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Protein transduction as a means of effective manipulation of Cdc42 activity in primary T cells

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Abstract

The Rho family GTPase Cdc42 is a critical regulator of cellular polarization from yeast to man. An analysis of its function in T cell activation is therefore of interest. This analysis poses two substantial challenges, similar to the analysis of many other critical T cell signaling intermediates. First, Cdc42 is required for development and cell survival, necessitating short-term manipulation of its activity. Second, Cdc42 is likely involved in multiple signaling pathways, requiring approaches to distinguish multiple roles. To address these challenges, we first determined and quantified spatio-temporal patterns of Cdc42 activity using live cell video fluorescence microscopy. This generates hypotheses at which times and locations Cdc42 might play possibly distinct roles. Second and as the focus of this manuscript, we employed protein transduction to manipulate Cdc42 activity for the generation of causality. Protein transduction allows such manipulation to be short-term, quantitative, and with multiple reagents. Here, we characterize uptake, retention, and subcellular distribution of protein transduction reagents. We describe how a more quantitative single cell analysis of Cdc42 activity provides superior distinction between experimental conditions. And we show how we have used dose responses of the protein transduction reagents to minimize side effects while retaining efficacy. We suggest that our strategy is an important complement to more established techniques to study protein function in primary T cells, in particular in the investigation of signaling intermediates that are essential for cell survival and regulate multiple aspects of T cell activation.

Keywords

protein transduction; T cell activation; Cdc42

1. Introduction

The Rho family GTPase Cdc42 is a critical regulator of cellular polarization from yeast to man. Its function in T cell activation is therefore of interest. However, a detailed analysis has been largely inaccessible for two reasons. First, Cdc42 is essential. Cdc42-deficiency results in embryonic lethality (Chen et al., 2000) and even overexpression of an active mutant interferes with cell survival (Lin et al., 1999). Second, as a suspected central regulator of T cell polarization, Cdc42 is likely to play multiple roles at different stages and intracellular locations

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in T cell activation that need to be distinguished. Importance in development or survival and likely involvement in multiple pathways are not unique to Cdc42, but are shared with other central T cell signaling intermediates, such as SLP-76 (Jordan et al., 2003) or PKB (Jones et al., 2000; Kane and Weiss, 2003; Song et al., 2004). Here we outline a strategy to address Cdc42 and describe in detail the role of protein transduction in it. This strategy should be applicable to other similarly central signaling intermediates and thus of general interest.

Our strategy contains two elements. First, we used a biosensor to assay Cdc42 activity in its spatio-temporal patterns by live cell fluorescence microscopy (Tskvitaria-Fuller et al., 2006). Such patterns allow the generation of hypotheses about different functions of Cdc42. Second as the focus of this manuscript, we used protein transduction (Wadia and Dowdy, 2002) to manipulate Cdc42 activity, thus generating causality. Various approaches can be used to manipulate protein activity in live cells. Widely used methods are the generation of gene-deficient mice, siRNA, and transfection or retroviral transduction with the gene or mutants thereof. All of these methods have significant limitations when used for the investigation of Cdc42 in T cell activation. Cdc42 is essential for cell survival, making the generation of gene-deficient mice or even siRNA problematic, as both approaches require survival of primary T cells with diminishing amounts of Cdc42 over at least days. Transfection of primary T cells is inefficient, making delivery of siRNA or of the Cdc42 cDNA and mutants thereof problematic. In retroviral transduction, adjustment of expression levels to optimize concentrations of reagents to manipulate Cdc42 activity is difficult and primary T cells are required to survive in the presence of these reagents for days. In protein transduction (Wadia and Dowdy, 2002), addition of a 10 amino acid peptide from the HIV tat protein allows uptake of peptides or proteins into live cells within minutes in a dose-dependent fashion, enabling short-term and quantitative manipulation of protein function. Protein transduction can easily be applied to multiple reagents. Here we describe, how we have taken advantage of these strengths of the protein transduction approach to optimize manipulation of Cdc42 activity in primary T cells.

2. Materials and methods

2.1. Tat fusion proteins

Plasmids for the expression of the constitutively active and dominant negative mutants of human Cdc42 and Rac1 as protein transduction reagents were a kind gift of Dr. Steve Dowdy (Soga et al., 2001). The sequences were changed to the mouse sequences as required. The proteins were expressed in BL21(DE3) E. coli at room temperature. Bacteria were lysed under native conditions in the EmulsiFlex C5 cell disruptor (Avestin, Ottawa, Canada) in the presence of 0.01 % IGEPAL (Sigma-Aldrich, St. Louis, MO) and 5 % Glycerol. The Rho GTPase mutants were purified over Nickel-NTA immobilized metal affinity chromatography columns by imidazole elution according to the instructions of the manufacturer (Qiagen, Valencia, CA). If necessary, the proteins were further purified by FPLC on a HiTrapSP cation exchange column (GE Healthcare) at one pH unit below the isoelectric point. Proteins were eluted in a single step with 1 M NaCl. Finally, a buffer exchange to PBS was performed. Protein identity was verified by mass spectrometry. To investigate protein transduction reagent uptake and retention, tat Cdc42dn and tat Rac1dn were conjugated to fluorescein using fluorescein isothiocyanate (Molecular Probes, Eugene, OR) according to the instructions of the manufacturer.

The tat-tagged version of the WASP GTPase-binding domain (GBD) contains the WASP GBD (amino acids 230–289) with the same N-terminal extension as the previously described tat-tagged WASP verprolin, cofilin-homology, acidic domain (Tskvitaria-Fuller et al., 2003) containing six histidines followed by the tat and HA tags. It was expressed in BL21 (DE3).pLysS E. coli at 37 °C from a pET15b plasmid (Novagen/EMS, La Jolla, CA). Bacteria were lysed under native conditions by sonication in the presence of 0.01 % IGEPAL and tat

WASP GBD was purified over a Nickel NTA column by Imidazole gradient elution according to the instructions of the manufacturer (Qiagen, Valencia, CA). The protein was further purified over a MonoS HR 10/10 anion exchange column using elution in a NaCl gradient from 50 mM to 1M. Finally, a buffer exchange to PBS was performed. Protein identity was verified by mass spectrometry. To investigate tat WASP GBD uptake, tat WASP GBD was conjugated to Alexa 488 (Molecular Probes, Eugene, OR) according to the instructions of the manufacturer.

