Modulation of DRAK2 Autophosphorylation by Antigen Receptor Signaling in Primary Lymphocytes*1

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Death-associated protein-related apoptotic kinase-2 (DRAK2), a member of the death-associated protein-like family of serine/threonine kinases, is highly expressed in lymphoid organs and is a negative regulator of T cell activation. To investigate the regulation of DRAK2 activity in primary lymphocytes, we employed mass spectrometry to identify sites of autophosphorylation on DRAK2. These studies have revealed a key site of autophosphorylation on serine 12. Using a phospho-specific antibody to detect Ser12 phosphorylation, we found that autophosphorylation is induced by antigen receptor stimulation in T and B cells. In Jurkat T cells, resting B cells and thymocytes, DRAK2 was hypophosphorylated on Ser12 but rapidly phosphorylated with antigen receptor ligation. This increase in phosphorylation was dependent on intracellular calcium mobilization, because BAPTA-AM blocked DRAK2 kinase activity, whereas the SERCA inhibitor thapsigargin promoted Ser12 phosphorylation. Our results show that DRAK2 kinase activity is regulated in a calcium-dependent manner and that Ser12 phosphorylation is necessary for optimal suppression of T cell activation by this kinase, suggesting a potential feedback loop may act to modulate the activity of this kinase following antigen receptor signaling.

DRAK2, a member of the death-associated protein (DAP) kinase family, has recently emerged as a regulator of immune function. The DAP family of pro-apoptotic serine/threonine kinases consists of five members; all are capable of inducing apoptosis upon overexpression in cell culture (1). Indeed, the prototype DAP kinase (DAPK1) was identified in a screen for positive mediators of apoptosis. Sequence homology between the five family members is restricted to the N-terminal kinase domain, whereas the C-terminal regions are unique and are required for the regulatory functions of each kinase, linking individual family members to specific signal transduction pathways. The fifth member, DRAK2, is expressed primarily in hematopoietic tissues. DRAK2-deficient mice have no defects in apoptosis, and we have shown that retroviral expression of DRAK2 in NIH3T3 cells does not induce overt apoptosis. Rather, recent data have demonstrated an important role for DRAK2 in negative regulation of T cell activation (2, 3). In response to suboptimal T cell antigen receptor (TCR) stimulation, T cells from mice deficient in DRAK2 hyperproliferate, produce higher levels of cytokines, and mobilize greater levels of calcium when compared with wild-type T cells.

DRAK2 is developmentally regulated, with low levels of expression in immature thymocytes and high levels in mature thymocytes, and analysis of DRAK2 knock-out mice has shown that this kinase negatively regulates the T cell activation threshold in developing thymocytes (3). Although double positive (immature) thymocytes from Drak2−/− mice mobilize calcium in a manner similar to thymocytes from wild-type mice, mature, single positive thymocytes from Drak2−/− mice have enhanced calcium responses to suboptimal TCR stimulation. Drak2−/− mice have also been reported to possess B cells with a similarly diminished activation threshold (2). Recent data have also shown that Drak2−/− mice have defective germinal center formation, demonstrating a critical role for DRAK2 in adaptive humoral immunity.5

Like other members of the DAP kinase family, DRAK2 is capable of both auto- and trans-phosphorylation (4). Kinase activity is abolished in a C-terminal truncation mutant and when the critical lysine of the ATP-binding site is mutated (4). The first two members of the DAP kinase family, DAPK1 and DRP-1 kinase, both have autophosphorylation sites at Ser2028 (Refs. 5 and 6, respectively). These autophosphorylation sites exist within their calmodulin regulatory domains, a domain absent from DRAK2. Additionally, six autophosphorylation sites have been defined in ZIP kinase (7), the third member of...
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the DAP kinase family. In many cases, these sites of autophosphorylation have been shown to play key roles in modulating the function of these DAP kinases (8).

Engagement of the TCR or B cell antigen receptor results in rapid recruitment of various signaling molecules to the receptor complex to transmit signals generated at the cell surface. Importantly, a cascade of early intracellular activation events results in calcium mobilization (9). When inositol trisphosphate is generated subsequent to TCR and B cell antigen receptor stimulation, it binds to receptors in the endoplasmic reticulum and opens Ca\(^{2+}\) channels that release Ca\(^{2+}\) into the cytosol (9). Once intracellular stores have been depleted, calcium channels in the plasma membrane (calcium release-activated calcium channel) open, resulting in sustained calcium influx into the cell, facilitating store refilling. Recently, this process has been shown to involve the participation of the endoplasmic reticulum protein Stim1 and the plasma membrane protein Orai1 during T cell activation (10–13).

Because calcium mobilization plays a central role in controlling lymphocyte function, and because DRAK2 modulates thresholds for calcium mobilization in peripheral T cells and post-selection thymocytes, we sought to examine the physiological relevance and the mechanism that regulates DRAK2 function. In an effort to understand the regulation of DRAK2 kinase activity during lymphocyte activation, we have employed mass spectrometry to identify sites of autophosphorylation within the sequence of this kinase. Thus far, two sites have been identified, and one has been analyzed in primary lymphocytes using a phospho-specific Ab. In the present study, we show that DRAK2 autocatalytic activity is induced rapidly following antigen receptor signaling, and that its phosphorylation status is regulated by intracellular calcium mobilization. We further demonstrate that mutation of one of these sites to prevent autophosphorylation diminishes the biological activity of DRAK2 in primary T cells.

#### EXPERIMENTAL PROCEDURES

**Plasmid Constructs** — pGEX-2TK-DRAK2 (GST-DRAK2) was cloned using the pGEX-2TK vector backbone from Amersham Bioscience. Site-specific mutation at serine 12 to alanine was made using the oligonucleotide (5’-GCGAAGCTTGGGGCTTGCTAACT-3’) and to aspartic acid using the oligonucleotide (5’-GCGAAGCTTGGGGCTTGCTAAC-3’). The Ser to Ala mutation at AA348 was made using the oligonucleotide (5’-CCTGAAGATGGCGCTTAGTATTCTATAA-3’). pEGFP-N1 vector was obtained from Clontech. MSCV-IRE-3'-DRAK2 was cloned as described (3).

