

# Rac1-mediated Bcl-2 induction is critical in antigen-induced CD4 single-positive differentiation of a CD4<sup>+</sup>CD8<sup>+</sup> immature thymocyte line

Hiroyo Oda,\* Harumi Suzuki,\*<sup>1</sup> Kouhei Sakai,\* Seiji Kitahara,\* Michael S. Patrick,\* Yoshinao Azuma,\* Kazuro Sugi,<sup>†</sup> Toshio Kitamura,<sup>‡</sup> Jonathan Kaye,<sup>§</sup> and Mutsunori Shirai\*

\*Department of Microbiology and Immunology, Yamaguchi University School of Medicine, Ube, Japan; <sup>†</sup>Department of Clinical Research, National Sanyo Hospital, Ube, Japan; <sup>‡</sup>Division of Cellular Therapy, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan; and <sup>§</sup>Department of Immunology, The Scripps Research Institute, La Jolla, California, USA

**Abstract:** Rac1, one of the Rho family small guanosine triphosphatases, has been shown to work as a “molecular switch” in various signal transduction pathways. To assess the function of Rac1 in the differentiation process of CD4 single-positive (CD4-SP) T cells from CD4CD8 double-positive (DP) cells, we used a DP cell line DPK, which can differentiate into CD4-SP cells upon TCR stimulation *in vitro*. DPK expressing dominant-negative (dn)Rac1 underwent massive apoptosis upon TCR stimulation and resulted in defective differentiation of CD4-SP cells. Conversely, overexpression of dnRac2 did not affect differentiation. TCR-dependent actin polymerization was inhibited, whereas early ERK activation was unaltered in dnRac1-expressing DPK. We found that TCR-dependent induction of Bcl-2 was suppressed greatly in dnRac1-expressing DPK, and this suppression was independent of actin rearrangement. Furthermore, introduction of exogenous Bcl-2 inhibited TCR-dependent induction of apoptosis and restored CD4-SP generation in dnRac1-expressing DPK without restoring TCR-induced actin polymerization. Collectively, these data indicate that Rac1 is critical in differentiation of CD4-SP from the DP cell line by preventing TCR-induced apoptosis via Bcl-2 up-regulation. *J. Leukoc. Biol.* 81: 500–508; 2007.

**Key Words:** T lymphocytes · TCRs · apoptosis · thymus

## INTRODUCTION

Rac1 belongs to the Rho family of small guanosine triphosphatases (GTPases), which play critical roles in actin cytoskeletal rearrangement in many cell systems. Among the Rho family GTPases, Rac1 has a broad range of guanine nucleotide exchanging factors (GEFs) and effectors so that the molecule acts as a molecular switch in many aspects of signal transduction pathways. Recent studies using transgenic technology have revealed that Rho family GTPases play crucial roles in

thymocyte development and TCR-mediated signal transduction [1]. Ectopic expression of bacterial C3T, which inhibits RhoA, -B, and -C, resulted in decreased numbers of CD4CD8 double-positive (DP) cells in the thymus [2], and transgenic expression of constitutively active RhoA resulted in enhanced positive selection [3]. Constitutively active cdc42 induced massive apoptosis in DP thymocytes [4], suggesting that cdc42 is also involved in T cell development.

Rac consists of three independent genes: Rac1, -2, and -3. Rac1 is expressed ubiquitously, whereas expression of Rac2 is restricted to hematopoietic cells. Rac2-deficient mice showed normal T cell development in the thymus, defective Th1 differentiation caused by decreased IFN- $\gamma$  production [5], perturbed chemotaxis [6], and defective T cell activation accompanied by decreased ERK activation [7]. Rac2, a component of NADPH oxidase, plays a critical role in reactive oxygen species in phagocytes [8], and recently, Rac1 was shown to play a similar role in human macrophages [9]. Transgenic expression of constitutively active Rac1 (L61) generates DP thymocytes in a RAG<sup>-/-</sup> background [10] and converts positive selection to negative selection [11], indicating that Rac1 regulates the strength of TCR-mediated signal transduction. Rac1-deficient mice are embryonic-lethal, and neutrophil-specific disruption of Rac1 was reported [12]. Recently, conditional knockout mice for Rac1 in a Rac2<sup>-/-</sup> background were generated, and differential, critical roles of Rac1 and Rac2 in growth and engraftment of hematopoietic stem cells [13–15] as well as in B cell development [16] were reported. However, the effect of each Rac in T cell development is still unknown. As all three Rac proteins are expressed in T cells, we have studied the role of Rac in T cell development using a dominant-negative (dn) strategy. Using DPK, a DP thymic lymphoma capable of differentiation into CD4 single-positive (SP) cells upon antigenic stimulation *in vitro* [17], we demonstrate that activation of Rac1 is required

<sup>1</sup> Correspondence at current address: Department of Pathology, International Medical Center of Japan, 1-21-1, Toyama, Shinjuku-ku, Tokyo, 162-8655, Japan. E-mail: hsuzuki@ri.imcj.go.jp

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for generation of CD4-SP T cells. CD4-SP generation was blocked by dnRac1, but not by dnRac2, suggesting that Rac2 is not involved in this differentiation process and also indicating the independent regulation of upstream GEFs for Rac1 and Rac2 in DP cells. We demonstrate further that Rac1 is critical in TCR-mediated Bcl-2 induction, indicating that Rac1 is important in antiapoptotic signal transduction in developing T cells as well as inducing actin cytoskeletal reorganization.

