Transgenic Drak2 Overexpression in Mice Leads to Increased T Cell Apoptosis and Compromised Memory T Cell Development*

Received for publication, January 17, 2006, and in revised form, February 24, 2006 Published, JBC Papers in Press, March 3, 2006, DOI 10.1074/jbc.M600497200

Jianning Mao‡1, Xiaoying Qiao‡1, Hongyu Luo‡2, and Jiaping Wu‡3,‡4

From the ‡Laboratory of Immunology and §Nephrology Service, Centre Hospitalier de l’Université de Montréal, Notre Dame Hospital, Montreal, Quebec H2L 4M1, Canada

Drak2 is a death-associated protein family serine-threonine kinase. Its expression and roles in the immune system were investigated in this study. According to in situ hybridization, Drak2 expression was ubiquitous at the mid-gestation stage in embryos, followed by more focal expression in various organs in the perinatal period and adulthood, notably in the thymus, spleen, lymph nodes, cerebellum, suprachiasmatic nuclei, pituitary, olfactory lobes, adrenal medulla, stomach, skin, and testes. Drak2 transgenic (Tg) mice were generated using the human β-actin promoter. These Tg mice showed normal T cell versus B cell and CD4 versus CD8 populations in the spleen, but their spleen weight cellularity was lower in comparison with wild type mice. After TCR activation, the proliferation response in Drak2 Tg T cells was normal, although their interleukin (IL)-2 and IL-4 but not interferon-γ production was augmented. Activated Drak2 Tg T cells demonstrated significantly enhanced apoptosis in the presence of exogenous IL-2. At the molecular level, Drak2 Tg T cells manifested a lower increase of anti-apoptotic factors during activation; such a change probably rendered the cells vulnerable to subsequent IL-2 insults. The compromised apoptosis in Drak2 Tg T cells was associated with reduced numbers of T cells with the memory cell phenotype (CD62Llo) and repressed second- ary T cell responses in delayed type hypersensitivity. Our study demonstrates that Drak2 expresses in the T cell compartment but is not T cell-specific; it plays critical roles in T cell apoptosis and memory T cell development.

To elucidate the molecular mechanisms of T cell activation and differentiation, we conducted DNA microarray analysis employing the mouse 15,000 cDNA panel of the NIA, National Institutes of Health, to compare gene expression patterns of resting versus activated T cells (anti-CD3 and anti-CD28 stimulation for 24 h). Drak2 was one of the genes found to undergo significant changes after activation and was thus selected for further investigation.

Drak2 is a serine/threonine kinase belonging to a family of death-associated protein (DAP)3 kinases that consists of DAP (1), DRP-1 (2), ZIP kinase (3), DAPK2 (4), Drak1, and Drak2 (5). Drak1 and Drak2 share 67.1% identity in their kinase domain and 24.2% identity in their non-catalytic regions (5). Drak2 also shares about 50% identity in the kinase domain with other members of the family (2). Although DAP, DRP-1, and DAPK2 have a calmodulin regulatory domain in their C terminus, ZIP, Drak1, and Drak2 do not (1–5). DAP, DAPK2, and DRP-1 are localized in the cytosol (1, 2, 4), ZIP kinase and Drak1 resides mainly in the nuclei (3, 5), and Drak2 is found in both the cytosol and nuclei (5, 6), suggesting different mechanisms of action. When DAP family kinases are overexpressed in various cells, apoptosis ensues, either directly or after cytokine stimulation (1–5), involving their inhibition in apoptosis.

The mechanism of action and regulation of the prototype DAP family kinase, DAP, at the molecular level have been better studied. During apoptosis, DAP can associate with tumor necrosis factor α receptor and Fas-associated death domain, whereas in surviving cells, it binds to 14-3-3 (7). It phosphorylates myosin light chain II in vivo and is necessary for serum-induced stress fiber formation (8); it also phosphorylates p190 (4) and suppresses fibroblast oncogenic transformation (9). DAP is capable of autophosphorylation, which is inhibitory to its kinase activity (10). It also interacts with DAP-interacting protein-1, which is an E3 ubiquitin ligase and regulates the cellular level of DAP (11). Much less is known about the mechanisms of action of Drak2. It autophosphorylates and phosphorylates myosin light chains as an exogenous substrate (5), although its endogenous substrates have not been identified. Drak2 interacts with a calcineurin homologous protein (6), but the biological significance of this interaction is not clear. According to DNA microarray (12) and real time reverse transcription (RT)-PCR analysis (13) of different tissues, Drak2 is considered exclusively expressed in the T cell compartment; yet such analyses are not precise, because these methods cannot reveal possible focal Drak2 expression in certain organs.