**Recombinant Protein Purification** — GST-tagged recombinant protein was purified from bacterial lysate using GSH-agarose beads (Sigma). Isopropyl 1-thio-β-D-galactopyranoside-induced BL21 cells were lysed by sonication in phosphate-buffered saline followed by incubation in phosphate-buffered saline, 1% Tween, 1% Triton X-100. The lysate was subsequently spun for 30 min at 11,000 rpm and subjected to overnight binding with GSH-agarose beads. The beads were then washed with phosphate-buffered saline. Recombinant GST-DRAK2 was eluted by incubating the beads for 20 min in 10 mM reduced glutathione (Sigma) diluted in 50 mM Tris-HCl, pH 8.

**In Vitro Kinase Assays** — Eluted GST-DRAK2 was incubated at 30 °C with kinase reaction buffer (10 mM Tris-HCl, pH 7.5, 10 mM MgCl\(_2\), 3 mM MnCl\(_2\), 20 mM ATP, 2 mM dithiothreitol, and 5 μCi of \(\gamma\)-\(^{32}\)P]ATP (Amersham Biosciences). Some reactions included 5 μg of rabbit myosin light chain (Sigma) as a trans-phosphorylation substrate. Kinase reactions were terminated by the addition of Laemmli sample buffer. After boiling, the samples were separated by SDS-PAGE and visualized by autoradiography.

For analysis of DRAK2 kinase activity in mammalian cells, HEK293T cells were transiently transfected with EGFP-tagged DRAK2 (wt or mutants) by the calcium phosphate method. At 24 h post-transfection, cells were lysed in high salt buffer (250 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 20 mM Hepes, 1% Triton X-100) containing 300 μg/ml phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM sodium vanadate. EGFP-DRAK2 was isolated from pre-cleared whole cell extracts using anti-GFP polyclonal antibody (BD Bioscience) for immunoprecipitation in IP buffer 3 (30 mM Tris-HCl, pH 6.8, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) in the presence of Protein G-agarose beads (Amersham Biosciences). Following extensive washing, immunoprecipitates were subjected to in vitro kinase assays as above, in some cases with non-radioactive ATP added for subsequent mass spectrometric characterization.

**Liquid Chromatography-Tandem Mass Spectrometry** — Proteins were separated by one-dimensional SDS-PAGE and visualized with Coomassie Blue staining. The selected bands were cut, reduced, alkylated, and digested by trypsin (Promega Corp, Madison, WI), and the resulting tryptic digests were subjected to LC-MS/MS analysis as described (14). Briefly, the experiments were carried out by nanoflow reversed-phase liquid chromatography (RPLC, Ultimate LC Packings, Dionex) coupled on-line to a quadrupole orthogonal time-of-flight tandem mass spectrometer (QSTAR XL, Applied Biosystems/MDS Sciex). The QSTAR MS was operated in an information-dependent mode in which each full MS scan was followed by three MS/MS scans where the three most abundant peptide molecular ions were dynamically selected for collision-induced dissociation that generates tandem mass spectra.

**Data Base Searching** — The acquired MS/MS spectra were subsequently submitted for database searching and protein identification using the development version of Protein Prospector (14). The mass accuracy for parent ions and fragment ions were set as ±100 ppm and 300 ppm, respectively. The cysteine was set as modified by iodoacetamide and phosphorylation of serine, threonine, and tyrosine was chosen as default modification during Batchtag searching. The Search Compare program within the developmental version of Protein Prospector (15) was used to summarize, validate, and compare the results from different experiment samples. The identified phosphorylated peptides were further confirmed by manual inspection of the MS/MS spectra. Protein sequence alignments were performed using ClustalW from the European Bioinformatics Institute (available at www.ebi.ac.uk/clustalw). Searches to determine the computed molecular weight and pI ranges of
each phosphorylated isoform were conducted using ScanSite (scansite.mit.edu) using an algorithm developed by Hochstrasser and colleagues (16, 17).

**Antibody Production**—The anti-phospho-Ser12 polyclonal antibodies were produced by immunizing rabbits with a key-hole limpet hemocyanin-coupled synthetic peptide corresponding to residues surrounding Ser12 of mouse DRAK2. The antibodies were purified by protein A and peptide affinity chromatography.

**Western Blotting and Enzyme-linked Immunosorbent Assays**—Western blotting was performed as described previously (3). Briefly, protein lysates were resolved by SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore). Primary antibody incubation was performed overnight at 4 °C in Tris-buffered saline/Tween 20 (TBST) containing 5% bovine serum albumin. Secondary antibody incubation was performed for 1 h at room temperature in TBST plus 5% bovine serum albumin. IL-2 enzyme-linked immunosorbent assays were conducted as described previously (18).

**Calf Intestinal Phosphatase Treatment and Peptide Blocking**—Splenocytes from C57BL/6 mice were treated with 100 nm Calyculin A (Cell Signaling Technologies) or vehicle only for 30 min. Cells were lysed in 150 mM low salt lysis buffer (100 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 20 mM HEPES, 1% Triton X-100) followed by incubation with 10–40 units/ml calf intestinal phosphatase (Fisher Scientific) and 1× phosphatase buffer for 30 min at 37 °C. To assess the specificity of the phospho-Ser12 Ab, Western blots were produced using lysates from 293T cells transfected with EGFP-DRAK2 or EGFP only. 1 µg of anti-phospho-Ser12 was incubated with 5, 10, or 30 µg of either non-phosphorylated or phosphorylated Ser12 peptide for 18 h prior to probing Western blots. Western blots were stripped and reprobed with anti-DRAK2 to verify equivalent loading.

**Mouse—Dra2−/−** (Stk17b−/−) mice have been described previously (2). Dra2−/− and C57BL/6 mice were bred and maintained in accordance with the regulation of the Institutional Animal Care and Use Committee at the University of California, Irvine. Mice were used between 8 and 12 weeks of age.

