DRAK2, a lymphoid-enriched DAP kinase, regulates the TCR activation threshold during thymocyte selection

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Abstract

DAP kinases are a family of serine/threonine kinases known to regulate intrinsic apoptotic processes. DAP-related apoptotic kinase-2 (DRAK2) is highly expressed in lymphoid organs, with differential expression during thymocyte development. Low levels of transcript were observed in CD4/CD8 double-positive (DP) and double-negative populations, whereas single-positive thymocytes possessed elevated levels. Ex vivo stimulation of DP thymocytes with phorbol myristate acetate or antibodies that activate the TCR complex led to the accumulation of DRAK2 in a protein kinase C- and MAP Kinase-dependent fashion. Although DAP kinase family members are thought to potentiate apoptosis, ectopic expression of DRAK2 using retroviral transduction of primary T cells and NIH3T3 fibroblasts failed to decrease rates of survival, suggesting that DRAK2 expression is not sufficient to promote apoptosis. Rather, our results demonstrate that DRAK2 is a primary response gene activated by TCR stimulation in DP thymocytes. Further, we observed that DRAK2 controlled the threshold for calcium signaling in the thymus since positively selected Drak2-deficient thymocytes displayed a reduced requirement for TCR cross-linking. These findings are consistent with a role for DRAK2 in thymocyte selection and lymphoid maturation, and demonstrate that DRAK2 transduces non-apoptotic signals during thymocyte differentiation.

Introduction

The prevention of autoimmunity and the preservation of lymphocyte homeostasis depend on mechanisms that block inappropriate stimulation by auto-antigens. Although protein kinases play an essential role in lymphocyte activation, surprisingly little is known about the regulation of intracellular signaling pathways important for immune tolerance. Disruptions in various signaling cascades that depend on protein phosphorylation have been shown to play key roles in the development of numerous diseases, including many forms of cancer and immune system dysfunction. Recently, DAP-related apoptotic kinase-2 (DRAK2) has emerged as a potential regulator of immune tolerance (1). DRAK2 is highly enriched in lymphoid tissues and belongs to the DAP-like family of serine/threonine kinases (DAPKs), which have been shown to universally induce apoptosis upon ectopic expression in a kinase-dependent fashion (2), yet its role in modulating immune function remains poorly understood.

There are five known mammalian members of the DAPK family; additional homologs have also been found in Caenorhabditis elegans and Drosophila melanogaster. All family members share a characteristic serine/threonine kinase domain at the extreme amino-terminus with high primary amino acid sequence homology to myosin light chain kinases, and all are known to possess intrinsic kinase activity (2).
However, these proteins share little homology outside of this kinase domain, suggesting that these factors regulate, or are regulated by, disparate processes in cells. In addition to this conserved kinase domain, the prototype DAP kinase (DAPK1) contains a calmodulin-binding domain, an ankryin repeat region, a cytoskeletal-binding domain and a carboxy-terminal death domain (3). The latter is thought to physically link DAPK1 to death receptor-mediated apoptosis, though the means by which DAPK1 potentiates this form of apoptosis is unclear (4–6). DRAK2 was originally cloned as a result of its sequence homology with DAPK1, and was shown to promote apoptosis upon expression in murine NIH3T3 cells (7, 8). While DAP kinase family members are thought to be involved in both extrinsic and intrinsic pathways of apoptosis, a physiological role for DRAK2-dependent apoptosis has not been firmly established. DRAK2-deficient mice have no defects in apoptosis, and as described here, retroviral expression of DRAK2 in NIH3T3 cells did not induce apoptosis. Rather, we have recently described a role for DRAK2 in negative regulation of T cell activation (1); T cells from mice deficient in DRAK2 are hypersensitive to TCR stimulation, proliferate in response to sub-optimal stimulation and produce higher levels of cytokines (especially IL-2 and IL-4) when compared with wild-type T cells.

With an interest in characterizing a potential role for DRAK2 in thymocyte development, we investigated the expression of this gene in distinct thymocyte subsets. We found that DRAK2 expression was regulated differentially during lymphoid development, with increasing expression corresponding to lymphocyte maturation. In the thymus, DRAK2 was expressed at low levels in immature double-negative (DN) cells, whereas CD4/CD8 double-positive (DP) cells lacked appreciable expression of DRAK2 mRNA. In contrast, DRAK2 mRNA was found to exist at the highest levels in CD4 and CD8 single-positive (SP) thymocytes. In DP thymocytes, treatment with phorbol myristate acetate (PMA) strongly induced DRAK2 mRNA expression, and this occurred in a protein synthesis-independent fashion, suggesting that DRAK2 is a primary response gene in this thymocyte subset. Stimulation of purified DP cells with TCR cross-linking antibodies, especially with cross-linking of CD4 or CD8 co-receptors, similarly enhanced DRAK2 expression. Pharmacologic inhibition with agents that block non-classical protein kinase C (PKC) isoforms abrogated the PMA-dependent up-regulation of DRAK2, suggesting that non-classical PKCs are involved in DRAK2 transcription in activated DP thymocytes. Similar to previous reports using ectopically expressed PKCs, endogenous DRAK2 was primarily localized in the nucleus. This nuclear localization required an 80-residue domain carboxy-terminal to the kinase domain. However, in Jurkat T cells and thymocytes, TCR stimulation led to export of a large fraction of DRAK2 out of the nucleus.