The immune system of Drak2 null mutant mice was investigated recently by McGargill et al. (13). Unexpectedly, in vitro, Drak2−/− T cells have no apparent defect in activation-induced apoptosis, after stimulation with anti-CD3 and anti-CD28; this leads to a conclusion by the authors that Drak2 does not play significant roles in T cell apoptosis. Interestingly, Drak2−/− T cells have a lowered threshold to stimulation, compared with wild type (WT) T cells; the mechanism of such a phenomenon has not been explored.

‡ This work was supported by Canadian Institutes of Health Research Grants MOP57697 and MOP69089 and funds from the Kidney Foundation of Canada, funds from the Heart and Stroke Foundation of Quebec, Juvenile Diabetes Research Foundation USA Grant 1-2005-197, and funds from the J.-Louis Levesque Foundation (to J. W.). This work was also supported by grant grants from the Canadian Institutes of Health Research for New Emerging Teams in Transplantation and from Fonds de la Recherche en Santé du Québec for Transfusional and Hemovigilance Medical Research. 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.

1 These authors contributed equally to this work.

2 To whom correspondence may be addressed: Laboratory of Immunology, Research Centre, CHUM, Notre Dame Hospital, Pavilion DeSe`ve, Rm. Y-5616, 1560 Sherbrooke St. East, Montreal, PQ H2L 4M1, Canada. Tel.: 514-890-8000 (ext. 27421); Fax: 514-412-7596; E-mail: hongyu.luo@umontreal.ca.

3 National Scholar of the Fonds de la Recherche en Santé du Québec. To whom correspondence may be addressed: Laboratory of Immunology, Research Centre, CHUM, Notre Dame Hospital, Pavilion DeSe`ve, Rm. Y-5616, 1560 Sherbrooke St. East, Montreal, PQ H2L 4M1, Canada. Tel.: 514-890-8000 (ext. 25164); Fax: 514-412-7596; E-mail: jianping.wu@umontreal.ca.

© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
In this study, we mapped the Drak2 expression pattern during ontogeny and conducted a detailed in vitro and in vivo investigation of its functions in the immune system, using transgenic (Tg) mice with actin promoter-driven Drak2 expression. The novelty of our study is as follows. We corrected two misconceptions that Drak is expressed only in the T cell compartment and that it does not involve T cell apoptosis. We also provided evidence that Drak2 controlled T cell memory response.

MATERIALS AND METHODS

In Situ Hybridization (ISH)—A 1516-bp ApaI/KpnI mouse Drak2 cDNA fragment (positions 1150–2676) derived from MGC-6721 (American Type Culture Collection, Manassas, VA) was cloned in pCMV-SPORT6 and was named pCMV-SPORT6-Drak2 (American Type Culture Collection, Manassas, VA) was cloned in pAC between the human actin poly(A) signal and 35S-UTP incorporation (14). Anatomic ISH was conducted with x-ray films. ISH microscopy was undertaken with photographic emulsion followed by 8-day exposure. The slides were developed and then counterstained with hematoxylin.

Northern Blot Analysis—A partial Drak2 cDNA fragment (clone H3022H03; NIA, National Institutes of Health; mouse 15K cDNA clone set; positions 17–2554, according to the sequence in GenBank®) was pre-sented as controls (A–F). CNS, central nervous system; SpC, spinal cord; Th, thymus; BM, bone marrow; Cb, cerebellum; H, heart; K, kidney; Int, intestine; Li, liver; Lu, lung; Sk, skin; SMax, submaxillary gland; Spl, spleen; St, stomach. Bar, 1 cm. Ages are indicated.

FIGURE 1. Drak2 expression during ontogeny according to in situ hybridization. X-ray autoradiography (dark field) of Drak2 in situ hybridization in mice from E12 to adulthood, as indicated, is shown (A–F). Hybridization with the sense probe (G) is presented as controls (A’–F’). CNS, central nervous system; SpC, spinal cord; Th, thymus; BM, bone marrow; Cb, cerebellum; H, heart; K, kidney; Int, intestine; Li, liver; Lu, lung; Sk, skin; SMax, submaxillary gland; Spl, spleen; St, stomach. Bar, 1 cm. Ages are indicated.

