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A Network of PDZ-Containing Proteins Regulates T Cell Polarity and Morphology during Migration and Immunological Synapse Formation

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Summary

T cell shape is dictated by the selective recruitment of molecules to different regions of the cell (polarity) and is integral to every aspect of T cell function, from migration to cytotoxicity. This study describes a mechanism for the regulation of T cell polarity. We show that T cells contain a network of asymmetrically distributed proteins with the capacity to dictate the subcellular localization of both cell surface receptors and morphological determinants in T cells. Proteins from the Scribble, Crumbs3, and Par3 complexes, previously shown to regulate epithelial polarity, were polarized in T cells containing either uropods or immunological synapses. Reduction in Scribble expression prevented the polarization of cell surface receptors and prevented morphological changes associated with uropod formation, migration, and antigen presentation. By dynamically coordinating molecular distribution throughout the T cell, this network provides a mechanism by which T cell function and polarity are linked.

Introduction

T cell migration, proliferation, homotypic interaction, activation in response to antigen presentation, and cytotoxicity all require cell polarization (Davis and Dustin, 2004; Montoya et al., 2002; Stinchcombe et al., 2004; Wei et al., 1999). T cell polarization involves morphological changes that are dictated by the recruitment of surface receptors, signaling complexes, and cellular organelles to discrete functional domains (Friedl and Storim, 2004; van der Merwe, 2002; Vicente-Manzanares et al., 2002; Wei et al., 1999). Two such domains are the immunological synapse (IS), formed during antigen presentation and T cell cytotoxicity, and the uropod, a single protrusion formed during cell-cell interactions and migration. IS formation involves both molecular scaffolding, by proteins such as CD2AP and Ezrin, and localized intracellular signals that affect cytoskeletal and membrane activities (Cullinan et al., 2002; del Pozo et al., 1999; Dustin et al., 1998; Etienne-Manneville and Hall, 2002; Ivetic and Ridley, 2004; Lee et al., 2004). IS formation is accompanied by a coordinated reorganization of the whole T cell, with recruitment of proteins such as Ezrin and CD43 to the distal pole of the cell and translocation of the microtubule organizing center (MTOC) to the IS (Cullinan et al., 2002; Das et al., 2002; Faure et al., 2004; Kuhn and Poenie, 2002; Samstag et al., 2003). Although many of the scaffolds and signals involved in T cell polarization have been elucidated, it is not yet known how particular subdomains of a polarized T cell are defined or how the polarized T cell integrates activities in different regions of the cell.

In contrast, the regulation of epithelial polarity is well understood. We speculated that, despite the striking morphological and functional differences between epithelial cells and T cells, the fundamental principles of polarity determination might be conserved across these cell types. Experiments on *C.elegans*, *Drosophila*, and mammals have recently shown that epithelial polarity is regulated by a network of proteins organized into spatially distinct clusters that can influence the localization and function of each other. The epithelial network comprises three functional units, the Scribble, Par3, and Crumbs complexes, containing many proteins with protein binding motifs called PDZ domains (Etienne-Manneville and Hall, 2002; Macara, 2004; Nelson, 2003). The Scribble functional unit in *Drosophila* comprises Scribble, Lethal giant larvae (Lgl), and Discs large (Dlg) (Bilder, 2004). Each of these proteins has functional mammalian homologs, including four isoforms of Dlg (Dlg1–4) (Humbert et al., 2003). Together, these proteins regulate epithelial polarity by (1) recruiting surface receptors and signaling molecules to the basolateral domain, (2) interacting with the cytoskeleton and other structural elements of the cell, and (3) influencing the positioning and activity of the other PDZ-containing polarity complexes (Humbert et al., 2003).

Of the epithelial polarity proteins, only Dlg1, Dlg4, and PKCζ have previously been identified in T cells, where they have been implicated in T cell signaling (Giagulli et al., 2004; Hanada et al., 1997; Ludford-Menting et al., 2002; San-Antonio et al., 2002). Dlg1 in T cells interacts with the surface receptors CD2, Kv1.3, and PTA-1 as well as with the tyrosine kinase Lck and the kinesin-like protein GAKIN (Hanada et al., 1997; Hanada et al., 2000; Ralston et al., 2004). Dlg1 has been observed in the T cell uropod (Cullinan et al., 2002) and transiently in the IS upon antigen presentation (Xavier et al., 2004). Reduction in Dlg1 expression resulted in increased NFAT activation, leading to the proposal that Dlg1 acts as a negative regulator of T cell signaling (Xavier et al., 2004). We proposed that these seemingly disparate activities might all relate to the existence of a polarity network in T cells. Indeed, we demonstrate

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here that PDZ-containing polarity proteins are differentially localized throughout polarized T cells and regulate polarity and function. This provides a framework on which T cell polarity can be coordinated throughout the cell and can regulate the morphological changes that occur during IS formation and migration.

Results

Polarity Proteins Are Expressed and Asymmetrically Localized in Uropod-Containing T Cells

The hallmark of many polarity proteins is their asymmetric localization, therefore we investigated the expression and localization of key polarity proteins in activated murine T cells (Figure 1 A, panel i), which comprise both spherical cells (either resting or undergoing mitosis) and cells with a distinctive uropod (identified by arrows). In all uropod-containing cells, Scribble and Dlg (detected with a pan-Dlg antibody to multiple Dlg isoforms) were concentrated in and immediately below the uropod, with little or no fluorescence in the cell body. Similar results were obtained with activated human T cells (Figure 1A, panel ii) and with the uropod-containing murine T cell line MD45 (Figure 1A, panel iii, and Figure S1A, panel i, available in the Supplemental Data with this article online). The localization of Dlg and Scribble was similar to the uropod marker Ezrin (Figure 1A, panel ii) and contrasted with Talin (Figure 1A, panel iii), with the other member of the Scribble complex, Lgl (Figure 1A, panel iii), and with EGFP (Figure S1A, panel ii), which were not polarized. To address the possibility that the polarized staining was an artifact of uneven distribution of cytoplasm, we performed immunohistochemistry and overlayed the images onto differential interference contrast (DIC) images to visualize the cytoplasm (Figure 1B). Dlg and Scribble preferentially stained the uropod even when cytoplasm was clearly visible at the opposite end of the cell, whereas Talin stained both ends of the cell even where very little cytoplasm was evident. Each of the four Dlg isoforms was polarized to the uropod, although with subtle differences in distribution (Figure S1B) indicating that the panDlg antibody staining represents the aggregate distribution of Dlg1, Dlg2, Dlg3, and Dlg4. All antibodies identified bands of the appropriate size by immunoblot (Figure S2), indicating that the staining was specific. Thus, Dlg, Scribble, and Lgl are expressed in T cells, and Dlg and Scribble are asymmetrically localized at the uropod.

In addition to Scribble, Dlg, and Lgl, Crumbs3 and Par3 were detected in T cells by immunoblot (Figure S2). In MD45 cells, Crumbs3 was distributed throughout the T cell but concentrated at the base of the uropod (Figure 1C, panel i). Par3 was preferentially localized in the cell body, with little or no expression in the uropod, but PKCζ was not clearly polarized (Figure 1C, panel i). Two-color staining (Figure 1C, panel ii) indicated that Dlg localized with Scribble and with the uropod markers CD44 and Ezrin at the uropod. The localization of Crumbs3 and Dlg partly overlapped, but Par3 and Dlg were mutually exclusive. These data indicate the existence of a polarity network that is asymmetrically distributed in uropod-containing T cells.

