Deficiency of Small GTPase Rac2 Affects T Cell Activation

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Abstract

Rac2 is a hematopoietic-specific GTPase acting as a molecular switch to mediate both transcriptional activation and cell morphological changes. We have examined the effect of Rac2 deficiency during T cell activation. In $Rac2^{-/-}$ T cells, proliferation was reduced upon stimulation with either plate-bound anti-CD3 or T cell receptor–specific antigen. This defect is accompanied with decreased activation of mitogen activated protein kinase extracellular signal–regulated kinase (ERK)1/2 and p38, and reduced Ca^{2+} mobilization. TCR stimulation–induced actin polymerization is also reduced. In addition, anti-CD3 cross-linking–induced T cell capping is reduced compared with wild-type T cells. These results indicate that Rac2 is important in mediating both transcriptional and cytoskeletal changes during T cell activation. The phenotypic similarity of $Rac2^{-/-}$ to $Vav^{-/-}$ cells implicates Rac2 as a downstream mediator of Vav signaling.

Key words: T cell activation • Rac2 • VAV • MAPK • cytoskeleton

Introduction

After the engagement of TCR and its specific ligand, peptide/MHC complexes, a cascade of biochemical changes occur leading to transcriptional activation and cell cytoskeletal reorganization. Engagement of TCRs cause the activation of Src and Syk/ZAP-70 family proteins and the tyrosine phosphorylation of various cellular substrates, calcium (Ca^{2+}) mobilization, and activation of mitogen-activated protein kinases (MAPKs)* including extracellular signal–regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38, thus translating the receptor signal to transcriptional activation of effector genes. Simultaneously, TCR engagement also causes cytoskeleton reorganization, results in T cell polarization toward APCS, enhanced T cell APC interaction, and supramolecular activation clusters (SMACs) formation (1). These biochemical and cytoskeletal changes are well orchestrated and ensure sustained TCR signaling, leading to T cell proliferation, differentiation, and cytokine production. Compromising either biochemical or cytoskeletal changes results in dampened T cell response to stimulation (2). Thus, molecules orchestrating these two aspects play a key role in T cell activation.

Rac proteins are members of the Rho GTPase family and cycle between the active GTP-bound form and the inactive GDP bound form. Activated Rac binds to a variety of down stream effector molecules thereby mediating multiple cellular functions in different cell types (3). Specifically, Rac is well known for its involvement in cytoskeleton reorganization. Activation of Rac is associated with changes in actin polymerization, causing lamellipodia formation and membrane ruffles in fibroblasts (4, 5), morphological changes in platelets (6), and facilitating cell adhesion and chemotaxis response in macrophages and neutrophils (7–9). Rac also participates in signal transduction via mitogen-activated kinase pathways. In vitro studies indicate that by activating PAK1, a well known Rac target, Rac can activate ERK in T cells upon TCR stimulation (10), and in NK cells upon interaction with the target cell (11). Rac also activates c-Jun NH_2-terminal kinase and p38 kinase pathways through PAK1 (12, 13). Moreover, Rac GTPases play an important role in cell cycle progression and survival in response to mitogenic stimulation (14, 15), and are essential for cellular transformation by Ras (16, 17). Furthermore, Rac is essential for the activation of a multiprotein complex that produces superoxide in phagocytic cells, the NADPH oxidase (9, 18–20).

While the function and biochemical properties have been

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**Abbreviations used in this paper:* ERK, extracellular signal–regulated kinase; JNK, c-Jun NH_2-terminal kinase; KO, knockout; MAPK, mitogen-activated protein kinase; SMAC, supramolecular activation cluster; GEF, guanine nucleotide exchange factor.

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well studied in many cell types, the role of Rac GTPases during T cell activation is less clear. Studies from Vav-deficient mice suggest that Rac could play an important role in T cell activation (21–23). Vav is a guanine nucleotide exchange factor (GEF), enhancing the exchange of GDP to GTP, thus promoting the activation of Rho GTPase. In vitro, Rac GTPase has been shown to be a major substrate of Vav GEF activity (24). Upon T cell stimulation, Vav is rapidly activated through phosphorylation. In the absence of Vav, T cells exhibit a profound activation defect, with decreased proliferation in response to anti-CD3 in the presence and absence of anti-CD28, and decreased IL-2 production (21–23). Biochemical analysis of $Vav^{-/-}$ T cells showed that although early tyrosine phosphorylation of Zap70, Cbl-c, SH2 domain–containing leukocyte protein of 76 kD (SLP-76), and linker for activation of T cells (LAT) are normal, ERK and nuclear factor (NF)-κB activation are defective upon TCR stimulation. TCR induced Ca^{2+} flux is also defective. Moreover, $Vav^{-/-}$ T cells showed defects in actin polymerization and cap formation in response to TCR stimulation (21–23). As the Rac GTPase is a major substrate for Vav GEF activity and has demonstrated its function in both transcriptional activation and cytoskeleton reorganization, it is conceivable that at least part of the phenotype of Vav knockouts may derive from the defective activation of Rac. Most recently, using an in vitro system, Arrieumerlou et al. have demonstrated that dominant negative Rac affects Ca^{2+} mobilization and actin polymerization in response to TCR cross-linking (25). These results indicate that Rac could play an important role in T cell activation. However, as dominant negative small GTPases are pleiotropic in action, it is possible that this effect is mediated through other proteins such as Ras or Rho. Moreover, there are three members within the Rac subfamily, Rac1, Rac2, and Rac3. While Rac1 is universally expressed, the expression of Rac2 is restricted to the hematopoietic lineage (26). Rac3 is a relative newly described member of Rac family and is highly expressed in brain (27). As Rac proteins are highly homologous to each other, sharing 89–92% sequence homology, it is plausible that there are functional overlaps between Rac proteins.

In this report, we studied the role of the hematopoietic-specific GTPase Rac2 in T cell activation using Rac2-deficient mice (9). T cell activation was compromised in response to either anti-CD3 or TCR-specific antigen. Costimulation with anti-CD28 or adding IL-2 partially compensates for the proliferation defect. Biochemically, $Rac2^{-/-}$ T cells showed decreased phosphorylation of ERK1/2 and p38. Antigen-induced Ca^{2+} flux is also reduced. Moreover, actin polymerization upon TCR cross-linking or antigen stimulation is decreased. This defective cytoskeletal change is accompanied by decreased cap formation. These results indicated that deficiency of Rac2 affects both transcriptional activation and cytoskeleton reorganization during T cell stimulation, and suggests that defective Rac activation is partially responsible for the observed defects in $Vav^{-/-}$ mice.

