The Role of SPECs, Small Cdc42-binding Proteins, in F-actin Accumulation at the Immunological Synapse*

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SPEC1 and SPEC2 are structurally similar Cdc42-binding proteins of 79 and 84 amino acid residues, respectively. We investigated the role of SPEC2 in T cell function due to its high mRNA expression in lymphocytes. Western blot analysis revealed abundant SPEC2 protein in lymphocytes, which in glutathione S-transferase-capture experiments specifically interacted with only GTP-bound Cdc42. Immunofluorescence experiments revealed that the SPEC2 protein was diffusely localized in the cytoplasm and at the cell membrane in unstimulated Jurkat T cells and Raji B cells. Recruitment of SPEC2 within Jurkat T cells to the antigen-presenting cell interface occurred following incubation with staphylococcal enterotoxin E superantigen-loaded B cells and colocalized there with F-actin and Cdc42. T cell receptor (TCR) activation studies using anti-CD3 antibody-coated polystyrene beads showed that SPEC2 was recruited to the site of bead contact, which was not observed with anti-major histocompatibility complex antibody-coated beads. Accumulation of SPEC2 following TCR engagement occurred as early as 5 min, before obvious F-actin accumulation. Biochemical studies with Jurkat T cells demonstrated that N-terminal cysteine residues in SPEC2 were palmitoylated. Overexpression studies of the related SPEC1 showed that it also was recruited to the activated TCR. Mutational analysis revealed that localization of SPEC1 to the TCR required two N-terminal cysteine residues. Furthermore, a SPEC1 Cdc42 Rac-interacting binding mutant, containing an intact N terminus but defective in Cdc42 binding, completely blocked F-actin accumulation at the activated TCR. Taken together these results suggest that SPECs may play important roles in Cdc42-mediated F-actin accumulation at the immunological synapse.

Cytoskeletal rearrangements play an important role in T cell activation following engagement of the T cell receptor (TCR)†

* This work was supported by funding through the Susan G. Komen Foundation (Grant 9851) (to P. D. B.) and a DOD breast cancer predoctoral fellowship (to K. H. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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† The abbreviations used are: TCR, T cell receptor; BMCC, 1-biotin-(maleimidomethyl)cyclohexanecarboxamido) butane; SEE, staphylococcal enterotoxin E; HRP, horseradish peroxidase; GSST, glutathione S-transferase; GTPγS, guanosine 5'-3-O-(thio)triphosphate; NEM, N-ethylmaleimide; APC, antigen-presenting cell; WASP, Wiskott-Aldrich syndrome protein; CRIB, Cdc42 Rac-interacting binding.
teins, the SPECs are small and contain no encoded enzymatic/signalizing domains. Here we report that SPEC2 protein is highly expressed in lymphocytes and immune tissues and is recruited to the immunological synapse at the site of T cell/APC contact in an antigen-dependent manner. This recruitment was associated with TCR activation, because anti-CD3 antibody-coated polystyrene beads were sufficient for endogenous SPEC2 or overexpressed SPEC1 accumulation. Significant and rapid SPEC2 recruitment occurred at the site of TCR activation in advance of marked F-actin accumulation. Biochemical analysis using Jurkat T cell lysates revealed that SPEC2 was palmitoylated in the N-terminal region. Although we had difficulty detecting Jurkat T cells overexpressing SPEC2, studies with overexpressed SPEC1 revealed that it was also recruited to the activated TCR. SPEC1 recruitment to the activated TCR did not require the Cdc42-binding sequence, but was blocked by a SPEC1 CRIB mutant, still containing an intact N terminus, acting as a dominant negative and blocked F-actin accumulation at the activated TCR. Taken together, these results suggest that SPECs play an important role in F-actin dynamics at the immunological synapse.

**Experimental Procedures**

**Antibodies and Biochemical Reagents—**Texas Red-conjugated donkey anti-rabbit antibody was obtained from Amersham Biosciences. Polyclonal sheep anti-Cdc42 antibody and anti-sheep secondary antibodies conjugated fluorescein isothiocyanate were obtained from Cytoskeleton Inc. (Denver, CO). Monoclonal anti-Cdc42 antibody and biotin-conjugated mouse monoclonal anti-human CD3 antibody (clone UCHT1) were purchased from BD Biosciences (San Diego, CA). Polyclonal rabbit anti-Myc antibody recognizing the Myc tag was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Biotin-conjugated monoclonal antibodies directed against HLA-ABC or anti-CD3 (25) were purchased from Celltech Laboratories (Westbury, NY). Goat anti-rabbit antibody conjugated-peroxidase was purchased from Amersham Biosciences. Streptavidin-coated polystyrene beads (6.7 μm) were obtained from Spherotech Inc. (Libertyville, IL). EZ-Link Biotin-BMCC (1-biotinamido-4-(4’-maleimidomethylcyclohexanecarboxamido) butane) and streptavidin-conjugated horseradish peroxidase were from Pierce (Rockford, IL). Alexa Fluor 488-labeled phalloidin, Texas Red-labeled phalloidin, Texas Red fluorophoreconjugated 7-amino-4-chloromethylcoumarin Cell-Tracker Blue were obtained from Molecular Probes (Eugene, OR). Staphylococcal enterotoxin E (SEE) was purchased from Toxin Technologies (Sarasota, FL).