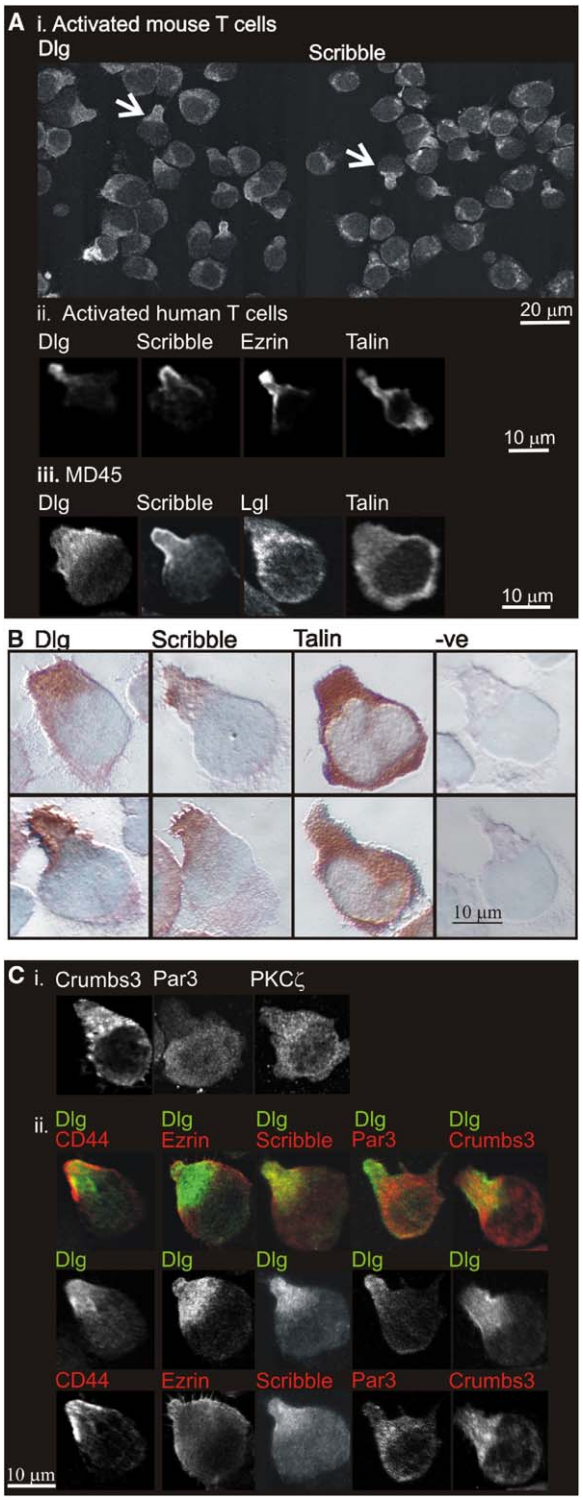

Figure 1. Polarity Proteins Are Asymmetrically Distributed in T Cells Containing Uropods

T cells were stained for the proteins indicated above each image and analyzed by confocal immunofluorescence microscopy (A and C) and immunohistochemistry (B). OT-1 mouse T cells activated with peptide-pulsed splenocytes (A, panel i). Examples of uropod-containing cells are labeled with arrows. Human T cells activated with phytohemagglutinin (A, panel ii). MD45 cells are in (A) panel iii, (B), and (C). Merged images ([C], panel ii, first row) were pseudocolored according to the coloring of the labels above each image.

IS Formation Correlates with the Asymmetrical Distribution of Polarity Proteins

To test whether Dlg and Scribble were polarized during IS formation, conjugates of activated OT-1 splenocytes with peptide-pulsed dendritic cells (DCs) were immunolabeled. Live cell imaging of this interaction indicated that T cells migrated toward and docked onto the DCs with their uropod at the rear of the cell and then rounded up within minutes (Movie S1 and Figure S3). Cells at different stages of this process were stained for CD3 (a component of the TCR and a marker of IS) and Dlg (representative images in Figure 2A). Unconjugated T cells, and T cells that had been incubated with DCs for 2 min, contained a uropod to which Dlg and CD3 was localized (Figure 2A, panels i and ii). By 5 min (Figure 2A, panel iii), the uropod was dismantled, and Dlg was at the contact site between the T cell and the DC. At this time, CD3 was still concentrated away from the contact site. At 10, 15, and 25 min, CD3 was clearly polarized at the IS, but Dlg was relocated away from the contact site (Figure 2A, panel iv) and then concentrated at the distal pole (Figure 2A, panels v and vi). The average intensity of fluorescence at the T cell perimeter (quantitation of Figure 2A, panel vi in panel vii) showed Dlg excluded from the site of the IS. Again, this pattern represented the combined localization of multiple Dlg isoforms, which all showed similar distribution during IS formation (data not shown). A similar pattern was observed for Scribble (Figure 2B), which was concentrated at the uropod in unconjugated cells (Figure 2B, panel i), recruited to the interface at 5 min while CD3 was still at the rear of the cell (Figure 2B, panel ii), and enriched at the distal pole at 15 and 25 min when CD3 was at the IS (Figure 2B, panels iii and iv, and quantitation of panel iv in panel v). In control experiments with unpulsed DCs, CD3 and Dlg were each spread throughout the cell (Figure 2C, panel i), and the nonpolarized localization of $p120^{Cbl}$ (Figure 2C, panel ii) indicates that these results were not an artifact of the staining process. Dlg colocalized with Scribble, consistent with a cooperative role for these two proteins (Figure 2C, panel iii). The recruitment of Dlg and Scribble distal to the synapse is reminiscent of the localization of Ezrin to the distal pole (Cullinan et al., 2002), and indeed, Dlg colocalized with Ezrin (Figure 2C, panel iii, quantitation of panel iv in panel v). Blind scoring indicated that, with peptide-pulsed DCs, CD3 accumulated at the synapse in 56% ± 4% of the cells, and Dlg accumulated at the distal pole in 67% ± 7% of the cells (Figure 2D). In contrast, polarization of CD3 and Dlg in the absence of peptide was only 5% ± 5% and 17% ± 6%, respectively. Similarly, polarization of Scribble and Ezrin to the distal pole was 77% ± 3% and 69% ± 2%, respectively, in the presence of peptide and 16% ± 2% and 21% ± 1%, respectively, in the absence of peptide (Figure 2D). Thus, all four proteins were polarized as a consequence of specific TCR signaling.

We next investigated the localization of other polarity proteins during antigen presentation. Par3 staining was diffuse but concentrated at the synapse by 40 min (Figure 3A). Costaining with Dlg (Figure 3B) indicated that Par3 was excluded from the cell cortex, and this was particularly evident at sites where Dlg was concentrated at the cell surface (Figure 3B, panel iv). Crumbs3 staining overlapped with CD3 (Figure 3C) and was localized posterior to the DC interface at 5 min but moved to the IS with time (see also Figure S5). Like Par3, in regions of the cell where Dlg was concentrated at the cell surface, Crumbs3 was excluded from the cortex (Figure 3D). These data combined indicate that Crumbs3 and Par3 undergo dynamic trafficking separate to Dlg and Scribble during the formation of the IS (see Figure 7), raising the possibility that the surface localization of Par3 and Crumbs3 might be restricted by the Scribble functional unit, as previously reported for *Drosophila* epithelia (Bilder and Perrimon, 2000; Bilder et al., 2003).