Materials and Methods

Mice. B10. BR mice were obtained from The Jackson Laboratory. AND TCR transgenic mice with $CD4^+$ T cells expressing a TCR specific for the COOH terminus of pigeon cytochrome c have been described (28) and were maintained on the C57BL/6 background in our facility. Rac2 knockout mice (Rac2KO) on the C57BL/6 background have been described (9). Rac2KO mice were further bred with AND TCR transgenic mice. All mice were maintained in pathogen-free condition and were used at 6–12 wk of age.

Preparation of APC, $CD4^+$, $CD8^+$ Mature T Cells and Thymocytes Subpopulations. T cell–depleted APC were prepared by incubating B10BR splenocytes with anti–Thy-1, anti-CD8, anti-CD4, and anti-NK1.1 antibody, followed by complement mediated lysis. $CD4^+$ T cells from spleens and lymph nodes were isolated by immunomagnetic negative selection using antibodies against CD8, NK1.1, and MHC class II, followed by incubation with anti–mouse and anti–rat Ig-coated magnetic beads (PerSeptive Biosystems). Mature $CD8^+$ T cells were isolated in the similar manner as $CD4^+$ T cells. $CD4^+CD8^+$ and $CD4^+$ thymocytes were isolated by flow cytometry sorting. $CD4^-CD8^-$ and $CD8^+$ thymocytes were isolated by depleting $CD4^+$ cells in total thymocytes first, followed by separation of $CD8^+$ and $CD4^-CD8^-$ cells using MACs column according to the manufacturer's suggestion (Miltenyi Biotec).

T Cell Proliferation and IL-2 Production. 10^5 purified $CD4^+$ T cells were cultured in triplicate in round-bottom plate precoated with anti-CD3 antibody (clone 145–2C11) with or without anti-CD28. Alternatively, 10^5 AND TCR transgenic $CD4^+$ cells were cocultured with 10^5 mitomycin C treated I-E^{k+} CH27 cells or B10BR APCs in the presence of different doses of MCC peptide (VFAGLKKANERADLIAYLKQATK). T cells were pulsed with 1 μCi/well [^{3}H]thymidine 48 h after stimulation and were harvested 12 h later. The level of [^{3}H]thymidine incorporation was determined by scintillation counting. IL-2 production was measured by ELISA (BD PharMingen) 24 h after stimulation.

Immunoblotting. AND TCR transgenic T cells were stimulated with either fresh or paraformaldehyde fixed B10BR APCs prepulsed with MCC peptide. At the indicated time point, cells were harvested and lysed in ice-cold lysis buffer (20 mM Tris, pH 7.2, 1% NP-40, 150 mM NaCl, 1 mM $MaCl_2$, 1 mM EDTA) containing protease and phosphatase inhibitors. Nuclear material was removed by centrifuging at 13,000 rpm for 15 min. Supernatant of the cell lysate was electrophoresed on a 12.5% SDS-PAGE gel, transferred to immunoblot membrane, and blotted with the indicated antibody. Antibody against phospho-tyrosine 4G10 was obtained from Upstate Biotechnology. Antibodies against phospho-ERK, ERK, phospho-p38, p38, Phospho-JNK, and JNK were obtained from New England Biolabs, Inc. and were used as directed. To check for rac protein expression in different population of mature T cell and thymocytes, 50 μg of cell lysates from each cell population were used for Western blotting. Rac1 antibody was from Santa Cruz Biotechnology, Inc., and Rac2 antibody was provided by Gary M. Bokoch from the Scripps Research Institute, La Jolla, CA.

ERK kinase assay AND TCR transgenic T cells were stimulated with fresh B10BR APCs prepulsed with MCC peptide for the indicated time. Cells were then lysed in the aforementioned lysis buffer. ERK kinase activity were assayed using MAPK Immunoprecipitation Kinase Assay Kit (Upstate Biotechnology) following the manufacturer's protocol.

Ca^{2+} Mobilization. Calcium signaling after Ag-specific stimulation was monitored as described previously (29). Briefly, $CD4^+$

T cells loaded with 5 μM fluo3/AM ester (Molecular Probes) were plated by centrifugation in flat 96-well plates at a concentration of 5×10^5 cell/50 μl. The cells were then scanned using the ACAS 570 video laser cytometer (Meridian Instruments). After initiation of scanning, 4×10^6 T cell–depleted splenocytes pulsed with peptide was added to the $CD4^+$ T cells. To examine antibody cross-linking–induced Ca^{2+} flux, fluo3/AM loaded T cells were incubated with the indicated amount of anti-CD3 antibody for 5 min, then recovered by centrifugation in a microcentrifuge (2,000 rpm for 3 min), resuspended, and plated in a flat 96-well plate. The cells were then scanned for 1 min followed by addition of cross-linking antibody anti–hamster IgG (100 μg/ml). The initial average fluorescence of each cell was digitized and normalized to 1, and the results are expressed as changes in normalized fluorescence intensity of individual cells over time. The percentage of responding cells was determined by dividing the number of cells demonstrating an increase in intracellular calcium of >50% by the total number of scanned cells. The time from the addition of the antibody to the start of Ca^{2+} flux was recorded among 100 $Rac2^{-/-}$ and $Rac2^{+/+}$ cells studied.

Actin Polymerization. To measure changes in actin polymerization upon TCR cross-linking, $CD4^+$ T cells were incubated with anti-CD3 antibody (1 μg/ml) on ice for 30 min, followed by washing and cross-linking with anti–hamster IgG (3 μg/ml) for the indicated time. Alternatively, AND TCR transgenic T cells were mixed with equal number of CH27 APCs preloaded with various amounts of MCC peptide. T cell APC interaction was initiated by a quick spin of the cell mixture followed by incubation at 37°C for the indicated time. Activation was terminated by addition of 4% paraformaldehyde. After fixation, cells were incubated with FITC-conjugated phalloidin (Sigma-Aldrich) for 30 min, washed three times in PBS, and analyzed using a FACSCalibur™ flow cytometer.