FIGURE 2. Drak2 expression in selected adult tissues. A–N, in situ hybridization of selected adult tissues for Drak2 expression. Hybridization with the sense probe (G) on various tissues (B and D, thymus; F and H, spleen) is included as negative controls. A–D, thymus (Me, medulla; Cx, cortex); E–H, spleen (WP, white pulp; RP, red pulp); I, brain (OL, olfactory lobe; V2, ventricular zone; CA1, hippocampal area CA1; DG, dentate gyrus; Cb, cerebellum); J, pituitary gland (AL, anterior lobe; IL, intermediate lobe; PL, posterior lobe); K, brain suprachiasmatic nuclei and vicinity (V III, third ventricle; SCH, suprachiasmatic nuclei); L, adrenal gland (long arrow, medulla; short arrow, cortex); M, stomach (N GI, nonglandular region; GI, glandular region); N, testis (large arrow, Sertoli cells; thin arrows, interstitial Leydig cells; Sp, spermatocytes). A, B, E, F, and I–M are dark field x-ray autoradiography; panels C, D, G, H, and N are bright field emulsion autoradiography with hematoxylin counterstaining. O, Northern blot analysis of Drak2 expression. Thymocytes and spleen T cells were activated by soluble anti-CD3 (0.1 μg/ml, right panel). The duration of activation is indicated. Bands of 28 and 18 S ribosomal RNA are presented to show RNA loading.

sion number NM_133810) was labeled with a digoxigenin labeling kit from Roche Applied Sciences (Laval, Canada). Total RNA was extracted from cultured cells with TRizol (Invitrogen). RNA (20 μg/lane) was resolved in 0.9% formaldehyde agarose gels and transferred to N-Hydrogen and methylamine membranes (Amersham Biosciences). The membranes were hybridized with digoxigenin-labeled probes, and the signals were revealed by a digoxigenin detection kit (Roche Applied Sciences). Band intensities of 18 and 28 S ribosome RNA were indicators of even RNA loading.

Generation of Drak2 Tg Mice—Mouse full-length Drak2 cDNA (MGC-6721) was excised from the vector with XbaI and Sall and cloned into XbaI and BamHI sites in vector pAC between the human β-actin promoter and β-actin poly(A) signals. The resulting construct was named pAC-Drak2. The 4090-bp Clal/ClaI fragment containing the β-actin promoter, Drak2 cDNA, and β-actin poly(A) signal was ligated and injected into fertilized C3HxC57BL/6 eggs. Genotyping of the Tg mice was performed by Southern blot analysis. Tail DNA of the founders (10 μg/each) was digested with PstI and resolved by 1% agarose gel electrophoresis. The DNA was transferred onto N² Hybridblot.
necessarily

nylon membranes after denaturation. A 2-kb band specific to the transgene was detected by the same digoxigenin-labeled Drak2 probe as was employed in the Northern blot analysis. PCR was conducted for subsequent genotyping. The 5’ and 3’ primers were CTGGTGCTCATGCCCGTCAGG, respectively, for the detection of a 264-bp band; and the 5’ and 3’ primers were TGGAGTAAACTGGGGTCGCATC and AGCCA-CAGACTTC and CAGAGGACCTGAGAGTCAG, respectively. A 160-bp product was detected with the following amplification program: 94 °C for 15 min for one cycle and 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 1.5 min for 30 cycles; and 72 °C for 5 min for one cycle. Real Time RT-PCR—Drak2 mRNA in Tg and WT cells was measured by real time RT-PCR; the 5’ and 3’ primers were CACACGTGGCCACAGACTTC and CAGAGGACCTGAGACTTCG and GTGGCCCAGGTATGCACCCAG, respectively, to detect a 800-bp band. The following PCR conditions were employed as internal controls; the 5’ and 3’ primers were GCTAGGCTTCTGTCCACTATGGC and GAAATGCAATTGTTGGTGAACATTTG, respectively, to detect a 800-bp band. The means ± S.D. of ratios of Drak2 versus β-actin signals are shown; samples are in triplicate. D, Immunofluorescent staining of Drak2 in WT versus Tg T cells. Permeablized lymph node T cells were double-stained with rabbit anti-Drak2/sheep anti-rabbit Ig-FITC and anti-Thy1.2-PE monoclonal antibody, and signals were registered by confocal microscopy. The cell surface Thy1.2 is shown in red, and intracellular Drak2 is in green. Flow Cytometry—T cell, B cell, CD4 cell, and CD8 cell populations in the spleen were analyzed by flow cytometry. CD25, CD69, and CD62L expression in T cells was investigated by gating on Thy1.2 cells, as described in our previous publication (15). Annexin V staining was conducted in Thy1.2-gated T cells or transfected CTEV cells, according to a procedure described before (16). T cell Lymphokine Secretion and Proliferation—T cells were purified according to established methods (15). They were stimulated with solid phase anti-CD3, or solid phase anti-CD3 plus anti-CD28. In some experiments, total spleen cells were stimulated with mitomycin C-treated BALB/c spleen cells in mixed lymphocyte culture. Cell supernatants were collected and assayed for IL-2, interferon-γ, and IL-4 by enzyme-linked immunosorbent assay. Proliferation of T cells and transfected CTEV cells was measured with [3H]thymidine uptake. All of these methods have been detailed in our previous publication (15). Transfection of CTEV Cells—CTEV cells (17) were transfected with pCMV-SPORT6-Drak2 (MGC-6721) using Lipofectamine (Invitrogen) according to the manufacturer’s instructions; pCMV-SPORT6-Drak2Δ, which contains the Drak2 3′-untranslated region, served as a control. Twenty-four h after transfection, dead cells were removed by Lympholyte-M gradient; test and control samples with similar viability of about 80% were cultured overnight and stained with annexin V to assess cell apoptosis.