Dlg and Scribble Regulate Uropod Formation

To test the hypothesis that these proteins regulate T cell polarity, we targeted Scribble (a member of the *Drosophila* epithelial polarity network with a single mammalian homolog [Dow et al., 2003]) by using shRNA-mediated inhibition of protein expression and screened single cell clones for efficient knockdown. Densitometric quantitation (Figure 4A) indicated a 40% reduction in Scribble expression in clones MD45.3 and MD45.8 and normal levels of expression in the control clone (MD45.12) transduced with an shRNA targeting an irrelevant transcript (EGFP). Control clones had extensive irregularity in shape (Figure 4B, panel i, top row), but clones in which Scribble expression had been reduced were spherical in shape (bottom row). Blind scoring of the morphology (Figure 4B, panel ii) indicated uropods in 89% ± 2% and 83% ± 5% of MD45 and MD45.12, respectively, but only 27% ± 4% and 5% ± 3% of MD45.3 and MD45.8, respectively. A number of other clones with varying degrees of Scribble knockdown demonstrated a dose-dependent effect on uropod formation (data not shown), which is in agreement with the functional dependence on Scribble levels in *Drosophila* epithelium (Zeitler et al., 2004). To test that this effect was specific for Scribble, we attempted to rescue the loss of uropod by overexpression of human Scribble (the sequence of which differs from that targeted by the shRNA sequence). Indeed, overexpression of Scribble in the MD45.3 knockdown cells (Figure 4C, panel i) resulted in a reversion of the rounded shape, with 90% ± 3% and 89% ± 3% polarization in MD45.3 knockdown cell lines transduced with Scribble, compared with 35% ± 3% and 38% ± 11% in MD45.3 knockdown cell lines transduced with a vector control (Figure 4C, panel ii). We investigated whether incubation with Antp-CRIPT peptides, which have previously been shown to block Dlg4 function (Niethammer et al., 1998; Passafaro et al., 1999), affected the uropod. Similar to the results with Scribble shRNA, abrogation of uropod production was observed when cells were incubated with Antp-CRIPT (Figure S4). These data combined indicate that Dlg and Scribble are required for uropod formation and support the notion that Dlg and Scribble act together in T cells.

Dlg and Scribble Regulate the Asymmetric Distribution of Proteins in T Cells

Although the loss of uropod described above might result from defects in morphological determinants (for ex-

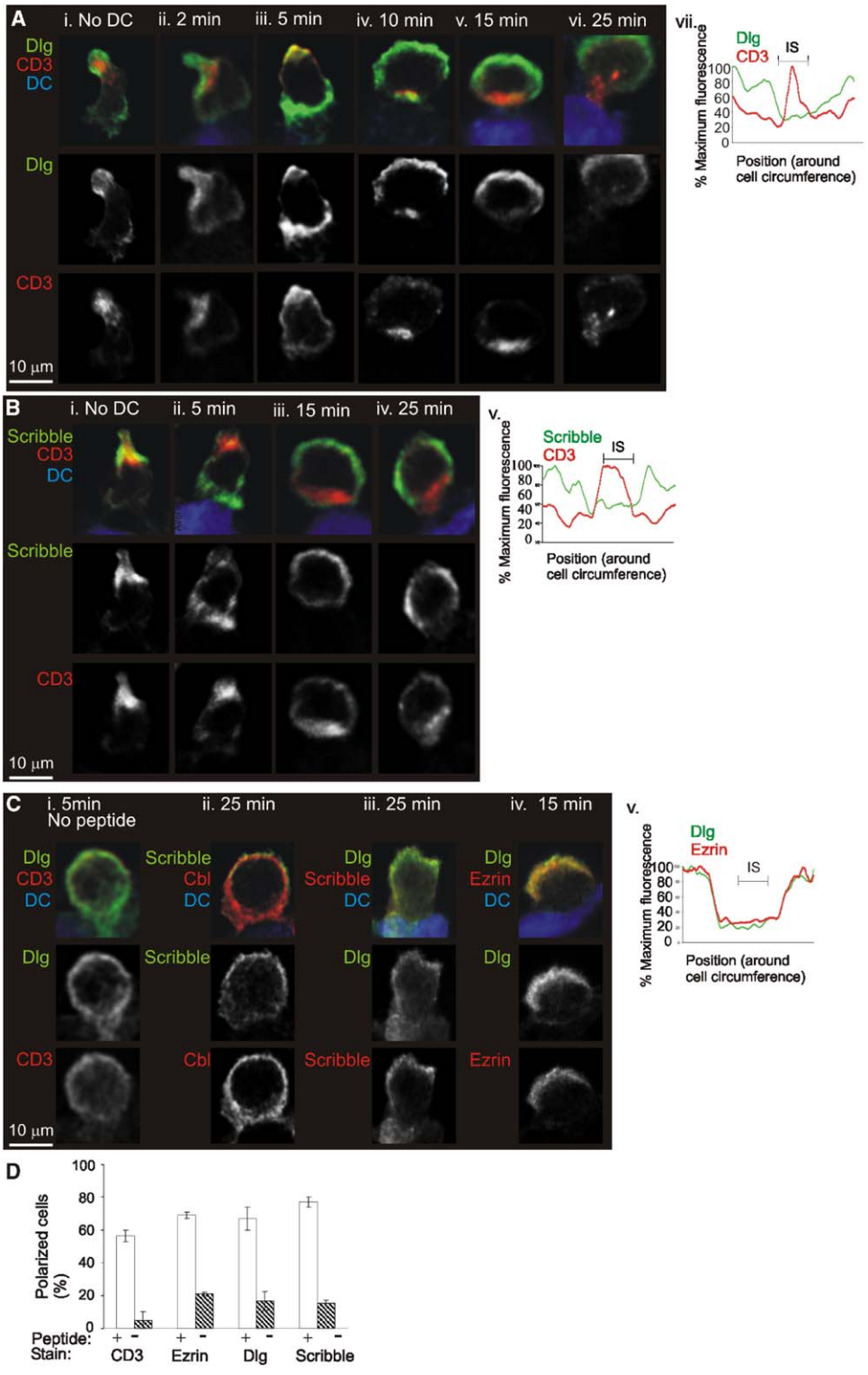

Figure 2. Dlg and Scribble Are Rapidly Recruited to the IS and then Polarize to the Distal Pole

(A–C) T cells from OT-1 mice were incubated with adhered dendritic cells (DCs) pulsed with the SIINFEKL peptide (except [C], panel i, which had not been pulsed with peptide), fixed after defined times, and immunolabeled with Dlg and CD3 (A), Scribble and CD3 (B), Dlg and CD3 ([C], panel i), Scribble and Cbl ([C], panel ii), Dlg and Scribble ([C], panel iii), and Dlg and Ezrin ([C], panel iv). Times after addition of T cells at which cells were fixed and analyzed are indicated above each merged panel. Images are representative of the localization at these time points in at least eight separate experiments. Pseudocoloring of merged images in the first row is blue (DCs), green (as per middle rows), and red (as per third rows). Scale bars representing 10 μm are indicated at the bottom left of each panel. Quantitation of Avi, Biv, and Civ is displayed in Avii, Bv, and Cv, respectively. In these histograms, the fluorescence of the circumference of the T cell was calculated as the