The tat-tagged version of the ZAP-70 tandem SH2-GFP fusion protein contains the ZAP-70 tandem SH2-GFP fusion protein (Purtic et al., 2005) with the same N-terminal extension as the previously described tat-tagged WASP verprolin, cofilin-homology, acidic domain (Tskvitaria-Fuller et al., 2003). It was expressed in BL21(DE3).pLysS E. coli at room temperature from a pET15b plasmid (Novagen/EMS, La Jolla, CA). Bacteria were lysed under native conditions by sonication and tat ZAP-70 tandemSH2-GFP was purified over a Nickel NTA column by Imidazole gradient elution according to the instructions of the manufacturer (Qiagen, Valencia, CA). Purity was > 95 % at this point.

Tat-tagged peptides and proteins were loaded into primary T cells for 30 min at 37 °C by simple incubation in standard cell culture media. For the determination of tat fusion peptide retention and intracellular distribution, excess protein transduction reagent was washed away. For the investigation of T cell polarization, protein transduction reagent was also added to the imaging cover slip at the same concentration as used during loading.

2.2. Cells and imaging

In vitro primed primary T cells from 5C.C7 (Seder et al., 1992) TCR transgenic mice were used in all experiments and were generated as described (Wülfing et al., 2002). The use of these mice has been reviewed and approved by the UT Southwestern Institutional Animal Care and Use Committee. As APCs, CH27 B cell lymphoma cells were used. Agonist peptide concentrations were adjusted by dilution into the null peptide MCC D93E/K99T/T102S (Wülfing et al., 2002). Costimulation blockade with antibodies against ICAM-1 or B7-1/B7-2 was as described (Tskvitaria-Fuller et al., 2003). The live cell Cdc42 sensor consists of amino acids 230–289 of WASP, directly followed by EGFP, and the last 14 amino acids of Cdc42 containing the Caax box. A control sensor is described elsewhere (Tskvitaria-Fuller et al., 2006). The Cdc42 sensor was introduced into primary T cells by retroviral transduction using a Moloney murine leukemia virus-derived system as described (Tskvitaria-Fuller et al., 2003). The microscopy system and image acquisition have been described in detail (Tskvitaria-Fuller et al., 2003). Briefly, primary T cells and peptide incubated APCs were allowed to interact at 37 °C on the microscope stage. Every 20 seconds a differential interference contrast (DIC) bright field image and 21 GFP images spaced 1 µm in z covering the entire cell were acquired. For all image analysis, we generated 3-dimensional reconstructions of productive cell couples. Time points were every 20 seconds between 20 sec before and 100 sec after cell couple formation plus $\geq 5'$ after cell couple formation. Time 0 was set to the first time point with a maximally spread T cell/APC interface. This results 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 sec prior to the maximal spreading of the interface. Cdc42 sensor accumulation was analyzed with accumulation and centrality indices, as described in the results section. For a comparison with more conventional analyses, % cell couples with accumulation in any or in a central pattern were also determined, as described before (Wülfing et al., 2002). Briefly, interface accumulation at > 40 % above the cellular fluorescence background in any pattern was classified as 'any accumulation'. Any such accumulation where the accumulation area only covered < 25 % of the interface area and was more than 20 % of the interface diameter away from the edge of the interface was classified as 'central'.

For the characterization of the intracellular location of the protein transduction reagents, 10⁶ 5C.C7 T cells were incubated in T cell growth medium for 30 min at 37 °C with 10 µM fluorescein-conjugated tat Cdc42dn or tat Rac1dn. As a positive control for fluid phase endosomal uptake, T cells were incubated 10 min at 37 °C with 100 µM Oregon Green 488 (Molecular Probes, Eugene, OR). To label endosomes in parallel with protein transduction reagents of Oregon Green 488, T cells were concomitantly incubated for 10 min at 37 °C with 25 µg/ml Tetramethylrhodamine-conjugated Transferrin (Molecular Probes, Eugene, OR). After incubations, T cells were washed twice with ice-cold PBS, kept on ice, and immediately imaged by spinning disk confocal microscopy on a Perkin Elmer Ultraview ERS microscope with a 63x oil immersion objective. 42 z planes spanning 20 µm were acquired. In two-color experiments, both colors were acquired at one z-plane before moving to the next. Pearson's coefficients of colocalization were calculated in 3D volumes containing individual background-corrected cells using the Imaris software (Bitplane AG, Zürich, Switzerland). We verified that uptake patterns for fluorescein-conjugated protein transduction reagent and Oregon Green 488 did not differ upon variation of the incubation time between 10 and 30 min. 30 min incubation was chosen for transduction reagents to match their application in investigating T cell polarization, 10 min was chosen for Oregon Green 488 to minimize extracellular fluorescence background.

Cell couple formation was determined in bright field images. For every T cells in contact with an APC for ≥ 2 frames (i.e. > 20 sec) it was determined, whether a tight cell couple formed or not.

The significance of differences between experimental conditions in analyses based on % cell couples with a particular phenotype was calculated using a 2-sample proportion z-test. In analyses based on accumulation and centrality indices, a Student's t-test was used, as described elsewhere (Tskvitaria-Fuller et al., 2006).