Capping and Immunofluorescence Study. 10^6 $CD4^+$ T cells were incubated with anti-CD3 at the indicated concentrations on ice for 30 min. After washing, cells were further incubated with biotinylated anti–hamster Ig (3 μg/ml) at 37°C for 30 min, followed by addition of 4% paraformaldehyde to fix cells. Fixed cells were stained with Avidin-Texas Red (BD PharMingen) and were cytospun onto slides and mounted with Vectorshield (Vector Laboratories). The cells were visualized with a ZEISS fluorescence microscope. The percentage of capped cells was obtained by counting cells from 10–15 random fields and a total of more than 100 cells were counted per experimental condition in each experiment.

Results

Rac2 Deficiency Affect T Cell Activation. Rac2 is a GTPase specifically expressed in hematopoietic lineage cells. To study the role of Rac2 during T cell activation using

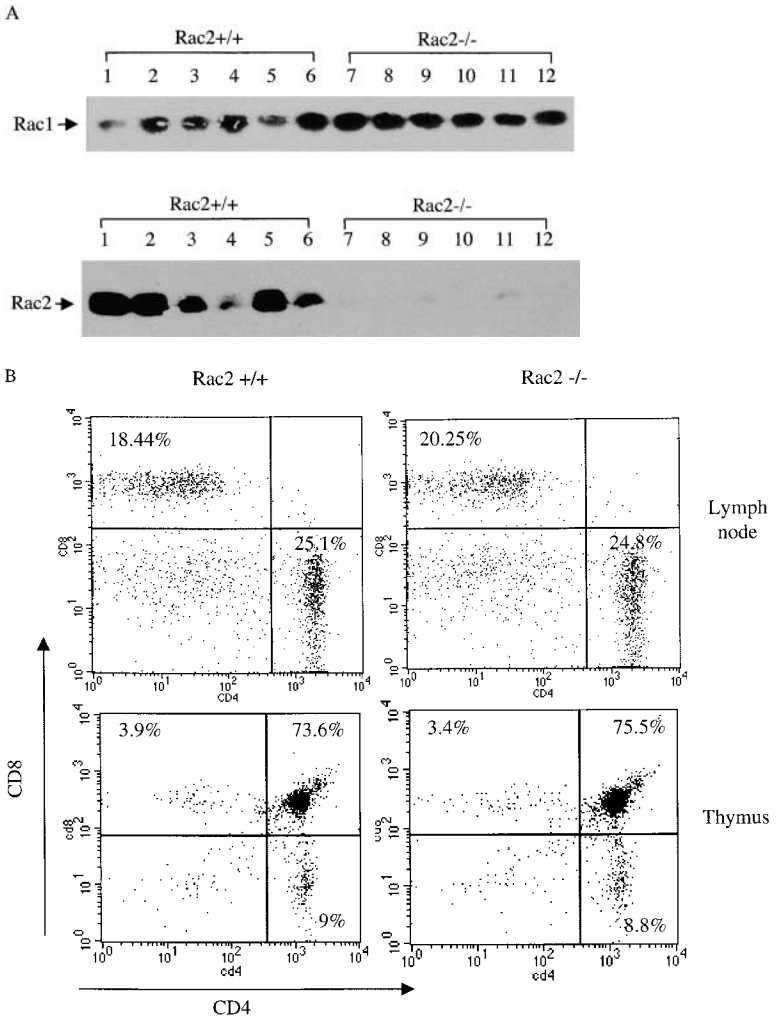

Figure 1. Rac protein expression and T cell development in $Rac2^{-/-}$ and $Rac2^{+/+}$ mice. (A) Rac1 and Rac2 protein expression in subpopulation of thymocytes and mature peripheral T cells. 50 μg of cleared cell lysate from each cell population was used for Western blotting. The same blot probed for Rac1 was stripped and reprobed for Rac2. Lane 1 and 7, mature $CD4^+$ cells; lane 2 and 8, mature CD8 cells; lane 3 and 9, $CD4^+CD8^+$ thymocytes; lane 4 and 10, $CD4^-CD8^-$ thymocytes; lane 5 and 11, $CD4^+$ thymocytes; lane 6 and 12, $CD8^+$ thymocytes. The rac2 antibody weakly cross reacts with another protein in lysates from $Rac2^{-/-}$ cells and the control cells (reference 9). Data represents one of two experiments. (B) Lymph node cells or thymocytes were isolated from $Rac2^{-/-}$ mice and its littermate $Rac2^{+/+}$ mice. The expression of CD4 and CD8 was examined by two-color flow cytometry.

Rac2KO mice, we first examined the expression pattern of Rac proteins among subpopulation of thymocytes and mature peripheral T cells in wild-type and Rac2KO mice. As shown in Fig. 1 A, while Rac1 showed lower level of expression in $CD4^+$ cells, Rac2 showed higher expression in $CD4^+$ thymocytes and mature CD4 and CD8 single-positive cells. Upon disruption of Rac2, there is compensatory overexpression of Rac1 in $CD4^+$ thymocytes and mature T cells (Fig. 1 A). Analysis of peripheral T cell subsets demonstrated no significant alterations in cell number or distribution. Thymocyte cellularity and subsets were also similar to wild-type mice (9). Thus, T cell development was not grossly altered in $Rac2^{-/-}$ mice (Fig. 1 B). This normal T cell development may partly due to the compensatory effect of Rac1 in the Rac2KO mice. To study the functional consequences of the Rac2 deficiency in T cell activation, we first examined the proliferation of total splenocytes stimulated with a dose range of plate bound anti-CD3. As shown in Fig. 2 A, decreased proliferation was observed in $Rac2^{-/-}$ total splenocytes. Purified $CD4^+$ T cells were further studied by stimulating with a dose range of plate-bound anti-CD3. $Rac2^{-/-}$ CD4 T cell proliferation was reduced about twofold upon stimulation with plate-bound anti-CD3 (Fig. 2 B). The addition of IL-2 partially compensated for the defect in T cell activation, suggesting that IL-2 production is compromised in the $Rac2^{-/-}$ T cells and is partially responsible for the proliferation defect (Fig. 2 C). Costimulation with anti-CD28 also compensated for the T cell proliferation defect (Fig. 2 C). As anti-CD28 stimulation alone can cause Vav phosphorylation (30, 31), it is plausible that costimulation of anti-CD28 further activates other Rho GTPases such as Rac1 and Rac3, thus compensating for the Rac2 deficiency. Interestingly, Rac2-deficient B cells also showed reduced proliferation upon stimulation with anti-IgM plus IL-4 ($P < 0.01$; Fig. 2 D).