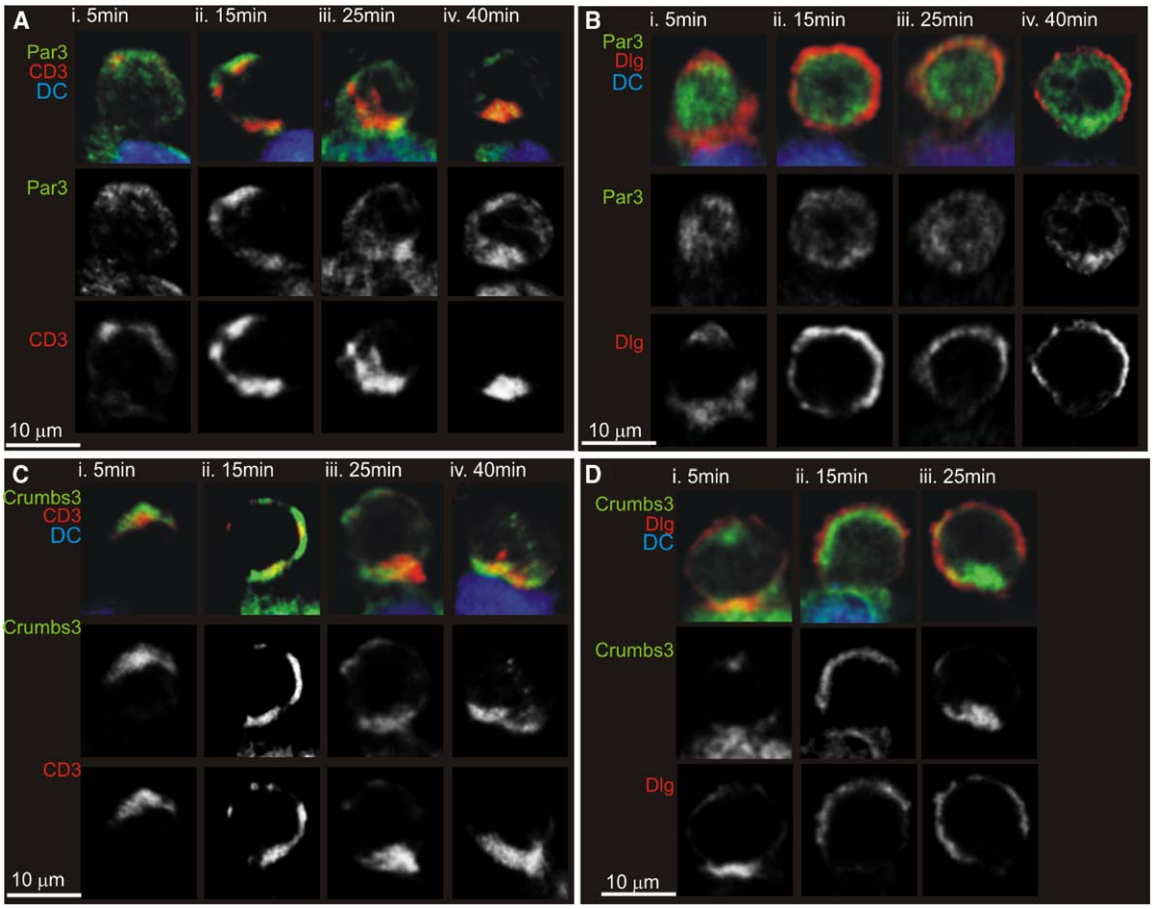

Figure 3. Crumbs3 and Patj Are Recruited to the IS

SIINFEKL-specific T cells undergoing antigen presentation were analyzed as in Figure 2 for the localization of Par3 and CD3 (A), Par3 and Dlg (B), Crumbs3 and CD3 (C), and Crumbs3 and Dlg (D).

ample, defects in actin polymerization or vesicular trafficking), we speculated that an alternative cause might be a reduced polarization of proteins, preventing specification of the site of uropod extrusion (analogous to the failure of axon specification when the Par3 complex is disrupted in mammalian neurons [Shi et al., 2003]). To test whether Scribble regulates the polarization of T cell proteins, we investigated whether reduced Scribble expression resulted in loss of asymmetry of proteins that normally localize to the uropod. Scribble itself, as well as being reduced in intensity as a direct consequence of the knockdown, was evenly distributed throughout the cells (Figure 5A, panel i). Three other proteins that normally polarize to the uropod, Dlg, Ezrin, and the cell surface protein CD44 (Figure 5A, panels ii–iv), also showed no asymmetry in Scribble knockdown cells, indicating that Scribble is not only required for the morphological changes associated with uropod formation but also for the polarization of T cell proteins.

This observation does not, however, indicate whether the role of Scribble and Dlg in protein polarization is direct or indirect, so to explore this further we investigated the polarization in T cells of the human cell surface marker CD46 (Russell, 2004). CD46 is alternatively spliced to yield two cytoplasmic tails, one of which (CD46-Cyt1) binds to Dlg4 via its C-terminal four amino acids, and in epithelial cells, CD46 is basolaterally targeted via the Dlg binding site (Ludford-Menting et al., 2002). Firstly, we showed that CD46 in primary human T cells is localized to the uropod and costains with Dlg (Figure 5B, panel i). Secondly, to determine whether this polarization depended upon Scribble, CD46-Cyt1

rolling (n = 15) average number of fluorescent pixels (y axis) relative to their position along the region (x axis) and plotted as the percentage of maximum fluorescence for each protein. The number of points on the x axis corresponds to the resolution of the pictures. Bar indicates the position of the IS. Each graph is representative of the fluorescent pattern observed for at least 25 conjugates per experiment (n = 4).
(D) Conjugates were scored for polarization at 25 min and plotted as a percentage of the total cells conjugated to a DC (polarization was at the IS for CD3 and at the distal pole for Dlg, Scribble, and Ezrin; white bars represent counts in the presence of peptide, hashed in the absence of peptide, error bars represent the SEM). A total of at least 100 cells from four separate experiments were scored. ***$p < 10^{-10}$ determined by using additive logistic regression.