The transfected cells were also selected with G-418 for 20 days. Then a similar number of viable cells (4 × 10^5 cells/200 μl/well) from the test and control samples were cultured for 16 h in the presence of [3H]thymidine and harvested for the measurement of thymidine uptake. DTH Assay—Mice were first primed by painting their shaved abdominal skin with FITC. On day 6, ear thickness was measured, and then the ears were challenged by FITC painting; ear thickness was again measured after 24 h on day 7, and any increases were registered. This method has been described elsewhere (15). To assess the secondary DTH response, these mice were reprimed with FITC on the abdominal skin on day 21; on day 24, ear thickness was measured, and ears were rechallenged by FITC painting; ear thickness was quantified after 24 h on day 25, and any increases were recorded.
FIGURE 4. Characterization of Drak2 Tg lymphoid organs and cells. A, spleen weight and cellularity in Drak2 Tg mice. Ten pairs of Tg mice and their WT littermates were compared for their spleen weight and cellularity. The difference was highly significant (p = 0.0003 for weight, and p = 0.01 for cellularity, paired Student’s t test). B, spleen cell subpopulations for Drak2 Tg mice. T cell (CD3^+^) and B cell (B220^+^) populations and CD4 and CD8 T cell populations in Drak2 Tg and WT spleens were analyzed by two-color flow cytometry. The percentages are indicated in the histograms. C, CD69 and CD25 expression on activated Drak2 Tg T cells. Drak2 Tg and WT T cells were stimulated overnight by solid phase anti-CD3 (4 μg/ml) or anti-CD3 plus anti-CD28 (0.57 μg/ml and 2.86 μg/ml, respectively) (concentration used during coating). CD69 and CD25 expression on Thy-1.2-gated T cells was measured by two-color flow cytometry (CD69/Thy1.2 and CD25/Thy1.2). The experiments described in B and C were repeated more than three times, and representative data are shown.
RESULTS

Drak2 Expression during Ontogeny and Lymphocyte Activation—Drak2 expression during ontogeny is not known. Its expression was studied previously with DNA microarray and with real time RT-PCR in various tissues (12, 13). The limitation of these methods is that they are not able to identify focal expression in a given organ. We therefore employed ISH to map the Drak2 expression pattern during ontogeny, starting from embryonic day 12 (E12), and in adult organs, using an antisense probe derived mainly from the Drak2 3'-untranslated region, which does not share significant homology with other members of the DAP kinase family.

In the mid-gestation stage on E12 and E14, Drak2 presented ubiquitous expression in most organs (Fig. 1, A and B). During the perinatal period on E18 and postnatal day 1, Drak2 expression diminished in most organs but was very high in the thymus (Fig. 1, C and D). Further expression modulation occurred after birth. On postnatal day 10 (Fig. 1E) as well as in adulthood (Fig. 1F), Drak2 was prominently expressed in lymphoid organs, such as the thymus, spleen, and lymph nodes; in addition, high level signals were detected in the cerebellum, skin, intestine, testes, and vertebrae, which probably reflected the signals from bone marrow. ISH of individual adult organs revealed that in the thymus, Drak2 signals were concentrated in the cortex, whereas in the spleen, in the white pulp (Fig. 2, A–H). In the brain (Fig. 2, I and J), olfactory lobe, ventricular zone, hippocampal area CA1, dentate gyrus, cerebellum, and pituitary intermediate and anterior lobes presented high level expression. Most interestingly, the suprachiasmatic nuclei were positive for Drak2 mRNA, suggesting the involvement of Drak2 in the circadian rhythm (Fig. 2K). Drak2 was also expressed in the adrenal gland medulla (Fig. 2L), nonglandular and glandular regions of the stomach (Fig. 2M), and testes spermatocytes, Sertoli cells, and Leydig cells (Fig. 2N). These results clearly demonstrate that Drak2 expression is not restricted to the