To study Rac2 function during T cell activation in a more physiologically relevant context, $Rac2^{-/-}$ mice was crossed with AND TCR transgenic mice on the C57B/6 background. The AND TCR transgenic mouse expresses $V\alpha11/V\beta3$ TCR recognizing a peptide of cytochrome c (pigeon cytochrome c fragment) presented on IE^k-bearing APCs (28). Although the AND TCR can be positively selected on $I\text{-}A^b$ in the thymus, it cannot recognize MCC peptide on $H\text{-}2^b$ APC (32), therefore effectively eliminating any contribution of APC contaminants in the T cell preparation. The expression of TCR transgene was assessed by flow cytometry using $V\alpha11$ and $V\beta3$ antibody. A similar percentage of $V\alpha11/V\beta3$ $CD4^+$ cells in the lymph node and spleen was observed in $Rac2^{-/-}$ TCR transgenic compared with the wild-type mice (data not shown), indicating that Rac2 deficiency does not significantly affect T cell–positive selection in the thymus. $Rac2^{-/-}$ $CD4^+$ TCR transgenic cells were stimulated with $I\text{-}E^{K+}$ splenic APCs with varying amount of MCC peptide. Proliferation was two- to threefold lower in $Rac2^{-/-}$ T cells compared with the control (Fig. 3 A). $I\text{-}E^{K+}$ CH27 cells, which constitutively express both CD80 and CD86 molecules, were also used to stimulate TCR transgenic CD4 cells. Although the proliferation was much more vigorous compared with the stimulation with resting splenic APCs, $Rac2^{-/-}$ T cells still showed a two- to threefold lower level of proliferation (Fig. 3 B), accompanied by a lower level of IL-2 production (Fig. 3 C). Similar defects were also observed when stimulating T cells with fibroblast APCs (DCEK-Hi7; reference 33) expressing $I\text{-}E^k$ and B7 molecules (data not shown). This result suggests that the activation defect in

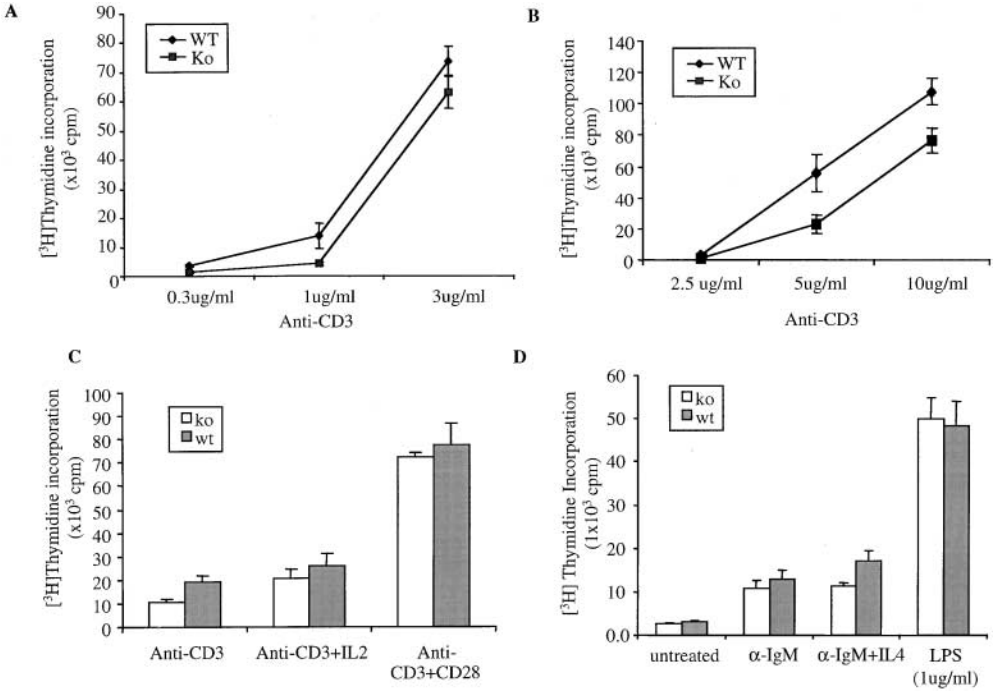

Figure 2. Defective T cell proliferation upon stimulation of plate-bound anti-CD3. (A) Splenocytes from $Rac2^{-/-}$ and control mice were stimulated with varying amounts of plate-bound anti-CD3. WT, wild-type. (B) Purified CD4 T cells stimulated with a dose range of plate-bound anti-CD3. (C) Purified T cells were stimulated with plate-bound anti-CD3 (5 μg/ml) alone, in the presence of IL-2 (50 μ/ml), or plate-bound anti-CD28 (2.5 μg/ml). (D) Purified B cells were stimulated with anti-IgM (10μg/ml), anti-IgM (10 μg/ml) + IL-4 (1,000 U/ml), or LPS (1 μg/ml). [3H]Thymidine was pulsed 48 h after stimulation and harvested 12 h later. Data represent one of three separate experiments.