## MATERIALS AND METHODS

### Construction of retroviral vectors

To construct pMXs-PREP retroviral vector, a *Cla*I fragment of woodchuck post-transcriptional regulatory element (wPRE) sequence [18] and a *Sal*I fragment of puromycin-resistance gene were inserted into the *Cla*I and *Sal*I sites of pMXs-IRES-GFP IRES-GFP [19], respectively. PCR-cloned Rac1N17 cDNA was inserted into pMXs-PREP to produce pMXs-PREP-dnRac1. Retroviral vector pML2, which contains the IRES-hCD2 sequence, was a kind gift of Dr. Stephen Levin (University of Washington, Seattle, WA), and PCR-cloned human Bcl-2 cDNA was inserted into the multicloning site of pML2 to construct pML2-Bcl-2. HR2MU, a retroviral expression vector containing human mutant dnRac2 (D57N), was a kind gift of Dr. David A. Williams (University of Cincinnati Medical Center, OH).

### Retroviral transduction

Retrovirus-containing supernatants from vector pMXs-PREP, pMXs-PREP-dnRac1, or HR2MU-transfected 293gp packaging cells [20] with vesicular stomatitis virus-envelope plasmid, were used for infection of DPK cells. Retrovirally transduced cells were selected with 1  $\mu$ g/ml puromycin and sorted for GFP<sup>hi</sup> cells without single-cell cloning using a FACSVantage SE (Becton Dickinson, Palo Alto, CA).

### Detection of activated Rac1 and Rac2

Activation of Rac1 and Rac2 was evaluated by the standard p21-activated kinase (PAK)-binding domain assay. Briefly,  $2 \times 10^7$  control- and dnRac1-transduced DPK cells were activated with anti-CD3 and anti-CD28 mAb (5  $\mu$ g/ml each), followed by goat antihamster polyclonal antibody (20  $\mu$ g/ml, Jackson ImmunoResearch Lab, West Grove, PA) for 5 min at 37°C. Then, activated Rac protein was precipitated with p21-binding domain (PBD) beads (Upstate Biotechnology, Lake Placid, NY) and was subjected to Western blotting analysis using Rac1-specific mAb (23A8, Upstate Biotechnology) and Rac2-specific antibody (Santa Cruz Biotechnology, CA).

### In vitro T cell differentiation culture

DPK differentiation assay was carried out as described elsewhere [17] with some modifications. Briefly,  $9 \times 10^5$ -irradiated DC-I (E<sup>k</sup> and ICAM-1-transfected murine fibroblasts) per well in six-well plates were precultured for 24 h, with or without 100 ng/ml staphylococcal enterotoxin A (SEA; Toxin Technology, Sarasota, FL) during the last 2 h of the culture period. Then,  $4.5 \times 10^5$  DPK cells were added, and the culture was continued for 3 more days at 37°C. The cells were harvested after the indicated culture periods and stained with anti-CD4-PE (GK1.5) and anti-CD8 $\alpha$ -biotin (53-6.7) antibodies, followed by Streptavidin-TruRed for flow cytometry. All antibodies and staining reagents were purchased from PharMingen (Palo Alto, CA).

### Cell cycle analysis

DPK cells were activated with plate-bound anti-CD3 $\epsilon$  mAb (50  $\mu$ g/ml) for 16 h, fixed with 70% ethanol, and treated with RNaseA (1 mg/ml). Fixed cells were stained with 50  $\mu$ g/ml propidium iodide (PI) for 3 h at room temperature and analyzed on a FACSCalibur (Becton Dickinson).

### Measurement of ERK activation

DPK cells were incubated with 10  $\mu$ g/ml anti-CD3 $\epsilon$  antibody at 4°C and cross-linked with antihamster secondary antibody for the indicated time at 37°C. After the indicated period (min) of incubation, cells were lysed and Western blotted with antiphospho-ERK and anti-ERK antibodies (Transduction Lab, Palo Alto, CA).

### Staining of polymerized actin

DPK cells were added onto anti-CD3 $\epsilon$  plus anti-CD28 mAb-coated (20  $\mu$ g/ml each) coverslips and cultured for 30 min at 37°C. After removing the supernatant, cells were fixed with 4% paraformaldehyde, followed by permeabilization with 0.1% Triton X-100 and stained with phalloidin-Alexa 594 (Molecular Probes, Eugene, OR) and 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes).

### Flow cytometry

Thymocytes or DPK cells were stained with various combinations of FITC-conjugated anti-CD45.2 (Clone 104), PE-conjugated anti-CD4 (GK1.5), anti-CD5 (53-7.3), biotin-conjugated anti-CD8 $\alpha$  (53-6.7), anti-TCR- $\beta$  (597-H57), Annexin V, anti-CD69 (H1.2F3), anti-CD45.1 (A20), and APC-conjugated anti-CD8 $\alpha$  (53-6.7). Stained cells were analyzed by two laser FACSCalibur (Becton Dickinson) for four-color FACS analysis.

### Real-time RT-PCR analysis

Total RNA was isolated from cells using the RNeasy kit (Qiagen, Hilden, Germany), and real-time PCR was carried out using the QuantiTect SYBR Green RT-PCR kit (Qiagen) with specific primers for Bcl-2 $\alpha$  (5'cctgtggatcactgagtacct3'/5'gagcaggtcttcagagaca3') and Nor1 (5'aaggctcttcaagagaac3'/5'tgaaatctgcagtactgacac3').

### Western blotting

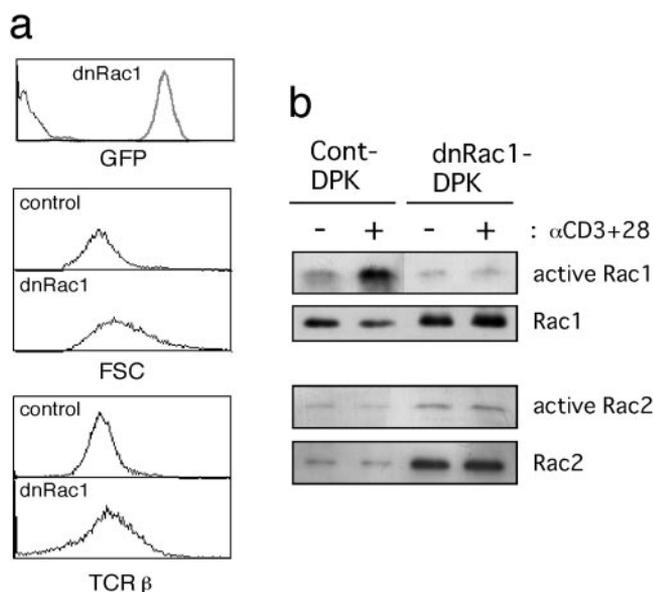
Bcl-2-transduced DPK cells ( $5 \times 10^5$  cells) were lysed and applied to SDS-PAGE. Transferred membranes were Western-blotted with rabbit polyclonal anti-Bcl-2 antisera (PharMingen), and reactive proteins were visualized with ECL chemiluminescent substrates (Cell Signaling Technology, Beverly, MA).