Since DRAK2 was expressed at low levels in DP cells, we hypothesized that its increased expression in SP cells might serve as a mechanism to interfere with activation as cells transit through this developmental stage. With evidence indicating that DRAK2 raises the threshold for T cell activation in naive peripheral T cells, we used Drak2-deficient thymocytes to examine the role of DRAK2 in thymocyte activation. An increase in the concentration of free Ca2+ is an essential signal during T cell development and activation, leading to the activation of downstream transcriptional pathways (9). Our experiments using Drak2-deficient thymocytes demonstrate that this DAP kinase sets a threshold during selection for TCR signal strength, as measured by Ca2+ release following TCR cross-linking. Thus, in addition to negatively regulating the activation of mature T cells, DRAK2 also acts as a rheostat to alter the outcome of TCR signaling in developing thymocytes.

Methods

Isolation of DRAK2 from a murine thymocyte cDNA library

We originally identified DRAK2 (clone F3.4) in an interaction screen for FADD-binding partners, although subsequent assays for interactions between clone F3.4 and FADD have failed to substantiate a physiological interaction (10). Following three sequential rounds of purification, positive clones were in vivo excised and used for subsequent sequencing analyses. For sequencing, single-stranded oligonucleotides were used to prime cycle-sequencing reactions for each strand. The products were analyzed on an Applied Biosystems model 390 Automated DNA sequencer. In our analyses (roughly three million clones), we obtained numerous clones encoding truncated or complete forms of Fas/CD95, TRADD and RIP. One clone (F3.4) was obtained that was distinct from these, and we decided to study this gene further. The complete 3.2-kb sequence of clone F3.4 has been deposited to GenBank with the accession number AY092028.

Northern analysis of tissue-specific DRAK2 expression

To study the tissue-specific expression of DRAK2, we generated a multiple tissue northern blot. Indicated organs were isolated from adult C57BL/6 mice and total cellular RNA was produced using Trizol (Invitrogen, San Diego, CA, USA). For northern blotting, 20 µg of total RNA for each sample was loaded onto a 1.0% agarose–6% formaldehyde gel, separated electrophoretically and transferred to a GeneScreen (NEN/Perkin Elmer, Boston, MA, USA) nylon membrane. The membrane was hybridized with radiolabeled cDNA probes for DRAK2 (the entire 3.2-kb cDNA) or GAPDH, and analyzed by autoradiography.

Northern analyses of T and B cell developmental subsets and activated DP thymocytes

To characterize DRAK2 expression in thymocyte subsets, pooled C57BL/6 thymocytes were incubated with anti-CD4–FITC and anti-CD8–PE antibodies, washed in staining buffer (PBS with 2% FCS) and separated on a cell sorter (MoFlow). Four populations were recovered consisting of DP (CD4+CD8+), CD4 SP, CD8 SP and CD4/CD8 DN cells. Cellular RNA was extracted from these thymocytes using Trizol and 15 µg per condition was used for northern analyses with DRAK2 or GAPDH probes. B cell developmental subsets were assayed for DRAK2 expression following sequential separations using magnetic cell sorting (Miltenyi Biotech, Auburn, CA, USA). Briefly, total bone marrow (8M) cells were isolated from C57BL/6 mice and pooled prior to incubation with antibodies to mouse IgM and purification on a MACS column following...
binding of paramagnetic beads specific for the primary antibody. These cells were then separated on a MACS column (Miltenyi Biotech); cells retained on this column were labeled ‘IgM’. The flow-through from this column was then incubated with antibodies to BP-1, followed by incubation with isotype-specific antibody-conjugated paramagnetic beads, and these cells were isolated again on a second MACS column. These cells were labeled ‘BP-1’. Finally, the flow-through from this purification step was incubated with antibodies to murine B220, followed by anti-isotype binding to beads and column separation. These cells were labeled ‘B220’. Following isolation, total cellular RNA was purified as described above and subjected to electrophoresis, followed by northern blotting and hybridization with radiolabeled DRAK2 cDNA or radiolabeled GAPDH probes.

To characterize DRAK2 mRNA expression patterns in activated DP thymocytes, we isolated cells from AND. Rag2<sup>−/−</sup>/H-<sup>2<sup>d</sup></sup> TCR transgenic mice, which lack positive selection (see below) and treated these cells under a variety of conditions, as described previously (11). Although these thymocytes are contaminated with some DN cells, most of the cells (>90%) are DP. A total of 2.5 × 10<sup>6</sup> thymocytes were used for each sample, and following the activation period, total cellular RNA was isolated. In some cases, cells were treated with PMA at 1 ng ml<sup>−1</sup> for the indicated times, with or without PD98059 (90-min pre-treatment). A total of 10 μg ml<sup>−1</sup> cycloheximide (CHX) was added to some samples 90 min prior to addition of PMA. For TCR-induced expression, 5 × 10<sup>5</sup> cells were incubated for 4–8 h with plate-bound anti-CD3 (145-2C11, 1 μg ml<sup>−1</sup>) and/or anti-CD4 (GK1.5, 1 μg ml<sup>−1</sup>) antibodies prior to northern blotting.