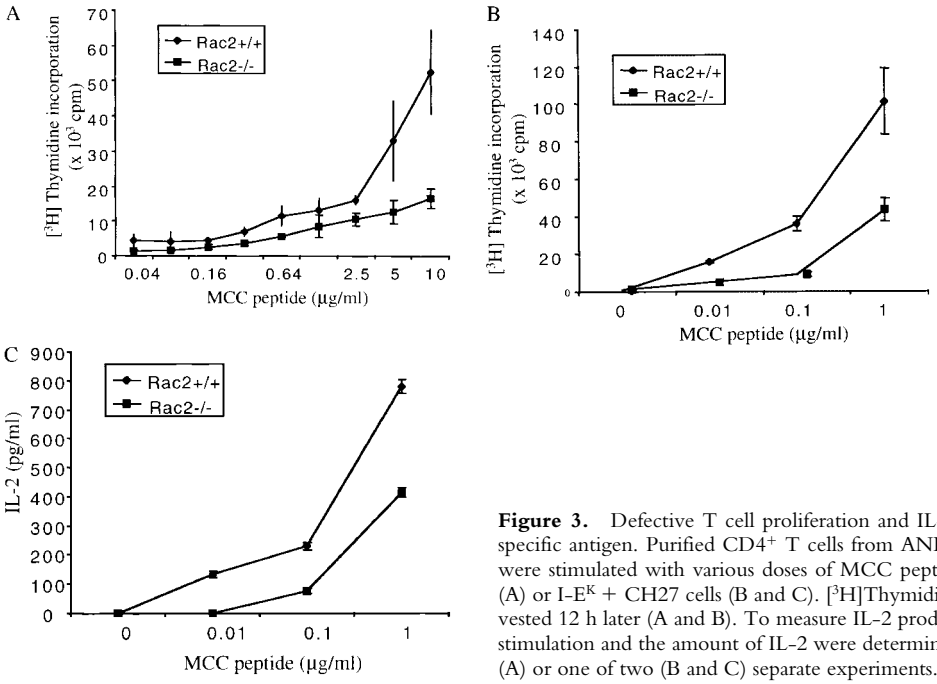

Figure 3. Defective T cell proliferation and IL-2 production upon stimulation with TCR-specific antigen. Purified $CD4^+$ T cells from AND TCR transgenic $Rac2^{-/-}$ or control mice were stimulated with various doses of MCC peptide in the presence of B10BR splenic APCs (A) or $I\text{-}E^K$ + CH27 cells (B and C). [^{3}H]Thymidine was pulsed 48 h after stimulation and harvested 12 h later (A and B). To measure IL-2 production, supernatant were harvested 24 h after stimulation and the amount of IL-2 were determined by ELISA (C). Data represent one of four (A) or one of two (B and C) separate experiments.

$Rac2^{-/-}$ T cells cannot be overcome using activated APCs. It is also consistent with previous findings that plate-bound anti-CD28 stimulation is different from stimulation with nature B7 ligand (30).

Decreased ERK, p38 Activation upon T Cell Stimulation. To understand the mechanism that accounts for the T cell functional defect in $Rac2^{-/-}$ mice, we first studied the early biochemical changes upon T cell stimulation. Total tyrosine phosphorylation was examined in $CD4^+$ T cells after stimulation with antigen pulsed APCs for the indicated time. There was no significant change in the total tyrosine phosphorylation pattern in $Rac2^{-/-}$ T cells compared with the wild-type controls (Fig. 4 A). Further examination of the phosphorylation pattern of Zap70 and PLC-γ1 by immunoprecipitation showed no significant difference between $Rac2^{-/-}$ and control T cells (data not shown). TCR stimulation has been reported to cause activation of the MAPK pathway (34–37). As a signaling mol-

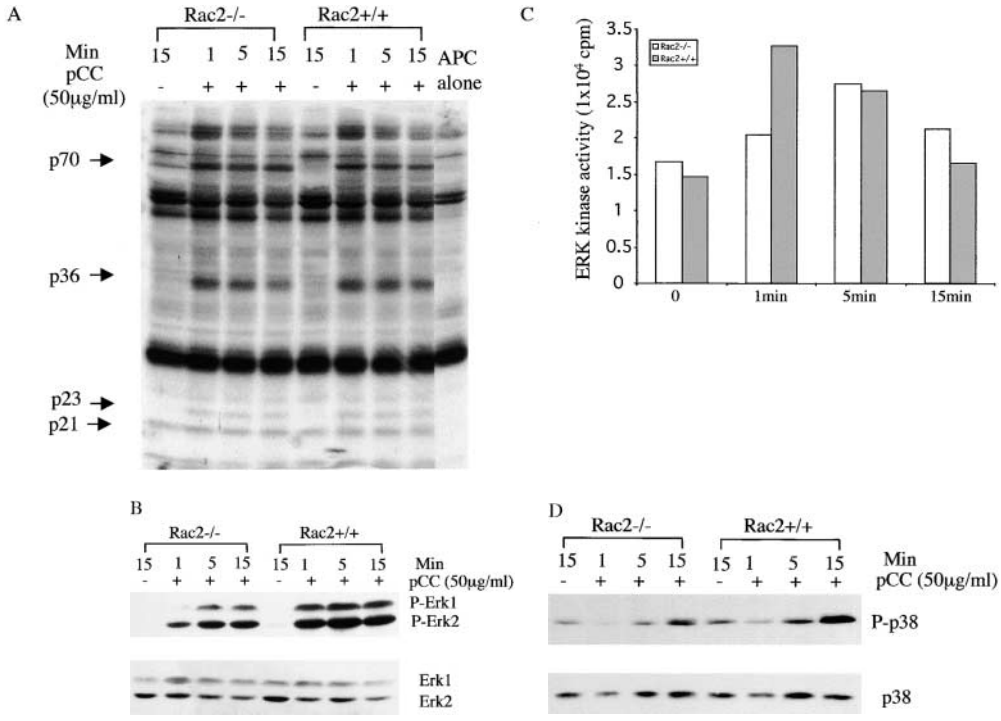

Figure 4. Reduced MAPK activation upon TCR stimulation. Purified $CD4^+$ T cells (2×10^6) from AND TCR transgenic $Rac2^{-/-}$ or control mice were stimulated with B10BR splenic APCs (2×10^6) pulsed with 50 μg/ml MCC peptide for 1, 5, and 15 min at 37°C. Cell lysates were separated by 12.5% SDS-PAGE and subject to immunoblot analysis. (A) Analysis of total phosphotyrosine proteins after stimulation using anti-phosphotyrosine antibody. (B) Analysis of phosphorylation of ERK1/2 using phospho-ERK antibody. The same blot was stripped and reprobed with ERK kinase antibody to confirm equal protein loading. (C) Analysis of ERK kinase activity. (D) Analysis of phospho-p38. Protein loading was confirmed by reprobing with antibody against total p38 (data represent one of four separate experiments except ERK kinase analysis represent one of two separate assays).

ecule, Rac has been shown to contribute to the activation of the Ras pathway through PAK (10, 11). In addition, overexpression of Rac has been demonstrated to activate the JNK and p38 pathways (12). Thus, we further examined the activation of ERK, JNK, and p38 in $Rac2^{-/-}$ T cells during antigen-specific stimulation. As shown in Fig. 4 B, although $Rac2^{-/-}$ T cells showed increased ERK1/2 phosphorylation upon stimulation, the level of ERK1/2 activation is reduced compared with the control. We further examined ERK kinase activity upon stimulation. Consistent with what was observed in ERK1/2 phosphorylation, decreased ERK activity was observed 1 min after stimulation (Fig. 4 C). A slight decrease in p38 phosphorylation was also observed in $Rac2^{-/-}$ T cells (Fig. 4 D). Little phosphorylated JNK was detected upon stimulation in both wild-type and $Rac2^{-/-}$ T cells (data not shown). This is consistent with previous studies from our laboratory which showed that JNK protein is only expressed at low levels in naive T cells, and is upregulated during T cell activation and therefore functions during the differentiation rather than activation of T cells (38, 39).