**Western Blotting with Anti-SPEC1 and Anti-SPEC2 Antibodies—** Two unique peptides, SPEC1-C, corresponding to 17 amino acid residues derived from the C terminus of SPEC1 (AMTGVQVMQR-SKGRND) and SPEC2-C, corresponding to 15 amino acid residues from the C terminus of SPEC2 (MPAVNQMQLVDTKAG) were synthesized (Research Genetics, Huntsville, AL) with a cysteine residue at the N terminus. These peptides were conjugated to maleimide-activated keyhole limpet hemocyanin (Pierce) according to the manufacturer's instructions. The two different C-terminal peptide-keyhole limpet hemocyanin conjugates were then used as immunogens in rabbits. The anti-SPEC1 antibody worked poorly in Western blotting and could not detect endogenous SPEC1 by immunofluorescence. In contrast, the SPEC2 antibody easily detected SPEC2 protein in both Western blotting and in immunofluorescence. Studies examining SPEC2 protein using cultured cells (i.e. HeLa cells, COS1 cells, Jurkat T cells, CCRF-CEM, and Raji B cells) involved first preparing 1% Triton-soluble cell extracts and then mixing equal protein amounts of these extracts with SDS-PAGE sample buffer. Mouse lung, spleen, and thymus tissues from a 10-week-old animal were also harvested, chopped finely, and sonicated in SDS-PAGE buffer. Equal amounts of protein were resolved on an 18% Tris-glycine SDS-PAGE gel. Following transfer to nitrocellulose, SPEC2 protein expression was examined using the rabbit anti-SPEC2 antisera diluted at 1:5,000. Identical dilutions of preimmune antiserum from the rabbit were used as a control. Following washing, a goat anti-rabbit antibody HRP conjugate was used followed by ECL reagents (Pierce) to detect SPEC2 protein. For detection of SPEC2 protein in blood cells, we first immunoprecipitated SPEC2 protein from soluble lysates and then performed Western blotting because whole blood extracts displayed some endogenous peroxidase activity around 10 kDa. Briefly, whole human blood (0.1 ml) was allowed to clot, and then cell membranes were extracted by centrifugation using a hypotonic buffer containing 10 mM Tris (pH 7.5). The pellet obtained was resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM MgCl2, 1% Triton X-100, and a protease inhibitor mixture). The soluble lysates were then pre-cleared with protein A/G-agarose beads, then incubated with either preimmune rabbit sera or anti-SPEC2 antibody and protein A/G-agarose beads at 4 °C. Next, captured proteins were run on an 18% SDS-PAGE gel and subjected to Western blotting and immunoblotting as described above.

**Cdc42 Binding Experiments—** The GST-capture experiments were performed as previously described (25). Briefly, recombinant glutathione-S-transferase (GST), or GST-Cdc42-V12 fusion proteins were expressed in *Escherichia coli*, purified on glutathione-agarose resin, and loaded with 10 μM GTP-γS or 10 μM GDP. Jurkat T cells were lysed in 500 μl of 1% Triton X-100 lysis buffer, pre-cleared with GST bound to glutathione-agarose beads, and then added to the purified GST fusion protein immobilized on gel. Protein incubation at 4 °C for 40 min, the beads were washed with lysis buffer. Bound proteins were then resolved by SDS-PAGE on an 18% Tris-glycine gradient gel, and SPEC2 protein was detected with the polyclonal anti-SPEC2 antibody and processed with secondary antibody-HRP conjugates and ECL reagents.

**Conjugate Formation and Analysis—** For B and T cell conjugations, Raji B lymphocytes were first incubated by incubation in media containing 10 μg/ml streptavidin-coated polystyrene beads for 20 min at 37 °C, fixed, and processed for immunofluorescence. SPEC2 polarization was determined by counting 30 random B-T cell conjugates in three independent experiments. Positions of the conjugated B cells were scored by reverse PCR: Protein incubation at the B-T cell contact site in the absence and presence of SEE. The recruitment of SPEC2 to unstimulated cells not treated with SEE antigen was likely overestimated due to high SPEC2 membrane staining in some Jurkat T cells. For bead conjugation assays, streptavidin-coated polystyrene beads were first incubated with 10 μg/ml biotin-conjugated anti-CD3 antibody or 10 μg/ml biotin-conjugated anti-HLA-ABC antibody for 20 min at 37 °C. Protein conjugation was then washed, incubated with Jurkat T cells for 5 min at room temperature then transferred to poly-l-lysine-coated glass coverslips and incubated for an additional 5 or 20 min. Cells were then fixed and processed for immunofluorescence. SPEC2 polarization was determined by counting 20 conjugates formed with either anti-HLA-ABC or anti-CD3-coated beads in three independent experiments.