## RESULTS

### dnRac1 inhibits CD4-SP differentiation

To analyze the detailed function of Rac in TCR-mediated signal transduction during positive selection, we decided to use an in vitro experimental system of CD4-SP differentiation. DPK is a naturally occurring DP thymic lymphoma from AND-TCR transgenic mice [17, 21]. This cell line has been shown to differentiate into CD4-SP cells when cocultured with antigen-loaded APC in vitro [17] and thus, has been used to study signal transduction during positive selection of immature thymocytes [22–24]. A dnRac1 (N17) gene was PCR-cloned into the newly established pMXs-PREP retroviral vector, which contains an IRES-GFP cassette and a mRNA stabilizing element (wPRE) sequence [18] for effective gene expression [25]. Although dnRac1-introduced DPK showed some increase in cell size, expression levels of CD4, CD8, and TCR were indistinguishable from the vector control-infected cells (**Figs. 1a** and **2a**).

As we expected, TCR-induced activation of Rac1 was decreased severely in dnRac1-expressing DPK cells (**Fig. 1b**). Conversely, TCR stimulation did not induce Rac2 activation in



**Fig. 1.** TCR-induced activation of Rac1 is blocked by introduction of dnRac1. (a) DPK cells infected with pMX-PREP-dnRac1, express dnRac1 protein as well as GFP. Sorted GFP<sup>hi</sup> cells were analyzed by flow cytometer [forward scatter (FSC)]. (b) TCR stimulation induced Rac1 activation but not Rac2 activation. Control (Cont-DPK; pMXs-PREP) and pMXs-PREP-dnRac1-transduced DPK cells were stimulated with anti-CD3 and -CD28 antibody for 5 min, and active Rac protein was pulled down with PBD beads and subjected to Western blotting analysis using anti-Rac1- and -Rac2-specific antibodies.

vector control-transduced DPK or dnRac1-expressing DPK (Fig. 1b).

As shown in Figure 2a, vector control-transduced DPK cells decreased CD8 expression gradually, and almost 80% of cells differentiated into CD4-SP cells 3 days after antigenic stimulation. In contrast, the generation of CD4-SP cells was abolished almost completely in dnRac1-DPK (Fig. 2a). Addition of Rac1 inhibitor NSC23766 in control DPK also suppressed CD4-SP generation, whereas introduction of dnRac2 (D57N mutation [26]) did not inhibit CD4-SP generation (Fig. 2b).

Lack of CD4-SP generation in dnRac1-expressing DPK could be a result of defective signal transduction or massive apoptosis after antigenic stimulation. We thus examined apoptotic cell death in cultured DPK cells, with or without TCR stimulation. Apoptotic death after 16 h incubation was evaluated by the proportion of cells in the sub-G1 fraction in cell cycle analysis using PI staining (Fig. 3a). Control vector expressing DPK showed 21% cell death without stimulation, and this did not increase significantly after TCR stimulation. Conversely, TCR stimulation induced strong cell death in dnRac1-expressing DPK (56%), although it showed augmented cell death without stimulation. At the same time, the number of Annexin V-positive apoptotic cells upon TCR stimulation increased dramatically in dnRac1-DPK compared with control DPK cells (Fig. 3b). Similar results were obtained using the Trypan blue dye exclusion method for determination of cell viability (data not shown). Collectively, expression of dnRac1 in DP thymocytes leads to inhibition of CD4-SP differentiation and augmentation of TCR-induced apoptosis.

## Early activation events are intact in dnRac1-expressing DPK

To investigate the function of Rac1 in TCR-dependent signal transduction during positive selection, early activation of ERK was determined. DPK cells were stimulated by anti-CD3 $\epsilon$  mAb-coated beads, and activation of ERK was evaluated by phosphorylation of ERK1 and -2 (Fig. 4a). The dnRac1 mutant had no effect on ERK activation, indicating that TCR-stimulated activation of ERK was independent of Rac1. We also found that TCR-mediated up-regulation of CD69 and CD5 in dnRac1-DPK cells was indistinguishable from that seen in control DPK cells (Fig. 4b).