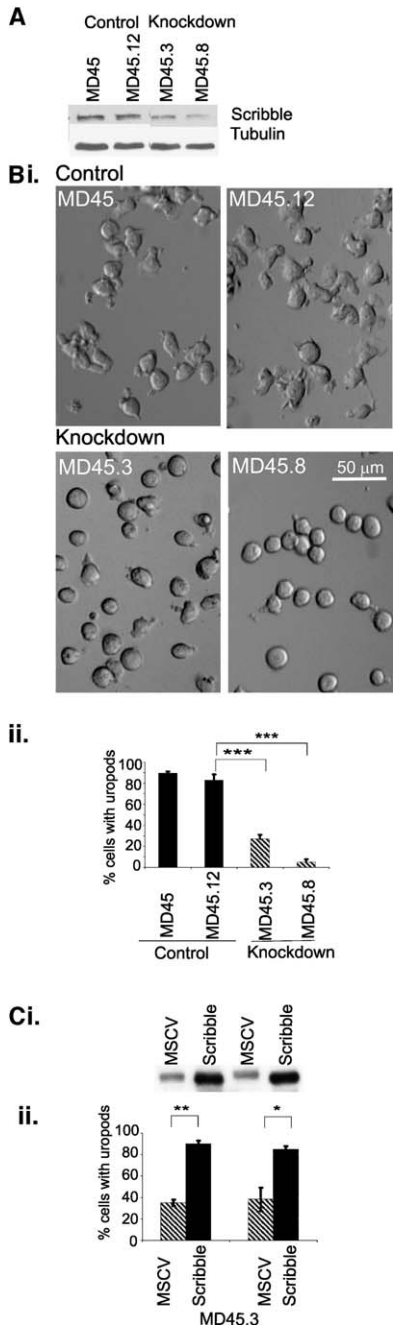

Figure 4. Scribble Is Required for Formation of the Uropod

(A) Immunoblot of MD45 parental cells (lane 1) and of single cell clones of MD45 cells transduced with control shRNA (lane 2) or with Scribble shRNA (lanes 3 and 4), probed with antibody to Scribble (top) or α-tubulin (bottom). The ratio of Scribble to tubulin was determined by using densitometric analysis of three representative immunoblots and was normalized to the ratio of Scribble to tubulin in parental cells.

(B) Transmitted light images of MD45 subclones acquired during live cell imaging (panel i). Cells were scored blind for uropod formation, and the number of polarized cells (expressed as a percentage of total cells) is represented in panel ii. Results are the average of five separate experiments, and SEMs are shown, and two-tailed t tests indicate ***p < 0.0001, **p < 0.001, and *p < 0.05. Black bars are control cells, and hashed bars represent Scribble knockdown cells.

(C) The MD45.3 Scribble knockdown cells were transduced with either MSCV or Scribble, and sorted for EGFP expression. Two independent lines expressing MSCV or Scribble as shown (panel i) were first assessed for Scribble expression by immunoblot (the antibody crossreacts with both human and mouse Scribble) and then (panel ii) analyzed for uropod formation by blind scoring. Results are the average of three separate experiments, SEMs are shown, and two-tailed t tests indicate **p < 0.001 and *p < 0.05. Hashed bars represent Scribble knockdown cells transduced with vector control, and black bars represent Scribble knockdown cells transduced with human Scribble.

was expressed in control and Scribble knockdown cells. Similar to the endogenous proteins shown in Figure 5A, CD46-Cyt1 was asymmetrically distributed in control MD45 cells, but not in Scribble-depleted cells (Figure 5B, panel ii). Similarly, blocking Dlg interactions with the Antp-CRIPT peptide abrogated CD46 polarization (Figure S4). To determine the role of the Dlg binding site in CD46 polarization, we compared the localization of different isoforms of CD46 in MD45 cells (Figure 5C, panel i, quantitation in panel ii). As previously observed for MDCK epithelial cells (Ludford-Menting et al., 2002), CD46-Cyt1 was polarized in 84% ± 3% of cells, but CD46-Cyt2 (which cannot bind Dlg and is not polarized in MDCK cells) was distributed evenly throughout the cell surface (polarized in 2% ± 1% of cells). A mutant of CD46-Cyt1 with a single amino acid substitution at the C terminus, CD46-Cyt1L358R (which cannot bind Dlg and is not polarized in MDCK cells) gave an intermediate pattern, with 35% ± 7% of cells showing polarization of CD46. Although not completely abrogated, the polarization of CD46 was significantly reduced ($p = 0.0001$) when the Dlg binding site was mutated. Thus, Dlg and Scribble both regulate T cell morphology and protein asymmetry in T cells.

Scribble Is Required for T Cell Migration

The uropod has been implicated in T cell migration, so we tested whether the loss of Scribble might affect the nondirected migration of T cells in culture. Cells were imaged by time-lapse microscopy over 30 min, and the migration pathways were traced for quantitation (path traces in Figure 6A, panel i, Movies S2–S5, and quantitated in Figure 6A, panel ii). Control cells exhibited migration of ~4 μm/min that after 10 min resulted in an average distance from origin of 9.1 ± 1.2 μm. In contrast, the MD45.8 Scribble knockdown cells showed substantially less migration, with an average distance from origin after 10 min of 2.1 ± 0.5 μm. MD45.3 knockdown cells transduced with the control vector (MSCV) also showed reduced migration compared to MD45 cells, with an average distance from origin after 10 min of 2.3 ± 0.4 μm. However, expression of human Scribble in the MD45.3 knockdown cells significantly increased the migration, resulting in an average distance from origin after 10 min of 5.9 ± 0.9 mm. Thus, loss of Scribble correlated with significantly reduced migratory capacity in the MD45 T cell line.

Scribble Regulates Morphological Changes during Antigen Presentation

To investigate the role of Scribble in IS formation, we established a protocol for generating surrogate IS for-

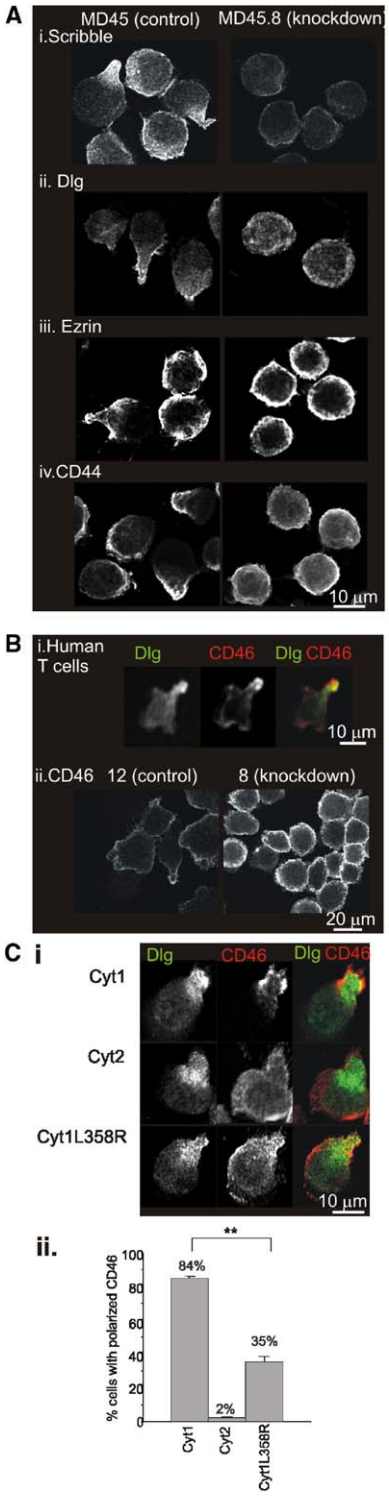

Figure 5. Scribble and Dlg Are Required for the Recruitment of Cell Surface Proteins to the Uropod

(A) MD45 cells (either MD45.12 shRNA control cells or MD45.8 Scribble knockdown cells) were immunolabeled in Scribble (panel i), Dlg (panel ii), Ezrin (panel iii), and CD44 (panel iv). In the left panels showing control cells, groups with both uropod-containing cells and round cells were chosen to demonstrate the polarization.