FIGURE 5. Lymphokine production and proliferation of Drak2 Tg T cells. Drak2 Tg and WT spleen T cells were stimulated by solid phase anti-CD3 (4 µg/ml) or solid phase anti-CD3 (0.57 µg/ml) plus anti-CD28 (2.86 µg/ml) (concentrations used during plate coating) as indicated. In mixed lymphocyte culture, total spleen cells from Drak2 Tg or WT mice were stimulated by mitomycin C-treated allogeneic BALB/c spleen cells. Supernatants were harvested on the days indicated and assayed for lymphokines by enzyme-linked immunosorbent assay (A). [3H]Thymidine uptake of the remaining cells was measured in triplicate (B). The experiments were repeated more than three times, and representative data with the means ± S.D. are shown.
FIGURE 6. Drak2 T cells are prone to apoptosis. A, augmented apoptosis of activated Drak2 Tg T cells in the presence of exogenous IL-2. Drak2 Tg and WT spleen T cells were stimulated with solid phase anti-CD3 plus anti-CD28, as described in the legend to Fig. 5, in the absence (first column) or presence (second column) of exogenous IL-2 (50 units/ml), which was added to the culture at 24 h. Some T cells (third column) were transferred from anti-CD3- and anti-CD28-coated wells to uncoated wells at 24 h, and exogenous IL-2 was then added to the culture. The cells were harvested at 72 h, and their annexin V expression was assessed by flow cytometry. B, increased apoptosis and reduced proliferation of Drak2 Overexpression Compromises Memory T Cell Development.
Drak2 Overexpression Compromises Memory T Cell Development

T cells, and its expression in several brain regions, where no active apoptosis occurs, suggests that it has functions other than the regulation of apoptosis.

Drak2 expression during T cell activation was investigated by Northern blot analysis. Thymocytes and spleen T cells were stimulated with anti-CD3 (clone 2C11) and were harvested at 5 and 24 h. As shown in Fig. 2C, two discrete bands representing differential splicing products of Drak2 above the 18 S ribosome marker were detected in unstimulated cells; their intensity decreased after 5 and 24 h, indicating down-regulation after T cell activation.

*Generation of Drak2 Tg Mice—* Tg mice expressing human β-actin promoter-driven Drak2 were generated to study Drak2 in vivo function in the immune system. The plasmid construct for Tg mice generation is illustrated in Fig. 3A. Three Tg founders were identified by Southern blot analysis, which revealed 2-kb bands specific to Tg mice (lines 877 and 907 were shown in Fig. 3B). Increased Drak2 mRNA expression in mature spleen T cells was confirmed by real time RT-PCR in line 887 Tg mice (Fig. 3C); similar data were obtained from line 907 (data not presented). Drak2 protein in line 907 Tg and WT lymph node T cells were verified by confocal microscopy employing rabbit anti-Drak2 antibody and anti-thy1.2 monoclonal antibody double staining (Fig. 3D); Drak2 overexpression (in green) in nuclei and cytosol of Tg T cells (according to cell surface staining of Thy1.2, in red) was obvious compared with WT T cells, in which weak Drak2 signals (in green) were still detectable; control rabbit antibody did not stain the cells (data not shown).

Lines 877 and 907 were expanded for detailed study. These mice were fertile and manifested no gross anomalies upon visual inspection except for their spleen weight. Both lines had a similar phenotype in *vivo* and *in vitro*. Therefore, in most of the cases, unless specified otherwise, representative data for line 887 are reported.

*Lymphe kinase Production and Proliferation of Drak2 Tg T Cells—* Spleen weight and cellularity (Fig. 4A) were moderately but consistently reduced in Drak2 Tg mice. T versus B cell ratios and CD4 versus CD8 cell ratios in the spleen of Tg mice were comparable with those of WT controls (Fig. 4B). Expression of the T cell activation markers CD25 and CD69 was normal in Drak2 Tg T cells stimulated by anti-CD3, anti-CD3 plus anti-CD28, or phorbol 12-myristate 13-acetate plus ionomycin (Fig. 4C).

We next examined the cytokine production and proliferation of Tg T cells (Fig. 5). Compared with WT T cells, IL-2 and IL-4 but not interferon-γ secretion after anti-CD3 or anti-CD3 plus anti-CD28 stimulation in Tg T cells was augmented (Fig. 5A). On the other hand, despite the augmented IL-2 and IL-4 secretion, Tg T cell proliferation stimulated by anti-CD3, anti-CD3 plus anti-CD28, or alloantigen was comparable with that of WT T cells, suggesting that IL-2 and IL-4 were not rate-limiting factors for proliferation.