**Immunofluorescence Microscopy—** Jurkat T cells and Raji B cells, grown in RPMI 1640 supplemented with 10% fetal calf serum, in 5% CO2 at 37 °C, were plated on poly-l-lysine-coated coverslips and fixed with 3.7% (w/v) formaldehyde in phosphate-buffered saline for 15 min, washed, and permeabilized for 15 min with 0.05% Triton X-100 in phosphate-buffered saline. Fixed cells were then blocked with 1% goat serum. Cells were then incubated with primary antibodies diluted in phosphate-buffered saline for 45 min at room temperature, washed, and then incubated with the appropriate secondary antibody for 45 min at room temperature. F-actin was stained using Alexa Fluor 488-labeled phalloidin. Coverslips were mounted on glass slides using the ProLong Antifade Kit (Molecular Probes, Eugene, OR). Images were obtained using a Nikon E600 Epi-Fluorescence microscope with a 60× Plan Apo oil lens mounted with a Hamamatsu digital camera connected to Metamorph software (Universal Imaging, Downingtown, PA).

**SPEC2 Protein Palmitoylation—** A non-isotopic method to study protein palmitoylation was followed with some modification (26). Briefly, Jurkat T cells were harvested using L buffer (50 mM Tris (pH 7.5), 100 mM NaCl, 5 mM MgCl2, 1% Triton X-100, and a mixture of protease inhibitors) containing 50 mM N-ethylmaleimide (NEM). These lysates were then incubated for 30 min with preimmune antiserum or anti-SPEC2 antisera. Following the antibody incubation, protein A/G-coupled agarose beads were added to each sample for an additional 30 min. Following 3 washes (5 min per wash) using the L buffer containing NEM, the captured proteins were incubated for an additional 20 min in medium containing 50 μg/ml biotin-containing 0.1 M N-ethylmaleimide. The captured proteins were then washed two times with L buffer without NEM and treated with 1 μM hydroxylamine in 1 μl Tris or 1 μl Tris alone at room temperature for 1 h. Next the samples were washed 3 times with buffer D (50 mM Tris (pH 7.5), 50 mM NaCl, and 10 mM MgCl2) and then incubated with 2 μM BMCC for 2 h at room temperature. The captured proteins were resolved by 18% Tris-glycine PAGE electrophoresis and submitted to Western blotting. HRP-conjugated streptavidin was used to detect
biontinated proteins and the anti-SPEC2 antibody was used to detect SPEC2 protein. The blots were developed using ECL reagents and exposed to x-ray film.

**Mammalian Expression Vectors for SPEC1 Mutants, Transient Transfections, and Immunofluorescence—**Using several different SPEC2 mammalian expression vectors, including FLAG-tagged, EGFP-tagged, and untagged constructs, we were unable to detect significant numbers of Jurkat T cells expressing SPEC2. In contrast, significant expression of SPEC1 and mutant SPEC1 constructs was observed with the different SPEC1 mammalian expression vectors. In these studies, we utilized a pcDNA-III-C-terminal Myc-tagged mammalian expression vector for studying SPEC1 and its mutants. Specific mutants were generated using sequence-specific oligonucleotides and the QuiKChange mutagenesis kit (Stratagene). The two SPEC1 mutants utilized included SPEC1-C10A,C11A containing two alanine substitutions for the two N-terminal cysteines and SPEC1-P33A,H38A,H41A containing three alanine substitutions within the CRIB sequence involved in Cdc42 binding. The integrity of all constructs was confirmed by DNA sequencing. For Jurkat T cell transfection by electroporation, 5 x 10^6 cells in log-phase growth were first washed twice in phosphate-buffered saline, then resuspended in 0.5 ml of Opti-MEM (Invitrogen) and subjected to electroporation at 250 V, 960 microfarads using a Bio-Rad Gene Pulser™ on ice for 10 min. Following this incubation, Jurkat T cells were electroporated at 250 V, 960 microfarads using a Bio-Rad Gene Pulser™ and then placed back on ice for 10 min before replating in complete RPMI. Fifteen hours post-transfection, transfected cells were analyzed for SPEC1 accumulation using the anti-CD3 coated bead system described above. Detection of Myc-tagged SPEC1-expressing cells was performed with the polyclonal anti-Myc antibody that recognizes this epitope tag.

**RESULTS**

**SPEC2 Is Expressed in Immune Tissue and Cells—**Previous gene array mRNA expression analysis of human cells and tissues^2⁠ reveals that both SPEC1 and SPEC2 mRNA were ubiquitously expressed, but that the highest levels of human SPEC1 and SPEC2 mRNA expression occurred in T lymphocytes, dendritic, and whole blood cells (27). To characterize SPEC2 protein expression, we generated a polyclonal anti-SPEC2 antibody by synthesizing a peptide corresponding to a unique sequence in the C terminus of SPEC2 and immunized a rabbit against this peptide. Western blot analysis with this antibody revealed that it was specific for SPEC2 protein and reacted with full-length SPEC2 bacterial recombinant protein and not full-length SPEC1 protein (data not shown). Solubility studies also revealed that SPEC2 protein was easily extractable in 1% Triton X-100 buffer from a variety of cell types but was not extractable in buffers lacking detergents, suggesting a potential association with cellular membranes or other insoluble components (data not shown). Western blot analysis of equal amounts of protein from each of several different cell types revealed that the 10-kDa SPEC2 species was highly expressed in CCRF-CEM T lymphocytes, Jurkat T lymphocytes, and Raji B lymphocytes compared with COS1 cells or HeLa cells (Fig. 1A). In addition, the 10-kDa SPEC2 protein was also detected in mouse spleen, thymus, and lung (Fig. 1B). Reactivity to the 10-kDa SPEC2 protein species was not observed with preimmune rabbit serum (data not shown). These results suggest that SPEC2 protein may be important in lymphocyte signaling.