2.3. Biochemical determination of Cdc42 activity

The total amount of active Cdc42 in a T cell was determined by precipitation of active Cdc42 from T cell lysates with a p21-activated kinase-1 GBD GST fusion protein coupled to agarose according to instruction from the manufacturer (Chemicon, Temecula, CA) followed by Western blotting with an anti-Cdc42 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Stimulation of the primary T cells with APC and peptide resulted in a prohibitive background of active Cdc42. Therefore, T cells were stimulated with anti-CD3 ϵ (2C11) and anti-CD28 antibodies (both BD Pharmingen) at 10 µg/ml and separate cross-linking secondary antibodies at 10 µg/ml for 5' at 37 °C prior to lysis.

2.4. IL-2 production

IL-2 production was determined in primary T cell/APC couples by intracellular cytokine staining as published (Purtic et al., 2005).

3. Results

3.1. Protein transduction as an approach to manipulate Cdc42 activity

We used three reagents to manipulate Cdc42 activity. The use of multiple reagents proved instrumental in assessing how Cdc42 can regulate multiple aspects of T cell polarization and activation as described elsewhere (Tskvitaria-Fuller et al., 2006). Here we focus on how the application of individual reagents was established and optimized. To interfere with Cdc42 function, we used the dominant-negative (dn) N17 mutant of Cdc42 that blocks Cdc42 activation and the GTPase binding domain (GBD) of WASP that competes with endogenous effectors for access to active Cdc42. A protein transduction reagent based on the WASP GBD

has also been used by others (Haddad et al., 2001). As specificity control for Cdc42dn, we used N17 Rac1dn (Soga et al., 2001). To enhance Cdc42 function, we used the V12 constitutively active (ca) mutant (Soga et al., 2001).

For short-term, quantitative application of all of these proteins we used protein transduction. In protein transduction, addition of a 10 amino acid peptide from the HIV tat protein allows the uptake of peptides or proteins into live cells. To determine the suitability of protein transduction in T cells, we initially characterized uptake and retention of protein transduction reagents. As immortalization of cells in the generation of cell lines likely changes the regulation of cellular polarization, we used primary, in vitro primed T cells. As a T cell model, we used 5C.C7 TCR transgenic T cells, where polarization has been extensively studied before (Wülfing et al., 2002; Tskvitaria-Fuller et al., 2003; Purtil et al., 2005; Singleton et al., 2006; Tskvitaria-Fuller et al., 2006). The 5C.C7 TCR recognizes the moth cytochrome C 92–103 peptide (MCC) presented by I-E^k (Seder et al., 1992). We expressed our protein transduction reagents in *E. coli*, purified them under native conditions, as detailed in the methods section, and stored them at concentrations of 20 – 100 μ M in PBS. Using conjugation of these proteins to fluorescein, we could establish that they were effectively taken up into primary 5C.C7 T cells by 30 min incubation at 37 °C (Fig. 1A). In comparison to protein expression by retroviral transduction, where expression levels can span several orders of magnitude (Fig. 1B), the distribution of expression levels was tight (Fig. 1A). This limits cell-to-cell variability in single cell analyses. To determine retention of protein transduction reagents, the primary protein-transduced 5C.C7 T cells were washed and incubated further at 37 °C. Loss of tat Cdc42dn was slow with a half-life of 3.75 h (Fig. 1A). This was slower than the loss of the tat peptide by itself (half life of 1.5 h, C.W., data not shown), possibly because prenylation of tat Cdc42dn (Roskoski, 2003) improved retention through membrane anchorage. In summary, in primary T cells uptake of protein transduction reagents was fast and dose-dependent, and loss of tat Cdc42dn was slow on the time scale of the experiments to monitor T cell polarization.

Next, we assessed the subcellular localization of protein transduction reagents. Of particular interest is the question, whether protein transduction reagents can gain access to the cytoplasm or become trapped in endocytic vesicles. Endosomal uptake is the standard uptake route for fluid phase material and a specific form of fluid phase uptake, macropinocytosis, has been implicated in the uptake of protein transduction reagents (Wadia et al., 2004). To assess the subcellular localization of protein transduction reagents under the same conditions as used in the investigation of T cell polarization, we incubated 5C.C7 T cells with fluorescein-conjugated tat Cdc42dn at 37 °C for 30 min, washed the T cells, and immediately imaged them on a spinning disk confocal microscope. To concurrently label endosomes, we incubated the T cell with Tetramethylrhodamine (TMR)-conjugated Transferrin in parallel. As a control for a compound that is taken up by fluid phase uptake without known subsequent access to the cytoplasm we used Oregon Green 488, a more photostable derivative of fluorescein. As expected, Oregon Green 488 and TMR-transferrin remained predominantly vesicular (Fig. 2A). They were well colocalized with a Pearson's coefficient of 0.6 (Fig. 2B). In contrast, the distribution of fluorescein-conjugated tat Cdc42dn was more even (Fig. 2A). Colocalization with TMR-transferrin was significantly ($p < 0.001$) less than that of Oregon Green 488 with a Pearson's coefficient of 0.25 to 0.3 (Fig. 2B). Fluorescein-conjugated tat Rac1dn behaved similar to tat Cdc42dn (Fig. 2B). These localization data strongly suggest access of a substantial portion of tat Cdc42dn and tat Rac1dn to the cytoplasm. Corroborating access of tat Cdc42dn to the cytoplasm, we show below that tat Cdc42dn could interfere with the recruitment of a Cdc42 biosensor, a retrovirally expressed cytoplasmic fluorescent protein, to the T cell/APC interface.

To verify that protein transduction reagents can localize to established locations within the cytoplasm, we used a tat-tagged version of a ZAP-70 tandem SH2 domain-GFP fusion protein.

In polarized primary T cells, the kinase Syk that is highly related to ZAP-70 is recruited to the tip of the uropod through SH2 domain-mediated binding to Ezrin/Radixin/Moesin proteins (Yonemura et al., 1998; Urzainqui et al., 2002). Such localization was indeed found with the tat-tagged version of a ZAP-70 tandem SH2 domain-GFP fusion protein (Fig. 2C). This localization is distinct from that of endosomes that are located at the base of the uropod, as determined by staining for active Arf6 (C.W., unpublished). In summary, data using three different protein transduction reagents strongly suggest that a majority of the material can gain access to cytoplasm. Our data do not exclude localization of a part of the protein transduction material in endosomal vesicles. A minority of the primary T cells protein transduced with tat Cdc42dn or tat Rac1dn actually showed some colocalization with TMR-transferrin consistent with such localization (C.W., data not shown). But even in these cells, substantial staining outside the endosomal vesicles (as shown in Fig. 2A) was always present.

