The EMBO Journal (2004) 23, 1112–1122 | © 2004 European Molecular Biology Organization | All Rights Reserved 0261-4189/04
www.embojournal.org

THE EMBO JOURNAL

SPAK kinase is a substrate and target of PKCθ in T-cell receptor-induced AP-1 activation pathway

Yingqiu Li, Junru Hu, Randi Vita[1], Binggang Sun, Hiroki Tabata and Amnon Altman*

Division of Cell Biology, La Jolla Institute for Allergy and Immunology, San Diego, CA, USA

Protein kinase C-θ (PKCθ) plays an important role in T-cell activation via stimulation of AP-1 and NF-κB. Here we report the isolation of SPAK, a Ste20-related upstream mitogen-activated protein kinase (MAPK), as a PKCθ-interacting kinase. SPAK interacted with PKCθ (but not with PKCα) via its 99 COOH-terminal residues. TCR/CD28 costimulation enhanced this association and stimulated the catalytic activity of SPAK. Recombinant SPAK was phosphorylated on Ser-311 in its kinase domain by PKCθ, but not by PKCα. The magnitude and duration of TCR/CD28-induced endogenous SPAK activation were markedly impaired in PKCθ-deficient T cells. Transfected SPAK synergized with constitutively active PKCθ to activate AP-1, but not NF-κB. This synergistic activity, as well as the receptor-induced SPAK activation, required the PKCθ-interacting region of SPAK, and Ser-311 mutation greatly reduced these activities of SPAK. Conversely, a SPAK-specific RNAi or a dominant-negative SPAK mutant inhibited PKCθ- and TCR/CD28-induced AP-1, but not NF-κB, activation. These results define SPAK as a substrate and target of PKCθ in a TCR/CD28-induced signaling pathway leading selectively to AP-1 (but not NF-κB) activation.
The EMBO Journal (2004) **23,** 1112–1122. doi:10.1038/sj.emboj.7600125; Published online 26 February 2004
Subject Categories: signal transduction; immunology
Keywords: AP-1; PKCθ; signaling; SPAK; T cell

Introduction

Protein kinase C-θ (PKCθ), a member of the novel, Ca^{2+}-independent PKC subfamily (nPKC), plays an important role in mature T-cell activation (Altman *et al*, 2000; Arendt *et al*, 2002; Isakov and Altman, 2002), as evidenced by the failure of peripheral T cells from PKCθ-deficient T cells to proliferate and produce interleukin-2 (IL-2) upon stimulation with anti-CD3 plus anti-CD28 antibodies (Sun *et al*, 2000; Pfeifhofer *et al*, 2003). These defects have been attributed to deficient activation of two transcription factors that are essential for induction of the *IL-2* gene promoter, that is, AP-1 and NF-κB, consistent with the demonstrated important and selective role of PKCθ in the activation of these transcription factors, as well as the CD28 response element (RE), in Jurkat T cells (Baier-Bitterlich *et al*, 1996; Coudronniere *et al*, 2000; Khoshnan *et al*, 2000; Lin *et al*, 2000; Pfeifhofer *et al*, 2003). More recent evidence has also pointed out a role for PKCθ in the activation of NFAT (Pfeifhofer *et al*, 2003).

While the signaling pathways leading from PKCθ to NF-κB activation have been studied in some detail (Coudronniere *et al*, 2000; Khoshnan *et al*, 2000; Lin *et al*, 2000; Sun *et al*, 2000; Wang *et al*, 2002), little is known about the PKCθ signaling pathway(s) leading to AP-1 activation, a transcription factor complex whose regulation is exerted both at the transcriptional and translational levels via multiple, complex mechanisms (Karin *et al*, 1997). This complexity is illustrated by the finding that mature T cells from PKCθ$^{-/-}$ mice display impaired AP-1 activation but intact JNK activation (Sun *et al*, 2000), thereby implying an alternative, JNK-independent pathway for AP-1 activation by PKCθ.

Using a yeast two-hybrid screen of a T-cell cDNA library, we isolated a PKCθ-interacting upstream mitogen-activated protein kinase (MAPK), originally termed Ste20/SPS1-related proline- and alanine-rich kinase (SPAK or PASK) (Ushiro *et al*, 1998; Johnston *et al*, 2000). Here, we characterize SPAK and demonstrate that it selectively interacts with, and is phosphorylated by, PKCθ, and is involved in PKCθ-mediated activation of AP-1 but not NF-κB. The biological relevance of SPAK is further demonstrated by our findings that activation of this kinase by CD3/CD28 costimulation is impaired in T cells from PKCθ-deficient mice, and that a SPAK-specific RNAi inhibited PKCθ-mediated AP-1, but not NF-κB, activation.

Results

SPAK associates with PKCθ

To identify PKCθ-interacting proteins, we performed a yeast two-hybrid screen of a cDNA library constructed from Jurkat T cells, using a kinase-inactive mutant of PKCθ (PKCθ-K/R) fused to the LexA DNA-binding domain (pBD–PKCθ-K/R) as bait. Of 2×10^7 transformants, 70 formed colonies, which were positive for both *LEU2* and *LacZ* reporter genes. We ultimately identified and characterized one clone, C51, which interacted strongly with PKCθ (Figure 1A and B). Sequencing of this 297-nucleotide cDNA fragment, termed SPAK-2h, revealed that it encodes a sequence identical to the 99 COOH-terminal amino acids of human SPAK/PASK, a Ste20-related Ser/Thr kinase, which was originally isolated from rat brain (Ushiro *et al*, 1998) and a transformed rat pancreatic β cell line or human brain (Johnston *et al*, 2000). Based on the known nucleotide sequence of human SPAK (GenBank accession number AF099989), 5′ and 3′ primers were designed and used in an RT–PCR to obtain the full-length cDNA of human SPAK from a Jurkat T-cell cDNA library. Transfection of 293T cells with a wild-type PKCθ plasmid in the absence or presence of an epitope-tagged SPAK expression vector,

*Corresponding author. Division of Cell Biology, La Jolla Institute for Allergy and Immunology, 10355 Science Center Drive, San Diego, CA 92121, USA. Tel.: +1 858 558 3527; Fax: +1 858 558 3526; E-mail: amnon@liai.org
[1]Current address: Androclus Therapeutics, San Diego, CA 92121, USA

Received: 14 July 2003; accepted: 19 January 2004; published online: 26 February 2004

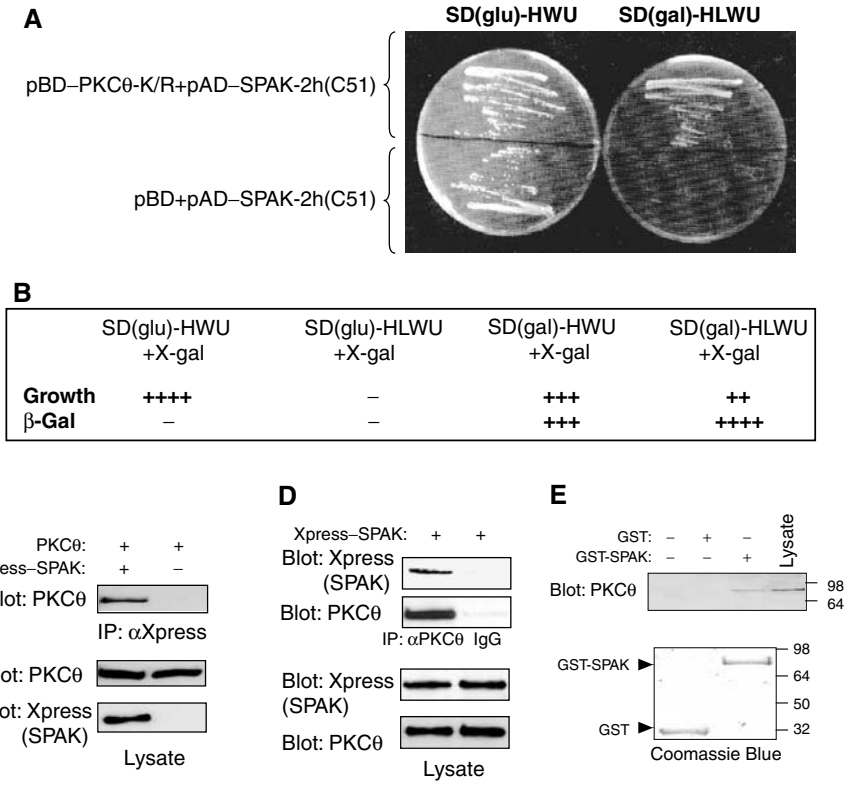

Figure 1 Interaction of PKCθ with SPAK. (**A, B**) EGY48(p8op–lacZ) yeast cells were cotransformed and assayed for growth and β-Gal activity following X-gal induction as described in Materials and methods. (**C**) SPAK was immunoprecipitated with an anti-Xpress mAb from 293T cells cotransfected with pEF–PKCθ plus pEF–Xpress–SPAK or empty vector. Immunoprecipitates (upper panel) or whole-cell lysates (two lower panels) were subjected to immunoblotting with anti-PKCθ or -Xpress antibodies. (**D**) Anti-PKCθ antibody or normal IgG was used to immunoprecipitate proteins from Jurkat-TAg cells transfected with Xpress–SPAK. The immunoprecipitates or lysates were probed with the indicated antibodies. (**E**) Jurkat T-cell lysates were incubated with empty glutathione–sepharose beads, GST-conjugated beads or GST–SPAK-conjugated beads, followed by SDS–PAGE analysis of bead-bound proteins and anti-PKCθ immunoblotting (upper panel). The lower panel shows the input of GST and GST–SPAK proteins determined by Coomassie blue staining.

followed by immunoprecipitation, confirmed the interaction between SPAK and PKCθ in mammalian cells, and demonstrated that either anti-Xpress (Figure 1C) or anti-PKCθ (Figure 1D) antibodies co-immunoprecipitated the reciprocal protein. Using a recombinant glutathione *S*-transferase (GST)–SPAK fusion protein in a pull-down assay, we further demonstrated that the SPAK fusion protein, but not the control GST protein, bound endogenous PKCθ from lysates of unstimulated Jurkat cells (Figure 1E).