In light of a study showing high levels of SPEC2 mRNA from human whole blood cells (27), we also examined SPEC2 protein expression in whole blood cells. Because of endogenous peroxidase-like activity in cell extracts from whole blood observed around the 10-kDa size of SPEC2 protein, we used a combined protocol of immunoprecipitation and Western blotting to examine SPEC2 protein expression (Fig. 1C). In these experiments, human blood cells were allowed to clot, washed, lyzed in hypotonic buffer, and extracted with buffer containing 1% Triton X-100. These 1% Triton X-100 extracts of human blood cells were then subjected to immunoprecipitation with preimmune or SPEC2 antisera. Western blotting revealed that the SPEC2 antibody specifically reacted with a 10-kDa species only in the anti-SPEC2 immunoprecipitation sample (Fig. 1C, lane 4). SPEC2 protein reactivity was not detected in the preimmune immunoprecipitates (Fig. 1C, lane 3) or when Western blotting was performed with preimmune antisera (Fig. 1C, lanes 1 and 2). These results confirm previous SPEC2 mRNA studies and suggest that SPEC2 protein is abundant in cells of hemopoietic origin.

Previously, SPEC1 and SPEC2 were shown by yeast two-hybrid to interact specifically with the constitutively active mutant Cdc42-V12 and not wild type Cdc42 (24), similar to yeast two-hybrid experiments examining WASP and Cdc42 interactions (12). To test whether endogenous SPEC2 protein extracted from Jurkat T lymphocytes could interact with Cdc42 in a GTP-dependent manner, we used a GST capture approach. Briefly, beads containing immobilized recombinant, constitutively active Cdc42 were loaded with 10 μM GTPγS or 10 μM GDP and then incubated with Jurkat T cell lysates. Using this GST-capture assay, SPEC2 was found to interact only with GST-Cdc42-GTPγS and not with GST-Cdc42-GDP or GST (Fig.

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^2 The data for SPEC1 and SPEC2 mRNA expression can be found at the Genomics Research Institute Novartis Foundation Gene Expression Atlas database (expression.gnf.org) under CDC42SE1 and CDC42SE2, respectively.
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1D). Furthermore, recombinant Cdc42 and SPECs produced from bacteria, which lack post-translational modifications, interact in vitro (data not shown). These results indicate that SPEC2 protein specifically interacts with activated Cdc42 and may function in Cdc42 signaling in lymphocytes.

SPEC2 Colocalizes with Cdc42 and F-actin at the Immunological Synapse—Staining for endogenous SPEC2 using the anti-SPEC2 antibody in Jurkat T cells and Raji B cells adherent to poly-l-lysine-coated coverslips revealed that the SPEC2 protein was diffusely localized in the cytoplasm and weakly at the plasma membrane in Jurkat T cells (see unstimulated Jurkat T cells in Fig. 3B and data not shown). A similar pattern of staining was also observed in lymphocytes isolated from normal blood, but it should be noted no staining of SPEC2 was observed in erythrocytes (data not shown). This immunofluorescent staining was not observed with preimmune antisera and could be completely blocked by incubation with excess SPEC2 peptide (data not shown). In Raji B lymphocytes, SPEC2 was localized primarily in the cytoplasm with less obvious membrane staining and showed little colocalization with Cdc42 staining in these unstimulated cells (data not shown). In light of previous studies showing that WASP and Cdc42-GTP are recruited to the immunological synapse (18, 19), we tested whether SPEC2 protein was recruited to the B-T cell interface in response to TCR stimulation. In these studies we labeled Raji B lymphocytes with 7-amino-4-chloromethylcoumarin cell tracker dye and then incubated them with Jurkat T lymphocytes in the absence or presence of SEE superantigen (staphylococcal enterotoxin E). In the absence of SEE superantigen, no enrichment of SPEC2, Cdc42, or F-actin occurred at the site of contact between the Raji B lymphocytes and Jurkat T lymphocytes (data not shown). In contrast, 10 min following contact of a Jurkat T cell with a SEE-loaded Raji B cell, marked SPEC2 recruitment was observed in Jurkat cells at the B-T cell interface (Fig. 2, B and E). At this interface, SPEC2 localized to a discrete band specific to the Jurkat T cell and was not altered in the Raji B cell (Fig. 2, B and E). Cell counting in three independent experiments confirmed that 73% of B-T cell conjugates showed significant recruitment of SPEC2 to the cell-cell interface in the presence of the SEE superantigen in comparison to 17% in the absence of the antigen (Fig. 2, lower panel). Double labeling experiments also showed that SPEC2 colocalized with F-actin (Fig. 2F) and endogenous Cdc42 (Fig. 2C) at the immunological synapse and again was specifically associated with the activated T cell and not the Raji B cell as previously reported (18, 19). These observations show that SPEC2 is recruited to the immunological synapse in T cells following TCR stimulation.