3.2. Single cell analysis of Cdc42 activity

Having established protein transduction as a suitable means to deliver reagents to manipulate Cdc42 activity in primary T cells, we needed to determine the effect of these reagents on Cdc42 activity. Traditionally, Cdc42 activity is determined through specific precipitation of active Cdc42 from cell extracts and quantification of the precipitate by Western blotting. However, this assay has substantial limitations for the investigation of Cdc42 activity in T cell/APC couples. Activation by APC was not feasible, as APCs contributed a high background of active Cdc42. Spatio-temporal patterns of Cdc42 activity cannot be determined. As an alternate approach to assay Cdc42 activity, we have therefore generated a live cell biosensor for active Cdc42, as described in detail elsewhere (Tskvitaria-Fuller et al., 2006). Briefly, the GTPase-binding domain (GBD) of WASP binds to GTP-bound Cdc42 with reasonable affinity ($K_d \sim 100$ nM) and high selectivity (Symons et al., 1996; Rudolph et al., 1998; Abdul-Manan et al., 1999). 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. This sensor was expressed in primary in vitro primed 5C.C7 T cells by retroviral transduction. These T cells were then activated by CH27 B cell lymphoma APCs in the presence of a high concentration (10 μ M) of agonist peptide. For clarity, we would like to emphasize that the GFP-labeled and prenylated version of the WASP GBD used as the biosensor is expressed in primary T cells without a tat tag by retroviral transduction. The generally modest expression levels achieved in such a manner in primary T cells (less than 1/10th of what is commonly seen in cell lines) allowed us to image active Cdc42 without significant interference with T cell activation (Tskvitaria-Fuller et al., 2006). In contrast, that tat-tagged version of the WASP GBD could be used at high concentrations due to the quantitative nature of protein transduction. Interference with Cdc42 function (see below) and T cell activation, as e.g. measured by IL-2 secretion (see below), could thus be achieved.

For our single cell analysis of Cdc42 activity, we distinguished two spatial and two temporal patterns. We addressed whether Cdc42 is active over the entire T cell/APC interface or selectively at its center (Fig. 3). 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 (Monks et al., 1997; Monks et al., 1998). We analyzed 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 towards the edges of the interface (Tskvitaria-Fuller et al., 2003), the uropod retracts (Montoya et al., 2002), and a deep invagination forms at the center of the interface (Singelton et al., 2006). In the subsequent about four minutes, proximal T cell signaling peaks. Our analysis of Cdc42 activity within the second minute after cell couple formation is meant to represent this phase. To quantify Cdc42 sensor accumulation, we have defined two indices (Tskvitaria-Fuller et al., 2006). For convenience, we repeat these definitions here. 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 intensity of the Cdc42 sensor within the area of interface accumulation was $\geq 40\%$ above the cellular fluorescence background. Such thresholding was required, as specific interface accumulation becomes difficult to distinguish from random intensity fluctuations below this threshold. The percent T cells above threshold under various experimental conditions for the most part varied little and are given in Fig. 4A. When the interface intensity was above this threshold, the fraction of total fluorescence that was translocated to the interface was determined from our 3-dimensional imaging 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 without any Cdc42 enrichment), and we expressed the difference as fraction of the total integrated fluorescence of the T cell. The accumulation index was then defined as

$$\text{Accumulation Index} = \text{Fraction of cell couples above threshold} \times \text{Average fraction of sensor translocation to the interface}$$

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. 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

$$\text{Centrality Index} = \frac{\text{Fraction of cell couples above threshold} \times \text{Average fraction of sensor translocation to the interface}}{\text{Area of accumulation as fraction of the interface area}}$$

Examples, how T cells with high/low centrality/accumulation indices look are given in Fig. 3.

Conventionally, the questions of how much of a protein accumulates at the interface or at the center thereof is answered by determining the percentage of cell couples with accumulation above an intensity threshold without or with additional geometrical constraints, respectively. When analyzing the same imaging data, index- and percentage-based analysis gave qualitatively comparable data, as shown in the comparison between figures 3 and 4. However, in the index-based analysis, to gain statistical significance with $0.005 < p \leq 0.05$ for comparisons between experimental conditions, the index-based value changed an average of 26 %, in the percentage-based analysis this average change was significantly ($p < 0.05$) larger at 39 %. Similarly, to gain statistical significance with $p \leq 0.005$, the average change in an index value was 50 %, in the percentage-based analysis 67 %. The index-based analysis thus provided superior distinction. We will use this distinction to assess localized Cdc42 activation across different experimental conditions, as detailed below (Fig. 3, table I). Furthermore, we have already used these indices to relate specific patterns of Cdc42 activity to distinct elements of Cdc42 activation (Tskvitaria-Fuller et al., 2006).

3.3. Substantial Cdc42 activity is required to allow efficient T cell/APC couple formation

To reliably analyze polarization during T cell activation, T cell/APC couples have to form efficiently. To directly test an involvement of Cdc42 activity in cell couple formation, we used increasing amounts of tat Cdc42dn in the interaction between 5C.C7 primary T cells with CH27 APC in the presence of a full stimulus (10 μM MCC peptide). Upon addition of no, 100 nM, or 350 nM tat Cdc42dn, 50 – 60 % of T cells contacting an APC formed a cell couple. However, adding 1 μM tat Cdc42dn significantly ($p < 0.05$) reduced cell coupling efficiency to 27 %

(Tskvitaria-Fuller et al., 2006)(Fig. 5). Interference with Cdc42 activity thus does not necessarily but can impede cell couple formation.