Reduced Ca^{2+} Mobilization upon TCR Stimulation. Ca^{2+} mobilization is an early event during T cell activation and is responsible for the activation of Ca^{2+} sensitive phosphatase Calcineurin and nuclear factor of activated T cells (NFAT) mobilization (40). We further examined Ca^{2+} mobilization during antigen-specific stimulation. As shown in A, upon addition of antigen-pulsed APCs, the number of $Rac2^{-/-}$ $CD4^+$ T cells to undergo Ca^{2+} flux was decreased compared with the CD4 T cells from wild-type mice at both high and low antigen doses tested (Fig. 5 A). To rule out that the decreased number of Ca^{2+} responsive cells was due to decreased T cell–APC interaction rather than defective Ca^{2+} mobilization, we also examined Ca^{2+} flux in response to TCR cross-linking. At a low dose of anti-CD3, there is lower Ca^{2+} flux among the responding cells in $Rac2^{-/-}$ T cells compared with the wild-type control (Fig. 5 B). In addition, $Rac2^{-/-}$ cells exhibited a longer lag time to Ca^{2+} increase compared with $Rac2^{+/+}$ cells, which were more synchronized in their response. The average responding time was 217 $\pm$ 72 s for $Rac2^{-/-}$ T cells versus 155 $\pm$ 60 s for wild-type stimulated with 0.1 μg/ml anti-CD3 ($P < 0.01$). At higher doses of anti-CD3, $Rac2^{-/-}$ T cells showed similar pattern of Ca^{2+} mobilization as wild-type T cells (Fig. 5 B, and data not shown).

Defective Cytoskeleton Changes. TCR activation leads to cytoskeletal reorganization in T cells. As Rac proteins have been shown to mediate actin polymerization and cell shape changes (3–5), we further investigated whether deficiency of Rac2 affects TCR-induced actin polymerization. Purified $CD4^+$ cells were cross-linked with anti-CD3 antibody and actin polymerization was analyzed quantitatively using phalloidin labeled with FITC. Phalloidin preferentially binds to polymerized filamentous actin (F-actin). Although a significant increase in actin polymerization was observed in $Rac2^{-/-}$ T cells, the level of polymerization was at a reduced level in comparison to the wild-type control (Fig. 6 A). Similar results were obtained when TCR transgenic T cells were stimulated with APC pulsed with various amount of antigen (Fig. 6 B, and data not shown). These results indicate that Rac2 plays a role in mediating TCR-induced cytoskeleton changes.

Defective Cap Formation and T Cell APC Interaction. We further investigated an actin dependent event upon TCR stimulation: TCR capping. Upon antibody cross-linking, TCR molecules cluster into a cap structure (1). Capping has been demonstrated to require actin polymerization and is blocked by Cytochalasin D (22, 41). To investigate whether antibody cross-linking–induced TCR capping is affected in $Rac2^{-/-}$ cells, purified T cells were incubated with a range of concentrations of anti-CD3 at 4°C, and was subsequently cross-linked with anti–hamster IgG and incubated at 37°C for 30 min. At all doses of anti-CD3 antibody, a reduced number of cells with capped TCR was observed in $Rac2^{-/-}$ T cells (Fig. 7).

Discussion

Rac GTPase plays important roles in both transcriptional activation and cytoskeletal reorganization in a variety of cell types. In this study, we examined the function of hematopoietic-specific Rac2 GTPase during T cell activation. In the absence of Rac2, T cell activation stimulated by either antibody or TCR-specific antigen was defective. This defective T cell activation was accompanied by aberrant biochemical changes manifested as decreased ERK, p38 activation, Ca^{2+} mobilization, and defective actin polymerization which affects TCR capping. Consistent with these in vitro observations, reduced delayed-type hypersensitivity response was observed (data not shown).

Upon T cell stimulation, the small GTPase Ras is rapidly activated and leads to Raf activation, which in turn activates MEK1, and ERK. ERK activation is involved in mediating cellular proliferation, transformation, and differentiation. Although Rac GTPase is well known for its ability to activate the JNK and p38 pathways, activated Rac alone has little effect in activating the ERK pathway (12, 42). However, accumulating evidence suggests that Rac can also influence ERK activation through its target PAK (10, 11, 43). In 293 cells, overexpression of either a dominant negative Rac2 or PAK inhibits Ras-mediated activation of ERK2, whereas constitutively active Rac synergizes with active Raf to increase ERK2 activity (43). In T cells, dominant negative PAK also inhibits TCR-mediated ERK2 activation (10). Activated PAK may influence ERK activation through phosphorylation of MEK1 and/or Raf1 (44). Similar to the findings in $Vav^{-/-}$ T cells and $Rac2^{-/-}$ neutrophils (9, 23), we have observed decreased ERK1/2 phosphorylation upon TCR stimulation. As in many other studies on early T cell activation (45–47), biochemical changes were observed in T cells stimulated with live antigen-pulsed APCs. To rule out the possibility that decreased ERK phosphorylation reflects changes in APCs rather than in T cells, we used fixed APCs prepulsed with antigen and obtained the same result (data not shown). Furthermore, reduced ERK activation was also observed in $Rac2^{-/-}$ T