TCR Engagement Is Sufficient for SPEC2 Recruitment—To investigate if TCR engagement alone is sufficient to drive recruitment of SPEC2 to the immunological synapse, we analyzed endogenous SPEC2 localization following TCR activation using anti-CD3 stimulatory antibodies. In these experiments, biotin-conjugated anti-human CD3 monoclonal antibodies or biotin-conjugated anti-HLA antibodies were immobilized to streptavidin-coated 6.7-μm-polystyrene beads. When Jurkat T cells contacted the control anti-HLA-ABC-coated beads (Fig. 3A), SPEC2 remained in a diffuse cytosolic pool and did not localize to the site of bead contact (Fig. 3B). Contrast, Jurkat T cell binding to the anti-CD3 antibody-coated beads (Fig. 3C) resulted in marked recruitment of SPEC2 to the site of contact where it appeared as a thick band of fluorescent staining at the bead-cell interface (Fig. 3D). Cell counting revealed that ~76% of Jurkat T cells engaged by anti-CD3 antibody-coated beads showed recruitment of SPEC2 to the site of contact as compared with 26% for the anti-HLA-coated beads (Fig. 3, right panel). As a positive control, we also stained CD3-engaged cells for endogenous WASP, known to be located at activated T cell receptors (18, 19). Because both anti-WASP and anti-SPEC2 antibodies were polyclonal reagents derived from rabbits, we were unable to perform dual immunofluorescence staining for endogenous WASP and endogenous SPEC2 in the same cell. Nevertheless, WASP protein displayed a similar staining pattern to SPEC2 when Jurkat T cells were stimulated with anti-CD3 coated beads under similar conditions (Fig. 3F). These observations demonstrate that TCR engagement alone is sufficient for SPEC2 recruitment to the activated TCR and suggest that multiple Cdc42 effector proteins may reside at the site of activated T cell receptors.

We also carefully monitored SPEC2 and F-actin accumulation at different time points following T cell receptor activation using the anti-CD3-coated polystyrene beads. At 5 min following addition of the anti-CD3-coated beads, we almost always observed substantial SPEC2 recruitment to the site of bead contact (Fig. 4B) without significant F-actin accumulation (Fig. 4C). SPEC2 accumulation at the activated TCR appeared maximal at the 5-min time point. In contrast, F-actin accumulation continued to increase, reaching a plateau in fluorescence intensity at the 15- to 20-min time point (Fig. 4F). At this time point, both SPEC2 and F-actin appeared as thick, bright bands of staining at the bead-T cell interface. These observations from four independent experiments suggest that the dramatic SPEC2 recruitment to sites of activated TCR is an early event.
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FIG. 3. SPEC2 is recruited to regions associated with activated TCR. A and B, streptavidin beads coated with biotin-conjugated monoclonal anti-human HLA or C–F, biotin-conjugated monoclonal anti-CD3 receptor were incubated with Jurkat T cells, plated on poly-L-lysine-coated coverslips for 20 min, then fixed. A, C, and E, location of the antibody-coated polystyrene bead and contacting cells was visualized by phase contrast. B and D, endogenous SPEC2 was visualized using polyclonal anti-SPEC2 antibody, and F, endogenous WASP was visualized using a polyclonal anti-WASP antibody. The bar represents 10 μm. Right panel, activated TCR-dependent recruitment of SPEC2. The bar graph represents the percentage of conjugates showing SPEC2 accumulation at the bead-T cell interface using beads coated with anti-HLA ABC or anti-CD3 antibody. Cells were scored positive for SPEC2 polarization by the recruitment of SPEC2 to the bead-T cell interface, as described under “Experimental Procedures.” The graph is representative of three independent experiments.

FIG. 4. SPEC2 is recruited to the activated TCR prior to significant actin polymerization. Jurkat T cells were incubated with streptavidin beads coated with biotin-conjugated monoclonal anti-CD3 antibody as described previously. Cells were incubated for 5 min (A–C) or 20 min (D–F) then processed for immunofluorescence. B and E, endogenous SPEC2 was visualized using polyclonal anti-SPEC2, and C and F, F-actin was stained with phalloidin. A and D, bead location was visualized by phase contrast. White arrows highlight SPEC2 and F-actin accumulation at the site of contact. The bar represents 10 μm.

that occurs before the marked accumulation of F-actin.