**Lymphocyte Purification, Stimulation, and Lysis—**Jurkat T cells were stimulated with anti-CD3 (clone C305, Upstate Biotechnology, Lake Placid, NY) for the indicated times followed by lysis and Western blotting. Thymocytes were harvested from 8- to 10-week-old C57BL/6 mice and stimulated with biotinated anti-CD3 (1 µg/ml) plus anti-CD4 (1 µg/ml), followed by cross-linking with streptavidin (1 µg/ml) for the indicated times. Immature and mature B cells were purified from total splenocytes using MACS CD43-conjugated beads to deplete non-B cells (Miltenyi Biotec, Auburn, CA). Typical purity was between 95 and 98%. Stimulation was performed using 20 µg/ml soluble anti-IgM F(ab)2 (Jackson ImmunoResearch) for the times indicated. Cells were lysed in radioimmunoprecipitation assay buffer with protease and phosphatase inhibitors.

**Analysis of Calcium Signaling—**Purified lymphocytes were stimulated as described above in the presence or absence of 4 mM EGTA (Fisher Scientific), 40 µM BAPTA-AM (Calbiochem), 1 mM thapsigargin (Calbiochem), 1 µM ionomycin, and/or 50 ng/ml phorbol 12-myristate 13-acetate (Sigma). For experiments with inhibitors or calcium chelators, stimulation was performed following a 20-min pretreatment.

**Retroviral Transduction and Calcium Mobilization Assays**—MiT, MiT-DRAK2, -S12A, and -S12D constructs were transfected into 293T cells, and retroviral supernatants were collected as described (3). Purified T cells from Dra2−/− mice were stimulated for 24 h using plate-bound anti-CD3 (200 ng/ml), soluble anti-CD28 (200 ng/ml), and human recombinant IL-2 (100 units/ml). Plates were spun at 24 and 48 h following primary stimulation using retroviral supernatants in the presence of 4 µg/ml Polybrene (Specialty Media). Cells were rested in RPMI containing 10 ng/ml each IL-7 and IL-15 (eBioscience). After a 2-day rest, calcium mobilization assays were performed essentially as described (3), except that cells were pre-bound with anti-CD3 (0.3 µg/ml) and cross-linked with 40 µg/ml anti-Hamster IgG (Vector Labs).

**RESULTS**

**Identification of in Vitro Autophosphorylation Sites**—With an interest toward investigating the regulation of DRAK2 kinase activity following antigen receptor stimulation, we sought to determine its sites of autophosphorylation. Our rationale was that such information would aid in the development of reagents to interrogate the phosphorylation status of DRAK2 in primary lymphocytes. Because the known autophosphorylation sites of DAPK1 and DRP-1 are not conserved in the DRAK2 sequence, we employed mass spectrometry to evaluate unique sites of autophosphorylation in DRAK2. We first demonstrated catalytic activity for recombinant DRAK2 by *in vitro* kinase assays. Recombinant GST-tagged DRAK2 kinase preparations (wild-type, the C-terminal truncation consisting of amino acid residues 1–290, and the kinase-inactive mutant DRAK2-K62A) were purified from BL21(DE3)pLysS E. coli using GSH-agarose beads and eluted using reduced glutathione. Catalytic activity was verified using eluted samples subjected to *in vitro* kinase assays in the presence of [γ32P]ATP (Fig. 1A). Although the wild-type kinase was highly active, this activity depended upon the presence of the C-terminal regulatory domain (291–372) and the ATP-binding lysine 62 in the ATP-binding loop of the kinase domain. These results concur with previously published data using Myc-tagged protein purified from transiently transfected COS7 cells (4, 19).

To determine the sites of autophosphorylation using a mass spectrometric approach, GST-tagged wild-type and kinase-inactive DRAK2 were affinity-purified and subjected to *in vitro* kinase assays using non-radioactive ATP. The protein products were separated by one-dimensional SDS-PAGE (Fig. 1A), followed by in-gel digestion and subsequent liquid chromatography LC-MS/MS analysis. The identified phosphorylated peptides are summarized in Table 1. Nine phosphorylated peptides were identified in the GST-DRAK2 protein, of which two phosphorylation sites were located in the regions of the GST fusion protein (Ser196) and the linker (Ser226), whereas the other seven sites were present in the DRAK2 protein. An example of the mass spectrometry result is shown in Fig. 1B. A doubly charged ion (MH+2 870.39) from the tryptic digest of GST-wild-type DRAK2 was detected and was determined as a phosphorylated...
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A

DRAK2wt  DRAK2(1-290)  K62A

Autorad: Phospho-DRAK2
Coomassie

B

EDKENIPEDGbpLVEK

C

SVSLTLTTTPQTPIK

D

SVpSLTLTTTPQTPIK

E

pSVSLTLTTTPQTPIK

Ion Counts

m/z
form of the peptide (338EDKENIPEDGSLVSK352) based on the fragment ions obtained in the MS/MS spectrum (Fig. 1B). A series of y ions (y3 ~ y12) was obtained, among which the y3 ~ y12 ions were identified as phosphorylated (i.e. 80 Da higher than the non-phosphorylated mass), and y1 ~ y4 ions were observed in a non-phosphorylated state. In addition, a loss of 98 Da (due to the loss of H3PO4) was observed for y5 ~ y12 ions, the characteristic loss of phosphorylated serine or threonine during collision-induced dissociation analysis. These results clearly demonstrate that the phosphorylation occurred on Ser at position 5 from the C terminus of this peptide, which corresponds to Ser348 in the full-length DRAK2 sequence.