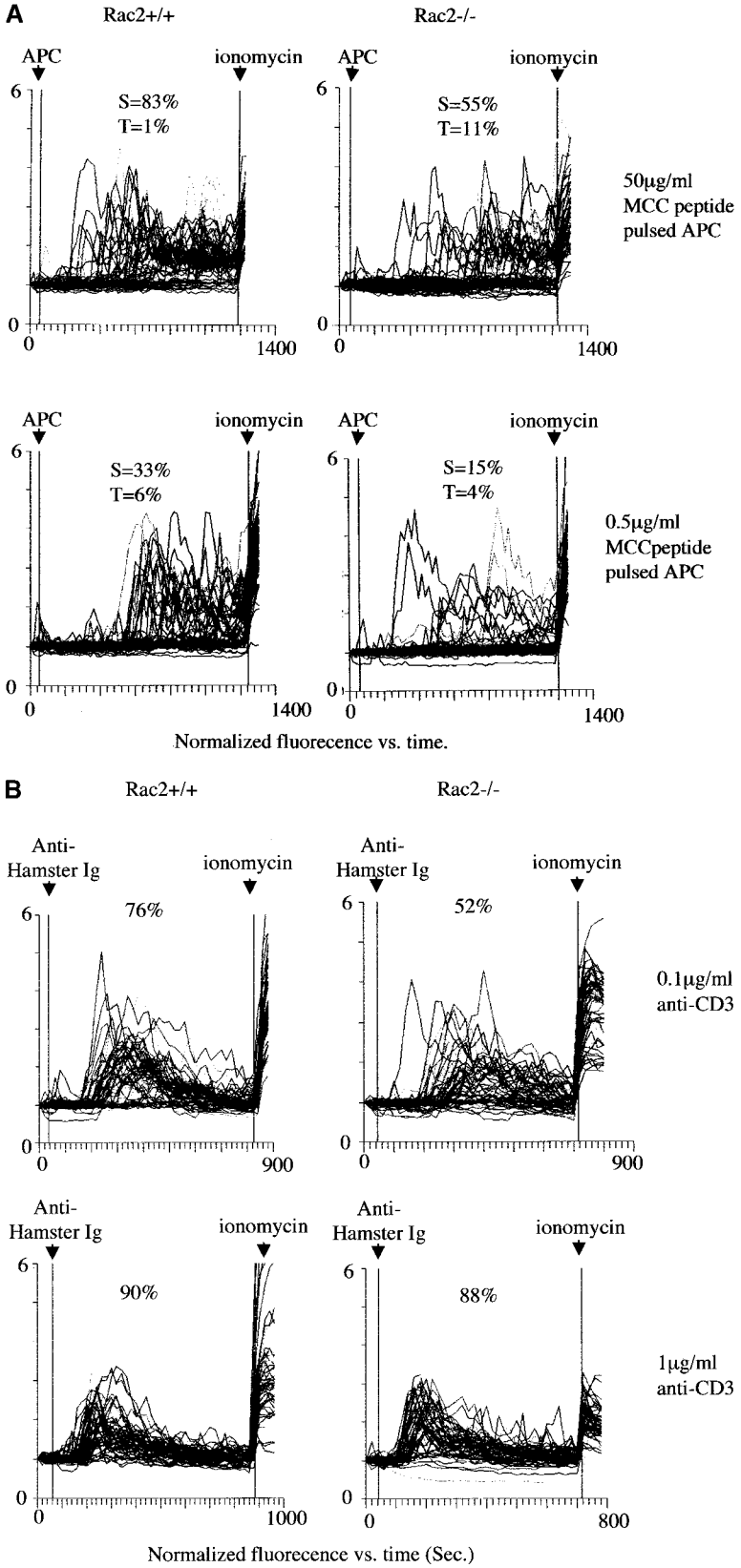

Figure 5. Reduced calcium mobilization in Rac2$^{-/-}$ T cells. (A) AND TCR transgenic CD4 cells were loaded with the Ca^{2+} sensitive fluorochrome, fluo-3, and stimulated with splenic APCs prepulsed with 50 μg/ml or 0.5 μg/ml MCC peptide. (B) CD4 T cells loaded with fluo-3 were stimulated with 0.1 or 1 μg /ml anti-CD3. The percentage of responding cells and the pattern of response in individual T cells was examined using a video laser cytometer. Each graph indicates the pattern of Ca^{2+} mobilization in a field of 40 to 50 cells, in which each line represents the average fluorescent intensity of an individual T cell over time. The first arrow indicates the addition of APC pulsed with peptide or cross-linking antibody, and the second arrow indicates the addition of ionomycin (666 ng/ml). S stands for sustained response, i.e., increased calcium concentration lasting >5 min. T stands for transient response, i.e., calcium increase lasting less than 5 min.

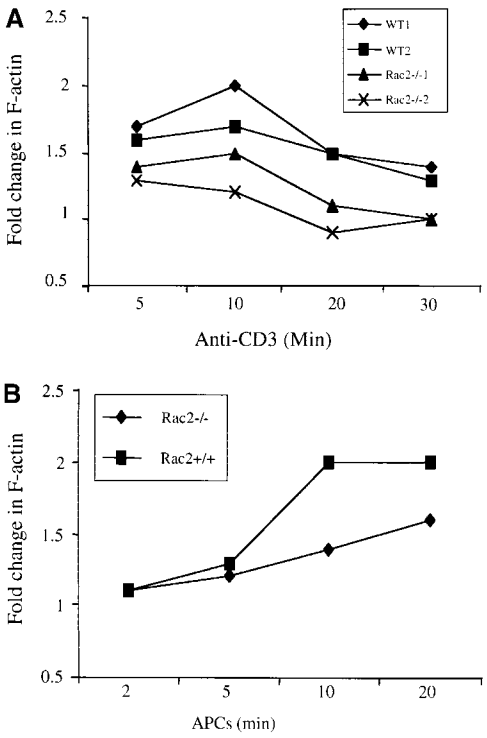

Figure 6. Impaired actin polymerization upon T cell stimulation. $CD4^+$ T cells were stimulated by anti-CD3 cross-linking (A) or with CH27 cells (B) prepulsed with 0.5 μg /ml of MCC peptide. Cells were fixed after stimulation for the indicated time and polymerized actin was measured by phalloidin staining. Data showing one of three representative experiments.