To obtain an estimate how much Cdc42 activity was required to allow efficient cell couple formation, we related cell couple formation to Cdc42 activity under a variety of physiological T cell activation conditions. Using 10 $\mu\text{g/ml}$ of anti-B7-1 plus anti-B7-2 antibodies in the interaction of primary 5C.C7 T cells with CH27 APC in the presence of a full stimulus (10 μM MCC agonist peptide) ('B7 blockade') did not significantly interfere with cell couple formation (Fig. 5). $42 \pm 17\%$ of T cells that contacted an APC formed a cell couple, as compared to $49 \pm 13\%$ in the presence of 10 μM MCC agonist peptide (Fig. 5). Nevertheless, localized Cdc42 activation was significantly ($p < 0.05$) reduced in the second minute after cell couple formation. The accumulation index was reduced by 36 %, the centrality index by 31 % (Fig. 3, table I). A 100-fold reduction of the MCC agonist peptide concentration ('0.1 μM MCC') led to a modest interference with cell couple formation. Only $32 \pm 14\%$ of T cells contacting an APC formed a cell couple ($p = 0.005$ against 10 μM MCC)(Fig. 5). Localized Cdc42 activity was more severely diminished ($p \leq 0.01$ versus 10 μM MCC). The accumulation index at 1 min and 2 min after cell couple formation was reduced by 19 % and 31 %; the centrality index at 1 min and 2 min was reduced by 31 % and 51 % (Fig. 3, table I). Using a B7 blockade plus 10 $\mu\text{g/ml}$ anti-ICAM-1 antibodies and 0.1 μM MCC agonist peptide in the interaction of primary 5C.C7 T cells with CH27 APC ('limiting'), only $12 \pm 6\%$ of the T cell formed cell couples ($p < 0.001$ versus 10 μM MCC)(Fig. 5). Localized Cdc42 activation was dramatically diminished ($p < 0.001$ versus 10 μM MCC). The accumulation/centrality indices at 1 min and 2 min after cell couple formation were reduced by 63 %/85 % and 60 %/81 % (Fig. 3, table I). In summary, upon B7 blockade or 0.1 μM agonist peptide, conditions with at most mild inhibition of cell couple formation, substantial Cdc42 activity remained, about 70 – 80 % of that of a full stimulus (10 μM MCC agonist peptide). Limiting T cell activation conditions led to severe interference with cell couple formation and with little Cdc42 activity remaining (about 20 – 35 % of that of a full stimulus). These relations support the notion that substantial Cdc42 activity, about $\frac{3}{4}$ of maximal, was required to achieve unimpeded cell couple formation. As corroboration, 70 – 95 % of Cdc42 activity remained upon addition of 100 nM or 350 nM tat Cdc42dn to a full stimulus (10 μM MCC peptide)(as detailed in the next paragraph), conditions where cell couple was barely unimpeded (Fig. 5).

The investigation of T cell polarization in T cell/APC couples requires effective cell couple formation. The analysis presented in this paragraph illustrates that this requirement can only leave a rather modest interference with the activity of a signaling intermediate as the appropriate experimental conditions. In such case, the ability to employ dose-responses of inhibitors of the activity of a signaling intermediate becomes particularly important.

3.4. Optimized doses of tat Cdc42dn and tat WASP GBD

Once a concentration window with unimpeded cell couple formation was established, reagent concentrations has to be optimized within this window. Reasonable concentrations of the interfering reagents Cdc42dn and WASP GBD would achieve a strong blockade of their targets, Cdc42 guanine nucleotide exchange factors (GEFs) that activate Cdc42 and active Cdc42 itself, respectively. To achieve a strong blockade, we wanted to use Cdc42dn and WASP GBD at at least 10 times the affinity constants of the Cdc42/Cdc42GEF (≤ 5 nM, based on data on Ras (Lenzen et al., 1998)) and WASP GBD/active Cdc42 interactions (20 nM in vitro (Seth et al., 2003)), respectively. As minimal concentrations we used 100 nM tat Cdc42dn and 1 μM tat WASP GBD. The initial concentration of tat WASP GBD is relatively higher, as the WASP GBD is devoid of any secondary targeting motifs that could enrich it in the vicinity of active Cdc42, as opposed to endogenous Cdc42 effectors it competes with (such as WASP with

additional proline-rich regions (Cannon et al., 2001)). WASP GBD thus is likely at a competitive disadvantage.

Saturation of phenotypes indicates reagent concentration in excess of those minimally needed. Therefore, we tested whether increasing the minimal reagent concentrations up to those that interfere with cell couples formation would lead to more effective interference with Cdc42 activity. In blocking Cdc42 activity, 350 nM tat Cdc42dn did not show a more severe phenotype than 100 nM (Fig. 3, table I). Using 100 nM and 350 nM tat Cdc42dn in the interaction of primary 5C.C7 T cells with CH27 APC in the presence of a full stimulus (10 μ M MCC agonist peptide) led to modest but mostly significant interference with localized Cdc42 activation. The accumulation index at 1 min and 2 min after cell couple formation was reduced by 6 % and 18 % by addition of 100 nM tat Cdc42dn, and by 23 % and 12 % by addition of 350 nM tat Cdc42dn. Similarly, the centrality index at 1 min and 2 min was reduced by 27 % and 23 % by the addition of 100 nM tat Cdc42dn and by 20 % and 9 % by addition of 350 nM tat Cdc42dn. Importantly, these reduction were not much smaller than those of a B7 blockade or of reducing the agonist peptide concentration 100-fold (Fig. 3, Table I), establishing a physiologically significant extent of inhibition. These data suggest that 100 nM or 350 nM tat Cdc42dn blocked the majority of the Cdc42 activity that could be blocked without interfering with cell couple formation. 350 nM tat Cdc42 was also not more effective in slowing MTOC reorientation and blocking T cell cycle entry (Tskvitaria-Fuller et al., 2006). 100 nM tat Cdc42dn thus was as effective as 350 nM in all assays tested, most importantly in the interference with Cdc42 activation. To maximize specificity, we used 100 nM as the optimized concentration of tat Cdc42dn.