In addition, we have observed two different, singly phosphorylated forms of the same peptide. The non-phosphorylated peptide was detected as MH2$^+$ 771.92, whereas the two phosphorylated peptides (p-peptides) were observed with the same nominal masses as MH2$^+$ 811.92. Their MS/MS spectra are illustrated in Fig. 1 (C–E, respectively). As shown in Fig. 1C, a series of y ions (y1', y3', y6', and y6' ~ y12') and b ions (b2 ~ b6') were obtained in the MS/MS spectrum of the non-phosphorylated peptide (MH2$^+$ 771.92), which determined the peptide sequence as 10SVSGLTTTPQTPK24. This unambiguously matches the N terminus of the DRAK2 protein. Comparing the three MS/MS spectra in Fig. 1 (C–E), the y ions (y1', y3', y6', and y6' ~ y12') were the same as the non-phosphorylated form, whereas b ions in these three spectra were different, suggesting that the Thr residues before position 11 from C terminus were not phosphorylated, and phosphorylation most likely occurred close to the N terminus. In Fig. 1D, except for the b1 ion, b2 and b4 ions were 80 Da higher than their corresponding non-phosphorylated b ions shown in Fig. 1C, indicating that these ions were phosphorylated. The detection of a series of b$i^+$ ions (i.e. b$i^+$ ~ b$n^+$) due to the neutral loss (~98 Da) of their phosphorylated b ions determined the phosphorylation of Ser at position 3 from N terminus. The unique internal ion (338.14, pSGL) and its neutral loss ion (240.15, pSGL$^*$) further confirmed the identification. In Fig. 1E, all of the observed b ions (b2 ~ b7) were 80 Da higher than the corresponding b ions in Fig. 1C. The b2 ion in Fig. 1E was 80 Da higher than the b1 in Fig. 1 (C and D), suggesting that phosphorylation occurred before Ser at position 3 from N terminus. In addition, the detection of b$i^+$ ions (i.e. b$i^+$ ~ b$n^+$), and the non-phosphorylated internal ion (258.17, SGL) in Fig. 1E established that the Ser at the N terminus, instead of the Ser at position 3 from N terminus, was phosphorylated. Therefore, the phosphorylation of two different Ser (i.e. Ser10 and Ser12 in the full sequence of DRAK2) in the same peptide was identified.

As summarized in Table 1, a total of seven phosphorylation sites were found in the full-length sequence of DRAK2: i.e. Ser10, Ser12, Ser232, Ser333, Ser348, Ser351, and Ser362. To confirm these results, LC-MS/MS analysis of a kinase-inactive mutant (K62A) was carried out after an in vitro kinase reaction and in-gel digestion. Data base searches of the MS/MS spectra did not reveal any phosphorylated peptides. To further verify that there was no phosphorylation in the K62A mutant digest after the in vitro assay, we attempted to extract both non-phosphorylated and the corresponding p-peptide ions from the entire LC-MS run and compare the extracted ion chromatogram (XIC) trace of each particular ion in both wild-type and mutant samples. If no phosphorylation occurred in the mutant sample, the non-phosphorylated peptides would be detected, but not the p-peptides. We focused our analysis on Ser10 and Ser12 to discern potential phosphorylation in the mutant sample. The XIC traces of the non-phosphorylated DRAK2 peptide (MH2$^+$ 771.92) were not observed in the MS/MS spectra of the non-phosphorylated DRAK2 peptide (MH2$^+$ 771.92).

**TABLE 1** Summary of autophosphorylation sites detected in recombinant wild-type and mutant forms of GST-DRAK2

Peptide sequences are shown at the left for peptides derived from GST and murine DRAK2; numbers represent the amino acid residues within the start methionine in each sequence. Columns under wild-type (DRAK2 WT) and mutant forms represent the detection of phosphorylation within the listed peptides for each GST fusion. For each peptide, the phosphorylated serine is designated by "pS" within the sequence listed.

| Peptides identified by MS/MS | DRAK2 WT | K62A | DeltaC | S12A | S348A |
|-----------------------------|---------|------|--------|------|-------|
| 102YLYKspsk197               | +       | -    | -      | -    | -     |
| 2115DSLVPRGpsRRR228          | +       | -    | -      | -    | -     |
| b4 ions identified in DRAK2 sequence |         |      |        |      |  
| 10pVSGLTTTPQTPK24            | +       | -    | -      | +    | +     |
| 10pVSGLTTTPQTPK24 NA         | +       | -    | -      | NA   | +     |
| 281RTAECSLHSSWLQGWDFGLHFPEETSspsSSLQDTRL199 | +       | -    | -      | NA   | -     |
| 325TShpSSCNGSCGAR137         | +       | -    | -      | NA   | -     |
| 325TShpSSCNGSCGAR137 NA      | +       | -    | -      | NA   | -     |
| 325E49KpSSCNGSCGAR135       | +       | -    | -      | NA   | -     |
| 325E49KpSSCNGSCGAR135 NA     | +       | -    | -      | NA   | -     |
| 354FRFDSSLPspHELVPDFLC572    | +       | -    | -      | NA   | -     |

\* NA, not applicable for this clone.