293T and DP thymocyte DRAK2 western blots

To determine the specificity of the anti-DRAK2 antibody, we transfected 293T cells with DRAK2 expression constructs encoding full-length and a C-terminal deletion mutant lacking the amino acids 351–365 epitope. The 293T cells were transfected as above with the indicated constructs. Following transfection, supernatants were isolated at various times and subsequently titered by immunofluorescence on murine NIH3T3 cells. To transduce T cells, spleens and lymph nodes were harvested from 6- to 8-week-old mice, and single-cell suspensions were incubated with plate-bound anti-CD3, soluble anti-CD4 (eBioscience, San Diego, CA, USA) (low: 25 ng ml<sup>−1</sup> αCD3 and 2 μg ml<sup>−1</sup> αCD4; high: 2 μg ml<sup>−1</sup> αCD3 and 10 μg ml<sup>−1</sup> αCD4; PMA 40 ng ml<sup>−1</sup>, ionomycin 2 μM) for 1 h. Cells were fractionated into cytoplasmic and nuclear fractions by homogenization of cells in hypotonic buffer (10 mM MgCl<sub>2</sub>, 10 mM KCl, 10 mM HEPES pH 7.0) followed by low-speed centrifugation (800 × g).

Retroviral expression of DRAK2 and survival/apoptosis studies of 3T3 cells and primary mouse T cells

The open reading frame of full-length DRAK2 was subcloned into MSCV-ires-Thy1.1 (MIT) (12, 13). This construct (or empty MIT) was co-transfected with the pIRES helper virus construct into 293T cells. Following transfection, supernatants were isolated at various times and subsequently titered by infection of murine NIH3T3 cells. To transduce T cells, spleens and lymph nodes were harvested from 6- to 8-week-old mice, and single-cell suspensions were incubated with plate-bound anti-CD3, soluble anti-CD28 and IL-2 to initiate cell cycle. After 24 h, retroviral supernatants were added, along with polybrene, and the cells were spun for 1 h to enhance infection. At various times following transduction, cells were isolated and stained for FACS. Cells were stained with anti-CD8–PerCP (BD Biosciences, San Diego, CA, USA), anti-CD4 aliphycocyanin–thrin (APC) (eBioscience, CA, USA) and Annexin-V–FITC (Caltag, Burlingame, CA, USA). In each case, mock-transduced cells served as negative controls for Thy1.1. For 3T3 retroviral transduction, cells were incubated overnight with polybrene and retroviral supernatant at the indicated dilutions. Following infection, cells were maintained for >40 days in culture. To demonstrate expression, some cells were harvested at 5-day post-infection, lysed...
and analyzed by western blotting with anti-DRAK2. For investigation of apoptotic potential, cells were harvested at 5-day post-infection, left unstimulated or treated with tumor necrosis factor (TNF) α (20 ng ml⁻¹) and CHX (10 μg ml⁻¹) for 18 h, and then harvested for FACS. Cells were stained with anti-Thy1.1–PE and AnnV–FITC, followed by data acquisition using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). All FACS data were analyzed using FlowJo software (Treestar, Inc., Ashland, OR, USA).

Mice

DRAK2⁻/⁻ mice have been described previously (1). All mice used were back-crossed onto a C57BL/6 background a minimum of six generations, and were used between 6 and 12 weeks of age. Mice were housed in pathogen-free conditions in accordance with regulations of the Institutional Animal Care and Use Committee at University of California, Irvine.

Calcium mobilization assays

Following harvest, thymocytes were re-suspended at 10 × 10⁶ ml⁻¹ and were labeled in RPMI + 2% FCS with 4.6 μM Fura red (Molecular Probes), 3.4 μM Fluo-3 (Molecular Probes) and 0.02% Pluronic (Molecular Probes) for 1 h at 37°C. Cells were washed with cold (unsupplemented) RPMI, then incubated on ice for 15 min with biotinylated αCD3 and αCD4 (PharMingen and eBioscience) as well as αCD4–APC and αCD8–PE (eBioscience and Caltag), and then washed and re-suspended in cold RPMI. Samples were pre-warmed for 15 min prior to analysis, then stimulated by cross-linking with 20 μg ml⁻¹ streptavidin (Sigma). Calcium mobilization was plotted as a ratio of Fluo-3 : Fura red using the kinetics suite of FlowJo Software (Treestar, Inc.).

Results

Developmental regulation of DRAK2 during lymphoid development

To determine if DRAK2 mRNA expression is regulated during thymopoiesis, thymocytes were pooled from a number of mice, stained with antibodies to CD4 and CD8 and sorted. Cellular RNAs from these populations were probed by northern blot hybridization with the DRAK2 cDNA (Fig. 1a). DRAK2 was expressed at low levels in CD4⁻CD8⁻ DN cells, and was almost undetectable at the CD4⁺CD8⁻ DP stage. However, message was present at high levels in the CD4⁺CD8⁺ SP thymocytes. Thus, DRAK2 mRNA is developmentally regulated during thymocyte maturation, consistent with previous results using western blotting (1). This pattern of expression was provocative since DP thymocytes readily undergo either apoptosis or positive selection in response to TCR ligation. Indeed, recent gene array studies of DP cells isolated from non-selecting versus selecting TCR transgenic backgrounds have revealed that DRAK2/STK17b is one of a handful of genes induced during thymocyte selection (14). Since DRAK2 expression was maintained in naive peripheral T cells (data not shown and ref. 1), we believe that it is developmentally expressed, and not simply a transient marker of activation.