Selective interaction of SPAK with PKCθ

In order to determine whether the interaction of SPAK with PKCθ is selective, we examined whether SPAK can associate with PKCα, a member of the conventional, Ca^{2+}-dependent PKC subfamily (cPKC). Transfected SPAK co-immunoprecipitated with PKCθ (Figure 2A), but not with PKCα (Figure 2B), despite the fact that both kinases were abundantly expressed. Immunoblotting with an anti-epitope antibody confirmed the proper and similar expression of the transfected SPAK in the respective groups.

Next, we analyzed the endogenous interaction between SPAK and the same two PKC isotypes and the effects of activating agents on this association. We generated rabbit anti-SPAK polyclonal antibodies, which recognized an ~70 kDa protein (not recognized by the corresponding pre-immune sera) expressed in Jurkat T cells, as well as transfected SPAK isolated by immunoprecipitation with an anti-epitope tag antibody (Supplementary Figure 1A and B). These antibodies were also capable of immunoprecipitating SPAK, and reacted with a protein of the expected size present in the thymus, lymph nodes, spleen, peripheral blood lymphocytes (PBL) and, at much lower levels, the in bone marrow (Supplementary Figure 1C and D, respectively).

Endogenous SPAK co-immunoprecipitated with PKCθ from unstimulated Jurkat T cells (Figure 2C, upper panel). Anti-CD3 stimulation for 5–10 min increased this association, which returned to the basal level after 30 min. Anti-CD3/CD28 costimulation caused a more pronounced and sustained enhancement of this association, which reached a maximum after 30 min. Phorbol myristate acetate (PMA) stimulation also enhanced the association at 5 min, with a return to the basal level at 10 min. This rapid decline may be due to the PMA-induced degradation of PKCθ. In contrast, no association of SPAK with endogenous cPKCs (α, β or γ) was detected (Figure 2C, second panel from top). Control immunoblots demonstrated that PKCθ, cPKC or SPAK were expressed at abundant and similar levels in the different lysates (three lower panels). Endogenous PKCθ could also be co-immunoprecipitated with SPAK from primary T cells (Figure 2D). The association was slightly augmented by anti-CD3 stimulation, and more so by CD3/CD28 costimulation, with peak increase observed after 5 min of stimulation. The

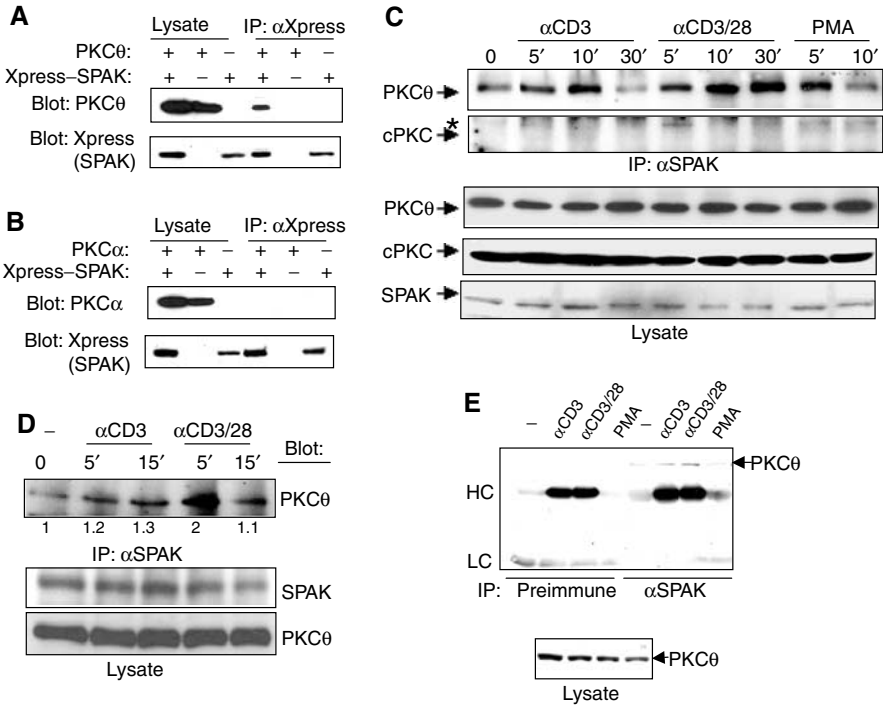

Figure 2 Specific interaction of SPAK with PKCθ and its enhancement by CD3/CD28 costimulation. (**A, B**) Proteins immunoprecipitated with anti-Xpress antibody or cell lysates prepared from 293T cells transfected with the indicated combinations of wild-type PKCθ (A) or PKCα (B) with or without Xpress-tagged SPAK, were subjected to SDS–PAGE and immunoblotted with anti-PKC antibodies (upper panels), followed by reprobing with an anti-Xpress antibody (lower panels). (**C**) Endogenous SPAK was immunoprecipitated from unstimulated or stimulated Jurkat E6.1 cells. Immunoprecipitates (two upper panels) or lysates (three lower panels) were immunoblotted with anti-PKC or -SPAK antibodies. Arrows indicate the position of PKC or SPAK, and the asterisk corresponds to a nonspecific band recognized by the anti-cPKC antibody. (**D**) SPAK immunoprecipitates (8×10^5 cell equivalents; upper panel) or cell lysates (1×10^6 cell equivalents; two lower panels) from unstimulated or stimulated primary C57BL/6 mice were immunoblotted with the indicated antibodies. The numbers under the upper panel represent the fold increase in PKCθ–SPAK association compared to unstimulated cells ($=1$) as determined by densitometry. (**E**) Jurkat E6.1 cell stimulated as indicated for 10 (anti-CD3/CD28) or 5 (PMA) min were immunoprecipitated with preimmune serum or anti-SPAK serum, and immunoprecipitates (upper panel) or lysates (lower panel) were subjected to anti-PKCθ immunoblotting. The heavy chain (HC) and light chain (LC) of the immunoprecipitating antibody are indicated.

finding that only the anti-SPAK serum, but not the preimmune serum, co-immunoprecipitated PKCθ (Figure 2E) validates the specificity of the co-immunoprecipitation.

The 99 COOH-terminal residues of SPAK interact with PKCθ

In order to map the region(s) of SPAK, which mediates the interaction with PKCθ, we generated different GST fusion constructs of mutated or truncated SPAK (Figure 3A) and used them in pull-down assays with Jurkat T-cell lysates (Figure 3B). Similar levels of the recombinant SPAK fusion proteins were used for each pull-down assay (Figure 3B, lower panel). Full-length wild-type or kinase-inactive mutated (K/E) SPAK bound endogenous PKCθ (Figure 3B, upper panel). Thus, the kinase activity of SPAK is not essential for this interaction, a result confirmed in intact cells by co-immunoprecipitation analysis of transfected cells (data not shown). Similarly, deletion of the PAPA box, that is, the proline/alanine-rich N-terminal region (SPAKΔPA) or the first 347-amino-acid residues including the catalytic domain (SPAK-R), did not have a significant effect on the level of PKCθ associated with SPAK. In contrast, deletion of 487 (SPAK-PA), 200 (SPAKΔR) or 99 (SPAKΔ2h) COOH-terminal residues abolished the interaction, indicating that the 99 terminal residues of SPAK are critical for interaction with PKCθ. Indeed, a fusion protein consisting of the 99 COOH-terminal residues of SPAK (SPAK-2h; corresponding to the cDNA fragment isolated in the initial yeast two-hybrid screen) bound PKCθ even more strongly than full-length SPAK or the other positive SPAK fusion proteins. Interestingly, the isolated kinase domain of SPAK (SPAK-K), but not the same domain with the additional 61 NH_2-terminal residues of SPAK (SPAKΔR), also bound PKCθ weakly.