For WASP GBD the situation was more complex. Interference with localized Cdc42 activity was comparable with 1 μ M and 3 μ M tat WASP GBD (Fig. 3, table I). Using 1 μ M and 3 μ M tat WASP GBD in the interaction of primary 5C.C7 T cells with CH27 APC in the presence of a full stimulus (10 μ M MCC agonist peptide), the centrality index at 1 min and 2 min after cell couple formation was reduced by 49 % and 29 % by the addition of 1 μ M tat WASP GBD and by 31 % and 15 % by addition of 3 μ M tat WASP GBD. These numbers were again comparable to those upon B7 blockade and a 100-fold reduction in the concentration of agonist peptide, establishing a physiologically significant extent of inhibition. However, 3 μ M tat WASP GBD was more effective than 1 μ M tat WASP GBD in blocking a critical downstream effector function of T cell activation, IL-2 secretion. Using 1 μ M and 3 μ M tat WASP GBD in the interaction of primary 5C.C7 T cells with CH27 APC in the presence of a high concentration (10 μ M) agonist peptide, IL-2 secretion was reduced by 15 ± 13 % and 33 ± 5 % ($p = 0.005$), respectively. For comparison B7 blockade reduced IL-2 secretion by 47 ± 19 %. Deciding between 1 μ M and 3 μ M as the most appropriate concentration, we had to weigh stronger biological effects against an increased risk of reduced specificity. We chose 3 μ M tat WASP GBD to account for the likely competitive disadvantage of WASP GBD with respect to endogenous effectors of Cdc42, as discussed above.

Corroborating the utility of reagent titration, addition of 1 μ M tat Cdc42dn surprisingly yielded increased Cdc42 sensor accumulation versus buffer only at the T cell/APC interface (Fig. 3) immediately after cell couple formation. In the interaction of primary 5C.C7 T cells with CH27 APCs in the presence of a full stimulus (10 μ M MCC agonist peptide), the accumulation index at 1 min after cell couple formation was increased by 53 % by addition of 1 μ M tat Cdc42dn ($p < 0.01$). The centrality index at 1 min was increased by 29 % ($p = 0.07$). 1 μ M tat Cdc42dn at the same time reduced the amount of Cdc42.GTP in antibody-activated primary 5C.C7 T cells by 57 % as determined biochemically (Tskvitaria-Fuller et al., 2006). Most likely direct binding of the GBD in the Cdc42 sensor to Cdc42dn, as described for the p21-activated kinase GBD (Hoppe and Swanson, 2004), was detected at 1 μ M tat Cdc42dn. The Cdc42 biosensor containing the WASP GBD thus could possibly be recruited to the Cdc42 GEFs at the T cell/

APC interface via Cdc42dn, yielding increased interface accumulation. Binding of the p21-activated kinase GBD to Cdc42dn in the biochemical pull-down assay could possibly compete with binding to endogenous active Cdc42, yielding decreased Cdc42-GTP precipitation. Without the ability to titrate Cdc42dn, we might have well concluded that Cdc42dn is not a useful reagent to interfere with Cdc42 activity in primary T cells.

In summary, protein transduction allowed the determination of the minimal concentrations of Cdc42dn and WASP GBD, where suppression of Cdc42 activity was maximized without leading to interference with cell couple formation. Such adjustment of reagent concentrations is an important element in balancing reagent efficacy and specificity. In addition as shown elsewhere (Tskvitaria-Fuller et al., 2006), protein transduction allowed dose responses in the enhancement of T cell/APC interface actin accumulation by the constitutively active mutant of Cdc42. Such dose response provided more convincing evidence for a role of Cdc42 in regulating T cell/APC interface actin accumulation than a single dose experiment.

3.5. Rac1dn interferes with global but not localized Cdc42 activation

Protein reagents as tools to manipulate protein function often have inherent limitations in their specificity. Here we describe how the combination of a live cell biosensor with protein transduction allowed the use of biochemically similar protein reagents with substantial specificity in live cells. Cdc42dn and Rac1dn function by blocking access of endogenous GTPases to guanine nucleotide exchange factors (GEF) (Rossman et al., 2005). As it is likely that Cdc42 and Rac1 share GEFs, some overlap between the effects of Cdc42dn and Rac1dn is expected. To assess the specificity of Cdc42dn at its optimized concentration, we compared interference with Cdc42 activation by 100 nM tat Cdc42dn and 100 nM tat Rac1dn. Both interfered with Cdc42 activation comparably when assessed biochemically by Cdc42/GTP pulldown (Fig. 6). Adding 100 nM tat Cdc42dn or 100 nM tat Rac1dn to primary 5C.C7 T cells in the presence of a full stimulus (10 µg/ml anti-CD3 and anti-CD28 antibodies) reduced the cellular Cdc42/GTP content by 50 % and 52 %, respectively ($p \leq 0.05$). Interestingly however and in contrast to Cdc42dn, Rac1dn could not interfere with the accumulation of active Cdc42 at the T cell/APC interface (Fig. 3, table I). Using 100 nM tat Rac1dn in the interaction of primary 5C.C7 T cells with CH27 APC in the presence of a full stimulus (10 µM MCC agonist peptide) led to only insignificant changes in localized Cdc42 activation. The accumulation index at 1 min and 2 min after cell couple formation was increased by 3 % and 6 % by addition of 100 nM tat Rac1dn. The centrality index at 1 min was increased by 1 % and at 2 min it was reduced by 6 % by the addition of 100 nM tat Rac1dn. These data suggest that overlap in GEF usage by Rac and Cdc42, while substantially affecting overall GTP loading of Cdc42, did not interfere with its localized activation. Accordingly, in biological assays, effects of 100 nM tat Rac1dn in comparison to those of 100 nM tat Cdc42dn ranged from comparable to no effect at all (Tskvitaria-Fuller et al., 2006), suggesting that for some elements of T cell activation global Cdc42 activity is more important, for others local activity. The determination of Cdc42 activity with respect to its spatio-temporal patterns and, likely, the use of optimized reagent concentrations thus allowed the use of reagents that are biochemically very similar with substantial specificity inside live cells.

