one Cdc42 pattern, accumulation at the center of the T cell/APC interface in the second minute after cell couple formation (measured with the centrality index), vs one element of T cell activation, sustained actin accumulation (measured with the actin accumulation index) is shown. Data points for five conditions as indicated were plotted. Anti-B7 indicates 10 µg/ml anti-B7-1 and anti-B7-2 Abs. A linear regression curve was generated with its slope and correlation coefficient (shown) with SE. For each other pair-wise combination of one Cdc42 pattern with one element of T cell activation, a similar linear regression analysis was performed (data not shown). In B, the normalized (Materials and Methods) slopes are given; in C, the correlation coefficients, as listed. For correlation coefficients $>0.5$, all SEs were $\leq 0.08$. Centrality and accumulation indices are abbreviated as "cen" and "acc," respectively. Because no significant relations exists between IL-2 production and any pattern of Cdc42 activity, slopes of the correlation graphs are meaningless and not included in B. The relative slope and the correlation coefficient for the relation between the Cdc42 centrality index at 1 min and sustained actin dynamics are not visible. They are 0.264 and 0.18, respectively.](image)
Cdc42 centrality rather than accumulation were related to particularly large changes in T cell activation, suggesting that Cdc42 activity at the center of the T cell/APC is its most important pattern.

Discussion
In this study, we have addressed the role of Cdc42 in T cell activation. Previous work using transfection of Cdc42dn into T cell hybridoma cells suggested that Cdc42 is required for actin accumulation at the T cell/APC interface and reorientation of the MTOC toward it (9). We largely confirm these findings here. However, MTOC reorientation was substantially less sensitive to interference with Cdc42 activation in our study. We found only moderately slowed but no blocked MTOC reorientation. We suggest that considering the nature of the T cells used can reconcile the data. With primary T cells in the presence of a high concentration of Cdc42dn (1 μM), MTOC reorientation was slowed and cell couple formation was reduced (Fig. 6). This suggests that a primary T cell, rather than forming a cell couple with grossly defective polarization, does not form a cell couple at all. A relaxed requirement for cell couple formation of the 2B4 hybridoma cells likely allows the observation not only of slowed but also of blocked MTOC reorientation. Other than this study, an important role for Cdc42 in the regulation of T cell activation was deduced by studying its activators, in particular Vav, and its effectors, in particular WASP. The function of the Rho family GEF Vav (as recently reviewed in Ref. 33) was mostly analyzed using Vav-deficient mice. Clear defects in cytoskeletal regulation emerged: integrin-mediated T cell spreading was impaired; actin recruitment in capping experiments was less efficient, Cdc42 activation in the SLP-76/WASP/Vav complex was reduced; MTOC reorientation and TCR clustering might be diminished (33). However, Vav is a multifunctional protein, and it is not clear whether such defects in cytoskeletal regulation are caused through diminished Cdc42 activation or through reductions in calcium signaling, dynamin 2 activation, or ezrin/radixin/moesin protein dephosphorylation (33). A principal function of the Cdc42 effector WASP (as reviewed in Ref. 34) is its ability to directly activate the Arp2/3 complex through its C-terminal verprolin, cofilin homology, acidic domain. Similar to Vav-deficient cells, WASP-deficient T cells show reduced actin recruitment in capping experiments (35). The segregation of talin and protein kinase C-θ in the immunological synapse was less efficient at reduced agonist peptide concentrations (6). However, it is unclear how much of T cell actin regulation is mediated by WASP, because recently described T cells deficient in the WASP homologue WAVE2 have more drastic phenotypes (36, 37). From a methodological point of view, it is unclear to which extent T cells deficient in Vav or WASP have adapted to these deficiencies by more heavily relying on alternate pathways of cytoskeletal regulation.

Previous data thus provide strong suggestions that Cdc42 should be a critical regulator of T cell polarization/activation. We have confirmed this. However, it was unclear to which extent various elements of T cell activation depend on Cdc42 and how Cdc42 could regulate multiple elements of T cell activation simultaneously. Using quantitative analysis of the effects of interference with Cdc42 activity, we have shown that direct interference with Cdc42 activity was as potent as B7 blockade or only limiting TCR or CD28 engagement. This suggests that Cdc42 is a sufficient member of this particular signaling network. Using quantitative relations between patterns of Cdc42 activity and elements of T cell activation, we provide first support for the hypothesis that Cdc42 can regulate multiple elements of T cell activation simultaneously by selectively using distinct spatiotemporal patterns of its activity. Specifically, the most influential pattern of Cdc42 activity was activation at the center of the T cell/APC interface, as mediated by the TCR and CD28 and inhibited by Cbl-b. Such Cdc42 activity was related to hallmarks of the mature immunological synapse, central TCR accumulation, and sustained actin accumulation, as well as to T cell cycle entry. Together with previous work that has linked sustained actin accumulation and central TCR clustering to T cell cycle entry and IL-2 secretion in the same experimental system (16, 18), these data suggest that Cdc42 might use localized regulation of actin and receptor patterning to affect downstream T cell activation. Cdc42 activity spread over the interface ≥1 s after its formation was related to sustained actin accumulation, too, as possibly required for maintaining a dynamic cellular interface. Central Cdc42 accumulation within 1 s of cell couple formation was also related to actin spreading to the edge of the forming interface with concomitant clearing of the center. We suggest that this allows a specific T cell invagination event that we speculate serves to reset TCR signaling upon interface formation (K. Singleton, N. Parvaze, K. R. Dama, K. S. Chen, P. Jennings, B. Purtic, M. D. Sjaastad, C. Gilpin, M. M. Davis, and C. Wülfing, submitted for publication). We have thus demonstrated that Cdc42 is a critical regulator of multiple elements of T cell polarization and activation. Notably, specific localization at specific times seems most critical in its regulation of multiple structural and functional elements of the immunological synapse.

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Disclosures
The authors have no financial conflict of interest.

References