SPAK is a specific substrate of PKCθ

To further address the relationship between SPAK and PKCθ, we examined whether SPAK is a substrate of PKCθ by subjecting GST fusion proteins of wild type or kinase-inactive (K/E mutant) of SPAK to an *in vitro* kinase assay with purified PKC enzymes. Surprisingly, PKCθ, but not PKCα, phosphorylated SPAK *in vitro* (Figure 4A). This difference did not reflect poor or absent activity of the PKCα preparation, as both PKC isotypes phosphorylated myelin basic protein (MBP) equally well (Figure 4B). The apparently stronger phosphorylation of wild-type SPAK as compared to SPAK-K/E most likely reflects the endogenous autophosphorylating activity of SPAK, which was documented previously (Johnston *et al*, 2000). Consistent with the ability of PKCθ to phosphorylate SPAK *in vitro*, a cotransfected constitutively active PKCθ mutant

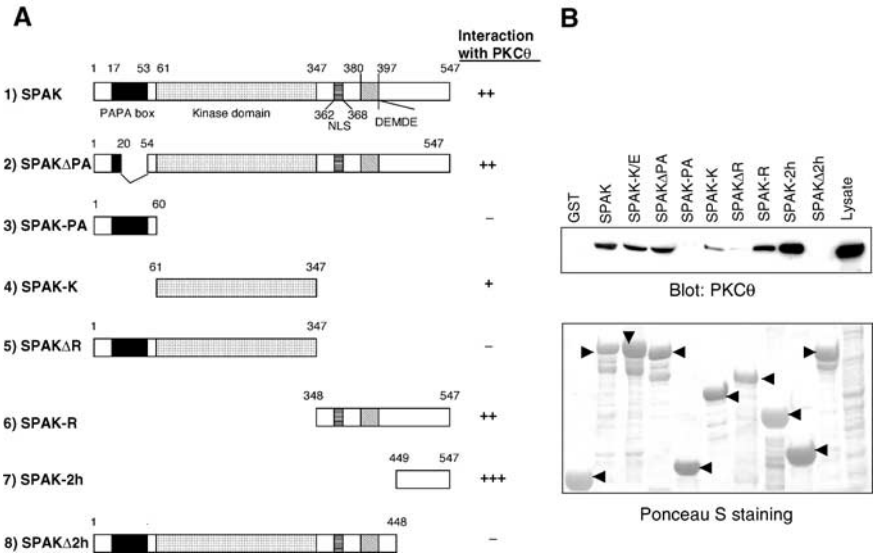

Figure 3 Mapping of the PKCθ-interacting domain of SPAK. (**A**) Schematic representation of the SPAK constructs expressed as GST fusion proteins. The proline/alanine-rich region (PAPA box), kinase domain, potential nuclear localization signal (NLS) and caspase cleavage site (DEMDE) are indicated. SPAK-2h corresponds to the cDNA insert from C51 identified in the yeast two-hybrid screen (Figure 1A). The numbers above the bars correspond to the amino-acid positions at the boundaries of each region. (**B**) Jurkat E6.1 cell lysates were incubated with the indicated GST or GST–SPAK proteins immobilized on glutathione–sepharose beads. Bead-bound proteins were analyzed as described in Materials and methods (upper panel). The lower panel shows the input of GST or GST–SPAK proteins (indicated by arrowheads) determined by Ponceau S staining.

(PKCθ-A/E) enhanced the phosphorylation of SPAK in transfected, $^{32}P_i$-labeled 293T cells (Figure 4C).

In order to map the region of SPAK, which is phosphorylated by PKCθ, we subjected the SPAK fusion proteins described above to similar *in vitro* kinase assays with recombinant PKCθ (Figure 4D). PKCθ strongly phosphorylated full-length SPAK as well as its ΔPA, Δ2h and kinase domain constructs. A much weaker phosphorylation of the C-terminal fragment (R) was also observed, but the NH_2-terminal PA or COOH-terminal 2h fragments were not phosphorylated. These findings indicate that PKCθ phosphorylates SPAK *in vitro* predominantly in its catalytic domain, and perhaps very weakly in the region included within residues 348–448.

Sequence analysis of SPAK using the ScanProsite program (http://us.expasy.org/tools) revealed five potential consensus PKC phosphorylation sites, that is, serine (S) residues 311 and 325 in its catalytic domain, and S residues 407 and 463 plus threonine (T) residue 520 in its COOH-terminal putative regulatory domain. We used the kinase-inactive (K/E) mutant of SPAK as a template to generate alanine replacement point mutations of each of these residues. Consistent with the poor phosphorylation of the COOH-terminal fragment (SPAK-R; Figure 4D), mutation of the three potential phosphorylation sites in this region (S407A, S463A and T520A) did not reduce the phosphorylation of SPAK by purified PKCθ (Figure 4E and data not shown). The S325A mutation reduced phosphorylation by 30% whereas the S311 mutation reduced it by 90%, a reduction similar to that observed with the double mutant (S2A), in which both S311 and S325 were mutated. These results identify S311 as the major *in vitro* phosphorylation site by PKCθ, possibly with a lesser contribution by S325.

TCR/CD28 costimulation activates SPAK

As CD28 costimulation enhances the TCR-induced membrane translocation and activation of PKCθ in T cells (Coudronniere *et al*, 2000; Villalba *et al*, 2000; Bi *et al*, 2001), we wished to determine whether CD28 costimulation would have a similar effect on SPAK. First, we used an *in vitro* kinase assay to analyze the activity of transfected SPAK from Jurkat-TAg cells. As these cells do not express CD28, they were additionally cotransfected with a CD28 expression vector in order to determine the effect of CD28 costimulation. The basal activity of SPAK isolated from unstimulated cells was barely detectable, but anti-CD3 stimulation caused an increase in the activity of SPAK, which peaked at 10 min and declined to a low level by 30 min (Figure 5A, upper panel). Anti-CD3/CD28 costimulation caused a markedly higher activation of SPAK at each time point. PMA stimulation also induced marked activation of SPAK at 5 min. All immunoprecipitates contained similar levels of transfected SPAK as determined by anti-Xpress immunoblotting (lower panel).

We also assessed the effect of CD3/CD28 costimulation on endogenous SPAK in Jurkat E6.1 cells (which express CD28). Similar to transfected SPAK, this costimulation activated SPAK with a similar time course (Figure 5B). This time course paralleled that of PKCθ activation under similar stimulation conditions (Figure 5C), with peak PKCθ activation slightly preceding that of SPAK. The possibility that co-immunoprecipitated PKCθ contributed significantly to the total *in vitro* kinase activity measured in this (Figure 5) and other (see below) experiments was ruled out by demonstrating that a specific pan-PKC inhibitor, which completely blocked the autophosphorylation of PKCθ, had only a small inhibitory effect (~30%) on the *in vitro* autophosphorylating activity of immunoprecipitated SPAK (Supplementary Figure 2).

Costimulation-dependent activation of SPAK requires its PKCθ-interacting domain and S311

The above results demonstrate that the 99 COOH-terminal residues of SPAK associate with PKCθ (Figure 3B) and, furthermore, that S311 represents a major PKCθ phosphoryla-