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**FIGURE 1. Determination of sites of GST-DRAK2 autophosphorylation.** A, in vitro kinase reactions of GST-tagged wild-type DRAK2, a C-terminal truncation mutant (retaining residues 1–290), and an ATP binding site mutant (K62A). Recombinant proteins were affinity-purified from E. coli lysates and subjected to in vitro kinase assays as described under “Experimental Procedures.” The reaction products were subjected to SDS-PAGE, followed by Coomassie staining and autoradiography. B, electrospay ionization-MS/MS spectrum of a doubly charged phosphopeptide (MH2$^+$ 870.39) obtained from the tryptic digest of wild-type GST-DRAK2. The peptide sequence was determined as 338EDKENIPEDGSLVSK352, where Ser348 was phosphorylated. b$i^*$, b$n^*$, b1~H2O. C–E, electrospray ionization MS/MS spectra of three related tryptic peptides from GST-wt DRAK2 digests. C, the non-phosphorylated peptide with MH2$^+$ 771.92. The sequence was determined as 10SVSGLTTTPQTPK24. D, a phosphorylated form of the peptide in C with MH2$^+$ 811.92. The sequence was determined as 10pVSGLTTTPQTPK24, where Ser10 was phosphorylated. E, another phosphorylated form of the peptide in C with MH2$^+$ 811.92. The sequence was determined as 10pVSGLTTTPQTPK24, where Ser10 was phosphorylated. P5 represents the phosphorylated serine. (MH2$^+$ 870.39) M+2H2O; y$i^*$ +2; y$i^*$ +3; y$i^*$ +4; y$i^*$ +5; y$i^*$ +6; b1~H2O; b$i^*$, b$n^*$, b$i^*$, b$n^*$, b$i^*$, b$n^*$; pSGL-; pSGL-H2O; b$i^*$, b$n^*$, b$i^*$, b$n^*$, b$i^*$, b$n^*$, b$i^*$, b$n^*$.
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771.92, 19SVGLTTTPQTPIK24) were first generated from the LC MS runs of both samples to determine its presence (supplemental Fig. S1, A and B). Due to the presence of another peptide with the same m/z that eluted slightly earlier, there were two peaks present in the XIC traces. Previous MS/MS analysis shown in Fig. 1 identified that the peptide with m/z at 771.92 eluted at 46.5 min was the non-phosphorylated peptide, which is present in both samples. To determine whether Ser10 or Ser12 were phosphorylated in the mutant, we then compared the XIC traces of the p-peptides (MH2+ 811.92) from both samples (supplemental Fig. S1, C and D). Although these two p-peptides have the same nominal masses, they actually eluted at different times during LC separation. These eluted after the non-phosphorylated peptide and were well separated chromatographically, giving two distinct elution peaks (supplemental Fig. S1C). Based on the MS/MS analyses (Fig. 1, D and E), peak I represents the Ser10 p-peptide and peak II represents the non-phosphorylated peptide and were well separated chromatographically, giving two distinct elution peaks (supplemental Fig. S1C). Based on the MS/MS analyses (Fig. 1, D and E), peak I represents the Ser10 p-peptide and peak II represents the non-phosphorylated peptide (supplemental Fig. S1, E and F). Although the non-phosphorylated peptide was obtained from the GST-K62A mutant, no visible corresponding phosphorylated peptides were detected in either the XIC or its time-of-flight MS traces (supplemental Fig. S1, D and G). Therefore, we conclude that neither Ser10 nor Ser12 were phosphorylated in the K62A mutant. In addition, no other phosphorylation was detected in this mutant after in vitro phosphorylation, indicating that this mutant indeed completely lacks kinase activity. Similarly, no phosphorylation was found in the c-terminal truncation (Delta-C) mutant (data not shown).

To investigate the potential function of two of the identified phosphorylation sites, mutagenesis of Ser to Ala was performed at Ser12 and Ser348. The single mutants were further expressed, purified, and assayed as described above. Autophosphorylation was observed in the two mutants and mass spectrometric analysis was used to identify additional phosphorylation sites. Four phosphorylation sites were identified for the S12A mutant, including Ser10, Ser348, Ser351, and Ser362 (Table 1). For the S348A mutant, a new phosphorylation site at Ser310 was observed in addition to the phosphorylation of Ser10, Ser12, Ser348, and Ser351. However, phosphorylation of Ser328 and Ser333 was not observed in either mutant. The basis for decreased phosphorylation at these two residues in the Ser → Ala mutants is currently unclear. While autophosphorylation is often necessary for full protein kinase activity, mutation of Ser12 or Ser348 to alanine failed to block autophosphorylation kinase activity (Fig. 2A). Further, mutation of both Ser12 and Ser348 in combination also failed to diminish DRAK2 autophosphorylation (Fig. 2B), likely due to phosphorylation at other sites in the kinase. Further, alanine substitutions at these putative autophosphorylation sites did not diminish the ability of DRAK2 to phosphorylate myosin light chain. Taken together, these results demonstrate phosphorylation of Ser12 and Ser348 is dispensable for DRAK2 auto- and trans-catalytic activity.

Identification of in Vivo Phosphorylation Sites—To evaluate in vivo phosphorylation of DRAK2, constructs containing EGFP fused to wild-type DRAK2 or the K62A mutant were overexpressed in 293T cells, followed by immunopurification using a monoclonal anti-GFP antibody. Although we detected autophosphorylation of wild-type DRAK2 using in vitro kinase reactions with [γ-32P]ATP, the K62A mutant was almost completely catalytically inactive (Fig. 3A), consistent with previously reported results (4, 19). The purified EGFP-DRAK2 and EGFP-K62A mutant proteins were then subjected to in vitro kinase reactions using non-radioabeled ATP, and the products were separated by one-dimensional SDS-PAGE. Following excision and in-gel tryptic digestion, both Ser10 and Ser12 phosphorylation were identified in wild-type DRAK2 by tandem mass spectrometry. To determine whether phosphorylation occurred in the K62A mutant, we extracted the XIC traces of the p-peptides. Two elution peaks representing pSer10 (peak I) or pSer12 (peak II) peptides were observed in the XIC traces of MH2+ 811.92 from both the K62A mutant and wild-type DRAK2 digests (Fig. 3B). However, the intensities of the two peaks were about four times higher in EGFP-wild-type DRAK2 than those in EGFP-K62A mutant, suggesting that DRAK2 can phosphorylate itself on these two sites.

Given that a small amount of phosphorylation on Ser10 and Ser12 was observed for the K62A mutant in 293T cells that lack endogenous expression of DRAK2, it is possible that one or more other kinases may also phosphorylate DRAK2 on the two sites in vivo. To test this hypothesis, transfected cells were treated with the phosphatase inhibitor calyculin A for 30 min prior to lysis to block potential in vivo dephosphorylation. Treatment with calyculin A resulted in a profound mobility shift for both wild-type and K62A mutant DRAK2, suggesting that other kinases present within 293T cells are capable of phosphorylating DRAK2 in vivo (Fig. 3C). To determine if Ser10 and Ser12 may be subject to trans-phosphorylation by other kinases in 293T cells, these DRAK2 proteins were immunoprecipitated with anti-GFP, and directly analyzed without further
in vitro phosphorylation. XIC traces of Ser\textsuperscript{10} and Ser\textsuperscript{12} p-peptides demonstrated that both p-peptides were observed in the two samples following calyculin A treatment; however the intensities of the two p-peptide peaks were only ~14% higher for wild-type DRAK2 as compared with those of the K62A mutant (Fig. 3D). This result further suggests that one or more other kinases may phosphorylate these two residues in DRAK2 in 293T cells. Of note, we failed to detect Ser\textsuperscript{10} or Ser\textsuperscript{12} p-peptides above background noise in untreated 293T cells transfected with the K62A mutant, demonstrating that our detection of in vitro DRAK2 phosphorylation in the absence of calyculin A depends on the autocatalytic activity of the kinase. With the exception of the Ser\textsuperscript{10} and Ser\textsuperscript{12} p-peptides, no other phosphorylation events were detected in wild-type DRAK2 or in the K62A mutant. It is possible that the other phosphorylation events may be too rare to be detected, or that the non-physiologically high concentration of recombinant GST-DRAK2 described in Fig. 1 may have led to spurious phosphorylation during in vitro kinase reactions as described above.