SPEC2 Is Palmitoylated—Although the recruitment of WASP to the immunological synapse is independent of Cdc42 binding and involves its proline-rich sequence (18), a number of important T cell signaling molecules, including Fyn (28, 29), Lek (28, 29), and LAT (30), require palmitoylation and/or other post-translation modifications. Inspection of the SPEC2 amino-acid sequence revealed a pair of N-terminal cysteine residues (cysteine 10 and cysteine 11) that are shared with SPEC1 (Fig. 5A). Furthermore, this pair of N-terminal cysteine residues is evolutionarily conserved in all known eukaryotic species harboring SPEC homologs, including mice, Xenopus, Drosophila, and sea urchins (24, 31). We investigated SPEC2 palmitoylation using a new, more sensitive approach than radioactively labeling with palmitic acid, which utilizes immunoprecipitation in conjunction with a biochemical labeling method (26). In brief, this novel method consists of first covalently blocking free thiol groups on amino acid residues utilizing NEM. Immunoprecipitated proteins are then treated with hydroxylamine to cleave any palmitoylated groups and liberate free thiol groups, which are subsequently labeled with a molecular probe, biotin-conjugated BMCC, and detected via streptavidin-HRP. Using Jurkat T cells, we did not observe any biotinylated 10-kDa SPEC2 protein when using preimmune rabbit sera to immunoprecipitate proteins and treat without (Fig. 5B, lane 1) or with hydroxylamine (Fig. 5B, lane 2). In contrast, a 10-kDa biotinylated species representing endogenous SPEC2 protein was detected in the lysate from the anti-SPEC2 antibody immunoprecipitates that had been treated with hydroxylamine (Fig. 5B, lane 3). Parallel Western blotting analysis with the anti-SPEC2 antibody confirmed that the 10-kDa biotinylated species migrated exactly at the same molecular weight as SPEC2 (data not shown). These results confirm that SPEC2 is palmitoylated and strongly suggest that the pair of evolutionarily conserved cysteines in the N terminus may represent a palmitoylation site that plays an important role in SPEC2 function and/or localization.

Two N-terminal Cysteines, but Not the CRIB Domain, Are Required for SPEC1 Targeting to the Activated TCR—To characterize the localization and function of SPEC2 at activated TCR in Jurkat cells, we tested a number of mammalian expression vectors for SPEC2 protein expression. Unfortunately, using these constructs we were unable to detect significant numbers of SPEC2-expressing cells possibility, due to the fact that overexpression of SPEC2 constructs may be toxic in Jurkat T cells (data not shown) and may be related to our finding that overexpression of SPEC1 or SPEC2 induces membrane blebbing in NIH fibroblasts (24). In contrast to these overexpression studies with SPEC2, we were able to readily detect SPEC1-overexpressing cells. In un-stimulated Jurkat T cells, overexpressed SPEC1 remained diffusely cytoplasmic and at the membrane and had no obvious effect on cell morphology (Fig. 6A). However activation of Jurkat T cells with anti-CD3 antibody-coated-beads caused a dramatic recruitment of SPEC1 protein to the bead-cell interface forming a thick band at the activated TCR (Fig. 6D). These findings with overex-
pressed SPEC1 are essentially the same as those observed with endogenous SPEC2 and SPEC1 are shown. Potential sites of palmitoylation in SPECs on cysteine residues are underlined. It should also be noted that, although there are only three cysteine residues present in SPEC2, all of which are located in the N terminus of the molecule, SPEC1 only has two cysteines in the entire molecule, which are the pair of cysteines shared with SPEC2 at cysteines 10 and 11. Known N-terminal palmitoylation sites on other signaling molecules, including G-α12 (37), G-α13 (37), AKAP18 (38), and GAP-43 (39), are bold and underlined. B, SPEC2 is palmitoylated. Jurkat T cells were harvested in lysis buffer containing 1% Triton X-100 and 50 mM NEM as described under "Experimental Procedures." Immunoprecipitations were performed with preimmune sera (lanes 1 and 2) or with anti-SPEC2 antibody (lanes 3 and 4). Lysates were then treated with 1 M hydroxylamine-1 M Tris (lanes 2 and 4) or with only 1 M Tris (lanes 1 and 3), followed by incubation with biotin-conjugated BMCC. Western blotting with streptavidin-conjugated HRP-detected biotinylated proteins. Results are representative of four experiments.

**FIG. 5. SPEC2 is palmitoylated.** A, potential palmitoylation of SPECs on N-terminal cysteine residues. The N-terminal sequences of human SPEC2 and SPEC1 are shown. Potential sites of palmitoylation in SPECs on cysteine residues are underlined. It should also be noted that, although there are only three cysteine residues present in SPEC2, all of which are located in the N terminus of the molecule, SPEC1 only has two cysteines in the entire molecule, which are the pair of cysteines shared with SPEC2 at cysteines 10 and 11. Known N-terminal palmitoylation sites on other signaling molecules, including G-α12 (37), G-α13 (37), AKAP18 (38), and GAP-43 (39), are bold and underlined. B, SPEC2 is palmitoylated. Jurkat T cells were harvested in lysis buffer containing 1% Triton X-100 and 50 mM NEM as described under "Experimental Procedures." Immunoprecipitations were performed with preimmune sera (lanes 1 and 2) or with anti-SPEC2 antibody (lanes 3 and 4). Lysates were then treated with 1 M hydroxylamine-1 M Tris (lanes 2 and 4) or with only 1 M Tris (lanes 1 and 3), followed by incubation with biotin-conjugated BMCC. Western blotting with streptavidin-conjugated HRP-detected biotinylated proteins. Results are representative of four experiments.