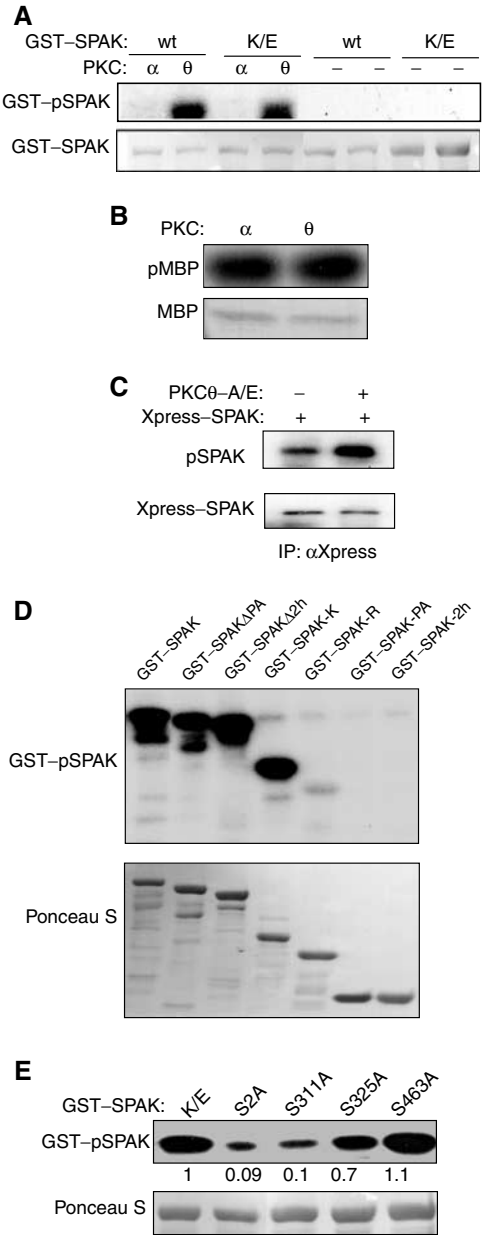

Figure 4 Selective SPAK phosphorylation by PKCθ. (**A**) Recombinant wild-type (wt) or kinase-inactive (K/E) SPAK fusion proteins were used as substrates in *in vitro* kinase assays in the absence (−) or presence of recombinant PKCθ or PKCα enzymes. Phospho-SPAK (pSPAK) was detected by autoradiography (upper panel), and Ponceau S staining of the membrane shows the SPAK protein band (lower panel). (**B**) To normalize the activity of recombinant PKCθ or PKCα, a parallel kinase reaction was performed using MBP as a substrate. Phospho-MBP (pMBP) determined by autoradiography (upper panel) or the input MBP protein revealed by Ponceau S staining (lower panel) is shown. (**C**) 293T cells transfected with Xpress–SPAK in the absence or presence of constitutively active PKCθ were labeled with $^{32}P_i$ and SPAK was immunoprecipitated with an anti-Xpress antibody. Immunoprecipitates were analyzed by SDS–PAGE and autoradiography (pSPAK; upper panel) or by anti-Xpress immunoblotting (lower panel). (**D**) PKCθ *in vitro* kinase assays were conducted using the indicated recombinant SPAK proteins as substrates, and analyzed as in (A). (**E**) GST fusion proteins of kinase-inactive SPAK (K/E) or SPAK-K/E with the indicated serine-to-alanine point mutations were used as substrates in PKCθ kinase reactions, and analyzed as in (D). The numbers refer to the phosphorylation level of each substrate relative to the phosphorylation of SPAK-K/E (=1).

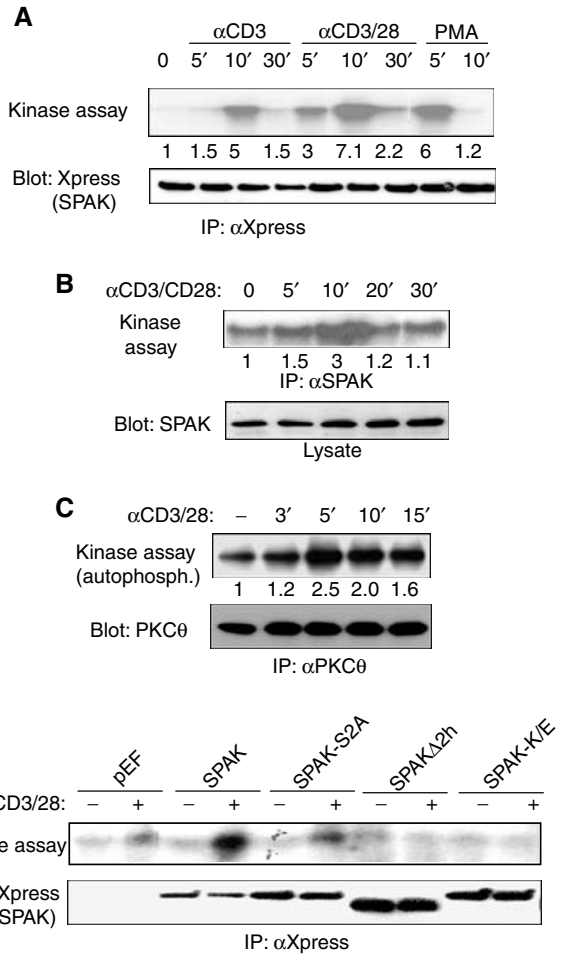

Figure 5 Activation of SPAK by TCR/CD28 costimulation. (**A**) Transfected SPAK was immunoprecipitated from unstimulated (0′) or stimulated Jurkat-TAg cells cotransfected with a CD28 plasmid, and subjected to an *in vitro* kinase assay (upper panel). The membrane was reprobed with anti-Xpress antibody (lower panel). (**B**) Endogenous SPAK was immunoprecipitated from unstimulated or stimulated Jurkat E6.1 cells, and subjected to an *in vitro* kinase reaction (upper panel) as in (A). Lysates from the same cells were immunoblotted with an anti-SPAK antibody (lower panel). (**C**) Endogenous PKCθ was immunoprecipitated from unstimulated or stimulated Jurkat E6.1 cells, and subjected to an *in vitro* autophosphorylation reaction (upper panel) or anti-PKCθ immunoblotting (lower panel). (**D**) Jurkat-TAg cells were cotransfected with a CD28 plasmid plus empty vector (pEF) or the indicated pEF–SPAK constructs. After 20 h, the cells were left unstimulated (−) or stimulated (+) with crosslinked anti-CD3 plus anti-CD28 antibodies for 10 min. SPAK kinase activity (upper panel) or transfected SPAK expression (lower panel) was determined as in (A).

tion site in SPAK (Figure 4E). In order to determine the biological significance of these two events, we assessed the ability of anti-CD3/CD28 costimulation to activate a SPAK mutant lacking the PKCθ-interacting region (SPAKΔ2h), or the doubly mutated (S2A) SPAK. As positive or negative controls, CD3/CD28 costimulation activated wild-type SPAK, but failed to activate a kinase-inactive (K/E) SPAK mutant, respectively (Figure 5D, upper panel). The S2A mutant was activated to a significantly lower degree than wild-type SPAK, indicating that PKCθ-mediated transphosphorylation of SPAK on S311 (and S325?) is required for maximal receptor-mediated activation of SPAK. In contrast, the SPAKΔ2h

mutant was not activated at all by CD3/CD28 costimulation, indicating that association of SPAK with PKCθ is critical for its CD3/CD28-induced activation.

Impaired SPAK activation in PKCθ$^{-/-}$ T cells

In order to establish the biological significance of the interaction between SPAK and PKCθ, we compared the activity of endogenous SPAK in wild-type versus PKCθ$^{-/-}$ T cells. CD3/CD28 costimulation of wild-type T cells induced activation of SPAK, which was first observed at 10 min, reached a maximum at 30 min and remained strongly elevated after 50 min (Figure 6A, upper panel). In contrast, PKCθ-deficient T cells displayed the first increase in SPAK activity after 10–30 min, and this activity declined to a nearly basal level by 50 min. Integration of the phosphorylation signals over the stimulation time course by densitometry revealed that the overall inducible phosphorylation of SPAK in PKCθ-deficient T cells represented only $26 \pm 2\%$ of the corresponding activity in wild-type T cells ($n = 2$). As noted earlier, the peak of PKCθ autophosphorylation slightly preceded that of SPAK activation (second and third panels from top). As a negative control, the activation of Akt was not reduced in the same cells (Figure 6A, two lower panels). Similarly, the PMA-induced activation of SPAK was also impaired in PKCθ-deficient T cells (Figure 6B). Thus, the *PKCθ* mutation impairs the activation of SPAK both in terms of its magnitude and duration. These results indicate that, although the TCR/CD28-mediated activation of SPAK does not absolutely depend on PKCθ, PKCθ is nevertheless required for maximal and sustained activation of SPAK, indicating that the physical and functional interaction between PKCθ and SPAK is biologically relevant.

SPAK and its S311 are involved in PKCθ- and receptor-induced AP-1, but not NF-κB, activation

The transcription factors NF-κB and AP-1 represent two major targets of PKCθ in T cells (Baier-Bitterlich *et al*, 1996; Coudronniere *et al*, 2000; Lin *et al*, 2000; Sun *et al*, 2000; Isakov and Altman, 2002; Pfeifhofer *et al*, 2003). Therefore, we wished to determine whether SPAK participates in PKCθ-dependent signaling pathways leading to activation of these transcription factors. We used an RNAi-based approach to inhibit SPAK expression in T cells and found that one SPAK-specific RNAi (RNAi-2) and, to a lesser extent, another RNAi (RNAi-1), but not the corresponding empty vector, substantially reduced the mRNA and protein expression of SPAK in transfected Jurkat T cells (Figure 7A). In parallel, RNAi-2 inhibited by ~70% the activation of a cotransfected AP-1 reporter gene induced by a constitutively active PKCθ mutant (Figure 7B, upper panel), but had no effect on an NF-κB reporter gene (Figure 7B, lower panel). These results indicate that SPAK functions downstream of PKCθ in a pathway leading to AP-1, but not NF-κB, activation in T cells. This specificity was supported by experiments measuring the effects of a dominant-negative, kinase-inactive SPAK mutant on CD3/CD28-costimulated AP-1 versus NF-κB activation (Supplementary Figure 3), or on the ability of SPAK to synergize with a limiting amount of a cotransfected PKCθ-A/E mutant to activate AP-1, but not NF-κB, reporter gene (Supplementary Figure 4).