## Rac-mediated actin polymerization

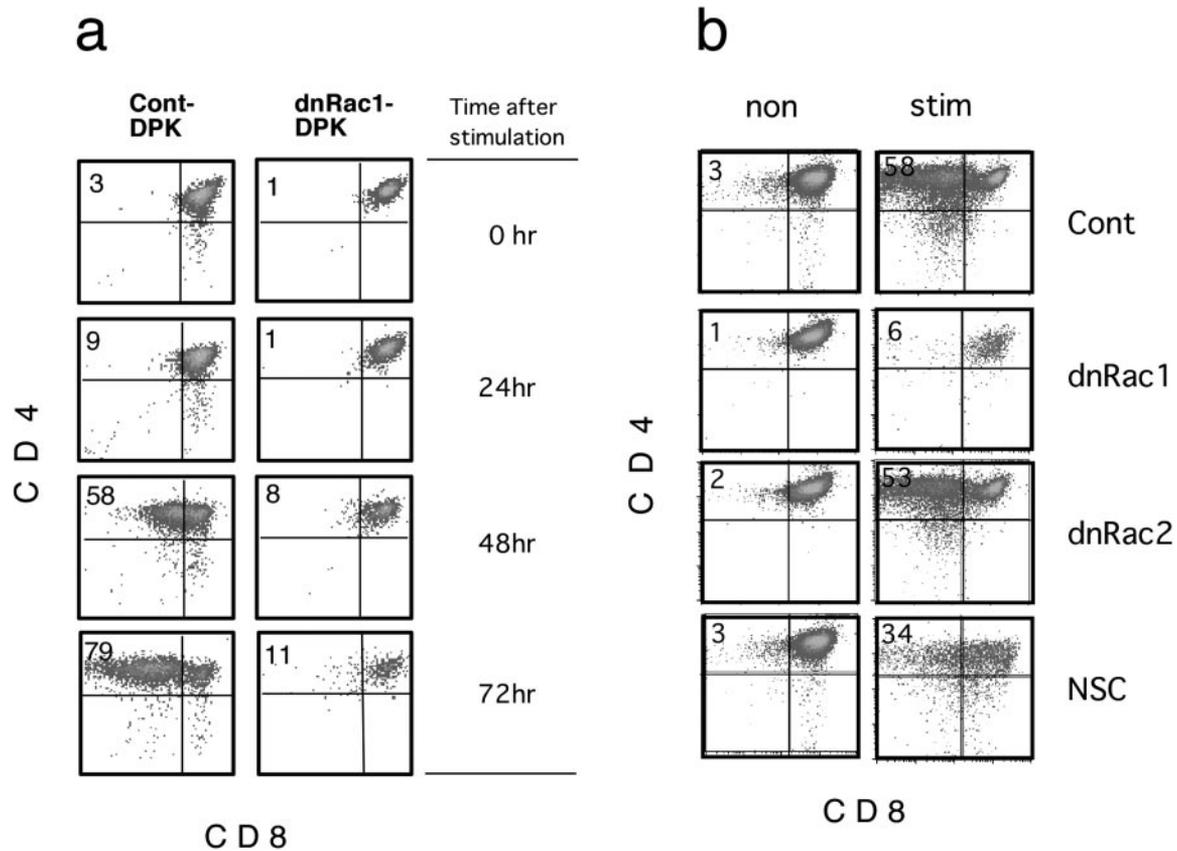
We next examined TCR-dependent actin polymerization in dnRac1-DPK, as Rac is widely known to play a critical role in cytoskeletal reorganization. After 30 min of TCR stimulation, control DPK cells showed accumulation of polymerized actin and induction of vigorous membrane ruffling, as detected by phalloidin staining, whereas TCR-dependent actin polymerization was abrogated in dnRac1-DPK (Fig. 5a). A requirement for actin reorganization during thymocyte development has not been determined directly. Thus, we examined the effect of an inhibitor of actin polymerization on differentiation of DPK cells. Latrunculin A, an inhibitor of actin polymerization [27], blocked antigen-induced CD4-SP cell generation completely in the DPK in vitro differentiation system (Fig. 5b).

## Suppression of TCR-mediated induction of Bcl-2

The massive apoptosis in stimulated dnRac1-DPK cells could be a result of an increase in death effectors or a decrease in expression of antiapoptotic proteins, as TCR ligation on DP thymocytes induces death effectors such as Nur77 and Nor1 [28], as well as antiapoptotic molecules such as Bcl-2 and Bcl-xL [29]. To this end, we examined TCR-dependent induction of these molecules in the DPK system. By real-time RT-PCR analysis, a two- to three-fold increase of Bcl-2 mRNA was observed in control DPK cells upon TCR stimulation, whereas stimulation-dependent induction of Bcl-2 was not seen in dnRac1-DPK cells (Fig. 6a). Furthermore, induction of bcl-2 was independent of actin polymerization, as Latrunculin A treatment of DPK cells did not inhibit TCR-dependent bcl-2 induction (Fig. 6a). In contrast, TCR-dependent induction of Nor1 in dnRac1-DPK was comparable with that of control cells (Fig. 6b). These data strongly suggest that increased susceptibility to TCR-induced apoptosis in dnRac1-DPK cells is a result of failed induction of antiapoptotic molecule Bcl-2 and not a result of increased induction of death effector molecules.

## Restoration of CD4-SP generation by Bcl-2

If the defective positive selection observed in dnRac1-DPK is mainly a result of massive apoptosis caused by insufficient induction of Bcl-2, overexpression of Bcl-2 in dnRac1-DPK should restore positive selection. To this end, we introduced Bcl-2 cDNA retrovirally into dnRac1-DPK cells (Fig. 7). Introduction of Bcl-2 enhanced the stimulation-dependent generation of CD4-SP (Fig. 7a), but it is more important that



**Fig. 2.** TCR-induced generation of CD4-SP in vitro is inhibited by dnRac1 but not dnRac2. (a) Vector-only (Cont-DPK) and pMXs-PREP-dnRac1-transduced DPK cells were cocultured with DC-1 and 100 ng/ml SEA. After the indicated culture periods, cells were harvested, and expression levels of CD4 and CD8 were analyzed by flow cytometry. Shown is the phenotype of GFP-positive cells. (b) Cont and dnRac1- and dnRac2-transduced DPK cells were stimulated with or without Rac1 inhibitor NSC23766 (NSC; 20  $\mu$ g/ml) for 3 days in the same way.

introduction of Bcl-2 restored the generation of CD4-SP in dnRac1-DPK almost completely (Fig. 7, a and c). At the same time, introduction of Bcl-2 attenuated the stimulation-dependent apoptosis observed in dnRac1-DPK (Fig. 7c, lower panel). On the contrary, introduction of Bcl-2 did not restore TCR-induced actin polymerization (Fig. 5a, bottom panels). These results indicate that defective CD4 generation in dnRac1-DPK is mostly a result of the lack of TCR-dependent Bcl-2 up-regulation.