Phylogenetic Comparison of DRAK2 Sequences to Confirm Sites of Autophosphorylation—To narrow down the possible sites of bona fide autophosphorylation, we examined the conservation of the identified serines by alignment of known DRAK2 sequences across various species. Our rationale was that any physiologically significant autophosphorylation site should be conserved among species. Indeed, all three sites identified by MS are conserved in zebrafish, chicken, rat, mouse, dog, chimp, and human DRAK2 orthologs (Fig. 4A). None of these sites are located within the kinase domain, but rather, are present within an ~30-amino acid N-terminal domain and in the ~80-amino acid C-terminal regulatory domain of the kinase (Fig. 4B). Although the identified sites are not known to be phosphorylated in other DAP family kinases with defined autophosphorylation sites (DAPK1, ZIPK, and DRP-1), Ser\textsuperscript{12} on DRAK2 shows sequence conservation with known DAP kinase substrates. There are two known endogenous substrates of DAPK1 identified by yeast two-hybrid interaction studies: Syntaxin-1A, and CaMKK\textbeta, at Ser\textsuperscript{188} and CamKK\textbeta, at Ser\textsuperscript{511} (21). Additionally, ZIP kinase, the second member of the DAP family of S/T kinases, was found to be phosphorylated by DAPK1 in vitro and in vivo upon coexpression in 293T cells (22). Analysis of the residues surrounding the DAP kinase phosphorylation sites of all three substrates revealed a conserved sequence preceding Ser\textsuperscript{12} on DRAK2 (Fig. 4C). The Ser\textsuperscript{12} residue and the phosphorylation sites of CaMKK\textbeta, ZIP kinase, and Syntaxin-1A are each preceded by a serine and one small hydrophobic residue (Leu or Ile for CaMKK\textbeta and Syntaxin1A) or a basic residue (His for ZIP kinase). This comparison lends credence to our belief that
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autophosphorylation at Ser<sup>12</sup> is likely physiologically significant. Although this is far from an exhaustive mapping of the substrate specificity, these results suggest that DRAK2 has at least an overlapping preference for other DAP kinase phosphorylation motifs.

Development of a Phospho-Ser<sup>12</sup>-specific DRAK2 Antibody—Following identification of the autophosphorylation sites described above, phospho-specific Abs to Ser<sup>12</sup> and Ser<sup>348</sup> were produced using synthetic phospho-peptides corresponding to residues surrounding these mapped phosphorylation sites. To verify that both putative phospho-specific Abs were specific for DRAK2, we analyzed lysates from the BaF3 B cell line and splenocytes of DRAK2<sup>+/−</sup> and DRAK2<sup>−/−</sup> mice by Western blotting (Fig. 5A). Both the phospho-Ser<sup>12</sup> and phospho-Ser<sup>348</sup> Abs detected DRAK2 in wild-type splenocytes and BaF3 cells but failed to detect these bands in DRAK2<sup>−/−</sup> splenocytes, demonstrating that both Abs are highly specific for DRAK2. Treatment of wild-type splenocytes and BaF3 cells with calyculin A induced a dramatic mobility shift as observed in Fig. 3 (5A). This enhanced phosphorylation also occurred on the Ser<sup>12</sup> site, because the signal relative to the unstimulated sample increased with this treatment. To verify the phospho-specificity of these Abs, 293T cells were transfected with EGFP-DRAK2, EGFP-S12A, or EGFP-S348A. Immunoblots were probed with anti-phospho-Ser<sup>12</sup>, anti-phospho-Ser<sup>348</sup>, or anti-DRAK2 antibodies. Figs. 3C and 5A, phospho-Ser<sup>348</sup> antibody did not appear to be phospho-specific, because it was capable of binding to the S348A mutant DRAK2. As an alternative approach to demonstrate the phospho-specificity of the anti-phospho-Ser<sup>12</sup> Ab, we performed a peptide blocking experiment using dilutions of non-phospho- or phospho-peptides used to generate the Ab (Fig. 5C). Preincubation of the anti-phospho-Ser<sup>12</sup> Ab with phospho-peptide prior to probing immunoblots containing EGFP-DRAK2 transfected

FIGURE 4. DRAK2 autophosphorylation sites are conserved across species. A, protein sequence alignment of various species expressing DRAK2 using ClustalW. Arrows indicate three sites of autophosphorylation identified by mass spectrometry. B, schematic representation of sites of autophosphorylation within DRAK2 structural domains. C, protein sequence alignment of DRAK2 and three known DAP kinase substrates (CaMKII, ZIPK, and Syntaxin-1A). The arrow indicates Ser<sup>12</sup> of DRAK2, which is flanked by residues conserved in other DAP kinase substrates. Shaded residues are conserved among all these substrates.