To further elaborate this apparent specificity in the effects of SPAK and evaluate the role of its S311/S325 residues in AP-1 activation, we compared wild-type SPAK with its kinase-inactive mutant, the S2A mutant, or the interaction-deficient mutant (Δ2h) with regard to their ability to synergize with active PKCθ in AP-1 activation. PKCθ-A/E synergized with wild-type SPAK to activate AP-1 and, conversely, the kinase-inactive mutant (K/E) functioned in a dominant-negative manner to inhibit the PKCθ-A/E-induced AP-1 activation (Figure 7C). Interestingly, the S2A mutant, which displayed a marked reduction in its phosphorylation (Figure 4E) or CD3/CD28-costimulated activation (Figure 5D), did not significantly enhance reporter activation by PKCθ-A/E, further

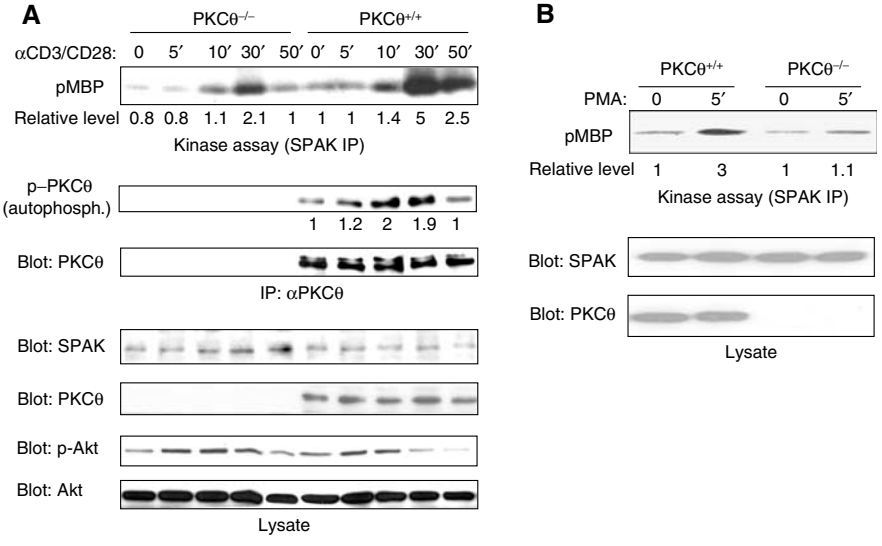

Figure 6 Deficient SPAK activation in PKCθ$^{-/-}$ T cells. Primary T cells purified from wild-type or PKCθ$^{-/-}$ mice were stimulated for the times as indicated. Endogenous SPAK was immunoprecipitated and assayed for kinase activity as in Figure 5. Lysates from the same cells were immunoblotted with anti-SPAK or -PKCθ antibodies. The numbers under the panels represent the fold increase in SPAK activity compared to unstimulated cells (= 1) as determined by densitometry. In (**A**), the time course of PKCθ *in vitro* autophosphorylation (second panel from top) or its expression (third panel from top), as well as the activation and expression of Akt in the same cells (two lower panels), were also determined. (**B**) This experiment is representative of another, similar experiment.

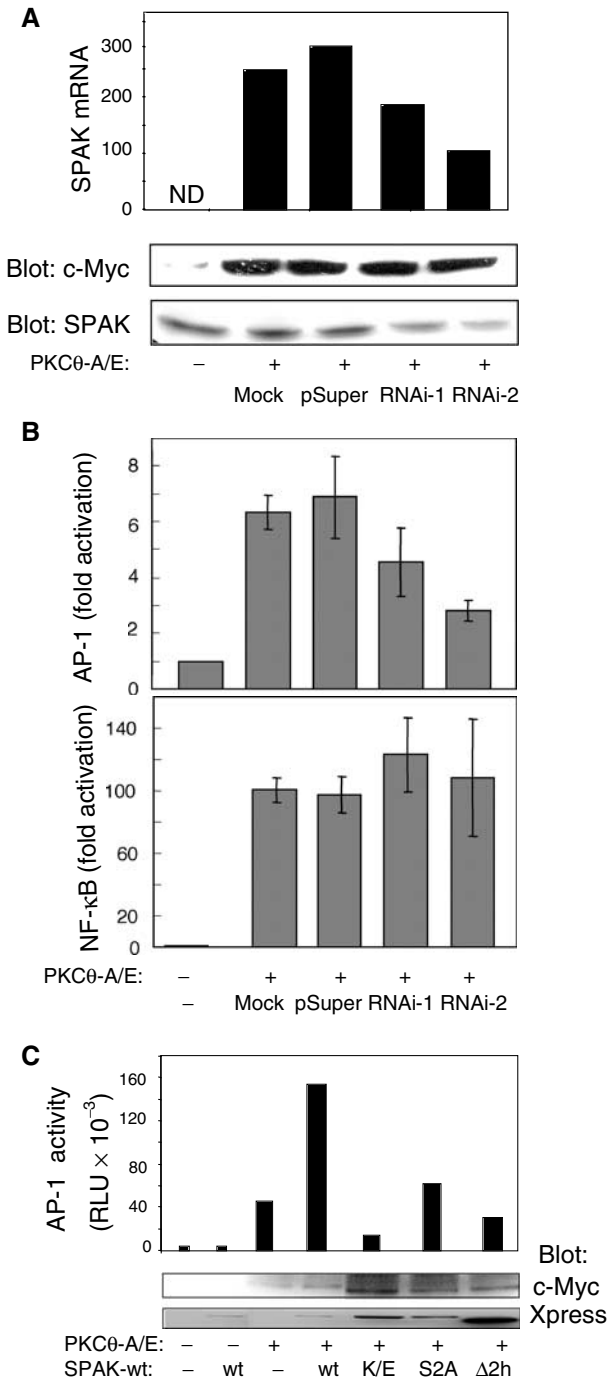

Figure 7 SPAK is required for PKCθ-A/E-induced AP-1, but not NF-κB, activation. (**A, B**) Jurkat E6.1 cells were transfected with empty vector (left lane) or c-Myc–PKCθ-A/E (four right lanes) plus the indicated pSuper, pSuper–RNAi-1 or pSuper–RNAi-2 vectors, β-Gal reporter plasmid and AP-1 (B; upper panel) or NF-κB (B; lower panel) reporter genes. (A) Expression levels of SPAK mRNA (measured by real-time RT–PCR and expressed as arbitrary units), and SPAK or PKCθ protein (measured by immunoblotting with anti-SPAK or anti-c-Myc antibodies, respectively). (B) Normalized AP-1 (upper panel) or NF-κB (lower panel) activities in the same cells. (**C**) Jurkat-TAg cells were cotransfected with the indicated combinations of empty vector, c-Myc–PKCθ-A/E and/or different SPAK constructs together with AP-1–Luc and β-Gal reporter genes. Cell extracts were prepared 24 h later, and normalized AP-1 activity (upper panel) or protein expression levels (two lower panels) were determined. Mean values of duplicate determination ± s.e. are shown. Similar results were obtained in two additional experiments.

attesting to the biological relevance of S311 (and S325?) in the functional interaction between PKCθ and SPAK. The interaction-deficient mutant (Δ2h) of SPAK also failed to synergize with PKCθ-A/E, indicating that the association of SPAK with PKCθ is essential for its biological activity, that is, AP-1 activation.

Discussion

The central role of PKCθ in mature T-cell activation is clearly established (Sun *et al*, 2000; Isakov and Altman, 2002; Pfeifhofer *et al*, 2003). This role reflects the fact that several transcription factors essential for IL-2 induction, that is, NF-κB, AP-1 and NFAT, are regulated by PKCθ. Thus, considerable effort is currently centered on defining the molecular pathways leading from the early activation of PKCθ in the immunological synapse (Monks *et al*, 1997, 1998) to AP-1 and NF-κB activation and identifying the relevant intermediate components. In general, more progress has been made in characterizing PKCθ signaling pathways involved in NF-κB activation. Following earlier demonstrations that PKCθ regulates IκB degradation (Sun *et al*, 2000) and IKKβ activation (Coudronniere *et al*, 2000; Lin *et al*, 2000), and associates with the IKK signalsome (Khoshnan *et al*, 2000), more recent work has focused on the CARD-domain-containing proteins, Bcl10 and CARMA-1/CARD11, as adapters that potentially link PKCθ to NF-κB activation (Gaide *et al*, 2002; Pomerantz *et al*, 2002; Wang *et al*, 2002). Interestingly, these two adapters do not play an apparent role in AP-1 activation (Pomerantz *et al*, 2002; Wang *et al*, 2002; Hara *et al*, 2003; Jun *et al*, 2003). In contrast, less progress was made in understanding the signaling pathway mediating AP-1 activation by PKCθ. Our earlier work implicated Ras in this pathway (Baier-Bitterlich *et al*, 1996). However, the biological relevance of the documented PKCθ-mediated JNK activation in Jurkat T cells (Avraham *et al*, 1998; Werlen *et al*, 1998; Ghaffari-Tabrizi *et al*, 1999) for AP-1 activation is questionable, as JNK activation was reported to be intact in PKCθ-deficient T cells (Sun *et al*, 2000) and, moreover, JNK1 and JNK2 are not required for primary T-cell activation (Dong *et al*, 2000).