*Enhanced Apoptosis in Activated Drak2 Tg T Cells in the Presence of Exogenous IL-2—* Kinases of the DAP family, including Drak2, have been implicated in apoptosis of various cell types, despite a controversial report that Drak2−/− T cells do not seem to undergo abnormal apoptosis after TCR ligation (13). Using Drak2 Tg mice, we set out to examine the effect of Drak2 on T cell apoptosis not only after simple TCR ligation but also under the influence of IL-2, which is critical for activated T cell apoptosis (18). WT and Drak2 spleen T cells were activated by solid phase anti-CD3 and anti-CD28; 24 h later, exogenous IL-2 was added; at 72 h, apoptosis was assessed by annexin V staining. As shown in Fig. 6A, without exogenous IL-2, WT and Tg T cells presented similar rates of apoptosis (37.6% versus 33.5%, first column). In the presence of exogenous IL-2, Tg T cells demonstrated a drastic increase of apoptosis over WT T cells (63.1% versus 27.6%, second column). Persistent TCR ligation is known to favor T cell apoptosis; under physiological or pathophysiological conditions, TCR ligation is probably a lot shorter than 72 h. We wondered whether the survival of WT and Tg T cells in the presence of IL-2 was still different if the TCR ligation duration was shortened. To this end, WT and Tg T cells were transferred to uncoated wells after 24-h stimulation with anti-CD3 and anti-CD28, and IL-2 was then added as before. As illustrated in the third column (Fig. 6A), apoptosis was reduced in WT and Tg T cells compared with continuous T cell ligation (column 2), as expected, but Tg T cells still had a much higher apoptosis rate than WT T cells (48.3% versus 17.5%).

We next employed *in vitro* Drak2 transfection to confirm our findings on the proapoptotic role of Drak2 in Tg T cells. An IL-2-dependent T cell line CTEV (17) was transfected with a Drak2 expression construct, pSPORT6-Drak2 (MGC-6721, ATCC), in which full-length Drak2 cDNA was placed after a cytomegalovirus promoter; the majority of dead cells were removed after 24 h with Lympholyte-M gradient. The remaining cells were of similar viability (about 80%) in the test sample (transfected with pSPORT6-Drak2) and the control (transfected with pSPORT6-Drak2Δ). After an additional 16-h culture, cell apoptosis was measured with annexin V staining. Compared with cells transfected with the control construct, CTEV cells transiently transfected with pSPORT6-Drak2 showed significantly increased apoptosis, as measured by annexin V-positive percentage.

We tried to establish stable pSPORT6-Drak2-transfected CTEV cells using standard G418 selection. However, compared with cells transfected with the control construct, pSPORT6-Drak2-transfected cells displayed a significantly slower proliferation rate according to visual inspection, and only a small amount of cells survived after a long term selection process of about 20 days. These cells constantly had low viability according to trypan blue staining. On day 20 after transfection, an equal number of viable cells from the test and control samples were pulsed with [3H]thymidine for 16 h, and thymidine uptake was measured with annexin V staining. As shown in Fig. 6B, indeed, pSPORT6-Drak2-transfected cells presented drastically reduced growth, most likely because of their high apoptosis rate. Thus, Drak2 overexpression led to increased CTEV cell apoptosis. It is to be emphasized that in this CTEV cell model, IL-2 was always present, and this model was quite similar to that of Fig. 6A, in which enhanced apoptosis was observed in activated Tg T cells in the presence of IL-2.

To understand the molecular mechanisms of T cell apoptosis associated with Drak2 overexpression, we surveyed the expression levels of a group of anti-apoptotic factors in Tg versus WT T cells. Bcl-2, Bcl-xL, and Flip were expressed at low levels in resting WT and Tg T cells and were significantly induced 24 h after activation in WT T cells (Fig. 6C); however, such induction was compromised in Tg T cells. In the follow-
Four pairs of Tg and WT mice were examined, and (total, responses in Drak2 Tg mice. Drak2 transgenic mice normal primary but compromised secondary DTH responses of Drak2 Tg mice.