4. Discussion

We have outlined a strategy to address the role of Cdc42 in primary T cell activation. The strategy takes into account an essential role of Cdc42 for development/survival and a likely involvement in multiple signal transduction pathways. We used a live cell Cdc42 biosensor to distinguish different spatio-temporal patterns of Cdc42 activity and protein transduction to manipulate Cdc42 activity short-term, quantitatively, and with multiple reagents. Here, we have established the utility of protein transduction for this purpose. Uptake of tat fusion proteins

was rapid, with access of a majority of the material to the cytoplasm, and stable on the time scale of the experiments to assess T cell polarization. Protein transduction allowed dose responses and the careful titration of reagents to manipulate Cdc42 activity to optimize specificity. The principal disadvantage of protein transduction is that it can only be as specific as the proteins used. This is mitigated by two elements in our strategy. First, using multiple reagents with independent mechanisms of action allows non-specific side effects to be singled out. Second, analysis of Cdc42 activity with respect to its spatio-temporal patterns revealed substantially higher levels of reagent specificity than whole cell biochemical analysis. Nevertheless, the generation of gene-deficient mice or siRNA will yield specific interference with protein function without having to determine optimized conditions of multiple reagents. One therefore has to weigh, whether the ability to manipulate protein activity short-term, quantitatively, and with multiple reagents or inherent specificity are more important. This will vary from protein to protein. For Cdc42 and similarly critical signaling intermediates that are characterized by a central role for T cell development and survival and by involvement in multiple signaling pathways, we feel that short-term, quantitative, and multiple interference can be more critical. Ideally, both types of approaches can be used together to complement each other. In any case, we suggest that the strategy presented here is a valuable addition to existing methods to study protein function, in particular if the protein under investigation is essential for cell survival and involved in multiple signaling pathways, i.e. of likely critical importance for the regulation of cellular activation.

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Fig. 1. tat Cdc42dn uptake and retention in primary T cells

A Fluorescein-conjugated tat Cdc42dn was incubated for 30 min at 37 °C with in vitro primed primary 5C.C7 T cells. Protein uptake and retention were analyzed by FACS with traces displayed on top. As a filled purple trace, autofluorescence of untreated cells is shown. As filled pink and green traces uptake after incubation with 2 or 10 μM tat Cdc42dn is given, respectively. Open traces denote remaining tat Cdc42dn fluorescence after transduction of 5C.C7 T cells with 10 μM tat Cdc42dn and subsequent incubation in the absence of tat Cdc42dn for 1 (dark pink), 2 (blue), 3 (orange), or 4 h (brown) at 37 °C. On the bottom, mean fluorescence of the traces is plotted as indicated. Data for 0 – 5 h are after initial transduction with 10 μM tat Cdc42dn. Exponential fitting of these data (with a correlation coefficient of $R^2 = 0.87$) was

used to determine the half life of tat Cdc42dn. In **B**, the distribution of expression levels upon retroviral transduction of primary 5C.C7 T cells with actin-GFP is shown for comparison. The large peak on the left is cellular autofluorescence.

Fig. 2. Subcellular distribution of protein transduction reagents

In **A**, 5C.C7 T cells were incubated with fluorescein-conjugated tat Cdc42dn or Oregon Green 488, each together with Tetramethylrhodamine-conjugated Transferrin. Representative spinning disk confocal slices of these two-color experiments are shown. Images are scaled comparably throughout. Each column contains images from one cell, as labeled at the right. The Pearson's coefficient of colocalization ('Rp') for the entire cells is given under the images of each cell. In **B**, a quantitative analysis of the imaging data shown in **A** is given. 5C.C7 T cells were labeled with TMR-Transferrin together with the reagent listed on the left. Protein transduction reagents were fluorescein-conjugated. Average Pearson's coefficients with standard deviations are given. An asterisk indicated statistical significance with $p < 0.001$

against the matching Oregon Green data. 19 – 26 cells (on average 24) were analyzed per experiment. In **C**, 5C.C7 T cells were incubated with 10 μ M tat ZAP-70 tandem SH2-GFP. Matching bright field and fluorescence images (as individual 3D slices) are shown for three T cells. Fluorescence intensity is encoded in a rainbow false color scale (increasing from blue to green, yellow, and red). The uropod is visible as a posterior extension of the T cell. tat ZAP-70 tandem SH2-GFP accumulation at the tip of the uropod can be seen.

Fig. 3. Inhibition of Cdc42 activation at the T cell/APC interface without concomitant blockade of cell couple formation is saturated at 100 nM tat Cdc42dn and 1 μ M tat WASP GBD

Cdc42 biosensor-transduced 5C.C7 T cells were activated by CH27 APCs under the indicated conditions. The agonist peptide concentration was 10 μ M MCC (full stimulus) unless noted otherwise. 'anti-B7' indicates 10 μ g/ml anti-B7-1 plus anti-B7-2, 'limiting' indicates 0.1 μ M MCC plus anti-B7 plus 10 μ g/ml anti-ICAM-1 antibody. Accumulation (A) and centrality (B) indices are given within the first (1') and second (2') minute after cell couple formation. For the 1 min time point, data from 0:00/0:20/0:40 after tight cell couple formation were pooled, for the 2 min time point, data from 1:00/1:20/1:40. Within the first minute distinct and dramatic morphologically changes occur. In the subsequent about four minutes, represented here by the