FIGURE 5. Development and characterization of phospho-specific anti-DRAK2 Abs. A, determination of DRAK2 specificity of phospho-Ser<sup>12</sup> and phospho-Ser<sup>348</sup> Abs. Phospho-specific Abs were developed using phospho-peptides derived from the sequences surrounding Ser<sup>12</sup> or Ser<sup>348</sup> as described under “Experimental Procedures.” Unfractionated splenocytes are from C57BL/6 or Drak2<sup>−/−</sup> mice, or BaF3 cells were left untreated or treated for 30 min with 10 or 100 nM Calyculin A, or phorbol 12-myristate 13-acetate (50 ng/ml) plus ionomycin (2 μM). Lysates were analyzed by immunoblot analysis using anti-phospho-Ser<sup>12</sup>, anti-phospho-Ser<sup>348</sup>, anti-DRAK2, or anti-ERK1/2 Abs. B, phospho-Ser<sup>12</sup> Ab is specific for DRAK2 phosphorylated on Ser<sup>12</sup>. 293T cells were transfected with EGF-P-DRAK2, EGFP-DRAK2-S12A, or EGFP-DRAK2-S348A. Lysates were subjected to Western blotting using anti-phospho-Ser<sup>12</sup>, anti-phospho-Ser<sup>348</sup>, or anti-DRAK2 antibodies. C, phospho-peptide blocking reveals that the anti-phospho-Ser<sup>12</sup> Ab is highly specific for DRAK2 phosphorylated on Ser<sup>12</sup>. 293T cells were transfected with EGF-P-DRAK2 or EGF-P empty vector (Ctrl), followed by immunoblotting. Prior to probing, 100 ng of anti-phospho-Ser<sup>12</sup> Ab was preincubated with a 5-, 10-, or 30-fold excess (by weight) of phosphorylated or non-phosphorylated peptides containing sequences surrounding Ser<sup>12</sup>. Following this blocking, anti-phospho-Ser<sup>12</sup> Abs were then added to immunoblots and visualized using ECL. Blots were stripped and reprobed with anti-DRAK2 to demonstrate equivalent loading. D, in vitro phosphatase treatment of primary T cell lysates demonstrates phospho-Ser<sup>12</sup> phospho-specificity. Splenocytes from a C57BL/6 mouse were treated with Calyculin A (100 nm) for 30 min, followed by lysis and incubation with increasing amounts of calf intestinal phosphatase (10–40 units/ml), and subsequent immunoblot analysis with anti-pSer<sup>12</sup> and anti-DRAK2.
293T cell lysates completely blocked detection of EGFP-DRAK2 by the phosho-Ser12 Ab at all peptide dilutions. However, except when incubated at a 30-fold excess of peptide to Ab (by weight), the non-phosphorylated peptide failed to block detection of EGFP-DRAK2 by the phosho-Ser12 Ab. As a final validation of the phospho-specificity of this Ab, we treated calyculin-treated splenocyte lysates with increasing doses of calf intestinal phosphatase to determine if such treatment would reduce or eliminate the detection by the phosho-Ser12 Ab. Increasing amounts of calf intestinal phosphatase decreased the amount of phosho-DRAK2 detected by Western blotting, further verifying antibody specificity of the anti-phosho-Ser12 antibody (Fig. 5D). It should be recognized here that the phosho-Ser12 Ab clearly detects both the lower and higher molecular weight bands in this blot (and above in Fig. 5A) following calyculin treatment. It is notable that phosphatase treatment reduced detection of both bands by the phosho-Ser12 Ab but only the higher molecular weight band by the total anti-DRAK2 Ab. This suggests that, although calyculin treatment promotes Ser12 phosphorylation in splenocytes, it also likely leads to phosphorylation at a number of other sites. As described below, such hyperphosphorylation has not been observed to occur during the activation of lymphocytes.

Analysis of DRAK2 Phosphorylation Status in Lymphocytes—Because DRAK2 has been found to modulate T and B cell activation in response to antigenic stimulation, we wished to determine if DRAK2 autophosphorylation is altered by antigen receptor stimulation. First, we evaluated its phosphorylation status in Jurkat T cells following TCR cross-linking. Unstimulated Jurkat cells possessed a low level of autophosphorylated DRAK2, whereas treatment with soluble anti-CD3 (an IgM isotype) greatly enhanced DRAK2 Ser12 autophosphorylation (Fig. 6A). As a control for T cell activation, the blot was stripped and reprobed with the monoclonal Ab 4G10 to detect tyrosine-phosphorylated species. Although we found that unstimulated primary splenic T cells had a high level of basal DRAK2 phosphorylation, thymic T cells possessed near undetectable basal phosphorylation (Fig. 6B). However, stimulation of these thymocytes with cross-linking anti-CD3 plus anti-CD4 led to induction of DRAK2 phosphorylation. Similarly, these blots were reprobed with anti-phosphotyrosine to evaluate TCR proximal signaling. Although the kinetics of DRAK2 phosphorylation in Jurkat cells and mouse thymocytes appears distinct, we caution that the activation conditions in these experiments are quite different. Despite potential differences in the magnitude and kinetics of activation in different T cells, these data demonstrate that DRAK2 phosphorylation on Ser12 is induced by TCR stimulation.

Because DRAK2 is also highly expressed in B cells (2, 3), we wished to evaluate its phosphorylation status during B cell activation. Following 2-min B cell antigen receptor cross-linking with anti-IgM, DRAK2 was phosphorylated at this site, with highest levels observed after 15 min of stimulation (Fig. 6C). After 4 h, DRAK2 autophosphorylation returned to levels observed in resting B cells. In contrast, ERK1/2 phosphorylation was maximal after 2 min of stimulation. These results demonstrate that DRAK2 is rapidly activated in B cells following antigen receptor cross-linking, although this process of activation occurs more slowly than for the ERK mitogen-activated protein kinase pathway.

Mobilization of Intracellular Calcium Is Necessary for DRAK2 Autophosphorylation in Lymphocytes—DRAK2 catalytic activity has been shown to be regulated by Ca2+ via the calcineurin B homolog calcineurin B-homologous protein (23). Because it is known that DRAK2 regulates calcium mobilization in peripheral T cells (2) and thymocytes (3), we sought to understand how autophosphorylation might be affected by the high intracellular calcium levels that persist following lymphocyte activation. First, we wished to evaluate the consequence of acute Ca2+ mobilization following treatment with thapsigargin, a potent inhibitor of sarcoplasmic-endoplasmic reticulum calcium ATPase pumps (24). Although purified splenic T cells
had a high level of basal phospho-DRAK2, treatment with thapsigargin enhanced DRAK2 phosphorylation (Fig. 7A). This treatment also enhanced ERK phosphorylation, consistent with previous results demonstrating that release of stored Ca\(^{2+}\) promotes mitogen-activated protein kinase activation (25). In purified splenic B cells, thapsigargin similarly led to high level DRAK2 phosphorylation on Ser\(^{12}\) (Fig. 7B). In both cell types, the effect of thapsigargin was dependent on mobilization of Ca\(^{2+}\), because the intracellular Ca\(^{2+}\) chelator BAPTA-AM blocked the induction of DRAK2 and ERK1/2 phosphorylation. To determine if depletion of intracellular Ca\(^{2+}\) stores was necessary for DRAK2 phosphorylation induced by antigen receptor signaling, purified B cells were stimulated with anti-IgM in the absence or presence of the calcium chelators BAPTA-AM (40 \mu M) and/or EGTA (4 mM). In all cases, lysates were subjected to serial immunoblotting with anti-phospho-Ser\(^{12}\), anti-DRAK2, anti-phospho-ERK, and anti-ERK1/2.