**FIG. 6. SPEC1 mutants alter F-actin accumulation at the activated TCR.** Jurkat T cells were electroporated with Myc-epitope-tagged wild type SPEC1 (A–E), SPEC1-C10A,C11A (F–H), or SPEC1-P33A,H38A,H41A (I–K). Lymphocytes were then conjugated with anti-CD3-coated beads and processed for immunofluorescence. A, D, G, and J, overexpressed SPEC1 and SPEC1 mutants were visualized with a polyclonal anti-Myc antibody. B, E, H, and K, F-actin was visualized with Alexa Fluor 488-labeled phalloidin. C, F, and I, bead location is shown by phase contrast. White arrows highlight SPEC1, SPEC1 CRIB mutant, or F-actin accumulation. The bar represents 10 μm. Upper right panel, SPEC1 and SPEC1 mutant localization at the activated TCR. Polarization of wild type SPEC1, SPEC1-C10A,C11A, or SPEC1-P33A,H38A,H41A expressed in Jurkat T cells conjugated to anti-CD3 coated beads were scored as positive by recruitment of tagged protein to the activated TCR as described under “Experimental Procedures.” The bar graphs are representative of three independent experiments. Lower right panel, F-actin accumulation at the TCR in Jurkat T cells expressing wild type SPEC1, SPEC1-C10A,C11A, or SPEC1-P33A,H38A,H41A. Positive F-actin accumulation at the bead-T cell interface was scored by the polarization of F-actin at the bead-T cell interface. The bar graphs represent three independent experiments. Values indicate mean and standard deviation from three independent experiments.
in the N terminus and the Cdc42-binding region (SPEC1-P35A,H38A,H41A) to determine whether these mutants altered SPEC1 localization to the activated TCR. Jurkat T cells expressing the SPEC1-C10A,C11A mutant, a mutant that still contains an intact CRIB sequence, rarely showed recruitment to the activated receptor, but was still able to associate with the plasma membrane (Fig. 6G). Cell counting experiments showed that 20% of the cells expressing this mutant had SPEC1 staining at the TCR (Fig. 6A). F-actin accumulation at the activated TCR in cells expressing SPEC1-C10A,C11A mutant was also often defective (Fig. 6H). Cell counting revealed that only 47% of cells expressing the SPEC1-C10A,C11A mutant had normal F-actin accumulation (Fig. 6, lower right panel). A different result was found when we tested SPEC1-P33A,H38A,H41A, a mutant protein that still contains an intact N terminus but is defective in Cdc42 binding (24). Similar to overexpressed wild type SPEC1, the SPEC1-P33A,H38A,H41A CRIB mutant still localized to the bead-cell interface in activated T cells in 75% of the cells (Fig. 6J and upper right panel). However, Jurkat T cells expressing the SPEC1-C10A,H38A,H41A mutant were dramatically affected in their ability to accumulate F-actin at the activated TCR (Fig. 6K). Only 6% of the conjugates expressing this mutant showed significant F-actin accumulation at the activated receptor (Fig. 6, lower right panel). Taken together, these results suggest that SPEC1, and likely SPEC2, do not require interaction with Cdc42 for recruitment to the activated TCR. Furthermore, these results suggest the dual cysteine residues conserved between SPEC1 and SPEC2 are involved in targeting to the activated TCR, and that this region in both molecules may play an important role in regulating F-actin accumulation at the immunological synapse.