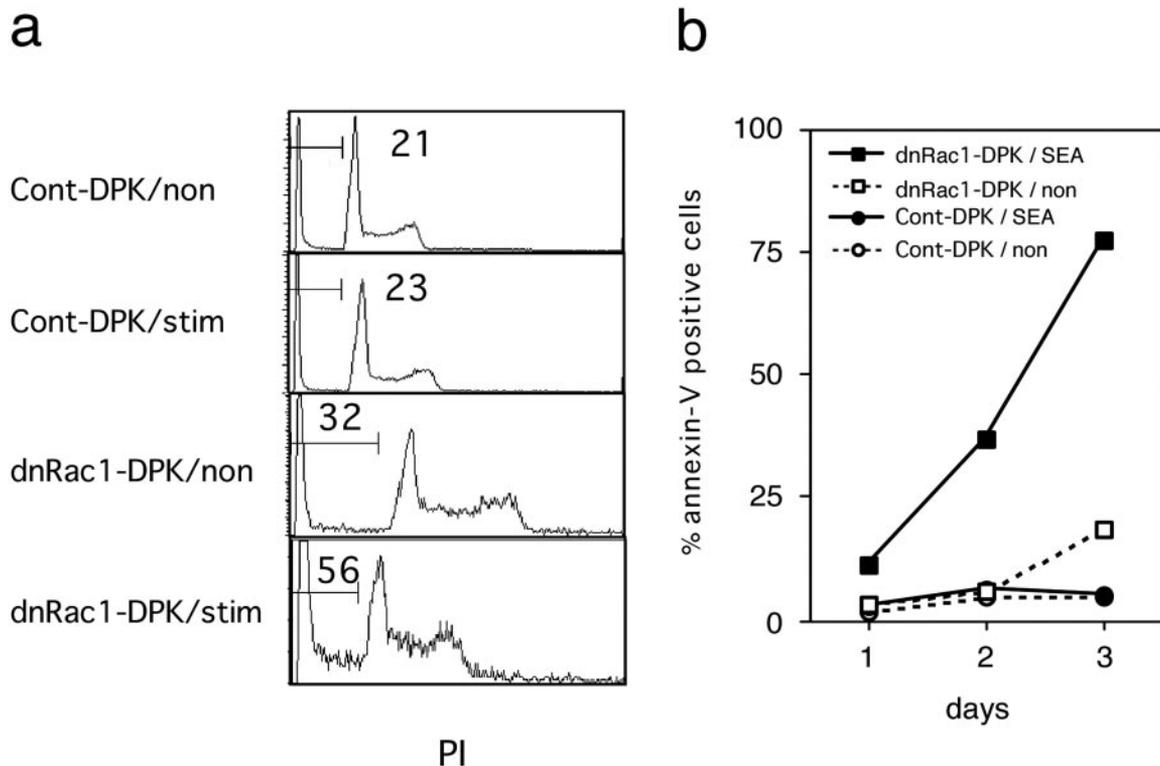
## DISCUSSION

Although the effect of constitutively active Rac1 mutant on T cell development has been studied extensively by Cantrell's groups [1, 10, 11], a loss-of-function-type Rac1 mutant study about T cell development has not been reported. Constitutively active Rac1-transgenic mice showed conversion from positive selection to negative selection [11], restoration of the T cell defect in *Vav*<sup>-/-</sup> mice, and generation of DP cells in *RAG*<sup>-/-</sup> mice [10] suggesting that active Rac1 enhances TCR signal transduction. In the current report, we have shown directly that Rac1 is required for positive selection in an in vitro model system by using dnRac1 and Rac1 inhibitor (Fig. 2). Indistinguishable TCR expression on dnRac1-DPK cells (Fig. 1a)

indicates that Rac1 is not involved in assembly and traffic of TCR components to the cell surface.

The inhibitory effect of dnRac1 was stronger than Rac1 inhibitor NSC23766, which inhibits Rac-GEFs Tiam1 and Trio specifically [30] (Fig. 2b). Therefore, these GEFs may not be important in activation of Rac1 in DP cells. As a matter of fact, dedicator of cyto-kinesis 2 (DOCK2)/engulfment and cell motility 1 (ELMO 1) complex is reported to work as a critical Rac1-GEF in TCR-mediated signal transduction [31]. Different from normal DP thymocytes [32], TCR stimulation did not induce Rac2 activation in DPK cells (Fig. 1b). Consistent with the lack of Rac2 activation, overexpression of dnRac2 (D57N mutation [26]) did not inhibit CD4-SP generation (Fig. 2b). It also indicates differential GEF use for Rac1 and Rac2 in TCR-mediated activation in DPK cells.

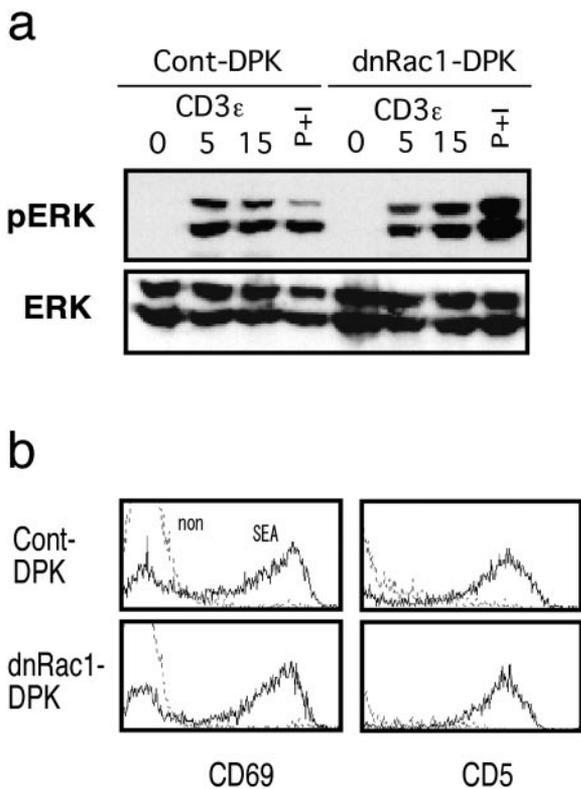
We found that early ERK activation was not inhibited by the presence of dnRac1 (Fig. 4a). The requirement of ERK activation in positive selection has been well established [33, 34], and PAK1, a major downstream target of Rac1, has been shown to be involved in ERK activation [35, 36]. However, normal ERK activation in dnRac1 DPK indicates that TCR-mediated activation of ERK is independent of Rac1. Thus, the inhibitory effect of dnRac1 on positive selection cannot be attributed simply to inhibition of ERK activation.