(B) Activated human peripheral lymphocytes were labeled for Dlg (green) and CD46 (red) (panel i). MD45 cells (either MD45.12 shRNA control cells or MD45.8 Scribble knockdown cells) were transduced with CD46-Cyt1 and labeled for CD46 (panel ii).

(C) MD45 cells transduced with CD46 variants as shown were immunolabeled for Dlg (green) and CD46 (red), and images representing the degree of polarization of the CD46 variants are shown in panel i and quantitated in panel ii. For each MD45-transduced cell line, at least 100 cells in which Dlg was polarized to the uropod were scored for polarization of CD46 to the uropod. Scoring was performed blind, error bars represent SEM, and Mann-Whitney comparison of Cyt1 and Cyt1-L358R in five experiments indicated $p < 0.01$.

mation in MD45 cells by incubation with beads coupled to anti-CD3 and anti-CD28 antibodies (see validation of the IS in Figure S5). Comparison of Scribble knockdown cells revealed a striking defect in the ability of these cells to form stable conjugates with the coated beads (Figure 6B, panel i). Although control cells formed 188 ± 9 conjugates per experiment, Scribble knockdown cells formed only 40 ± 6 conjugates per experiment. Of the conjugates that were formed, neither PKCθ (a marker of the IS) nor CD3 were polarized to the site of contact with the beads, indicating a complete lack of polarization in response to the TCR signal (Figure 6B, panel ii). These data indicate that Scribble is required for the conformational changes that are triggered by TCR signaling and for the formation of a stable conjugate and IS.

Discussion

We provide here evidence that T cell shape is regulated by PDZ-containing proteins. In addition to Dlg1, Dlg4, and PKCζ (previously identified in T cells), other polarity proteins such as Scribble, Lgl, Par3, and Crumbs3 are expressed in T cells, and the subcellular distribution of these proteins in T cells shares important features with a polarity network that has previously been defined in epithelial cells (Figure 7). The dramatic loss of polarization caused by a reduction in Scribble expression supports the hypothesis that this network of PDZ-containing proteins regulates T cell polarity. Furthermore, the failure of cells with reduced Scribble expression to either migrate or form conjugates upon TCR signaling demonstrates the functional importance of the polarity network in T cells. The identification of a polarity network in T cells, and the wealth of information related to this network in other cell systems, means that we can now predict with some confidence the mechanisms by which the PDZ network influences T cell polarization.

Interactions between the Polarity Network and Structural Components of the Cell

T cell events such as the translocation of the MTOC and cytotoxic granules during IS formation (Kuhn and Poenie, 2002; Stinchcombe et al., 2001), ERM-mediated morphological changes in conjugate formation (Faure et al., 2004), and myosin II-mediated motility and arrest (Jacobelli et al., 2004) all require a coordinated integration of signals throughout the cell. Molecular interactions between polarity proteins and the MTOC (Etienne-Manneville and Hall, 2003), Ezrin (Medina et al., 2002), myosin II (Barros et al., 2003; Ohshiro et al.,

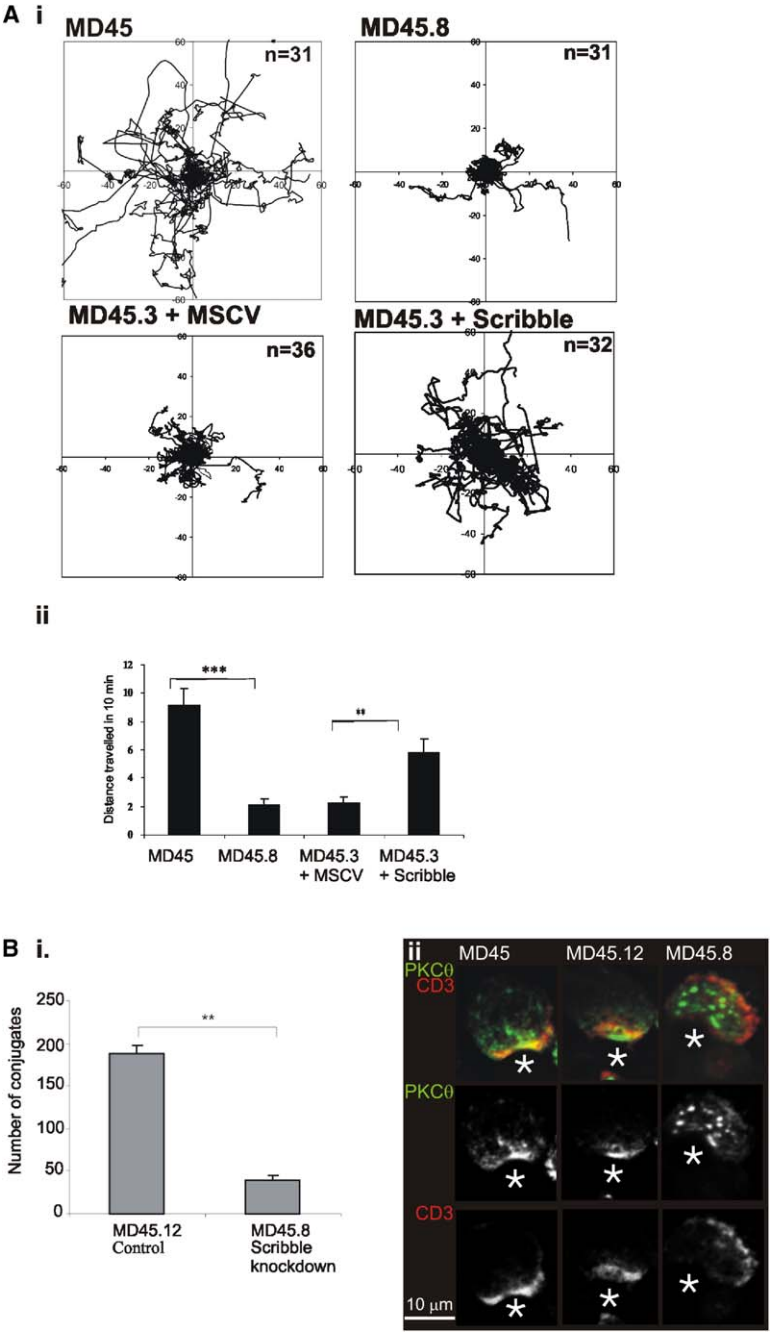

Figure 6. Scribble Is Required for T Cell Migration and Conjugate Formation

(A) MD45 parental cells, MD45.8 Scribble knockdown cells, and MD45.3 Scribble knockdown cells transduced with either MD45 vector control or human Scribble were analyzed for cell migration by time-lapse microscopy (panel i). Migration paths were assessed by using MetaMorph, and each was assigned a starting coordinate of 0 on the x and y axes. The number of cells tracked for each cell type is indicated on the top right of each plot. Panel ii shows the average distance from origin after 10 min of the cells in panel i. SEMs are shown, and the number of cells analyzed is indicated in the top right corner of panels i–iv; $^{**}p < 0.001$ and $^{***}p < 0.0001$ as determined by a nonparametric Mann-Whitney test.