A similar developmental pattern of expression existed in developing B cells derived from the BM (Fig. 1b). Different B cell developmental subsets can be isolated using antibodies to B220, BP-1 and IgM (15). In this case, pre-B (B220⁺BP-1⁺/IgM⁻ BM cells) are the most developmentally immature. Cells expressing BP-1 but lacking IgM (BP-1⁺) represent a mixed population of pro-B and pre-B cells (pre/pro-B). Following productive rearrangements of the heavy and light chain Ig genes, cells express IgM on the surface before moving into the lymphoid system as immature B cells. These IgM⁺ B cells constitute a population of immature (IgD⁻) and mature (IgD⁺) B cells. Analysis of DRAK2 expression in developing BM B cells demonstrated an increase in the expression in the most mature B cells, following presentation of the B cell receptor on the surface. Thus, DRAK2 mRNA expression was developmentally regulated in both T and B lymphocytes, with the highest levels present in the most mature populations.

DRAK2 is a primary response gene activated by TCR ligation in DP thymocytes

As described above, DRAK2 mRNA was expressed at low levels in the DP population, whereas it was found at high levels in positively selected, mature CD4⁺ and CD8⁺ thymocytes. It is known that many signals derived through the TCR complex are necessary for the maturation of DP cells to become CD4⁺ and...
CD8+ thymocytes. Thus, we sought to determine if signaling through the TCR complex might increase the levels of DRAK2 mRNA in isolated DP thymocytes. Treatment of isolated DP thymocytes with PMA resulted in the accumulation of DRAK2 mRNA (Fig. 2a). This induction of DRAK2 message accumulation occurred rapidly (within 30 min) and in a PMA dose-dependent fashion, with maximal levels achieved between 0.1 and 1.0 ng ml⁻¹. This induction occurred in the presence of the MEK inhibitor PD98059, demonstrating that the activation of the Erk MAP kinase (MAPK) pathway (downstream of Ras-GRP) was not involved in the induction of DRAK2 mRNA accumulation in DP thymocytes (Fig. 2b). Furthermore, the ability of PMA to up-regulate DRAK2 mRNA was found to be independent of protein synthesis since the elongation factor inhibitor CHX failed to inhibit this (Fig. 2c).

To determine whether signaling through the TCR complex might also up-regulate expression of DRAK2 mRNA, DP thymocytes were treated with plate-bound anti-CD3 antibodies. With this treatment, we observed a modest induction of DRAK2 mRNA (Fig. 2d). However, including anti-CD4 or anti-CD8 cross-linking antibodies with CD3 ligation significantly enhanced the expression of DRAK2 message. This result is consistent with the notion that the participation of these co-receptor molecules is crucial to this stage of development (16). As expected, addition of the MEK inhibitor PD98059 did not block this up-regulation. Interestingly, the calcineurin inhibitor cyclosporin A caused a very minor decrease in DRAK2 mRNA in the presence of PD98059; calcineurin acts as a phosphatase activated by calcium/calmodulin and dephosphorylates NFAT (17–19). Thus, DRAK2 is likely a primary response gene activated by TCR complex and co-receptor ligation. The participation of the MEK1–Erk and calcineurin–NFAT signaling pathways does not appear to be essential for the accumulation of DRAK2 mRNA following TCR cross-linking.

Regulation of DRAK2 in DP thymocytes by non-classical PKC pathways

To characterize the regulation of DRAK2 protein levels in DP thymocytes, we utilized a polyclonal antibody produced to a peptide corresponding to human DRAK2 amino acids 351–365. In this region, murine and human DRAK2 differ by only a single amino acid. To test the specificity of this antibody against murine DRAK2, we transfected 293T cells with 3× HA-tagged versions of full-length and carboxy-terminal-deleted DRAK2 and probed these by western blot using antibodies to HA or the polyclonal DRAK2 antibody (Fig. 3a). Anti-DRAK2 detected a 43-kDa band in wild-type DRAK2 transfected cells, but failed to identify the lower molecular weight mutant form since this mutant lacks the epitope recognized by the polyclonal antibody. The anti-DRAK2 antibody detected an artifactual high-molecular weight band, as this band, but not the 43-kDa band, was present in DRAK2-deficient thymocytes (data not shown). Thus, this antibody is highly specific for DRAK2.

To investigate the regulation of DRAK2 in DP thymocytes, we utilized MHC class I/class II double-deficient thymocytes, which lack SP cells by virtue of the absence of positive selection in these mice; >90% of the cells in the thymi of these mice are DP thymocytes. Therefore, we sought to determine if signaling through the TCR complex might increase the levels of DRAK2 mRNA in isolated DP thymocytes. Treatment of isolated DP thymocytes with PMA resulted in the accumulation of DRAK2 mRNA (Fig. 2a). This induction of DRAK2 message accumulation occurred rapidly (within 30 min) and in a PMA dose-dependent fashion, with maximal levels achieved between 0.1 and 1.0 ng ml⁻¹. This induction occurred in the presence of the MEK inhibitor PD98059, demonstrating that the activation of the Erk MAP kinase (MAPK) pathway (downstream of Ras-GRP) was not involved in the induction of DRAK2 mRNA accumulation in DP thymocytes (Fig. 2b). Furthermore, the ability of PMA to up-regulate DRAK2 mRNA was found to be independent of protein synthesis since the elongation factor inhibitor CHX failed to inhibit this (Fig. 2c).