**Fig. 3.** Expression of dnRac1 increases TCR-induced apoptosis. (a) Stimulation-dependent apoptotic cell death in suspension culture. Vector control or dnRac1-expressing DPK were cultured for 16 h with (stim) or without (non) plate-bound, anti-CD3 $\epsilon$  mAb, and cell death was evaluated by DNA content analysis using PI. Numbers show the proportion of cells in the sub-G1 fraction (percent) in cell cycle analysis. (b) GFP-positive dnRac1 or vector control-transduced DPK cells at indicated culture periods were stained with Annexin V, and the proportion of Annexin V-stained cells in each group was plotted.

Rac1 is generally recognized as a key molecule in actin reorganization processes [37]. It is thus not surprising that introduction of dnRac1 prevents TCR-mediated actin polymerization (Fig. 5a). The role of actin cytoskeletal reorganization in T cell development is still not clear, although complete abrogation of positive selection in dn Wiskott-Aldrich syndrome protein transgenic mice [38] and in *Vav*<sup>-/-</sup> mice [39] is certainly consistent with a requirement for actin polymerization during positive selection. We demonstrate that actin polymerization is required for positive selection in the DPK system by using an inhibitor of actin polymerization, Latrunculin A (Fig. 5b). However, it is obvious that Rac1-dependent actin polymerization is not required in the differentiation of DPK, as overexpression of Bcl-2 successfully restored CD4-SP differentiation of dnRac1-expressing DPK (Fig. 7) without rescuing the failure of TCR-mediated actin assembly (Fig. 5a). Therefore, a Rac1-independent mechanism of actin polymerization might be required in DPK cell differentiation, or actin polymerization might be critical for antigen presentation in DC-I cells in this experimental system. Recently, TCR-mediated Rac activation and immunological synapse formation have been shown to be dependent on DOCK2 [32]. Impaired positive selection observed in DOCK2-deficient mice [32] is consistent with our finding that Rac1 is critical in positive selection. Rac1 is also reported to be involved in integrin-mediated cell adhesion in thymocytes [40], which is dependent on RhoA [41]. Therefore, it would be interesting to investigate the activity of the integrin in dnRac1-expressing DPK cells.

In the present study, we observed increased TCR-mediated apoptosis in dnRac1-expressing DPK cells (Fig. 3b). Introduction of dnRac1 also affected spontaneous cell death, as we observed increased cell death in dnRac1-expressing DPK, even without stimulation (Fig. 3b). However, this slight increase of spontaneous cell death could not explain the complete loss of stimulation-dependent CD4-SP generation observed in Figure 2b. We therefore don't think that the increase in spontaneous cell death is the major cause of defective CD4-SP generation. At the same time, dnRac1-DPK did not show increased susceptibility to steroid-induced cell death (data not shown). In Jurkat cells, expression of dnRac1 (Rac1-N17) has been shown to protect the cell from Fas-mediated apoptosis [42]. The balance between TCR-mediated induction of proapoptotic and antiapoptotic mediators is a key discriminating factor in positive and negative selection. TCR stimulation of DP thymocytes induces orphan transcription factors Nur77 and Nor1 [28], which play major roles in negative selection [43, 44] by inducing Bim [45, 46] and Fas ligand [47] expression. Induction of the *Nur77* gene is controlled positively by myocyte enhancer factor 2 (MEF2) and negatively by Cabin1 [48] and histone deacetylase 7 [49] via histone deacetylation. TCR signaling releases these repressors from MEF2 to activate *Nur77* transcription. We observed no effect of dnRac1 on induction of these proapoptotic mediators in the DPK system (Fig. 6b). In fact, TCR-mediated Nur77 induction was even lower in dnRac1-DPK compared with control cells (data not shown). These results indicate clearly that increased apoptosis



**Fig. 4.** dnRac1 does not inhibit TCR-dependent, early MAPK activation. (a) DPK cells were activated by anti-CD3 $\epsilon$  antibody. After the indicated time (min), cell lysates were prepared and analyzed by Western blotting with antiphospho-ERK (pERK) or anti-ERK antibodies as indicated. P+I, 10 ng/ml PMA and 1  $\mu$ g/ml A23187. (b) Expression of CD5 and CD69 on dnRac1-DPK cells was analyzed by FACS analysis after 16 h culture with APC in the absence (dotted line) or presence (solid line) of SEA.

in activated dnRac1-DPK cells is not a result of increased expression of proapoptotic mediators.