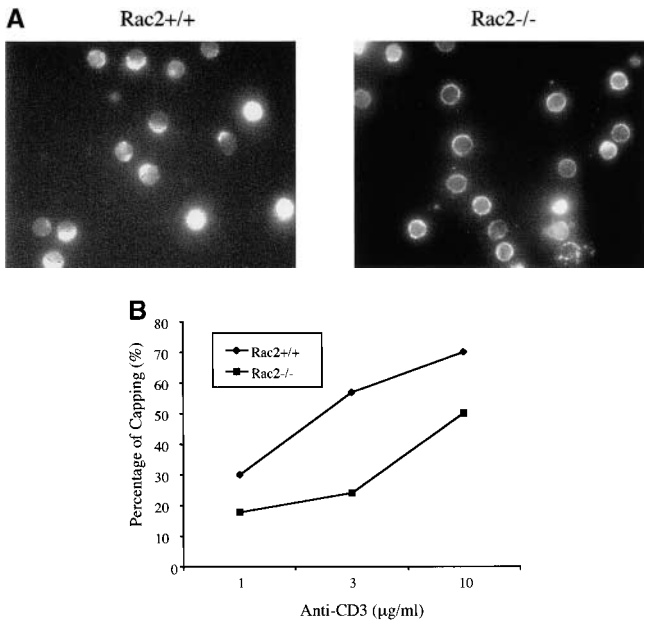

Figure 7. Reduced cap formation in $Rac2^{-/-}$ T cells. Purified CD4 T cells coated with a dose range of anti-CD3 were cross-linked with biotinylated anti–hamster antibody and further incubated at 37°C for 30 min followed by fixation. TCR cap formation was visualized by staining with Texas-red-Avidin and analyzed by immunofluorescence microscopy. (A) Representative T cell capping in $Rac2^{-/-}$ and control cells. (B) Percentage of capping at different dose of anti-CD3. Data represents at least three separate experiments.

cells stimulated in the absence of APCs, by anti-CD3 cross-linking (data not shown). This result is consistent with the aforementioned in vitro findings that Rac influences the activation of the ERK pathway. In reference to results from $Vav^{-/-}$ T cells showing decreased ERK activation (23), our result is also consistent with the hypothesis that TCR-stimulated tyrosine phosphorylation of Vav promotes its GEF activity toward Rac, which in turn activates PAK and ERK (10). As the activation of the ERK pathway plays an important role in TCR-mediated AP-1 induction and cell cycle progression, defective ERK activation may be partially accountable for the observed proliferation defect in $Rac2^{-/-}$ T cells.

MAPK p38 is activated by cellular stresses, inflammatory cytokines, LPS, and G protein–coupled receptors (48–51). During T cell activation, p38 is activated with either anti-CD3 or anti-CD28 in primary lymphocytes and its activation correlates closely with T cell proliferation (37). However, how p38 influence primary T cell activation is less clear. As T cell proliferation is normal in response to anti-CD3 or ConA in dominant-negative p38 transgenic mice, activated p38 may mainly affect T helper 1 cell differentiation rather than directly affect T cell activation (52). Consistent with these studies, we observed decreased p38 activation in $Rac2^{-/-}$ T cells, accompanied with decreased T cell proliferation and T helper 1 cell differentiation (53).

Calcium mobilization is a crucial event during T cell activation. This process is achieved through the activation of phospholipase C-γ (PLC-γ) to catalyze the hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP2) to diacylglycerol and inositol 1,4,5-triphosphate (IP3), which activate protein kinase C and elevates intracellular calcium, respectively. In this study, we have observed decreased Ca^{2+} flux upon TCR stimulation by antigen or antibody cross-linking. Several potential mechanisms may explain this Ca^{2+} mobilization defect. First, Rac may affect Ca^{2+} mobilization by modulating the level of PIP2, the substrate for PLC-γ. Previously, Rho and Rac have been implicated in the activation of phosphatidylinositol 4-phosphate 5-kinase (PIP5K), which in turn phosphorylates phosphatidylinositol 4 phosphate (PIP), resulting in the production of PIP2 (6, 54). Decreased activation of PIP5K may leads to reduced level of PIP2, decreased production of IP3, hence decreased Ca^{2+} mobilization. Alternatively, Rac may affect the activation of PLC-γ through phosphatidylinositol 3-kinase (PI3K) activation. Recent studies suggests that phosphatidylinositol 3,4,5-trisphosphate (PIP3), a catalytic product of PI3K, can activate and synergize with phosphorylated PLC-γ to achieve full-scale Ca^{2+} mobilization (55, 56). As PI3K may act downstream of Rac (57), Rac may affect Ca^{2+} flux through PI3K activation. Lastly, inhibition of actin polymerization in thymocytes and human T cell lines also reduces Ca^{2+} flux during activation mediated by spe-

cific peptide/MHC complex on APCs (58, 59). We have observed decreased actin-polymerization in $Rac2^{-/-}$ T cells upon stimulation, which may account for the decreased Ca^{2+} mobilization.

T cell activation is accompanied by rapid cytoskeletal changes and formation of a cap upon antibody cross-linking, or SMACs at the interfaces of physical contact between T cells and APCs (1, 60). It has been proposed that receptor clustering could favor sustained T cell signaling in three ways: first, by increasing the likelihood of contact between the TCR and MHC-bound ligand; second, by increasing the concentration of cytosolic signaling molecules and second messengers at regionally organized focal points in the proximity of TCRs; and last, by excluding negative regulatory molecules such as phosphatases from the zone of antigen receptor signaling (1). The formation of cap or SMAC requires actin polymerization in T cells (61). Rac has long been recognized to play a role in receptor-mediated cytoskeletal changes (62). We found that in the absence of Rac2, T cells showed decreased level of actin polymerization upon stimulation, accompanied with reduced antibody cross-linking–induced capping. These defects are similar to the findings in $Vav^{-/-}$ T cells, and are further evidence of the importance of actin in TCR signaling.

In summary, our results indicate that Rac2 affects ERK and p38 activation, Ca^{2+} mobilization, and the cytoskeleton reorganization related event, capping, during T cell activation. These functional defects are similar in nature but lesser in magnitude compared with the $Vav^{-/-}$ T cell phenotype (21–23). In light of the high homology among Rac proteins (sharing 89–92% sequence homology), functional overlap among Rho family GTPases (3), and the compensatory overexpression of Rac1 in $Rac2^{-/-}$ $CD4^+$ T cells, it is conceivable that the moderate phenotypic defect could be due to the compensatory effect of Rac1, Rac3, and other Rho family GTPases such as CDC42. In any event, our results corroborate the concept that Rac is a major substrate of Vav GEF activity, and may relay Vav signaling during T cell activation. The correlation between defective T cell activation, defective actin polymerization, and defective capping in $Rac2^{-/-}$ T cells confirms the importance of Rac as a signaling molecule connecting the TCR signal to cytoskeleton reorganization.

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