**DISCUSSION**

Recent data suggest an increasingly important role of Cdc42 signaling pathways in T cell activation (2). Although numerous Cdc42 effector proteins are known, few have been studied in detail in the context of the immunological synapse. In this study we have investigated the role of two small Cdc42-binding proteins, SPEC1 and SPEC2, in TCR signaling. Analysis of SPEC2 mRNA expression in humans and mice indicates that it is ubiquitously expressed in all cells and tissues but shows unusually high levels of expression in thymus, spleen and lymphocytes (27). Although we have only analyzed SPEC2 protein levels in a small number of cell lines and normal mouse tissue extracts, our results are consistent with these previously published mRNA profiles. It should be noted that, although SPEC2 does show the highest expression in lymphocyte cell lines and tissues, many other tissues and cell types show significant SPEC2 mRNA and protein expression. Interestingly, SPEC1 mRNA expression studies also reveal that the highest level of expression of SPEC1 is in T lymphocytes and hematopoietic cells, but the relative difference in expression of SPEC1 between cells of hematopoietic origin and other cells and tissues is not as dramatic as observed for SPEC2 (27). Although SPEC proteins are widely expressed across different tissue types, these differing mRNA expression patterns between SPEC1 and SPEC2 are reminiscent of that observed for WASP and N-WASP, in which WASP is highly expressed in cells of hematopoietic origin (11), whereas N-WASP is ubiquitously expressed (32). Using models of T cell activation, we found that endogenous SPEC2 protein is recruited to the immune synapse in T cell-APC conjugates and to the site of receptor engagement on anti-CD3 antibody-coated beads. Similarly, overexpressed wild type SPEC1 in T cells also localized to the site of receptor engagement with anti-CD3 antibody-coated beads. Both endogenous SPEC2 and overexpressed SPEC1 localized with significant F-actin accumulation at the bead-cell interface. Studies with SPEC1 mutants revealed that the Cdc42 binding sequence was not required for localization to the activated TCR, but that the two evolutionarily conserved N-terminal cysteine residues (Cys-10 and Cys-11) are required for targeting. These results are consistent with a seminal study on WASP showing that Cdc42 binding is not required for its cellular localization to the immunological synapse (18). One possible mechanistic role of these dual cysteine residues in targeting SPECs to activated TCR comes from our biochemical studies showing that SPEC2, and likely SPEC1, are S-palmitoylated. S-Palmitoylation is a post-translational event involving the addition of palmitate onto cysteine residues, increasing a protein’s hydrophobicity and thereby affecting protein localization and/or activity (33). S-Palmitoylation of a number of signaling molecules, including Fyn (28, 29), Lck (28, 29), and LAT (30) are important for their function in synapse formation, because treatment of T cells with 2-bromopalmitate, a reagent that blocks protein palmitoylation, inhibits formation of the immunological synapse (34). Additional support for an important role for palmitoylation in Cdc42 signaling comes from studies showing that 2-bromopalmitate treatment inhibits the formation of Cdc42-mediated filopodia (35). Studies using palmitoylation mutants of LAT demonstrate that palmitoylation is required for its association with lipid rafts and for lipid raft aggregation following TCR engagement, but not for association with the plasma membrane in unstimulated cells (30, 34, 36). Because palmitoylation is a reversible and regulated event (33), it is possible that the activation state of the T cell influences the palmitoylation status of SPECs. Although technical limitations prevented us from biochemically analyzing the palmitoylation status of SPEC1 and SPEC2 mutants, future studies with more efficient expression systems, including adenoviral expression vectors, may allow us to analyze the role of palmitoylation in SPEC function in more detail. Studies with SPEC1 mutants in Jurkat T cells demonstrated that SPEC1 likely plays an important role in the F-actin changes associated with TCR signaling. In contrast to fibroblast studies in which SPEC overexpression-induced membrane blebbing (24), overexpression of wild type SPEC1 had no obvious effect on the morphology of Jurkat T cells or F-actin accumulation at the activated TCR. However, overexpression of both the double cysteine (SPEC1-C10A,C11A) and the CRIB (SPEC1-P33A,H38A,H41A) mutants blocked F-actin accumulation at the activated TCR. The ability of the SPEC1-C10A,C11A mutant to block F-actin accumulation is not surprising based on the ability of this mutant to still bind and sequester active Cdc42 (24). The effects of this SPEC1 mutant are also consistent with studies showing that dominant negative Cdc42 blocks F-actin and tubulin changes associated with the immune synapse (3). Interestingly, a more dramatic inhibition of F-actin accumulation at the bead-cell interface occurred with the SPEC1 CRIB mutant, which still contains an intact N terminus and is still recruited to the activated TCR. These results suggest that the N terminus, when overexpressed, may act as a dominant negative mutant, blocking F-actin accumulation at the activated TCR possibly by preventing the accumulation of additional signaling protein(s) directly involved in actin polymerization. Interestingly, the importance of these dual cysteines is consistent with our finding that they are required for SPEC1-induced membrane blebbing in fibroblasts (24). Alternatively, it is possible that the normal mechanistic function of SPECs is to bind, stabilize, and concentrate GTP-bound Cdc42 at polarized sites in different cell types, including the immunological synapse in T cells. In this model, again the dominant negative N terminus of SPEC1 may block Cdc42 localization to specific sites at the synapse, thereby inhibiting F-actin accumulation. In agreement with
this model, endogenous SPEC2 colocalized with Cdc42 at the APC interface in T cells and following TCR activation. T cell polarization following TCR activation is just one example of induced cellular asymmetry that requires activated Cdc42. Migrating cells, another example of cell polarity, accumulate activated Cdc42 at their leading edges, and Cdc42 is required for filopodia formation (37). Interestingly, SPEC2 is recruited from the cytoplasm to the leading edge of migrating HeLa cells in a scratch assay where it colocalizes with Cdc42. These observations suggest that SPECs may generally be involved in Cdc42-mediated polarity establishment in other cell types besides lymphocytes. Finally, because it has required over 10 years of research to establish that WASP is not involved in initiating F-actin polymerization at the immunological synapse (22), the exact in vivo functional significance of SPECs will require many more experiments using more sophisticated reagents such as SPEC knock-out mice.

Acknowledgment—We thank Dr. V. S. Shapiro (University of Pennsylvania) for helpful discussions and advice.

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