Peripheral blood T cells from Drak2 Tg or WT mice (6–8 months old) were double-stained with Thy-1.2-PE and CD62L-FITC. The histograms showed CD62L expression on Thy1.2-gated cells; percentages of CD62Llo populations are indicated. Four pairs of Tg and WT mice were examined, and data from a representative pair are presented. B, normal primary but compromised secondary DTH responses in Drak2 Tg mice. Drak2 transgenic mice (total, n = 9; n = 5 for line 887 and n = 4 for line 907), and their WT littermates (total, n = 10; n = 6 for line 887 and n = 4 for line 907) were primed by FITC painting on abdominal skin on day 0, and challenged by FITC painting of the ear on day 6. Primary DTH was determined on day 7 by measuring the increase in ear thickness. The mice was reprimed on day 21 on abdominal skin and rechallenged on the ear on day 24. Secondary DTH was measured on day 25 according to the increase of ear thickness. The increase in ear thickness of each mouse is presented. The differences between the two groups are not significant in their primary response (p = 0.96) but highly significant (p < 0.01, 2-tailed Student’s t test) in their secondary response.

**DISCUSSION**

In this study, we investigated the role of Drak2 in mature T cell apoptosis and memory T cell development. Several issues are worth discussing. We have corrected a misconception that Drak2 expression is T cell-specific. Two previous publications using DNA microarray and RT-PCR failed to detect Drak2 expression outside the T cell compartment (12, 13). The limitation of those methods is that they could not identify localized gene expression in organs; moreover, Drak2 expression during embryonic development was not studied before. EmployingISH, we found that Drak2 was ubiquitously expressed during the mid-gestation stage, around E12 and E14, indicating the participation of this gene in embryonic development. Drak2 expression in the suprachiasmatic nuclei (19); Drak2 is one of a limited number of genes (excluding housekeeping genes) with strong expression in the central nerve system indicates that this molecule has important roles not related to apoptosis, which do not occur significantly in a normal brain. Drak2 expression in the suprachiasmatic nuclei is especially intriguing, because this region is essential in controlling the behavioral circadian rhythm (19); Drak2 is one of a limited number of genes (excluding housekeeping genes) with strong expression in the central nerve system.
suprachiasmatic nuclei. We monitored the Tg mouse circadian rhythm in 12-h dark/12-h light cycles according to their wheel running activity (20), but abnormalities were not found compared with WT mice. It would be very interesting to examine whether Drak2−/− mice have an impaired circadian rhythm. The Drak2 gene expression pattern revealed by in situ hybridization provides a useful guidance for further exploration of Drak2 functions in different organs. Actin promoter-driven Drak2 Tg mice were generated for such explorations; however, in this paper, our attention was focus on the role of Drak2 in mature T cells.

Two previous publications failed to discover a role of Drak2 in T cell apoptosis (13, 21). We have proven that Drak2 served a critical function in T cell apoptosis, but such an effect was only obvious in the presence of exogenous IL-2. IL-2 is not only a critical lymphokine for T cell activation, but paradoxically, it is also essential in activated T cell apoptosis, as first reported by Lenardo in 1991 (18). Physiologically, IL-2-dependent T cell apoptosis could be important in clonal shrinkage after clonal expansion during an immune response, and high in vivo IL-2 concentration could be achieved locally in the vicinity of activated T cells either as an autocrine or paracrine; consequently, the degree of such apoptosis will decide the pool size of surviving T cells, which further differentiate into memory T cells. This could be one of the explanations for the severe autoimmune diseases in mice deficient in IL-2 production (IL-2 gene null mutant) or IL-2 signal reception (IL-2 receptor β gene null mutant) (22, 23). Indeed, we have found that secondary but not primary T cell DTH responses were diminished in Drak2 Tg mice, and middle-aged Drak2 Tg mice had elevated CD62Llo populations; all of these observations are consistent with the notion that Drak2 influences memory T cell development, which was compromised in the presence of Drak2 overexpression. It is noteworthy that Drak2 expression was normally down-regulated 24 h after T cell activation (Fig. 2O), after a surge within 90 min (21). Although the meaning of the rapid surge is not clear, the down-regulation after 24 h probably represents a mechanism to prevent excess apoptosis of activated T cells by raising their threshold of vulnerability toward IL-2 and allowing some of the activated T cells to survive and develop into memory cells. Taken together, our data suggest that Drak2 is essential in regulating memory T cell development.

How Drak2 affects apoptosis is not currently known. We observed that Drak2 Tg T cells had intrinsic defects in up-regulation of anti-apoptotic factors, such as Bcl-2, Bcl-xL, and Flip, 24 h after activation, although at that time point before addition, IL-2, Tg, and WT T cells had no difference in their survival rates. This suggests that Drak2 renders T cells susceptible to IL-2-induced apoptosis induction by reducing their protection by anti-apoptotic factors. Further studies will be required to elucidate the links between Drak2 and diminished apoptotic factor expression.

In adult Drak2 Tg mice, there was a small but consistent reduction of spleen weight and cellularity that might reflect compromised ongoing secondary immune responses to environmental antigens. Excessive T cell apoptosis likely contributes to such an anomaly; whether this also involves abnormal B cells apoptosis is currently under investigation.