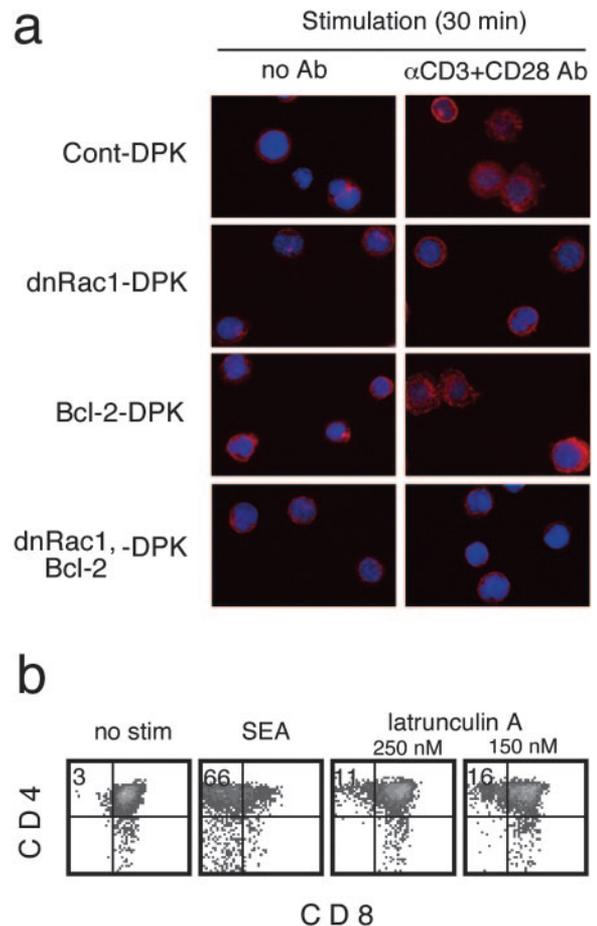
In contrast, expression of dnRac1 did affect expression of the antiapoptotic mediator Bcl-2 (Fig. 6a). Involvement of Rac1 in the Bcl-2-mediated survival response could be critical in positive selection of thymocytes. Rac2-deficient mast cells were defective in Akt activation and Bcl-xL expression, resulting in impaired survival [50]. Thus, the involvement of Rac proteins in mediating cell survival may be a more general phenomenon. In DP thymocytes, one of the antiapoptotic signals is the exclusion of Nur77 from the nucleus by Akt-dependent phosphorylation of Nur77 [51, 52]. Although we have not examined the phosphorylation status of Nur77 in dnRac1-DPK cells, we think the involvement of Rac1 in regulation of Nur77 phosphorylation is unlikely. TCR-mediated activation of Akt is dependent on PI-3K, and generation of CD4-SP cells in the DPK in the in vitro differentiation system is resistant to PI-3K inhibitors wortmannin and Ly294002 (unpublished observation).

Although Bcl-2-deficient mice showed unimpaired T cell development [53], Bcl-2 is a major antiapoptotic molecule in thymocytes and has been reported to play important roles in their development and maintenance [54]. Bcl-2 expression increases immediately after positive selection [55], and TCR stimulation induces its transcription in vitro as well [29]. Two

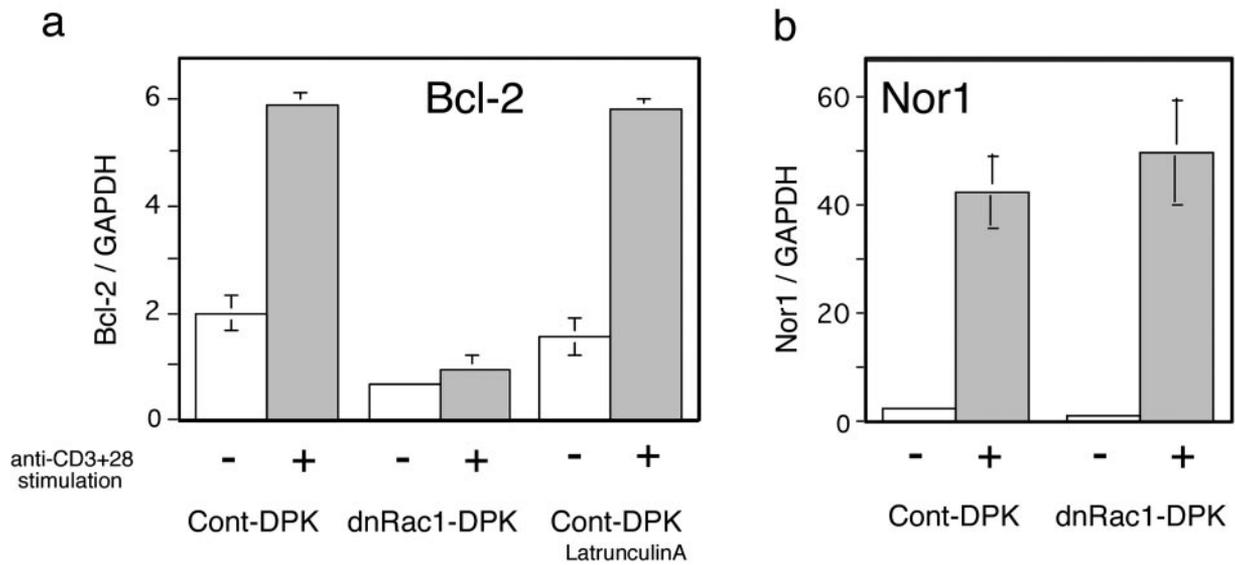
promoter regions have been identified in the 5' regulatory region of the Bcl-2 gene, and NF- $\kappa$ B [56] was shown to bind one of these promoter regions. Other studies showed that the Bcl-2 gene is positively regulated by NFAT4 [57] and NF- $\kappa$ B2 [58]. Thus, Rac1 may be involved in TCR-mediated activation of NF-AT or NF- $\kappa$ B to induce Bcl-2 transcription. Consistent with this idea, Rac1 has been shown to be involved in activation of NFAT4 in FcR-mediated signal transduction in mast cells [59].

TCR-dependent induction of Bcl-2 was not inhibited in the presence of Latrunculin A, an inhibitor for actin polymerization (Fig. 6a). At the same time, Latrunculin A did not inhibit TCR-dependent up-regulation of CD69 and CD5 (data not shown). These data suggest that Rac1 affects actin reorganization and Bcl-2 induction independently in DPK cells.