Here, we identify SPAK, a Ste20-related kinase isolated by several groups (Ushiro *et al*, 1998; Johnston *et al*, 2000), as an immediate target of PKCθ in a selective pathway involved in AP-1 activation. The findings that support this conclusion include (1) the selective interaction of SPAK with PKCθ, but not PKCα, and the enhancement of this association induced by TCR/CD28 costimulation; (2) the enhanced *in vivo* phosphorylation of SPAK in cells coexpressing constitutively active PKCθ and, more importantly, the direct *in vitro* phosphorylation of recombinant, kinase-inactive SPAK by purified PKCθ (but not PKCα), which occurs predominantly at Ser-311. The sequence surrounding this residue (KYGK$\underline{S}^{311}$FRKL) resembles the pseudosubstrate sequence found in PKCθ (RRGAIKQAK, where the serine residue is replaced by an isoleucine) in that it also contains the requisite basic residues surrounding the phosphorylation sites. Although it remains to be proven that S311 is phosphorylated by PKCθ in intact T cells in response to CD3/CD28 costimulation, our finding that mutation of this site reduces both the CD3/CD28-induced activation of SPAK and its ability to activate AP-1 in T cells does lend biological significance to this site; (3) the enhancement

of SPAK activation by CD28 costimulation, similar to what was found for PKCθ (Coudronniere *et al*, 2000; Villalba *et al*, 2000; Bi *et al*, 2001); (4) the requirement of the PKCθ-interacting domain of SPAK (residues 449–547) for its maximal TCR/CD28-induced activation and its ability to activate synergistically AP-1 when combined with PKCθ; (5) the markedly impaired (in terms of both magnitude and duration) of SPAK activation in PKCθ$^{-/-}$ T cells; and (6) the synergistic activation of AP-1, but not NF-κB, by SPAK and PKCθ and, conversely, the ability of SPAK-specific RNAi or a dominant-negative SPAK mutant to inhibit AP-1, but not NF-κB, activation. This selectivity indicates that SPAK lies downstream of PKCθ in a signaling pathway leading to AP-1, but not NF-κB, activation. Thus, SPAK defines a point at which the PKCθ signaling pathways involved in AP-1 versus NF-κB activation have diverged.

The ability of PKCθ, but not PKCα, to phosphorylate SPAK *in vitro* is intriguing as protein kinases tend to be more promiscuous *in vitro*, and the consensus phosphorylation sites for different members of the PKC family are quite similar, albeit not identical (Nishikawa *et al*, 1997). However, a recent study reported that moesin is phosphorylated *in vitro* by PKCθ, but not by other PKC isotypes (Pietromonaco *et al*, 1998). The residual SPAK activation in PKCθ$^{-/-}$ T cells indicates that a PKCθ-independent pathway, for example, autophosphorylation or transphosphorylation by some other kinase, may contribute to SPAK activation, although this level of activation is apparently insufficient for full AP-1 activation given the impaired receptor-induced AP-1 activation in PKCθ-deficient peripheral T cells (Sun *et al*, 2000). In particular, the shorter duration of SPAK activation in the absence of PKCθ may be relevant as, for example, the duration (in addition to the magnitude) of ERK activation can have a major impact on positive selection in the thymus (Hogquist, 2001).

Our work demonstrates that the 99 COOH-terminal amino acids of SPAK, corresponding to the SPAK fragment isolated in the initial yeast two-hybrid screen, are necessary and sufficient for binding PKCθ. Interestingly, this COOH-terminal fragment was required for activation of SPAK by TCR/CD28 costimulation in intact cells and for SPAK-mediated AP-1 activation, suggesting that receptor-mediated SPAK activation depends on its association with PKCθ. Although in most cases kinases do not stably associate with their substrate, the association between PKCθ and SPAK and its apparently important role in proper SPAK activation is reminiscent of the stable interaction of JNK with its substrate, c-Jun, which is important for efficient c-Jun phosphorylation and activation (Kallunki *et al*, 1996). The isolated COOH-terminal fragment of SPAK appeared to bind PKCθ more effectively than the other SPAK constructs containing this region, suggesting that region(s) of SPAK outside the COOH-terminus may exert a negative regulatory influence on the interaction with PKCθ. Our results also revealed a weak interaction of the kinase domain of SPAK with PKCθ, consistent with the report of a SPAK-co-immunoprecipitated unidentified Ser/Thr kinase, which associated with the catalytic domain of SPAK (Johnston *et al*, 2000). This interaction could reflect a weak kinase-substrate interaction between PKCθ and SPAK. However, inclusion of the 61 NH_2-terminal residues of SPAK reduced this weak interaction to a barely detectable level, suggesting that these residues may negatively regulate the SPAK–PKCθ interaction.

Taken together, our findings suggest the following scenario for the regulated activation of SPAK in T cells. In resting cells, SPAK associates constitutively with PKCθ via its COOH-terminal region. TCR/CD28 costimulation significantly enhances this association, perhaps by inducing additional interaction(s) between the two enzymes and/or localizing the SPAK–PKCθ complex to a compartment, which stabilizes this interaction, for example, membrane lipid rafts (Bi *et al*, 2001). When PKCθ is activated by TCR/CD28 costimulation, it phosphorylates the associated SPAK, allowing its activation. Full activation of SPAK may involve its autophosphorylation at additional site(s), consistent with the report that SPAK autophosphorylates (Johnston *et al*, 2000). The reported association of SPAK with an unknown, SPAK-phosphorylating Ser/Thr kinase in mammalian cells (Johnston *et al*, 2000) raises the possibility that SPAK, which is ubiquitously expressed (Ushiro *et al*, 1998; Johnston *et al*, 2000), associates with different members of the PKC family (or with other kinases) in other cell types. However, we could not detect an interaction between SPAK and PKCα in T cells.

Little is known about the physiological functions of SPAK, which belongs to the SPS1 subfamily of STE20 kinases and is considered to be an upstream MAP4K or MAP3K (Johnston *et al*, 2000). SPAK undergoes stress-induced translocation to the cytoskeleton, and it associates in the brain with protein complexes that include actin and tubulin, and *in vitro* with actin (Tsutsumi *et al*, 2000). SPAK specifically activates p38 stress-activated protein kinase in cotransfection assays (Johnston *et al*, 2000), and associates with the cation chloride cotransporters, KCC3, NKCC1 and NKCC2, which are activated during osmotic and oxidative stress in order to maintain fluid/ion homeostasis (Piechotta *et al*, 2002). Lastly, androgen stimulation was found to upregulate SPAK expression in prostate tissue (Qi *et al*, 2001). Based on these findings and the close relationship of SPAK to yeast Ste20 and Sps1 (Ushiro *et al*, 1998; Johnston *et al*, 2000), it has been suggested that SPAK mediates stress-activated signals (Johnston *et al*, 2000; Tsutsumi *et al*, 2000; Piechotta *et al*, 2002) and regulates the cytoskeleton in response to cellular stress (Tsutsumi *et al*, 2000).

The above studies and our results raise the possibility that in T cells, PKCθ may participate in mediating stress signals. Note that TCR/CD28 costimulation induces cytoskeletal translocation of PKCθ (Bi *et al*, 2001; Villalba *et al*, 2001) as well as the activation of JNK and p38, two stress-activated protein kinases (Su *et al*, 1994; Salojin *et al*, 1999; Schafer *et al*, 1999; Zhang *et al*, 1999). It would be interesting to determine whether SPAK selectively activates p38 in T cells and whether PKCθ plays some role in p38 activation. Although stimulated PKCθ$^{-/-}$ primary T cells reportedly display intact phosphorylation of p38 (Sun *et al*, 2000), it is possible that compensatory p38-activating mechanisms operate under conditions of chronic PKCθ deficiency in the respective knockout mice. Studies are currently in progress to address this potential link and to determine whether SPAK-mediated AP-1 activation depends on JNK. These and other studies aimed at identifying the targets of SPAK in T cells will shed light on the role of this kinase in T cells.

Materials and methods

Mice, antibodies and enzymes

PKCθ-deficient mice were a kind gift from Dr Dan Littman (Sun *et al*, 2000). Normal C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). The anti-human (OKT3) or -mouse (2C11) CD3 monoclonal antibodies (mAb) and the anti-mouse CD28 mAb (37.51) were affinity purified from culture supernatants of the corresponding hybridomas as described (Liu *et al*, 1997). An anti-human CD28 mAb was obtained from Pharmingen (San Diego, CA), and the PKCθ-specific mAb (clone 27) was from Transduction Laboratories (Lexington, KY). An anti-PKC mAb (MC5), which crossreacts with the three cPKC isotypes (α, β and γ), and the anti-c-Myc mAb (9E10) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Xpress mAb was from Invitrogen Life Technologies (Carlsbad, CA). Anti-Akt or phospho-Akt antibodies were from Cell Signaling Technology (Beverly, MA). Polyclonal rabbit anti-SPAK antibodies were obtained from Dr L Harrison (Walter and Eliza Hall Institute of Medical Research, Parkville, Australia) (Johnston *et al*, 2000), or generated by immunizing two rabbits with a purified GST–SPAK fusion protein coupled to keyhole limpet hemocyanin. Recombinant PKCθ was prepared and purified as described (Liu *et al*, 1999). Recombinant PKCα was purchased from Calbiochem (San Diego, CA).