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mice are DPs. These cells were treated with PMA, ionomycin or apoptogenic levels of anti-Fas/CD95 cross-linking antibody. Following this incubation, these cells were lysed and analyzed by western blotting using anti-DRAK2 polyclonal antibodies. Under these conditions, PMA enhanced the expression of DRAK2 protein (Fig. 3b), consistent with studies described above. However, treatment with calcium ionophore or agonistic anti-Fas/CD95 antibodies did not lead to DRAK2 up-regulation. Thus, DRAK2 was found to be transcriptionally and translationally up-regulated by PMA, but not by alternative signals known to be active in DP thymocytes following TCR ligation or via death receptor signals known to directly induce apoptosis. Curiously, inhibition of the MEK-Erk pathway using either PD98059 or U0126 (a second MEK-selective inhibitor) blocked the accumulation of DRAK2 protein in PMA-treated thymocytes, whereas the p38 MAPK inhibitor SB203580 did not (Fig. 3c). The specificity of these inhibitors was demonstrated by reprobing this western blot with anti-phospho-Erk1/2; it can be seen that both MEK inhibitors blocked the phosphorylation of Erk1/2, whereas the p38 inhibitor did not. These findings indicate that levels of DRAK2 mRNA and protein are both controlled by signaling pathways induced by PMA, but only the latter depends on MEK activity.

Given that PMA induced the accumulation of DRAK2 in DP thymocytes and that this process did not explicitly involve MEK (at least at the mRNA level), we wanted to know if PKC activity in thymocytes was involved in its up-regulation, since PKC function is thought to be crucial for T cell activation and thymocyte selection (20–22). To test this notion, we utilized cell-permeable inhibitors of classical and non-classical PKC isoforms (Fig. 3d). PKC inhibitors Ro31-8425 and Ro31-8220 blocked the PMA-dependent accumulation of DRAK2 protein in DP thymocytes; these inhibitors are only moderately isoform selective and are known to inhibit the activities of both classical and non-classical PKC isoforms (23). However, addition of Go-6976, a potent and selective inhibitor of classical PKC isozymes (IC50 = 2.3 nM, PKCα IC50 = 6.2 nM), did not block the up-regulation of DRAK2 protein (24). Reprobing this blot with anti-phospho-Erk1/2 demonstrated that the blockade in DRAK2 up-regulation correlated with the phosphorylation status of the upstream MAPKs. These results suggest that the PMA-dependent up-regulation of DRAK2 in DP thymocytes requires the activation of a signaling pathway downstream of a non-classical PKC isoform(s). Taken together, we conclude that this pathway is comprised, at least partly, by a Raf-MEK-Erk signaling cascade.

DRAK2 expression alone is not sufficient to induce apoptosis in 3T3 cells or primary T cells

To determine if murine DRAK2 could promote apoptosis upon ectopic expression, we employed retroviral transduction of 3T3 cells and primary T cells. The DRAK2 open reading frame was subcloned into MiT, a murine stem cell retroviral construct useful for ectopic expression in primary lymphocytes (12, 13). In this retroviral system, DRAK2 expression alone is not sufficient to induce apoptosis in 3T3 cells or primary T cells.
293T cells transfected with MiT-DRAK2 or MiT (empty vector) were used to infect 3T3 cells at different dilutions. After 5 days of infection, the cells were lysed and analyzed by western blotting with an anti-DRAK2 antibody. Mock- and MiT-infected cells lacked any detectable DRAK2 expression, whereas MiT-DRAK2-infected cells expressed DRAK2 in a dose-dependent fashion (Fig. 4a). To determine if such ectopic expression of DRAK2 induced apoptosis in 3T3 cells, cells were infected with a 1:1 dilution of MiT or MiT-DRAK2, and then stained with anti-Thy.1.1 and Annexin-V to detect apoptosis in uninfected and infected cells (Fig. 4b). Treatment of uninfected and MiT- and MiT-DRAK2-infected cells with TNFα and CHX (TNF + CHX) induced appreciable apoptosis, as assessed by Annexin-V+ events. However, no difference in the proportion of apoptotic cells was observed with MiT-DRAK2 infection alone. Concerned that DRAK2 might manifest apoptotic effects on a longer time-scale, we maintained MiT-DRAK2-infected 3T3 cells over 40 days in culture. Our rationale was that if DRAK2 was capable of inducing apoptotic events slowly, the proportion of cells expressing the retrovirally maintained transgene would decrease over time. However, even at the highest concentration of MiT-DRAK2 supernatant, no appreciable difference in the proportion of cells expressing the transgene was observed (Fig. 4c).

We also wished to ascertain the effect of ectopically expressing DRAK2 in primary mouse T cells following mitogenic stimulation. Should DRAK2 decrease the survival of primary T cells following mitogenic stimulation, the proportion of Thy1.1+ cells should be dramatically decreased. Splenocytes from Thy1.2+ C57BL/6 mice were harvested, activated with an overnight stimulation using plate-bound anti-CD3 plus soluble anti-CD28 and IL-2 and then spin transduced with MiT or MiT-DRAK2 supernatants. Although the proportion of Thy1.1+ cells was slightly lower in the MiT-DRAK2-transduced cells after 7 days culture (Fig. 4d), this proportion did not significantly change over time (data not shown). These results demonstrate that while human DRAK2 has been reported to induce apoptosis upon over-expression, retrovirally enforced expression of DRAK2 in murine 3T3 fibroblasts and primary T cells failed to induce any detectible apoptosis, nor did it decrease the survival or growth rate by any other means. Although DRAK2 may play a role in apoptosis, particularly when expressed at supra-optimal levels, its expression alone does not appear sufficient to promote apoptosis. For this reason, we conclude that DRAK2 does not directly promote apoptosis, but may alter the signaling capacity of certain cell types, ultimately leading to cell death.