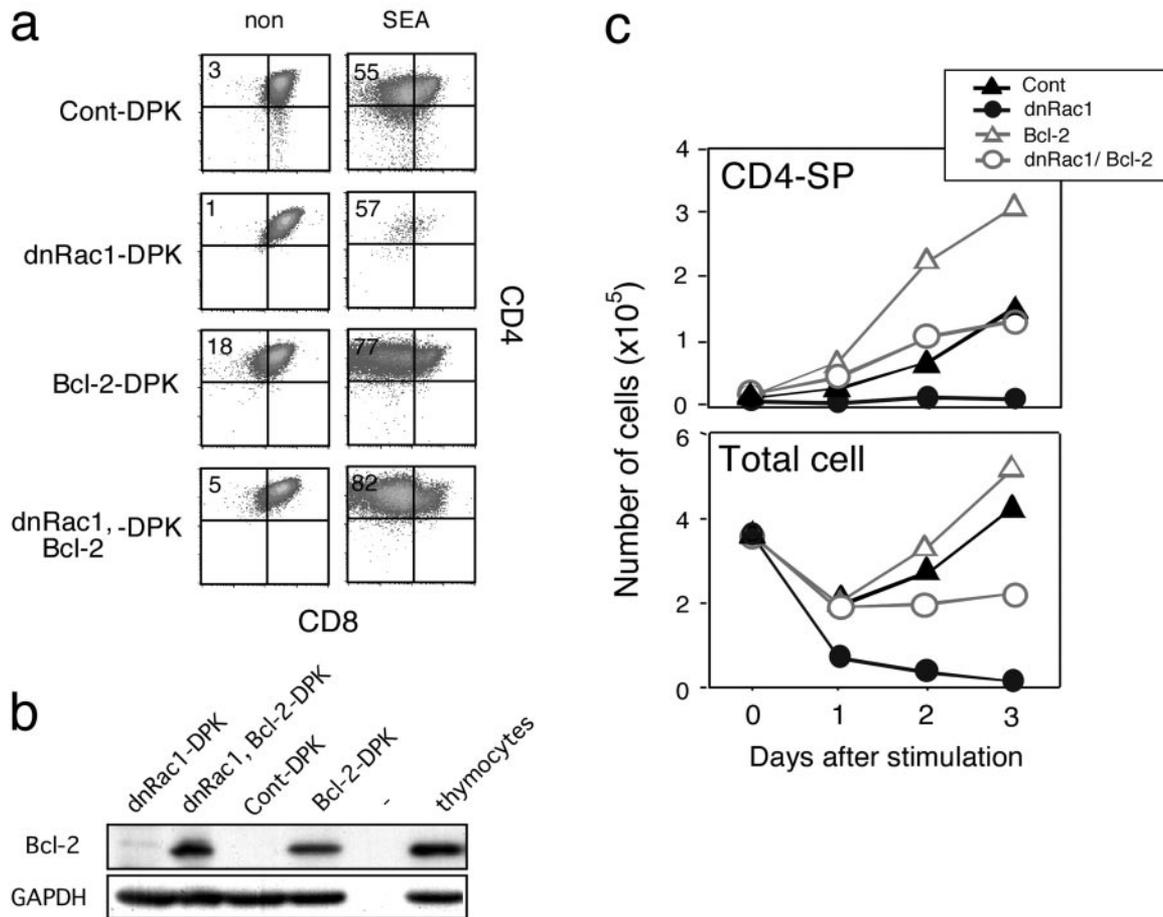
In the present study, we demonstrated that Rac1, but not Rac2, is required for positive selection of a DP cell line. Using the DPK in vitro positive selection model system, Rac1 was shown to be critical in TCR-mediated actin cytoskeletal reor-



**Fig. 5.** Rac1 is critical in TCR-dependent actin polymerization. (a) TCR-mediated actin polymerization was inhibited by dnRac1. Vector control or dnRac1-transduced DPK cells were cultured on anti-CD3 and -CD28 mAb-coated coverslips for 30 min and then fixed, permeabilized, and stained with Alexa 594-conjugated phalloidin (red) to detect polymerized actin fibers and DAPI (blue) to visualize nucleus. (b) Generation of CD4-SP cells requires actin polymerization. DPK cells were cocultured with DC-I and SEA for 3 days in the presence of the indicated concentration of Latrunculin A, an inhibitor of actin polymerization.



**Fig. 6.** Effect of dnRac1 on the expression of apoptosis-related genes. Changes in expression of (a) Bcl-2 and (b) Nor1 were determined by quantitative real-time RT-PCR in DPK cells activated with plate-bound anti-CD3 and -CD28 antibodies for 16 h, with or without 150 nM Latrunculin A. Results are presented as a ratio of expression of the indicated gene and the control housekeeping gene GAPDH.



**Fig. 7.** Overexpression of Bcl-2 rescues CD4-SP generation and attenuates TCR-mediated apoptosis in dnRac1-DPK. (a) DPK cells were transduced with pMXs-PREP vector-only control (Cont-DPK), pMXs-PREP-dnRac1, pMI.2-Bcl-2, or dnRac1 + pMI.2-Bcl-2 and cocultured with DC-1 (non) and 100 ng/ml SEA. After 3 days, cells were harvested and analyzed by flow cytometry. Shown are the CD4 and CD8 profiles of GFP<sup>+</sup> (panels in top two rows and bottom row) and hCD2<sup>+</sup> (panels in bottom two rows) cells. (b) Protein expression of Bcl-2 in transduced cells. Cell lysates of each transformant were subjected to SDS-PAGE and Western blotted with anti Bcl-2 antibody. (c) Absolute number of CD4-SP (upper panel) and total cell (lower panel) after indicated culture periods. Number of GFP-positive cells was counted.

ganization as well as induction of the antiapoptotic mediator Bcl-2. A Rac1-dependent pathway of Bcl-2 induction could be a critical process during positive selection by preventing TCR-mediated apoptosis, and thus, a detailed mechanism of Bcl-2 induction needs to be elucidated in future studies.

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