Yeast two-hybrid screening

The yeast pGilda plasmid encoding a kinase-inactive mutant of human PKCθ (PKCθ-K/R) fused to the LexA DNA-binding domain (pBD–PKCθ-K/R) has been described (Witte *et al*, 2000). A human Jurkat T-cell cDNA library in the pJG4-5 vector, which expresses the *Escherichia coli* B42 transactivating domain (pAD) (Ma and Ptashne, 1987), was obtained from Origen Technologies, Inc. (Rockville, MD). Library screening by the yeast two-hybrid method was performed according to the Origen manual. Briefly, the yeast EGY48 strain of *Saccharomyces cerevisiae* (MAT, *trp1, ura3, his, leu2:plexApo6-leu2*) was first transformed with the lacZ reporter plasmid, p8op–lacZ, to create EGY48[p8op–lacZ]. The latter yeast was transformed with the cDNA library (0.1 mg) and denatured salmon sperm DNA (5 mg) using LiCl. Colonies that formed on leucine-deficient SD-plates containing 2% galactose and 1% raffinose were removed and grown in fresh SD-plates for 2–4 days, then transferred to a Whatman filter paper, and tested for activation of the *lacZ* reporter gene using the β-galactosidase (β-Gal) colony-lift filter assay. From an initial screen of $\sim 2 \times 10^7$ transformants, 70 colonies that transactivated the *LEU2* reporter gene were identified. Of these, 50 colonies were also positive for β-Gal when color was scored after 1 h. Isolated plasmid DNAs from these $LEU^+/lacZ^+$ clones were again cotransformed with the PKCθ-K/R bait (or with empty pGilda as a negative control) to eliminate false positives and confirm two-hybrid interactions.

Cloning of full-length SPAK

Messenger RNA from Jurkat T cells was isolated using the Trizol–phenol method and reverse transcribed using an oligo(dT) primer and Superscript II reverse transcriptase (Invitrogen). The cDNAs were amplified by PCR using the Advantage-GC2 PCR kit from BD Biosciences Clontech (Palo Alto, CA). Briefly, 5′ and 3′ primers (2 μM each) specific for human SPAK (GenBank accession number AF099989) were dissolved in 50 μl of Tricine–KOH (40 mM, pH 9.2 at 25°C) containing 1 μl of Advantage-GC2 polymerase mix (Clontech), 0.5 M GC-Melt™, 15 mM potassium acetate, 3.5 mM magnesium acetate, 5% (v/v) DMSO and 3.75 μg/ml BSA. The PCR was carried out by incubating the sample at 94°C for 1 min, followed by 35 cycles at 94°C for 30 s, 56°C for 4 min and 68°C for 3 min, and soaking in buffer at 15°C. The PCR product was analyzed on a 1% agarose gel and sequenced.

Expression plasmids

c-Myc-epitope-tagged mammalian expression vectors of wild-type PKCθ or constitutively active PKCθ (A/E mutant) were constructed by PCR using a pair of PKCθ-specific primers, of which the 5′ primer also included a sequence encoding a c-Myc epitope. After sequencing, the PCR fragments were cloned into the *Bam*HI/*Xba*I sites of the mammalian expression vector pEF4/Myc-His C (Invitrogen), which expresses an additional c-Myc epitope tag downstream of the cDNA insert. cDNAs corresponding to full-length SPAK or fragments thereof (Figure 3A) were generated by standard PCR and cloned into the *Eco*RI/*Xho*I sites of the bacterial expression vector pGEX–4T-1 (Amersham Pharmacia Biotech, Piscataway, NJ) or into the *Eco*RI/*Xba*I sites of pEF4/His C. A point mutation inactivating the catalytic activity of SPAK (K94E or K/E) was generated by site-directed mutagenesis using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and a wild-type SPAK–GST fusion construct in pGEX–4T-1 or pEF4–SPAK as templates. The AP-1, NF-κB and β-Gal reporter plasmids have been described (Coudronniere *et al*, 2000; Villalba *et al*, 2000; Bi *et al*, 2001).

GST fusion protein purification and binding assay

Bacterial expression vectors were used to transform competent BL21 *E. coli* cells, which were grown on LB/ampicillin plates overnight. IPTG (isopropyl-1-thio-β-D-galactosidase) induction, recombinant protein immobilization on glutathione–sepharose beads, binding assays and analysis of bound proteins were conducted as described (Liu *et al*, 1997, 1999). When necessary, the bound proteins were eluted from the beads by adding 50 mM Tris–HCl (pH 8.0) and 5 mM reduced glutathione.

Cell culture, transfection, labeling and reporter assays

Primary T cells were isolated from the spleens or lymph nodes of wild-type C57BL/6 mice or $PKC\theta^{-/-}$ mice by standard procedures, and enriched to >85% purity using a mouse T-cell enrichment column (R&D Systems, Minneapolis, MN). The cells were stimulated for the indicated times with anti-CD3 (10 μg/ml) and/or -CD28 mAbs (2 μg/ml each), which were crosslinked using a goat anti-Syrian hamster Ig (10 μg/ml), or with PMA (50 ng/ml). Culture and transfection of human leukemic Jurkat (E6.1), Jurkat-TAg cells or 293T cells, as well as reporter assays, were described (Coudronniere *et al*, 2000; Villalba *et al*, 2000; Bi *et al*, 2001). Reporter assays were performed at least three times with similar results. In some experiments, transfected 293T cells were starved in phosphate-free medium for 3 h, washed ×3 in serum-free, phosphate-free DMEM, and then metabolically radiolabeled by overnight incubation in serum-supplemented phosphate-free DMEM containing 0.3 mCi/ml $^{32}P_i$ (ICN Biochemicals Inc., Costa Mesa, CA). The cells were washed, lysed and subjected to SDS–PAGE and immunoblotting as described below.

Immunoprecipitation, immunoblotting and kinase assays

These procedures were performed as described (Coudronniere *et al*, 2000; Villalba *et al*, 2000; Bi *et al*, 2001). MBP (1 μg) or the indicated recombinant GST–SPAK proteins (1 μg) were used as substrates in kinase reactions. Titration experiments using different amounts of recombinant PKCθ or PKCα and MBP as substrate were initially performed in order to determine the amounts of recombinant enzymes that yielded equivalent phosphorylation of MBP. These amounts were then used to determine the phosphorylation of recombinant SPAK. Kinase reactions were analyzed by SDS–PAGE and autoradiography, and substrate phosphorylation was quantitated using the NIH Image 1.61 densitometry software.

RNAi experiments and real-time RT–PCR

The mammalian RNAi expression vector, pSuper.retro.neo + gfp (OligoEngine, Seattle, WA) was used for siRNA expression in Jurkat-TAg cells. Two *SPAK* gene-specific oligonucleotides, which specify 19-mer sequences corresponding to nucleotides 255–273 (acccagg caagaacgtgta; RNAi-1) or 334–352 (attcaagccatgagtcagt; RNAi-2) downstream of the transcription start site of *SPAK*, were inserted into the *Bgl*II/*Hin*dIII-digested circular pSuper vector.

Real-time RT–PCR was performed on a GeneAmp 5700 Sequence Detector (Applied Biosystems, Foster City, CA) using SYBR Green technology. PCR primers were designed using Primer Express 1.0 software with the manufacturer's default settings. The *SPAK*-specific primers were: aggaggttatcggcagtgga (forward) and tgcatagggctgcct gaac (reverse). Amplification of human ribosomal protein L32-3A gene was used in the same reaction as an internal control, and the expression level of SPAK mRNA was normalized to that of L32-3A mRNA.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank Drs Dan Littman and Leonard Harrison and Mr Pat Fitzgerald for providing PKCθ$^{-/-}$ mice, anti-SPAK antibody and the Jurkat cDNA library, respectively. This work was supported by grants CA35299, CA95332 and AI49888 from the National Institutes of Health. This is publication number 560 from the La Jolla Institute for Allergy and Immunology.