Subcellular localization of DRAK2 in T cells and thymocytes
Since DRAK2 lacks any apparent homology with other proteins outside of its kinase domain, we endeavored to characterize its cellular function. One important clue to the function of a protein of interest can be obtained by the investigation of its subcellular localization. Analysis of the primary amino acid sequence using the Swiss Prot PSORT II server predicts that DRAK2 is either a nuclear (score = 43.5%) or mitochondrial (score = 43.5%) protein (25). This algorithm detects a putative nuclear localization signal (NLS) near

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![Fig. 4.](https://academic.oup.com/intimm/article-abstract/17/11/1379/813253) Retrovirally enforced expression of DRAK2 in 3T3 cells and primary mouse T cells fails to reduce survival or promote apoptosis. (a) Ectopic expression of DRAK2 in retrovirally transduced murine 3T3 cells. Cells that were mock infected or infected with MiT-empty or MiT-DRAK2 were left untreated or treated with TNF + CHX, then stained with anti-Thy1.1–PE and Annexin-V–FITC. (b) Five days following infection of cells with the indicated dilutions of retroviral supernatant, cells were harvested, lysed and analyzed by western blotting using anti-DRAK2 antibodies. The position of DRAK2 is indicated. The blot was stripped and reprobed with anti-β-actin to demonstrate equivalent protein loading.
position 65 (KKRRR) within the kinase domain. To assay the localization of DRAK2, we produced an amino-terminal fusion between GFP and the DRAK2 open reading frame. This protein fusion migrated with an apparent molecular mass of ~70 kDa on western blots probed with anti-GFP polyclonal antibodies (data not shown). COS7 cells were transiently transfected with expression vectors encoding GFP–DRAK2 or GFP alone and cells were analyzed by fluorescent microscopy (Fig. 5a). To identify nuclear morphology, the cells were counterstained with the nucleic acid-intercalating Hoechst 33342 dye, and composite images were produced. Transfection with a GFP-only vector yielded green fluorescence throughout the cell, whereas the GFP–DRAK2 fusion was found to be highly concentrated in the nucleus; similar results have been reported for human and rat DRAK2 (7, 8). Closer inspection of GFP–DRAK2 localization revealed that the fusion was excluded from nuclear areas characteristic of nucleoli. Although mutation of residues in the putative NLS sequence failed to reliably alter the nuclear localization of DRAK2 (data not shown), deletion of the 80-residue carboxyl-terminus of DRAK2 (1–290) completely inhibited nuclear accumulation in COS7. However, in this case, DRAK2 GFP fluorescence

![Subcellular localization of DRAK2. (a) The carboxyl-terminus of DRAK2 is essential for nuclear localization in COS7 cells. Cells were transiently transfected with a GFP, a GFP–DRAK2 fusion, or a GFP–DRAK2(1–290) truncation mutant, and analyzed by fluorescent microscopy. Cells were first stained with Hoechst 33342, and a UV filter was used to capture these images. To determine the subcellular localization of GFP in these transfectants, images were collected using a filter set for green fluorescence. Bottom panels are composites of the Hoechst and GFP images electronically superimposed. (b) DRAK2 exits the nucleus of Jurkat cells following TCR activation. Jurkat cells transfected with GFP or GFP–DRAK2 were left untreated or stimulated with PMA plus PHA, then stained with Hoechst dye and analyzed by confocal microscopy. (c) Western blot showing localization of DRAK2 in fractionated thymocytes. Thymocytes from a C57BL/6 wild-type mouse were treated under various conditions and fractionated into cytoplasmic and nuclear fractions. To confirm that the fractions were pure, the blot was stripped and reprobed with anti-poly [ADP-ribose] polymerase (PARP) (nuclear) and anti-tubulin (cytoplasmic).](https://academic.oup.com/intimm/article-abstract/17/11/1379/813253)
appeared punctate, presumably due to accumulation in perinuclear organelles.

We also wished to characterize the localization of DRAK2 in T cells. In stably transfected Jurkat cells, GFP–DRAK2 was also primarily localized within the nucleus (Fig 5b). However, stimulation of these cells with PMA plus PHA to mimic the antigenic stimulation of the TCR resulted in the export of a large fraction of DRAK2 from the nucleus to the cytoplasm. We also examined the endogenous DRAK2 localization in murine thymocytes using subcellular fractionation and western blotting. In SP thymocytes, DRAK2 accumulated in the cytoplasmic fraction following stimulation with PMA plus ionomycin; a lesser degree of export was observed when the thymocytes were stimulated with a high dose of anti-CD3 and anti-CD4 (Fig. 5c). Thus, as in Jurkat clones, DRAK2 is similarly shuttled from the nucleus to cytoplasmic compartments following TCR stimulation or with treatment using agents that mimic TCR signaling. These results demonstrate that DRAK2 is a likely nuclear protein in quiescent T cells, possibly as a consequence of a non-canonical nuclear targeting sequence present in its carboxyl-terminus. An alternative hypothesis is that another protein capable of shuttling DRAK2 into the nucleus interacts with this carboxyl-terminal domain. Following T cell stimulation, DRAK2 is rapidly re-distributed to cytoplasmic compartments and likely plays a role in regulating T cell function in this context.