(B) MD45.12 shRNA control and MD45.8 Scribble knockdown cells were incubated with beads coupled to antibodies against CD3 and CD28 for 90 min, adhered to microscope slides by centrifugation, and the number of cells attached to beads on each slide was counted. An average of three experiments is represented, SEMs are shown, and $p < 0.01$ as determined by a two-tailed t test (panel i). Conjugates from panel i were immunostained with antibodies to PKCθ and CD3. The images shown represent the consistent polarization of PKCθ and CD3 to the site of contact with the bead (indicated by an asterisk) in MD45 and MD45.12 control cells and the lack of polarization of either CD3 or PKCθ in the few conjugates that had been formed by the MD45.8 knockdown cells (panel ii).

2000), and vesicular trafficking (Lee et al., 2003; Musch et al., 2002; Roche et al., 2002; Sans et al., 2003) have all been identified in other cell types, suggesting that the polarity network in T cells has the capacity to coordinate T cell polarity and morphology at a number of levels. Indeed, the loss of Ezrin asymmetry, reduction in conjugation efficiency, and reduced migration observed in Scribble knockdown cells are all consistent with this notion.

The Integration of Signaling with Cell Shape by PDZ-Containing Proteins in T Cells

Our observations suggest a mechanism not only for the precise compartmentalization implicit in the regulation of T cell function but also for the reorganization of the entire T cell in response to signals in a single region of the cell. This is particularly relevant to interactions between polarity proteins and Rho GTPases, which often determine the localization of subcellular domains during the initiation of polarity. For example, Cdc42 dictates polarity in many diverse cells (Etienne-Manneville and Hall, 2002; Etienne-Manneville and Hall, 2003; Macara, 2004; Stowers et al., 1995), and the site of action of Cdc42 is often determined by cooperation with atypical PKC. In turn, the activity of atypical PKC is localized by Par3 and Par6, and therefore dictated by the spatial and functional antagonism between the Scribble, Par3, and Crumbs complexes (Bilder et al., 2003;

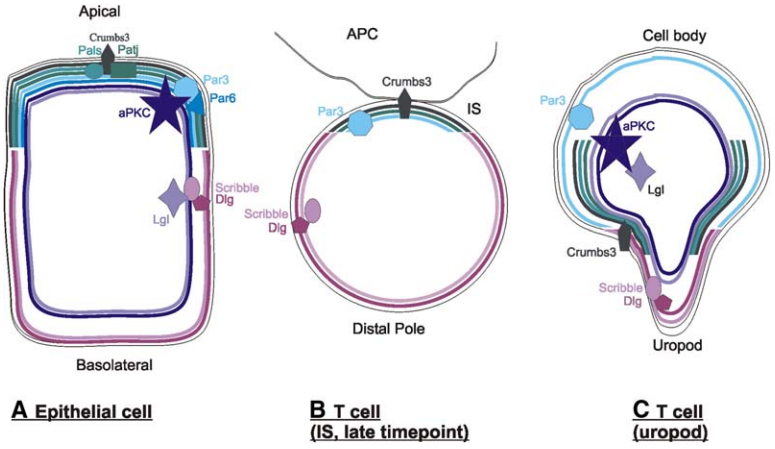

Figure 7. The Regulation of T Cell Shape by a PDZ Network

Schematic representation of the distribution of polarity proteins in a polarized epithelial cell (A), a T cell undergoing antigen presentation (B), and a uropod-containing T cell (C). Colored lines corresponding to each protein (as labeled) demarcate the regions of the cell in which the protein has been observed by immunostaining (proteins whose localization has not been determined are absent). Note that the recruitment of Dlg and Scribble to the IS at early stages of antigen presentation is not depicted here. All proteins investigated at later times of IS formation showed a number of similarities with their distribution in polarized epithelial cells. Firstly, Scribble is copolarized with multiple Dlg family members, and loss of Scribble gives a similar phenotype to disruption of Dlg binding, suggesting a functional cooperation similar to epithelial cells and neuroblasts. Secondly, Lgl is expressed throughout the T cell, similar to epithelial cells and consistent with an interaction of Lgl with multiple components of the polarity network (Betschinger et al., 2003; Plant et al., 2003; Strand et al., 1994). Thirdly, an overlap of Crumbs and Par3, and their exclusion from regions in which Scribble and Dlg are enriched, suggests cooperation and antagonism between these proteins that again parallel observations in epithelial cells (Bilder and Perrimon, 2000). Although individual Dlg isoforms (1–4) are not depicted here, the localization of each is represented by Dlg in both uropod-containing and IS-containing T cells.

Hurd et al., 2003; Plant et al., 2003; Roh and Margolis, 2003; Tanentzapf and Tepass, 2003). Our identification of a T cell polarity network suggests that the polarization of the Scribble complex in the uropod, and in the distal pole of T cells during antigen presentation, might restrict the cooperation of Cdc42 and the atypical PKC PKCζ to the opposite pole of the cell (for example, the IS or the leading edge).

In addition to direct effects on T cell shape, the polarity network is ideally suited to integrate cell shape with signaling by influencing the localized activity of signaling molecules. It is already clear that Dlg1 coordinates signaling in T cells (see Introduction) and negatively regulates the activation of NFAT (Xavier et al., 2004). This activity might result from a transient signaling role for Dlg1 at the IS (Xavier et al., 2004) or from an indirect role for Dlg1 as part of a polarity network that, for instance, affects NFAT activation through GSK3β (Etienne-Manneville and Hall, 2003; Ohteki et al., 2000).

A PDZ Network as a Means of Reshaping T Cells

The importance of Scribble and Dlg in T cell shape and the interaction of Dlg with T cell surface molecules suggest that this complex might orchestrate the rapid shape changes of T cells in response to extracellular cues. Lymphocyte surface receptors that physically interact with PDZ-containing proteins would therefore have the capacity, upon binding to extracellular cues, to trigger a redistribution of the PDZ polarity network and subsequent changes in T cell shape. CD2, which participates in the early phase of conjugate and IS formation, can mediate patching of Dlg1 when crosslinked (Hanada et al., 2000; Tibaldi et al., 2002). This activity, as well as the interaction of LFA-1 with Dlg1 (Ralston et al., 2004), could trigger a reorganization of the polarity network to arrest leukocyte trafficking prior to the formation of the immunological synapse. Another example is CD46, which signals to alter T cell activation, cytokine production, and the induction of regulatory T cells. These effects have been correlated with alterations in T cell shape (Kemper et al., 2003; Marie et al., 2002; Russell, 2004; Zaffran et al., 2001), and the association of CD46 with Dlg4 (Ludford-Menting et al., 2002) provides a mechanism for this activity.

In summary, we describe here a network of proteins that influences T cell polarity and that is comprised of polarized clusters of PDZ-containing proteins. Disruption of this network not only prevents cell polarity but also prevents important functions of the T cell such as migration and cell remodeling during antigen presentation. These findings provide a framework for the elucidation of mechanisms by which multiple extracellular inputs are integrated by the T cell to orchestrate cell polarity.