2' data, proximal T cell signaling peaks. The concentrations of tat Cdc42dn and tat WASP GBD added are listed. '100/350 nM Cdc42dn' and '1/3 μ M WASP GBD' refer to pooled data for 100 nM and 350 nM tat Cdc42dn and for 1 μ M and 3 μ M tat WASP GBD, respectively. Data for the presence of 100 nM tat Rac1dn are shown to allow assessment of the overlap in Cdc42dn and Rac1dn function. p-values (Student's t-test) versus 10 μ M MCC are given at the right of each panel in parentheses. The left number refers to the 1 min data, the right number to the 2 min data. 'n.s.' indicates $p > 0.05$. In C, representative images of Cdc42 biosensor-transduced 5C.C7 T cells are given. T cells were activated with a CH27 B cell lymphoma APC in the presence of 10 μ M MCC agonist peptide (full stimulus). Top down maximum projections of 3-dimensional Cdc42 sensor fluorescence data are shown. The Cdc42 sensor fluorescence intensity is displayed in a rainbow false color scale (increasing from blue to green, yellow, and red). APCs are not visible. In the four panels from left to right, the T cell/APC interfaces are on the bottom right, bottom left, right, and bottom, respectively. The fraction of sensor translocated to the interface ('% accumulation') and the ratio of (the fraction of sensor translocated to the interface) to (the area of accumulation as a fraction of the interface area) ('% acc/area'), i.e. the two single cell measures that are part of the accumulation and centrality indices are given under each cell. The first and fourth image from the left represent high/low accumulation, the second and third high/low centrality.

Fig. 4. Inhibition of Cdc42 activation at the T cell/APC interface without concomitant blockade of cell couple formation is saturated at 100 nM tat Cdc42dn and 1 μ M tat WASP GBD

An alternate analysis of the data presented in Fig. 3 is shown. % cell couples with sensor accumulation in any pattern (A) or in a central pattern (B) are given within the first (1') and second (2') minute after cell couple formation as in Fig. 3. ' % cell couples with accumulation in any pattern ' is the same number as the 'fraction of cell couples above threshold' in the calculation of the accumulation and centrality indices.

Fig. 5. Limiting T cell activation conditions interfere with T cell couple formation

The percentage of primary 5C.C7 T cells contacting a CH27 APC under the indicated conditions (top four bars as Fig. 3; bottom four bars 10 μ M MCC with increasing concentrations of tat Cdc42dn) that form a tight cell couple is given with standard deviations. One/two asterisks indicate statistical significance against the matching 10 μ M MCC data set with $p \leq 0.05/0.001$, respectively (Student's t-test). For the top four conditions, on average 242 (205 – 294) T cells were analyzed per condition from at least two independent groups of experiments. The bottom four conditions are a duplicate of the data presented in (Tskvitaria-Fuller et al., 2006) to facilitate comparison. On average 22 cells from at least two independent experiments were analyzed.

Fig. 6. Cdc42dn and Rac1dn block Cdc42 activity to the same extent when analyzed biochemically Cdc42 GTP was precipitated from 5C.C7 T cell extracts with the PAK GBD and quantified by Western blotting. Data are given as the average amount of precipitated Cdc42 GTP $\pm$ standard deviation relative to a full stimulus. The T cell activation conditions were: 10 μ g/ml anti-TCR plus 10 μ g/ml anti-CD28 (full stimulus), 10 μ g/ml anti-TCR only, 0.1 μ g/ml anti-TCR plus 10 μ g/ml anti-CD28 (low anti-TCR), and 10 μ g/ml anti-TCR plus 10 μ g/ml anti-CD28 plus 100 nM tat Cdc42dn or 100 nM tat Rac1dn. 2 - 4 independent experiments were performed per condition.

Table I

Inhibition of Cdc42 activation at the T cell/APC interface without concomitant blockade of cell couple formation is saturated at 100 nM tat Cdc42dn and 1 μ M tat WASP GBD

Reductions	Accumulation index 1'	Accumulation index 2'	Centrality index 1'	Centrality index 2'	Average
B7 blockade	9 %	36 %	6 %	31 %	21 %
0.1 μ M MCC	19 %	31 %	31 %	51 %	33 %
100 nM Cdc42dn	6 %	18 %	27 %	23 %	19 %
350 nM Cdc42dn	23 %	12 %	20 %	9 %	16 %
1 μ M WASP GBD	- 4 %	- 6 %	49 %	29 %	17 %
3 μ M WASP GBD	- 13 %	- 5 %	31 %	15 %	7 %
Average	7 %	14 %	27 %	26 %	
100 nM Rac1dn	- 3 %	- 6 %	- 1 %	6 %	- 1 %
limiting	63 %	60 %	85 %	84 %	73 %

Cdc42 biosensor-transduced 5C.C7 T cells were activated by CH27 APCs under the indicated conditions. The agonist peptide concentration was 10 μ M MCC (full stimulus) unless noted otherwise. 'anti-B7' indicates 10 μ g/ml anti-B7-1 plus anti-B7-2, 'limiting' indicates 0.1 μ M MCC plus anti-B7 plus 10 μ g/ml anti-ICAM-1 antibody. The concentrations of tat Cdc42dn and WASP GFP added are listed. Data for the presence of 100 nM tat Rac1dn are shown to allow assessment of the overlap in tat Cdc42dn and Rac1dn function. Accumulation and centrality indices were determined within the first (1') and second (2') minute after cell couple formation and are given in Fig. 3. For the 1 min time point, data from 0:00/0:20/0:40 after tight cell couple formation were pooled, for the 2 min time point, data from 1:00/1:20/1:40. Here the reductions in the indices in comparison to a full stimulus (10 μ M MCC agonist peptide) are given. Averages across different localization patterns for individual experimental conditions (last, bolded column) are given to allow an assessment of changes of the overall localization of Cdc42 activity between experimental conditions. Averages across experimental conditions for individual indices (bolded row for the values above) are given to allow an assessment of the sensitivity of individual indices to experimental manipulation.