**DRAK2 raises the threshold for calcium mobilization in SP thymocytes**

We sought to determine a functional role for DRAK2 in developing thymocytes. It has been previously shown that peripheral naive T cells from Drak2-deficient mice receive a TCR signal of greater intensity and have an enhanced calcium flux (1). To determine if DRAK2 modulates TCR signaling within distinct stages in the thymus, we examined calcium flux in developing thymocytes. Freshly isolated thymocytes from C57BL/6 wild-type and Drak2-deficient mice were labeled with the calcium-binding indicators Fluo-3 and Fura red, stimulated with biotinylated antibodies to CD3 and CD4 and cross-linked with streptavidin. To discern the effect of a Drak2 deletion in specific developmental subsets, these thymocytes were first labeled with fluorescently tagged anti-CD4 and anti-CD8. CD4+, DP and transitional cells were electronically gated for subsequent analyses (Fig. 6a). In the DP population, the extent of calcium mobilization following TCR/CD4 cross-linking in Drak2−/− thymocytes was similar to that seen in wild-type cells (Fig. 6b). At various sub-optimal doses of anti-CD3 tested, no obvious difference between Drak2−/− and wild-type DP cells was observed (data not shown). In contrast, CD4+ thymocytes from Drak2−/− mice generated a maximal calcium response following cross-linking with sub-optimal levels of anti-CD3 and -CD4. These results confirm that, similar to the situation in naive peripheral T cells, the presence of DRAK2 is required for negatively regulating TCR signaling in positively selected thymocytes. We also wished to determine how DRAK2 might affect the TCR activation threshold of transitional thymocytes (CD4loCD8lo DP cells), a subset thought to have undergone recent selection events (26). At sub-optimal levels of anti-CD3, there

![Fig. 6. Calcium flux is enhanced in CD4+ thymocytes from Drak2-deficient mice. Thymocytes were isolated from Drak2+/+ or Drak2−/− mice, labeled with Fluo-3 and Fura red and stained with αCD4–APC and αCD8–PE. For stimulation, cells were incubated with biotinylated antibodies to CD3 and CD4, then cross-linked with streptavidin and analyzed by flow cytometry. (a) Thymocyte CD4/CD8 profile, indicating the specific populations analyzed. (b) Calcium release induced in Drak2+/+ and Drak2−/− thymocyte subsets by anti-CD3 plus anti-CD4 antibodies. These biotinylated antibodies were first bound to Fura red- and Fluo-3-loaded thymocytes on ice. The cells were shifted to 37°C, and sampled on a FACSCalibur cytometer to establish baseline Ca2+ levels. After ~60 s of sampling, streptavidin was added to promote cross-linking (indicated by the arrow labeled ‘SA’). Maximal calcium release was measured by the addition of ionomycin near the end of each experiment (indicated by the arrow labeled ‘iono’). Calcium mobilization was determined by loading the cells with Fura red and Fluo-3, and plotted as a ratio of FL1 (Fluo-3) to FL3 (Fura red). Shown are the kinetics of calcium mobilization in DP thymocytes, CD4+ thymocytes and transitional (CD4loCD8lo) cells as indicated in (a).](https://academic.oup.com/intimm/article-abstract/17/11/1379/813253)
was a modest but reproducible release of $Ca^{2+}$ in this population in Drak2$^{-/-}$ cells, but not in wild-type thymocytes. These results are consistent with the pattern of expression of DRAK2 within distinct thymocyte subsets described above, and suggest that DRAK2 participates in setting the threshold for TCR signaling during thymocyte selection.

Discussion

In this work, we have identified a member of the DAP kinase family that is highly enriched in lymphoid organs. Further, we demonstrated that DRAK2 modulates the sensitivity of SP and transitional thymocytes following selection events that lead to its induction during this stage of differentiation. Although DRAK2 is a distant member of the DAP kinase family, we surmise that its role is not for modulating apoptosis but, rather, for regulating lymphocyte activation. DRAK2 was originally cloned by Akira and colleagues, and, along with DRAK1, was shown to be most closely related to the DAP kinase family (7). A DRAK2 ortholog has also been identified in rats; in this work, the authors also found very high expression levels in the thymus, consistent with our findings (8). Curiously, searches of the EST and non-redundant databases have yielded no obvious murine DRAK1 homolog. However, a DRAK1 isoform has been identified in rabbits (8, 27). DRAK2 orthologs have also been found in other species, including from horse, dog, chicken, gorilla and chicken cDNA libraries, the latter of which was isolated from the bursa of Fabricius. A search of the non-redundant EST database yielded a number of clones from non-mammalian organisms that have high homology with DAPKs, including clones from D. melanogaster and C. elegans. However, such homology was restricted to the kinase domain and was not maintained in the N- or C-terminal domains of DRAK2, suggesting that these are not DRAK2 orthologs. Notably, a large number of ESTs displaying extended homology with DRAK2 have been found in frog, salamander, zebrafish and pufferfish libraries, consistent with the presence of lymphocytes in these distantly related non-mammalian vertebrates.