References

Altman A, Isakov N, Baier G (2000) PKCθ: a new essential superstar on the T cell stage. *Immunol Today* **21:** 567–573

Arendt CW, Albrecht B, Soos TJ, Littman DR (2002) Protein kinase C-θ: signaling from the center of the T-cell synapse. *Curr Opin Immunol* **14:** 323–330

Avraham A, Jung S, Samuels Y, Seger R, Nen-Neriah Y (1998) Co-stimulation-dependent activation of a JNK-kinase in T lymphocytes. *Eur J Immunol* **28:** 2320–2330

Baier-Bitterlich G, Überall F, Bauer B, Fresser F, Wachter H, Grünicke H, Utermann G, Altman A, Baier G (1996) PKCθ isoenzyme selective stimulation of the transcription factor complex AP-1 in T lymphocytes. *Mol Cell Biol* **16:** 1842–1850

Bi K, Tanaka Y, Coudronniere N, Hong S, Sugie K, van Stipdonk MJB, Altman A (2001) Antigen-induced translocation of PKC-θ to membrane rafts is required for T cell activation. *Nat Immunol* **2:** 556–563

Coudronniere N, Villalba M, Englund N, Altman A (2000) NF-κB activation induced by CD28 costimulation is mediated by PKCθ. *Proc Natl Acad Sci USA* **97:** 3394–3399

Dong C, Yang DD, Tournier C, Whitmarsh AJ, Xu J, Davis RJ, Flavell RA (2000) JNK is rquired for effector T-cell function but not for T-cell activation. *Nature* **405:** 91–94

Gaide O, Favier B, Legler DF, Bonnet D, Brissoni B, Valitutti S, Bron C, Tschopp J, Thome M (2002) CARMA1 is a critical lipid raft-associated regulator of TCR-induced NF-κB activation. *Nat Immunol* **3:** 836–843

Ghaffari-Tabrizi N, Bauer B, Altman A, Utermann G, Uberall F, Baier G (1999) Protein kinase Cθ, a selective upstream regulator of JNK/SAPK and IL-2 promoter activation in Jurkat T-cells. *Eur J Immunol* **29:** 132–142

Hara H, Wada T, Bakal C, Kozieradzki I, Suzuki S, Suzuki N, Nghiem M, Griffiths EK, Krawczyk C, Bauer B, D'Acquisto F, Ghosh S, Yeh WC, Baier G, Rottapel R, Penninger JM (2003) The MAGUK family protein CARD11 is essential for lymphocyte activation. *Immunity* **18:** 763–775

Hogquist KA (2001) Signal strength in thymic selection and lineage commitment. *Curr Opin Immunol* **13:** 225–231

Isakov N, Altman A (2002) Protein kinase C-θ in T cell activation. *Annu Rev Immunol* **20:** 761–794

Johnston AM, Naselli G, Gonez LJ, Martin RM, Harrison LC, DeAizpurua HJ (2000) SPAK, a STE20/SPS1-related kinase that activates the p38 pathway. *Oncogene* **19:** 4290–4297

Jun JE, Wilson LE, Vinuesa CG, Lesage S, Blery M, Miosge LA, Cook MC, Kucharska EM, Hara H, Penninger JM, Domashenz H, Hong NA, Glynne RJ, Nelms KA, Goodnow CC (2003) Identifying the MAGUK protein Carma-1 as a central regulator of humoral immune responses and atopy by genome-wide mouse mutagenesis. *Immunity* **18:** 751–762

Kallunki T, Deng T, Hibi M, Karin M (1996) c-Jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. *Cell* **87:** 929–939

Karin M, Liu Z, Zandi E (1997) AP-1 function and regulation. *Curr Opin Cell Biol* **9:** 240–246

Khoshnan A, Bae D, Tidell CA, Nel AE (2000) The physical association of protein kinase Cθ with a lipid raft-associated inhibitor of κB factor kinase (IKK) complex plays a role in the activation of the NF-κB cascade by TCR and CD28. *J Immunol* **165:** 6933–6940

Lin X, O'Mahony A, Geleziunas R, Greene WC (2000) Protein kinase C θ participates in NF-κB/Rel activation induced by CD3/CD28 costimulation through selective activation of IκB β (IKKβ). *Mol Cell Biol* **20:** 2933–2940

Liu Y, Liu Y-C, Meller N, Giampa L, Elly C, Doyle M, Altman A (1999) PKC activation inhibits tyrosine phosphorylation of Cbl and its recruitment of SH2 domain-containing proteins. *J Immunol* **162:** 7095–7101

Liu YC, Liu Y, Elly C, Yoshida H, Lipkowitz S, Altman A (1997) Serine phosphorylation of Cbl induced by phorbol ester enhances its association with 14-3-3 proteins in T cells via a novel serine-rich 14-3-3-binding motif. *J Biol Chem* **272:** 9979–9985

Ma J, Ptashne M (1987) A new class of yeast transcriptional activators. *Cell* **51:** 113–119

Monks CR, Freiberg BA, Kupfer H, Sciaky N, Kupfer A (1998) Three-dimensional segregation of supramolecular clusters in T cells. *Nature* **395:** 82–86

Monks CRF, Kupfer H, Tamir I, Barlow A, Kupfer A (1997) Selective modulation of protein kinase C-θ during T-cell activation. *Nature* **385:** 83–86

Nishikawa K, Toker A, Johannes F-J, Songyang Z, Cantley LC (1997) Determination of the specific substrate sequence motifs of protein kinase C isozymes. *J Biol Chem* **272:** 952–960

Pfeifhofer C, Kofler K, Gruber T, Ghaffari-Tabrizi N, Lutz C, Maly K, Leitges M, Baier G (2003) Protein kinase Cθ affects Ca^{2+} mobilization and NFAT cell activation in primary mouse T cells. *J Exp Med* **197:** 1525–1535

Piechotta K, Lu J, Delpire E (2002) Cation chloride cotransporters interact with the stress-related kinases Ste20-related proline-alanine-rich kinase (SPAK) and oxidative stress response 1 (OSR1). *J Biol Chem* **277:** 50812–50819

Pietromonaco SF, Simons PC, Altman A, Elias L (1998) Protein kinase C-θ phosphorylation of moesin in the actin-binding sequence. *J Biol Chem* **273:** 7594–7603

Pomerantz JL, Denny EM, Baltimore D (2002) CARD11 mediates factor-specific activation of NF-κB by the T cell receptor complex. *EMBO J* **21:** 5184–5194

Qi H, Labrie Y, Grenier J, Fournier A, Fillion C, Labrie C (2001) Androgens induce expression of SPAK, a STE20/SPS1-related kinase, in LNCaP human prostate cancer cells. *Mol Cell Endocrinol* **182:** 181–192

Salojin K, Zhang J, Delovitch T (1999) TCR and CD28 are coupled Via ZAP-70 to the activation of the Vav/Rac-1-/PAK-1/p38 MAPK signaling pathway. *J Immunol* **163:** 844–853

Schafer PH, Wadsworth SA, Wang L, Siekierka JJ (1999) p38a mitogen-activated protein kinase is activated by CD28-mediated signaling and is required for IL-4 production by human $CD4^+CD45R0^+$ T cells and Th2 effector cells. *J Immunol* **162:** 7110–7119

Su B, Jacinto E, Hibi M, Kallunki T, Karin M, Ben Neriyah Y (1994) JNK is involved in signal integration during costimulation of T lymphocytes. *Cell* **77:** 727–736

Sun Z, Arendt CW, Ellmeier W, Schaeffer EM, Sunshine MJ, Gandhi L, Annes J, Petrzilka D, Kupfer A, Schwartzberg PL, Littman DR (2000) PKC-θ is required for TCR-induced NF-κB activation in mature but not immature T lymphocytes. *Nature* **404:** 402–407

Tsutsumi T, Ushiro H, Kosaka T, Kayahara T, Nakano K (2000) Proline- and alanine-rich Ste20-related kinase associates with F-actin and translocates from the cytosol to cytoskeleton upon cellular stresses. *J Biol Chem* **275:** 9157–9162

Ushiro H, Tsutsumi T, Suzuki K, Kayahara T, Nakano K (1998) Molecular cloning and characterization of a novel Ste20-related protein kinase enriched in neurons and transporting epithelia. *Arch Biochem Biophys* **355:** 233–240

Villalba M, Bi K, Rodriguez F, Tanaka Y, Schoenberger S, Altman A (2001) Vav1/Rac-dependent actin cytoskeleton reorganization is required for lipid raft clustering in T cells. *J Cell Biol* **155:** 331–338

Villalba M, Coudronniere N, Deckert M, Teixeiro E, Mas P, Altman A (2000) Functional interactions between Vav and PKCθ are required for TCR-induced T cell activation. *Immunity* **12:** 151–160

Wang D, You Y, Case SM, McAllister-Lucas LM, Wang L, DiStefano PS, Nunez G, Bertin J, Lin X (2002) A requirement for CARMA1 in TCR-induced NF-κB activation. *Nat Immunol* **3:** 830–835

Werlen G, Jacinto E, Xia Y, Karin M (1998) Calcineurin preferentially synergizes with PKC-θ to activate JNK and IL-2 promoter in T lymphocytes. *EMBO J* **17:** 3101–3111

Witte S, Villalba M, Liu Y, Hernandez J, Isakov N, Altman A (2000) Inhibition of the c-Jun N-terminal kinase/AP-1 and NF-κB pathways by PICOT, a novel protein kinase C-interacting protein with a thioredoxin-homology domain. *J Biol Chem* **275:** 1902–1909

Zhang J, Salojin KV, Gao JX, Cameron MJ, Bergerot I, Delovitch TL (1999) p38 mitogen-activated protein kinase mediates signal integration of TCR/CD28 costimulation in primary murine T cells. *J Immunol* **162:** 3819–3829