**DISCUSSION**

Although a role for DRAK2 in negatively regulating suboptimal signals that follow TCR ligation has been established, the mechanisms that control DRAK2 function remain to be elucidated. Using mass spectrometry, we have identified three sites of in vivo phosphorylation, two of which clearly depend on the intrinsic kinase activity of DRAK2. We have used a phospho-specific antibody to analyze autophosphorylation in vivo, and our data show that DRAK2 autophosphorylation is dependent on release of Ca\(^{2+}\) following antigen receptor ligation in lymphocytes. Finally, we have demonstrated that phosphorylation at Ser\(^{12}\) has important functional consequences, because a mutation at this site reduced the ability of DRAK2 to interfere with Ca\(^{2+}\) mobilization following TCR stimulation. Because our data suggest that DRAK2 is activated by intracellular Ca\(^{2+}\) mobilization, and because DRAK2 limits Ca\(^{2+}\) release in T cells and thymocytes, our data promote a model in which this kinase acts as a governor to limit TCR signal transduction.
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In resting Jurkat T cells, primary thymocytes and splenic B cells, DRAK2 is phosphorylated at low levels (Fig. 5). Treatment of these lymphocytes with antigen receptor cross-linking Abs led to rapid phosphorylation on Ser\textsuperscript{12}, as detected by the anti-phospho-Ser\textsuperscript{12} Ab. Curiously, multiple experiments using purified splenic T cells demonstrated a high level of basal Ser\textsuperscript{12} phosphorylation (Fig. 6A). At present, it is unclear if this difference is due to an experimental artifact, or if it may indicate a distinct mode of activation in resting mature T cells freshly isolated from secondary lymphoid tissue. Regardless of this caveat, it is clear that DRAK2 Ser\textsuperscript{12} phosphorylation is modulated in some fashion by antigenic stimulation of lymphocytes.

To better understand the regulation of DRAK2 function, we found that calcium release from intracellular stores was sufficient for inducing phosphorylation at Ser\textsuperscript{12} in primary B and T cells. Treatment of purified lymphocytes with thapsigargin, which selectively inhibits sarcoplasmic-endoplasmic reticulum calcium ATPase calcium pumps in the endoplasmic reticulum and results in leakage of calcium into the cytosol, led to an increase in Ser\textsuperscript{12} phosphorylation, whereas BAPTA-AM prevented this increase. Ser\textsuperscript{12} phosphorylation following treatment with thapsigargin increased in the presence of calcium-free media via the divalent cation chelator EGTA (data not shown). Thus, we conclude that, although extracellular calcium mobilization does not alter DRAK2 phosphorylation, intracellular calcium is both necessary and sufficient for inducing maximal DRAK2 autophosphorylation on Ser\textsuperscript{12} in primary lymphocytes. In vitro studies with recombinant DRAK2 have demonstrated that its only known interaction partner calcineurin B-homologous protein blocks kinase activity in response to increasing Ca\textsuperscript{2+} concentrations, a key factor in reg-
ulating calcineurin activity in T cells (23, 28, 29). Based on our analysis using anti-phospho-Ser\(^{12}\), we conclude that Ca\(^{2+}\) acts to positively regulate DRAK2 autophosphorylation in vivo. How calcineurin B-homologous protein might negatively regulate DRAK2 activity in response to Ca\(^{2+}\) mobilization, and whether this regulation is physiologically significant in lymphocytes, remains to be determined.

While DRAK2 is subject to phosphorylation on at least two distinct sites, phosphorylation of Ser\(^{12}\) has important functional consequences. The S12A mutation diminished the ability of DRAK2 to limit Ca\(^{2+}\) mobilization following TCR cross-linking (Fig. 7B). This suggests that this site may be partially responsible for the ability of DRAK2 to restrain Ca\(^{2+}\) mobilization following T cell activation. These apparent defects in DRAK2 function were not due to diminished kinase activity, because the S12A mutant retained catalytic activity as assessed by in vitro kinase assays (Fig. 2). We speculate that phosphorylation at Ser\(^{10}\), and perhaps at other sites, may be necessary for DRAK2 to be fully active in restricting lymphocyte Ca\(^{2+}\) mobilization following antigen receptor cross-linking. Because Ser\(^{12}\) mutation did not interfere with kinase activity, it is likely that this site serves to assist in docking of potential substrates to the kinase or may alternatively be involved in its subcellular localization. A more thorough understanding of the significance of DRAK2 autophosphorylation at Ser\(^{12}\) awaits discovery of its physiologically relevant substrates in lymphoid cells.

Like other DAP kinase family members, DRAK2 is clearly subject to autophosphorylation, and, based on data provided here, this autophosphorylation is regulated in lymphocytes. As with other kinases in this family, we have demonstrated that such autophosphorylation modulates the biological function of DRAK2. Although we have found differences in the magnitude of phosphorylation in B cells versus T cells, it will be of interest to determine the level of DRAK2 activity in distinct B and T cell subsets. Further, it is possible that other immune stimuli may alter the activity of DRAK2, leading to important immunological consequences. Because DRAK2 plays an important role in restricting T cell activation, the evaluation of its regulation in distinct primary lymphocyte populations should yield additional insight into how it participates in modulating immune tolerance.

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