We have found DRAK2 to be expressed primarily in the nucleus of COS7 and Jurkat cells (Fig. 5), a similar site of localization reported for the human and rat isoforms. Furthermore, we found that DRAK2 was excluded from regions of high Hoechst staining; we believe these data indicate that DRAK2 is prevented access to nucleoli, and thus, it is unlikely that it regulates rDNA transcription. DRAK1 and DRAK2 both contain strong consensus sequences for NLSs present near the kinase active site. DAPK3 and DAPK1 also contain such NLS motifs in this region, though DAPK1 is not normally localized in the nucleus as a result of retention by cytoskeletal proteins outside of this organelle (3, 28). However, this DAPK1 NLS is likely a functional one, since deletion mutants of DAPK1 retaining only the kinase domain do accumulate in the nucleus. DAPK3/ZIP/Dlk is also known to traffic to the nucleus, but appears to be concentrated in pro-myelocytic leukemia nuclear bodies, multiprotein compartments thought to be involved in the suppression of oncogenic transformation (28–30). Other DAPKs exist elsewhere in cells, with DAPK1 associating with the cytoskeleton and DAPK2 expressed primarily as a soluble protein in the cytoplasm (31). The nuclear localization of DRAK2 suggests it may play a role in transcriptional regulation in resting cells, whereas it appears to function in the cytoplasm of activated T cells. Although the potential involvement of the putative canonical NLS has not yet been tested, we demonstrated that the 80-residue C-terminal domain was required for DRAK2 nuclear localization. Thus, DRAK2 may utilize an alternative nuclear localization mechanism via the action of this domain, and this process may be subject to regulation by TCR signaling.

Ectopic expression of DRAK2 in murine 3T3 fibroblasts promoted an apoptotic nuclear morphology and decreased the recovery of colonies stably expressing the transgene (7). However, using transduction with a bicistronic retroviral system to track cells expressing the transgenic mRNA, we failed to detect any evidence of apoptosis or decreased survival in primary T cells or murine 3T3 cells ectopically expressing DRAK2 (Fig. 4). This discrepancy may be due to the reduced levels of expression of the transgene driven by retroviral long terminal repeats when compared with CMV promoter-driven expression reported. However, we and others have found that cell lines such as 293T, HeLa, COS7 and SV40 large T antigen-transformed Jurkat T cells are resistant to death following high-level DRAK2 expression (Fig. 5, also data not shown) (7). It has been speculated that this discrepancy in apoptotic sensitivity is dependent upon the presence of the large T antigen in certain transformed cell lines, though it should be noted that DRAK1/2-insensitive HeLa cells are not transformed with large T antigen (2). Given the high expression of DRAK2 in lymphoid tissues, particularly in non-apoptotic differentiated subsets, we believe that such cells would be, of necessity, similarly resistant to any pro-apoptotic function of DRAK2 due to a requisite induction of inhibitory pathways or via the down-modulation of pro-apoptotic mediators. Differential apoptotic sensitivity during development, or following activation, is certainly a common mechanism for regulating cell fate. However, our attempts to define an apoptotic role for DRAK2 have not revealed any clear requirement for this DAP kinase in promoting physiological apoptosis (Fig. 4 and ref. 1).

DRAK2 expression is developmentally regulated during thymocyte development. Additionally, as demonstrated here, DRAK2 exists as a primary response gene induced by TCR stimulation of DP thymocytes. This pattern of expression is intriguing since it suggests that DP cells are subject to counter-regulation by DRAK2 as a consequence of the TCR-dependent signals received during the process of selection. We found that the TCR-dependent increase in DRAK2 expression was greatly enhanced by co-receptor cross-linking (Fig. 2d). DRAK2 mRNA accumulation did not depend on protein synthesis, suggesting that it is a direct target of transcription factors induced by TCR cross-linking (Fig. 2c). Although we found little effect of blocking calcineurin or MEK/Erk signaling on the induction of DRAK2 mRNA (Fig. 2b and e), inhibition of MEK using PD98059 or U0126 blocked the synthesis of DRAK2, as detected by western blotting (Fig. 3c). Although we are presently unclear about the significance of these findings, these results suggest that both DRAK2 transcription and translation are subject to control by signals emanating from the TCR complex. Additionally, we have found that a non-classical PKC is essential for PMA-induced up-regulation of DRAK2 protein in T cells (Fig. 3d). We favor...
a model in which DRAK2 transcription depends on the activity of a PKC-dependent transcription factor, perhaps via the action of NF-κB. DRAK2 mRNA possesses very large 5’ and 3’ untranslated regions. It may be that these sequences subject DRAK2 mRNA to translational regulatory mechanisms that depend on the action of the Raf→MEK→Erk cascade. Although additional regulatory factors may be involved in the maintenance of DRAK2 expression in quiescent, naïve T cells, our results demonstrate that DRAK2 serves as a negative feedback loop for TCR signaling during DP thymocyte selection.

We have found that DRAK2 raises the threshold for TCR signaling in developing thymocytes. Deficiency of certain negative regulators of TCR signaling, including c-Cbl, SLAP and Csk, results in enhanced selection (32); similarly, we have recently shown that in Drak2−/− mice positive selection of CD4+ T cells was enhanced, as demonstrated by increased numbers of CD4+ thymocytes and increased levels of CD5 and CD69 expression (1). We hypothesize that DRAK2 is involved in regulating the signal strength that controls the fate of developing thymocytes. As its expression dramatically increases in DP thymocytes following TCR cross-linking (Figs 2 and 3), DRAK2 negatively regulates calcium mobilization, a critical signaling pathway for the activation of T cells. Once the TCR is activated, phospholipase Cγ hydrolyzes phospholipids at the cell membrane to produce two crucial second messengers at the cell membrane to produce two crucial second messengers, diacylglycerol and inositol 1,4,5-triphosphate.

Elevated intracellular Ca2+ results in the activation of the messengers, diacylglycerol and inositol 1,4,5-triphosphate. lipids at the cell membrane to produce two crucial second messengers. Calcineurin affected negative selection (38, 39). These results confirm a specific role for DRAK2 in the negative regulation of thymocyte activation. These results confirm a specific role for DRAK2 in the negative regulation of thymocyte activation.

References

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