In summary, our study suggests that Drak2 plays critical roles in mature T cell apoptosis and memory T cell development, and this molecule also has functions related and unrelated to apoptosis in a variety of organs. One of its mechanisms in inducing T cell apoptosis is to down-regulate anti-apoptotic factors, which makes the cells vulnerable to apoptotic insults.

Acknowledgments—We thank Ovid Da Silva for editorial assistance, Dr. Martin Marcinkiewicz for in situ hybridization analysis, and the Core Facility of the New Emerging Team of Transplantation for DNA microarray analysis.

REFERENCES

1. Deiss, L. P., Feinstein, E., Berissi, H., Cohen, O., and Kimchi, A. (1995) Genes Dev. 9, 15–30
2. Inbal, B., Shani, G., Cohen, O., Kissil, J. L., and Kimchi, A. (2000) Mol. Cell. Biol. 20, 1044–1054
3. Kawai, T., Matsumoto, M., Takeda, K., Sanjo, H., and Akira, S. (1998) Mol. Cell. Biol. 18, 1642–1651
4. Kawai, T., Nomura, F., Hoshino, K., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Akira, S. (1999) Oncogene 18, 3471–3480
5. Sanjo, H., Kawai, T., and Akira, S. (1998) J. Biol. Chem. 273, 29066–29071
6. Matsumoto, M., Miyake, Y., Nagita, M., Inoue, H., Shitakubo, D., Takemoto, K., Ohtsuka, C., Murakami, H., Nakamura, N., and Kanazawa, H. (2001) J. Biochem. (Tokyo) 130, 217–225
7. Henshall, D. C., Araki, T., Schindler, C. K., Shinoda, S., Lan, J. Q., and Simon, R. P. (2003) J. Neurochem. 86, 1260–1270
8. Kuw, J. C., Lin, J. R., Staddon, J. M., Hosoya, H., and Chen, R. H. (2003) J. Cell Sci. 116, 4777–4790
9. Raveh, T., Droguett, G., Horwitz, M. S., DePinho, R. A., and Kimchi, A. (2001) Nat. Cell Biol. 3, 1–7
10. Shani, G., Henis-Korenblit, S., Iona, G., Gileadi, O., Eisenstein, M., Ziv, T., Admon, A., and Kimchi, A. (2001) EMBO J. 20, 1099–1113
11. Jin, Y., Blue, E. K., Dixon, S., Shao, Z., and Gallagher, P. L. (2002) J. Biol. Chem. 277, 46980–46986
12. Su, A. L., Cooke, M. P., Ching, K. A., Hakak, Y., Walker, J. R., Wiltshire, T., Orth, A. P., Vega, R. G., Sapinioso, L. M., Moqrich, A., Patapoutian, A., Hampton, G. M., Schultz, P. G., and Hogenesch, J. B. (2002). Proc. Natl. Acad. Sci. U. S. A. 99, 4465–4470
13. McGarrill, M. A., Wen, B. G., Walsh, C. M., and Hedrick, S. M. (2004) Immunity 21, 781–791
14. Marcinkiewicz, M. (2002) J. Neuropathol. Exp. Neurol. 61, 815–829
15. Luo, H., Yu, G., Tremblay, I., and Wu, J. (2004) J. Clin. Invest. 114, 1762–1773
16. Luo, H., Wan, X., Wu, Y., and Wu, J. (2001) J. Immunol. 167, 1362–1370
17. Hu-Li, J., Ohara, J., Watson, C., Tsang, W., and Paul, W. E. (1989) J. Immunol. 142, 800–807
18. Lenardo, M. J. (1991) Nature 353, 858–861
19. Silver, R., LeSauter, J., Tesoro, P. A., and Lehman, M. N. (1996) Nature 382, 810–813
20. Laemle, L. K., and Ottenweller, J. L. (1989) Physiol. Behav. 46, 145–171
21. Friedrich, M. L., Wen, B. G., Rain, G., Kee, B. L., Katayama, C., Murre, C., Hedrick, S. M., and Walsh, C. M. (2005) Int. Immunol. 17, 1379–1390
22. Sadlack, B., Merz, H., Schorle, H., Schimpl, A., Feller, A. C., and Horak, I. (1993) Cell 75, 253–261
23. Suzuki, H., Kundig, T. M., Furlonger, C., Wakeham, A., Timms, E., Matsuyama, T., Schmits, R., Simon, J. J., Ohashi, P. S., Griesser, H., Hingubi, T., Poi, C., and Mak, T. W. (1995) Science 268, 1472–1476