Experimental Procedures

Reagents

Antibodies and detection reagents were mouse IgG2a to multiple Dlg isoforms (pan-Dlg, PSD-95 family, Upstate Biotechnology, NY, USA); rabbit and mouse IgG_1 (SAP97, Santa Cruz Biotechnology, Santa Cruz, CA) to Dlg1; goat (PSD-93, Zymed Laboratories Inc, CA) to Dlg2 and rabbit to Dlg3 (SAP102, Synaptic Systems, Germany); mouse IgG2a (PSD-95, Upstate Biotechnology, NY) to Dlg4; goat (C20) to Scribble (Santa Cruz Biotechnology, CA) and mouse IgG1 to Scribble (Dow et al., 2003); mouse to Lgl1 provided by Tony Pawson (Plant et al., 2003) and rabbit to Crumbs3 provided by B. Margolis (Roh et al., 2003); rabbit to Par3 (Upstate Biotechnology, NY); rabbit to PKCζ (Upstate Biotechnology, NY); rabbit to Ezrin (Andreoli et al., 1994) and mouse IgG1 to Talin (Sigma); mouse IgG2a (E4.3) and rabbit (1840) to CD46 (Crimeen-Irwin et al., 2003; Russell et al., 1992); mouse IgG1 to $p120^{cbl}$ (BD Transduction Laboratories, CA); hamster monoclonal to CD3 and CD28 (BD Pharmingen, CA); rabbit to PKCθ (Santa Cruz Biotechnology, CA); rat IgG2a to LFA-1 (CD11a, BD Pharmingen, CA); rabbit to GFP (Molecular Probes); and Alexa Fluor 488- and 594-conjugated secondary antibodies, biotinylated secondary antibodies, and streptavidin-coupled Alexa Fluor 488, 594, or 647 (Molecular Probes, OR).

Mice, Cells, and Constructs

C57Bl/6 mice or OT-1 mice (C57Bl/6 background) transgenic for a TCR that recognizes the ovalbumin peptide SIINFEKL in the context of $H\text{-}2K^b$ (Hogquist et al., 1994) of 8–12 weeks of age were used in all experiments. OT-1 splenocytes were activated by incu-

bation for 6 days with interleukin-2 (Chiron Corp, CA) and with irradiated (2000 rad) C57Bl/6 splenocytes previously pulsed with 1 μM SIINFEKL (Auspep, Australia). These cells were $CD8^+IL\text{-}2R\alpha^{high}$ and responded specifically to peptide-pulsed DCs. Bone marrow cells from hind limbs of C57BL/6 mice were cultured in 10 ng/ml granulocyte macrophage colony-stimulating factor (Peprotech) and 5 ng/ml interleukin 4 (Peprotech) for 6 days to generate immature DCs ($CD11c^+$, $CD86^{low}$, and $MHC\text{-}II^{low}$). Human $CD4^+$ and $CD8^+$ T cells were purified from buffy coats by using Ficoll gradients (Crimeen-Irwin et al., 2003), removal of monocytes by adherence on gelatin-coated flasks, and negative selection with MACS magnetic bead separation kits (Miltenyi Biotec). Human and murine T lymphocytes were cultured in RPMI 1640 with 50 IU/ml recombinant interleukin-2 (Chiron) and 2 μg/ml phytohemagglutin (Sigma). CD46-Cyt1, CD46-Cyt2, and CD46-Cyt1L358R (Ludford-Menting et al., 2002) were subcloned into pMSCV-GFP for expression in the MD45 T cell line as previously described (Haynes et al., 1999). Short hairpin oligos corresponding to sense and antisense mouse Scribble and EGFP (shRNA control) were cloned into the pRetro-SUPER (Brummelkamp et al., 2002) (bold indicates sequences corresponding to mouse Scribble): shEGFP (1):5′-GATCCCC**GCTGGAGTACAACTACAAC**TTCAAGAGA**GTTGTAGTTGTACTCCAGC**TTTTTGGAAA-3′, shEGFP (2):5′-GCTTTTCCAAAAA**GCTGGAGTACAACTACAAC**TCTCTTGAA**GTTGTAGTTGTACTCCAGC**GGG-3′, shScrib3.1(1):5′-GATCCCC**AAGCTGACTAACCTCAATGTG**TTCAAGAGA**CACATTGAGGTTAGTCAGC**TTTTTTTGGAA-3′, and shScrib3.1(2):5′-AGCTTTTCCAAAAA**GCTGACTAACCTCAATGTG**TCTCTTGAA**CACATTGAGGTTAGTCAGCTT**GGG-3′. Full-length human Scribble cDNA (Dow et al., 2003) was cloned into pMSCV-GFP.

Imaging

Cells were attached to slides by centrifugation (250 rpm, 10 min). For synapse experiments, DCs (2.5×10^4/well) were labeled with CFSE (Molecular Probes, OR), adhered overnight onto 8-well chamber slides (Nalgene Nunc, IL, USA), and incubated with 1 μM SIINFEKL (1 hr, 37°C). T cells were overlayed (10^5/well) for the indicated times (0 represents the time of cell addition) and nonadherent cells washed off. Cells were fixed with 3.7% (w/v) paraformaldehyde in 100 mM Pipes, 5 mM $MgSO_4$, 10 mM EGTA, and 2 mM DTT (10 min, RT), then washed twice, permeabilized in 0.1% Triton X-100 in 50 mM Tris-HCl (pH 7.6) (5 min, RT), and labeled with primary antibodies as described. Antibodies were detected with Alexa Fluor- or HRP-conjugated secondary antibodies or biotin-conjugated secondary antibodies and streptavidin-Alexa Fluor and mounted in Prolong antifade (Molecular Probes) or counterstained with 0.5% Methyl Green. Samples were examined by using a Leica microscope attached to an MRC-1024 Bio-Rad laser-scanning confocal microscope or a Zeiss Axioskop2 microscope. Digital images acquired with Bio-Rad Lasersharp 2000 software were processed with Image J (NIH, MD) and MetaMorph (Universal Imaging Corporation, PA). For live cell imaging, cells were seeded in individual wells of an 8-well chamber slide (Nalge Nunc International, IL) and maintained at 37°C on an Olympus IX-81 microscope fitted with a temperature controlled chamber (Solent Scientific, UK) and a computer controlled XY stage (Prior Proscan, MA). DIC images were captured at specified intervals by using an ORCA-ER CCD camera (Hamamatsu, Japan) controlled by MetaMorph software (Universal Imaging Corporation, PA). Movies were compiled from individual images and play at four frames/sec. To quantitate T cell shape, clones were observed by light microscopy and assigned a score according to the proportion of cells with clear uropods or an elongated shape.

T Cell-Bead Conjugation

Polystyrene beads (10 μm, Polysciences) were coated with 2 μg/ml anti-CD3 and anti-CD28 in 0.05 M Tris (pH 9.0) (5 hr, RT), then washed with PBS containing 0.5% FCS. 5×10^4 cells were incubated with 2×10^5-coated beads in 100 μl (2 hr, RT). Conjugates were adhered to glass slides by centrifugation (250 rpm, 10 min), immunostained, and visualized on the Leica confocal microscope powered by Bio-Rad Lasersharp 2000 software.

Supplemental Data

Supplemental Data include five figures and five movies and are available with this article online at http://www.immunity.com/cgi/content/full/22/6/737/DC1/.

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Note Added in Proof

A recent report demonstrated a transient recruitment of Dlg1 to the IS, and a role for Dlg1 in morphological and functional aspects of T cell biology (Round et al., 2005), in agreement with our studies.